Reviewer's report

Title: Prolyl-4-hydroxylase alpha subunit 2 promotes breast cancer progression and metastasis by regulating collagen deposition

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Reviewer: Franziska Baenke

Reviewer's report:

The manuscript ‘Prolyl-4-hydroxylase alpha subunit 2 promotes breast cancer progression and metastasis by regulating collagen deposition’ by Gaofeng Xiong and colleagues aimed to determine the role of P4HA2 in breast cancer. They investigated P4HA2 expression in normal and breast cancer tissue, and also differentiated P4HA2 expression according to ER status and stage of breast cancer. The authors showed that aberrant P4HA2 expression using RNAi decreased proliferation and phenotypically changed 3D cultures of four breast cancer cell lines compared to its controls. This was supported in vivo when breast cancer cells transduced with shP4HA2 displayed reduced tumor growth in mice and also lacked lung metastasis after tail vein injection.

Overall, this is a fair study question, which tries to understand the relevance of P4HA2 in breast cancer. Although potentially interesting, there are numerous leaps of claims, which were not corroborated by the data presented. A number of crucial experiments to provide direct evidence of the conclusions drawn are missing, including in vitro invasion studies. The manuscript was difficult to follow as it was written poorly and the methodology was in a very sketchy fashion that the reader is unable to critically review the results and to reproduce the experiments described in this manuscript. Moreover, two studies from the Semenza group that the authors included in their conclusion section recently investigated the role of P4HA2. In one study they show that P4HA2 is important for breast cancer metastasis. This is in line with the idea of this manuscript.

Major Compulsory Revisions

1. In Fig 1A-C, the authors claim there is a significant correlation between different collagen and P4HA2. The Spearman correlation used here, however, shows a weak correlation (everything below 0.4 is weak or very weak). Moreover, the authors state that they have used normal and cancerous tissues for this analysis. Please indicate how the normal tissue is distributed in this graph! Please clarify which published microarray datasets have been used, as this is unclear from the text/methods!

2. The numbers to establish the Kaplan-Meier survival analysis are inconsistent. In the method section, it is 1413 ER-positive breast cancer and 457 ER-negative breast cancer, whereas in Fig 1G 1452 ER-positive breast cancer and 473 ER-negative breast cancer are used. Please clarify!
3. Fig 1G shows survival analyses of patients with breast cancer and also the survival analysis depending on ER-status regarding to low and high P4HA2 expression. However, the authors labeled the y-axis as probability. This is not the right definition as it is speculative if these graphs mean overall survival or relapse-free survival. Please clarify in the text and figure! In addition, explain in more detail how these graphs were generated as the webtool for these KM plots have different versions available ranging from year 2010 to 2012.

4. The panel of cell lines used here should be displayed in Fig 2A as this is described in the text. However, this panel is missing. Please add the missing cell lines ZR-75-?, MDA-MB-231, and MBA-MD-157 and the silencing effect of P4HA2.

5. The cell line ZR-75 is not a breast cancer cell line. Please clarify if you have used ZR-75-1 or ZR-75-30 and add this detail to every figure! Does cell line ZR-75-? change its polarity too?

6. In Fig 2B, 3D cultures have been stained with integrin #6 and DAPI. The authors do not mention, which cell line has been used and why they have used these markers. It would be beneficial to add a sentence why this staining was performed. And maybe close-up images can be used to underline the change in polarity.

7. The proliferation figures in Fig 2E, 3C and 4E have been generated by using the EdU click it system. It seems that the shControl or control cells show an effect on proliferation as none of them reach 100%. It is unclear to the reader how these numbers have been established. Please explain and also add the graph for the missing cell line MDA-MB-157 regarding proliferation to Fig 3C and Fig 4E.

8. The silencing of P4HA2 has an effect on proliferation. Please explain the change in proliferation and add some information if this is caused by cell death, decrease in cell viability and/or growth arrest.

9. The cell lines have been cultured with 3D lrECM and Matrigel to form 3D cultures. However, the authors claim that ‘reduced invasive branches in P4HA2-silenced cells indicate that P4HA2 contributes to malignant tissue morphogenesis and cancer cell invasion in 3D culture.’ The changes in morphology after silencing of P4HA2 can’t be linked to invasion as an appropriate invasion assay is missing. This 3D culture only shows a change in morphology. However, if the change in morphology affects invasion is not further elucidated. The same applies to the inhibitor studies (Fig 3A and Fig 4A). Please clarify!

10. The authors claim that the P4HA2 is a potential target for treating breast cancer (last paragraph of 2nd result subheading). They use the compound 1,4-DPCA as proof of principle. However, 1,4-DPCA is a general inhibitor against Prolyl-4-hydroxylases and also FIH (Cayman website information). Can the authors exclude the involvement of P4HA1, PH4A3 or FIH in the observed phenotypes for Fig 4 and Fig 5?

11. The authors claim that P4HA2 is required for collagen secretion and
deposition by breast cancer cells implying breast cancer progression and metastasis (last part of 3rd result subheading and 4th result subheading). Does this imply that overexpressing P4HA2 in a non-malignant breast cell line e.g. MCF10A would change a) its morphology and b) produce more collagen and c) show invasive potential?

12. Can the authors please include a staining for P4HA2 to Fig 6 to see if expression of P4HA2 is diminished and also to establish if there is a correlation of P4HA2 expression and the invasive front? Please add a line or bigger image to clearly identify the invasive front. Please quantify the collagen staining for Fig 6E!

Minor Essential Revisions

1. Please include a sentence of the function of P4HA3 as the authors list the function of P4HA2 and P4HA1 in paragraph 2 of the introduction.
2. Please use one abbreviation of HMT-3522 T4-2 as authors switch between T4 and T4-2.
3. How many days have the cells been cultured on matrigel? Please include time frame of 3D culture studies in methods and in figure legends!
4. Please change font in methods for xenograft experiment.
5. Please use a subheading for statistical analysis in the methods section as this is currently combined with the Kaplan Meier survival analysis. It is very confusing.
6. Please change figure labels in text as Fig 2E is actually Fig 2D and Fig 2D in text should be Fig 2E (first paragraph of 2nd result subheading).
7. Spelling mistake in figure legend of Fig 2E, needs to be receptor!
8. Could the authors please follow the Gene/Protein Nomenclature Guidelines.
9. Please add loading control for western blot in Fig 5A and clarify which shRNA (1 or 2) has been used for the western and add second shRNA.
10. The HMT-3522 T4-2 cells are grown under collagen-coated conditions (see Anders M et al 2003 or http://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=9810221). Could this be stated in the text! Why was this cell line chosen when it is cultured under very different culture conditions?
11. Please specify which shRNA (1 or 2) was used for the in vivo study!

Discretionary Revisions

1. Please provide a table of the cell lines used in this study regarding their origin as well as their ER/PR/HER2 status.
2. Please add the information about the relevant shRNA sequences used in this.
3. For Fig 1E, Mackay et al Oncogene 2003 could be added as this study has shown that P4HA2 is regulated by ERBB2 in human mammary luminal epithelial cells.
**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**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