THE EMBO JOURNAL

Manuscript EMBO-2011-77619

MICRO-RNA-34C IS A NOVEL TARGET TO TREAT DEMENTIAS

Athanasios Zovoilis, Hope Y Agbemenyah, Roberto C Agis-Balboa, Roman M Stilling, Pooja Rao, Dieter Edbauer, Pooja Rao, Laurent Farinelli, Ivanna Delalle, Andrea Schmitt, Peter Falkai, Sanaz Bahari-Javan, Susanne Burkhardt, Farahnaz Sananbenesi and Andre Fischer

Corresponding author: Andre Fischer, European Neuroscience Institute

Review timeline:

Submission date:	17 March 2011
Editorial Decision:	11 April 2011
Revision received:	21 July 2011
Accepted:	09 August 2011

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 April 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by tworeferees and their comments are provided below.

As you can see both referees find the analysis interesting, but that they also indicate that additional controls and further experiments supporting a role for miR-34c in memory formation is needed. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript addressing the concerns raised. I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to address the main concerns raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

© European Molecular Biology Organization

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the present manuscript, Zoviolis et al. identify a hippocampus-enriched microRNA, miR-34c, as a potentially negative regulator of memory formation in mice. The authors further show that miR-34c is elevated in a mouse model of Alzheimer's and in Alzheimer's patients, and that inhibiting miR-34c restores memory in the Alzheimer's mouse model.
Overall, these are interesting findings that could significantly advance our understanding of microRNA function in memory formation and neurodegenerative diseases. However, in my opinion, the presented dataset is too preliminary and does not fully support the authors`s claims, in particular with regard to the physiological role of this microRNA as a putative memory constraint. As outlined in more detail below, a number of additional experiments/controls are needed before this manuscript can be considered for publication in the EMBO Journal.

Major concerns:
1. The physiological role of miR-34c during memory formation is not sufficiently addressed. If miR-34c is indeed a constraint on memory formation, then blocking miR-34c in the hippocampus of wild-type animals should lead to improved memory (and an associated increase in Sirt1 protein levels). However, this important experiment was not performed. In addition, all the conclusions regarding memory rest on one single behavioral task („contextual fear conditioning"), which might even not be strictly hippocampus-dependent. Data from at least one alternative memory test should be presented.

2. The specificity of the tools used to study miR-34c loss-/gain-of-function was not sufficiently demonstrated. Injection of the oligos led to expected changes in the putative target Sirt1 (see 3.), but this is purely correlative. A more direct way to show effects on miR-34c activity would be to monitor expression of miR-34c responsive fluorescent transgenes („sensors") and analogous constructs with mutated miR-34c target sites. These controls are especially important since the detailed protocol for delivery of these oligos is not presented due to „ ... currently pending patent application."

3. Sirt1 is assumed to be a physiologically relevant, direct miR-34c target due to a previous publication (Yamakuchi and Lowenstein, 2009; a review rather than the primary research paper is cited here). However, colon cancer cell lines were used in this study. The authors should therefore at the very least demonstrate a direct regulation of Sirt1 translation by endogenous miR-34c in hippocampal neurons, using i.e. standard luciferase reporter assays. Showing that Sirt1 re-expression is able to rescue memory defects upon miR-34c would convincingly demonstrate the physiological relevance of this interaction.

Minor concerns:

1. Figures 1 and 2 contain little information that is necessary to support the conclusions of the paper. These figures should be condensed.
2. The regulation of miR-34c during memory formation is very confusing. miR-34c targets are enriched among mRNAs induced by contextual fear conditioning (Fig. 2). Therefore, one would expect downregulation of miR-34c under these conditions, but apparently the opposite is the case (suppl. Fig. S4b). Finally, mRNA levels of the putative miR-34c target Sirt1 do not change at all upon miR-34c perturbation (Fig. 6a, Fig. 7c), arguing that this microRNA might act primarily at the level of translation. This part need further clarification.
3. There are very few references in the main body of the manuscript to the supplementary data. This makes it difficult to judge which part oft he suppl. Data is actually required to support the main conclusions of the paper.

Referee #2 (Remarks to the Author):

Review of the manuscript: "MicroRNA-34c is a novel target to treat dementias"

In this manuscript, the authors used massive parallel sequencing to analyze miRNAome of mouse

© European Molecular Biology Organization

hippocampus. They found expression of miR-34c in the hippocampus. There was also increased expression (3X) in 24 months old mouse compared to 3 months and they found 1.7X increased expression in APPPS1-21 mouse compared to wild-type. This was correlated with decreased learning and inversely correlated with expression of SIRT1 protein. By injecting miR-34c mimics in the APPPS1-21 mouse brain, the authors recapitulated learning impairment and injection of seed inhibitors rescued memory function.

These findings are of great interest however additional experiments are needed to improve the quality of the manuscript.

1. The 1.7X fold change of miR-34c in AD patients is quite modest and the number of patients is really limited. Minimally a series of other miRNA should be analyzed to allow to appreciate the significance of this change.

2. The same holds true for the miRNAome of 24 months old mouse: how is expression of other miRNA affected (needed to evaluate the 3X fold change reported here).

3. What is the effect of miRNA34c injection in mouse brain on the targets of miR34c (preferably to be expanded from Sirt1 to other identified targets as well)

4. Importantly, the observations that injecting seed inhibitors rescued learning in APPPS1-21 mouse and thus miR-34c could represent a novel target to treat dementias, is shortcut. The experiment should be repeated in other models of AD to rule out an effect specific of APPPS1-21 strain. I am surprised about the spectacular effects shown in the figure documenting these experiments, and would like to receive more details eg about the down regulation of other putative targets of miR34c analyzed by western blotting. What happens when the same treatment is done in wild type mice? Is there also improved learning?

5. The number of independent investigations needs to be indicated in every figure (eg fig 3, Sirt-1 determination).

1st Revision - authors' response 21 July 2011

RESPONSE TO REFEREES:

Referee #1 (Remarks to the Author):

Referee #1, point 1:

He/she says: "*The physiological role of miR-34c during memory formation is not sufficiently addressed. If miR-34c is indeed a constraint on memory formation, then blocking miR-34c in the hippocampus of wild-type animals should lead to improved memory (and an associated increase in Sirt1 protein levels). However, this important experiment was not performed."*

This experiment has been now performed. We now show that inhibition of miR-34c activity significantly facilitates learning in wild type mice, further supporting that miR-34c acts as a molecular constraint of memory formation. This novel data has now been included within the revised version of our manuscript. See Fig. S9 and page 7, last 3 lines. We also discuss this issue in greater detail on page 10, lines 5-11.

This referee further comments that: *"all the conclusions regarding memory rest on one single behavioral task („contextual fear conditioning"), which might even not be strictly hippocampus-dependent. Data from at least one alternative memory test should"*

That miR-34c acts as constraint of memory formation is now further supported by positive data in two additional behavioral tests. This novel data is now presented as Figure S7 and described on page 7, lines 2-4.

Referee #1, point 2:

© European Molecular Biology Organization

He/she states that *"The specificity of the tools used to study miR-34c loss-/gain-of-function was not sufficiently demonstrated. Injection of the oligos led to expected changes in the putative target Sirt1 (see 3.), but this is purely correlative. A more direct way to show effects on miR-34c activity would be to monitor expression of miR-34c responsive fluorescent transgenes („sensors") and analogous constructs with mutated miR-34c target sites. These controls are especially important since the detailed protocol for delivery of these oligos is not presented due to „ ... currently pending patent application."*

We apologize for any confusion. We have now included all the necessary information regarding transfection oligos, protocols and reagents. See supplement material page 15. The transfection protocol is based on modifications on standard miRNA transfection procedures and reagents as presented by the manufacturer. Successful insertion into the cell and load of oligos on RISC complex is shown by the successful reduction of expression levels of a *mapk1* by using the control oligo provided by the company Qiagen against *mapk1* (Fig S6).

To further prove the specificity miR-34c on the 3' UTR binding sites of Sirt-1 *in vivo* we now established an *in vivo* miRNA target protection assay. We used so-called miRNA target protectors that hybridize to the specific mRNA target sites of the mRNA and do not act as miRNAs themselves, but rather specifically block interaction of miR-34c with the two miR-34c-*Sirt1* 3' UTR binding sites, without affecting binding sites of other miRNAs in *Sirt1* 3'UTR or binding sites of miR-34c on UTRs of any other gene.
Protection of miR-34c binding sites on *Sirt1* 3UTR was able to reverse the negative effect of miR-34c mimic on SIRT protein levels and memory function. This data provides strong evidence that the effect of miR-34c on learning and SIRT1 levels *in vivo* is mediated through translational repression and it is not an indirect effect. Most importantly, it confirms that selection of SIRT1 as an *in vivo* read out for miR-34c action is reliable. This data is now presented as a novel Figure 6 and described on page 6, lines 1-5 from bottom.

Referee #1, point 3:

He/She says: "*Sirt1 is assumed to be a physiologically relevant, direct miR-34c target due to a previous publication (Yamakuchi and Lowenstein, 2009; a review rather than the primary research paper is cited here). However, colon cancercell lines were used in this study. The authors should therefore at the very least demonstrate a direct regulation of Sirt1 translation by endogenous miR-34c in hippocampal neurons, using i.e. standard luciferase reporter assays. Showing that Sirt1 re-expression is able to rescue memory defects upon miR-34c would convincingly demonstrate the physiological relevance of this interaction*."

In the revised version of the manuscript we now cite the corresponding primary research paper. Moreover, we provide novel data using a luciferase reporter assay to show that miR-34c regulates SIRT1 in cultured neurons depending on the predicted seed regions in its 3'UTR. This data is now included as novel panel (d) within Fig S4 and description on text page 5, lines 1-3 from bottom.

We also provide novel data indicating that Sirt-1 re-expression through target protects co-injected with miR-34c mimics is able to rescue memory defects. We found that the miR-34c-*Sirt1* target protectors leads to increased levels of SIRT1 and rescue memory deficits (see response to point 2).

Minor concerns:

He/she states: *"Figures 1 and 2 contain little information that is necessary to support the conclusions of the paper. These figures should be condensed"*

We thank the referee for this comment. However since the referee 2 had no similar concerns, we did not to remove this data from the main paper since we consider it essential.

He/She says: "*The regulation of miR-34c during memory formation is very confusing. miR-34c targets are enriched among mRNAs induced by contextual fear conditioning (Fig. 2). Therefore, one would expect downregulation of miR-34c under these conditions, but apparently the opposite is the case (suppl. Fig. S4b). Finally, mRNA levels of the putative miR-34c target Sirt1 do not change at all*

© European Molecular Biology Organization

upon miR-34c perturbation (Fig. 6a, Fig. 7c), arguing that this microRNA might act primarily at the level of translation. This part need further clarification."

We thank this referee for these comments. We now discuss this issue in greater detail on page 9, bottom line and page 10, lines 1-19.

He/She mentions: *"There are very few references in the main body of the manuscript to the supplementary data. This makes it difficult to judge which part of the suppl. Data is actually required to support the main conclusions of the paper."*

We apologize if there has been any confusion. We have now included detailed text references for all supplemental material.

Referee #2 (Remarks to the Author):

Referee #2, point 1:

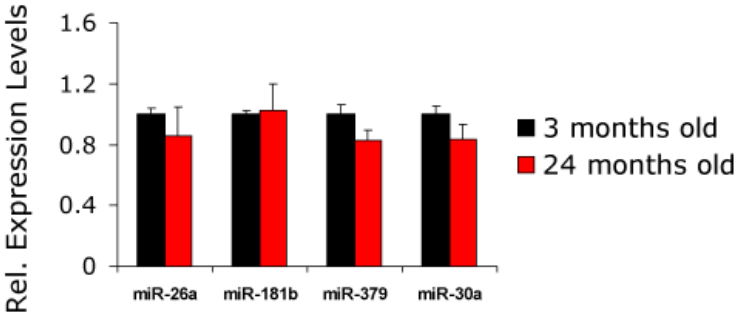
This referee says: *"The 1.7X fold change of miR-34c in AD patients is quite modest and the number of patients is really limited. Minimally a series of other miRNA should be analyzed to allow to appreciate the significance of this change."*

We now analyzed the levels of miR-124 that has been previously linked to learning and memory and miR-379 which has been tested in our study. This data is now included as novel Fig S5 and discussed on page 6, lines 8-10. Please note that the amount of RNA obtained from patients is limited which hinders the analysis of further miRs at this point.

Referee #2, point 2:

He/She says: "*The same holds true for the miRNAome of 24 months old mouse: how is expression of other miRNA affected (needed to evaluate the 3X fold change reported here)"*

We now analyzed the expression levels of miR-26a, miR-30a, miR-379 and miR-181b. No changes were detected (See below)



The figure depicts expression levels of selected miRNAs by quantitative real time PCR. Levels in old mice (24 months) are compared to those of younger ones (3 months). In contrast to miR-34c none of the selected miRNAs is de-regulated in aged mice (n=4).
Since the supplemental data is already extensive (e.g. see last comment by referee 1) we did not include this data in the revised version of the manuscript because we feel that this information is not crucial.

Referee #2, point 3:

He/She says: *"What is the effect of miRNA34c injection in mouse brain on the targets of miR34c (preferably to be expanded from Sirt1 to other identified targets as well)."*

© European Molecular Biology Organization

This is an interesting point. Although, SIRT1 had already been implicated with learning impairment, we initially used SIRT1 levels as a readout of miR-34c action *in vivo*. We clearly state that the action of miR-34c on SIRT1 in unlikely to be the only reason for the observed effects. The fact that a target protector against the action of miR-34c specifically on *Sirt1* mRNA was able to reverse miR-34c mediated memory impairment, nevertheless suggests a crucial role in miR-34c mediated memory function (See response to referee 1, point 2).

In addition we now provide novel data showing the effect of miR-34c mimic injection on c-MYC levels, another known target of miR-34c (Christoffersen et al, 2010; Cannell et al, 2010). As expected expression levels of c-MYC are reduced upon application of miR-34c mimic. Although c-MYC has not been directly implicated with memory consolidation, we previously observed increased *c-myc* expression in response to contextual fear conditioning (Peleg et al, Science 2010). This data is now presented as novel Fig S 10 and discussed on page 10. Lines 10-12.

Moreover, we now discuss in even greater detail that the mechanisms underlying the action of miR-34c on memory function are complex and are likely to involve other target genes than *Sirt1*. See page 10, lines 5-11.

Referee #2, point 4:

He/She mentions: *"Importantly, the observations that injecting seed inhibitors rescued learning in APPPS1-21 mouse and thus miR-34c could represent a novel target to treat dementias, is shortcut. The experiment should be repeated in other models of AD to rule out an effect specific of APPPS1-21 strain. I am surprised about the spectacular effects shown in the figure documenting these experiments, and would like to receive more details eg about the down regulation of other putative targets of miR34c analyzed by western blotting. What happens when the same treatment is done in wild type mice? Is there also improved learning?"*

We performed the requested experiments. We have now applied the miR-34 seed inhibitor to aged mice. Inhibition of miR-34c in aged mice was able to reinstate memory function and rescued SIRT1 levels. Administration of miR-34 seed inhibitor also facilitated learning behavior in 3-month-old wild type mice, although the underlying molecular mechanisms are likely to be different. This data is now presented within novel Fig S8 & S9 and discussed on page 7, lines 1-4 from bottom; page 10, lines 5-11.

Referee #2, point 5:

The referee mentions: *"The number of independent investigations needs to be indicated in every figure (eg fig 3, Sirt-1 determination)"*

We have now checked the manuscript thoroughly and included any missing number.

2nd Editorial Decision 09 August 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referees to review the revised manuscript and referee #1 was available to do so. I have now received the comments back from this referee and as you can see below the referee appreciates the introduced changes and supports publication in the EMBO Journal. I am therefore very pleased to proceed with the acceptance of the paper for publication here.

You will receive the formal acceptance letter shortly

Editor
The EMBO Journal

© European Molecular Biology Organization

REFEREE REPORT

Referee #1

The authors sufficiently addressed all my concerns and I can now recommend publication.

© European Molecular Biology Organization