June 25, 2021

Lian-Sheng Ma
Science Editor and Company Editor-in-Chief
World Journal of Gastroenterology

Dear Dr. Lian-Sheng Ma:

Thank you for your letter of June 12, 2021, informing that the Manuscript NO: 67536 titled “Involvement of PTHrP in the aggressive phenotype of colorectal cancer cells” has been found to be potentially publishable in the journal pending appropriate revision.

We have carefully reviewed the comments of both reviewers and the Science Editor and have modified the manuscript in response to their suggestions. These modifications are highlighted in red in the revised manuscript. We appreciate this criticism which has contributed to improving the presentation of our work.

Detailed in the enclosed sheet there is an enumeration of the changes made in our manuscript. We hope that it will be possible for you to find our paper fully acceptable for publication in the World Journal of Gastroenterology.

Yours sincerely,

Dr. Claudia Gentili

Address for correspondence:

Dr. Claudia Gentili
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Comments to the Editorial Office

As indicated below, we revised the manuscript according to the Editorial Office's comments and indications.

- We have carefully checked the entire manuscript in order to improve the language.

- To avoid abbreviations, we have now modified the title. Therefore, the new title of the manuscript is the following: “Involvement of Parathyroid Hormone - related Peptide in the Aggressive Phenotype of Colorectal Cancer Cells”.

- We have shortened the running title, as follows: PTHrP role in CRC aggressive phenotype

- We have numbered the sections.

Comment to the Reviewers and the Editor:

We thank the Reviewers and Science Editor for taking the time to revise our work. The paragraphs that we have made the changes (in red) are included in each response to both Reviewers.

Reviewer #1:

Thank you very much for your valuable suggestions to improve our study. As indicated below, appropriate changes have been made to meet the comments of the reviewer.

Reviewer #1 comment 1:

Fig 2a and 2b should be merged. ERK should be ERK1/2.

Author response 1:

We appreciate your suggestion and observation. We have now merged Figures 2A and 2B as a single figure (Figure 2). In addition, we replaced ERK with ERK1/2 in
the figure. The changes made in the Figure and the corresponding legend are shown below:

**Figure 2. Molecular mechanisms involved in PTHrP effects on CRC cells.**


PTHR1: parathyroid hormone receptor 1; PTHrP: parathyroid hormone-related peptide; Src: non-receptor tyrosine kinase Src; PKC: protein kinase C; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; ERK 1/2 MAPK: ERK 1/2 mitogen activated protein kinases; p38 MAPK: p38 mitogen-activated protein kinases; RSK: p90 ribosomal S6 kinase; FAK: focal adhesion kinase; CREB: cAMP response element binding protein; ATF-1: activating transcription factor 1.
Reviewer #1 comment 2:

2. It is note that c-myc is one of the main targets of Wnt canonical pathway.

Author response 2:

We appreciate your observation. There is strong evidence that the expression of c-Myc is strictly regulated by the canonical Wnt/β-catenin pathway in CRC (Ren et al., Int J Biol Sci 2020; 16(12):2051–62). Our data revealed that c-Myc protein expression and β-catenin activation are modulated by PTHrP, but the link between these two proteins in our experimental conditions is still unknown. The regulation of the expression of c-Myc and other transcription factors by PTHrP in CRC-derived cell lines through the β-catenin signaling pathway is currently being explored by our research group and the results obtained will be included in a future manuscript for their publication. However, in agreement with the Reviewer’s comment, we think it's appropriate to add a comment in Section 1 of the manuscript, suggesting that β-catenin may be the responsible of the increased of c-Myc protein expression induced by PTHrP in our experimental model. Moreover, we have added an arrow in Figure 2 with a dotted line and a question mark in concordance with this hypothesis. The changes made are shown below (see Section 1, page 10 in the manuscript):

Additionally, as shown in Figure 2, the signaling pathways regulated by PTHrP are responsible for modulating several cell cycle regulators. Our data demonstrated that PTHrP increases the protein expression of Cyclin D1, Cyclin D-dependent kinase 6 (CDK6) and c-Myc. As PTHrP treatment in CRC cells increases β-catenin protein expression and its subsequent nuclear translocation (Martin et al., J Cell Biochem 2014; 115(12):2133–45) and as it is known that c-Myc is a target gene of β-catenin (Ren et al., Int J Biol Sci 2020; 16(12):2051–62), we suggest that the positive modulation of c-Myc by PTHrP in CRC cells may be via β-catenin pathway. More experiments are needed to confirm this hypothesis.
Reviewer #1 comment 3:

3. The crosstalk among pathways should be explored in detail.

Author response 3:

We really understand the concern of the reviewer. According to this, we incorporated in Section 1 (see page 8) of the revised manuscript our findings regarding the molecular mechanisms and the complex cross-talk among the pathways when PTHrP exerts its effects on CRC cells with a paracrine mode of action, as shown below:

1. **PTHRP PROMOTES CELL CYCLE PROGRESSION, PROLIFERATION AND MIGRATION OF CRC CELLS**

As mentioned in the Introduction section, we previously observed that PTH exerts anti-tumor effects in Caco-2 cells through PTHR1[32]. Given that PTHrP (1-34) also binds to the same receptor on the plasma membrane[19,21,33] our first objective was to analyze the actions of this fragment in cell lines derived from colorectal tumors and the associated molecular mechanisms. As previously stated, PTHrP is a factor whose mode of action is mainly paracrine and for this reason all our in vitro experiments were performed with the addition of exogenous PTHrP to cells in culture. Regarding the selection of the concentration of this cytokine, we decided to start our investigations employing doses similar to those used with PTH[32] and considering studies carried out in other experimental models[34]. Since we observed that PTH (1-34) (10^{-8} M) induced apoptosis in Caco-2 cells[32], we investigated whether PTHrP employed at the same dose (10^{-8} M) is able or not to induce this response in this cell line. Surprisingly, PTHrP exerts the opposite effect to PTH since we obtained evidence that PTHrP through a paracrine pathway increases the survival of Caco-2 cells under apoptotic conditions[35]. According to our findings, it was observed in other tumor cells such as breast, renal and prostate cancer cells that PTHrP also increases the resistance to die through the inhibition of apoptosis[20] It is known that the malignant cell transformation involves enhanced cell proliferation, enhanced cell survival by evasion of apoptosis or a combination of both processes[36]. Taking into account this important concept and based on our initial and interesting result concerning the PTHrP effect on Caco-2 cells, the following goal was to further study the role of this cytokine employing the same concentration in these tumor cells.
By the implementation of multiple assays, we found that PTHrP (1-34) stimulates the cell cycle progression and proliferation of Caco-2 cells\cite{37,38}. In line with these results, we reported similar effects induced by the cytokine in HCT116 cells, a CRC-derived cell line more undifferentiated and aggressive concerning Caco-2 cells, which also expresses PTHR1\cite{39}. Despite the notable differences between Caco-2 cells and HCT116 cells phenotypes, the molecular mechanisms leading to these responses to the peptide in both CRC cells were similar and implied the activation of well-known deregulated pathways in CRC. Specifically, we found the activation by PTHrP of non-receptor tyrosine kinase Src, protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), protein kinase B (PKB or Akt), ERK 1/2 mitogen activated protein kinases (ERK 1/2 MAPK), p38 mitogen-activated protein kinases (p38 MAPK), p90 ribosomal S6 kinase (RSK) and β-catenin signaling pathways in CRC cells\cite{37–40}.

\textbf{Figure 2} shows the molecular mechanisms modulated by PTHrP and the complex cross-talk among the pathways when this cytokine acts on CRC cells. The upstream/downstream relations between the proteins of these cascades were analyzed employing specific inhibitors that block the protein activity. So, using Ro-318220, PP1 and LY294002, which are the inhibitors of PKC, Src and PI3K, respectively, we found that the activity of these three kinases converge in the phosphorylation/activation of Akt in CRC cells exposed to PTHrP. Furthermore, the specific inhibitor of Akt, GSK690693, suppressed the phosphorylation/activation of ERK1/2 MAPK induced by PTHrP, suggesting the role of Akt in the activation of this MAPK\cite{38,39}.

Although the upstream cascades which regulate the activation of p38 MAPK by PTHrP is still unknown, we think that the mechanisms involved are triggered immediately after the activation of PTHrP receptor. We suppose this hypothesis because in CRC cells exposed to PTHrP, we observed that p38 MAPK is phosphorylated and subsequently activated faster than Src, PKC and Akt. More studies are needed to confirm our idea.

RSK is a serine/threonine kinase associated with several types of cancer, including CRC. Its activation is complex and involves various signaling pathways such as MAPKs\cite{41}. We found that RSK is activated by PTHrP and to investigate the involvement of ERK1/2 MAPK and p38 MAPK in this activation, we used PD98059 (a specific inhibitor of MEK1/2 which are the upstream kinases of ERK1/2 MAPK) and SB203580 (a p38 MAPK inhibitor). Experimental data revealed that ERK1/2 inhibitor totally blocked the phosphorylation of RSK induced by PTHrP whereas the inhibition of p38 MAPK did not reverse the effect
of PTHrP over RSK phosphorylation. These results indicate that PTHrP activates RSK via ERK1/2 MAPK signaling pathway but not through p38 MAPK [40].

Additionally, as shown in Figure 2, the signaling pathways regulated by PTHrP are responsible for modulating several cell cycle regulators. Our data demonstrated that PTHrP increases the protein expression of Cyclin D1, Cyclin D-dependent kinase 6 (CDK6) and c-Myc. As PTHrP treatment in CRC cells increases β-catenin protein expression and its subsequent nuclear translocation[38] and as it is known that c-Myc is a target gene of β-catenin[42,43], we suggest that the positive modulation of c-Myc by PTHrP in CRC cells may be via β-catenin pathway. More experiments are needed to confirm this hypothesis. On the other hand, PTHrP paracrine action diminishes the expression of the following negative cell cycle regulators: p27Kip1, p15INK4B, and p53. The inhibition of ERK1/2 MAPK, p38 MAPK, PI3K, Akt and RSK pathways suppressed the changes in the protein expression of all the mentioned molecular markers[37–40]. Other transcription factors related to cell proliferation were also activated by exogenous PTHrP, such as cAMP response element binding protein (CREB) and activating transcription factor 1 (ATF-1). The pre-incubation of CRC cells with the specific MAPK inhibitors suppressed the activation of these transcription factors induced by PTHrP[38]. Taken together, our results demonstrated that in CRC cells, PTHrP positively modulates cell cycle progression and proliferation through the modulation of several mitogenic pathways such as PI3K, Akt, ERK1/2 MAPK, p38 MAPK and RSK.

In order to assess PTHrP effects in a more complex CRC model, we also performed in vivo investigations. The studies employing subcutaneous murine xenografts of HCT116 cells revealed that the intratumor administration of PTHrP also stimulates ERK 1/2 MAPK pathway among other mitogenic markers. These data validated part of the results that we observed in vitro[39,40]. One difficulty encountered when implementing the study of in vivo models was that xenografts of HCT116 cells grew rapidly in the subcutaneous area of the mice and this situation led to an insufficient blood irrigation in the center of the tumors. This caused some of the tumors showed internal areas of necrosis that was detrimental from the experimental point of view, since we were unable to observe differences in tumor volume growth due to treatment with PTHrP. Due to this, and in order to preserve the welfare of the animals, all of the in vivo assays were forced to finish at 20 days of the initial administration of PTHrP or vehicle solution. Although at the end of the trials the differences between the volumes and weights of tumors from
untreated and treated animals were not significant, we are sure that if we had continued the assays the size of the tumors in the mice treated with PTHrP would be higher than that observed in control mice. We support this idea because we observed that the continued administration of PTHrP in nude mice xenografts increased the protein levels not only of the mentioned ERK 1/2 MAPK but also of Ki67, which is a marker of CRC cell proliferation, and the following markers: cyclin D1, CREB/ATF-1, RSK\[^{39,40}\] and others\[^{44,45}\] which we will mention in the next sections due to their relation with the tumor-associated angiogenesis and invasion.

As several of these studied signaling pathways are also involved in cell migration\[^{46}\], we then decided to study this process. As shown in Figure 2, we observed that PTHrP enhances the motility of CRC-derived cell lines\[^{40}\]. However, contrary to the results obtained studying tumor proliferation, the effect of this cytokine on migration was higher in the more aggressive cell line, HCT116, than in Caco-2 cells. Furthermore, our investigations revealed that under PTHrP action, ERK 1/2 MAPK and RSK pathways have a relevant role in the increased expression of the focal adhesion kinase (FAK) and in the migration of CRC-derived cells (Figure 2)\[^{40}\].

**Reviewer #2:**

We appreciate the reviewer's opinion regarding that the manuscript is interesting and well written. We also appreciate the comment considering that our group has done important contributions in the field of colon cancer biology. As indicated below, appropriate changes have been made to meet the comments of the reviewer.
The only concern that this reviewer has, is that concerning the reported actions of PTHrP against tumor progression, particularly at the early disease stage, and how these protective effects change over the progression of the disease. This new will improve not only the quality of the manuscript but also provide a more comprehensive landscape for the reader.

Author response:

For the reviewer information: In our initial study (Lezcano et al., Biochim Biophys Acta - Mol Cell Res 2013; 1833(12):2834–43) we observed that Caco-2 cells died due apoptosis when they were exposed to an oxidative insult in the form of hydrogen peroxide. However, the death induced by this apoptotic inductor was prevented when Caco-2 cells were pre-treated with PTHrP prior to H₂O₂ incubation.

Therefore, the concept “protective” mentioned in our manuscript in Section 1 (page 8) does not refer to the protection exerted by the cytokine PTHrP against tumor progression, but rather explains the protective effect of PTHrP under apoptotic conditions in intestinal tumor cells. In this scenario, the number of death cells diminished and the number of viable cells increased because PTHrP favors cell survival. According to our findings, it was observed in other tumor cells such as breast, renal and prostate cancer cells that PTHrP also increased the resistance to die through the inhibition of apoptosis (Soki et al., Futur Oncol 2012; 8(7):803–17).

It is well established that the accumulation of neoplastic cells can occur through enhanced cell proliferation, enhanced cell survival due to the evasion of apoptosis or a combination of both processes (Kaufmann and Gores, BioEssays 2000; 22:1007–17). We observed both responses of tumor cells to PTHrP because this cytokine has an anti-apoptotic effect favoring the survival of CRC-derived cells (Lezcano et al., Biochim Biophys Acta - Mol Cell Res 2013; 1833(12):2834–43) and also induces cell cycle progression and cell proliferation (Calvo et al., Biochem Cell Biol 2014; 92(4):305–15; Martin et al., J Cell Biochem 2014; 115(12):2133–45.).

Based on the reviewer's concern and to avoid confusion for the readers, we have added an appropriate comment in Section 1 (see page 8 in the manuscript) that includes the term “survival” instead of the word “protective”. These changes in the manuscript are shown below:

As mentioned in the Introduction section, we previously observed that PTH exerts anti-tumor effects in Caco-2 cells through PTHR1[32]. Given that PTHrP (1-34) also
binds to the same receptor on the plasma membrane\cite{19,21,33} our first objective was to analyze the actions of this fragment in cell lines derived from colorectal tumors and the associated molecular mechanisms. As previously stated, PTHrP is a factor whose mode of action is mainly paracrine and for this reason all our in vitro experiments were performed with the addition of exogenous PTHrP to cells in culture. Regarding the selection of the concentration of this cytokine, we decided to start our investigations employing doses similar to those used with PTH\cite{32} and considering studies carried out in other experimental models\cite{34}. Since we observed that PTH (1-34) (10^{-8} M) induced apoptosis in Caco-2 cells\cite{32}, we investigated whether PTHrP employed at the same dose (10^{-8} M) is able or not to induce this response in this cell line. Surprisingly, PTHrP exerts the opposite effect to PTH since we obtained evidence that PTHrP through a paracrine pathway increases the survival of Caco-2 cells under apoptotic conditions\cite{35}. According to our findings, it was observed in other tumor cells such as breast, renal and prostate cancer cells that PTHrP also increases the resistance to die through the inhibition of apoptosis\cite{20} It is known that the malignant cell transformation involves enhanced cell proliferation, enhanced cell survival by evasion of apoptosis or a combination of both processes\cite{36}. Taking into account this important concept and based on our initial and interesting result concerning the PTHrP effect on Caco-2 cells, the following goal was to further study the role of this cytokine employing the same concentration in these tumor cells.

Comments from the Science editor:

1 Scientific quality: The manuscript describes a Frontier of the PTHrP in the aggressive phenotype of colorectal cancer cells. The topic is within the scope of the WJG.

(1) Classification: Grade A and Grade B;

(2) Summary of the Peer-Review Report: This is an interesting and well-written manuscript on a very emerging topic on cancer research. The crosstalk among pathways should be explored in detail. The questions raised by the reviewers should be answered;

Author response: We have carefully answered the comments of both reviewers and have modified the manuscript in response to their suggestions. The responses
to the reviewers were previously listed in this attachment and the modifications were highlighted in red in the revised manuscript.

**Comments from the Science editor:**

(3) Format: There are 5 figures;

(4) References: A total of 80 references are cited, including 19 references published in the last 3 years;

**Author response:** Due to modifications made to the manuscript, we have added the following references:


(5) Self-cited references: There are 10 self-cited references. The self-referencing rates should be less than 10%. Please keep the reasonable self-citations (i.e. those that are most closely related to the topic of the manuscript) and remove all other improper self-citations. If the authors fail to address the critical issue of self-citation, the editing process of this manuscript will be terminated.

**Author response:** We carry out an evaluation of our self-citations keeping those closely related to the topic of the manuscript. Changes made to references are detailed below:

Of the four self-cited references that corresponded to antecedents of previous results, we have kept only the next: “Calvo N, de Boland AR, Gentili C. PTH inactivates the AKT survival pathway in the colonic cell line Caco-2. *BiochimBiophys Acta - Mol Cell Res* 2010; 1803:343–51. DOI:10.1016/j.bbamcr.2009.11.011”.

And we have removed the other three self-cited references:


We have chosen a manuscript that summarizes the previous works carried out by members of the group and thus includes part of the background on which the investigations described in Frontier article were based. Furthermore, due to the elimination of the last reference (Calvo et al., 2011), we have removed the following sentence from the **Introduction section** in the revised manuscript: “Moreover, the hormone affects cell cycle biomarkers expression; we observed that PTH upregulates the expression of p27Kip1 protein, an inhibitor of the cell cycle, and downregulates the expressions of cyclin-dependent kinase 6 (Cdk6) and of both cyclins, D1 and D3, in Caco-2 cells”.

Based on these changes, we now include in the revised manuscript a total of 81 references and 8 of them are self-citations.

Comments from the Science editor:

(1) The "Author Contributions" section is missing. Please provide the author contributions;

Author response: We have now incorporated in the revised manuscript the "Author Contributions" section in the first page.

Comments from the Science editor:

(2) The authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s);

Author response: The corresponding documents have been uploaded to the F6 Publishing system along with the rest of the revision files.

Comments from the Science editor:

(3) The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor;

(4) Please confirm if the figures are original. If an author of a submission is re-using a figure or figures published elsewhere, or that is copyrighted, the author must provide documentation that the previous publisher or copyright holder has given permission for the figure to be republished; and correctly indicating the reference source and copyrights. For example, "Figure 1 Histopathological examination by hematoxylin-eosin staining (200 ×). A: Control group; B: Model group; C: Pioglitazone hydrochloride group; D: Chinese herbal medicine group. Citation: Yang JM, Sun Y, Wang M, Zhang XL, Zhang SJ, Gao YS, Chen L, Wu MY, Zhou L, Zhou YM, Wang Y, Zheng FJ, Li YH. Regulatory effect of a Chinese herbal medicine formula on nonalcoholic fatty liver disease. World J Gastroenterol 2019; 25(34): 5105-5119. Copyright ©The Author(s) 2019. Published by Baishideng Publishing Group Inc[6]]. And please cite the reference source in the references list. If the author fails to properly cite the published or copyrighted picture(s) or table(s)
as described above, he/she will be subject to withdrawal of the article from BPG publications and may even be held liable;

**Author response:** The following comment answers concerns to the Issues raised (3) and (4) of the editor.

All figures are original for this work.

**Figure 1** was modified to provide more information. This figure is original for this work and is based in data published in Soki FN, Park SI, McCauley LK. The multifaceted actions of PTHrP in skeletal metastasis. Futur Oncol 2012; 8(7):803–17; Wysolmerski JJ. Parathyroid hormone-related protein: An update. J Clin Endocrinol Metab 2012; 97(9):2947–56; Goltzman D. Nonparathyroid Hypercalcemia. Front Horm Res 2018; 51:77–90.

Given that the results presented in this article derived from a large amount of original data already published, we have made the decision to create new figures that schematically represent and compile the information that our research group has obtained over the last ten years.


**Figure 4** is original for this work and it is created based on the results from Calvo N, Carriere P, Martin MJ, Gigola G, Gentili C. PTHrP treatment of colon cancer cells promotes tumor associated-angiogenesis by the effect of VEGF. *Mol Cell Endocrinol* 2019; 483:50–63.

**Figure 5** is original for this work and it is created based on the results from Carriere P, Calvo N, Novoa MB, Lopez-Moncada F, Riquelme A, Torres MJ, et al.
Role of SPARC in the epithelial mesenchymal transition induced by PTHrP in human colon cancer cells. *Mol Cell Endocrinol* 2021; 530:111253.

The references were added in the corresponding figure legend and were cited in the references list.

In addition, we prepared and arranged the figures using PowerPoint.

**Comments from the Science editor:**

(5) For PMID and DOI numbers of references from English-language journals, please ensure there is a space between the PMID and DOI numbers in the square brackets.

**Author response:** As described in the Guidelines for Manuscript Preparation and Submission for Frontier articles and in the Format for references guidelines we have included in the references the PMID number, and the CrossRef DOI® written in square brackets with a space between them as [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

**Comments to the Editorial Office**
We attach the Biography Section that had previously been requested along with the manuscript.

**BIOGRAPHY**

Claudia Gentili (Top image) achieved the degree of Ph.D. in Biochemistry in 2004 (National University of South, UNS, Bahia Blanca city, Argentina). Her Thesis work provided evidence of why calcitropic hormones function related to intestinal cell physiology is impaired with age. She is an Independent Researcher of the National Council for Scientific and Technical Research (CONICET), Titular Professor of Structure and Function of the Human Body (for Medicine and University Graduate in Nursing careers at UNS) and Professor of courses belonging to the postgraduate study program of the UNS. She supervises several research projects and is advisor of Doctoral Thesis, Degree Thesis and Fellowships. Dr. Gentili is author of thirty-one original publications in international scientific journals; and eighty presentations in national and international scientific events. Currently, she leads her group (Bottom image) in Institute of Biological and Biomedical Sciences of the South (INBIOSUR-CONICET-UNS), where their investigations are based on the analysis of the effects of several factors and the microenvironment on tumor cells in vitro, in vivo and in human patients. The contributions of this research group to the knowledge of the biology of CRC and the molecular mechanisms related to the aggressive phenotype of CRC cells have been recognized by oncologists, researchers and other specialists in the field of basic and clinical research; thus, by collaborations with doctors from argentine hospitals it was possible to identify PTHrP as a factor associated with the pathogenesis of other malignancies. The expertise of Dr. Gentili and her group in the management of tumor cell models also made it possible to collaborate with researchers in the Nanotechnology area of the UNS-CONICET with the aim to design biocompatible nanoparticles as potentially useful tools in CRC therapy.