Re-expression of ABI3-binding protein suppresses thyroid tumor growth by promoting senescence and inhibiting invasion

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Abstract

Loss of ABI gene family member 3-binding protein (ABI3BP) expression may be functionally involved in the pathogenesis of cancer. Previous reports have indicated a loss of expression in lung cancer and a presumed role in inducing cellular senescence. We show here that ABI3BP expression is significantly decreased in most malignant thyroid tumors of all types. To better understand ABI3BP’s role, we created a model by re-expressing ABI3BP in two thyroid cancer cell lines. Re-expression of ABI3BP in thyroid cells resulted in a decrease in transforming activity, cell growth, cell viability, migration, invasion, and tumor growth in nude mice. ABI3BP re-expression appears to trigger cellular senescence through the p21 pathway. Additionally, ABI3BP induced formation of heterochromatin 1-binding protein γ-positive senescence-associated (SA) heterochromatin foci and accumulation of SA β-galactosidase. The combination of a decrease in cell growth, invasion, and other effects upon ABI3BP re-expression in vitro helps to explain the large reduction in tumor growth that we observed in nude mice. Together, our data provide evidence that the loss of ABI3BP expression could play a functional role in thyroid tumorigenesis. Activation of ABI3BP or its pathway may represent a possible basis for targeted therapy of certain cancers.

Introduction

We have previously identified biomarkers that distinguish malignant follicular thyroid carcinoma (FTC) from benign follicular thyroid adenoma (FTA) with high sensitivity and specificity (Cerutti et al. 2004). These four genes also help to distinguish malignant tumors in a wide range of thyroid tumors (Cerutti et al. 2006). The expression studies, performed originally to locate clinical markers, also revealed genes expressed in non-malignant tissues (normal thyroid and FTA), but not expressed or expressed at lower levels in the malignant FTC. One of these genes, ABI3-binding protein (ABI3BP), formerly named TARSH, was of particular interest because it was expressed in nearly all FTAs and normal thyroid samples, with consistent downregulation in all FTCs (Cerutti et al. 2004).

Although the biology of ABI3BP is largely unknown, it has been suggested that ABI3BP interacts with an SH3 domain in Abl-interactor member 3 (ABI3; Matsuda et al. 2001). ABI3, along with the Abl interactors E3B1/Abi2/Argbp1, belong to the family of cytoplasmic molecular adaptors containing SH3, which interact with Abl-family tyrosine kinases. Although speculative, it seems that members of Abl-interactors family might negatively regulate cell growth and transformation by suppressing certain tyrosine kinase-mediated signaling in mammalian cells (Ichigotani et al. 2002a). Overexpression of these family members was associated with inhibited cell proliferation, reduced transforming potential, and suppression of motility and metastasis dissemination (Ichigotani et al. 2002a,b). These findings suggest that Abl interactors may act as tumor suppressor
molecules. Therefore, if ABI3BP binds to the Abl interactor ABI3, it is predicted to result in inhibited cell growth. Circumstantial evidence consistent with a function for ABI3BP that suppresses cell proliferation is the report that its expression is greatly reduced in lung cancers and other primary tumors (Terauchi et al. 2006).

In the present study, we sought to determine whether the loss of ABI3BP expression plays a role in thyroid tumorigenesis. We expanded our analysis and investigated the expression of ABI3BP in a larger series of thyroid tumors, which includes, in addition to a larger number of FTAs and FTCs, Hürthle cell adenomas, Hürthle cell carcinomas, papillary thyroid carcinomas, and normal thyroids. ABI3BP is lost in most of the malignant lesions while it is expressed in a large number of benign lesions and normal thyroid tissues. We next explored functionally the effects of ABI3BP re-expression into two thyroid carcinoma cell lines in which ABI3BP expression was lost. Additionally, we suggest a possible pathway in which ABI3BP is involved. Finally, we show that ABI3BP re-expression has an effect on s.c. tumors of thyroid origin grown in nude mice.

Materials and methods

Tissue samples

A total of 79 thyroid tissue specimens obtained from patients undergoing thyroid surgery for thyroid disease at Hospital São Paulo, Federal University of São Paulo, Brazil were used for this study. Samples were frozen immediately after surgical biopsy and stored at −80 °C and included 36 benign thyroid lesions (21 FTAs and 15 Hürthle cell adenomas), 36 malignant thyroid tumors (17 FTCs, 6 Hürthle cell carcinomas, and 13 papillary thyroid carcinomas), and 7 normal thyroid tissues. All tissue samples were obtained with informed consent according to an established Human Studies Protocols at Federal University of São Paulo. The study of patient materials was conducted according to the principles expressed in the Declaration of Helsinki.

Cell lines

A follicular (WRO; UCLA RO-82W-1) and an undifferentiated (ARO; UCLA RO-81A-1) thyroid carcinoma cell lines were used in this study (generous gifts of Dr Alfredo Fusco, University Federico II, Naples, Italy). WRO and ARO were grown in DMEM (Invitrogen Corp.) supplemented with 10% FBS (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator containing 5% CO2 at 37 °C. ARO and WRO cell lines are known to have a number of specific genetic abnormalities including RAS (Rat sarcoma viral oncogene homolog), p53, and BRAF (V-raf murine sarcoma viral oncogene homolog B) mutation (Ouyang et al. 2002, Kimura et al. 2003).

RNA extraction, cDNA synthesis, and quantitative PCR (qPCR) of ABI3BP in human tissues

Total RNA and cDNA synthesis were performed as previously described (Cerutti et al. 2004). An aliquot of cDNA was used in a 15 µl PCR containing 1× TaqMan Universal PCR Master Mix, 10 µmol of each specific primer and FAM-labeled probe for the target gene (ABI3BP) or VIC-labeled probe for reference gene (RS8; TaqManGene Assays on Demand; Applied Biosystems, Foster City, CA, USA). Quantitative PCRs (qPCRs) were performed in triplicate and the threshold cycle (Ct), obtained using Applied Biosystems software (Applied Biosystems), was averaged (s.d. ≤1). Gene expression was calculated as described (Cerutti et al. 2004).

Plasmid constructs

To obtain the full-length human ABI3BP coding sequence, cDNA was synthesized from RNA isolated from normal thyroid tissue and used as template for PCR amplification using the sense 5′-TAAATGCAGCTCCTAGGGTTG-3′ and antisense 5′-CTCCTCCGCAAAGCATTCTCA-3′ primers. PCR product was purified and cloned into the mammalian expression vector pcDNA3.1 using a directional TOPO expression kit (Invitrogen) according to the manufacturer’s recommendations. This DNA construct was sequenced to confirm the sense orientation as described (Guimaraes et al. 2006).

Stable expression in thyroid carcinoma cell lines

To establish cell lines expressing ABI3BP, 10 µg DNA construct were transfected into 5 × 10⁶ WRO and ARO cells by electroporation using a Gene Pulser II (Bio-Rad Laboratories, Inc). ARO and WRO cells transfected with pcDNA3.1 vector were used as controls. Clones were isolated after 3 weeks of selection with G418 (800 µg/ml). At least, six G418-resistant clones from each transfection were isolated, expanded, maintained on G418 (400 µg/ml), and tested for ABI3BP expression by qPCR. To this end, total RNA extracted from each clone was used for cDNA synthesis as described (Cerutti et al. 2004). An aliquot of cDNA was used in 20 µl PCR containing SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of each primer for ABI3BP or reference gene (ribosomal protein S8). Primer sequences for ABI3BP and reference gene were as follows: ABI3BP,
Focus formation assay

WRO cells (5 × 10⁶) were transfected with 10 μg of either pcDNA3.1 or DNA construct coding ABI3BP as described previously. After 3 weeks of selection with G418 (800 μg/ml), WRO cells were fixed in 10% acetic acid and 10% methanol, stained with 1% crystal violet, and colonies were counted (Iervolino et al. 2006).

MTT assay

ARO cells were analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. In brief, 2 × 10⁴ ARO cells were seeded in 35 mm plates on day 0. Cell growth was measured daily from days 1 to 5 by adding 0.5 mg/ml MTT (Sigma–Aldrich) in medium at 37°C for 3 h. The medium was removed and the purple formazan crystals were dissolved in acid isopropanol at room temperature for 10 min. The spectrophotometric absorbance was measured at 560 nm.

Cell viability assay

ARO cells (2 × 10⁴) were seeded in 35 mm plates. Cells were mixed with Guava ViaCount reagent and allowed to stain for 10 min (Guava Technologies, Hayward, CA, USA). The Guava System differentiates viable from non-viable cells by detecting fluorescent signals from two fluorescent DNA-binding dyes: one membrane-permeable dye stains all nucleated cells while the second dye enters cells with compromised membrane integrity, i.e., non-viable cells. Viable cells were quantified using a Guava Personal Analyzer (PCA) flow cytometer (Guava Technologies) following the manufacturer’s specifications. Experiments were performed in quintuplicate.

Quantification of apoptotic cells by annexin V labeling

ARO cells (2 × 10⁴) were seeded in 35 mm plates. For the detection of apoptosis, the cells were double stained with annexin V and necin 7-AAD according to the manufacturer’s recommendations (Guava Nexin method; Guava Technologies). Cell-associated fluorescence was analyzed by the Guava PCA flow cytometer (Guava Technologies). The results are expressed as the percentage of apoptotic-positive cells. Both early apoptotic (annexin V positive) and late apoptotic (annexin V and 7 AAD positive) cells were included in the analysis. Experiments were performed in quintuplicate.

Cellular senescence

Senescence-associated (SA) β-gal staining was performed as described (Severino et al. 2000). Briefly, ARO cells (2 × 10⁴) were seeded in 35 mm plates. On referred days, cells were washed twice with PBS, fixed for 15 min, and stained with 1 mg/ml of 5-bromo-4-chloro-3-indolyl-b-D-galactosidase (X-gal) in buffer (dimethylformamide, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferri cyanide, 150 mM NaCl, and 2 mM MgCl₂). Cells were incubated at 37°C in 5% CO₂ for 18 h and washed twice with PBS. Cells were examined using a light microscope and counted in five optical fields (100 ×). Data represent the mean of an experiment performed in quintuplicate.

Senescence associated heterochromatin foci (SAHF)

Another feature of senescence cells is formation of Senescence associated heterochromatin foci (SAHF; Narita et al. 2003, Collado et al. 2005). The formation of such structures coincides with the recruitment of protein complexes containing the heterochromatin 1-binding protein γ (HP1γ). The HP1γ expression was analyzed using immunofluorescence. Cells were plated at a density of 1.7 × 10⁵ cells per well in an eight-well glass slide (Nalge Nunc International, Rochester, NY, USA). At day 5, cells were washed with PBS and fixed in methanol. Plates were blocked using 20% normal goat serum in PBS/Tween-20 and incubated for at least 16 h at 4°C with HP1γ primary antibody (1/500; MAB3450; Chemicon, Temecula, CA, USA). Cells were counterstained using DAPI.

Expression of p21, E2F1, MMP-1 and Ki67 by qPCR

Expression analysis was performed at days 3 and 5. Total RNA isolation and cDNA synthesis were performed as described (Cerutti et al. 2007). An aliquot of cDNA was

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used in 20 μl PCR containing SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of each primer for target gene or reference gene (ribosomal protein S8). Primer sequences were as follows: p21, sense: 5'-TGGAGACTCTCTAGGTGAAA-3' and antisense: 5'-GGCTTCCTCTTTGGAGAAGATCA-3', yielding a product of 90 bp; Ki67, sense: 5'-GTCGACGCTCCC-CACCTTTC-3' and antisense: 5'-GACACACAGGAA-GCTGGTACAG-3', yielding a product of 92 bp; E2F1, sense: 5'-TTGAGGAGCTCACGTCATT-3' and antisense: 5'-CCTGGGTCAACCCCTCAAG-3', yielding a product of 105 bp; and matrix metalloproteinase-1 (MMP-1), sense: 5'-TGTGGCTCAGTTTGTCCTCA-3' and antisense: 5'-GGCAAATCTGGCGTAAT-3', yielding a product of 90 bp.

**Protein extraction and western blotting analysis**

Protein extraction and western blotting analysis were performed according to the standard procedures. Briefly, total protein was isolated from clones expressing ABI3BP and vector-transfected clones using an extraction buffer containing 50 mM Tris–HCl (pH 7.4), 100 mmol/l NaCl, 50 mM NaF, 1 mM NaVO4, 0.5% NP-40, and complete protease inhibitor cocktail (Roche). The whole cell extract was clarified by centrifugation at 10 000 g. Protein concentration was estimated with manufacturer’s recommendation. Experiments were performed in quintuplicate.

**Anchorage-independent growth**

Anchorage-independent growth was assessed by a double-layer soft agar assay. Initially, 60 mm dishes were layered with 0.5% agar and 1× complete medium. Subsequently, ARO cells (1.5×10⁴) were suspended in 1× complete medium and 0.35% agar and seeded in triplicate over a bottom layer of solidified agar. The dishes were incubated at 37 °C in 5% CO₂. After 3 weeks, colonies greater than 20 μm in diameter were counted. Colony formation rate was calculated as percentage of total seeded cells. Two independent experiments were performed.

**Nude mouse xenograft model**

Four- to five-week-old male athymic nude (nu/nu) mice were maintained according to the guidelines of the Division of Animal Resources at the Federal University of São Paulo. ARO stable cell clones were suspended in sterile PBS to 2×10⁶/200 μl and injected subcutaneously into the flank of individual nude mice. Mice were then monitored biweekly during 3 weeks. If a tumor was present, volume was calculated by the rotational ellipsoid formula: \( V = A \times B^2 / 2 \) (A, axial diameter; B, rotational diameter). Tumor tissues were collected and were either embedded in paraffin for conventional histology or stored at −80 °C. Additionally, we also extracted RNA from formed tumors, performed cDNA synthesis and tested by qPCR ABI3BP, p21, and MMP-1 expressions as described above.

**Matrigel invasion assay**

Cell invasion was analyzed using BioCoat Matrigel Invasion Chamber according to the manufacturer’s recommendation (Becton Dickinson, Bedford, MA, USA). FBS was used as chemotaxtractant. WRO cell clones were added to the invasion or control chamber at a density of 2.5×10⁵ and, after 24 h, cells remaining above the insert membrane were removed by gentle scraping with a sterile cotton swab. Cells that had invaded through the Matrigel to the bottom of the insert were fixed, stained in rapid panoptic LB (Laborclin, Pinhais, PR, Brazil), and mounted. Cells were examined using a light microscope and counted in three optical fields (100×). All of the experimental and control groups were done in triplicate. The percentage of invasion cells was determined as the mean of cells invading through Matrigel insert membrane/mean of cell migrating through control insert membrane 100×.
Migration assay

To assess cell migration, WRO stable cell clones were suspended in 10 ml DMEM supplemented with 10% FBS, seeded onto 0.75% agar base-coated plates prepared in DMEM and cultured until spheroids were formed. At least, 12 spheroids of similar diameter were placed in the middle of uncoated 24-well plates. The area covered by cells spreading out from the spheroid was measured at the beginning of the experiment and daily for an observation period of 6 days. The areas of spheroids were calculated as described (Vajkoczy et al. 2006).

Statistical analysis

The qPCR values were log transformed and submitted to Student’s t-test (unpaired). In vitro results were log transformed and submitted to Student’s t-test (paired). In vivo results were submitted to Wilcoxon test and were declared significant the results with $P < 0.05$.

Results

**ABI3BP is underexpressed in malignant thyroid lesions**

Among the genes, we previously analyzed as underexpressed in FTCs, *ABI3BP* stood out because of its consistent loss in malignant follicular tumors (Cerutti et al. 2004). We extended our previous analysis by also including Hürthle cell carcinomas, papillary thyroid carcinomas, and the benign Hürthle cell adenomas in our assay. As demonstrated by qPCR, *ABI3BP* expression was either reduced or absent in most thyroid carcinomas (FTCs, Hürthle cell carcinomas, and papillary thyroid carcinomas), when compared with normal thyroid and most of benign lesions (FTAs and Hürthle cell adenomas; $P = 0.0001$; Fig. 1A). Surprisingly, *ABI3BP* was upregulated in few adenomas, compared with normal samples. Although samples were not matched to one another, it may suggest that these pre-malignant lesions may express *ABI3BP* as a mechanism to prevent proliferation of cells at risk for neoplastic transformation. Additionally, *ABI3BP* was consistently underexpressed in thyroid carcinomas. Therefore, we hypothesized that *ABI3BP* is functionally involved in suppressing either thyroid tumor growth or tumor progression.

**Re-expression of ABI3BP in thyroid carcinoma cell lines**

To explore *ABI3BP*’s potential pathogenic role, we generated an in vitro thyroid cancer model. As previously demonstrated, ARO thyroid carcinoma cell lines do not express *ABI3BP*. WRO expresses *ABI3BP* at very low levels (Cerutti et al. 2004). A construct expressing *ABI3BP* and an empty vector were transfected into both cell lines. Several stable clones were obtained from each transfected cell line and qPCR was used to assess the levels of *ABI3BP*. Two representative clones from each group were chosen for our studies. As shown in Fig. 1B, the qPCR data demonstrated that *ABI3BP* transcripts were induced about 2000-fold in ARO transfected with a construct encoding *ABI3BP*. WRO transfected with a construct encoding *ABI3BP* had an average twofold increase in *ABI3BP* (Fig. 1B). This fold induction was obtained when *ABI3BP* expression was normalized to

Figure 1  Relative expression levels of *ABI3BP* in thyroid tumors and cell lines and its ability to decrease foci formation. (A) Symbols correspond to the relative expression values for *ABI3BP* in normal thyroid tissues (NT; $n = 7\pm^\text{m}$), follicular thyroid adenomas (FTA; $n = 21\pm^\text{m}$), Hürthle cell adenomas (HCA; $n = 15\pm^\text{m}$), follicular thyroid carcinomas (FTC; $n = 17\pm^\text{m}$), Hürthle cell carcinomas (HCC; $n = 6\pm^\text{m}$), and papillary thyroid carcinomas (PTC; $n = 13\pm^\text{m}$). The qPCR values were log transformed before the application of statistical test ($P = 0.0001$). (B) qPCR analysis of stably transfected clones selected for the in vitro and in vivo experiments. The analysis demonstrated that *ABI3BP* expression was induced about 2000-fold in ARO transfected with a construct encoding *ABI3BP* when normalized to vector-transfected cells. WRO cells had an average twofold increased basal level expression when transfected with a construct encoding *ABI3BP*. (C) Transforming potential of a construct encoding *ABI3BP* in WRO cell line compared with a pCDNA3.1 empty vector control. *ABI3BP* decreased dramatically the transformation ability of WRO cells ($0.8\ foci/\mu g\ plasmid\ DNA$) compared with control ($32.7\ foci/\mu g\ plasmid\ DNA$). Black bar corresponds to WRO transfected with control clones and white bar corresponds to the cells transfected with *ABI3BP* expression vector. The data represent the mean $\pm$ s.d. of the experiment performed in triplicate.
vector-transfected clones (endogenous expression). Importantly, when the clones re-expressing ABI3BP were normalized to the expression observed in normal thyroids, the relative expression observed in ARO–ABI3BP-transfected cells was similar to the biological range observed in most normal thyroid and adenomas. The WRO–ABI3BP-transfected cells had expression similar to that observed in the few adenomas with greater ABI3BP expression (data not shown). Based on the ability to obtain physiologic levels of ABI3BP in ARO cells, and that the ARO line before transfection has a high tumorigenic potential (Cerutti et al. 1996), ARO clones were used in most of the experiments in this work. WRO clones were chosen for migration and invasion assays and foci formation, because ARO does not aggregate into spheroids, does not migrate through Matrigel within 24 h, and it was difficult to measure foci formation as ARO has a high growth rate.

Expression of ABI3BP reduces colony formation in thyroid cancer cells

To see whether ABI3BP could inhibit foci formation, the vector only or the expression construct encoding ABI3BP was transfected into the WRO cell line. When WRO was transfected with a vector containing wild-type human ABI3BP, the number of colonies was significantly reduced (0.8 foci/μg plasmid DNA) when compared with the number observed with control vector (32.7 foci/μg plasmid DNA; P < 0.05; Fig. 1C). This significant reduction in the number of colonies suggests that ABI3BP reduces the malignant potential of the WRO cells.

Expression of ABI3BP inhibits cell growth

To determine whether ABI3BP re-expression decreases proliferation, the stably transfected clones were compared with controls by an MTT assay. As showed in Fig. 2A, ABI3BP was able to significantly suppress the growth of ARO cells in comparison with the cells transfected with empty vector (P < 0.05).

Effect of ABI3BP on cell viability and apoptosis

To ascertain if re-expression of ABI3BP would decrease thyroid cancer cell’s viability, the Guava ViaCount reagent was used. The Guava ViaCount reagent contains two fluorescent dyes, one cell permeable and the other impermeable to cells with an intact plasma membrane. As determined by ViaCount, re-expression of ABI3BP led to a lower percentage of viable cells (P < 0.05; Fig. 2B). This finding suggests that re-expression of ABI3BP might induce apoptosis. To quantify the apoptotic rate of ARO cells expressing ABI3BP, we analyzed annexin V binding using a Guava Nexin kit, which reveals phosphatidylserine externalization in apoptotic cells. Re-expression of ABI3BP led to a moderate increase in apoptosis (Fig. 2C), which suggest that another mechanism may also be involved in inhibition of cell growth and decrease in cell viability. Since ABI3BP was previously associated with senescence process (Uekawa et al. 2005) and it was previously suggested that senescence may interfere with cell membrane integrity (Xin et al. 2003), a percentage of non-viable cells observed may represent senescent cells.

ABI3BP expression induces senescence in ARO cells

Senescent cells stain positive for SA-β-gal and a distinct heterochromatic structure designed as SAHF formation. We first observed that SA-β-Gal-positive cells, indicated by blue staining, were increased when ABI3BP is re-expressed (Fig. 2D and E). Secondly, immunofluorescence of HP1γ showed an expression pattern concentrated in DNA foci of cells expressing ABI3BP compared with controls, which suggested that the re-expression of ABI3BP was associated with the nuclear changes that occur during senescence. Conversely, in vector-transfected cells, the staining for HP1γ gave a weak signal and it was disperse through the nucleoplasm (Fig. 2F).

ABI3BP reduces expression of the proliferation marker Ki67 in ARO cells

As aforementioned, ABI3BP significantly suppresses the growth of ARO cells. In order to measure cell proliferation, beyond the MTT test, the expression levels of the proliferation marker Ki67 were tested at days 3 and 5. As measured by Ki67 expression, a lower proliferation rate was observed in ARO re-expressing ABI3BP when compared with control cells (Fig. 3A).

ABI3BP induces senescence markers in ARO cells

We have shown that ARO cells undergo senescence process following ABI3BP re-expression, although the underlie mechanism is still unclear. Several genes/pathways were associated with senescence process and elucidation of the pathway that controls senescence is complex. To start to build a model, we here investigate the potential role of a number of previously identified senescence markers (p21, E2F1, and MMP-1; Brown et al. 1997, Collado et al. 2000, Narita et al. 2003, 1996, 1997, 2000, 2003).
Collado & Serrano 2006, Park et al. 2006). We used qPCR to quantify mRNA levels of tested markers at days 3 and 5, a time that cells re-expressing ABI3BP became positive for SA-β-Gal staining, have decreased the expression of proliferation marker and nuclei were positive for HP1γ. At day 5, a time in which senescence process was more evident, p21 mRNA levels were threefold increased in ARO cells following ABI3BP re-expression (P < 0.05; Fig. 3A). The expression of MMP-1 was significantly increased at days 3 and 5 (P < 0.001; Fig. 3A). On the other hand, E2F1 mRNA levels were reduced in ARO cells expressing ABI3BP (Fig. 3A). Of note, E2F1-responsive genes were stably repressed in senescent cells. It was demonstrated that HP1γ mediates SAHF formation and E2F gene silencing occurs during senescence (Narita et al. 2003).

**ABI3BP re-expression alters AKT and ERK phosphorylation**

The effect of ABI3BP re-expression was further substantiated by investigation of AKT and ERK signaling pathways. The re-expression of ABI3BP resulted in a slight decreased ERK phosphorylation mainly at day 5 compared with vector-transfected cells. Meanwhile, a significant increase in AKT phosphorylation was observed at day 5 (Fig. 3B).

**ABI3BP causes a decrease in cells in S and G2/M**

Cell cycle distribution was analyzed by flow cytometry. G0/G1 arrest occurred in ARO cells expressing ABI3BP as indicated by an increase in the percentage number of cells at this phase (Fig. 4A). Although cell
formulation, represented as mean of tumor volume, is demonstrated in mice with cells expressing $ABI3BP$ when compared with controls ($P=0.027$; Fig. 4D). When presented, an aliquot of tumor was stored at $-80\,^\circ$C and a fraction was processed for routine histology and immunohistochemical analysis. H&E staining revealed the presence of tumors within the flank of 100% of controls. Neither lung nor lymph node metastases were found in any mice.

**Senescence markers on tumor growth in nude mice**

Since we observed an increased expression of senescence markers in cells re-expressing $ABI3BP$, they were also investigated in tumor samples that were taken from tumors growing on the flanks of nude mice. The expression of $p21$, MMP-1, and $ABI3BP$ was assayed by qPCR. A higher expression of $p21$ and MMP-1 was detected in tumors formed after injection of cells expressing $ABI3BP$ compared with tumors formed upon injection of control cells (Fig. 4E). These findings corroborate with our in vitro analysis and suggest that $ABI3BP$ induces senescence-like growth arrest through modulation of the $p21$ pathway.

**$ABI3BP$ inhibited invasion and migration of WRO cells**

Given these in vivo and in vitro findings, we sought to determine invasion and migration potential of cells expressing $ABI3BP$. Cellular invasiveness was quantified using BioCoat Matrigel Invasion Chamber. WRO cells expressing $ABI3BP$ significantly attenuated cellular invasiveness (30.92%) when compared with cells transfected with empty vector (62.86%; Fig. 5A and B). The migration ability was demonstrated by a spheroid growth assay. We observed the reduction in spheroid area in $ABI3BP$-expressing cells. A more significant effect on migration was observed in WRO cells expressing $ABI3BP$ at day 6 compared with control (Fig. 5C).

**Discussion**

Our previous work suggested a consistent loss of $ABI3BP$ expression in malignant FTC (Cerutti et al. 2004). In this study, we expanded our analysis of follicular thyroid tumors and also included other thyroid tumors subtypes. $ABI3BP$ expression was either reduced or absent not just in FTCs, but in most malignant lesions and thyroid carcinoma cell lines compared with benign lesions and normal thyroid tissues. Therefore, the loss of $ABI3BP$ expression is not
restricted to a specific malignant histological subtype. Recently, others have showed that $ABI3BP$ expression was significantly decreased in several tumor types when compared with the expression in corresponding non-neoplastic tissue specimens (Terauchi et al. 2006). This expression pattern hints that $ABI3BP$ might play a role in maintaining a normal phenotype in several types of cancer.

We next sought to determine whether $ABI3BP$ was functionally involved in thyroid malignancy. We tested the effects of re-expression of $ABI3BP$ on thyroid carcinoma cells in vitro and in vivo. Several lines of evidence from this research support the hypothesis that $ABI3BP$ reduces tumor growth and progression.

We first demonstrated that the ectopic expression of $ABI3BP$ inhibits foci formation and reduces cellular proliferation and viability. Furthermore, the results show that $ABI3BP$ markedly affected tumor growth in vivo. Since growth inhibitory effects may be caused by the re-establishment of programmed cell death, we next investigated the role of $ABI3BP$ in apoptosis. Although forced $ABI3BP$ expression increased apoptosis, the small percentage of apoptotic cells could not fully explain the larger reduction on cell proliferation and viability. Another mechanism that controls cell proliferation and restricts tumor growth is cellular senescence.

Since senescent cells may remain viable, one could argue that senescence could not be the mechanism by
cellular senescence, suggesting that senescence might be the preferential mechanism associated with growth inhibitory effect observed in cells expressing ABI3BP. It is an important aspect because prevention of tumor progression can be done either by inducing apoptosis or senescence (Collado & Serrano 2006). Since cellular senescence is an important tumor suppression process, the characterization of genetic pathways to senescence is needed.

It has been observed that various forms of stress including DNA-damaging agents, oncogenic activation, oxidative stress, and inadequate culture conditions induce a state that is indistinguishable from replicative senescence that was called premature or stress-induced senescence (Campisi 2001, Serrano & Blasco 2001, Lowe et al. 2004). Although the molecular mechanism underlying the senescence process remains poorly understood, recent studies have provided mechanistic insights into the senescence process. There is no doubt that constitutively active mutants of RAS/RAF provoke premature senescence in vivo and in vitro, additional alteration may be necessary to maintain an irreversible state of growth arrest. Investigation of the signaling pathways showed that inactivation of one of the effectors of the p53 or RB pathways is required to bypass senescence (Itahana et al. 2001, Narita et al. 2003, Mason et al. 2004, Michaloglou et al. 2005). How p53 and RB induce senescence is not completely understood and may be cell type and context dependent. As suggest by recent report, RB contributes to senescence by promoting SAHF and by silencing E2F target genes (Itahana et al. 2001, Narita et al. 2003).

Although reliable, molecular markers for the senescent state are still missing, some characteristics such as SA β-galactosidase (SA-β-gal) activity, Ki67 downregulation, expression of cell cycle inhibitors such as p21 and chromatin remodeling have proven useful as indicators of cellular senescence (Braig & Schmitt 2006). Therefore, the investigation of these SA phenotypes will help to clarify the role of ABI3BP.

ARO cell line exhibits a number of specific genetic changes that may be a mechanism of tumor cells to evade senescence, including BRAF V600E and p53 mutation (Fagin et al. 1993, Kimura et al. 2003). Interestingly, when the ARO cell line was stably transfected with wild-type p53, effects similar to those seen when we re-expressed ABI3BP were observed (Nagayama et al. 2000). Whether the re-expression of ABI3BP induces senescence by restoring p53 pathway and/or interfering with RAS/RAF/MAPK/ERK pathway may lead to a better and novel understanding on how ABI3BP induces senescence.

A rational approach would be investigating the level of expression of p21, which is downstream of p53 and...
appears to be essential for induction of senescence (Itahana et al. 2001). We here demonstrated that p21 mRNA was upregulated following ABI3BP re-expression compared with control cells, in both in vitro and in vivo analyses. Although the mechanism that underlies the p21 induction is unknown, ectopic expression of ABI3BP induces changes in gene expression, which resemble the gene expression changes that occur in senescent cell.

Since several other proteins have been implicated in this complex process, a question that immediately comes to our minds is: what other proteins may be involved in senescence process induced by ABI3BP?

It was demonstrated that constitutive activation of AKT promotes senescence-like growth arrest via p21-dependent pathway (Miyachi et al. 2004). Recently, it was demonstrated that AKT-dependent inactivation of FOXO3a (forkhead box) induced many senescence phenotypes such as SA-β-Gal staining and upregulation of p53 and p21 (Kyoung Kim et al. 2005). Additionally, it has been suggested that AKT participates in the regulation of both p53-dependent and p53-independent p21 expressions (Mitsuuchi et al. 2000). A markedly increased expression of pAKT was observed in ARO cells re-expressing ABI3BP, mainly at day 5 when most of the SA phenotype was observed, such as SA-β-Gal staining, SAHF formation, p21 overexpression, Ki67 downregulation, and in vitro and in vivo growth inhibition.

E2F1 is among the growth regulatory genes that are repressed during senescence (Dimri et al. 1994), although this is controversial (Dimri et al. 2000, Park et al. 2006). The contradictory activities of E2F1 may be due to cell context. We here demonstrated that E2F1 was underepressed in cells re-expressing ABI3BP when compared with controls. This result corroborates with previous findings in which lower E2F1 expression was associated with a favorable prognosis in breast cancer (Vuaroqueaux et al. 2007) and a higher E2F1 expression was found in papillary and anaplastic thyroid cancers (Onda et al. 2004).

Regarding the RAS/RAF/MAPK/ERK pathways, it has been suggested that RAF-induced senescence appears to have no obvious requirement for either p53 or p21 (Zhu et al. 1998). Although we have found a slight decrease in pERK, our findings suggest that it may occur through independent mechanism.

However, the senescence phenotype is not only mainly characterized by irreversible arrest of cell division and gene expression changes aforementioned, but senescent cells also express genes that have no obvious direct role in cell proliferation but are associated with remodeling. For example, cells may develop a constitutive matrix-degrading phenotype by secreting large amounts of matrix metalloproteinases such as MMP-1 (interstitial collagenase; Linskens et al. 1995, Collado & Serrano 2006). We here found an increased expression of MMP-1 in ARO cells re-expressing ABI3BP compared with control cells in both in vivo and in vitro.

Furthermore, besides its role in growth suppression and the determination of some components of the signaling pathways through which ABI3BP induces senescence, we here investigated the role of ABI3BP in invasion and migration. In this report, we show that ABI3BP significantly reduces spheroid growth and the percentage of invading cells in Matrigel. These findings, together with the in vivo results, strongly suggest that ABI3BP impairs migration and invasive ability of thyroid carcinoma cell line.

It is worth noting that ABI3BP was described to be an ABI3BP, as it was isolated from a human placenta cDNA library using the SH3 domain of human ABI3, formerly named NESH (new molecule including SH3; Matsuda et al. 2001). ABI3 is an adapter protein that, through the SH3 domain, interacts with Abl-family tyrosine kinases and suppresses the transforming activity in mammalian cells. It was also suggested that the loss of ABI3 expression could be involved in the mechanism of cell motility and metastasis (Ichigotani et al. 2002b). One can postulate that ABI3BP may interact with ABI3 or other Abl interactors to regulate actin cytoskeleton (Ichigotani et al. 2002b, Hirao et al. 2006). However, the mechanism by which ABI3BP inhibits migration and invasion remains to be understood.

The mechanism by which ABI3BP is underexpressed is unknown. PCR amplification and karyotype analysis did not detect the loss of heterozygosity at 3q12 where ABI3BP is located (data not shown). Methylation analysis of the region could find an epigenetic alteration responsible for its expression loss, although using the CpG plot program, we did not find CpG islands in the promoter region of ABI3BP (www.ebi.ac.uk/emboss/cpgplot). Another likely explanation is that an activated tyrosine kinase could lead to downregulation of ABI3BP, as usually occurs with Abl interactors (Ichigotani et al. 2002b).

Interestingly, we previously identified five isoforms of ABI3BP in thyroid tissues, most of them expressed in normal thyroid tissues and not in malignant lesions (Guimaraes et al. 2006). One specific isoform, an insertion of 265 bp in intron 24, which generates a premature stop codon, was found only in FTC. These findings suggest that alternative splicing could be a mechanism responsible for ABI3BP loss of expression. Interestingly, alternative splicing of ABI3BP was also described in murine (Uekawa et al. 2005).
Based on our findings, we suggest that ABI3BP inhibits cell growth, reduces cell viability, promotes senescence, and decreases migration and invasion in vitro. All these features are consistent with the fact that ABI3BP behaves to reduce tumor growth when re-expressed in thyroid cancer cells.

Of note, many of the chemotherapeutic agents used in clinical are assumed to exert their anti-tumor effect through induction of apoptosis. Since ABI3BP may act to suppress growth in thyroid and possibly other tumor types, understanding its molecular mechanism of suppression is of great interest. The possibility is raised if increasing ABI3BP expression levels through pharmacological or other means may be useful to inhibit malignant growth and invasion. Chemotherapy-induced premature senescence could represent an alternative approach to treat tumors that are resistant to conventional therapies.

Declaration of interest
The authors declare that they have no affiliations that would constitute a financial conflict of interest relating to the subject matter of this study.

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