RSK activation via ERK modulates human colon cancer cells response to PTHrP

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Abstract
Parathyroid hormone-related peptide (PTHRp) is associated with several human cancers such as colon carcinoma. This disease is a complex multistep process that involves enhanced cell cycle progression and migration. Recently we obtained evidence that in the human colorectal adenocarcinoma Caco2 cells, exogenous PTHrP increases the proliferation and positively modulates cell cycle progression via ERK1/2, p38 MAPK and PI3K. The purpose of this study was to explore if the serine/threonine kinase RSK, which is involved in the progress of many cancers and it is emerging as a potential therapeutic target, mediates PTHrP effects on cancer colon cells. Western blot analysis revealed that PTHrP increases RSK phosphorylation via ERK1/2 signaling pathway but not through p38 MAPK. By performing subcellular fractionation, we found that the peptide also induces the nuclear localization of activated RSK, where many of its substrates are located. RSK participates in cell proliferation, in the upregulation of cyclin D1 and CDK6 and in the downregulation of p53 induced by PTHrP. Wound healing and transwell filter assays revealed that cell migration increased after PTHrP treatment. In addition, the hormone increases the protein expression of the focal adhesion kinase FAK, a regulator of cell motility. We observed that PTHrP induces cell migration and modulates FAK protein expression through ERK/RSK signaling pathway but not via p38 MAPK pathway. Finally, in vivo studies revealed that the hormone activates RSK in xenografts tumor. Taken together, our findings provide new insights into the deregulated cell cycle and migration that is characteristic of tumor intestinal cells.

Introduction
The parathyroid hormone-related protein (PTHRP), originally identified as the factor responsible for malignant hypercalcemia, was later found to be widely expressed in fetal and adult tissues. Today it is recognized for its endocrine, paracrine and autocrine modes of action (Maioli & Fortino 2004, McCauley & Martin 2012). Full-length PTHrP undergoes posttranslational processing, which gives rise to a family of mature secretory peptides (Wysolmerski 2012). Peptides with the N-terminal PTH-like region, such as PTHrP (1–34), bind to and activate the PTH/PTHrP receptor (PTH1R), whereas midregion and C-terminal peptides are functionally active through uncharacterized receptors (Wysolmerski 2012). Cytosolic PTHrP can also use a bipartite multibasic nuclear localization signal to translocate to the nucleus and act through an intracrine pathway (McCauley & Martin 2012). There is
evidence that the hormone is implicated in different cancers such as colon carcinoma (McCauley & Martin 2012), one of the major causes of cancer death worldwide (Curtin 2013, Siegel et al. 2015). Although great progress has been made in the diagnosis and therapy of the colon cancer, the survival rate is still low (Chan et al. 2012). Therefore, investigation of the underlying mechanisms for the tumorigenesis of colon cancer will facilitate the diagnosis and therapy of the colon cancer. The pathogenesis of this disease involves various processes including enhanced cell survival, cell cycle progression, proliferation, migration and angiogenesis. PTHrP can participate in these processes, promoting tumor growth and dissemination by the regulation of different signaling pathways (Soubrier & Massfelder 2006).

The p90 ribosomal S6 kinase (RSK) comprises a family of serine/threonine kinases that are activated by MAPK family. Four isoforms, RSK1–4, have been reported in mammalian cells (Anjum & Blenis 2008, Lara et al. 2013). The best functionally characterized isoforms are RSK1 and RSK2. Each RSK isoform contains two nonidentical kinase domains, one at the N-terminus and one at the C-terminus. Phosphorylation of RSKs at serine/threonine residues, which occurs at multiple sites, is required for its activation (Anjum & Blenis 2008) and the N-terminal kinase domain is primarily responsible for substrate phosphorylation. RSKs phosphorylate many proteins, both cytosolic and nuclear (Romeo et al. 2012). The many effects of these kinases on various proteins may contribute to the observations that RSKs mediate wide-ranging cellular processes, including cell cycle and migration (Lara et al. 2013, Wu et al. 2014, Zhou et al. 2015).

RSK signaling is involved in many cancers, controls oncogenic processes through the regulation of transcription factors and/or cellular modulators (Romeo et al. 2012, Lara et al. 2013, Sulzmaier & Ramos 2013). These kinases also regulate protein synthesis, and therefore, RSK-mediated translational control may play a critical role in the regulation of tumorigenic cellular events.

As RSK has been shown to contribute to the etiology of numerous cancers, new therapeutic strategies could be included in anti-cancer chemotherapy (Hecht et al. 2015, Poomakkoth et al. 2016). Thus, RSKs emerge as potential therapeutic targets in various types of human cancer (Romeo et al. 2012, Lara et al. 2013, Sulzmaier & Ramos 2013).

In SNU-407 colon cancer cells, carbachol treatment induced RSK activation in an atropine-sensitive manner, and this RSK activation was decreased by the inhibition of either the epidermal growth factor receptor (EGFR) or protein kinase C (PKC). Moreover, the inhibition of RSK almost completely blocked carbachol-stimulated cell proliferation (Park & Cho 2012). However, the molecular mechanisms leading to RSK activation as well as its role in colon cancer cells is not fully studied.

Recently, we obtained evidence that in Caco2 cells and HCT116 cells, two cell lines from human intestine tumors, exogenous PTHrP activates ERK1/2 as well as the α isoform of p38 MAPK. The hormone also increases cell proliferation, modulates cell cycle progression and exerts a protective effect under apoptotic conditions via MAPK signaling pathways in these tumor intestinal cells (Lezcano et al. 2013, Calvo et al. 2014, Martin et al. 2014; Martín MJ, Gigola G, Carriquiroborde M, Gentili F & Gentili C, unpublished observations).

Based on our observations and given the relevance of signaling pathways associated with cell cycle and cell migration in the spread of cancer and the complex inputs capable of modulating these processes, the aim of the present study was to investigate whether in Caco2 cells and HCT116 cells, PTHrP activates RSK and if so, to explore whether this kinase mediates the effects of PTHrP on colon cancer cells and the molecular mechanisms that are involved in these processes. To validate the results observed from in vivo studies, we also evaluated the hormone effect in vitro.

Materials and methods
Materials
Human PTHrP (1–34) and high glucose Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Sigma-Aldrich Chemical. Fetal bovine serum (FBS) was from Natocord (Córdoba, Argentina). Antibodies were from the following sources: anti-RSK1/2/3, anti-FAK, anti-cyclin D1, anti-CDK-6 and anti-p53 were from Cell Signaling Technology. Anti-phospho RSK, anti-GAPDH, anti-phospho tyrosine antibody, goat anti-rabbit peroxidase-conjugated secondary antibody and goat anti-mouse peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology. PD 98059 and SB 203580 were from Calbiochem. SL0101 was from Santa Cruz Biotechnology. SB 202190 was from Tocris Bioscience (United Kingdom). Crystal violet was from MERCK. Protein size markers were from Amersham Biosciences, PVDF (Immobilon polyvinylidene difluoride) membranes and ECL chemiluminescence detection kit were from Amersham. Transwell filters were with the following features: polyethylene terephthalate (PET), 8μm pores,
24-well format, were from JETBIOFIL. All other reagents used were of analytical grade.

Cell culture and treatment

The human colon cell lines Caco2 and HCT 116 (from the American Type Culture Collection, Manassas, VA, USA) were cultured at 37°C in DMEM containing 10% FBS, 1% non-essential acids, 100IU/mL penicillin, 100mg/mL streptomycin and 50mg/mL gentamycin in a humid atmosphere of 5% CO2 in air. Cultures were passaged every 2 days with fresh medium. All experiments were performed using passages less than 15. Experimental cultures were grown to 80% confluence in serum-containing medium, and then cells were serum-deprived 24h before the addition of 10⁻⁸ mol/L PTHrP (1–34) for different times. This dose of exposure was selected because we previously studied the effects of PTHrP (1–34) 10⁻⁸ mol/L in Caco2 cells and HCT 116 cells expressing PTH/PTHrP receptor (Lezcano et al. 2013, Calvo et al. 2014, Martín et al. 2014). Where indicated, cells were pretreated for 30 min with SL0101 (an inhibitor of RSK), PD 98059 (an inhibitor of MEK, which is the upstream kinase of ERK1/2), SB 203580 (an inhibitor of p38 MAPK) or SB 202190 (another inhibitor of p38 MAPK). Control conditions were performed by addition of an equivalent volume of DMSO (the vehicle of the inhibitors). In previous works, we confirmed the effectiveness of the kinases inhibitors employed, SB 203580 and PD 98059, by determining phosphorylated protein levels of CREB/ATF1 or ERK, respectively, by Western blot analysis (Lezcano et al. 2013, Calvo et al. 2014, Martín et al. 2014). The kaempferol glycoside SL0101 is an ATP-competitive inhibitor of the kinase domain N-terminal (NTKD) and was in fact the first identified specific inhibitor of RSK. SL0101 is a natural product obtained from the tropical plant Forsteronia refracta. The EC₅₀ of SL0101 was found to be approximately 50μmol/L in intact cells (Smith et al. 2005).

Trypan blue dye exclusion assay

Caco2 cells were seeded at a density of 2 x 10⁴ cells/well in 24-well plate. Cells were pre-incubated with or without RSK inhibitor SL0101 (50μmol/L) for 30 min and then treated with PTHrP in triplicates for 5 days. Then, the cells were washed with PBS buffer, released from the cultured dish using trypsin-EDTA, incubated with 0.4% of Trypan blue stain and counted in a Neubauer chamber observed in a microscope. Cells were counted per field, and the number of cells that excluded the stain (viable cells) was determined in each condition.

Wound healing assay

Cells were seeded in a 6-well plate and grown to confluency. The cell monolayer was wounded by scratching, using a 10μL pipette tip. After washing with a phosphate-buffered saline solution (PBS), the cells were incubated with or without PTHrP (10⁻⁸ mol/L). At time points 0, 4, 24 and 48 h, the same positions along the scratch wound were photographed using a microscope (NIKON Eclipse Ti-S) and ImageJ (MRI Wound Healing Tool) was used for quantification of the scratch wound. Three measurements per scratch were performed (2 replicates/condition, n=3 experiments).

Transwell migration assay

Cells were seeded on the top of transwell filters and were grown for 48 h followed by serum starvation for another 24 h in medium without FBS. Then, the medium in the lower chamber was replaced by medium containing 5% FBS while the medium in filter inserts was replaced by serum-free medium with or without PTHrP (10⁻⁸ mol/L). Cells were incubated with the hormone for 18 h in HCT 116 cells or for 24 h in Caco2 cells and the treatment was finalized when they were washed twice with PBS followed by fixation using methanol for 15 min at −20°C. Then, the cells on the upper side of the transwell filters were removed with a cotton swab while the cells on the lower side were stained with 0.1% crystal violet for 30 min at room temperature. Where indicated, cells were pretreated for 30 min with RSK or MAPKs inhibitors. Migrated cells were counted using an inverted microscope (2 replicates/condition, n=3 experiments).

Western blot analysis

Cells were washed with PBS buffer plus 25 mmol/L NaF and 1 mmol/L Na₃VO₄, and lysed in buffer containing 50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L EDTA, 1% Tween-20, 1% Nonidet P-40, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 25 mmol/L NaF and 1 mmol/L Na₃VO₄. The lysates were incubated on ice for 10 min, vortexed for 45 s and maintained on ice for another 10 min. After centrifugation at 14,000 x g and 4°C for 15 min, the supernatant was collected and proteins were quantified by the Bradford method (Bradford 1976).
Lysate proteins dissolved in 6× Laemmli sample buffer were separated (30μg/lane) using SDS-polyacrylamide gels (8–12% acrylamide) and electrotransferred to PVDF membranes. After blocking with 5% nonfat milk in TBST buffer (50 mM Tris pH 7.2–7.4, 200 mM NaCl, 0.1% Tween-20), the membranes were incubated overnight with the appropriate dilution of primary antibody in TBST with 1% nonfat milk. After washing, membranes were incubated with the appropriate dilution of horse radish peroxidase-conjugated secondary antibody in TBST with 1% nonfat milk. Finally, the blots were developed by ECL with the use of Kodak BioMax Light film and digitalized with a GS-700 Imaging Densitomer (Bio-Rad).

### Stripping and reprobing membranes

The complete removal of primary and secondary antibodies from the membranes was achieved by incubating the membranes in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS and 50 mM β-mercaptoethanol) at 55°C for 30 min with agitation. Then, membranes were washed for 10 min in TBST (1% Tween-20) and blocked, as indicated previously, for 1 h at room temperature. After that, membranes were ready to reprobe with the corresponding antibodies.

### Subcellular fractionation

Cells were washed with PBS, resuspended in ice-cold TES buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose-containing inhibitors) homogenized in a Teflon-glass hand homogenizer (30 stokes) and then centrifuged at 80 g for 20 min at 4°C. The supernatant was centrifuged at 1400 g for 20 min at 4°C to pellet the nuclei. The resulting supernatant was used as cytosolic fraction. The pellet was washed twice in TES buffer, resuspended in the lysis buffer described previously for Western blot and used as nuclear fraction. The purity of each isolated fraction was assessed by assaying for proteins known to be associated with cellular components. Proteins from each fraction were quantified by the Bradford method (Bradford 1976). Equal proteins from each fraction were dissolved in 6× Laemmli sample buffer and separated (30 μg/lane) using SDS-polyacrylamide gels (8% acrylamide).

### Xenograft in nude mice

1 × 10⁶ human colorectal carcinoma (HCT116) cells were subcutaneously injected into the left dorsal flanks of 6-week-old BALB/c male nude mice. Four days after inoculation, animals were randomized (blindly) into control and treatment groups (n=6/group). Mice were administered daily with PTHrP (40 μg/kg in 200 μL PBS) or an equal volume of vehicle solution, as control, by intratumoral injection (2 groups in total; each group represents an experimental unit). The mice were killed and tumors were removed and weighted after the 20 days of PTHrP treatment. Tumor size was calculated according to the following formula: tumor volume (mm³) = tumor width (mm²) × tumor length (mm)/2 (Wang et al. 2002). All experiments with animals were approved by a local animal committee for ethics. One piece of each tumor was fixed with 4% neutral buffered formaldehyde solution for immunohistochemistry assay.

### Immunohistochemistry

Paraffin-embedded sections were deparaffinized, re-hydrated and pre-treated using heat-mediated antigen retrieval (using a pressure cooker) with sodium citrate buffer (10 mM, pH 6) for 15 min. After the antigen retrieval step, sections were washed and blocked in 30% H₂O₂, and the primary antibody (anti-phospho RSK) was added for overnight incubations at 4°C. Immunohistochemical staining was carried out manually using ABCAM Detection IHC Kit (ABCAM) according to the manufacturer’s instructions. Finally, the slides were monitored under microscope and the reaction was stopped with distilled water, counterstained with hematoxylin, dehydrated and coverslipped. Staining was visualized using a light microscope.

### Statistical analysis

The statistical significance of the data was evaluated using Student’s t test (Snedecor & Cochran 1989), and probability values below 0.050 (P<0.050) were considered significant. Quantitative data are expressed as means ± S.D. from the indicated set of experiments.

### Results

PTHrP increases RSK phosphorylation via ERK1/2 in tumor intestinal cells

RSK signaling is involved in many cancers, including colon cancer (Park & Cho 2012). Phosphorylation of this kinase at serine/threonine residues, which occurs at multiple sites, is required for its activation (Anjum & Blenis 2008).
With the aim of elucidating whether exogenous PTHrP is able to activate RSK in the tumor intestinal cells, we investigated the changes in the phosphorylation state of this kinase in Caco2 cells and HCT 116 cells. To that end, the cells were exposed for different times to PTHrP, whole cell lysates were subjected to SDS-PAGE and then immunoblotted using a phospho-specific antibody against the Ser-363 and Thr-359 residues of RSK. We observed that PTHrP markedly increased RSK phosphorylation in a time-dependent manner in both Caco2 cells and HCT 116 cells (Fig. 1, left panel and right panel, respectively). The maximal stimulation was achieved at 1 h and declined at 24 h of treatment with the peptide. The protein levels of total RSK1/2/3 were no different in the absence or presence of PTHrP demonstrating a true increase in their phosphorylation status.

We found that PTHrP activates ERK1/2 and p38 MAPK in Caco2 cells (Martin et al. 2014) and in HCT 116 cells (Martin et al. unpublished work). RSK can be phosphorylated by ERK1/2 on threonine 573, threonine 359 and serine 363 residues (Lara et al. 2013) and by p38 MAPK indirectly on tyrosine 529 residue (Czaplinska et al. 2014). In order to investigate the involvement of MAP kinases in PTHrP-dependent RSK phosphorylation, tumor intestinal cells were pre-incubated in the presence or absence of MAPKs inhibitors for 30 min and then treated for 1 h with or without the hormone. As shown in Fig. 2A, PD 98059 (20 µmol/L), a specific inhibitor of ERK1/2 (the upstream kinases of ERK1/2), totally blocked the phosphorylation of RSK induced by PTHrP while SB 203580 (20 µmol/L) and SB 202190 (10 µmol/L) and the results obtained exclude the possibility that p38 MAPK contributes to RSK activation through the phosphorylation of its tyrosine residues (Fig. 2B). Taken together, these findings suggest that PTHrP increases RSK phosphorylation through ERK1/2 signaling pathway but not via p38 MAPK signaling pathway in both tumor intestinal cell lines.

**Figure 1**

Effects of PTHrP on RSK phosphorylation. Caco2 cells and HCT116 cells were treated with or without PTHrP (1–34) 10⁻⁸ mol/L for different intervals (15 and 30 min and 1, 4, 8 and 24 h) as shown in left and right panels, respectively. Western blot analysis of cell lysates was carried out using an anti-phospho RSK antibody. Total RSK1/2/3 was measured in the same immunoblot by stripping the membrane and re-incubating with anti-RSK1/2/3 antibody. The membranes were stripped and reblotted with anti-GAPDH antibody to ensure the equivalence of protein loading. Representative immunoblots and the quantification by scanning densitometry of three independent experiments are shown; means ± s.d. are given. *P < 0.05.

**RSK signaling in PTHrP-treated tumor cells**

These results were confirmed employing SB 202190 (10 µmol/L), which is other selective p38 MAPK inhibitor (Fig. 2A). As RSK can be phosphorylated by p38 MAPK indirectly on tyrosine 529 residue (Czaplinska et al. 2014), and then we also evaluated the levels of phospho-tyrosine RSK after PTHrP treatment in the absence or presence of the p38 MAPK inhibitors SB2 03580 (20 µmol/L) and SB 202190 (10 µmol/L) and the results obtained exclude the possibility that p38 MAPK contributes to RSK activation through the phosphorylation of its tyrosine residues (Fig. 2B). Taken together, these findings suggest that PTHrP increases RSK phosphorylation through ERK1/2 signaling pathway but not via p38 MAPK signaling pathway in both tumor intestinal cell lines.

**PTHRP induces nuclear localization of activated RSK in tumor intestinal cells**

Activated RSK proteins remain associated with the membrane, remain in the cytosol or translocate to the nucleus, and therefore, can phosphorylate substrates throughout the cell (Anjum & Blenis 2012). The translocation to the nucleus correlates with the induction of immediate-early gene expression (Chen et al. 1992). As the regulation of subcellular localization is important for the function of RSK (Gao et al. 2012), we investigated the subcellular distribution of this kinase in cells exposed to PTHrP for 1 and 4 h. We observed that under basal conditions, activated RSK localized in both, cytosolic and nuclear compartments and that after PTHrP treatment, the amount of the activated enzyme increased in the nuclear fraction of Caco2 cells and HCT 116 cells (Fig. 3, top panel and bottom panel, respectively).
the hormone induces nuclear localization of activated RSK in tumor intestinal cells. Also, in the same figure, the immunoblots showing protein levels of total RSK revealed that in both cell lines, the treatment with PTHrP for 1 h increases total nuclear RSK. Taken together, these results indicate that, at least at 1 h of hormone exposure, the increase of phospho-RSK in the nucleus may be due to the fact that the enzyme is phosphorylated in the nucleus and/or is translocated to the nucleus.

The effects of PTHrP on cell proliferation and in the expression of cyclin D1, CDK6 and p53 are dependent on the ERK/RSK signaling pathway

We previously reported that PTHrP increases the expression of cyclin D1 and CDK6, two cell cycle regulatory proteins, which are essential for cell cycle progression, and diminishes the amount of p53, a negative cell cycle regulator, via ERK1/2, p38 MAPK and PI3K signaling pathways in tumor intestinal cells (Calvo et al. 2014, Martin et al. 2014). It has been shown that RSK is involved in the regulation of cell cycle progression and cell proliferation (Lara et al. 2013). Based on these previous findings and as we observed that PTHrP induced the phosphorylation of RSK through ERK 1/2 signaling pathway in tumor intestinal cells, we first perform experiments employing trypan blue dye exclusion assay to confirm that RSK regulates cell proliferation in these cells treated with PTHrP. To that end, Caco2 cells were pre-incubated with a specific inhibitor of RSK, SL0101 (50 µmol/L) and then treated for 5 days with PTHrP. As shown, counting live cells in a Neubauer chamber by means of trypan blue dye exclusion revealed that Caco2 cells response to PTHrP was partially abolished in the presence of RSK inhibitor. We selected this time of treatment because in previous studies we observed that the maximum response in cell proliferation is achieved at 5 days of PTHrP exposure in Caco2 cells (Martin et al. 2014). In addition, we observed that the combination of the inhibitor of RSK, SL0101 (50 µmol/L) with the chemotherapeutic drug 5-fluorouracil (5-FU) (100 µM) potentiated the inhibition of cell growth observed in tumor intestinal cells treated with 5-FU alone (Fig. 4A).

Then, we investigated the role of this kinase in cell cycle progression induced by PTHrP. To that end, Caco2 cells were pre-incubated with SL0101 (50 µmol/L), and then incubated with PTHrP for 1 and 24 h. Western blot analysis revealed that the effect of PTH analog on p53, cyclin D1 and CDK6 protein levels is reversed by the RSK inhibitor, suggesting that PTHrP-mediated expression of these cell

Figure 2
Involvement of ERK1/2 but not of p38 MAPK signaling in PTHrP-dependent RSK phosphorylation. (A) Cells were pre-incubated with MEK inhibitor PD 98059 (20 µmol/L) or p38 MAPK inhibitors (SB 203580, 20 µmol/L or SB 202190, 10 µmol/L), and then treated with or without PTHrP 10⁻⁸ mol/L for 1 h. Western blot analysis of cell lysates was carried out using an anti-phospho RSK antibody. Total RSK1/2/3 was measured in the same immunoblot by stripping the membrane and re-incubating with anti-RSK1/2/3 antibody. The membranes were stripped and reblotted with anti-GAPDH antibody to ensure the equivalence of protein loading. Representative immunoblots and the quantification by scanning densitometry of three independent experiments are shown; means±s.d. are given (* P<0.05). Caco2 cells and HCT 116 cells are shown in left and right panels, respectively. (B) Tumor intestinal cells were exposed to PTHrP (10⁻⁸ mol/L) for 1 h in the presence or absence of the p38 MAPK inhibitors SB 203580 (20 µmol/L) or SB 202190 (10 µmol/L). Then, in cell lysates RSK was immunoprecipitated with anti-RSK1/2/3 antibody, resolved onto SDS-PAGE gels and immunoblotted with anti-phospho tyrosine antibody. The membranes were dyed using Coomassie brilliant blue to demonstrate equal loading.
cycle regulatory proteins is dependent, at least in part, on ERK/RSK signaling pathway in tumor intestinal cells (Fig. 4B and C). According to the results showed in Fig. 1, in Fig. 4B we also observed an increment of phospho- RSK at 1 h of PTHrP treatment, whereas the levels of total RSK were not different in all experimental conditions in Fig. 4B as well as Fig. 4C.

**PTHRP enhances tumor intestinal cell migration through ERK/RSK signaling pathway but not via p38 MAPK**

It has been demonstrated that MAPK activity is also essential for cell migration (Huang et al. 2004). Also, there is increasing evidence indicating that RSK is strongly implicated in cell motility (Czaplinska et al. 2014). In order to obtain further investigation of the functional consequences of RSK activated by PTHrP, we assessed whether the hormone induces changes on migration behavior of tumor intestinal cells employing two methods: wound healing assay and transwell migration assay. A scratch was performed in culture of confluent Caco2 cells using a 10 µL pipette tip and then we observed the wound healing to compare the migration between untreated cells and cells treated with PTHrP. Representative photographs, taken at time points 0, 4, 24 and 48h of the identical location and the quantification of the results of three separate experiments are shown in Fig. 5A. While under all conditions a closing of the wound was observed, a significant enhancement in wound closure was detected in cells exposed to PTHrP compared to control values at 24h with a maximal effect at 48h. As the wound-healing assay is a method that measures combined effects on cell migration and proliferation, then we performed a transwell migration assay to exclude the contribution in the measurements due to proliferative effects. Caco2 cells and HCT 116 cells were cultivated on 8µm pore size filters in a 24-well culture plate with the same conditions as used for the wound healing assay. Migrated cells were found under all conditions, but a significant increase in migration was evidenced after stimulation with PTHrP for 24 h in Caco2 cells and for 18 h in HCT 116 cells compared to control values (Fig. 5B, left panel and right panel, respectively). These results indicate that PTHrP enhances migration of these tumor intestinal cells.

To gain insight into the signaling events that link PTHrP to cell migration process and to analyze whether MAPK and RSK are involved in PTHrP-stimulated motility, cells were pretreated with MAPK inhibitors or with the inhibitor of RSK, SL0101 (50 µmol/L) and then treated with PTHrP followed by transwell migration assays. As shown in Fig. 5C, ERK1/2 and RSK inhibitors prevented the effect of PTHrP on cell migration, whereas p38 MAPK inhibitor did not reverse the response of Caco2 cells and HCT116 cells to the hormone (left panel and right panel, respectively). These results were confirmed employing SB 202190 (10 µmol/L), which is other selective p38 MAPK inhibitor (data not shown). Taken together, these results suggest that effect of PTHrP on cell migration is dependent on ERK 1/2 and RSK activation but is independent of the p38 signaling pathway.

**PTHRP increases the protein expression of FAK via ERK1/2 and RSK**

The focal adhesion kinase (FAK) is a tyrosine kinase that localizes in focal adhesions acting as a regulator of cell migration (Provenzano & Keely 2009). For further clarification of the mechanisms and the molecular effectors
that may be involved in PTHrP regulation of tumor intestinal cells migration, we evaluated the expression of FAK after PTHrP treatment for 15 min to 24 h. As shown in Fig. 6, western blot analysis revealed that PTHrP increased the protein levels of FAK in both Caco2 cells and HCT116 cells (left and right panel, respectively). The maximal stimulation was achieved at 1 h and declined at 24 h of treatment with the peptide.

To investigate the involvement of RSK and MAP kinases in the upregulation of FAK expression induced by PTHrP, cells were pre-incubated in the presence or absence of the RSK or MAPKs inhibitors and then treated with or without PTHrP. Figure 7, top panel showed that in Caco2 cells RSK and ERK1/2 inhibitor reversed FAK upregulation induced by the hormone, whereas p38 MAPK inhibitor did not reverse PTHrP effect. Similar results were observed in HCT116 cells (Fig. 7, bottom panel), suggesting that PTHrP increases FAK protein expression via ERK1/2-RSK signaling pathway in both tumor intestinal cell lines. We also evaluated the protein levels of FAK after PTHrP treatment in the absence or presence of SB 202190 (10 µmol/L), which is other selective p38 MAPK inhibitor and the results obtained exclude totally the possibility that p38 MAPK participates in the upregulation of FAK expression induced by PTHrP (Fig. 7).

**PTHRP activates RSK in xenografts tumor**

In view of the results observed in both Caco2 cells and HCT116 cells, the next objective was to investigate whether the hormone also activates RSK in colorectal tumor tissues. Nude mice xenografts of HCT116 cells were established to investigate PTHrP effects in vivo as it has been shown that these cells are tumorigenic in nude mice its tumorigenic capacity being much greater than that of Caco2 cells (Dunn et al. 2011). Immunohistochemistry analysis of these xenografts tumor showed increased levels of p-RSK in tumors treated with PTHrP with respect to the levels observed in tumor treated with PBS, which is the vehicle of the hormone (Fig. 8).
Figure 5
The migration of Caco2 cells and HCT 116 cells is increased by PTHrP. (A) Representative phase micrographs of Caco2 tumor intestinal cells treated with or without PTHrP 10^{-8} mol/L at various times after monolayer wounding and the quantification of the results expressed as percentage of wound closure relative to control are shown. (B) Transwell migration assay. Cells were treated in the presence or absence of PTHrP 10^{-8} mol/L, fixed with methanol and stained with violet crystal. The migrated cells were counted, and the quantification of the results expressed as percentage of migrated cells relative to control is shown. Data are representative of three independent experiments performed in triplicate. *P<0.05. Caco2 cells and HCT 116 cells are shown in left and right panels, respectively. (C) Involvement of ERK1/2 and RSK but not of p38 MAPK in tumor intestinal cells migration induced by PTHrP. Cells were pre-incubated with RSK inhibitor SL0101 (50 μmol/L), MEK inhibitor PD 98059 (20 μmol/L) or p38 MAPK inhibitor SB 203580 (20 μmol/L) for 30 min, treated with or without PTHrP 10^{-8} mol/L and the cell migration was evaluated using a transwell migration assay. Cells were fixed with methanol and stained with violet crystal. The migrated cells were counted and the quantification of the results expressed as percentage of migrated cells relative to control is shown. Data are representative of three independent experiments performed in triplicate. *P<0.05. Caco2 cells and HCT 116 cells are shown in left and right panels, respectively.
Discussion

PTHrP was found to be expressed in numerous types of cancer (McCauley & Martin 2012) and in more than 90% of colon cancer patients (Soki et al. 2012). Differences in its prognostic applicability may reflect temporal aspects and/or downstream events that have been difficult to elucidate in the context of cancer. Moreover, PTHrP is a polyhormone with multiple biologically active domains, which may explain the variability seen in cancer prognosis and the necessity to further elucidate PTHrP actions in cancer. Alternative splicing and post-translational proteolysis generate different PTHrP isoforms and fragments that can elicit various cellular responses. The variety of PTHrP fragments and different actions (autocrine, paracrine, endocrine and intracrine) portrays the complexity of PTHrP-induced responses. PTHrP can participate in cell autonomous processes such as tumor cell cycle progression, apoptosis, survival and migration, by the regulation of different signaling pathways, which enhance the capacity for tumor growth and dissemination (Sourbier & Massfelder 2006).

Recently, we obtained evidence that in Caco2 cells, a cell line from human colorectal adenocarcinoma, exogenous PTHrP (1–34) increases cell proliferation,
promotes cell cycle progression and exerts a protective effect under apoptotic conditions via ERK1/2 and p38 MAPK (Lezcano et al. 2013, Calvo et al. 2014, Martin et al. 2014).

A huge variety of intracellular and extracellular signaling molecules have been implicated in colon cancer. In the intracellular signaling networks, one of the most fundamental pathways is the mitogen-activated protein kinase (MAPK) cascades. The best known are the extracellular signal-regulated kinases (ERK1/2); the c-Jun NH2-terminal or stress-activated protein kinases (JNK or SAPK) and p38 MAPK (Huang et al. 2004). Activation of ERK1/2 signaling is very common in colon cancer (Setia et al. 2014). It has been reported that small molecular inhibitors targeting ERK signaling have shown anti-tumor effects on the colon cancer (Peng et al. 2013). However, mutations of core components in the ERK signaling pathway have been observed in samples, which lead to the drug resistance (Hirschi et al. 2014, Mori et al. 2015). Thus, the study of target of the ERK signaling will facilitate the development of drugs. Recently we observed that PTHrP induces the phosphorylation/activation of ERK 1/2 and α p38 MAPK in Caco2 cells and HCT 116 cells (Martin et al. 2014, Martin et al. unpublished work). Therefore, we propose to further delineate the molecular mechanism involved in tumor intestinal cells with response to PTHrP.

MAPKs phosphorylate and activate downstream protein kinases such as the ribosomal S6 kinase (RSK) that can target proteins involved in cell cycle progression and cell migration (Lara et al. 2013). RSKs kinases emerge as potential therapeutic targets in various types of human cancer (Romeo et al. 2012, Lara et al. 2013, Sulzmaier & Ramos 2013). In this work, we proposed to address whether PTHrP modulates RSK phosphorylation status in Caco2 and HCT 116 cells. To that end, we used an antibody that recognizes RSK1 and RSK2 isoforms with their residues phosphorylated on threonine 359 and serine 363. The function of the different isoforms and their subcellular localization vary depending on cell system. The results presented indicate that in these tumor intestinal cells, PTHrP increases the phosphorylation of RSK via ERK1/2. Several studies show that RSK2 promotes the pro-metastatic function of ERK1/2 (Lara 2013), whereas RSK1 and RSK4 isoforms may have the opposite role in lung cancer (Lara 2011). The fibroblast growth factor increases the phosphorylation of RSK2 on tyrosine 529 indirectly through the p38 MAPK pathway in breast adenocarcinoma cells, regulating the formation of focal adhesions and cell migration. This activation by p38 MAPK possibly facilitated the phosphorylation mediated by ERK1/2 in threonine 359 and serine 363 (Czaplinska et al. 2014). In contrast to these observations, our results suggest that the phosphorylation of RSK induced by PTHrP in these tumor intestinal cells is independent of p38 MAPK.

Activated RSK proteins remain associated with the membrane, remain in the cytosol or translocate to the nucleus, and therefore, can phosphorylate substrates throughout the cell (Anjum & Blenis 2008). Chen and coworkers (Chen et al. 1992) demonstrated that a fraction of activated RSKs enter to the nucleus. Moreover, the regulation of subcellular localization is important for the function of RSK (Gao et al. 2012). In line with these observations, our results show that, in Caco2 cells and HCT 116 cells, PTHrP induces the nuclear localization of activated RSK.

RSKs have been involved in the regulation of cell cycle in various malignancies through indirect (modulation of transcription factors) or direct effects on the cell-cycle machinery (Lara et al. 2013). RSK phosphorylates serum response factor (SRF) and contributes to the transcriptional activation of c-FOS. Activation of c-FOS results in the activation of cyclin D1, promoting G1–S phase progression (Anjum & Blenis 2008). RSK phosphorylates and inhibits glycogen synthase kinase (GSK3), which has been suggested to promote stabilization of cyclin D1 and MYC, resulting in cell cycle progression and cell survival (Anjum & Blenis 2008). However, the roles of RSKs in cell cycle progression of colon cancer cells still remain
unclear. In this study, we provide evidence that ERK/RSK signaling pathway is involved in cell proliferation and cell cycle progression of tumor intestinal cells induced by PTHrP, regulating the expression of cell cycle regulatory proteins. In line with our observations, in the breast cancer cell line MCF-7, inhibition of RSKs using the pan-inhibitor SL-0101 arrests the cell cycle in G1 by preventing the expression of cyclin D1 (Smith et al. 2005).

It has been reported that PTHrP overexpressed in LoVo cells increases the migration of these human colon tumor cells (Shen et al. 2007). This experimental model is used to evaluate mainly an autocrine and/or intracrine action of endogenously expressed PTHrP; however, until now, it was unknown whether exogenous PTHrP could regulate, through a paracrine pathway, the migration of intestinal tumor cells. Using wound healing and transwell migration assays, we now demonstrate, and for the first time, that exogenous PTHrP also increases the migration of Caco2 cells and HCT 116 cells. In agreement with our findings, PTHrP modulates the migration of bone and breast tumor cells (Shen et al. 2004, Mak et al. 2013).

Several lines have implicated the importance of ERK signaling pathway in cancer cell migration (Davies et al. 2014, Li et al. 2014, Zhang et al. 2014, Sever & Brugge 2015). The present study shows that in Caco2 and HCT 116 cells, ERK1/2 inhibition prevented the effect of PTHrP on cell migration, indicating that the hormone regulates this process through an ERK1/2-dependent pathway. According to our results, activation of the ERK1/2 cascade by galectin-3 promotes colon cancer cell migration (Wu et al. 2013). Moreover, ERK1/2 signaling pathway has been involved in the migration of the colon cancer cell SW620 induced by TF/FVIIa/PAR2 (Guo et al. 2011). p38 MAPK has also been implicated in the movement of various cell types (Ray et al. 2003, Yu et al. 2004, An et al. 2015, Lv et al. 2015, Yang et al. 2015). However, here we show that the migration of tumor intestinal cells induced by PTHrP is independent of p38 MAPK pathway.

Figure 9
Signal transduction pathways associated with the migration and cell cycle progression of human colon cancer cells induced by exogenous PTHrP.
RSK signaling is involved in the regulation of cell migration (Zhou et al. 2015). Using an inhibitor of RSK, SL0101, we observed that RSK is implicated in cell migration of Caco2 cells and HCT 116 cells induced by PTHrP. In line with our observations, among RSK family members, RSK1 and RSK2 have been reported to promote cancer cell motility in various cancer cell types, including head and neck squamous cell carcinoma, colon adenocarcinoma and prostate cancer cell lines, through cancer-specific mechanisms (Doehn et al. 2009, Kang et al. 2010, Gawcka et al. 2012).

Cell migration is a highly coordinated complex process that involves the reorganization of the actin cytoskeleton, formation of plasma membrane projections and turnover of cell adhesions. The focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes in focal adhesions acting as a regulator of cell migration. This kinase is overexpressed in multiple human cancers, and it is known to be directly involved in the fundamental adhesion and in many growth factor signaling pathways that are relevant in cancer (Proenzano & Keely 2009). Most studies have focused on understanding the activation of FAK at the posttranslational level. However, increasing evidence demonstrates the importance of small changes in FAK mRNA and protein levels of migrating intestinal epithelial cells (Yu et al. 2000, Basson et al. 2006) and in human colon cancer cells (Nakagawa et al. 1998, Agochiya et al. 1999). So we decided to investigate whether the expression of FAK in our experimental model is modulated by PTHrP and if so, the possible involvement of RSK, ERK1/2 and p38 MAPK in this hormone-induced response. The results showed that the protein expression of FAK is increased in Caco2 cells and HCT 116 cells stimulated with PTHrP through ERK 1/2 and RSK but not via p38 MAPK, suggesting that increased availability of FAK could play a prominent role in the migration process. FAK protein can functionally interact with the MAPK pathways, including ERK1/2 and p38 MAPK, and with RSK (Yu et al. 2000, Walsh et al. 2008). RSK2 is involved in the relocalization of FAK to the focal adhesions (Czapinska et al. 2014). Recent studies have suggested a role of ERK1/2 as an upstream activator to regulate FAK expression via the transcription factor PEA3 in highly metastatic melanoma cancer cells (Li et al. 2013). On the other hand, in disagreement with our findings, the transforming growth factor-beta stimulates intestinal epithelial FAK synthesis via p38-dependent mechanisms (Walsh et al. 2008). Understanding in the functional interaction between FAK, RSK and MAPKs may provide new insights into the deregulated cell migration that is characteristic of cancer.

Conclusions

In conclusion, the present investigation provides, to our knowledge, the first direct evidence demonstrating that exogenous PTHrP increases the phosphorylation of RSK via ERK1/2 but not through p38 MAPK signaling pathway in Caco2 cells and HCT 116 cells. The peptide induces the nuclear localization of activated RSK. RSK is involved in cell proliferation and cell cycle progression of tumor intestinal cells induced by PTHrP through the regulation of the expression of cell cycle regulatory proteins. The hormone also increases the migration and the protein expression of FAK via ERK1/2 and RSK but not through p38 MAPK signaling pathway (Fig. 9). In addition, PTHrP activates RSK in xenografts tumor demonstrating the relevance of the results observed from in vitro studies.

Therefore, the results obtained in this study expand our knowledge on the signaling pathways that are involved on Caco2 cells and HCT 116 cells response to PTHrP. RSK could emerge as potential therapeutic target in colon cancer. Furthermore, this work clearly illustrates a specific context in which ERK1/2 and RSK have been shown to play a key role in the stimulation of intestinal tumor cell migration by PTHrP, while p38 MAPK is not involved in this process.

We consider that our findings may provide new insights into the deregulated cell cycle and cell migration that is characteristic of cancer.

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

Funding
This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) (PICT-2013-1441), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP112-201101-00409), Instituto Nacional del Cáncer (Resolución Ministerial 493/14; RESOL-2016-1006-E-APN-MS) and Universidad Nacional del Sur (PGI: 24/B188; PGI: 24/B230), Argentina.

Acknowledgements
The authors thank Dr Ariel Zwenger for providing us the chemotherapeutic drug 5-FU.
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Received in final form 2 April 2017
Accepted 6 April 2017
Accepted Preprint published online 6 April 2017