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# TGFβ induces epithelial-mesenchymal transition of thyroid cancer cells by both the BRAF/MEK/ERK and Src/FAK pathways

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**Short title:** $^{V600E}$BRAF and Src/FAK are involved in TGFβ-induced EMT

**Keywords:** $^{V600E}$BRAF; TGFβ; Src/FAK; EMT; thyroid cancer

***Abbreviations:*** ATC, Anaplastic thyroid cancer; DMSO, Dimethyl sulfoxide; EGF, Epidermal growth factor; EMT, epithelial-mesenchymal transition; FAK, Focal adhesion kinase; GSK3β, Glycogen synthase kinase 3 beta; LNM, lymph node metastasis; MAPK, Mitogen-activated protein kinase; NFκB, Nuclear factor κB; PTC, Papillary thyroid cancer; TGFβ, Transforming growth factor; TβRII, TGFβ receptor type II.

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**Abstract**

The epithelial-mesenchymal transition (EMT) is a crucial process in tumour progression, by which epithelial cells acquire a mesenchymal phenotype, increasing its motility and the ability to invade distant sites. Here, we describe the molecular mechanisms by which $^{V600E}$BRAF, TGFβ and the Src/FAK complex cooperatively regulate EMT induction and cell motility of anaplastic thyroid cancer cells. Analysis of EMT marker levels reveals a positive correlation between TGFβ and Snail expression, with a concomitant downregulation of E-cadherin, accompanied by an increase of cell migration and invasion. Furthermore, we show that $^{V600E}$BRAF depletion by siRNA or inhibition of its activity by treatment with its inhibitor PLX4720 reverses the TGFβ-mediated effects on Snail, E-cadherin, migration and invasion. Moreover, $^{V600E}$BRAF induces TGFβ secretion through a MEK/ERK-dependent mechanism. In addition, TGFβ activates the Src/FAK complex, which in turn regulates the expression of Snail and E-cadherin as well as cell migration. The inhibition of Src with the inhibitor SU6656 or abrogation of FAK expression with a specific siRNA reverses the TGFβ-induced effects. Interestingly, we demonstrate that activation of the Src/FAK complex by TGFβ is independent of $^{V600E}$BRAF signalling, since inhibition of this oncogene does not affect its phosphorylation. Our data strongly suggest that TGFβ induces EMT and aggressiveness of thyroid cancer cells by parallel mechanisms involving both the $^{V600E}$BRAF/MEK/ERK and Src/FAK pathways independently. Thus, we describe novel functions for Src/FAK in mediating the EMT program and aggressiveness regulated by TGFβ, establishing the inhibition of these proteins as a possible effective approach in preventing tumour progression of $^{V600E}$BRAF-expressing thyroid tumours.

## Introduction

Papillary thyroid carcinoma (PTC) accounts for 80% of thyroid malignancies. The large majority of PTCs generally exhibits an excellent prognosis with conventional therapy [1]. However, 10-15% of cases progress to more aggressive forms of thyroid cancer, including poorly differentiated thyroid carcinoma (PDTC) and undifferentiated (anaplastic) thyroid cancer (ATC), both associated with local invasion, distant metastases, treatment resistance and poorer clinical outcome [2]. At molecular level, PTCs and ATCs show a high incidence of the activating mutation $^{V600E}$BRAF, which in turn increases the activity of the MAPK-ERK pathway [3]. This mutation has been associated with increased aggressiveness, extrathyroidal extension and a high risk of relapse [3]. Thus, targeted therapies directed toward this oncogene are currently in phase II trials in metastatic thyroid cancer (NCT01286753).

The aggressive behaviour of PTCs and ATCs is mainly the result of an increase of the motility and invasiveness of tumour cells [4-6], which are features related to the so-called epithelial-mesenchymal transition (EMT). EMT is a process in which epithelial cells switch to a mesenchymal phenotype by losing their polarity and acquiring increased motility [7]. Many evidences have shown that this process is abnormally activated during thyroid cancer development. It has been found that thyroid tumour cells from PTCs and ATCs constitutively display an active EMT process as compared to normal thyrocytes, with loss of polarity/cohesiveness, decreased expression of epithelial markers and increased expression of mesenchymal markers, both *in vitro* and *in vivo* [4,6,8-10].

Two hallmarks of the EMT are the loss of the E-cadherin expression and the overexpression of Snail, a zinc finger transcription factor that directly represses E-cadherin expression [7,11]. E-cadherin is commonly observed in normal thyroid gland, benign thyroid lesions and differentiated thyroid cancer [12,13], whereas the loss of its expression is characteristic of thyroid cancer cell lines and invasive human PTCs [4,14]. By contrast, Snail is not expressed in normal thyroid tissue, but it is overexpressed in thyroid cancer cell lines and human PTCs [15,16]. These changes correlate with aggressiveness, lymph node metastasis (LNM), tumour recurrence and poor prognosis [6,13,15-17].

We and others have shown that $^{V600E}$BRAF plays an important role on EMT induction and aggressiveness of tumoral cells [18,19]. Thus, we demonstrated that $^{V600E}$BRAF induces EMT in thyroid cancer cells through changes in Snail and E-cadherin expression levels, which in turn, increase migration and invasion of these cells [18]. Moreover, inhibition of

$^{V600E}$BRAF significantly decreases invasion of thyroid cancer cells, tumour volume and metastasis in a mouse model of ATC [19-22].

TGFβ regulates many biological processes involved in cancer growth and metastasis through activation of receptor-regulated Smad2 and Smad3 downstream proteins or by noncanonical Smad-independent signalling pathways [23]. TGFβ is basally expressed in normal thyrocytes where it has a potent antitumor activity [24]. However, it is overexpressed in a high number of thyroid cancers [16,25], acting as a tumour-promoting factor associated to EMT induction, increased invasion, extra-thyroid extension and LNM [14,16,26]. An association has been proposed between BRAF and TGFβ on aggressiveness induction of PTCs. In fact, it has been shown in rat thyroid cells overexpressing $^{V600E}$BRAF that this oncogene stimulates TGFβ secretion, and both proteins exert the same effect on both E-cadherin expression and invasion [26]. Moreover, TGFβ-induced EMT during progression from PTC to PDTC in an animal model requires BRAF activity [14]. However, different roles for BRAF and TGFβ in infiltrative PTCs have also been reported [27].

TGFβ signalling and its coupling to EMT have also been associated with modulation of Src and focal adhesion kinase (FAK). FAK is activated in response to many extracellular signals that lead to its autophosphorylation at Y397 and its binding to Src, which mediates further phosphorylations of tyrosine residues of FAK [28]. The activated Src/FAK complex transduces signals through different signalling pathways, thus regulating cell proliferation, survival, adhesion, migration and invasion [28,29].

Src and FAK are present in all cells at low basal levels; however their expression or activities are increased in different cancer cells types [28,30], connecting them to EMT-mediated tumour cell migration and invasion [31]. Regarding thyroid cancer, they are overexpressed in a subset of malignant PTCs and ATCs compared to benign thyroid lesions, and their expression is directly associated with the most aggressive phenotypes [30,32,33]. Moreover, inhibition of Src/FAK complex reduces tumour growth in a mouse model of ATC [34-36], indicating that might be considered as a novel therapeutic target in thyroid cancer. The suggestion that Src and FAK might be involved in TGFβ-mediated EMT in cancer cells comes from evidences showing that TGFβ increases the interaction of Src and FAK and that these kinases mediate the EMT induced by this cytokine [37,38].

We have therefore studied the relationship between $^{V600E}$BRAF, TGFβ and Src/FAK complex on EMT induction and tumour invasion in thyroid cancer cells. We found that the

presence of $^{V600E}$BRAF mutation increased TGFβ secretion, which in turn, induced EMT and invasion through activation of Src/FAK signalling. The present study shows the potential therapeutic effectiveness of inhibiting TGFβ and Src/FAK activity, either alone or in combination with BRAF inhibitors, in aggressive $^{V600E}$BRAF-driven thyroid tumours.

## Material and methods

### *Cell lines and in vitro treatments*

The human ATC cell lines 8505C and BHT101, harbouring the $^{V600E}$BRAF mutation, were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and the human FTC cell line WRO carrying $^{wt}$BRAF, was kindly provided by Dr. A. Fusco (Institute of Endocrinology and Experimental Oncology, Naples, Italy). All cell lines were authenticated using standard sequencing techniques and identity was confirmed *vs.* published data [39]. WRO-mock and WRO-VE cells were generated by lentiviral infection as described in Baquero *et al*. [18].

For *in vitro* treatments, cells were incubated with 5 ng/mL human recombinant TGFβ (R&D Systems, Minneapolis, MN, USA) for various periods of time as specified in the text. Where appropriated, cells were treated with either vehicle (DMSO), 5 μM PLX4720 (Axon MedChem, Groningen, The Netherlands), 10 μM U0126 (Promega, Madison, WI, USA) or 10 μM SU6656 (Sigma, St. Louis, MO, USA) for 24 hours.

### *siRNA transfection*

$2.5x10^5$ cells/35-mm well were seeded and transfected the day after using LipofectAMINE (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to manufacturer's protocols. Cells were incubated for 6 h in 1 mL of OPTIMEM medium with 100 nM BRAF (5'-CAGUCUACAAGGGAAAGUG-3'), Snail (5´-GAAUGUCCCUGCUCCACAA-3´), FAK (5´-GGGAGAAGUAUGAGCUUGC-3´), or Silencer™ negative control#1 specific siRNA (Ambion, Life Technologies, Carlsbad, CA, USA). Medium was replaced with 2 mL of fresh medium containing 10% FBS and cells were treated and harvested at the indicated times, as stated in figure legends.

***Western blot and immunoprecipitation analysis***

Total cell extracts preparation and Western blot analysis were performed as previously described [18]. The antibodies used were anti-BRAF and anti-Fibronectin (Santa Cruz Biotechnology, Dallas, TX, USA); anti-E-cadherin, anti-Smad2/3 and anti-FAK (BD Biosciences, Franklin Lakes, NJ, USA); anti-Snail, anti-Src, anti-phospho-Smad2 and anti-phospho-GSK3β (Cell Signalling Technology, Danvers, MA,USA); anti-β−Tubulin (Sigma, St. Louis, MO, USA), phosphorylation site-specific (Y397, Y407, Y576, Y577 and Y861) anti-FAK antibodies (Biosource, Life Technologies, Carlsbad, CA, USA) and peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark). For immunoprecipitation experiments, 1 mg protein was bound to specific antibody or the correspondent IgG control antibody and Western blot was performed using standard protocols.

***Immunofluorescence staining***

Cells cultured on coverslips were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, permeabilized with 0,1% Triton-X-100 and blocked with 1% BSA. After several PBS washes, cells were stained for E-cadherin and Snail with specific antibodies followed by the appropriated anti-mouse Alexa Fluor® 488 and 633 secondary antibodies (BD Biosciences, Franklin Lakes, NJ, USA). Samples were mounted using ProLong® Gold Antifade Mountant with DAPI (Invitrogen, Life Technologies, Carlsbad, CA, USA) and fluorescence was visualized in a confocal microscope Leica TCS-SP5 (Leica microsystem, Wetzlar, Germany).

***RNA extraction and Quantitative RT–PCR (qRT-PCR)***

Total RNA extraction and real-time qRT-PCR analysis were performed as previously described [18]. Fluorescein-based probes and primer sequences for qRT-PCR assays were designed using the Human Universal ProbeLibrary Set and the ProbeFinder Assay Design Software (Roche, Basel, Switzerland). The gene-specific primers were as follows: E-cadherin: forward (5´-GAATGACAACAAGCCCGAAT-3´), reverse (5´-GACCTCCATCACAGAGGTTCC-3´); Snail: forward (5´-GCTGCAGGACTCTAATCCAGA-3´), reverse (5´-ATCTCCGGAGGTGGGATG-3´) and GAPDH: forward (5´-TCCACTGGCGTCTTCACC-3´), reverse (5´-GGCAGAGATGATGACCCTTTT-3´). ΔΔCt method was used to calculate the relative

changes in gene expression; results were normalized by comparison to GAPDH gene expression.

### *Cell migration and invasion assays*

Migration and invasion were examined in transwell cell culture chambers using polycarbonate membranes (Corning, Corning, NY, USA) coated with 0.1 mg/mL collagen type I or Matrigel-coated transwells (BD Biosciences, Franklin Lakes, NJ, USA), respectively. These assays were performed using the same protocol as previously described [18]. Migrated cells were stained with crystal violet and three different cell fields of each well cells were photographed under a phase contrast microscope (Nikon eclipse Ti-S) at 10x magnification. Cells were counted using the ImageJ software. The number of migrated cells in each condition was normalized with the number of migrated cells of the respective controls and expressed as fold increase.

### *ELISA for TGFβ*

Secreted TGFβ protein levels were analysed by ELISA with the Human TGFβ Emax Immunoassay Kit from Promega (Promega, Madison, WI, USA). 2,5x$10^4$ cells/well were seeded into 6-well plates and incubated as described in figure legends. Culture supernatants were collected and TGFβ levels were determined following the manufacturer's instructions.

### *Statistical analysis*

All data are expressed as means ± SEM. In statistical analysis, the student´s *t* test was performed using the SSCStat software (V2.18, University of Reading, United Kingdom).

## Results

### *TGFβ downregulates E-cadherin expression by induction of Snail in thyroid cancer cells*

To investigate the role of TGFβ in thyroid tumour progression as well as the pathways used by this cytokine to induce EMT, we first analysed its effects on Snail and E-cadherin expression in the ATC-derived cell lines 8505C and BHT101. As we have shown before, both cell lines expressed low basal levels of Snail, whereas only the BHT101 cell line expressed high basal levels of E-cadherin [18]. Besides these differences, the treatment with

TGFβ significantly increased Snail expression reaching the peak levels after 24 hour. These levels were maintained for at least 72 hours in both cell lines (Figure 1A). Moreover, this sustained Snail expression is parallel to a time-dependent decrease of E-cadherin expression after 24 h of TGFβ treatment in BHT101 cells (Figure 1A). By contrast, due to the almost undetectable E-cadherin protein basal levels in the 8505C cell line, we could not notice any effect of TGFβ on this protein by Western blot (Figure 1A).

However, by immunofluorescence assays we observed a clear decrease of E-cadherin in plasma membrane and an increase of nuclear Snail expression induced by TGFβ in both cell types (Figure 1B). Moreover, TGFβ induced morphological changes in these cells and increased stress fiber formation, typical features of EMT (data not shown). We then studied whether Snail induction by TGFβ also directly regulated E-cadherin expression in our cells. To this purpose, we silenced Snail expression using a specific siRNA and analysed the protein and mRNA levels of E-cadherin in BHT101 cells. We observed that treatment of BHT101 cells with Snail siRNA decreased both the protein (Figure 1C) and the mRNA levels (supplementary Figure S1) of Snail by about 80%, demonstrating the higher efficiency of this treatment. As expected, the inhibition of Snail expression induced both E-cadherin protein (Figure 1C) and mRNA levels (Figure 1D) when compared to control cells, as we have shown before [18]. On the other hand, TGFβ treatment significantly decreased these levels when compared to untreated cells (Figures 1C, 1D), and Snail abrogation reversed these effects (Figures 1C, 1D). The quantification of TGFβ-regulated E-cadherin protein (supplementary Figure S2) and mRNA (supplementary Figure S3) levels, in the absence or presence of Snail, are similar when compared to their respective controls, demonstrating that E-cadherin expression appears to be not only dependent on Snail. However, we should note that in TGFβ-treated cells, the basal levels of E-cadherin are lower and the Snail levels are higher than those observed under basal conditions, indicating that TGFβ regulates, at least in part, E-cadherin through Snail induction. Consistently with the effect of TGFβ on Snail and E-cadherin expression, we also observed that this cytokine increased the levels of another mesenchymal marker, Fibronectin, in both cell lines (Figure 1E). Together, these data show that TGFβ regulates the expression of EMT markers in thyroid cancer cells.

### ***$^{V600E}$BRAF mediates TGFβ–induced expression of Snail in thyroid cancer cells***

We previously demonstrated that $^{V600E}$BRAF behaves as TGFβ inducing E-cadherin down-regulation through up-regulation of Snail in thyroid tumour cells. This prompted us to study whether the regulation of these EMT markers by TGFβ was related with this oncogene.

We first determined the levels of these proteins in cells treated with TGFβ, in which we previously inhibited the BRAF expression with a specific siRNA. Importantly, the lack of BRAF not only decreased the basal levels of Snail in both cell lines, but also repressed the induction of Snail expression by TGFβ (Figure 2A). As expected, BRAF abrogation increased the basal levels of E-cadherin in 8505C and BHT101 cell and also reversed the inhibition caused by TGFβ in BHT101 cells (Figure 2A). Furthermore, although we could not determine the downregulation of E-cadherin protein expression in 8505C cells treated with TGFβ, due to the low levels of this protein at basal conditions; this cytokine did not decrease the upregulated level of E-cadherin achieved after BRAF silencing (Figure 2A). On the other hand, as expected, the treatment with TGFβ did not modify the levels of ERK phosphorylation in any condition (Figure 2A), since its activation is BRAF-dependent in these cells [18]. We then analysed the Snail and E-cadherin mRNA levels by qRT-PCR under the same conditions and observed similar results (Figure 2B). TGFβ treatment increased the mRNA levels of Snail in both cell lines, whereas this effect was decreased 2-fold after BRAF depletion (Figure 2B). In addition, BRAF silencing increased E-cadherin mRNA in both untreated and TGFβ-stimulated 8505C and BHT101 cells (Figure 2B). However, the treatment of 8505C cells with TGFβ did not affect the expression of this gene since this was already very low in the absence of this cytokine (Figure 2B). Regarding BHT101 cells, the basal levels of E-cadherin mRNA were very high and were decreased by incubation with TGFβ; moreover, despite these high basal levels, silencing of BRAF increased the amount of E-cadherin mRNA by about 50% in control cells and prevented the decrease achieved upon TGFβ treatment (Figure 2B).

To confirm the role of $^{V600E}$BRAF on TGFβ-mediated changes in the EMT markers Snail and E-cadherin, we next determined their levels of both protein and mRNA in 8505C and BHT101 cells treated with the BRAF inhibitor PLX4720 or the MEK inhibitor U0126, either in the absence or presence of TGFβ. Similar to the effect observed with BRAF depletion, PLX4720 treatment decreased both basal and TGFβ-induced Snail protein levels in 8505C and BHT101 cells (Figure 2C). Moreover, this inhibitor exerted similar effects on

Snail mRNA expression (Figure 2D). Likewise, PLX4720 increased the levels of E-cadherin in both unstimulated cell lines and reversed the decrease induced by TGFβ in BHT101 cells (Figures 2C and 2D). Similar results were obtained in the experiments using the MEK inhibitor, U0126 (Figures 2C and 2D).

Several evidences have demonstrated that the MEK/ERK pathway can modulate the canonical TGFβ/Smad signalling pathway, thus we studied whether BRAF abrogation affected TGFβ-regulated levels of Snail and E-cadherin by decreasing the activation of the transcription factor Smad2. 8505C and BHT101 cells displayed undetectable levels of phosphorylated Smad2 in basal conditions and, as expected, TGFβ significantly increased them, whereas BRAF inhibition, achieved by either siRNA or PLX4720 treatment, did not alter those (Figures 2A, 2C). These results indicate that Smad2 activation by TGFβ in these cells is likely to be BRAF independent.

Collectively, these results demonstrate that TGFβ−dependent regulation of Snail and E-cadherin is partially exerted through the $^{V600E}$BRAF/MEK/ERK pathway.

***$^{V600E}$BRAF/MEK/ERK pathway but not TGFβ mediates GSK3β phosphorylation***

GSK3β is a Snail kinase that can bind to and phosphorylates this transcription factor, facilitating its proteasomal degradation. To address whether, in addition to a transcriptional regulation, GSK3β was a convergence point of $^{V600E}$BRAF and TGFβ signalling to regulate Snail, we examined the activity of GSK3β in BHT101 cells by measuring the phosphorylation level of its Ser9 residue, which is indicative of an inactive state. As shown in figure 3A, inhibition of $^{V600E}$BRAF signalling either by abrogation of BRAF expression with siRNA or by treatment with the PLX4720 or U0126 inhibitors, decreased the levels of Ser9 phosphorylation of GSK3β, indicating an increase on its activity (Figure 3A). As expected, GSK3β activation was parallel to a decrease of Snail levels and an increase of E-cadherin expression (Figure 3A). We then examined Ser9 phosphorylation of GSK3β in TGFβ-treated cells, with or without BRAF activity, by treatment with PLX4720 inhibitor. The incubation of BHT101 cells with TGFβ did not affect the Ser9 phosphorylation status of GSK3β neither in control cells nor in PLX4720-treated cells (Figure 3B). These data indicate that $^{V600E}$BRAF can increase Snail expression both at a transcriptional level and through GSK3β inhibition, while TGFβ specifically regulate the expression of this transcription factor by a GSK3β-independent mechanism.

### *TGFβ cooperates with $^{V600E}$BRAF to increase migration and invasion of thyroid cancer cells*

Since TGFβ induces EMT in 8505C and BHT101 cells, and this process is linked to a higher invasiveness of many cancer cells, we next tested whether this cytokine also affected the migration and invasion of these cells. As expected, TGFβ treatment increased by about 50% both the migration and invasion of 8505C and BHT101 cells compared to untreated control cells (Figures 4A, 4B). Given the fact that TGFβ regulates EMT in a BRAF-dependent manner (Figure 2), we next studied whether this oncogene was also involved in the induction of both migration and invasion driven by this cytokine. To this purpose, we performed these assays in cells pretreated with PLX4720 and incubated with TGFβ. Consistent with the data observed with the EMT markers; $^{V600E}$BRAF inhibition decreased both migration and invasion in basal conditions and abolished the increase of these processes achieved upon TGFβ treatment (Figures 4A and 4B). Moreover, the abrogation of BRAF expression with specific siRNA decreased both migration and invasion in basal conditions, as well as repressed the increase achieved by TGFβ treatment (Figure 4C). These results suggest that TGFβ and $^{V600E}$BRAF cooperate to induce higher levels of migration and invasion of thyroid cancer cells.

### *$^{V600E}$BRAF induces TGFβ secretion in thyroid cancer cells*

Because TGFβ is a secreted cytokine and exerts similar effects than $^{V600E}$BRAF on thyroid tumour progression by inducing EMT, cell migration and invasion, we, therefore, studied the possibility that this oncogene increases TGFβ secretion.

First, we analysed the basal levels of TGFβ secreted by WRO cells, which express $^{WT}$BRAF, and the 8505C and BHT101 cells, which harbour the $^{V600E}$BRAF mutant. As shown in Figure 5A, the levels of TGFβ secreted by 8505C and BHT101 cells were increased by 6-fold compared to those corresponding to the WRO cells, suggesting a relationship between $^{V600E}$BRAF expression and TGFβ secretion. To confirm this, we studied the role of $^{V600E}$BRAF on TGFβ secretion in both 8505C and BHT101 cells. In all cases, we observed that silencing of BRAF expression decreased TGFβ protein level in the medium by approximately 30% when compared to control cells (Figure 5B). Moreover, inhibition of either $^{V600E}$BRAF or MEK by incubation with the PLX4720 or U0126 inhibitors,

respectively, also decreased the levels of TGFβ secreted by about 30-50% in both cell lines (Figure 5C). To further confirm that $^{V600E}$BRAF increased TGFβ secretion, we performed similar experiments in WRO-mock and WRO-VE cells, in which we stably overexpressed $^{V600E}$BRAF by lentiviral infection. ELISA assays showed that WRO-VE cells secreted 2-fold more TGFβ to the medium than WRO-mock control cells. In addition, we observed that treatment with PLX4720 or U0126 reversed the increase of TGFβ secretion induced by overexpression of $^{V600E}$BRAF in WRO-VE cells, without affecting the levels of secreted TGFβ in WRO-mock control cells (Figure 5D).

These results demonstrate that $^{V600E}$BRAF increases TGFβ secretion through the MEK/ERK pathway in thyroid tumour cells.

***TGFβ induces EMT through a Src/FAK-dependent mechanism***

Studies in other cell types have linked Src and FAK to the TGFβ-induced EMT. Therefore, we next studied whether TGFβ could exert some of its effects on EMT in our cells beyond $^{V600E}$BRAF through the Src/FAK pathway.

We first analysed the activation of FAK in response to TGFβ in both 8505C and BHT101 cells by measuring the phosphorylation status of the relevant tyrosines Y397, Y407, Y576, Y577 and Y861 involved on its activation. As shown in figure 6A, the autophosphorylation Y397 residue of FAK was constitutively phosphorylated and TGFβ did not induce any change on it in these cells. However, TGFβ treatment for 24 hours increased the phosphorylation at Y407, Y576, Y577 and Y861 residues of FAK and this increase persisted for at least 72h (Figure 6A).

After Y397 phosphorylation, Src associates with FAK and subsequently phosphorylates tyrosine residues Y576 and Y577. Therefore, we examined whether Src was involved in FAK activation by TGFβ. To this purpose, we performed co-immunoprecipitation assays in 8505C cells and found that Src interacted with FAK at basal conditions (Figure 6B). Moreover, TGFβ significantly increased this association as well as enhanced the phosphorylation of Y576 of FAK (Figure 6B). Additionally, we analysed the Src involvement on TGFβ-induced phosphorylation of Y576 and Y577 residues in BHT101 cells treated with the Src inhibitor SU6656. Our data showed that this inhibitor decreased the TGFβ-mediated phosphorylation of both residues (Figure 6C), thus demonstrating that FAK is activated by TGFβ in a Src-dependent manner.

In order to determine the role of Src/FAK complex in TGFβ-induced EMT, we inhibited the expression of FAK by siRNA and measured both the Snail and E-cadherin levels in 8505C and BHT101 cells (Figure 6D). FAK silencing decreased Snail protein basal levels and reversed the higher levels induced by TGFβ in both cell lines. Moreover, abrogation of FAK expression increased basal levels of E-cadherin and prevented the decrease produced by TGFβ in BHT101 cells (Figure 6D). Similar results were obtained when we inhibited Src with SU6656 inhibitor; Src inhibition decreased both basal and TGFβ up-regulated Snail levels in both cell lines and increased E-cadherin levels in both unstimulated and TGFβ-treated BHT101 cells (Figure 6E). We further evaluated the effect of FAK on TGFβ-induced migration. FAK knock-down reversed the increased motility induced by TGFβ (Figure 6F). These results all together demonstrate the involvement of Src/FAK signalling in both EMT and migration induced by TGFβ in thyroid cancer cells.

Since we have observed that TGFβ regulates Snail and E-cadherin expression through BRAF/MEK/ERK and Src/FAK signalling pathways, we finally studied whether there was any relationship between them. First, we analysed whether $^{V600E}$BRAF was involved in the phosphorylation of FAK by TGFβ in 8505C and BHT101 cells observing that inhibition of BRAF by PLX4720 treatment did not alter either the basal or the increased phosphorylation of Y576 and Y577 produced by TGFβ (Figure 7A). Then, we studied the levels of Snail and E-cadherin in cells in which we inhibited $^{V600E}$BRAF and Src simultaneously. As shown in figure 7B, the simultaneous inhibition of $^{V600E}$BRAF and Src resulted in a greater reduction of Snail levels compared to single inhibition in both unstimulated and TGFβ-stimulated cells (Figure 7B). We also observed that the levels of E-cadherin in 8505C cell were restored at the same extent only after treatment with PLX4720 alone or in combination with SU6656 (Figure 7B). However, in the case of BHT101 cells, the joint $^{V600E}$BRAF and Src inhibition further increased the expression of E-cadherin than inhibition of each of these proteins individually (Figure 7B). Moreover, all these treatments did not inhibit TGFβ-induced Smad2 phosphorylation (Figure 7B). Finally, we determined cell migration and invasion of these cells under the same conditions (Figure 7C). We observed that PLX4720 or SU6656, when incubated alone, reversed the increased cell migration and invasion induced by TGFβ. In addition, the simultaneous inhibition of $^{V600E}$BRAF and Src decreased cell motility similarly to that observed after the inhibition of $^{V600E}$BRAF alone (Figure 7C). All these results indicate that $^{V600E}$BRAF and the Src/FAK pathway act independently in the TGFβ-mediated

EMT in thyroid cancer cells through non-canonical pathways that do not involve Smad2 activation.

## Discussion

$^{V600E}$BRAF and TGFβ have been related with higher aggressiveness of certain thyroid cancers. Here, we show, for the first time, that TGFβ induces migration and invasion of thyroid cancer cells by promoting an EMT signature that requires two independent pathways: MAPK activation by $^{V600E}$BRAF and activation of the Src/FAK signalling complex.

TGFβ increases the expression of the mesenchymal marker Fibronectin and the transcriptional repressor Snail, which in turn, promotes the down-regulation of E-cadherin expression. These changes are accompanied by an increase in cell migration and invasion of ATC-derived cell lines. Our data are similar to those showing that TGFβ partially regulates EMT in both thyroid tumoral cell lines and animal models of thyroid tumours [9, 14, 26]. The higher levels of TGFβ and the mesenchymal phenotype observed in the invasive front of thyroid tumors, compared with their central regions, suggest a key role of TGFβ as inductor of EMT and metastasis [16,26,27]. Furthermore, the cells within the invasive front of human thyroid cancer also display a different expression pattern of genes involved in EMT in comparison to the central part of the tumour [4]. In this sense, thyroid tumours associated with higher aggressiveness showed an increased expression of Snail, mainly detected in the invasive front, compared to normal thyroid tissue [15,17]. Most recently, Wan et al. have shown a significant positive correlation between higher expression levels of TGFβ and Snail in PTCs regarding to normal thyroid samples, which are also associated with LNM [16]. Additionally, the loss of E-cadherin expression in the invasive tumour front has also been identified as a risk factor associated with increased aggressiveness of PTCs [6]; thus being considered a hallmark of progression from poorly differentiated PTCs to undifferentiated ATCs [40]. In our study we used the 8505C and BHT101 cells lines derived from ATC, which show different basal levels of E-cadherin. These differences could be due to the different origin of these cells lines; 8505C cells were established from a primary undifferentiated ATC, whereas BHT101 cells were established of a lymph node metastasis derived from an ATC. Therefore, these cells could be in different stages of the reversible EMT/MET, necessary for the invasion and colonization of new tissues by tumoral cells. Alternatively, the differences in E-cadherin expression may be a consequence of the different

mutational status of these cells: 8505C are homozygous for $^{V600E}$BRAF, whereas BHT101 are heterozygous.

Here, we also demonstrate that the effects of TGFβ on Snail and E-cadherin levels, as well as cell migration and invasion are mediated by a $^{V600E}$BRAF signalling pathway-dependent mechanism. These results are consistent with those obtained by Riesco-Eizaguirre et al., who demonstrated that TGFβ and MEK cooperate to increase the invasion of rat thyrocytes cells overexpressing $^{V600E}$BRAF [26]; as well as with those showing that TGFβ requires MEK activation to induce EMT in a mouse model of $^{V600E}$BRAF-PTC [14]. Thus, although cooperation between TGFβ and $^{V600E}$BRAF with the higher aggressiveness of cancer cells has been previously proposed, this is the first study demonstrating the relationship of molecular effects between TGFβ and $^{V600E}$BRAF on EMT of human thyroid tumour cells. We first demonstrated that $^{V600E}$BRAF and TGFβ regulate Snail expression at transcriptional level, since both modulated its mRNA levels in these cells. It has been previously observed that overexpression of $^{V600E}$BRAF in thyroid cells affect the TGFβ transcriptional activity in PTCs developed in mice [14]. However, our observations differ in that $^{V600E}$BRAF inhibition failed to suppress Smad2 activation by TGFβ, indicating that this oncogene is involved in TGFβ-induced EMT acting either downstream of Smad2 activation or independently of the TGFβ canonical pathway. Secondly, we have shown that $^{V600E}$BRAF, but not TGFβ, regulated Snail also at post-transcriptional level through inactivation of GSK3β, which phosphorylated Snail and induced its degradation [41]. Given that inhibition of either $^{V600E}$BRAF or MEK increased it activity by decreasing the levels of its inhibitory phosphorylation at Ser9. However, TGFβ does not have any effect on the GSK3β activity, opposite to that reported by Lan et al., who demonstrated that TGFβ induced EMT in human proximal tubular epithelial cells HK-2 by inhibiting this kinase through the activation of AKT2 [42]. Consistently with the absence of effect of TGFβ on GSK3β activity in our cells, we have not observed an increase in AKT phosphorylation in TGFβ-treated cells (data not shown). All these results, together with the fact that TGFβ was able to further increase the Snail expression, migration and invasion in the presence of $^{V600E}$BRAF suggest that this cytokine is not exerting its functions only through this oncogene, but also through another signalling pathway. In this sense, we here demonstrate that human thyroid cancer cells harbouring $^{V600E}$BRAF mutation secrete much higher levels of TGFβ than cells carrying $^{WT}$BRAF and that this increased secretion is dependent on the activity of this oncogene.

Similar data described that overexpression of $^{V600E}$BRAF in rat thyroid cells induced an increase of TGFβ secretion, which was associated with invasion and nodal metastasis by an autocrine loop [26]. Thus, the existence of a possible autocrine TGFβ loop reinforces the idea that this cytokine has a strong cooperative effect on $^{V600E}$BRAF-induced EMT, migration and invasion through activation of another signalling pathway.

The Src/FAK complex is related to changes associated with the EMT in cancer [28,31], being proposed that these kinases could mediate the EMT induced by TGFβ [37,38,43]. Here, we provide the first demonstration of a Src/FAK functional role in TGFβ-mediated EMT and migration in human thyroid cancer cells. Importantly, we found that TGFβ activated the Src/FAK complex by increasing the phosphorylation of both proteins, without affecting their expression levels. Moreover, we demonstrated that Src/FAK signalling was required for TGFβ-mediated Snail activation, E-cadherin down-regulation and increased migration. The Src/FAK activation by TGFβ could be explained by the cooperation of these kinases with the EGF/ErbB receptor system. In this sense, it has been proposed that this cytokine enhances ErbB-initiated signal transduction [43,44], and a mutual regulation of TGFβ, TβRII and EGFR expression has also been observed in human thyroid carcinomas [45].

The functional role of Src/FAK pathway on expression of Snail and E-cadherin is not fully understood. For instance, the reexpression of FAK drives Snail-induced EMT in FAK-null embryonic cells [46]. Moreover, the ability of FAK to induce EMT and its association with the aggressive phenotype of thyroid carcinomas has been linked to its overexpression [30]. Despite these data, the absence of an effect of this kinase on TGFβ-mediated Snail and E-cadherin regulation has also been reported [37]. In this study, we have demonstrated that TGFβ did not up-regulate FAK protein levels but only increased its phosphorylation. On the other hand, contradictory results regarding Src have also been shown; while some authors demonstrated that Src does not play a role in TGFβ-induced EMT [47,48], others have reported that Src promoted it [34,49,50]. Thus, it remains to be clarified whether the involvement of Src/FAK in TGFβ-induced EMT is cell type specific, or whether it is associated with the stage of malignancy within a given tumour context.

Different mechanisms can be proposed by which Src/FAK signalling regulates Snail expression in thyroid cancer cells. In our model, inhibition of Src did not affect Smad2 activation by TGFβ, reinforcing the idea that this cytokine regulates Snail independently of

its canonical pathway. Thus, a possible candidate could be the NFκB pathway, considering that this transcription factor regulates Snail expression [51] and that the Src/FAK complex has been associated with its activation [52-54]. Alternatively, TGFβ-induced EMT and cell migration may be mediated through a Src/FAK/p38-MAPK-dependent pathway. In this regard, it has been shown that FAK activates p38-MAPK [52] and that Src regulates stimulation of this kinase by TGFβ during invasion and proliferation of cancer cells [47,55-57]. Lastly, it is also possible that Src/FAK up-regulate Snail expression through the FAK substrate p130CAS, since it has been recently shown that this protein is required for TGFβ-mediated EMT in lung cancers [58].

Interestingly, our study demonstrated that $^{V600E}$BRAF did not affect the TGFβ-induced Src/FAK activation and the Snail induction achieved by this complex. Surprisingly, here we also found that Src was involved in the up-regulation of E-cadherin only in BHT01 cells, independently of $^{V600E}$BRAF. Similar results were shown by Schweepe et al, demonstrating that FAK is phosphorylated and regulated by Src in PTC and ATC cells and that Src induction of invasion was independent of MAPK [34]. Moreover, these data reinforces our previous hypothesis considering that E-cadherin expression in thyroid cancer cells is not only dependent on its repressor Snail, but also on other different molecular alterations that are required to restore its expression [18].

Our findings undoubtedly demonstrate that TGFβ-mediated effects on EMT and cell motility requires of $^{V600E}$BRAF-signalling pathway and Src/FAK complex activation. These observations suggest that in human thyroid cancer cells $^{V600E}$BRAF induces TGFβ secretion, which in turn activates Src/FAK complex by an autocrine loop, leading to EMT induction to cooperate with this oncogene on neoplastic progression and acquisition of invasive properties.

The use of RAF inhibitors has revealed the complexity of this oncogene´s signalling pathway in cancer cells. In this sense, different studies have demonstrated that inhibition of BRAF with RAF inhibitors resulted in a “paradoxical activation” of the MEK/ERK pathway in cells with $^{WT}$BRAF or kinase-impaired BRAF mutant, as well as those with RAS mutation, promoting that some patients treated with the BRAF inhibitor, Vemurafenib, experience squamous cell carcinomas and/or keratoacanthomas, as well as other secondary cutaneous lesions [59]. Moreover, despite the encouraging results obtained, the duration of the response to BRAF inhibitors is limited because tumors quickly develop resistance via molecular alterations with other pathway components [59]. Similar facts have been observed after using

TGFβ signalling inhibitors, which have shown efficacy in preclinical models, abrogating EMT *in vivo* and the formation of metastasis, but also led to biochemical resistance of tumor cells to the drug, inducing undesirable and opposite effects in driving EMT in a mouse model of skin carcinogenesis [23, 60]. Regarding Src, although Src inhibitors have shown their potential to reduce tumor growth and metastasis in mouse models, some evidence have shown that they play a role in resistance to $^{V600E}$BRAF inhibition [36, 61].

Thus, despite being very beneficial for tumor suppression, the use of BRAF, TGFβ or Src inhibitors as monotherapy shows undesirable effects under some circumstances. For these reasons, understanding the mechanisms responsible for the unexpected effects of these cancer therapies is very important and could contribute to the development of effective new anticancer therapies. In addition, many studies show that regardless of the agent chosen, it might be necessary to use a combination of drugs to effectively treat BRAF-mutant tumors. Therefore, although a full characterization of the role of TGFβ and Src/FAK on the aggressiveness of thyroid tumour cells bearing the $^{V600E}$BRAF mutation needs to be addressed, we propose that inhibition of TGFβ and the Src/FAK complex alone or in combination with $^{V600E}$BRAF inhibition could underlie the development of novel therapeutic approaches in advanced thyroid cancer with very aggressive phenotypes, that currently lack an effective treatment.

## Conflict of Interest

No potential conflicts of interest are disclosed by the authors.

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**Figure legends**

**Figure 1.** TGFβ induces EMT in thyroid cancer cells. (A) 8505C and BHT101 cell were incubated with TGFβ for different times. Snail and E-cadherin protein expression are shown from a representative Western Blot. Membranes were reprobed with an anti-β-Tubulin antibody as a loading control. (B) Immunofluorescence assay of Snail (red) and E-cadherin (green) expression in 8505C and BHT101 cells incubated with TGFβ for 24 h. Nuclear staining with DAPI was used as control. Photographs were taken at 10X magnification. (C) Representative Western Blot for E-cadherin, Snail and β-Tubulin expressions 72 h after transfection of BHT101 cells with siRNA oligonucleotides specific for Snail (siSnail) or a scrambled oligo control (sc). (D) E-cadherin mRNA levels measured in BHT101 cells transfected as in (C), RNA was isolated and used in qRT-PCR for E-cadherin and GAPDH, used as control, genes. The mRNA levels were normalized to GAPDH and the results were expressed as the changes in mRNA expression. Data showing the mean ± SEM are compiled from three independent experiments performed in triplicate. (E) Fibronectin levels detected by Western Blot in 8505C and BHT101 cell treated as in (B). Blots are representative of experiments performed three times with similar results. Significant differences compared to the corresponding controls: ***$p<0.001$ siSnail *vs* sc and siSnail+TGFβ *vs* sc; $^{\#\#\#}p<0.001$ siSnail+TGFβ *vs* siSnail.

**Figure 2.** $^{V600E}$BRAF inhibition repressed TGFβ-induced EMT. (A) Western blot analysis of Snail, E-cadherin, phospho-ERK (p-ERK) and phospho-Smad2 (p-Smad2) in 8505C and BHT101 cells transfected with siRNA oligonucleotides specific for BRAF (siBRAF) or a scrambled oligo control (sc) for 72 h, and incubated the last 24 h in the absence or presence

of TGFβ. For each pair of rows, images of the reprobed membranes with anti-BRAF and anti-β-Tubulin antibody as controls are shown. (B) Snail and E-cadherin mRNA levels measured in 8505C and BHT101 cells treated as in (A). Total RNA was prepared and qRT-PCR was carried out using specific primers for Snail, E-cadherin and GAPDH, used as control, genes. The mRNA levels were normalized to GAPDH and the results were expressed as the changes in mRNA expression. The data shown represent the mean ± SEM of three independent experiments performed in triplicate with similar results. (C) 8505C and BHT101 cells were incubated for 24 h with DMSO (-), PLX4720 (PLX) or U0126 (U0) alone or with TGFβ, and Snail, E-cadherin, p-ERK and p-Smad2 expression were detected by Western Blot. Blots were reprobed with anti-β-Tubulin as control. (D) 8505C and BHT101 cells were incubated as in (C), RNA was isolated and used in qRT-PCR for detection of Snail, E-cadherin and GAPDH transcripts. Data showing the mean ± SEM are compiled from three independent experiments performed in triplicate. Blots are representative of experiments performed three times with similar results. Significant differences compared to the corresponding controls: *p<0.05, **p<0.01 and ***p<0.001 (siBRAF, sc+TGFβ or siBRAF+TGFβ *vs* sc in panel B) (PLX, U0, TGFβ, PLX+TGFβ or U0+TGFβ *vs* DMSO in panel D); $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001(siBRAF+TGFβ *vs* sc+TGFβ sc in panel B) (PLX+TGFβ and U0+TGFβ *vs* TGFβ in panel D).

**Figure 3.** $^{V600E}$BRAF/MEK/ERK pathway but not TGFβ increases GSK3β phosphorylation. (A) Phosphorylation of GSK3β (p-GSK3β) at serine 9 (S9), expression of Snail, E-cadherin and β-Tubulin, as control, detected by Western blot in BHT101 cells transfected with siRNA oligonucleotides specific for BRAF (siBRAF) or a scrambled oligo control (sc); or treated

with DMSO (-), PLX4720 (PLX) or U0126 for 24 h. (B) BHT101 cells were incubated for 24 h with DMSO (-) or PLX4720 (PLX) alone or with TGFβ and phospho-GSK3β(S9), Snail and E-cadherin expression was detected by Western Blot; β-Tubulin was used as control. Blots are representative of experiments performed three times with similar results.

**Figure 4.** Increased migration and invasion induced by TGFβ depends on $^{V600E}$BRAF signalling. (A) Migration and (B) invasion of 8505C and BHT101 cells treated with DMSO (-) or TGFβ, in the absence or presence of PLX4720 (PLX), for 24 h and measured in transwell cell culture chambers coated with Collagen I (A) or Matrigel (B). (C) Cell migration and invasion of 8505C and BHT101 cells transfected with siRNA oligonucleotides specific for BRAF (siBRAF) or a scrambled oligo control (sc) for 72 h, and incubated the last 24 h in the absence or presence of TGFβ. Pictures taken at 10X magnification are from one representative experiment. The data shown represent the mean ± SEM of three independent experiments performed in duplicate and results expressed as relative units referred to each control. Significant differences compared to the corresponding controls: **p<0.01 and ***p<0.001 (PLX, TGFβ or PLX+TGFβ *vs* DMSO in panel A and B) (siBRAF, sc+TGFβ or siBRAF+TGFβ *vs* sc in panel C); $^{###}$p<0.001 (PLX+TGFβ *vs* TGFβ in panels A and B) (siBRAF+TGFβ *vs* sc+TGFβ sc in panel C).

**Figure 5.** $^{V600E}$BRAF induces secretion of TGFβ in thyroid tumour cells. (A) Active TGFβ was detected by ELISA in supernatants of WRO, 8505C and BHT101 cells incubated for 24h in serum-free medium. (B and C) Secreted active TGFβ was detected by ELISA in supernatants collected from 8505C and BHT101 cells incubated for 24 h in serum-free

medium after transfection with siRNA control (sc) or specific BRAF siRNA (siBRAF) for 48 h (B), or incubated for 24 h with DMSO as vehicle control (-), PLX4720 (PLX) or U0126 (U0) in medium without serum (C). (D) TGFβ levels secreted from WRO-mock and WRO-VE cells treated as in C. TGFβ levels were normalized with protein concentration and the ratios expressed as fold induction over control. Results shown are the means ± SEM of three independent experiments performed in triplicate. Significant differences compared to the corresponding controls: **p<0.01, treated *vs* untreated cells, and $^{###}$p<0.001, control WRO-VE *vs* control WRO-mock.

**Figure 6.** TGFβ regulates EMT and cell migration by a FAK/Src-dependent mechanism. (A) Phosphorylation of different FAK residues detected by western blotin 8505C and BHT101 cells incubated with TGFβ for different times, using the appropriate phospho-specific antibodies. The membrane was reprobed with anti-FAK as control. (B) Endogenous FAK was immunoprecipitated from 8505C control and TGFβ-treated cells for 24h and bound Src was analysed by Western blot. Expression of precipitated FAK and phospho-FAK (Y576) in the immunoprecipitated, and Src levels in cell lysates were assessed as controls. (C) FAK phosphorylation at Y576 and Y577 of BHT101 cells treated with TGFβ for 24 h, in the absence or presence of the Src inhibitor SU6656 (SU), evaluated using Western blot analysis. (D) Snail, E-cadherin, FAK and β-Tubulin expressions 72 h after transfection of 8505C and BHT101 cells with siRNA oligonucleotides specific for FAK (siFAK) or a scrambled oligo control (sc) and treated with TGFβ for the last 24 h. (E) Representative western blots for Snail and E-cadherin expressions of 8505C and BHT101 cells treated as in C. Blots are from one representative experiment performed three times with similar results. (F) Cell migration of 8505C and BHT101 cell treated with TGFβ for 24 h, in which the FAK expression was

depleted with specific siRNA (siFAK). Data showing the mean ± SEM are compiled from three independent experiments performed in triplicate. Significant differences compared to the corresponding controls: ***$p<0.001$, sc+TGFβ *vs* sc; $^{###}p<0.001$, siFAK+TGFβ *vs* sc+TGFβ.

**Figure 7.** $^{V600E}$BRAF and Src/FAK act independently in TGFβ-mediated EMT. (A) Western blot analysis of phospho-FAK at Y576 and Y577 of both 8505C and BHT101 cells treated with TGFβ for 24 h, in the absence or presence of PLX4720 (PLX). (B) Expression of Snail, E-cadherin and phospho-Smad2 in 8505C and BHT101 cells incubated with PLX4720 (PLX), SU6656 (SU) or both (PLX/SU), alone or with TGFβ for 24 h. For each pair of rows, an image of the reprobed membrane with anti-Smad 2/3 as control is shown. Blots are representative of experiments performed three times with similar results. (C) Cell migration of 8505C and BHT101 cell treated as in (B). Results shown are the means ± SEM of three independent experiments performed in triplicate. Significant differences compared to the corresponding controls: **$p<0.01$ and ***$p<0.001$, PLX, PLX+SU, TGFβ, PLX+TGFβ or PLX+SU+TGFβ vs DMSO; ###$p<0.001$, PLX+TGFβ, SU+TGFβ or PLX+SU+TGFβ vs TGFβ.

FIG 1. Baquero P. *et al.*

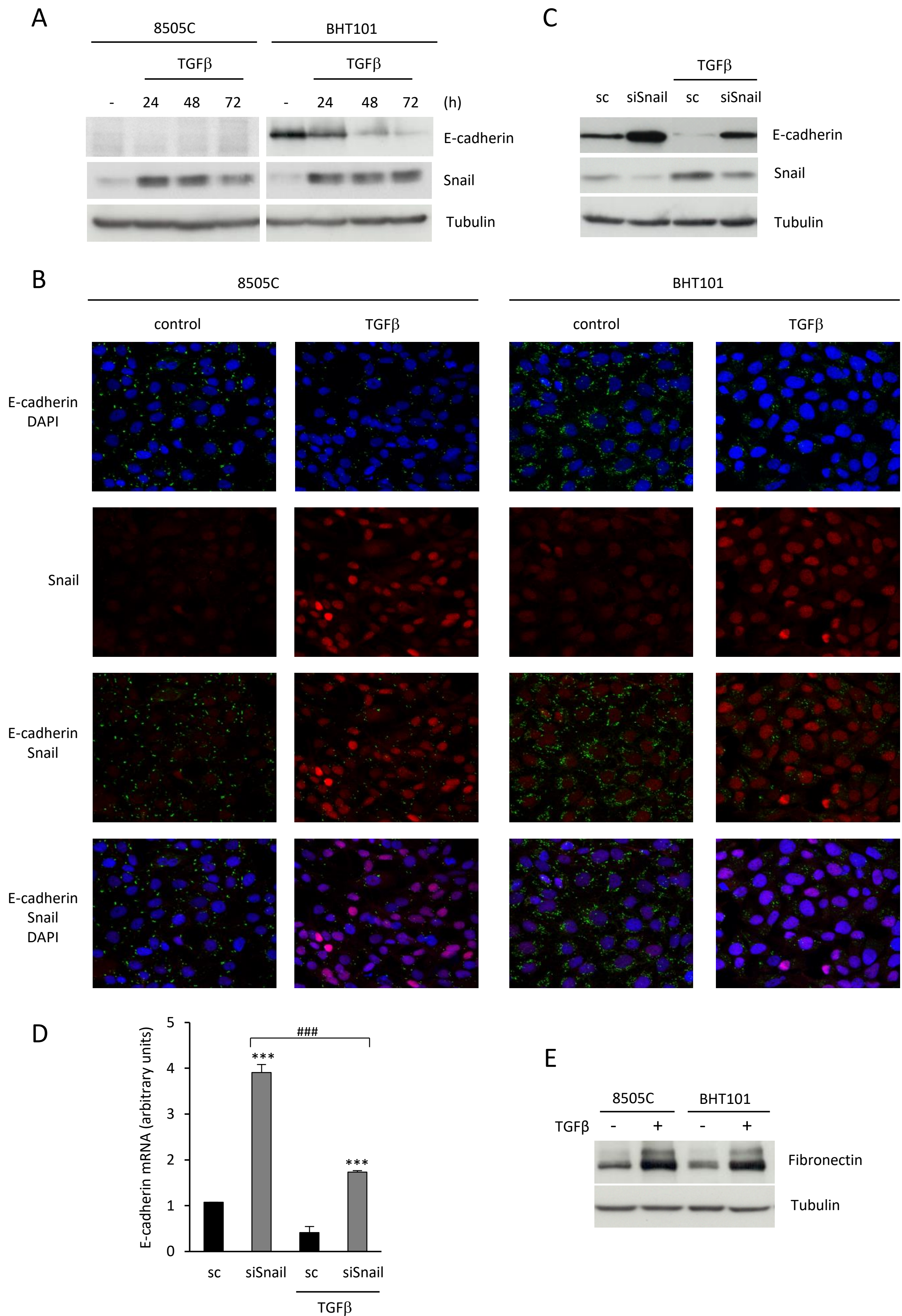

FIG 2. Baquero P. *et al.*

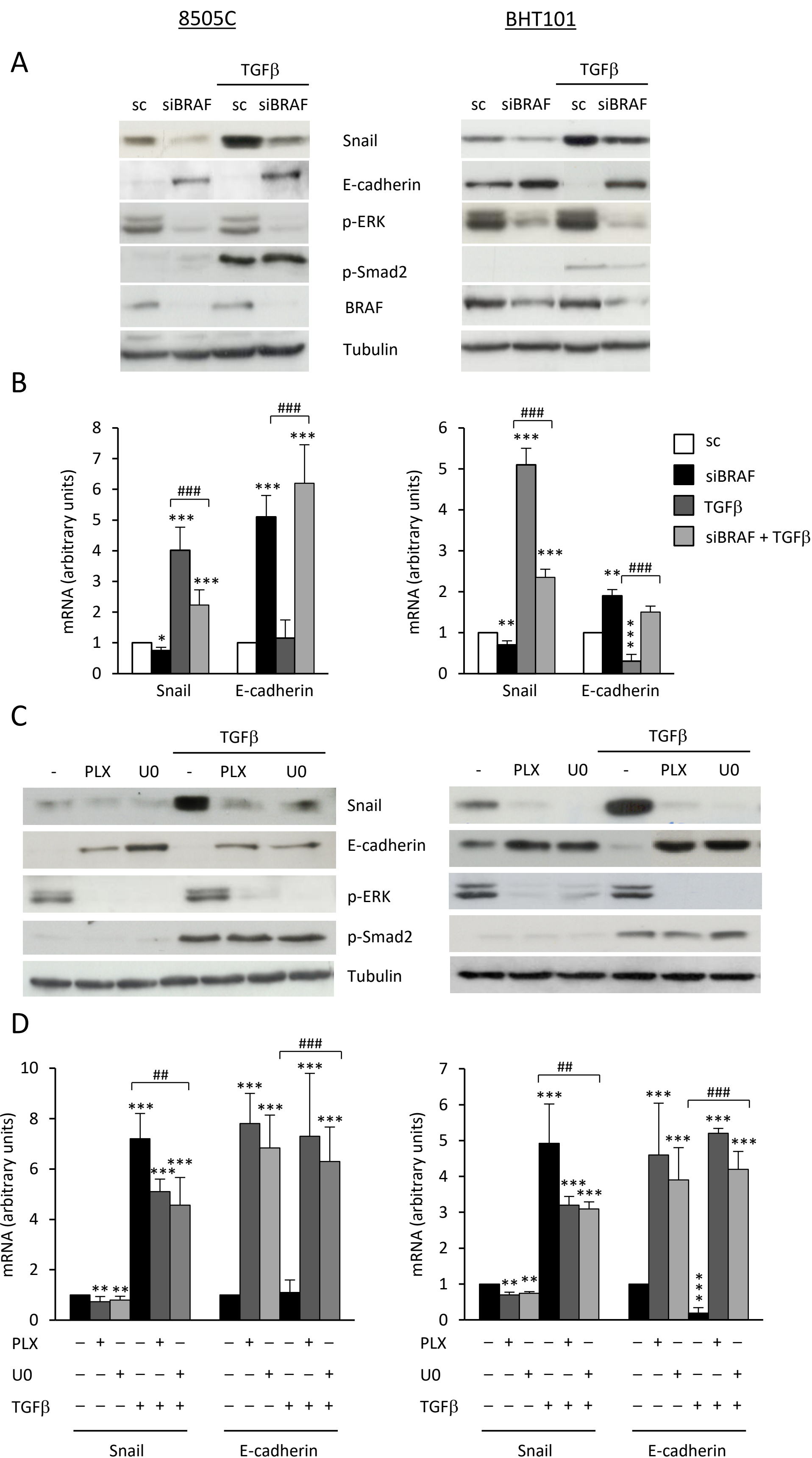

FIG 3. Baquero P. *et al.*

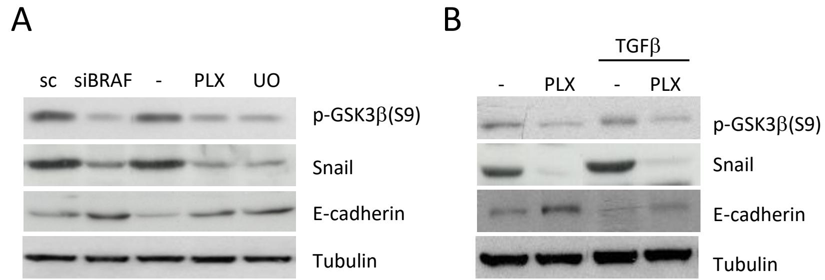

FIG 4. Baquero P. *et al.*

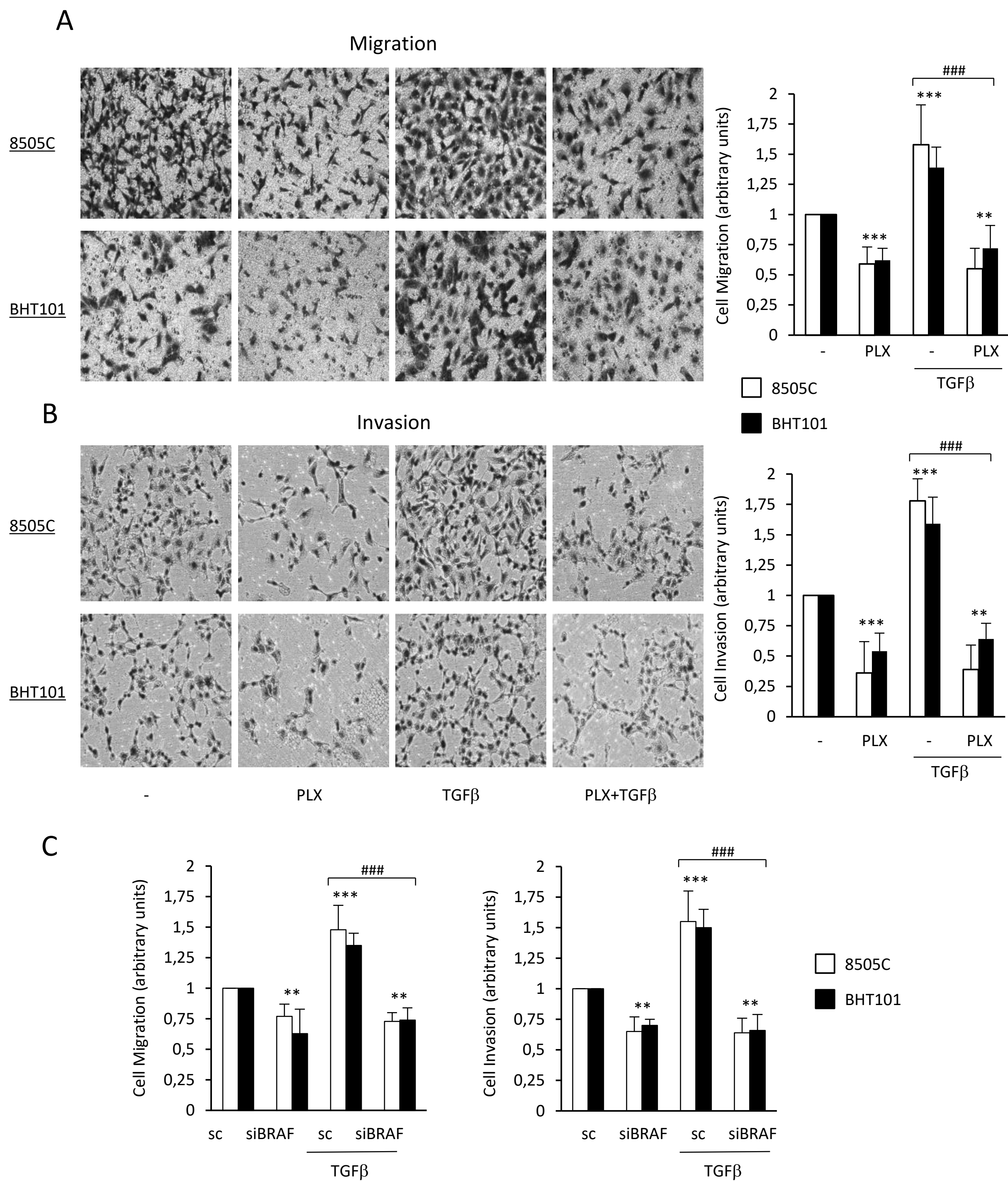

FIG 5. Baquero P. *et al.*

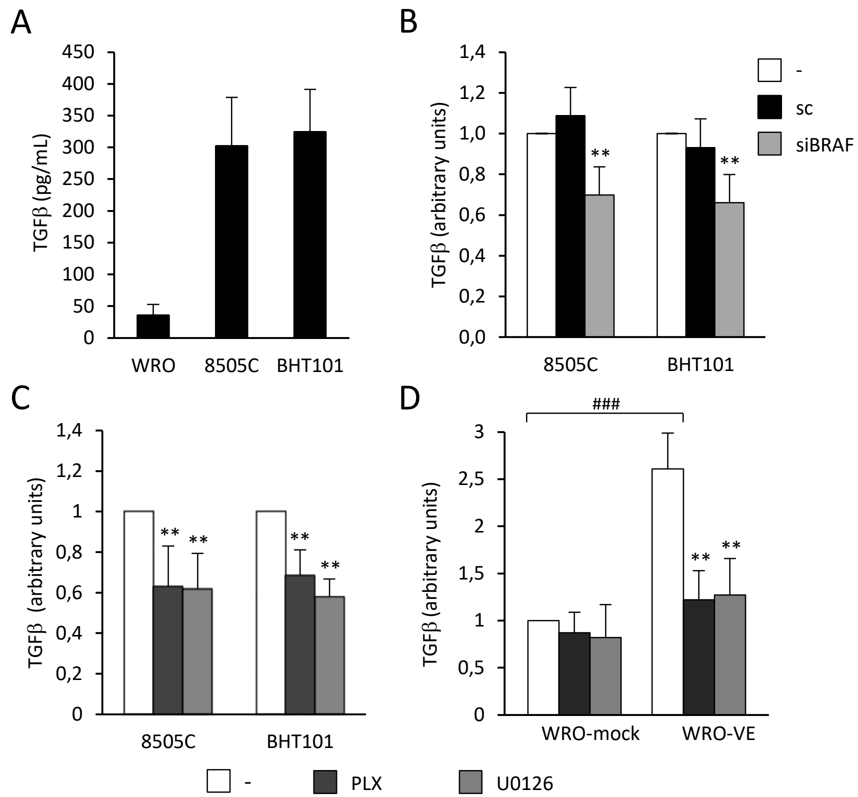

FIG 6. Baquero P. *et al.*

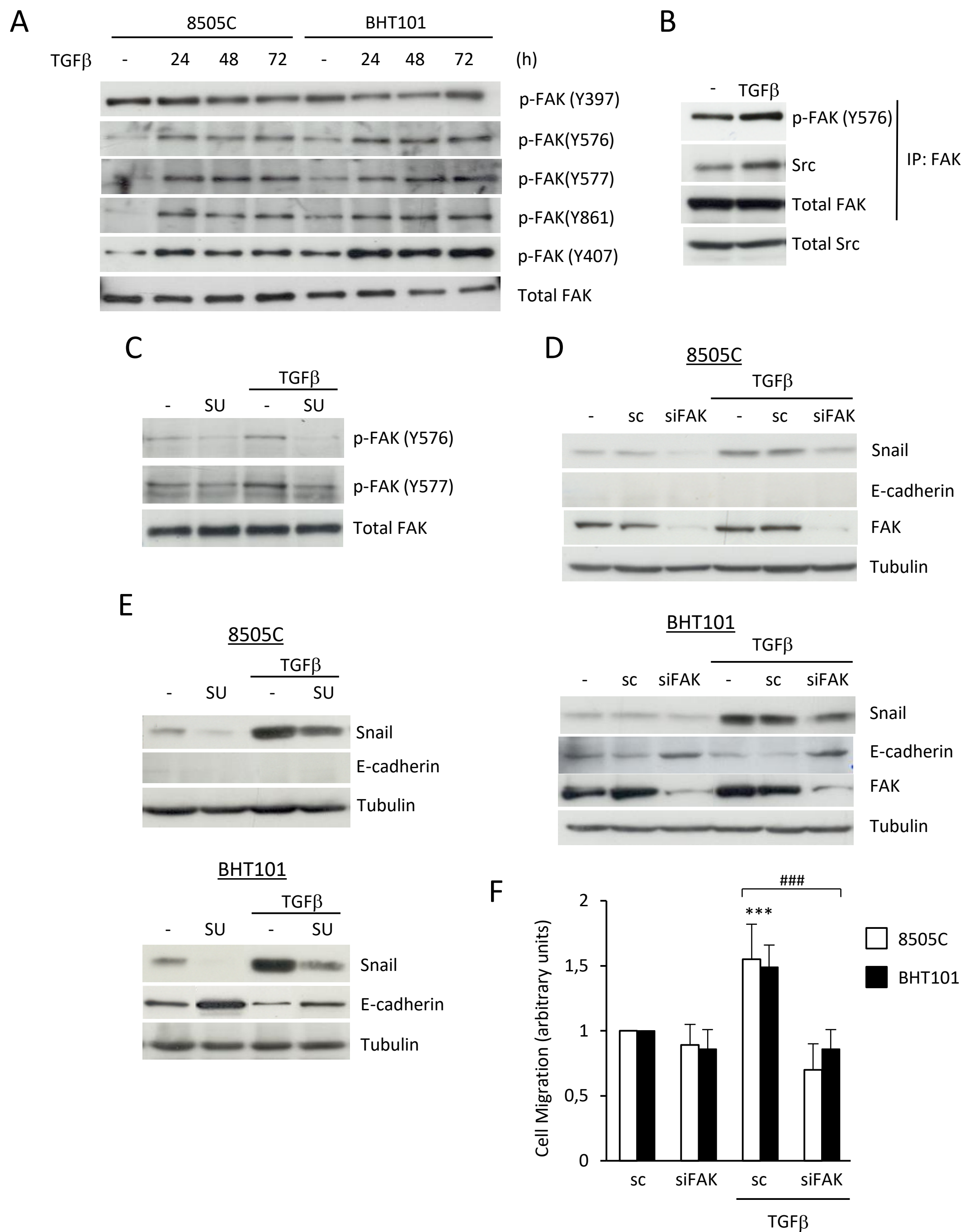

FIG 7. Baquero P. *et al.*