Reviewer’s report

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

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Reviewer: Kathleen Shiverick

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In this manuscript, human bladder carcinoma cells UMUC-3 (PTEN negative) and UMUC-14 (PTEN positive) were studied to identify the signaling pathways downstream of PI3-kinase that regulate p21 expression. For background, the authors have previously shown that deletion of the Pten tumor suppressor gene in murine urothelial cells in vivo results in upregulation of p21, a cyclin dependent kinase inhibitor. In the mouse model, p21 expression appears to block an increase in urothelial cell proliferation due to Pten deletion.

Strengths:

1) Western blot data show that EGF induced p21 expression in both UMUC-3 (PTEN -) and UMUC-14 (PTEN +) cells. p21 induction was dependent on PI3-kinase and AKT activity based on effects of the inhibitors LY294002 (PI3-kinase) or AKTi-1/2 (AKT), which blocked signaling via AKT phosphorylation at the critical activation site, serine 473. In addition, p21 induction caused by the PI3-kinase pathway is partially mediated by mTOR.

2) Treatment of both UMUC-3 and UMUC-14 cells with EGF further resulted in increased phosphorylation of GSK-3β and α at serine 9 and 21 which was PI3-kinase and AKT dependent.

3) Subsequent experiments with a GSK-3 inhibitor (SB216763) showed that p21 protein levels increased, evidence that GSK-3 activity negatively regulates p21 expression.

In transfection experiments, exogenous expression of GSK-3β in UMUC-14 cells resulted in a massive decrease in p21 levels (Fig. 4B). This evidence indicates that GSK-3β downregulates p21, but that activity is inhibited by an active PI3-kinase pathway.

4) In the mouse model, GSK-3β is also inhibited in mice that are conditionally deficient for Pten (Fabpl-Cre;Ptenloxp/loxp) in bladder epithelium. These mice were previously shown to have elevated levels of nuclear p21 in urothelium [ref 19]. Immunohistochemical staining of Pten deficient mouse bladders and their wild-type littermates showed not only higher levels of p21 positive cells, but also greatly increased cytoplasmic staining of phospho-GSK3β and α at serines 9 and 21, respectively. This finding is consistent with the idea that GSK-3 inhibition contributes to elevated p21 levels in the Pten-deficient mouse bladder.
In summary, these experiments have defined two pathways, GSK-3# and mTOR, by which p21 levels are regulated in bladder cells during PI3-kinase/AKT signaling.

Concerns and Major Compulsory Revisions:

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.

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.

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.

4) The authors need to provide some data on whether p21 is subject to proteasomal degradation in theses cell lines. While this study presents evidence that GSK-3# 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-3# is known to directly phosphorylate substrates such as #-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-3# 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.

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.
Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests.