Author's response to reviews

Title: Dysregulated miR-183 Inhibits Migration in Breast Cancer

Authors:

Aoife J Lowery (aoife.lowery@gmail.com)
Nicola Miller (nicola.miller@nuigalway.ie)
Roisin M Dwyer (roisin.dwyer@nuigalway.ie)
Michael J Kerin (michael.kerin@nuigalway.ie)

Version: 2 Date: 1 June 2010

Author's response to reviews: see over
Dear Professor Norton,

We recently submitted a manuscript for review entitled “Dysregulated miR-183 inhibits migration in breast cancer” which was found to be potentially acceptable for publication in BMC Cancer pending satisfactory revisions. In accordance with the reviewer’s recommendations, have amended the manuscript and responded to the reviewers’ comments as described below:

**Reviewer 1 - Aaron Sarver**

**Major Compulsory Revisions**

1) The fold changes in miR-183 observed in the different intrinsic subtypes of tumor tissues appear to be very modest (less than 1.5 fold average change) and the average fold change between groups is not reported anywhere. This is essential to determine whether or not "Statistically significant" changes are diagnostically useful and is currently not reported in the text. Gene profiling experiments usually disregard miRNA with fold changes less than a certain threshold.

**Response**

The relative quantity of miR-183 expression was calculated using the comparative Ct (ΔΔCt) method with the lowest expressed sample being used as a calibrator, using q-basePLUS software. Data that were not normally distributed required log transformation to produce an adequate approximation to a normal distribution prior to parametric analysis – the RQ values for miR-183 were not normally distributed and were log transformed prior to analysis using parametric t-tests to compare means. Admittedly this may be not highlighted appropriately in the manuscript and in figure 1 the x-axis of the graphs for miR-183 expression actually represents log10 RQ values. Using non-
parametric statistical analysis (Mann Whitney U) to compare the mean RQ values (not log transformed) for miR-183 expression there are statistically significant differences in miR-183 expression between distinct tumour phenotypes as follows:

- **miR-183** expression is significantly lower in ER positive tumours (mean RQ 82.73 +/-SEM 16) compared to ER negative tumours (260.58 +/-91) p=0.013 Mann Whitney U.

- **miR-183** expression is significantly higher in HER2/neu positive tumours (329.79 +/-150) compared to HER2/neu negative tumours (93.33 +/-15) p=0.012, Mann Whitney U.

- The expression of **miR-183** is significantly lower in Luminal A breast tumours (76.26 +/-13) compared to the other subtypes which include Luminal B (199.27 +/-71), Basal (171.90 +/-52) and HER2/neu overexpressing (421.15 +/-253), p=0.019, Kruskall Wallis.

These data may be presented using this non parametric statistical analysis and in amended graphs which have the actual RQ value on the x-axis rather than the log transformed value (see below), alternatively we can present the log transformed data (as recommended by our institutional statistician) and re-label the existing graphs to clarify that the values have been log10 transformed, this is what we have done in the resubmitted manuscript, but can be changed to the graphs in this response letter if deemed appropriate.
2) The authors used a type of normalization in their RQ-PCR normalization that is non standard for miRNA measurement. For example, U6 is commonly used for normalization (Cancer Res. 2005 Aug 15;65(16):7065-70.) The authors do not describe why they deviate from standard U6 based normalization for RQ-PCR. A possible interpretation of the data is that the normalization method used (normalization to Let7a and miR-16) may be leading to the changes observed. In reference 12 Figure 5 and 6 Let7A is shown to have differential expression in breast tumors dependent on clinical features and appears inappropriate as a
control.

Response
We agree with the reviewer that accurate and reliable interpretation of RT-qPCR results is heavily dependent on the use of suitable reference genes for normalisation to eliminate or minimise non-biological variation between test samples. However, it is recognised that a single universal reference gene for all tissue types is unlikely to exist (1-3) and the use of a single reference gene for normalisation can lead to large errors and may therefore be inappropriate (3, 4). To date there are few miRNA RT-qPCR studies detailing the validation of reference genes for normalisation and we disagree that U6 can be accepted as a standard endogenous control for miRNA normalisation. In fact, despite being commonly used normalisers, U6 and 5S RNAs have been shown in a study by Peltier et al (5) to be the two least stable RNA species in cancerous and non cancerous tissue and thus far from ideal as endogenous controls in normalisation of miRNA data. In reality, there is currently no consensus on the most suitable endogenous controls for quantitative analysis of miRNA by RQ-PCR in human breast tissue and recognising the importance of validating candidate endogenous controls in the samples we work with we commenced our miRNA expression studies with the careful selection of appropriate endogenous controls for normalisation. This data has been published and is referenced in the manuscript (6). Briefly, the expression of five miRNA genes (let-7a, miR-10b, miR-16, miR-21 and miR-26b) and three small nucleolar RNA genes (RNU19, RNU48 and Z30) was examined across malignant, benign and normal breast tissues to determine the most appropriate normalisation strategy. The combination of Let7a and miR-16 was found to be the most stable combination of endogenous controls for normalisation of miRNA expression data in our breast tissue samples.

As it is recognised that a universal, invariably expressed miRNA/gene is unlikely to exist, the goal should be to identify the most reliable set of miRNAs/genes as endogenous controls for a particular experiment. We believe that we have adequately addressed this issue in the above experiment, and indeed we have repeated the same process when working with other tissues and have identified appropriate endogenous controls for use in colorectal tissue which are distinct from those used in breast (7).
3) The current title "Dysregulated miR-183 Inhibits Migration in Breast Cancer" is only supported by data in T47D cells and is NOT supported in SKBR-3 and MDA-MB-231 cells thus the use of the term Breast Cancer is inappropriate. A more accurate title would be 'Overexpression of miR-183 in T47D cells decreases in vitro migration'. This comment also applies to results header on page 11 'MiR-183 represses breast cancer cell migration in-vitro'

Response
We would be happy to change the title if the editors think this is appropriate to “Over expression of miR-183 in ER-positive, HER2/neu negative breast cancer cells decreases in-vitro migration”. This would also apply to the results header on page 11.

4) The work surrounding VIL2 presents more questions then answers. Does miR-183 overexpression lead to decrease in VIL2 protein levels in T47D cells as measured by western blot? What happens in the other cell lines?

Response
We agree that the work surrounding VIL2 does present questions that we would aim to answer through ongoing work on the functional role of miR-183 in breast cancer, which is outside the scope of this manuscript but would form the basis of a follow-up manuscript including in-vivo analysis in animal systems.

Regarding the specific points on western blot and what happens in other cell lines;
More than 50% downregulation of the VIL2 gene in T47D cells transfected with miR-183 was confirmed using RQ-PCR which is the gold standard for measuring gene expression. This experiment was performed and reported in T47D cells following phenotypic analysis in a range of breast cancer cell lines, as they had the lowest endogenous miR-183 levels and had shown the greatest phenotypic change in response to miR-183 modulation. We have also performed this experiment in SKBR3 cells and shown a more modest decrease in VIL2 gene expression of <40%, which did not translate to a functional effect as illustrated in our migration assays, possibly due to the fact that SKBR3 cells did not have a low endogenous level of miR-183 to begin with. Immunocytochemistry was selected as the method for protein analysis as it can be used to analyse the localisation of proteins within a cell or tissue section while western blotting only analyses protein expression. This was important in the case of VIL2 as the expression of VIL2/Ezrin protein in breast cancer cells can be both cytoplasmic and membranous, and the differential localization, rather than total
VIL2/Ezrin protein levels has been linked to the malignant potential of breast cancer cells (8).

We analysed VIL2/Ezrin protein expression in both T47D and SKBR3 cells based on their differential ER and HER2/neu receptor status, which are the clinicopathological parameters which were associated with miR-183 expression and having shown no phenotypic change in the other cell lines following miR-183 modulation did not feel that it would add anything to the study to evaluate VIL2/Ezrin expression in all cell lines.

Immunocytochemical analysis of T47D cells for VIL2/Ezrin protein expression following transfection with miR-183 demonstrated more membranous staining in the transfected cells, which is in keeping with less metastatic potential. These were the cells that demonstrated decreased migration on functional analysis. In the SKBR-3 cells which did not exhibit decreased migration, there appeared to be decreased membranous and cytoplasmic expression of Ezrin in the transfected cells (figure 6b), this suggests that the overexpression of miR-183 in these cells had disrupted Ezrin expression but this had not translated to a measurable functional effect. We feel that this addresses the issue of “what happens in other cell lines” adequately.

Finally, we would like to comment on the appropriateness of using immunocytochemistry in the setting of breast cancer research; immunohistochemistry is a well established tool in breast cancer clinical setting for quantitating protein expression for the ER, PR and HER2/neu receptors. It is the standard in clinical practice and the protein expression determined by this method is the parameter upon which decisions regarding adjuvant therapy are based, thus it would appear to be an appropriate strategy for analysing protein expression in the research setting also.

**Minor Essential Revisions**

1) The model for miR-183 mediated inhibition of migration is not clear in T47D. Is it transcriptional control, translational control, localization control or a combination of the above that is hypothesized as the mechanism?

**Response**

Having shown a down-regulation in VIL2 gene expression by RQ-PCR, it seems likely that the miR-183 mediated inhibition of migration is likely to be a result of transcriptional control of this gene. However, there were obvious changes in localization of the VIL2/Ezrin protein on immunocytochemical analysis of cells
transfected with miR-183 indicating that a combination of the above may be the mechanism through which miR-183 mediates migration.

2) The statement "firstly T47D cells were shown to express the lowest endogenous levels of miR-183, thus the effect of overexpression in these cells would be expected to result in a more dramatic or measurable phenotypic effect than in cells where miR-183 is expressed at higher levels endogenously." Is misleading as MDA-MB-231 cells are shown in FIGURE 2 to have almost identical miR-183 levels as T47D.

Response
T47D cells were the cells with the lowest endogenous levels of miR-183 expression, although, admittedly the MDA-MB-231 cells appear to also have low endogenous levels of miR-183. The statement can be amended to read, “T47D (ER+ve/HER2/neu-ve) and MDA-MB-231(ER-ve/HER2/neu-ve) cells expressed lower levels of miR-183 SKBR3 (ER-ve/HER2/neu +ve) and ZR751(ER+ve/HER2/neu+ve) cells. The lowest endogenous miR-183 levels were expressed in T47D cells”.

The second part of the statement is not misleading, as one would expect a more measureable phenotypic change in low endogenous miR-183 expressing cells following transfection with this miRNA.

3) The following statement is not understandable. "Downregulation of miR-183 expression was validated using RQ-PCR, indicating that regulation occurred at the mRNA level, this is in contrast to the findings of Wang et al[13] who found regulation of Ezrin to be at the posttranscriptional level in lung cancer." Possibly the authors mean Downregulation as a result of miR-183 expression?

Response
This statement has been amended to read “Downregulation of VIL2 as a result of miR-183 overexpression was validated using RQ-PCR, indicating that regulation occurred at the mRNA level, this is in contrast to the findings of Wang et al[13] who found regulation of Ezrin to be at the posttranscriptional level in lung cancer.

4) In the following sentence it is unclear which SKBR-3 cells exhibited decreased migration as Figure 3 shows miR-183 overexpression leading to increased migration in SKBR. "In the SKBR-3 cells which did exhibit decreased migration, there appeared to be decreased membranous and cytoplasmic expression of Ezrin in the transfected
cells (figure 6b), this suggests that the overexpression of miR-183 in these cells had disrupted Ezrin expression but this had not translated to a measurable functional effect.”

Response
This was an error and has been corrected to read “In the SKBR-3 cells which did not exhibit decreased migration, there appeared to be decreased membranous and cytoplasmic expression of Ezrin in the transfected cells (figure 6b), this suggests that the overexpression of miR-183 in these cells had disrupted Ezrin expression but this had not translated to a measurable functional effect”.

Discretionary Revisions
1) One potential way to turn this into an article of general interest would be to determine whether VIL2 decrease via rnaI alone is sufficient to recapitulate the miR-183 overexpression migration phenotype in T47D cells. In light of the previously published work, presentation of this data would show a clear advance in understanding.

Response
This is a very interesting point and certainly one that we plan to address in our ongoing analysis of miR-183 expression in breast cancer, but at present we feel that it is outside the scope of this particular manuscript.

References
Reviewer 2 – Pedro Gonzalez

Major Compulsory Revisions
1) The main problem with the manuscript is that that Ezrin has been already validated as a miR-183 target by Wang et al 2008. The paper by Wang et al provided convincing evidence of the targeting of Ezrin as well as the associated effects on cancer cells. Although this paper is cited in the manuscript (Reference 13), there is no mention to the targeting of Ezrin or the resulting effects in cancer cells reported by Wang et al.

Response
We have referenced the paper by Wang et al (1) in both the introduction and the discussion of our manuscript where we comment on the targeting of Ezrin and the resulting effects in cancer cells in the following statement on pg. 15:

“During the course of these experiments, the first functional study of miR-183 in malignancy was reported; Wang et al[13] identified miR-183 as a potential metastasis inhibitor in lung cancer and demonstrated that over expression of miR-183 inhibited migration of lung cancer cells. They reported miR-183 induced dysregulation of expression of genes involved in migration and invasion, including VIL2”

We have added a reference to the effects in cancer cells reported by Wang et al (1) to the introduction section also in the following statement:

“downregulation of miR-183 has been shown to be associated with metastasis in lung cancer, and its ectopic expression inhibits the invasiveness of cancer cells [13], suggesting that miR-183 plays a role in carcinogenesis or the metastatic cascade, possibly having a tumour suppressor role.

While it is clear that Wang et al (1) were the first to report on the targeting of Ezrin by miR-183, we believe that this is not necessarily problematic for our findings, as suggested by Reviewer 2. Firstly, ours is the first report of miR-183 dysregulation in breast cancer. It is known that miRNA expression and function are tissue specific, so our finding that miR-183 targets Ezrin and inhibits migration in breast cancer cells not only supports the findings of Wang et al (1) but is novel to breast cancer.

Furthermore, we have shown downregulation of miR-183 at the transcriptional level with mRNA downregulation which was not shown by Wang and colleagues in lung cancer. There has been limited functional analysis of miR-183 to date, however the potential anti-metastatic role of miR-183 is an exciting hypothesis and warrants
further investigation in both in-vitro and in-vivo environments to build evidence and develop the potential of miR-183 as a therapeutic agent in cancer. Indeed, since the submission of this manuscript we note that Li et al (2) have also demonstrated that miR-183 transfection led to a significant decrease in cell invasion and migration capacities of HeLa cells via down regulation of integrin beta 1, indicating that miR-183 is likely to have multiple miRNA targets through which it mediates biological effects, these targets may differ according to tissue type and so our investigation in breast cancer is warranted. This point has been added to the discussion.

It is important to note that the functionality of miRNAs that are now well established as having a role in carcinogenesis, for example miR-21 have been investigated and reported in numerous cancer types, adding to the evidence of the role for this miRNA as an oncomir (3-7), similarly the potential role of miR-183 in the metastatic cascade, and its targeting of VIL2/Ezrin warrants investigation in different tumour types.

2) The information about the genes present in the PCR arrays used in the study should be made available. The complete list of the genes included in the PCR array should be provided since information about the cancer related genes that do not change with miR-183 is also important.

Response

We agree with the reviewer, and the information about the genes present in the PCR arrays can be made available as supplementary data.

3) The title is appropriate, but the abstract should not present Ezrin as a novel target of miR-183.

Response

The abstract has been amended to state that we confirm VIL2/Ezrin as a target of miR-183 rather than identify it as a target, which has already been reported in the literature.

4) Since reporting Ezrin a novel miR-183 target is not appropriate, the manuscript would strongly benefit from the inclusion of some new potential mechanism of action of miR-183. The manuscript should probably focus on the new data related to the expression of miR-183 in cancer cells and the association with
other variables such as ER, PR, and HER2/neu status. If possible, some specific mechanism for these effects should be provided.

**Response**

We agree with the reviewer that the data related to the expression of miR-183 in breast cancer cells and the association with other variables such as ER, PR and HER2/neu status is an important finding and have increased the focus on this data in the discussion; ER, PR and HER2/neu status are used as indicators of prognosis in breast cancer management, as are the intrinsic breast cancer subtypes, however there is significant heterogeneity even within the subtypes which may be due to dysregulation of miRNAs such as miR-183. It is important to functionally characterise miRNAs that are found to be dysregulated in breast cancer as they may be responsible for the heterogeneity of breast tumour behaviour.

We feel that reporting the targeting of miR-183 by Ezrin in breast cancer is appropriate in this context, particularly as ours is the first report of this in breast cancer, however we also report dysregulation of a number of other genes identified using the TLDA, the functions of these genes were analysed using NetAffx Gene Ontology Mining Tool available at the Affymetrix website (www.affymetrix.com/) and a number of the genes were found to have roles in migration and maintenance of the actin cytoskeleton integrity. These data support the hypothesis that regulation of metastasis requires coordinated expression of multiple genes. We focused protein analysis on VIL2/Ezrin as this was identified as having a complementary binding site for miR-183 on in-silico analysis, the confirmation of Ezrin as a target of miR-183 supports the findings of other authors (1) and will add to the evidence linking miR-183 to the metastatic pathway. Further analysis of the other dysregulated genes, and indeed the metastatic pathway is warranted and underway and will be the subject of a further manuscript.

**References**


We hope that these revisions and responses are in keeping with the reviewers’ suggestions and are resubmitting this manuscript to be considered for publication in BMC Cancer

Yours Sincerely
Dr Aoife Lowery
Dr Nicola Miller (Corresponding Author)
Department of Surgery
Rm 311 Clinical Science Institute
University College Hospital, Galway, Ireland
t: +353 91 544637
f: +353 91 494509
e: nicola.miller@nuigalway.ie