Author's response to reviews

Title: siRNA Inhibition of Telomerase Enhances the Anti-Cancer Effect of Doxorubicin in Breast Cancer Cells

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Author's response to reviews: see over
Dear Editor:

We greatly appreciate the careful review of the previous submission. We have done additional experiments suggested by the reviewers and revised our manuscript accordingly. Our point-by-point responses to the reviewers are as follows:

**Response to Referee 1:**

For the Major Compulsory Revisions:

“The authors nicely demonstrated that their hTERT si-RNA knock-down strategy efficiently reduces hTERT at the mRNA and protein levels. However, they do not measure levels of telomerase activity and they do not assess telomere lengths. Instead they reference a previously published paper that states hTERT levels correlate with activity. While this is true, **it is important to determine whether their level of knock-down is sufficient to cause a marked reduction in telomerase activity. If not, it is unlikely that this is a telomere length-mediated cell death.**”

Response: This is an excellent suggestion. We have conducted a TRAP assay to ascertain the effect of the hTERT siRNA knockdown on telomerase activity level. The result is shown in Figure 1E and 1F. For both cell lines, we found that under our experimental conditions, the level of telomerase activity was reduced to about 30% of those of the blank control and the control siRNA-treated cells.

“The authors fail to cite and discuss an important publication: Cerone, et al. Molecular Cancer Therapy 2006; 5(7): 1669-1675 ("Telomerase inhibition enhances the response to anticancer drug treatment in human breast cancer cells"). In many ways this publication undermines the novelty of the paper under review. **Other papers that should at least be cited include Tauchi et al, Methods Mol Bio 2007, 405:181-189 and Ludwig et al, Cancer Res 61, 3053-3061, 2001. Both of these papers relate to telomerase inhibition and sensitization of breast cancer cells to topoisomerase inhibitors.**”

Response: We have added these publications to our references accordingly.

“Since multiple papers have been already been published on telomerase inhibition enhancing anti-cancer effects of doxorubicin in breast cancer cells, **Dong et al need to clearly articulate how their study advances the field.**”

Response: While we agree that our results are not entirely novel, our goal was to show that direct application of siRNA to knock down telomerase combined with doxorubicin is a valid approach in breast cancer cells as well.

For the Minor Essential Revisions:

“Several studies have reported that a similar dose of AdR causes senescence rather than apoptosis in MCF-7 cells. At a minimum, these studies should be briefly described and cited.”
Response: We appreciate the reviewer’s calling of our attention to this important facet of tumor biology; these studies have been included in the references and are succinctly discussed in conjunction to our results.

“There is an inconsistency in the number of cells inoculated into nude mice. Is it 2 million or 20 million? If it is 20 million, this is an unusually high number and needs to be justified.”

Response: We have revised our manuscript to make sure that the 2 million cells used for the inoculation is clearly stated.

“There is mention that MDA-MB453 cells are not tumorigenic in nude mice. I believe these cells are tumorigenic, but they are not invasive or metastatic.”

Response: We first got the information that MDA-MB-453 being not tumorigenic from the following source: http://icbp.lbl.gov/breastcancer/viewline.php?id=50. We nevertheless conducted the inoculation experiment with this cell line. The result is shown in Figure 2C and 2D. We found that this cell line is indeed tumorigenic. The tumors formed by the MDA-MB-453 cells seemed smaller than those formed by MCF-7, but did not reach statistical significance.

“Numerous places in the text the authors conclude that there are “statistically significant” differences, yet no statistical test was performed. Inclusion of a statistical test, such as a student’s t-test, is appropriate with the p-values and statistical differences being noted on the figures and/or in the legends.”

Response: We have included p-values from t-tests in figure legends (Fig.1, 3) where they are needed.

“The gel in Figure 1D needs to be replaced with a better quality image.”

Response: The image for Figure 1D has been replaced.

For the Discretionary Revisions:

“This study limited the AdR concentration to a single acute dose at 0.5µM. If a range of concentrations (i.e., 25-500nM) + hTERT inhibition was included one could determine whether knock-down by siRNA sensitizes breast cancer cells to AdR, which would be encouraging pre-clinical data since AdR is highly toxic to patients.”

Response: That is very good point. The actual plasma concentration of free doxorubicin that is reached upon intravenous bolus administrations into average sized persons can get up to 50µM [26]. We had done preliminary dose-dependent study (data not shown). 0.5µM of AdR was found to be the minimal dose and still sensitive effective.
“This study would be greatly strengthened by inclusion of a cytogenetic analysis (i.e., GTG-banding showing a high frequency of end-to-end fusion, rings and radials, which would indicate telomere dysfunction).”

Response: We agree that the showing the chromosomal abnormalities caused by the hTERT knock down will strengthen our claim in the efficacy of the method. We hope to include these analyses in our future studies. However, at this point, we have little experience in karyotyping, and we feel that the priority of our study at the present is to show that the siRNA treatment can cause cells to undergo apoptosis.

“To complement the apoptosis data, inclusion of senescence (i.e., SA-β-galactosidase activity) as an end-point would strengthen the manuscript.”

Response: This is an excellent suggestion. We did the β-gal staining as an end-point as you have suggested. The result is shown in Figure 3B. We found a large portion of MCF-7 cells in senescence, which is expected based on previously published data [28]. We, however, did not expect the relatively low level of senescence seen with the MDA-MB-453 cells. The differential senescence of these two cell lines from the AdR treatment will certainly give insights to how cell fate is determined, and we are developing research plans to investigate further.

“Rather than include gross images of the xenografts (Figure 3A), including a tumor section revealing apoptotic cell death would integrate nicely with the in vitro data. This could include a TUNEL assay as simply visualization of apoptotic bodies on an H&E stained tumor section.”

Response: This is a great suggestion. We have been encountering technical difficulties and are still struggling to obtain a staining that is of good enough quality. Moreover, the injection study was to demonstrate that telomerase inhibition can interfere with cell growth in an in vivo setting.

“In Figure 1, it would be helpful to directly label the lanes rather than include 1, 2 and 3.”

Response: We changed the labeling in Figure 1 accordingly.
Response to Referee 2:

1. Major compulsory

“The author must do some Immunofluoresence or Immunohistochemistry experiments to confirm that hTERT has been knocked down by siRNA in breast cancer cell lines and xenograft tumor samples”.

Response: This is an excellent suggestion. Figure 1 showed that the hTERT-siRNA has knocked down hTERT expression at both mRNA and protein levels, and we have also assayed and confirmed that the telomerase activity are reduced by the siRNA as well in both cell lines (Fig.3). Immunohistochemical staining of hTERT has shown that siRNA blocked about 55% of hTERT expression in xenograft tumor samples, indicating that hTERT siRNA inhibits tumor cell viability in vitro and in vivo.

2. Minor Essential Revisions

“The Fig 1a looks like Fig 1b. The cell line name should be labeled on them, respectively. The X axle notes in Fig 2a and Fig 2b should be ”days”, rather than ”day”.

Response: Thank you for pointing that. We labeled the cell line names above each figure now for better clarity, and have also changed the x-axis in Figures 2A, 2B and 2C to day(s).