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^{V600E}BRAF promotes invasiveness of thyroid cancer cells by decreasing E-cadherin expression through a Snail-dependent mechanism

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Abstract

BRAF is a main oncogene in human thyroid cancer. Here, we show that BRAF depletion by siRNA or inhibition of its activity by treatment with BRAF inhibitor PLX4720 decreases migration and invasion in thyroid cancer cells expressing oncogenic ^{V600E}BRAF through a MEK/ERK-dependent mechanism, since treatment with the MEK inhibitor U0126 exerts the same effect. Moreover, over-expression of ^{V600E}BRAF increases migration and invasion of wild-type BRAF thyroid cells. Using the same strategies, we demonstrate that these effects are mediated by upregulation of the transcriptional repressor Snail with a concomitant decrease of its target E-cadherin, both hallmarks of EMT. These results reveal a novel ^{V600E}BRAF-induced mechanism in thyroid tumours progression and provides a rationale for using the PLX4720 inhibitor to target ^{V600E}BRAF signalling to effectively control progression of thyroid cancer.

1. Introduction

Papillary thyroid cancer (PTC) is the most common type of thyroid cancer, accounting for 80% of all cases. Patients displaying PTCs have a good prognosis with appropriated therapy, whereas a small percentage of them develop a progressive and aggressive disease that fail standard treatments [1]. Anaplastic thyroid cancer (ATC) is relative uncommon, accounting for less than 2% of all thyroid cancer, but is extremely aggressive, with a dismal prognosis despite various therapeutic modalities, and a high mortality rate [2].

^{V600E}BRAF is the most common mutation found in PTCs; present in 44% of cases, and in 24% of ATCs derived from PTCs, but not in other types of thyroid tumours such as follicular thyroid cancers (FTCs) [3]. This ^{V600E}BRAF mutant has constitutive protein kinase activity promoting sustained activation of its direct downstream target, MEK, and of the ERK1/2 kinases in turn [4,5]. ^{V600E}BRAF plays an important role in thyroid tumours. Thus, overexpression of ^{V600E}BRAF in rat thyroid cells increases their proliferation and invasiveness, and transgenic mice overexpressing ^{V600E}BRAF in the thyroid gland develop invasive PTCs [6,7]. On the other hand, abrogation of ^{V600E}BRAF expression prevents the transformation of cell lines derived from human PTCs, inhibiting proliferation and invasiveness, and in a orthotopic thyroid tumours its inhibition decreases tumour growth and aggressiveness [3,8]. These results are consistent with the clinico-pathological data found in human PTCs and ATCs harbouring this mutation, where a close relationship has been shown between the presence of ^{V600E}BRAF and the progression to advanced stages of tumour, extrathyroidal invasion, lymph node metastases and tumour recurrence [3,8].

Recent studies have identified that PTCs developed in mice by overexpression of ^{V600E}BRAF are susceptible to TGF-β–induced epithelial to mesenchymal transition (EMT) through an ERK-dependent mechanism [9]. EMT is a developmental process defined by the loss of epithelial-specific characteristics, the acquisition of a fibroblast-like morphology, reduced cellular adhesion and increased motility [10]. In addition, EMT is often associated with the invasion and metastatic ability of tumour cells. Thus, the disruption of the intercellular junctions causing dissociation from surrounding cells and the acquisition of a migratory mesenchymal-like characteristic enables tumoral cells to migrate away from the original tissue, invading the stromal components [10,11]. From a molecular point of view, EMT is characterized by the loss of epithelial markers, including E-cadherin and cytokeratins and up-regulation of mesenchymal markers, such as fibronectin, vimentin and N-cadherin [10].

Cadherins are transmembrane proteins involved in cell-cell interaction and the loss of the prototypical member, E-cadherin, is considered the hallmark of EMT. The loss of E-cadherin has been associated with increased invasion, metastatic dissemination and poor prognosis in several solid tumours [10,12], while its re-expression in tumour cells lacking this protein decreases migration and induces a more epithelial-like phenotype [13,14]. E-cadherin is expressed in normal thyroid gland and in benign thyroid lesions [15], whereas its expression is decreased in poorly differentiated thyroid carcinomas from transgenic mice overexpressing ^{V600E}BRAF and in human PTCs [9,15,16].

The E-cadherin gene (CDH1) is frequently down-regulated by specific transcriptional repressors including zinc finger proteins of the Snail family Snail (SNAI1) and Slug (SNAI2), Twist and ZEB1/2, and the basic helix-loop-helix factor E12/E47 [10,17-19]. Among them, Snail plays a fundamental role in EMT and its expression is associated with invasiveness, metastasis, tumour recurrence and poor prognosis [20-22]. Snail has been found overexpressed in the invasive fronts of several human tumours derived from epithelial cells [20,23]. Interestingly, hyperactivation of ^{V600E}BRAF mutant in melanoma cells results in Snail overexpression and increases their metastatic potential [24]. Regarding thyroid gland, Snail is not expressed in normal thyroid tissue, whereas is overexpressed in thyroid cancer cell lines, human tumours and their metastases [25].

PLX4032 and PLX4720, two selective ^{V600E}BRAF inhibitors, have been shown to potently inhibit proliferation and migration of melanoma, colorectal and thyroid cancer cells harbouring the ^{V600E}BRAF mutation, but much less so in wild-type BRAF expressing cells [8, 26,27]. Consistent with the high degree of selectivity of these inhibitors, recent clinical studies have reported exciting results on the therapeutic effect of PLX4032 in melanoma and thyroid tumours [28,29].

To investigate the role of ^{V600E}BRAF mutation in thyroid tumour progression, we analysed the effects of this mutant and the therapeutic potential of PLX4720 on EMT, examining E-cadherin and Snail expression together with the migration and invasion of thyroid cancer cells. We found that ^{V600E}BRAF induces EMT by increasing Snail expression and inhibits the expression of E-cadherin, with a concomitant increase of migration and invasion of thyroid cancer cells. Moreover, we demonstrate that PLX4720 inhibits the ^{V600E}BRAF-induced effects, showing the potential role of this inhibitor for the treatment of patients with thyroid cancer carrying this mutation.

2. Material and methods

2.1. Materials and expression vectors

Antibodies included anti-BRAF and anti-ERK2 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-tubulin (Sigma); anti-HA tag 12CA5 (Abcam) anti-E-cadherin (BD Biosciences), anti Snail (Cell Signalling Technology) and peroxidase-conjugated secondary antibodies (DAKO). The BRAF inhibitor PLX4720 was from Axon Medchem and the MEK inhibitor U0126 was from Promega. The expression construct pcDNA3-Snail1 was kindly provided by Dr. Amparo Cano (IIB, CSIC-UAM, Madrid). Lentiviral vectors FG2-GFP and FG2-GFP-^{V600E}BRAF were a generous gift of Dr. M. Soengas (CNIO, Spain) and are described in Denoyelle *et al*. [30].

2.2. Cell culture

Human thyroid cancer cells WRO were kindly provided by Dr. A. Fusco (Institute of Endocrinology and Experimental Oncology, Naples, Italy). Human thyroid cancer cells 8505C and BHT101 were kindly provided by Dr. M. Santoro (Universita Federico II, Naples, Italy). All cells were cultured in DMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37ºC in a 5% CO_2 atmosphere. Identity was confirmed *vs.* published data [31], using standard sequencing techniques.

2.3. Cell transfection

For expression vector, cells were seeded in 6-well plates ($2.5x10^5$ cells/35-mm well) and transfected after 18-20 h with the appropriate construct using LipofectAMINE (Invitrogen), according to manufacturer's protocols. After 24 h, cells were treated as stated in figure legends and harvested. For siRNA silencing, cells were seeded at $2.5x10^5$ cells/35-mm well the day before transfection. Cells were transfected using LipofectAMINE in 1 mL of OPTIMEM with 100 nM BRAF-specific (5'-CAGUCUACAAGGGAAAGUG-3'), Snail (5-GAAUGUCCCUGCUCCACAA-3´) or Silencer^{TM} negative control#1 siRNA (Ambion, Inc.). After 6 h incubation with the RNA-complex, medium was replaced and 2 mL of fresh medium containing 10% FBS was added. Cells were treated and harvested at the indicated times after the transfection as stated in figure legends.

2.4. Generation of lentiviral supernatants and infection conditions

Lentiviral vectors used in this study are described above. Viral supernatants were generated by calcium phosphate transfection of 293T cells [30]. For lentiviral infection, $5x10^4$ WRO cells/35-mm well were infected with lentiviral supernatants in conditions that lead to transduction efficiency close to 100%. Infection efficiencies were estimated by imaging of GFP by fluorescence microscopy 48 h after transfection and before each experiment. All transduced cell lines used stably expressed high levels of ^{V600E}BRAF and GFP.

2.5. Cell lysis and Western blot analysis

After incubation, cells were harvested into 200 μL lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 10% glycerol, 100 mM KCl, 1% Triton X-100, 0.3% 2-mercaptoethanol, 5 mM NaF, 0.2 mM Na_3VO_4, 5 mM $MgCl_2$) supplemented with protease inhibitors. Cell extracts were normalized for protein content. Proteins were separated by SDS-PAGE, and Western blot analysis was performed following standard protocols using the indicated antibodies.

2.6. Migration and invasion assays

Invasion and migration were examined in transwell cell culture chambers using 8 μm pore polycarbonate membranes (BD Bioscience). Migration assays were performed in collagen type I-coated transwells at a final concentration of 0.1 mg/mL in 0.1 N acetic acid. Briefly, $2.5x10^5$ cells were seeded in 35-mm plates and after treatments were harvested using trypsin/EDTA. Trypsin was neutralized with 1 mL of DMEM with 10% FBS, cells were then centrifuged at 1,500 x g for 5 min, resuspended in 2 mL of DMEM without serum and counted. $2.5x10^4$ cells were placed in the upper chamber. The lower chamber contained 600 μL of DMEM 10% FBS as chemoattractant factor. Cells were allowed to migrate for 24 h at 37° C and 5% CO_2. Nonmigrated cells on the upper chamber were removed; filters were fixed with 10% trichloroacetic acid (TCA) and stained with crystal violet. The number of migrated cells to the lower surface of the filter was determined by counting the cell number in the field at 100× magnification.

Invasion assays were performed in Matrigel-coated transwells (BD Biosciences), a complex mixture of extracellular matrix components, using the same protocol described for migration.

2.7. RNA extraction and Quantitative RT–PCR (qRT-PCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's protocols and was retrotranscribed to cDNA by oligo(dT) primer using SuperScript II reverse transcription kit (Invitrogen).

qRT–PCR was performed using the Universal ProbeLibrary Set, Human (Roche, Basel, Switzerland) and the following primers, E-cadherin: forward (5´- GAA TGA CAA CAA GCC CGA AT-3´), reverse (5´-GAC CTC CAT CAC AGA GGT TCC-3´); Snail: forward (5´-GCT GCA GGA CTC TAA TCC AGA-3´), reverse (5´-ATC TCC GGA GGT GGG ATG-3´) and GAPDH: forward (5´-TCC ACT GGC GTC TTC ACC-3´), reverse (5´-GGC AGA GAT GAT GAC CCT TTT-3´). qPCR assays were done in a final volume of 20 µL with 5 µL of cDNA, 1 µM forward and reverse primers, 0,25 µL probe and 7.5 µL of TaqMan master mix (Applied Biosystems, Foster City, CA, USA). The quantitative PCR reaction was performed in a *7500 Fast Real Time PCR System* (Applied Biosystems), with the following parameters: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. To quantify changes in gene expression, the ΔΔCt method was used to calculate the relative changes normalized against the GAPDH gene.

2.8. 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).

3. Results

To study the role of BRAF in thyroid tumour progression, we used three cell lines derived from human thyroid tumours. The WRO cell line, derived from FTC, which express wild-type BRAF protein, and the cell lines 8505C and BHT101, both derived from ATCs, harbouring the ^{V600E}BRAF mutant.

3.1. PLX4720 blocks the migration and invasion induced by ^{V600E}BRAF in thyroid cancer cells

We first analyzed the effect of the presence of ^{V600E}BRAF on migration and invasion of the three thyroid tumour cell lines by inhibiting the expression of this oncogene with specific small interfering RNA (siRNA). As control, we examined the BRAF protein levels after transfection and found a highly significant silencing of BRAF expression in all cell lines (Figure 1A). Next, we determined cell migration and invasion after inhibiting the expression of BRAF. The results showed that BRAF abrogation significantly reduced cell migration and invasion compared to cells transfected with a control siRNA (Figures 1B and 1C). Thus, silencing of BRAF slightly reduced cell migration by about 15% in WRO cells, however a statistically significant decrease of about 30% and 45% was observed in 8505C and BHT101 cells, respectively (Figure 1B). Moreover, BRAF silencing only significantly decreased invasion of the 8505C and BHT101 cell lines by approximately 30%, but not of WRO cells (Figure 1C).

Numerous studies have linked the increased invasiveness of different cancers with the activation of ERK proteins, which is induced by C-RAF or BRAF, depending on the cellular context. Therefore, we tested whether activation of ERK1/2 in our cells was mediated by ^{V600E}BRAF, as well as the role of the MEK-ERK pathway in cell migration and invasion. We observed that the three cell lines had constitutive ERK phosphorylation, and that BRAF silencing completely suppressed it in 8505C and BHT101 cells, whereas it was only partially decreased in WRO cells (Figure 1A). These data are consistent with findings showing that cells harbouring BRAF mutations are significantly more dependent on BRAF signalling for ERK activation than cells where BRAF is not mutated [32,33].

Next, we determined the levels of ERK1/2 phosphorylation after the inhibition of the BRAF-MEK-ERK pathway by either the selective inhibitor of ^{V600E}BRAF, PLX4720, or the MEK inhibitor, U0126. As shown in figure 1D, ERK phosphorylation was completely abolished by both inhibitors in 8505C and BHT101 cells. By contrast, in WRO cells the levels of ERK phosphorylation where only blocked by the U0126 inhibitor, whereas the treatment with PLX4720 inhibitor slightly increased them. These data are consistent with the finding showing the opposite effect of the PLX4720 inhibitor on ERK1/2 activation in wild-type BRAF cells and in cells bearing the ^{V600E}BRAF mutant. We followed studying the effect on cell migration and invasion of ^{V600E}BRAF and MEK inhibition using the PLX4720 and U0126 inhibitors, respectively. Cell migration was decreased by approximately 40% in 8505C and BHT101 cells treated with both inhibitors, whereas they did not have a significant effect in

WRO cells (Figure 1E). Likewise, treatment with PLX4720 or U0126 also decreased cell invasion by about 60% and 50% in 8505C and BHT101 cells, respectively, and did not exert any effect in WRO cells (Figure 1F).

To further confirm the participation of ^{V600E}BRAF in cell migration and invasion of thyroid tumour cells, we overexpressed ^{V600E}BRAF in WRO cells by lentiviral transduction. We generated two stable cell lines expressing either the FG12 lentiviral empty vector (WRO-mock) or FG12-HA-^{V600E}BRAF (WRO-VE). We observed very significant morphological changes in WRO-VE cells *versus* WRO-mock control cells, which included the acquisition of a mesenchymal phenotype characterized by a more elongated form, fibroblast-like phenotype and less intercellular contacts (Figure 2A). The overexpression of ^{V600E}BRAF in WRO-VE cells also resulted in an increased ERK phosphorylation when compared to control WRO-mock cells (Figure 2B). Moreover, WRO-VE migrated and invaded approximately 40% more than control WRO-mock cells (Figures 2C and 2D).

To demonstrate that increased cell migration and invasion was indeed due to ^{V600E}BRAF, we analyzed the effects of PLX4720 inhibitor on these processes. As expected, the treatment with this inhibitor decreased ERK phosphorylation in WRO-VE cells, whereas a slight increase was observed in WRO-mock cells (Figure 2B). Furthermore, the treatment with PLX4720 inhibited both the ^{V600E}BRAF-induced migration and invasion of WRO-VE cells, while no effect on these processes was observed in WRO-mock cells (Figures 2C and 2D).

All these results indicate that ^{V600E}BRAF increases migration and invasion in thyroid cancer cells through a MEK/ERK-dependent mechanism and that these processes can be inhibited by using the ^{V600E}BRAF specific inhibitor PLX4720.

3.2. ^{V600E}BRAF induces down-regulation of E-cadherin expression in thyroid cancer cells

One of the key processes for the primary tumour cells to acquire migratory and invasive capacity is EMT. The hallmark of this process is the down-regulation of E-cadherin expression. For this reason, we studied whether the observed effect of ^{V600E}BRAF on the migration and invasion of thyroid tumour cells was mediated by E-cadherin down-regulation. First, we analyzed whether ^{V600E}BRAF regulated E-cadherin protein levels in 8505C and BHT101 cells. Surprisingly, we observed a significant difference between the E-cadherin basal levels in both cell lines; in 8505C cells the protein was almost undetectable, whereas BHT101 cells expressed high levels (Figure 3A). Despite these differences, we observed that

the inhibition of BRAF expression with specific siRNA increased protein levels of E-cadherin in both cell lines (Figure 3A). As expected, due to the high basal levels, the increase of E-cadherin expression was lower in BHT101 cells than in 8505C cells. We then analyzed the E-cadherin mRNA levels by qRT-PCR and observed similar results (Figure 3B). The abrogation of BRAF expression induced E-cadherin mRNA expression about 5-fold in 8505C cells, while this increase was only about 0.5-fold in BHT101 cells (Figure 3B). To verify the role of ^{V600E}BRAF on E-cadherin expression, we measured its levels after treatment with PLX4720 or U0126. As shown in figure 3C, both inhibitors increased E-cadherin protein levels in both cell lines, with a higher effect in the case of 8505C cells. After, we examined the E-cadherin mRNA levels in 8505C treated with both inhibitors and observed an increase of about 5-fold compared to untreated cells (Figure 3D).

These results demonstrate that silencing or inhibition of BRAF increases E-cadherin expression in thyroid cancer cells through ERK activation, but also indicate that constitutive activation of ERK is necessary but not sufficient for E-cadherin loss of expression in 8505C cells.

3.3. ^{V600E}BRAF increases Snail expression through an ERK-dependent mechanism

One of the main regulators of E-cadherin expression is the transcription factor Snail. To check whether ^{V600E}BRAF regulates E-cadherin expression through this protein, we analyzed the Snail expression levels in our cellular models and their regulation by this oncogene. First, we analyzed by Western blot the effect of BRAF silencing on the protein levels of Snail in 8505C and BHT101 cells, and we observed that BRAF depletion induced a significant decrease of Snail expression in both cell types (Figure 4A). Moreover, BRAF abrogation also decreased the levels of Snail mRNA in these cell lines by about 30% (Figure 4B), suggesting that ^{V600E}BRAF has an effect on Snail expression at the transcriptional level.

In addition, we analyzed the protein levels of Snail after treatment of the cells with PLX4720 and U0126 inhibitors, and observed that both inhibitors significantly reduced Snail protein levels (Figure 4C). We also showed that the decreased Snail expression was due to transcriptional regulation since Snail mRNA levels in 8505C and BHT101 cells treated with the PLX4720 inhibitor were lower than in control cells (Figure 4D). We further investigated this regulation by analysing the effects of ^{V600E}BRAF overexpression on Snail levels in WRO cells. As expected, we demonstrated that WRO-VE cells have significantly increased Snail protein levels compared to WRO-mock cells (Figure 4E). This induction was also observed in

Snail mRNA levels, which increased by 40% in WRO-VE cells when compared to WRO-mock cells (Figure 4F). Moreover, treatment with PLX4720 restored both protein and mRNA levels of Snail to basal values in WRO-VE cells, although the inhibitor did not significantly change the basal levels of Snail in WRO-mock cells (Figures 4E and 4F). These data clearly demonstrate that the presence of ^{V600E}BRAF mutation increases Snail expression in thyroid cancer cells.

3.4. Snail mediates the ^{V600E}BRAF-induced E-cadherin down-regulation

Given that ^{V600E}BRAF decreased E-cadherin levels and increased Snail levels, we examined whether Snail also regulated E-cadherin expression in our cells. To do this, we inhibited Snail expression using a specific siRNA and analyzed the protein and mRNA levels of E-cadherin. Silencing of Snail in BHT101 cells induced both E-cadherin protein (Figure 5A) and mRNA levels (Figure 5B), reaching an increase of about 4-fold of E-cadherin mRNA level in cells transfected with Snail specific siRNA when compared to control cells. Surprisingly, silencing of Snail expression did not increase E-cadherin protein levels in 8505C cells (Figure 5A); however, an increase of about 3-fold in E-cadherin mRNA levels was observed (Figure 5B), demonstrating the involvement of Snail in the transcriptional regulation of E-cadherin in these cells. Moreover, since BHT101 cells express high levels of E-cadherin, we next examined whether Snail overexpression could decrease E-cadherin in these cells. As expected, Snail overexpression significantly decreased E-cadherin level (Figure 5C). Thus, these data demonstrate that Snail down-regulates E-cadherin expression at transcriptional level in thyroid cancer cells, but that a different regulation mechanism, independent of this transcriptional repressor, may be operating in 8505C.

The ectopic overexpression of Snail also induced very significant morphological changes in both cells lines and a decrease of the intercellular junctions (data not shown). These morphological changes, together with the decreased E-cadherin expression observed, correspond to the characteristic changes of EMT. Thus, we analyzed the effects of overexpression of Snail on cell migration and invasion (Figures 5D and 5E). Snail expression significantly increased cell migration and invasion in both cell lines; cell migration increased by about 80% in the case of 8505C cells and 50% in BHT101 cells, when compared to their corresponding controls (Figure 5D), and cell invasion increased by approximately 40% in both cell lines (Figure 5E), thus confirming the role of Snail on induction of tumour progression.

Finally, taking into account that both ^{V600E}BRAF and Snail increase migration and invasion and down-regulate E-cadherin expression of thyroid tumour cells, we studied whether ^{V600E}BRAF decreased the levels of E-cadherin through Snail. For this purpose, we examined whether overexpression of Snail could prevent the increase of E-cadherin levels in 8505C cells achieved by inhibition of either ^{V600E}BRAF or MEK activity upon treatment with PLX4720 and U0126 inhibitors (Figure 5F). As we have shown above, these cells do not express detectable E-cadherin levels at basal conditions, so Snail overexpression did not have any effect in these circumstances. However, Snail overexpression reversed the increase of E-cadherin induced by PLX4720 and U0126 treatment in these cells (Figure 5F), indicating that ^{V600E}BRAF suppresses the expression of E-cadherin through the activation of Snail expression.

4. Discussion

Here, we show that ^{V600E}BRAF induces migration and invasion of thyroid cancer cells and for the first time we demonstrate that ^{V600E}BRAF regulates the expression of genes involved in EMT in thyroid cancer cells, considered a crucial step in cancer progression. Specifically, ^{V600E}BRAF increases the expression of Snail protein, which in turn, promotes the down-regulation of E-cadherin expression. We demonstrate that either BRAF depletion with siRNA or treatment with the specific ^{V600E}BRAF inhibitor PLX4720 decreases migration and invasion in two cell lines bearing the ^{V600E}BRAF mutant, 8505C and BHT101 cells. Conversely, the overexpression of ^{V600E}BRAF in WRO cells harbouring wild-type BRAF confers to these cells a higher capacity to migrate and invade than control cells. Moreover, this gain of functions is effectively inhibited by PLX4720. The shown effects are dependent of MEK/ERK pathway since the inhibitor U0126 exerts the same effects on migration and invasion. The role of MEK/ERK is also demonstrated by showing that PLX4720 only decreases ERK activation in cells with the ^{V600E}BRAF mutation but not in the WRO wild-type BRAF cells. These results are in agreement with the finding reporting that thyroid cancer cells carrying the ^{V600E}BRAF mutation respond far more effectively to another ^{V600E}BRAF inhibitor, PLX4032, in the inhibition of ERK activation than thyroid cancer cells harbouring the wild-type BRAF gene [27,34]. These data indicate that ^{V600E}BRAF plays an important role in thyroid tumour progression and demonstrate that PLX4720 may be used for BRAF mutation-targeted therapy in thyroid cancer. In this sense, Nucera *et al.* have shown that inhibition of ^{V600E}BRAF with PLX4720 decreases migration and invasion of thyroid tumour

cells in vitro and inhibits tumour aggressiveness of an orthotopic mouse model of ATC harbouring ^{V600E}BRAF [35]. Thus, PLX4720 and PLX4032 have been recently developed with great promise as anti-cancer drugs for melanoma and thyroid cancer. In fact, current clinical trials with PLX4032 (vemurafenib) as both mono- or combination therapy with other kinase inhibitors in advanced melanoma patients have been very successful [29].

E-cadherin plays an important role as an invasion suppressor, since either its loss of expression or abnormal function leads to an increased ability of cancer cells to invade neighbouring tissues [10,23,36]. On the other hand, it is well accepted that cells within the invasive front of human thyroid cancer have a different expression pattern of genes involved in EMT compared to the central part of the tumour [37]. Thus, the loss of E-cadherin in the invasive tumour front has been identified as a risk factor in PTCs associated with increased aggressiveness of these tumours [38] and is a hallmark of progression from poorly differentiated PTCs to undifferentiated ATCs [39,40,41]. However, although a correlation between the presence of ^{V600E}BRAF and the higher aggressiveness in cancer cells has been described, no previous studies have demonstrated the molecular effects of ^{V600E}BRAF on EMT of thyroid tumour cells. Here, we demonstrate that ^{V600E}BRAF mutant represses the E-cadherin expression in thyroid tumour cells and that silencing of BRAF or treatment with PLX4720 and U0126 inhibitors recovers it, which is associated with significant morphological changes that result in a phenotype with epithelial characteristics. Our data are consistent with those obtained by Riesco-Eizaguirre et al., who demonstrated that overexpression of ^{V600E}BRAF in rat thyrocytes decreases E-cadherin levels [6]. They are also in agreement with data reported in a mouse model showing a relationship between the lack of E-cadherin and loss of cell differentiation during the progression of ^{V600E}BRAF-induced thyroid cancer [9].

Snail has emerged as one of the most important identified transcriptional repressors of E-cadherin. Significantly, Snail expression is associated to the down-regulation of E-cadherin in melanoma and other carcinoma cell lines [20-22], but not in thyroid cancer cells. Moreover, in primary tumour, Snail is associated to the invasive regions of different carcinomas [20,23], supporting a central role for Snail in the induction of EMT and invasiveness. Our results show that ^{V600E}BRAF upregulates Snail levels in thyroid cancer cells. This conclusion is based on the experiments performed in the ^{V600E}BRAF mutant cell lines, 8505C and BHT101, which shown a decrease in Snail protein and mRNA levels after BRAF knockdown or treatment with the PLX4720 or U0126 inhibitors. They are also based on the results obtained after V600EBRAF overexpression in WRO cells showing an increase

in Snail protein and mRNA and that PLX4720 blocks these increases. Similar data were reported by Massoumi et al. who also observed that expression of ^{V600E}BRAF in melanoma cells correlates with the levels of Snail [24]. Although there are no data in the literature on the relationship of ^{V600E}BRAF and Snail gene expression, it has been shown that thyroid tumours display an increased expression of Snail compared to normal thyroid tissue, thus associating this transcription factor with thyroid cancer development [25]. We also demonstrate that overexpression of Snail in cells derived from ATC results in an increased cell migration and invasion, accompanied by a phenotypic change characterized by a mesenchymal morphology, as previously shown in cell lines derived from keratinocytes and squamous cell carcinoma [19,42]. These results support the central role this transcription factor plays in the induction of EMT and invasiveness of thryroid cancer cells. Although, we still do not know the mechanism by which ^{V600E}BRAF regulates Snail expression in these cells, a possible candidate would be the NF-κB pathway, since this transcription factor also regulates Snail expression [43] and ^{V600E}BRAF has been associated with hyperactivation of NF-κB in thyroid cells [44,45]. These data, together with the high activity of NF-κB found in some thyroid cancer cells [45], further supports the hypothesis that ^{V600E}BRAF may regulate Snail expression through this transcription factor. On the other hand, TGF-β is a main regulator of Snail expression. It has been shown that ^{V600E}BRAF overexpression induces TGF-β expression in a thyroid rat cell line and that both ^{V600E}BRAF and TGF-β cooperate to induce EMT in thyroid cancer cells [6,9], hence ^{V600E}BRAF could exert its effect on Snail through this cytokine signalling. It is also possible that ^{V600E}B-RAF acts through GSK3-β. GSK3-β phosphorylates Snail and induces its subsequent polyubiquitination and proteasomal degradation. The stimulation of the Ras-RAF-MEK-ERK pathway in breast cancer cells pathway stabilises Snail through inhibitory phosphorylation of GSK3-β at serine 9 [46]. Thus, ^{V600E}BRAF may decrease the activity of GSK3-β and therefore the degradation of Snail. In fact, we have preliminary results, not shown in this study, indicating that U0126 increases the activity of GSK3-β by decreasing the levels of its inhibitory phosphorylation.

We also demonstrate that Snail represses the E-cadherin expression in our cell model. Snail silencing increases the expression of E-cadherin and the opposite, overexpression of Snail decreases it. Furthermore, we demonstrate that expression of Snail impairs the recovery of E-cadherin expression induced by PLX4720 and U0126 inhibitors, indicating that ^{V600E}BRAF and MEK-ERK pathway act through Snail to suppress E-cadherin. However, we believe that this is not the only mechanism of regulation of E-cadherin in these cells. The fact that the

inhibition of Snail in 8505C cells does not recover E-cadherin expression suggests that the repression of E-cadherin is not only dependent of Snail and that additional molecular alterations produced by ^{V600E}BRAF are required to completely suppress the expression of E-cadherin. The activation of RTKs have been associated with increased expression of EMT inducers such as the repressors of E-cadherin, ZEB1, ZEB2 and bHLH factors [10,47], therefore it would not be surprising that ^{V600E}BRAF, through hyperactivation of the MEK-ERK pathway, could regulate the expression of these transcription factors. On the other hand, the presence of ^{V600E}BRAF in PTC is associated with hypermethylation of tumour suppressor genes and subsequent transcriptional repression [48]. Smith JA et al. shown that E-cadherin gene is methylated more frequently in patients with PTC than in control patients [49]. These data, together with the results showing that the inhibition of the MEK-ERK pathway, results in demethylation and recovery of some of the expression of tumour suppressor genes [50], justifies the hypothesis that probably ^{V600E}BRAF is also suppressing the expression of E-cadherin through other Snail-independent mechanisms.

To our knowledge, this is the first report showing that ^{V600E}BRAF induces migration and invasion of thyroid cancer cells through regulation of the gene expression program involved in EMT; in particular, increasing the expression of Snail with a concomitant repression of E-cadherin. Moreover, we also demonstrate that ^{V600E}BRAF targeting with the PLX4720 inhibitor decreases aggressiveness of thyroid tumours harbouring this mutation. This may mean that this inhibitor could be a good tool in the clinic to decrease the progression to more aggressive phenotypes and poorer prognosis of thyroid tumours.

Conflict of Interest

No potential conflicts of interest are disclosed by the authors.

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Figure legends

Fig. 1. Inhibition of BRAF decreases migration and invasion in ^{V600E}BRAF mutant thyroid cells. (A) Representative western blot for BRAF, phospho-ERK (p-ERK) and ERK2 expressions 72 h after transfection of WRO, 8505C and BHT101 cells with siRNA oligonucleotides specific for BRAF (siBRAF) or a scrambled oligo control (sc). The results show a representative experiment repeated three times with similar results. (B and C) Cells were transfected as in (A) for 72 h; after this time cells were collected, counted and migration (B) and invasion (C) were measured after 24 h in transwell cell culture chambers in which collagen I (B) or matrigel (C) were place as a barrier on the upper side of the transwells. Data shown represent the mean ± SEM of three independent experiments performed in duplicate and results expressed as relative units referred to each control. (D) Phosphorylation of ERK1/2 (p-ERK) and ERK2 expression, as control, detected by western blot in thyroid cancer cells treated with DMSO (-) as vehicle control, PLX4720 (PLX) (5 μM) or U0126 (10 μM) for 24 h. Blots are from one typical experiment performed three times with similar results. (E) Migration and (F) invasion of cells treated as in (D) and examined as in (B and C). Results shown are the mean ± SEM of three independent experiments performed in duplicate and results expressed as relative units referred to each control. **$0.001<p<0.01$, ***$p<0.001$, significant differences compared to the corresponding control.

Fig. 2. ^{V600E}BRAF increases cell migration and invasion of WRO thyroid cancer cells by an ERK-dependent mechanism. (A) Immunofluorescence assay done in WRO cells infected with the empty lentivirus FG2-GFP (WRO-mock) or the lentiviral vector FG2-GFP-^{V600E}BRAF (WRO-VE) to determine GFP expression, as infection efficiency control, and morphological changes induced by ^{V600E}BRAF. (B) Western blot analysis of HA-^{V600E}BRAF, phospho-ERK (p-ERK) and ERK2 in both WRO-mock and WRO-VE treated with DMSO (-), as control, or PLX4720 (PLX) (5 μM) for 24 h. (C) Migration and (D) invasion of WRO-mock and WRO-VE treated with DMSO (-) as vehicle control, or PLX4720 (PLX) (5 μM) for 24 h and examined in transwell cell culture chambers coated with collagen I (C) or matrigel (D) after 24 h. Data shown represent the mean ± SEM of three independent experiments performed in duplicate with similar results expressed as relative units referred to each control. $^{\#\#}0.001<p<0.01$, significant differences compared to WRO-mock control. ***$p<0.001$, significant differences compared to the corresponding control.

Fig. 3. ^{V600E}BRAF down-regulates E-cadherin expression in thyroid cancer cells. (A) 8505C and BHT101 cells were transfected with siRNA oligonucleotides specific for BRAF (siBRAF) or a scrambled oligo control (sc) for 72 h and E-cadherin expression was detected by western blot. For each pair of rows, an image of the E-cadherin blot is shown with the reprobed membrane with anti-BRAF and anti-β-tubulin antibody as controls. Blots are representative of experiments performed three times with similar results. (B) E-cadherin mRNA levels were measured in 8505C and BHT101 cells transfected as in (A). Total mRNA was prepared and qRT-PCR was carried out using primers against E-cadherin and GAPDH. The mRNA levels were normalized by 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) (5 μM) or U0126 (10 μM) and E-cadherin expression was detected by western blot. Blots were reprobed with anti-phospho ERK (p-ERK) and ERK2 as controls. (D) 8505C cells were incubated as in (C), RNA was isolated and used in qRT-PCR for E-cadherin and GAPDH transcripts. Data showing the mean ± SEM are compiled from three independent experiments performed in triplicate. **$0.001<p<0.01$, ***$p<0.001$, significant differences compared to the corresponding control.

Fig. 4. Snail expression is activated by ^{V600E}BRAF in thyroid cancer cells. 8505C and BHT101 cells were transfected with siRNA oligonucleotides specific for BRAF (siBRAF) or a scrambled oligo control (sc) for 72 h and Snail expression was detected by western blot. For each pair of rows, an image of the Snail blot is shown with the reprobed membrane with anti-BRAF and anti-β-tubulin antibody as controls. Blots are representative of experiments performed three times with similar results. (B) Snail mRNA levels measured in 8505C and BHT101 cells transfected as in (A). Total RNA was prepared and qRT-PCR was carried out using primers against Snail and GAPDH. The mRNA levels were normalized by 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) (5 μM) or U0126 (10 μM) and Snail expression was detected by western blot. Blots were reprobed with anti-phospho ERK (p-ERK) and ERK2 as controls. (D) 8505C and BHT101 cells were incubated in the absence, DMSO (-), or presence of PLX4720 (5 μM) for 24 h,

RNA was isolated and used in qRT-PCR for detection of Snail and GAPDH transcripts. Data showing the mean ± SEM are compiled from three independent experiments performed in triplicate. (E) Western blot analysis of Snail, phospho-ERK (p-ERK) and ERK2 in both WRO-mock and WRO-VE treated with DMSO (-) or treated with PLX4720 (PLX) (5 μM) for 24 h. (F) Snail mRNA levels of WRO-mock and WRO-VE treated and processed as in panel D. Data shown represent the mean ± SEM of three independent experiments performed in triplicate with similar results expressed as relative units referred to each control. *0.01<p<0.05, **0.001<p<0.01, ***p<0.001, significant differences compared to the corresponding control. $^{\#\#}$0.001<p<0.01, significant differences compared to WRO-mock control.

Fig. 5. ^{V600E}BRAF represses E-cadherin expression through Snail. (A) Representative western blot for Snail, E-cadherin and β-tubulin expressions 72 h after transfection of 8505C and BHT101 cells with siRNA oligonucleotides specific for Snail (siSnail) or a scrambled oligo control (sc). The results show a representative experiment repeated three times with similar results. (B) E-cadherin mRNA levels measured in 8505C and BHT101 cells transfected as in (A), RNA was isolated and used in qRT-PCR for E-cadherin and GAPDH transcripts. Data showing the mean ± SEM are compiled from three independent experiments performed in triplicate. (C) E-cadherin levels detected by western blot 48 h after transfection of BHT101 cells with an empty vector (-) or HA-Snail expression vector. (D) Migration and (E) invasion assays of 8505C and BHT101 cells transfected with empty vector or a HA-Snail construct for 48 h and examined in transwell cell culture chambers coated with collagen I (D) or matrigel (E) after 24 h. Data shown represent the mean ± SEM of three independent experiments performed in duplicate with similar results expressed as relative units referred to each control. (F) Snail level detected by western blot in 8505C cells transfected with empty vector or HA-Snail and then incubated with PLX4720 (PLX) (5 μM) or U0126 (10 μM) for 24 h. Representative blots of ectopically expressed HA-Snail and β-tubulin are shown as controls in panels C and F. The arrow indicates HA-Snail and the asterisk the endogenous Snail. Similar results were obtained in three independent experiments. **0.001<p<0.01, ***p<0.001, significant differences compared to the corresponding control.

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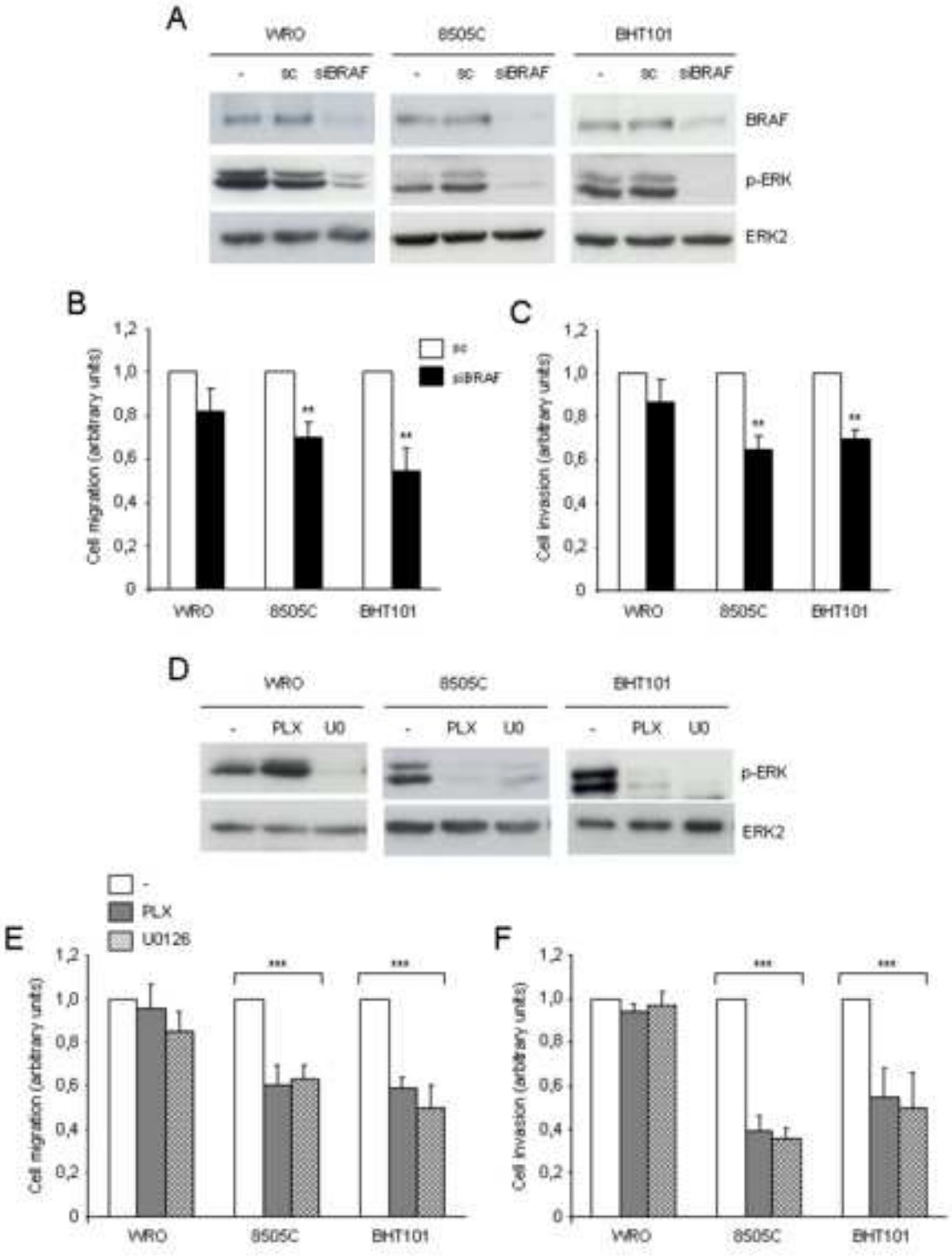

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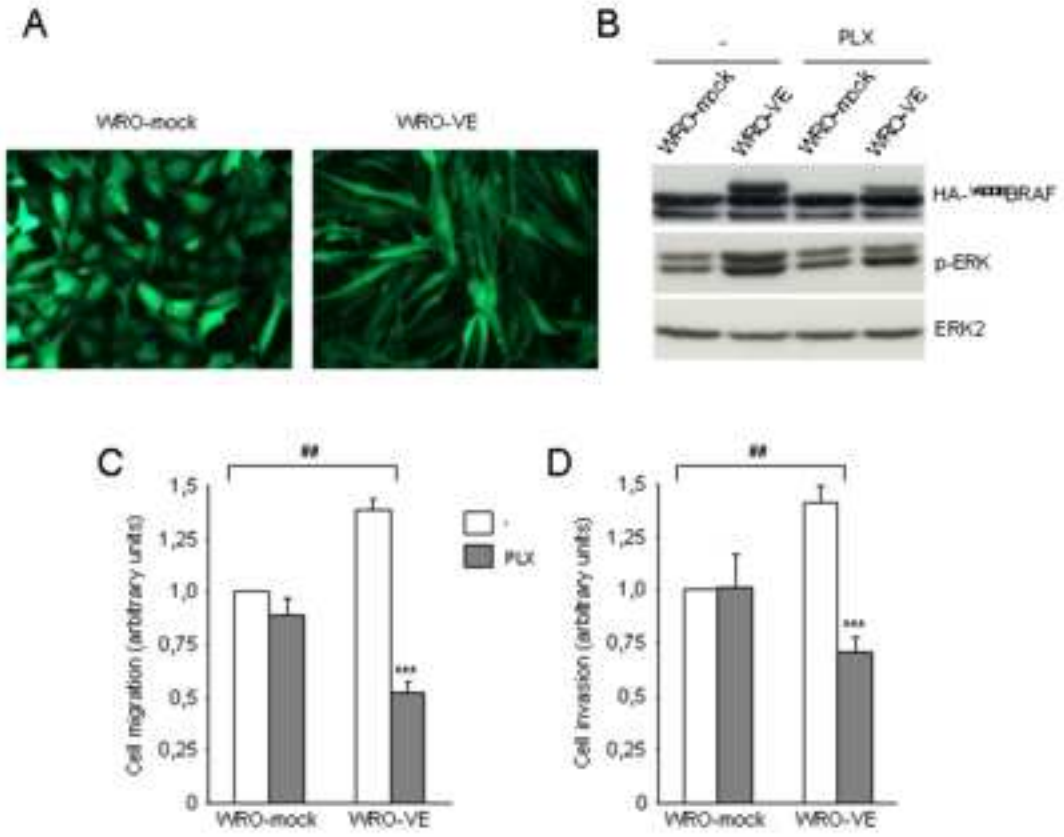

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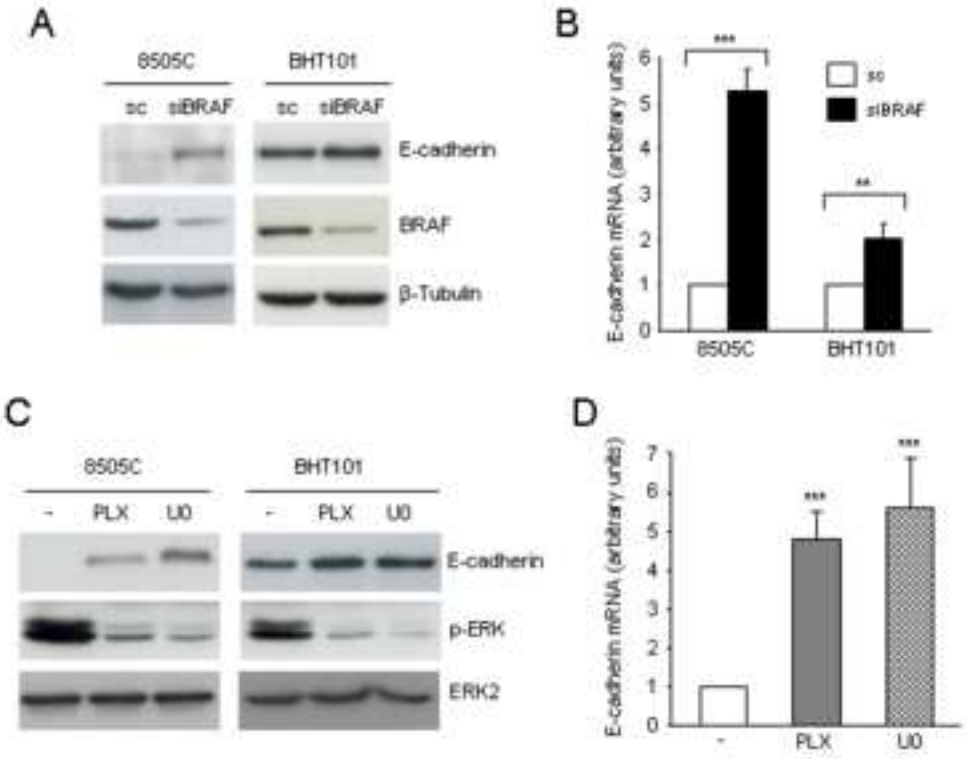

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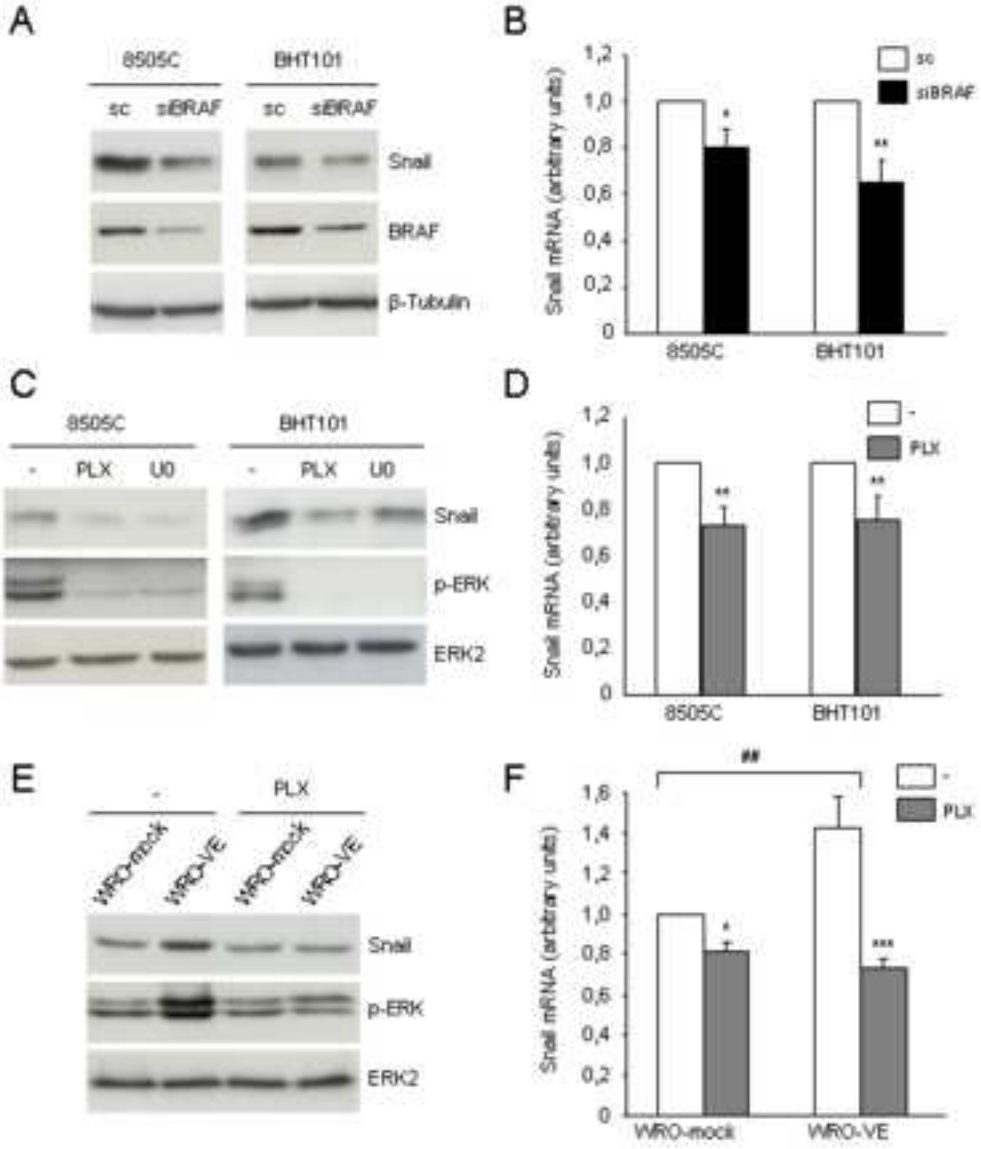

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