Author’s response to reviews

Title: Peripheral T-lymphocytes express WNT7A and its restoration in leukemia-derived lymphoblasts inhibits cell proliferation

Authors:

Alejandra B Ochoa-Hernández (bereniceqfb@hotmail.com)
Moisés Ramos-Solano (leboyfr@gmail.com)
Ivan D Meza-Canales (jdavidmc@hotmail.com)
Beatriz García-Castro (gacas.bet@hotmail.com)
Mónica A Rosales-Reynoso (mareynoso@hotmail.com)
Judith A Rosales-Aviña (judithabisag1@gmail.com)
Esperanza Barrera-Chairez (vivibarrera2010@hotmail.com)
Pablo C Ortiz-Lazareno (paulcesar05@hotmail.com)
Georgina F Hernández-Flores (geodic1967@yahoo.com.mx)
Alejandro Bravo-Cuellar (abravocster@gmail.com)
Luis F Jave-Suarez (lfjave@yahoo.com)
Patricio Barros-Núñez (pbarros_gdl@yahoo.com.mx)
Adriana Aguilar-Lemarroy (adry.aguilar.lemarroy@gmail.com)

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Author’s response to reviews: see over
Dear Editorial Board Members,

BMC Cancer

We have now revised our manuscript (Peripheral T-lymphocytes express WNT7A and its restoration in leukemia-derived lymphoblasts inhibits cell proliferation) according to the comments and suggestions raised by the reviewers, which we have found very helpful for the improvement of the manuscript.

We have attempted to address all of the concerns addressed by the reviewers carefully. We added new information that supports the affirmations we have made. Additionally, we attempted to clarify the presentation of the manuscript. We have made corrections in the writing throughout the manuscript. Furthermore, the manuscript was proofread by a native English speaker.

We hope that we have responded to all of the questions generated by the reviewers and we are grateful for all of the effort that the reviewers generously invested in this paper.

Thanks in advance,

Adriana Aguilar Lemarroy, PhD
(Corresponding Autor)
Sierra Mojada No. 800,
Col. Independencia,
C.P. 44340, Guadalajara, Jalisco,
México
Tel: +52 33 36 17 00 60 ext. 31926
Fax: +52 33 36 54 08 54
email: adry.aguilar.lemarroy@gmail.com
We are thankful for the helpful suggestions and comments that all reviewers made. We have worked again on the manuscript considering these important observations.

**Referee 1:**

*The proposed question is very clear, properly boarded and well answered. The methods are appropriate and the written is also clear and correct. The results and the discussion are well connected, and the current knowledge properly integrated. However, two major revisions should be done in order to make the study suitable for publication.*

**Major Compulsory Revisions**

- It is very interesting the fact that restoration of WNT7A in Jurkat cells inhibits proliferation rates, but it arise the very immediate and important question that is what happens to the other leukemia-derived cell lines. And if it exists any differential response to WNT7A exposure between at least the four cell lines used in the present study. This is a very easy question to answer that should be included in the results section, or at least in the discussion, in the case the results are not easy to integrate.

Thank you for your suggestion, to answer this important question we modified the leukemia-derived cell lines K562, BJAB and CEM to express WNT7A in an inducible manner (only in the presence of doxycycline) and made the proposed experiment. We observed that all tested cell lines inhibits its proliferation after WNT7a restoration. These results are shown in Figure 8 and the methodology used in the inducible system was also added in the Methods section.

- In the final section of the results the authors try to investigate the mechanism by which WNT7A exerts its function in this model. They analyze the levels of expression of putative targets of the canonical and non-canonical wnt signalling pathway, and conclude that it does not exert its function through beta-catenin activation. Although this result is properly discussed, I think that the authors could be further in this analysis. It is not easy nowadays to asses any non-canonical Wnt function but they could address the question weather it modulates the canonical signal, for instances acting as an inhibitor, as argued in the discussion, by using a TOP-FLASH reporter or analyzing the nuclear localization of beta-catenin. In the case they do not succeed in detailing a bit more the mechanism, they should explain and discuss the new results.
We have shown in the previous version of the manuscript that c-Jun and Fra-1 gene expression was not induced in the Jurkat-pLVX-WNT7a when compared with Jurkat-pLVX cells; in contrast, expression of both genes was clearly decreased. Since JUN and FRA-1 are well described direct target genes of the canonical pathway (Mann B., 1999), we suppose that in our model WNT7A does not induce the canonical pathway. In the actual version of the manuscript, we included additional data supporting the non-induction of the canonical pathway. First, we measured again by qRT-PCR the expression of the four target genes analyzed (AXIN2, MYC, JUN and FRA-1) in Jurkat-pLVX and Jurkat-pLVX-WNT7a cells treated with LiCl (it is known that LiCl induces stabilization of B-catenin by impairing the activity of GSK3B). A restoration of the expression levels of JUN and FRA-1 was observed, which indicates that LiCl antagonize the WNT7A activity in these cells. Additionally, as you recommended, we have made Western blots to detect beta-catenin in cells expressing or not WNT7a. As control, we included also LiCl treated cells. Interestingly, accumulation of B-catenin was only observed in the LiCl treated cells and not in the WNT7A expressing cells. All these results are now included in Figure 6.

Minor Essential Revisions
- The readers would be grateful to an explanation of what are PBMCs, CD3 and CD19 positive cells. Readers not directly related with this model for sure know nothing about the meaning of these antigens.
We agree, we have now integrated an explanation about PBMCs, CD3 and CD19 in the methods section.

- In the background section, when explaining the number of Wnt members, the authors should specify that they refer to humans.
We have made this modification in the introduction/background section.

Referee 2:
The manuscript is well written and the data are presented in a clean manner. The finding that leukemic cell lines as well as leukemic cells isolated from patients with ALL display remarkably low levels of WNT7A mRNA is interesting. Strikingly, re-expression of WNT7A by lentiviral gene transfer into Jurkat cells or addition of recombinant human WNT7A slows cellular proliferation. The authors conclude that this may point out to a potential role of
WNT7A in the regulation of leukemic cell growth, as has been proposed for lung cancer cells by others before (Winn et al., JBC 2005 & 2006).

However, the study has some drawbacks: Firstly, the authors exclusively investigated WNT7A. Secondly, analyses were performed at the mRNA level by qPCR for the most part. Thirdly, the mechanism by which WNT7A blocks cellular proliferation is hardly addressed and remains enigmatic.

Major remarks:
1. It would be interesting to know whether the expression of other WNT family members, such as WNT3, WNT4, WNT5A, or WNT10B, is unaltered in leukemia cell lines or primary samples from patients with ALL. This would strengthen the author’s hypothesis that WNT7A plays a prominent role in regulating proliferation of lymphocytes or leukemia-derived lymphoblasts.

We have made these experiments from the beginning of the study in leukemia derived cell lines versus normal peripheral blood cells; we checked by real time PCR the majority of WNT ligands and receptors and some of them gave very interesting results. Thanks to this study, we could detect that WNT7A is differentially expressed in normal cells compared with immature cells (leukemic cells); similar observations were noted for other WNT ligands and also FZD receptors. However, we preferred to focus on the role that WNT7A is playing in the proliferation of these cells and not only to report the expression of all ligands and receptors that we analyzed, since we don’t have evidences of their participation in the biology of the leukemic cells. We are developing further studies involving other WNT ligands and receptors that we observed modulated in leukemic cells. For these reasons in this paper we concentrated in the study of WNT7a.

2. Figure 1B: I assume that the bars represent data +/- SD obtained by analyzing samples of blood, PBMCs, CD3+ or CD19+ cells of several individuals. If so, it would be more adequate to show box (as in Figure 3A) or scatter plots. How many individuals were analyzed?

Thank you for your comment; we forgot to explain in the Figure legend that the bars represent data ± SD. We have now included this in the legend. For Figure 1B, maybe we were not sufficiently clear describing and showing our data. We obtained blood samples from 5 healthy volunteers and isolated the different cell groups. After RNA extraction and retro-transcription, we mixed the samples of the 5 individuals in each group. The SD is
given because we sorted cells from the same volunteers twice and because we used 2 different reference genes for relative expression analysis. In this case we cannot show the results like in Figure 3, since the samples were not individually analyzed. We have now included more detailed information in the results section.

3. Figure 5A: The authors use the optical density (OD) of cell cultures as a surrogate for cellular proliferation over a prolonged period of time, i.e. up to 6 days. Was there any difference in cell viability between empty-vector transduced vs WNT7A-expressing cells? Does expression of WNT7A abrogate the clonogenic growth of the cells?

We have seen that WNT7a-expressing cells growth lower than empty-vector transduced cells, but we agree with the referee that it is a prolonged period of time. To avoid this inconvenience, we seeded equal number of Jurkat cells and stable puromycin selected cells (Jurkat-pLVX and Jurkat-pLVX-WNT7a) and measured the cell proliferation rate after 48 h cell culture. Figure 5A has been modified to include these results. Additionally, we succeeded in obtaining BJAB, K562 and CEM cells that express WNT7a in an inducible manner (by adding doxycycline). With this system we could shown now a most clearly effect on the cell proliferation inhibition by restoring WNT7a after 24 and 48 h doxycycline addition. The results mentioned above are now included in Figure 8.

4. Figure 5B: A control, i.e. vehicle-treated PBMCs, is missing in this experiment. Do cells treated with exogenous WNT7A lose viability?

Wnt7a recombinant protein was resuspended in PBS at a high concentration and later diluted in medium (only 1 µl of PBS in 100 microliter medium), for this reason we have not included vehicle-treated cells. We have now specified this in the methods section. To dilucidate whether cells lose viability after WNT7a expression, we measured apoptosis by flow cytometry utilizing Annexin-V-Fluos after 48 h of WNT7a over-expression induction. Results are shown in Figure 8.

5. Was there a decrease in the expression of c-Jun or Fra-1 at the protein level in Jurkat cells designed to express WNT7A? Is there a change in phosphorylation of c-Jun upon expression of WNT7A or addition of rhWNT7A to the culture medium?
Unfortunately, we could not perform these experiments suggested by reviewer 2. The antibodies didn’t arrive on time. However, we measured again by qRT-PCR the expression of the four target genes analyzed (Axin 2, c-Myc, c-Jun and Fra-1) in Jurkat-pLVX and Jurkat-pLVX-WNT7a cells treated or not with LiCl (it is known that LiCl induces stabilization of β-catenin by impairing the activity of GSK3B). As can be seen in the new modified Figure 6, significant different expression levels of c-Jun and Fra-1 were observed between LiCl treated and not treated cells (compare Figure 6A vs 6B), which indicates that LiCl antagonize the WNT7A activity in these cells.

Minor remarks:

1. The authors should provide more information on the ALL samples used in their experiments: Adults or infants? What subtype of ALL, e.g. B vs T lineage? Blast count in peripheral blood? Were the samples taken at initial diagnosis?

All blood samples were obtained from adults at initial diagnosis without previous treatment. We are unable to include additional clinical information because in our protocol, patients were managed anonymously.

2. Figure 5A/B: What pLVX-WNT7A-clone was used in these experiments?

For a better understanding this figure was modified. In Figure 5A was used the clone 1 and in Figure 5B PBMCs or parental Jurkat cells were used.

Referee 3:

Major Compulsory Revisions:

1. The authors used GAPDH and RPL32 as reference transcripts throughout the manuscript and compared T-cells, B-cells, a number of cell lines, cells that are quiescent and cells that are proliferating, cells from healthy patients, and cells from patients with ALL. The assumption is that in all of these various cell lines and conditions is that these transcripts are expressed at the same level. No data supports this assumption. It would be surprising if GAPDH and RLP32 were not expressed at very different levels in quiescent vs proliferating cells. Comparing WNT7 expression based on these reference transcripts without validating them genes is incorrect.
This is a good observation, we are in accordance with you about that the utility of reference genes must be experimentally validated for particular tissue or cell types; this was a serious concern at the beginning of our study since data without an appropriate normalization strategy is meaningful less. The reference genes used in this study were validated and evaluated in: “Evaluation and validation of housekeeping genes in response to ionizing radiation and chemical exposure for normalizing RNA expression in real-time PCR” (Banda M. et al., Mutat Res. 2008 Jan 8;649(1-2):126-34). We also develop some experiments to demonstrate that these reference genes were confident. We tested 5 of the most used reference genes (ACTB, GAPDH, RPL32, RPS18 and B2M) and select GAPDH, RPL32 and RPS18, since they gave very similar results. These experiments are now included in Additional Files 1 and 2.

2. The authors use of delta CP values is questionable. These data are all exponential and must be log-transformed before valid statistics can be performed. It would be much more appropriate to use the delta delta Ct technique. However, this technique still requires validated reference transcripts.

In Figure 3A we used delta CP values because in this case is recommended for a better evaluation of the data outliers as referred in “Standarization of data for real-time quantitative PCR methods-evaluation of outliers and comparison of calibration curves”, Burns et al., 2005, BMC Biothechnology. Delta CP values are also used for example in “Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR”, Silver et al, BMC Molecular Biology, 2006. However, Fig. 3B shows also the data in delta delta CPs. We have included a better description of the manner in which ∆CPs are calculated in Materials and Methods and cited the 2 mentioned references in the manuscript.

The linear representation of the exponential data on the ordinant in nearly all the figures is inappropriate.

The figures in which we show the “relative expression” are not exponential data; they were previously normalized and relativized and are showing as “Fold”. These kinds of data are usually presented as we have done, as shown for example in:

The authors should take a look at The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments (Clinical Chemistry 55:611-622, 2009) details on what should be included in the manuscript.

We have revised in detail the MIQE guidelines. In order to present the results as the guidelines propose we included more information about primers (now included in Table 2) and add an exemplification of the amplification curves, melting peaks and standard curves (please see Figure 7 and Additional Files 1 and 2).

3. The authors have used Q-PCR and RNA quantification in numerous experiments to assess "expression" of Wnt7A and conclude that the changes they have observed have biological effect. This must be supported by quantification of the protein. This would be especially important in the ALL patients where changes in translation efficiency might be suspected. Clearly they have a Wnt7a antibody and can perform the appropriate Western-blot type of experiment.

We are unable to perform experiments for protein expression in these patients because we didn’t obtain at that time protein, now they are already treated or unfortunately many of them have died.

However, we suppose that WNT7A have an effect in the biology of leukemic cells, since addition of human recombinant WNT7A to leukemia-derived cells inhibits cell proliferation, exogenous expression of WNT7A also decrease the proliferation. In this new version of the manuscript we added results with an inducible system in which the expression of WNT7A is regulated by the availability of Doxycyclin. With this system we could shown that WNT7A decrease the proliferation of all tested leukemia cells. The indicible WNT7a expression at protein level was also corroborated by western blot (Figure 8).

An important question that rises from these observations is whether WNT7A can perform the same effect in leukemic cells freshly isolated from patients, if so, this molecule could have a therapeutic potential. We are already developing new studies to address this and
other important questions regarding the biological effect of WNT7A in leukemic and normal cells.

4. The data in Figure 6 is not convincing as there is no positive control. Either expressing for example Wnt3a or adding Wnt3a to these cells could act as a positive control.

Thank you for the suggestion. WNT3A could be a good positive control only if Jurkat cells respond to WNT canonical ligands. To analyze whether WNT7A induce stabilization of β-catenin and consequently activates the canonical pathway, we made experiments by adding LiCl to the cells. We observed stabilization of β-Catenin only in LiCl treated cells. These new results are now included in Figure 6.

**Minor Essential Revisions**

1. There are numerous examples of poor grammar that need to be corrected. For example in the first paragraph of the Abstract "Moreover, the expression of this gene and the role that plays" should be "Moreover, the expression of this gene and the role that IT plays". In the next sentence "the aim of this study was to seeking" should be the aim of this study was to seek". Please have an editor help on the manuscript.

   Sorry, we have sent again the manuscript to a native English speaker to try to avoid these mistakes.

2. On page 7 cDNA synthesis "8 ul" should probably be 8 ug

   In the case of cell lines the synthesis of cDNA was realized from 5 µg of total RNA; for patient and healthy volunteers we isolated mRNA, not total RNA. Quantifying mRNA is difficult because its low concentration, for this reason, we used the maximum volume permitted (8µL) in the kit (SuperScript III first-Strand Synthesis System) for the cDNA synthesis.

3. p 14 "a pull of cDNAs" means what?

   Sorry, maybe this word was not correctly used, “a pull of cDNAs” means mix of cDNA, but to avoid confusions we change this word in the paper.