Author's response to reviews

Title: Wnt/beta-Catenin Pathway Regulates ABCB1 Transcription in Chronic Myeloid Leukemia.

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Version: 7 Date: 5 June 2012

Author's response to reviews: see over
To Dr. Paul Ekert, Associate Editor of the “BMC Cancer Journal”

Dear Dr. Ekert,

The authors are extremely grateful for the contributions of all the reviewers to the manuscript. We included all suggestions asked in the first round of revisions and we are glad to resubmit for your consideration our response to reviewer Frank Thevenod questions.

1- “Figure 1: Competition experiments with 200x excess wild-type oligos do not show any competition in all EMSAs. Moreover, the “unrelated” Smad8 shows a super shift as well. Both results cast doubts on the specificity of the performed experiments”.

Authors’ response

The electrophoretic mobility shift assay (EMSA) is an in vitro strategy, performed with synthesized oligonucleotides, that by itself, can show non specific bindings together with specific bands. For this reason competition assays are necessary to elucidate which bands are specific. In the experiments shown in Figure 1 the authors demonstrated the specificity of the ligation of the protein extract complex with the TCF consensus binding site and indicated the specific bands with black arrows. To confirm this for all assays we performed competition reactions not only with the same oligo unlabelled but also with a shorter oligo containing the core binding site for TCF.

In the lanes that contain protein extracts with labelled oligonucleotide it is possible to observe two distinct bands (an upper band and a lower band). The competition with the entire unlabeled probe reduce almost completely both bands in all experiments indicating that in this promoter region distinct protein complexes are found. However when the competition assays were performed with 200 fold excess of the unlabeled probe that contains the main TCF binding site core (named Opt) only the lower band showed a reduction in its intensity indicating that this lower band correspond to the specific TCF binding.

We choose to use this second competition assay with oligonucleotide Opt with the aim to discriminate which band were related to TCF binding site and these results are clearly demonstrated in the Figure 1.
The Smad8 antibody was selected to super shift assays because in the labelled oligonucleotide used there is no consensus binding site for this transcriptional factor, moreover in all lanes it is possible to observe unspecific bands in the same position of the unspecific band observed in the lane with Smad8 antibody. For this reason we considered that only with beta-catenin antibody we were able to observe a super shift band that is different from the unspecific ones.

Moreover as EMSA experiments are in vitro assays that need proper validation, we confirmed our results with ChIP assays on Native chromatin followed by its quantification by RT-qPCR. For this we immuno precipitated the chromatin with beta-catenin antibody and amplified DNA with primers for ABCB1 promoter as shown in Figure 2.

2- “Figure 4: It is striking that LiCl has no effects whatsoever on the nuclear translocation of beta-catenin. This is particular prominent in Lucena Cells, where beta-catenin is excluded from the nucleus despite a significant increase in beta-catenin protein (Figure 3). In spite of the obvious result the authors claim the contrary (line 278)”.

Authors’ response

Several works take advantage of LiCl treatment to induce beta-catenin nuclear translocation by inhibiting GSK3 activity and therefore mimics Wnt Signalling activation. Below the authors have compiled previously published data from high quality journals concerning the well-established use of LiCl treatment for this purpose: Klein and Melton, 1996; Goentoro and Kirschner, 2009; Stambolic et al., 1996; Hedgepeth et al., 1997.

Also the authors have already documented the beta-catenin nuclear translocation using LiCl treatment in K562 cell line in the published manuscript: Pizzatti L, Binato R, Cofre J, Gomes BE. et al., SUZ12 is a candidate target of the non-canonical WNT pathway in the progression of chronic myeloid leukemia. Genes Chromosomes Cancer. 2010, (49) 2: 107-18.

In this present manuscript we used this strategy to verify if ABCB1 expression could be regulated by WNT/beta-catenin and this result is shown in Figure 5 where we could demonstrate that after LiCl treatment the expression of ABCB1 measured by RT-qPCR increased in both cell lines with a more pronounced increase in K562.

In Figure 4 we used immunofluorescence staining and confocal laser microscopy to show that in our hands LiCl treatment has an effect on the translocation of beta-catenin (as asked by reviewer
Michelle Perugini) and it is unquestionably the nuclear translocation of beta-catenin in K562 treated with LiCl (10mM) in the DAPI merged panel compared with the control (the two upper panels).

The nuclear translocation of beta-catenin is more pronounced in the K562 cell line and this result is consistent with all data showed in Figures 3 and 4. As in the Lucena cell line there is already a higher expression of beta-catenin in both cytoplasm and nucleus, documented by RT-qPCR, Western blot, flow cytometry and immunofluorescence and also a beta-catenin higher occupancy of the ABCB1 promoter (ChIP assays, Figure 2) the effect of LiCl is less pronounced probably because there is a saturation in the process.

However in Figure 4 after LiCl treatment in Lucena cell line (lower panel) it’s possible to observe a beta-catenin aggregation in both compartments. Comparing the number of beta-catenin foci in the nucleus before and after treatment we found approximately the same number but a more intense fluorescence of the foci after treatment. So although less pronounced there is a translocation of beta-catenin to the nucleus of Lucena cells.

To make this point clearer we changed line 278 (actual 278-282 lines).

3- “Figure 5: Lithium blocks GSK3-alpha and –beta with similar affinities (Ki 3.5 vs 2.0 mM) by competing with co-factor Mg2+ and /or activating Akt-mediated phosphorylation of GSK3. Why is GSK3-alpha expression increased, but GSK3-beta expression abolished by lithium? No change in expression should be expected”.

The authors included the data of Figure 5 as a simple control to, one more time; demonstrate that LiCl treatment in our hands is working as a tool to investigate the changes in the expression of ABCB1 gene mediated by Wnt/beta-catenin pathway in chronic myeloid leukemia.

In Figure 5 results, the authors showed that K562 cell line presented both phosphorylated isoenzymes of GSK3 (alpha and beta) while Lucena cell line only presents phosphorylated GSK3-beta isoenzyme. After LiCl treatment we demonstrated that phosphorylated GSK3-beta was abolished in both cell lines, indicating that inhibition of GSK3-beta avoid beta-catenin phosphorylation and its degradation via phosphorylation-dependent ubiquitination. These events promote beta-catenin accumulation and its nuclear translocation leading to activation of Wnt/beta-catenin gene targets. All these results are in accordance with all our other results.
The alterations in GSK3-alpha were not taken into account and they were not discussed because only GSK3-beta is relevant to beta-catenin nuclear translocation (Goentoro and Kirschner, 2009). The antibody that we used shows both pGSK3 isoenzymes (alpha and beta) and we choose to show the entire experiment. However the regulation of GSK3-alpha and -beta activity is a very complicated issue that is not still clear for most of the purposes (Metcalf and Bienz, 2011). Inactivating GSK3 by phosphorylation is accomplished by two different signalling pathways, the insulin receptor that leads to GSK3 inactivation through P13K/AKT and follows exactly what is described by the reviewer (Freland and Beaulieu, 2012); and the WNT signalling pathway that inactivates GSK3-beta through DVL (dishevelled)/FRAT (Cohen and Goedert, 2004). Although the two isoenzymes of GSK3 have similar biochemical properties they are not identical so the inhibition by LiCl may not work identically (Phiel and Klein in 2001). This seems to be the case in our cell lines where in Lucena cell line the LiCl treatment leads to a phosphorylated GSK3 alpha while the phosphorylation of the GSK3-beta was abolished in the two cell lines.

We think that the reviewer was confused because we have not informed in the legend and in the figure that a phosphorylated antibody was used although this information was described in material and methods. We included now in the new version this information in the Figure 5 legend and in the result section.

4- “Figure 9: It is unclear why increased nuclear translocation of beta-catenin induced by lithium (which is actually not the case; see Figure 4) should decrease reporter gene activity. Lines 312-314 do not make sense: A reduction of luciferase activity does not indicate activation of ABCB1 through TCF binding sites, rather inhibition of ABCB1 transactivation”.

The reviewer forgot that in transfection assays with the Luciferase reporter vector the construction is located in the cytoplasm. So when cells are treated with LiCl with consequent beta-catenin nuclear translocation less beta-catenin are available to bind in the report vector. Thus, as our results showed, the activity of the reporter gene after treatment presented a slight reduction. However all constructions that included the TCF consensus binding site showed increased in the luciferase activity, compared with the construction with the basal promoter only. Together these results indicate that activation of ABCB1 through TCF binding sites is mediated by beta-catenin.
In the revised manuscript we corrected the names of the constructs that were wrong although described correctly in the text. Also we changed lines 312-314 to make our point more clear (actual 315-319 lines).

Please contact me if you have any further question. Thank you.

Sincerely yours,

Stephany Cristiane Corrêa.