Title: Expression profiles of ANXA1 in human gastrointestinal cancers and downregulation of ANXA1 in gastric cancer

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Author's response to reviews: see over
Point by point Response

General Responses

We would like to thank Editors and Reviewers for their thorough review, and constructive and helpful comments and suggestions. We hope we have adequately and carefully addressed all the points raised to their satisfactions.

Responses to REFEREE #1

#1. **Major Compulsory Revisions 1.** “Figure 5C and 5D: I assume pcDNA3.1 vector transfection should not alter cell proliferation compared to un-transfected cells, however, if the two figures (5C and 5D) are superimposed together, one will find pcDNA3.1 transfected N87 cells are actually having higher proliferation rate compared to pcDNA3.1 transfected AGS cells. This contradicts the observations made in Figure 4D, where N87 cells having lower proliferation rate compared with AGS cells. Please explain.”

**Answer:** Thanks. I agreed that pcDNA3.1 vector transfection would not alter cell proliferation compared to un-transfected cells a lot. Although pcDNA3.1 transfected N87 cells seem to have higher proliferation rate than pcDNA3.1 transfected AGS cells when we superimposed the two figures (5C and 5D) together, this is not the fact since the starting densities of tumor cells of AGS and N87 cells were totally different. If we used graphical figures to compare the proliferation rates, the difference is remarkable (Figure R1).

![Figure S1](image)

Figure S1. The difference of proliferation rate between AGS and N87 cells at 96h compared to 24h.

#2. **Major Compulsory Revisions 2.** “Second paragraph, page 11: The authors claimed that “these data suggested tumor suppressor function of ANXA1 to inhibit proliferation partly through regulating the production of COX-2.”. This is not
convincing to me if the evidence only includes the negative correlation of ANXA1 and COX-2 in vitro and in vivo. Further experiments are needed for this claim, for example, by showing that overexpression both ANXA1 and COX-2 simultaneously abolish the effect of inhibition of cell proliferation by ANXA1 alone.”

**Answer:** Yes. I agree. We purchased a Plasmid containing full-length COX-2 and co-transfected ANXA1 and COX-2 plasmids into AGS cells and we found that COX-2 overexpression could inhibited ANXA1-induced cell proliferation (Figure C).

**Minor Essential Revisions:**

#1. “Methods, Statistical analysis section: It’s not very clear what the “in vitro”, “in vivo” or “within group correlations” data are. I suggest explicitly specify for those experiments listed in the figures which statistical tests are used. As a standard way to report statistical tests result, please specify what kind of test is used in the figure legend rather than simply report the p value.”

**Answer:** Yes. We specified the statistical tests in the figure legend.

#2. “Figure 1B, figure 3A, figure 4A: The authors said that the y axis is “fold of changes….. divided by log”, what does it mean by “divided by log”? Does it mean the fold of change in log scale?”

**Answer:** Yes. The y axis in Figure 1B, figure 3A, figure 4A means “the fold of change in log scale”.

#3. “Figure 1c and figure 4B: What are the two bands observed in the blots? Are the 42KD bands the beta-actin? And the 37 KD bands ANXA1? These needs to be stated in the figure legend and result description.”

**Answer:** Yes. The 37 KD bands are ANXA1, which had been stated in the figure legends and result section.

#4. “Figure 4C: What are subcellular locations of ANXA1 based immunofluorescence staining in the two cell types? Are there any difference between them? Please also show an image for GES-1 cell staining so we can compare the immunofluorescence difference with mRNA and protein expression result as shown in figure 4A and 4B.”

**Answer:** Immunofluorescence staining showed that ANXA1 staining mainly located on the membrane of the two cell types. Interestingly, it seems that ANXA1 staining...
could be observed in the nuclei of AGS cells, not in N87 cells. This is another story. We would like to provide GES-1 cell staining for ANXA1. However, we couldn’t provide this figure right now because of the quality of new purchased antibody.

#5. “Figure 4D: According to the description in the method section, there are three repetitions for this proliferation measurement, please show the standard deviations of different repetitions as error bars for each time points. Looks to me this could be modeled as a repeated measure experiment and please perform the statistical test (for example, repeated measure ANOVA) to check whether there is overall difference of the proliferation rate between two cell types. In addition, post hoc analysis needs to be done.”

**Answer:** Yes. We used two-way ANOVA to perform the statistical test. We asked a statistician who said that post hoc analysis is not necessary for this experiment.

#6. “Figure 4E: Are the invasion cell numbers between the two cell types significantly different? Please perform a statistical test and describe the test result.”

**Answer:** Unpaired t test was carried out to analyze the difference of the invasion cell numbers between the two cell types and the $P$ value was less than 0.001.

#7. “Figure 5A and paragraph 3 in page 10: “Enforced expression of ANXA1 in AGS cells induced markedly changes of morphology of AGS cells and ANXA1 translocated from the cytosol to the plasma membrane”: It is not very clear to me based on the figure what the morphological changes are? Is the bright green patch in ANXA1 transfection image a single cell or a cluster of cells? The membrane translocation is also difficult to distinguish. Please show an enlarged and distinguishable image. Does the same membrane translocation also occurred when the transfection is done in N87 cells? What is the biological implications or significance for this membrane translocation?”

**Answer:** I agree. We observed that a small number of AGS cells, not a cluster of cells, had the morphological changes after enforced expression of ANXA1. And this phenomenon was not observed in N87 cells. Since it has been reported that membrane translocation of ANXA1 could induce apoptosis, we assumed that the morphological changes of some AGS-ANXA1 cells must have certain biological implications or
significance. However, it is difficult to distinguish this kind of membrane translocation and explain its significance. Here, we changed the picture and revised our description. We will design another experiment to prove the membrane translocation and to explore its biological significance.

# 8. “Figure 5B: In theory, we should expect a much stronger fluorescence signal when we do overexpression of ANXA1. It is hard to see that comparing ANXA1 transfected cells with pcDNA3.1 transfected cells even though the western blot did show mild increase.”

Answer: Yes. Since the fundamental level of ANXA1 expression in N87 is very high, it is hard to compare ANXA1 transfected cells with pcDNA3.1 transfected cells only through fluorescence signal. Here, we changed Figure 5B (left).

#9. “Figure 5C and 5D: what is the statistical test is used to get the p value here as marked by the asterisk? Again this is a repeated measure experiment. Please report the details of statistical analysis result in the figure legends.”

Answer: Yes. We reported the details of statistical analysis result in the figure legends.

#10. “Figure 6A: the authors mentioned “silencing of ANXA1 by ANXA1-shRNA promoted cell viability in N87 cells”. Have authors examined whether silencing ANXA1 using shRNA affects clone formation and cell migration compared to scramble shRNA in N87 cells?”

Answer: Sorry. We didn’t perform the experiment about whether silencing ANXA1 using shRNA affects clone formation and cell migration compared to scramble shRNA in N87 cells.

#11. “Figure 6D: Are the first two columns are for ANXA1 and the last two columns for COX-2? Please also state it clearly in the figure legend.”

Answer: Yes. We demonstrated it clearly in the figure6D and in the figure legend.

Discretionary Revisions:

#1. “Figure 2, third panel, tumor image: Please label which part is “well-differentiated squamous cell carcinomas” and which part is “weak or not in poorly differentiated carcinomas” as described in first paragraph, page9. The same for cholangiocarcinoma and gastric carcinoma.”
**Answer:** Yes. Since the page is limited, we provided these pictures in supplemental Figure 1.

**#2.** “Figure 6B: have the authors examined whether Cyclin D1 expression is boosted in ANXA1 knocked-down N87 cells? The authors did not make any points by showing Cyclin D1 data here. If not relevant, there is no need to show.”

**Answer:** Yes. Cyclin D1 is not the main point of this study and we didn’t show it.

**Responses to REFEREE #2**

**# 1.** “The title is “Expression profiles of ANXA1 in human gastrointestinal cancers and downregulation of ANXA1 in gastric cancer”. As the authors not only discuss the downregulation of ANXA1 in gastric cancer but also in other gastrointestinal cancers. I would suggest them to change the title.”

**Answer:** I agree. We change the title: Differential expression of ANXA1 in benign human gastrointestinal tissues and cancers

**#2.** “Fig.1 c: There are 2 bands, which band is ANXA1 band? They also need to add the actin blots as loading controls. And from this figure, I cannot draw a same conclusion as them, “ANXA1 protein expression was down-regulated in CHO, ESC and GC.” I think the expression levels are totally different between sample1 and sample2.”

**Answer:** 37KD band is ANXA1 band. Figure 1d is the Graphical representation of the ratio of T/N from (C). ANXA1 expression was down-regulated in sample 1 of CHO, ESC and GC, but not changed, at least increased, in sample2.

**#3** “Fig. 2, Fig. 3 B-H: The quality of the images is not good. The authors should show the high quality high magnification images.”

**Answer:** Yes. We made new arrangement.

**#4.** “Fig.2 3rd panel. The authors conclude that “Immunohistochemistry for ANXA1 revealed high expression level of this protein in well-differentiated squamous cell carcinomas, but weak or not in poorly differentiated carcinomas (Fig.2 third panel).” Where is the evidence?”
**Answer:** We provided the evidences in supplemental Figure 1.

**#5.** “Fig.3H, the authors propose “immunohistochemistry for ANXA1 in liver metastases displayed a significant reduction of this protein in tumor cells compared with surrounding benign liver cells……. ANXA1 is a differentiation marker” However, this does not make sense as liver metastases and surrounding benign liver tissue are totally different. The tumor cells are from gastrointestinal tissues, and Fig. 1 already shows that ANXA1 has higher expression level in liver cells than that of gastrointestinal tissues.”

**Answer:** I agree. Although we can use ANXA1 expression level in liver cells as internal positive control, it shouldn’t compare the difference of ANXA1 levels in these two tissues. Here, we changed our description.

**# 6.** “Fig.4 B: There are 2 bands, which band is ANXA1 band? Also Actin blots are needed.”

**Answer:** The 37 KD bands are ANXA1. The 42kd bands are β-actin.

**# 7.** “Fig. 4 D, E: N87 and AGS are different cell lines, so this does not make sense to compare their proliferation and invasion.”

**Answer:** Although N87 and AGS are different cell lines, the levels of ANXA1 expression are extremely different. Our aim is to present the correlation between ANXA1 levels and cell proliferation and invasion.

**#8.** “Fig. 5A, B: The authors suggest “ANXA1 translocated from the cytosol to the plasma membrane” However, I think ANXA1 is still in the cytosol according to their images.”

**Answer:** Yes. It is hard to get the conclusion “ANXA1 translocated from the cytosol to the plasma membrane” from the existed evidences. So we made a change in the figure and description.

**#9.** “Why do the ANXA1 western blots here have only one band?”

**Answer:** Actually, ANXA1 western blots do have only one band. The other band in other figures is Actin. Since the two bands are too close, we presented in the figures together.

**#10.** “Fig. 5 C-H: The authors need to check the transfection efficiency first, and the
efficiency must be very high. Otherwise, they can’t use these cells to do such experiments.”

**Answer:** Thanks. Figure 5 A and B showed that ANXA1 was increased in gastric cancer cells transfected with pcDNA3.1-ANXA1 than those transfected with pcDNA3.1 vector, which proved the transfection efficiency.

#11. “Fig. 6: The authors conclude that “tumor suppressor function of ANXA1 to inhibit proliferation partly through regulating the production of COX-2.” I do have some problems with this conclusion. If the authors really want to show that COX2 is the downstream effector. They need to get COX2 knocking down and overexpression data, other than only correlation between ANXA1 and COX2.”

**Answer:** Yes. We purchased GV147-COX2 plasmid and cotransfected ANXA1 and COX-2 into AGS cells and explored the cell proliferation rate. The results had been presented in Figure 6.

**Minor points**

#1. “Page 13: The authors mention “selected two anti-ANXA1 antibodies after antibody specificity confirmation by Western blotting.” Where is the 2nd antibody’s data?”

**Answer:** The second antibody was from *LifeSpan Biosciences*. The pictures were presented in supplemental Figure 2.

#2. “Page 14: “ANXA1 expression is “tumor-specific”.” What does this sentence mean? It is very confusing.”

**Answer:** Yes. We changed the description in Discussion section.