Let-7 and miR-125 cooperate to prime progenitors for astrogliogenesis

Archana Shenoy, Muhammad Danial and Robert H. Blelloch

Corresponding author: Robert H. Blelloch, UCSF

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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: Anne Nielsen

1st Editorial Decision 13 August 2014

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript; however, they also raise a number of concerns that will have to be addressed before they can support publication of your work here.

I would particularly ask you to focus your efforts on the following points:

-> please extend the analysis and provide data on the statistical significance of miR125 and Let7b as specific regulators of astrocyte differentiation as requested by both ref #1 and #3 (also including analysis on the significance of miRNA enrichment and target gene regulation)

-> please strengthen the characterization of the astrocyte cell fate and survival as outlined by ref #1

-> please also address the numerous minor control points raised by all three refs (experimentally where needed).

Regarding points 2 and 7 raised by ref#1 - delineating a network of relevant target(s) of miR125 and let7b - we would encourage you to include any data that you may have that could shed further light
on this point. However, it will not be an absolute requirement from our side to delineate this downstream regulatory network. In addition, we find that point 2 from ref #3 would be beyond the scope of the present work.

Given the referees’ overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

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REFEREE COMMENTS

Referee #1:

Blelloch lab has published very exciting papers in recent years on miRNA in embryonic stem cell differentiation and Dr. Blelloch is highly appreciated in the field. In a work entitled 'Let-7 and miR-125 cooperate to prime progenitors for astrogliogenesis' by Archana Shenoy, Muhammad Danial and Robert Blelloch the authors suggest that miRNAs are involved in the molecular mechanisms underlying the specification and differentiation of glial precursors into astrocytes.

The key findings of the current work are: glial progenitor cells lacking microRNAs failed to differentiate into astrocytes, reintroduction of let-7 or miR-125 rescued differentiation and functioned with JAK-STAT signaling, a known regulator of astrogliogenesis. Overexpression of specific miRNA targets in wild type GPCs blocked differentiation.

The main concerns are:
(1) Let-7 and miR-125 are broadly expressed in many differentiated tissues and were not sufficient to introduce differentiation in proliferating Dgcr8Δ/Δ GPCs. Therefore, authors should seek ways to show miR-125 and Let-7 specificity for astrocytosis/astrocyte lineage and hence to demonstrate that they are not acting as general and non-specific differentiation promotion factors.

(2) Many efforts have been put in the work into target analysis including a big knockdown screen. While these efforts are appreciated, the successful analysis of relevant targets that will actually establish a 'network' downstream of two miRNAs requires additional work with more satisfactory experimental target endpoint.

(3) There is no evidence for cooperative miRNA or target activity throughout the manuscript. Authors should rephrase text from the title down without this claim or add data that supports cooperative activity. In fact by comparing the rescue of GFAP with introduction of let-7 and miR-125 into Bax/Bak/Dgcr8 TKO GPCs (Fig.4C) to adding let-7 + miR-125 together (Fig.E4A) the effect seems negative and counterproductive.

(4) To substantiate the view of astrocyte differentiation, characterize the astrocytes gained by miRNA overexpression better:
(4.1) immune-detect additional common marker(s) at protein level, e.g., S100beta. Additional potential markers might be considered: vimentin, brain-lipid-binding protein; astrocyte-specific glutamate transporter
(4.2) Explain better or revise particular mRNA markers used in Figure 4D vs. 1F: Of the four mRNA markers in 4D, two do not change or are downregulated (Cyp4f14, Atp1a2), two markers from Figure 1 are not shown (AldoC, Pla2g7) and two that are up-regulated x200-300 fold in 1F are up-regulated only 5-25x (Aqp4, GFAP). Please discuss these differences and provide one consistent set of mRNA markers.
(4.3) Perform similarity / nearest neighbor search to show that your cells display higher similarity to astrocytes then other cells reported by in Cahoy et al., including neurons, and oligodendrocytes (2008): a new resource for understanding brain development and function. J. Neurosci. 28: 264-278. Compare your profile also to Ditte Lovatt and Maiken Nedergaard (The Astrocyte Transcriptome, in
Neuroglia 2013 Neuroglia edited by Helmut Kettenmann, Bruce R. Ransom Oxford University press

4.4) Prelude neuronal or oligodendrocyte fate: Are the cells β-III-tubulin-neg (non-neurons) and O4-neg (non oligodendrocyte)?

4.5) suggested only: (4.4) Classify by revisiting your mRNA expression data the gained astrocytes e.g., Type 1: Ran2+, GFAP+, FGFR3+, A2B5-, OR Type 2: A2B5+, GFAP+, FGFR3-, Ran 2-.

6) Bioinformatics

6.1) Figure 3: If "levels of the most abundant miRNAs were largely unchanged following 48 hours of differentiation (Fig.3A, 1C-D)"; then how/why targets of expressed miRNAs respond (by upregulation)? Why is the mRNA of Dgcr8Δ/Δ be different from control Dgcr8f/Δ GPCs? at what time after induction of Dgcr8Δ/Δ was RNA harvested?

6.2) Figure 3C: Among upregulated genes, enriched motifs were uncovered (p<0.01). Four of the most highly expressed miRNA families, let-7, miR-9, miR-181 and miR-30 were within the 104 enriched motifs that perfectly matched miRNA seed region (Fig.3C, Table E1). Provide statistical significance to these data and reject the null hypothesis that the motifs of highly expressed miRNAs can be found at random within the 104 motifs.

6.3) Target informatics: provide direct global analysis of targets that were upregulated in the Dgcr8Δ/Δ GPCs and could be reversed by simple addition of either let-7 or miR-125 by analyzing together data in Figure 5 relative to data in Figure 3B. Are the correlated changes statistically significant?

7) targets

7.1) As way of supporting cooperativity, consider the overexpression and knockdown of several candidates: N-myc; Hmga2; Tlx; Igfbp1; Lin28 based on published works:
Sanosaka et al identified N-myc and Hmga2, two important targets of Let-7, to restrict astrocyte differentiation (Neuroscience. 2008 Aug 26;155(3):780-8).
Zhaoa et al shown that let-7 increased astroglial differentiation by targeting nuclear receptor Tlx (Neuroscience. 2008 Aug 26;155(3):780-8).
Nishino et al, 2013 shown that let-7 promote the switch between neurogenic and gliogenic potential of neural stem cells during fetal development by repressing Igfbp1, Hmga2 and Lin28.

7.2) explicitly reveal the position of N-myc; Hmga2; Tlx; Igfbp1; Lin28 at mRNA scatter plots in Figure 3B and Figure 5A,B (or in a new supplementary figure).

7.3) As way of supporting cooperativity, consider trying knocking down more than a single target e.g., knockdown both Plagl2 and Imp2, as they have effect if overexpressed.

8) Textual changes and suggested points for discussion:

8.1) Discuss: Wen Hao Neo et al., MicroRNA miR-124 Controls the Choice between Neuronal and Astrocyte Differentiation by Fine-tuning Ezh2 Expression ( 2014 The Journal of Biological Chemistry, 289,20788-20801) in the context of your Fig.6C and Fig.E5.

8.2) Discuss: Zhaoa et al as these authors have shown that Transfection of let-7b increased astroglial differentiation in control neural stem cells, by targeting nuclear receptor TLX signaling, PNAS 2010 107 1876-81.

8.3) Discuss: STAT3 signaling importance to both pluripotency and astrocytosis. Might let-7 and miR- 125 be contributing to uncoupling of stemness cues (Niwas et al Genes & Dev. 1998. 12: 2048-2060) from astrocyte differentiation cues (Bonni 1997 Science 278 477-483)?

8.4) Discuss or rephrase JAK-STAT/miRNAs: The introduction of JAKi to the differentiation media along with transfection of let-7 and miR-125 completely blocked the ability of the miRNAs to rescue GFAP expression in Dgcr8Δ/Δ cells. Since blocking JAK signaling overrode miRNA activity, these data suggest that the two molecular pathways converge in GPC differentiation. One possibility is that let-7/miR-125 introduction is upstream of activation of JAK-STAT and hence JAKi abrogates the effect of the miRNAs. The molecular components do not necessarily act in parallel.
Discuss: use of Bax/Bak in the context of Chang et al., 2007 Mol Cell Biol. 274293-305 Bcl-XL/Bax proteins direct the fate of embryonic cortical precursor cells.

The bioinformatics of tumors (Figure 6E) is not contributing much to the main theme of the work and in fact diffuses the focus

(9) Minor:
(9.1) Rephrase: "In this study, we sought to overcome this roadblock by using the in vitro counterpart to in vivo astrocyte differentiation, allowing the production of homogenous populations of glial progenitor MicroRNAs in astrocyte differentiation cells (GPCs) that could then be induced to differentiate in synchrony into astrocytes under defined culture conditions (Brüstle & McKay, 1999)." Since in fact the roadblock to using in vitro counterpart was overcome by Brüstle & McKay, 1999

(9.2) Rephrase: "This work provides the first evidence for the role of specific miRNAs and their target gene networks in astrogliogenesis" and "provide the first detailed characterization of how miRNAs promote the GPC to astrocyte" and refer to previous works including Wen Hao Neo et al., 2014 The Journal of Biological Chemistry, 289,20788-20801 and Zhao et al PNAS 2010 107 1876-81

(9.3) Ref "5" is different format: "While the neural progenitors derived using this protocol are initially restricted to a neurogenic fate, continued passaging in the presence of FGF and EGF leads to loss of neurogenic potential and gain of glial specification (GPCs) as previously described5"

(9.4) Play down interpretation on Page 9 "These results suggest that overexpression of many of the miRNA targets is likely to block differentiation" as only four genes were overexpressed and you present only two positive hits.

(9.5) Figure 4B - explain in legend what the dots are?

(9.6) In methods: A library of 570 miRNA mimics described previously was transfected into Dgcr8 KO GPCs plated in 96 well format in proliferation conditions (+EGF/FGF). What are the differences in the transfection protocol relative to transfection of ESCs as in (Wang et al, 2008)

(9.7) "Surprisingly, levels of the most abundant miRNAs were largely unchanged following 48 hours of differentiation, even though the cells have taken on an astrocyte morphology and express GFAP and other markers of differentiated astrocytes (Fig.3A, 1C-D)." GFAP is the only marker presented.

Referee #2:
Shenoy et al investigated the involvement of miRNA in astrocyte differentiation. They performed a series of elegantly designed experiments and showed the importance of let7 and mir125 miRNAs in repressing a network of inhibitory genes for astrocyte differentiation. Although all the experiments are performed in vitro, they carried out bioinformatical analysis of let7 and mir125 target genes in the in vivo differentiation of astrocytes and in glioma. The amazing correlation of let7 and mir125 target gene changes in astrocyte development and in glioma supports a role of let7 and mir125 in astrocyte differentiation in vivo. The strength of this manuscript includes the importance of the question, the novel finding of the involvement of miRNAs in astrocyte differentiation, and the painstaking screen that identified 2 miRNAs regulating astrocyte differentiation programs. The experiments are nicely designed, the data are convincing, and the text is clearly written. This manuscript has my full support.

Minor issues:
1. The acronym KD (knock down?) appeared for the first time in the abstract, where it should be spelled out.
2. Introduction, 1st paragraph, "very little is known about molecular mechanisms underlying the specification and differentiation from glial precursors during development". It is necessary to cite and briefly mention a few important publications on this topic, for example, previous work on JAK/STAT, Sox9, NF1a/b from Anderson, Deneen, etc.
3. The authors wrote "The Dgcr8Δ/Δ GPCs showed a striking inability to upregulate GFAP or develop the characteristic astrocytic morphology" but Fig.1C is too small to show the morphology of the cells. A zoomed-in figure is needed.
4. The species of the embryonic stem cells used in this study should be noted. Is it from human?
5. Figure 3A, in addition to the proportion of specific miRNAs in total miRNA, whether the levels of the specific miRNAs change during astrocyte differentiation should be described.
6. Figure 4D, how does let7 and mir125 rescued cells compare with Dgcr8 wildtype cells? Is it a full or partial rescue? This information should not be neglected.
7. Page 9, 2nd paragraph, Fig.6E should be Fig.6D. 3rd paragraph, Fig.6F should be Fig.6E.

Referee #3:

In this manuscript, Showy et al investigate the role of microRNAs during astrocyte differentiation in vitro, using a loss of function in the processing machinery DGCR8. These defects can be in part rescued by activation of the JAK,STAT pathway. Interestingly, in an unbiased screening, they identify miR-125 and let-7 as microRNAs that can rescue part of the defects observed during differentiation, although they are not able to rescue the loss of the JAK-SAT signaling and are not able to induce astrocyte differentiation, indicating that they are required but not sufficient to induce differentiation. The authors identify a set of targets misregulated in the loss of function for DGCR8, that are downregulated when adding back miR-125 or let 7 and have miRNA seed sequences for these miRNAs, although the statistical significance of this overlap is unclear in the current manuscript. In summary this manuscript provide insight into the role of miRNAs during astrocyte differentiation, and uses a clever approach previously develop by the Blelloch laboratory to identify miRNAs that can rescue these defects and their putative targets. Finally, they link the set of targets regulated by these miRNAs in vitro to differentiation in vivo and misregulation of gene expression during malignant glioma. In summary, this manuscript provides interesting insights into the function of miRNAs during astiocyte differen

1) At the moment it is unclear whether the overlap between different groups in the various comparisons (Fig 6a, 6c) and the enrichment (fig 3c) arc statistically significant, different from what one might expect by chance. The authors should calculate what the overlap would be on randomly selected set of genes, showing the number of genes that fall within each part of the venn diagram. For target site enrichment, it is also unclear whether enrichment for seed regions is different from what would be expected by chance, this could be done using Sylamer (Enright Lab) or calculating the pValue for (seed for specific miRNA in the transcriptome, compared to the seed for all miRNAs in the transcriptome and the seeds identified in the experimental set for a given miRNA vs the seeds identified in the same experimental set for all miRNAs). This will provide two values, an enrichment and a pvalue associated to it. In particular, it seems that the seed for miR-125 is not enriched?

2) it is unclear how these findings relate to the role of the miRNA pathway during astrocyte differentiation in vivo. 'lb address this, the authors could analyze the phenotype of DGCR8 Knock out in astnacyte precursors using a specific Cre.

3) it is unclear how the microRNAs that are expressed before and after differentiation are actually modulating this process. Are they primarily regulating a new set of targets that are only induced upon differentiation?

Minor points:

4) For the non-afficionado, it would be useful to include the seed sequence of the miRNAs being discussed.

5) The authors state "Globally, mRNAs upregulated by let'? and miR-125 in Dger8D/D GPCs were upregulated during normal astrocyte maturation (Fig.6E)." what is the overlap and non overlapping and what is the significance, this should be shown in a ven diagram?

6) The authors need to include additional references for this statement "Previous studies have shown that the addition of single miRNAs is sufficient to rescue individual phenotypes seen with global miRNA loss" For example, the Schier Laboratory was one of the first ones that showed a rescue of a global loss of dicer function by adding miR-430 (Giraldez et at 2005).
7) The authors state "In sum, these miRNAs are functioning through multiple targets simultaneously to regulate astrocyte differentiation" Given the current results, I would recommend the authors to soften that statement: the miRNAs in question do not change during differentiation, and it could still be one other target that single handily causes the observed phenotype. It might be more appropriate to phrase it "is likely or our results are consistent with a model where these miRNAs function to regulate multiple targets simultaneously to allow astrocyte differentiation, but we cannot exclude that..."

8) this sentence should probably be corrected "it has been proposed that misregulation of astrocytes can lead to the formation of malignant glioma." misregulation of astrocyte what? proliferation? differentiation? both?

Referee #1:

Blelloch lab has published very exciting papers in recent years on miRNA in embryonic stem cell differentiation and Dr. Blelloch is highly appreciated in the field. In a work entitled 'Let-7 and miR-125 cooperate to prime progenitors for astrogliogenesis' by Archana Shenoy, Muhammad Danial and Robert Blelloch the authors suggest that miRNAs are involved in the molecular mechanisms underlying the specification and differentiation of glial precursors into astrocytes.

The key findings of the current work are: glial progenitor cells lacking microRNAs failed to differentiate into astrocytes, reintroduction of let-7 or miR-125 rescued differentiation and functioned with JAK-STAT signaling, a known regulator of astrogliogenesis. Overexpression of specific miRNA targets in wild type GPCs blocked differentiation.

The main concerns are:
(1) Let-7 and miR-125 are broadly expressed in many differentiated tissues and were not sufficient to introduce differentiation in proliferating Dgcr8Δ/Δ GPCs. Therefore, authors should seek ways to show miR-125 and Let-7 specificity for astrocytosis/astrocyte lineage and hence to demonstrate that they are not acting as general and non-specific differentiation promotion factors.

We did not mean to suggest that let-7 and miR-125 are specific to the astrocytosis/astrocyte lineage. Very few miRNAs are specific to a single lineage. We find the fact that let-7 and miR-125 are used over and over again in development to regulate cell fate decisions very exciting. It is also very interesting that these miRNAs can have very different targets in different developmental contexts. As this reviewer points out in comment 7.1, let-7 has been shown to target N-myc, Tlx, Igfbp1, Hmga2 and Lin28 in other contexts. These genes are basically not even expressed in the GPC context. We have changed the text to make these interesting points more clear. It should be noted that in some contexts, miR-125 has been shown to promote stemness (Zhang et al, 2011; Guo et al, 2010). Thus, while these miRNAs are used in many different settings, they are used in different ways.

(2) Many efforts have been put in the work into target analysis including a big knockdown screen. While these efforts are appreciated, the successful analysis of relevant targets that will actually establish a 'network' downstream of two miRNAs requires additional work with more satisfactory experimental target endpoint.

We agree that this would require much additional work that is beyond the scope of this manuscript. We have edited the text to soften the claim that the effect of the miRNAs is mediated through a network of targets. We did try some combinatorial siRNAs experiments as we had done in previous work on miRNA roles in reprogramming (Judson et al, 2013). However, unfortunately the GPCs were very sensitive to transfection of multiple siRNAs making the experiments technically challenging. Going forward, we agree dissecting the functional interactions between targets is an important and exciting area of pursuit and is becoming increasingly feasible with new technologies such as CRISPR.
(3) There is no evidence for cooperative miRNA or target activity throughout the manuscript. Authors should rephrase text from the title down without this claim or add data that supports cooperative activity. In fact by comparing the rescue of GFAP with introduction of let-7 and miR-125 into Bax/Bak/Dgcr8 TKO GPCs (Fig. 4C) to adding let-7 + miR-125 together (Fig.E4A) the effect seems negative and counterproductive.

We apologize for not providing clear evidence regarding the cooperativity of let-7 and miR-125. The data in Fig.4C and E5A (previously E4A) cannot be compared as transfection efficiency is inherently variable across experiments and cell lines. We have added data in Figure 4 showing that when comparing within experiments, the addition of let-7 and miR-125 together leads to increased percentage of GFAP positive cells relative to let-7 or miR-125 alone (>2-fold increase). Issues of target cooperativity were discussed in previous point.

(4) To substantiate the view of astrocyte differentiation, characterize the astrocytes gained by miRNA overexpression better:

(4.1) immune-detect additional common marker(s) at protein level, e.g., S100beta. Additional potential markers might be considered: vimentin, brain-lipid-binding protein; astrocyte-specific glutamate transporter

We have included data in Figure E5 showing the expression of Vimentin in both the control cells as well as Dgcr8 KO cells rescued by let-7 and miR-125. We have also added qPCR data showing two additional markers are increased with addition of let-7 and miR-125 in Dgcr8 KO GPCs.

(4.2) Explain better or revise particular mRNA markers used in Figure 4D vs. 1F: Of the four mRNA markers in 4D, two do not change or are downregulated (Cyp4f14, Atp1a2), two markers from Figure 1 are not shown (AldoC, Pla2g7) and two that are up-regulated x200-300 fold in 1F are up-regulated only 5-25x (Aqp4, GFAP). Please discuss these differences and provide one consistent set of mRNA markers.

We apologize for not being clear in our explanation of the qPCR. The markers that are not affected by loss of miRNAs (Figure 1, AldoC and Pla2g7) are also unchanged with addition of let-7 and miR-125 relative to Mock treatment as expected. The fact that Cyp4f14 and Atp1a2 are unchanged/downregulated by addition of let-7 and miR-125 suggests that the miRNAs do not rescue all aspects of the differentiation program. Similarly, the incomplete rescue of Aqp4, GFAP as well as other markers we have added in this resubmission (Figure 1 and Figure 4, ApoE, Clu) may be due to low transfection efficiency resulting in <100% of cells expressing GFAP or due to the inability of let-7 and miR-125 to completely rescue differentiation. All these points have been clarified in the text.

(4.3) Perform similarity / nearest neighbor search to show that your cells display higher similarity to astrocytes then other cells reported by in Cahoy et al., including neurons, and oligodendrocytes (2008): a new resource for understanding brain development and function. J. Neurosci. 28: 264-278. Compare your profile also to Ditte Lovatt and Maiken Nedergaard (The Astrocyte Transcriptome, in Neuroglia 2013 Neuroglia edited by Helmut Kettenmann, Bruce R. Ransom Oxford University press)

see answer to 4.4

(4.4) Preclude neuronal or oligodendrocyte fate: Are the cells β-III-tubulin-neg (non-neurons) and O4-neg (non oligodendrocyte)?

We appreciate the reviewer’s comments regarding comparison of in vitro-derived astrocyte transcriptome to data from oligodendrocyte and neurons. Due to the inherent difficulties of performing nearest neighbor analysis with data from multiple array platforms, we have chosen instead to show expression levels of astrocyte genes relative to oligodendrocyte and neuronal genes in in vitro-derived cells (Figure E1). The list of astrocyte-enriched genes was created by combining the two sources listed by the reviewer. The data shows higher expression of astrocyte markers relative to oligodendrocyte or neuronal markers in WT ESC-derived GPCs.
Furthermore, we have included data in Figure E5B showing that let-7/miR-125-rescued Dgcr8 KO GPCs do not express MBP (oligodendrocyte) or Tuj1 (neuronal) markers.

(4.5) suggested only: (4.4) Classify by revisiting your mRNA expression data the gained astrocytes e.g., Type 1: Ran2+, GFAP+, FGFR3+, A2B5-, OR Type 2: A2B5+, GFAP+, FGFR3-, Ran 2-, EAAT1/SLC1A3+, EAAT2/SLC1A2+ OR mGluR+, AMPA+.

The mRNA data shows that they are GFAP+, FGFR3+, and highly express SLC1A3. However, we do not feel comfortable overstating this data and subclassifying the type of astrocytes.

(5) Cell death: Add quantification to the moderate/variable amount of cell death during differentiation of Dgcr8Δ/Δ GPCs. In Figure 1E are cell numbers the same after astrocyte differentiation (+serum). If Bax/Bak are blocking apoptosis, cells might still die by necrosis / autophagy etc.

We have added data in Figure E2 to quantify increased levels of apoptosis in Dgcr8 KO cells during differentiation using Annexin/Sytox Blue flow cytometry. As for alternative forms of cell death, we do not see any visual evidence and if anything, as shown in Figure 1E, there is an increase in the number of TKO cells after differentiation, just as in Bax/Bak double knockout cells. We make this more clear in the text.

(6) Bioinformatics
(6.1) Figure 3: If "levels of the most abundant miRNAs were largely unchanged following 48 hours of differentiation (Fig 3A, IC-D)", then how/why targets of expressed miRNAs respond (by upregulation)? Why is the mRNA of Dgcr8Δ/Δ be different from control Dgcr8f/Δ GPCs? at what time after induction of Dgcr8Δ/Δ was RNA harvested?

We apologize for any confusion in the text. The upregulation of targets of expressed miRNAs is already seen in the undifferentiated state upon loss of miRNAs as they are functional in GPCs. We have clarified.

(6.2) Figure 3C: Among upregulated genes, enriched motifs were uncovered (p<0.01). Four of the most highly expressed miRNA families, let-7, miR-9, miR-181 and miR-30 were within the 104 enriched motifs that perfectly matched miRNA seed region (Fig.3C, Table E1). Provide statistical significance to these data and reject the null hypothesis that the motifs of highly expressed miRNAs can be found at random within the 104 motifs.

We appreciate the feedback and have performed a fisher's exact test to determine the probability of the top miRNA seed matches appearing at random (p<2.2E-16). The corresponding text has been updated.

(6.3) Target informatics: provide direct global analysis of targets that were upregulated in the Dgcr8Δ/Δ GPCs and could be reversed by simple addition of either let-7 or miR-125 by analyzing together data in Figure 5 relative to data in Figure 3A. Are the correlated changes statistically significant?

This data is presented in Figure 5C.

(7) targets
(7.1) As way of supporting cooperativity, consider the overexpression and knockdown of several candidates: N-myc; Hmga2; Tlx; Igfbp1; Lin28 based on published works: Sanosaka et al identified N-myc and Hmga2, two important targets of Let-7, to restrict astrocyte differentiation (Neuroscience. 2008 Aug 26;155(3):780-8).
Zhaoa et al shown that let-7 increased astroglial differentiation by targeting nuclear receptor Tlx (Neuroscience. 2008 Aug 26;155(3):780-8).
Nishino et al, 2013 shown that let-7 promote the switch between neurogenic and gliogenic potential of neural stem cells during fetal development by repressing Igfbp1, Hmga2 and Lin28.

Interestingly, these mRNAs are largely lowly/not expressed in GPCs suggesting that miRNAs are acting via separate targets to promote astrocyte differentiation in the context of glial-specified
progenitors. See response to point 1.

(7.2) explicitly reveal the position of N-myc; Hmga2; Tlx; Igfbp1; Lin28 at mRNA scatter plots in Figure 3B and Figure 5A,B (or in a new supplementary figure).

A number of the let-7 targets listed by the reviewer identified in early stem cell populations (N-myc; Hmga2; Igfbp1; Lin28) are excluded from Figure 3B and Figure 5A,B because they are undetected based on a detection p-value cutoff of 0.05. We have included a table in supplementary figure 5 showing the average detection p-values for these probes. However, we appreciate the need to show the positions of known targets. To this end, we have added data in Supplementary Figure 5 showing that with the exception of TLX, a number of other known let-7 targets identified in more differentiated cell types such as skeletal muscle and fibroblasts are expressed in GPCs and downregulated in KO cells upon addition of let-7.

(7.3) As way of supporting cooperativity, consider trying knocking down more than a single target e.g., knockdown both Plagl2 and Imp2, as they have effect if overexpressed.

As described above, multiple siRNA transfections induced too much toxicity. Therefore, alternative strategies such as CRISPR will be needed, which is beyond the scope of this paper.

(8) Textual changes and suggested points for discussion:
(8.1) Discuss: Wen Hao Neo et al., MicroRNA miR-124 Controls the Choice between Neuronal and Astrocyte Differentiation by Fine-tuning Ezh2 Expression (2014 The Journal of Biological Chemistry, 289, 20788-20801) in the context of your Fig.6C and Fig.E5.

Discussion of Ezh2 has been removed based on other changes in text.

(8.2) Discuss: Zhaoa et al as these authors have shown that Transfection of let-7b increased astroglial differentiation in control neural stem cells, by targeting nuclear receptor TLX signaling. PNAS 2010 107 1876-81.

added

(8.3) Discuss: STAT3 signaling importance to both pluripotency and astrocytosis. Might let-7 and miR-125 be contributing to uncoupling of stemness cues (Niwas et al Genes & Dev. 1998. 12: 2048-2060) from astrocyte differentiation cues (Bonni 1997 Science 278 477-483)?

We do reference the Bonni paper. The Niwas paper is in embryonic stem cells. We feel uncomfortable speculating on the possible uncoupling of stemness cues as we have no data directly relevant to this question.

(8.4) Discuss or rephrase JAK-STAT/miRNAs: The introduction of JAKi to the differentiation media along with transfection of let-7 and miR-125 completely blocked the ability of the miRNAs to rescue GFAP expression in Dgcr8Δ/Δ cells. Since blocking JAK signaling overrode miRNA activity, these data suggest that the two molecular pathways converge in GPC differentiation. One possibility is that let-7/miR-125 introduction is upstream of activation of JAK-STAT and hence JAKi abrogates the effect of the miRNAs. The molecular components do not necessarily act in parallel.

We originally really liked the idea that let-7/miR-125 is upstream of JAK-STAT. That would have led to a simple model. However, introduction of the miRNAs did not lead to increased pStat3. For this reason, it appears that the two pathways are acting in parallel and are interdependent. We make this more clear in the text.

(8.5) Discuss: use of Bax/Bak in the context of Chang et al., 2007 Mol Cell Biol. 274293-305 Bcl-XL/Bax proteins direct the fate of embryonic cortical precursor cells.

We thank the reviewer for bringing this study to our attention. Interestingly, another study using Bax/Bak double knockout cortical precursor cells showed no obvious defect in generation of astrocytes or neurons (Thomson et al. 2003. J. Neurosci). Comparing the two studies the major
difference is the Thomson study added factors known to promote astrocyte differentiation in vitro (FBS, which induces BMP signaling), similar to our studies. We are unsure of the basis of the contradicting phenotype of the Chang study, but given their use of different conditions, we did not feel it worthwhile discussing in this paper. It may have something do with developmental state of their neural progenitors, but we can not be sure and would hate to hand-wave. Importantly, differentiation appears normal in our Bax/Bak knockout GPCs (Fig. 1E).

(8.6) The bioinformatics of tumors (Figure 6E) is not contributing much to the main theme of the work and in fact diffuses the focus

While we agree that the biology of tumors is not directly relevant to the main theme of the work, we feel that this data provides important added relevance for both the study of differentiation-promoting miRNAs as well as for the in vitro model shown here. Furthermore, the other reviewers identify these analyses as an added strength of the paper. Therefore, we would hate to have to remove it.

(9) Minor:
(9.1) Rephrase: "In this study, we sought to overcome this roadblock by using the in vitro counterpart to in vivo astrocyte differentiation, allowing the production of homogenous populations of glial progenitor MicroRNAs in astrocyte differentiation cells (GPCs) that could then be induced to differentiate in synchrony into astrocytes under defined culture conditions (B्रustle & McKay, 1999). Since in fact the roadblock to using in vitro counterpart was overcome by Brűstle & McKay, 1999"

We have rephrased this sentence to make it clear that we are using the protocol established by Brustle and McKay.

(9.2) Rephrase: "This work provides the first evidence for the role of specific miRNAs and their target gene networks in astrogliogenesis " and "provide the first detailed characterization of how miRNAs promote the GPC to astrocyte" and refer to previous works including Wen Hao Neo et al., 2014 The Journal of Biological Chemistry, 289,20788-20801 and Zhaoa e et al PNAS 2010 107 1876-81

The above mentioned work is performed in the setting of adult multipotent neural stem cells (Zhao et al) and the neurogenic to gliogenic switch (Wen Hao Neo). Like work from the Morrisson and Lowry labs, we believe that these studies provide important insight into the balance between neurogenesis and gliogenesis. However, our work represents the first setting of studying astrocyte differentiation post-specification. That is, the loss of miRNAs and destabilization of astrocyte fate does not lead to activation of neuronal fate. We adjusted the text to make this point more clear.

(9.3) Ref "5" is different format: "While the neural progenitors derived using this protocol are initially restricted to a neurogenic fate, continued passaging in the presence of FGF and EGF leads to loss of neurogenic potential and gain of glial specification (GPCs) as previously described5"

We have edited the text to correct this.

(9.4) Play down interpretation on Page 9 "These results suggest that overexpression of many of the miRNA targets is likely to block differentiation" as only four genes were overexpressed and you present only two positive hits.

We agree with this point and have edited the text to reflect the fact that we show two positive hits.

(9.5) Figure 4B - explain in legend what the dots are?

Each dot represents a well in the screen. We apologize for the missing information and have updated the figure legend.

(9.6) In methods: A library of 570 miRNA mimics described previously was transfected into Dger8 KO GPCs plated in 96 well format in proliferation conditions (+EGF/FGF). What are the differences in the transfection protocol relative to transfection of ESCs as in (Wang et al, 2008)
The main difference in the transfection protocol is the use of the transfection reagent DharmaFECT3 which we found to have maximal transfection efficiency in GPCs (vs DharmaFECT1 in ESCs) and the use of mimics at 50 nM concentration (vs 100 nM). We have updated the text in the Methods to include these details.

(9.7) "Surprisingly, levels of the most abundant miRNAs were largely unchanged following 48 hours of differentiation, even though the cells have taken on an astrocyte morphology and express GFAP and other markers of differentiated astrocytes (Fig.3A, 1C-D)." GFAP is the only marker presented.

We have updated the text to reference additional figures that show upregulation of astrocyte markers at the RNA level in addition to GFAP.

Referee #2:

Shenoy et al investigated the involvement of miRNA in astrocyte differentiation. They performed a series of elegantly designed experiments and showed the importance of let7 and mir125 miRNAs in repressing a network of inhibitory genes for astrocyte differentiation. Although all the experiments are performed in vitro, they carried out bioinformatical analysis of let7 and mir125 target genes in the in vivo differentiation of astrocytes and in glioma. The amazing correlation of let7 and mir125 target gene changes in astrocyte development and in glioma supports a role of let7 and mir125 in astrocyte differentiation in vivo. The strength of this manuscript includes the importance of the question, the novel finding of the involvement of miRNAs in astrocyte differentiation, and the painstaking screen that identified 2 miRNAs regulating astrocyte differentiation programs. The experiments are nicely designed, the data are convincing, and the text is clearly written. This manuscript has my full support.

Minor issues:
1. The acronym KD (knock down?) appeared for the first time in the abstract, where it should be spelled out.

This has been corrected.

2. Introduction, 1st paragraph, "very little is known about molecular mechanisms underlying the specification and differentiation from glial precursors during development". It is necessary to cite and briefly mention a few important publications on this topic, for example, previous work on JAK/STAT, Sox9, NFIa/b from Anderson, Deneen, etc.

We agree with the reviewer that it is important to mention these publications. We have updated the introduction to highlight these studies. Due to space limitations, we selected an excellent review that covers these topics. However, if the editor and reviewer prefer the original references, we are happy to update the text. The only problem is that if we cover all of papers, it would be a long reference list.

3. The authors wrote "The Dgcr8Δ/Δ GPCs showed a striking inability to upregulate GFAP or develop the characteristic astrocytic morphology" but Fig.1C is too small to show the morphology of the cells. A zoomed-in figure is needed.

We have edited this statement as we do not have immunofluorescence data to compare detailed morphology of Dgcr8 KO cells to control. However, we have added zoomed in images of control and let7/125-rescued knockout cells in Fig.E5E to show that both GFAP expression and localization are faithfully recapitulated with addition of miRNAs.

4. The species of the embryonic stem cells used in this study should be noted. Is it from human?

The embryonic stem cells used here are derived from mouse. We apologize for the confusion and have updated the text.

5. Figure 3A. in addition to the proportion of specific miRNAs in total miRNA, whether the levels of the specific miRNAs change during astrocyte differentiation should be described.
The reviewer brings up a very important point. We normalized the miRNA reads to total reads mapping to tRNAs, repeat RNAs and mRNAs and added a new Figure with the data (Figure E4). The data is consistent with the conclusions from the main figure.

6. Figure 4D, how does let-7 and mir125 rescued cells compare with Dgcr8 wildtype cells? Is it a full or partial rescue? This information should not be neglected.

Based on the qPCR data in Figure 4, we believe that let-7 and miR-125 do not fully rescue all aspects of astrocyte differentiation as all markers are not rescued during differentiation. In terms of the extent of upregulation of markers such as GFAP, it is difficult to compare directly to wild-type cells due to differences in confluency and transfection. We make these issues more clear in the text.

7. Page 9, 2nd paragraph, Fig.6E should be Fig.6D. 3rd paragraph, Fig.6F should be Fig.6E.

This is corrected.

Referee #3:

In this manuscript, Showy et al investigate the role of microRNAs during astrocyte differentiation in vitro, using a loss of function in the processing machinery DGCR8. These defects can be in part rescued by activation of the Jak,STAT pathway. Interestingly, in an unbiased screening, they identify miR-125 and let-7 as microRNAs that can rescue part of the defects observed during differentiation, although they are not able to rescue the loss of the JAK-SAT signaling and are not able to induce astrocyte differentiation, indicating that they are required but not sufficient to induce differentiation. The authors identify a set of targets misregulated in the loss of function for DGCR8, that are downregulated when adding back miR-125 or let-7 and have miRNA seed sequences for these miRNAs, although the statistical significance of this overlap is unclear in the current manuscript. In summary this manuscript provide insight into the role of miRNAs during astrocyte differentiation, and uses a clever approach previously develop by the Blelloch laboratory to identify miRNAs that can rescue these defects and their putative targets. Finally, they link the set of targets regulated by these miRNAs in vitro to differentiation in vivo and misregulation of gene expression during malignant glioma. In summary, this manuscript provides interesting insights into the function of miRNAs during astrocyte differentiation, providing insight into the defects observed in vivo.

1) At the moment it is unclear whether the overlap between different groups in the various comparisons (Fig 6a, 6c) and the enrichment (fig 3c) are statistically significant, different from what one might expect by chance. The authors should calculate what the overlap would be on randomly selected set of genes, showing the number of genes that fall within each part of the venn diagram. For target site enrichment, it is also unclear whether enrichment for seed regions is different from what would be expected by chance, this could be done using Syamer (Enright Lab) or calculating the pValue for (seed for specific miRNA in the transriptome, compared to the seed for all miRNAs in the transcriptome and the seeds identified in the experimental set for a given miRNA vs the seeds identified in the same experimental set for all miRNAs). This will provide two values, an enrichment and a p value associated to it. In particular, it seems that the seed for miR-125 is not enriched?

We appreciate the insightful comments regarding the bioinformatics. Upon further analysis, we found a statistically significant overlap of mRNAs downregulated by let-7 and miR-125, further strengthening our argument for cooperative activity and using overlapping mRNAs as one of the criteria in Fig.6A. We have edited the text to better explain the rationale for criteria used for overlap in Figure 6 and added a panel in Fig.E7 to show that the overlap between let-7 and miR-125 downregulated genes is statistically significant.

For target site enrichment in Figure 3C, we have performed a Fisher’s exact test to determine the probability that the seeds of highly expressed miRNAs would randomly occur in the enriched motifs. As the reviewer pointed out, the seed for miR-125 is not enriched in mRNAs upregulated in Dgcr8 knockout. The reasons for this are unclear – for example, miR-125 may have few direct targets, thus rendering it difficult to see an enrichment in a global analysis. The addback experiment
shows that enrichment for miR-125 seed is not as high as enrichment for let-7 seed (Figure 6C).

2) it is unclear how these findings relate to the role of the miRNA pathway during astrocyte differentiation in vivo. ‘lb address this, the authors could analyze the phenotype of DGCR8 Knock out in astrocyte precursors using a specific Cre.

While we appreciate the insight that could be gained from in vivo experiments, these experiments are complicated by a lack of astrocyte precursor-specific Cre. We hope to pursue these experiments in the future as new cre lines or alternative means of glial progenitor specific deletion are developed, but believe they are beyond the scope of the current paper.

3) it is unclear how the microRNAs that are expressed before and after differentiation are actually modulating this process. Are they primarily regulating a new set of targets that are only induced upon differentiation?

We apologize if the text was unclear. Our data suggests that let-7 and miR-125 are expressed abundantly prior to differentiation suggesting that they serve to downregulate a set of genes in GPCs that are inhibitory to differentiation when overexpressed. Many of these targets are further downregulated in vivo during differentiation likely through a combination of transcriptional and post-transcriptional mechanisms. We have made this more clear in text.

Minor points:
4) For the non aficionado, it would be useful to include the seed sequence of the miRNAs being discussed.

We apologize that this relevant information is absent and have included it in Figure E7A.

5) The authors state that "Globally, mRNAs upregulated by let? and miR-125 in Dgcr8D/D GPCs were upregulated during normal astrocyte maturation (Fig.6E)." what is the overlap and non overlapping and what is the significance, this should be shown in a ven diagram?

Figure 6E essentially shows the overlap of the genes. We believe that a venn diagram would be less informative in this regard due to genes with small increases in expression not passing statistical cutoffs. In Figure 6E, we see how the whole population changes when subsetted according to target information.

6) The authors need to include additional references for this statement "Previous studies have shown that the addition of single miRNAs is sufficient to rescue individual phenotypes seen with global miRNA loss" For example, the Schier Laboratory was one of the first ones that showed a rescue of a global loss of dicer function by adding miR-430 (Giraldez et at 2005).

We apologize for overlooking this reference and have added it to the current version of the text.

7) The authors state "In sum, these miRNAs are functioning through multiple targets simultaneously to regulate astrocyte differentiation" Given the current results, I would recommend the authors to soften that statement: the miRNAs in question do not change during differentiation, and it could still be one other target that single handily causes the observed phenotype. It might be more appropriate to phrase it "is likely or our results are consistent with a model where these miRNAs function to regulate multiple targets simultaneously to allow astrocyte differentiation, but we cannot exclude that..."

We agree that we do not provide sufficient data to make such as strong conclusion. We have changed the text as per the reviewer’s suggestion.

8) this sentence should probably be corrected "It has been proposed that misregulation of astrocytes can lead to the formation of malignant glioma." misregulation of astrocyte what? proliferation? differentiation? both?

We have corrected the text to avoid ambiguity.
Thank you for submitting a revised version of your manuscript to The EMBO Journal. Your study has now been seen by two of the original referees whose comments are shown below. As you will see, they both find that all main criticisms have been sufficiently addressed and consequently recommend your study for publication here. However, before we can proceed to officially accept your manuscript, I have to ask you address the following editorial concerns:

- Please provide a short paragraph stating author contributions
- Please provide database accession numbers for microarray and RNA seq data
- Please include scale bars in all IF images as well as an indication of the scale bar size in the relevant figure legends

We also noticed that several bar charts display error bars based on two replicas only (figs 2 and 6, as well as several supplemental figures) and to avoid a depiction of underpowered statistics we would ask you to present this data as two separate sets of data points rather than an average with the error bar indicating range. Please refer to our author guidelines for a more detailed outlined of our requirements for statistics. We would also ask you to indicate the nature of the error bars displayed in figs 4 and 5.

I would like to invite you to submit a final revision of the manuscript incorporating the points listed above. Please feel free to contact me with any questions regarding this.

Thanks again for sending this work to The EMBO Journal, I look forward to receiving the final revision.

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REFEREE COMMENTS

Referee #1:

The work reads well and my concerns are addressed.

It is EMBOJ policy to allow for one major round of revision only and therefore because I support its acceptance, I leave some concerns aside. I think that overall the work should be published in EMBO J.

Nonetheless, as there is little evidence for cooperative miRNA or target activity throughout the manuscript (data in Figure 4D, E7D), it may be better to avoid the term and rephrase the claims.
Referee #3:

The authors have satisfied the reviewers criticisms and uncover an interesting biological problem in astrocyte differentiation. The relevance in vivo remains a question, but it is understandable that is beyond the scope of this study.

One stylistic feedback, the frames in the plots seem unnecessary, the panels within the figures are misaligned and the shadow in plot 4E is unnecessary.

2nd Revision - authors’ response 15 January 2015

We thank the reviewers for their helpful comments throughout the review process. The result is a stronger manuscript.

Referee #1:

The work reads well and my concerns are addressed.

It is EMBOJ policy to allow for one major round of revision only and therefore because I support its acceptance, I leave some concerns aside. I think that overall the work should be published in EMBO J.

Nonetheless, as there is little evidence for cooperative miRNA or target activity throughout the manuscript (data in Figure 4D, E7D), it may be better to avoid the term and rephrase the claims.

We agree that we have little evidence of cooperativity between targets. Thus, we included text to make it clear that there exist alternative explanations for the failure of knockdown of any one target to rescue differentiation. We are also very cautious in our wording when suggesting the possibility of cooperation.

Referee #3:

The authors have satisfied the reviewers criticisms and uncover an interesting biological problem in astrocyte differentiation. The relevance in vivo remains a question, but it is understandable that is beyond the scope of this study.

Yes, we look forward to performing future in vivo experiments. As the in vivo glial progenitor population becomes better defined and tools are developed, these experiments should become more straightforward.

One stylistic feedback, the frames in the plots seem unnecessary, the panels within the figures are misaligned and the shadow in plot 4E is unnecessary.

We have reformatted figures to remove frames around the graphs and removed the shadow in Plot 4E. We have also attempted to realign figures that we noticed contained misaligned panels. However, we imagine the editorial process will move the panels around and realign.

3rd Editorial Decision 16 January 2015

Thank you for submitting the final revision of the manuscript, I am pleased to inform you that your study has now been officially accepted for publication in The EMBO Journal.

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