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SMAD3 is essential for transforming growth factor- β 1-induced urokinase type plasminogen activator expression and migration in transformed keratinocytes

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ARTICLE INFO

Article history:

Available online 26 July 2011

Keywords:

TGF- β 1
uPA
PAI-1
Smad3
E-cadherin
Migration

ABSTRACT

Transforming growth factor- β 1 (TGF- β 1) stimulates the extracellular matrix degrading proteases expression and cell migration in order to enhance cancer cells malignancy. In the present study, we analysed the role of TGF- β 1-induced Smad3 activation in the urokinase type plasminogen activator (uPA) production, as well as in cell migration and E-cadherin downregulation in transformed PDV keratinocyte cell line. TGF- β 1 signalling was interfered by the chemical inhibitor of the TGF- β 1-receptor 1 (ALK5), SB505124, and the specific Smad3 inhibitor, SiS3. Our results showed that TGF- β 1 stimulates uPA expression directly through ALK5 activation. The inhibition of Smad3 strongly reduced the capacity of TGF- β 1 to stimulate uPA expression, in parallel decreasing the uPA inhibitor plasminogen activator inhibitor type 1 (PAI-1) expression. In addition, the transient expression of dominant negative Smad3 mutant inhibited the TGF- β 1-induced uPA promoter transactivation. Moreover, Smad3^{-/-} mouse embryonic fibroblasts were refractory to the induction of uPA by TGF- β 1. The inhibition of both ALK5 and Smad3 dramatically blocked the TGF- β 1-stimulated E-cadherin downregulation, F-actin reorganisation and migration of PDV cells. Taken together, our results suggest that the TGF- β 1-induced activation of Smad3 is the critical step for the uPA upregulation and E-cadherin downregulation, which are the key events preceding the induction of cell migration by TGF- β 1 in transformed cells.

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1. Introduction

Transforming growth factor- β 1 (TGF- β 1) has been postulated to have a dual role in tumour progression, acting as a tumour suppressor in early stages of carcinogenesis, and exerting a pro-oncogenic role in the last steps of the metastatic disease.¹ TGF- β 1 induces the epithelial mesenchymal transition (EMT) of transformed cells, which contributes to tumour invasion and metastasis, and is frequently over-expressed in carcinoma cells.^{2–5} TGF- β 1 binds at cell surface

receptors to activate Smads and non-Smads dependent signal pathways.⁶

To invade and metastasise, cancer cells traverse the surrounding extracellular matrix (ECM) expressing a set of ECM degrading proteases, such as urokinase type plasminogen activator (uPA), which play a key role in cells' invasion and metastasis. uPA converts plasminogen to plasmin, which in turn can degrade a wide variety of ECM components and enable the tumour cells to penetrate the basement membrane.^{7,8} In addition, uPA also modulates cell adhesion,

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doi:10.1016/j.ejca.2011.06.043

proliferation and migration.^{9,10} Consistent with their role in cancer dissemination, a high level of uPA correlates with the adverse patient outcome.^{11,12}

The signalling pathways through which TGF- β exerts its effects on cancer cell migration and invasion are gradually being elucidated. As previously reported, both Smad2 and Smad4 act as dominant tumour suppressive factors in skin carcinogenesis. Smad3 is implicated in both EMT induction and pro-metastatic effects of TGF- β 1 through facilitating TGF- β 1-induced activation of ERK1,2 and JNK, and participating in TGF- β 1-induced keratinocytes migration.^{13–15} Although it has been reported that TGF- β 1 stimulates uPA expression through a plethora of signal transduction pathways, such as ILK, NF κ B, ERK1,2 and JNK,^{16–19} the involvement of Smad signalling is not well elucidated yet. There is divergent information about the participation of Smad4 in the regulation of uPA expression by TGF- β 1. In breast cancer cells, Smad4 is required for TGF- β 1-induced uPA, whereas exogenous expression of Smad4 in colon cancer cells reduces uPA production.^{20,21}

We investigated the signalling events involved in the response of transformed epidermal keratinocytes (PDV cell line) to TGF- β 1. In contrast to normal keratinocytes, PDV cells are refractory to TGF- β 1-induced terminal differentiation. Under chronic TGF- β 1 exposure, PDV cells undergo an epithelial-mesenchymal conversion, which can be associated with the transition to a poorly differentiated tumour phenotype and its increased metastatic ability *in vivo*.² The early response of PDV cells to TGF- β 1 is enhanced expression/secretion of uPA, concomitant with the increment in cell migration and invasion.^{18,22} In this report, we examined whether ALK5/Smad3 axis mediates the stimulation of uPA synthesis, E-cadherin expression and cell migration by TGF- β 1 in transformed PDV keratinocytes.

2. Material and methods

2.1. Plasmids and antibodies

The p-4.8 uPA-Luc luciferase reporter plasmid (–4.8 kb of murine uPA promoter) was provided by Dr. Munoz-Canoves (CRG, Spain). The proximal mouse E-cadherin-Luc (–178 to +92) promoter was kindly provided by Dr. Cano (IIB-CSIC, Spain). Dominant negative mutant Smad3 was kindly provided by Dr. Bishop (University of Auckland, New Zealand). Dominant negative Smad2 was kindly provided by Dr. Balmain (Cancer Research Institute, UCLA, United States of America). Anti-E-cadherin, anti-tubulin and secondary antibodies coupled with HPO or FITC were from Sigma (St. Louis, Mo). Anti-p-Smad2 and anti-p-Smad3 were purchased from Calbiochem (Darmstadt, Germany). Anti Smad2,3 (sc-8332) was from Santa Cruz Biotechnology (CA, USA). Phalloidin-coupled to Alexa Fluor 594 to detect F-actin was from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture and transfection procedures

PDV cells, kindly provided by Dr. Quintanilla (IIB-CSIC, Spain), were cultured in Ham's F-12 medium in the presence of 10% foetal bovine serum (FBS) and antibiotics. For TGF- β treatments, human recombinant TGF- β 1 (R&D Systems GmbH, Germany) was used as described. The Smad3 inhibitor, SiS3,

and ALK5 inhibitor, SB505124 from Sigma-Aldrich (St. Louis, Mo) were dissolved in DMSO and used at 2.5 and 1.0 μ m, respectively. Wild-type and Smad3-deficient (Smad3 $^{-/-}$) mouse embryonic fibroblasts (MEFs)²³ were cultured in DMEM with 10% FBS and antibiotics. Transient transfections to analyse uPA and E-cadherin promoters' transactivation were performed as previously described.²⁴ Firefly luciferase activity (Promega, Adison, WI, USA) was standardised for β -galactosidase activity (Tropix, Bedford, MA, USA).

2.3. Migration and zymography assays

The motility of PDV cells was analysed by *in vitro* wound healing assay. 'Wounded' cell cultures were allowed to grow for 24 h in the absence or presence of TGF- β 1, SiS3 and SB505124. uPA activity was assayed in serum-free medium conditioned for 24 h in cell cultures treated or not with TGF- β 1, SiS3 and SB505124, subjected to SDS-PAGE and casein-zymography, as reported.^{18,22}

2.4. Immunofluorescence and immunoblotting

Detection of E-cadherin and F-actin by fluorescence analysis was performed as previously reported.^{24,25} E-cadherin expression and Smad3 activation were analysed by Western blot assays as previously described.²⁴

2.5. RT-PCR

Two micrograms total RNA isolated from PDV cells was reverse transcribed with Superscript II (Invitrogen, Carlsbad, Ca). PCR products were obtained after 30–35 cycles of amplification with an annealing temperature of 55–60 °C. uPA, PAI-1, E-cadherin, Snail1 and GAPDH primer sets were reported previously.²⁴ Smad2 and Smad3 primers sets were reported.²⁶

2.6. Densitometry analysis

The gels bands obtained by Zymography, Western blot and RT-PCR were quantified using NIH-Image J software. Values expressed are relative to untreated cells, to which an arbitrary value of one was given.

2.7. Statistics

Data are given as means (\pm SEM) from at least three independent experiments. Asterisks (*) denote significant differences at a value of $p < 0.05$ for experimental groups being compared with control in the absence of TGF- β 1, while (#) denote significant differences at value $p < 0.05$ for experimental groups being compared with cells in the presence of TGF- β 1 only, as determined by student's t-test.

3. Results

3.1. TGF- β 1 enhances uPA expression and activates Smad3 in PDV cells

We previously demonstrated that TGF- β 1 potently stimulates uPA production in PDV cells after 48 h²² by transcriptional

activation of the uPA mRNA. It is shown in Fig. 1A that TGF- β 1 provokes a rapid increment of uPA mRNA, visible already after 6 h of treatment, as determined by RT-PCR. Since the mRNA expression remained high up to 4 d after TGF- β 1 treatment (data not shown), we chose a 24 h TGF- β 1 treatment to determine uPA mRNA in the subsequent experiments.

Next, we determined the capacity of TGF- β 1 to activate Smad2 and Smad3 in our cell model. Western blot analysis revealed that TGF- β 1 induces the phosphorylation of both Smad2 and Smad3 15 min after treatment onwards (Fig. 1B). Activation of Smad3 in response to TGF- β 1 was strongly

inhibited by the Smad3 inhibitor (SiS3), while Smad2 phosphorylation was not affected. The treatment with ALK5 inhibitor (SB505124) provoked a dramatic inhibition of TGF- β 1-induced Smad2 and Smad3 activation (Fig. 1C).

3.2. Smad3 and ALK5 mediate TGF- β 1-induced uPA expression

Since Smad3 plays a critical role in the cells malignancy induced by TGF- β , we further analysed whether ALK5–Smad3 axis modulates uPA expression induced by TGF- β 1.

PDV cells were therefore treated with SiS3 and SB505124. As shown in Fig. 2A and B, TGF- β 1-induced uPA activity and mRNA production were highly reduced in the presence of Smad3 or ALK5 inhibitor. Interestingly, the expression of uPA inhibitor PAI-1 mRNA was also inhibited by both inhibitors. Additionally, the transactivation of the uPA promoter was also reduced by both mentioned inhibitors, as well as by transient ectopic expression of the Smad3 dominant negative mutant, while Smad2 negative mutant did not modify TGF- β 1-induced uPA transactivity (Fig. 2C). Finally, the requirement of Smad3 for TGF- β 1-induced uPA expression was confirmed by using Smad3 $^{-/-}$ MEFs (Fig. 3B). No uPA expression was noticed in Smad3 $^{-/-}$ cells in response to TGF- β 1 when compared to normal MEFs (Fig. 3A).

3.3. Cell migration and E-cadherin delocalisation/downregulation are Smad3 dependent

Considering that uPA improves the capacity of tumour cells to penetrate the basement membrane, and then facilitates migration and invasiveness of cancer cells,⁸ we next tested whether Smad3 signalling is required for TGF- β 1-induced cell motility, using a wound healing assay. TGF- β 1-stimulated control cells to almost completely close the 'wound' made 24 h before, whereas TGF- β 1-stimulated cell motility was strongly inhibited by SiS3 or SB505124 (Fig. 4A and B).

The disruption of cell-cell contacts, such as E-cadherin dependent cell interaction occurring during cell spreading,³ is strongly induced by TGF- β 1, and interestingly uPA has also been implicated in EMT and in E-cadherin shedding from extracellular cell membranes.^{27,28} We finally investigated whether Smad3 is required for E-cadherin downregulation induced by TGF- β 1. As shown in Fig. 4C, TGF- β 1 provoked E-cadherin loss in cell-cell contacts while Smad3 and ALK5 inhibitors blocked this effect of TGF- β 1 as demonstrated by strongly visible E-cadherin immunostaining. In addition, TGF- β 1-induced F-actin reorganisation to transcellular stress fibers was strongly disabled by Smad3 inhibitor and ALK5 inhibitor, as cells displayed cortical actin similar to control cells. Furthermore, both E-cadherin promoter transactivity, mRNA and protein expression inhibited by TGF- β 1 were counteracted by both SiS3 and SB505124 (Fig. 4D–F). Moreover, the expression of E-cadherin transcriptional repressor Snail1,²⁹ which is highly expressed in PDV cells alongside, and concomitant with the reduced expression of E-cadherin in response to TGF- β 1, was strongly inhibited by both inhibitors (Fig. 4E).

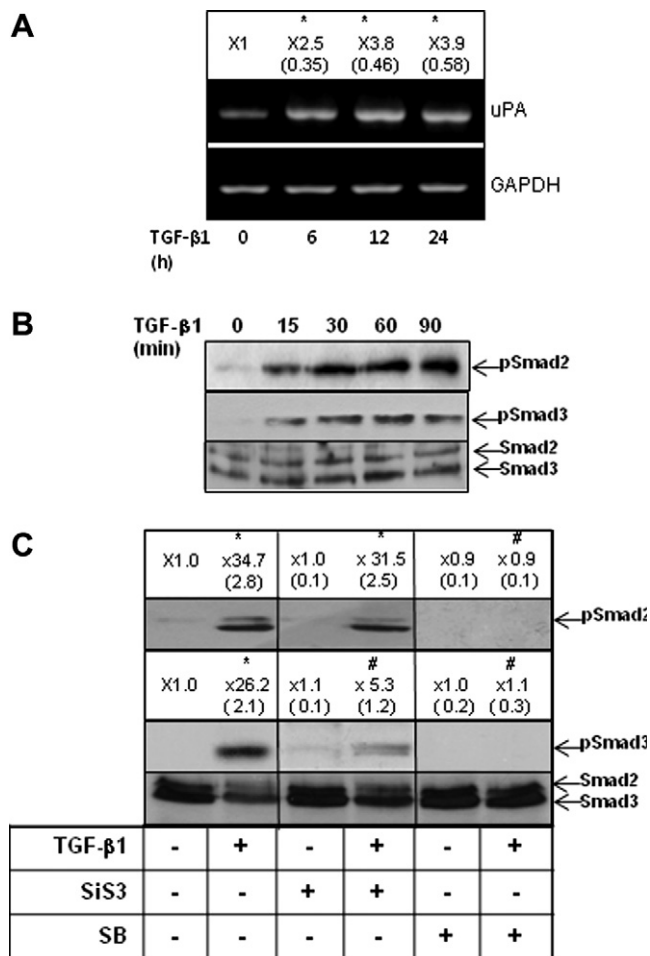


Fig. 1 – Transforming growth factor- β 1 (TGF- β 1) induces urokinase type plasminogen activator (uPA) expression and activates Smad2, 3 in PDV cells. (A) Expression of uPA mRNA determined by RT-PCR after treatment with TGF- β 1 at 5 ng/ml at times indicated, (h) hours. GAPDH was used as a control for mRNA loading. (B) Western blot analysis of the Smad2 and Smad3 activation by TGF- β 1. Cells were treated with 5 ng/ml of TGF- β 1 during times indicated. Total Smad2, 3 was used to confirm the same amount of protein in each sample. (C) Western blot analysis of Smad2 and Smad3 activation in PDV cells treated with TGF- β 1 for 30 min in the presence or absence of Smad3 inhibitor, SiS3, or ALK5 inhibitor, SB505124 (SB).

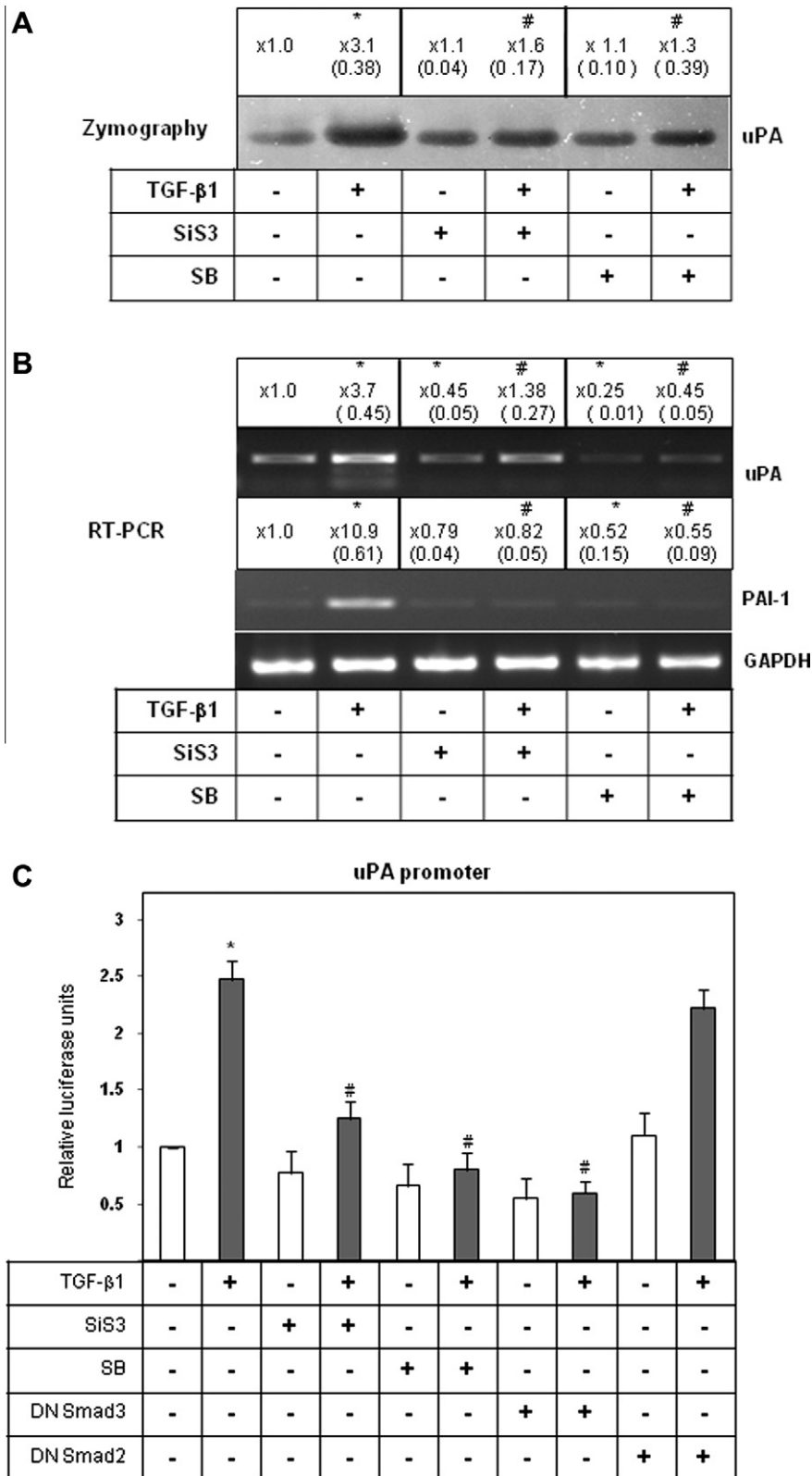


Fig. 2 – Smad3 mediates TGF-β1-induced uPA expression. (A) uPA activity determined by zymography in the serum-free conditioned media of cells treated or not with TGF-β1 for 48 h in the presence or absence of Smad3 inhibitor SiS3 or ALK5 inhibitor SB505124 (SB). **(B)** Expression of uPA and PAI-1 mRNA in cells treated for 24 h with TGF-β1, determined by RT-PCR. **(C)** uPA promoter transactivity in transiently transfected PDV cells treated with TGF-β1 for 48 h, in the presence of SiS3 or SB505124 (SB), or cotransfected with plasmids encoding Smad3 or Smad2 dominant negative mutants (DN Smad3 or DN Smad2).

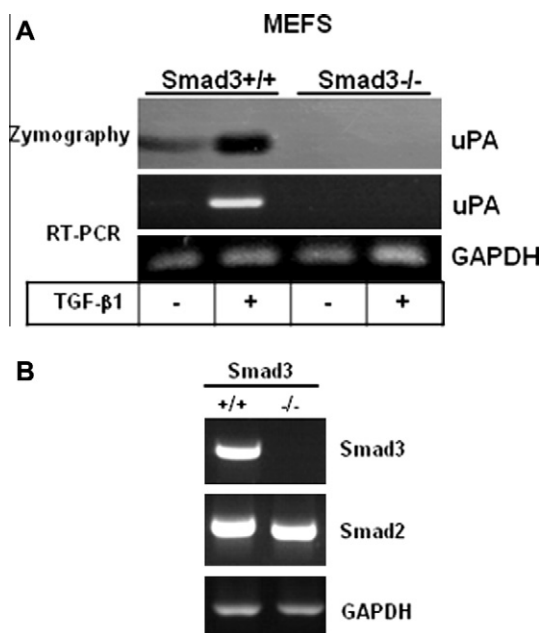


Fig. 3 – Smad3 knockout cells do not increase uPA expression in response to TGF-β1. (A) Zymography and RT-PCR analysis of uPA expression in Smad3^{+/+} or Smad3^{-/-} MEFs. Cells were treated with TGF-β1 for 24 h. GAPDH was used as a control of mRNA loading. (B) Characterisation of MEFs. The expression of Smad2 and Smad3 mRNA was determined in Smad3^{+/+} or Smad3^{-/-} MEFs. GAPDH was used as a control for mRNA loading.

4. Discussion

We have previously demonstrated that TGF-β1 increases migration, invasiveness and EMT of transformed PDV keratinocytes, concomitantly with the stimulation of uPA expression and secretion^{18,19,22} and this report. The present study examines the role of Smad3 activation, through TGF-β1 receptor (ALK5), on the induction of uPA expression by TGF-β1.

The individual roles of Smads in skin cancer have been recently documented. Smad4 deletion in keratinocytes results in spontaneous SCC formation, whereas mice with keratinocytes-specific Smad2 deletion exhibited accelerated formation and malignant progression of chemically induced skin tumours associated with an enhancement of EMT.^{13,14} These data indicate a dominant tumour suppressive effect of Smad4 and Smad2 in skin carcinogenesis. However, Smad3-knockout mice are resistant to skin chemical carcinogenesis due to abrogation of TGF-β1-mediated inflammation and gene expression critical for tumour promotion.¹⁵ Intriguingly, Smad2 has been implicated in the expression of matrix metalloproteinase (MMP) type 2 by TGF-β1 in human ovarian cancer SKOV3 cells.¹⁶ P-Smad2 also has been associated to malignant phenotype of advanced gastric cancer,³⁰ as well as in advanced breast cancer where knockdown of Smad2 can reverse the EMT phenotype.³¹ These data suggest that activated Smad2 may be involved in the malignance of cancer types other than skin cancer.

Although the importance of Smad3 in TGF-β1-induced cell malignance is known, its role on uPA expression is still not

elucidated. Our results demonstrate that in PDV cells, TGF-β1 greatly induces the activation of both Smad2 and Smad3 (Fig. 1B). To determine the role of Smad3, we used SiS3, a potent selective inhibitor of Smad3 function with no effect on Smad2 activation, in parallel with ALK5 kinase inhibitor, SB431542, which strongly blocked the activation of both Smad2 and Smad3, showing the functionality of both inhibitors (Fig. 1C).

Furthermore, Smad3 or ALK5 inhibition, by SiS3 and SB505124 respectively, decreased TGF-β1-induced uPA expression (Fig. 2A). Also, Smad3 knockout mouse embryonic fibroblasts were refractory to the induction of uPA by TGF-β1 (Fig. 3A), suggesting the essential requirement of the Smad3 signal in TGF-β1-induced uPA expression. Although TGF-β1 also activates Smad2, our results suggest that this signalling protein is not implicated in the TGF-β1-induced uPA expression (Fig. 2C).

In addition to Smads, TGF-β1 activates other intracellular signalling pathways, such as ILK, ERK1,2 and JNK,⁶ the last two also being implicated in the elevation of uPA expression in PDV cells.^{18,19} Moreover, Smad3 deficiency suppresses TGF-β1 activation of ERK1,2 and JNK.³² At this point, the possibility that Smad3 inhibition may disturb the activation of ERK1,2 and JNK in PDV cells, thus allowing a broader inhibition of TGF-β1 signalling involved in the reduction of uPA expression, can not be excluded.

The enhancement of uPA production improves the capacity of cancer cells to migrate and invade surrounding tissues and organs. uPA activates the latent zymogen plasminogen by converting it to plasmin, this way enormously enhancing the proteolytic machinery of cancer cells, as plasmin can cleave a wide variety of ECM components and also activate MMPs promoting matrix degradation, cell migration and invasion.^{7,8} Previous *in vitro* analysis showed reduced migration of Smad3^{-/-} keratinocytes,³³ suggesting a pivotal role of Smad3 in the induction of cell migration by TGF-β1. Current study demonstrates that Smad3 is required for the TGF-β1-induced cell motility, as the inhibition of ALK5–Smad3 axis reduced the migration of PDV cells. Furthermore, our results imply the possibility that this reduction in cell migration may also be due to the impairment of uPA production. By binding to its receptor at the cell surface, uPA triggers signals which enhance cell migration,³⁴ required for TGF-β1-induced PDV cells migration as well.²² Additionally, PAI-1^{-/-} keratinocytes have been shown to lose their ability to migrate in an *in vitro* scratch assay, while TGF-β1 has been shown to stimulate the attachment and invasion of cells by up-regulating PAI-1.^{35,36} Thus, our results imply that Smad3 inhibition may produce a general decrease in TGF-β1-induced uPA system with profound effects on cell migration. It was recently reported that TGF-β1-induced migration of breast cancer MDA-MB-231 cells is also dependent of Smad2 activation.³⁷ As we cannot exclude a possible participation of Smad2 in the migration of PDV cells in response to TGF-β1, further experimental analysis is required to determine the role of Smad2 in the increment of PDV cell malignance by TGF-β1.

Cell migration is also a consequence of the transition of epithelial cells to a more mesenchymal phenotype during tumourigenesis.³ In this aspect, TGF-β1 strongly induces EMT of PDV cells.² TGF-β1-induced E-cadherin downregulation

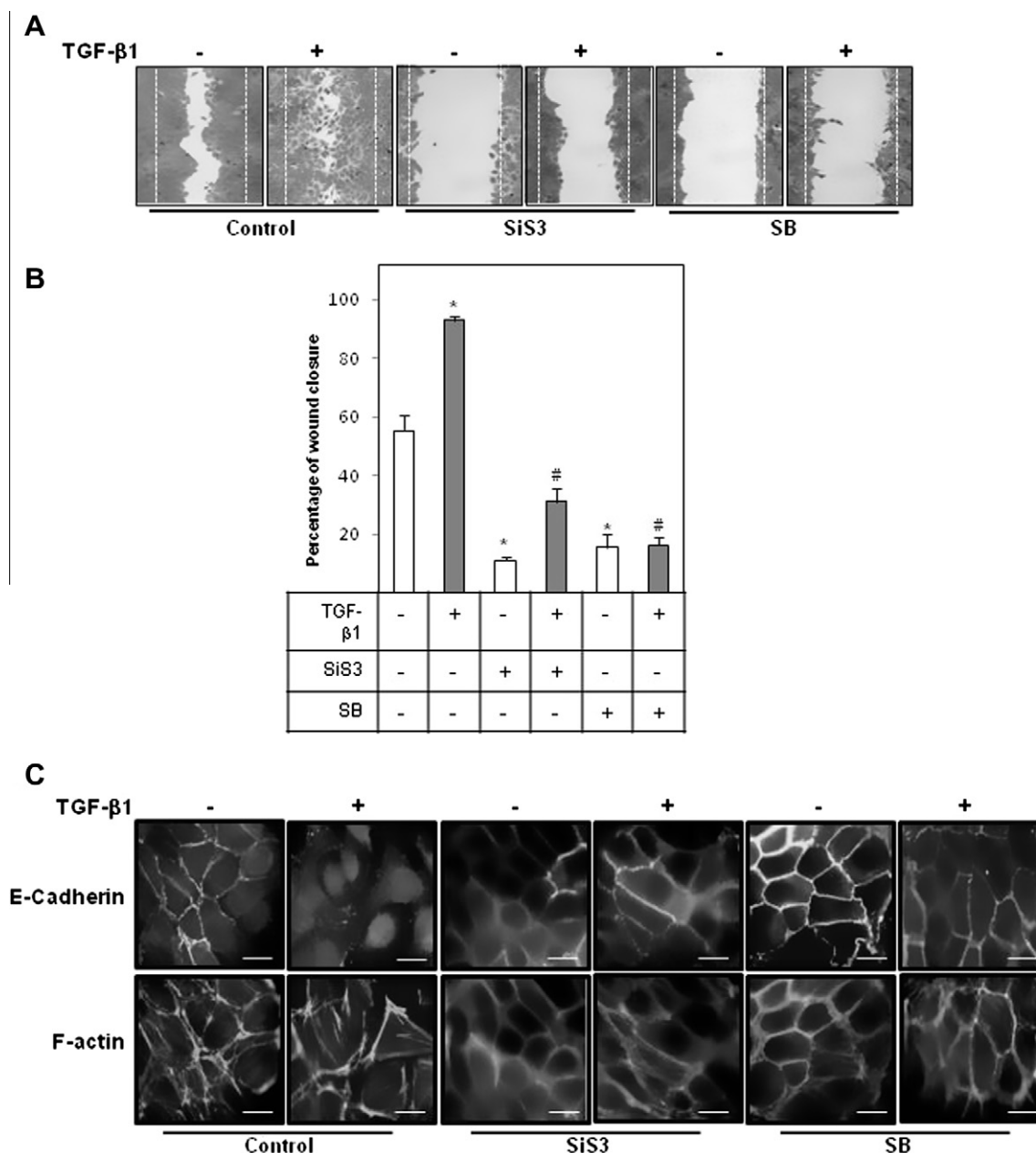


Fig. 4 – Smad3 and ALK5 inhibition impairs TGF- β 1-induced cell migration, E-cadherin downregulation and F-actin reorganisation. (A) Wound healing assay. Areas free of cells were examined after 24 h of TGF- β 1 treatment in the presence of SiS3 or SB505124. White dashed line shows the wound area at zero time. Magnifications 250 \times . (B) Quantitative analysis of cell migration shown in (A). Areas free of cells were measured and expressed as the mean (\pm SEM) percentage of the area at zero time ($n = 9$). (C) Immunofluorescence analysis of E-cadherin and F-actin cytoskeleton in PDV cells after stimulation (72 h) with TGF- β 1 and SiS3 or SB505124. Scale bar represents 10 μ m. (D) Luciferase inducible activity determined in PDV cells transfected with E-cadherin promoter treated for 48 h with TGF- β 1 in the presence of Smad3 inhibitor SiS3 or ALK5 inhibitor SB505124 (SB). (E) and (F) Expression of E-cadherin and Snail1 mRNAs determined by RT-PCR, and E-cadherin by Western blot, (ND) not detected. Cells were treated as above except the TGF- β 1 treatment for the RT-PCR lasted 24 h. GAPDH and alpha-tubulin (α -tub) were used as controls for mRNA and protein loading, respectively.

and F-actin reorganisation are blocked by ALK5/Smad3 inhibition (Fig. 4C). The repression of E-cadherin by Snail1 in PDV cells was previously documented,²⁹ and here the expression of Snail1 was inverted after Smad3 or ALK5 inhibition (Fig. 4E).

Intriguingly, we observed Snail expression without full E-cadherin downregulation at the protein and mRNA level.

In PDV cells full E-cadherin downregulation, at protein and mRNA level, requires a chronic treatment, necessary to stabilise the mesenchymal phenotype.² Several mechanisms have been implicated in the regulation of E-cadherin expression during tumour progression, including epigenetic, such as hypermethylation of E-cadherin promoter, and transcrip-

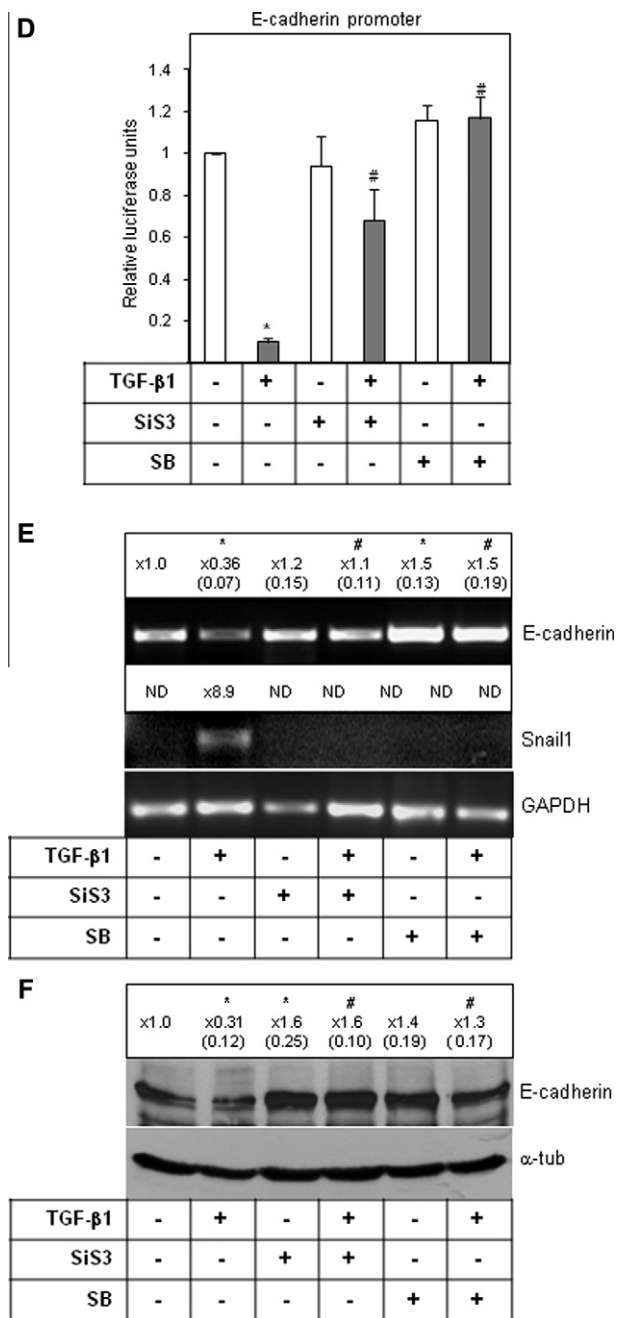


Fig 4. (continued)

tional changes, including the zinc finger factor Snail, beta-catenin and Smad3.^{38,39} We found that, in PDV cells, E-cadherin expression after TGF-β1 treatment is approximately 30% of that expressed by control cells (Fig. 4E and F). This amount of E-cadherin appears to be enough to enhance cell migration (Fig. 4A, B, D and E). These results are in agreement with those reported by Vicente et al.³⁹ where even though Snail is increased by TGF-β1, E-cadherin is not totally repressed, as shown in NMuMG cells. Additionally, the expression of Snail protein, which was not determined in this study, could be helpful in the understanding of E-cadherin and Snail coexistence in PDV cells.

The regulation of E-cadherin is complex; several events are needed in order to culminate the repression of E-cadherin

gene, involving several repressor complexes and/or promoter gene hypermethylation.³⁸ In addition, the stabilisation of Snail by GSK-3b inhibition may play a role, which in conjunction with beta-catenin, Smad3 and other transcription factors may produce a strong repression of E-cadherin expression.³⁹ In PDV cells, a full repression of E-cadherin expression may require sequential processes and increased duration of TGF-β1 treatment to fully produce the mesenchymal phenotype of the cells. Further research is necessary to elucidate the mechanisms implicated in the downregulation of E-cadherin by TGF-β1 in PDV cells.

In addition, it was recently demonstrated that the blockage of Snail1 reduces the expression of uPA/uPA-receptor and PAI-1 in breast cancer cells,⁴⁰ suggesting Smad3 and Snail1 as strategic components in TGF-β-induced uPA expression system.

The expression of uPA has also been implicated in EMT as well as in the E-cadherin shedding.^{27,28} We may speculate that the elevation of uPA is necessary not only for TGF-β1-induced cell migration, but may also participate in the enhancement of E-cadherin downregulation, thus facilitating the development of EMT stimulated by TGF-β1. Further studies are required to elucidate the collaboration of uPA in TGF-β1-induced EMT in transformed cells.

The present work demonstrates that the TGF-β1 signalling through ALK5-Smad3 axis is crucial for the induction of uPA expression and cell migration by TGF-β1, with important implications in the TGF-β1-dependent E-cadherin downregulation in PDV cells. Additionally, our data support Smad3 as a therapeutic target in the regulation of cell malignancy by TGF-β1 in transformed cells.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by Grants FONDECYT No. 1050476, Chile and No. 175062 from The Ministry of Science and Technological Development, Republic of Serbia.

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