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A Regulatory Circuitry Comprised of miR-302 and the Transcription Factors OCT4 and NR2F2 Regulates Human Embryonic Stem Cell Differentiation

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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.)

1st Editorial Decision 24 August 2010

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. I did enclose the comments of three scientists that have assessed your work. These indicate some interest in the described miR302/Oct4/Nrf2 circuitry. At the same time however, ref #1 and #2 request to go further by analyzing and integrating potential mechanisms that enable the switch from pluripotency to neuronal differentiation. As such extensions would significantly improve the current state of the study, I urge you to address this topic experimentally as much as you can during major revisions of your study.

Please be reminded that it is EMBO_J policy to allow a single round of revisions only and that the final decision will entirely depends on the content within the last version of your manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this paper, authors tried to address i) a regulatory circuitry among OCT4, NR2F2 and miR-302 that are involved in both pluripotency and differentiation of human ES cells, ii) expression of NR2F2 and its roles in the activation of neural genes during early differentiation of human ES cells. The reviewer found that early part of this MS characterizing a regulatory circuitry among OCT4, NR2F2 are very well described and solid enough. On the other hand, in the later part characterizing

expression of NR2F2 and its roles in the activation of neural genes during early differentiation of human ES cells, the presented data are verbose and their novelty is not strong enough. Important message is already presented in mouse ES cells' system (Masui et al., Nature Cell Biol., 2007). From these aspects, the reviewer would urge authors to address the following issues.

1. Is germ cell nuclear factor (GCNF) involved in a regulatory circuitry among OCT4, NR2F2 and mir-302?
2. The data presentation in Figs. 5 and 6 is verbose and redundant. The reviewer suggests that authors should combine these Figures by consolidating the results of immunostainings of NR2F2, PAX6 and OCT4 in three time points including i) ES cells (in conditioned medium), ii) EBs (in non-conditioned medium) and iii) EBs (in N2M medium). Furthermore, the overall immunostainings of these Figs. 5 and 6 is very poor. It is very difficult to judge whether the cells are double-positive or not. Authors should represent the data of higher magnification and higher quality.
3. Is the regulatory roles of NR2F2 upon Pax6, Six3, Zic1, N-cad and Lhx2 expression (shown in Fig. 7A) direct? Authors should address issue by ChIP analysis or other method.
4. In later part, the expression of miR-302 is NOT characterized at all. The in situ hybridization should be examined.

Referee #2 (Remarks to the Author):

In their article, Rosa and Brivanlou describe the regulatory circuit composed of miR-302, OCT4, and NR2F2, operating in human ES cells. Authors, in a clear and concise way, present evidence of interrelation of the three molecules in both the maintenance of pluripotent state of hES but also in the process of differentiation into the neuronal lineage precursors. The conclusions drawn in this paper are adequately supported by well presented experimental data.

A few points should be addressed:
MAJOR:
- In Figure 1 authors say that "levels of miR-302 HT faithfully mirrored the expression of OCT4" - I would like to see the quantification of that and also other experiments represented in this figure - judging the relative expression levels from the pictures might be somewhat problematic.

- In Figure 2D, overexpression of miR-302 in ES cells leads to the general increase of OCT4 mRNA - authors do not comment how this may influence the expression of NR2F2, especially that in their model in Figure 3 they show that OCT 4 has the direct inhibitory effect on NR2F2. One could possibly speculate that miR-302 have no direct effect on the observed lack of NR2F2 induction.

- In my opinion, the separation of pluripotency and neural differentiation circuits of OCT4, miR302, and NR2F2 is somewhat artificial. I think the two processes should be included in one model - and possibly discussed more.

MINOR:
- In figure 3A, legend is missing (it seems that it is placed by mistake in Fig. 3B)

- Figure 3B, should rather have the log scale for mRNA expression

- I think it would make more sense to put into one graph panels C & D of Figure 4

- Authors tend to present panels in the figures in the order which does not correspond to the order in which they cite it -¬ it does make the reading more difficult.

Ref#3 (remarks to the authors)

This is a well conducted study that addresses the mechanistic implication of Oct-4 and miRNA302 in ES cell differentiation. The authors show convincingly that OCT4 and miRNA302 are linked in a regulatory circuit to determine the balance between differentiation and pluripotency at least in part by reciprocal regulation of the expression of the transcription factor NR2F2. Oct4 and NR2F2 are shown to mutually repress each other: Oct4, aided by MiRNA302 represses NR2F2 to maintain pluripotency whereas NR2F2 represses Oct4 and miRNA302 during differentiation. The authors

provide evidence that NR2F2 plays a potentially important role in the induction of genes implicated in early neural development. They also suggest that miRNA302 may provide a failsafe mechanism to counteract potential leakiness in Oct-4-mediated repression of NR2F2. Overall this is a relevant study to the field of differentiation and pluripotency that addresses the timely issue of miRNA-ependent fine-tuning of genes implicated in cell fate determination. The experiments are well performed and the authors have taken care not to overinterpret their results.

1st Revision - Authors' Response 28 October 2010

RESPONSE TO THE REFEREES' COMMENTS

Here we addressed all of the referee's comments and suggestions, whom we thank for their interest. We believe that their comments and suggestions have substantially improved our paper.

Referee #1

1. Is germ cell nuclear factor (GCNF) involved in a regulatory circuitry among OCT4, NR2F2 and mir-302?

While Germ Cell Nuclear Factor (GCNF), an orphan nuclear receptor not related to NR2F2, has previously been shown to repress OCT4 transcription in mouse teratocarcinoma and ES cells (Gu et al., MCB 2005; Zechel, Mol. Repr. Dev. 2005; Ben-Shushan et al., Mol Cell Biol 1995; Schoorlemmer et al., Mol Biol Rep 1995; (Furhmann et al, Dev. Cell 2001), its activity has never been shown to be regulated by miRNA. Thus collectively, the involvement of GCNF in early embryogenesis, neural differentiation and regulation of OCT4 (taking also in consideration NR2F2) has been addressed elsewhere and it is not in the scope of our paper. However, following the referee's suggestion, we have now addressed whether GCNF could be regulated by miR-302. Three independent inquiries using bioinformatic software for miRNA-target prediction (TargetScan, PicTar and Miranda) indicated no potential miR-302 binding sites on the GCNF 3'UTR. We have experimentally validated these predictions and the presence of possible cryptic sites by taking advantage of the same luciferase assay previously used for NR2F2 (described in figure 2). We found that GCNF 3'UTR is not responsive to miR-302 (new supplemental figure 6). A paragraph about GCNF has been included in the discussion (pages 14-15). Therefore GCNF is not part of the mir-302 circuitry.

2. The data presentation in Figs. 5 and 6 is verbose and redundant. The reviewer suggests that authors should combine these Figures by consolidating the results of immunostainings of NR2F2, PAX6 and OCT4 in three time points including i) ES cells (in conditioned medium), ii) EBs (in non-conditioned medium) and iii) EBs (in N2M medium). Furthermore, the overall immunostainings of these Figs. 5 and 6 is very poor. It is very difficult to judge whether the cells are double-positive or not. Authors should represent the data of higher magnification and higher quality.

As suggested by the referee, we have now combined figures 5 and 6 in a single new figure 5. Due to space limitations, some of the data previously included in the old figure 6 have now been moved to supplemental material (new supplemental figure 3). Furthermore, as requested by the referee, we have also included higher resolution images taken at higher magnification for differentiated cells.

3. Is the regulatory roles of NR2F2 upon Pax6, Six3, Zic1, N-cad and Lhx2 expression (shown in Fig. 7A) direct? Authors should address issue by ChIP analysis or other method.

Following the referee's suggestion we have used three different antibodies that are commercially available for NR2F2 (Abcam ab66662; Abcam ab64849; R&D H7147) to perform ChIP experiments. Our positive control: the promoter of OCT4 containing a binding site for NR2F2 (Ben-Shushan et al., Mol Cell Biol 1995; Schoorlemmer et al., Mol Biol Rep 1995) failed to result to any specific enrichment for binding, demonstrating that the commercially available antibodies are not adequate for this assay. We decided to overcome this limitation by generating Flag-tag at the N-terminal of our inducible NR2F2. Surprisingly, tagging NR2F2 displayed significant toxicity leading to massive cell death (whereas the untagged construct used in experiments of figure 6 had no toxic effect leading to a specific phenotype). We therefore have used different strategies to address the important point raised by the referee, but are unfortunately due to technical limitation of reagents this issue cannot be resolved at this point. The text has been changed accordingly to reflect the referee's important point that presently the directness of the effect remains unknown (see Discussion, page 15).

4. In later part, the expression of miR-302 is NOT characterized at all. The in situ hybridization should be examined.

We had previously described the in situ hybridization patterns for miR-302 family members in our previous paper (Rosa et al., Dev Cell 2009). We showed that both in mouse and frog embryos the miRNA is highly abundant around gastrulation, in cells that are also OCT4+, declining at later stages. Interestingly, in both systems we observed a restriction of miR-302 expression after gastrulation, at neurula stage, when the miRNA is specifically enriched in the neural territory, supporting our model in which miR-302 may play a role during neural differentiation. A paragraph has been added in the Results section (page 9).

We hope that our additional experiments, improvements of the image qualities and our genuine attempt to address the direct versus indirect effect, as well as the characterization of the final point satisfies this referees constructive criticism.

Referee #2

We thank this referee for her/his comments mentioning that we have "in a clear and concise way, presented evidence of interrelation of the three molecules in both the maintenance of pluripotent state of hES but also in the process of differentiation into the neural lineage precursors", and that "The conclusions drawn in this paper are adequately supported by well presented experimental data".

The referee however also wants to see three major and four minor points to be addressed which we have done below.

Major points:

1- In Figure 1 authors say that "levels of miR-302 HT faithfully mirrored the expression of OCT4" - I would like to see the quantification of that and also other experiments represented in this figure - judging the relative expression levels from the pictures might be somewhat problematic.

As per the referee's advice, we have now added the quantification of the experiments represented in figure 1A-C, included real-time PCR analysis of the samples used for panels A and B and densitometric analysis of panel C (see new figure 1D). This analysis has helped to confirm our observations regarding: (i) reciprocal expression of OCT4/miR-302 and NR2F2; (ii) the levels of miR-302 Host Transcript (miR-302 HT) mirror the expression of OCT4; (iii) the decline of the mature miR-302 is delayed when compared to miR-302 HT; and (iv) NR2F2 protein accumulation is delayed when compared to its mRNA.

2- In Figure 2D, overexpression of miR-302 in ES cells leads to the general increase of OCT4 mRNA - authors do not comment how this may influence the expression of NR2F2, especially that in their model in Figure 3 they show that OCT 4 has the direct inhibitory effect on NR2F2. One could possibly speculate that miR-302 have no direct effect on the observed lack of NR2F2 induction.

The referee is right to point out that the observed increase of OCT4 levels upon miR-302 overexpression can lead to inhibition of NR2F2 transcription. The existence of multiple levels of control of NR2F2 expression is indeed one of the main points of the present work. As suggested by the referee we have now included a speculation about the possible indirect effect of miR-302 overexpression on NR2F2 lack of induction (see discussion, page 14).

3- In my opinion, the separation of pluripotency and neural differentiation circuits of OCT4, miR302, and NR2F2 is somewhat artificial. I think the two processes should be included in one model - and possibly discussed more.

In full agreement with the referee we now present a unique model to illustrate the OCT4/miR-302/NR2F2 molecular circuitry during pluripotency and differentiation. The model is depicted in the new figure 7. We also provide a more detail discussion of the model in page 16 of the Discussion section.

Minor:

1- In figure 3A, legend is missing (it seems that it is placed by mistake in Fig. 3B)

The figure legend has now been corrected.

2- Figure 3B, should rather have the log scale for mRNA expression

The panel has been replaced with a new panel with the log scale for the y-axis.

3- I think it would make more sense to put into one graph panels C & D of Figure 4

While they both represent luciferase assays, they represent two different experiments: in panel C cells were transfected with the NGFI-A:Luc reporter + GFP or the NR2F2 variants, whereas in panel D the NGFI-A:Luc reporter is always co-transfected with the NR2F2-001 isoform + GFP or the other NR2F2 variants. Therefore for clarity we would rather keep these two panels separated.

4- Authors tend to present panels in the figures in the order which does not correspond to the order in which they cite it; and it does make the reading more difficult.

As suggested by the referee the order of presentation has now been changed in the text to facilitate reading.

We thank again this referee for his/her positive outlook and hope that our changes have accommodated the criticism

Referee #3

We are grateful for the comments of this referee, which is entirely positive and complementary about our work citing that:

" This is a well conducted study that addresses the mechanistic implication of Oct-4 and miRNA302 in ES cell differentiation. The authors show convincingly that OCT4 and miRNA302 are linked in a regulatory circuit to determine the balance between differentiation and pluripotency at least in part by reciprocal regulation of the expression of the transcription factor NR2F2. Oct4 and NR2F2 are shown to mutually repress each other: Oct4, aided by MiRNA302 represses NR2F2 to maintain pluripotency whereas NR2F2 represses Oct4 and miRNA302 during differentiation. The authors provide evidence that NR2F2 plays a potentially important role in the induction of genes implicated in early neural development. They also suggest that miRNA302 may provide a failsafe mechanism to counteract potential leakiness in Oct-4-mediated repression of NR2F2. Overall this is a relevant study to the field of differentiation and pluripotency that addresses the timely issue of miRNA-dependent fine-tuning of genes implicated in cell fate determination. The experiments are well performed and the authors have taken care not to over interpret their results."

We like to thank again all three referees as their constructive criticism has helped improving the quality of our submitted studies on the regulatory circuitry underlying mir-302, Oct4 and NR2F2 in the context of pluripotency and neuronal specification.

