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MiR-133 Promotes Cardiac Reprogramming by Directly Repressing Snai1 and Silencing Fibroblast Signatures

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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 21 January 2014

Thank you for the submission of your manuscript entitled "MiR-133 Promotes Cardiac Reprogramming by Directly Repressing Snai1 and Silencing Fibroblast Signatures" to The EMBO Journal. We have just now received the reports from the two referees asked to evaluate your work, which I copy below. As both referees agree on the interest of your manuscript and their comments are in general positive, I would like to invite you to revise it.

Without going into details that you will find below rather explicitly, both referees consider that your manuscript should be published in The EMBO Journal. In both cases, most of their concerns refer to further discussion points that can be, in their view, better explained or clarified in the manuscript. I would like to draw your attention, however, to point 3 of Referee #2. After further communication with the reviewer, we believe that an extra effort to reproduce the results in human fibroblasts is not out of the scope of the manuscript, as it would essentially answer the question of pathophysiological relevance of your findings. We would therefore like to encourage you to perform the necessary experiments for your revised manuscript.

Please be aware that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will essentially depend on the completeness of your responses included in the next version of the manuscript. Do not hesitate to contact me by e-mail or on the phone in case you have any questions, you need further input or you anticipate any problems during the revision process.

We generally allow three months as standard revision time. As a matter of policy, competing

manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). However, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://emboj.msubmit.net/html/emboj_author_instructions.html#a2.12

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

REFEREE REPORTS

Referee #1:

Muraoka et al convincingly show that miR-133 enhances the ability of transcription factors to convert mouse fibroblasts to cardiomyocyte-like (iCM) cells. Importantly, this also results in a much higher proportion of beating cells, a key limitation of previous studies. Mechanistically the authors show that miR-133 targets Snai1, which is then implicated directly in the resistance of fibroblasts to reprogramming. While Snai1 is clearly not the only factor responsible for the block to reprogramming, pinpointing its role is interesting and brings welcome mechanistic insights into the field of cellular reprogramming. A puzzling aspect of the data is the very low level of reprogramming from adult cardiac fibroblasts; previous studies by this group used neonatal cardiac fibroblasts, but it is not clear why there should be such a large difference. This should be touched upon in the text. Overall this study is clear and the data support the conclusions. It might have been nice to know more than a small handful of genes affected by the addition of Snai1, to establish which portion of the transcriptional changes effected by GMT+miR-133 were actively mediated via Snai1.

Referee #2:

This is a very well done, rigorous, and intellectually honest study that takes another look at the original reprogramming study published by the senior authr, who was first author on the original Cell paper announcing the principle of cardiac cell reprogramming from fibroblasts. The role of Mirs in enhancing reprogramming has been established, and this study, while well done, largely confirms this previous finding. At the same time, it does help to clarify a very confusing story line about the level of robustness of the reprogramming, the level of differentiation of the few ells that are reprogrammed, and initial reclarification of the original Cell study by the first author where now it appears the robust conversion is really "inefficient" and the cells poorly differentiated. Mysteriously, the in vivo reprogramming is more robust than in vitro, implying missing factors, although it is entirely possible that the effect seen is due to decreased fibrosis or some other indirect effect rather than producing more fully functional myocytes. A myriad of papers have appeared by multiple independent groups that now have begun to tell the same story, that the level of reprogramming is inefficient regardless of the factors used, and the level of differentiation is still not optimal. Nevertheless, the core finding that it is possible to reprogram fibroblasts to a cardiomyocyte like phenotype is intriguing and worthy of pursuit and clearly supports the view that the cardiac program is combinatorially driven rather than by a single master transcription factor, as opposed to the MyoD story in skeletal muscle. This study helps to clarify the story. Also, it presents a plausible, testable hypothesis for the enhanced reprogramming my Mir 133,

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via Snail repression, and the data is internally consistent with this notion. Taken together, the study has value to the CV community at large.

Specific Comments

1) A table that lists all the studies and their various disparate findings on cardiac reprogramming (level of efficiency of conversion, assay for end phenotype, degree of maturation, factors used, fibroblast used (MEFS, versus embryonic, versus adult cardiac etc.) etc. would enhance the current study as a was to clarify the confusing literature. Openly stating that the original Cell paper that claimed robust conversion was an overstatement would be helpful step forward, given that the first author of the original cell paper is the senior author on this paper which appears to be completely well done and rigorous and fairly presented

2) The efficiency even in this paper appears relatively low in terms of spontaneously beating myocytes...while there is a several fold increase only a few cells (e.g., 6-7) appears per well...this is out of how many cells in the well?

3) Does the protocol work equally well with human adult cardiac fibroblasts? If not the clinical relevance of the study is still an open question.

4) The in vivo reprogramming effect with the current cocktail....what is level of increased efficiency and does it have any effect on the level of maturation and functionality of the end cardiomyocyte phenotype?

5) Is there a list of core criteria that can be presented that constitutes the minimal level of phenotypic conversion that would allow the claim of efficient conversion to a mature differentiated cardiomyocyte phenotype by direct reprogramming?

7) There is a confusing literature emerging on the precise role of Mir-1/133...the original KO appears to be a bit divergent with subsequent reports of the no phenotype with the KO of mir 1 alone. there are both mir 133a and 133b genes in distinct locations....some comment would be made about there role in cardiogenesis per se, and any clarification of the specific role of 133a/133b and Mir1/2, which all are clustered closely together, would be helpful for those outside the immediate field.

1st Revision - authors' response 18 April 2014

To Reviewer 1

Thank you very much for your positive review, and also for your constructive comments on our manuscript submitted to *The EMBO Journal*. According to your comments, we have revised the manuscript as follows.

1. A puzzling aspect of the data is the very low level of reprogramming from adult cardiac fibroblasts; previous studies by this group used neonatal cardiac fibroblasts, but it is not clear why there should be such a large difference. This should be touched upon in the text.

We agree with the reviewer's opinion and discussed this point in the revised manuscript. Given that the reprogramming efficiency of adult CFs was low compared with MEFs in this study and our previous results using neonatal CFs, differences among mouse lines used and the transcriptional and epigenetic differences between neonate and adult fibroblasts might have contributed to the lower reprogramming efficiency in adult CFs. We revised the manuscript accordingly.
Pages 12, lines 27- pages 13, lines 3 in the revised manuscript (Discussion)

2. It might have been nice to know more than a small handful of genes affected by the addition of Snai1, to establish which portion of the transcriptional changes effected by GMT+miR-133 were actively mediated via Snai1.

Thank you for your suggestion. We have performed microarray analyses for GMT-, GMT/miR-133-, and GMT/miR-133/Snai1-iCMs, and analyzed the global transcriptional changes mediated by miR-133/Snai1. These additional experiments showed that addition of Snai1 downregulated 85% (39/46) of the genes that were upregulated by miR-133, while it upregulated 81% (105/129) of the genes that were downregulated by miR-133, suggesting most portions of the transcriptional changes effected by GMT+miR-133 were mediated via Snai1 suppression (Figure 5B, Figure E3). These new results were incorporated into the revised manuscript.
Pages 8, lines 27- pages 9, lines 6 in the revised manuscript (Results)

To Reviewer 2

Thank you very much for your positive review, and also for your constructive comments on our manuscript. According to your comments, we have revised the manuscript as follows.

1. A table that lists all the studies and their various disparate findings on cardiac reprogramming (level of efficiency of conversion, assay for end phenotype, degree of maturation, factors used, fibroblast used (MEFS, versus embryonic, versus adult cardiac etc.) etc. would enhance the current study as a way to clarify the confusing literature.

We have now provided the detail data of FACS analyses, immunocytochemistry, Ca imaging, and number of beating cells in MEFs, adult CFs, and human CFs induced with the several combinations of cardiac reprogramming factors (Table E2). We also discussed that the iCM population was heterogeneous with respect to differentiation level, and that a majority of the cells were still partially reprogrammed iCMs. We revised the manuscript accordingly.
Pages 12, lines 27- pages 13, lines 3 in the revised manuscript (Discussion)

2. The efficiency even in this paper appears relatively low in terms of spontaneously beating myocytes...while there is a several fold increase only a few cells (e.g., 6-7) appears per well...this is out of how many cells in the well?

We apologize for not being clear about the counting beating iCMs. For counting beating cells, we seeded 50,000 fibroblasts per well on 12-well plates, performed cell transductions, and then monitored cell contraction. The number of spontaneously contracting cells was manually counted in each well in at least three independent experiments in a blinded manner, and the number of beating cells per well is shown.
Pages 17, lines 2-5 in the revised manuscript (Materials and Methods)

3. Does the protocol work equally well with human adult cardiac fibroblasts? If not the clinical relevance of the study is still an open question.

Thank you for your comment. We have performed several experiments using human cardiac fibroblasts (HCFs), and showed that miR-133-mediated Snai1 suppression was also crucial for human cardiac reprogramming (Figure 7, Figure E4). These new results were incorporated into the revised manuscript.
Pages 10, lines 15 - Pages 11, lines 12 in the revised manuscript (Results)

4. The in vivo reprogramming effect with the current cocktail....what is level of increased efficiency and does it have any effect on the level of maturation and functionality of the end cardiomyocyte phenotype?

We appreciate this question, but such investigations in vivo are perhaps beyond the timeline of the revisions. We would like to work on the in vivo reprogramming by the current cocktail in the near future to facilitate application of this strategy in regenerative medicine. We discussed this in the revised manuscript.

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Pages 13, lines 3-10 in the revised manuscript (Discussion)

5. Is there a list of core criteria that can be presented that constitutes the minimal level of phenotypic conversion that would allow the claim of efficient conversion to a mature differentiated cardiomyocyte phenotype by direct reprogramming?

Thank you for your comment. The iCM population was heterogeneous with the majority remaining as partially reprogrammed cells, and few iCMs were functional in culture (Table E2). Nevertheless, we found that addition of miR-133 to the cardiac reprogramming factors increased cardiac reporter and gene expression, shifted the global gene expression profile of the iCMs toward a cardiac fate, and generated more functional iCMs, all partly mediated by Snai1 suppression. We revised the manuscript accordingly.
Pages 11, lines 23-26 in the revised manuscript (Discussion)
Pages 12, lines 27- pages 13, lines 3 in the revised manuscript (Discussion)

6. There is a confusing literature emerging on the precise role of Mir-1/133...the original KO appears to be a bit divergent with subsequent reports of the no phenotype with the KO of mir-1 alone. There are both mir 133a and 133b genes in distinct locations....some comment would be made about their role in cardiogenesis per se, and any clarification of the specific role of 133a/133b and Mir1/2, which all are clustered closely together, would be helpful for those outside the immediate field.

Thank you for your advice. We explained the miR-133 family members and the phenotype observed in the mice lacking both miR-133a-1 and miR-133a-2, and discussed the roles of miR-133a in cardiogenesis in the revised manuscript.
Pages 11, lines 20-23 in the revised manuscript (Discussion)

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