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MiR-135b is a direct PAX6 target and specifies human neuroectoderm by inhibiting TGF /BMP signaling

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Editor: Thomas Schwarz-Romond

1st Editorial Decision 13 November 2013

Thank you very much for submitting your study describing a novel circuit for neuroectoderm development for consideration to The EMBO Journal editorial office.

I enclose comments from two referees that encourage significant further experimentation before being able to offer their crucial support for potential publication in our title.

As you will recognize, ref#1 asks for much better integration into lineage determining signaling events that would have to involve stringent loss-of function as well as an assessment how general the contribution of miR-135b actually appears to be.

Ref#2 similarly expects functional corroboration applying loss-of function strategies and strongly encourages delineation of the Pax6/miRNA enhancer/repressor relationship as well as their putative connection in a regulatory loop.

On face value, and assuming you might be in a strong position to be able to address these important points in a relatively timely and satisfactorily manner, I am happy to offer the chance for a single round of major and significant revisions.

Please do not hesitate to get in touch in case of further questions/to outline feasibility and timeline of planned experiments and/or to request additional time beyond the usual three month for requested experimental work (preferably via E-mail).

I am looking forward to receive your revised version and would be delighted to work with you towards eventual publication of your data.

REFEREE REPORTS:

Referee #1 (Report):

In this work, the authors investigate the genome-wide binding of the neural induction-driving gene PAX6 in hPSC-derived neuroectoderm. They then focus on one novel target, miR-135b, and show that it is positively regulated by PAX6, and that miR-135b overexpression in turn promotes neuroectoderm induction to some degree. It is proposed that miR-135b acts though inhibiting Activin and Bmp signaling, thereby promoting neural induction. The manuscript is well-written, nicely structured, and would be of interest to the stem cell readership of EMBO J. However, there are several concerns about the role and importance of miR-135b that would need to be clarified.

Major

- Looking at the authors' model of Fig. 5D, it seems like there is a sort of chicken and egg problem here. The pre-requisite and cause for neual induction is the lack of signaling pathway activation - which is true for the anterior pre-gastrulation epiblast in vivo as well as for in-vitro models such as the protocol used by the authors. Besides other mechanisms, this e.g. leads to OCT4 downregulation to release PAX6 from repression in the undifferentiated state (e.g. Rosa and Brivanlou, EMBO J 2011, 30, 237 - 248; Greber et al., EMBO J 2011, 30, 4874 - 4884). Hence, in principle, neuroectoderm is formed once PAX6 becomes upregulated and OCT4 is diminished. What would be the significance of the authors' model in which Smad inhibition - which is most UPSTREAM of the neural induction process - is a result of mirR-135 action DOWNSTREAM of PAX6? I do not agree that PAX6-positive cells would still have the option to differentiate e.g. do mesendoderm as the authors' model suggest. Perhaps miR-135b could be part of a feedforward loop reinforcing neural induction - but not causing it. However, in an in vivo or in vitro environment that is already characterized by lack of Smad pathway activation (or pathway inhibition), it seems questionable that miR-135b would actually be required. To clarify this, the authors should perform miR-135b loss-of-function experiments and see whether this compromises overall neural induction in their protocol - and/or if this causes medendoderm etc. differentiation as the model predicts.

- The authors do not seem to be too consistent in showing data of more than one cell line. However, in Fig. S4B, the induction of miR-135b upon neuroectoderm formation appears to be cell line-dependent. To rule out cell line-dependent effects, key experiments must be carried out with at least one more independent cell line - PAX6 overexpression and silencing, correlation of miR-135b and PAX6 induction (also see comment below), miR-135b overexpression. Does miR-135b overexpression have a consistent effect on repressing pluripotency genes, as it would be expected from its downregulation of Activin signaling?

Minor essential

- Fig. 1 In the text, I think the authors should tone down on the novelty of the neural induction protocols they used. Simultaneous inhibition of Activin and Bmp signaling using small molecules has been employed by several groups by now.

- Fig. S3 What about the PAX6 binding motif itself? Please clarify / discuss.

- Fig. 3E The authors used PAX6 overexpressin in NE cells to test whether miR-135b becomes induced. There are several points here that are not clear to me. First, PAX6 is already expressed in

NE cells - what is the point in overexpressing it there? Secondly, the fold expression of PAX6 seems very high considering the fact that PAX6 is already expressed in these cells. Compared to that, the induction of miR-135b is very modest - just ~80%. The authors should also test the effect of PAX6 overexpression on miR-135b in hPSCs which do not yet express PAX6 and miR-135b.

- In Fig. 4A, it cannot be well appreciated that PAX6 is striktly correlated with (or preceeding) miR-135b induction. The time-points shown seem arbitrary. The comparison should be performed at daily intervals from d0 until d8.

- Fig. 4B,C It is said that in Fig. 4B,C the cells were differentiated in an undirected fashion. Please indicate how exactly and for how long the cells were treated. Moreover, it is known that OCT4 is a strong repressor or PAX6. However, in the real-time PCR data of 4A, OCT4 is not downregulated at all. How can, in general, a strong induction of PAX6 under these conditions be explained?

- In Fig. 5B, it is unclear to me how the Luc data has been normalized. In Fig. S5 all controls were set to 1 but apparently this is not the case in Fig. 5B.

- Fig. 5: Mutagenesis of the miR-135b seed sequences suggested that it regulates mRNA levels of its targets. However, there is a discrepancy between hPSC RNA and protein data (Figs. 5C vs. D). This is particularly striking in light of the data in Fig. S5 (non-hESCs). This is confusing and should be discussed.

- Several figure legends could contain more information about the experiments. For example, in Fig. 5B and C it is not immediately clear which cell type has been used. Throughout the manuscript, information on the cell type, cell line name, time-point of analysis, and treatment conditions should be included in the figure legends.

- The work by Rosa and Brivanlou published in EMBO J should to be discussed (The EMBO Journal, 2011, 30, 237 - 248).

- Abbreviations, e.g. NECs (p6), should be defined upon first occurence

Referee #2 (Report):

This manuscript provided by Bhinge A et al introduces an additional regulatory network involved in human neuroectoderm development through the use of hESC-derived NECs in vitro. The finding suggests the synergistic function of transcription factors and miRNAs in lineage specification of hESC. Through the use of ChIP-sequencing, the authors further filtered the potential direct Pax6 targeted genes, including miRNAs, through the overlap of H3K27Ac and H3K27Me3 occupied regions. Amongst the many Pax6 activated miRNAs, miR-135b is verified to be a direct target by enhancer luciferase assay. The authors confirmed that the targets of miR-135b seem to be involved in BMP and Activn/Nodal signaling, and overexpression of mir-135b can promote NE specification, likely through the suppression of non-neural lineage differentiation. It is still unknown why Pax6 has a unique early function for human neuroectoderm induction, and this study provides evidence implicating the role of miR-135b in promoting neural specification and differentiation, and the possibility of this microRNA accounting for Pax6 function in human ESC derived neuroepithelium. However additional support should be included to strengthen this proposed TF-miRNA regulation network.

Major concerns:

1. All of the functional analyses of mir-135b and its targets are based on the overexpression approach. It is essential to reveal the role of mir-135b by knock-down approach to demonstrate that mir-135b/TGFb-Activin is required to specify neuorectoderm lineage.
2. As the major conclusion of this manuscript is that mir-135b is a direct target of Pax6, it not clear to what extent Pax6 function to promote neuroectoderm is via mir-135b. It will be interesting to see

if overexpression of mir-135b in Pax6off hESCs can still induce neuroectoderm,or if knocking down of mir-135b can suppress neuroectodem differentiation under 2i (or 3i) condition.
3. The authors argue that Pax6 can function either as an enhancer or a repressor and suggested that Pax6 functions as an enhancer to activate mir-135b expression. However, in fig 3E, the overexpression of Pax6 only induces the level of mir-135b mildly. It is possible that Pax6 might function as a repressor to derepress the mir-135b expression level. With the Pax6-VP16 and Pax6-En2 fusion protein, the authors should be able to dissect the relationship of Pax6 activity and mir-135b induction.
4. Overexpression of miR-135b led to increase Pax6 expression (Figure 4C). Is miR-135b and Pax6 in a regulatory loop? Or is this increase in Pax6 a consequence of the inhibitory function of miR-135b? In addition, the expression of mir-135b is upregulated from day 2 of differentiation, while activation of Pax6 starts from day 3 of differentiation (Fig4A). The authors should consider the reciprocal regulation between Pax6 and mir-135b during NE development.

Minor points:

Fig2A
With the focus on miRNA, it would be fit to incorporate a category or annotation of non-coding RNAs or miRNAs into the pie chart regarding the distribution of Pax6 binding sites.

Fig4B
The author proposed a model that mir-135b prevent differentiation towards mesoendoderm, trophectoderm or ectoderm through the inhibition of BMP and Activin signaling, but in Fig4B, overexpression of mir-135b did not suppress markers for endoderm (Lama3), mesoderm (brachyury and Acta2), endoderm (Sox17) and trophectoderm (Cdx2). The authors should explain this contradiction.

Fig4C
The resolution of immunostaining is poor. A higher magnification figure with quantification should be used.

In materials and methods, the new protocol for human ES neural induction is not clear. The authors should describe it in further detail. Also, all " " are mislabeled as "u" in this section.

Zhang et al (2010) demonstrated that Pax6 is required for the specification of human NE by downregulating pluripotent genes. Moreover, they found that Pax6a and not Pax6b is responsible for inducing NE-related genes and the overexpression of only Pax6a could direct hESCs towards a neural identity. It is not clear which Pax6 isoform or a non-discriminative Pax6 antibody was used in this study. Therefore, whether the binding sites of Pax6 are isoform-specific is uncertain and whether miR-135b is activated by Pax6a or Pax6b is also not addressed.

1st Revision - authors' response 14 March 2014

Referee #1 (Report):

In this work, the authors investigate the genome-wide binding of the neural induction-driving gene PAX6 in hPSC-derived neuroectoderm. They then focus on one novel target, miR-135b, and show that it is positively regulated by PAX6, and that miR-135b overexpression in turn promotes neuroectoderm induction to some degree. It is proposed that miR-135b acts though inhibiting Activin and Bmp signaling, thereby promoting neural induction. The manuscript is well-written, nicely structured, and would be of interest to the stem cell readership of EMBO J. However, there are several concerns about the role and importance of miR-135b that would need to be clarified.

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here. The pre-requisite and cause for neual induction is the lack of signaling pathway activation - which is true for the anterior pre-gastrulation epiblast in vivo as well as for in-vitro models such as the protocol used by the authors. Besides other mechanisms, this e.g. leads to OCT4 downregulation to release PAX6 from repression in the undifferentiated state (e.g. Rosa and Brivanlou, EMBO J 2011, 30, 237 - 248; Greber et al., EMBO J 2011, 30, 4874 - 4884). Hence, in principle, neuroectoderm is formed once PAX6 becomes upregulated and OCT4 is diminished. What would be the significance of the authors' model in which Smad inhibition - which is most UPSTREAM of the neural induction process - is a result of mirR-135 action DOWNSTREAM of PAX6? I do not agree that PAX6-positive cells would still have the option to differentiate e.g. do mesendoderm as the authors' model suggest.
Perhaps miR-135b could be part of a feedforward loop reinforcing neural induction - but not causing it. However, in an in vivo or in vitro environment that is already characterized by lack of Smad pathway activation (or pathway inhibition), it seems questionable that miR-135b would actually be required. To clarify this, the authors should perform miR-135b loss-of-function experiments and see whether this compromises overall neural induction in their protocol - and/or if this causes medendoderm etc. differentiation as the model predicts.

We performed the experiment that the reviewer has suggested where we knocked down miR-135b and assayed for neuroectodermal and non-neural lineage markers. We found that inhibition of miR-135b significantly impairs neuroectoderm differentiation while promoting non-neural differentiation (Fig 4D and 4E) (pg. 17). This suggests that miR-135b is required for neuroectoderm development. This data is consistent with the model the reviewer has suggested where Pax6 and miR-135b are part of a feed-forward loop that is required to reinforce neural induction and inhibition of any one component is enough to impair neural induction. The revised model has been depicted in Fig 5E.

- The authors do not seem to be too consistent in showing data of more than one cell line. However, in Fig. S4B, the induction of miR-135b upon neuroectoderm formation appears to be cell line-dependent. To rule out cell line-dependent effects, key experiments must be carried out with at least one more independent cell line - PAX6 overexpression and silencing, correlation of miR-135b and PAX6 induction (also see comment below), miR-135b overexpression. Does miR-135b overexpression have a consistent effect on repressing pluripotency genes, as it would be expected from its downregulation of Activin signaling?

The suggested experiments have now been performed in the H9 ES cell line. The results shown in Expanded View Figures E4, E5 and E6) and discussed on pgs. 15,16, 17, are consistent to those we reported based upon experiments using the H1 ES cells.

As suggested by the reviewer, we assayed the effect of miR-135b overexpression on pluripotency in H1 as well as H9 ES cells. MiR-135b overexpression caused downregulation of Nanog when overexpressed in H1 ES cells while miR-135b overexpression in H9 ES cells lead to downregulation of both Oct4 and Nanog (Fig. 4B and Expanded view Figure E6). This indicates that miR-135b does downregulate pluripotency signals when overexpressed. These results have been included in the Results section (pg 16).

Minor essential

- Fig. 1 In the text, I think the authors should tone down on the novelty of the neural induction protocols they used. Simultaneous inhibition of Activin and Bmp signaling using small molecules has been employed by several groups by now.

We agree with the reviewer and the suggested changes have been made in the document (pg. 9).

- Fig. S3 What about the PAX6 binding motif itself? Please clarify / discuss.

A section on Pax6 motif analysis has now been added to the results (pg. 13, 14).

- Fig. 3E The authors used PAX6 overexpressin in NE cells to test whether miR-135b becomes induced. There are several points here that are not clear to me. First, PAX6 is already expressed in NE cells - what is the point in overexpressing it there? Secondly, the fold expression of PAX6 seems

very high considering the fact that PAX6 is already expressed in these cells. Compared to that, the induction of miR-135b is very modest - just ~80%. The authors should also test the effect of PAX6 overexpression on miR-135b in hPSCs which do not yet express PAX6 and miR-135b.

We agree with the reviewer that Pax6 overexpression in NE cells was not an optimal experiment. As suggested by the reviewer, we overexpressed Pax6 in human ESCs (both H1 and H9) using lentiviral expression vectors and allowed cells to differentiate by withdrawal of growth factors. At the time cells were harvested (96 hours after initiating differentiation), Pax6 expression in the controls cells was low (as was evident by the high Ct values in the quantitative PCR data). Pax6 was induced about 200 fold using lentiviruses which activated miR-135b greater than 2-fold (Fig. 3E and Expanded view Figure E4) (pg. 15). A 2-fold activation of miR-135b under these conditions is not surprising in the context of Pax6 and miR-135b upregulation using the 2i method (Fig. 4A). When hESC were induced by the 2i method, Pax6 was activated more than 2000 fold whereas miR-135b was activated to a maximum of 15-fold (Fig. 4A). This indicates that a strong upregulation of Pax6 is required to activate miR-135b.

The revised experimental details on Pax6 overexpression in H1 and H9 ESC instead of NEC have been included in the Materials and Methods section. The relevant changes have also been made in Fig. 3E that now shows data for Pax6 overexpression in H1 ESC and Expanded View Figure E4A that displays data for Pax6 overexpression in H9 ESC. We have also modified the Results section (pg. 15) to include these changes.

- In Fig. 4A, it cannot be well appreciated that PAX6 is striktly correlated with (or preceeding) miR-135b induction. The time-points shown seem arbitrary. The comparison should be performed at daily intervals from d0 until d8.

As suggested, we repeated the experiment with more detailed time points in both H1 and H9 ES lines to highlight the correlation between Pax6 and miR-135b activation (Fig. 4A and Expanded view Figure E5) (pg. 15).

- Fig. 4B,C It is said that in Fig. 4B,C the cells were differentiated in an undirected fashion. Please indicate how exactly and for how long the cells were treated. Moreover, it is known that OCT4 is a strong repressor or PAX6. However, in the real-time PCR data of 4A, OCT4 is not downregulated at all. How can, in general, a strong induction of PAX6 under these conditions be explained?

ES cells were plated as single cells at a density of 50,000 cells/cm2, transfected and differentiated by culturing them in ES cell media without any growth factors i.e. DMEM:F12, 20% knock-out serum, 2mM L-glutamine, β-mercaptoethanol, 10uM ROCK inhibitor. Cells were harvested at day 6 and day 8. The additional details have now been included in the Materials and Methods section (pg. 7,8).

The reviewer has raised an important concern regarding the miR-135b overexpression data. We agree that Pax6 activation would require Oct4 being released from the Pax6 promoter leading to de-repression of Pax6. In the course of neural differentiation, Pax6 levels are strongly activated accompanied by downregulation of Oct4 levels. It may be possible that, in the time frame of our experiment, Pax6 activation is not yet accompanied by a detectable down-regulation of Oct4 transcript levels though the promoter has been de-repressed, possibly through a yet unknown post-transcriptional mechanism. The loss of pluripotency upon miR-135b overexpression is evident from downregulation of Nanog transcript levels. Also, stronger induction of Pax6 maybe required to downregulate Oct4 transcript levels as is evident in the miR-135b overexpression data for H9 cells. The effect of miR-135b on Pax6 induction and repression of Oct4 and Nanog is more evident in the H9 ES line. We have addressed this issue in the Results section (pg. 16).

- In Fig. 5B, it is unclear to me how the Luc data has been normalized. In Fig. S5 all controls were set to 1 but apparently this is not the case in Fig. 5B.

The data displayed was not normalized and showed the de-repression of luciferase activity upon mutating the miR recognition site. We have replaced Fig 5B with a graph that shows the same data normalized to the mutant UTR for each gene.

- Fig. 5: Mutagenesis of the miR-135b seed sequences suggested that it regulates mRNA levels of its targets. However, there is a discrepancy between hPSC RNA and protein data (Figs. 5C vs. D). This is particularly striking in light of the data in Fig. S5 (non-hESCs). This is confusing and should be discussed.

The 3'UTR luciferase assay used in this study quantifies the effect of the microRNA on the luciferase enzyme protein levels. The UTR of the potential target gene is cloned downstream of the luciferase enzyme. If the miRNA targets the UTR, luciferase protein translation is inhibited resulting in lower enzyme activity and hence lower luminescence values that are displayed in Expanded view Figure E7 (original Fig. S5). If the miR recognition site is mutated, the miRNA can no longer bind the UTR to suppress translation, so luminescence values increase (displayed in Fig. 5B). Both these datasets are in 293T cells and do not quantify the effect of the miR on mRNA levels. Fig 5C displays the effect of miR-135b overexpression in H1 ESC on the transcript levels of its target genes, which do not change significantly. This is commonly observed for miRNA targets, especially in animals where protein levels of the target gene are reduced without a significant reduction in transcript levels. To enhance the clarity of our experimental design additional details have now been added to the Results section (pg. 19).

- Several figure legends could contain more information about the experiments. For example, in Fig. 5B and C it is not immediately clear which cell type has been used. Throughout the manuscript, information on the cell type, cell line name, time-point of analysis, and treatment conditions should be included in the figure legends.

We agree with the reviewer and the recommended changes have been made to the figure legends wherever possible. Further experimental details have been added to the Materials and Methods section.

- The work by Rosa and Brivanlou published in EMBO J should to be discussed (The EMBO Journal, 2011, 30, 237 - 248).

The recommended changes have been made. The work of Greber et. al. EMBO J 2011 has also been cited (pg. 20).

- Abbreviations, e.g. NECs (p6), should be defined upon first occurence

The recommended changes have been made.

Referee #2 (Report):

This manuscript provided by Bhinge A et al introduces an additional regulatory network involved in human neuroectoderm development through the use of hESC-derived NECs in vitro. The finding suggests the synergistic function of transcription factors and miRNAs in lineage specification of hESC. Through the use of ChIP-sequencing, the authors further filtered the potential direct Pax6 targeted genes, including miRNAs, through the overlap of H3K27Ac and H3K27Me3 occupied regions. Amongst the many Pax6 activated miRNAs, miR-135b is verified to be a direct target by enhancer luciferase assay. The authors confirmed that the targets of miR-135b seem to be involved in BMP and Activn/Nodal signaling, and overexpression of mir-135b can promote NE specification, likely through the suppression of non-neural lineage differentiation. It is still unknown why Pax6 has a unique early function for human neuroectoderm induction, and this study provides evidence implicating the role of miR-135b in
promoting neural specification and differentiation, and the possibility of this microRNA accounting for Pax6 function in human ESC derived neuroepithelium. However additional support should be included to strengthen this proposed TF-miRNA regulation network.

Major concerns:

1. All of the functional analyses of mir-135b and its targets are based on the overexpression

approach. It is essential to reveal the role of mir-135b by knock-down approach to demonstrate that mir-135b/TGFb-Activin is required to specify neuorectoderm lineage.

We have performed the experiment that the reviewer has suggested where we knocked down miR-135b and assayed for neuroectodermal markers. We found that inhibition of miR-135b significantly impairs neuroectoderm differentiation (Fig 4D and 4E) (pg. 17). This suggests that miR-135b is required for neuroectoderm development.

2. As the major conclusion of this manuscript is that mir-135b is a direct target of Pax6, it not clear to what extent Pax6 function to promote neuroectoderm is via mir-135b. It will be interesting to see if overexpression of mir-135b in Pax6off hESCs can still induce neuroectoderm,or if knocking down of mir-135b can suppress neuroectodem differentiation under 2i (or 3i) condition.

The contribution of miR-135b to Pax6-mediated neuroectoderm specification can be assayed by the effect of miR-135b knockdown on neuroectoderm development. Knockdown of miR-135b significantly impairs neuroectoderm differentiation while promoting differentiation towards non-neural lineages. Thus, as suggested by both reviewers, Pax6 and miR-135b may be acting in a feed-forward loop to cause irreversible neuroectoderm specification, once Pax6 levels are de-repressed. These results have been added to the Results (pg 17) and Discussion sections (pg. 22).

We tried knocking down miR-135b under 2i conditions but failed to get a significant difference. The main reason behind this being the small molecules used in the 2i method act on the same pathways as the miRNA, essentially substituting the role of the miRNA. Additionally, induction of Pax6 under the 2i conditions is highly robust and antagomir mediated inhibition of miRNA levels were not sufficient to bring about any observable changes.

We overexpressed miR-135b in human ESCs and cultured these cells under conditions that maintain pluripotency (i.e. cells were cultured in mTeSR1). Cells were harvested 120 hours post-transfection. However, no detectable changes in pluripotency or neuroectodermal markers could be discerned (Expanded view Figure E8) (pg. 22). This suggests either the microRNA alone is not able to override pluripotency signaling pathways or the time frame of the experiment is too short to observe any effect i.e. sustained miR expression over a longer period maybe required to suppress pluripotency.

3. The authors argue that Pax6 can function either as an enhancer or a repressor and suggested that Pax6 functions as an enhancer to activate mir-135b expression. However, in fig 3E, the overexpression of Pax6 only induces the level of mir-135b mildly. It is possible that Pax6 might function as a repressor to derepress the mir-135b expression level. With the Pax6-VP16 and Pax6-En2 fusion protein, the authors should be able to dissect the relationship of Pax6 activity and mir-135b induction.

The reviewer has raised an interesting alternative to the model we have proposed. Since our ChIP-Seq data revealed that Pax6 can also act as a repressor, we agree that the activation of miR-135b may be via an indirect mechanism where Pax6 represses a repressor of miR-135b. Additionally, either mechanisms may play a role in miR-135b activation i.e. Pax6 directly activates miR-135b as well inhibits a suppressor of miR-135b. Though, it would be very interesting to dissect the exact mechanism, the experiments suggested by the reviewer are technically challenging. We would like to present the following arguments to support our claim that miR-135b upregulation, at least in part, is due to a direct activation by Pax6:

1. Pax6 directly binds to enhancers in the proximity of miR-135b (Fig. 3B, 3C, 3D).
2. Knockdown of Pax6 prevents activation of the miRNA (Fig. 3F).
3. Overexpression of Pax6 in differentiating ES cells upregulated miR-135b more than 2-fold (Revised Fig 3E). When hESC were induced by the 2i method, Pax6 was activated more than 2000 fold whereas miR-135b was activated to a maximum of 15-fold (Fig. 4A). So in the context of Fig. 4A, a 2-fold upregulation of miR-135b is not surprising. This indicates that high levels of Pax6 may be required to activate miR-135b.

Taken together, we feel that our data supports the case that Pax6 acts as an activator, at least in part, to upregulate miR-135b expression. We have included the above discourse in the Discussion section (pg 21).

4. Overexpression of miR-135b led to increase Pax6 expression (Figure 4C). Is miR-135b and Pax6 in a regulatory loop? Or is this increase in Pax6 a consequence of the inhibitory function of miR-135b? In addition, the expression of mir-135b is upregulated from day 2 of differentiation, while activation of Pax6 starts from day 3 of differentiation (Fig4A). The authors should consider the reciprocal regulation between Pax6 and mir-135b during NE development.

Pax6 overexpression increases miR-135b expression while Pax6 knockdown downregulates miR-135b (Fig. 3E, F). Reciprocally, miR-135b overexpression induces Pax6 expression while knockdown of miR-135b leads to reduced Pax6 levels as well as other neuroectoderm markers (Fig. 4B, C, D, E) (Discussed on pgs. 21,22,23). In light of this data, we propose that Pax6 and miR-135b act in concert in a feed-forward regulatory loop to enhance neural differentiation. MiR-135b mediated increase in Pax6 is likely due the inhibitory effect of miR-135b on pluripotency maintaining or neural suppressing signaling pathways such as TGFβ and BMP.

The original Fig 4A panel that showed Pax6 expression during neural differentiation, displayed the y-axis as a multiple of 1000. This perhaps made interpretation of the data cumbersome. The figure has been replaced with a more detailed time course where Pax6 expression is displayed on a log10 scale. In the revised Fig 4A, it is clear that Pax6 expression is activated at Day 1, while miR-135b levels start to increase by day 2.

Minor points:

Fig2A
With the focus on miRNA, it would be fit to incorporate a category or annotation of non-coding RNAs or miRNAs into the pie chart regarding the distribution of Pax6 binding sites.

As suggested, we have updated the distribution of Pax6 binding sites to include lincRNAs and miRNAs (Fig. 2A) (pg. 11).

Fig4B
The author proposed a model that mir-135b prevent differentiation towards mesoendoderm, trophectoderm or ectoderm through the inhibition of BMP and Activin signaling, but in Fig4B, overexpression of mir-135b did not suppress markers for endoderm (Lama3), mesoderm (brachyury and Acta2), endoderm (Sox17) and trophectoderm (Cdx2). The authors should explain this contradiction.

We agree with the reviewer that according to our model, miR-135b overexpression should have suppressed expression of the non-neural markers. However, as we have used transfection to deliver miRNA mimics into ESCs, our experimental timeframes had to be short. It's possible that in the short time frame of the experiment, the non-neural markers are not strongly induced in the control transfections. Secondly, transfection efficiencies of hESC are usually less than 30%. So low transfection efficiencies combined with minimal induction of the non-neural markers might be the reason why we could not detect a significant downregulation of the non-neural markers. It must be noted that, in a previous study on the role of Pax6 in NE development, overexpression of Pax6a-GFP in human ESCs strongly upregulated neural markers without significant changes in the non-neural markers (Zhang et. al. Cell Stem Cell 2011). The main reason we assayed for the non-neural markers was to test whether miR-135b overexpression leads to a pan-lineage differentiation of ES cells. Specific upregulation of the neural markers indicated that this was not the case and the miRNA was specifically promoting neural conversion of ESCs. We have included this discourse in the Discussion section (pg. 22).

Fig4C
The resolution of immunostaining is poor. A higher magnification figure with quantification should be used.

The recommended changes have been introduced. Details on the quantification have been included in the materials and methods section (pg. 5).

In materials and methods, the new protocol for human ES neural induction is not clear. The authors should describe it in further detail. Also, all "μ" are mislabeled as "u" in this section.

The neural induction protocol has been described in greater detail with the recommended changes incorporated (pg. 5).

Zhang et al (2010) demonstrated that Pax6 is required for the specification of human NE by downregulating pluripotent genes. Moreover, they found that Pax6a and not Pax6b is responsible for inducing NE-related genes and the overexpression of only Pax6a could direct hESCs towards a neural identity. It is not clear which Pax6 isoform or a non-discriminative Pax6 antibody was used in this study. Therefore, whether the binding sites of Pax6 are isoform-specific is uncertain and whether miR-135b is activated by Pax6a or Pax6b is also not addressed.

The antibody we are using cannot distinguish between different isoforms of Pax6, hence we cannot address the issue whether Pax6a or Pax6b binds proximal to miR-135b. However, the overexpression studies were carried out with the Pax6a isoform that was able to upregulate miR-135b levels. Additionally, given that our data shows a neural-specific role of miR-135b combined with the fact that only Pax6a promoted neural differentiation, it is likely that only the Pax6a isoform is responsible for miR-135b activation. We have included these details in the Materials and Methods (pg. 7) as well as the Discussion section (pg. 23).

2nd Editorial Decision 28 March 2014

Your revised study has been assessed by the two original referees. While both are essentially supportive, ref#2 raises a few points that would need your further attention:

-Please minimally discuss potential implications that may arise from the expression data presented (ref#1 point 1);

-I carefully checked particularly Fig 4 C, E and have to agree that the control/starting point should roughly be identical; inclusion of quantitative row data as well as the indicated control for kd/overexpression would be helpful to appreciate better the indicated changes.

-I noticed another NEP (instead of NEC) in Fig 3A in addition to the refs mention in Fig 1A.

Overall, I would be delighted to receive an ultimate version for eventual publication from you shortly.

REFEREE REPORTS:

Referee #1:

The authors have adequately addressed my previous points and this has made this work significantly stronger. I would hence recommend publication of this article in EMBO J.

Referee #2:

The authors have meticulously answered and clarified reviewers' questions, and performed the recommended experiments. Their results demonstrated that the induction of miR-135b by PAX6, and together promote NE differentiation in hESC. The authors performed additional assays, particularly the miR-135b knockdown experiments, to strengthen the role of miR135b in neuroectoderm differentiation. Despite some remaining concerns, the manuscript is much more improved.

Major concerns

1. It is still quite intriguing that miR135b is indeed indispensible for Pax6 induced neuron differentiation via inhibition of non-neuronal cell fat. Interestingly, in Fig. 4A the expression of miR-135b continues to increase until D8, indicating additional roles of miR-135b for neuroectoderm differentiation at a later stage, in addition to the proposed inhibition of pluripotency mechanism. Although overexpression and knockdown experiments of miR-135b reveal the role of the microRNA in mediating neural differentiation, whether miR-135b is necessary for NE differentiation remains puzzling. Do hESC still differentiate into different neuronal and glial types (TUJ1+, GFAP+, OLIG2/HB9+ neurons) from NEC with equivalent efficiency and robustness compared to WT in the absence of miR-135b? The reduction of PAX6 could be a delay of differentiation due to transfection.

2. Fig. 4 B to 4 E, PAX6 protein levels shown in the representative figure of Pax6 immunostaining in both control cells (Fig. 4C and Fig. 4E) are not comparable and is inconsistent to the quantitative result (Y axis in %). Both the efficiencies of miR-135b knockdown and overexpression should be included.

Minor concerns:

1. What does the N number represent in all experiments here? Triplicate or independent experiments?
2. All genes names should be capitalized as the experiments were all performed using human ESCs. In Fig1A, NEP should be changed to NEC. Also, figure legends "H1 human embryonic stem cells into Oct4-negative, Sox2-, Pax6-
and Nestin-positive human NECs in 8 days" is inconsistent to the Fig1A.

2nd Revision - authors' response 03 April 2014

Major concerns

1. It is still quite intriguing that miR135b is indeed indispensible for Pax6 induced neuron differentiation via inhibition of non-neuronal cell fat. Interestingly, in Fig. 4A the expression of miR-135b continues to increase until D8, indicating additional roles of miR-135b for neuroectoderm differentiation at a later stage, in addition to the proposed inhibition of pluripotency mechanism. Although overexpression and knockdown experiments of miR-135b reveal the role of the microRNA in mediating neural differentiation, whether miR-135b is necessary for NE differentiation remains puzzling. Do hESC still differentiate into different neuronal and glial types (TUJ1+, GFAP+, OLIG2/HB9+ neurons) from NEC with equivalent efficiency and robustness compared to WT in the absence of miR-135b? The reduction of PAX6 could be a delay of differentiation due to transfection.

Reviewer #2 raises an interesting point as it has been shown that Pax6 levels influence the timing of NEC differentiation into neurons and astrocytes as well as specification of NEC into specific regional progenitors such as spinal motor- and inter-neuron progenitors. Hence, it is possible that miR-135b mediated regulation of Pax6 could also be involved in the differentiation of NEC into specific neuronal subtypes as well as glial cells. Certainly we would be interested to compare and contrast the roles of miR-135b at different stages of neurectodermal development, but we feel it is beyond the scope of this paper. Given the interesting nature of this point, it is now raised in the discussion section (pg. 24)

2. Fig. 4 B to 4 E, PAX6 protein levels shown in the representative figure of Pax6 immunostaining in both control cells (Fig. 4C and Fig. 4E) are not comparable and is inconsistent to the quantitative result (Y axis in %). Both the efficiencies of miR-135b knockdown and overexpression should be included.

The immunostaining results were included to provide a representative visual rendering of the accompanying quantitative data, which was generated by averaging at least 8 different images for the overexpression and knockdown experiments, thereby making the quantitation reliable and robust. We have revised Fig 4E with a more representative immunostaining photo. Additionally, we have confirmed that the difference between the control and experiment is statistically significant by performing a t-test on the original raw data. To make this point more clearly we have revised Figure 4E, to show the P-values as < 0.05.

We quantified efficiencies of miR-135b knockdown and overexpression by the TaqMan RT-PCR assay. In the overexpression experiment, miR-135b was found to be 7-fold more than the control at the time cells were harvested while antagomir tranfections reduced miR-135b levels by 2-fold in the knockdown experiments. However, it must be cautioned that these expression levels do not reflect the functional levels of the miRNA per cell for 2 main reasons: 1. When cells are transfected, the amount of miRNA/antagomir per cell gets diluted as cells divide. Additionally, ES transfection efficiencies are low to begin with. Hence, the final levels of the miRNA in the average population is not reflective of its levels inside any given cell. 2. It is difficult to determine how much of the transfected miR actually gets incorporated into the RISC complex to enable a functional effect. The same is true when an antagomir is used (Thomson, DW et.al. 2013)

For these two reasons, we feel that including the efficiency of miR overexpression and knockdown would not add significantly towards understanding the results.

Daniel W. Thomson, Cameron P. Bracken, Jan M. Szubert and Gregory J. Goodall. PLoS One. 2013; 8(1): e55214, Jan 24, 2013. On Measuring miRNAs after Transient Transfection of Mimics or Antisense Inhibitors

Minor concerns:

1. What does the N number represent in all experiments here? Triplicate or independent experiments?

These are 3 independent experiments. This has now been emphasized in all figure legends.

2. All genes names should be capitalized as the experiments were all performed using human ESCs. In Fig1A, NEP should be changed to NEC. Also, figure legends "H1 human embryonic stem cells into Oct4-negative, Sox2-, Pax6-
and Nestin-positive human NECs in 8 days" is inconsistent to the Fig1A.

The recommended changes have been made throughout the document as well as in Figure 1 legend (pg. 28) and in the Results section (pg.9). NEP has been changed to NEC throughout the document including Fig. 1 and Fig. 3.