Author's response to reviews

Title: FOXA1 promotes tumor cell proliferation through AR involving the Notch pathway in endometrial cancer

Authors:

Meiting Qiu (xiaomei727@hotmail.com)
Wei Bao (forever_chipper@hotmail.com)
Jingyun Wang (fishann0303@gmail.com)
Tingting Yang (y402115432@163.com)
Xiaoying He (hexiaoying06@gmail.com)
Yun Liao (liao.yun@aliyun.com)
Xiaoping Wan (wanxp@smtu.edu.cn)

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Author's response to reviews: see over
Cover Letter

Title: FOXA1 promotes tumor cell proliferation through AR involving the Notch pathway in endometrial cancer
MS ID: 9379103581163720

Dear Ms. Cherry Battad,

Thank you very much for having our manuscript (FOXA1 promotes tumor cell proliferation through AR involving the Notch pathway in endometrial cancer. MS ID: 9379103581163720) peer reviewed and for giving us the opportunity to revise the manuscript for your further consideration of publication in BMC Cancer.

We are very encouraged by the positive comments of the reviewers who have also pointed out the problems and deficiencies in the manuscript, but most of all they recognized the value of our work, the publication of which will advance our understanding into the mechanism of cell proliferation of endometrial cancer. We have revised the manuscript in accordance with the suggestions by the reviewers. All changes are highlighted in red in the revised manuscript. Point-by-point responses to the comments are listed below this letter.

Again all the authors have read the final manuscript and agreed to its publication if accepted by the journal. No duplicate publication or submission of the manuscript has been made elsewhere.

Each of the comments of the two reviewers has been addressed, and a point-by-point response detailing the revisions is provided. We hope you agree that our revisions have improved the manuscript to sufficient quality for publication in BMC Cancer. We would like to express our great appreciation to you and to the reviewers for the helpful comments on our paper.

If you or the reviewers have any questions, please do not hesitate to contact us.
Thank you for your consideration of our manuscript.

Best regards.

Yours sincerely,

Mei-Ting Qiu, MD, PhD
Department of Obstetrics and Gynecology, International Peace Maternity & Child Health Hospital Affiliated to Shanghai Jiao Tong University School of Medicine
Shanghai 200030, China

Corresponding author: Xiao-Ping Wan, MD, PhD
E-mail address: wanxp@sjtu.edu.cn
Department of Obstetrics and Gynecology, Shanghai First People’s Hospital Affiliated to Shanghai Jiao Tong University School of Medicine
Shanghai 201620, China.
Point-by-point Response to the Reviewers’ Comments:

Reviewer #1: Professor Diego Sisci

In this manuscript, the authors demonstrate that FOXA1 (FA1) promotes cell proliferation of endometrial cancer by AR. The work is well done and the data are interesting. They demonstrated the effects elicited of FA1 by overexpressing or silencing either FA1 or AR. Interesting results are also presented in vivo (animals and IHC). The results are interesting and convincing.

Response: Thank you for your time and efforts in reviewing our manuscript and for your very helpful and insightful comments.

Minor comments

1. In Fig. 2B FA1 seems not silenced enough. It is more evident in WB.

Response: Thanks for the insightful comment. The primer of shFOXA1 used in our study is according to the reference (Hurtado A, Holmes KA, Ross-Innes CS et al. Nat Genet 2011. PMID: 21151129). Before we establish stably transfected MFE-296/shFOXA1 cells to perform follow-up experiments, we examined the efficiency of silence of transient transfecting shFOXA1 to MFE-296 cells, getting the silencing efficiency to 81% compared with negative control (data not shown), which suggests high silencing efficiency of shFOXA1.

However, as caution by the reviewer, we have re-examined the efficiency of silence of stably transfected MFE-296/shFOXA1 cells in qRT-PCR assays by three independent experiments, and re-evaluated the data and obtained identical results: although our qRT-PCR assays revealed less evident than WB, the efficiency of silence in FOXA1 mRNA in qRT-PCR assays was over 70% in MFE-296/shFOXA1 as compared to the negative control (MFE-296/NC), which provided sufficient

Nevertheless, as caution about vagueness of our data, we have shortened the major ticks interval of Y axis from 0.5 to 0.25 in the cartogram of qRT-PCR in order to show the effect of FOXA1 shRNA on FOXA1 expression more clearly (new Figure 2C).

2. To reinforce the functional difference of FA1 in relation to the expression of ER, you can cite a recent paper published on cell cycle demonstrating the functional role of FoxO3a in ER positive and negative breast cancer cells.

**Response:** Thanks for your valuable suggestion. We have cited an additional paper according to your comment. As we speculated that FOXA1 acted as a tumor suppressor in ER-positive endometrial cancer (EC) cells but a tumor activator in ER-negative EC cells, in order to clarify the possibility of the functional difference of FOXA1 in relation to the expression of ER, apart from citing the study of FOXA1 in relation to ER in breast cancer, we have, as suggested by the reviewer, cited a recent paper published on Cell Cycle (Sisci D, Maris P, Cesario MG et al. *Cell Cycle* 2013. PMID: 24047697) in the Discussion section explaining another FOX member (FoxO3a) with inhibiting effects of motility and invasiveness in ER+ breast cancer cells but promoting effects of motility and invasiveness in ER- breast cancer cells (page 23).

We thank you once again for all your excellent comments and suggestions, which we believe have improved the manuscript greatly.
The paper by Qiu et al shows that the expression of FOXA1 and androgen receptor (AR) in endometrial carcinomas (EC) is significantly higher than in normal tissue or atypical hyperplasia. FOXA1 expression is significantly correlated with AR expression in pathological tissue specimens and high levels of both FOXA1 and AR positively correlate with pathological grade in EC. Furthermore, expression of downstream AR targets such as XBP1, MYC, ZBTB16 and UHRF1 is promoted by FOXA1 up regulation. The data reported also indicate that overexpression of Notch1 and Hes1 induced by FOXA1 can be reversed by AR depletion, which also attenuates FOXA1 stimulated cell proliferation. This apparently is mediated by an interaction between FOXA1 and AR. The AR seems involved only in proliferative effects of FOXA1 whereas seems not involved in FOXA induced cell migration. On this basis the Authors suggest that FOXA1 and AR could represent potential target in EC therapy.

Response: Thank you for spending your time on reviewing our manuscript and for your good evaluation and suggestions to our manuscript.

This paper is rather interesting but as it stands it is predominantly descriptive. The Authors suggest that FOXA1/AR interaction could play a main role in the cooperative effect of these proteins but they do not provide molecular insight into the mechanism of this cooperation. If the Authors hypothesize that it occurs at transcriptional level a ChIP assay on the promoters of AR target genes could provide useful informations.

Response: It is a valid point that the conclusion would be strengthened by analyzing the effect of FOXA1/AR on AR target genes at transcriptional level. In consideration
of your suggestion, we examined whether FOXA1 and AR bind to the same regions upstream of TSS (transcription start sites) of AR target genes. As MYC, which is an AR target gene, is a known oncogene involving in cell proliferation in EC (Zhao ZN, Bai JX, Zhou Q et al. PLoS One 2012. PMID: 23028803). We had a ChIP assay on the promoters of MYC to seek useful information. We showed that both FOXA1 and AR bound to the promoters of MYC, and furthermore, we found that FOXA1 and AR also bound to the enhancer regions of MYC, with the greatest binding to Enh-1 (enhancer 1) region, to which FOXA1 and AR had even greater binding than to promoters of MYC (new Figure 4C and 4D). Our finding is consistent with the study that FOXA1 and AR have overlapping sites upstream of TSS of MYC in breast cancer, with greater binding to enhancer regions than to promoter regions (Ni M, Chen Y, Fei T et al. Genes Dev 2013. PMID: 23530127). The reason might be that another protein such as TCF7L2 is involved in FOXA1/AR interaction to MYC, thus mediating DNA looping for long-distance interactions of distal enhancers to proximal promoters (Ni M, Chen Y, Fei T et al. Genes Dev 2013. PMID: 23530127).

Moreover, as a feedback of our data in ChIP assays, we have re-written abstract in "page 2" and "page 3", have added information of “Chromatin immunoprecipitation (ChIP)-PCR” in Methods in "page 10" and "page 11", described experimental results in Results in "page 17" and "page 18", and have instead added several sentences to the Discussion explaining the possible reason why FOXA1/AR binds to enhancer regions even more greatly than the promoter regions of MYC and further study directions ("page 24" and "page 25").

*The role of FOXA1 is rather debated as this protein has been reported to act in many cases as a tumor suppressor. The Authors should better discuss this issue.*

**Response:** Thanks for your suggestion. We have supplemented information of FOXA1 as a tumor suppressor in Discussion section according to your comments.
Apart from FOXA1 as a tumor activator, other researchers have shown that FOXA1 also acts as a tumor suppressor in hepatocellular carcinoma, pancreatic, and estrogen receptor (ER)-positive breast cancer (Coulouarn C, Factor VM, Andersen JB et al. Oncogene 2009. PMID: 19617899) (Song Y, Washington MK, Crawford HC. Cancer Res 2010. PMID: 20160041) (Hurtado A, Holmes KA, Ross-Innes CS et al. Nat Genet. PMID: 21151129). Through investigating the concrete mechanisms of FOXA1 in these cancers, researchers have suggested that FOXA1 positively regulates miRNA-122, which is correlated with favourable prognosis in human hepatocellular carcinoma (Coulouarn C, Factor VM, Andersen JB et al. Oncogene 2009. PMID: 19617899). In addition, FOXA1 acts as an important antagonist of the epithelial-to-mesenchymal transition (EMT) in pancreatic ductal adenocarcinoma through their positive regulation of E-cadherin and maintenance of the epithelial phenotype (Song Y, Washington MK, Crawford HC. Cancer Res 2010. PMID: 20160041). It is critical to note that the role for FOXA1, as a tumor oncogene or a tumor suppressor gene, has been reported to vary in prostate and breast cancers depending on multiple cancer subtypes and states of hormone dependence or independence (Robinson JL, Macarthur S, Ross-Innes CS et al. EMBO J 2011. PMID: 21701558) (Hurtado A, Holmes KA, Ross-Innes CS. Nat Genet 2011. PMID: 21151129). We added this point in revised manuscript ("page 22").

Similarly the effect of AR and FOXA1 alone or in combination on Notch pathway should be investigated in deeper detail.

Response: We agree with you about the importance of investigating the relation of FOXA1-AR-Notch pathway in deeper detail. We investigated the effect of FOXA1 alone or in combination with AR on Notch pathway in our original manuscript. Our results showed that FOXA1 alone activated Notch pathway in EC cells, and moreover, AR depletion attenuated the activation of Notch pathway caused by upregulation of FOXA1. These results have suggested that the effects of FOXA1 on Notch pathway
activation are at least partly mediated by AR. However FOXA1 can target a series of transcription factors representing anywhere from several to hundreds of genes (Nakshatri H, Badve S. *Expert Rev Mol Med* 2009. PMID: 19261198), it is valid to investigate whether the effects of FOXA1 on Notch pathway are primarily through up-regulating AR. In consideration of your suggestion, we have performed additional experiments to assess the effect of AR alone and in combination with FOXA1 on Notch pathway. The results indicated that FOXA1 depletion could not rescue the over-expression of Notch1 and Hes1 proteins, which are common targets in Notch pathway, caused by AR over-expression (New additional file 3: Figure S1), providing further evidence that AR might be a necessary medium in FOXA1-enhanced Notch pathway activation in EC cells ("page 18" and "page 19").

Thank you once again for your many helpful comments, which we believe have improved our manuscript greatly.