Accepted Manuscript

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PII: S0303-7207(19)30005-X

DOI: https://doi.org/10.1016/j.mce.2019.01.005

Reference: MCE 10367

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 5 October 2018

Revised Date: 30 December 2018

Accepted Date: 7 January 2019

Please cite this article as: Calvo, N., Carriere, P., Martín, Marí.Julia., Gigola, G., Gentili, C., PTHrP treatment of colon cancer cells promotes tumor associated-angiogenesis by the effect of VEGF, *Molecular and Cellular Endocrinology* (2019), doi: https://doi.org/10.1016/j.mce.2019.01.005.

PTHrP treatment of colon cancer cells promotes tumor associated-angiogenesis by the effect of VEGF

Natalia Calvo*[1], Pedro Carriere[1], María Julia Martín[1], Graciela Gigola[2], Claudia Gentili[1]

[1]Dept. Biología Bioquímica y Farmacia-INBIOSUR, Universidad Nacional del Sur, Bahía Blanca, Argentina

[2]Dept. Biología Bioquímica y Farmacia, Universidad Nacional del Sur, Bahía Blanca, Argentina.

***Adress for Correspondence:**

Dr. Natalia Calvo

Dept. Biología, Bioquímica y Farmacia-INBIOSUR

Universidad Nacional del Sur

San Juan 670

(8000) Bahía Blanca- Argentina

Tel.: 54-291-4595100, ext. 2430

Fax: 54-291-4595130

e-mail: ncalvo@criba.edu.ar

Abstract

We showed that Parathyroid Hormone-related Peptide (PTHrP) induces proliferation, migration, survival and chemoresistance via MAPKs and PI3K/AKT pathways in colorectal cancer (CRC) cells. The objective of this study was to investigate if PTHrP is also involved in tumor angiogenesis. PTHrP increased VEGF expression and the number of structures with characteristics of neoformed vessels in xenografts tumor. Also, PTHrP increased mRNA levels of VEGF, HIF-1α and MMP-9 via ERK1/2 and PI3K/Akt pathways in Caco-2 and HCT116 cells. Tumor conditioned media (TCMs) from both cell lines treated with PTHrP increases the number of cells, the migration and the tube formation in the endothelial HMEC-1 cells, whereas the neutralizing antibody against VEGF diminished this response. In contrast, PTHrP by direct treatment only increased ERK1/2 phosphorylation and the HMEC-1 cells number. These results provide the first evidence related to the mode of action of PTHrP that leads to its proangiogenic effects in the CRC.

Highlights:

PTHrP has indirect proangiogenic effects in the CRC via VEGF.

PTHrP mediates the interaction of colon tumor cells with the endothelial cells.

PTHrP does not stimulate directly migration neither tube formation of endothelial cells.

Keywords: PTHrP; colon cancer; tumor angiogenesis; VEGF

Abbreviations: PTHrP: Parathyroid Hormone-related Peptide; CRC: colorectal cancer; TCMs: tumor conditioned media.

1. Introduction

Parathyroid hormone-related protein (PTHrP) has a high homology with parathyroid hormone (PTH) in its N-terminal amino acid sequence, so it activates the PTH/PTHrP receptor (PTHR1) mimicking the action of this hormone (Nikitovic et al., 2016). Interestingly, this peptide has wide spread distribution in fetal and adult tissues with physiologic or pathologic functions through endocrine, paracrine, autocrine or intracrine signaling (McCauley and Martin, 2012).

With respect to its role in pathologies, PTHrP was initially involved with cancers, mainly as inductor of hypercalcemia but is also associated with osteoporosis and osteoarthritis, so this hormone can be keeping in mind to be used in therapy (Wysolmerski , 2012). Furthermore, recent research indicates that the peptide has also a critical role in the progression of skeletal metastasis (Zheng et al., 2018) and other different critical features that massively contributes to malignant behavior of different cancers such as colorectal cancer (CRC) (McCauley and Martin, 2012; Hong et al., 2016), CRC is one of the main causes of cancer death in the world (Siegel et al., 2017) where angiogenesis has a critical role (Battaglin, 2018).

One attractive target in cancer therapy is the process of angiogenesis, which is the formation of new blood vessels from a pre-existing network of capillaries, because it allows the supply of oxygen and nutrients to the tumor cells and it contributes to the metastasis. Thus, it is important for the growth and metastasis of various types of cancers, such as CRC. The wide process of angiogenesis involves the activation, proliferation and migration of vascular endothelial cells and a fine

equilibrium between molecules with pro-angiogenic or anti-angiogenic effects, which leads to the subsequent reconstruction and formation of vascular structures (Loizzi et al., 2017; Kong et al., 2017). The loss of this balance affects the progression of the CRC (Battaglin et al., 2018). A pro-angiogenic factor secreted by endothelial and tumor cells is the vascular endothelial cell growth factor (VEGF), which interacts with three subtypes of the vascular endothelial growth factor receptor (VEGFR), numbered 1, 2 and 3. VEGFR2 mediates a wide range of VEGF physiological responses in endothelial cells by activating downstream signaling pathways, such as ERK1/2, c-Src, Akt, endothelial nitric oxide and p38 MAPK (Koch et al., 2011; Greenberg et al., 2008). Despite the use of therapeutic agents directed to the inhibition of angiogenesis in the treatment of metastatic CRC (Battaglin et al., 2018), it has been observed the resistance of many patients leading to the treatment failure and the progression of the disease. Therefore, the knowledge of the mechanisms involved in angiogenesis is of great importance to provide new therapeutic alternatives.

In previous studies, we observed that PTHrP activates ERK1/2 and p38 MAPK, as well as the serine-threonine kinase AKT in the two cell lines from human colon tumors, Caco-2 and HCT116 cells. In these cells, the hormone increases cell proliferation and promotes survival under apoptotic conditions via MAPKs and PI3K/AKT signaling pathways (Lezcano et al., 2013; Martín et al 2014; Calvo et al., 2014).

In both cell lines the hormone also activates p90 ribosomal S6 kinase (RSK) and increases cell migration via ERK1/2-RSK but independently of p38 MAPK signaling

pathway (Calvo et al., 2017). Recently, we found that PTHrP induces chemoresistance in Caco-2 and HCT116 cells through mitogenic signaling pathways such ERK and Akt (Martin et al., 2018). Also, the administration of PTHrP in HCT 116 xenografts of nude mice, increased the expression of RSK and others markers related to tumorigenic events (Calvo et al., 2017; Martín et al., 2018).

PTHrP is involved in different types of tumors, however, its precise and direct role in angiogenesis is controversial and it is not clear (Bakre et al., 2002; Akino et al., 2000). Furthermore, it is unknown if this hormone is involved in tumor-associated angiogenesis in the CRC. It is well established that the signaling pathways regulated by PTHrP in the cell lines derived from CRC and in xenografts of nude mice may participate in angiogenesis (Xu et al., 2015; Hammoud et al., 2016; Dong et al., 2017). Therefore, the objective of the present study was to investigate, both *in vitro* and *in vivo* models, whether PTHrP has a role in Tumor-Associated Angiogenesis and if so, the molecular mechanisms that are involved in this process. In this work, we hypothesized that PTHrP is an important factor that stimulates the interaction between the microenvironment endothelial cells and colon cancer cells mainly through the secretion of pro-angiogenic factors by colon cancer cells treated with the hormone.

We suppose that tumor angiogenesis is subsequent to this interaction and therefore it is also facilitated by the hormone.

2. Materials and methods

116 **2.1. Materials**

117 Human PTHrP (1-34), high glucose Dubelcco's modified Eagle's medium (DMEM)
118 and Trypan blue dye were obtained from Sigma-Aldrich Chemical Co. (St. Louis,
119 Missouri, USA). Fetal bovine serum (FBS) was from Natocord (Córdoba,
120 Argentina). Anti-phopho ERK1/2 and antiCD-31 were from Cell Signaling
121 Technology (Beverly, Massachusetts, USA). Anti-VEGF, anti-GAPDH, goat anti-
122 rabbit peroxidase conjugated secondary antibody and goat anti-mouse peroxidase
123 conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz,
124 California, USA). Mouse monoclonal antibody against PTH receptor (opossum
125 kidney) was from BAbCO (Richmond, California). PD 98059 and LY 294002 were
126 from Calbiochem (San Diego, California, USA). GSK690693 was from Santa Cruz
127 Biotechnology (Santa Cruz, California, USA). Crystal violet was from MERCK
128 (Buenos Aires, Argentina). Protein size markers were from Amersham Biosciences
129 (Piscataway, New Jersey, USA), PVDF (Immobilonpolyvinylidene difluoride)
130 membranes and ECL chemiluminescence detection kit were from Amersham (Little
131 Chalfont, Buckinghamshire, England). Geltrex was from Invitrogen (Carlsbad,
132 California, USA). Transwell filters of polyethylene terephthalate (PET), 8 μm pores,
133 24-well format, were from JETBIOFIL. All other reagents used were of analytical
134 grade.
135

136 **2.2. Xenograft in nude mice**

137 We injected subcutaneously 1×10^6 human colorectal carcinoma HCT116 cells
138 into the left dorsal flanks of 6-week-old N:NIH(S)_nu mice. The number of mice in

each group, the dose of PTHrP(1-34) (for treatment group) and its vehicle PBS (for control group) and the administration scheme were chosen according our previous studies (Calvo et al., 2017; Martín el al., 2018). The animals were sacrificed and tumors were removed after 20 days of PTHrP treatment (Wang et al., 2002). All experiments with animals were approved by a local animal committee for ethics. One piece of each tumor was immediately frozen in liquid nitrogen and maintained at -80 ºC until the corresponding analysis by real-time quantitative RT-PCR assay and another piece was fixed with 4% neutral buffered formaldehyde solution for immunohistochemistry assay. Total RNA from three piece of each tumor was isolated; following with the RNA quantification, the synthesis of the cDNA and the PCR reaction carried out in a real-time PCR system.

2.3. Colon cancer cells culture and treatment

The human colon cell lines Caco-2 and HCT 116 (from the American Type Culture Collection, Manassas, Virginia) were cultured at 37 °C in DMEM that contains 10% FBS, 1% non-essential acids, 100 UI/mL penicillin, 100 mg/mL streptomycin and 50 mg/mL gentamycin in a humid atmosphere of 5% CO_2 in air. We used cells with 80% confluence. Cells were FBS-deprived 24 h for Caco-2 cells and 2 h for HCT 116 cells and then treated with PTHrP (1-34) at different times, using a dose of 10^{-8} M which was chosen in previous studies (Lezcano et al., 2013; Martín et al., 2014; Calvo et al., 2014). In some experiments, cells were pretreated for 30 min with PD 98059 (an inhibitor of MEK, which is the upstream kinase of ERK1/2), LY 294002

(an inhibitor of PI3kinase), GSK690693 (an inhibitor of Akt kinase) or with an equivalent volume of the vehicle of the inhibitors (DMSO) as control. The inhibitors doses employed were obtained according to previous studies (Lezcano et al., 2013; Martín et al., 2014; Calvo et al., 2014; Mahfouz et al., 2017).

2.4. Tumor conditioned media (TCMs) preparation

We obtained colon tumor conditioned media (TCMs) from cultured media of Caco-2 or HCT 116 cells after 24 h incubation with or without 10^{-8} M PTHrP (1-34), always following the same cells/volume ratio. TCMs were collected, centrifuged for 10 min at 10,000 rpm to eliminate cell debris and the supernatants were stored at −80 °C until assayed. The protein content was measured to normalize the results.

2.5. Endothelial Cell culture, treatment and co-culture

HMEC-1 is an immortalized cell line which retains the morphology, phenotype and function of normal human microvascular endothelial cells. HMEC-1 cells (from the American Type Culture Collection, Manassas, Virginia) were cultured at 37 °C in high glucose DMEM supplemented with 10% FBS, 1% non-essential acids, 100 UI/mL penicillin, 100 mg/mL streptomycin and 50 mg/mL gentamycin in a humid atmosphere of 5% CO_2 in air. We used this experimental model because the microvascular endothelial cell line is mainly involved in clinically relevant angiogenesis capillary sprouting *in vivo (*Folkman, 2006), and the effect of PTHrP(1-34) on these cells has not yet been studied.

The treatments were according to the assay. For measuring of endothelial cells number, HMEC-1 cells were treated with the corresponding TCMs or with PTHrP(1-34). For endothelial cell migration assay, in some experiments HMEC-1 cells were cultured with DMEM without serum and the corresponding TCMs or the hormone were used as chemoattractants and in others, endothelial cells were directly exposed with PTHrP(1-34) and DMEM with serum was used as chemoattractant. In co-culture experiments, endothelial cells and colon cancer cells were seeded using transwell filters. Finally, in tube formation assay, HMEC-1 cells were cultured with the corresponding TCMs or with the hormone which was directly applied to the cells seeded on geltrex matrix.

2.6. RNA Isolation and cDNA Synthesis

First, we isolate the total RNA from all pieces of the tumor employing the *EasyPure*® RNA Kit (TRANS, Beijing, China) and from all samples of colon cancer cell lines employing the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) taking into account the manufacturer's instructions in both isolations. Then, we synthesize the cDNA using High Capacity cDNA Reverse Transcription Kits following the manufacturer's instructions (Applied Biosystems, USA) and we store it at −20 °C for real-time quantitative RT-PCR (qRT-PCR).

2.7. Real-Time Quantitative RT-PCR

The PCR reaction was performed in a real-time PCR system (Applied Biosystems, model 7500), using SYBR green master mix (No. 4309159, Applied Biosystems)

for PCR reactions according previous works (Calvo et al., 2014). The primers used were the following: 5´-CACCTCTGGACTTGCCTTTC-3´ (forward) and 5´-GGCTGCATCTCGAGACTTTT-3´(reverse) for HIF-1α; 5´-TGCAGATTATGCGGATCAAACC-3´ (forward) and 5´-TGCATTCACATTTGTTGTGCTGTAG-3´(reverse) for VEGF; 5´-CCTTCACTTTCCTGGGTAAG-3´ (forward) and 5´-CCATTCACGTCGTCCTTATG-3´(reverse) for MMP-9; 5´-ACCACAGTCCATGCCATCA-3´ (forward) and 5´- TCCACCACCCTGTTGCTGTA-3´(reverse) for GAPDH. We obtained mRNA levels according to the $2^{-\Delta CT}$ equation and with respect to the corresponding control.

2.8. Immunohistochemistry

First, we deparaffinised the paraffin embedded sections and re-hydrated them. Then, we performed the antigen retrieval using heat and a sodium citrate buffer (10 mM, pH 6) for 15 minutes. The sections were washed with PBS, blocked in 30% H_2O_2 and incubated with the primary antibody (anti-VEGF or anti-CD31) overnight at 4ºC. We employed ABCAM Detection IHC Kit (ABCAM, Cambridge, MA, USA) according to the manufacturer's instructions. Finally, the reaction was stopped with distilled water according to microscopic observation and, counterstained with hematoxylin, dehydrated, and coverslipped. The slides were visualized using a light microscope.

2.9. Measuring of the number of cells

2.9.1. Crystal violet staining. A 96-well plate seeded with HMEC-1 cells (20,000 cells/ well) and cultured with the corresponding TCMs or the hormone at 37 °C for 24 h. Cells were washed with PBS, fixed with methanol for 10 min at -20 °C and stained with 0.1% crystal violet for 30 min at room temperature. Then, we solubilized the dye that stained the cells with10% acetic acid and measured the solution absorbance, which is proportional to the number of cells, at 595nm. Each experiment was carried out with independently obtained TCM.

2.9.2. Trypan blue dye exclusion test. Endothelial cells were washed with PBS, trypsinized to lift them from the plates and stained with 0.4% of Trypan Blue. We counted the number of viable cells that excluded the stain in a microscope using a Neubauer chamber. Each experiment was carried out with independently obtained TCM.

2.9.3. Resazurin Cell Viability assay. Cell viability was evaluated by Resazurin Cell Viability Kit (Cell Signaling Technology, Beverly, Massachusetts, USA). Endothelial cells were plated for triplicate in 96- well plates. After each treatment, 10 µl of the reagent was added on each well followed by 1 hour of incubation at 37 °C. Viable cells retain the ability to reduce resazurin, which is blue and non-fluorescent, into resorufin, which is red and brightly fluorescent. After the incubation step, the relative fluorescent units were measured.

2.10. Endothelial cell migration assay

We performed migration assays employing cell culture inserts (8 µm pore size). A total of 20,000 HMEC-1 cells were seeded in medium without FBS in the upper

chamber (on the top of transwell filters). Then, in some experiments, TCMs prepared as previously described or PTHrP (10^{-8} M) in FBS free medium was added to the bottom chambers so that the HMEC-1 cells on the top of transwell filters migrate towards the lower chamber and to evaluate the indirect or direct PTHrP effect, respectively. In others studies, to further analyze the direct PTHrP effect, the medium in filter inserts was replaced by FBS free medium with or without PTHrP (10^{-8} M) and medium containing 5% FBS was added in the lower chamber. For co-culture assays, Caco-2 or HCT 116 cells (15,000 cells) were previously plated into the lower chamber, grown for 48 h, serum starved for another 24 hours followed by the treatment with or without the hormone to induce secretion of angiogenic factors. Next, the inserts with HMEC-1 cells were placed in the wells with colon cancer cells. After 16 hours, the endothelial cells were washed with PBS and fixed using methanol for 10 minutes at -20 °C. This time was selected according to results of pilot experiments using as a negative control FBS free medium and as a positive control medium with 5% FBS. We removed with a cotton swab the cells on the top side of the transwell filters and then stained the cells on the botton side (migrated cells) with 0.1% crystal violet for 30 min at room temperature. In some experiments, TCMs were pre-incubated for 2 h at 37 °C prior to their use in the migration assay with a specific neutralizing antibody directed against human VEGF (0.1 µg/ml; Santa Cruz) or with an appropriate isotype-matched control rabbit IgG. The antibody concentration was chosen according to literature data (Shtivelband et al., 2003). Finally, the cells that have migrated were

counted using a microscope (2 replicates/ condition, n=3 experiments). Each experiment was carried out with independently obtained TCM.

2.11. Tube formation assay using geltrex matrix

We employed the tube formation assay using geltrex matrix according to previous studies (Chim et al., 2011) and to evaluate the ability of endothelial cells to form an organized tubular network. Growth factor-reduced geltrex basement membrane matrix was thawed at 4 °C, added to 96well-plates (50 µL/well), and left at 37 °C for 1 h to allow gelification. Then, 20,000 HMEC-1 cells/well were seeded on geltrex and incubated with the corresponding TCMs or serum-free medium with or without PTHrP at 37 °C. After 24 h cells were examined and photographed under an inverted light microscope at 100× magnification (NIKON Eclipse Ti-S). Tube formation was quantified by measuring the number of nodes, junctions and branching points and the total branching length using ImageJ (NIH) program with the tool to analyze angiogenesis as described Carpentier (Carpentier, 2012).

In some experiments, TCMs were pre-incubated for 2 h at 37 °C and then used in the geltrex assay with a specific neutralizing antibody directed against human VEGF (0.1 µg/ml; Santa Cruz) or with an appropriate isotype-matched control rabbit IgG. (2 replicates/ condition, n=3 experiments). Each experiment was carried out with independently obtained TCM.

2.12. Western blot analysis

We washed the cells with PBS plus 25 mM NaF and 1 mM Na_3VO_4, and lysed them in buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 3 mM KCl, 1 mM EDTA,1% Tween-20, 1% Nonidet P-40, 20 µg/mL aprotinin, 20 µg/mL leupeptin,1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF, and 1 mM Na_3VO_4. The lysates obtained were vortexed for 45 s, and centrifuged at 14 000*g* and 4 °C for 15 min to collect the supernatant were we performed the quantification of the proteins by the Bradford method (Bradford, 1976). Then, we performed western blot analysis according to the protocol described in our previous studies (Calvo et al., 2014; Calvo et a., 2017).

2.13. Statistical analysis

Statistical analysis of the data was performed using the Student's test (Snedecor and Cochran, 1989), and probability values below 0.050 ($p < 0.050$) were considered significant. The data are expressed as the means ± SD of at least three independent experiments.

3. Results

3.1. PTHrP increases VEGF expression and the number of structures with characteristics of neoformed vessels in xenografts tumor

Several of the signaling pathways that may participate in the regulation of pro-angiogenic factors (Xu et al., 2015; Hammoud et al., 2016; Dong et al., 2017) are modulated by PTHrP in cells derived from CRC (Lezcano et al., 2013; Martín et al., 2014; Calvo et al., 2014; Calvo et al., 2017; Martín et al., 2018). So, to evaluate the

possible role of the hormone in tumor-associated angiogenesis, we initially investigated the effect of PTHrP in the expression of the angiogenic factor VEGF in colorectal tumor tissues. RT-qPCR analysis of nude mice xenografts of HCT116 cells exhibited increased levels of mRNA VEGF in tumors treated with PTHrP respect to the levels observed in control tumor (**Figure 1A**). Furthermore, we also observed increased protein levels of VEGF by immunohistochemistry analysis of these xenografts tumor (**Figure 1B**). According to the results showed by others authors (Domigan et al., 2015; Bhattacharya et al., 2016), the localization of VEGF was observed near the nuclear membrane and cytoplasmic, suggesting also the possible involvement of this factor in an intracrine signaling.

In view of these results, we then evaluated the expression of CD31 by immunohistochemistry in HCT116 xenograft tumors treated or not with PTHrP because this marker is expressed in vascular endothelial cells and it is widely used to highlight intra-tumoral vessels and the degree of neoangiogenesis (Avdalyan et al., 2012). This protein shows the vascular status and it marks both neoformed vessels and normal, preexistent vessels in neoplastic and nonneoplastic tissues (Gee et al., 2003). So, to avoid mistakes, we choose to asses only CD31 positive stain of structures with characteristics of neoformed vessels, which correlates with a more proliferation status of endothelial cells. Both large vessels with hyalinized walls and inflammatory cells with CD 31 positive stain were not counted. The last cells can be distinguished from endothelial cells on the basis of morphological differences. The quantification was performed by counting the number of cells or structures with characteristics of neoformed vessels which stained positively for

CD31 in 10 consecutive fields at x200 magnification. We detected that tumors resulting from subcutaneously implanted HCT 116 cells treated with PTHrP showed increased the number of cells or structures with characteristics of neoformed vessels (CD31+, neoformed structures) (**Figure 1C**). Taken together, these results suggest the pro-angiogenic effects of PTHrP in xenografts *in vivo.*

3.2. PTHrP increases mRNA levels of the factors involved in angiogenesis, VEGF, HIF-1α and MMP-9, in Caco-2 and HCT 116 cell lines.

In view of the results observed *in vivo*, the next goal was to study if the hormone also increases mRNA levels of VEGF in the colon cancer Caco-2 and HCT116 cells. According to the results obtained *in vivo*, RT-qPCR assays revealed that mRNA levels of the angiogenic factor VEGF are increased after PTHrP treatment (10^{-8} M) for 20 h in both cell lines (**Figure 2**). Moreover, the hormone also increased the mRNA levels of others molecules involved in angiogenesis as HIF-1α and MMP-9 (**Figure 2**).

3.3. Colon cancer cells exposed to PTHrP increases the number of endothelial cells.

Tumor angiogenesis is established and maintained by a complex molecular and cellular crosstalk between tumor cells and endothelial cells. Thus, we employed HMEC-1 cells, an immortalized cell line of human microvascular endothelial cells, and TCMs to evaluate the effect on endothelial cells of the factors released from colon cancer cells exposed to PTHrP and so to test tumor angiogenic potential of

366 the hormone *in vitro.* To that end, we first carried out studies to test whether TCMs
367 from colon cancer cells treated with PTHrP modify the number of endothelial cells.
368 The evaluation of endothelial cells number by staining with the basic crystal violet
369 dye revealed that TCMs from colon cancer cells treated with PTHrP for 24 hours
370 increased the absorbance by 23.2 % (TCMs from PTHrP-treated Caco-2 cells) and
371 27.4 % (TCMs from PTHrP-treated HCT 116 cells) with respect to cells exposed to
372 control TCMs (**Figure 3A**). Also, counting live cells (which are not stained with
373 trypan blue in a Neubauer chamber) revealed an increase of 53% and 62% in
374 endothelial cell number after 24 hours of exposure to TCMs from Caco-2 or HCT
375 116 cells treated with PTHrP, respectively (**Figure 3B**). Finally, endothelial cells
376 treated with TCMs were incubated with resazurin and, upon treatment; the
377 metabolic capacity of these cells was measured. We found that TCMs from colon
378 cancer cells treated with PTHrP increase the bioreduction of resazurin, which was
379 accompanied by a corresponding increment in fluorescent measurement by 49.8 %
380 (TCMs from PTHrP-treated Caco-2 cells) and 57.3 % (TCMs from PTHrP-treated
381 HCT 116 cells) with respect to cells exposed to control TCMs (**Figure 3C**). Taking
382 together, these results indicate that TCMs from colon cancer cells treated with
383 PTHrP increases the number of endothelial cells. Based on these findings, then we
384 studied whether the increased number of HMEC-1 cells occurs from an increased
385 rate of proliferation or a decreased rate of cell death or both. So we evaluated the
386 expression of relevant molecular markers associated with apoptosis and
387 proliferation regulation by Western blot analysis. We observed that TCMs from
388 CRC cells treated with the hormone increase the protein levels of PCNA

(proliferating cell nuclear antigen), which is a well-known cell proliferation indicator; however, the expression levels of poly-ADP ribose polymerase (PARP), which is an apoptotic marker, did not change (data not shown). Although these results suggest a proliferative effect, more research is required to confirm this hormonal action.

3.4. Colon cancer cells exposed to PTHrP enhance the migration of endothelial cells.

As during tumor angiogenesis the endothelial cells migrate to generate tumor vasculature, then we evaluated the migratory properties of HMEC-1 cells using transwell inserts and TCMs, as is described in materials and methods. Under these conditions, the endothelial cells were allowed to migrate for 16 h. As shown in **Figure 4A**, TCMs collected from colon cancer cells treated with the hormone for 24 h markedly increase the endothelial cells mobility. Similar data were obtained when the endothelial cells were co-cultured employing transwell inserts with colon cancer cells which were pre-treated with PTHrP (**Figure 4B**). The HMEC-1 cell migration in the control groups (TCMs or cells without PTHrP treatment, **Figure 4A** and **B**, respectively) is probably due to endogenous expression of proangiogenic molecules by the colon cancer cells independently of PTHrP treatment.

3.5. Colon cancer cells exposed to PTHrP induce the formation of tube-like structures in endothelial cells.

To additionally investigate the PTHrP role in the tumor-associated angiogenesis process, we employed the tube formation assay using geltrex matrix to evaluate the capacity of endothelial cells to form an organized tubular network. HMEC-1 cells were seeded on geltrex and they were incubated with the corresponding TCMs. After 24 hours cells were photographed under an inverted light microscope. As shown in **Figure 5A**, TCMs from colon cells treated with PTHrP induced the formation of tube-like structures in endothelial cells seeded on geltrex and also formed a net structure composed of connected HMEC-1 cells. The quantification of the structures from three independent experiments by the Image J program, demonstrated a remarkable increase in the number of nodes, junctions, branching points and in the total branching length (**Figure 5B**).

3.6. PTHrP promotes tumor angiogenesis mainly through VEGF

As the number, migration and formation of tube-like structures of HMEC-1 cells were increased in response to a hormone indirect effect (Caco-2 or HCT 116-dependent), we presumed that the hormone promotes tumor angiogenesis mainly through the secretion of proangiogenic factors from stimulated colon cancer cells. Normally, VEGF would be secreted by cancer cells into the surrounding environment, acting on endothelial cells to drive their proliferation, survival, chemotaxis and migration, and leading to tumor angiogenesis (Greenberg et al., 2008). As PTHrP increases mRNA levels of this angiogenic factor in colon cancer cells, so we sought to investigate if the hormone has an indirect action on endothelial cells through VEGF performing studies with a neutralizing antibody

435 against VEGF. We evaluated if the response of HMEC-1 cells observed previously
436 by us is the same or is different when these cells were exposed to TCMs which
437 were pre-incubated for 2 h at 37 °C with the anti-VEGF antibody or with an
438 appropriate isotype-matched control rabbit IgG. As shown in **Figures 6A**, **B** and **C**,
439 anti-VEGF antibody attenuated the stimulatory effects of TCMs on endothelial cells
440 *in vitro*. Collectively, these findings indicate that PTHrP increases the expression of
441 the angiogenic VEGF in Caco-2 and HCT 116 cells, which in turn is secreted to the
442 culture medium and thus the hormone exerts its effects on endothelial cells in a
443 colon cancer cells dependent manner.
444
445 **3.7. PTHrP increases mRNA levels of VEGF, HIF-1α and MMP-9 in Caco-2 and**
446 **HCT 116 cell lines via ERK1/2 and PI3K/AKT signaling pathways.**
447
448 In previous studies, we obtained evidence that the hormone increases the
449 proliferation, migration, survival and chemoresistance via MAPKs and PI3K/AKT
450 signaling pathways in the colon cancer Caco-2 cells and HCT116 cells (Lezcano et
451 al., 2013; Martín et al., 2014; Calvo et al., 2014; Calvo et al., 2017; Martín et al.,
452 2018). ERK1/2 and AKT are central proteins in many cellular pathways leading to
453 angiogenesis (Xu et al., 2015; Dong et al., 2017). So, to investigate the relation
454 between ERK 1/2 MAPK and PI3K/AKT signaling pathways triggered by PTHrP
455 and the tumor associated-angiogenesis process, colon cancer cells were
456 pretreated with PD 98059 (20 μM) (an inhibitor of the upstream kinase of ERK1/2,
457 MEK), LY294002 (50 μM) (an inhibitor of PI3K) or GSK 690693 (0.1 μM) (an

458 inhibitor of AKT kinase) and then treated with PTHrP followed by RT-qPCR
459 analysis. As shown in **Figure 7A**, ERK1/2 and PI3K/AKT inhibitors reversed the
460 increase of mRNA levels of VEGF, HIF-1α and MMP-9 in Caco-2 and HCT 116 cell
461 lines exposed to PTHrP. Taken together, these results suggest that the effect of
462 PTHrP on the expression of these proangiogenic factors is dependent on ERK 1/2
463 and PI3K/AKT pathways.
464 Then, to determine if ERK1/2 and AKT mediate the angiogenic potential of colon
465 cancer cells induced by PTHrP, we employed TCMs from colon cancer cells
466 pretreated for 30 min with PD 98059, LY 294002 or GSK 690693, to inhibit ERK
467 1/2 and AKT activity, following with the incubation with PTHrP for 24 hours as
468 described in materials and methods. The inhibition of ERK1/2 and PI3K/AKT
469 signaling pathways in colon cancer cells abrogated the stimulatory effects of Caco-
470 2 and HCT 116 cells on HMEC-1 migration (**Figure 7B**). Taken together, these
471 results suggest that the activation of Akt and ERK1/2 by PTHrP in both cell lines is
472 an early and upstream event leading to the induction of VEGF expression and
473 subsequent angiogenic behavior of HMEC-1 cells mediated by secreted VEGF.
474

475 **3.8. PTHrP by direct treatment increases the phosphorylation of ERK1/2 and**
476 **the cells number but not stimulates migration neither tube formation of**
477 **endothelial cells**

478 Results showed that TCMs from colon cancer cells exposed to PTHrP induce an
479 increase in the number of cells, migration and the formation of tube-like structures
480 in HMEC-1 cells. In order to verify if PTHrP also exerts direct effects (colon cancer

cells-independent) on endothelial cell angiogenic behavior, HMEC-1 cells were exposed directly to PTHrP and the cell number, migration and tube formation of these cells were assessed. First, lysates from HMEC-1 cells was tested for the presence of PTH/PTHrP receptor, PTHR1. Western blot analysis showed the presence of a PTH binding component of 90 KDa which is the size for the mature PTHR1 (Kaufmann et al., 1994) (**Figure 8A**). Then, we investigate if the hormone is able to activate MAP kinase signaling in endothelial cells, studying the phosphorylation of ERK1/2. So, HMEC-1 cells were exposed with PTHrP (10^{-8} M) for different times and then western blot analyses were performed with an antibody that recognizes the active form of the ERK1/2. As shown in **Figure 8B**, PTHrP increased the phosphorylation on tyrosine residue of ERK1/2 at 1 hour of treatment but no effects were observed for longer periods. Then, we added the hormone directly to the endothelial cells and then we evaluated the number of HMEC-1 cells by three different methods: crystal violet staining, Trypan blue dye exclusion assay and resazurin reagent. Also, we evaluated their migratory properties using transwell inserts and the formation of tube-like structures employing tube formation assay using geltrex matrix. By staining the cell with the basic crystal violet dye we observed that the treatment with the hormone for 24 hours at a dose of 10^{-7} and 10^{-8} M increased the absorbance by 27% and 42% respectively with respect to untreated cells (**Figure 8C**). Counting live cells that are not stained with trypan blue in a Neubauer chamber revealed that PTHrP at a dose of 10^{-7} and 10^{-8} M for 24 hours of treatment also increase endothelial cell number by 44% and 60% respectively with respect to untreated cells (**Figure 8C**). Furthermore, we found

that PTHrP at a dose of 10^{-8} M for 24 hours of treatment increase the bioreduction of resazurin, which was accompanied by a corresponding increment in fluorescent measurement by 27 % with respect to cells exposed to control (**Figure 8C**). This endothelial cells response to PTHrP was completely reversed in the presence of the ERK1/2 inhibitor (data not shown). Taken together, these results indicate that PTHrP by direct action increases the number of endothelial cells. PTHrP added directly to the cells increased the protein levels of PCNA but did not promote PARP-degradation (data not shown); these findings suggest that the hormone has a proliferative effect but more research is required to confirm this hormonal action. Despite this PTHrP effect on cell accumulation, we found that the hormone (10^{-8} M) directly added to the cells or as chemoattractant did not modify migration of HMEC-1 cells (**Figure 8D**). Moreover, direct treatment with PTHrP (10^{-7} - 10^{-8} M) for 24 hours did not stimulate the tube formation of endothelial cells (**Figure 8E**). Neither was observed when HMEC-1 cells were exposed with the hormone for 48 hours (data not shown). Overall, these results suggest that PTHrP exerts its effects on endothelial cell angiogenic behavior through a colon cancer cells-dependent manner.

4. Discussion

CRC is one of the main causes of death for cancer in the world (Siegelet al., 2017). At the time of diagnosis several patients have metastatic CRC (mCRC) and approximately half of those who have undergone surgery for CRC at an early stage

526 will develop mCRC (Young et al., 2014). Despite improvements in treatments for
527 mCRC, there are still deficiencies and it is necessary more efficient and tolerable
528 alternatives in mCRC therapy.
529 Recently we obtained evidence that PTHrP in HCT 116 xenografts of nude mice
530 increased the expression of markers related to tumorigenic events and it positively
531 modulates RSK, ERK1/2, p38 MAPK and PI3K/AKT signaling pathways in cell lines
532 from human colorectal adenocarcinoma. The hormone also increases cell
533 proliferation, promotes cell cycle progression, enhances cell migration, induces
534 chemoresistance and has a protective effect in conditions of apoptosis by the
535 regulation of these signaling pathways (Lezcano et al., 2013; Martín et al., 2014;
536 Calvo et al., 2014; Calvo et al., 2017; Martín et al., 2018). We think that other
537 possible PTHrP action mode on cells derived from CRC is through the release of
538 bioactive factors induced by the hormone which facilitate tumor growth. The
539 successful tumor growth and establishment of metastasis relies on angiogenesis
540 (Ronca et al., 2017). Several studies have been carried out to elucidate the
541 process of angiogenesis, studying its role in the growth of the primary tumor and
542 the metastasis; they provided promising results and allowed the development of
543 proangiogenic inhibitors such as bevacizumab (a humanized antibody against
544 VEGF) used in combination with chemotherapy for the treatment of tumors such as
545 mCRC (Sánchez-Gundín et al., 2018). However, due to the generation of
546 resistance, it is important to review and continue with the investigation of this
547 process and its regulation to outline new alternative therapies for cancer.

Within the vasculature, endothelial cells produce PTHrP, while there are different reports regarding the expression of the PTH/PTHrP receptor. Rian and collaborators (Rian et al., 1994) showed for the first time that PTHrP, but not the PTH/PTHrP receptor, is produced by human endothelial cells. As smooth muscle cells express PTH/PTHrP receptors (Funk et al., 2002), these investigators suggest that the hormone produced by endothelial cells may has a paracrine action on these muscle cells (Diamond et al., 2006), however, they cannot rule out an autocrine effect of PTHrP.

Curiously, there are controversial results about the effects of PTHrP on angiogenesis. Despite several works suggested that the peptide has a role in this process, the data are contradictory (Akino et al., 2000; Bakre et al., 2002). So, further research is needed to establish if PTHrP may stimulate or not tumor angiogenesis.

An inhibitory role of PTHrP on angiogenesis was first found by Bakre and collaborators (Bakre et al., 2002). They observed that the hormone inhibits tumor-associated angiogenesis in prostate tumors (Bakre et al., 2002). Consistent with the inhibitory effect, the impact of the expression pattern of PTHrP on hair growth also suggest an inhibitory role in the angiogenesis (Diamond et al., 2006; Skrok et al., 2015). Furthermore, Deckers and collaborators showed that the treatment with the hormone significantly decreased VEGF levels in a model of osteoblast differentiation (Deckers et al., 2000).

In the other hand, other authors demonstrated that PTHrP rapidly and transiently stimulates the expression of VEGF in osteoblasts (Esbrit et al., 2000; de Gortazar

et al., 2006; Alonso et al., 2011), suggesting a role of the hormone as an angiogenesis stimulator. Esbrit and collaborators also observed that CMs from osteoblastic cells treated with the hormone increase the growth of bovine aortic endothelial cells (BAEC) (Esbrit et al., 2000). Subsequent reports indicated that PTHrP stimulated bone angiogenesis mainly by its effects on osteoclasts (Cackowski et al., 2010; Zhu et al., 2013). A stimulatory effect of PTHrP on angiogenesis was also reported by Akino and collaborators (Akino et al., 2000). They observed that rat pituitary malignant tumor cells, mGH3, that overexpress PTHrP compared to original GH3 cells, show hypervascularization in xenografts *in vivo.* Moreover, they reported that PTHrP increased capillary formation by BAECs endothelial cells (Akino et al., 2000). Another reported role of PTHrP in the regulation of angiogenesis is important in the prostate cancer (Park and McCauley, 2012). The hormone can induce the expression of IL8, an angiogenic factor, in PCa prostate cancer cells by an intracrine manner independent of its classical nuclear localization sequence (Gujral et al., 2001). In addition, prostate cancer-derived PTHrP has a pro-angiogenic role indirectly, by stimulating of different angiogenic factors in bone marrow stromal cells (BMSCs) (Liao et al., 2008) and increasing the recruitment and angiogenic potential of the bone marrow-derived cells, $CD11b^{+}Gr1^{+}$ cells (Park et al., 2013). There is also a link between PTHrP and VEGF in breast cancer bone metastasis. The hormone modulates breast tumor cell angiogenesis by the regulation of expression levels of critical factors such as VEGF (Isowa et al., 2010), factor VIII (Li et al., 2011), and the connective tissue growth factor (CTGF/CCN2) (Shimo et al., 2006).

Contradictory data may due to the diverse interactions in the microenvironment where there are different target cells of PTHrP. The differences in the expression of the PTHR1 receptor in endothelial cells may also contribute. Although, it is unclear if PTHrP can directly inhibit or stimulate endothelial cells, in most reports, the hormone has a role in tumor angiogenesis as a key mediator for communication and interactions between cancer cells and the microenvironment by stimulating the production of a number of angiogenic factors. Moreover, different fragments of PTHrP may have differing effects on endothelial cells.

In this study, we investigated, for the first time, the roles of PTHrP(1-34) on tumor angiogenesis using colon cancer and endothelial cells and a mouse model. We observed that PTHrP treatment increases both mRNA and protein levels of the pro-angiogenic factor VEGF and the number of structures with characteristics of neoformed vessels in HCT 116 xenografts tumor. Other authors showed a correlation between PTHrP and key markers of angiogenesis process in human tumors such as human prostate cancer and clear cell renal cell carcinoma (Liao et al., 2008; Feng et al., 2013). These previous results provide support for the potential clinical relevance of our observations. Studies with biopsies of CRC patients are necessary to perform to evaluate the significance of our work.

The hormone also increases the mRNA levels of VEGF and others factors involved in angiogenesis, HIF-1α and MMP-9, in Caco-2 and HCT 116 cell lines. The use of specific inhibitors of ERK1/2, PI3K, and Akt suggest that these signaling pathways participate in this response to PTHrP (1-34). Despite pharmacological inhibitors can be useful tools to explore the involvement of signaling pathways in cellular

617 responses to a given hormone, the limitations and off-target effects of employed
618 inhibitors need to be carefully considered in the interpretation of experimental data.
619 Therefore, further researches are required to support the involvement of these
620 signaling pathways in this cellular response to PTHrP.
621 TCM from cultured media of Caco-2 and HCT 116 cells treated with the hormone
622 markedly increased the number and the migration of HMEC-1 endothelial cells.
623 Similar data were obtained using co-culture assays. In addition, TCM from colon
624 cancer cells exposed to PTHrP induced the formation of tube-like structures in
625 endothelial cells. Studies with a neutralizing antibody against VEGF diminished the
626 response of endothelial cells exposed to TCMs suggesting that this response is
627 associated with enhanced production of VEGF.
628 Herein we employed two CRC cell lines with phenotypic differences (Caco-2 cells
629 and HCT116 cells) with the aim to evaluate whether their response to PTHrP are
630 similar or different in these two types of tumor intestinal cells. It is known that
631 mutations in KRAS and PIK3CA genes are reflected in the HCT116 cell line (KRAS
632 and PIK3CA mutant) but not in Caco-2 cell line (KRAS and PIK3CA WT)
633 (Botchkina et al., 2009). In other hand, it has been reported that the mutations of
634 APC gene is very frequent in several CRC cell lines as Caco-2 (APC mutant),
635 however, this mutation is not present in HCT116 cell line (APC WT) (Ilyas et al.,
636 1997). Although mutations of KRAS, PIK3CA and APC correlate with VEGF
637 expression and angiogenesis (Yeh et al., 2017; Chen et al., 2018; Lai et al., 2015;
638 Zhang et al., 2003; Baudino et al., 2002; Yekkala and Baudino, 2007), the fact that
639 both cell lines have similar response to PTHrP support the idea about that the

induction of tumor angiogenesis by PTHrP is independent of these mutation statuses.

In conclusion, the studies carried out in this work show, for the first time, that PTHrP signaling stimulates the production of VEGF in colon cancer cells which acts in the tumor microenvironment promoting the angiogenesis. These results agree with the hypothesis that PTHrP mediates the communication between colon cancer cells and the endothelial cells by stimulating the production of angiogenic factors.

In the other hand, PTHrP by direct treatment only increased the phosphorylation of ERK1/2 and the cells number but not stimulated the migration neither tube formation of HMEC-1 cells and these findings rule out a proangiogenic effect of the hormone by its direct action on these endothelial cells and also indicate that PTHrP alone is not enough to regulate tumor angiogenesis.

Taken together, these results provide new insights of colon tumor cell behavior induced by PTHrP (1-34); also, this work provides the first evidence related to the paracrine/autocrine mode of action of PTHrP (1-34) that leads to its proangiogenic effects in the CRC which is indirect and involves the interaction of colon tumor cells with the microenvironment endothelial cells.

The elucidation of the indirect regulation of the hormone on this process is very important and of great interest for the understanding of the different roles of PTHrP in the colon cancer and its relationship with the angiogenesis. Further researches are required to evaluate the possible role of the other PTHrP fragments in the CRC, and if the hormone also acts in an intracrine manner.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding and acknowledgements

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) (PIP11220150100350), Instituto Nacional del Cáncer (Asistencia Financiera III-2016-2017, RESOL-2016-1006-E-APN-MS) and Universidad Nacional del Sur (PGI: 24/B188; PGI: 24/B230), Argentina.

References

Akino K, Ohtsuru A, Kanda K et al. Parathyroid hormone-related peptide is a potent tumor angiogenic factor. Endocrinology. 2000;141:4313–4316.

Alonso V, de Gortázar AR, Ardura JA et al. Parathyroid hormone-related protein (107-139) increases human osteoblastic cell survival by activation of vascular endothelial growth factor receptor-2. J Cell Physiol. 2008;217:717-727.

686 Avdalyan A, Bobrov I, Klimachev V et al. Prognostic Value of Microvessel Density
687 in Tumor and Peritumoral Area as Evaluated by CD31 Protein Expression and
688 Argyrophilic Nucleolar Organizer Region Count in Endothelial Cells in Uterine
689 Leiomyosarcoma. Sarcoma. 2012;2012:594512.
690
691 Bakre MM, Zhu Y, Yin H et al. Parathyroid hormone-related peptide is a naturally
692 occurring, protein kinase A-dependent angiogenesis inhibitor. Nat Med.
693 2002;8:995–1003.
694
695 Battaglin F, Puccini A, Intini R et al. The role of tumor angiogenesis as a
696 therapeutic target in colorectal cancer. Expert Rev Anticancer Ther. 2018;19:1-16.
697
698 Baudino TA, McKay C, Pendeville-Samain H, et al. c-Myc is essential for
699 vasculogenesis and angiogenesis during development and tumor progression.
700 Genes Dev. 2002;16(19):2530-43.
701
702 Bhattacharya R, Ye XC, Wang R et al. Intracrine VEGF Signaling Mediates the
703 Activity of Prosurvival Pathways in Human Colorectal Cancer Cells. Cancer Res.
704 2016;76:3014-3024.
705
706 Botchkina IL, Rowehl RA, Rivadeneira DE et al. Phenotypic subpopulations of
707 metastatic colon cancer stem cells: genomic analysis. Cancer Genomics
708 Proteomics. 2009;6(1):19-29.

Bradford M. A rapid and sensitive method for quantification of microgram quantities of proteins utilizing the principle of protein binding. Anal Biochem. 1976; 72:248–254.

Cackowski FC, Anderson JL, Patrene KD et al. Osteoclasts are important for bone angiogenesis. Blood. 2010;115:140-149.

Calvo N, Carriere P, Martin MJ et al. RSK activation via ERK modulates human colon cancer cells response to PTHrP. J Mol Endocrinol. 2017;59:13-27.

Calvo N, Martín MJ, de Boland AR et al. Involvement of ERK1/2, p38 MAPK, and PI3K/Akt signaling pathways in the regulation of cell cycle progression by PTHrP in colon adenocarcinoma cells. Biochem Cell Biol. 2014;92:305-315.

Carpentier G. Angiogenesis analyzer, Image. J. News. 2012. Available from: http://image.bio.methods.free.fr/ImageJ/?Angiogenesis-Analyzer-for-ImageJ

Chen M, Lin M, Wang X. Overexpression of miR-19a inhibits colorectal cancer angiogenesis by suppressing KRAS expression. Oncol Rep. 2018;39(2):619-626.

730 Chim SM, Qin A, Tickner J et al. EGFL6 promotes endothelial cell migration and
731 angiogenesis through the activation of extracellular signal-regulated kinase. J Biol
732 Chem. 2011;286:22035-22046.
733
734 de Gortazar AR, Alonso V, Alvarez-Arroyo MV et al. Transient exposure to PTHrP
735 (107-139) exerts anabolic effects through vascular endothelial growth factor
736 receptor 2 in human osteoblastic cells in vitro. Calcif Tissue Int. 2006;79:360-369.
737
738 Deckers MM, Karperien M, van der Bent C et al. Expression of vascular endothelial
739 growth factors and their receptors during osteoblast differentiation. Endocrinology.
740 2000;141:1667-74.
741
742 Diamond AG, Gonterman RM, Anderson AL et al. Parathyroid hormone hormone
743 related protein and the PTH receptor regulate angiogenesis of the skin. J Invest
744 Dermatol. 2006;126:2127–2134.
745
746 Domigan CK, Ziyad S, Iruela-Arispe ML. Canonical and noncanonical vascular
747 endothelial growth factor pathways: new developments in biology and signal
748 transduction. Arterioscler Thromb Vasc Biol. 2015;35:30-39.
749
750 Dong Y, Wu G, Zhu T et al. VEGF promotes cartilage angiogenesis by phospho-
751 ERK1/2 activation of Dll4 signaling in temporomandibular joint osteoarthritis

caused by chronic sleep disturbance in Wistar rats. Oncotarget. 2017;8:17849-17861.

Esbrit P, Alvarez-Arroyo MV, De Miguel F et al. C-Terminal parathyroid hormone-related protein increases vascular endothelial growth factor in human osteoblastic cells. J Am Soc Nephrol. 2000;11:1085-1092.

Feng CC, Ding GX, Song NH, et al. Paraneoplastic hormones: parathyroid hormone-related protein (PTHrP) and erythropoietin (EPO) are related to vascular endothelial growth factor (VEGF) expression in clear cell renal cell carcinoma Tumour Biol. 2013;34:3471-6

Folkman J. Angiogenesis. Annu Rev Med. 2006;57:1–18.

Funk JL, Wei H, Downey KJ et al. Expression of PTHrP and its cognate receptor in the rheumatoid synovial microcirculation. Biochem Biophys Res Commun. 2002;297:890–897.

Gee MS, Procopio WN, Makonnen S et al. Tumor vessel development and maturation impose limits on the effectiveness of anti-vascular therapy. Am J Pathol. 2003;162:183-93.

774 Greenberg JI, Shields DJ, Barillas SG et al. A role for VEGF as a negative
775 regulator of pericyte function and vessel maturation. Nature. 2008;456:809–813.
776
777 Gujral A, Burton DW, Terkeltaub R et al. Parathyroid hormone-related protein
778 induces interleukin-8 production by prostate cancer cells via a novel intracrine
779 mechanism not mediated by its classical nuclear localization sequence. Cancer
780 Res. 2001;61:2282-2288.
781
782 Hammoud L, Adams JR, Loch AJ et al. Identification of RSK and TTK as
783 Modulators of Blood Vessel Morphogenesis Using an Embryonic Stem Cell-Based
784 Vascular Differentiation Assay. Stem Cell Reports. 2016;7:787-801.
785
786 Hong N, Yoon HJ, Lee YH et al. Serum PTHrP Predicts Weight Loss in Cancer
787 Patients Independent of Hypercalcemia, Inflammation, and Tumor Burden. J Clin
788 Endocrinol Metab. 2016;101:1207-1214.
789
790 Ilyas M, Tomlinson IP, Rowan A, et al. Beta-catenin mutations in cell lines
791 established from human colorectal cancers. Proc Natl Acad Sci U S A.
792 1997;94(19):10330-4.
793
794 Isowa S, Shimo T, Ibaragi S et al. PTHrP regulates angiogenesis and bone
795 resorption via VEGF expression. Anticancer Res. 2010;30:2755-2767.
796

797 Kaufmann M, Muff R, Born W et al. Functional expression of a stably transfected
798 parathyroid hormone/parathyroid hormone related protein receptor complementary
799 DNA in CHO cells. Mol Cell Endocrinol. 1994;104:21-27.
800
801 Koch S, Tugues S, Li X et al. Signal transduction by vascular endothelial growth
802 factor receptors. Biochem J. 2011;437:169–183.
803
804 Kong DH, Kim MR, Jang JH et al. A Review of Anti-Angiogenic Targets for
805 Monoclonal Antibody Cancer Therapy. Int J Mol Sci. 2017;18: pii: E1786.
806
807 Lai K, Killingsworth MC, Lee CS. Gene of the month: PIK3CA. J Clin Pathol.
808 2015;68(4):253-7.
809
810 Lezcano V, Gentili C, de Boland AR. Role of PTHrP in human intestinal Caco-2 cell
811 response to oxidative stress. Biochim Biophys Acta. 2013;1833:2834-2843.
812
813 Li J, Karaplis AC, Huang DC et al. PTHrP drives breast tumor initiation,
814 progression, and metastasis in mice and is a potential therapy target. J Clin Invest.
815 2011;121:4655-4669.
816
817 Liao J, Li X, Koh AJ et al. Tumor expressed PTHrP facilitates prostate cancer-
818 induced osteoblastic lesions. Int J Cancer. 2008;123:2267–2278.
819

820 Loizzi V, Del Vecchio V, Gargano G et al. Biological Pathways Involved in Tumor
821 Angiogenesis and Bevacizumab Based Anti-Angiogenic Therapy with Special
822 References to Ovarian Cancer. Int J Mol Sci. 2017;18:pii: E1967.
823
824 Mahfouz N, Tahtouh R, Alaaeddine N et al. Gastrointestinal cancer cells treatment
825 with bevacizumab activates a VEGF autoregulatory mechanism involving
826 telomerase catalytic subunit hTERT via PI3K-AKT, HIF-1α and VEGF receptors.
827 PLoS One. 2017;12:e0179202.
828
829 Martín MJ, Calvo N, de Boland AR et al. Molecular mechanisms associated with
830 PTHrP-induced proliferation of colon cancer cells. J Cell Biochem. 2014;115:2133-
831 2145.
832
833 Martín MJ, Gigola G, Zwenger A et al. Potential therapeutic targets for growth
834 arrest of colorectal cancer cells exposed to PTHrP. Mol Cell Endocrinol.
835 2018;478:32-44.
836
837 McCauley LK, Martin TJ. Twenty-five years of PTHrP progress: from cancer
838 hormone to multifunctional cytokine. J Bone Miner Res. 2012;27:1231-1239.
839
840 Nikitovic D, Kavasi RM, Berdiaki A et al. Parathyroid hormone/parathyroid
841 hormone-related peptide regulates osteosarcoma cell functions: Focus on the
842 extracellular matrix (Review). Oncol Rep. 2016;36:1787-1792.

Park SI, Lee C, Sadler WD et al. Parathyroid hormone-related protein drives a CD11b+Gr1+ cell-mediated positive feedback loop to support prostate cancer growth. Cancer Res. 2013;73:6574-6583.

Park SI, McCauley LK. Nuclear localization of parathyroid hormone-related peptide confers resistance to anoikis in prostate cancer cells. Endocr Relat Cancer. 2012;19:243-254.

Rian E, Jemtland R, Olstad OK et al. Parathyroid hormone-related protein is produced by cultured endothelial cells: a possible role in angiogenesis. Biochem Biophys Res Commun.1994;198:740–747.

Ronca R, Benkheil M, Mitola S et al. Tumor angiogenesis revisited: Regulators and clinical implications. Med Res Rev. 2017;37:1231-1274.

Sánchez-Gundín J, Fernández-Carballido AM, Martínez-Valdivieso L et al. New Trends in the Therapeutic Approach to Metastatic Colorectal Cancer. Int J Med Sci. 2018;15:659-665.

Shimo T, Kubota S, Yoshioka N et al. Pathogenic role of connective tissue growth factor (CTGF/CCN2) in osteolytic metastasis of breast cancer. J Bone Miner Res. 2006;21:1045–1059.

Shtivelband MI, Juneja HS, Lee S et al. Aspirin and salicylate inhibit colon cancer medium- and VEGF-induced endothelial tube formation: correlation with suppression of cyclooxygenase-2 expression. J Thromb Haemost. 2003;1:2225-2233.

Siegel RL, Miller KD, Fedewa SA et al. Colorectal cancer statistics, CA. Cancer J Clin. 2017;67:177-193.

Skrok A, Bednarczuk T, Skwarek A et al. The effect of parathyroid hormones on hair follicle physiology: implications for treatment of chemotherapy-induced alopecia. Skin Pharmacol Physiol. 2015;28:213-225.

Snedecor G, Cochran W. Statistical. Methods, 8th edition. Ames, IA, USA: Iowa. State. University. Press.1989.

Wang Q, Li N, Wang X et al. Augmentation of sodium butyrate-induced apoptosis by phosphatidylinositol 3′-kinaseinhibition in the KM20 human colon cancer cell line. Clin Cancer Res. 2002,8:1940–1947.

Wysolmerski JJ. Parathyroid hormone-related protein: an update. J Clin Endocrinol Metab. 2012;97:2947-2956.

889 Xu J, Yi Y, Li L et al. Osteopontin induces vascular endothelial growth factor
890 expression in articular cartilage through PI3K/AKT and ERK1/2 signaling. Mol Med
891 Rep. 2015;12:4708-12.
892
893 Yeh YW, Cheng CC, Yang ST, et al. Targeting the VEGF-C/VEGFR3 axis
894 suppresses Slug-mediated cancer metastasis and stemness via inhibition of
895 KRAS/YAP1 signaling. Oncotarget. 2017;8(3):5603-5618.
896
897 Yekkala K, Baudino TA. Inhibition of intestinal polyposis with reduced angiogenesis
898 in ApcMin/+ mice due to decreases in c-Myc expression. Mol Cancer Res.
899 2007;5(12):1296-303.
900
901 Young P, Womeldorph C, Johnson E et al. Early detection of colorectal cancer
902 recurrence in patients undergoing surgery with curative intent: current status and
903 challenges. J Cancer. 2014;5:262–271.
904
905 Zhang L, Yang N, Katsaros D, et al. The oncogene phosphatidylinositol 3'-kinase
906 catalytic subunit alpha promotes angiogenesis via vascular endothelial growth
907 factor in ovarian carcinoma. Cancer Res. 2003;63(14):4225-31.
908
909 Zheng X, Kang W, Liu H et al. Inhibition effects of total flavonoids from Sculellaria
910 barbata D. Don on human breast carcinoma bone metastasis via downregulating
911 PTHrP pathway. Int J Mol Med. 2018;41:3137-3146.

Zhu K, Jiao H, Li S et al. ATF4 promotes bone angiogenesis by increasing VEGF expression and release in the bone environment. J Bone Miner Res. 2013;28:1870-1884.

Legends

Figure 1. PTHrP increases mRNA levels and the expression of VEGF and the number of structures with characteristics of neoformed vessels *in vivo*. HCT 116 xenograft tumors removed from nude mice were analyzed by: **(A)** RT-qPCR to evaluate the levels of mRNA VEGF in tumors treated with PTHrP respect to the levels observed in tumor treated with PBS. Bar graphs represent the mRNA levels of VEGF of three independent experiments; means ± s.d. are given. *P < 0.05 with respect to the corresponding control. We obtained the ΔCT according the following equation: ΔCT = CT of the gene of interest − CT GAPDH and mRNA levels according to the $2^{-\Delta CT}$ equation and with respect to the corresponding control. **(B)** Immunohistochemistry. Tumor sections were stained with anti-VEGF antibody. Images (400X) are from tumor treated with PTHrP (right) and tumor untreated (left). Arrow indicates localization of VEGF near the nuclear membrane. **(C)** Immunohistochemistry. Tumor sections were stained with anti-CD31 antibody. Images (200X) are from tumor treated with PTHrP (right) and tumor untreated (left).

Figure 2. PTHrP increases mRNA levels of the factors involved in angiogenesis, VEGF, HIF-1α and MMP-9, in Caco-2 and HCT 116 cell lines. Colon cancer cells were treated with or without PTHrP 10^{-8} M for 3 and 20 h and the mRNA levels of these pro-angiogenic factors were analyzed by quantitative real-time RT-PCR (RT-qPCR) as described in Materials and methods. Bar graphs represent the mRNA levels of VEGF, HIF-1α and MMP-9 of three independent experiments; means ± s.d. are given. *P < 0.05 with respect to the corresponding control.

Figure 3. Colon cancer cells exposed to PTHrP increases the number of endothelial cells. (A) Crystal violet staining. A 96-well plate were seeded with HMEC-1 cells (20,000 cells/ well) and cultured with the corresponding TCMs at 37 °C for 24 h. Then, the number of viable cells was determined by Crystal violet staining as described in materials and methods. Results were expressed as percentage relative to control of three independent measurements performed in triplicate. *P <0.05 with respect to the corresponding control. **(B) Trypan blue dye exclusion.** Cell counts were performed in a Neubauer chamber by means of trypan blue dye exclusion. The effects of each treatment have been compared with the control. The data shown are the average of cell number respect to control of three independent experiments. *P <0.05 with respect to the corresponding control. **(C) Resazurin assay.** The number of viable cells was determined by Resazurin staining. Results were expressed as percentage relative to control of three

956 independent measurements performed in triplicate. **P <0.01 with respect to the
957 corresponding control.
958
959 **Figure 4. Colon cancer cells exposed to PTHrP enhance the migration of**
960 **endothelial cells.** We evaluated the migratory properties of HMEC-1 cells using
961 transwell inserts and: **(A)** TCMs as chemoattractant. A total of 20,000 HMEC-1
962 cells were seeded in medium without FBS in the upper chamber (on the top of
963 transwell filters). Then, TCMs prepared as previously described in Materials and
964 methods were added to the bottom chambers. After 16 h, HMEC-1 cells were fixed
965 with methanol and stained with violet crystal. **(B)** Co-culture assays. Caco-2 and
966 HCT 116 cells were plated into the lower chamber, grown for 48 h, serum starved
967 for another 24 hours followed by the treatment with or without PTHrP. Then, a total
968 of 20,000 HMEC-1 cells were seeded in medium without FBS in the upper
969 chamber (on the top of transwell filters). After 16 h, HMEC-1 cells were fixed with
970 methanol and stained with violet crystal. **(C)** The migrated cells were counted, and
971 the quantification of the results expressed as percentage of migrated cells relative
972 to control is shown. Data are representative of three independent experiments
973 performed in triplicate. *P < 0.05.
974
975 **Figure 5. TCMs from colon cancer cells exposed to PTHrP induce the**
976 **formation of tube-like structures in endothelial cells.** A 96-well plate was
977 coated with cold geltrex 50 µL/well and incubated at 37 °C to solidify the geltrex.
978 HMEC-1 cells (20,000 cells/well)were seeded on geltrex-precoated wells and

cultured with the corresponding TCMs at 37 °C for 24 h. Cells were photographed under an inverted light microscope. **(A)** Representative photomicrographs of tube-like formations by HMEC-1 cells are shown (100x). **(B)** Tube formation was quantified by using Angiogenesis Analyzer for ImageJ (NIH) by measuring the number of nodes, junctions and branching points and the total branching length. Data are representative of three independent experiments performed in triplicate. *P < 0.05.

Figure 6. PTHrP promotes tumor angiogenesis mainly through VEGF. (A) Measuring of the cell number. HMEC-1 cells were cultured at 37 °C for 24 h with the corresponding TCMs which were pre-incubated for 2 h at 37 °C with a specific neutralizing antibody directed against human VEGF or with an appropriate isotype-matched control rabbit IgG. Then, the number of viable cells was determined by Crystal violet staining, Trypan blue dye exclusion and Resazurin assays as described in materials and methods. Results were expressed as percentage relative to control of three independent measurements performed in triplicate. *P <0.05, **P <0.01 with respect to the corresponding control. **(B) Transwell migration assay.** A total of 20,000 HMEC-1 cells were seeded in medium without FBS in the upper chamber (on the top of transwell filters). Samples of TCMs prepared as previously described in Materials and methods were pre-incubated for 2 h at 37 °C with an specific neutralizing antibody directed against human VEGF (0.1 µg/ml). Appropriate isotype-matched control rabbit IgG were included. Then, TCMs were added to the bottom chambers. After 16 h, cells were fixed with

1002 methanol and stained with violet crystal. The migrated cells were counted, and the
1003 quantification of the results expressed as percentage of migrated cells relative to
1004 control is shown. Data are representative of three independent experiments
1005 performed in triplicate. *P < 0.05. **(C) Tube formation assay.** A 96-well plate was
1006 coated with cold geltrex50 µL/well and incubated at 37 °C to solidify the geltrex.
1007 TCMs were pre-incubated for 2 h at 37 °C with an specific neutralizing antibody
1008 directed against human VEGF or with an appropriate isotype-matched control
1009 rabbit IgG. Then, HMEC-1 cells (20,000 cells/well) were seeded on geltrex-
1010 precoated wells and cultured with the corresponding TCMs at 37 °C for 24 h. Cells
1011 were photographed under an inverted light microscope. Representative
1012 photomicrographs of tube-like formations by HMEC-1 cells are shown (100x).
1013
1014 **Figure 7. PTHrP increases mRNA levels of VEGF, HIF-1α and MMP-9 in Caco-**
1015 **2 and HCT 116 cell lines via ERK1/2 and PI3K/AKT signaling pathways. (A)**
1016 Colon cancer cells were pre-incubated for 30 min with PD 98059 (20 µM),
1017 LY294002 (50 µM) or GSK 690693 (50 µM) and then exposed to PTHrP 10^{-8} M for
1018 20 h followed by RT-qPCR analysis as described in Materials and methods. Bar
1019 graphs represent the mRNA levels of VEGF, HIF-1α and MMP-9 of three
1020 independent experiments; means ± s.d. are given. *P < 0.05 with respect to the
1021 corresponding control. **(B) Transwell migration assay.** A total of 20,000 HMEC-1
1022 cells were seeded in medium without FBS in the upper chamber (on the top of
1023 transwell filters). Then, TCMs prepared from colon cancer cell lines treated or not
1024 with PTHrP either alone or combined with the corresponding inhibitors were added

to the bottom chambers. After 16 h, cells were fixed with methanol and stained with violet crystal. The migrated cells were counted, and representative photographs of three independent experiments performed in triplicate are shown.

Figure 8. **Role direct of PTHrP in endothelial cells. (A)** Western blot analysis of HMEC-1 cell lysates was carried out using an anti-PTHR1 antibody. The membranes were stripped and reblotted with anti-GAPDH antibody to ensure the equivalence of protein loading. Caco-2 and HCT 116 cell lines were used as positive control. A representative immunoblot is shown. **(B)** Time course of PTHrP-induced phosphorylation of ERK 1/2 in endothelial cells. HMEC-1 cells were treated with PTHrP (10^{-8} M) for different time intervals. Whole cell proteins were extracted and Western blot was done using specific anti-MAPKs antibodies. The membranes were stripped and re-blotted with anti-GAPDH antibody to ensure the equivalence of protein content among the different experimental conditions. A representative immunoblot and the quantification by scanning densitometry of three independent experiments are shown; means±S.D. are given. *P<0.05 with respect to the control. **(C) Measuring of the cells number.** HMEC-1 cells were cultured with PTHrP (10^{-7} - 10^{-8} M) at 37 °C for 24 hours. Then, the number of viable cells was determined by Crystal violet staining, Trypan blue dye exclusion and Resazurin assays as described in materials and methods. Results were expressed as percentage relative to control of three independent measurements performed in triplicate. *P <0.05 with respect to the corresponding control. **(D) Transwell migration assay.** We evaluated the migratory properties of HMEC-1

cells using transwell inserts and PTHrP as chemoattractant or directly applied to the cells in top panel and bottom panels, respectively. A total of 20,000 HMEC-1 cells were seeded in medium without FBS in the upper chamber (on the top of transwell filters). Then, in some experiments, PTHrP was added to the bottom chambers in PBS free medium as possible chemoattractant using FBS free medium as a negative control. In others experiments the medium in filter inserts was replaced by FBS free medium with or without PTHrP (10^{-8} M) and medium containing 5% FBS was added in the lower chamber. After 16 h, HMEC-1 cells were fixed with methanol and stained with violet crystal. The migrated cells were counted, and representative photographs of three independent experiments performed in triplicate are shown. **(E) Tube formation assay** using geltrex matrix and the hormone directly applied to the cells to evaluate the formation of tube-like structures. A 96-well plate was coated with cold geltrex 50 µL/well and incubated at 37 °C to solidify the geltrex. HMEC-1 cells (20,000 cells/well) were seeded on geltrex-precoated wells and treated with PTHrP (10^{-7} - 10^{-8} M) in SFB-free medium at 37 °C for 24 h. We used medium without FBS without PTHrP and TCM from HCT116 cells treated with PTHrP as control negative and positive, respectively. Cells were photographed under an inverted light microscope. Representative photomicrographs of tube-like formations by HMEC-1 cells are shown (100x).

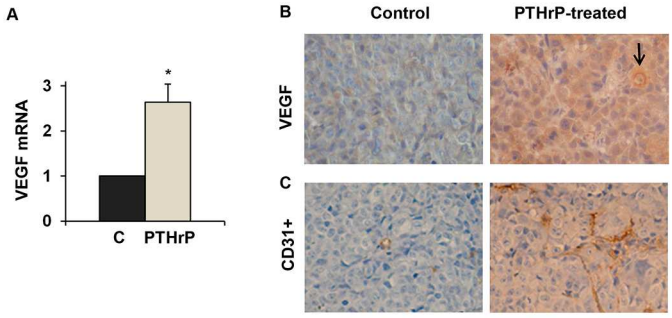


Figure 1

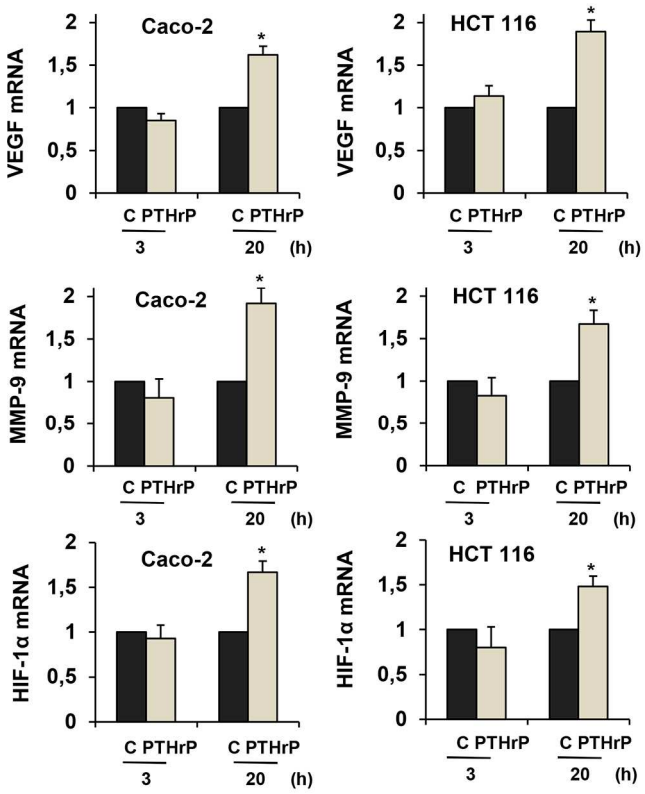
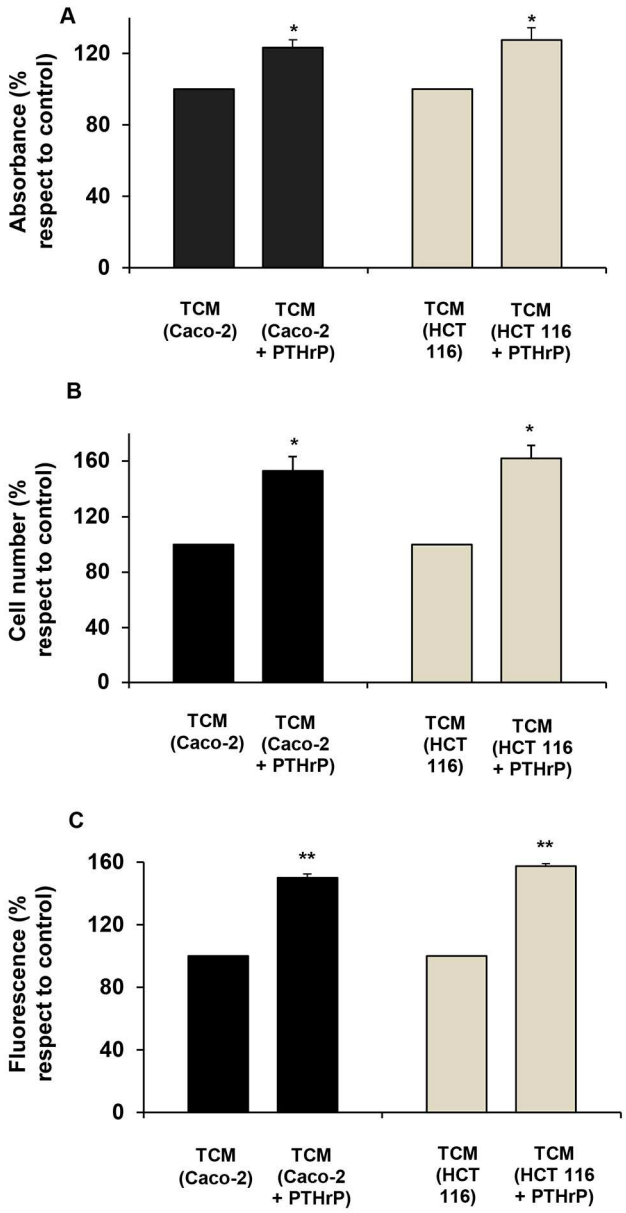


Figure 2



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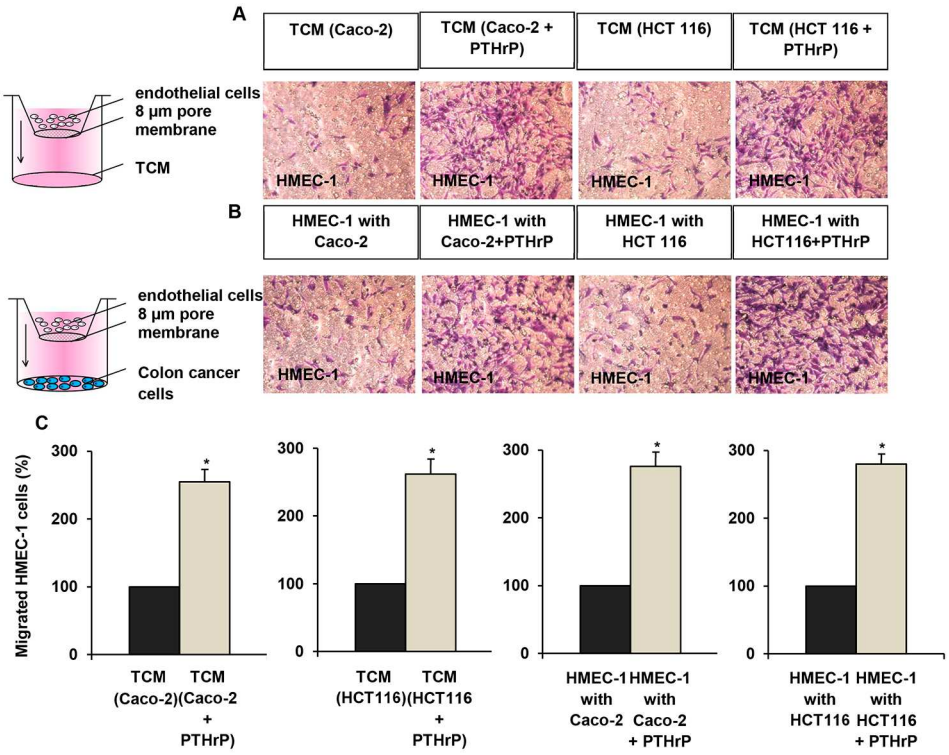
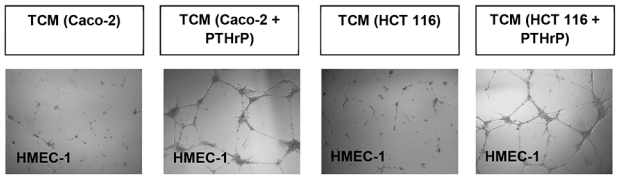


Figure 4

A



B

	Nb nodes	Nb Junctions	Nb branches	Tot. branches lenght
TCM (Caco-2)	0	0	0	0
TCM (Caco-2 + PTHrP)	21	6.5	2.5	739
TCM (HCT 116)	0	0	0	0
TCM (HCT 116 + PTHrP)	24	6.5	6	735.5

Figure 5

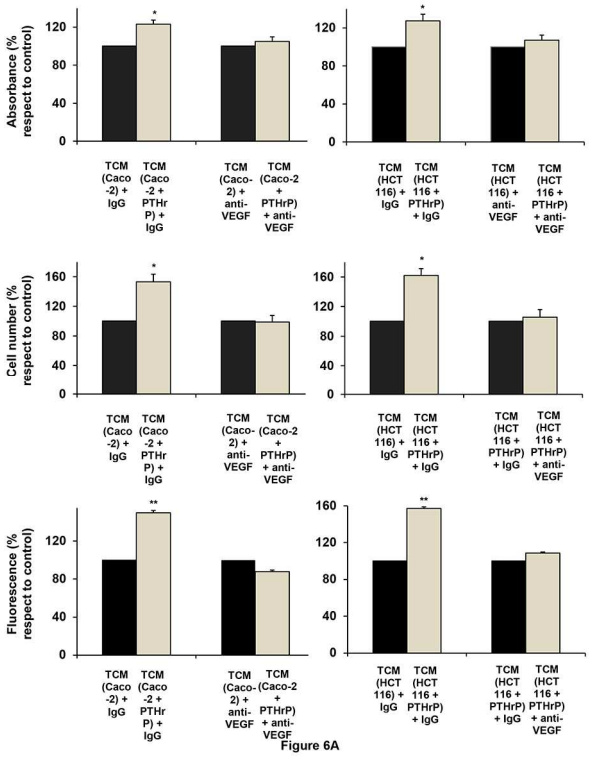


Figure 6A

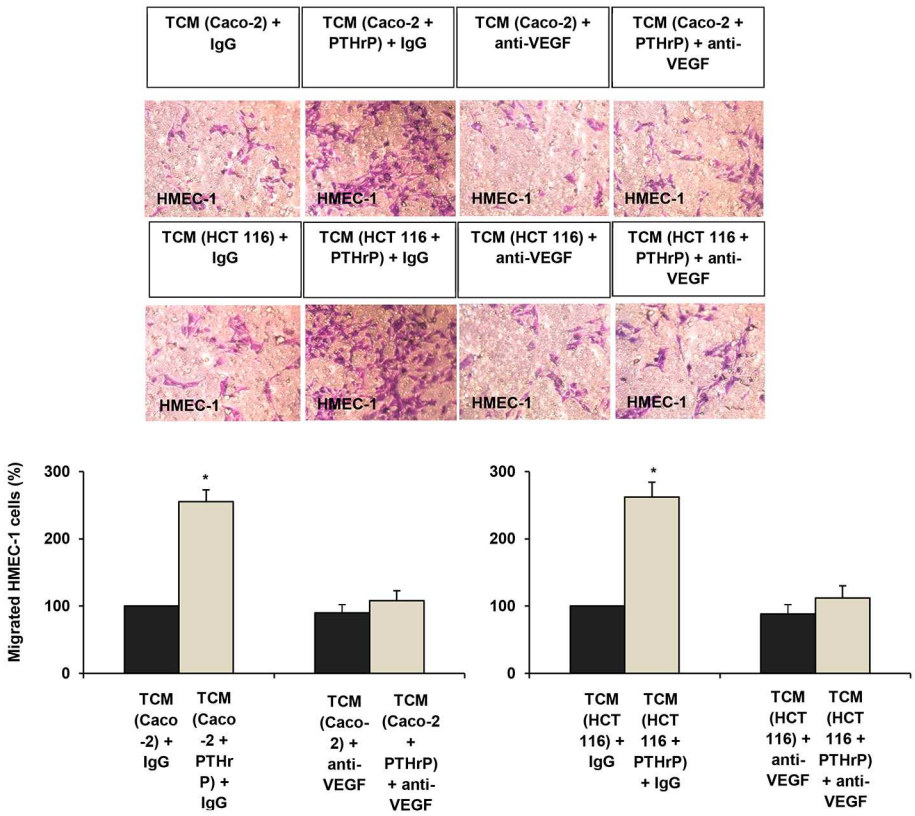


Figure 6B

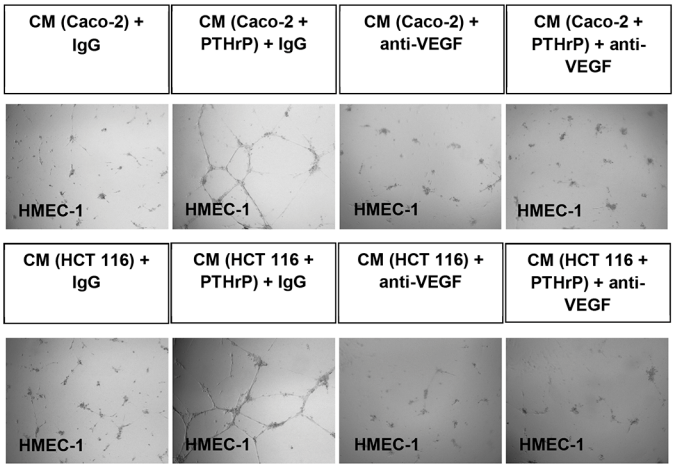


Figure 6 C

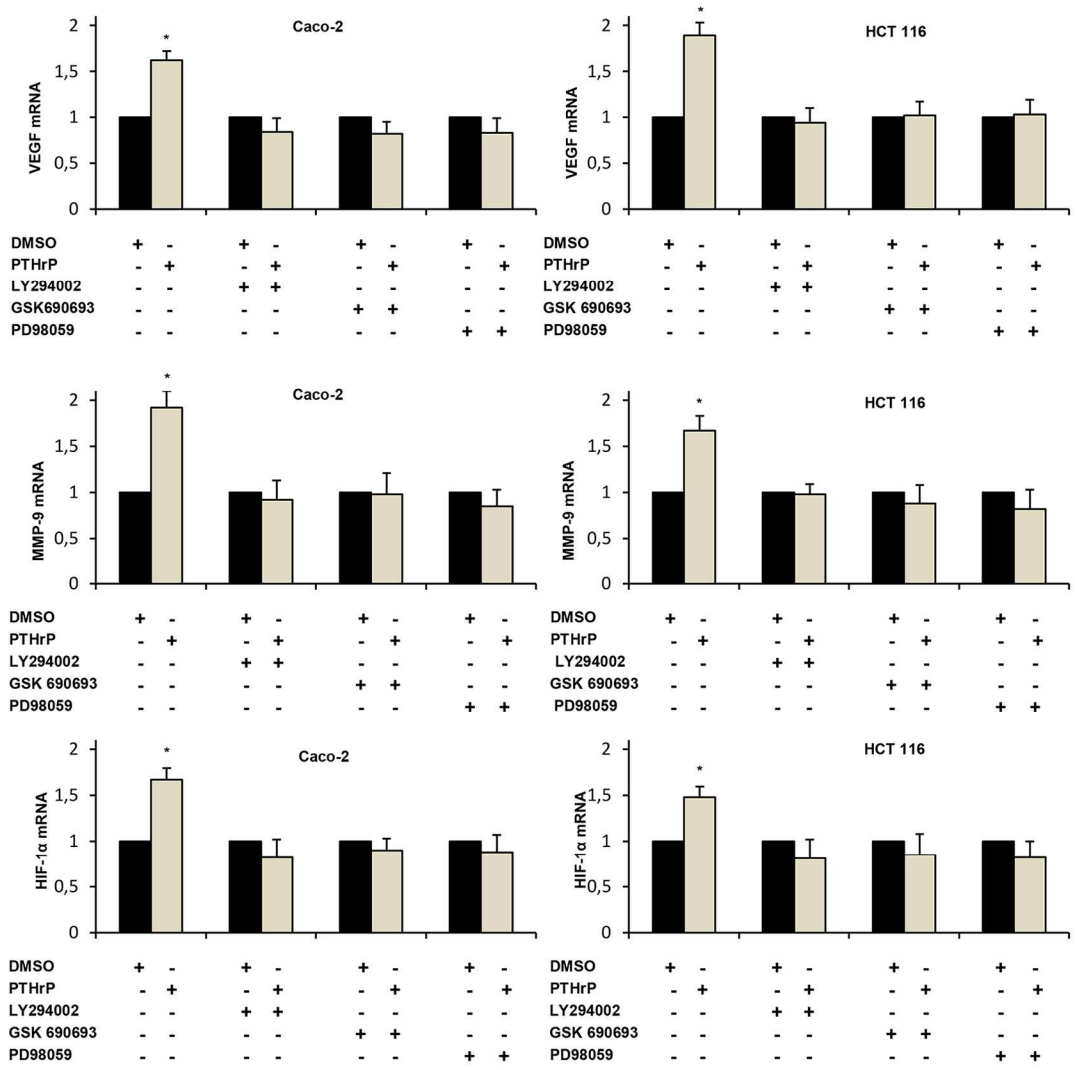


Figure 7A

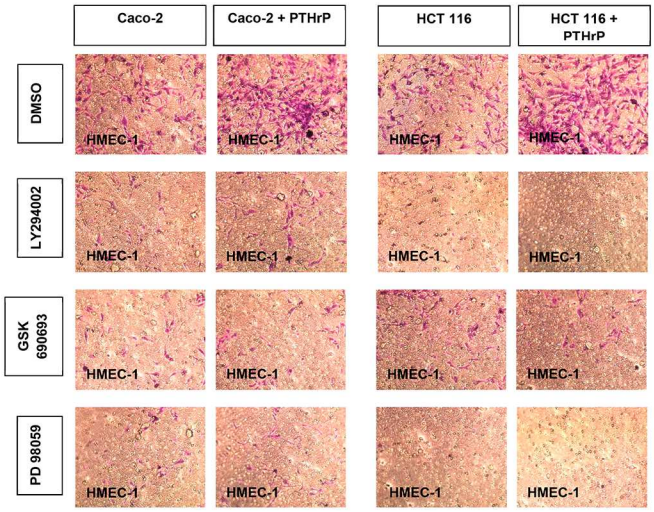


Figure 7 B

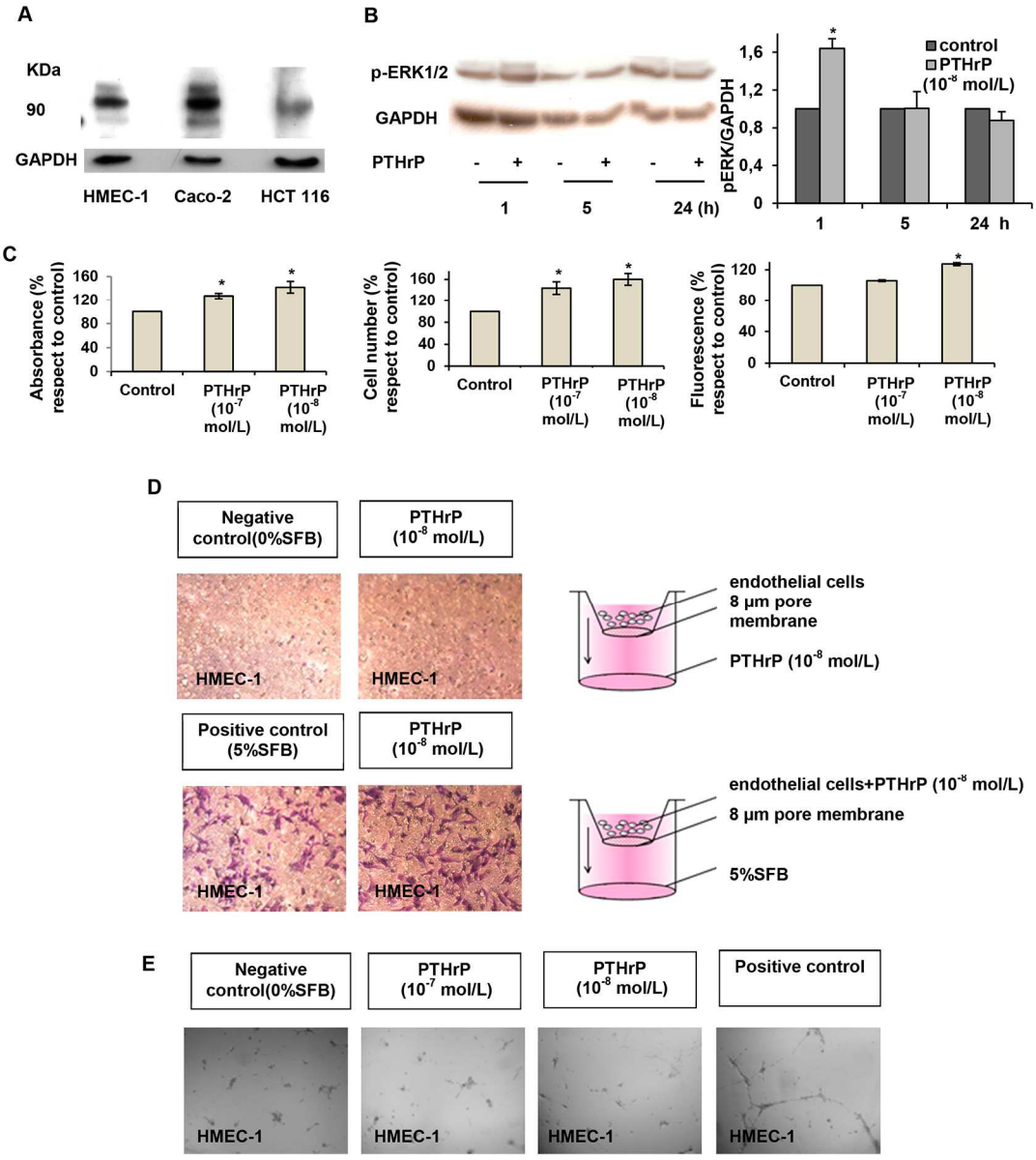

A
KDa
90
GAPDH
HMEC-1
Caco-2
HCT 116
B
p-ERK1/2
GAPDH
PTHrP
- + - + - +
1
5
24 (h)
pERK/GAPDH
1,6
1,2
0,8
0,4
0
control
PTHrP (10-8 mol/L)
1
5
24 h
C
Absorbance (% respect to control)
Cell number (% respect to control)
Fluorescence (% respect to control)
Control
PTHrP (10-7 mol/L)
PTHrP (10-8 mol/L)
D
Negative control(0%SFB)
PTHrP (10-8 mol/L)
HMEC-1
endothelial cells
8 µm pore membrane
PTHrP (10-8 mol/L)
Positive control (5%SFB)
endothelial cells+PTHrP (10-8 mol/L)
5%SFB
E
Negative control(0%SFB)
PTHrP (10-7 mol/L)
PTHrP (10-8 mol/L)
Positive control

Figure 8

Highlights:

PTHrP has indirect proangiogenic effects in the CRC via VEGF.

PTHrP mediates the interaction of colon tumor cells with the endothelial cells.

PTHrP does not stimulate directly migration neither tube formation of endothelial cells.