Author's response to reviews

Title: Combination of hepatocyte specific delivery and transformation dependent expression of shRNA inducing transcriptional gene silencing of c-Myc promoter in hepatocellular carcinoma cells

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Author's response to reviews: see over
To  
The Editor-in-Chief,  
BMC Cancer.

Subject: Submission of revised manuscript titled “Combination of hepatocyte specific delivery and transformation dependent expression of shRNA inducing transcriptional gene silencing of c-Myc promoter in hepatocellular carcinoma cells” for consideration for publication in BMC Cancer.

Dear Sir,

We are submitting the revised manuscript titled “Combination of hepatocyte specific delivery and transformation dependent expression of shRNA inducing transcriptional gene silencing of c-Myc promoter in hepatocellular carcinoma cells” by Zakaria et al. for your kind consideration for publication in BMC Cancer. Kindly find the response to the individual reviewer’s comments described below.

We request you to kindly consider the manuscript for publication in BMC Cancer.

With regards,

Yours sincerely,

Professor Subrata Sinha,  
Director, National Brain Research Centre (NBRC),  
Manesar, Haryana, 122050, India
Response to the Reviewers:

General Reply:
We are grateful to the reviewers for their valuable comments. We have tried to address the issues mentioned by them in this revision and have conducted most of the required experiments under stringent quality control.

Reviewer 1

Major Revisions:
1) Figure 1. HCC specific expression of AFP promoter/enhancer system and Figure 2. AFP promoter/enhancer +2 driven shRNA against c-Myc P2 promoter. Author used the CHO cells for non-liver cells it would be more appropriate to use only human cells rather than Chinese hamster ovary cells (CHO cells).

Reply: Our main aim was to check the delivery of the generated shRNA system through the Sendai F-virosomes. Sendai F-virosole binds to the hepatocytes through the asialoglycoprotein receptors (ASGPR), which are present exclusively on the surface of liver cells. CHO cells lack surface ASGPR, hence served as negative control. This was also tested by the fluorescence de-quenching assay (Figure 6A in revised manuscript), where Sendai F-virosomes did not fuse with the CHO cells. Additionally, the shRNA system is active in the CHO cell line. When shRNA under the control of CMV promoter was transfected in the CHO cells via non-virosomal system, it was able to suppress c-Myc expression (Figure 2G and H). This demonstrated that c-Myc shRNA mediated suppression works in both mouse and human cells. Hence, this humbly suggested that CHO cells may be considered as a valid negative control for Sendai virosome mediated internalization.

Minor Revisions:
1) In Figure 2E, Huh7 cells of NFκBEn-Pr+2- myc expression ratio did not correlate with figure 2H of Huh7 cells western blot. The lanes of scrambled, test and untreated all were showing the same band intensity.

Reply: As stated by the reviewer that there is no correlation in the expression of c-myc by Real-Time PCR and Western Blotting through NFκBEn-Pr+2 construct (Figure 2E and 2H), this could have been due to overexposure during development of the blot. We therefore repeated the western blotting under stringent experimental conditions. We have revised the figure with the new blot and it shows visible significant difference in the scrambled and test lanes, and there is a correlation with real time PCR.
Major Revisions:

1) The authors should determine the level of expression of the shRNA in cells upon transfection with their delivery systems and monitor it over time. Is the expression of the shRNA constant or changes over time? They claim that there is a prolonged effect on myc transcription due to chromatin and DNA modifications. Could the prolonged effect be due to (and require) continuous expression of the shRNA? If so, the interpretation of their results and the relevance of the epigenetic changes may need to be re-examined.

Reply: We thank the reviewer for the suggestion. In a new experiment, we transfected various c-Myc shRNA in HepG2 cells, as mentioned in the methodology (Page 21 of revised manuscript), and monitored the shRNA expression by anti c-Myc dsRNA specific PCR (Additional file 1: Figure S7). We were able to follow up the cells for up to 6 days post transfection. After 6 days, the extensive cell death was observed in the test shRNA treated cells but not in the scrambled control. Furthermore, cells treated with the test shRNA were almost completely detached on the 7th day, making it impossible to do shRNA quantitation. The level of shRNA as seen by Real Time PCR was high for 3 days post transfection (Maximum in 48 hours) and started decreasing thereafter. The shRNA expression by AFPEn–Pr+2 – myc was at about 18% of the maximum on day 6. However, all the molecular features of TGS were maintained on day 6 (Figure 8 in revised manuscript). This supports the possibility that TGS continues even after the reduction of shRNA, even though because of cell death we were unable to reach zero expression. This is also described in the results section (Page 12-13 in revised manuscript). The discussion has been modified to incorporate the referee’s comments (Page 17 of revised manuscript).

However, in the published literature (Hawkins et al., 2009) as well as in another study from our laboratory on the TGS of HIV long terminal repeat (LTR; Singh et al., 2014), TGS has been shown for over a period of 20-30 days.

2) What would be the effect of a shRNA targeting the myc mRNA post-transcriptionally in the same conditions of cell specific promoter and delivery? Would be any different than their promoter targeting construct in term of efficiency, selectivity and persistence of the effects?

Reply: In published literature, possible advantages of TGS over PTGS has been mentioned (Morris, 2009; Morris et al., 2004). It is indicated that while PTGS would require sustained presence of effector siRNA molecule, TGS would not. Additionally, since TGS induces epigenetic changes, it could be heritable and long lasting. Hence, it is expected that PTGS would behave in a similar manner in this cell specific promoter/delivery system. However, we have not demonstrated the same experimentally. This has been included in the discussion (Page 16 of revised manuscript). The primary purpose of this paper is to demonstrate the combination of cell
specific delivery with tumor specific expression of the shRNA. Also, the previous literature on PTGS/TGS in other systems and discussion of such literature may be accepted as a response to this comment.

3) The authors define as “long term” the observation made after 5-6 days in culture following transfection. Was any longer interval of time tested? Did the effects on chromatin marks, DNA methylation and cell proliferation persist behind the 5-6 days time point?

Reply: As per the esteemed reviewer’s suggestion, we tried to look for the effects of TGS on the 6th day and beyond. In the earlier experiments, we were not able to perform analysis beyond 5-6 days as almost all the cells (HepG2) detached and died on the 7th day post AFPEn–Pr+2 – myc treatment. Similarly, in a new experiment involving Trypan blue based cell counting (Figure 4 in the revised manuscript), post transfection of shRNA constructs, of HepG2 cells showed similar results which corroborates with the MTT assay and cells were almost killed and detached even upon changing the media from time to time. This indicates that epigenetic modifications might be persistent; ultimately leading to the death of majority of cells (HepG2).

4) It would be important to support their data on cell proliferation, survival and apoptosis with additional assays. For instance, cell proliferation could be monitored by cell counting at different time points (and behind the 5-6 days) after transfection. Flow cytometry data should be analyzed to determine also the state of cell cycle in control and shRNA transfected cells. Myc downregulation may induce cell cycle arrest in addition to apoptotic cell death. It will be important to know what happens and compare also the delivery systems.

Reply: Besides MTT assay and Flow Cytometry, as per reviewer’s suggestion, cell survival was also determined by trypan blue cell counting method in HepG2, Huh7 and Chang Liver cells post transfection of all shRNA constructs along with their appropriate controls (Figure 4 in revised manuscript). The cell viability, estimated by trypan blue method, corroborated with the previous MTT and Flow cytometry results. Also, we analyzed the flow cytometry data, post virosomal delivery of c-myc shRNA, by WinMDI software (http://winmdi.software.informer.com/2.8) and various cell cycle phases were determined. c-Myc suppressed cells (HepG2 and Huh7), in addition to apoptosis (subG1 proportion), were found to be within the G0-G1 phase with decreased S and G2M phase (Figure 5 in revised manuscript and Additional file 1: Figure S4). This has been mentioned in the results and discussion (Page 9 and 15 of revised manuscript respectively).
5) Please clarify the time of the experiments shown in the figures and state it in the figure legends (e.g., figure 4A-B, 6D, 7A, etc.). This information is missing in many cases. When was OAS1 measured (Fig. 6A)? Was it done also at earlier times (24-48 h post transfection) or only after 5-6 days?

Reply: Time period of various experiments (especially Figure 4A-4B, 6D and 7A) have been mentioned in the legends. OAS1 (Figure 7A in revised manuscript) was measured post 5 days of shRNA treatment through F-virosomes. Additionally, OAS1 levels were also evaluated after 24, 48, 72, 96 hours post virosomal treatment in HepG2 cells (Additional file 1: Figure S5) and we observed no significant increase in its expression on each day. This further ruled out the interferon response being generated even at the earlier time points, following F-virosomal delivery of c-Myc shRNA constructs.

6) The experiments in Figure 7 are not very informative. What was the effect of TSA on histone acetylation and the other chromatin marks shown in Figure 7A? Moreover, what was the effect of the single and combined treatments with TSA or AZA shown in Figure 7 on myc transcription?

Reply: In order to make figure 7 more informative, legends have been revised and Figure 7 is now Figure 8 in the revised manuscript. Earlier, we had checked the effect of c-Myc shRNA on heterochromatization markers (H3K9me2 and H3K27Me3) and it was observed that c-Myc suppression led to increase in the methylation of these markers. TSA treatment abrogated the enrichment around the target locus, in the presence of test shRNA, indicating the likely involvement of histone deacetylases in gene silencing. Also, as per reviewer’s suggestion, we evaluated the effect of shRNA on histone acetylation in HepG2 cells treated/untreated with TSA (Figure 8B in revised manuscript). Significant decrease in the acetylation status of the shRNA target region was observed by Chip assay and quantitative PCR. This effect was nearly abolished in the presence of TSA, indicating the failure of shRNA to induce deactylation through HDACs. Moreover, in a new experiment, the effect of single and combined treatments with TSA or AZA was also evaluated on the 6th day post shRNA transfection in HepG2 cells (Additional file 1: Figure S6). Significant decrease in the c-Myc levels were observed in AZA + AFPEn – Pr+2 – myc and TSA + AFPEn – Pr+2 – myc treated HepG2 cells (p < 0.05 for both) justifying that both heterochromatization and DNA methylation are responsible for c-Myc suppression. Combined treatment of both AZA+TSA along with AFPEn – Pr+2 – myc showed no significant decrease in c-Myc levels (p > 0.05). This confirmed that shRNA induces recruitment of both HDACs and DNMTs which ultimately results in c-Myc down-regulation. These have been incorporated in the results section (Page 12 of revised manuscript). The same has also been mentioned in the discussion (Page 17 of revised manuscript).
Minor Revisions:

1) There is no clear explanation of the molecular mechanism by which the shRNA construct inhibits myc transcription or attempt to reconcile their results with previous published work on this subject. What is the shRNA targeting? DNA elements, promoter transcripts? How are the chromatin and DNA changes induced?

Reply: The shRNA is designed to be complementary to the ME1a1 binding site (required for the activity of P2 promoter) between the P1 and P2 promoter region of c-Myc. By the current RNA: RNA model of dsRNA induced TGS (RITS: RNA induced initiation of transcriptional silencing), it is expected that the shRNA probably acts via promoter associated transcripts. This eventually induces histone and DNA methylation since we observed changes in these epigenetic marks by ChIP and Bisulfite PCR-DNA sequencing respectively. This was accompanied by decrease in the acetylation of the target region following shRNA delivery through F-virosomes (Figure 8 of the revised manuscript). Previously published reports on TGS, indicating the mechanism by which it occurs in various cell types, has been mentioned in the revised manuscript discussion (Page 16, line 359).

2) The authors should discuss previous work in which similar approaches targeting the same gene (myc) were used. This would be useful to put their present data in the proper context. Sites in the myc promoter identical or adjacent to the site that they target with shRNA have been targeted previously using oligonucleotides, small interfering RNAs, small molecule compounds. It would be important to discuss the relevant literature and compare the results in terms of efficiency and molecular mechanism of the different approaches.

Reply: The previous reports, targeting c-Myc, have been included and briefly described in the manuscript discussion section with their references. Majority of the relevant literature on the same has also been added in the discussion (Page 16 line 362) and introduction (Page 5, line 106) of the revised manuscript.
**Reviewer 3**

**Major Revisions:**
1) The previous study by the authors stated that TGS of c-myc by test siRNA is dependent on DNMT activity, but not on HDAC activity (on page 2306, ref #34). However, in the current manuscript, it is stated that both HDACs and DNMTs are involved (page 10). Please reconcile.

**Reply:** In our earlier report, we were successfully able to induce TGS in glioma cell line U87. This was shown to be by DNA methylation (Mehndiratta et al., 2011). The current study involves hepatocellular carcinoma cells. It is possible that the variation in HDAC involvement is related to the cell type. As the primary message in the paper is related to the internalization of cargo via the asialoglycoprotein receptors (ASGPR), we have not explored the subtle differences in the mechanism of c-Myc TGS. This has also been mentioned in the discussion (Page 17 of revised manuscript).

2) References are sometimes over cited and other times under cited. For example (A) Ref#11 and 12 are from the same group and about the same topic. My suggestion is to delete the older reference (ref#11). (B). in discussing about the specificity of gene transfer, especially at the level of promoter, it is appropriate to cite one review article very relevant to the topic in Introduction: Guo ZS et al., Gene transfer: the challenge of regulated gene expression. Trends Mol Med. 2008; 14:410-8.

**Reply:** The error of reference over and under citation has been resolved in the revised manuscript. A) Reference number 11 has been omitted while number 12 has been retained. B) As the reviewer indicated the importance of a review article very relevant to the topic, we have incorporated Guo ZS et al., Gene transfer: the challenge of regulated gene expression Trends Mol Med. 2008; 14:410-8 within the revised manuscript’s introduction (page 5 of revised manuscript).

**Minor Revisions:**
1) There are quite a few typos throughout the manuscript, especially in the reference. For example, (A). Line 642: “23” was repeated twice; (B). line 723: “J Int Cancer” should be deleted.

**Reply:** Typographic errors throughout the manuscript have been corrected including references. A) Line 642: “23” was repeated twice and has been rectified. B) Line 723 is deleted in the revised manuscript.