Journal of Cell Science

The miR-26 family regulates neural differentiation-associated microRNAs and mRNAs by directly targeting REST

Mark Sauer, Nina Was, Thomas Ziegenhals, Xiantao Wang, Markus Hafner, Matthias Becker and Utz Fischer
DOI: 10.1242/jcs.257535

Editor: Giampietro Schiavo

Review timeline
Original submission: 19 November 2020
Editorial decision: 19 January 2021
First revision received: 7 April 2021
Accepted: 11 May 2021

Original submission

First decision letter

MS ID#: JOCES/2020/257535

MS TITLE: The miR-26 family controls a regulatory RNA network required for neurogenesis via REST/CTDSP

AUTHORS: Mark Sauer, Nina Was, Thomas Ziegenhals, Xiantao Wang, Markus Hafner, Matthias Becker, and Utz Fischer
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. Instead please use yellow shading or different colour font to highlight changes in your manuscript.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this new and interesting study, the authors are investigating miR-26a/b function in neurogenesis and provide important and novel insight into the underlying molecular mechanism. Using a neuronal differentiation system of murine embryonic stem cells, they show that miR-26a/b KO results in arrest of differentiation on the neural progenitor cell level. Underlining the robustness of the KO model, phenotypes could be rescued through exogenous overexpression of miR-26a/b. Previous publications already indicated an impact of miR-26 on progenitor proliferation and a connection of miR-26 to REST and Ctdsp. The strength of this new study lies in the detailed dissection of the molecular network underlying miR-26 driven neurogenesis. The data are well presented and the manuscript is well written and easy to follow. Together, I think the findings will be of wide interest as it completes our picture of the role of miR-26 in neuronal differentiation.

Comments for the author

In this new and interesting study, the authors are investigating miR-26a/b function in neurogenesis and provide important and novel insight into the underlying molecular mechanism. Using a neuronal differentiation system of murine embryonic stem cells, they show that miR-26a/b KO results in arrest of differentiation on the neural progenitor cell level. Underlining the robustness of the KO model, phenotypes could be rescued through exogenous overexpression of miR-26a/b.

Previous publications already indicated an impact of miR-26 on progenitor proliferation and a connection of miR-26 to REST and Ctdsp. The strength of this new study lies in the detailed dissection of the molecular network underlying miR-26 driven neurogenesis. The data are well presented and the manuscript is well written and easy to follow. Together, I think the findings will be of wide interest as it completes our picture of the role of miR-26 in neuronal differentiation. Before this interesting and timely manuscript shall be published, the authors could consider the following points to improve their study.

In Fig. 5, the authors show that deletion of miR-26 target site in REST partially phenocopies the miR-26a/b KO. It would be interesting to see, whether a combination of both Ctdsp2 and REST ts deletion will result in a complete and full phenocopy of the phenotype. Do the authors already have experimental insight on this point? This study would certainly benefit from this important information.

Furthermore, the authors state that differentiation into neurons and astrocytes is regulated by miR-26. Here, it would be helpful to better interpret the data and discuss whether proliferation of neural precursor cells is more likely to be affected compared to differentiation (Supp. Fig. 4B,C). Currently, some figures are not displayed in high quality, most likely due to compression, resulting in pixelated Western Blots and images (Fig. 3, Fig. 4A, Fig.6).

Minor comments:
- While the authors cited that Lambert et al found Ddx17 to transcriptionally regulate miR-26, they do not comment on its role on miR-26 processing. Could this helicase also be responsible for the stalling of pre-miR-26 processing?
- Fig. 1D: Are size/scale bars of WT and KO images the same? WT images appear to have lower magnification.

Reviewer 2

Advance summary and potential significance to field

The repressor element silencing transcription factor (REST) is a key transcriptional regulator in stem cells and represses neuronal gene expression.
REST activity is regulated by its cofactors, such as Ctdsp2. Previous studies have demonstrated that miR-26b represses Ctdsp2 in zebrafish neurogenesis. The present study demonstrates that miR-26 family is part of the neuronal gene expression program during the transition from neural precursor cells (NPCs) to neurons by inhibiting the transcription factor REST. The authors found that miR-26 family inactivation (by CRISPR/Cas9) arrests neurogenesis specifically at the neuronal progenitor (NPC) level which interrupts the neurogenesis and astrogenesis.
Interestingly, expression of miR-26a and miR-26b rescued the neuronal differentiation ability of miR-26 deficient NPCs. Further, ablation of the miR-26 site on the Rest 3Â'UTR leads to reduced neuronal differentiation mimicking the miR-26b/21/a2 deficient cultures. MiRnoma experiments in the miR-26 deficient cultures identified decreased expression of miR-9 and miR-124, previously reported as REST target genes (PMID: 19561591). These data propose that the miR-26 family controls neurogenesis via a REST/miRNAs dependent pathway. This study is novel characterizing, in mammals, the miR-26/REST-miRNAs pathway during the transition from NPCs to neurons specifically.

Comments for the author

Even though the study is novel, there are some questions to be solved.

1. Since the title of this article includes CTDSP as part of the mechanism by which miR-26 controls neurogenesis, it is important to further explain why the miR-26 ts KO in CTDSP2 had no effect on neuronal differentiation (Fig. 5).

2. Previously, it has been demonstrated that REST is a target of miR-26a and miR-26b in the neuroblastoma cell line SH-SY5Y. This interaction is independent of the most conserved miR-26 binding site present in the 3'UTR, indicative of additional miR-26 binding sites (PMID: 29931089). Therefore, it is important to include in this study a detailed analysis of the Rest 3'UTR, how many miR-26 sites are present, their conserved level and the specific site mutated in present study that abolished luciferase activity.

3. In order to demonstrate that miR-26 family is part of the neuronal gene expression program by inhibiting the REST/CTDSP pathway during the transition from NPCs to neurons, chromatin immunoprecipitation assays for actively transcribed RNA polymerase (RNA pol II CTD phosphoSer5) on the promoter region of the neuronal (Tubb3, Neurod1, Neurog1, Ncam1) and miRNA (miR-9, -124, 218 and -135a) genes identified by RNAseq and miRnoma, respectively must be performed.

4. The protein levels of REST and CTDSP2 must be included in the luciferase assays as indicative of miR-26 mediated repression.

5. For the statistical analysis, authors should consider to apply analysis of variance (ANOVA) instead of t test to compare the means among different groups (e.g. expression/luciferase activity/frequencies respect to stages of differentiation/miR-26a/miR-26b, etc.).

6. Authors should reconsider the title since at the present is not accurate/focus enough of the scientific work presented in the article.

First revision

Author response to reviewers' comments

Reviewer #1

1. Fig. 5, the authors show that deletion of miR-26 target site in REST partially phenocopies the miR-26a/b KO. It would be interesting to see, whether a combination of both Ctdsp2 and REST

ts deletion will result in a complete and full phenocopy of the phenotype. Do the authors already have experimental insight on this point? This study would certainly benefit from this important information.

We are grateful for this suggestion and agree with the referee that the proposed experiment would further support the conclusion of our study.
However, given the complexity of the proposed experiment, its completion in the time frame of the revision of this manuscript appears to us not feasible. In addition, because the observed effect of the Ctdsp2 ts deletion is rather small (see Fig.5E), its combination with the Rest ts deletion will most likely only slightly enhance this phenotype. Given the time constrains of the revision and our assumption that the gain from this experiment is only incremental we feel that these analyses are beyond the current scope of the manuscript.

2. Furthermore, the authors state that differentiation into neurons and astrocytes is regulated by miR-26. Here, it would be helpful to better interpret the data and discuss whether proliferation of neural precursor cells is more likely to be affected compared to differentiation (Supp. Fig. 4B,C).

We are grateful for bringing up this important point, which we addressed in a set of additional experiments that we now present in Supplemental Fig. 4D that shows that the increased fraction of cells in cycle in the $tKO^{26b/a1/a2}$ cultures are indeed NPCs. Based on our new data we now changed the text of the Results section on page 12/13 as follows:

"To ask if the cycling population in $tKO^{26b/a1/a2}$ cultures consists indeed of NPCs, we next determined the frequencies of $BrdU^+$ cells as well as the $BrdU^+/Msi1^+$ cell ratio at day 10 and day 12 of differentiation in $tKO^{26b/a1/a2}$ and WT cultures (Supplementary Fig. 4D). Consistent with an increased frequency of NPCs, these analyses revealed that $MSI1^+$ cell frequencies were also increased at day 12 of differentiation in $tKO^{26b/a1/a2}$ cultures. At this time point, also frequencies of $BrdU^+$ cells were increased in $tKO^{26b/a1/a2}$ cultures. Our analyses further show that the ratio $BrdU^+/MSI1^+$ is at the same level in WT and $tKO^{26b/a1/a2}$ cultures, indicating that most of the cells in cycle in WT and $tKO^{26b/a1/a2}$ cultures are indeed NPCs."

We also included a paragraph in the revised Discussion (page 19) where we address the new findings as follows:

"BrdU labeling of WT and $tKO^{26b/a1/a2}$ cells indicates that most of the BrdU positive cells are indeed NPCs in both cultures. Published data show that neuronal differentiation of adult NSCs can be promoted by cell cycle inhibition (Roccio et al., 2013). It is therefore tempting to speculate that differentiation of miR-26 KO cells is impaired due an incapability to exit the cell cycle. The effects on the cell cycle progression are in agreement with earlier observations where ectopic miR-26a expression suppressed cell proliferation by inducing a G1- phase arrest (Lu et al., 2011). Likewise, a G1/S transition block was shown following overexpression of miR-26a or -26b (Zhu et al., 2012). During neurogenesis a reduction of G1-phase length results in inhibition, while an extension of G1-phase promotes differentiation (Artegiani et al., 2011; Lange et al., 2009; Lim and Kaldis, 2012). Future work should therefore address the question whether miR-26 KO results in a reduced G1-phase duration"

3. Currently, some figures are not displayed in high quality, most likely due to compression, resulting in pixelated Western Blots and images (Fig. 3, Fig. 4A, Fig.6).

We are sorry for the low resolution of parts of our figures in our original submission. In our revised manuscript, we have now uploaded high quality figures. The Western blots are acquired using a digital imaging device (BioRad, Chemidoc XRS low light imager) and the

quality of the images has been set to the highest output level. We hope that the images are now suitable for the Journal.

4. While the authors cited that Lambert et al found Ddx17 to transcriptionally regulate miR-26, they do not comment on its role on miR-26 processing. Could this helicase also be responsible for the stalling of pre-miR-26 processing?

   We agree with the referee that the excellent paper about Ddx17 should be discussed in more detail in the context of our study. We have therefore included the following paragraph in the Discussion section (page 21):

   "We speculate that the miR-26 family binds to a subset of protein factors that either suppress processing in non-neuronal cells or activate maturation upon transition from NPCs to NCs. Interestingly, a recent study implicated DDX17 in miR-26a biogenesis (Lambert et al., 2018). The authors of this study found that DDX17 is required for pri-miR-26a2 processing. In contrast, our data and also data from Dill et al. show that miR-26 processing is arrested during differentiation at the pre-miR-26 level, i.e. downstream from the reported role of DDX17 (Dill et al., 2012). It is hence an interesting possibility that DDX17 and another factor, yet to be identified regulate miR-26 maturation at different levels. Recent advances in the analysis of miR-interacting proteins (Treiber et al., 2017) will help to identify and functionally characterize potential factors involved in miR-26 during neural differentiation."

5. Fig. 1D: Are size/scale bars of WT and KO images the same? WT images appear to have lower magnification.

   Both images indeed have the same magnification. However, based on our own measurements, NPC nuclei have an average surface area of 172 μm^2, whereas neuronal nuclei are much smaller and display only an average surface area of 35 μm^2. Nuclei of cells in WT cultures consist predominantly of mature neurons and thus have small nuclei. The predominant cell type in miR-26 KO cultures, however, are NPCs with larger nuclei. The apparent difference in magnification is therefore a reflection of a difference in size of nuclei and hence of different cell types in the respective cultures.

Reviewer#2

1. Since the title of this article includes CTDSP as part of the mechanism by which miR-26 controls neurogenesis, it is important to further explain why the miR-26 ts KO in CTDSP2 had no effect on neuronal differentiation (Fig. 5).

   As requested, we further explained why the miR-26 ts KO in CTDSP2 had no effect on neuronal differentiation and now have modified the Discussion at page 20as follows:

   "Deletion of the miR-26 target site in the REST complex co-factor *Ctdsp2* only affected the expression of *Ctdsp2* but had neither an effect on the expression of *Rest* or other *Ctdsp* family members, nor did it affect neural differentiation. This is in contrast to earlier studies in zebrafish that showed a rescue of the miR-26 knockdown mediated reduction of neurons when Ctdsp2 was reduced simultaneously (Dill et al., 2012) and suggests species-specific differences in the function of CTDSP2. In contrast, the deletion of the Rest miR-26 target site almost completely phenocopied the $tKO^{26b/a1/a2}$ situation and also affected *Ctdsp2* and *CtdspL* mRNA expression. This is consistent with a key role for REST in the miR-26-mediated regulation of neuronal gene expression and suggests a regulation of the REST complex on the network level rather than on the level of individual genes. Our own bioinformatics analyses further revealed that the miR-26 target sites in the Rest 3´UTR are conserved in human and mouse, but not in zebrafish. This notion is in agreement with previously published data on miR-125-mediated regulation of the p53 network, which is also conserved on a network level but not on the level of individual miRNA target pairings between human, mouse and zebrafish (Le et al., 2011)."

2. Previously, it has been demonstrated that REST is a target of miR-26a and miR- 26b in the neuroblastoma cell line SH-SY5Y. This interaction is independent of the most conserved miR-26 binding site present in the 3'UTR, indicative of additional miR-26 binding sites (PMID:

29931089). Therefore, it is important to include in this study a detailed analysis of the Rest 3'UTR, how many miR-26 sites are present, their conserved level and the specific site mutated in present study that abolished luciferase activity.

As requested, we have extended our analyses of putative miR-26 binding sites in the 3`UTR of *Rest*. By TargetScan analysis we identified an additional potential target site. This site is less conserved as compared to the first site that we focused on in our studies. We have now included luciferase assays addressing the functionality of this additional site in a revised Fig. 5A. The results show that mutational inactivation of this so far not studied miR-26 target site had no effect on the luciferase activity. This new finding has now been mentioned in the Results section (page15/16):

„Bioinformatics analyses revealed one potential miR-26 target site at position 2971-2977 of the *Ctdsp2* 3´ UTR and two potential miR-26 target sites in the 3' UTR of Rest at positions 346-353 (8mer target site) and 2519-2525 (7mer target site), respectively. Upon co-transfection with miR-26a or miR-26b mimics into HEK 293T cells both *Ctdsp2*- and *Rest*- WT-reporter constructs yielded reduced luciferase activity in comparison to control transfected cells (Fig. 5A). In contrast, the reporter constructs harboring the mutated miR-26 binding site in the *Ctdsp2* 3´UTR and the mutated 8mer binding site in the *Rest* 3`UTR showed no significant change in luciferase activity, indicating that these binding sites are direct miR-26 targets. A reporter construct harboring the mutated 7mer target site in the *Rest* 3´UTR yielded the same reduction in luciferase activity as the WT Rest 3`UTR, indicating that this site is not targeted by miR-26. These data show that only the 8mer site is a functionally active miR-26 target in the *Rest* 3´UTR. "

3. In order to demonstrate that miR-26 family is part of the neuronal gene expression program by inhibiting the REST/CTDSP pathway during the transition from NPCs to neurons, chromatin immunoprecipitation assays for actively transcribed RNA polymerase (RNA pol II CTD phosphoSer5) on the promoter region of the neuronal (Tubb3, Neurod1, Neurog1, Ncam1) and miRNA (miR-9, -124, 218 and -135a) genes identified by RNAseq and miRnoma, respectively must be performed.

We agree with the reviewer, that the suggested experiments would further support the conclusions of our paper. However, given the time constrains of the revision, we strongly feel that these analyses would be beyond the scope of this study and should be included in a comprehensive systems-biology based investigation focusing on the miR-network in neurogenesis.

4. The protein levels of REST and CTDSP2 must be included in the luciferase assays as indicative of miR-26 mediated repression.

As requested, we performed the analysis of the REST and CTDSP protein levels (see Fig. R1 at the bottom of this point-by-point response letter). These analyses show that the endogenous REST and CTDSP2 protein levels in the HEK293T cells that were used in the luciferase analyses are slightly reduced upon miR-26 transfection. We would prefer not to show these data as we feel that they would not significantly contribute to our key findings showing the involvement of miR- 26 in mouse neural differentiation. We would be prepared, however, to include them if this Referee and/or the Editor would request so.

5. For the statistical analysis, authors should consider to apply analysis of variance (ANOVA) instead of t test to compare the means among different groups (e.g. expression/luciferase activity/frequencies respect to stages of differentiation/miR- 26a/miR-26b, etc.).

As requested, we now applied analysis of variance (ANOVA) instead of t-test to compare the means among different groups in our study.

6. Authors should reconsider the title since at the present is not accurate/focus enough of the scientific work presented in the article.

We are grateful to the reviewer for bringing this point up. We have now changed the title of the article to better represent the scientific work and now write: “The miR-26 family regulates neural differentiation-associated microRNAs and mRNAs by directly targeting REST”

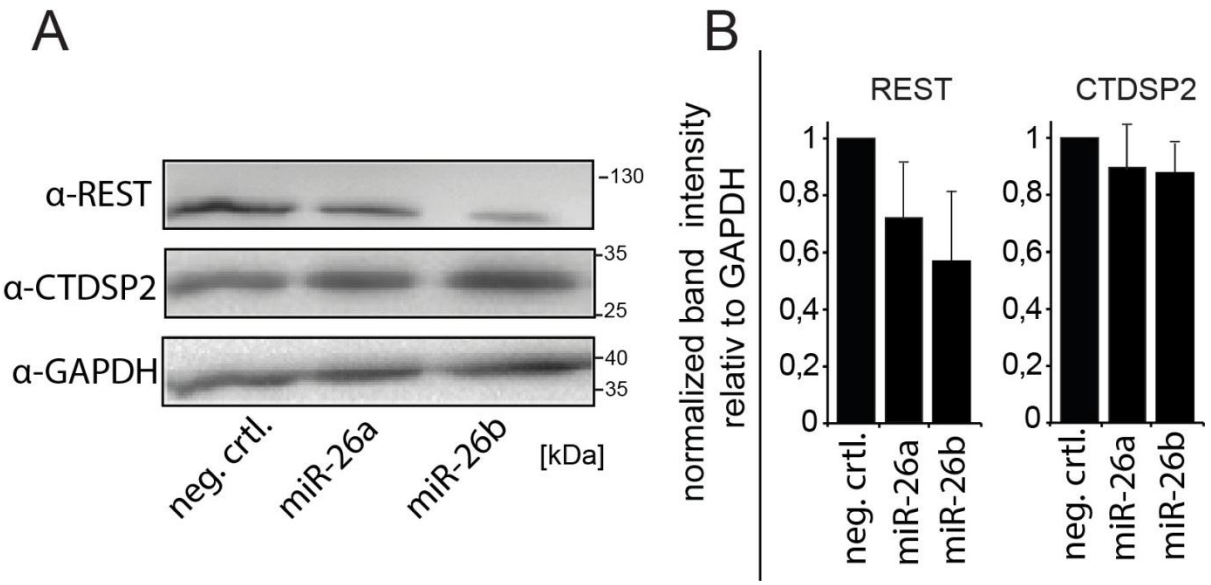

Figure. R1 (A) Representative Western blots of REST, CTDSP2 and GAPDH (loading control). HEK293T cells were transfected with either miR mimic negative control / cel-miR-67-3p mimic, miR-26a or miR-26b mimics. (B) Quantification of Western blot band intensities relative to GAPDH, n=3 biological replicates, mean ± SD.

Materials and Methods:

Transfection

$2{,}5x10^5$ HEK 293T cells were seeded in a 6-well plate 16h prior to transfection. Cells were transfected with 50 nM miR mimics (Dharmacon, GE Healthcare) using Lipofectamin 2000 (Thermo-Fisher). miR mimics were: miR mimic negative control / cel-miR-67-3p mimic (CN-001000-01-05), mmu-miR-26a-5p mimic (C-310519-07-0002) and mmu-miR-26b-5p mimic (C-310520-07-0002). Medium was changed 6h post transfection. Cells were harvested 24h after transfection and prepared for Western blot analysis.

Western blotting

Western blots of lysates prepared from transfected HEK 293T cells were incubated with primary antibodies diluted in blocking solution overnight at 4°C. Antibodies used in this study were rabbit anti-REST (bs-2590-R, Bioss), rabbit anti-CTDSP2 (#PA5-21624, Invitrogen) and mouse anti-GAPDH (MAB 374, Merck Millipore). Secondary antibodies (goat anti-rabbit secondary antibody HRP, #32460 (ThermoFisher) or goat anti-mouse secondary antibody HRP, #32430 (ThermoFisher)) were diluted in blocking solution and incubated (1h, room temperature). Quantification was done by comparison of band intensities and normalization to loading controls using the ImageJ software (NIH).

Second decision letter

MS ID#: JOCES/2020/257535

MS TITLE: The miR-26 family regulates neural differentiation-associated microRNAs and mRNAs by directly targeting REST

AUTHORS: Mark Sauer, Nina Was, Thomas Ziegenhals, Xiantao Wang, Markus Hafner, Matthias Becker, and Utz Fischer
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

see initial review

Comments for the author

Dear authors very carefully and thoughtfully responded to my concerns and suggestions. Most of the experiments worked out and the paper clearly improved. I sympathize with the authors' wish that my first comment reg. ctdsp2 and REST may be beyond the scope of this revision to be addressed experimentally. It would be good to include the sentence from the rebuttal in the revision. In conclusion, I congratulate the authors to a fine study and propose to publish this important study.