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miR379-410 cluster miRNAs regulate neurogenesis and neuronal migration by fine-tuning N-cadherin

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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.)

Editor: David del Alamo

1st Editorial Decision 17 September 2013

Thank you for the submission of your manuscript entitled "miR379-410 cluster miRNAs regulate neurogenesis and neuronal migration by fine-tuning N-cadherin" to The EMBO Journal. It has been sent to three referees, and we have so far received reports from two of them, which I copy below. As both referees are convinced about the interest and quality of your study, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and I will forward you the third report, probably including further requests, as soon as I receive it.

Without going into details that you will find below, both referees are very positive and, besides a more detail analysis of the miRNAs expression patterns requested by referee #2, ask mainly for minor text and technical clarifications. 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 initiative, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact me as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a

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problem in meeting this three-month deadline, please let me know in advance and we may be able to grant an extension.

Do not hesitate to contact me by e-mail or on the phone in case you have any questions or need further input.

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

REFEREE REPORTS:

Referee #1

In this manuscript, Rago and Winter present a number of evidences indicative that N-cadherin expression in the developing cortex is regulated by 3 micro RNAs which bind directly to the UTR of N cadherin and repress translation. In general, this is a well-performed study and in most ways an original contribution. I have however a number of requests that need to be addressed before I can recommend acceptance.

1) Figure 2B: Authors should explain the rationale behind the selection of the different mutations and on the different binding sites. These would help to explain by which mechanism miR369 and miR496 bind cooperatively: Figure 2B does not necessarily indicate that both bind to site 1.
2) Figure 3A (and other figures): The location of the area shown in the insets should be indicated in the overview picture. Moreover, the apical side of the transfected region over-expressing miRs is often destroyed, making difficult to raise conclusions. Authors should show areas at the edge of the transfected regions where the density of transfected cells is usually lower yet the effects still visible and therefore possible to evaluate. Authors should make comparisons within the same section (eg. ipsi versus contralateral) rather than between different slices.
3) Suppl Fig 2E: Beta catenin is extremely reduced, but this is also the case in non-transfected cells. Analysis of adjacent regions would help.
4) In Figure 3E and 4A authors show that miR inhibition induces N cadherin increase. However, in Figure 3E it is not evident that N cadherin expression in basal progenitors is reduced compared to Radial Glia progenitors. Sowing N cad expression together with Tbr2 or Pax6 staining would help.
5) Figure 5A. The examples are not well chosen. It is difficult to see for instance the decrease in the RG pool when miRs are over-expressed. Moreover, the distribution of Pax6+ cells and Tbr2+ is rather different in those miR over-expressing areas, they seem to be more spread and intermingled. Is that a true phenotype?
6) Figure 5C: Again the Tbr2+ cells seem to be much more apically located. Is that also true in the adjacent sites?
7) Figure 6: this figure needs more consistency: do authors suggest glia-guided migration or VZ to IZ migration. The data in this figure should be more concise and condensed to a single point. Moreover if these miRs do not affect N-cadherin expression in neurons (see before), how do authors explain the migration defects?

Minor comments:

The statement in the summary that over-expression of the miRNAs increased migration is later contradicted. Clarification is needed here.
Figure 1: What is the reason to select 4 out of 5 miRNAs?
Figure 1C: How was the normalization done, if it was normalized to the levels of miRNA in the Hes negative fraction, this number should be always one?
Figure 1C: quite different regions are chosen. It would be good to see the transfected cells in the other channel in order to see the functionality of the approach in single cells (neighbor transfected versus non-transfected cells).

Referee #2

The manuscript from Rago and Winter describes the regulation of N-cadherin expression by microRNAs belonging to the miR379-410 cluster and its effect on the development of the cortex during embryogenesis. Most of the experiments performed by the authors are in vivo and soundly controlled, and the results provided with these elegant approaches are of interest both for the microRNA and the neuroscience community. On one hand, this study provides novel information on the function of the still poorly characterized biggest microRNA cluster identified so far, emphasizing its importance for coordinately fine tuning gene expression in the nervous system. On the other hand it identifies a new layer of regulation of an essential gene for neuronal differentiation and migration, thereby opening new avenue of investigations. I am supportive of publication provided that my concerns outlined below are adequately addressed.

-The expression pattern of miR-543,-369 and 495 should be analyzed in greater detail. ISH for at least one of the less expressed microRNAs should be performed, and the analysis should not be limited to E15.5 but also include later time points. Alternatively the authors could use FACS sorting and determine the miRNA expression levels in different cell populations (Pax6,Tbr1 and and Tbr2 positive cells). These experiments (in combination with the one suggested below) would begin to shed some light on the different phenotypical consequences observed when microRNAs are mis-expressed at different times and places (Fig 5 and 6).
-Does overexpression of Ncad under the control of the NeurD1 promoter phenocopy the microRNA gain of function at E17.5?
-Fig. 2A to C: The term Fold Induction as label for the y-axis is confusing, They could use instead either % of repression or relative luciferase activity.
-Fig 2d. Quantification and statistical analysis of the expression of Ncad should be shown.
-Fig. 3c: How was the statistical analysis performed? A T-test between two samples where one is set to 1 does not appear to be appropriate.
-Fig. 4A: Is the profile of the Tbr1 panel representative? It is surprising that the quantification reveals no difference between the Anti-miR and Anti-scr conditions given that the profiles are quite separated from each other.
-Fig. 7 does not add much to the main message of the paper and could be moved to the supplementary material.

Additional Editorial Correspondence: 23 September 2013

Thank you again for the submission of your research manuscript to The EMBO Journal. As I mentioned in my previous letter, your manuscript was sent to three referees and we have just received the third report, which I copy below.

As you can see below, referee #3 is also positive towards the publication of your manuscript in The EMBO Journal. Although perfectly clear in his/her report, I would like to point out to the two major issues that will require your attention during the revision process. First, referee #3 considers that the experiments analyzing the rescue of over-expression of miRNAs by N-cad over-expression are not conclusive and secondary effects due to other miRNA targets could be the reason. And second, the referee finds that the differential effects of miRNAs in RGCs vs. neurons have not properly been explained.

Again, do not hesitate to contact me in case you have any questions or need further input.

REFEREE REPORT:

Referee #3

This manuscript by Rago and Winter describes an analysis of the regulation of neurogenesis and neural migration in the mammalian cortex via fine-tuning of the levels of N-cadherin (N-Cad). Previous studies have shown that precise regulation of the levels of N-Cad is important for proper

neurogenesis and neuronal migration. By using immunohistochemistry methods following manipulations of mouse embryonic brains in vivo, as well as luciferase reporter assays, the authors identify three microRNAs (of the miR379-410 cluster) that down-regulate the levels of N-Cad. This fine-tuning of N-Cad levels has a role in regulation of neuronal differentiation and migration. In addition, the work identifies other targets of these miRNAs. The findings that miRNAs modulate of N-Cad levels in cortical development provides with novel insight on mechanisms regulating neurogenesis and neuronal morphology. The study is thorough and data is well presented. There are nevertheless few issues that must be addressed to substantiate and extend the conclusions.

Major comments:

1) The authors convincingly demonstrate that over expression of specific combination of miRNAs results in down regulation of N-Cad and that this reduction can be rescued by over expression of N-Cad-HA. Furthermore, they state (Fig. S2) that over expression of the miRs results in a phenocopy of N-Cad mutant phenotype including altered morphology, reduced PKCζ and reduced beta-catenin. It is further claimed that this phenotype can be partly rescued by over expression of N-Cad-HA. The data demonstrating this rescue however is only partially presented in Fig. S2.

To substantiate that the observed phenotype is indeed due to reduced N-Cad and not due to other effects of miRs, it is required to further demonstrate rescue of the levels of PKCζ and beta catenin in the N-Cad-HA rescue experiment. The one figure presented in Figure S2 is not convincing due to low quality of PKCζ staining and lack of quantification. Rescue of beta-catenin levels should be included. It is suggested to include the rescue of the phenotype in the main figures of the article.

2) Figure 4A: according to the presented FACS results, knockdown of the three miRNAs results in elevation of N-Cad levels in a significant fraction of the Tbr1-positive cells. Despite this it is concluded that reduced levels of these miRNAs leads to elevated N-Cad levels only in RGCs. This needs to be clarified.

The authors conclude that miR369-3p, miR496 and miR543 are partly redundant and function in regulation of N-Cad expression in RGCs but the results are ambiguous relating to the regulation of N-Cad in neurons. Yet, the title claims that these miRNAs regulate both neurogenesis and migration by regulation of N-Cad. This should be changed in the interpretation of the results, explained in the discussion also the title should be revised.

3) In page 14 it is stated that "N-Cad is an important target of these miRNAs in migrating Neurons". To demonstrate this it is required to add quantification of N-Cad levels in neurons following over expression or reduced levels of the miRNAs.

Minor comments:
1) Figure S2A: it seems that some panels are switched. Based on the current figure the miR transfection does not seem to result in reduced levels of N-Cad.

2) Figure 3: The HA-N-Cad rescue experiments, it is assumed that the miRNAs plasmids and the HA-N-Cad overexpression construct are expressed in the same cell, based on the mCherry reporter. Add the staining for HA to demonstrate that co-expression of all plasmids in the same cells. The rescue shown may be at the level of the tissue and not at the level of a single cell.

3) Page 13: revise this sentence "..in agreement with the FACS results that show higher N-Cad level in RGCs but not in IPCs. We observe similar effects when weoverexpress N-Cad." This should be separate sentence and the effect mentioned should be explained.

4) Correct:
page 18 - "oppocite"
page 18 - "While in RGCs is required'

1st Revision - authors' response 15 December 2013

Please find attached a revised version of our manuscript and a detailed point-by-point response to the reviewer's comments. We appreciate the comments of the three reviewers and the constructive criticism. We have performed several additional experiments during the last three months. As you will see our manuscript has improved substantially and we are now able to address all of the reviewer's concerns.

Referee #1

In this manuscript, Rago and Winter present a number of evidences indicative that N-cadherin expression in the developing cortex is regulated by 3 micro RNAs which bind directly to the UTR of N cadherin and repress translation. In general, this is a well-performed study and in most ways an original contribution. I have however a number of requests that need to be addressed before I can recommend acceptance.

1) Figure 2B: Authors should explain the rationale behind the selection of the different mutations and on the different binding sites. These would help to explain by which mechanism miR369 and miR496 bind cooperatively: Figure 2B does not necessarily indicate that both bind to site 1.

To make these things clearer we have now included a schematic showing the predicted location of miRNA binding sites in the N-cadherin 3'UTR and indicating the mutated bases.

2) Figure 3A (and other figures): The location of the area shown in the insets should be indicated in the overview picture.

We have indicate the location of the area in the overview picture in our new version of the manuscript.

Moreover, the apical side of the transfected region over-expressing miRs is often destroyed, making difficult to raise conclusions. Authors should show areas at the edge of the transfected regions where the density of transfected cells is usually lower yet the effects still visible and therefore possible to evaluate. Authors should make comparisons within the same section (e.g. ipsi versus contralateral) rather than between different slices.

We have now included overviews of the electroporated regions including areas at the edge of the transfected regions in Supplementary Figure 3A and C and in Figure 3C.

3) Suppl Fig 2E: Beta catenin is extremely reduced, but this is also the case in non-transfected cells. Analysis of adjacent regions would help.

We have repeated the staining and exchanged the pictures in the Figure. We now also show adjacent regions.

4) In Figure 3E and 4A authors show that miR inhibition induces N cadherin increase. However, in Figure 3E it is not evident that N cadherin expression in basal progenitors is reduced compared to Radial Glia progenitors. Sowing Ncad expression together with Tbr2 or Pax6 staining would help.

We have now included pictures showing Ncad expression together with Tbr2.

5) Figure 5A. The examples are not well chosen. It is difficult to see for instance the decrease in the RG pool when miRs are over-expressed. Moreover, the distribution of Pax6+ cells and Tbr2+ is rather different in those miR over-expressing areas, they seem to be more spread and intermingled. Is that a true phenotype?

We agree that this might be misleading. The spreading occurs because of the disruption of the ventricular surface upon down regulation of Ncad. The differentiation phenotype is independent of the spreading. We now show pictures of areas of lower transfection efficiency without spreading.

6) Figure 5C: Again the Tbr2+ cells seem to be much more apically located. Is that also true in the adjacent sites?

This was also the case in the adjacent sites. Other slices did not show this. We have exchanged the picture.

7) Figure 6: this figure needs more consistency: do authors suggest glia-guided migration or VZ to IZ migration. The data in this figure should be more concise and condensed to a single point.

We have now added a paragraph in the results (page 15) and discussion (page 20) where we comment on the different modes of migration that might be affected. In addition, we have made the data in Figure 6 more concise.

Moreover if these miRs do not affect N-cadherin expression in neurons (see

before), how do authors explain the migration defects?

We agree that this is an important point. In Tbr1+ cells that have already reached the CP we don't see a change in N-cadherin expression. We have now analysed possible changes in N-cadherin expression in new-born neurons in the IZ which are NeuroD1+ but Tbr1-. In these neurons we see clear shifts in N-cadherin expression upon the knockdown of the miRNAs. Therefore, we suggest that the regulation in neurons is restricted to those new-born neurons that migrate from the IZ to the CP which may lead to the migration defects. Moreover, we have now expressed Ncad under control of the NeuroD1 promoter which leads to a phenocopy of the migration defect observed upon the miRNA knockdown.

Minor comments:

The statement in the summary that over-expression of the miRNAs increased migration is later contradicted. Clarification is needed here.

To clarify this we have pointed out in the new version of the summary that we first interfered with miRNA expression in RGCs and then in neurons and that two different modes of migration may be affected (VZ to IZ vs. IZ to CP).

Figure 1: What is the reason to select 4 out of 5 miRNAs?

We did not include miR329 because we could not confirm the regulation of Ncad by this miRNA in subsequent experiments. We have now stated this in the text of the manuscript.

Figure 1C: How was the normalization done, if it was normalized to the levels of miRNA in the Hes negative fraction, this number should be always one?

It changes because we have related all values to the first measurement.

Figure 1C: quite different regions are chosen. It would be good to see the transfected cells in the other channel in order to see the functionality of the approach in single cells (neighbour transfected versus non-transfected cells).

Unfortunately, due to technical issues we lose the signal of the transfected cells during the procedure of the in situ hybridization and we were also not able to get a double in situ/immunostaining protocol to work. To further confirm our results of the in situ we have now included more stages in Supplementary Figure 1D as well as a scramble control. Moreover we have now FACS sorted neural progenitors and neurons to confirm the miRNA expression (Figure 1D) and have also confirmed the miRNA expression in neural progenitors and neurons in vitro (Supplementary Figure 1B).

Referee #2

The manuscript from Rago and Winter describes the regulation of N-cadherin expression by microRNAs belonging to the miR379-410 cluster and its effect on the development of the cortex during embryogenesis. Most of the experiments performed by the authors are in vivo and soundly controlled, and the results provided with these elegant approaches are of interest both for the microRNA and the neuroscience community. On one hand, this study provides novel information on the function of the still poorly characterized biggest microRNA cluster identified so far, emphasizing its importance for co-ordinately fine tuning gene expression in the nervous system. On the other hand it identifies a new layer of regulation of an essential gene for neuronal differentiation and migration, thereby opening new avenue of investigations. I am supportive of publication provided that my concerns outlined below are adequately addressed.

-The expression pattern of miR-543,-369 and 495 should be analysed in greater detail. ISH for at least one of the less expressed microRNAs should be performed, and the analysis should not be limited to E15.5 but also include later time points.

We have now added in situs of miR543 of E13.5 and E17.5 embryos as well as a scramble probe as an additional negative control (Supplementary Figure 1D). Unfortunately, we were not able to get the in situ to work for the other miRNAs.

Alternatively the authors could use FACS sorting and determine the miRNA expression levels in different cell populations (Pax6,Tbr1 and Tbr2 positive cells). These experiments (in combination with the one suggested below) would begin to shed some light on the different phenotypical consequences observed when microRNAs are mis-expressed at different times and places (Fig 5 and 6).

Determining the miRNA expression in Pax6+, Tbr1+ and Tbr2+ sorted cells does unfortunately not work because as Pax6, Tbr2 and Tbr1 are nuclear proteins the cells have to be fixed and permeabilized for sorting. During this procedure most of the miRNAs are lost.

As an alternative we have searched for suitable surface markers to distinguish neural progenitors from neurons which would enable us to sort living cells. We found CD29 and CD24 suitable to sort progenitors and neurons and have analysed the miRNA expression in these cell populations. Moreover, we have taken neocortical cells into culture under non-differentiating or differentiating conditions and have confirmed the in vivo expression patterns of the miRNAs in vitro. We have now included these results in Figure 1D and Supplementary Figure 1A-C.

-Does overexpression of Ncad under the control of the NeurD1 promoter phenocopy the microRNA gain of function at E17.5?

We agree that this is an important point. We have now cloned Ncad into the NeuroD1-plasmid and performed this experiment. The overexpression of Ncad from the NeuroD1 promoter phenocopies the microRNA gain of function at E17.5. These new data are now included in Figure 6B.

-Fig. 2A to C: The term Fold Induction as label for the y-axis is confusing, They could use instead either % of repression or relative luciferase activity.

We have changed this to relative luciferase activity.

-Fig 2d. Quantification and statistical analysis of the expression of Ncad should be shown.

We have now performed the quantification and show this in the new Figure 2D.

-Fig. 3c: How was the statistical analysis performed? A T-test between two samples where one is set to 1 does not appear to be appropriate.

We have changed this and have now added error bars for all samples.

-Fig. 4A: Is the profile of the Tbr1 panel representative? It is surprising that the quantification reveals no difference between the Anti-miR and Anti-scr conditions given that the profiles are quite separated from each other.

We have not found a statistically significant difference between the Anti-miR and Anti-scr conditions. We have also realized that the profile of the Tbr1 panel is not representative and have exchanged the picture by a better one.

-Fig. 7 does not add much to the main message of the paper and could be moved to the supplementary material.

We have moved Figure 7 to the Supplementary (now Supplementary Figure 8).

Referee #3

This manuscript by Rago and Winter describes an analysis of the regulation of neurogenesis and neural migration in the mammalian cortex via fine-tuning of the levels of N-cadherin (N-Cad). Previous studies have shown that precise regulation of the levels of N-Cad is important for proper neurogenesis and neuronal migration. By using immunohistochemistry methods following manipulations of mouse embryonic brains in vivo, as well as luciferase reporter assays, the authors identify three microRNAs (of the miR379-410 cluster) that down-regulate the levels of N-Cad. This fine-tuning of N-Cad levels has a role in regulation of neuronal differentiation and migration. In addition, the work identifies other targets of these miRNAs. The findings that miRNAs modulate of N-Cad levels in cortical development provides with novel insight on mechanisms regulating neurogenesis and neuronal morphology. The study is thorough and data is well presented. There are nevertheless few issues that must be addressed to substantiate and extend the conclusions.

Major comments:

1) The authors convincingly demonstrate that over expression of specific combination of miRNAs results in down regulation of N-Cad and that this reduction can be rescued by over expression of N-Cad-HA. Furthermore, they state (Fig. S2) that over expression of the miRs results in a phenocopy of N-Cad mutant phenotype including altered morphology, reduced PKCz and reduced beta-catenin. It is further claimed that this phenotype can be partly rescued by over expression of N-Cad-HA. The data demonstrating this rescue however is only partially presented in Fig. S2. To substantiate that the observed phenotype is indeed due to reduced N-Cad and not due to other effects of miRs, it is required to further demonstrate rescue of the levels of PKCz and beta catenin in the N-Cad-HA rescue experiment.

The one figure presented in Figure S2 is not convincing due to low quality of PKCz staining and lack of quantification. Rescue of beta-catenin levels should be included. It is suggested to include the rescue of the phenotype in the main figures of the article.

We have repeated the stainings and added the rescue and have now included the PKCz staining and the quantification in Figure 3C. We have included the rescue of beta-catenin in Supplementary Figure 3C.

2) Figure 4A: according to the presented FACS results, knockdown of the three miRNAs results in elevation of N-Cad levels in a significant fraction of the Tbr1-positive cells. Despite this it is concluded that reduced levels of these miRNAs leads to elevated N-Cad levels only in RGCs. This needs to be clarified.

We have not found a significant effect in the Tbr1+ cells. We agree that the representative picture shown is not good. We have exchanged this in the new version of the paper.

The authors conclude that miR369-3p, miR496 and miR543 are partly redundant and function in regulation of N-Cad expression in RGCs but the results are ambiguous relating to the regulation of N-Cad in neurons. Yet, the title claims that these miRNAs regulate both neurogenesis and migration by regulation of N-Cad. This should be changed in the interpretation of the results, explained in the discussion also the title should be revised.

We agree that this is an important point. To clarify this we have now analysed the regulation of Ncad expression in NeuroD1+ neurons in the IZ. In contrast to the Tbr1+ neurons of the CP we find a clear regulation of Ncad in the migrating NeuroD1+ neurons. We have included these new data in Figure 4C and D. Moreover we have now found that overexpressing Ncad from the NeuroD1 promoter phenocopies the migration defect observed upon the miRNA knockdown. We have included these new data in Figure 6B.

3) In page 14 it is stated that "N-Cad is an important target of these miRNAs in migrating Neurons". To demonstrate this it is required to add quantification of N- Cad levels in neurons following over expression or reduced levels of the miRNAs.

As stated above we have now added the analyses of Ncad levels in migrating NeuroD1+ neurons where we see a miRNA-dependent regulation of Ncad expression (new Figure 4C,D).

Minor comments:

1) Figure S2A: it seems that some panels are switched. Based on the current figure the miR transfection does not seem to result in reduced levels of N-Cad.

We have exchanged these pictures.

2) Figure 3: The HA-N-Cad rescue experiments, it is assumed that the miRNAs plasmids and the HA-N-Cad overexpression construct are expressed in the same cell, based on the mCherry reporter. Add the staining for HA to demonstrate that co-expression of all plasmids in the same cells. The rescue shown may be at the level of the tissue and not at the level of a single cell.

To show this we have added the staining for HA in the new version of the manuscript (Supplementary Figure 3E).

3) Page 13: revise this sentence "..in agreement with the FACS results that show higher N-Cad level in RGCs but not in IPCs. We observe similar effects when weoverexpress N-Cad." This should be separate sentence and the effect mentioned should be explained.

We have revised this sentence to: "Furthermore, there is a significant increase in apical, but not basal, mitoses, which suggests an increase in RGCs, but not IPCs proliferation (Supplementary Figure 6C, D). This is in agreement with the FACS results that show higher Ncad level in RGCs but not in IPCs. In addition, the overexpression of Ncad results in an increase in apical, but not basal, mitoses and therefore phenocopies the lack of the miRNAs."

4) Correct:

page 18 - "oppocite"

page 18 - "While in RGCs is required'

We have corrected these mistakes.