Manuscript 19822: Reply to reviewers' comments

Reviewer 1:

Data from this manuscript added evidence that downregulation of YES1 is a mechanism by which miRNA 210 inhibits hepatocellular carcinoma (HHC) cell proliferation.

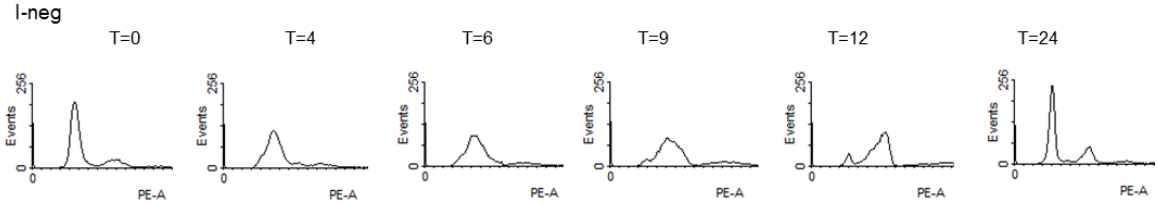
1. Change "affects" to "inhibits" in the title.
2. Combine the methods for quantification of miRNA 210 and Yes1 mRNA, subtitle as 'Quantitative RT-PCR'. Present the four pairs of PCR primers, including the miRNA 210, 5S rRNA, Yes1 and GAPDH primers, in a Table.
3. If the expression levels of miRNA 210 in primary hepatocytes, HepG2 and HuH7 are significantly different, mark them in Fig. 1B.
4. In Fig. 2C, it is important to add an experiment for studying the effect of miRNA 210 inhibitor (210-I) on cell cycle, since miRNA 210 is already up-regulated in HHC cells.
5. The abstract says: "overexpression of miRNA 210 led to significant inhibition of cell proliferation (68.9 and 53.6%)". This is misleading. The inhibited proliferation is 31.1 and 47.4%.
6. In Figs. 2 and 5, add a sentence to describe the cell proliferation assay: cell proliferation was determined using a MTS cell proliferation assay kit.
7. Correct typos in the text.

Response to reviewer 1:

1. The title had been changed and the word "affects" has been replaced with "inhibits".
2. As suggested by the reviewer, the methods for the quantification of miR-210, 5S rRNA, Yes1 mRNA and GAPDH mRNA have been combined as one section subtitled "***Quantitative RT-PCR***" in the revised manuscript. The sequences of the primers used for 5S rRNA, Yes1 mRNA and GAPDH mRNA are presented in Table 1 (new table).

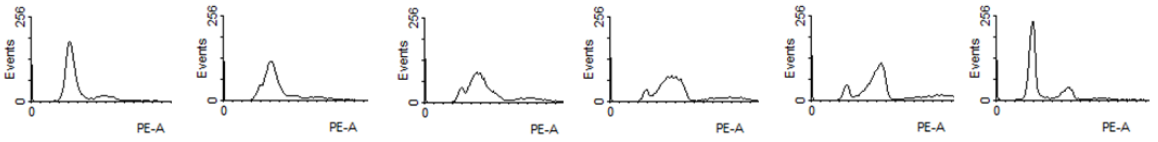
3. For Fig. 1B, the * mark has been added to indicate that there is significantly different expression of miR-210 in primary hepatocytes when compared to HepG2 or HuH7 cells.
4. With reference to Fig 2C, flow cytometry analysis of HuH7 cells at various time points (T = 0 h – T = 24 h) following transfection with either I-neg or 210-I and synchronization of the cells was done (see figure below for data). It was observed that cells transfected with 210-I took a shorter time to enter the S-phase at T=4h (48.4% in S-phase) compared to the cells transfected with I-neg (22.1% in S-phase). However, at subsequent time points (T=6h up to T=24h), there were no significant differences in the percentages of cells at G1, S or G2/M phases between the two treatments. As the transfection of the 210-I was transient, the effect of 210-I on cell cycle progression might be diluted as the cells would have multiplied in between the two thymidine blocks.

I-neg



	T=0	T=4	T=6	T=9	T=12	T=24
G1	74.3	64.2	29.0	14.3	13.3	65.3
S	8.0	22.1	56.4	57.4	29.6	8.8
G2/M	17.7	13.7	14.6	28.4	57.1	25.9

210-I



	T=0	T=4	T=6	T=9	T=12	T=24
G1	71.8	***37.6***	24.4	16.8	17.6	71.4
S	12.8	***48.4***	58.8	51.5	37.2	9.0
G2/M	15.4	***14.1***	16.7	31.6	45.2	19.7

5. Descriptions of the effects miR-210 mimic, miR-210 inhibitor and siRNA for Yes1 on cell proliferation have been corrected and are now described as "significantly reduced compared to mock-treated cells" in the abstract and results sections, with the mock-treated cells set as 100%.

6. The sentence "Cell proliferation was determined using the MTS assay." has been added to the figure legends for Figs. 2 and 5.
7. Typos in the text have been corrected.

Reviewer 2:

This paper titled," Up-regulation of microRNA-210 affects proliferation of hepatocellular carcinoma cells by targeting YES1" is focusing to determine the expression of microRNA-210 (miR-210) in hepatocellular carcinoma and to examine its role using hepatocellular carcinoma cells. Authors said that miR-210 is significantly up-regulated in hepatocellular carcinoma and over-expression of miR-210 decreased cell proliferation and delayed cell cycle progression of hepatocellular carcinoma cells via down-regulation of Yes1. This paper has been well described to suggest that Yes1 is a target of miR-210 and affects cell proliferation in hepatocellular carcinoma. My minor comments are as follows; - It will be better to show cell proliferation pictures of Fig 2A and B to strongly support the data if possible. - I recommend you to describe what happen to HepG2 cells proliferation when treated with 210-I in Figure 2A.

Response to reviewer 2:

Fig. 2A and B: We did not show any cell proliferation pictures. In addition to the MTS cell proliferation assay, similar experiments where HuH7 or HepG2 cells were treated followed by counting of viable cells after trypan blue staining were also carried out (see table below for data). The number of HuH7 or HepG2 cells following treatment with 210-M was significantly different ($P<0.05$) from the control (M-Neg).

	HuH7 ($x10^4$ cells)	% live cells	HepG2 ($x10^4$ cells)	% live cells
M-Neg	3.750 + 0.357	98.7	6.188 $\pm$ 0.344	98.0
210-M	2.613 + 0.291	95.9	4.988 $\pm$ 0.650	98.5

However, there was no change to the proliferation of HepG2 cells when treated with 210-I. Similar results were also obtained with the cell count experiment. The difference in the effect of 210-I between the two cell lines may be due to the fact that

HepG2 cells are fast growing cells and may be at the maximal rate of proliferation and hence cannot proliferate any faster in the presence of 210-I.