

# Role of reduced expression of SMAD4 in papillary thyroid carcinoma

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## Abstract

It has been demonstrated that transforming growth factor- $\beta$  (TGF $\beta$ ) and other members of TGF $\beta$  superfamily play an important role in thyroid proliferative diseases. The deficiencies of SMAD4 are responsible to accelerate the malignant progression of neoplastic lesions in several types of tumors. Therefore, the objective of the present study was to determine the functional role of reduced expression of SMAD4 in human papillary thyroid carcinogenesis. For this purpose, we examined the TGF $\beta$  response in two cell lines, TPC-1 and BCPAP. Our data demonstrated for the first time that these cells showed a strong reduction in the level of SMAD4 protein, which was responsible for an alteration of TGF $\beta$  signaling and for some of the TGF $\beta$ -mediated biological effects. The overexpression of SMAD4, restoring TGF $\beta$  transduction, determined a significant increase of antiproliferative response to TGF $\beta$ , and reduced the invasive behavior of these cells. Therefore, our data indicated that reduction of SMAD4 may play a significant role in thyroid carcinogenesis.

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## Introduction

Transforming growth factor- $\beta$  (TGF $\beta$ ) belongs to a large superfamily of growth factors and controls a plethora of cellular responses, including cell proliferation, differentiation, motility, adhesion, extracellular matrix protein expression, apoptosis, and specification of developmental fate, during embryogenesis as well as in mature tissues, in species ranging from flies and worms to mammals (Patterson & Padgett 2000, Shi & Massagué 2003). Hence, in normal cells, TGF $\beta$  acts as a tumor suppressor by inhibiting cell growth or by promoting cellular differentiation or apoptosis.

At some time during the stepwise transition towards malignancy, human cancer cells acquire several abilities that most normal cells do not have, including the ability to become, at least partially, resistant to growth inhibition, to proliferate without dependence on growth factors, to replicate without limit, to invade, and to metastasize. TGF $\beta$  is one of the complex signal transduction pathways that control normal cellular homeostasis regulating these processes. TGF $\beta$  signaling is propagated via cell surface Ser/Thr kinases, TGF $\beta$  type I receptor (T $\beta$ RI, listed in the HGNC database as TGFBR1), and TGF $\beta$  type II receptor (T $\beta$ RII, TGFBR2), followed by an intracellular cascade of events involving receptor-regulated SMADs (R-SMADs), SMAD2 and

SMAD3, and their interacting partner, common-mediator SMAD, SMAD4. Once formed the complexes, the SMADs translocate in the nucleus where they regulate target genes (Attisano & Wrana 2000). In contrast, the inhibitory SMAD (I-SMAD), SMAD7, binds to T $\beta$ RI and prevents the phosphorylation of R-SMADs, resulting in the inhibition of TGF $\beta$  signaling (Imamura *et al.* 1997, Nakao *et al.* 1997). A SMAD ubiquitin regulatory factor 1 (SMURF1), being a HECT-type E3 ubiquitin ligase, interacts with I-SMAD7 and enhances the turnover of T $\beta$ RI (Suzuki *et al.* 2002). TGF $\beta$  elicits context-dependent and cell-specific effects that often appear conflicting. It is puzzling how a seemingly direct transduction scheme, dependent on shuttling of SMAD proteins from the cytoplasm to the nucleus, can mediate such diverse array of responses.

TGF $\beta$  is normally expressed and secreted in epithelial follicular thyroid cells. In thyroid cell lines, the differentiated phenotype is controlled by TGF $\beta$ , which inhibits iodide trapping (Taton *et al.* 1993, Pekary & Hershman 1998) and thyroglobulin synthesis (Colletta *et al.* 1989); some of these effects are exerted through SMAD signaling (Nicolussi *et al.* 2003, Costamagna *et al.* 2004). TGF $\beta$  is also the negative regulator of thyrocyte proliferation: it antagonizes the mitogenic effects of the main growth factors in thyroid cells of rat (Morris *et al.* 1988, Colletta *et al.* 1989, Coppa *et al.* 1995, Carneiro

*et al.* 1998), of porcine (Franzén *et al.* 1999), and of humans (Wyllie *et al.* 1991), and delays progression during the mid-late G<sub>1</sub> phase (Carneiro *et al.* 1998, Depoortere *et al.* 2000).

Several studies on surgical samples and on thyroid cell cultures have been performed showing the role of TGFβ and other members of TGFβ superfamily in thyroid proliferative diseases: benign cases (Morosini *et al.* 1994) and malignant ones (Heldin *et al.* 1999, Kimura *et al.* 1999, Cerutti *et al.* 2003). These data support the hypothesis that this growth factor is important in thyroid cell physiology, modulating thyroid functions and proliferation, and justify the need to study also the proteins involved in signaling from the cytoplasm to the nucleus.

The objective of this study was to determine the functional role of TGFβ pathway in human papillary thyroid carcinogenesis. For this purpose, we examined the TGFβ response in two cell lines, TPC-1 and BCPAP, and in a group of 23 cases of human papillary thyroid carcinomas (PTCs). Our data demonstrated for the first time that these cells showed a strong reduction in the level of SMAD4 protein, which was responsible for an alteration of TGFβ signaling and for some of the TGFβ-mediated biological effects. The overexpression of SMAD4, restoring TGFβ transduction, determined a significant increase of antiproliferative response to TGFβ, and reduced the invasive behavior of these cells. Therefore, these data indicate that reduction of SMAD4 may play a significant role in thyroid carcinogenesis.

## Materials and methods

### Reagents and constructs

DMEM, Coon's modified Ham's F-12 medium, PBS, FBS, trypsin-EDTA, L-glutamine 100× (200 mM), and the six-hormone mixture (6H) containing TSH (10 mU/ml), insulin (10 µg/ml), hydrocortisone (10<sup>-8</sup> M), transferrin (5 µg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 µg/ml) were purchased from Sigma-Aldrich, Co.

The human recombinant TGFβ1 isoform was purchased by Peprotech (Rocky Hill, NJ, USA). MG132 was provided by Calbiochem (La Jolla, CA, USA).

Phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail containing 1 mg/ml leupeptin, 2.5 mg/ml aprotinin, and 1 mg/ml benzamide hydrochloride, and phosphatase inhibitor cocktail were purchased from Sigma-Aldrich, Co. Antibodies to TβRI and TβRII, green fluorescent protein (GFP), SMURF1 (H60), SMAD7 (H79), SMAD4 (B8), SMAD4 (H552), N-cadherin (H63), β-actin (C4), and Lamin B (C20) were obtained from Santa Cruz Biotechnology (Santa Cruz,

CA, USA). Antibodies to the phosphorylated form of SMAD2 (SMAD2-P) and SMAD3 (SMAD3-P) were obtained from Cell Signaling Technology (Beverly, MA, USA), to SMAD2-P from Millipore (Billerica, MA, USA), to E-cadherin from Immunological Sciences (Rome, Italy), to SMAD3 from Zymed Laboratories (San Francisco, CA, USA), and to SMAD2/ SMAD3 from BD Biosciences PharMingen (San Diego, CA, USA). Texas Red-conjugated goat anti-mouse IgG was obtained from Jackson Laboratories (Bar Harbor, ME, USA); FITC-conjugated goat anti-mouse and goat anti-rabbit IgG was obtained from Cappel (Organon Teknica Corp., West Chester, PA, USA).

Transfections were performed using lipofectin kit provided by LipofectAMINE PLUS (Gibco BRL, Life Technologies).

GFP-tagged *SMAD4* wt was constructed subcloning the human *SMAD4* gene in CLONTECH pEGFPC3 vector, which allows in-frame fusion to the C terminus of GFP. pCMV5 3TP-Lux, pCMV5-βGal, and pCMV5 FLAG-SMAD2 wt have been gently provided by Prof. Liliana Attisano.

### Cell cultures and transient transfection analysis

TPC-1 and BCPAP (human PTC cell lines), and FRO (human anaplastic thyroid carcinoma cell lines) were kindly provided by Prof. Alfredo Fusco and were maintained in culture as previously described (Iuliano *et al.* 2003). The FRTL-5 cells were kindly provided by Dr L D Kohn. This cell line, diploid between their 5th and 25th passage, maintains the functional characteristics of iodide uptake, thyroglobulin synthesis, and cyclic nucleotide metabolism over prolonged periods of culture and doubling time of ~36 h (Ambesi-Impimbatto *et al.* 1980). These cells were grown as previously described (Nicolussi *et al.* 2003) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> air in w/o supplemented with 5% calf serum and 6H mixture. FTC133, human follicular thyroid tumor cell line (Goretzki *et al.* 1990), kindly provided by Prof. Salvatore Ulisse, MCF7 and MDA MB468 (Schutte *et al.* 1996), breast cancer cell lines, HepG2, human hepatoma cell line, and HEK293, derived from human embryonic kidney cells, purchased from American Type-Culture Collection (ATCC, Rockville, MD, USA), were grown at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> air in DMEM supplemented with 10% calf serum.

TPC-1 and BCPAP were transiently cotransfected with empty vectors or pEGFPC3-SMAD4 wt and/or pCMV5 FLAG-SMAD2 wt using lipofectin technique following the manufacturer's instructions. After 24 h of incubation, transfected cells were used in immunofluorescences, luciferase assays, proliferation assays, wound-healing assays, and western blot assays.

### Proliferation assays

The cell proliferation was assessed by cell counting. Briefly,  $5 \times 10^4$  cells were seeded into 35 mm plates and allowed overnight at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Cell numbers were determined using a hemacytometer both at time zero (T0), to determine the number of cells in the plates before the TGFβ1 treatment, and after 24 and 48 h with or without 10 ng/ml of TGFβ1 in serum-free medium. Cells were counted three times by two independent investigators. Inter-observer variation was below 5%. Values represent the mean of triplicate determination  $\pm$  s.d. of three experiments, and the results were also presented as a percentage of growth inhibition with respect to relative untreated control for each time point.

### Luciferase assays

These assays were carried out as described previously (Nicolussi *et al.* 2003, 2006). After transfection, cells were treated or untreated with 10 ng/ml of TGFβ1 in serum-free medium. Luciferase activities were normalized for transfection efficiency using the β-galactosidase-expressing control vector. The experiments were repeated four times in triplicate.

### Protein extraction and western blotting analysis

Protein extracts were obtained as previously described (Nicolussi *et al.* 2006). Subconfluent cells, transfected or not, were treated or untreated with 10 ng/ml of TGFβ1 for 30, 60, or 120 min or with MG132 (30 μM) for 24 h.

To obtain the cytosolic and nuclear fractions, cells were lysed in 0.4 ml hypotonic buffer A (10 mM Hepes, pH 7.4, 42 mM KCl, and 5 mM MgCl<sub>2</sub>) supplemented with protease inhibitor cocktail. After centrifugation of lysate at 1200 g for 10 min, the supernatant containing the cytosolic fraction was clarified by centrifugation at 15 000 g for 1 h at 4 °C. The pellet containing nuclear fraction was repeatedly washed in a specific buffer B (10 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.1 mM EDTA) and resuspended in extraction buffer C (20 mM Hepes, pH 7.4, 25% glycerol, 0.4 M NaCl, 1.5 mM MgCl<sub>2</sub>, and 0.2 mM EDTA), supplemented with protease inhibitor cocktail, PMSF, and dithiothreitol. Protein extracts were subjected to immunoblotting as previously described (Nicolussi *et al.* 2006) using primary antibodies to TβRI, TβRII (1:600), SMAD2-P (1:1000), SMAD3-P (1:1000), SMAD3 (1:1000), GFP (1:500), E-cadherin (1:1000), N-cadherin (1:500), Lamin B (1:1000), SMAD2/SMAD3 (1:500), SMAD4 (1:500), and β-actin (1:5000). SMAD4, E-cadherin, SMAD2-P, and SMAD3-P bands

were analyzed using Bio-Rad Laboratories software. Data, obtained from three different protein extracts, were collected in terms of average intensity of bands of each proteins per average intensity of bands of β-actin or SMAD2.

### RNA isolation and analysis

RNA was extracted using Tri Reagent (Sigma–Aldrich, Co.), following the manufacturer's instructions. Using 1 μg RNA, cDNAs were synthesized using MuLV reverse transcriptase (Applied Biosystems, Hammon, NJ, USA) and random primers (Roche) according to the manufacturer's instructions. The primers used in the amplification of human *SMAD4* (*SMAD4A* forward 5'-CCTTGCAACGTTAGCTGTTG-3', reverse 5'-CTTC-AGTGGACAACGATG-3'; *SMAD4B* forward 5'-CACTG-CAGAGTAATGCTCC-3', reverse 5'-GTCCACGTATCC-ATCAAC-3'; *SMAD4C* forward 5'-GATGTTCCAGGTAG-GAGAG-3', reverse 5'-CCAACGGTAAAAGACCTCAG-3') and *GAPDH* genes as internal control (*GAPDH* forward 5'-ACCACAGTCCATGCCATCAC-3', reverse *GAPDH* 5'-TCCACCACCCTGTTGCTGTA-3') were designed to cross intron–exon junctions. The amplified cDNAs were purified with a gel cleanup kit, and subjected to sequencing reactions (MWG Biotech AG, Martinsried, Germany). All the obtained sequences corresponded to the expected ones (data not shown).

Real-time reverse transcription (RT)-PCR was performed three times in six replicates on a 7500 RT-PCR System (Applied Biosystems) using the SYBR Green detection system. mRNA levels were compared with reference curves and normalized to *GAPDH* mRNA. Each experiment was repeated three times using different total RNA extracts.

### Immunofluorescence experiments

Immunofluorescences were carried out as described previously (Nicolussi *et al.* 2006). Cells were incubated with or without 10 ng/ml of TGFβ1 in serum-free medium for 1 h and then fixed with 4% paraformaldehyde/PBS for 30 min, followed by treatment with 0.1 M glycine/PBS for 20 min and permeabilized with 0.1% Triton X-100 for additional 5 min. For detection of SMAD2/SMAD3 and SMAD2-P, cells were incubated with anti-SMAD2/3 antibody (1:50) and SMAD2-P antibody (1:50) for 1 h respectively, followed by Texas Red-conjugated goat anti-mouse IgG (1:50). For detection of SMAD4, cells were incubated with anti-SMAD4 antibody (1:100), followed by FITC-conjugated goat anti-mouse IgG (1:10). Nuclei were stained with 10 μg/ml 4',6'-diamidino-2-phenylindole (Sigma–Aldrich, Co.).

Immunofluorescences in cells transiently transfected with pEGFPC3-SMAD4 wt or pEGFPC3 empty vector were carried out as described above. The anti-GFP antibody (1:100) was used to detect GFP-tagged SMAD4 protein. Fluorescence was observed with an Olympus BX51 microscope and photographed at 40× optical magnification. Each experiment was repeated three times.

### Wound-healing assay

TPC-1, BCPAP, and FRTL-5 cells, native or transiently transfected, were plated in 35 mm dish and cultured to confluence of 90%. Cell layers were then scratched with a pipette tip, and cellular debris has been removed washing gently. Some plates were fixed and photographed immediately after scratching (T0 control). The cells that were allowed to incubate with serum-free medium for 24 h were fixed with 4% paraformaldehyde for 30 min, and dishes were plated under a phase contrast microscope Axio Observer (Carl Zeiss, Inc., Oberkochen, Germany) and photographed to measure the gap distance using the computing software Axio Vision (Carl Zeiss, Inc.). Each result is the mean of three independent experiments.

### Tissue samples and immunohistochemistry analysis

A group of 23 PTCs, collected at the Anatomic Pathology of Department of Experimental Medicine of Sapienza University of Rome from 2008 to 2010, were used. Thyroid tissue sections were studied using the catalyzed signal amplification protocol (DAKO A/S, Glostrup, Denmark; [Lazzereschi et al. 1997](#)). Slides were incubated in a humidified chamber overnight at 41 °C with 1:100 dilution of polyclonal serum to SMAD4 (H552). The peroxidase-based LSAB2 Detection kit (DAKO A/S), followed by hematoxylin counterstaining, was used to visualize the reactions. Written informed consent was obtained from each patient according to Helsinki Declaration and approved by the local ethics committee.

### Statistical analysis

All statistical analyses have been performed using JMP Software purchased by Statistical Discovery SAS Institute (Cary, NJ, USA). Data were analyzed by the following tests: Student's *t*-test; Fisher test across groups (*P* value referred to *F* value); non-parametric one-way ANOVA Wilcoxon/Kruskal–Wallis test (*P* value referred to  $\chi^2$ -value). *P* < 0.05, statistical significance; *P* < 0.001, high statistical significance.

## Results

### TGFβ signaling in TPC-1 and BCPAP

Expression of members of TGFβ pathway has been studied in TPC-1 and BCPAP cells. These cells represent a model of human papillary thyroid cell lines, whose thyroid origin has been confirmed by RT-PCR for the presence of *RET/PTC* rearrangement and *PAX8* expression in TPC-1 and *PAX8* and *TTF1* expression in BCPAP (data not shown).

The control cells used in this study were a human follicular cell line, FTC133, and a normal rat thyroid cell line, FRTL-5. FTC133 retains the differentiated thyrocyte functions like responsiveness to TSH for the presence of TSH receptor, *TTF1*, and *PAX8* confirmed by RT-PCR; FRTL-5 was widely used as a normal control ([Pang et al. 1989](#), [Shimura et al. 2001](#), [Pomérance et al. 2006](#), [Visconti et al. 2007](#), [Kogai et al. 2008](#)).

Western blot analyses performed on total cell lysates, using polyclonal antibodies anti-TβRII and anti-TβRI, demonstrated that both receptors were present in all examined cell lines ([Fig. 1A](#)), and they were not modified by TGFβ1 treatment (data not shown). Since the expression of TβRI was lower in TPC-1 and in BCPAP with respect to the control cells, we assayed the level of SMAD7 and SMURF1 to assess whether there was an alteration in the mechanism of TβRI degradation. The expression of these proteins was comparable in all cell lines ([Fig. 1A](#)). Consistent with the finding that the expression of SMAD7 is induced by TGFβ, the level of this protein was increased by TGFβ1 treatment, demonstrating that this branch of signaling was functioning in PTC cell lines (data not shown). As an indication of the ability of the lower level of TβRI to phosphorylate SMAD2 and SMAD3, we performed western blot analyses with or without 10 ng/ml of TGFβ1 treatment for 30 min. Our data demonstrated that SMAD2 and SMAD3 were phosphorylated both in TPC-1 and in BCPAP, as in FTC133 and FRTL-5 control cells ([Fig. 1B and C](#)).

The transcriptional activity of the TGFβ cascade was evaluated using a TGFβ-responsive reporter construct, p3TP-Lux. The p3TP promoter contains three consecutive 12-O-tetradecanoyl phorbol 13-acetate (TPA) response elements, which are involved in the transcriptional responses of several genes to TGFβ, and a portion of the plasminogen activator inhibitor-1 promoter region ([de Groot & Kruijer 1990](#), [Keeton et al. 1991](#), [Wrana et al. 1992](#)). The results showed ([Fig. 1D](#)) that the increase of transcriptional activity, after 24 h of TGFβ1 treatment, was not significant both in TPC-1 (3.5-fold) and in BCPAP (3.1-fold), while it was significant both in FTC133 (10.3-fold, *P* < 0.001) and in FRTL-5 (25-fold, *P* < 0.001).



Altogether, our data demonstrated an important impairment of TGF $\beta$  signaling in papillary thyroid cell lines.

### SMAD4 expression in TPC-1 and BCPAP

Based on the observation of frequent inactivation of SMAD4 in several types of tumors, we analyzed SMAD4 expression in TPC-1, BCPAP, and in a set of different human cell lines: MDA MB468, FRO, and human-immortalized B lymphocytes. FTC133 and FRTL-5 were used as a positive control, whereas MDA MB468, in which *SMAD4* was deleted in homozygosis, was used as a negative control. Western blot in Fig. 2A shows that SMAD4 expression was strongly reduced in all PTC cells with respect to FTC133 and FRTL-5. Densitometric analysis of bands confirmed a significant ( $P < 0.05$ ) reduction with respect to FTC133 both in TPC-1 (66%) and in BCPAP (72%), and with respect to FRTL-5 both in TPC-1 (69%) and in BCPAP (75%).

The expression of *SMAD4* gene was studied by RT-PCR. As shown in Fig. 2B, all the primers, used for RT-PCR, amplified fragments of the expected size both in PTC cells and in human controls (FTC133, FRO, human-immortalized B lymphocytes, and MCF7). Since mutational inactivation of *SMAD4* in different carcinomas has been reported, we sequenced the CDS region of *SMAD4* cDNA to look for nucleotide

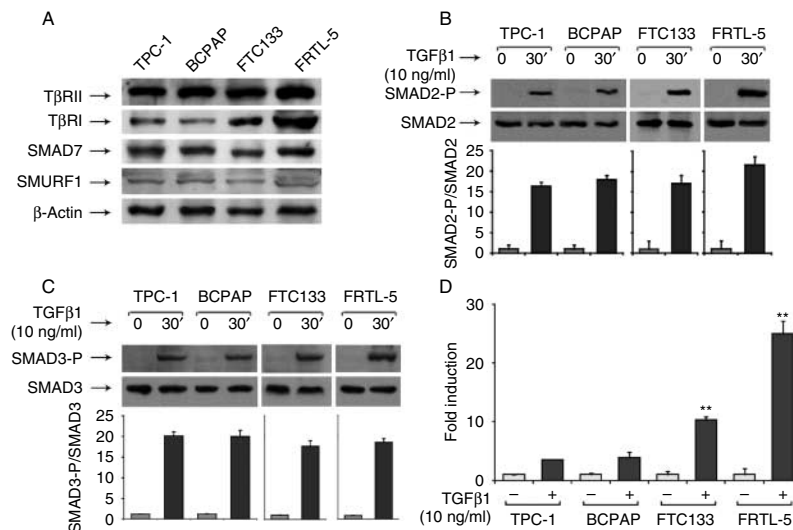
alterations. Nucleotide sequencing did not show any changes in all examined cell lines (data not shown).

*SMAD4* mRNA levels were evaluated by semiquantitative real-time PCR on cDNA of TPC-1 and BCPAP and a set of positive controls (FTC133, HepG2, and HepG2 transfected with pEGFPC3-SMAD4 wt) and a negative control (MDA MB468). The results obtained demonstrated that all cell lines showed a comparable level of *SMAD4* transcript (Fig. 2C). Therefore, these data suggested that the cause of hypoexpression of SMAD4 protein in these cells could be an alteration in one of the post-transcriptional mechanisms.

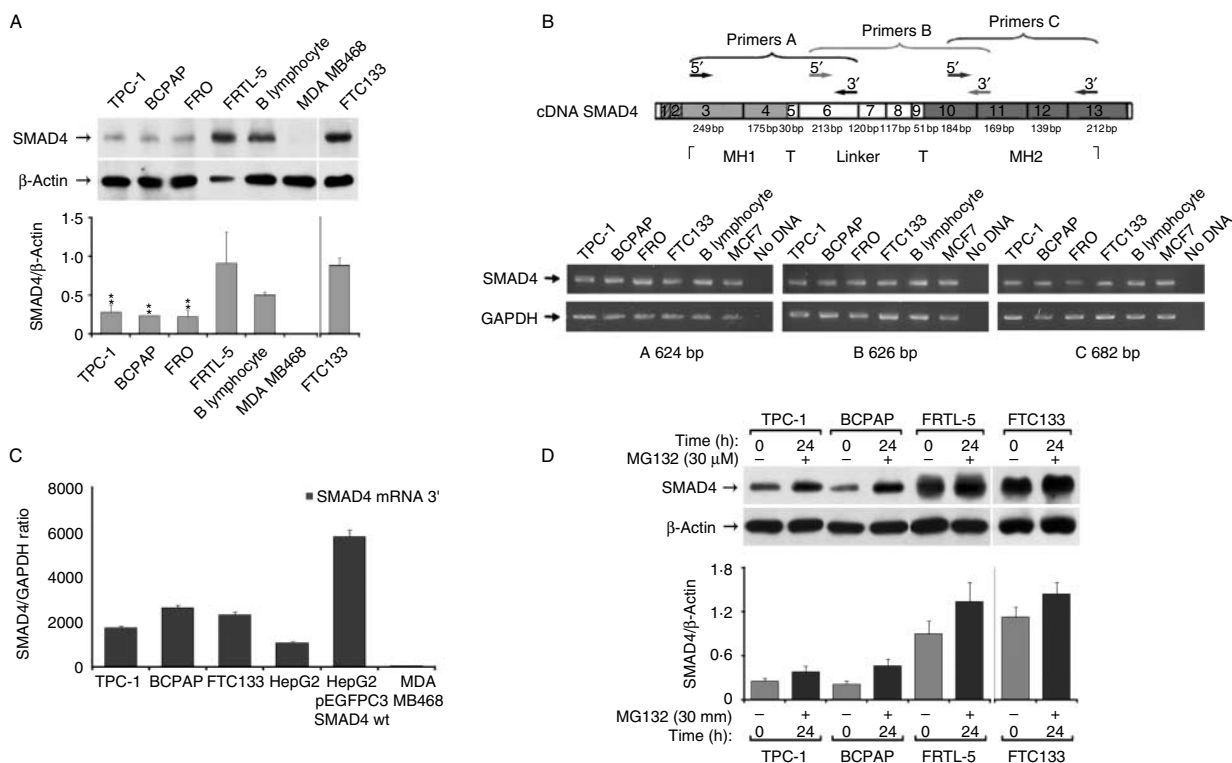
To analyze the implications of proteasome in SMAD4 degradation, we treated cell lines for 24 h with MG132, an inhibitor of proteasome activity. As showed in Fig. 2D, MG132 treatment determined an increase of SMAD4 level in both cell lines, likewise in FRTL-5 and FTC133 (about 1.5-fold). These results indicated that the ability of proteasome to degrade SMAD4 in these cells was preserved, but not enhanced.

### Shuttling of SMAD proteins

The molecular mechanism that controls subcellular localization and activation of SMAD proteins is crucial for transduction of TGF $\beta$  signal from transmembrane receptors into the nucleus, and it is not yet fully understood. The immunofluorescence experiments



**Figure 1** TGF $\beta$  signaling in TPC-1 and BCPAP. (A) Whole cell extracts (40  $\mu$ g/lane) were analyzed by western blot, using antibodies against T $\beta$ RII, T $\beta$ RI, SMAD7, SMURF1, and  $\beta$ -actin as equal loading control. (B and C) Phosphorylation of SMAD2 and SMAD3 after treatment with 10 ng/ml of TGF $\beta$ 1 for 30 min. Histograms of densitometric analysis of the SMAD2-P and SMAD3-P bands normalized to the respective unphosphorylated forms. (D) Cells were transiently transfected with 3TP-Lux reporter and grown for 24 h in the presence or the absence of 10 ng/ml of TGF $\beta$ 1. Luciferase activity was expressed in fold induction  $\pm$  s.d. Each result is the mean of four independent experiments (\*\* indicates a high statistical significance (Student's *t*-test,  $P < 0.001$ ) correlate to untreated cells).



**Figure 2** Expression of SMAD4. (A) Whole protein lysates (80 μg/lane) from TPC-1, BCPAP, FTC133, FRTL-5, and a set of different human cell lines MDA MB468, FRO, and human-immortalized B lymphocytes were analyzed by western blot using an antibody against SMAD4. Densitometric evaluation of the SMAD4 signals was performed normalizing to the levels of β-actin (\* indicates a statistical significance (Student's *t*-test,  $P < 0.05$ ) correlate to FTC133 and FRTL-5). (B) The upper part of panel shows a schematic representation of specific primers for SMAD4 used for the amplification. *GAPDH* was used as a housekeeping gene. (C) Semiquantitative real-time PCR analysis of *SMAD4* mRNA levels. Data reported represent the mean of three independent experiments. FTC133, HepG2, and HepG2 transiently transfected with pEGFPC3-SMAD4 wt construct and MDA MB468 samples were used as controls in the experiments. (D) Total protein lysates (80 μg/lane) from TPC-1, BCPAP, FTC133, and FRTL-5, treated or untreated with MG132 (30 μM) for 24 h, were analyzed by western blot using an anti-SMAD4 antibody. Histogram of densitometric analysis of SMAD4 levels normalized to the levels of β-actin.

demonstrated that in unstimulated cells, SMAD2 was predominantly cytoplasmic, whereas after 1 h of TGFβ1 treatment, it was localized throughout the cytoplasm and the nucleus, as a result of its shuttling between these two compartments (Fig. 3A). The localization of SMAD2-P, clearly cytoplasmic in basal condition, became nuclear after TGFβ1 treatment in all cell lines (Fig. 3A).

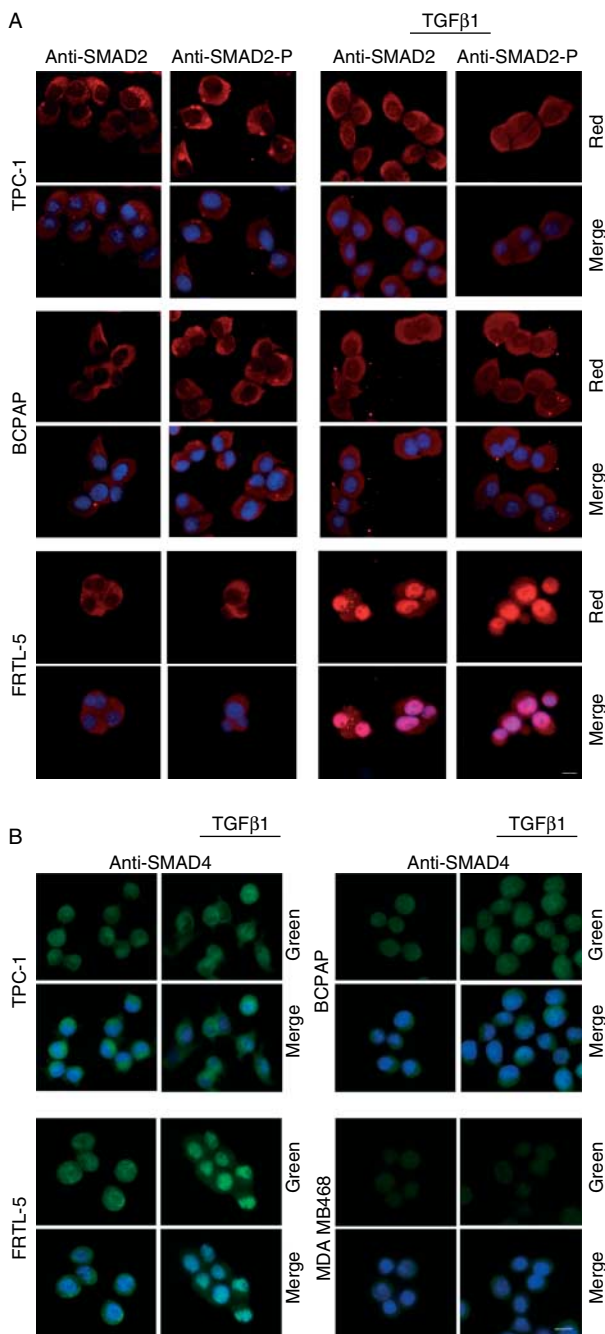
The nuclear translocation of SMAD4 was investigated in the same cells and in the negative control represented by MDA MB468. SMAD4 not only accumulates in the nucleus in association with activated R-SMADs, but also undergoes continuous nucleocytoplasmic shuttling on its own, independently of TGFβ signaling (Pierreux *et al.* 2000, Watanabe *et al.* 2000). In accordance with these findings, the data presented in Fig. 3B demonstrated the presence of continuous nucleocytoplasmic shuttling both in basal condition and after TGFβ1 treatment. Therefore, the SMAD4 ability to translocate to the nucleus was preserved, and

the weak cellular fluorescence signal observed in TPC-1 and in BCPAP was due to the hypoexpression of this protein.

Western blot performed on the cytoplasmic and nuclear extracts of TPC-1, BCPAP, and FRTL-5 demonstrated a clear nuclear translocation of SMAD4 and SMAD2-P, after 30 and 60 min of TGFβ1 treatment in all cell lines. The presence of SMAD2 and SMAD4 in the cytoplasmic fractions in all cell lines, after TGFβ1 treatment, confirmed the continuous nucleocytoplasmic shuttling of these proteins (Fig. 4).

#### SMAD4 overexpression in TPC-1 and BCPAP

In order to study the effects of the SMAD4 overexpression, the cells have been transiently transfected with a construct containing human *SMAD4* wt, tagged with GFP (pEGFPC3-SMAD4), or with a construct containing human *SMAD2* wt tagged with FLAG (pCMV5 FLAG-SMAD2) or cotransfected with both



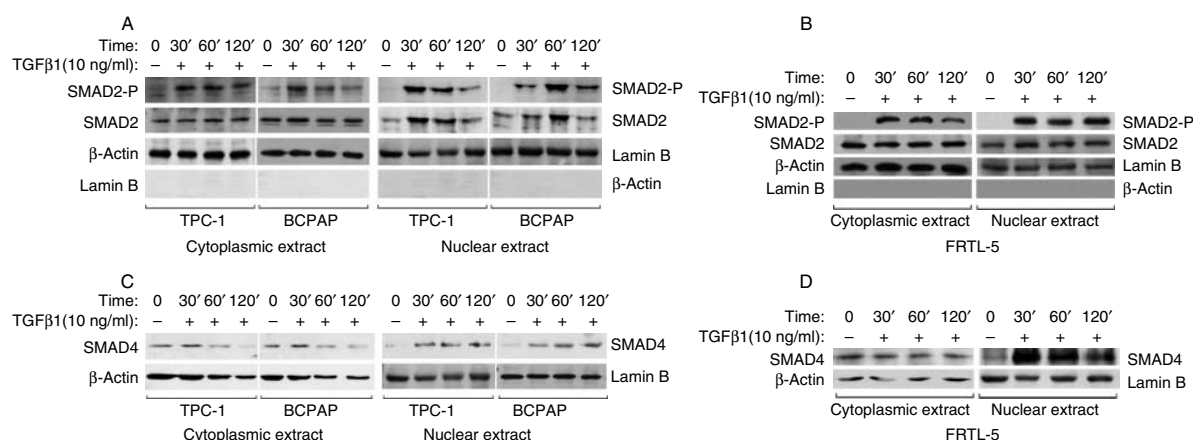
**Figure 3** Subcellular localization of SMAD2 and SMAD4. (A and B) TPC-1, BCPAP, and FRTL-5, treated or untreated with 10 ng/ml of TGFβ1 for 1 h, were fixed and immunolabeled for SMAD2, SMAD2-P, and SMAD4. Nuclei were counterstained with DAPI. Images are representative of three separate experiments. Scale bar, 20 μm.

plasmids. The transcriptional activity of *SMAD4* was studied using the p3TP-Lux reporter construct in the presence or in the absence of 10 ng/ml of TGFβ1 for 24 h. The results, evaluated by Student's *t*-test,

demonstrated a significant ( $P < 0.05$ ) increase of 3TP-Lux activity in TPC-1 transfected with *SMAD4* or *SMAD4*/*SMAD2*-cotransfected cells versus cells transfected with reporter construct. In BCPAP, the increase was highly significant ( $P < 0.001$ ) in *SMAD4*-transfected cells versus cells transfected with reporter construct, and the increase was significant ( $P < 0.05$ ) in *SMAD4*/*SMAD2*-cotransfected cells versus cells transfected with reporter construct (Fig. 5A).

The response to TGFβ1 treatment between each group and across groups was evaluated by matched pair test. The Student's *t*-test between groups demonstrated that the transcriptional activity was significantly increased ( $P < 0.001$ ) in all conditions examined (Fig. 5A). Fisher test across groups (*P* value referred to *F* value) demonstrated that the luciferase induction after TGFβ1 treatment was statistically significant in all of the following conditions: TPC-1 cells transfected with *SMAD4* versus cells transfected with reporter construct ( $P = 0.0204$ ) or versus cells transfected with *SMAD2* ( $P = 0.0034$ ) and *SMAD4*/*SMAD2*-cotransfected cells versus cells transfected with reporter construct ( $P = 0.0144$ ); BCPAP cells transfected with *SMAD4* versus cells transfected with reporter construct ( $P = 0.0010$ ) or versus cells transfected with *SMAD2* ( $P = 0.0015$ ). The results obtained in *SMAD4*/*SMAD2*-cotransfected cells versus cells transfected with reporter demonstrated an increase of luciferase activity ( $P = 0.0516$ ; Fig. 5A). The Fisher test across groups in a matched pair test evaluates the difference in the response to TGFβ1, but does not take into account the baseline values induced by the overexpression of *SMAD4*. To determine whether the fold induction in 3TP-Lux reporter activity for TPC-1 and BCPAP was significantly increased by *SMAD4* overexpression, we examined the data using the non-parametric one-way ANOVA Wilcoxon/Kruskal-Wallis test (*P* value referred to  $\chi^2$ -value). The results obtained demonstrated that the mean of fold induction of transcriptional activity, after TGFβ1 treatment in non-transfected TPC-1, was 3.6 vs 4.2 ( $P < 0.0076$ ) in *SMAD4*-transfected cells, and the mean of fold induction in non-transfected BCPAP was 3.1 vs 4.1 ( $P < 0.0466$ ) in *SMAD4*-transfected cells (Fig. 5A). These results showed that in transfected cells, the fold induction of luciferase activity was significantly increased after TGFβ1 treatment: the level of SMAD4 in the cells was causing this response.

To verify whether the reduction of SMAD4 shuttling was caused by hypoexpression of this protein, we performed immunofluorescences in transfected cells, using an antibody specific for GFP. The results shown in Fig. 5B demonstrated that the overexpression of SMAD4 determined a very strong nuclear translocation of SMAD4 after TGFβ1 treatment (upper panel), with respect to the control cells transfected with empty vector (lower panel). Western blot confirmed the



**Figure 4** Subcellular fractionation of SMAD2, SMAD2-P, and SMAD4. (A–D) Cells, treated with 10 ng/ml of TGFβ1 for indicated time periods, were separated into cytosol and nuclear fractions before analysis by immunoblotting with the SMAD2, SMAD2-P (A and B), and SMAD4 (C and D) antibodies. Western blots, performed using antibodies against the β-actin, cytoplasmic protein, and against Lamin B, nuclear protein, indicated that the extracts are virtually free from cross-contamination.

overexpression of GFP-tagged SMAD4 protein in transiently transfected cells (Fig. 5C).

Taken together, these data indicated that the attenuated signaling pathway observed in TPC-1 and BCPAP could result from the low levels of SMAD4, and the hyperexpression of this protein was responsible for a significant enhancement of TGFβ responsiveness.

### TGFβ growth inhibition in TPC-1 and BCPAP

The responsiveness to TGFβ growth inhibition has been evaluated in PTC cells by proliferation assay. TPC-1, BCPAP, and FRTL-5 control cells were grown with or without 10 ng/ml of TGFβ1, and counted at time 0 and at 48 h. The results, expressed as a percentage of growth inhibition with respect to relative untreated control, showed that TPC-1 was significantly inhibited by 32% ( $P < 0.05$ ), BCPAP was inhibited only by 8%, and FRTL-5 was highly significantly inhibited by 72% ( $P < 0.001$ ; Fig. 6A). Therefore, BCPAP was resistant to the TGFβ-mediated growth inhibition, while TPC-1 showed a significant inhibition of proliferation, although lower than FRTL-5.

To investigate the role of SMAD4 in the control of cell growth, we performed proliferation assay on cells overexpressing SMAD4. TPC-1 and BCPAP, transiently transfected with GFP-tagged SMAD4 wt or with the empty vector, were counted at time 0, at 24 h, and at 48 h in the presence or in the absence of 10 ng/ml of TGFβ1. In basal conditions, SMAD4 overexpression did not cause a significant difference in growth rate when comparing SMAD4-transfected cells to empty vector-transfected cells (Fig. 6B and C). TGFβ1 treatment, instead, determined a considerable reduction of proliferation.

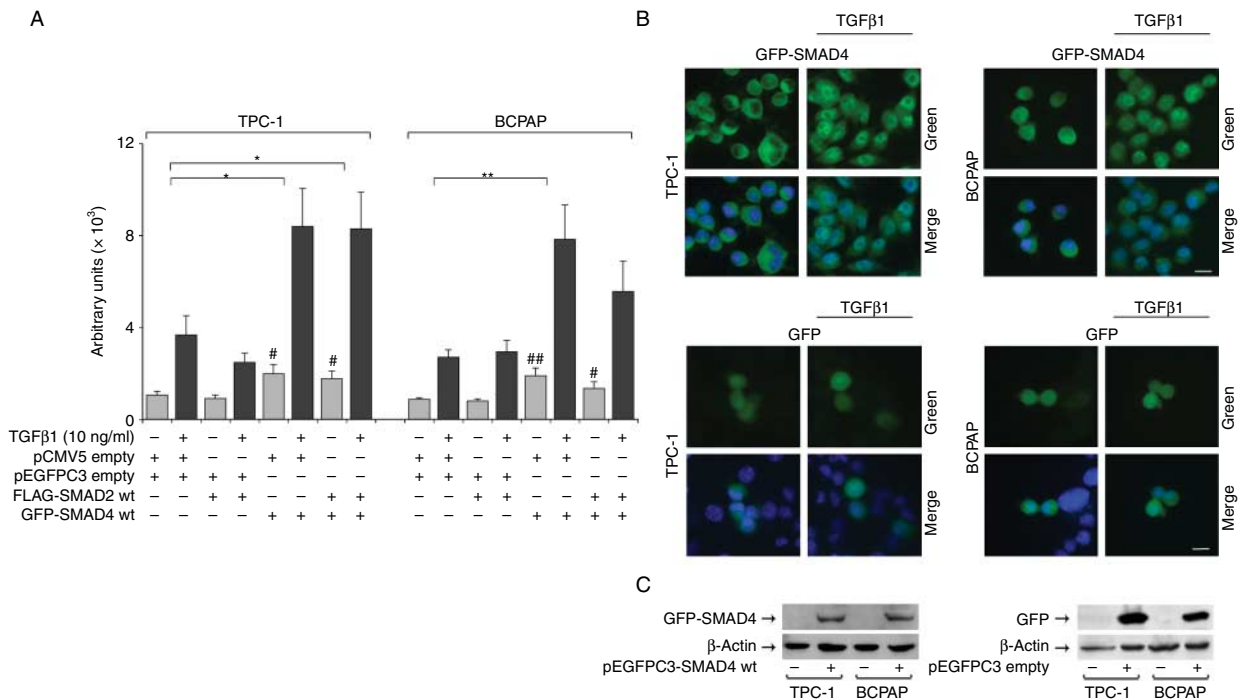
In TPC-1, the inhibition of proliferation was significant ( $P < 0.05$ ) at 24 h (59%) and highly significant ( $P < 0.001$ ) at 48 h (71%), while in the cells transfected with empty vector, the inhibition was 19% at 24 h and 35% at 48 h ( $P < 0.05$ ), as in untransfected cells (Fig. 6D). The results obtained in BCPAP overexpressing SMAD4 demonstrated a significant ( $P < 0.05$ ) recovery of TGFβ-mediated inhibition of proliferation only at 48 h (23%), when compared to the empty vector-transfected cells (6%; Fig. 6E). Student's *t*-test, which was performed comparing the percentage of growth inhibition of SMAD4-transfected PTC cell lines versus empty vector-transfected cells, demonstrated a significant increase ( $P < 0.05$ ) at 24 h, 59 vs 19%, and a highly significant increase ( $P < 0.001$ ) at 48 h, 71 vs 35%, in TPC-1, and it also demonstrated a significant increase ( $P < 0.05$ ) only at 48 h, 23 vs 6%, in BCPAP. The trypan blue test performed in all proliferation assays (data not shown) demonstrated that the growth inhibition observed in these cells was not due to reduction of cellular viability.

The above results provided evidence that the overexpression of SMAD4 caused a strong increase of the antiproliferative effect of TGFβ in human PTC cell lines.

### SMAD4 overexpression in the control of migratory behavior of TPC-1 and BCPAP

Migration of tumor cells has an important role in specific processes for metastases formation (Chiang & Massagué 2008). The ability of TPC-1 and BCPAP to migrate was studied through the wound-healing assay. The experiments were performed in basal condition and after transient transfection either with pEGFPC3-SMAD4 wt or with pEGFPC3 empty vector.



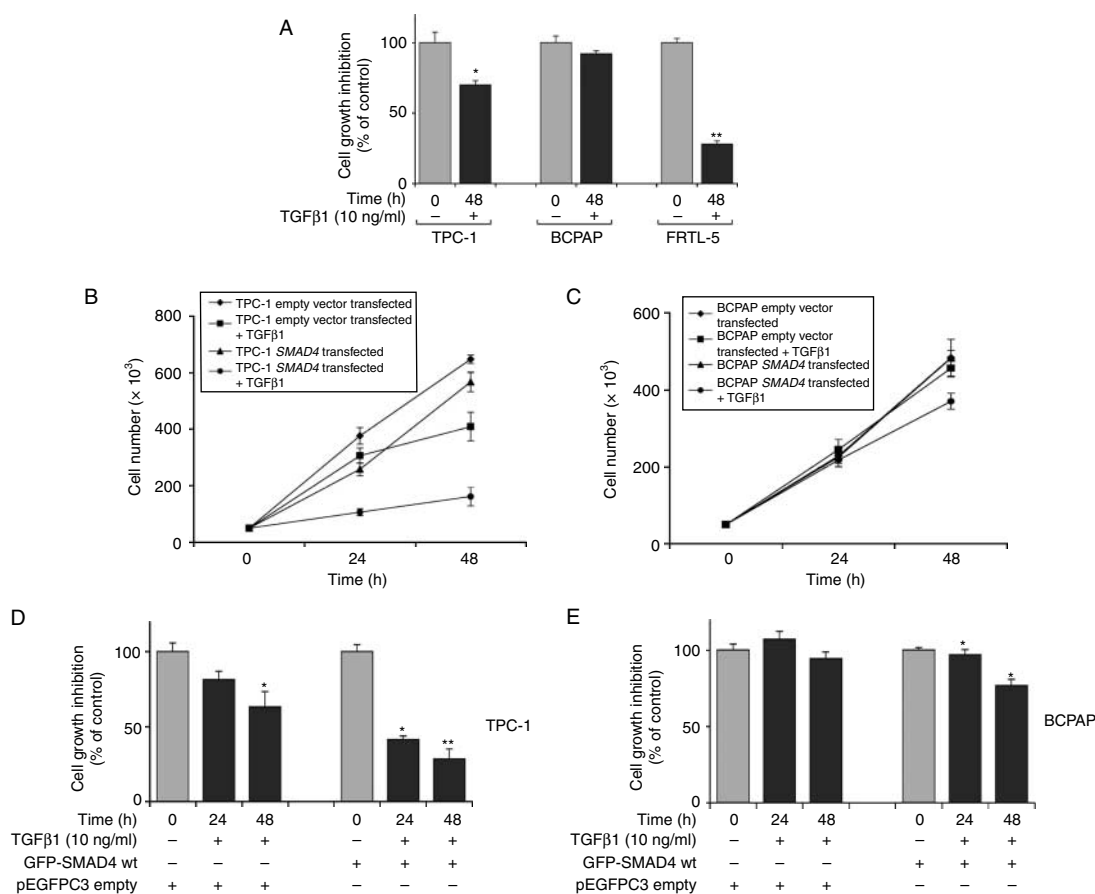


**Figure 5** TGFβ signaling in TPC-1 and BCPAP overexpressing *SMAD4*. (A) TPC-1 and BCPAP overexpressing *SMAD4* wt and/or *SMAD2* wt were transiently transfected with 3TP-Lux reporter. Cells were stimulated with 10 ng/ml of TGFβ1 for 24 h, and the luciferase activity was expressed as arbitrary units. # and ## indicate a statistical significance ( $P < 0.05$  and  $P < 0.001$  respectively,  $P$  value referred to Student's  $t$ -test) of TPC-1 and BCPAP overexpressing *SMAD4* versus cells transfected with reporter construct. \* and \*\* indicate a statistical significance ( $P < 0.05$  and  $P < 0.001$  respectively,  $P$  value referred to  $F$  value, Fisher test across groups) of cells transfected with *SMAD4* versus cells transfected with reporter construct and *SMAD4*/*SMAD2*-cotransfected cells versus cells transfected with reporter construct. Each result is the mean of four independent experiments in triplicate  $\pm$  S.E.M. (B) Immunofluorescence assays of GFP-tagged *SMAD4* protein in TPC-1 and BCPAP overexpressing GFP-tagged *SMAD4* wt and cells transfected with empty vector, treated or untreated with 10 ng/ml of TGFβ1 for 1 h. Scale bar, 20 μm. (C) Representative western blots performed using specific antibody anti-GFP in total extracts from transiently transfected cells.

The cells were grown to confluence in monolayers, and their ability to migrate into and across a denuded area of the monolayer has been evaluated after 24 h. As shown in Fig. 7A, TPC-1 and BCPAP had a highly significant ( $P < 0.001$ ) migratory attitude, compared to the FRTL-5, which migrated quite slowly. The overexpression of *SMAD4* strongly reduced the migratory behavior of these cells (Fig. 7B). The analysis of the data demonstrated that over a 24 h time course, TPC-1 overexpressing *SMAD4* consistently migrated at a slower rate (21%;  $P < 0.001$ ) than empty vector control cells (50%). BCPAP overexpressing *SMAD4* showed a similar behavior, migrating at a slower rate (14%;  $P < 0.001$ ) than empty vector control cells (38%). Stimulation with TGFβ1 did not cause any significant modification in motility in transfected cells, since *SMAD4* overexpression alone was responsible for maximal activation of SMAD signaling (Fig. 7B). The inhibition of migration at 24 h was independent of the proliferative response because the overexpression of *SMAD4* did not influence the growth rate in the same gap (Fig. 6B and C).

It has been demonstrated that E-cadherin and N-cadherin have an important role in progression-promoting pathway in invasive and migratory behavior in many human carcinomas (Christofori 2006, Shiou *et al.* 2007). The expression levels of E-cadherin and N-cadherin have been evaluated performing western blot analyses on total lysates of TPC-1, BCPAP, FTC133, and FRTL-5. MCF7 has been used as a positive control of E-cadherin and a negative control of N-cadherin; HEK293, instead, has been used as a positive control of N-cadherin and a negative control of E-cadherin (Hogan *et al.* 2004). The results obtained demonstrated that E-cadherin expression was lower in PTC cell lines with respect to FTC133, and N-cadherin expression was comparable in all thyroid carcinoma cell lines (Fig. 7C). TPC-1 and BCPAP overexpressing *SMAD4* showed a highly significant ( $P < 0.001$ ) increase of the E-cadherin level (Fig. 7D).

In conclusion, our results strongly supported that, in thyroid papillary carcinomas, the overexpression of *SMAD4* was responsible both for the increase of antiproliferative effect of TGFβ and for the reduction of cellular motility *in vitro*.



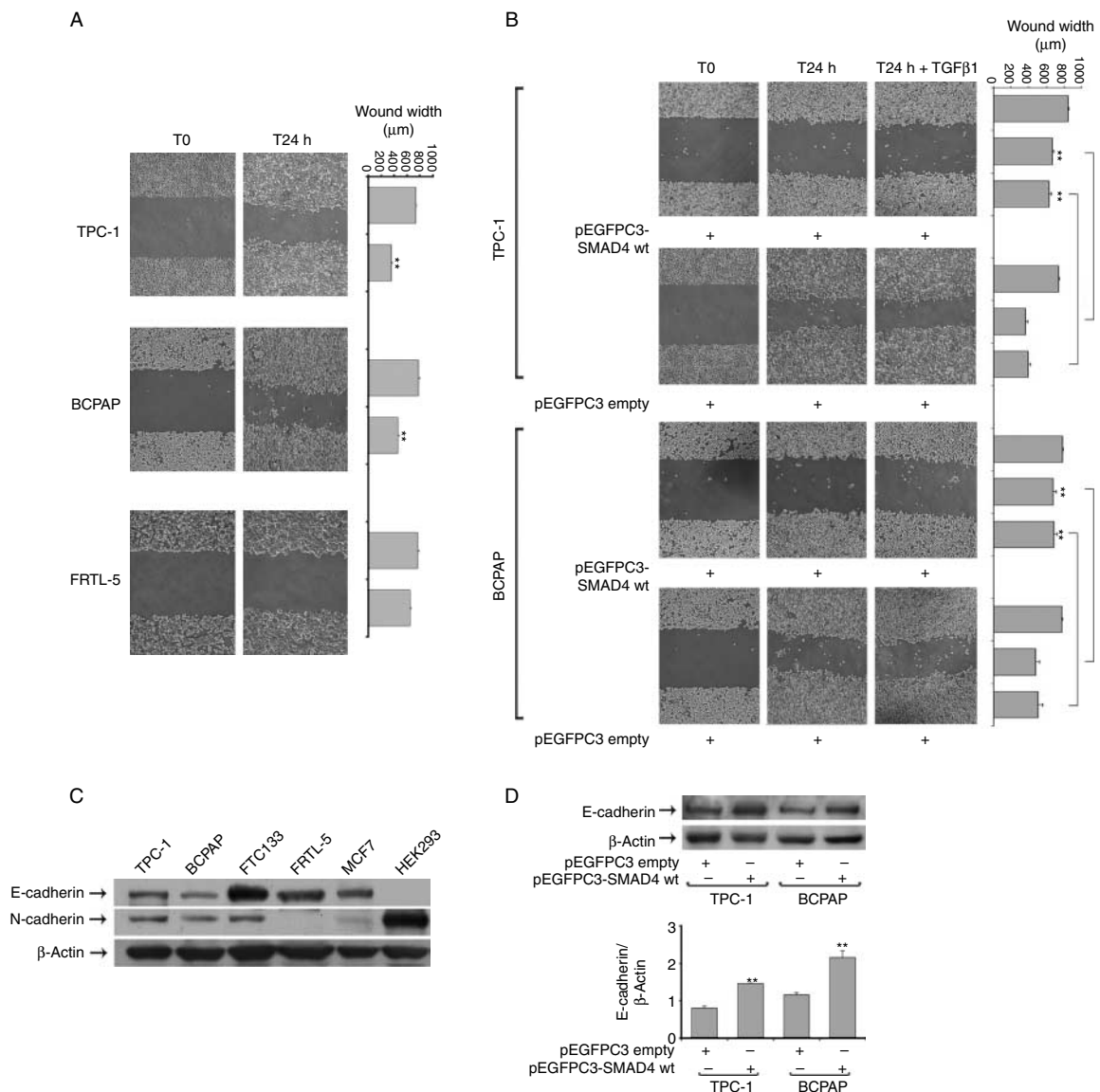
**Figure 6** Effect of TGFβ1 on cell growth in thyroid cell lines. (A) Cells were grown in the presence or the absence of 10 ng/ml of TGFβ1 for 48 h and were counted three times by two independent investigators. Inter-observer variation was below 5%. Values represent the mean of triplicate determination  $\pm$  s.d. of three experiments, and the results were expressed as a percentage of growth inhibition (\* and \*\* indicate a statistical significance (Student's *t*-test),  $P < 0.05$  and  $P < 0.001$  respectively) of treated versus untreated cells. (B and C) Cells transiently transfected with GFP-tagged SMAD4 wt or with the empty vector, grown in the presence or the absence of 10 ng/ml of TGFβ1 for 24 and 48 h, were counted three times by two independent investigators. Inter-observer variation was below 5%. Values represent the mean of triplicate determination  $\pm$  s.d. of three experiments. (D and E) Histograms of the results expressed as a percentage of growth inhibition (\* and \*\* indicate a statistical significance (Student's *t*-test),  $P < 0.05$  and  $P < 0.001$  respectively).

### SMAD4 expression in human PTCs

To investigate whether the findings observed in cell lines were relevant to human thyroid carcinogenesis, a group of 23 human PTCs were examined for SMAD4 expression by immunohistochemistry and compared to normal tissues from the opposite lobe. Our results showed that 7 out of 23 (30%) PTC tumor samples, including 1 case of follicular variant of PTCs (Fig. 8), presented a weak and focal intensity of SMAD4 staining with SMAD4-negative areas compared to normal tissue, where a diffuse staining of SMAD4 was observable. In remaining samples, SMAD4 expression appeared to be retained and comparable to the normal tissues. This result was consistent with the data obtained in PTC cell lines, outlining the important role of SMAD4 in thyroid carcinogenesis.

### Discussion

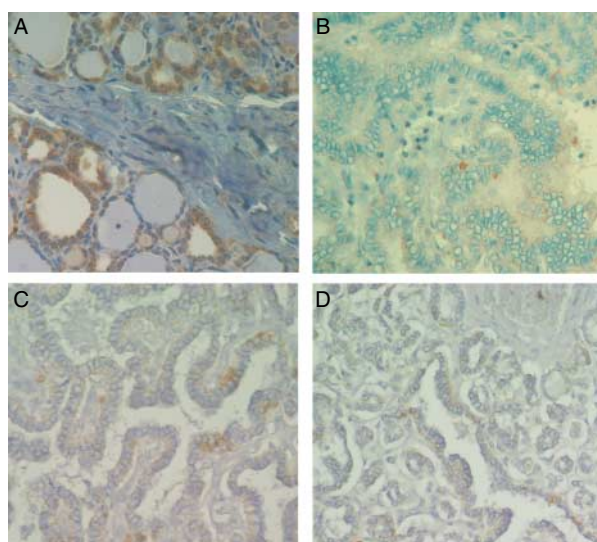
It is known that a lack of response to TGFβ inhibitory action in thyroid cells may lead to a loss of controlled growth and is responsible for some cases of multinodular thyroid goiter (Grubeck-Loebenstien *et al.* 1989, Bidey *et al.* 1999). In the last year, the role of TGFβ in the thyroid carcinogenesis has received a significant attention. Experimental evidences demonstrate that thyroid cancer cells can escape to tumor-suppressive action of TGFβ for the quantitative reduction of receptors and transduction elements. In a model of KRAS-transformed rat thyroid cells, the overexpression of TβRII reverts their malignant phenotype and strongly reduces the number of metastases, when transplanted in athymic nude mice



**Figure 7** Migratory behavior of TPC-1 and BCPAP. (A) Wound-healing assay was performed in confluent monolayers for 24 h, and the extension of the area colonized by the cells was estimated using Axio Vision, Zeiss software (\*\* indicates a statistical significance (Student's *t*-test,  $P < 0.001$ ) compared to FRTL-5). (B) Wound-healing assay in TPC-1 and BCPAP overexpressing *SMAD4* wt, untreated or treated with 10 ng/ml of TGFβ1 for 24 h (\*\* indicates a high statistical significance (Student's *t*-test,  $P < 0.001$ ) compared to empty vector control cells). Images are representative of three separate experiments. (C) Whole protein lysates (40 μg/lane) from TPC-1, BCPAP, FTC133, and FRTL-5 control cells, MCF7, and HEK293, were analyzed by western blot using antibodies against E-cadherin and N-cadherin. (D) Western blot of whole protein lysates (40 μg/lane) from TPC-1 and BCPAP transiently transfected with pEGFPC3-SMAD4 wt or pEGFPC3 empty vector performed using an antibody against E-cadherin. Bottom, densitometric evaluation of the E-cadherin bands normalized to the levels of β-actin (\*\* indicates a high statistical significance (Student's *t*-test),  $P < 0.001$ ).

(Turco *et al.* 1999). A deregulated expression with a reduced TBR11 RNA level has been identified in a large number of thyroid carcinomas and correlates with a more undifferentiated histotype (Lazzereschi *et al.* 1997). Moreover, mutations and/or imbalances in expression due to alternative RNA splicing and altered localization of the SMAD4 protein have been

demonstrated in a large group of thyroid cancer with different histotypes (Lazzereschi *et al.* 2005). It is known that TGFβ's role in human cancer appears both complex and context dependent. Depending on the tumor type and the stage of tumor progression, it can exhibit strong tumor-suppressive or tumor-promoting functions. More recently, it has been demonstrated that



**Figure 8** SMAD4 expression in human papillary carcinomas. Immunohistochemistry, using antibody against SMAD4, was performed in thyroid samples from normal tissue (A), primary PTCs (B and C), and in one case of PTC follicular variant (D). Original magnification was 150 $\times$  (A–C) and 100 $\times$  (D).

also in thyroid cells, as well as in the skin tumors, or in metastatic colon cancer (Padua & Massagué 2009), TGF $\beta$  can act as a tumor-promoting factor. The expression of BRAF V600E in normal rat thyrocytes and in 50 cases of human PTCs determines a reduction of NIS expression and an increase of TGF $\beta$  secretion, suggesting an overactivation of TGF $\beta$  signaling, responsible for the pro-tumorigenic activity (Riesco-Eizaguirre *et al.* 2009).

The present study focuses on the role of TGF $\beta$  pathway as tumor suppressor in two human PTC cell lines, TPC-1 and BCPAP, and in a group of 23 cases of human papillary carcinomas. In PTC cells, which were widely used by different groups (Baldini *et al.* 2004, Motti *et al.* 2005, Meireles *et al.* 2007, Visone *et al.* 2007, Schweppe *et al.* 2008, Salerno *et al.* 2010), we have studied the TGF $\beta$  pathway, demonstrating that the hypoexpression of SMAD4 protein is responsible for alteration in TGF $\beta$  signal transduction, as supported by the strong reduction of transcriptional activity of 3TP-Lux. The p3TP-Lux reporter is most commonly used to measure TGF $\beta$  responsiveness (Wrana *et al.* 1992, Carcamo *et al.* 1994). SMAD4 has been shown to be required for this transcriptional activity, since SMAD4-deficient cell lines are not responsive but can be rescued with SMAD4 expression (Lagna *et al.* 1996, de Caestecker *et al.* 1997). The reduction of 3TP-Lux activity observed in PTC cell lines could be explained by the low threshold of activation of TGF $\beta$  signaling, depending on T $\beta$ RI or SMAD levels (Yingling *et al.* 1995). The T $\beta$ RI level in TPC-1 and in BCPAP is lower than the control cells, but the ability of the receptor to

phosphorylate SMAD2 and SMAD3 is fully maintained, as demonstrated in Fig. 1B and C. The stability of T $\beta$ RI represents an important regulatory mechanism for TGF $\beta$  signaling, both in cell culture studies and *in vivo* models. TGF $\beta$  receptors are ubiquitinated and degraded through the action of several cooperating protein complexes containing E3 ligases, as well as other important regulators of protein degradation. The I-SMADs seem to have an important role in regulating many of these complexes, orchestrating both ubiquitination and de-ubiquitination (Lönn *et al.* 2009). The levels of SMURF1 and SMAD7, overexpressed in the anaplastic thyroid carcinoma cell line (Cerutti *et al.* 2003), are also unaltered in our model. Therefore, the lower level of T $\beta$ RI observed in PTC cell lines cannot be attributed to SMAD7 and SMURF1 alterations. However, whether the receptors are degraded via the lysosomes, proteasomes or via both of these pathways is still an open question (Lönn *et al.* 2009). Thus, the hypoexpression of SMAD4 is responsible for the reduction of the threshold of induction of the signaling cascade, resulting in partial loss of TGF $\beta$ -transcriptional response. In fact, the overexpression of SMAD4 significantly increases the transcriptional stimulation of 3TP-Lux in the absence or in the presence of TGF $\beta$ 1 treatment. Altogether, these data demonstrate that, despite the lower level of T $\beta$ RI, the hypoexpression of SMAD4 is mainly responsible for the impairment of TGF $\beta$  signaling.

Since we previously demonstrated that SMAD4 mutations are frequent in PTCs (Lazzereschi *et al.* 2005), sequencing of the entire coding part of the *SMAD4* gene was performed. It did not find any mutations in the *SMAD4* gene; also mRNA levels are not modified; consequently, the alteration responsible for the hypoexpression of SMAD4 protein does not involve the transcriptional mechanism. Our data, in agreement with Heldin *et al.* (1999), led us to hypothesize that the cause of hypoexpression could be found in alteration of major components of translational machinery, which are frequently altered in human neoplasms (Rosenwald 2004). Moreover, recent observations demonstrate an important role of microRNA in the development of a wide range of human diseases, including cancer. Therefore, in our cells, we could hypothesize an alternative mechanism in the regulation of SMAD4 levels, involving an alteration in miRNA expression profiles (Erson & Petty 2008).

One of the important effects of SMAD4 hypoexpression in TPC-1 and BCPAP is the drastic reduction of nuclear accumulation of this protein. The molecular mechanism that controls subcellular localization and activation of SMAD proteins is crucial for TGF $\beta$  signaling, and it is not yet fully clarified. SMAD4 accumulates in the nucleus by association with activated R-SMADs (Liu *et al.* 1997, Hoodless *et al.* 1999,



Watanabe *et al.* 2000). However, SMAD4 also undergoes continuous nucleocytoplasmic shuttling on its own, independently of TGF $\beta$  signaling (Pierreux *et al.* 2000, Watanabe *et al.* 2000, Hill 2009). Our results demonstrate that the SMAD4 ability to translocate into the nucleus is preserved, and the weak cellular fluorescence signal observed in TPC-1 and in BCPAP was due to the hypoexpression of this protein. Work over the past few years has emphasized the importance of SMAD nucleocytoplasmic shuttling in TGF $\beta$  signaling regulation. Tumorigenic SMAD4 alterations, as demonstrated in pancreatic human cancer, can eliminate nuclear accumulation of SMAD4, suggesting that defects in nuclear import may contribute to tumorigenicity (Morén *et al.* 2000). The overexpression of SMAD4 in TPC-1 and in BCPAP clearly reinstates the translocation of SMAD4 from the cytoplasm to the nucleus. The levels and the duration of residence in the nucleus of SMAD4 are important events for the response to TGF $\beta$  in the cells. Consistent with the notion that TGF $\beta$  can act as a morphogen that induces distinct cell fates along a concentration gradient in embryonic development, many studies demonstrate that the intensity and duration of the TGF $\beta$ -SMAD response are important determinants for signaling specificity and, therefore, the activity of SMADs is carefully regulated (Gurdon & Bourillot 2001, Hill 2001, ten Dijke & Hill 2004). Enhanced proteasomal degradation of SMAD4 is responsible for TGF $\beta$  resistance in breast cancer (Dupont *et al.* 2005). In our cells, the ability of proteasome to degrade SMAD4 is maintained and comparable to that of control cells. Therefore, different mechanisms of protein degradation seem to be involved.

Lack of responsiveness to TGF $\beta$  antiproliferative effects is a hallmark for the cancer, where TGF $\beta$  and its pathway function as oncosuppressors. Our data show that BCPAP is resistant to growth inhibition of TGF $\beta$ , while TPC-1, in accordance with the data of Matsuo *et al.* (2006), is still sensitive to its antiproliferative response, although lower than control cells. Here, we demonstrate that the level of SMAD4 protein is low both in TPC-1 and in BCPAP, and it is responsible for the weakening of TGF $\beta$  responsiveness in these cell lines. The overexpression of SMAD4 significantly increases the TGF $\beta$  growth inhibition in TPC-1 and partially restores this response in BCPAP. We think that the behavior observed in BCPAP could be caused by the different genetic and molecular profile, recently well characterized in these cell lines (Meireles *et al.* 2007, Schweppe *et al.* 2008).

Although the majority of thyroid cancers have an excellent prognosis, there are a small percentage of cases that show an extensive local invasion and distant metastases, which frequently do not respond to standard treatments and have a worsened prognosis.

The genetic basis for thyroid cancer initiation and development is well characterized. It has been demonstrated that the activation of oncogenes like *RAS*, *BRAF*, *RET/PTC*, and *PI3K/AKT* plays an important role in thyroid tumorigenesis (Vasko *et al.* 2007), but the molecular mechanism involved in the thyroid cancer invasion needs clarification. The acquisition of a migratory phenotype is an essential property of invading and metastasizing cancer cells. TPC-1 and BCPAP cells have a strong and significant ability to migrate (Sancho *et al.* 2006, Scarpino *et al.* 2007). Genetic data indicate that loss of SMAD4 in cancer cells may correlate not only with the specific loss of TGF $\beta$  cytostatic response, but also with acquisition of an invasive phenotype (Takaku *et al.* 1998). In SW480 colon carcinoma cells, the re-expression of SMAD4 results in loss of tumorigenicity in nude mice, and is accompanied by the restoration of a more epithelioid morphology and induction of E-cadherin (Müller *et al.* 2002, Tian *et al.* 2009). Here, we demonstrate that the overexpression of SMAD4 in PTC cell lines induces a strong suppression of motility, as well as a significant increase of E-cadherin expression, indicating that the level of SMAD4 is a critical regulator of these processes.

Finally, our results obtained in the model *in vitro* are strongly supported by the preliminary finding that 30% of human PTCs, examined by immunohistochemistry, show a strong reduction of the SMAD4 staining.

All together, our data demonstrate that the level of SMAD4 protein plays an important role in thyroid carcinogenesis; although the mechanism by which tumor cells achieve this hypoexpression needs further investigation, we think it could be considered as a new possible therapeutic target for thyroid cancer.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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