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Transient exposure to miR-203 enhances the differentiation capacity of established pluripotent stem cells

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1st Editorial Decision 5th Feb 2020

Dear Marcos,

Thank you for your interest and for the submission of your manuscript (EMBOJ-2019-104324) to The EMBO Journal, as well as for your patience with our response, which got delayed due to protracted referee input and detailed discussions in the team. Your study has been sent to three reviewers, and we have received reports from all of them, which I enclose below.

The referees acknowledge the potential interest and relevance of your results, although they also express major concerns. In particular, referee #1 raises substantial issues regarding generality of your findings and requests extensive additional experimentation to corroborate your human iPSC work (ref#1, pts.1-4). Referee #2 agrees in that a better characterization of the human iPSC as well as expanded annotation will be required to increase robustness and unequivocally support the claims made (ref#2, pt.4). This reviewer also asks you to discriminate miR-203's effects on cellular plasticity vs. proliferation (ref#2, pt.2) and to address the kinetics of the exposure (ref#2, pt.5; see also ref#3, pt.4). Further, referee #3 requests you to explore whether miR-203 is also relevant for complementary reprograming schemes (ref#3, pt.5). Finally, the referees raise a number of issues related to related to missing controls, methods annotation, statistics and data illustration that would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I need to stress though that we do need strong support from the referees on a revised version of the study in order to move on to publication of the work and as to the open outcome of the revisional work suggest to keep EMBO Reports in mind for this work as an alternative venue.

Please feel free to contact me if you have any questions or need further input on the referee comments.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, 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.

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

Kind regards,

Daniel

Daniel Klimmeck, PhD
Editor
The EMBO Journal

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Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method) that follows the model below (see also https://www.embopress.org/page/journal/14602075/authorguide#availabilityofpublishedmaterial). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at

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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (http://emboj.embopress.org/authorguide).
- Expanded View files (replacing Supplementary Information)
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 5th May 2020.

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Referee #1:

In this study, Salazar-Roa et al. find that transient induction of miR-203 promotes pluripotency and differentiation capability of both mouse and human iPSCs and ESCs. The authors identify Dnmt3a/b as targets of the miR-203, and direct modulation of Dnmt3a/b can mimic or rescue the effects of miR-203. While this observation is interesting and relevant, I have several points that the authors should address before it can be considered for acceptance.

Major comments:

1, The EB differentiation potency of the control mouse iPSCs used in this study is much lower than the mouse ESCs. They totally cannot differentiate into cardiomyocytes. The authors seem to use only one clone. In the Methods, the reprogramming readout used in this study is AP staining, which is not a stringent marker, so the genuine pluripotency of the iPSCs is questionable. It is well known that many AP+ iPSC clones are only partially reprogrammed (pre-iPSCs). More appropriately, the

authors should immuno-stain iPSCs with Nanog or Oct4 antibody and compare with ESCs to confirm the pluripotency state. Or, the authors could compare several rather than one to see whether miR-203 works only in pre-iPSCs, but not full-iPSCs. Based on this idea, what if OE miR-203 in reprogramming? Could it promote full-iPSC efficiency?

2, The differentiation potency of the mESCs seems not stable. Fig1E,F show inferior to miR-203 exposed PSCs, but in Fig2E show superior to both iPSC and miiPSC. Is this inconsistency a technical variation because of the serum culture condition? Along this line, it will be interesting to see whether induction of miR-203 in mESCs also promotes 2C-like state transition, like in Fig1D.

3, Human iPSCs could be more problematic because lack of gold standard like in the mouse. The authors should provide some basic pluripotency characterization and compare with standard human ESC line which have been shown to have normal differentiation potency. Ideally, like in the mouse, comparing several human iPSC clones to discriminate good and bad will be very informative, considering that miR-203 may only improve the bad but not the good ones and Dnmt3a/b mediated DNA methylation could be the difference.

4, The human iPSC part of Fig3 is still preliminary. First, it seems that miR-203 greatly promotes OCT4 expression in Fig3B, could the authors show the difference of pluripotency mRNA and protein expression between control and miR-203 exposed iPSCs? Second, like in the mouse, whether 8C markers are unregulated by miR-203 in the same iPSCs? Ideally, RNA-seq analysis could be done.

5, For Fig4A, DNMT3A/B protein expression, both short and long term after miR-203 exposure, should be amended to confirm they are downregulated by miR-203. Similarly, Western blot confirmation of Dnmt3a/b expression is necessary for Fig4G. It will also be relevant to compare Dnmt3a/b expression between iPSCs and ESCs. The authors could make effort to repeat E and G in human iPSCs in order to show the mechanistic generality.

6, For Fig5, first, it will be relevant to check the global DNA methylation levels using dot-blot with anti-5mC or DNA methylation sensitive restriction enzyme assay between Ctrl and miR-203 exposed iPSCs. Ideally, 2i treatment could be included as a positive control because 2i induces a global hypomethylation through Dnmt1-Uhrf1 but not Dnmt3a/b downregulation. Second, in 5F&G, it is interesting that short-term (t=10) of miR-203 exposure doesn't induce TSS hypomethylation both genome-wide and on 2C genes, while long-term (t=25) does. However, in Fig1D, 2C genes show mainly a short-term induction. Could the authors explain the discrepancy? Or are there other mechanism or miR-203 targets may be involved? Third, in EV5A, the kinetics of Dnmt3a/b expression anti-correlate almost perfectly with miR-203 and resume on T=10, however, DNA demethylation only gradually happens after T=10 (5F,G and EV5B,C). Could the authors explain why there is a lag? Again, is there other mechanism involved? Forth, what is the correlation of DNA hypomethylation and gene expression change? In particular, could the author analyze deeper to see how DNA hypomethylation explain the gain of potency? For example, is Elf5 or Sirt6 induced in miiPSCs or during differentiation to improve differentiation? Any effort along this direction would be a great addition to this study.

Minor comments:

1, In the Methods, the gender and passage number for all the PSC lines need to be stated. I don't find the use of 2i in any experiment. Typo: "250.000 cells".
2, Fig5 is confusing: 5B, DMV is not explained clearly in the text. I assume it to be methylation

downregulated region. 5C seems to show an increase of methylation in miR-203 samples. Is this global level rather than TSS, as in F? 5I, how the top 100 DMRs are selected? Among all or between which groups?
3, Fig6 is blurred, contains a yellow line and needs a rotation.

Referee #2:

In this manuscript, Salazar-Roa et al. describe the effect of the transient expression of miR-203 on the developmental potential of iPSCs. Transient expression of miR-203, which is normally expressed in murine early cleavage embryos, increases the tumor volume and outgrowth frequency of iPSCs towards teratomas. In human-mouse interspecies chimerism experiments, a transient exposure of human iPSCs to miR203 expression increased the frequency and level of interspecies chimerism in mouse blastocyst and E9.5 embryos. The effects of miR-203 is at least in part mediated by the downregulation of the de novo DNA methyltransferases Dnmt3a and Dnmt3b, which causes a global and reversible DNA hypomethylation.

This manuscript is of general interest for the field. It aims at improving the differentiation potential of already-established iPSC (and not the efficiency of iPSC generation, as already addressed by several other groups). Though the authors used multiple and stringent developmental potency assays, we have some fundamental concerns regarding the experimental conditions and genomics presented in this manuscript, that should be addressed by the authors.

Major concerns

1) The major concern relates to the question whether transient miR-203 expression generally potentiates cellular plasticity and thereby promotes the development of treated cells towards different germ layer lineages in response to intrinsic and extrinsic cues (e.g. in teratomas, developing embryos). Alternatively, transient miR-203 expression may promote proliferation and/or survival of such cells, which indirectly would contribute to teratoma and interspecies chimerism formation. It is not clear whether one, the other or both contribute to the observed results.

2) A second concern relates to the molecular characterization of the iPSC cells. While many clones have been tested in the cellular assays, it is not clear which and how many iPSC and ESC clones have been tested in the RNA and DNA methylation sequencing assays. In other words, how reproducible are these results? To support this concern please review the data shown in Figure 5B (and 5D): the methylation level of wildtype control cells at t=0 strongly differs from those of the un-induced transgenic miR-203 iPSC cells. Are the differences observed between wildtype and miR-203 transgenic cells at later time points due to the variability of methylation that was present at the original t=0 timepoint? Are the greater dynamic changes observed for the miR-203 transiently expressing cells reproducible between different iPSC clones? On Page 9, log2 fold changes for Dnmt3a and Dnmt3b of -0.24 and -0.22 are described. These are remarkably minor changes in expression to explain the rather larger changes in DNA methylation reported. It might be useful to measure expression of these de novo Dnmts as well as those of proteins involved in maintenance methylation in the time course experiments.

3) Technically, to what extend are the differences in the number of DMVs and PMDs between conditions dependent on the criteria that were used to call them? When using slightly different criteria, do the main findings still hold up?

4) Regarding the RNA sequencing experiment shown in Figure 1C, what does the X-axis represent? Have these cells been used in the DNA methylation experiments?

5) One related concern is the variability in experimental setups. The variability in time course experiments could have a large impact on data interpretation. As shown in Figure EV5, DNA methylation is reduced between t=0 and t=25 but increases at t=35. Therefore, we can expect to see changes in transcription and maybe in developmental potency at different time points. To be able to compare transcription and methylation, we suggest performing a more precise time-course analysis. Moreover, authors should take into consideration that cells could have different developmental potency depending on the incubation time (as developmental potency assays were performed on variable conditions) and discuss this.

6) One concern relates to the use of different control settings (either wildtype or transgenic non-induced iPSC). Why was this done? Does it affect the interpretation of the results?

7) For Figures 2 and 3 (and related EVs): how reproducible are the results obtained by using different iPSC clones as starting cells? Please quantify. Further, please describe the origin of the different iPSC and Esc clones. Finally, the IF images are very difficult to interpret. When showing results of one single color channel, we highly recommend presenting the data in B/W, preferably in a higher magnification.

8) Another concern relates to the cellular composition of embryoid bodies upon miR-203 stimulation. In Figure 1, authors showed that miPSC-derived embryoid bodies show beating behavior at a higher frequency compared to controls. This could suggest that miPSC are more prone to differentiate towards the cardiomyocyte cell fate, without improving differentiation into any other cell lineage. Indeed, Figure 6 shows that miPSC differentiate more efficiently compared to control into mature cardiomyocytes. To exclude that miR-203 expression primes cells towards the cardiomyocyte cell fate only, we suggest testing differentiation towards a different cell fate, such as of neural stem/progenitor cells (NPCs).
Is it possible to assess the cellular composition (heterogeneity) of the embryoid bodies using IF or RNA-seq approaches?

Minor concerns

9) In Figure 1C, authors showed that miPSC are more similar to ESC rather than to regular iPSC. It would be interesting to expand this analysis, including the relationship to 2C embryos, blastocysts and mESC with different developmental potency, such as 2C-like cells and EPSC.

10) MiESC showed a phenotype similar to that of miPSC. Is thus the developmental potency of miESC increased similarly to miPSC? How does this relate to genome wide DNA methylation patterns?

11) It would be interesting, if possible, to know the expression of miR-203 in human embryos and whether miR-203 is conserved between human and mice. Does miR-203 modulate DNMT3A and DNMT3B expression in human cells?

12) In Figure 2C, 2E and 3A statistical tests are required. Increasing the number of embryos could improve statistical significance (particularly in Figure 3A).

13) In Figure 3A, it is not clear what the difference is between the miPSC P29+0 and miPSC P36+2.

Can these conditions be pooled? If not, why?

14) In Figure 4D, iPSC and iPSC-siC don't overlap in the principal component analysis. Indeed, it seems as PC2 captures differences between siRNAs treated and non-treated cells. Similarly, iPSC-siDnmt3a/3b and miPSC are separated in PC2. To improve the comparison of Dnmt3a/3b knock down conditions to miPSC cells we propose treating miPSC cells with control siRNAs as well (and possibly with siDnmt3a/3b as well).
How much of the variance is covered by the different PC dimensions? This should be indicated in the figure (legend).

15) Figure 5C is difficult to interpret. A different format or color choice might improve clarity.

Referee #3:

In current manuscript, Salazar-Roa, Trakala et al. identified miR-203, which is expressed at morula stage, as an augmenter of differentiation ability of pluripotent stem cells. Authors claim that transient exposure of pluripotent stem cells with miR-203 enhances their differentiation into cardiomyocytes and other lineages. To support their claims, they utilized tetraploid complementation and chimera formation and demonstrated improved differentiation potential upon exposure to miR-203. Authors further show that regulation of Dnmt3a and Dnmt3b via miR-203 contributes to increased differentiation ability.

Authors address an important issue of inadequate differentiation properties after long-term culture of pluripotent stem cells. Therefore, strategies that are able to improve differentiation of pluripotent stem cells are focus of several laboratories world wide. Overall, the manuscript is well written and most of the results support authors' claims. I have following suggestions to improve manuscript further.

Major
1. It is unclear why only miR-203 out of more than 1000 known miRNAs was selected. Is it the only one that is preferentially expressed in 2 cells stage?

2. The results presented in Figures 1B-G are obtained upon overexpression of miRNA. As stated on page 5 miR-203-encoding sequence was inserted downstream of.. it seems premir sequence was cloned. If it is correct, and the fact that a mature miRNA can be derived either from -5p or -3p and therefore, authors should mention clearly which of those mature miRNA cause the effect shown in 1B-G. Expression analyses of both mature miR-203-3p and 5p should be presented in Figure EV1.

3. miR-203 mimics in Figure 1G-H are used? I assume, it is miR-203-3p (due to the fact only -3p targets Dnmt3a and Dnmt3b). Authors should also test mimics of miR-203-5p in EB formation assay and all other experiments to ascertain the enhanced differentiation is indeed due to miR-203-3p.

4. Authors investigated transient exposure of miR-203 in figure 2. Will longer induction time further improve the developmental potential of iPSCs and ESCs? The data should be included in the manuscript.

5. Is this effect of miR-203 specific to OKSM induced iPSCs? Can it also be applied in other

combination of transcription factors-induced iPSCs?

Minor
In the methylation heatmaps (fig.5l) the differences between patterns in iPSC and miiPSC embryonic bodies, both for differentially methylated regions and imprinting control regions are not obvious even though the morphology of EBs is very different. Authors should discuss the reasons for this. Could it be explained by a mechanism other than epigenetic control?

Answers to the reviewers

We thank the reviewers for their insightful comments and recommendations. We have addressed all their comments in this revised version of the manuscript, in some cases adding new experiments data or new analyses. Unfortunately, the current shutdown of the laboratories has prevented us from performing a couple of additional assays, that we would like to perform in the future. However, we are confident that the new data included in this version of the manuscript has greatly improved the experimental support of the conclusions of the study. In particular, we have included the following new figures:

New Panel E in Figure 1, where we have assessed the transcriptomes of different clones of *mii*PSCs, comparing them with previously-published mESCs, 2-cell-like mESCs subpopulations (MacFarlan et al., 2012; GSE 33923) and epiblast stem cells (Najm et al., 2011; GSE 26814) (Fig. 1B, E).

Expanded View 4E, showing the miR-203-mediated regulation of Dnmt3a/b also at protein level, both in mouse and human iPSCs.

Appendix Figure S1, showing the effect of transient exposure to miR-203 in embryoid body formation, in 5 representative independent clones of mouse iPSCs. Also, we include a more comprehensive analysis of gene expression on *mii*PSC-derived EBs, showing the improved differentiation to the three germ layers.

Appendix Figure S2, showing the effect of a transient exogenous expression of miR-203 in PSCs cultured upon 2i/LIF conditions. This figure includes also RNAseq analysis of the *mii*PSCs (2i/L) versus control iPSCs (2i/L) and the miR-mediated outcomes on differentiation, as determined by EBs assays.

Appendix Figure S3, showing how the transient exposure to miR-203 induces 2-cell-like markers in mESCs and human PSCs. We have tested the expression of the mouse and human retrotransposons (MERVL and HERVH, respectively), associated to the 2C-like stage in mouse and naïve pluripotency in human cells.

Appendix Figure S4, showing in detail the differentiation properties in teratomas generated from *mii*PSCs. In this figure we include a broader analysis of teratomas, at the transcriptomic level by RNAseq and also a deep immunohistochemistry analysis, to further validate that miR-203 induces the differentiation to the three germ layers, proliferation and expression of unusual markers found in mouse teratomas.

Appendix Figure S5, showing how transient exposure to miR-203 in vitro improves the in vivo developmental potential of iPSCs and ESCs in 2n chimera assays. This new figure shows the efficiency of miR-203 transient exposure to increase both iPSCs and ESCs contribution to 2n mouse chimeras. Please, note that we also include the data of adult chimeras with germline transmission.

Appendix Figure S6, showing how human *mii*PSCs efficiently contribute to chimerism in 8C-stage mouse embryos (when 15 human cells are injected). This original data (not included in the first version of the manuscript) undoubtedly reinforces the data on Figure 3, where only one human cell was injected in mouse embryos.

Appendix Figure S7, showing how human *mi*iPSCs efficiently contribute to chimerism in post-implantation mouse conceptuses (when 15 human cells are injected). This original data (not included in the first version of the manuscript) undoubtedly reinforces the data on Figure EV3, where only one human cell was injected in mouse embryos.

Appendix Figure S8, showing how DNA methyltransferases 3a and 3b are miR-203 targets involved in its induction of the 2C-like stage. We have now tested the expression of the 2C-like characteristic retrotransposon MERVL in mouse ESCs when miR-203 is co-expressed with cDNAs of Dnmt3a/b and even more, we have mimicked the miR-203-mediated effects by silencing the Dnmt3a/b in these settings.

Appendix Figure S9, showing the common genes up-regulated and hypomethylated in *mi*iPSC 20 days after Dox withdrawal. We have generated new RNAseq analysis on the same samples tested for DNA methylation in Figure 5, and crossed those data with the previous DNA methylation data. This new figure is accompanied by two **new Appendix Tables: S9 and S10**, that complement the information.

Appendix Figure S10, showing improved embryoid body formation after transient exogenous expression of miR-203a-3p versus miR-203a-5p.

We have also re-formatted some of our figures, like Figure 5C, to show the data more clearly and effectively. We have taken care of the organization of figure panels, generally improving the esthetic of the manuscript and therefore, illustrating more carefully the messages.

Finally, we have also included two new Figures specifically for the reviewers in the Answers-to-Reviewers section.

Specific answers to the reviewers' comments

Referee #1:

1, The EB differentiation potency of the control mouse iPSCs used in this study is much lower than the mouse ESCs. They totally cannot differentiate into cardiomyocytes. The authors seem to use only one clone. In the Methods, the reprogramming readout used in this study is AP staining, which is not a stringent marker, so the genuine pluripotency of the iPSCs is questionable. It is well known that many AP+ iPSC clones are only partially reprogrammed (pre-iPSCs). More appropriately, the authors should immuno-stain iPSCs with Nanog or Oct4 antibody and compare with ESCs to confirm the pluripotency state. Or, the authors could compare several rather than one to see whether miR-203 works only in pre-iPSCs, but not full-iPSCs. Based on this idea, what if OE miR-203 in reprogramming? Could it promote full-iPSC efficiency?

> We appreciate the reviewer´s comment related to the differentiation potency of the mouse iPSCs tested. As expected, ESCs are more proficient than iPSCs in the Embryoid Body differentiation assays. For instance, the EBs beating and cavities formation represent a grade of differentiation rarely observed in iPSC-derived EBs. We are sorry if we did not specify in the figure legend that, in all the experiments, we have used a wide number of clones. In the case of the EBs experiments, more than 10 independent clones have been used, both for iPSCs and ESCs. In the new Appendix Figure S1, we show and quantify the

effect of transient miR-203 expression on 5 representative independent clones of iPSCs throughout time. It is important to highlight that, in all the clones tested and experiments performed, the miR-203 transient treatment improves the differentiation potency of the PSCs tested.

As explained in the Methods, we have reprogrammed the mouse model-derived MEFs to iPSCs using OSKM transcription factors, and we have evaluated AP staining. Apart from that approach, we indeed performed several PCRs and even a RNAseq, as shown in Figure 1 for Reviewers. In particular, in panel E, 562 genes from a consensus pluripotency signature are significantly overexpressed in two representative clones of our iPSCs, derived by OSKM-reprogramming from MEFs. In panel C, other concrete pluripotency markers were validated by PCR. Therefore, according to the pluripotency markers expression, these iPSCs clones are genuine pluripotent.

Finally, the reviewer´s curiosity about the effects of miR-203 on reprogramming is very interesting and we have some data on this particular issue indeed. We have in fact analyzed the effect of miR-203 in reprogramming and we have characterized new mechanism of action of miR203 during this process, that are different from the ones shown in this manuscript. As shown in Figure 1-2 for Reviewers, miR-203 blocks indeed the reprogramming from MEFs to pluripotent cells, while lack of miR-203 seems to accelerate the process. As shown in the PCA depicted in Figure 1 for Reviewers, reprogramming is completely impeded when miR-203 is expressed during the process. We have actually characterized the reasons for these observations, that are multiple: including an anti-proliferative effect of miR-203 in somatic cells, partially as a component of a p53-mediated stress response, and additional miR-203 targets critical during the reprogramming process that are not shared for its function in ES cells or already-reprogrammed iPSCs.

We have actually analyzed miR-203 during reprogramming not only in vitro but also in vivo (Figure 2 for Reviewers), with similar outcome confirming the in vitro data. These two pieces of data are not included in the present manuscript since the cellular scenario and the miR-203 targets are different from the pathways analyzed here. These data will be reported in an independent manuscript dedicated particularly to the reprogramming process.

2, The differentiation potency of the mESCs seems not stable. Fig1E,F show inferior to miR-203 exposed PSCs, but in Fig2E show superior to both iPSC and miiPSC. Is this inconsistency a technical variation because of the serum culture condition? Along this line, it will be interesting to see whether induction of miR-203 in mESCs also promotes 2C-like state transition, like in Fig1D.

Thank you for the observation. In all our EBs experiments, iPSCs exposed to miR-203 have always been superior than ESCs. See for instance the Figure EV1G, where WT iPSCs and WT ESCs have been exposed to miR-203 mimics or miR-203 expression vectors, and assayed in EBs differentiation experiments. miR-203 increases cavity formation and EBs beating in all cases tested, at a higher extent than control ESCs. These in vitro assays are of course more limited than chimeras, to assess pluripotency and developmental potency of PSCs. In general, our data point always in the same direction although data from two different methodologies are always difficult to compare.

In Figure 2E (tetraploid complementation test), the usual percentage of success for standard iPSCs is zero. In our case, we get a proportion of all-iPSC mice born and more importantly (something very rarely mentioned in the literature) those all-iPSC mice reached the adulthood and were proficient at germline transmission. In any case, to better convince the reviewer about the potential of transient miR-203 expression to improve differentiation and developmental capacity of PSCs in vivo, we have included chimerism experiments in the new Appendix Figure S5. As shown in this table, the two independent iPSC clones tested improved significantly their contribution to chimerism (chimeras born, chimerism efficiency and adult chimeras reaching the adulthood with germline transmission) when exposed to miR-203. The results obtained for *mi*iPSCs are better than those obtained with ESCs. Again, ESCs exposed to miR-203 improve their capacity to contribute to chimeras.

To further explore the role of miR-203 on ESCs, we have also performed new assays on mouse ESCs stably expressing the *2C::tdTomato* reporter, and transiently transfected with either control mimics, miR-203 mimics, miR-203 mimics + Dnmt3a and Dnmt3b cDNAs or siRNA against both Dnmt3a and Dnmt3b transcripts. As depicted in the new Appendix Figure S3, miR-203 exposure induces a transient 2C-like stage in mESCs and in human iPSCs (as determined by the expression of endogenous retroviruses characteristic of the 2C-like stage). Importantly, as shown in the new Appendix Figure S8, this effect can be somehow partially mimicked by silencing of both Dnmt3a/3b and rescued by co-transfection with cDNAs for the two DNA methyltransferases.

3, Human iPSCs could be more problematic because lack of gold standard like in the mouse. The authors should provide some basic pluripotency characterization and compare with standard human ESC line which have been shown to have normal differentiation potency. Ideally, like in the mouse, comparing several human iPSC clones to discriminate good and bad will be very informative, considering that miR-203 may only improve the bad but not the good ones and Dnmt3a/b mediated DNA methylation could be the difference.

For a more extensive characterization of human pluripotent cells, we have started a new collaboration with Anna Veiga, scientific coordinator and director of the stem cell bank at the Centre of Regenerative Medicine, in Barcelona (Spain), to analyze in detail the effects of miR-203 transient exposure on a variety of human iPS reprogrammed through different protocols and ES cell lines from different origins. Among other experiments, we intend to perform RNAseq analysis and functional comparisons in these human *mi*iPSCs/miESCs generated to gold-standard human ESCs cell lines. We understand that these extensive studies will take time and would hopefully be part of a future manuscript, mainly due to the management and proceedings of licenses and ethical permissions required to work with human material (especially, with human pluripotent cells). Moreover, the COVID-19 crisis has irremediably blocked all the experiments and plans initiated in this particular aspect. We are still interested in performing these assays but we are afraid that it will take too long in the present situation.

Yet, in the meantime, we have been able to analyze the expression of human endogenous retroviruses (HERVs) associated to human pluripotency, in a similar way that the retrotransposon MERVL for mouse counterparts (see for instance Wang, J. et al, 2016)

already included in the manuscript. As shown in the new Appendix Figure S3C-D, miR-203 mimics induced the expression of the HERVH-GFP reporter in a significant number of colonies, in many cases not only in the periphery but in almost the totality of the cells of the colony, and such expression was sustained for 7-10 days in culture. We are aware that these assays with specific reporters do not report the whole effect of miR-203 in human cells, but at least they suggest that the effect we have described in mouse cells is maintained in human cells.

4, The human iPSC part of Fig3 is still preliminary. First, it seems that miR-203 greatly promotes OCT4 expression in Fig3B, could the authors show the difference of pluripotency mRNA and protein expression between control and miR-203 exposed iPSCs? Second, like in the mouse, whether 8C markers are unregulated by miR-203 in the same iPSCs? Ideally, RNA-seq analysis could be done.

Data in Figure 3 are actually not intended to show differences in expression of OCT4 or other pluripotency markers but to show that the number of human iPSCs (all OCT4+) maintained in the mouse embryos is higher when cells have been previously treated with miR-203, suggesting functional improvement, rather than differences in expression markers.

To make these data more robust, we and our collaborators have notably increased the number of embryos tested in panels A and B. Please, see figure 3A, where we have amplified the numbers with very interesting results. Importantly, we can even reach the 100% efficiency in interspecies chimerism when the human cells are injected in the mouse embryos at longer time points (human *mi*iPSCs "P36 + 4 passages after miR-203 transient transfection" is the most efficient condition tested). In panel B we have presented the images of human-mouse blastocyst chimeras with higher magnification, to better show the presence of human *mi*iPSCs on 8C-stage mouse blastocysts, 48 hours after the injection of one single human cell per embryo. The images clearly show the higher contribution to chimerism achieved by miR-203 exposure.

To further convince about the strength of these data, we are now showing additional results that we obtained in interspecies chimeras. As a first approach, we injected 15 human cells (either iPSCs or *mi*iPSCs) in mouse embryos and tested their contribution to human-mouse chimeras. As depicted in the new Appendix Figure S6-7, our first results were very encouraging and indeed demonstrated that human *mi*iPSCs were significantly more proficient than their control counterparts (defined as Enhanced Pluripotent Stem cells; EPS) at these stringent assays. Afterwards, to challenge even more the system, we decided to repeat the assays injecting only one single human cell (either iPSCs or *mi*iPSCs) in the mouse embryos. Those data are presented in the Figures 3 and EV3 of the manuscript.

5, For Fig4A, DNMT3A/B protein expression, both short and long term after miR-203 exposure, should be amended to confirm they are downregulated by miR-203. Similarly, Western blot confirmation of Dnmt3a/b expression is necessary for Fig4G. It will also be relevant to compare Dnmt3a/b expression between iPSCs and ESCs. The authors could make effort to repeat E and G in human iPSCs in order to show the mechanistic generality.

We appreciate the reviewer´s comments. We show RNA data to demonstrate that Dnmt3a and 3b levels are being regulated by miR-203 at the mRNA level (please, see figures 4F, 4H, EV5A). Also, RNAseq data confirmed the relevance of Dnmt3a/3b as miR-203 targets (see figure 4A), which was meticulously validated in Figures 4B, 4C, 4D, 4E and 4G. Now, in the new Figure EV4E, we have included Dnmt3a/b protein modulation, in 5 different clones of mouse iPSCs and one human iPS cell line, exposed or not to miR-203, to further demonstrate the regulation of the Dnmts at the protein level as well and importantly, that the miR-203-mediated downregulation of Dnmt3a/b is a general mechanism, that also applies to human PSCs.

We have addressed the relevance of the mechanism through rescue assays presented in the new Appendix Figure S8. In these experiments we have actually rescued to a significant level the induction of the 2C-like stage in miESCs by co-trasfecting with Dnmt3a/3b cDNAs.

It is important to highlight here that the message of our work is that transient expression of miR-203 can notoriously improve the differentiation capacity of any PSC tested. That implies also (and importantly) transient downregulation of Dnmt3a/b so that the levels of these proteins can recover later thus allowing functionality. Indeed, one of the most important observations in this methodology is how the Dnmt3a/3b levels are recovered when the cells differentiate, as shown for instance in Figure EV5A and also is implied in Figure 5B, where we can observe that the methylation levels on *mii*PSC-derived EBs are indeed higher than those on control EBs.

6, For Fig5, first, it will be relevant to check the global DNA methylation levels using dot-blot with anti-5mC or DNA methylation sensitive restriction enzyme assay between Ctrl and miR-203 exposed iPSCs. Ideally, 2i treatment could be included as a positive control because 2i induces a global hypomethylation through Dnmt1-Uhrf1 but not Dnmt3a/b downregulation.

We appreciate the reviewer´s comments. Our study shows changes in DNA methylation using two independent approaches: genome-wide bisulphite sequencing and clonal bisulphite sequencing for some loci. Whole genome bisulphite sequencing is actually one of the gold standards and state-of-the-art methods for the analysis of genome-wide DNA methylation with the highest CpG coverage, sensitivity and specificity. While we appreciate the additional value of using other techniques, we are afraid that given the current situation in which we will not able to perform these assays (neither in our collaborator laboratories in USA or Australia) these experiments cannot be incorporated in the present manuscript. Yet, we are confident that the extensive genome-wide bisulphite sequencing studies and locus-specific assays included in the manuscript actually support the major conclusions described in the manuscript.

Regarding the differences with 2i conditions, we now include new data in the new Appendix Figure S2, describing the differences between miR-203 and 2i treatments, using EB formation analysis and RNAseq profiling as readouts. These experiments indicate that miR-203 exerts its effects independently of the 2i and still improve significantly the quality of 2i-exposed iPSCs, suggesting (as the reviewer thoughtfully remarks) different mechanisms of action.

Second, in 5F&G, it is interesting that short-term (t=10) of miR-203 exposure doesn't induce TSS hypomethylation both genome-wide and on 2C genes, while long-term (t=25) does. However, in Fig1D, 2C genes show mainly a short-term induction. Could the authors explain the discrepancy? Or are there other mechanism or miR-203 targets may be involved?

We are sorry if the data were not represented to sufficient quality in the original version of the manuscript. Figures 5F and G do show hypomethylation of the genes involved in the 2C signature at the short time point (t=10 days). When t=10 and t=0 are compared, the dot cloud is under the line, as observed in panel G. This is also true for global genome-wide hypomethylation, shown in panel F. Such hypomethylation is indeed more pronounced at longer time points (t=25 days) in both analyses. In panel 1D, the expression of the 2C signature is very dramatic at shorter time points and, although is still significantly increased compared to the control counterparts, such induced expression is less accentuated at longer time points. Indeed, it makes sense that the hypomethylation detected at time "10 days" correlates with higher transcriptomic induction of the signature, which is progressively lost with time, probably due to additional compensatory mechanisms, apart from/independent of the TSS methylation levels, as the reviewer suggests.

Third, in EV5A, the kinetics of Dnmt3a/b expression anti-correlate almost perfectly with miR-203 and resume on T=10, however, DNA demethylation only gradually happens after T=10 (5F,G and EV5B,C). Could the authors explain why there is a lag? Again, is there other mechanism involved?

We apologize for the apparent confusion. On one hand, the experimental setups in Fig. 5 and EV5 are different. On the other hand, and assuming this comparison can be done, the reviewer is right and we have observed that demethylation is maintained and even enhanced with time, as far as the cells are maintained in pluripotency culture conditions. As the reviewer is aware, DNA demethylation is a passive process and requires cell division. Although we cannot explain entirely the dynamics of the process it is clear that Dnmt3a/b are not immediately active in inducing DNA methylation as far as cells are maintained in pluripotency conditions. However, they are very rapid to induce de novo-methylation as soon as the conditions change (e.g. embryoid body formation).

Forth, what is the correlation of DNA hypomethylation and gene expression change? In particular, could the author analyze deeper to see how DNA hypomethylation explain the gain of potency? For example, is Elf5 or Sirt6 induced in miiPSCs or during differentiation to improve differentiation? Any effort along this direction would be a great addition to this study.

The reviewer suggests interesting new ideas to further explore new links between hypomethylation and pluripotency. Our data support a model in which global DNA demethylation provides a window of opportunity for cell plasticity towards a stem phenotype. Indeed, this epigenetics process occurs during normal development from the 2-cell to

blastocyst state (https://doi.org/10.1016/j.gde.2017.02.003). Overall, it seems that the global demethylation induced by miR-203 may have a relevant effect in pluripotency. To what extent one or two specific genes (e.g. Elf5 or Sirt6) participate in this process or may explain the differences in the differentiation potential would require extensive investigation and very likely will depend on which differentiation processes are selected for the analysis. This is an area that we would like to explore more in detail in the future.

Nevertheless, we thank the reviewer for this interesting observation, and have performed a new analysis to answer this particular question. As depicted in the new Appendix Figure S9, we can find common genes up-regulated (data from RNA seq studies) and hypomethylated (data from genome-wide methylation studies) in *mi*PSC 20 days after Dox withdrawal. A total of 235 genes (shown in the new Appendix Table S9) were both DNA-hypomethylated and up-regulated in such conditions. Gene Ontology Analysis of this list is presented in the right panel of the new Appendix Figure S9 and extended in the new Appendix Table S10.

Minor comments:

1, In the Methods, the gender and passage number for all the PSC lines need to be stated. I don't find the use of 2i in any experiment. Typo: "250.000 cells".

We apologize for this error. The gender and passage number of the PSCs in our study is included in the Methods Section. We have used 2i in the culture media for cardiomyocyte differentiation experiments and in the new Appendix Figure S2.

2, Fig5 is confusing: 5B, DMV is not explained clearly in the text. I assume it to be methylation downregulated region. 5C seems to show an increase of methylation in miR-203 samples. Is this global level rather than TSS, as in F? 5I, how the top 100 DMRs are selected? Among all or between which groups?

We are sorry for these defects. The definition of DMV is identical to that of Hovestadt et al. 2014 (https://www.nature.com/articles/nature13268). The implementation of the DMV finder can be found here (https://github.com/astatham/aaRon/blob/master/R/findDomains.R). DMV meaning is explained in the text (line 7, page 11; DNA methylation valleys) and in the figure legend as well (panel B). PMDs were also called using this method.

Regarding panel 5C, it shows the methylation distribution of all CpG sites smoothing the data using 100kb windows, therefore, it shows global DNA methylation changes. We apologise if 5C was a bit confusing. We have followed Reviewer's recommendation and thus we have changed the colours and separate the plot into two plots, one for miR-203 PSCs and the other one WT PSCs. This new visualisation allows to better appreciate that miEBs show a slight higher global DNA methylation.

The top DMRs selected in panel 5I are unbiased, and represent the top Differentially Methylated Regions in the experiment, not comparing concrete experimental groups.

3, Fig6 is blurred, contains a yellow line and needs a rotation.

We apologize. We were aware of it and indeed the figure was changed during the submission, although apparently not corrected in the final version sent to the reviewers. It is now corrected.

Referee #2:

1) The major concern relates to the question whether transient miR-203 expression generally potentiates cellular plasticity and thereby promotes the development of treated cells towards different germ layer lineages in response to intrinsic and extrinsic cues (e.g. in teratomas, developing embryos). Alternatively, transient miR-203 expression may promote proliferation and/or survival of such cells, which indirectly would contribute to teratoma and interspecies chimerism formation. It is not clear whether one, the other or both contribute to the observed results.

The differentiation potency that miR-203 confers to the stem cells is very likely a combination of several aspects. Indeed, by definition, the proficiency of PSCs in such stringent assays we have shown in this work (human-mouse interspecies chimerism, tetraploid complementation) is heavily dependent on those three factors: proliferation skills, viability under stressful conditions and of course, differentiation skills *per se*. Our hypothesis is that miR-203 definitely contributes to boost the differentiation skills, to promote survival and also induce proliferation in this particular scenario. In our interspecies chimerism experiments, we detected how the transient expression of miR-203 in human PSCs makes them more proficient to survive in such stringent assay, in which the index of human cell death within the mouse embryo is usually extremely high. Not to mention, of course, the given capacity to differentiate to different lineages in the embryo, in vivo, as shown in Figure EV3C.

Since the reviewer finds this point very critical, we show in the new Appendix Figure S4 some further analysis on the *mi*PSCs-derived teratomas, compared to the control counterparts. The GO analysis of the teratomas (new Appendix Figure S4A), and the staining with markers specific of the different lineages and mature tissues (new Appendix Figure S4B-D) demonstrate how general this plasticity is. We truly believe that miR-203-mediated effects on differentiation potency are global and affect development in general. In the new Appendix Figure S4D, ki67 staining demonstrates that miPSCs-derived teratomas proliferate more, which is also obvious in Figure 2A.

The plasticity is also obvious in the in vivo experiments, tetraploid complementation, mouse chimerism (shown in the new Appendix Figure S5) and interspecies chimeras. The developmental capacity of the tested *mi*PSCs in these assays is manifest, since (i) *all-iPSC* mice are born, (ii) we obtain 100% chimerism contribution in some examples and (iii) human PSCs develop to the three germ layers in the mouse embryo.

2) A second concern relates to the molecular characterization of the iPSC cells. While many clones have been tested in the cellular assays, it is not clear which and how many iPSC and ESC clones have been tested in the RNA and DNA methylation sequencing assays. In other words, how reproducible are these results? To support this concern please review the data shown in Figure 5B (and 5D): the methylation level of wildtype control cells at t=0 strongly differs from those of the un-induced transgenic miR-203

iPSC cells. Are the differences observed between wildtype and miR-203 transgenic cells at later time points due to the variability of methylation that was present at the original t=0 timepoint? Are the greater dynamic changes observed for the miR-203 transiently expressing cells reproducible between different iPSC clones? On Page 9, log2 fold changes for Dnmt3a and Dnmt3b of -0.24 and -0.22 are described. These are remarkably minor changes in expression to explain the rather larger changes in DNA methylation reported. It might be useful to measure expression of these de novo Dnmts as well as those of proteins involved in maintenance methylation in the time course experiments.

Throughout the manuscript, different experimental settings, with different PSCs clones of different origins have been systematically tested. The number of clones tested in each particular panel is always depicted in the figure legend or in the panel itself (we have now revised all the panels and figure legends). At least three independent clones (in several cases more than that) have been analyzed in every single approach.

For DNA genome-wide DNA methylation analysis, due to the complexity of the assay and the high costs, we reduced our samples to 2 independent iPSC clones for each condition, 2 technical replicates per clone, as explained in the Figure legend. These numbers have been used in many papers in the literature for this sort of experiments.

Indeed, the methylation levels of wild-type control cells and un-induced transgenic miR-203 cells is very similar at time 0. In terms of methylation levels, it is recognized that the differences are counted in magnitude orders. In other means, 720 DMVs versus 556 DMVs is not considered a substantial difference. However, 2,034 DMVs versus 723 DMVs is a real difference to be considered. Again, 198417 DMPs versus 732 DMPs, or 12549 DMPs versus 136 DMPs are very significant differences.

Regarding the Dnmts levels, we appreciate the reviewer´s comments. We show RNA data to demonstrate that Dnmt3a and 3b levels are being regulated at the mRNA level, by miR-203 (please, see figures 4F, H, EV5A). Also, RNAseq data confirmed the relevance of Dnmt3a/3b as miR-203 targets (see figure 4A), which was meticulously validated in Figures 4B, C, D, E and G. Now, in the new Figure EV4E, we have included Dnmt3a/b protein modulation, in 5 different clones of mouse iPSCs and one human iPS cell line, exposed or not to miR-203, to further demonstrate the regulation of the Dnmts at the protein level as well.

3) Technically, to what extend are the differences in the number of DMVs and PMDs between conditions dependent on the criteria that were used to call them? When using slightly different criteria, do the main findings still hold up?

We understand that the reviewer is asking here whether we have tweaked the thresholds to suit our narrative - but this is not the case. In this work and many others from the field, we have used an independent and intuitively reasonable definition of DMV and PMD. DMV is where the average methylation is < 0.15 over the region, and a PMD is > 0.15 but < 0.6. (i.e. Hovestadt 2014 as a precendent). Since we have not defined these thresholds ourselves, we are encouraging reproducibility. We have clearly showed consistent demethylation in the miR-203-exposed cells as evidence to firmly believe that the final conclusions would not change at all using slightly different criteria.

4) Regarding the RNA sequencing experiment shown in Figure 1C, what does the X-axis represent? Have these cells been used in the DNA methylation experiments?

We apologize for this error. In Figure 1C, X-axis represents PCA2 and Y-axis represents PCA1. These particular clones have not been tested in the DNA methylation analysis, but we have performed RNAseq with the very same clones tested in genome-wide DNA methylation experiments. In the new Appendix Figure S9 we show the results of the RNAseq and how the genes upregulated and hypomethylated exhibit GO related to development, differentiation and morphogenesis. See also the new Appendix Tables S9 and S10.

5) One related concern is the variability in experimental setups. The variability in time course experiments could have a large impact on data interpretation. As shown in Figure EV5, DNA methylation is reduced between t=0 and t=25 but increases at t=35. Therefore, we can expect to see changes in transcription and maybe in developmental potency at different time points. To be able to compare transcription and methylation, we suggest performing a more precise time-course analysis. Moreover, authors should take into consideration that cells could have different developmental potency depending on the incubation time (as developmental potency assays were performed on variable conditions) and discuss this.

We thank the reviewer for this comment. We acknowledge the complexity of some of the figures, but we were very careful showing first our experimental schedules, to make the understanding easier for the readers. What we have shown in Figure 5 is an experimental setup detailed in 5A. What is shown in Figure EV5A is an experimental setup described in the same panel and what we have shown in panels EV5B and C is an experimental setup detailed in EV5B as well.

The longest time points in these figures correspond to EBs formation (see for instance t=32 days in Figure 5A-G; t=10 and t=20 in Figure EV5A; t=32 in Figure EV5B, C). Whenever the methylation levels have been tested on EBs formation, they increase, as expected during a differentiation process.

In Figure 5H, the DMRs at the Sirt6 locus have been tested, just as an example, and in Figure EV5D,E we have done the same for Elf5 TSS. Of course, there is variability on the level of methylation between different locus, that is obvious. Please, note that neither Figure 5H or EV5D,E are reflecting the global genome-wide DNA methylation levels, those are just two particular examples selected in a completely unbiased way to test Dnmt3a/b influence on DNA hypomethylation. Therefore, we cannot compare those time points with the ones depicted in Figure 5A-G.

The data showed throughout the manuscript clearly show that miR-203-mediated effects have similar outcomes, even changing minimally the time course conditions, the experimental settings, the PSCs clones used, the origin of the cells, even the laboratory where the miR-203 effects were tested. These data together give a global idea of the generality of this methodology.

6) One concern relates to the use of different control settings (either wildtype or transgenic non-induced iPSC). Why was this done? Does it affect the interpretation of the results?

In every new approach we tested the effect of Doxycycline alone, as indicated in the Methods, just to make sure that none of the observed effects were due to DOX treatment. In many of our panels we include this control, in some others we use the isogenic control also to demonstrate that the observed miR-203-mediated effects are clone-independent. Specifically, in methylation analysis, we found more appropriate to use wild type also exposed to DOX throughout the time course, and measure the methylation potential during time in miR-203-exposed cells and in DOX-exposed cells. It was very important to highlight that doxycycline treatment has not consequences on global methylation levels, and it was clearly depicted in the Figure 5. Using one or other control did not affect the main conclusions in any of the assays performed.

7) For Figures 2 and 3 (and related EVs): how reproducible are the results obtained by using different iPSC clones as starting cells? Please quantify. Further, please describe the origin of the different iPSC and Esc clones. Finally, the IF images are very difficult to interpret. When showing results of one single color channel, we highly recommend presenting the data in B/W, preferably in a higher magnification.

In Figure 2, we used multiple different iPSCs or ESCs clones, derived from the miR-203 transgenic mice. In panel B, for instance, we used 9 iPSCs and 15 *mi*iPSCs clones, with very similar outcomes. In panel C we include 8 iPSCs clones, 12 *mi*iPSCs clones, with very similar outcomes in each group. In tetraploid complementation the numbers are also indicated. We did not find significant differences among the clones in any of the experiments developed, and we have revised all figure legends to indicate the number of clones used.

In any case, we have included in the revised version of the manuscript additional assays performed with additional clones. For instance, in the new Figure 3, we have notably augmented the number of embryos tested, with very satisfactory results. We are also showing additional results that we obtained in interspecies chimeras. As a first approach, we injected 15 human cells (either iPSCs or *mi*iPSCs) in mouse embryos and tested their contribution to human-mouse chimeras. As depicted in the new Appendix Figure S6 and S7, our first results were very encouraging and indeed demonstrated that human *mi*iPSCs were significantly more proficient than their control counterparts (defined as EP stem cells) at these stringent assays. Afterwards, to challenge even more the system, we decided to repeat the assays injecting only one single human cell (either iPSCs or *mi*iPSCs) in the mouse embryos. Those data are exposed in the Figures 3 and EV3 of the manuscript.

We have also included new chimerism experiments in the new Appendix Figure S5 with two independent iPSC clones. The number of chimeras born, chimerism efficiency and adult chimeras reaching the adulthood with germline transmission were all improved when exposed to miR-203. The results obtained for *mi*iPSCs are better than those obtained with ESCs. Again, ESCs exposed to miR-203 improve their capacity to contribute to chimeras.

Regarding the quality of images in the Figure 3B, we apologize for that and now we have substituted the former figures by new ones with higher magnification. The reviewer´s suggestion has been very appreciated, since the new images in Figure 3B distinctly show the improved contribution to human-mouse blastocyst chimeras in the case of human *mi*iPSCs.

8) Another concern relates to the cellular composition of embryoid bodies upon miR-203 stimulation. In Figure 1, authors showed that miPSC-derived embryoid bodies show beating behavior at a higher frequency compared to controls. This could suggest that miPSC are more prone to differentiate towards the cardiomyocyte cell fate, without improving differentiation into any other cell lineage. Indeed, Figure 6 shows that miPSC differentiate more efficiently compared to control into mature cardiomyocytes. To exclude that miR-203 expression primes cells towards the cardiomyocyte cell fate only, we suggest testing differentiation towards a different cell fate, such as of neural stem/progenitor cells (NPCs).

Is it possible to assess the cellular composition (heterogeneity) of the embryoid bodies using IF or RNA-seq approaches?

This observation is interesting, and we have further analyzed our EBs in more detail. As depicted in the new Appendix Figure S1D-E, miPSCs give rise to the three germ layers when differentiated to EBs. We show this by IF and IHC in these new figures. To better exclude the possibility that miR-203 exposure primes PSCs towards the cardiac cell fate, new Appendix Figure S4 includes some further analysis on the miPSCs-derived teratomas, compared to the control counterparts. The GO analysis of the teratomas (Appendix Figure S4A), and the staining with markers specific of the different lineages and mature tissues (Appendix Figure S4B-D) demonstrate how general this plasticity is.

The reviewer suggests to apply our miRNA-based strategy on differentiation towards additional cell fates. First, our data suggest that the effect of miR-203 is not cell fate-specific given the large number of cell lineages and tissues observed in the embryoid body assays, teratomas etc. In addition, we are currently working on a comprehensive project, more translational, in which we are testing our miR-based thechnology on particular differentiation settings, with interesting outcomes. We hope this second project will be published within the next year.

Minor concerns

9) In Figure 1C, authors showed that miPSC are more similar to ESC rather than to regular iPSC. It would be interesting to expand this analysis, including the relationship to 2C embryos, blastocysts and mESC with different developmental potency, such as 2C-like cells and EPSC.

We really thank the reviewer for this comment. Following, her/his recommendations, now we have also assessed the transcriptomes of different clones of *mi*iPSCs, mESCs, 2-cell-like mESCs subpopulations (MacFarlan et al., 2012; GSE 33923) and epiblast stem cells (Najm et al., 2011; GSE 26814) (Fig. 1B, new 1E). Principal Component Analysis revealed a gene

expression pattern of *mii*PSCs clones 1 and 2 (analyzed 10 days after miR-203 exposure) similar to that observed for tomato-positive cells, characterized as 2C-like ESCs by MacFarlan and collaborators, while *mii*PSCs clones 3 and 4 (analyzed 25 days after miR-203 exposure) exhibited a profile more similar to ESCs clones, corroborating the observations showed in Fig. 1D.

10) MiESC showed a phenotype similar to that of miPSC. Is thus the developmental potency of miESC increased similarly to miPSC? How does this relate to genome wide DNA methylation patterns?

This is also an interesting observation. We have tested miR-203 impact on ESCs only in EBs formation. In the revised version, we have made the effort to include new assays to test the effects of miR-203 exposure on ESCs. In the new Appendix Figure S5, we have performed chimera assays, not only with iPSCs but also with ESCs. It is interesting to note that miR-203 exposure improves the contribution and the efficacy on chimera contribution, both in iPSCs and ESCs. Particularly, in the case of miESCs, we can even reach 100% chimera efficiency, with 7 out of 10 adult chimeras with germline transmission.

Following the reviewer´s suggestion, we have also included some new data regarding the molecular mechanisms. First, we were interested on reproducing the induction of the 2C-like stage also in ESCs. As depicted in the new Appendix Figure S3, miR-203 also induces the 2C reporter on mESCs. Even more, we can revert these effects by co-transfecting miR-203 and Dnmt3a+3b cDNA (new Appendix Figure S8). This data indicates that the miR-203-mediated effects on ESCs are also dependent (at least in part) on Dnmt3a/b.

11) It would be interesting, if possible, to know the expression of miR-203 in human embryos and whether miR-203 is conserved between human and mice. Does miR-203 modulate DNMT3A and DNMT3B expression in human cells?

Human miR-203 is expressed from chromosome 19. Its murine counterpart, mmu-miR-203 (Accession in miRbase MI0000246) is expressed from chromosome 14 of Mus musculus. The main mature sequence of mmu-miR-203, mmu-miR-203-3p (Accession in miRbase MIMAT0000236) seems to be identical to that of hsa-miR203a-3p. There are experimental evidences of a second mature sequence, mmu-miR-203-5p* (Accession in miRbase MIMAT0004547), which is shorter than hsa-miR-203a-5p (22 nucleotides instead of 25) and differs slightly from its human counterpart in the rest of the sequence (in position 11, G is replaced by A in hsa-miR203a-5p*).

Both miR-203a-3p/-5p were used in these assays, aiming to faithfully mimic the endogenous scenario in which both mature forms are expressed. However, the most abundant form (miR-203a-3p) appears to be responsible for the observed effects, as depicted in the new Appendix Figure S10.

Regarding the Dnmt3a/3b 3´-UTR sequences, Figure EV4A shows how those sequences are conserved in several representative species, including of course human and mouse. Furthermore, in the new Figure EV4E, we have included Dnmt3a/b protein modulation, in 5 different clones of mouse iPSCs and one human iPS cell line, exposed or not to miR-203, to further demonstrate the regulation of the Dnmts at the protein level as well, both in mouse and human samples.

12) In Figure 2C, 2E and 3A statistical tests are required. Increasing the number of embryos could improve statistical significance (particularly in Figure 3A).

We have included new assays to increase the numbers of embryo development in vivo. As shown in the new Appendix Figure S5, we have tested the effect of miR-203 on chimera contribution, using new clones of iPSCs and ESCs. Also, in interspecies chimeras, we have increased the numbers, as shown in Figure 3A, and include statistics.

13) In Figure 3A, it is not clear what the difference is between the miPSC P29+0 and miPSC P36+2. Can these conditions be pooled? If not, why?

We apologize for the lack of clarity in the legend of this figure. In the nomenclature of Figure 3A, "P" indicates the number of passages of human iPSCs or *mi*iPSCs and the number after "+" indicates the number of passages of these cells after miR-203 transient transfection. Indeed, these conditions might be pooled, but we consider that they are actually different and should be presented individually. It is interesting to note that the efficiency in chimera contribution increases with the passages after miR-203 exposure.

14) In Figure 4D, iPSC and iPSC-siC don't overlap in the principal component analysis. Indeed, it seems as PC2 captures differences between siRNAs treated and non-treated cells. Similarly, iPSC-siDnmt3a/3b and miPSC are separated in PC2. To improve the comparison of Dnmt3a/3b knock down conditions to miPSC cells we propose treating miPSC cells with control siRNAs as well (and possibly with siDnmt3a/3b as well).

How much of the variance is covered by the different PC dimensions? This should be indicated in the figure (legend).

In the PCA panel, iPSCs and iPSC-siC do not overlap in PC2 probably because they are different clones. However, it is important to note that they do overlap in PC1, which captures the most variation of the comparison (51.2 % by PC1 versus 19.6% by PC2).

Regarding *mi*iPSCs and iPSCs-siDnmt3a/b, we mention in the text that they have similar profiles (specially -again- from the PC1 perspective) but not identical. They indeed slightly differ also in PC1. Throughout our manuscript we never assume that the only targets responsible for miR-203 mediated effects are the Dnmt3a/3b. We do believe that those are, at least in part, responsible for the observed phenotype, as corroborated by the genome-wide DNA methylation data and the rescue experiments we show in the manuscript. But we do not expect to have the same profiles in *mi*iPSCs than in iPSCs-siDnmt3a/b. Following the reviewer comments, we have now indicated the specific variation represented by PC1 and PC2 in the figure legend.

15) Figure 5C is difficult to interpret. A different format or color choice might improve clarity.

We appreciate the observation. We have re-formatted the Figure 5C to show the data more effectively.

Referee #3:

1. It is unclear why only miR-203 out of more than 1000 known miRNAs was selected. Is it the only one that is preferentially expressed in 2 cells stage?

We really appreciate the reviewer´s comments.

We have been working on this microRNA for several years. As described in the first paragraph of the results section, miR-203 was originally proposed to limit the stemness potential of skin progenitors (Yi, Poy et al., 2008) and to display tumor suppressive functions in multiple cancers (Bueno, Perez de Castro et al., 2008, Michel & Malumbres, 2013), suggesting a role in the balance between stemness and differentiation. However, its expression during early development remained undefined. A first analysis of miR-203 levels during normal murine and bovine preimplantation development suggested a modest but specific wave of expression during the 2-cell stage and early blastocysts, whereas its expression was lost in cultured embryonic stem cells (Goossens, Mestdagh et al., 2013, Yang, Bai et al., 2008). In our settings, we corroborated such specific wave of expression of miR-203 in mouse embryos at the 2C stage, as shown in Figure 1A. We found this observation extremely interesting, and together with the previous connections found between miR-203 and stemness regulation by ours and other labs, we decided to further explore its role in pluripotency and differentiation potential of stem cells.

2. The results presented in Figures 1B-G are obtained upon overexpression of miRNA. As stated on page 5 miR-203-encoding sequence was inserted downstream of.. it seems premir sequence was cloned. If it is correct, and the fact that a mature miRNA can be derived either from -5p or -3p and therefore, authors should mention clearly which of those mature miRNA cause the effect shown in 1B-G. Expression analyses of both mature miR-203-3p and 5p should be presented in Figure EV1.

We really appreciate this reviewer´s observation, since it is true that this aspect is not clear in the original version of the manuscript. In the case of miR-203, the most abundant mature form is miR-203a-3p, while the low abundant form is called miR-203a-5p*. Human miR-203 is expressed from chromosome 19. Its murine counterpart, mmu-miR-203 (Accession in miRbase MI0000246) is expressed from chromosome 14 of Mus musculus. The main mature sequence of mmu-miR-203, mmu-miR-203-3p (Accession in miRbase MIMAT0000236) seems to be identical to that of hsa-miR203a-3p. There are experimental evidences of a second mature sequence, mmu-miR-203-5p* (Accession in miRbase MIMAT0004547), which is shorter than hsa-miR-203a-5p (22 nucleotides instead of 25) and differs slightly from its human counterpart in the rest of the sequence (in position 11, G is replaced by A in hsa-miR203a-5p*).

Both miR-203a-3p/-5p were used in these assays, aiming to faithfully mimic the endogenous scenario in which both mature forms are expressed. However, the most abundant form (miR-203a-3p) is responsible for the observed effects, as depicted for example in the new Appendix Figure S10.

Regarding the Dnmt3a/3b 3´-UTR sequences, Figure EV4A shows how those sequences are conserved in several representative species, including of course human and mouse. Importantly, only the miR-203-3p targets Dnmt3a/3b, reinforcing the fact that the 3p sequence is the responsible of the observed effects.

3. miR-203 mimics in Figure 1G-H are used? I assume, it is miR-203-3p (due to the fact only -3p targets Dnmt3a and Dnmt3b). Authors should also test mimics of miR-203-5p in EB formation assay and all other experiments to ascertain the enhanced differentiation is indeed due to miR-203-3p.

We appreciate this reviewer´s observation. The miR-203 mimics we have used in Figure 1G, H, Figure EV1G, Figure 6, Figure EV6 and several of the new Figures (Appendix Figure S1, S3, S8) are a mixture of the two sequences, aiming to faithfully mimic the endogenous scenario in which both mature forms are expressed. We do know that the most abundant form, and the one that targets Dnmt3a/3b is the miR-203-3p sequence. To further confirm it, we included the new Appendix Figure S10, where we have tested separately the miR-203a-3p mimics and miR-203a-5p mimics on embryoid bodies formation. These data show that it is the 3p version the one responsible for the observed effects on improved differentiation, as expected.

4. Authors investigated transient exposure of miR-203 in figure 2. Will longer induction time further improve the developmental potential of iPSCs and ESCs? The data should be included in the manuscript.

In the origins of this project, we indeed tested different schedules of miR-203 exposure. After several trials, we defined the 5 days-treatment as the shortest most effective exposure in PSCs, to improve the differentiation potency of such cells. Longer induction times were not more effective than the 5 days-induction. Indeed, very long treatments can be detrimental for the stem cells, probably because other additional effects mediated by this miRNA (such as proliferative as we have seen in other cell types) get involved and become unfavorable for the PSCs culture.

Regarding the mimics and transduction experiments, it is important to highlight that the transient transfection of miR-203 mimics gives rise to a miR-203 exposure of around 3-5 days and so do the viral vectors in the viral transductions: the expression is maintained for a few days before the promoters are silenced in the PSCs.

Altogether, the different approaches have this short time exposure in common, and in all the settings tested, this timing is enough to detect a remarkable differentiation improvement.

5. Is this effect of miR-203 specific to OKSM induced iPSCs? Can it also be applied in other combination of transcription factors-induced iPSCs?

This is an important point. Actually, the miR-203-mediated effects on differentiation, as the reviewer emphasizes, occur in already-established stem cells, not during the reprogramming process. In this project, we have tested OSKM-induced iPSCs, but also ESCs (not reprogrammed), EPS (enhanced-pluripotent cells, generated using a new cocktail of

chemicals as described in Yang et al., 2017), neonatal primary cardiomyocytes (in this particular case, not pluripotent cells, but committed cardiomyocytes that fully mature with the transient exposure to miR-203 – see complete Figure EV6-) and interestingly, iPSCs generated in other labs (Serrano´s, Wang´s, Pu´s etc), which were reprogrammed from different sources, with different protocols and even maintained with different culture medias (see, for instance, the effects of miR-203 over 2i-treated PSCs- Figure 6 and the new Appendix Figure S2) .

In any case, we thank that the recommendation of testing a wider variety of pluripotent cells is of great interest for future applications. Following the reviewer´s advice, we have decided to start a collaboration with the stem cell bank at the Centre of Regenerative Medicine, in Barcelona (Spain). There, they hold a collection of several human iPSCs, from different origins and reprogrammed through different protocols (not restricted to OSKM-mediated reprogramming). Our intention is to get a number of representative hIPSCs cell lines from the bank, and analyze in detail the effects of miR-203 transient exposure on all of them. Among other experiments, we intend to perform RNAseq analysis and compare the human *mii*PSCs generated to gold-standard human ESCs cell lines. However, we feel that these extensive studies will take time and would hopefully be part of a future manuscript, mainly due to the management and proceedings of licenses and ethical permissions required to work with human material (especially, with human pluripotent cells).

Minor

In the methylation heatmaps (fig.5I) the differences between patterns in iPSC and miiPSC embryonic bodies, both for differentially methylated regions and imprinting control regions are not obvious even though the morphology of EBs is very different. Authors should discuss the reasons for this. Could it be explained by a mechanism other than epigenetic control?

We thank the reviewer for this observation. Please, note that the heatmap for ICRs (Figure 5I, lower panel) does not show evident changes among the different conditions. Indeed, we claim in our manuscript that miR-203-induced demethylation of ICRs was very moderate, when compared to the stronger miR-203 effects detected on global DNA methylation (see Figure 5B, C, D, E) and even when only the top 100 DMRs are tested (Figure 5I, upper panel).

Therefore, we do not expect changes in imprinted regions, neither in iPSCs or EBs derived from those. We believe that observation is very relevant, given the recent findings showing that a widespread loss of methylation in PSC cultures might be deleterious when accompanied by massive erasure of genomic imprints. Data in the Figure 5 shows that it is not the case for miR-203-based strategy.

Regarding the upper panel in Figure 5I, here we show the methylation levels at the top 100 DMRs. Thus, they are representative of the global differences on methylation in this experiment but are not necessarily representative of the global differences on EBs DNA methylation. To have a clear idea of what the differences on EBS DNA methylation are, we need to check the global data shown in Figure 5B-E. For instance, DNA Methylation Valleys (DMVs) and Partially Methylated Domains (PMDs) are notably higher in *mii*PSCs compared

to the control iPSCs, at t=10 and t=25 days of culture. However, when differentiation occurs (t=32 days), the numbers of DMVs and PMDs of *mii*PSCs-derived EBs are lower than the ones of control iPSCs-derived EBs (Figure 5B). There is indeed a significant difference on EBs global DNA methylation. We discuss this observation in the manuscript (Page 11, last sentence): Interestingly, the EBs derived from *mii*PSCs displayed higher global levels of DNA methylation and lower numbers of PMDs (partially methylated domains) (Figure 5B-E) in agreement with the upregulation of Dnmt3a and Dnmt3b transcripts observed after induction of differentiation (Figure EV5A).

Figures for the reviewers

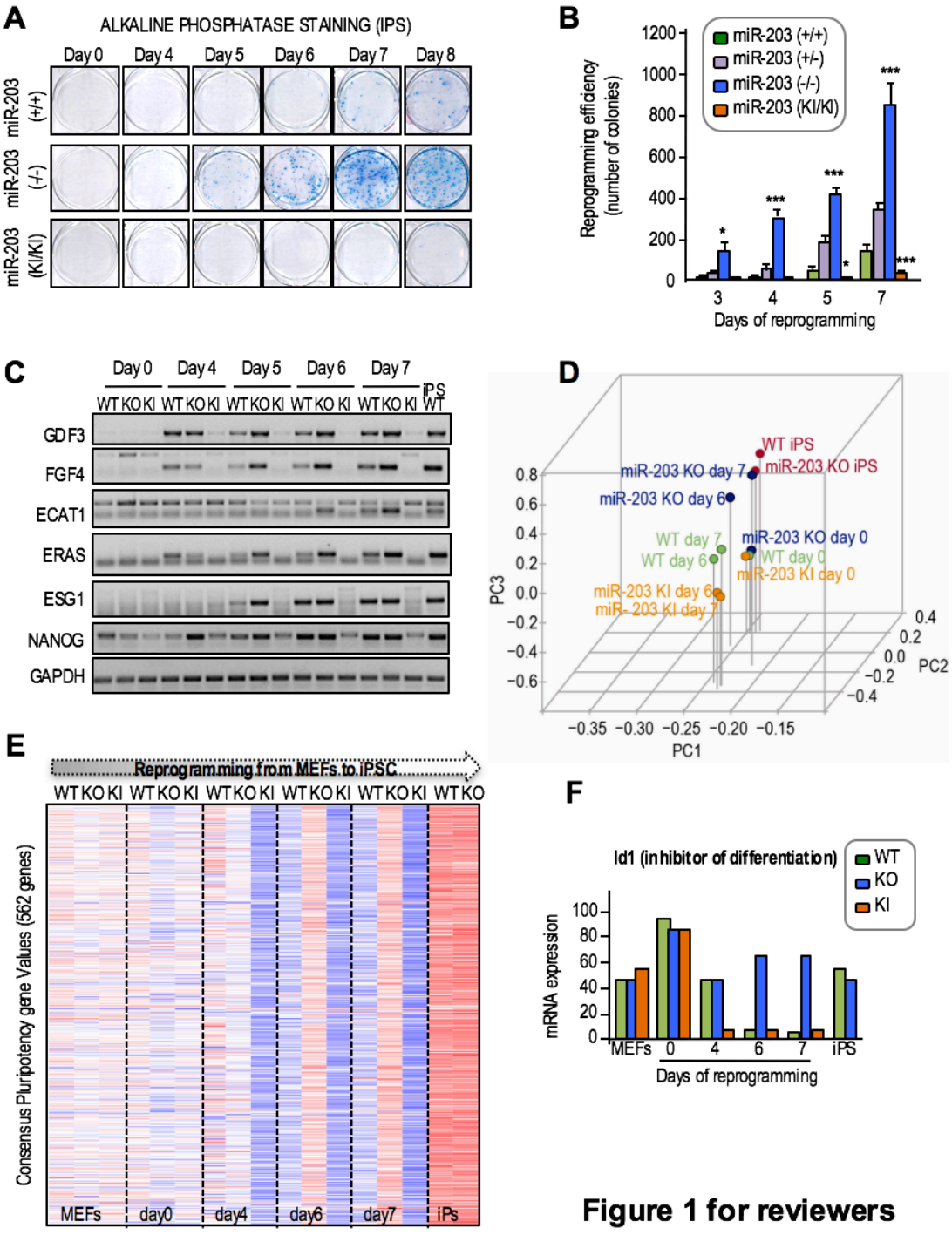
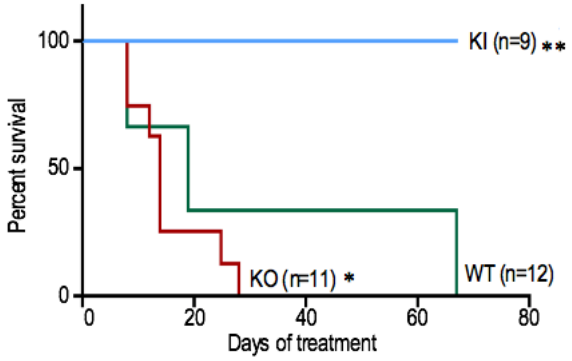


Figure 1 for reviewers

Figure 1. A, MEFs of the indicated mouse lines (miR-203 wild-type, knock-out and the miR-203 inducible model) were transduced with non-inducible Oct4-Sox2-Klf4-cMyc (OSKM) lentiviral vectors (FUW-OSKM, Addgene #20328). For inducing transient miR-203 over-expression in the *ColA1*(miR-203/miR-203*); Rosa26*(rtTA/rtTA) MEFs, cell cultures were treated with Dox (1 mg/ml; Invitrogen) during the complete reprogramming process. Doxycycline was added to wt and ko MEFs as well, as a control. Colonies of iPS cells were stained for alkaline phosphatase (AP) at different time points during the induction of pluripotency. Representative images of 8 independent MEF isolates per line are shown. **B**, Quantification of the number of colonies as assessed by AP

staining during the reprogramming process, for the indicated MEFs (miR-203 wild-type, heterozygous, knock-out and the miR-203 inducible model). Values correspond to the average and s.d. (n= 5 independent MEF isolates per line). Statistical significance was evaluated by the Student´s t-test (unpaired; two-tailed) *, P<0.05; **, P<0.01; ***, P<0.001. **C**, Real time PCR to determine the levels of different pluripotency markers, indicated in the panel, during the reprogramming process for the three MEF lines. A control of wild type iPSCs is included. **D**, Principal Component Analysis of RNAseq data from wild-type, miR-203 knock-out and miR-203 inducible model at different time points during the reprogramming process (day 0, day 6, day 7, IPSCs). **E**, Heatmap plot showing the comparative expression of 562 genes associated with a consensus pluripotency signature (Chung, Lin et al., 2012). **F**, mRNA expression levels of the Inhibitor of Differentiation (Id1) as determined by qPCR, during the reprogramming process for the three MEF lines (miR-203 wild-type, knock-out and the miR-203 inducible model).

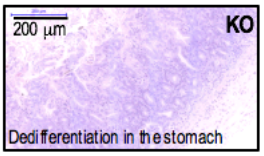
A

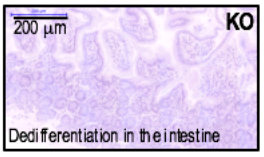


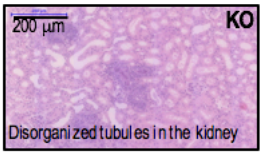
B

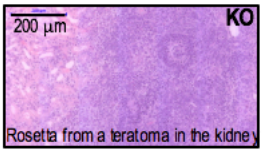
Percentage of mice affected by in vivo reprogramming (15 days-DOX treatment followed by DOX withdrawal)

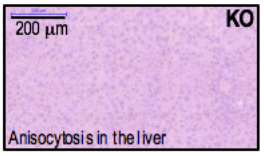
	WT N=12	KO N=11	KI N=9
Stomach/ intestine			
Dedifferentiation/ disorganized structures	20%	80%	0%
Displasia	20%	60%	0%
Hyperplasia	0%	40%	0%
Hyperproliferation/mitosis	20%	80%	0%
Apoptosis	20%	60%	0%
Inflammatory infiltration	20%	40%	0%
Enteritis	20%	40%	0%
Early teratomas	8%	20%	0%
Differentiated teratomas	0%	20%	0%
Kidney			
Abnormal tubules	20%	80%	0%
Dysplasia	20%	60%	0%
Early teratomas	0%	60%	0%
Differentiated teratomas	0%	40%	0%
Liver			
Inflammatory infiltration	0%	60%	0%
Apoptosis	20%	40%	0%
Degeneration	20%	60%	0%
Hyperplasia	0%	20%	0%
Pancreas			
Pancreatitis	0%	40%	0%
Dedifferentiation/ disorganized structures	20%	40%	0%
Early teratomas	0%	40%	0%
Differentiated teratomas	0%	20%	0%











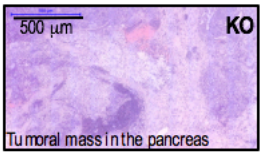


Figure 2. **A**, *ColA1*(miR-203/miR-203); *Rosa26*(rtTA/rtTA) mice were crossed with the i4F reprogrammable mice (Abad M et al., 2013), generously provided by Manuel Serrano, including the doxycycline inducible tetracistronic cassette with the four murine reprogramming factors (Oct4, Sox2, Klf4, c-Myc) (Tet-O-FUW-OSKM, obtained from Addgene #20321). Thus, miR-203 wild-type, knock-out and miR-203 inducible mice expressing the OSKM transcription factors upon doxycycline treatment were obtained. Doxycycline (Sigma) was administered in vivo in the drinking water supplemented with 7.5% of sucrose or alternatively in diet (Dox pellets, from Jackson laboratories) during 15 days, followed by Dox withdrawal during 65 more days. Dox treatment induced the OSKM

cassettes expression in the three strains as well as the miR-203 expression in the case of the miR-203 inducible mice. The graph shows the survival curve of the three mouse strains (miR-203 wild-type, n=12; miR-203 knock-out, n=11; miR-203 inducible mice, n=9) is represented in the panel. Experiments were performed indistinguishably with mice of both sexes and from 2 to 6 months of age. **B**, Histopathology analysis of all the mice subjected to the doxycycline treatment in vivo (15 days of Dox treatment followed by 65 days of Dox withdrawal). The percentages of mice affected by the in vivo reprogramming are represented in the table, specifying the different abnormalities observed by the pathologist in the tissues. Stomach, intestine, kidney, liver and pancreas were the main organs affected by the in vivo reprogramming, showing the listed tissue alterations. On the right, representative images of the most dramatic phenotypes (always observed in the miR-203 knock-out mice) are shown. Dedifferentiation processes in the stomach and intestine, disorganized tissues in the kidney, teratoma formation in the kidney or in the pancreas and anisocytosis in the liver were the most representative abnormalities detected in miR-203 knock-out mice after in vivo reprogramming. Scale bars are indicated in the images.

1st Revision - Editorial Decision 20th May 2020

Dear Marcos,

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your revised study was sent back to the three referees for re-evaluation, and we have received comments from all of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending the minor remaining issues and additional aspects related to formatting and data representation as detailed below are addressed at re-submission.

Regarding the remaining points of referee #1, we would ask you to evaluate whether you can address these with additional data or relativize claims where appropriate.

Please contact me at any time if you have further questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel

Daniel Klimmeck PhD
Editor
The EMBO Journal

****

Formatting changes required for the revised version of the manuscript:

>> Add up to five keywords to your study.

>> Please label the supplemental movie files as "Movie EV1" etc and zip each legend individually with the respective file. Add callouts in the text for movies EV1-3.

>> The appendix ToC table count includes Appendix Tables 1,4,5,6, which are uploaded separately. These tables should be renamed "Dataset EV1-4" and they should be removed from the appendix ToC. Appendix table numbering will need to be adjusted. There is an additional appendix file with figures only; this can be removed.

>>Provide an ORCID for all corresponding authors (M.SR.)

>> Add funding information SAF2014-60442-JIN for MAF to the manuscript.

>> Please consider additional changes and comments from our production team as indicated by attached .doc file and leave changes in track mode.

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 18th Aug 2020.

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Referee #1:

- general summary and opinion about the principle significance of the study, its questions and findings
This story is interesting for the researchers in the field of stem cells and RNA biology. The authors

reported that miR-203, a microRNA preferentially expressed in the 2C morula stages, to which transient exposure of induced pluripotent stem cells (iPSC) or ESCs enhances the differentiation ability of pluripotent stem cells.
Transient expression of miR-203 in PSCs (miPSCs) improves the efficiency using assays including embryoid body (EB) formation, small embryolike structure formation, human-mouse interspecies chimeras and cardiomyocyte differentiation. But more evidence should be provided in the tetraploid complementation assay. Mechanistically, these effects are partially mediated by Dnmt3a and Dnmt3b, resulting in global and reversible DNA hypomethylation in PSCs.

- specific major concerns essential to be addressed to support the conclusions
1. The microRNA profiling of 2C morula stage by sequencing is suggested in this study, since the cited paper of miR-203 expression used the imprecise rt-PCR and microarray analysis in bovine blastocyst and mouse embryos. Comparative experiments between other 2C morula specific microRNAs to miR-203 in the regulation of differentiation and pluripotency are suggested.
2. To confirm the enhancement of miR-203 in tetraploid complementation assay, more iPSC lines should be investigated since no"All-iPSC/ESC" mice were born using iPSC in this study.
- minor concerns that should be addressed
1. The title should be more accurate, since the study was focus on the pluripotent stem cells. Candidate title: Transient exposure to miR-203 expands the differentiation ability of mouse and human pluripotent stem cells.
2. Figure 3A, 54,5%, 80,9%, 96,7% should be 54.5%, 80.9%,96.7%. P=0,02123 should be 0.02123.
3. Transient exposure to synthetic miR-203 in mouse and human PSCs is suggested in the embryo body formation assay.
- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)
Konrad Hochedlinger and colleagues reported that the simple addition of ascorbic acid (vitamin C) to culture medium substantially improves the quality of reprogramming, facilitates generation of all-iPS cell mice from terminally differentiated B cells. It would be interesting to investigate whether vitamin C regulates the miR-203 expression.

Referee #2:

We thank the authors for the comprehensive revision of the manuscript including an improvement of the readability of the text. We think that the paper is now suitable for publication.
1. Our concern was clearly addressed by authors and it is now resolved.
2. Regarding methylation, we agree with the authors that the difference of 2,034 DMVs versus 723 DMVs (at t=25) is greater than the difference of 720 DMVs versus 556 DMVs (at t=0), nonetheless MDS analysis in Figure 5D shows a clear difference between wildtype control cells and un-induced transgenic miR-203 at t=0. It is possible that differences in the initial population cause (non-linear) differences upon miR-203 treatment.
3. Our concern was clearly addressed by authors and it is now resolved.
4. We thank the authors for correcting the axis labels.
5. We acknowledge the complexity of the biological system. Future experiments may reveal the critical targets of the Dnmt3 proteins.
6. Our concern was clearly addressed by authors and it is now resolved.
7. Our question was clearly addressed by the authors. We thank them for improving image quality of Figure 3B.
8. We are looking forward to see results that will be published in the next paper.

9. This is a very interesting observation. (Probably there is a mistake in axis labeling, as PCA3 % is higher that PCA1 %)
10. These are very interesting new results.
11. We thank the authors for this elucidation.
12. Thank you.
13. We thank the authors for this elucidation and for adding the specific variation represented by PC1 and PC2 in the figure legend.
14. We agree with authors that Figure 5C is clearer in the present format.

Referee #3:

Authors have addressed my comments and hence in my opinion this manuscript should be accepted for publication.

Answers to the reviewers

Referee #1:

1. The microRNA profiling of 2C morula stage by sequencing is suggested in this study, since the cited paper of miR-203 expression used the imprecise rt-PCR and microarray analysis in bovine blastocyst and mouse embryos. Comparative experiments between other 2C morula specific microRNAs to miR-203 in the regulation of differentiation and pluripotency are suggested.

The reviewer is right and we have modified the text to better discuss the literature. Whereas it would be very interesting to compare with other microRNAs, performing those assays properly would take long and we feel these data will not change the major message of the manuscript. As a matter of fact, miR-203 has never been identified before as a microRNA remarkably up-regulated at any stage of development with known functional consequences. Following the reviewer recommendation, we have included the following text in the corresponding section of Results: “A first analysis of miR-203 levels during normal murine and bovine development suggested a modest but specific wave of expression during early development (blastocyst stage in murine and more particularly hatched blastocysts in the case of bovine embryos), whereas its expression was lost in cultured embryonic stem cells (Goossens, Mestdagh et al., 2013, Yang, Bai et al., 2008). Interestingly, our quantitative PCR analysis in mouse embryos isolated at different early developmental stages showed that miR-203 expression was low in oocytes, slightly induced at the 2-cell stage and displayed a gradual reduction in morulas and blastocysts”.

2. To confirm the enhancement of miR-203 in tetraploid complementation assay, more iPSC lines should be investigated, since no"All-iPSC/ESC" mice were born using iPSC in this study.

We thank the Reviewer for this comment. As the Reviewer is aware, generating “all-iPSC” mice by tetraploid complementation is notably complicated as confirmed in the available literature. As a summary of representative publications during the last decade, we show here a table with a particular mention to the tetraploid complementation assays (considered one of the most stringent tests to demonstrate proper pluripotency):

Reference	Contribution to All-iPSC mice in 4N complementation assays
miPSCs, this work	*4 mice born/144 blastocysts (2.7% using miiPSCs).* ***2 survived to adulthood with germline transmission***
Yang et al. Cell 2017 (PMID: 28388409)	7 mice born/311 blastocysts injected (2.2% using ESCs). 4 survived to adulthood. Germline contribution not tested
Choi et al., Science 2017 (PMID: 28082412)	Not determined
Wu et al., Cell 2017 (PMID: 28129541)	Not determined
Choi et al., Nature 2017 (PMID: 28746311)	Prolonged 2i/LIF culture impairs contribution to all-ESC mice in 4n assays. Adulthood not

	dertmined.
Yagi et al., Nature 2017 (PMID: 28746308)	Prolonged 2i/LIF culture impairs contribution to all-ESC mice in 4n assays. Adulthood not determined.
Choi et al., Nat Biotech 2015 (PMID: 26501951)	Not determined
Wu et al., Nature 2015 (PMID: 25945737)	Not determined
Abad et al., Nature 2013 (PMID: 24025773)	Not determined
Stadtfeld et al., Nature 2010 (PMID: 20418860)	13-20% in ESCs; 0% in control iPSCs; 3.1% in iPSCs high for Gtl2. 3 out of 7 mice survived to adulthood. Germline transmission not tested.
Zhao et al., Nature 2009 (PMID: 19672241)	Upto 3.5% in 3 different clones. 0% in other 34 clones
Kang et al., Cell Stem Cell 2009 (PMID: 19631602)	0% and 0.53% in two different clones
Stadtfeld et al., Nature Genetics 2012 (PMID: 22387999)	0% in 2 different control (Gtl2 OFF) iPSCs clones; 6,25% in iPSCs Gtl2 ON. 6 out of 12 total mice survived to adulthood. Germline transmission not tested. (*reprogramming from differentiated B cells, not fibroblasts).

Please, note that in none of those publications, control standard iPSCs clones gave rise to "all-iPSC" mice (of course, ESCs exhibit different outcomes).

As the Reviewer mentions, *Stadfeld et al.* (Hochedlinger´s laboratory) have accurately tested the 4N-complementation contribution of their Gtl2-ON iPSCs clones. As shown in the table, in their papers they always obtained 0% contribution to 4N complementation when control iPSCs clones are tested. Whenever they get Gtl2 high expression, the contribution is notably improved. In the first paper, their numbers are very similar to ours, with the difference that we have demonstrated germline transmission in our "all-*mi*IPSCs" adult mice, and none of these papers mention that such germline contribution has been tested. In their second paper, as the Reviewer knowingly mentions, they show the positive effects of Ascorbic Acid on the reprogramming to iPSCs, and those iPSCs indeed contribute to 4N-complementation when Gtl2 is highly expressed. Again, germline contribution of the adult "all-iPSCs" mice is not mentioned in that paper. Please, notice that the reprogramming process is quite different in such work, since they do not obtain the iPSCs from fibroblasts but from terminally differentiated B cells.

Finally, we would like to emphasize here (as we do in the manuscript) that 4N complementation assays are performed on the very same iPSC clones with and without miR-203 exposure, which means that the very same clone that was not successful on tetraploid complementation, gave rise to "all-iPSC" mice when transiently exposed to miR-203. This is precisely the key message we want to communicate in the manuscript: how this microRNA converts a standard PSC clone into a PSC clone with a higher developmental and differentiation potency.

- minor concerns that should be addressed:

1. The title should be more accurate, since the study was focus on the pluripotent stem cells. Candidate title: Transient exposure to miR-203

expands the differentiation ability of mouse and human pluripotent stem cells.

We thank the Reviewer for this suggestion. We have now changed the title to focus on pluripotent stem cells.

2. Figure 3A, 54,5%, 80,9%, 96,7% should be 54.5%, 80.9%, 96.7%. P=0,02123 should be 0.02123.

We thank the Reviewer for detecting these typos. We have changed the Figure 3A accordingly. Indeed, we have noticed the same erratum in the Appendix Figure S6 and it is also corrected now.

3. Transient exposure to synthetic miR-203 in mouse and human PSCs is suggested in the embryo body formation assay.

We thank the Reviewer for referring to that technology. These assays are actually included in the manuscript in Figure 1H-I (transient exposure to synthetic miR-203 mimics in human PSCs differentiated to EBs) and Figure EV1G (same experiments on mouse PSCs).

4. Any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion). Konrad Hochedlinger and colleagues reported that the simple addition of ascorbic acid (vitamin C) to culture medium substantially improves the quality of reprogramming, facilitates generation of all-iPS cell mice from terminally differentiated B cells. It would be interesting to investigate whether vitamin C regulates the miR-203 expression.

We thank the Reviewer for this suggestion. Considering Hochedlinger's observations and the fact that miR-203 does not affect Gltl2 expression levels (indeed miR-203 in general does not affect the expression of imprinted genes) we assume that the mechanisms of action attributed to Gtl2 expression and the mechanisms governed by miR-203 are different. We find very interesting to test miR-203 effects over iPSCs reprogrammed using alternative tools, such as other terminally-differentiated adult cell types as source material (as happens in this paper) or various means of reprogramming factor expression such as Sendai, episomal factor, mRNA etc. Indeed, as explained above, we have just started a collaboration to do so in the near future. The use of Ascorbic Acid as part of the reprogramming cocktail is interesting and we will definitely include it in our new study.

We thank the Reviewer for his/her encouraging comments and insightful suggestions during the revisdion process.

Referee #2:

We thank the authors for the comprehensive revision of the manuscript including an improvement of the readability of the text. We think that the paper is now suitable for publication.

1. Our concern was clearly addressed by authors and it is now resolved.

Thank you.

2. Regarding methylation, we agree with the authors that the difference of 2,034 DMVs versus 723 DMVs (at t=25) is greater than the difference of 720 DMVs versus 556 DMVs (at t=0), nonetheless MDS analysis in Figure 5D shows a clear difference between wildtype control cells and un-induced transgenic miR-203 at t=0. It is possible that differences in the initial population cause (non-linear) differences upon miR-203 treatment.

We really thank the Reviewer for this comment. We realized that the PCA in Figure 5D can be confusing in that sense, even if the numbers of DMVs and PMDs are presented in the panels before (Figures 5A, B). In fact, PC1 contribution in Figure 5D is very high (90.58% of variance explained) compared to PC2 contribution (1.91% of variance explained). Please note also that PC1 axis show changes in a scale from -30 to +20, while PC2 axis shows a scale from -2 to +2.

We apologize for not including before the exact percentage of contribution of every PC in Figure 5D. In the new version, we have included PC contribution numbers which certainly facilitates the general understanding of Figure 5 and helps to avoid such confusion mentioned by the Reviewer.

3. Our concern was clearly addressed by authors and it is now resolved.

4. We thank the authors for correcting the axis labels.

5. We acknowledge the complexity of the biological system. Future experiments may reveal the critical targets of the Dnmt3 proteins.

6. Our concern was clearly addressed by authors and it is now resolved.

7. Our question was clearly addressed by the authors. We thank them for improving image quality of Figure 3B.

8. We are looking forward to see results that will be published in the next paper.

We thank the reviewer for all these positive comments.

9. This is a very interesting observation. (Probably there is a mistake in axis labeling, as PCA3 % is higher that PCA1 %)

The Reviewer is right and there was a mistake on the PC labeling which is now

corrected. We agree and thank the Reviewer for the suggestion to include this new analysis.

10. These are very interesting new results.

11. We thank the authors for this elucidation.

12. Thank you.

13. We thank the authors for this elucidation and for adding the specific variation represented by PC1 and PC2 in the figure legend.

14. We agree with authors that Figure 5C is clearer in the present format.

We thank the Reviewer for all the suggestions and positive comments during the revision process.

Referee #3:

Authors have addressed my comments and hence in my opinion this manuscript should be accepted for publication.

We thank the Reviewer for his/her encouraging comments and enriching suggestions and clarifications during the revision process.

2nd Revision - Editorial Decision 28th May 2020

Dear Marcos, dear María,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figures included in this file.

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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work.
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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

Finally, we have noted that the submitted version of your article is also posted on the preprint platform bioRxiv. We would thus appreciate if you could alert bioRxiv on the acceptance of this manuscript at The EMBO Journal in order to allow for an update of the entry status. Thank you in advance!

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

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Corresponding Author Name: Marcos Malumbres & Maria Salazar-Roa
Journal Submitted to: EMBO J
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- → the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- → figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- → graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- → a specification of the experimental system investigated (eg cell line, species name).
- → the assay(s) and method(s) used to carry out the reported observations and measurements
- → an explicit mention of the biological and chemical entity(ies) that are being measured.
- → an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- → the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- → a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- → a statement of how many times the experiment shown was independently replicated in the laboratory.
- → definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size in mice experiments was estimated based in previous published experiments. Sample sizes in general for in vitro data were estimated based on previously published experiments and the suggestions from our bioinformaticians at CNIO.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size in mice experiments was estimated based in previous published experiments. Sample sizes in general for in vitro data were estimated based on previously published experiments and the suggestions from our bioinformaticians at CNIO.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded. Therefore, no criteria were established to exclude any of the data generated.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples or mice were not randomized.
For animal studies, include a statement about randomization even if no randomization was used.	Samples or mice were not randomized.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Samples or mice were not randomized.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The investigators were not blinded to group allocation during experiments and outcome assessment, except in interspecies experiments in which investigators were blinded for quantification purposes.
5. For every figure, are statistical tests justified as appropriate?	Yes, they are.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, they do.
Is there an estimate of variation within each group of data?	It is indicated in the figures, when necessary.
Is the variance similar between the groups that are being statistically compared?	Yes, it is.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All the primary antibodies used in this study have been previously validated on the species tested here, according to the manufacturer´s websites and datasheets. A complete list of antibodies used in this work is included in the Experimental Procedures (See Appendix Table S7). The primary antibodies used were the following: for NANOG, Cell Signalling Biotechnology, 8822; for cytokeratin 8 (CK8), CNIO Monoclonal Antibodies Core Unit, AM-TROMA I; for GFP, Roche, 11814460001; for SOX2, Cell Signaling Technology, 3728 and R&D System, AF2018; SOX17, R&D Systems, AF1924; for alpha-fetoprotein (AFP), R&D Systems, AF5369; for OCT-4, Santa Cruz Biotechnology, sc-9081 and Cell Signalling; C30A3; for KI-67, Master Diagnostica, 0003110QD; for NESTIN, Millipore MAB353; for CD31, ABCAM ab28364; for CD34, ABCAM ab8158; for CD73, Cell Signaling Technology, 13160; for Collagenase type I, Rockland, 600-401-103S; for GATA-4, Santa Cruz Biotechnology, sc-1237; for SMOOTH MUSCLE ACTIN, Thermo Scientific RB-9010-PO;

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This information is included in the corresponding paragraph of the Experimental Procedures. All our cells are routinely tested for mycoplasma contamination. The cell lines used in this work were not contaminated. No commonly misidentified cells were used. ESCs G4 used in this study were kindly provided by the Transgenic Mice Unit (CNIO) and routinely tested for stemness potential by the unit. No commonly misidentified cells were used. In vivo-generated iPSCs were gifted by Dr. Manuel Serrano (Abad et al, Nature 2013). miR-203 WT and miR-203 KI IPSCs and MEFs were generated in our laboratory, following the procedures described in the Methods Section. WT IPSCs were also kindly provided by Dr. Manuel Serrano Serrano (Abad et al, Nature 2013) and the Transgenic Mice Unit (CNIO) and also routinely tested for stemness potential. HEK293 cells used in luciferase experiments were purchased from ATCC (CRL-3216). ESCs stably expressing the 2C::tomato reporter were kindly provided by Dr. Macfarlan (Macfarlan et al, Nature 2012). Human IPSCs expressing HERVH endogenous retroviruses were kindly provided by Zsuzsanna Izsvak (Wang et al., 2016). Experimentation with human cells was approved by the ISCIII Ethics Committee for Research (CEI; number PI 61_2017). Human iPSCs expressing Tomato reporter were used in J.C. Izpisua-Belmonte's lab (Yang et al., Cell 2017) following the ethical guidelines of the Salk Institute.
	Primary cardiomyocytes were isolated from rats at postnatal day 1, following the standard procedures in Dazhi Wang´s laboratory (Children´s Hospital, Boston, US). Cardiomyocytes differentiated in vitro from IPSCs were also generated in Wang´s laboratory, following the procedures described in the Methods Section.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mice (Mus musculus) were maintained in a C57BL6/J-129S mixed background. Mice were housed in the pathogen-free animal facility of the CNIO (Madrid) and maintained under a standard 12-h light-dark cycle, at 23ºC with free access to food and water. All animal work and procedures were approved by the ISCIII committee for animal care and research, and were performed in accordance with the CNIO Animal Care program following international recommendations, and approved by the Salk Institutional Animal Care and Use Committee (IACUC) and followed the ethical guidelines of the Salk Institute. Both sexes were used in the in vivo experiments, with the exception of athymic nude mice. Female athymic nude mice (obtained from Charles Rivers) were used for cell inoculation, to generate teratomas and embryo-like structures. Finally, for Mouse Embryonic Fibroblasts (MEFs) extraction, embryos were collected at day E13.5. The details of these protocols are included in the Experimental Procedures.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experimentation in this study was performed according to protocols approved by the CNIO-ISCIII Ethics Committee for Research and Animal Welfare (CEIyBA). All the interspecies chimeric experiments were reviewed and approved by the Salk Institutional Animal Care and Use Committee (IACUC) and followed the ethical guidelines of the Salk Institute.
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Human IPSCs expressing HERVH endogenous retroviruses were kindly provided by Zsuzsanna Izsvak (Wang et al., 2016). Experimentation with human cells was approved by the ISCIII Ethics Committee for Research (CEI; number PI 61_2017). Human iPSCs expressing Tomato reporter were used in J.C. Izpisua-Belmonte's lab (Yang et al., Cell 2017) following the ethical guidelines of the Salk Institute.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The data that support the findings of this study are available from the corresponding author upon reasonable request. RNAseq and methylation data has been deposited in the GEO repository under accession numbers GSE81571 and GSE86653.
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G- Dual use research of concern

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