Author's response to reviews

Title: Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer.

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Author's response to reviews: see over
Dear Dr. Diana,

Please find attached the revised manuscript entitled “Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer” (4014112012312690). We thank the reviewers for their insightful comments on our manuscript. We have addressed each of their comments as outlined below and modified the manuscript appropriately.

Reviewer 1

General Comments.

“...several studies have already investigated the effects of miR-143 and miR-145 reconstitution in in vitro models.” Although we agree with the reviewer that there have been in vitro studies analyzing the effects of miR-143 or miR-145 re-expression on cancer cell phenotypes, we believe that our data showing that re-introduction of miR-145 into an in vitro model for colorectal cancer metastasis promotes oncogenic-like phenotypes represents a novel finding. Whether this observation is cell line-specific or a more global effect will require further experimentation.

“As the authors state themselves the finding that miRNAs can be “reliably” measured in FFPE tissues has great perspectives. It would significantly strengthen the manuscript if FFPE tissues matched to all 49 fresh frozen samples were investigated ….. If the authors further collected clinical follow-up information for the tumor samples and could show that FFPE tissues could be used to identify prognostic miRNAs then this would greatly improve the news value of the manuscript.” We agree with the reviewer that the inclusion of data from a more extensive collection of matched FFPE and fresh frozen tissues, and associated clinical data, would further enrich the findings of the present study. However, we do not have access to additional FFPE samples or clinical outcome data for the tissues used in the current manuscript.

Major Compulsory Revisions.

Comment 1a: regarding “miR-145 has an oncogenic effect in metastatic CRC cells…… From figure 1 is its clear that nearly all cancer samples have very low levels of miR-145. The authors have provided no evidence that up-regulation of miR-145 occurs in late
stage tumors.” In response to the reviewer’s remarks, we have removed the comment regarding the higher expression level of miR-145 in late-stage tumor samples. A re-examination of this data did not substantiate this conclusion. However, it should be noted that the heat map in figure 1 represents the hypergeometric mean of signal intensity. The actual intensity values for miR-145 range from 10 to 15 log2 units (see Table 2). This is in contrast to other miRNAs such as miR-31 which was in the range of 6 to 12 log2 units. Therefore, compared to other miRs, miR-145 is not at background levels in all cancer samples.

Comment 1b: “The observation that miR-145 stimulates growth might very well be an artifact of the SW620 cell line. In particular when one considers that miR145 has been reported to repress growth in at least four other CRC cell lines SW480, DLD1, LS174T and HCT116.” We acknowledge that we have only examined miR-145 re-expression in a single colorectal cancer cell line. However, in contrast to the previous studies, which used non-metastatic cells, we performed our experiments in the SW620 colorectal cell line, an accepted in vitro model for studying metastasis. Although the effect observed with miR-145 could be an artifact, our observation that miR-143 re-expression produces the same effect on cell proliferation as observed in previous studies suggests that some aspects of miR-143 and miR-145 regulation are shared between these different in vitro cell models.

Comment 1c: “The authors should tone down the importance of the SW620/miR145 findings or provide data from clinical samples supporting the oncogenic function. It would also add significant strength to the manuscript if they could demonstrate the oncogenic function in at least one other cell line.” We agree with the reviewer that we have not provided evidence for an oncogenic function for miR-145 in clinical samples. However, our in vitro data using over-expression of miR-145 in the metastatic cell line model SW620 did produce cellular phenotypes consistent with a potential oncogenic role of this miRNA. This observation has major relevance for those that may be contemplating the use of miR-145 augmentation as a therapeutic strategy for colorectal cancer. Our data cautions those interested in this application to consider the possibility that specific miRNAs may have opposing functions at different stages of tumor development. We have added a sentence covering this latter comment in the discussion.

Comment 2a: “regarding: Figure 2. Correlation of miRNA expression comparing the mirVana Bioarray and ABI Taqman platforms ….. In Figure 2, the sample IDs are different from what is listed in Table 1.” Table 1 has been modified to include both the sample and the Ambion chip identifiers. The sample identifiers used in figures 1-3 are now consistent with those in Table 1.

Comment 2b: “The IDs of the 19 miRNAs should be provided as well as the ABI product numbers.” In response to the reviewer’s comment we have expanded the data obtained using 169 ABI Taqman miRNA assays that were available at the time of analysis (Figs 2 and 3). We have included the relevant identification numbers of these assays as an Additional File.
Comment 2c: “Why is the comparison only shown for 19 miRNAs? This seems strange considering that in figure 3 the authors have investigated 26 miRNAs in the same four samples using the type of ABI Taqman assays. The authors should show the data for all 26 miRNAs in Figure 2.” See reply to comment 2b.

Comment 2d: “Comparing expression levels of different transcripts by comparing the Ct values obtained with different real time assays (or array probes) is not so straightforward as is indicated. To convince the reader that the qRT-PCR and array platforms produce similar results the authors should show that the two platforms find the same miRNAs differentially expressed when the same samples are investigated. They could select 5-10 miRNAs that the array platform indicated were differentially expressed e.g. miR-93, miR-183, and miR30a-3p which according to Table 2 were differentially expressed between normal and cancer and miR-31, miR-7, miR-125a which according to Table 4 were differentially expressed between early and late stage tumors.” Although we are not able to perform the precise experiment suggested by the reviewer, we have expanded the number of miRNA-specific QPCR assays and compared this larger dataset with the array results. From this comparison, we still observe a reasonable correlation between the two platforms (median correlation of 0.81). Also see reply to comment 2b.

Minor Essential Revisions.

Comment 1a: regarding profiling miRNA in clinical CRC samples ….. On Page 6 the authors write “Hierarchical clustering showed that many of these miRNAs were coordinately expressed, including the miR-143-145 and miR-17-92 clusters, which were consistently down- or up-regulated in CRC, respectively (Fig. 1).” How were the miRNAs in the hierarchical cluster analysis selected? With other words is it a supervised or unsupervised cluster analysis? That information is critical for the reader to able to interpret the figure.” A supervised hierarchical clustering analysis was performed on the 37 miRNAs indicated in Table 2. The text in the Results section has been modified to clarify this point.

Comment 1b: regarding profiling miRNA in CRC cell lines ….. On page 7 the authors write ….. To validate the cell line microarray data, we performed Northern blot analyses on 22 of these miRNAs in these and an additional 4 cell lines (Fig. 4). MiRs 96, 221, 182, 27a, 103, let7a, and 155 from figure 4, are not in Table 5? According to table 5 only 14 miRNAs were confirmed by Northern, while in the text it says 22? The miRNAs selected for confirmation of expression levels by Northern analysis represented candidates showing increased, decreased and unchanged expression profiles compared with normal colonic epithelium. To make this point more clearly, we removed the Northern blots for those miRNAs that did not appear in Table 5. In addition, the text in the Results section was adjusted to indicate that 14 of the miRNAs were examined using Northern analyses.

Comment 2a: “Regarding the in vitro and in silico analysis of miR-143 and 145. The authors should motivate why they, out of the 37 miRNAs in Table 2, chose miR143 and 145 for in vitro studies?” The potential candidates for further functional analyses were
selected from the 11 miRNAs shown to be commonly de-regulated in CRC samples and CRC cell lines. MiR-143 and miR-145 consistently displayed low expression levels using different expression analysis platforms and provided a low background expression profile for our over-expression strategy. Furthermore, previous studies had also reported loss of expression of these miRNAs in different clinical samples. Given the above, we aimed to understand the biological significance of re-expressing these specific miRNAs in the metastatic cell line model SW620.

Comment 2b: “Regarding the in vitro and in silico analysis of miR-143 and 145. On Page 6 the authors write “However, it was noted that late stage CRC samples (Stage IIIB and IV) exhibited twice the level of miR-145 expression compared to miR-143 (slope of 1.92).” Please see the reply to comment 1a under the major compulsory revisions.

Comment 2c: On page 8 the authors write “The level of miR-145 approximated those observed in normal colonic epithelial tissue (data not shown).” The authors should describe how they did this comparison and show the data.” We performed a Northern analysis to compare the expression level of miR-145 in normal colonic epithelium and the SW620 cell line containing the re-delivered miR-145 expression vector. Small RNA-enriched samples from both biological samples were isolated and probed for miR-145 and U6 snRNA. For each sample, the expression signal for miR-145 was normalized to U6 snRNA. A ratio of 0.9 was calculated comparing the SW620/miR-145 pooled population to the normal colonic epithelium, indicating similar levels of miR-145 expression. These data can be visualized from the miR-145 Northern data in Figs 4 and 6a.

Comment 2d: The authors should motivate why they grow the cells with and without serum? In the present study we attempted to examine the impact of miR-143 or miR-145 re-expression in different cellular contexts. To this end, we tested the re-expressing cell lines in the presence or absence of serum to determine whether these different conditions would augment or repress any observed phenotypes. It has been reported that the expression or activity of some miRNAs can be affected by environmental cues. In addition, the use of the serum-free medium mimicked a subset of the conditions observed during tumor development.

Comment 2e: “Why is there so large a difference in the results obtained with the AS oligo on the vector control in figure 6C and 7B? What is the explanation for the growth suppression observed for the AS oligo on the vector control when cultured serum free? The authors should comment on this.” The observed growth suppression in vector alone cells treated with the miR-145-specific antisense oligonucleotide could be due to off-target effects or specific inactivation of residual miR-145 remaining in the vector-alone cells.

Comment 2f: On page 8 the authors write “As seen in the previous experiment (Fig. 7C), over-expression of miR-145 in the presence of sense control RNA resulted in increased cell proliferation in serum, and more markedly in serum-free medium (Fig. 7B). This sentence is confusing and should be reformulated. Moreover, should not the reference to
*Figure 7C have been to Figure 6C?* The requested changes have been introduced into the text.

**Comment 3:** In the discussion (page 10) the authors write “Interestingly, the muscle specific miRNAs, miR-1 and miR-133a, were highly expressed in normal colon mucosa but were significantly down-regulated in CRC, indicating these miRNAs are not confined to muscle [32].” Apparently the authors believe that the reduced levels of miR-1 and 133a observed in the tumor samples indicate that the expression of the two miRNAs is not confined to muscle cells. An alternative explanation could be that there are differences in the tissue composition (muscle cells) of the investigated cancer and normal mucosa specimens. The authors should comment on this. Alternatively, they could use in situ hybridization to demonstrate that miR-1 and 133a are predominantly expressed by epithelial and not muscle cells in normal mucosa samples.” In response to the reviewer’s comment on the potential expression of miR-1 and miR-133a from muscle cells contained within the clinical specimens, we have removed our discussion on the apparent altered expression of these specific miRNAs in normal colon mucosa and colorectal cancer samples.

**Comment 4a:** “Regarding the description of the clinical samples. On page 14 the authors write “In total, 49 fresh-frozen human tissue samples were obtained from Genomics Collaborative Inc. (Cambridge MA) or Clinomics Bioscience, Inc (Pittsfield, MA), including 4 normal colon, 4 Stage I, 19 Stage II, 20 Stage III and 2 Stage IV samples (Table 1). In addition, we obtained 8 matched formalin fixed paraffin embedded (FFPE) samples (3 Stage II, 4 Stage III and 1 Stage IV).” Table 1 contains 64 clinical samples and not 49. Even adding the 8 FFPE samples does not give 64. What is the explanation? The sample IDs in the table are not identical to those used in the figures, why is that?” This has been updated and clarified in the text. A total of 64 samples from 49 patients are included (15 replicates). Four FFPE samples were assayed.

**Comment 4b:** “It is well known that two major molecular subtypes of CRC exists the one being mismatch repair deficient (MSI) and the other proficient (MSS). Is the MSI/MSS status known for the investigated samples? Several papers have shown that miRNAs are differentially expressed between the MSS/MSI subtypes potentially this could confound the findings in the present study.” We agree with the reviewer that the MSI/MSS status of the clinical samples may be a confounding factor. However, we do not have access to the molecular subtype information related to these samples to address this question.

**Comment 4c:** “Thorough information about the four normal colon specimens is needed. As these specimens are the reference material for the entire study, at least this must include information on how the specimens were collected, under which circumstances (surgery, bowel endoscopy, rectoscopy), previous and/or concurrent diseases, are the specimens from non-cancer areas of cancer patients, etc.” These are matched adjacent normal colorectal tumors from CRC patients included in the study. This information has been added to the text.
Comment 5: “regarding Figure 1. Two-way hierarchical clustering of CRC and normal colorectal tissue using 37 differentially expressed miRNAs. The sample IDs in figure 1 are not consistent with Table 1 (listing the clinical characteristics of the clinical samples). This makes it nearly impossible for the reader to evaluate and interpret the figure.” Please see the reply to comment 2a in the major compulsory revisions.

Comment 6: “regarding Figure 3. miRNA expression in fresh frozen versus formalin-fixed paraffin embedded CRC samples ….. The sample IDs in figure 3 are different from those listed in Table 1. This should be changed. According to the description of the clinical samples (on page 14) eight FFPE samples were selected for analysis. However, according to legend to figure 3 only 6 FFPE samples were investigated - why is that? Why were not all eight samples investigated by both the mirVana Bioarray assay and the Taqman miRNA expression assays? ) The IDs of the 26 miRNAs should be provided as well as the ABI product numbers.” Table 1 has been updated to show the sample and Chip IDs. Only 4 FFPE samples were profiled and this has been clarified in the text. The IDs of the miRNAs profiled by ABI Taqman PCR have been added as an Additional File.

Discretionary Revisions.

“On page 4 the authors write “Several miRNAs have been identified as differentially expressed between normal and tumor tissues or cancer cell lines [20]. In CRC, there have been limited studies examining the expression patterns of miRNAs [21-25].” The authors should acknowledge the existence of larger miRNA transcription profiling studies in CRC than those cited. In both these studies more CRC samples were investigated than in the present study.” We agree with the reviewer’s comment and have added in the relevant references and indicated in the text that these substantial studies pre-existed our report.

Reviewer 2.

Reviewer’s Report.

“……The authors show an interesting phenomenon and the authors propose an interesting hypothesis but there is no mechanism behind the finding. Lack of mechanism is a weakness of this study and I would suggest the authors to characterize the mechanism of their hypothesis since at the moment their data do not justify their conclusions.” Please see the response below to comment 3.

Major Compulsory Revisions.

Comment 1: “The expression of deregulated miRNA in CRC cell lines is very heterogeneous. Only 11 miRNAs were commonly deregulated in CRC clinical samples and CRC cell lines. The authors suggest that the finding in clinical samples is not completely reproduced in cell line models. However, the authors should consider if altered miRNA in CRC tissues are consequence of epithelial cancer cells or of other cell types. In situ hybridization could help to elucidate this question.” We agree with the
reviewer that differences observed between clinical samples and cell lines could be related to the potential existence of other cell types in the tissue samples. However, the suggested experiments are beyond the scope of the present manuscript.

Comment 2: “......the authors performed Northern blot analysis on 22 of altered miRNAs in 8 CRC cell lines. However, there are several miRNAs that are validated by Northern but are not represented in Table 5 as differentially expressed in CRC cell lines (for instance miR-221, miR-96, miR-182, and miR-90b).” Please refer to the reply to comment 1b under minor essential revisions from reviewer 1.

Comment 3: “......The authors hypothesized that the differentially status between metastatic and non-metastatic cells might be the cause of different function of miR-145. In my opinion, this is a good hypothesis but it should be verified. However, the authors should demonstrate that miR-145 over-expression in the isogenically matched non-metastatic SW480 cell line induce the opposite effect than in the metastatic SW620 cell line. Actually, the genetic network analysis of miR-145 targets in metastatic versus non-metastatic cells is not enough to explain the potential dual-effect of miR-145.” We agree with the reviewer’s comment that testing miR-145 over-expression in the isogenic, non-metastatic cell line SW480 would an excellent way to test the hypothesis proposed in this study. However, these studies have not been completed to date and therefore could not be included in the present manuscript.

Discretionary Revisions.

“The introduction is very long. I think that it should be to shorter.” Following a review of other manuscripts published in BMC Cancer, we have elected not to modify the length of the introduction.

Reviewer 3.

The comments from this reviewer were positive and supportive of publication. There were no comments that required replies from the authors.

In all we believe the revised manuscript answers all of the reviewer’s concerns. We have provided specific details of which companies employ which authors in the “Competing Interests” section as requested. The microarray data has also been deposited in the NCBI GEO with accession no. GSE10259. If you require any further information, please contact me.

Yours sincerely,

Dr Mitch Raponi, PhD