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A miR-34a-SIRT6 axis in the squamous cell differentiation network

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(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: Thomas Schwarz-Romond

1st Editorial Decision 17 January 2013

Thank you very much for submitting your study on the role of miR-34a/SIRT6 in squamous cell differentiation for consideration to The EMBO Journal editorial office.

Please accept my apologies for the slight delay in providing comments, caused by the recent holiday season and thus late incoming reports.

You will easily recognize that the three referees appreciate the amount and technical quality of the experimental results. However, all referees also agree that the most novel aspect, namely the role of miR34a and particularly the identification of the miR34a/SIRT6 axis in regulating cellular differentiation, is currently not sufficiently established. It would thus need further experiments to (i) distinguish possible apoptotic/senescence effects from differentiation for both miR 34a and SIRT6 (ii) establish their causality by the recommended rescue experiments and (iii) conceptually integrate the proposed tumor suppressive role of miR34a with the reported downregulation in SCCs.

These are huge demands that certainly require significant further experimentation. I am convinced however, that you are in a very strong position to develop the study accordingly and that the general appeal of the study would indeed benefit from such expansions.

Please do not hesitate to get in touch (preferably via E-mail) in case you require further clarifications OR to discuss a timeline for necessary amendments/request additional time beyond our usual three-month revision period.

I am sorry that I cannot be more encouraging at this stage, but I hope that precise communication of essential demands for a more general title such as The EMBO Journal will facilitate efficient proceeds for this relatively interesting project.

REFEREE REPORTS:

Referee #1:

This manuscript presents a number of interesting findings highlighting for the first time the importance of miR-34a-SIRT6 link in the differentiation pathway of human skin keratinocytes. This is an elegant report well written, the data is well presented, with adequate controls. However few minor issues in my opinion should be addressed prior to accept this manuscript for publication

1. In figure 1F although it is clear the downregulation of miR-34a expression within the SSC tumour the authors should improve the information provided. It is correct and appropriate using the skin of SCC from the same patient. I would only suggest choosing another field of the SSC skin where more epidermis is shown and the reader could therefore appreciate the downregulation of miR-34a. More importantly nothing is mentioned about the differentiation state of the SCC shown in fig. 1F (i.e. well, moderate, poor differentiated). This is quite relevant as again would strengthened whether miR-34a expression correlates or changes according to the differentiation state of the tumours.

2. In figure 2 which is showing the contribution miR-34a in wtp53-dependent pro-differentiation function not a single western blot of p53 expression is shown. Most of the data even if well controlled are only showing that miR-34a is a p53 downstream target. How p53 expression (protein and RNA) is affected for instance by overexpression of miR-34a? Moreover the reader would appreciate knowing whether antigo-miR-34a has any effect on endogenous p53 expression either in HKC or in a SCC cell line at least to exclude any feedback loop.

3. The role of p63 in this signalling model represents my major concern in this entire story. The author states in the discussion: "...at least in human keratinocytes, persistently elevated p63 expression was not sufficient to prevent differentiation-dependent induction of miR-34a". First of all this statement is perhaps referred to unshown data which should be incorporate into the manuscript. As correctly referenced in the discussion the current knowledge of np63 role in SCC is that its expression is frequently increased. Antonini et al recently showed that miR-34a is also a p63 target in the opposite way it is of p53, i.e. that p63 repress miR-34a expression. In figures 3D-E it looks very clear that in HKC there must be a negative regulatory feedback loop, as increased expression of miR-34a also repress np63 at protein and RNA level. In light of this how can p63 be ruled out of the story-especially when the key experiment to rule it out (i.e. its knockdown) hasn't been shown? How miR-34a and p53 expression are affected by silencing np63 in HKC but more importantly in a SCC cell line? The authors should then rephrase or include this in the discussion and perhaps summarize their final model with a cartoon where even p63 is included.

Minor points:

- Figure 2D: although there are only 4 mice error bars should be shown.
- Figure 3C: likewise no error bars are shown.

Referee #2:

In their manuscript Lefort and colleagues describe mir-34a as regulator of squamous cell differentiation and identify SIRT6 as a novel target of this micro RNA. The identification of novel key regulators of squamous differentiation is highly important for the development of novel anti-cancer strategies. Overall, the manuscript is well-done and contains a tremendous amount of data. However, I am concerned that the manuscript lacks novelty in the field for many aspects. The micro RNA mir-34a has extensively been linked to cancer in many different tissues. The transcriptional regulation of mir-34a by both p53 and p63 has been established, and finally mir-34a has also been extensively linked to the Notch pathway.

However, the majority of the published work focused on the effects of mir-34a on apoptosis, senescence and regulation of the cell cycle. Thus, connecting mir-34a to keratinocyte differentiation is a novel functional aspect of this micro RNA. Unfortunately, the authors do not exclude the possibility that the observed effect of mir-34a deletion or over-expression only indirectly affects keratinocyte differentiation (see major comments for detail). For instance, how can the authors exclude the possibility that mir-34a simply regulated cell cycle arrest leading a selective enrichment of differentiated cell population over the proliferative one and vice versa. The provided transplantation assays to directly measure differentiation in response to mir-34a over-expression are not convincing.

The identification of SIRT6 as a novel target of mir-34a is interesting and novel but only very limited experiments are provided. Furthermore, the identification as SIRT6 seems a bit contradictory to the overall hypothesis of the manuscript since SIRT6 has been described as a tumor suppressor.

Major Comments:

1.The authors rightly point out that the role of mir-34a in skin down-stream of p63 has already been established. Antonini et al. 2010 does indeed focus on mir-34a-dependent cell cycle regulation. The authors further argue that the role of mir-34a on keratinocyte differentiation has not yet been established. Whereas, these statements are correct, many of the presented data (ie figure 1 and 2) can be coincidental and simply due to alteration of the cell cycle in keratinocytes.
2.The relevance of figures 1G-J is unclear to me. While DNA methylation is important for keratincytes differentiation, methylation of the mir-34a promoter does not make mir-34a a differentiation factor or tumor suppressor. To test whether methylation of the mir-34a promoter might be relevant for keratinocyte differentiation, at the very minimum the authors need to differentiate HKCs and measure the level of methylation during this process. As a minor point, it is also unclear to me why mRNA levels of pri-34a and involucrin are presented twice.
3.The authors need to cite Raver-Shipra et al. 2007, who showed that p53 can directly transcriptionally activate mir-34a, a fact that explains most observations in figure 2.
4.Figure 2E is unclear. According to the text primary keratinocytes isolated from the respective mice were transduced with Ras (+) and all cells were then treated to reactivate p53. Why is mir-34a expression increased in the Ras background versus non-transfected cells and why is this relevant to p53?
5.Figures 2F-H: Nutlin-3a and UVB exposure induce apoptosis and senescence. Thus, increased expression of involucrin can be indirectly due to a selective enrichment of the differentiated cell population over cell death in the proliferative population. Thus, these assays do not directly show pro-differentiation aspects as stated on page 9.
6.Details about generation, analysis and treatment of keratinocytes to obtain gene expression data in figure 3F-G are missing and thus the figures are not informative.
7.The conclusions that can be drawn from figure 4 are unclear to me. The statement "Within a week under these conditions, control cells formed small nests of proliferating cells with little evidence of keratinization" is not reflected in the figure.
8.In line with my above comments, down-regulated Notch1 in figure 5 might be due to senescence or apoptosis rather than differentiation (see figure 3a-c).
9.The identification of SIRT6 as mir-34a regulated mRNA is interesting and novel. SIRT6 has been recently identified as tumor suppressor (Sebastian et al. 2012) and is also related to senescence. However, at the very minimum the data in figure 7a-c need to be confirmed by rescue experiments using a SIRT6 construct that cannot be targeted by mir-34a. Again, the authors need to show that the observed effects are not caused by apoptosis or senescence.

10.It is also unclear how the repression of a tumor suppressor by mir-34a fits into the overall hypothesis of the paper that mir-34a is down-regulated in tumors and suggested by the authors to have tumor suppressor activities.

Minor Comments:

1. Page 1 abstract 3rd line; I suppose the authors mean "is a novel node in the...

Referee #3:

In this manuscript, the authors describe a new role of miR-34a in promoting epidermal differentiation by repressing SIRT6. This is a potentially interesting finding and should be of general interests for the EMBO Journal. However, I have some major concerns about the approach and thus the conclusion drawn from the study. In the past years, two studies have examined miR-34a null and miR-34a/b/c null mice (Choi YJ et al., Nature Cell Biology 2011; Concepcion CP et al., PLoS Genetics 2012). In both cases, they failed to report any significant developmental phenotypes. In particular, the second paper, in which the authors specifically examined the p53-dependent function in the miR-34a/b/c null animals, found p53-mediated responses are largely intact. These results begin to call into question the role of miR-34a as revealed by previous studies, which have been taking exclusively gain-of-function approaches. In this manuscript, many results were obtained by delivering miR-34a into cells and the authors didn't document the level of upregulation caused by such approaches. Thus, the potential risk exists for some observed phenotypes being a consequence of the very high miR-34a level. I recommend the authors to carefully document the efficacy of their overexpression approach and establish the link between the expression level of miR-34a and any observed phenotypes.

I have the following specific comments:

1. In Fig. 1H and I, what is the reason to measure pri-34a instead of mature 34a? Although the level of pri-34a could reflect the transcription activity of the locus, pri-miRNA or pre-miRNA is intrinsically unstable as they are processed to generate mature miRNA. I suggest the authors to measure mature miRNA level throughout the manuscript e.g. Fig. 1H-I, Fig. 2A-B.
In Fig. 1G, the authors show that the promoter of miR-34a is unmethylated in HKC and methylated in SCC13. However, upon 5-aza-dC treatment, both HKC and SCC13 show similar level of miR-34a upregualtion. If the repression of 34a is caused by DNA methylation in SCC13, how does the treatment enhance its expression in both HKC and SCC13? Does it suggest that the increased expression of miR-34a is a secondary instead of primary effect of the treatment?

2. In Fig. 2G, the authors show that the inhibition of miR-34a compromises p53-induced differentiation. With a scramble inhibitor, nutlin treatment upregulates inv ~3.4 fold. With a miR-34a inhibitor, the same treatment also upregulates inv ~3.5 fold (1.4 vs 0.4). Thus it seems the inhibition of miR-34a only reduces the base level of inv but does not interfere with the effect of p53 induction. Can the authors conclude that miR-34a is the mediator of p53's function in differentiation?

3. In Fig. 3, the authors should document the level of miR-34a overexpression in each experiment and compare it to the endogenous level in differentiated skin cells. In Fig. 3C, the authors shows that the overexpression of miR-34a causes senescence. However, it is clear that even differentiated skin cells, where miR-34a is highly expressed as shown in Fig. 1F, do not usually become senescent. Thus it suggests the level of miR-34a overexpression contributes to the phenotype.
In Fig. 3G, the authors should list the genes in each GO group. From this data, it is clear that the primary effect of miR-34a is directly linked to cell cycle control but indirectly linked to epidermal differentiation. The authors should provide more detailed analysis to document the direct link between miR-34a and epidermal differentiation.

4. In Fig. 4, the authors should document the level of miR-34a by in situ.

5. Results from Figs. 5-7 are confusing. The authors demonstrate that the inhibition of many miR-34a targets including Notch1 does not contribute to epidermal differentiation. And only downregulation of SIRT6 alone appears to enhance the differentiation as documented by marker induction (Fig. 7A-B) and reduced colony formation (Fig. 7C). However, if SIRT6 is largely responsible for miR-34a mediated differentiation, its function should be analyzed in the presence of miR-34a as well as miR-34a induced inhibition for all of its downstream targets. Instead of knocking down SIRT6 (it is to show the function of SIRT6 but not miR-34a), the authors should enhance SIRT6 expression in the presence of miR-34a and test if w/o repressing SIRT6 miR-34a would fail to promote differentiation.

Additional Author Correspondence 17 January 2013

Many thanks for forwarding the reviewers' comments so promptly. I have found their concerns quite reasonable, and we should be in a position to address them. Concerning the main points that you are making, I can already reply :

1) As for the link between cell cycle control and differentiation (whether one is a consequence of the other), I have had the same question in the past for Notch signaling. The answer in that case was that parallel ways are involved. I suspect it's the same for miR34 and Sirt6, but we'll look into this in detail.

2) To show a rescue of the miR34 pro differentiation effects by constitutive Sirt6 may be more tricky. We have already done the exp. with positive results in one case, but unfortunately not in another (which is why we did not include this work in the paper). Different rescue capability is likely to depend on many variables (like timing, strain of cells, etc.) and would not be very surprising, given that miR34 has multiple targets. We were careful in stating that our evidence points to Sirt 6 as one mediator of miR34 impact on differentiation, but not necessarily the only one. We will however further look into this and try to come to a more definitive conclusion.

3) Concerning a possible conceptual conflict between our findings and the recently reported tumor suppressive function of Sirt 6 (I guess you were referring to miR34 and not Sirt6 in your comments), a duality of function of this gene would be perfectly consistent with what is by now well established and accepted for Notch. In fact, we have other ongoing work pointing to other well known pro-oncogenic genes that in keratinocytes seem to have an opposite function. We will expand more on the Sirt 6 conjunction, which is undoubtedly the most interesting part of the paper.

In summary, we feel rather motivated to further develop this project and revise the paper accordingly. If it's OK with you, I would touch base again in a month from now, to let you know whether we can meet the 3 months deadline.

1st Revision - authors' response 06 June 2013

2nd Editorial Decision 17 June 2013

Thank you very much for the revised study that was rapidly assessed by one of the original referees with no further demands for modifications.

I am therefore happy to initiate formal acceptance of your paper.

Please notice that The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels and blots, with the aim to make primary data more accessible and transparent to the reader. This entails presentation of un-cropped/unprocessed scans for KEY data of published work. We would be grateful for one PDF-file per figure combining this information. These will be linked online as supplementary "Source Data" files. Please do let me know if you have any questions regarding this initiative.

Irrespective of this further request that I am sure you will efficiently handle, I like to congratulate to publication of this study!

