JNK mediates TGF-β1-induced epithelial mesenchymal transdifferentiation of mouse transformed keratinocytes

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Abstract In this study we analyzed the role of the c-Jun N-terminal kinases (JNK) pathway in the TGF-β1 stimulation of urokinase-type plasminogen activator (uPA), initial stages of epithelial-mesenchymal transdifferentiation (EMT) and cell migration. TGF-β1 induces JNK phosphorylation, c-Jun transactivation and AP1 activation. The involvement of JNK was evaluated using dominant negative mutants SEK-1 AL, JNK and cJun, depletion of JNK1,2 proteins by treatment of cells with antisense oligonucleotides, as well as the chemical inhibitor SP600125. Our results demonstrated that the JNK pathway is required in the TGF-β1 enhancement of uPA, fibronectin, E-cadherin delocalization, actin re-organization and vimentin expression, concomitant with the induction of cell migration. These results allow us to suggest a role of JNK in the TGF-β1 induction of EMT in relation with the stimulation of malignant properties of mouse transformed keratinocytes.

Keywords: JNK; TGF-β1; uPA; Keratinocytes; EMT

1. Introduction

The process of epithelial-mesenchymal transdifferentiation (EMT) is characterized by a set of transient phenotypic changes often associated with the acquisition of migratory properties by cancer cells and provides a means for cancer propagation through out the organism [1,2].

The TGF-β super-family is implicated in the regulation of cell proliferation, differentiation, migration, extracellular matrix production, apoptosis and tumorigenesis [3]. TGF-β binds to the functional complex of the TGF-β family of receptors at the cell surface [4,5], which, in turn, activate the Smads and MAP kinases pathways including Ras, Erk1/2, and JNK1 [6–12]. Transforming growth factor-β1 (TGF-β1) has been postulated to have a dual role in tumour progression by acting as tumor suppressor in the early stages of carcinogenesis, and as pro-oncogenic in the last stages of metastatic disease [13,14]; it also induces EMT of transformed cells [15,16].

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Abbreviations: EMT, epithelial-mesenchymal transdifferentiation; JNK, cJun N-terminal kinase; uPA, urokinase type plasminogen activator; TGF-β1, Transforming growth factor-β1; Fn, Fibronectin; E-cad, E-cadherin; vim, vimentin

TGF-β1 increases plasminogen activator like-urokinase (uPA) expression, which is regulated at the transcriptional level by the AP-1 transcription factor [17,18]. The transcription factor c-Jun (AP-1 component) is consequently activated by the c-Jun NH_2-terminal kinase (JNK) [19].

Although Ras-MAP kinases and the Smad signal pathways contribute to malignant enhancement by TGF-β1 [10,20,21], the mechanisms that mediate TGF-β1 transformed cell responses have not been fully elucidated. In the present study, we have evaluated the role of the JNK pathway in the stimulation of uPA, cell migration and EMT by TGF-β1. We found that TGF-β1 activates JNK MAP kinase and transactivates the c-Jun and AP1 complex. In addition, the inhibition of the JNK pathway was found to affect uPA and cell migration and consequently the initial step of malignant EMT of mouse skin transformed cells.

2. Material and methods

2.1. Cell cultures and treatment conditions

The PDV cell line [22] was cultured as described [10]. Cells were incubated with TGF-β1 (Calbiochem-Novabiochem, La Jolla, CA) at a final concentration of 10 ng/ml for the indicated period of time. The chemical JNK inhibitor SP600125 (10 μM) provided by Calbiochem was added 30 min before the addition of TGF-β1.

2.2. Plasmids

AP-1-Luc and p-c-Fos-luc were provided by Dr. A. Corbí (Centro de Investigaciones Biológicas, Madrid, Spain), GAL4-c-Jun was provided by Dr. JL. Jameson (Northwestern University, Chicago, IL). Vector pFA-Luc (5× GAL4-binding element) was purchased from Stratagene. The pcDNA3.1 blank vector was obtained from Invitrogen (Carlsbad, CA). The p-4.8 uPa-Luc luciferase reporter plasmid (−4.8 kb of murine uPA promoter) was provided by Dr. P Munoz-Canoves (Center for Genomic Regulation (CRG), Barcelona, Spain). Dominant negative SEK1 AL (MEK4 mutant) was kindly provided by Dr. J. R. Woodgett (York University, Toronto, Ontario, Canada). Dominant Negative JNK and cJun were kindly provided by J. M. Redondo (Instituto Severo Ochoa, UAM, Madrid, Spain).

2.3. Antibodies

The anti-phospho JNK monoclonal antibody, anti-JNK1 rabbit polyclonal antibody, anti-p38 and anti-fibronectin monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-cadherin-E rat monoclonal antibody was kindly provided by Dr. M. Quintanilla (Instituto de Investigaciones Biomédicas, Madrid, Spain). The anti-vimentin and anti α-tubulin monoclonal antibody (Sigma, St. Louis, Mo).

2.4. Immunofluorescence

Cells seeded on coverslips were fixed with 4% *p*-formaldehyde for 10 min at room temperature. For JNK1,2, F-actin and vimentin immu-

nostaining cell monolayers were permeabilized with 0.1% Triton-X 100 for 2 min. at room temperature. F-actin was stained using Phalloidin-Alexa Fluor (Molecular Probes, Eugene, OR), and secondary rabbit, mouse and rat antibodies were coupled to FITC (Sigma, St. Louis, Mo).

2.5. Immunoblotting

Proteins were separated by SDS–PAGE and then transferred to nitrocellulose membranes (BIORAD, Hercules, CA) which were blocked in 4% milk (diluted in Tris-buffered saline and 0.5% Tween 20) and incubated with the appropriate antibody at 4 °C overnight. The targeted proteins were detected by enhanced chemiluminescence as indicated by the manufacturers (Pierce).

2.6. Transient transfections and reporter gene measurements

For luciferase assays, PDV cells were transfected with superfect (Quiagen) following the manufacturer's instructions. Typically, 2×10^5 cells were plated in each well of a 24-well plate. The next day cells were transfected with 500 ng/well of each specific luciferase construction, together with 25 ng/well SV40-β-Gal RL (Promega) as internal control for transfection efficiency. After 24 h of TGF-β1-treatment cells were lysed and luciferase activity determined. For the GAL4 fusion transactivation luciferase determinations, cells were co-transfected with 0.5 μg of pFA-Luc and 0.2 μg of Gal4-cJun1.

2.7. Oligodeoxynucleotide treatments

JNK1 antisense (TCACGCTTGCTTCTGCTCAT) and JNK2 antisense (TCACATTTACTGTCGCTCAT) phosphorothioate-modified oligodeoxynucleotides [36] were synthesized and purified by Isogen Bioscience BV (Maarssen, The Netherlands). As control, scrambled S-oligo were used. A 1:1 mixture of antisense oligonucleotides for both JNK 1 and 2 was added to the cells (50% confluent) and treated as described by Santibanez et al. [10]. The media containing oligos was changed daily.

2.8. Zymographic and migration assay

The uPA secreted activity of cell cultures was determined by caseinolytic zymography and the migration by wounded assay as previously described [10].

3. Results

3.1. TGF-β1 induces JNK activation and c-Jun and AP1 transactivation in PDV cells

To examine whether TGF-β1 activates the JNK pathway, we performed JNK phosphorylation, AP1 and cJun transactivation assays. TGF-β1 increased JNK phosphorylation within 60 min. (Fig. 1A), reached its maximum (~5.0 fold time) at 120 min and started to decrease after 4 hours. This rapid and

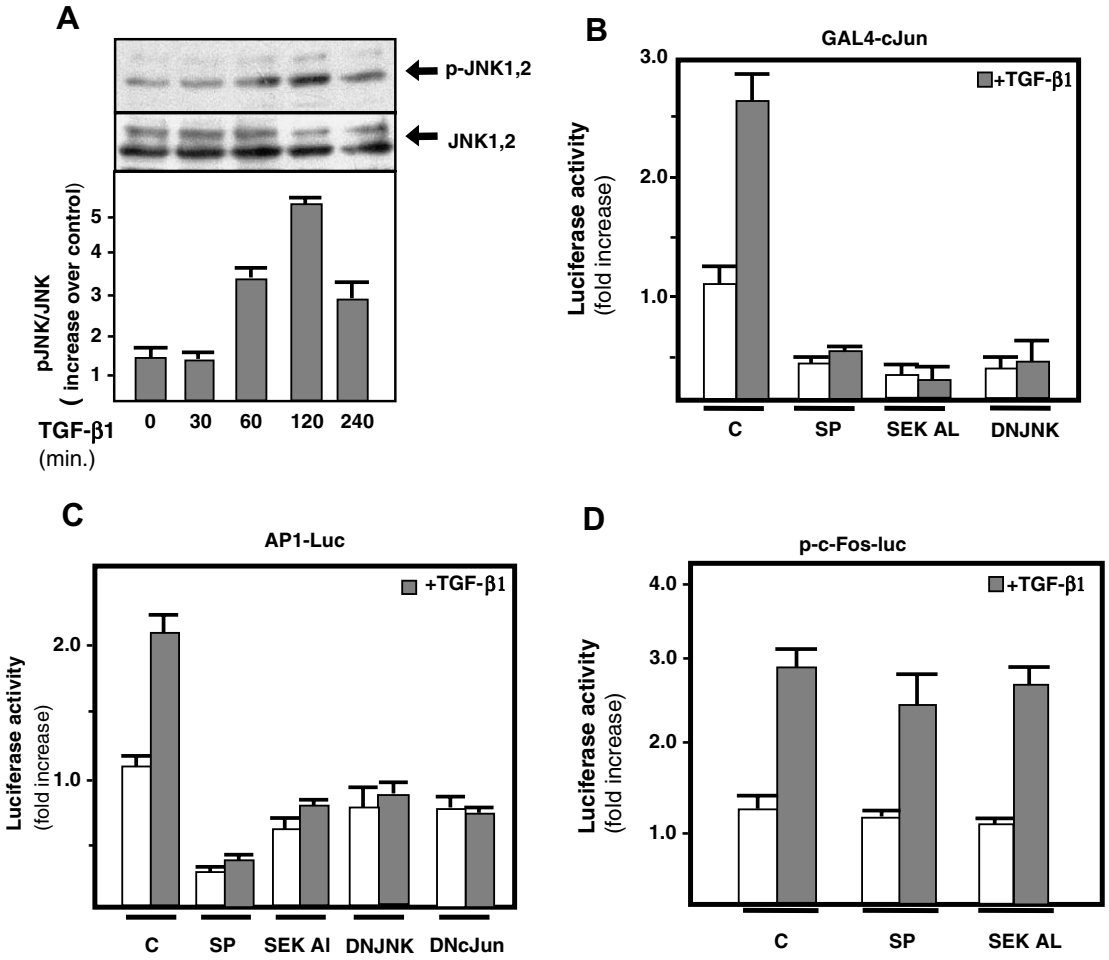

Fig. 1. TGF-β1 induces on JNK Phosphorylation, AP1 and c-Jun transactivation in PDV cells. (A) Cell lysates were immunobloted and revealed with either anti-p-JNK or JNK antibodies. Two independent experiments were performed and a representative is shown. Bottom part: densitometric scans of results shown in top part. Cells were transiently transfected with Gal-4-cJun/pRf-Luc (B), AP1-luc (C) or c-Fos-luc (D). Dominants negative SEK-AL, JNK or cJun were co-transfected, or pre-treated with JNK inhibitor SP600125 as in indicated points. Transfections and assays were performed independently three times, each run in triplicate.

transient change suggests a direct effect of TGF-β1 on the JNK pathway. Afterwards, we analyzed the capacity of TGF-β1 to transactivate the Gal-4-c-Jun and AP1 costructs. TGF-β1 enhanced significantly c-Jun transactivation, which was strongly inhibited by the dominant negatives SEK1 (SEK1-Al) and JNK (DNJNK) or JNK inhibitor SP600125 (Fig. 1B). A similar effect was observed in the TGF-β1-increased AP1 transactivation which in addition was inhibited by the dominant negative to c-Jun (DncJun) (Fig. 1C). To analyze the specificity of JNK activation by TGF-β1 in PDV cells we analyzed the c-Fos promoter, preferentially activated by the ERK1,2 pathway in PDV cells (data not shown). TGF-β1 stimulated the c-Fos promoter transactivation, and was not significantly inhibited by SEK-AL or SP600125 (Fig. 1D). Together, these results suggest that the TGF-β1 activates the JNK pathway in PDV cells.

3.2. TGF-β1-induced uPA expression is mediated by JNK pathway

To examine the role of JNK in the enhancement of cell malignant transformation by TGF-β1, we investigated the involvement of JNK in the regulation of *upa*, a gene known to be induced by TGF-β1, and highly involved in the invasion and migration of transformed cells [10,23]. To test whether JNK participates in TGF-β1-enhaced uPA, we transiently transfected PDV cells with mouse uPA promoter, and assayed uPA activities in conditioned media by caseinolytic zymography. After 24 h of TGF-β1 treatment of the uPA promoter the activation was strongly inhibited by co-transfection with either upstream SEK1 AL, dominant negative JNK or cJun constructs, as well as by the chemical JNK inhibitor SP600125 (Fig. 2A). As observed in Fig. 2B control cells secreted low levels of uPA and 24 h of TGF-β1 treatment of cells induced an approximately 2.5 fold increase of a single band Mr 45Kd corresponding to mouse uPA. The TGF-β1-increased uPA secreted activity was strongly suppressed by SP600125 (Fig. 2B). Thus, our results indicate that JNK activity was involved in the TGF-β1-induced upregulation of both uPA transcription and its enzimatic activity.

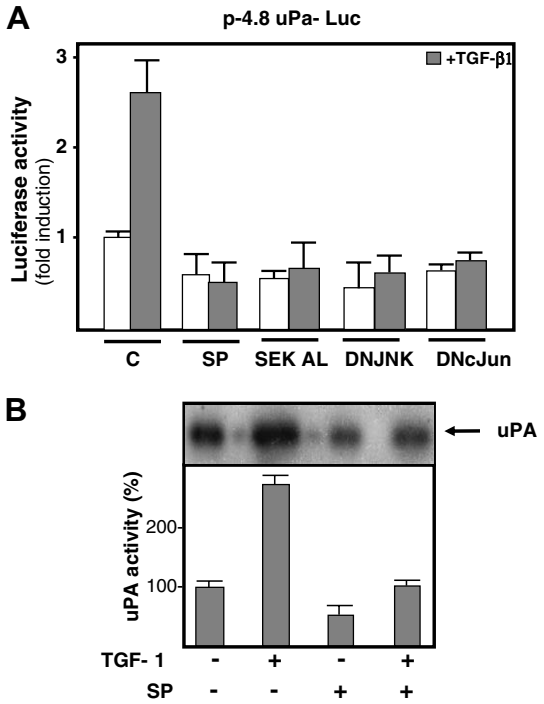

Fig. 2. The uPA promoter activation and secreted activity stimulated by TGF-β1 were blocked by JNK inhibition. (A) Cells were transiently transfected with p-4.8 Luc mouse uPA reporter gene. Dominants negative for SEK-AL, JNK and cJun were co-transfected. (B) Conditioned serum-free media from TGF-β1-stimulated cells (T) with or without SP600125 (SP) treatment were subject to caseinolysis zimographic analysis. Three independent experiments were performed and a representative one is shown here.

3.3. JNK mediates the TGF-β1-stimulated epithelial-mesenchymal transdifferentation and cell migration

TGF-β1 is a potent inductor of EMT; this process occurs associated with several changes in epithelial markers to the mesenchymal phenotype [24,25]. We analyzed the effect of JNK modulation on TGF-β1-induced EMT in PDV cells. For this, we used two experimental approaches by applying

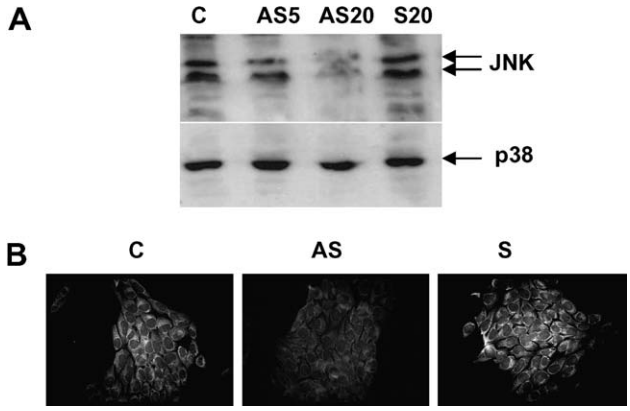

Fig. 3. JNK mediates TGF-β1-induced epithelial-mesenchymal transdifferentiation PDV cells and cell migration. (A) Cells seeded at 0.5 106 in six well plates were treated with JNK1,2 antisense-oligonucleotides, AS, (5 and 20 μM), or scramble oligonucleotides, S, (20 μM)n. After 48 h of treatment cells were lysates and subject to Western blot analysis for JNK1,2 and p38. Two independent experiments were performed and a representative one is shown. (B) Cells seeded in coverslips were treated as (A), except AS was only to 20 μM, and were immunostained to JNK1,2. Photograph is representative of two independent experiments. (C) Cells seeded in coverslips were immunostained to cellular-derived FN (a–e) (1000×), E-cadherin (e–h) (400×), stained for F-actin using phalloidin-Alexafluor 546 (i–l) (400×), and immunostained for vimentin expression (m–p) (400×). Cells were stimulated with TGF-β1 (T) for 48 h, except vimentin that was stimulated for 96 h. 10 μM of SP6001 (SP) and 20 μM JNK1,2 antisense oligonucleotides (AS) were used respectively. All photographs are representative fields of at least two or three independent experiments. (D and E) Statistical analysis for E-cadherine loss contact and vimentin expression. For E- cadherine the percentage of untreated cells was defined as 1, and the cells that lost E-cadherin contact are shown as relative value against the control. For vimentin a percentage of positive cells are represented. Values shown are the means (+/− S.E.M.) of ten different fields of two independent experiments. (F) Western blot for vimentin expression. Cells were seeded as (A), and treated for four days with TGF-β1 in presence or absence of 10 μM of SP6001 or 20 μM JNK antisense oligonucleotides, a lysates cells samples were subject to Western blot analysis for vimentin (Vm) and α-tubulin (α-Tub) as a control protein. Molecular weight markers are indicated on the left. Two independent experiments were performed and a representative is shown. (G) PDV cells monolayer were wounded and stimulated with TGF-β (T) in the presence or absence of SP600125 (SP) or AS JNK1,2 oligonucleotides (20 μM). Photograph is representative of two independent experiments.

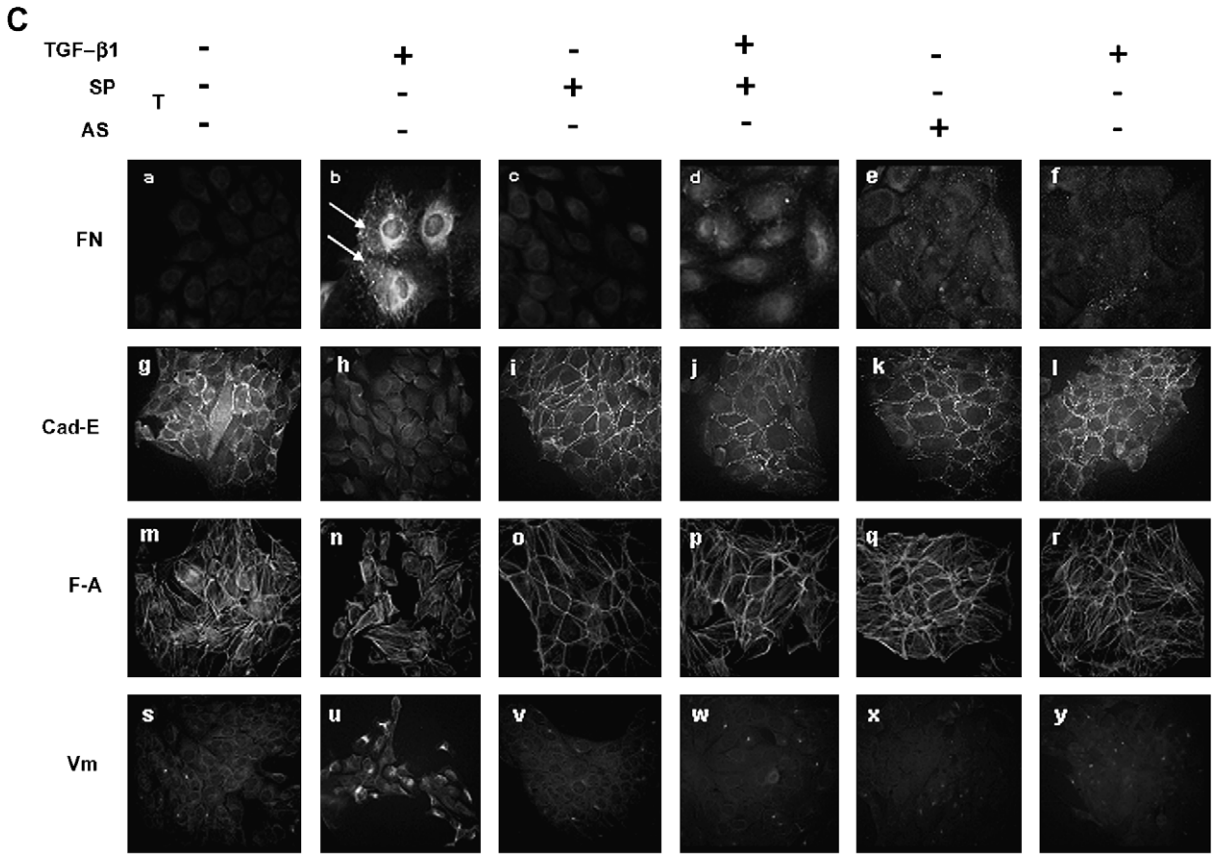

Fig. 3 *(continued)*

antisense S-oligo strategie to cells depleted of JNK1,2 protein, and JNK1,2 inhibition by using SP600125. As observed in Fig. 3A, antisense oligonucleotides inhibited the expression of JNK1,2 less intensely at 5 μM and strongly at 20 μM (about 90%), while the sense oligonucleotides had no effect on JNK expression. As a control we analyzed the expression of the p38 MAPK, and found no changes in p38 protein levels in cells pretreated with the JNK sense or antisense oligos. Similar results were observed by immunofluorescence analysis, where PDV cells treated with JNK antisense oligonucleotides displayed lower levels of immunoreactivity to the JNK1,2 antibody (Fig. 3B). We next tested whether JNK participates in fibronectin (FN) production and in fibrillogenesis in PDV cells stimulated by TGF-β1. After TGF-β1 treatment cells displayed an organized extracellular matrix of FN fibrils on their surface (arrows, Fig. 3C (b)); this effect was strongly inhibited by antisense oligos or SP600125 treatment (Fig. 3C (d and f)). PDV cells pre-treated with the sense oligos responded to TGF-β1 similarly to control cells (data not shown).

Cells treated with TGF-β1 displayed decrease of E-cadherin cell–cell adhesion, showing a punctuated pattern distributed along the cells surface (Fig. 3C (h)) as compared with control cells (Fig. 3C (e)); this effect did not occur with JNK depletion by antisense oligos or inhibition by SP600125, whereas the intercellular distribution of E-cadherin was maintained after TGF-β1 treatment, similarly to control cells (Fig. 3C (i and k)).

PDV cells showed strong F-actin staining distributed mainly along cortical structures; after TGF-β1 treatment F-actin was mainly organized in a network that resembles that of more motile cells (Fig. 3C (m and n)). Pre-treatment with JNK antisense oligonucleotides or SP600125 exerted an inhibitory effect on the F-actin reorganization in PDV cells, although these continued displaying cortical F-actin upon TGF-β1 treatment (Fig. 3C (p and r)).

TGF-β1 treatment for four days induced cells to display a vimentin cytoskeleton, unlike control cells that were mainly negative for vimentin staining (Fig. 3C (s and u)). This effect of TGF-β1 was strongly blocked by the JNK antisense oligonucleotides or SP600125 inhibitor (Fig. 3C (m–p)). The effect of interference on JNK by TGF-β1-induced EMT was confirmed by statistical analysis of the loss of E-cadherine cells contact and vimentin expression; with TGF-β1 treatment about 85% of the cells do not have E-cadherine cell contacts. JNK antisense oligos or the JNK inhibitor block the effect of TGF-β1 and cells remain in contact like the cells without growth factor addition (Fig. 3D). During TGF-β1-treatment about 76% of the cells expressed vimentin compared with control cells (4.7%); with antisense oligos or the inhibitor to JNK this effect was strongly blocked to level comparable to that of untreated cells (Fig. 3E). Furthermore, both antisense oligos and JNK inhibitor blocked TGF-β1-induced vimentin when analysed by Wester blotting (Fig. 3F), confirming the results showed in Fig. 3C and E. Thus, these results indicate that JNK1,2 play an important role in TGF-β1 induced EMT.

PDV cells were allowed to migrate in a wound healing assay using TGF-β1; this growth factor induced cells to quickly close the wound area after 24 h. However, cells treated with the antisense oligonucleotides or SP6001 did not respond to TGF-β1-induced motility, suggesting that JNK participates in TGF-β1-induced migration (Fig. 3G).

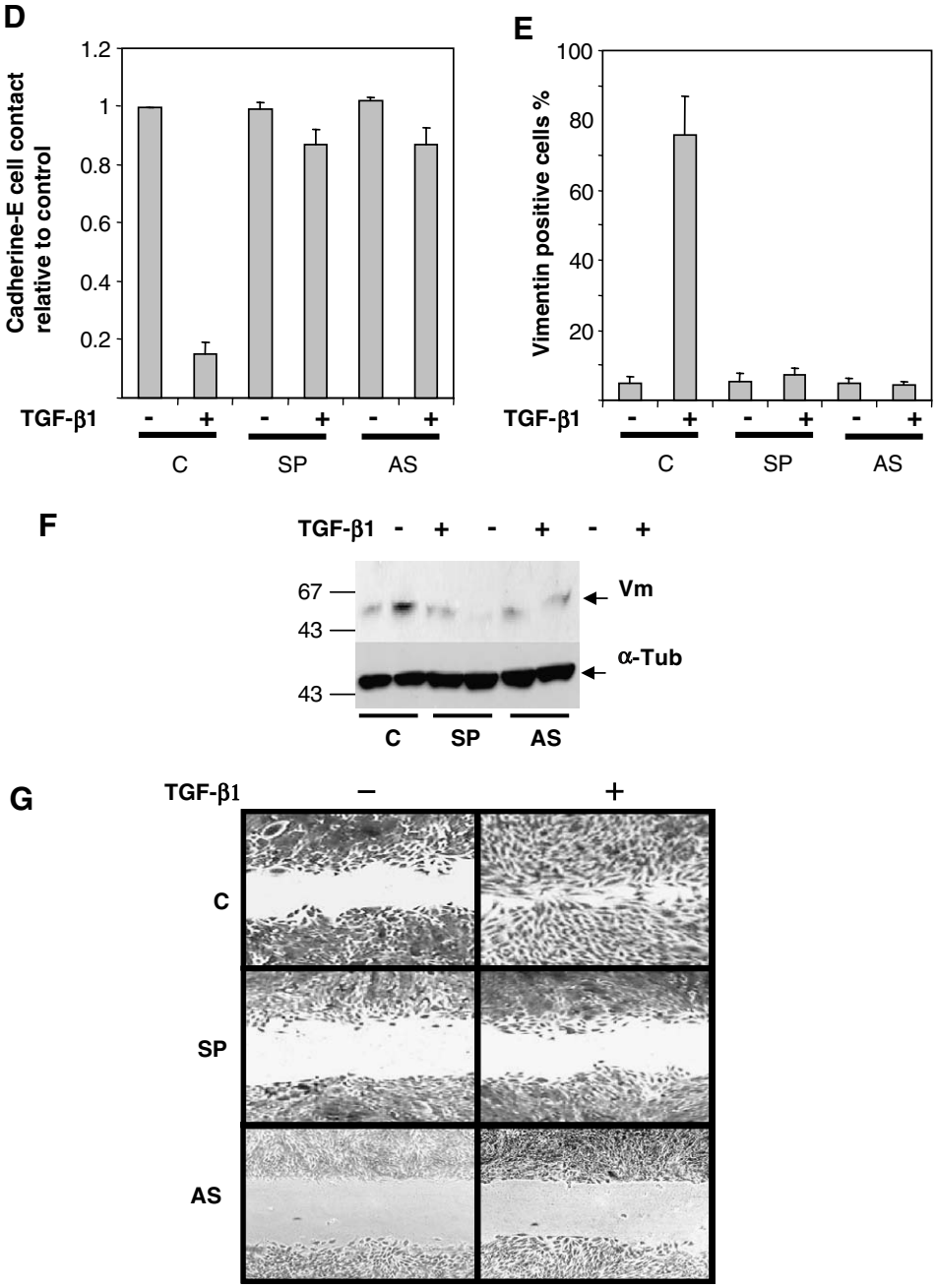

Fig. 3 *(continued)*

4. Discussion

In this study, we demonstrate that TGF-β1 induces uPA, EMT and cellular migration in transformed mouse keratinocytes *in vitro,* using the JNK pathway as mediator. Treatment of PDV cells with TGF-β1 activates the JNK pathway as evidenced by JNK phosphorylation and c-Jun and AP1 transactivation (Fig. 1A). The kinetics of JNK phosphorylation suggested its direct activation by TGF-β1; this is in agreement with a previous report by Yue and co-workers [26]. JNK activation by TGF-β1 is independent of TβRI-induced Smad activation, because ALK-5 mutant in the L45 loops of the kinase domain, disabled to activate Smads, retained the capacity to activate JNK [27].

We previously demonstrated that TGF-β1 induces uPA expression, a key component of cell malignant transformation [10,28]. The role of JNK on TGF-β1-induced uPA has not been fully understood. Our results indicate that the JNK pathway mediates the TGF-β1-induced uPA expression, because dominant negatives of SEK1, JNK, cJun, and the JNK inhibitor SP600125 inhibit uPA-promoter transactivation; Furthermore SP600125 inhibited the TGF-β1-stimulated uPA production. uPA expression is subject to regulation at the transcriptional level by AP1. In addition, as it has already been described c-Jun participates directly in uPA expression induced by TGF-β1, and ATF2/c-Jun heterodimer is the predominant form that promotes uPA expression [17]. The promoter construction used in this study has also been reported to be

effectively activated by overexpression of wild type c-Jun and inhibited by mutated c-Jun [29].

TGF-β1-enhanced FN expression was dramatically blocked by the JNK depletion using antisense oligonucleotides or **inhibition** by SP600125 (Fig. 3C (a–f)). This is in agreement with Hocevar et al. [30] who demonstrated that FN expression was stimulated by TGF-β1 and was JNK-dependent in human fibrosarcoma cells. TGF-β1-induced actin cytoskeleton reorganization was mediated by JNK pathway (Fig. 3C (m–r)). However a direct link between actin organization and JNK has not been clearly established. P150-Spir, which acts as an initiator of the actin polymerization and is a downstream target of JNK, may represent a direct link between JNK and actin organization [31].

At the initial stages of EMT, junctional protein complexes are remodelled and functionally disintegrated [24,32]. After a short interval TGF-β1 induces delocalization and loss of E-cadherin cell contact in about 85% in PDV cells. This effect of TGF-β1 on E-cadherin based cell-cell adhesion was abolished by antisense oligos or SP600125, and remained close to the actin cytoskeleton in the cell cortex (Fig. 3C (g–I) and D). The mechanisms by which JNK could mediate TGF-β1-induced E-cadherin delocalization remain unknown; it is possible that the JNK induced actin rearrangement could exert a destabilizing effect on E-cadherin interaction with β and β-catenin. E-cadherin plays a key role in epithelial integrity [23,33], and mey act as a tumour-suppressor protein because this downregulation plays a significant role in multistage carcinogenesis [34].

Associated with the decrease of E-cadherin-based cell–cell contacts, a concomitant increase in vimentin expression was observed after TGF-β1 treatment (Fig. 3C (m–p), E and F); this display of a mesenchymal type of cytoskeleton was dependent on JNK expression and activity. Most invasive and/or metastatic carcinomas are characterized by EMT, in which the epithelial phenotype, exhibiting strong cell–cell junctions and polarity, is replaced by a mesenchymal phenotype with reduced cell–cell interactions and increased motility [35]. The cell migratory capabilities induced by TGF-β1 were strongly dependent on JNK activity as shown in wound healing assays (Fig. 3G); this is probably the result of JNK inhibition upon TGF-β1-enhanced uPA, actin reorganization and loss of E-cadherin cell adhesion. In summary, in this study we show that JNK could mediate one of the early steps of malignant EMT induced by TGF-β1 concomitant with a more aggressive cell phenotype (uPA production and cell migration), and we further suggest that the JNK pathway is part of a network of signal pathways that mediate the TGF-β1 stimulated malignant transformation of epithelial cells.

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