Author's response to reviews

Title: SLUG and Human Chorionic Gondadotropin Induce Breast Cells With "Stem Cell-Like" Properties

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Author's response to reviews: see over
Response to review
Reviewer: Gianluca Storci

Major concerns
1) a major role for CSC generation is claimed--- data about this issue is scanty

Response: We have performed additional studies with a new batch of HCG that we purchased from Sigma. For unknown reason, there was a significant variation between experiments. Therefore, we have deleted data related to HCG. However, we have strengthened our observations with TNF with additional experiments. Title has been changed accordingly. We believe that studies related to HCG are a minor component of the manuscript and as such should not diminish the impact of this study.

2) Figure 2 data presentation should be ameliorated to allow the reader….
Response: We regret this error. Better explanation is provided including description of integrin beta 1 and ST-2. Furthermore, in response to reviewer 2, additional data on N-cadherin expression in different cell types by RT-PCR and Western blot analysis is presented as a part of this figure in the revised manuscript. Notch 3 was not part of our microarray results. Notch-3 RT-PCR has been deleted as we could not reproduce Notch-3 in a new batch of sorted cells that was used for generating the new Figure 2C. We have included the Sansone et al. publication while discussing results.

3) The effect of TNF alpha stimulation seems to be quite low compared to p65 overexpression-----
Response: Additional discussion is presented in the text. Again, the effect of TNF on CSC generation is comparable with that of TGFβ1 (Morel et al., PLOS One 3:e2888). We have repeated these experiments after treating cells for 15 days and the results are same. This figure has been replaced with a new figure showing 15% CSC after TNF treatment and statistical differences are also included (p=0.03, n=4). We had previously shown that TNF-induced expression of EMT-associated genes ZEB-1 and ZEB-2 attenuates after TNF withdrawal (Oncogene 26:711-724). In contrast, overexpression of constitutively active p65 leads to consistent overexpression of ZEB-1 and ZEB-2. The differences between TNF treatment and p65 overexpression could, therefore, be related to transient versus sustained induction of EMT-associated factors.

4) Figure 5A. It is not clear what data the graph refers to. Are these data reported by Liu et al?
Response: This is data derived from Liu et al study. They did not specifically state about Slug in their study but we extracted data from GEO (additional details are provided in the legend, GDS2617) and generated the diagram. We have also included discussion of a recent manuscript published in Breast Cancer Research (Lim et al., 2010 R21) while our manuscript was under review. That study also demonstrated enrichment of Slug by 3.16-fold in mammary stem cells of both human and mouse compared to luminal progenitor or mature cells (please see supplementary table 4 of that manuscript).

5) Figure 5D should report the timing of mammosphere formation and total number of mammosphere.
Response: Timing of mammosphere is included in the legend and number of mammosphere for number of cells plated is also included. In the original manuscript, we presented per field because mammosphere media contained methylcellulose to reduce movement of mammospheres while counting under microscope. We have repeated these experiments, collected mammospheres and counted only larger spheres; data regarding MCF-7Gli2, MCF10ASlug and MCF-10A cells are presented using this method.

6) Figure 6 on Gli overexpression may be included in the supplementary. 36B4 should be included.
Response: We have moved this figure to supplementary as Figure S11. 36B4 is inserted into figures. Data regarding mammospheres formation by parental and Gli2 overexpressing cells (as per suggestion from reviewer
2) are included in this figure. Similar studies with SATB1 overexpressing cells could not be performed because of unstable nature of these cells.

7) In various parts of the manuscript authors refer link between SLUG basal tumors and mammospheres. Such issue has been addressed in a previous paper, which may be included (Storci et al).

Response: Results of Storic et al is discussed in the context of this manuscript both in the introduction and discussion.

Minor concerns:
1) Scale bars may be appropriate in Figure 1B and Figure 5D.

Response: Scale bars for both of these figures have been inserted. Scale bar for Figure 1B was present in the original version and is located on top right. Figure 5D is now Figure 6.

2) Figure 2 should report the name of the cell line (MCF10A) above RT and WB
Response: Necessary corrections have been made.

3) In Figure 3B, EMSA should report the full name of the populations----
Response: Necessary corrections have been made to the figure. Additional description of EMSA is also included.
Review 2:
Reviewer: Gema Moreno-Bueno

Major comments:
1) The evidence showed in this manuscript for SNAI2 induction of stem cell-like characteristics is not sufficient. It should be confirmed with an in vitro (soft agar invasion) and in vivo (tumor formation in nu/nu mice)

Response: We have performed soft agar assay with MCF10A cells with or without SLUG overexpression but the results are negative as we expected. Since these cells did not show transformed phenotype in vitro, it is hard to justify studies in nude mice as these cells are less likely to form tumors and Institutional Animal Care Committee usually do not approve these studies without in vitro proof of transformation. MCF-7 cells are transformed cells and form tumors without additional manipulation. It is also important to note that two previous studies have shown that EMT induces stem-like cells but not cancer stem cells (Mani et al., Cell 133:704-715; Morel et al., PLOS one 3:e2888). Transformed phenotype of these cells in soft agar and nude mice requires oncogene overexpression (Moral et al). It is clear from these studies as well as our soft agar assay, CD44+/CD24- phenotype is not associated with transformation. It only represents stem cell phenotype of both normal or cancer cells. With respectfully disagree with reviewers comment stem cell-like should be of transformed phenotype. In addition, Shipistin et al., (Cancer Cell 11:259-273) reported the presence of CD44+/CD24- cells in the normal breast. For these reasons, title of our manuscript indicates SLUG generation breast cells (not breast cancer cells) with CD44+/CD24- phenotype. In addition, the abstract and the text have been modified to emphasize this view.

2) The M&M is very descriptive, there is not a specific paragraph----

Response: In the previous version, we had quoted references for the procedures that were used for matrigel and mammospheres assays in the very first section of M&M. If established procedures are used, it is customary to include citations but not description. In the revised version, a separate section for these two assays is included. Additional paragraph describing the expression array is included. We had included expression array method in the previous version but did not indicate it in a separate paragraph. As indicated earlier, the arrays were done with four independent biological replicates. Previously published statistical methods were used for the analysis; therefore, only a brief description with suitable reference has been included. In addition, more details of the analysis are available along with expression profile in GEO database. In addition, endogenous controls are included for figures with RT-PCR data.

3) and 4) page 10, first paragraph and reference to Sarrio et al study.

Response: Because of the previously described plasticity of these cells based on confluence, we were careful in our studies to maintain cells at similar confluency for all experiments. In addition, cells for a set a experiments were maintained with a same preparation of media because of our previous observation of the influence of serum on CD44+/CD24- phenotype (Sheridan et al., Breast Cancer Research 8:R59). These descriptions are included in the materials and methods.

5 Page 10, Figure 1B, in the 2D culture, authors did not show the proliferation----

Response: Growth rate of CD44+/CD24-, CD44+/CD24+ and CD44-/CD24+ cells are shown figure 1C. Matrigel assay followed by H&E staining is a qualitative assay but not a quantitative assay. In this assay, matrigel embedded with cells are scoped from 8-well chamber slides, embedded in histogel followed by paraffin. This procedure does not retain the structure of matrigel for quantitative measurement. In general, CD44+/CD24- cells formed few colonies than CD44-/CD24+ cells. However, the size of the colonies can be easily measured. Please see scale mark in the top right of the figure, which clearly shows larger size of acini-like structures by CD44+/CD24- cells compared to CD44-/CD24+ cells. Since matrigel gel assays are not
quantitative, statistical analysis cannot be performed. Statistical analysis of TNF-treated cells is included in Figure 4.

6) Page 10, when authors described.
Response: Corrected numbers are presented in the text. 07-sep and 12-sep are the errors of EXEL - whenever gene with a name SEP is inserted into EXEL, it automatically converts into September date. Genes in these two rows correspond to SEPTIN6 and SEPTIN11 - full-length name is provided to overcome this error.

7) In figure 1B the authors mentioned the vacuolated cells suggest the apoptosis----
Response: We have performed immunohistochemistry for active caspase 3 and found that these cells to be negative for this caspase. Therefore, suggestion regarding apoptosis is deleted from the text.

8) The MCF10 should be analyze as control cell in all of the showed-----
Response: We have included additional RT-PCR data and western blot data of select genes with parental MCF10A cells as well as in CD44+/CD24+ cells (Figure 2). Double positive cells are very difficult to grow after sorting; therefore, only limited experiments could be performed with these cells.

9) Figure 2A (upper left panel) in order to confirm the EMT related stem cell phenotype the authors should characterize---
Response: Western blots showing the expression pattern of E-cadherin, N-cadherin, Vimentin and alpha smooth muscle actin are presented in Figure 2.

10) Figure 2A (upper middle panel): the 36B4 gene used as endogenous control-----
Response: We performed additional controls including beta-actin and GAPDH but found 36B4 to be most appropriate control. Even if 36B4 levels are normalized after densitometric scanning, still genes that are upregulated in CD44+/CD24- cells remain up-regulated. In fact, figures under represent their upregulation. We did not observe a difference in the expression levels of BMI-1 between CD44+/CD24- and CD44-/CD24+ cells, which can also be considered as a control (see both central and right panel in Figure 2A). Also note that the analysis of newly sorted cells presented in Figure 2 shows similar 36B4 in CD44+/CD24-, CD44+/CD24+, and CD44-/CD24+ cells.

11, Figure 2A the control should be included;
Response: as discussed above BMI-1 serves as control here and additional 36B4 control is included.

12), page 13, Table 1, the authors should discuss more about the absent snail1----
Response: The absence of Snail in the list is mentioned in the result section.

13) Figure 3B, the MCF10A CD44-/CD24+ and wild type cells should----
Response: CD24+ cells indicated in the figure are CD44-/CD24+. Because of space only abbreviated version was given in the original figure. The figure has been modified to include complete description. Additional data comparing NF-kB in wild type cells, CD44+/CD24-, and CD44-/CD24+ cells are included in the figure.

Page 14: Authors indicate that HCG or FSH treatment-----
Response: We do not claim FSH inducing CD44+/CD24- cells. It is only HCG. However, with new batch of HCG, we were not able to obtain consistent results. Therefore, studies related to HCG have been removed. We
did mammosphere assay. In one experiment both TNF increased the number of mammosphere from 8000 to 26000. However, this data could not be reproduced. This could be because of instability of TNF in growth media or semi solid nature of the media preventing uniform access to TNF when cells are supplemented with new media. Therefore, data are not included. Note that we did not observe this discrepancy with cells modified to express a gene stably.

15) Figure 4B, the authors mentioned the Ras or TNF treatment-----
Response: The effect of Ras overexpression or TNF treatment on the expression of EMT markers including E-cadherin and Vimentin is included in the manuscript. We have previously shown TNF induced expression of EMT associated genes ZEB-1 and ZEB-2. Similarly, the expression levels of p65 in overexpressing cells and its effects on the expression of EMT makers have already been published and the that publication is referenced in the manuscript (Oncogene 26, 711-724, please refer to figures 1, 3 and 5). It is not appropriate to reproduce published data on characterization of these cells.

16) The authors suggest that overexpression of SNAI2 promotes EMT events in MCF-10A or MCF-7, this has been associated with stem cell------
Response: Expression levels of E-cadherin, Vimentin and alpha smooth muscle actin in control and SNAI2 overexpressing MCF10A and MCF-7 cells are included in the manuscript.

17) In the same way, the authors should analyze same markers in Gli-2 MCF10A and Gli-2 MCF-7 cells----
Response: We have done these studies in MCF-7 cells overexpressing Gli-2. While MCF-7-slug overexpressing cells showed increased expression of the EMT marker Vimentin, similar change was not observed in Gli-2 overexpressing cells. Similarly, only MCF-7-Slug overexpressing cells but not Gli-2 overexpressing cells showed increased number of mammosphere. Because of variability in number of mammospheres in control cells between experiments, statistical analysis is difficult but trend was same in all experiments. Therefore, data from two experiments are presented separately. As per suggestion of reviewer #1, studies related to Gli-2 are in supplementary (Figure S1). Similar experiments in MCF-10A-Gli-2 cells could not be performed because these cells are extremely difficult to grow and could not be maintained for prolonged period of time.

18) Figure 5B, the authors show the mRNA levels of SNAI2 in transfected cells-----
Response: We did do western blot with commercially available antibody from ProScience Incorporated. However, because of poor quality of the antibody, protein could not be detected.

19) Figure 5D, the authors should show the mammosphere analysis
Response: Mammosphere assay has been done four times; there was no statistically significant difference between control and slug overexpressing MCF10A cells. Slug overexpressing MCF10A cells appear to form larger mammosphere- a representative figure is included.

20) The additional figure should show----
Response: Data on MCF-7-gli-2, which are negative, is included in the supplementary figure 10. MCF-7 SATB-1 and MCF10A-Gli-2 cells are difficult to grow; therefore, mammosphere assays with these cells could not be performed.

Minor comments:
1) Actually, the accepted name for slug is snai2---
Response: Title is modified to indicate Snai2.
2) M&M: The authors should indicate the commercial origin—
Response: This information is included in the manuscript.

4) The authors should indicate-----
Response: This information has been added.

5) p16, the first paragraph, “SLUG is a potent repressor of p53-deregulation----
Response: The error in this sentence is corrected.....

6) The genes identify in the expression profile analysis used in protein-protein—network should show----
Response: Please see explanation in the text. Pathway analysis was done with upregulated and downregulated genes separately. Figures S1-S5 correspond to genes that are upregulated, whereas Figures S6-S10 correspond to down-regulated genes. Therefore, color-coding of differentially expressed genes are unnecessary and color-coding makes figures bit difficult to download.