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# Spred2 inhibits TGF- $\beta$ 1-induced urokinase type plasminogen activator expression, cell motility and epithelial mesenchymal transition

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TGF- $\beta$ 1 is a potent inducer of malignance in cancer cells. TGF- $\beta$ 1 stimulates the expression of extracellular matrix degrading proteases, cell migration and it is also involved in the epithelial-mesenchymal transition (EMT). In the present work, we analyzed the role of Spred2 in the urokinase-type plasminogen activator (uPA) stimulation, EMT and cell migration by TGF- $\beta$ 1. We found that both the expression of mRNA and the protein level of Spred2 were lower in transformed keratinocytes PDV compared with immortalized keratinocytes MCA-3D. The transient ectopic expression of Spred2 in PDV cells inhibited the TGF- $\beta$ 1-transactivated SRE-Luc reporter which is related with the ERK1,2 signal. The stable ectopic expression of Spred2 in PDV cells (SP cells) led to the loss of ERK1,2 activation by TGF- $\beta$ 1, although Smad2 activation was not affected, and the knockdown of Spred2 enhanced the activation of ERK1,2 signal by TGF- $\beta$ 1. The increment of uPA expression induced by TGF- $\beta$ 1 was suppressed in SP cells. In contrast, the stimulus on PAI-1 expression was not affected and comparable to parental PDV cells. SP cells under TGF- $\beta$ 1 treatment were unable to display the EMT, since the overexpression of Spred2 abolished the TGF- $\beta$ 1-induced disruption of the E-cadherin cell to cell interactions, reorganization of the actin cytoskeleton and upregulation of the mesenchymal marker vimentin. Finally, SP cells could not respond to the TGF- $\beta$ 1 stimulus on cell migration. Taken together, the data in the present study suggests that Spred2 is a regulator of TGF- $\beta$ 1-induced malignance in transformed keratinocytes.

The TGF- $\beta$  super-family of factors is implicated in the regulation of cell proliferation, differentiation, migration, extracellular matrix production, apoptosis and tumorigenesis.<sup>1</sup> TGF- $\beta$  binds to the functional complex of the TGF- $\beta$  family of receptors (T $\beta$ Rs) at the cell surface, which consist of 2 Type II and 2 Type I transmembrane serine/threonine kinase receptors,<sup>1,2</sup> which in turn activate downstream cellular components including Smads and members of the Ras/MAP kinases pathways.<sup>3</sup> TGF- $\beta$ 1 has been postulated to have a dual

role in tumor progression, by acting as tumor suppressor in early stages of carcinogenesis, and exerting a prooncogenic role in the last steps of metastatic disease.<sup>4</sup> TGF- $\beta$ 1 induces the epithelial mesenchymal transition (EMT) of transformed cells,<sup>5</sup> which contributes to the tumor invasion and metastasis.<sup>6</sup>

The urokinase type plasminogen activator (uPA) is a secreted serine proteinase that converts plasminogen to plasmin, a trypsin-like serine proteinase which in turn can degrade a wide variety of ECM components. In addition, the activity of uPA is finely regulated by PAI-1. The binding of uPA to its specific membrane receptor, uPAR, initiates a proteolytic cascade for degrading ECM which in turn regulates cell migration and invasiveness.<sup>7-9</sup>

The activation of Ras-ERK1,2 signal pathway is a relevant step in the induction of EMT and metastasis by TGF- $\beta$ 1.<sup>10-12</sup> It is important to understand the regulation of this signal in its attempt to modulate the effects of TGF- $\beta$ 1 on tumorigenesis. Recently, members of a Spred protein family (Spred1, 2 and 3) have been discovered as negative regulators of Ras-ERK1,2 pathway activation by receptor tyrosine kinases.<sup>13</sup> Spred-2 is expressed ubiquitously in adult tissues, whereas Spred1 and 3 are predominantly expressed in the brain.<sup>13</sup> Spreds block the ERK1,2 stimulation by inhibiting the RAF-1 activation by Ras.<sup>14</sup> New data has implicated Spreds in cancer since they inhibit tumor cell migration and cellular metastasis.<sup>15</sup> Also, the expression of Spreds has been deregulated in

**Key words:** TGF- $\beta$ 1, ERK1,2, Spred2, u-PA, EMT

**Abbreviations:** E-cad: E-cadherin; EMT: epithelial-mesenchymal transition; ERK: extracellular regulated kinase; PAI-1: plasminogen activator inhibitor type 1; shRNA: small hairpin RNA; TGF- $\beta$ 1: transforming growth factor- $\beta$ 1; u-PA: urokinase type plasminogen activator; vim: vimentin

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human hepatocellular carcinoma.<sup>16</sup> Although it is known that TGF- $\beta$ 1 activates Ras-ERK1,2 pathway, it is not clear whether Spred2 could modulate the TGF- $\beta$ 1 signal pathways; moreover, its implications in tumorigenesis have to be further studied. In this work, we evaluated the role of Spred2 on ERK1,2 MAPK activation, and its effect on the stimulation of uPA expression, cell migration and EMT by TGF- $\beta$ 1.

## Material and Methods

### Cell cultures and treatment conditions

The PDV and MCA-3D cell lines were cultured in Ham's F-12 medium supplemented with amino acids and vitamins (Gibco, Rockville MD), 10% fetal bovine serum (FBS; Gibco) and 80  $\mu$ g ml<sup>-1</sup> Gentamycin. Cells were maintained at 37°C in a 5% CO<sub>2</sub> in humidified atmosphere. The 293T cells were kindly provided by Dr. C. Bernabeu (CIB, Spain) and cultured in DMEM supplemented with 10% FBS. For TGF- $\beta$  treatments, human recombinant TGF- $\beta$ 1 (R&D, Minneapolis, MN) was used at a final concentration of 10 ng ml<sup>-1</sup>.

### Plasmids

Mouse Spred-2 cloned into pcDNA3 with a 6 repeated Myc tag was kindly provided by Dr. A. Yoshimura (ILS, KU, Japan). SRE-luc was provided by Dr. A. Corbí (CIB, Spain). The p-4.8 uPa-Luc luciferase reporter plasmid (-4.8 kb of murine uPA promoter) was provided by Dr. P. Munoz-Canoves (CRG, Spain). The promoter of PAI-I, p-800-luc, constitutively active ALK5 (ca-ALK5, T204D) and the Smad2-responsive pARE-lux reporter vector were kindly provided by Dr. C. Bernabeu (CIB, Spain). pUSEamp- Ha-Ras (Q61L) (Upstate Biotechnology, New York). Small hairpin RNA (shRNA) against Spred2 (pRS-Sp2, TI331300H) and pRS-shGFP noneffective were from Origene (Rockville, MD).

### Antibodies

The anti-Spred2, anti-Myc, anti- E-cadherin, anti-vimentin, anti- $\alpha$ -tubulin and secondary antibodies-coupled to HPO or FITC were from Sigma (St. Louis, Mo). The PAI-1 rabbit antibody, the anti-phospho monoclonal antibody and the rabbit polyclonal antibodies against ERK1,2 were purchased from Santa Cruz Biotechnology, CA. Anti p-Smad2 and anti Smad2 antibodies were purchased from Calbiochem, (Darmstadt, Germany).

### Immunofluorescences

Detection of E-cadherin, F-actin and vimentin by immunofluorescence was performed in cells grown on glass coverslips fixed with 4% p-formaldehyde in PBS. For F-actin and vimentin cells were permeabilized with 0.1% Triton-X100 for 2 min at room temperature. For F-actin staining, phalloidin coupled to Alexa Fluor 594 (Molecular Probes, Eugene OR) was used. Images were taken with a microscope equipped with epi-fluorescence.

### Immunoblotting

Proteins were separated by SDS-PAGE, then transferred to nitrocellulose membranes (BIORAD, Hercules, CA) and blocked with 4% of nonfat milk, 0.5% Tween 20 in TBS pH 8.5. Membranes were incubated with appropriate antibodies at 4°C overnight. The targeted proteins were detected by enhanced chemiluminescence to peroxidase as indicated by the manufacturer (Pierce, Dallas, TX).

### RT-PCR

Two micrograms of total RNA isolated from PDV cells was reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA). PCRs were performed using One-step PCR (Invitrogen, Carlsbad, CA) with the following settings: 95°C for 3 min, 26 cycles at 95°C for 10 sec, 55°C for 10 sec, and 72°C for 1 min. The primer sets used were as follows: 5'-TGT GAG CAC CGG AAG ATT TAT ACC-3', 5'-CG CGG CG GCT TTG TGC TT-3' (m-Spred-2); 5'TGC CCA AGGA AAT TCT GCC CAA GGA AAT TCC ACGC 3', 5'GCC AAT CTG CAC ATA GCA CC3' for m-uPA; 5'ATC CTG CCT AAG TTC TCT CTG 3', 5'ATT GTC TCT GTC GGG TTG TG3' for m-PAI-I, and 5'ACC ACA GTC CAT GCC ATC AC-3', 5'TCC ACC ACC CTG TTG CTG TA 3' for GAPDH.

### Stable transfection procedure

PDV cells ( $\sim 10^6$ ) seeded in 60-mm plates were transfected with 2  $\mu$ g of either pCDNA3-Spred2-Myc plasmid or empty pCDNA3 vector using Superfect (Qiagen, Hilden, Germany) following manufacturer's protocol. Transfected cells were selected for 2 weeks in medium containing 10% FBS and 400  $\mu$ g ml<sup>-1</sup> of G418. Resistant clones were isolated by cloning rings and regularly maintained in the presence of G418 (400  $\mu$ g ml<sup>-1</sup>).

### Transient transfections and reporter gene measurements

PDV cells seeded in T24 plates ( $\sim 2 \times 10^5$  cells/well) were transfected with 500 ng/well of each specific luciferase construction. A 25 ng/well of SV40- $\beta$ -Gal as internal control for transfection efficiency was used. Transfected cells were grown 24 hr in complete medium before treatment with TGF- $\beta$ 1. Cells were then treated with the growth factor for an additional 24 hr. For Spred2 knockdown experiments, cells were cotransfected with 100 ng/well of shRNA against Spred2 or noneffective shGFP as control, and 50 ng of ca-ALK5. A 25 ng/well of Ha-Ras Q61L was used at times indicated, and cells were cultured 48 hr after transfections. Firefly luciferase activity (Promega, Adison, WI,) was standardized for  $\beta$ -galactosidase activity (Tropix, Bedford, MA).

### Zymographic assay

uPA secreted activity was assayed in serum-free medium conditioned for 48 hr in PDV cell cultures treated or not with TGF- $\beta$ 1 subjected to caseinolytic zymography.<sup>17</sup> Briefly,

aliquots of conditioned serum-free medium were subjected to 10% SDS-PAGE under nonreducing conditions, the gels were washed with 2.5% Triton X-100, placed on 1% agarose gels containing 0.5% casein and  $2 \mu\text{g ml}^{-1}$  of plasminogen, and incubated at  $37^\circ\text{C}$  for 24 hr. uPA-dependent proteolysis was detected as a clear band. Quantification of these areas was performed by densitometric analysis.

### Wound healing assay

The motility property of cell transfectants was analyzed by an *in vitro* wound healing assay.<sup>17</sup> Wounded cell cultures were allowed to grow for 24 hr in the absence or presence of TGF- $\beta$ 1. The cells were then fixed with cold methanol and stained with crystal violet (0.1%) 20 min at room temperature.

### Statistics

Data are given as means  $\pm$  SD from at least 3 independent experiments. When necessary, statistical significance was evaluated using the Students' *t*-test. Differences were considered to be significant at a value of  $p < 0.01$ .

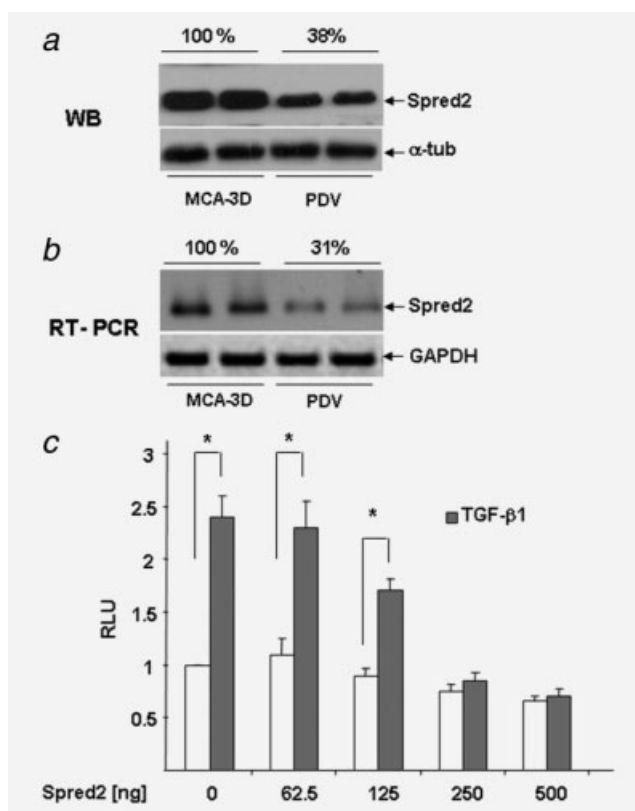
### Results

#### Spred2 expression is low in tumorigenic PDV cells, and the transient expression of Spred2 inhibits the activation of ERK1,2 pathway by TGF- $\beta$ 1

A previous study suggested a downregulation of Spred2 expression in hepatocellular carcinomas.<sup>16</sup> For further insight into Spred2 expression in normal and tumor cells, Spred2 was determined in immortalized, "normal," mouse keratinocytes, MCA-3D cells, and in tumorigenic PDV cells.<sup>18</sup> As seen in Figure 1*a*, the Western blot and RT-PCR analyses revealed low levels of Spred2 expression in PDV cells, (38 and 31%, respectively) when compared with MCA3D (100%). Since the expression of both housekeeper proteins ( $\alpha$ -tubulin and GAPDH) did not suffer changes, it was suggested that these results were owing to a difference in the expression of Spred2 in the both cell lines. Subsequently, it was analyzed whether the effect of the ectopic expression of Spred2 interfered with the activation of ERK1,2 pathway by TGF- $\beta$ 1 in PDV cells. For this purpose, a reporter plasmid with the SRE consensus sequence followed by the luciferase gene was used. It was found that the treatment of PDV cells with TGF- $\beta$ 1 produced 2.5 times higher activation of the reporter, and the transfection with increased amounts of Spred2 inhibited the transactivation of the ERK1,2 reporter by TGF- $\beta$ 1 (Fig. 1*c*).

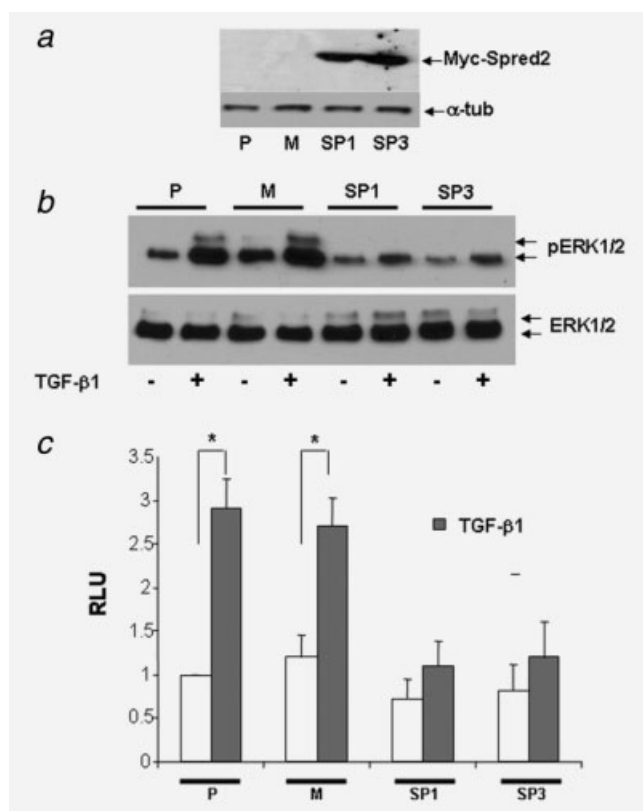
#### Expression of Spred2 inhibits the TGF- $\beta$ 1 stimulation of ERK1,2 but not the activation of Smad2 in PDV cells

PDV cells under TGF- $\beta$ 1 treatment undergo EMT and have enhanced malignance.<sup>5,18</sup> Therefore, the role of Spred2 in cell malignancy stimulated by TGF- $\beta$ 1 was studied. After a stable transfection with Spred2 performed in PDV cells, 2 clones with high levels of Spred2 expression were obtained, namely SP1 and SP3 (Fig. 2*a*). When the capacity of both SP1 and SP3 cells to respond to the TGF- $\beta$ 1 treatment was deter-



**Figure 1.** Spred2 expression in immortalized MCA3D and transformed PDV cell lines. Transient expression of Spred2 inhibits TGF- $\beta$ 1-induced ERK1,2 pathway activation. Spred2 expression was determined by Western blot (*a*) and RT-PCR analysis (*b*) in "normal" immortalized MCA3D and transformed PDV cells, using  $\alpha$ -tubulin ( $\alpha$ -tub) and GAPDH as a control respectively. (*c*) PDV cells were transiently transfected with SRE-luc reporter plasmid and cotransfected with increasing amounts of plasmid containing Spred2 cDNA and treated with TGF- $\beta$ 1 for 24 hr, and then the luciferase activity was determined. (RLU. Relative Luciferase Activity). The results shown are representative of 3 independent determinations. (\*  $p < 0.01$ ).

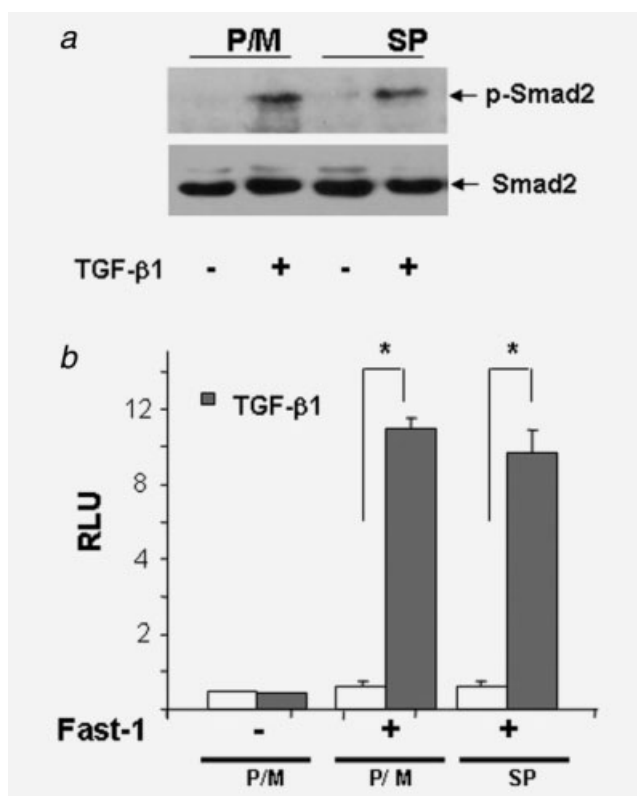
mined, it was observed that the growth factor was not able to induce the activation of ERK1,2 route, whereas in parental and mock cells, both ERK1,2 phosphorylation and SRE-reporter activity were enhanced by TGF- $\beta$ 1 (Figs. 2*b* and 2*c*). Since both SP1 and SP3 cells responded similarly to TGF- $\beta$ 1-induced ERK1,2 activation and showed a similar behavior in preliminary experiments, and to avoid artifacts resulting from the use of single clones of transfected cells, the cell clones were pooled for the subsequent experiments and renamed as SP cells. The effect of Spred2 on the activation of Smad2 by TGF- $\beta$ 1 was then analyzed. It was found that the treatment with TGF- $\beta$ 1 induced the phosphorylation of Smad2 as well as the transactivation of Smad2 dependent reporter in control conditions, and SP cells responded in a manner similar to that of control cells for the activation of Smad2 by TGF- $\beta$ 1 (Figs. 3*a* and *b*).



**Figure 2.** Stable expression of Spred2 inhibits TGF- $\beta$ 1-induced ERK1,2 activation. (a) Western blot analysis for cells expressing myc-Spred2: PDV cells were stably transfected with myc tagged Spred2 protein, and selected with G418. *P* (parental PDV), *M* Mock transfected with empty vector and 2 clones expressing Spred2 named SP1 and SP3, were subjected to Western blot determination of myc-tagged Spred2;  $\alpha$ -tubulin was used as housekeeping protein. (b) Western blot analysis of ERK1,2 activation. PDV/Mock (*P/M*), SP1 and SP3 in serum free media were treated during 30 min with TGF- $\beta$ 1, and protein samples were subjected to p-ERK1,2 and ERK1,2 immunoassay. (c) Determination of SRE-luc reporter. PDV, Mock, SP1 and SP2 cells were transiently transfected with SRE-luc reporter, treated with TGF- $\beta$ 1 for 24 hr, followed by the luciferase activity determination. The results shown are representative of at least 3 independent determinations. (\*  $p < 0.01$ ).

### Spred2 knockdown enhances the TGF- $\beta$ 1 stimulation of ERK1,2

Since the overexpression of Spred2 inhibited TGF- $\beta$ 1-induced ERK1,2 activation, experiments were carried out to elucidate whether the knockdown of Spred2 affected the TGF- $\beta$ 1 signaling. For this purpose, 293T cells were used, which are easily transfected and strongly express exogenous proteins. The knockdown of Spred2 enhanced ERK1,2 activation in the absence of constitutively active exogenous expression of ca-ALK5, and the presence of ca-ALK5 further enhanced the activation of SRE reporter. This activation was inhibited by the coexpression of Spred2-myc tagged (Fig. 4a). The Smad2



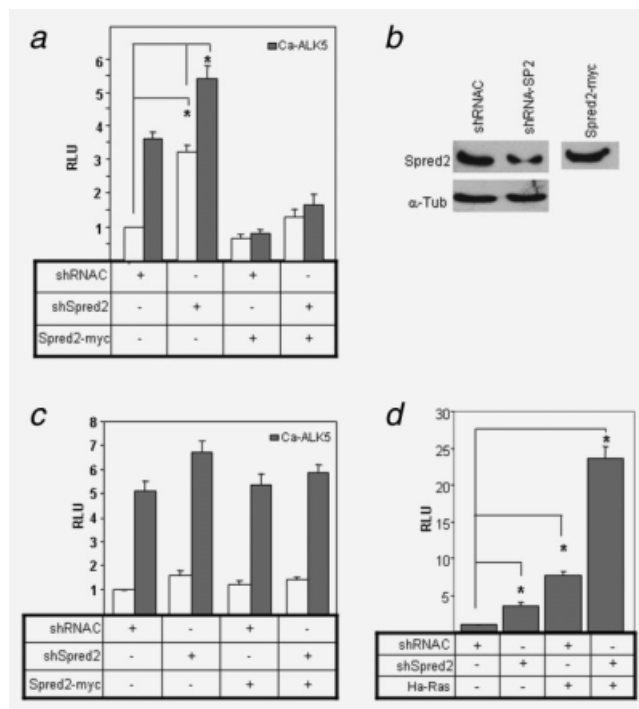
**Figure 3.** Spred2 does not affect the TGF- $\beta$ 1-induced Smad2 pathway (a) Western blot analysis of Smad2 activation. PDV/Mock (*P/M*) and SP in serum free media were treated for 30 min with TGF- $\beta$ 1, and protein samples were subjected to p-Smad2 and Smad2 immunoanalysis. (b) Determination of Smad2 reporter. PDV/Mock, SP cells were transiently transfected with pARE-luc/fast-1 reporter, treated with TGF- $\beta$ 1 for 24 hr and analyzed for luciferase activity. The results shown are representative of at least 2 independent determinations. (\*  $p < 0.01$ ).

activation was unaffected by the Spred2 knockdown (Fig. 4c). Also, the depletion of Spred2 tremendously enhanced the activation of ERK1,2 signaling in the presence of the oncogenic version of Ha-Ras (Fig. 4d).

### Spred2 overexpression inhibits TGF- $\beta$ 1-induced uPA without affecting PAI-1 expression

The expression of uPA by the tumoral cells is correlated with the malignancy and is considered to be a "bad prognosis" marker.<sup>19,20</sup> The uPA activity is controlled by its natural inhibitor PAI-1.<sup>21</sup> In PDV cells, TGF- $\beta$ 1 induces both uPA and PAI-1 expression.<sup>22</sup> Therefore, it was analyzed whether Spred2 expression affects TGF- $\beta$ 1-induced uPA and PAI-1 in PDV cells. The results showed that TGF- $\beta$ 1 significantly enhanced the expression of uPA in control cells; however, TGF- $\beta$ 1 did not increase the uPA expression, mRNA and promoter transactivation in SP cells (Figs. 5a–5c). At the same time, it was observed that Spred2 did not affect the induction of PAI-1 by TGF- $\beta$ 1, whereas the stimuli by TGF-



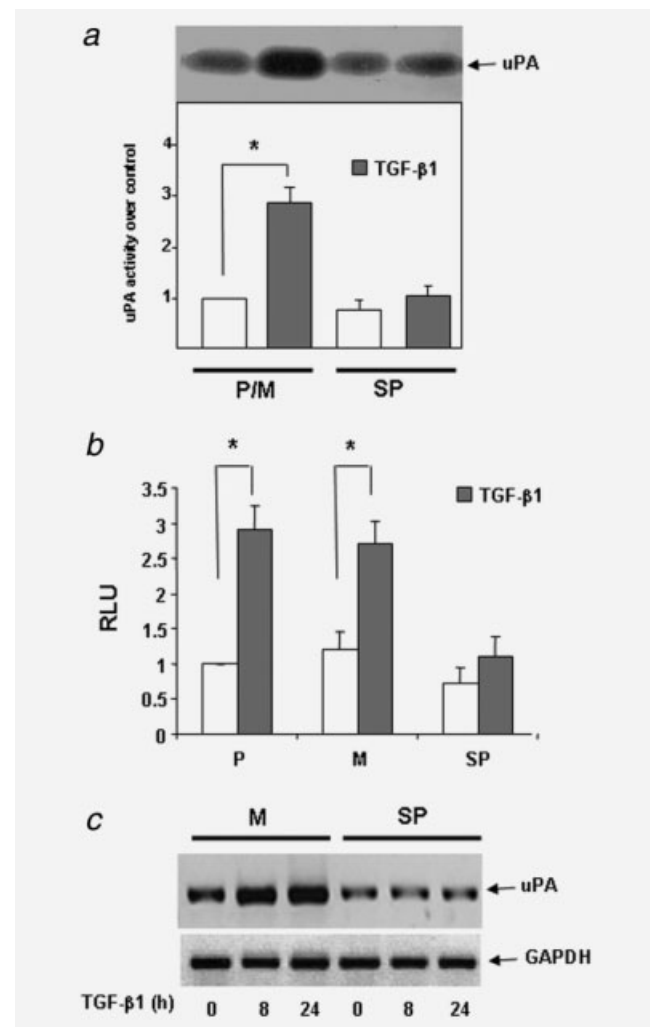


**Figure 4.** Spred2 knockdown enhances ERK1,2 signaling. (a) Determination of SRE transactivation. 293T cells were transiently transfected with shRNA against Spred2 or non-effective shGFP, with or without constitutively active ALK5 (Ca-ALK5) or Spred2-myc tagged. Cells were cultured 48 hr after transfection and then the luciferase activities were determined. (b) Western blot analysis of expression of Spred2 in cells transfected with noneffective shGFP, shRNA against Spred2 or Spred2-myc tagged. (c) Smad2 reporter, cells were transfected as in (a) and the activity of pARE-luc/fast-1 reporter was determined. (d) Determination of SRE transactivation by Ha-Ras. Cells were transiently transfected with SRE-luc reporter and with shRNA against Spred2 or noneffective shGFP with or without oncogenic Q61L Ha-Ras. The results shown are representative of at least 3 independent determinations. (\*  $p < 0.01$ ).

β1 on protein and mRNA expression as well as the promoter transactivation to PAI-1 were similar to those in control cells (Figs. 6a–6c), thereby indicating that Spred2 does not affect the PAI-1 expression induced by TGF-β1 in PDV cells.

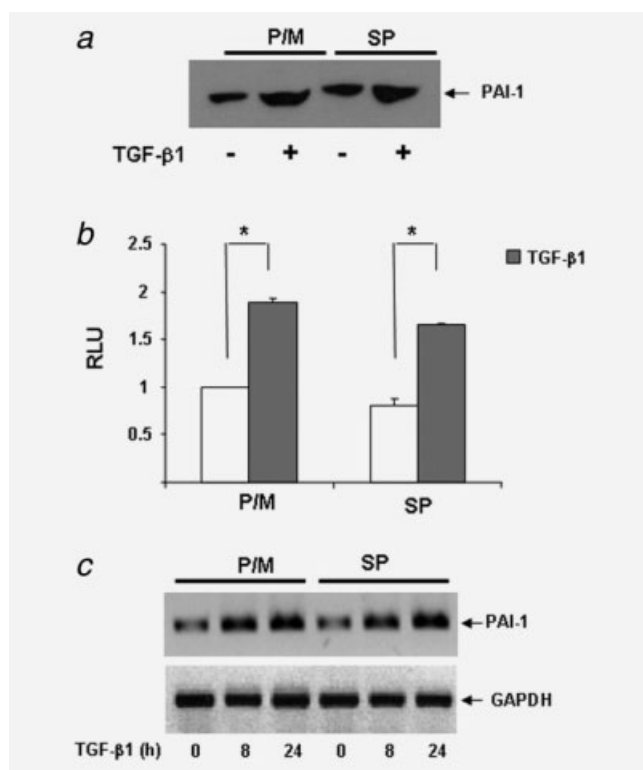
#### Spred2 inhibits TGF-β1-induced epithelial mesenchymal transition and cell migration

TGF-β1 is a potent inducer of EMT which allows tumor cells to acquire a more metastatic phenotype.<sup>3</sup> Several tests were carried out to determine whether Spred2 affects the induction of EMT by TGF-β1 in PDV cells. The analysis of E-Cadherin (E-Cad) by immunofluorescence indicated a strong signal of the protein in PDV cells, whereas the treatment with TGF-β1 for 48 hr produced a delocalization of E-Cad from the cell periphery with a punctuated pattern in the cellular cytoplasmic membranes; cells assumed an elongated shape, and several cells lost contact with the neighboring cells



**Figure 5.** Spred2 inhibits TGF-β1-induced uPA expression. (a) PDV/Mock (P/M) cells were treated for 48 hr with TGF-β1 in serum free serum; the conditioned media were subjected to zimographic analysis for uPA activity. (b) PDV/Mock and SP were transiently transfected with mouse uPA promoter, p-4.8 uPa-Luc, treated 48 hr with TGF-β1 and Luciferase activity was determined. (c) mRNAs from PDV and SP cells were purified at indicated times after TGF-β1 treatment, subjected to cDNA synthesis and PCR analysis for uPA; GAPDH was used as housekeeping gene. Three independent experiments were performed and a representative is shown. (\*  $p < 0.01$ ).

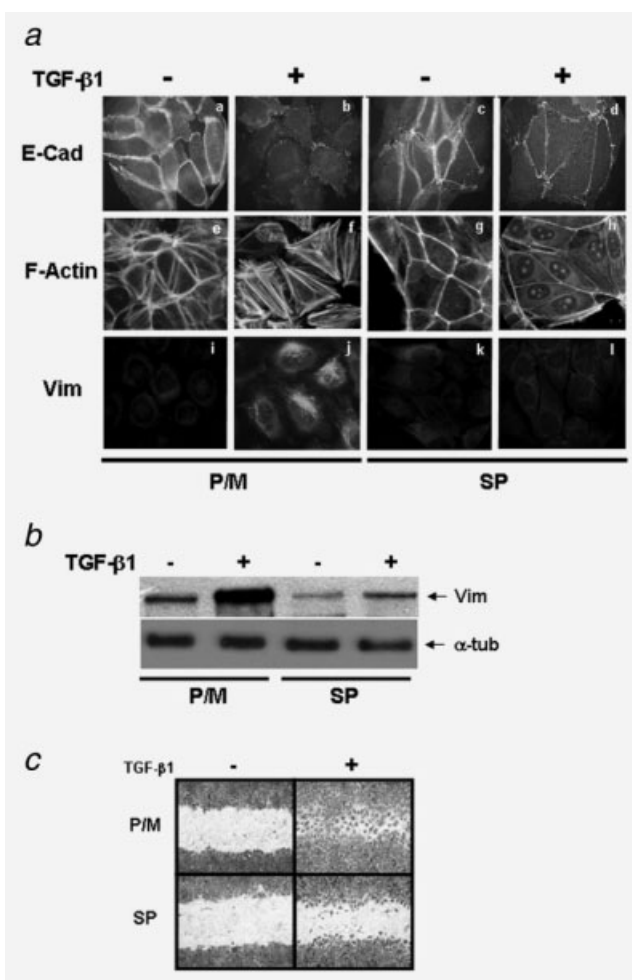
by reduction of E-Cadherin-dependent cell–cell adhesion, Figure 7a (a–b). In SP cells, the intercellular distribution of E-Cad after TGF-β1 treatment remained similar to that in the parental cells, Figure 7a (c–d). These results are related with the effect in the actin cytoskeleton, where control cells exhibit a typical cubic epithelial morphology characterized by cortical actin. The treatment with TGF-β1 for 48 hr produced spindle-like cell morphology with a decreased cell–cell adhesion and a conversion of the F-actin cytoskeleton from submembranous fibers to transcellular stress fibers which



**Figure 6.** Spred2 does not affect the TGF- $\beta$ 1-induced PAI-I expression. (a) PDV/Mock (P/M) and SP cells were treated for 48 hr with TGF- $\beta$ 1 in serum free medium. After the treatment conditioned media, normalized by monolayer cells protein amount, were subjected to Western blot analysis to PAI-1. (b) PDV/Mock and SP cells were transiently cotransfected with Spred2 and PAI promoter (p800-luc), treated for 24 hr with TGF- $\beta$ 1 and subjected to luciferase activity determination. (c) PDV/Mock cells and SP cells were treated with TGF- $\beta$ 1 at indicated times and mRNAs were purified, subjected to cDNA synthesis and PCR analysis; GAPDH was used as housekeeping gene. At least 3 independent experiments were performed and a representative is shown (\*  $p < 0.01$ ).

resemble a motile cellular phenotype, Figure 7a (e-f). TGF- $\beta$ 1 did not induce F-actin reorganization in SP cells, and the cells displayed cortical F-actin in the same way as control cells, Figure 7a (g-h).

One of the main events that characterize the EMT is the expression of vimentin.<sup>23</sup> It was noticed that TGF- $\beta$ 1 strongly enhanced the vimentin expression in PDV cells, Figure 7a (i-j), but not in SP cells, Figure 7a (k-l). These results were confirmed by Western blot analysis. PDV and SP cells were found to express very low levels of vimentin, while TGF- $\beta$ 1 increased the vimentin expression only in control cells (Fig. 7b). The changes observed (E-cadherin delocalization, actin rearrangement and upregulation of vimentin expression) are related to the migration capacity of the cells. As observed in Figure 7c, after 24 hrs of treatment with TGF- $\beta$ 1, the PDV cells were induced to close the wound area. However, SP cells did not migrate under the TGF- $\beta$ 1-stimulus. Thus, these data



**Figure 7.** Spred2 inhibits TGF- $\beta$ 1-induced epithelial mesenchymal transition and cell migration. (a) Parental (P) or Mock (M) PDV Cells and SP cells were seeded over coverslips and immunostained for E-cadherin (E-Cad), (a-d), stained for F-actin using phalloidin-Alexafluor 546, (e-h) (magnifications  $\times 400$ ), and immunostained for vimentin (Vim), i-l (Magnification  $\times 400$ ). Cells were stimulated with TGF- $\beta$ 1 for 48 hr for E-cad and F-actin and 96 hr for Vim. All photographs are representative fields of at least 2 or 3 independent experiments. (b) Cells were treated for 96 hr with TGF- $\beta$ 1, proteins samples were then analyzed by Western blot for vimentin (Vim) and  $\alpha$ -tubulin ( $\alpha$ -tub) as a control protein. Three independent experiments were performed and a representative is shown. (c) Cell monolayers were wounded and stimulated with TGF- $\beta$ 1 for 24 hr, fixed and stained with crystal violet. Photographs are representative of 3 independent experiments.

taken together suggest that Spred2, possibly by regulating the ERK1,2 activation, modulates the enhancement of the EMT and migration by TGF- $\beta$ 1.

## Discussion

The present study examines the role of Spred2 as a regulator of the cellular malignance stimulation by TGF- $\beta$ 1. The TGF-

$\beta 1$  pathway has been implicated in the metastatic process and has been shown to influence the ability of tumor cells to spread throughout the body.<sup>24</sup> It is of enormous interest to know the regulatory mechanisms that would allow modulating the effect of TGF- $\beta 1$  on cancer cells.

TGF- $\beta 1$  transduces its signal through a plethoric network of signal pathways, including Smads and MAPK such as ERK1,2.<sup>3</sup> Although the positive and negative regulatory mechanisms that can control TGF- $\beta 1$ -Smads pathway<sup>1</sup> are relatively known, there is not much information about the control of Ha-Ras-ERK1,2 signaling for TGF- $\beta 1$ .

In this aspect, it has been recently discovered that the Spred proteins negatively regulate the Ha-Ras-ERK1,2 route when activated by tyrosine kinases receptors.<sup>13</sup> At present, information is provided about the capacity of Spred2 to negatively control the activation of ERK1,2 by TGF- $\beta 1$  and the repercussion of this on the cellular malignance. Yoshida *et al.*<sup>16</sup> have reported that the Spred2 expression was frequently decreased in tumor areas of human hepatocellular carcinoma compared with the normal tissues, and they suggested that the low level of Spreds could be possibly related with the malignancy of cancer cells. The result obtained in this study supports this possibility since it was found that the level of Spred2 expression in transformed PDV cells was significantly lower than that in the "normal" immortalized MCA3D cells (Figs. 1a and 1b). However, there is no evidence about the mechanisms by which the expression of Spred2 is downregulated in transformed cells, and further studies must be carried out to elucidate these mechanisms implicated.

It was found that the transient expression of Spred2 inhibited the ability of TGF- $\beta 1$  to activate ERK1,2 pathways in PDV cells (Fig. 1c). Since PDV cells have a comparatively low expression of Spred2, this cell line was transfected with Spred2-myc tagged (Fig. 2a). The activation of ERK1,2 by TGF- $\beta 1$  was strongly inhibited in cells that over-expressed Spred2, thus demonstrating the usefulness of Spred2 to negatively modulate the activation of this route by TGF- $\beta 1$  (Figs. 2b and 2c). At present, 2 mechanisms by which TGF- $\beta 1$  activates RAS-ERK1,2 signaling have been described: Uchiyama-Tanaka *et al.*<sup>25</sup> have reported an indirect mechanism in which the transactivation of EGF receptor mediates the activation of Ras-ERK1,2 signal by TGF- $\beta 1$ ; and more recently, it has been demonstrated that TGF- $\beta$  Type-I receptor phosphorylates ShcA on tyrosine and serine, triggering its association with Grb2 and Sos, and thereby initiating the activation of ERK signal.<sup>26</sup> Although Ras activation was not demonstrated in the second study, it is well known that the resulting ShcA/Grb2/Sos complex activates Ras.<sup>27</sup>

In PDV cells TGF- $\beta 1$  activates Ha-Ras-ERK1,2 signal,<sup>17,28</sup> and the overexpression of Spred2 blocks the ERK1,2 signal pathway by inhibiting the activation of Raf-1 by Ras, as has been demonstrated by Wakioka *et al.*<sup>14</sup> for tyrosine kinase receptors. The effect of Spred2 appears to be specific to ERKs because it does not affect the activation of p38 and JNK.<sup>13</sup> Also, it was observed that Spred2 did not affect the TGF- $\beta 1$

activation of Smad2 (Figs. 3a and 3b) which is dependent on the serine-threonine kinase activity of TGF- $\beta 1$  receptors.<sup>29</sup> The effect of Spred2 on TGF- $\beta 1$  signaling was corroborated for Spred2 knockdown in 293T cells, where the activity of ERK1,2 was strongly enhanced by constitutively active ALK5 or oncogenic Ha-Ras exogenous expression, and nonaffected Smad2 (Fig. 4) suggested that the overexpression or Spred2 depletion mainly affected the ERK1,2 MAPK pathways rather than the Smad2 signal.

During the tumoral progression, the cells display extracellular matrix degradative systems which allow the cells to invade neighboring tissues.<sup>30</sup> In this aspect, uPA plays a pivotal role in determining the capacity of tumor cells for degrading extracellular matrix (ECM). uPA converts the plasminogen to the active plasmin; this proteinase activity is capable of degrading ECM and activates metalloproteinases. This system is controlled by the uPA inhibitor PAI-1.<sup>9,19</sup> It was found that the exogenous expression of Spred2 in PDV cells inhibited the stimulus of uPA expression by TGF- $\beta 1$  (Fig. 4), which is mediated in these cells by ERK1,2.<sup>17</sup> Also, it was observed that Spred2 did not affect the enhancement of PAI-1 induced by TGF- $\beta 1$  (Fig. 5). Thus, it is possible that Spred2 generates an imbalance between uPA and PAI-1 levels in PDV cells by displacing the uPA/PAI ratio toward an inhibition status under TGF- $\beta 1$  treatment. Also, Yoshida *et al.*<sup>16</sup> demonstrated that the ectopic expression of Spred1 reduced the expression of MMP-2 and MMP-9 in human hepatocarcinoma cells which was in relation with a decreased cell migration. It has not been studied whether Spred2 affects the MMP-9 expression reduced by TGF- $\beta 1$  in PDV cells; however, owing to the fact that TGF- $\beta 1$  enhances MMP-9 expression through ERK1,2,<sup>31</sup> an inhibition of TGF- $\beta 1$ -induced MMP-9 in this cell line can be anticipated, although further determinations are necessary to assess this possibility. Thus, Spred proteins could be negative regulators of the extracellular matrix proteases expression with harmful consequences during the invasiveness of cancer cells.

TGF- $\beta 1$  can also enhance cell migration and the invasive capacity of cancer cells.<sup>24</sup> During these processes, the epithelial cells lose cell-cell adhesion and acquire a mesenchymal phenotype related with the epithelial-mesenchymal transition.<sup>32</sup> The EMT is characterized by the rearrangement of several membrane-associated proteins, including E-cadherin and ZO-1, as well as by changes in the cytoskeleton organization. The upregulation of vimentin expression is a particularly well known marker of the EMT, and the expression of vimentin in human epithelial cancers has been frequently observed.<sup>32-34</sup> TGF- $\beta 1$  promotes the EMT in cancer cells,<sup>24</sup> and the results of this study have demonstrated that the overexpression of Spred2 in transformed PDV cells inhibits the induction of the EMT by TGF- $\beta 1$ . Spred2 inhibits the loss in the cell-cell adhesion as well as the delocalization of E-Cadherin induced by TGF- $\beta 1$  (Fig. 7a). E-Cadherin is commonly downregulated in several cancers, and it has been described that the repression of E-Cadherin expression is induced by



the zinc-finger transcription factor, Snail.<sup>35</sup> Also, it has been described that TGF- $\beta$ 1 induces Snail through the Ras-ERKs signal pathway,<sup>36</sup> and it is speculated that Spred2 might inhibit the TGF- $\beta$ 1 induction of Snail by blocking ERKs activation and thereby avoiding the downregulation of E-Cadherin expression.

The expression of exogenous Spred2 also inhibits the rearrangement of actin cytoskeleton induced by TGF- $\beta$ 1, Figure 7a (d-h). Although Spred2 can inhibit the changes in the actin network by repressing Ras-ERK1,2 signaling, owing to the recent demonstration that Spred2 can also inhibit Rho GTPases,<sup>15</sup> it is possible that Spred2 regulates the actin rearrangement induced by TGF- $\beta$ 1 either indirectly by inhibiting the activation of Rho GTPases by Ras or directly by inhibiting Rho GTPases. Also, during the EMT, cells upregulate the expression of vimentin and acquire a mesenchymal phenotype. PDV cells which represent a squamous carcinoma have a low level of vimentin expression and this protein is dramatically upregulated after TGF- $\beta$ 1 treatment.<sup>5</sup> It was found that Spred2 inhibits the up-regulation of vimentin induced by TGF- $\beta$ 1, Figures 7a (i-l) and 7b. PDV cells were obtained from mouse keratinocytes treated *in vitro* with DMBA.<sup>8</sup> Although these cells display an epithelial phenotype, they have the expression of mutated H-Ras which might confer a pre-committed state for beginning the EMT with an incipient vimentin expression, and the presence of TGF- $\beta$ 1 is necessary to complete the EMT. The collaboration between Ras and TGF- $\beta$ 1 in the induction of EMT has been well

described, pointing out that the enhancement in Smads2 and Ras is sufficient for the generation of EMT in transformed cells.<sup>37</sup> The inhibition of EMT by Spred2 can be attributed not only to the inhibition of ERK1,2 activation by TGF- $\beta$ 1, but also to a result of general inhibition of ERK1,2 activation by other sources such as the mutant Ras which contributes to the induction of EMT by TGF- $\beta$ 1.

There is considerable evidence that the gain of mesenchymal characteristics and the loss of epithelial features (EMT) are associated with the acquisition of migratory and invasive properties of the epithelial cells.<sup>32,38</sup> Also, Spred2 overexpression inhibits the TGF- $\beta$ 1-induced cell migration of PDV cells which is closely related with the effect of Spred2 on TGF- $\beta$ 1-induced EMT. It has been suggested that Spred2 plays a role in the tumorigenesis since a reduced level expression of Spreds has been observed in hepatocellular carcinoma.<sup>13,16</sup> The results of this study support this finding because Spred2 was expressed in a relatively low level in PDV transformed cells, and the ectopic expression of Spred2 inhibited the stimuli on uPA expression, EMT and cell migration by TGF- $\beta$ 1. Further studies are necessary to elucidate the molecular mechanism by which Spred2 expression is deregulated in cancer cells, as well as to analyze the mechanism by which Spred2 downregulation could affect the malignant characteristics of tumor cells.

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