Author's response to reviews

Title: Pim1 promotes human prostate cancer cell tumorigenicity and c-MYC transcriptional activity

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Author's response to reviews: see over
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Miss Deepali Singhal
The BioMed Central Editorial Team

Re: 1892804712308861 - Pim1 promotes human prostate cancer cell tumorigenicity and c-MYC transcriptional activity

Dear Miss Singhal,

We would like to thank you and the reviewers for considering our manuscript. We have performed several new experiments and revised the manuscript substantially. We address below specific questions raised by the reviewers.

**Comment 1:** Both referee 1 and referee 2 pointed out that c-MYC is only upregulated in Pim1-expressing DU145 cells, not in LNCaP cells. According to referee 1: “Upregulation of c-myc at the protein level in PIM1 transfected LNCaP cells it not visible in the western blot provided. Therefore the statement ‘was further increased’ should be excluded.”

**Response 1:** We observe that the change in c-MYC levels due to Pim1 expression is consistently more noticeable in DU145 than in LNCaP cells. In agreement with reviewer 1, we have now excluded the statement “was further increased”.

**Comment 2:** The authors need to indicate whether they use male or female nude mice for their xenograft experiments.

**Response 2:** Since we were asking the role of c-MYC and Pim1 in the prostate cells including the effect of androgen signaling, we used male nude mice in all xenograft experiments. This has been noted in the Methods.

**Comment 3:** In the context of androgen receptor signaling (Figure 5), did the authors determine the level of androgen receptor expression following Pim1 or its mutant (K67M) over-expression? The results shown in Figure 5 could very well be due to change in AR expression in Pim1-LNCaP cells.

**Responses 3:** We appreciate the reviewer’s comment. We have examined AR as suggested and found AR levels were elevated in Pim1-LNCaP cells (2.5x) and K67M-LNCaP cells (1.7x) relative to controls. So, increased AR levels could partially explain the increase in PSA levels, but not all of it, since K67M cells have increased AR levels but lower PSA expression than control Neo cells. We added these data to Figure 5 and discussed it in the text.

**Comment 4:** It will be better to mention the passage # following selection of Pim1 over-expressing cells used for FACS analysis.

**Response 4:** The passage numbers of the cells have now been added to the manuscript.

**Comment 5:** In the second section of results (Page 9: Pim1 promotes proliferation and attenuates apoptosis of RWPE1...): In lines 4-6, it is not clear what the authors are trying to say: verifying that in vitro tumorigenicity of Pim1-expressing RWPE1 cells is due to polyploidy cells driven by
chromosomal instability as shown previously? This sentence contradicts their experimental observations.

Response 5: In the soft agar assay, RWPE1-Pim1 cells could not form colonies. In xenograft experiment in nude mice, similarly, RWPE1-Pim1 cells were not tumorigenic. However, we did see more cells in the RWPE1-Pim1 xenograft sections than in the RWPE1-Neo sections. This may indicate that even though they do not form a tumor, these cells could survive better than RWPE1-Neo cells or undergo some proliferation in vivo. We have now clarified this point in the manuscript.

Comment 6: Page 9 (section: Pim1 promotes in vitro and in vivo tumorigenicity of LNCaP and DU145 cells): The sentence “We next asked whether Pim1 can enhance the tumorigenicity established malignant prostate cancer cells” should be changed to “We next asked whether Pim1 can enhance the tumorigenicity of established malignant prostate cancer cells”.

Response 6: We corrected the sentence as the reviewer suggested.

Comment 7: The authors must provide evidence for the conclusion that c-myc is an important PIM1 effector in the models investigated. Is a myc siRNA or the myc inhibitor 10058-F4 reducing colony formation in LNCaP and DU145 cells? Furthermore, increased c-myc transcriptional activity is only shown in PIM1 expressing RWPE1 cells. Is c-myc more active in PIM1 expressing LNCaP and DU145 cells? Also the PIM1/c-myc target genes, which were defined in an elegant way in RWPE1-mycER cells, should be validated in LNCaP and DU145 cells. Are some of the target genes indeed upregulated at the mRNA level in PIM1 expressing LNCaP and DU145 cells and is this upregulation responsive towards a c-myc siRNA or the myc inhibitor?

Response 7: We appreciate the reviewer’s comments and suggestion on using 10058-F4, a c-Myc inhibitor. We have validated several PIM1/MYC targets in DU145 and LNCaP cells, although not surprisingly, there was some variability probably due to the different genetic contexts of these malignant cell lines. These results are shown in Fig. 7B. We also used 10058-F4 and found that it inhibited soft agar tumorigenicity of PIM1-expressing LNCaP and DU145 cells (Fig. B). In addition, the inhibitor reversed the effects of PIM1 on PIM1/MYC target genes. Upon further analysis we found that, unexpectedly, 10058-F4 suppressed Pim1 protein levels as well without a noticeable effect on the mRNA levels. While this is an intriguing finding and suggests that this agent can target two cooperating oncogenes MYC and PIM1, it complicates the interpretation of our experiments with the inhibitor. We therefore went ahead and used shRNA to downregulate MYC in LNCaP-Pim1 cells. We showed that knocking down MYC reversed the effects of Pim1 on Pim1/MYC target genes. Overall these results support the notion that MYC is an effector for Pim1 in this system. The reviewer’s suggestions have also led us to make the unexpected finding that small molecule inhibitor 10058-F4 can target both MYC and PIM1.

Comment 8: Figure 2D, which is described in the text is not included in the figures?

Response 8: There is no Figure 2D, so that is a mistake. We corrected that part of the paragraph with corresponding figures.

We believe we have addressed all the issues raised by the reviewers resulting in an improved manuscript. Once again, thank you for considering our manuscript.
Sincerely,

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