TGF-β_1 and Smad4 overexpression induce a less invasive phenotype in highly invasive spindle carcinoma cells

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Received 5 February 2002; revised 13 April 2002; accepted 18 April 2002

First published online 8 May 2002

Edited by Veli-Pekka Lehto

Abstract We have examined the effect of transforming growth factor β_1 (TGF-β_1) and overexpression of the *Smad4* gene on the phenotype of Car C, a *ras* mutated highly malignant spindle carcinoma cell line. TGF-β_1-treated Car C cells overexpressing *Smad4* spread with a flattened morphology with membrane ruffles abundant in vinculin and show a reduction in their invasive abilities. TGF-β_1 treatment and overexpression of *Smad4* also enhanced the production of PAI-1 measured by the activation of the p3TP-lux reporter gene containing a PAI-1-related promoter. This activation was abolished with a dominant-negative *Smad4* construct. These results lead us to conclude that both TGF-β_1 and *Smad4* overexpression reduce the invasive potential of Car C cells, probably via the Smad pathway.

Key words: TGF-B_1; Smad4; Spindle; Cat C

1. Introduction

Transforming growth factors β (TGF-β) are a family of growth factors which express a wide range of biological activities including effects on cell proliferation, extracellular matrix deposition, fibrosis, angiogenesis, morphogenesis, and immune regulation [1,2]. Epithelial cells are particularly sensitive to TGF-β-induced growth arrest while most cells of mesenchymal origin show little or no growth inhibition [1]. During epithelial carcinogenesis, TGF-β_1 acts as a tumor suppressor at early stages of tumorigenesis by inhibiting cell proliferation and inducing terminal differentiation [3–5]. However, growth arrest is lost at later stages of carcinogenesis [6,7]. Thus, carcinoma cells respond to TGF-β_1 by eliciting an epithelial–mesenchymal transition (EMT) associated with the development of highly invasive and metastatic spindle cell carcinoma [8]. This role of TGF-β_1 in promoting malignancy has been demonstrated in the model of mouse skin carcinogenesis [9–11], as well as in human carcinoma cell lines [12–14]. Our previous work has demonstrated that in transformed keratinocytes TGF-β_1-dependent EMT proceeds through the activation of the Ras-Erk pathway or the blockage of Smad4 and includes the enhancement of urokinase-type plasminogen activator (uPA) production and invasiveness [15,16].

Smad4 is a candidate tumor suppressor gene whose protein product acts as a cofactor that binds TGF-β_1 receptor-activated Smad2 and Smad3 and generates transcriptional complexes [17]. Inactivating mutations in *Smad4* are the most important of the known TGF-β_1 post-receptor defects in colon and pancreatic cancers [18]. Also, it has been shown that re-expression of *Smad4* induces suppression of tumorigenicity [19].

Some authors have proposed a mechanism for the maintenance of the spindle phenotype through the autocrine action of TGF-β_1 overproduced by the tumor cells and activated by surrounding stromal cells [12]. However, very few reports have analyzed the effects of TGF-β_1 on spindle tumor cells [20,21].

In this report, we have examined the action of TGF-β_1 and the overexpression of *Smad4* (Smad4wt cells) on the phenotype and invasiveness of spindle Car C cells. The Car C cell line was derived from a spindle cell carcinoma [22] and contains the typical genetic alterations found in vivo in spindle tumors, such as a mutated H-Ras gene (and loss of the normal allele), inactivated p53, and deletion of the INK4 locus that encodes the cell cycle regulators p15 and p16 [23,24]. Furthermore, Car C cells are insensitive to the TGF-β_1 antiproliferative response [6]. We found that, in contrast to transformed keratinocytes, Car C cells respond to TGF-β_1 and overexpression of Smad4 by an enhancement of cell spreading and reduction of invasiveness. The mechanism appears to be due, in part, to the capacity of TGF-β_1 to stimulate the expression of PAI-1, a specific inhibitor of uPA, and to promote focal contacts that inhibit cellular motility. These results suggest that phenotypic changes induced by TGF-β_1 in a spindle carcinoma cell line are mediated mainly by the Smad pathway.

2. Materials and methods

2.1. Cell culture, treatment conditions and transfection assays

The origin of the Car C cell line has been described [6]. Cells were grown in Ham's F-12 medium supplemented with amino acids and vitamins (Gibco, Paisley, UK), 10% fetal bovine serum and antibiotics. For TGF-β_1 treatments, human recombinant TGF-β_1 (Calbiochem-Novabiochem, La Jolla, CA, USA) was added to the cell cultures at a final concentration of 10 ng/ml.

2.2. Generation of stably transfected cells

Car C cells (2×10^5) were stably co-transfected with an empty pcDNA3 containing a Neomicin-resistant gene (In Vitrogene, San Diego, CA, USA) and a pCMV5 expression vector containing a Flag tag full-length *Smad4* cDNA in a 1:10 ratio, using Lipofectamine Plus (Gibco) according to the manufacturer's protocols. After 2 weeks of selection with 400 μg/ml G-418 two overexpressing clones were obtained. Car C cells transfected with only pcDNA3 empty vector (EV) were used as a transfection control. Clones transfected with

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0014-5793/02/$22.00
PII: S0014-5793(02)02760-6

Smad4 gene were analyzed for expression of Smad4 and Flag tag with antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and M2 (Sigma, St. Louis, MO, USA) respectively.

2.3. Transient transfection and reporter gene measurements

For the invasive assay, Car C cells (10^6) were transiently transfected using the above-mentioned methods with 5 μg of C-terminal truncated dominant-negative *Smad4* (1–514) pCMV construct or 5 μg of pcDNA3 EV along with 1 μg of SV40 *lacZ* vector as an internal control. To measure the PAI-1 promoter activity Car C cells (3×10^4) were transiently co-transfected as above with 0.5 μg of full-length pCMV or 1–514 truncated Smad4, pCDNA3 as a control vector and 0.5 μg of 3TPLux-specific luciferase construct along with 200 ng of SV40 *lacZ* vector as an internal control for transfection efficiency. After 18 h, the medium was changed and TGF-β1 (10 ng/ml) was added for an additional 24 h, cells were lysed and luciferase activity was determined [25].

2.4. Invasion and uPA activity assays

The capacity of the cells to migrate through Matrigel-coated filters was measured by using Transwell chambers (Costar, Cambridge, MA, USA) as described [25]. uPA secreted activity, radial caseinolytic and zymographic assays were also measured as described [25].

2.5. Immunofluorescence analysis

Indirect immunofluorescence staining of vinculin and vimentin was performed in cells grown on glass coverslips and fixed in methanol using mouse monoclonal antibodies (Sigma) at a 1:100 dilution and a FITC-labeled secondary antibody. Double immunofluorescence staining of uPA and PAI-1 was performed in non-permeabilized cells fixed in paraformaldehyde (4% in phosphate-buffered saline (PBS)) using an anti-uPA mouse monoclonal antibody [25] at 10 μg/ml and a goat anti-PAI-1 polyclonal antibody (Santa Cruz Biotechnology) at 1:100 dilution. Secondary antibodies were FITC-labeled anti-goat IgG and TRITC anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA).

2.6. Western blot analysis

For p-ERK-1,2 (Santa Cruz Biotechnology) and p-Smad2 (Upstate, Lake Placid, NY, USA) determinations, cells were lysed in PBS buffer

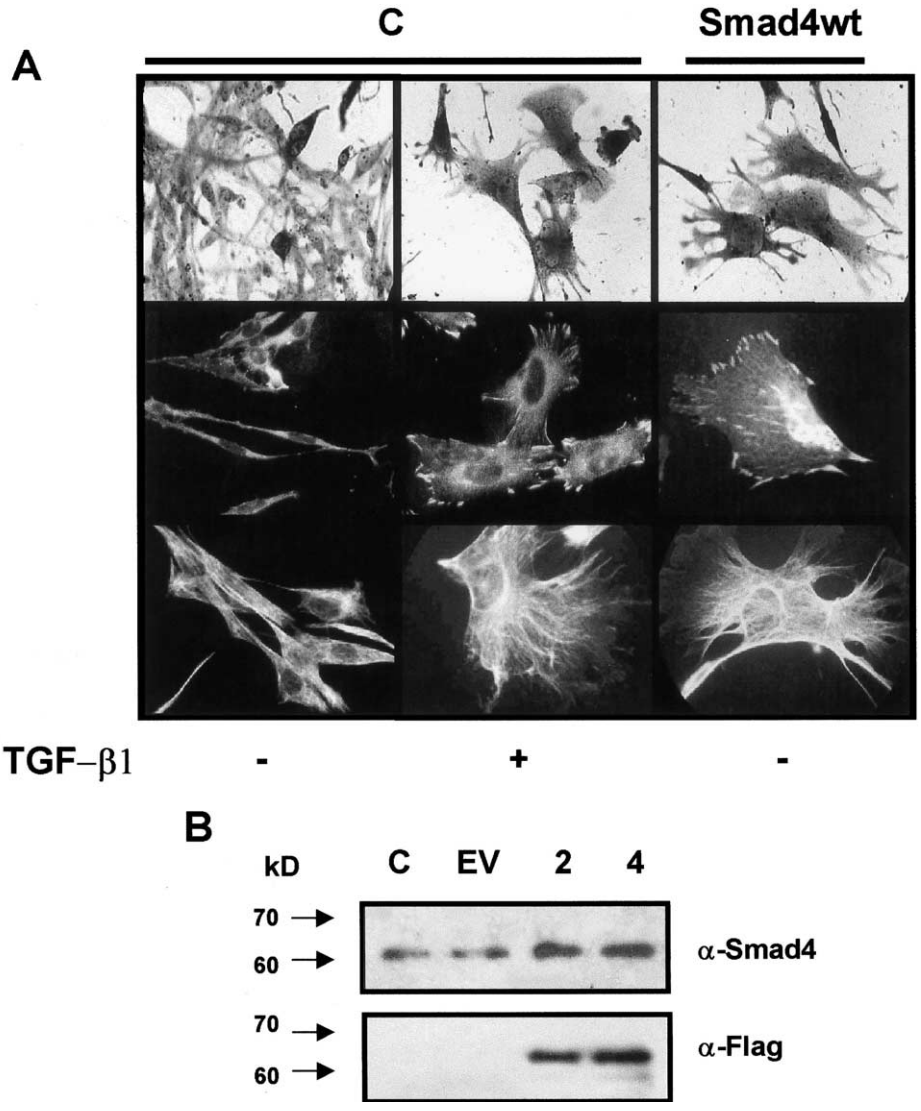

Fig. 1. Morphology, vinculin organization and vimentin network expression of TGF-β_1-treated and Smad4wt Car C cells. A: Upper row: Micrographs of methanol-fixed and crystal violet-stained Car C cells in culture. Left column: control cells. Center column: cells treated for 72 h with TGF-β_1. Right column: *wtSmad4* gene Car C transfected cells. Immunofluorescence detection of vinculin (middle row) and vimentin (lower row) in the three experimental conditions described. B: Expression of Smad4 (upper row) and Flag tag Smad4 (lower row) was determined by immunoblot analysis in: control Car C cells (C), cells transfected with EV, and two positive clones transfected with wtSmad4 gene (2 and 4). Arrows show molecular weight standards.

containing 150 mM NaCl, 1% Triton X-100, pH 7.4 and 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 2 mM *N*-ethylmaleimide, 1 mM orthovanadate and 1 mM NaF. For Smad4 analysis, cells were lysed in the same buffer mentioned above and in the absence of phosphatase inhibitors. Proteins (50 μg/lane) were resolved by 10% SDS–PAGE and transferred to Immobilon P membranes (Millipore, Bedford, MA, USA). PAI-1 was detected in the conditioned media of both intact and Smad4wt transfected cells untreated or treated for 48 h with TGF-β_1 in serum-free medium. Five-fold concentrated conditioned media were processed by Western blot analysis as above using a goat anti PAI-1 polyclonal antibody (Santa Cruz Biotechnology) at 1:500 dilution and developed using the enhanced chemoluminescent system (ECL, Amersham, Arlington Heights, FL, USA).

3. Results

3.1. TGF-β_1 and overexpression of wtSmad4 stimulate spreading and induce morphological changes in Car C cells

Car C are spindle-shaped fibroblastic cells that grow in culture forming a densely packed tubular-like network (Fig. 1). Treatment with TGF-β_1 or transfection with the wild type *Smad4* gene disrupted these structures and cells dissociated from each other adopting a flat and well spread morphology. Immunofluorescence analysis also revealed a poor organization of vinculin-containing adhesion plaques in untreated Car C cells while cells treated with TGF-β_1 or overexpressing the *Smad4* gene (Smad4wt clone 2) displayed a well developed adhesion plaque system suggesting that both treatments promoted increased adhesion to the substratum and reinforced focal adhesion (Fig. 1A, middle row). Morphological changes induced by TGF-β_1 or overexpression of the *Smad4* gene also included significant alterations of the microfilament cytoskeleton, generating a branched network as is shown in cells assayed by immunofluorescence to vimentin (Fig. 1A, lower row). The expression level of Smad4 protein and Flag tag Smad4 protein of control cells and cells transfected with EV and two different clones of Smad4wt+ cells is also shown (Fig. 1B).

3.2. TGF-β_1 and overexpression of Smad4 gene decrease invasiveness of Car C cells and stimulate PAI-1 expression and secretion

We examined the effect of TGF-β_1 on the invasiveness of Car C cells, which normally show extremely high invasive abilities through the reconstituted basement membrane Matrigel. We studied the effects of TGF-β_1 and the stable transfection with wild type (Smad4wt) and a transiently transfected dominant-negative (Smad4dn) version of Smad4 on the invasiveness of Car C spindle cells. As shown in Fig. 2A, TGF-β_1 significantly reduced invasiveness of the intact spindle Car C cells (C). A similar inhibition was observed in cells treated with 2 μg/ml PAI-1 (P). Cells transfected with EV behave as the controls parental cells. However, wtSmad4 cells show a strong inhibition of the invasiveness in the presence or the absence of TGF-β_1. In contrast, transfection with the dominant-negative version of Smad4 does not affect the invasiveness of Car C cells and block the TGF-β_1-induced inhibition of invasiveness.

To investigate if the diminished invasive capacity of TGF-β_1-treated and Smad4wt Car C was due to an alteration in the balance of the cellular proteolytic potential, we analyzed the expression of PAI-1, a specific inhibitor of uPA, in both experimental conditions. Fig. 2B shows that Smad4wt-2 cells express a higher amount of PAI-1 than control cells and

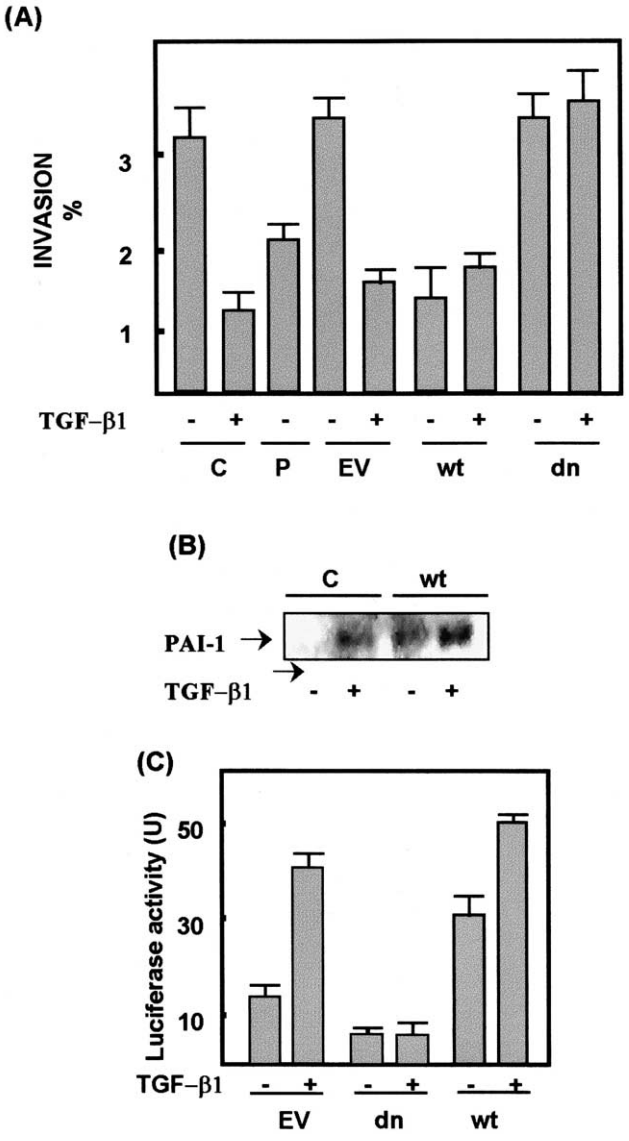

Fig. 2. Effect of TGF-β_1 and modification of Smad4 expression on Car C cell invasiveness, PAI-1 expression and PAI-1 promoter activity. A: Invasion analysis was assayed in the presence or absence of TGF-β_1. Parental Car C cells (C), PAI-1-treated cells (P), cells transfected with: EV, the full-length *wtSmad4* gene (wt), the dominant-negative mutant *Smad4* gene (dn). Results are expressed as a percentage of migrating cells in 24 h. B: Western blot analysis of PAI-1 production in the presence or absence of TGF-β_1 of control (C) and *wtSmad4* (wt) cells. C: TGF-β_1-dependent transcriptional activation of the p3TP-lux (PAI-1) reporter measured as luciferase activity in Car C cells transfected with: EV, full-length *wtSmad4* gene (wt) and dominant-negative mutant Smad4 gene (dn).

that TGF-β_1 was able to increase the production of PAI-1 in both control and Smad4wt cells. In a separate experiment, we transiently co-transfected Car C cells with *Smad4*, *Smad4dn* and pcDNA3 EV with the p3TP-lux reporter gene which contains a TGF-β_1-responsive PAI-1-related promoter. As Fig. 2C shows, TGF-β_1 stimulates PAI-1 promoter activities in cells harboring EV. Interestingly, Smad4dn Car C cells express a lower promoter activity (compared with control cells) and PAI-1 is not stimulated by the addition of TGF-β_1. The overexpression of Smad4wt in Car C cells results in a cellular phenotype with a potent promoter activity that retains the potential to be stimulated by TGF-β_1.

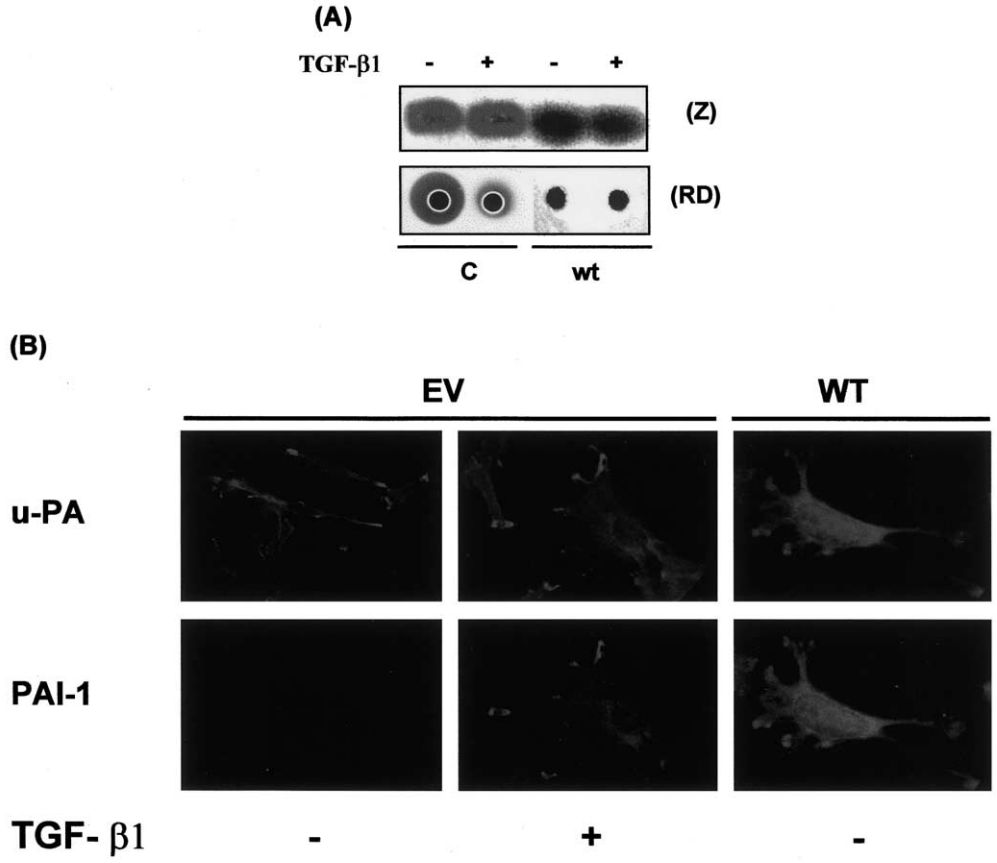

Fig. 3. TGF-β_1 effect of uPA production and immunofluorescence analysis of uPA and PAI-1 in Car C cells. A: uPA was analyzed in 24 h serum-free conditioned media of parental and Smad4wt transfected cells by zymographic assay (Z) and by radial diffusion assay (RD) according to Section 2. B: Parental cells transfected with EV and Smad4wt transfected cells (wt) were analyzed for uPA (upper row) and PAI-1 (lower row) expression by immunofluorescence in non-permeabilized cells.

3.3. *TGF-β_1 treatment and Smad4wt transfection do not affect uPA production. PAI-1 and uPA co-localize at the cell surface*

Measurements were also made to investigate if TGF-β_1 treatment and stable *Smad4wt* gene transfection affect the production of uPA. As Fig. 3A shows, neither TGF-β_1 treatment nor Smad4wt transfection affects the net secreted activity of uPA analyzed by zymography (Z). However, when enzymatic activity was assayed by radial diffusion (RD), inhibition of uPA activity was observed in parental control cells (C) treated with TGF-β_1. Smad4wt cells secreted a very low uPA activity in the presence or absence of TGF-β_1.

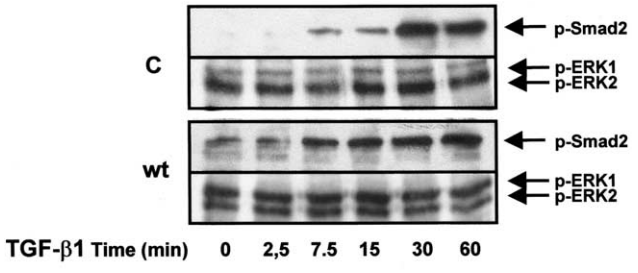

Fig. 4. Effect of TGF-β_1 on the activation of Smad2 and ERK-1,2 signaling routes. Parental (C) and *Smad4wt* transfected cells (wt) were stimulated with TGF-β_1 and phospho-Smad2 and phospho-ERK-1,2 were detected by Western blot analysis according to Section 2.

The interaction of uPA and PAI-1 was also analyzed by double immunofluorescence experiments in non-permeabilized cells. Fig. 3B shows that uPA was concentrated at plasma membrane extensions in untreated, TGF-β_1-treated and Smad4wt cells while no sign of PAI-1 was observed in untreated cells. Upon stimulation with TGF-β_1 PAI-1 was produced and co-localized with uPA in the same regions of the plasma membrane.

3.4. *TGF-β_1 treatment and Smad4wt transfection activate Smad signaling in Car C cells*

To assess if the phenotypic changes provoked by TGF-β_1 treatment or by transfection with the *Smad4* gene in Car C cells occur by activation of the Smad pathway, we measured the kinetics of phosphorylation of Smad2 in intact Car C cells and in Smad4wt-2 cells. As Fig. 4 shows, TGF-β_1 was able to activate Smad2 in parental as well as in Smad4wt-2 Car C cells with different kinetics. We also studied the possible activation by TGF-β of the Ras-MAPK signaling route in both cell types. As Fig. 4 shows, the same experimental conditions that induce the activation of Smad2 do not modify ERK-1,2 activity.

4. Discussion

In the present study, we show that TGF-β_1 treatment and overexpression of *Smad4* gene in Car C cells induce a set of

phenotypic changes that generate a less invasive phenotype. These results clearly show that these changes are not dependent on the activation of the Ras-ERK pathway but rather on Smad activation.

Smad4 plays a central role in TGF-β_1 signaling by serving as a common partner of other Smad proteins and accumulating in the nucleus in a highly regulated process [26]. The potential tumor suppressive activity of Smad4 has also been evaluated in Smad4-defective colon cancer cells by stable re-expression of Smad4, a phenomenon associated with reduced uPA expression [27]. In mouse cells, the *Smad4* gene is located on chromosome 18q [28]. Car C cells possess an intact pair of chromosome 18 and express a non-mutated version of *Smad4* (Quintanilla et al., unpublished results). In our data, clones transfected with *Smad4wt* express three to four times the amount of Smad4 protein of control cells and also express the Flag-tagged protein confirming the exogenous origin of the gene (Fig. 1B).

TGF-β_1-treated and Smad4wt Car C cells show changes in morphology illustrated in the expression of a network of vimentin and an increased peripheral localization of vinculin, an essential component of focal contact. This cellular shape is suggestive of a high cellular contact with the extracellular matrix and a less motile phenotype as has been previously demonstrated [29]. The vimentin network collaborates to maintain cellular mechanical stability, as has been demonstrated in other cellular phenotypes [30].

TGF-β_1-treated and Smad4wt Car C cells display a diminished invasive capacity as measured in a Matrigel assay, probably associated with the changes in cellular shape and the induction of the expression and secretion of PAI-1. The invasive capacity of spindle Car C was unaffected by transient transfection with *Smad4dn* or by treatment of these transformed cells with TGF-β_1 (see Fig. 2A). In contrast, cells transfected by the wild type version of Smad4 show inhibition of invasiveness even in the absence of added factor. These results permit us to suggest that the overexpression of *Smad4* has as a consequence the recruitment of Smad2/3 and the onset of a cellular response similar to those of stimulated parental TGF-β_1-treated cells. The inhibition of the invasive capacity by TGF-β_1 treatment or *Smad4wt* transfection in Car C cells can also be attributable to the stimulated expression of PAI-1. This hypothesis was confirmed in experiments where invasion was inhibited directly by the addition of PAI-1 (see Fig. 2A). Induction of PAI-1 appears to require Smad signaling, since Smad4wt cells show similar PAI-1 secretion to parental TGF-β_1-treated cells (Fig. 2B) and the transcriptional activation of the p3TP-lux reporter gene by TGF-β_1 was blocked by a dominant-negative construct (Fig. 2C). That the augmented production of PAI-1 inhibits the uPA-mediated proteolytic potential was suggested by the specific uPA–PAI-1 interaction observed on the surface of parental TGF-β_1-treated and Smad4wt cells (Fig. 3B). Also, it is important to note that TGF-β_1 induced the expression and secretion of PAI-1 in Car C cells without affecting the net uPA secreted activity. In a radial assay, where uPA and PAI-1 activities co-exist, a diminished uPA activity was observed in parental TGF-β_1-treated cells. In the same assay, Smad4wt showed a strong inhibition of uPA activity (Fig. 3A) probably attributable to the formation of a PAI-1–uPA complex. Interestingly, the exogenous addition of PAI-1 exerts similar effects to TGF-β_1 treatment or *Smad4wt* transfection on cell invasiveness but does not induce phenotypic changes (data not shown).

Results in Fig. 4 permit us to confirm that in intact Car C cells TGF-β_1 stimulates the Smad2 phosphorylation in a time-dependent manner while, in the same conditions, activation of ERK-1,2 is not observed. These results are in agreement with the assumption that Car C cells exhibit a Ras-ERK pathway permanently activated by the expression of a mutated version of H-Ras and the loss of the normal allele [23,24]. A similar result was obtained when Smad4wt cells were used, although, interestingly, in this case a basal activation of the pathway was observed. This finding can be compared with results of PAI-1 expression, where in the absence of added factor, Smad4wt cells express a two-fold increased stimulation in the TGF-β_1-responsive PAI-1 promoter activity which is increased further with the addition of the factor. It is important to note that other authors have reported that components of the JNK pathway can also regulate the TGF-β-dependent PAI-1 promoter activity but this possibility was not tested by us [31].

In conclusion, our results suggest that spindle Car C cells respond to TGF-β_1 stimulation oppositely than transformed keratinocytes because the expression of a different signaling behavior. In fact, while spindle Car C shows a very low response through its permanently active Ras-Erk pathway, transformed keratinocytes mount a robust EMT that depends specifically on this signaling pathway [15]. Our results also suggest that Car C cells conserve an intact Smad signaling pathway and that the loss of the TGF-β_1 antiproliferative response cannot be linked to inactivation of Smad signaling.

Acknowledgements: We thank Dr. J. Massagué for kindly providing Smad4 plasmids and Dr. Carmelo Bernabeu for the 3TP-lux reporter. This work was supported by Fondecyt 3000045 and post-graduate fellowship of Fundación Andes (to J.F.S.), 1010703 (to J.M.) and by the Comisión Interministerial de Ciencia y Tecnología and Comunidad Autónoma de Madrid of Spain (Grants SAF98-0085-CO3-02 and 8.1/22/97, to M.Q.).

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