Transforming growth factor- β 1 and activin A generate antiproliferative signaling in thyroid cancer cells

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Abstract

Transforming growth factor-beta 1 (TGF- β 1) and activin A (ActA) induce similar intracellular signaling mediated by the mothers against decapentaplegic homolog (SMAD) proteins. TGF- β 1 is a potent antimitogenic factor for thyroid follicular cells, while the role of ActA is not clear. In our study, the proliferation of TPC-1, the papillary thyroid carcinoma cell line, was reduced by both recombinant ActA and TGF- β 1. Due to the concomitant expression of TGF- β 1 and ActA in thyroid tumors, we investigated the effects of either TGF- β 1 or ActA gene silencing by RNA interference in TPC-1 cells in order to distinguish the specific participation of each in proliferation and intracellular signaling. An increased proliferation and reduced SMAD2,

SMAD3, and SMAD4 mRNA expression were observed in both TGF- β 1 and ActA knockdown cells. Recombinant TGF- β 1 and ActA increased the expression of inhibitory SMAD7, whereas they reduced *c-MYC*. Accordingly, we detected a reduction in SMAD7 expression in knockdown cells while, unexpectedly, *c-MYC* was reduced. Our data indicate that both TGF- β 1 and ActA generate SMADs signaling with each regulating the expression of their target genes, SMAD7 and *c-MYC*. Furthermore, TGF- β 1 and ActA have an antiproliferative effect on thyroid papillary carcinoma cell, exerting an important role in the control of thyroid tumorigenesis.

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Introduction

Transforming growth factor-beta (TGF- β) and activin are structurally related dimeric proteins belonging to the TGF- β superfamily, which includes other members such as bone morphogenetic protein, growth and differentiation factor, Müllerian-inhibiting substance, and inhibin (Chang *et al.* 2002, Miyazawa *et al.* 2002). While TGF- β , the first member of the superfamily to be isolated, was initially recognized by its capacity to induce anchorage-independent proliferation of rat fibroblasts (Roberts *et al.* 1981), activin was originally isolated from the gonads and identified by its ability to stimulate follicle-stimulating hormone secretion from the anterior pituitary (Ling *et al.* 1986, Vale *et al.* 1986). Lately, a diversity of biological processes has been attributed to the TGF- β superfamily, including control of cell proliferation, differentiation, apoptosis, and development. Much of this knowledge was acquired by studying TGF- β 1 and activin A (ActA), the most studied isoforms of each family. Despite its original transforming definition, TGF- β at present is best known as a potent growth inhibitor for epithelial cells (Roberts & Sporn 1991). In addition to its role in the reproductive system, activin also induces antiproliferative effects in a range of epithelial cells (Chen *et al.* 2002). TGF- β acts by binding to specific serine/threonine kinase transmembrane receptors, which induce

intracellular signaling by phosphorylation of receptor-regulated mothers against decapentaplegic homolog (R-SMADs), SMAD2 and SMAD3, allowing their interaction with the common-mediator SMAD (co-SMAD), SMAD4, and then this complex moves to the nucleus where it regulates gene transcription (ten Dijke *et al.* 2000). Although activin binds to its own specific receptors, the same set of SMADs is recruited for its signal transduction. This TGF- β /activin signaling is attenuated by SMAD7, an inhibitory SMAD (I-SMAD), that prevents phosphorylation of SMAD2/3 and interferes with the formation of complex SMAD2/3 and SMAD4 (Pangas & Woodruff 2000, Miyazawa *et al.* 2002). Although TGF- β and activins generate signaling by common intracellular mediators, represented by SMADs 2/3 and 4, they induce specific biological responses (Roberts & Sporn 1991, Vale *et al.* 1991). During embryony development, the distinct role exerted by each member of the superfamily becomes evident by observing mice deficient in TGF- β 1 or ActA. These studies reveal that the majority of the TGF- β 1 homozygote mutant mice died during embryogenesis and the survivors developed inflammatory infiltrate in multiple organs. Mice deficient in ActA, in turn, exhibited craniofacial defects and died during the perinatal period (Chang *et al.* 2002). These knockout mice models suggest that the lack of one superfamily member is not compensated by another, even though the common pathway

of SMADs is intact, supporting the specificity and independence of effects generated by each growth factor.

The TGF- β inhibitory effect in the proliferation of epithelial cells, such as gastric, pancreatic, mammary and intestinal cells, has been well characterized (Roberts & Sporn 1991, Siegel & Massague 2003). On the other hand, the expression of TGF- β 1 mRNA and protein are increased in tumors derived from epithelial cells when compared with normal cells, suggesting that these tumoral cells became unresponsive to TGF- β 1's growth inhibitory action (Asmis *et al.* 1996, Siegel & Massague 2003). In the thyroid, TGF- β promotes growth arrest in follicular cells derived from diverse animal species (Morris *et al.* 1988, Tsushima *et al.* 1988, Taton *et al.* 1993, Depoortere *et al.* 2000). Although activin propagates the same SMAD signaling, its role in the proliferation of thyroid follicular cells is not well clarified (Kotajima *et al.* 1995, Franzen *et al.* 1999, Schulte *et al.* 2000). Thyroid cancer is the most prevalent endocrine malignancy, papillary carcinoma being the most common thyroid carcinoma (DeLellis & Williams 2004). In previous studies, we observed TGF- β 1, ActA, and also SMADs expression in thyroid neoplasms (Kimura *et al.* 1999, Cerutti *et al.* 2003, Matsuo *et al.* 2003). The co-expression of TGF- β 1 and ActA does not permit us to distinguish the individual role of each of these growth factors in the thyroid. To investigate the contribution of each gene in SMADs signal transduction and in cell proliferation, we induced the silencing of the *TGF- β 1* and also of the *ActA* gene by RNA interference (RNAi) to analyze the effect of this knockdown upon SMADs gene expression and upon thyroid carcinoma cell proliferation.

Materials and Methods

Cell culture

TPC-1, a human papillary thyroid carcinoma cell line (Ishizaka *et al.* 1990), was kindly provided by Dr J A Fagin (University of Cincinnati College of Medicine, Cincinnati, OH, USA). TPC-1 (referred to as TPC) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 μ g/ml amphotericin at 37 °C in a humidified 5% CO₂ incubator.

Cell proliferation assays

Cell counting Cells were seeded into six-well plates at a density of 5×10^4 cells/well and cultured for 24, 48, and 72 h, when total confluence was reached. The number of cells was counted in a hemocytometer and the average cell number from quadruplicate measurements was determined.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, bromide (MTT) assay Cells were seeded into 96-well plates at a density of 10^4 cells/well. When the cells reached semi-confluence 15 h after seeding, MTT (Amresco,

Solon, OH, USA) was added to the medium at a concentration of 0.125 mg/ml. After 3 h, the medium was removed and the cells were solubilized in 100 μ l 0.04 M HCl in isopropanol and the reaction product was measured by a spectrophotometer at 595 nm.

BrdU labeling and immunocytochemistry Cells were seeded onto chamber slides, and at semi-confluence (24 h in culture) were incubated with 10 μ M bromodeoxyuridine (BrdU; Sigma) for 3 h. The cells were fixed in 4% paraformaldehyde for 10 min and incubated with 2 M HCl for 30 min. The immunocytochemical procedure was performed by an indirect three-stage immunoenzymatic method as described previously (Martins *et al.* 2002). Briefly, after endogenous alkaline phosphatase was blocked with 15% glacial acetic acid, cells were incubated with primary antibody mouse monoclonal anti-BrdU overnight. After incubation with biotin-streptavidin-alkaline phosphatase, the reaction was revealed by a mixture of naphthol ASMX phosphate, levamisole and fast-red TR salt diluted in veronal acetate buffer (pH 8.3). The cells were counterstained with Gill's hematoxylin. The immunopositivity of the reaction was detected as red color in the nucleus observed by light microscopy. The omission of the primary antibody resulted in a lack of positive staining. For analysis, the percentage of immunopositive cells was determined by counting three different areas in the slide in the total of 900 cells.

RNAi

Construction of small interfering RNA (siRNA) plasmids We used pSilencer 3.1-H1 puro small interfering RNA (siRNA) expression vector (Ambion, Austin, TX, USA), which contains the H1 RNA polymerase III promoter to drive siRNA expression and a puromycin resistant gene to enable antibiotic selection in mammalian cells. To design siRNA, three mRNA target sequences were selected for *TGF- β 1* RNAi (1, GGGCTACCATGCCAACTTC; 2, GACTATCGACATG-GAGCTG; 3, GTGGACATCAACGGGTTCA, GenBank accession no. NM_000660) and for *ActA* RNAi (1, GTTGCTGGATTATAGTGAG; 2, GACGCTGCACTTC-GAGATT; 3, GGAGCAGTCGCACAGACCT, GenBank accession no. M13436). For each target sequence, sense and antisense oligonucleotide siRNA were designed following Ambion's guidelines to encode a 19-mer hairpin structure with a loop and 3' overhanging ends. For annealing of siRNA duplexes, siRNA strands were heated for 3 min at 95 °C and then cooled slowly to room temperature. The annealed siRNA insert was ligated into the pSilencer vector according to the manufacturer's instructions. We performed sequencing as described previously (Martins *et al.* 2002) to confirm insertion of siRNA oligonucleotide into the plasmid.

siRNA cell lines

Transfection TPC cells were cultured to approximately 80–90% confluence. Cells were then transfected with *ActA* siRNA or *TGF- β 1* siRNA plasmid or with p*Silencer* negative control (Ambion) using Lipofectamine 2000 (Invitrogen). Stable cell lines *ActA* siRNA, *TGF- β 1* siRNA, and negative control siRNA were obtained by using 1 μ g/ml puromycin for 2–3 weeks. For each target sequence of *ActA* or *TGF- β 1*, one to three stable siRNA cells were generated. Clones of *TGF- β 1* or *ActA* siRNA that presented at least 60% inhibition of target genes by quantitative PCR (Fig. 1) were chosen for further analysis. For experimental procedures, third to fifth passages of each clone were used. The morphology of knockdown cells was monitored during culture under an inverted microscope. The *ActA* siRNA and *TGF- β 1* siRNA cell lines did not exhibit morphological differences when compared with nontransfected TPC observed by inverted light microscopy (Fig. 1C).

Quantitative PCR

Total RNA was isolated using Trizol (Invitrogen). The first strand of cDNA was generated from 3 μ g of total RNA as described previously (Matsuo *et al.* 2003). Primers were designed based on Primer Express Program (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and are listed in Table 1. Each set of sense and antisense primers was chosen in distinct exons to avoid amplification of genomic DNA. *ActA* or *TGF- β 1* primers of 600 nM and *SMAD2*, *SMAD3*, *SMAD4*, *SMAD7*, and ribosomal protein L19 (*RPL19*)

primers of 300 nM were optimized. PCR amplification was performed in duplicate using 50 ng of cDNA, 300 or 600 nM of each primer, and SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) in a Gene Amp 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems). PCR carried out without cDNA resulted in no amplification of any target gene. Each PCR reaction was repeated at least twice. The expression of each investigated gene was normalized with housekeeping gene *RPL19*. Data were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001) and are presented as the fold change in gene expression relative to the control sample. For the control sample, $2^{-\Delta\Delta C_T}$ is equal to 1.

The transfected TPC with p*Silencer* negative control, which encodes a hairpin siRNA whose sequence has no significant homology to known human genes, did not display significantly altered expression of any target gene when compared to nontransfected TPC (data not shown).

Treatment of cells with recombinant human TGF- β 1 or ActA

The cell lines were cultured until semi-confluence, when they were treated either with or without 10 ng/ml TGF- β 1 (PeproTech, Rocky Hill, NJ, USA) or 70 ng/ml ActA (PeproTech) and incubated for 3 h in DMEM 5% FBS. Total RNA was isolated and subjected to cDNA amplification.

The effect of TGF- β 1 or ActA addition was also assessed on cell proliferation. For this purpose, cells were seeded into 96-well plates at a density of 10^4 cells/well and treated either with or without 1 ng/ml TGF- β 1 (PeproTech) or 10 ng/ml ActA (PeproTech) in DMEM 5% FBS for 24 h. Subsequently, MTT was performed as described earlier.

Figure 1 RNAi targeting TGF- β 1 mRNA and ActA mRNA in the TPC cell line. (A) TGF- β 1 mRNA expression in TPC transfected with *TGF- β 1* siRNA evaluated by quantitative PCR. (B) Expression of ActA mRNA in TPC transfected with *ActA* siRNA by quantitative PCR. Columns in graphs (A) and (B) represent mean $\pm$ s.d. of two independent reactions performed in duplicate. (C) Cell morphology in non-transfected TPC, *TGF- β 1* siRNA, and *ActA* siRNA cell lines observed by inverted light microscopy. Scale bar = 50 μ m. * P < 0.05 vs TPC.

Table 1 Primers used in real-time PCR

	Sequence of primers (5'→3')	GenBank Accession no.
Genes		
<i>ActA</i>	Sense GGAGGGCAGAAATGAATGAA Antisense AATCTCGAAGTGCAGCGTCT	M13436
<i>TGF-β1</i>	Sense CGCGTGCTAATGGTGGAAA Antisense CGGAGCTCTGATGTGTTGAA	NM_000660
<i>SMAD2</i>	Sense CCGAAGGCAGACGGTAACA Antisense CCACTGTAGAGGTCCATTGATG	AF027964
<i>SMAD3</i>	Sense TCTCCAATGTCAACAGGAATGC Antisense GAGCCGACAGCCTCTTC	NM_005902
<i>SMAD4</i>	Sense CAGGTGGCTGGTCGGAAA Antisense AGATCAGGCCACCTCCAGAGA	U44378
<i>SMAD7</i>	Sense CGATGGATTTTCTCAAACCACT Antisense CCCTGTTTCAGCGGAGGAA	AF015261
<i>c-MYC</i>	Sense TTCGGGTAGTGGAAAACCAG Antisense TCCTGTTGGTGAAGCTAACG	X00196
<i>RPL19</i>	Sense TCTCATGGAACACATCCACAA Antisense TGGTCAGCCAGGAGCTTCTT	NM_000981

Statistical analysis

The results of proliferation assays and quantitative PCR are presented as the mean ± s.d. and were submitted to ANOVA followed by the Bonferroni *t*-test or Student Newman–Keuls-test to compare results between groups. Differences were considered significant at *P* < 0.05.

Results

We first examined the mRNA expression of TGF-β1, ActA, and SMADs in the thyroid cancer cell lines by quantitative PCR. As shown in Fig. 2, we detected expressions of TGF-β1, ActA, SMAD2, SMAD3, SMAD4, and SMAD7 in the TPC cell line. In relation to SMADs, the expression of SMAD4 was similar to SMAD7, while SMAD2 presented the lowest expression. The SMAD3 expression was significantly higher than that of SMAD2. However, the ActA mRNA expression was not detected in the NPA papillary thyroid carcinoma cell

Figure 2 Expression of ActA, TGF-β1, SMAD2, SMAD3, SMAD4, and SMAD7 mRNA in TPC cells by quantitative PCR. The gene expression levels were compared with SMAD2 expression in TPC, whose value was determined as 1.0. Columns in the graph represent mean ± s.d. of two independent reactions performed in duplicate.

line and in the ARO anaplastic thyroid carcinoma cell line (data not shown). The fact that TPC cell line expressed TGF-β1, ActA, and SMADs led us to choose this cell line as a suitable model for the investigation of the effects of TGF-β1 and ActA knockdown on cellular proliferation and on SMADs signaling.

TGF-β1 siRNA cell line

Role of TGF-β1 in TPC cell proliferation *TGF-β1* gene silencing resulted in an increase in cell proliferation at 24 ($2.9 \times 10^5 \pm 0.09$ vs $1.8 \times 10^5 \pm 0.09$, *P* < 0.05) and at 48 h ($7.5 \times 10^5 \pm 0.25$ vs $6.2 \times 10^5 \pm 0.36$, *P* < 0.05) during exponential growth by cell counting assay. At 72 h, when the cells reached total confluence, we did not observe any difference in proliferation rate between *TGF-β1* siRNA cell line ($16.1 \times 10^5 \pm 0.75$) and TPC ($15.9 \times 10^5 \pm 1.1$). Figure 3A shows representative results of cell counts.

The MTT assay also showed an increase in cell proliferation rate in *TGF-β1* siRNA cell line compared with control cells (0.14 ± 0.03 vs 0.08 ± 0.01 , *P* < 0.05; Fig. 3B). The percentage of BrdU labeling was also higher in *TGF-β1* knockdown cells than TPC (32 vs 15%, *P* < 0.05; Fig. 3C).

Role of TGF-β1 in SMADs expression To analyze the contribution of TGF-β1 to SMADs pathway generation, we verified the gene expression of SMAD2, SMAD3, and SMAD4 in *TGF-β1* siRNA cell by real-time PCR. The *TGF-β1* gene silencing reduced gene expression of SMAD2 by 73%, SMAD3 by 64% and SMAD4 by 72% (Fig. 4).

ActA siRNA cell line

Role of ActA in TPC cell proliferation Like the *TGF-β1* siRNA cell, a similar result was observed in the *ActA* siRNA line by cell counting. The proliferation rate in *ActA* siRNA line was

Figure 3 Effect of *TGF- β 1* gene silencing on TPC cell proliferation. (A) Cells were seeded at 5×10^4 cells/well in six-well plates and cell counting was determined at the indicated times. Points represent mean $\pm$ s.d. of four determinations. Data are representative of two independent experiments. (B) TPC and *TGF- β 1* knockdown cells were seeded at equal density and cultured until semi-confluence, and then incubated with MTT for 3 h. Data are presented as the mean $\pm$ s.d. of a single experiment performed in quadruplicate and are representative of three independent experiments. (C) TPC and *TGF- β 1* knockdown cells were seeded onto glass chamber slides and cultured until semi-confluence, and then incubated with 10 μ M BrdU for 3 h. Cells were fixed and then incubated with anti-BrdU antibody and the immunocytochemistry was performed by the biotin-streptavidin-alkaline phosphatase method. The percentage of immunopositive cells was obtained by counting three different areas in the slide in the total of 900 cells. Similar results were obtained in another independent experiment. * $P < 0.05$ vs TPC.

increased at 24 ($2.7 \times 10^5 \pm 0.17$ vs $1.8 \times 10^5 \pm 0.09$, $P < 0.05$) and at 48 h ($8.2 \times 10^5 \pm 0.43$ vs $6.2 \times 10^5 \pm 0.36$, $P < 0.05$), but unchanged at 72 h ($16.4 \times 10^5 \pm 1.0$ vs $15.9 \times 10^5 \pm 1.1$), when the cells became confluent (Fig. 5A).

By MTT assay, we also observed an increase in the proliferation rate of *ActA* siRNA cells when compared with control TPC (0.12 ± 0.01 vs 0.08 ± 0.01 , $P < 0.05$; Fig. 5B). The percentage of labeled cells by BrdU assay was also higher in *ActA* knockdown cells compared with control (22 vs 15%, $P < 0.05$; Fig. 5C).

Role of ActA in SMADs expression To investigate the involvement of SMADs in the activin pathway, we analyzed SMADs expression in TPC and *ActA* siRNA cells by quantitative PCR.

As seen in Figure 6, *ActA* siRNA cells had reduced mRNA expression of *SMAD2* by 51%, *SMAD3* by 58% and *SMAD4* by 58% in comparison to nontransfected TPC.

Figure 4 Effect of *TGF- β 1* gene silencing on SMADs expression in TPC cells. The graph shows mRNA expression of *SMAD2*, *SMAD3*, and *SMAD4* by quantitative PCR. The expression of each gene in non-transfected TPC (control) was determined as value 1. Columns in the graph represent mean $\pm$ s.d. of two independent reactions performed in duplicate. * $P < 0.05$ vs TPC.

Expression of target genes, SMAD7 and c-MYC, regulated by *TGF- β 1* and *ActA*

To examine the effect of *TGF- β 1* and *ActA* on regulation of target genes by SMAD signaling, we evaluated *SMAD7* and *c-MYC* mRNA expression in TPC and knockdown cells treated either with or without exogenous *ActA* or *TGF- β 1* by quantitative PCR.

The addition of recombinant *TGF- β 1* or *ActA* induced an increase in *SMAD7* mRNA levels in TPC (Fig. 7A and B). Inhibition of endogenous genes of *TGF- β 1* or *ActA* by RNAi resulted in a reduction in *SMAD7* expression in TPC cells (Fig. 7C and D).

While the addition of *TGF- β 1* and *ActA* induced a decrease of *c-MYC* expression in TPC (Fig. 8A and B), curiously, the silencing of *TGF- β 1* and *ActA* mRNA in TPC induced a decrease of *c-MYC* expression (Fig. 8C and D). *c-MYC* mRNA levels, already low in knockdown cells, were further decreased by *TGF- β 1* or *ActA* treatment (Fig. 8E and F).

Effects of *TGF- β 1* and *ActA* on TPC cell and siRNA cell proliferation

Cell proliferation rate was analyzed by MTT assay in TPC and in knockdown cell lines in the presence or absence of *TGF- β 1* (Fig. 9A) or *ActA* (Fig. 9B) treatment. Addition of *TGF- β 1* or *ActA* significantly reduced cell proliferation in TPC. Moreover, upon *ActA* or *TGF- β 1* treatment, *ActA* siRNA and also *TGF- β 1* siRNA cells exhibited decreased proliferation rates at similar levels to non-transfected TPC.

Discussion

We have shown previously that thyroid tumors express *TGF- β 1*, *ActA*, and SMAD proteins (Kimura *et al.* 1999, Cerutti

Figure 5 Effect of ActA gene silencing on TPC cell proliferation. (A) Cells were seeded at 5×10^4 cells/well in six-well plates and cell counting was determined at the indicated times. Points represent mean $\pm$ s.d. of four determinations. Data are representative of two independent experiments. (B) TPC and ActA knockdown cells were seeded at equal density into 96-well plates and cultured until semi-confluence, and then incubated with MTT for 3 h. Data are presented as the mean $\pm$ s.d. of a single experiment performed in quadruplicate and are representative of three independent experiments. (C) TPC and ActA knockdown cells were seeded on glass chamber slides and cultured until semi-confluence, and then incubated with 10 μ M BrdU for 3 h. Cells were fixed and then incubated with anti-BrdU antibody and the immunocytochemistry was performed by the biotin-streptavidin-alkaline phosphatase method. The percentage of immunopositive cells was obtained by counting three different areas in the slide in the total of 900 cells. Similar results were obtained in another independent experiment. * $P < 0.05$ vs TPC.

et al. 2003, Matsuo *et al.* 2003). It is well known that TGF- β 1 and ActA induce intracellular signaling pathways through the same set of SMADs (Pangas & Woodruff 2000, Miyazawa *et al.* 2002), but the concomitant expression of both factors raises the question of what is the individual contribution of these proteins in cell proliferation and induction of their mediator SMADs in thyroid cancer. Although the synthetic siRNA induces effective gene silencing, it is highly dependent on the transfection efficiency (Bantounas *et al.* 2004). In this study, the generation of stable cell lines with long-term gene silencing using siRNA expression vector approach (Sui *et al.* 2002) allowed us to investigate the effects of continuous TGF- β 1 or ActA knockdown. The role of TGF- β 1 as a potent antimitogenic factor is well characterized in rat thyroid follicular cell line as observed in the Fisher rat thyroid cell line (FRTL-5 cell (Morris *et al.* 1988, Cirafici *et al.* 1992, Pang *et al.* 1992), while the role of ActA in the proliferation of

follicular cells remains uncertain (Kotajima *et al.* 1995, Franzen *et al.* 1999, Schulte *et al.* 2000). In human thyroid cancer, the effect of TGF- β 1 or ActA upon cell proliferation is still controversial (Holting *et al.* 1994, Usa *et al.* 1994,

Figure 6 Effect of ActA gene silencing on SMADs expression in TPC cells. The graph shows mRNA expression of SMAD2, SMAD3, and SMAD4 by quantitative PCR. The expression of each gene in non-transfected TPC (control) was determined as value 1. Columns in the graph represent mean $\pm$ s.d. of two independent reactions performed in duplicate. * $P < 0.05$ vs TPC.

Figure 7 Effect of TGF- β 1 and ActA on SMAD7 expression. (A and B) Effect of recombinant TGF- β 1 and ActA on SMAD7 expression in TPC cells by quantitative PCR. TPC cells were incubated either in the absence or in the presence of TGF- β 1 (A) or ActA (B) in DMEM 5% FBS for 3 h. Columns in the graph represent mean $\pm$ s.d. of two independent reactions performed in duplicate. * $P < 0.05$ vs non-treated TPC. (C and D) Expression of SMAD7 in TGF- β 1 (C) and in ActA (D) knockdown cell lines by quantitative PCR. Columns in the graph represent mean $\pm$ s.d. of two independent reactions performed in duplicate. * $P < 0.05$ vs TPC.

Figure 8 Effect of TGF- β 1 and ActA on c-MYC expression in TPC. (A and B) Effect of exogenous TGF- β 1 and ActA proteins on c-MYC expression in TPC cell by quantitative PCR. TPC cells were incubated either in the absence or in the presence of TGF- β 1 (A) or ActA (B) in DMEM 5% FBS for 3 h. Columns in the graph represent mean $\pm$ s.d. of two independent reactions performed in duplicate. * $P < 0.05$ vs non-treated TPC. (C and D) Expression of c-MYC in TGF- β 1 (C) and in ActA (D) knockdown cell lines by quantitative PCR. Columns in the graph represent mean $\pm$ s.d. of two independent reactions performed in duplicate. * $P < 0.05$ vs TPC. (E and F) Effect of exogenous TGF- β 1 and ActA proteins on c-MYC expression in TPC/siRNA cell by quantitative PCR. TGF- β 1 (E) and ActA (F) knockdown cell lines were incubated either in the absence or in the presence of TGF- β 1 (E) or ActA (F) in DMEM 5% FBS for 3 h. Columns in the graph represent mean $\pm$ s.d. of two independent reactions performed in duplicate. * $P < 0.05$ vs non-treated siRNA cell.

Ohta *et al.* 1996, Schulte *et al.* 2001). Our study indicates that ActA has an antimitogenic role in the TPC cell line, since the addition of recombinant TGF- β 1 or ActA induced a reduction of cell proliferation in TPC as observed in Figure 9. To further understand the role of endogenous TGF- β 1 and ActA in thyroid cancer, we analyzed the gene silencing of TGF- β 1 and the gene silencing of ActA in TPC cells. The TGF- β 1 and ActA knockdown cell lines did not exhibit morphological alterations when compared to non-transfected TPC, as shown in Fig. 1C. Our study showed that ActA gene silencing led to an increase in cell proliferation of TPC, suggesting that endogenous ActA exerts an antiproliferative role. In relation to TGF- β 1, reducing its gene expression in TPC caused the cells to present a higher proliferation rate, indicating that TGF- β 1 also exerts antimitogenic effects in this tumor cell. Furthermore, exogenous ActA induced an antiproliferative response in ActA siRNA cells and also reduced proliferation in the TGF- β 1 siRNA cells (Fig. 9B), compensating the knockdown of TGF- β 1 in this cell. In addition, these data showed that ActA could induce antiproliferative effects independent of TGF- β 1. Similarly, the addition of TGF- β 1 compensated

the lack of ActA in ActA siRNA cells and restored the antimitogenic response in the TGF- β 1 siRNA cell line (Fig. 9A).

The TGF- β 1 pathway is well known as a tumor suppressor pathway, since TGF- β 1 is a potent growth inhibitor of most cell types. Inactivation of TGF- β 1 signaling components, TGF- β receptors, SMAD2, SMAD3, or SMAD4, has been found in a variety of cancer cells and loss of these components is associated with escape from TGF- β responsiveness (Derynck *et al.* 2001, Wolfrum *et al.* 2004). Our study showed that TPC expresses SMADs mRNA and that RNAi targeting TGF- β 1 mRNA and ActA mRNA inhibited gene expression of stimulatory SMADs, SMAD2, SMAD3, and SMAD4, thereby suggesting that both TGF- β 1 and ActA modulate the expression of their mediators and generate signaling that acts cooperatively in thyroid cancer. Although SMAD2 and SMAD3 share a high percentage of homology and both are stimulated by the complex TGF- β /receptor or activin/receptor, many *in vitro* studies have shown the differential participation of SMAD2 and SMAD3 in transducing the signal generated by TGF- β /activin in specific cell types (Kretschmer *et al.* 2003, Suszko *et al.* 2005). In SMAD knockout mice, this

Figure 9 Effect of TGF- β 1 and ActA on TPC, ActA, and TGF- β 1 knockdown cell proliferation. TPC and knockdown cells were plated at equal density and incubated either in the absence or in the presence of TGF- β 1 (A) or ActA (B) in DMEM 5% FBS for 24 h. Subsequently, cell proliferation rate was measured by MTT assay. Data are presented as the mean $\pm$ S.D. of a single experiment performed in hexaplicate. * $P < 0.05$ vs TPC.

differential role of each stimulatory SMAD is evident by observation that gene inactivation of either *SMAD2* or *SMAD3* produces distinct phenotypes (Chang *et al.* 2002). In our study, although we detected differential expression of *SMAD3* and *SMAD2* mRNA in TPC cell (Fig. 2), gene expression of either *SMAD2* or *SMAD3* was reduced in knockdown cells, suggesting an involvement of both R-SMADs in thyroid cancer.

TGF- β promotes cell growth arrest by induction of cyclin-dependent kinase (CDK) inhibitors p15 and p21 and inhibition of CDK2, CDK4, and CDK6, thereby preventing hyperphosphorylation of pRB and down-regulating *c-MYC* (Warner *et al.* 1999). Since TGF- β and activin act through the same SMADs, most of the known mechanisms involved in growth inhibition have been attributed to TGF- β action, while the antimitogenic mechanism induced by activin is poorly understood.

Similar to the previous report (Lin & Ying 1999), we observed a reduced expression of CDK inhibitor p16 in ActA siRNA cells using a membrane array assay, while p21 expression remained unchanged. Moreover, we did not detect p15 expression even in non-transfected TPC cells by the same approach (data not shown).

SMADs mediate several TGF- β and activin functions by modulating the transcription of target genes (Miyazawa *et al.* 2002). To analyze the integrity of TGF- β /SMAD and activin/SMAD signaling in the TPC cell line, we examined the expression of two target genes that are known to be regulated by these pathways: *SMAD7*, whose transcription is stimulated (Afrahkhte *et al.* 1998), and *c-MYC*, whose transcription is suppressed by TGF- β and activin (Miyamoto *et al.* 1990, Usa *et al.* 1994). In contrast to stimulatory SMADs (*SMAD2*, *SMAD3*, and *SMAD4*), *SMAD7* is a SMAD that inhibits TGF- β /activin signaling. Since transcription of *SMAD7* is induced by TGF- β /SMAD and also by activin/SMAD pathways, inhibitory SMAD may represent an auto-regulatory negative feedback mechanism in TGF- β superfamily signal transduction (Pangas & Woodruff 2000, ten Dijke *et al.* 2000, Miyazawa *et al.* 2002). In our study, the addition of TGF- β 1 and ActA induced an increase of *SMAD7* expression, while the knockdown of either TGF- β 1 or ActA genes in turn resulted in decrease of *SMAD7* mRNA levels, indicating that *SMAD7* is tightly regulated by TGF- β 1 and ActA in TPC cell. *c-MYC*, in turn, is closely implicated in cell proliferation and is one of the most well-known proto-oncogenes, whose activation is associated with several human tumors (Dang 1999). The *c-MYC* down-regulation mediated by the SMAD signaling pathway confers cell growth arrest in response to TGF- β 1 and activin (Miyamoto *et al.* 1990, Warner *et al.* 1999). In thyroid cancer, the inhibition of *c-MYC* induces a decrease in cell proliferation (Usa *et al.* 1994, Cerutti *et al.* 1996). When we analyzed the gene expression of *c-MYC* in TPC treated with TGF- β 1 or ActA, we observed a decrease in *c-MYC* gene expression (Fig. 8A and B). Despite increased proliferation rates induced by TGF- β 1 or ActA siRNA, we curiously observed that *c-MYC* expression was reduced in both TGF- β 1 and ActA knockdown cells (Fig. 8C and D). However, the addition of recombinant TGF- β 1 in TGF- β 1 siRNA cells and recombinant ActA in ActA siRNA cells led to a reduction of *c-MYC* expression (Fig. 8E and F). Although these results are contradictory in normal thyroid follicular cells, the suppression of *c-MYC* is not observed in response to TGF- β 1, even though these cells are responsive to the antimitogenic effect of TGF- β 1 (Carneiro *et al.* 1998, Depoortere *et al.* 2000). On the basis of these data, we would suggest that proliferation in TPC is not mediated by an increase in *c-MYC* levels. However, these findings remain to be further investigated, since *c-MYC* not only acts upon cell-cycle progression, but is also involved in several other cellular processes, including apoptosis (Dang 1999).

The loss of responsiveness to TGF- β 1 and the antiproliferative effects of ActA have been identified in several cancers

and implicated in tumoral progression (Derynck *et al.* 2001, Risbridger *et al.* 2001). Our data showed that the papillary thyroid carcinoma cell line, TPC-1, is still sensitive to the antiproliferative response generated distinctly by TGF- β 1 and ActA, possibly mediated through a functional SMADs pathway. The presence of this antiproliferative pathway is important to limit the unrestrained cell proliferation induced by mitogenic stimuli in thyroid cancer.

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