Author's response to reviews

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

Authors:

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Author's response to reviews: see over
Nov 10th, 2013  
Dr. Britta Weigelt  
Associate Editor  
BMC Cancer

Dear Britta,

Enclosed please find our revised manuscript (ID: 1104395371078333) entitled “Prolyl-4-hydroxylase α subunit 2 promotes breast cancer progression and metastasis by enhancing collagen deposition” to be considered for publication in BMC Cancer. We are grateful to the reviewers for their very thoughtful and helpful comments, and to you for allowing us to revise this manuscript for publication.

We are pleased that the reviewers found the study addressed an important topic in cancer research, and overall quality of the manuscript is satisfactory. Two reviewers commented that a Transwell invasion should be done to confirm the function of P4HA2 in regulating cancer invasion. We have performed the experiments and found that silencing P4HA2 significantly inhibits cancer cell invasion. In addition, we have quantified western blot results and Masson’s trichrome staining as suggested by reviewer. Below, we address the reviewers’ comments (in italics) point-by-point with exact changes. We hope you find the manuscript is significantly improved and ready for publication.

Included in the submission is: one file comprising the Manuscript and 6 Figures.

Best regards,

[Signature]

Ren Xu. Ph.D  
Assistant Professor, Department of Molecular and Biomedical Pharmacology  
Faculty, Markey Cancer Center  
University of Kentucky
Referee: 3

Comment: This manuscript addresses an important topic in cancer research. The experiments are well designed and well controlled, and the data convincing. Following the specific modifications listed below, this manuscript merits acceptance.
Response: We thank the reviewer for this and the following comments.

Major Compulsory Revisions

Comment 1: The experimental evidence showing reduced collagen-1 and collagen IV expression (Figure 5A) is not convincing, based on a single Western, and no quantification or statistical validation. The same is true for the *in vivo* assessment of collagen deposition in figure 6E. These data need to be strengthened in order to argue that P4HA2 down-regulation impairs collagen deposition.
Response: We have repeated the western blotting experiments and quantified the results (See Figure 5A). We have also quantified the Masson’s Trichrome staining in Figure 6E (See Figure 6E). Quantified results showed that silencing P4HA2 significantly reduced deposition of collagen I and IV.

Comment 2: Figure 6D is hard to interpret and needs improvement. The images appear to be of too low resolution to illustrate differences in invasiveness. The figure legend does not describe the arrows in the left panel.
Response: We have changed the images in Figure 6D to a high resolution one (100X) (See Figure 6D) and described the arrows in the figure legend (See Page 27).

Comment 3: The authors do not specify which TCGA datasets they used in their gene expression analyses, and this information must be added. Without these specifications, it is impossible for anyone to duplicate their analyses. Additionally, the TCGA datasets include microarray data and RNAseq data, but it appears that only the microarray data were analyzed. It will be useful to know if the same conclusions are produced using the RNAseq data.
Response: TCGA breast cancer microarray dataset was used for the analysis. It was downloaded from Oncomine. We have included this information in the results (page 10). The correlation between collagen I, III, IV and P4HA2 was also detected in another microarray dataset (Supplemental Figure 1). It would be interesting to analyze the RNAseq data, but currently we don’t have the expertise to do it.
Comment 4: The conclusion statement in the abstract exaggerates the conclusions permitted by the data. The data do not “reveal a critical role of P4HA2 in breast cancer progression”. The word “reveal” might be changed to “suggest”. The last sentence of the introduction is a more accurate statement.

Response: Thank the reviewer for pointing this out. We have changed “reveal” to “suggest” (See Page 3 “Conclusion”).

Minor Essential Revisions

Comment 1: The authors need to be more careful in their use of the word “collagen” in lieu of specifying collagen isoforms. Obviously, collagens are a large family of proteins, with diverse functions. Yet, throughout the manuscript, the authors rely mainly on the general term “collagen”, which results in many incorrect statements. Examples: the first sentence of the abstract is only demonstrated for certain collagens; the 2nd sentence of the introduction refers to collagen as a single protein; the 4th, 5th, 7th and 8th sentences of the introduction all refer to data that are specific to a subset of collagens, and not “collagen”.

Response: We are sorry for this oversight. We have used “collagen” and specific collagen names more carefully in the revised version.

Referee: 2

Comments: The overall quality of the manuscript is satisfactory. This manuscript has clearly conveyed the message regarding the association of P4HA2 and collagen deposition in human breast cancer tissues.

Response: We thank the reviewer for this and following comments.

Major Compulsory Revisions

Comment 1: Authors fail to describe the experimental details about the setup of 3D culture, e.g. what components/gel they used, how long they embedded the cells for subsequent analysis.

Response: Thank the review for bringing this to our attention. We used Engelbreth-Holm-Swarm (EHS) tumor extract (Matrigel: BD Biosciences, 354230). The cells were cultured in 3D matrigel for 4 days. We have included these and other details about the 3D culture in the materials and methods part (See Page 6 “Cell culture and virus preparation.”).

Comment 2: Figure 1. Authors categorize patients into P4HA2 low and high groups. What is the cut-off value for this categorization? Are the results consistent when different cut-off values are used?
Response: The patients were split evenly in the low and high groups. We used “median” cut-off in the webtool analysis. Similar results were obtained when “upper tertile” were used.

Comment 3: Authors should clearly explain how they grade the degree of polarization. It seems to me that this assay is only qualitative. Readers may not know how to interpret polarity of cells. Authors are suggested to provide a picture showing polarized cells and a picture of unpolarized cells. Or use arrows in the figures.

Response: Because the heterogeneity of T4-2 cells, silencing of P4HA2 could not reprogram all the cells to form the polarized acinus-like colonies in 3D culture. We quantified the percentage of polarized colonies in the total colonies. In Figure 2B, immunofluorescence staining of α6 integrin, a basal marker, showed un-polarized grape-like structure (see the lateral staining of α6 integrin) in control T4-2 cells (shCtrl) and polarized spheroid structure (basal staining of α6 integrin) of P4HA2-silenced T4-2 cells (shP4HA-1, shP4HA-2). These have been included in the figure legend. We have added arrows in Figure 2B to point the different α6 integrin staining in shCtrl and P4HA2-silenced T4-2 cells.

Comment 4: Typical transwell cell migration assay with Boyden chamber should be performed to examine the effect of -control and –shP4HA2 cells in breast cell migration.

Response: As reviewer suggested, we have done the Transwell invasion assay. The results is showed in Figure 3D and Figure 3E.

Comment 5: Can the authors observe lung metastasis from orthotopic model? A number of breast cancer cell lines can metastasize to the lungs when implanted into the mammary fat pad, why did the authors use tail vein injection for the last experiment?

Response: According to our animal protocol, the mice in this experiment had to be sacrificed when tumors reached 15 mm in maximum diameter. At the time point that the mice were euthanized, we did not observe any lung metastasis. Tail vein injection has been widely used to analyze lung colonization of breast cancer cells, even though it is not a perfect model to analyze cancer metastasis. We will use the orthotopic model to determine function of P4HA2 in metastasis in the future. These experiments will not be included in this study since it takes several months to get the results.

Comment 6: Discussion/conclusion is too short. Authors should discuss about the implications of the study, how this study is going to impact/benefit the current therapeutic regimen of breast cancer, any preclinical studies on collagen synthesizing and modifying enzymes, the importance of ECM, how the
ECM generated from P4HA4 positive breast cancer cells affect the motility of the cancer cells, how the branching/polarized properties of the cells affect cell movement, what future studies can be initiated from this work etc.....

**Response:** Thank the reviewer for this comment. We have included these discussions in the “Results and Discussion”.

**Minor Essential Revisions**

**Comment 7:** Figure 5B and 5C are mislabeled

**Response:** We have switched the labeling of Figure 5B and Figure 5C (See Figure 5).

**Referee: 1**

**Major Compulsory Revisions**

**Comment 1:** I am afraid that it has already been established that P4HA1 and P4HA2 are critical for collagen deposition by breast cancer cells (Gilkes et al., 2013, Cancer Res.). Also, it was shown by Gilkes et al., that the knockdown of P4HA2 in MDA-MB-231 cells inhibits tumor growth and metastasis and it was shown that P4HA2 gene expression levels are associated with decreased survival of breast cancer patients. Furthermore Gilkes et al., show and discuss the therapeutic potential of inhibitors of collagen prolyl hydroxylases. Thus, Gaofeng Xiong and co-authors should thoroughly work out the novelty of their findings and discuss the paper of Gilkes et al. in greater detail.

**Response:** Thank the review for these comments. We were aware of Gilkes’s work when we prepared this manuscript, and we cited their papers in the previous version. As the reviewer suggested, we have emphasized the following novel findings in the revised manuscript: (1) expression of P4HA2 is crucial for the breast cancer cells to form the aggressive and disorganized structures in 3D culture; (2) silencing P4HA2 or inhibiting P4HA activity reduced cell proliferation in 3D culture. In addition, we analyzed function of P4HA2 in multiple breast cancer cell lines (HMT-3522 T4-2, MDA-MB 231, ZR-75-1, and MDA-MB 157), including both ER positive and ER negative cancer cells, while they performed most of their experiments in MDA-MB 231 cells. We have included these discussions in Page 13 (Paragraph 2).

**Comment 2:** The authors should point out clearly that not only the activity of P4HA2 is inhibited upon 1,4-DPCA treatment and discuss their results accordingly.

**Response:** In the previous version, we mentioned that 1, 4-DPCA is an inhibitor of prolyl-4-hydroxylase (page 12, paragraph 3). In the revised version, we have further emphasized that 1,4-DPCA is not a
P4HA2-specific inhibitor. Recent study showed that P4HA1 and P4HA3 also contributed to breast cancer progression. Therefore, this inhibitor may suppress the malignant phenotypes of breast cancer cells in 3D culture by inhibiting all three P4HA isoforms. We have included this discussion on page 13 (paragraph 1)

**Comment 3:** Figure 2/3: P4HA2 knockdown efficiency is shown in T4-2 cells. Please also provide the immunoblot data for the other cell types (ZR-75, MDA-MB 157, MDA-MB 231).

**Response:** The knockdown efficiency is similar between different cell lines; we only showed the results in T4-2 cells. Following is the results in MDA-MB 231 cells.

![Immunoblot data for P4HA2 knockdown in T4-2 cells](image)

**Comment 4:** Figure 5A: Please show equal loading of control and knockdown cells (e.g. Ponceau S staining). Why was this experiment done with T4-2 cells and why is collagen deposition not shown for MDA-MB cells? Particularly with regard to the experiments in Figure 6 and the general conclusion drawn by the authors “our data demonstrate that P4HA2 is required for collagen secretion and deposition by breast cancer cells” please include a justification why the experiment in Fig. 5A was performed with T4-2 cells or include data on MDA-MB cells.

**Response:** The ponceau staining has been included as a loading control in the Figure 5A. Roles of P4HA2 in regulating collagen deposition in MDA-MB 231 cells have been shown by Gilkes, we also observed silencing P4HA2 reduced collagen deposition in tumors formed by MDA-MB 231 cells (Figure 6). Since we only tested the collagen deposition in T4-2 cells in 3D culture, we have changed our conclusion to “our data demonstrate that P4HA2 is required for secretion and deposition of collagen I and IV by T4-2 cells” (See Page 14, Paragraph 1).

**Comment 5:** It is unclear why the proliferation of T4-2 cells in Fig. 2 (~55 %) is different from the proliferation of control cells shown in Fig. 4 (~10%). What is the explanation?
Response: We are sorry that we did not provide enough details on these experiments. The cell proliferation was analyzed by 5-ethynyl-2’-deoxyuridine (EdU) incorporation assay. The incorporation of EDU into cellular DNA is DNA synthesis dependent. For these two experiments, T4-2 cells were incubated with EDU for different time (8h and 4h), which probably caused the different labeling ratio in the control group.

Minor Essential Revisions

Comment 1: Please provide more details in Materials and Methods on: - Sh-vector: was an empty control vector or a vector with a scrambled sequence used?  
- How was equal loading of protein for immunoblotting controlled?  
Response: As the reviewer suggested, we have provided this information in the Materials and Methods (the “shctrl-vector” is scramble shRNA vector (See Page 6-7 “Cell Culture and virus preparation”). Details about how was equal loading of protein have been included (See Page 7-8 “Western blot analysis”).

Comment 2: Where are the arrows in Fig. 6 D,E,G pointing to? Provide a short description.  
Response: We have provided a short description for the arrows in Fig. 6 D,E,G (See Page 27)

Comment 3: Data in Figures 5A, 6: Were the experiments performed with shP4HA2-1 or shP4HA2-2?  
Response: Experiments in Figure 5A and 6 were performed with shP4HA2-1, we have clarified this in the manuscript and figure legend (See Page 13-15 and Page 26-27).

Minor issues not for publication

Thank the reviewer for bring these issues to our attention

Comment 1: Figure 6B: labeling of the graph: (mm3) please use superscript 3  
Response: The (mm3) has been revised to superscript 3 in Figure 6B.

Comment 2: Introduction, 2nd paragraph: there is a comma missing between chondrocytes and osteoblasts  
Response: We have added a comma between chondrocytes and osteoblasts (See Page 5, Paragraph 2).

Comment 3: Materials and Methods, first and second heading: remove period at the end
Response: The periods have been deleted.

Comment 4: Results and Discussion: Please control spaces before the references
Response: We have paid attention to this issue.

Comment 5: Results and Discussion, second heading “Inhibition of P4HA2 suppresses the malignant phenotypes of breast cancer cells in 3D culture.”, first paragraph: reduced colony size is sown in Fig. 2B,C,D (not Fig. 2B, C, E) cell proliferation is shown in Fig. 2E (not in Fig. 2D).
Response: These errors have been fixed (See Page 11-12).

Comment 6: Figure legends: make uniform headings (either bold characters or standard characters)
Response: The headings in figure legends have been change to bold characters (See Page 24-27).

Referee: 4

Major Compulsory Revisions

Comment 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!
Response: We have consulted Dr. Chi Wang, a biostatistician at Markey Cancer Center. The r value between 0.3 to 0.5 is considered as moderate correlation. We have performed correlation analysis in another microarray dataset (Clin K. et al., 2006, Cancer cell), and the r vaules for CollA1 and Col3A1 are above 0.5. Based on these data, we conclude that mRNA levels of P4HA2, CollA1, Col3A1, and Col4A1 are significantly correlated. The expression of P4HA2 in normal and malignant tissues has been shown in Fig 1D. We have included information of the microarray dataset in the results.

Comment 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!
**Response:** We are sorry for this oversight. We have changed the number in the methods section with “1452 ER-positive breast cancer and 473 ER-negative breast cancer” (See Page 9 “Kaplan Meier survival analysis and other statistical analysis”).

**Comment 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.

**Response:** Thank the reviewer for bringing this to our attention. We have changed the labeling to “probability of overall survival”. We used 2012 dataset, and this information has been added in the method section.

**Comment 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.

**Response:** Please see the response to referee #1 (comment 3).

**Comment 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?

**Response:** We are sorry for this oversight. We have changed ZR-75 to ZR-75-1 in manuscript including figures. We did not examine the cell polarity of ZR-75-1 because the ZR-75-1 cells usually do not form polarized acinar structure in 3D culture, knockdown of P4HA2 or treatment with P4H inhibitor only inhibited colony size of ZR-75-1.

**Comment 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.

**Response:** T4-2 cells were stained with the antibody against integrin α6. Integrin α6 has been used as a basal marker to detect basal polarity mammary epithelial cells. We have added the following sentence to explain it (Page 11, Paragraph 2). As showed by the confocal images in Fig 2B, after P4HA2 is silenced
in T4-2 cells, the cells formed polarized acinar structure in 3D-culture (majority of integrin α6 (basal marker) staining is on the basal surface of the acinar, while the lateral staining was detected in the control T4-2 cells).

**Comment 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.

**Response:** We examined cell proliferation with 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay. The EDU will incorporate in the genome during the DNA synthesis. We quantified cell proliferation by analyzing the percentage of the Edu positive cells in the total cells (staining with DAPI). The cell proliferation in the control cells is not 100% because EDU cannot incorporate into all the cells in a short period of incubation time. We have included the proliferation data of MDA-MB-157 cell in Figure 4E (See Figure 4E).

**Comment 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.

**Response:** The EDU incorporation assay is DNA synthesis dependent, and it is a well-accepted and specific assay to analyze cell proliferation. We did not see cell and nuclei debris in DAPI staining, thus we don’t think silencing P4HA2 induces cell death or reduces cell viability.

**Comment 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!

**Response:** As reviewer suggested, we have done the invasion assay using P4HA2 knockdown cells (See Figure 3D,E).
**Comment 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?

**Response:** 1,4-DPCA inhibits FIH with an IC$_{50}$ value of 60 µM (Cayman website), in our experiments, we used 1,4-DPCA with concentration of 10 µM or 20 µM. At these concentrations, 1,4-DPCA has limited effect on FIH.

1,4-DPCA is a general inhibitor against Prolyl-4-hydroxylases, we cannot exclude the involvement of P4HA1 and P4HA3 in breast cancer progression. Recent study showed that P4HA1 and P4HA3 also contributed to breast cancer progression. Therefore, this inhibitor may suppress the malignant phenotypes of breast cancer cells in 3D culture by inhibiting all three P4HA isoforms. We have included this discussion on page 13 (paragraph 1)

**Comment 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?

**Response:** Collagen biosynthesis is a multistep process that involves a number of post-transcription modification enzymes. Thus collagen prolyl-4-hydroxylase may be necessary but not sufficient for collagen synthesis and deposition. It would be interesting to analyze whether overexpressing P4HA2 in a non-malignant breast cell line would change its morphology and produce more collagen in the future.

**Comment 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!

**Response:** Our antibody of P4HA2 is not work for IHC staining. But our western blotting result showed P4HA2 protein level was very low in sh-P4HA2-1 MDA-MB-231 cells (See Supplemental data). We have changed a bigger image (100X) to show the invasive front (See Figure 6D), and quantified the collagen staining of Figure 6E (See Figure 6E).
Comment 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.
Response: We have added a sentence of P4HA3 in paragraph 2 of the introduction (See Page 5).

Comment 2: Please use one abbreviation of HMT-3522 T4-2 as authors switch between T4 and T4-2.
Response: We have change all T4 to T4-2 as the abbreviation of HMT-3522 T4-2.

Comment 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!
Response: The cells were cultures on 3D matrigel for 4 days. We have added these in methods (See Page 6) and figure legends (See Page 25).

Comment 4: Please change font in methods for xenograft experiment.
Response: We have changed the font in the methods of xenograft (See Page 8).

Comment 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).
Response: We have changed the position of Figure 2D and Figure 2E in first paragraph of 2nd result subheading (See Page 12, Paragraph 1).

Comment 8: Could the authors please follow the Gene/Protein Nomenclature Guidelines.
Response: Prolyl 4-hydroxylase subunit alpha-2 is the protein name of the P4HA2 gene; the abbreviation (P4HA2) was used to make it easy to read for the audience. Gene symbol has been labeled italicised.

Comment 9: Please add loading control for western blot in Fig 5A and clarify which shRNA (1 or 2) has been used for the western.
Response: We have added a ponceau-s staining as the loading control in Fig 5A. Sh-P4HA2-1 was used for Western Blotting in Fig 5A, we have added the information in Page 13 Paragraph 3.
Comment 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?

Response: The HMT-3522 T4-2 cells were cultured on collagen-coated dishes, but when we examined the collagen expression after knockdown P4HA2 or 1,4-DPCA treatment, we plated these cells on none-coated flask. This cell line is malignant but non-metastatic, and form disorganized structures in 3D culture. These aggressive 3D malignant phenotypes of T4-2 cells can be reverted by a variety of microenvironmental signals (Weaver VM, et al., 1997, J Cell Biol; Wang F, et al., 1998, Proc Natl Acad Sci U S A), which provides a nice model to study roles of microenvironmental signals in breast cancer progression. That’s why we use this cell line for this study.

Comment 11: Please specify which shRNA (1 or 2) was used for the in vivo study!

Response: Sh-P4HA2-1 was used for in vivo study. We have included this information in the revised manuscript (See Page 13-15 and Page 26-27).

Discretionary Revisions

Comment 1: Please provide a table of the cell lines used in this study regarding their origin as well as their ER/PR/HER2 status.

Response: We have added the information about ER/PR status on page 6 “Cell Culture and virus preparation”.

Comment 2: Please add the information about the relevant shRNA sequences used in this.

Response: This information has been added in Methods on page 7 “Cell Culture and virus preparation”.

Comment 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.

Response: We have added this reference in results and discussions (See Page 10 Paragraph 1).