Author's response to reviews

Title: Phosphatidylinositol 3'-kinase, mTOR, and Glycogen synthase kinase-3 beta mediated regulation of p21 in human bladder carcinoma cells

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Version: 2 Date: 15 July 2011

Author's response to reviews: see over
Dear Editor,

Thank you very much for the opportunity to revise our manuscript, and we thank the reviewers for the helpful comments. We are very hopeful to publish in *BMC Urology* and we have added a substantial amount of data and information to the revised manuscript. We have incorporated the editorial and reviewer comments into this letter and added our point-by-point responses. Modified and additional text has been included in the manuscript itself using the “Track Changes” function.

**Editorial comments:**

1) We recommend that you copyedit the paper to improve the style of written English.

We have done this with the assistance of two skilled copyeditors.

Please note that the gene and protein symbol formatting, particularly for “PTEN” appears to be inconsistent in the manuscript because sometimes we are referring to the mouse gene, in which case it is appropriate to write “Pten” according to the International Committee on Standardized Genetic Nomenclature for Mice, and sometimes we refer to the human gene, in which case the convention is to write “PTEN” according to the HUGO Gene Nomenclature Committee. When we refer to the protein for mouse or human, we write “PTEN”.

2) Please describe your animal methods in the Methods section of your manuscript.

A new section entitled “Mice” has been added to the Methods section.

3) Ethics - Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. Research carried out on humans must be in compliance with the Helsinki Declaration ([http://www.wma.net/en/30publications/10policies/b3/index.html](http://www.wma.net/en/30publications/10policies/b3/index.html)), and any experimental research on animals must follow internationally recognized guidelines. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate.

All animal research was approved by the Harvard Medical Area Standing Committee on Animals, since the live animal work was done at Harvard Medical School. This
information, as well as the reference number, is now mentioned in the “Mice” section of the Methods.

Referee 1’s comments:

1) [4th paragraph in the Background section] The word ‘the early tumors’ is used. Do the authors mean ‘low stage tumors’ or ‘high grade tumors’? The authors should define it clearly. There are also the words ‘the early bladder tumors’ and ‘late tumors’ in 1st paragraph in the section Discussion.

We have clarified this information, both in the Background and the Discussion sections. Studies in the literature have shown that both low grade and early stage tumors have increased p21 levels compared to normal urothelium while high grade and late stage tumors have diminished p21 expression.

2) ['Western blotting’ in the Methods section] The concentrations of extracted protein were not measured in this study. Why did not the authors measure them? In general, various modifications and treatments lead to change of cell numbers and amount of extracted protein in each dish. How much protein was loaded in each well? In the Figure 3B, amount of #-tublin, a loading control, looks significantly different among the each other.

Protein concentrations were measured by BCA assay; 25µg was loaded in each lane. We have quantitated protein expression changes in this revised version of the manuscript (see Referee #3’s comment 2) and normalized all expression signals to tubulin to control for loading.

3) [last paragraph in the Discussion section] The authors describes that “It will be helpful to examine GSK-3b activation levels… GSK-3b inhibitor”. Why did not the authors investigate the cytotoxic assay of GSK-3b inhibitor or PI3K inhibitor using bladder cancer cell lines? It is important to assess the effect of these inhibitors for bladder cancer as well as colon cancer and ovarian cancer.

We have added a Figure (5C) with a cytotoxicity assay using the GSK-3 inhibitor SB216763 and the PI3K inhibitor LY294002 used alone and in combination on both cancer cell lines used in this paper. While it would certainly be of high interest to assess the effect of these inhibitors on colon cancer and ovarian cancer cells, we felt it would not be in the scope of this paper to include such an experiment, especially given the aims and audience of this journal.

4) [Figures 1B, 1C and 2] In some experiment shown by figures 1B, 1C and 2, the authors exhibited the results only using UMUC-3. As described in 1st paragraph of the Result section, UMUC-3 is PTEN negative, whereas UMUC-14 is positive. Why did not the authors perform the experiments using UMUC-14 as similar as UMUC-3? It is essential for the authors to compare the results between UMUC-3 and UMUC-14 and add the evaluation to the Discussion section. Moreover, in the experiments shown by
figure 1A, 3, and 4, there are no discussion in an aspect of comparing between UMUC-3 and UMUC14.

Experiments were done on both cell lines, and the results can be seen in the graphs added to Figures 1-6. We have added text comparing the two cell lines both briefly in the first paragraph of the Results section and in more detail in the second paragraph of the Discussion section.

5) [Figure 5] In figure 5A, urothelium is exhibited unclearly, leading to make it impossible to evaluate double staining IHC correctly. That's maybe due to too much PermountTM in mounting slides. Re-mounting and re-photographing is preferred. The authors should add a scale and/or information of original magnification to these figures.

New sections of tissue were stained, mounted in minimal Permount and re-photographed. A scale and information of original magnification were added to [new] figure 7.

6) The authors should describe the limitation of this work more clearly.

We added some text detailing the limitations of doing experiments with cancer cell lines as opposed to primary cell lines or explant cultures that may permit cellular differentiation. We also previously included in the discussion some commentary about the still unclear mechanistic connection between GSK-3 and p21 in these cells.

Referee #2’s comments:

No suggestions were made for improvement of this manuscript. We are humbly appreciative of the encouraging comments.

Referee #3’s comments:

1) The authors need to address that the initial hypothesis that PTEN status was critical to p21 induction was not verified, given the data in Figs 1 and 3 that both UMUC-3 and -14 cell lines induce p21 in response to EGF. In Fig 3, both cell lines respond to EGF with phosphorylation of GSK3a/B, which is blocked by the inhibitors of PI3 kinase and Akt.

The experiments with the PTEN positive and PTEN negative cell lines were not intended to show that PTEN status determines whether or not p21 can be induced. PTEN deficient cells tend to have overactive PI3-kinase signaling, and we believe that it is the PI3-kinase signaling that caused the increased p21 in the mouse model. In our cell culture model, the method of p21 induction in these cells was through PI3-kinase activation, and this should occur both in the presence and absence of
PTEN. One would predict, however, that both PI3-kinase activation and p21 induction would be weaker in the PTEN positive cell line. Our results suggested that this was in fact the case. We added a paragraph comparing the results in the two cell lines in the second paragraph of the Discussion section, as mentioned for Referee #1’s comment 4.

2) The authors need to provide quantitative analysis of changes in Western blot profiles, and information of the number of experiments, replicates, as well as some statistical analysis.

We quantitated protein level changes in the Western blots for Figures 1-6, and included standard error bars on all graphs. All experiments were done in quadruplicate.

3) The authors need to provide some functional data regarding the effects of EGF and the respective inhibitors on cell proliferation. A short time course of changes in phosphorylation patterns is suggestive, but not proof of changes in proliferation status.

We have added a figure (new Figure 1C) to show that EGF does not actually induce cell proliferation in these cells when it is given in isolation from other growth factors; this is at least partially because the EGF induces the p21 which inhibits cell cycle progression. It is also probable that other signals are needed in addition to the EGF in UMUC-3 and UMUC-14 cells in order to cause cell proliferation.

4) The authors need to provide some data on whether p21 is subject to proteasomal degradation in these cell lines. While this study presents evidence that GSK-3β may be an important mechanism by which p21 levels are regulated in the context of PI3-kinase/AKT signaling, no further data are presented re mechanism. The Discussion (p. 12), however, states that “GSK-3β is known to directly phosphorylate substrates such as β-catenin, cyclin D1, and cyclin D2, leading to ubiquitylation and subsequent proteasome dependent degradation of those proteins [references 38-40]. It is certainly possible that GSK-3β regulates p21 in this fashion [reference 41] in the bladder cells. We found that proteasome inhibition of the UMUC-3 cells leads to an increase in p21 (data not shown), suggesting that p21 levels are regulated by proteasomal degradation”. These preliminary results on p21 proteasomal degradation are tantalizing, but need to be extended and included in the Results.

We have shown the proteasome inhibition results, including quantitations, in the new Figure 6.

In this context, the authors should read a recent review (Mishra, Molecular Cancer, 2010, 9:144) on oral cancer that summarizes studies which show that GSK3B can play an active role as tumor suppressor or a tumor promoter. In oral cancer, active GSK3B kinase appears to be a tumor suppressor via activation of proteosomal
degradation of a number of cell cycle regulators, including cyclins D1/E, c-myc, c-Jun, B-catenin and p21, with the net effect being inhibition of cell cycle regulation. Phosphorylation (S9) of GSK3B inactivates the kinase activity which allows for the expression of Cyclins D1 and A, CDK4 and p21 proteins, with a net increase in S phase and cell proliferation. Thus focus in this manuscript only on p21 protein levels does not allow for consideration of the status of multiple other cell cycle regulators. Cells may have increased p21 expression, but the net balance of cell cycle regulators may in fact drive cell proliferation.

It is certainly the case that we have focused on p21, without considering many other cell cycle regulators that may have important effects on cell proliferation. This is a very interesting question, and in the future, we are highly interested in investigating the role of other cell cycle regulators on bladder cell proliferation, initially through the use of microarrays. However, in response to your comment that “the net balance of cell cycle regulators may in fact drive cell proliferation”, the evidence indicates that strong PI3-kinase signaling inhibits cell proliferation in bladder cells, rather than driving proliferation. The evidence is two-fold: 1) in the Pten deleted mouse bladder epithelium (our previously published work, Yoo LI et al. (2006) Cancer Res 66:1929-1939), there was diminished cell proliferation compared to wild-type bladder epithelium; and 2) EGF stimulation of the UMUC-3 and UMUC-14 human bladder carcinoma cell lines resulted in a decrease in cell proliferation compared to the untreated cells (Fig. 1C). We have therefore been investigating why this inhibition of proliferation occurs, and p21 appears to be an important component of the answer, because a p21 deficiency restores the PI3-kinase induced cell proliferation both in the murine bladder (Yoo et al., 2006) and in the human bladder cell lines (Fig. 1C).

Thank you for taking the time to read our cover letter and revised manuscript. We have attempted to address the concerns of the editor and reviewers with our additional experiments and extended discussion. We hope the manuscript is now acceptable for publication in BMC Urology.

Sincerely,

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