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Epigenetic predisposition to reprogramming fates in somatic cells

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Barbara Pauly

1st Editorial Decision 05 August 2014

Thank you for the submission of your research manuscript to our editorial offices. We have now received the three enclosed reports on it. As you will see, the referees agree on the potential interest of the manuscript, but they have also all raised a number of substantial concerns about the study that would need to be addressed prior to publication.

Both referees 1 and 2 (and to a slightly lower extent referee 3 as well) feel that the current dataset is not strong enough to support the claims made in the manuscript. They both point out that cell density and availability of doxycycline and reprogramming factors need to be analyzed and taken into account when interpreting the ability/readiness of different cells to be reprogrammed and reviewer 3 also comments along these lines (his/her point 3). Other issues of the referees also need to be addressed, including, but not limited to, points 4 and 5 of reviewer 1 and points 5, 7 and 8 of reviewer 2 and point 2 of referee 3. In addition, all referees also point out instances in which additional clarifications and/or controls are needed.

From the analysis of these comments it is clear that publication of your manuscript in our journal can only be considered after significant revision. But given the potential interest of your study and the reviewers' constructive suggestions on how to improve it, we would like to give you the opportunity to address the referee concerns and would be willing to consider a revised manuscript with the understanding that all main issues raised by our reviewers must be fully addressed.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

The manuscript "epigenetic predisposition to reprogramming fates in somatic cells" try to demonstrate that reprogramming potential is predetermined by preexisting epigenetic status of the cells, which of great interest. However, what authors claim is not supported strongly due to weak preliminary results and unclear description. Their approach is very interesting, but there are several parts that need revision. In addition, several methodologies are not clearly described, which causes much difficulty in understanding what authors performed.

Major comments:
1. Page 4: Although authors described "As long as a small number of cells per well (10-100) are ... to precisely measure per-cell efficiency", did author compare the precision of reprogramming efficiency between wells with a small number of cells (10 or 100 cells) and a large number of cells (1,000 cells)?

2. Page 4: Although "We seeded defined numbers of cells per well in 96-well plates (n=20)", does "n" represents the number of wells (20 wells of 96 well plate) or plates (20 of 96 well plates)? If it represents the number of plates, it is unclear how many plates of them were used to expand cells.

3. Page 5: "This suggests that starting cell count (day 0) ..., including a fully stochastic one". Since authors selected wells that the cell number is at least doubled, the cell density in each well is much different between day 0 (or their standard experiment (Fig1A top)) and day 5. It is possible that each cell at day 5 could not get enough dox for iPS reprogramming, and the efficiency is changed compared to the standard experiments. Authors should analyze the effect of the cell number on reprogramming efficiency. It is also possible that between 20 - 80 cells (Supp Table 1), there is no difference in reprogramming efficiency not because cells are predetermined for their fate in reprogramming, but because there are several factors limiting the reprogramming efficiency up to e.g. 200 cells. After which number, reprogramming efficiency is stochastic.

4. Authors classified lineage according to the final fates of daughter cells in the first division. We wonder how authors classified FD and iPSCs if their daughter cells are included in the same colony. In addition, we also wonder whether daughter cells in the second or third division maintain reprogramming potential or not. It is possible that the first division is just a filter of senescence cells, which cannot respond to OSKM induction.

5. Page 9: Between Ezh2 inhibitor and no treatment control, ratio of NR-NR is not different. It is possible that Ezh2 inhibitor changes FD-FD pairs to NR-FD or iPSC-iPSC pairs to NR-iPSC rather than increasing the number of cells generating iPSC forming lineage.

6. Page 9: Authors described "The treatment results in a small delay in divisions, and unlike in the non-treatment experiment, many responding progenitor cells (48%, 13/28) divide for the first time only after OSKM induction", but what is conclusion about this result? The delay of the first cell division was also happened in FD-FD and NR-NR as well as iPSC-iPSC, indicating that the delay of cell division is independent to response to OSKM induction.

7. Authors should expand the description of material and method.
• How did they seed defined number of cells to 96 well plate? Are cells manually picked up and moved to the plate?
• Authors seeded cells in two different types of plates (Gelatin-coated and feeder cell-coated plates), but it is unclear which plates were used in each experiment.
8. Authors need to give detailed description of the key statistical approach. Statistical meaning of the experiments due to lack of error bar in most of the figures.

Referee #2:

Summary
In the present paper the authors studied the epigenetic predisposition of somatic cells for reprogramming. In particular, the authors were interested by bringing evidence to either stochastic or deterministic models of reprogramming. First the authors show that the cells follow a deterministic model of reprogramming. Then they demonstrate that daughter cells share the same potential for reprogramming, further supporting a deterministic model and that reprogramming is independent of local environment. Finally the authors show evidence that global epigenetic alter reprogramming efficiency. However the manuscript is not suitable for publication in EMBO report. There are several points of concern

Major Concern:
1. In fig 1. Does the decrease in reprogramming efficiency of the '+dox count' is attributable to other factors? At 5 days post-seeding, one could expect the cells to be packed and more confluent than for the 'initial count' samples; this increase in confluence might decrease cell surface available for Dox to enter cells and this might lead to a reduced reprogramming efficiency.

2. Figure 1A: Condition of individual fibroblast differs significantly after 5 day of culture, cell division results in a cluster of cells around the same small area, the formation of these cluster affect the capacity to be reprogrammed, therefore the decrease in efficiency could be due to the overcrowding of some cluster (5 000 cells reprogrammed in a small cluster would give a different efficiency when 5000 cells are evenly spread). Since the starting cell number and the number on the day of induction are trivially the same, after 5 days of division, the same well could be split into another well for dox induction and the efficiency assayed.

3. Figure 2: It is established that there is a pre-disposition towards different reprogramming fate before the first cell division; the authors have to emphasize the context of prior to first cell division in the subheading as well as discussion as the fate of the cells can still be changed in subsequent division. Cells are capable of acquiring different fate after the first cell division and are evident in figure 2b where part of the circled colony are not positive for Nanog.

4. In fig 3.F; the basis for the experiment is that reprogramming results in decrease cell size (as mentioned in the Ref 15.). However, the authors choose to monitor the nucleus area (instead of the cell size). Is the nucleus area really correlated to cell size? In Ref 15 paper, the authors don't seem to study or mention nucleus size. A better experiment might want to study the correlation between OSKM intensity and cell size.

5. Figure 3: Replating CFP-labelled cells onto another plate does not remove effect of the lineage niche; after replating, subsequent division might recreate the self-supporting lineage niche or be embedded in similar niche. The experiment setups do not exclude signalling from neighbouring lineages do not play a significant role in determining lineage fate. Signalling could also be paracrine in nature. Replating onto another YFP-labelled plate of parallel reprogramming still provide signalling from neighbouring lineages. The conclusion drawn from this experiment is too far-fetch and the evidence are not evident.

6. Subtitle 'Perturbing chromatin pre-induction alters future lineage fates' should be changed for 'Reducing H3K27me3 epigenetic mark alters future lineage fates' as to make the title more accurate. The authors only studied 1 specific chromatin post-translational modification.

7. To further improve on Fig4., the authors should look at other epigenetic marks, more specifically activating epigenetic marks; or alternatively look at the effect of increasing the basal level of H3K27me3 mark (using Ezh2 overexpression for example).

8. One could argue that the effect observed on reprogramming increase following Ezh2 inhibition might be attributable to simply a higher expression of OSKM. It would be good if the author show

the mRNA or protein level of OSKM in the different conditions.

9. Figure S3: Replating the cells at later stage of reprogramming allows each reprogrammed colonies to be typsinized to single cell and each of these cell can potentially form a new colony after replating. This results in the higher number of iPSC colonies form after replating, but there is no evidence to suggest that the iPSCs cells are restricted in their route. It is also possible that some of these cells did not form iPSC colonies after replating, the authors did not have any experimental data to suggest otherwise.

10. Although the secondary system was derived from a single clone, heterogeneity in the starting secondary MEF was demonstrated in term of proliferation and other properties mentioned in the paper. This heterogeneity could be due to genetic variability acquired through differentiate of the iPSC clone, and could also contribute to the phenotypic variability.

Referee #3:

Comments to the authors:

Nachman and colleagues propose a pre-determined per-lineage model to explain somatic cell reprogramming, which contrasts with the prevalent view nowadays that the initial phase is stochastic. This is interesting work but I have the following comments:

1-The manuscript is very well written but in occasions is not clear enough.

2-Is it possible that a small Thy1- fibroblast population accounts for the pre-determined susceptibility described by the authors? This could be easily assessed by FACS. If this population exists then the authors should test their model with them and with the remaining Thy1+ population.

3-The results by the authors contrast with the Cell roadmap paper by Polo and Hochecdlinger. In that paper they show that additional expression of the reprogramming factors can turn refractory cells into iPSCs. I wonder whether the current findings are related to the specific threshold of expression of the reprogramming factors. By this I mean that with low/moderate expression (as it is common in some secondary systems) only a fraction of cells are sensitive/predetermined to become reprogrammed, but with higher expression the chances increase for other cells as well. It would be useful if the authors could try to prove this experimentally, if possible.

4-I think that with their current model the authors can not exclude that among the privileged population the chances to become reprogrammed still depend on stochastic events. This should be explained.

1st Revision - authors' response 14 November 2014

Referee #1:

The manuscript "epigenetic predisposition to reprogramming fates in somatic cells" try to demonstrate that reprogramming potential is predetermined by preexisting epigenetic status of the cells, which of great interest. However, what authors claim is not supported strongly due to weak preliminary results and unclear description. Their approach is very interesting, but there are several parts that need revision. In addition, several methodologies are not clearly described, which causes much difficulty in understanding what authors performed.

We thank the reviewer for the thoughtful comments and suggestions. We have addressed all of these issues and below we address each one of the points.

Major comments:

1. Page 4: Although authors described "As long as a small number of cells per well (10-100) are ... to precisely measure per-cell efficiency", did author compare the precision of reprogramming efficiency between wells with a small number of cells (10 or 100 cells) and a large number of cells (1,000 cells)?

This is a valid concern, which we have now sought to control by measuring a discretely labeled subpopulation of cells within a larger population of reprogramming cells to effectively eliminate density concerns. Specifically, we have repeated the efficiency estimates in Figure 1, while plating between 20-40 inducible CFP tagged cells in each well together with 1000 inducible YFP tagged cells. The results show the same efficiency of reprogramming for the CFP cells as in the low density experiment in Figure 1. The experiment is detailed in SI Figure **S3** and referenced in the revised text on page 4.

2. Page 4: Although "We seeded defined numbers of cells per well in 96-well plates (n=20)", does "n" represents the number of wells (20 wells of 96 well plate) or plates (20 of 96 well plates)? If it represents the number of plates, it is unclear how many plates of them were used to expand cells.

We apologize for the lack of clarity and have striven to provide more exact language describing the experimental design and results. *n* represents the number of plates. As noted, 13 plates were no-expansion (Day 0) plates. Out of the 7 expansion plates, 3 were expanded only for two days, and did not contain any wells that had at least doubled in cell number to contribute to our calculation efficiency statement. We have therefore corrected the stated plate number to 17, and explain the division between plate types more clearly.

3. Page 5: "This suggests that starting cell count (day 0) ..., including a fully stochastic one". Since authors selected wells that the cell number is at least doubled, the cell density in each well is much different between day 0 (or their standard experiment (Fig1A top)) and day 5. It is possible that each cell at day 5 could not get enough dox for iPS reprogramming, and the efficiency is changed compared to the standard experiments. Authors should analyze the effect of the cell number on reprogramming efficiency. It is also possible that between 20 - 80 cells (Supp Table 1), there is no difference in reprogramming efficiency not because cells are predetermined for their fate in reprogramming, but because there are several factors limiting the reprogramming efficiency up to e.g. 200 cells. After which number, reprogramming efficiency is stochastic.

To assess whether local density effects could either reduce dox availability, or hinder reprogramming efficiency in some other unforeseen way, we have repeated the Luria-Delbruck type experiment with a 5 days delay before dox, adding one change: just before addition of dox, cells were trypsinized and allowed to resettle in the same wells, dissociating any possible clumps that could have affected dox availability and eliminating any local relationship. The experiment is fully detailed in SI Figure S4, and referenced in the main text on page 5 as a critical control. The resulting reprogramming efficiency from this experiment is similar to the original experiment that proceeded without dissociation.

We further stress that the paired-lineages experiment alone suffices to conclude statistically that potential acquisition occurs before the first division. To test the effect of cell density in this context, we repeated our observations from Figure 2 at increased cell density by mixing YFP labeled cells with the dilute CFP population, and again only obtained same-fate pairs. We conclude that the predetermined effect we observe is not a result of low density reprogramming. The experiment is fully detailed in SI Figure S5, and referred from the main text on page 6.

4. Authors classified lineage according to the final fates of daughter cells in the first division. We wonder how authors classified FD and iPSCs if their daughter cells are included in the same colony. In addition, we also wonder whether daughter cells in the second or third division maintain reprogramming potential or not. It is possible that the first

division is just a filter of senescence cells, which cannot respond to OSKM induction.

We have now improved the definition of lineage classification in the Methods section (Cell fate classification). Specifically, a lineage containing both FD cells and iPS cells is classified as iPS. We note that the paired-lineages analysis ignores all cells that do not divide at all. From those that divide at least once, if only future cell senescence is determined following the 1st division, we would expect to get mixed iPS-FD pairs from lineages tracked prior to the time of factor induction, which we do not see. We managed to trace 36 pairs from the 2nd division, and these again showed strict same fate preferences (6 iPS pairs, 19 FD pairs, 11 NR pairs), indicating the potential is not lost over the 2nd division. For later divisions, it is harder to trace the fates of pairs, because of proximity and intermixing of the sub-lineages, and we have highlighted this limitation in the text on page 6.

5. Page 9: Between Ezh2 inhibitor and no treatment control, ratio of NR-NR is not different. It is possible that Ezh2 inhibitor changes FD-FD pairs to NR-FD or iPSC-iPSC pairs to NR-iPSC rather than increasing the number of cells generating iPSC forming lineage.

These changes are possible, and indeed logical from the pair counts, but do not explain the 2-fold increase in iPS-iPS pairs and overall 3-fold increase in the number of iPS containing wells observed. We now phrase more carefully the possible scenarios when comparing the pair statistics in the Ezh2 treatment to the no-treatment case.

6. Page 9: Authors described "The treatment results in a small delay in divisions, and unlike in the non-treatment experiment, many responding progenitor cells (48%, 13/28) divide for the first time only after OSKM induction", but what is conclusion about this result? The delay of the first cell division was also happened in FD-FD and NR-NR as well as iPSC-iPSC, indicating that the delay of cell division is independent to response to OSKM induction.

The term “responding” referred to iPS+FD fates - we apologize for the lack of clarity. The reviewer is indeed correct in that all types of pairs (except the mixed ones) show a similar delay in time of first division – we now clarify this in the text on page 10. We originally stated it as above, since in the untreated response, there are some NR cells that divide for the first time only after dox induction. We now removed that point in the text to avoid confusion.

7. Authors should expand the description of material and method.
• How did they seed defined number of cells to 96 well plate? Are cells manually picked up and moved to the plate?

The term “defined” was misleading – we have now changed it to “different low densities.” The exact number of cells that have landed in each well was counted after the fact by imaging. We now clarified this in the main text on page 4 and added more details on to the SI Methods section “Reprogramming and image acquisition”.

• Authors seeded cells in two different types of plates (Gelatin-coated and feeder cell-coated plates), but it is unclear which plates were used in each experiment.

To clarify this, we describe all the seeding details in the “Reprogramming and image acquisition” section in the Methods. Briefly, all 96-well experiments were done in feeder coated plates, while experiments in 6-, 12- or 24-wells, which use large numbers of secondary MEFs, were done in gelatin coated plates. The only exception for our experiments using large numbers of cells is the second part of the replating experiment (Figure S6) where we use both feeder-coated and gelatin-coated plates, as described in the main text on page 8.

8. Authors need to give detailed description of the key statistical approach. Statistical meaning of the experiments due to lack of error bar in most of the figures.

We describe all the statistical methods in detail in SI Text 2 and 3. We have further improved these descriptions to make them clearer. Error bars were missing in the original Figure S5, which has been moved to the new Figure 4b. We now show error bars wherever appropriate in the main and

supplementary figures.

Referee #2:

Summary
In the present paper the authors studied the epigenetic predisposition of somatic cells for reprogramming. In particular, the authors were interested by bringing evidence to either stochastic or deterministic models of reprogramming. First the authors show that the cells follow a deterministic model of reprogramming. Then they demonstrate that daughter cells share the same potential for reprogramming, further supporting a deterministic model and that reprogramming is independent of local environment. Finally the authors show evidence that global epigenetic alter reprogramming efficiency. However the manuscript is not suitable for publication in EMBO report. There are several points of concern

We thank the reviewer for the detailed comments and suggestions for follow-up experiments. Below we address each one of the points and describe the additional experiments and their results. These should address all the concerns and we hope the reviewer finds the revised manuscript now suitable for publication.

Major Concern:
1. In fig 1. Does the decrease in reprogramming efficiency of the '+dox count' is attributable to other factors? At 5 days post-seeding, one could expect the cells to be packed and more confluent than for the 'initial count' samples; this increase in confluence might decrease cell surface available for Dox to enter cells and this might lead to a reduced reprogramming efficiency.

As outlined above to Reviewer 1 (please see response to point 1 and 3 above for more details) this is a valid point and we have performed additional experiments to clarify this. We address both the mean density (confluence) of the cells as well as local density, showing that neither reduce efficiency in our experiment setup.

2. Figure 1A: Condition of individual fibroblast differs significantly after 5 day of culture, cell division results in a cluster of cells around the same small area, the formation of these cluster affect the capacity to be reprogrammed, therefore the decrease in efficiency could be due to the overcrowding of some cluster (5 000 cells reprogrammed in a small cluster would give a different efficiency when 5000 cells are evenly spread). Since the starting cell number and the number on the day of induction are trivially the same, after 5 days of division, the same well could be split into another well for dox induction and the efficiency assayed.

We thank the reviewer for this suggestion. We have implemented it in our resuspension+resettling experiment described for Reviewer 1 in point 3 above, with the slight variation that cells are resettled in the same wells to reduce variability and cell loss. See Figure S4 for full description of the experiment and results, which demonstrate that the reprogramming efficiency is similar to the original experiment where cells were not dissociated prior to factor induction. Figure S1 additionally shows that delaying induction by 5 days does not affect efficiency due to altered internal state of the cells during a conventional reprogramming experiment with high cell numbers.

3. Figure 2: It is established that there is a pre-disposition towards different reprogramming fate before the first cell division; the authors have to emphasize the context of prior to first cell division in the subheading as well as discussion as the fate of the cells can still be changed in subsequent division. Cells are capable of acquiring different fate after the first cell division and are evident in figure 2b where part of the circled colony are not positive for Nanog.

By adding analysis of second division pairs (point 4 of Reviewer 1), we have expanded the context of this section. We now emphasize in the section and its heading (page 5), as well as in the discussion, that our analysis is explicitly limited to the existence and maintenance of potential during the early observed divisions, mostly prior to factor induction. Cells can indeed acquire different fates by losing potential or partially realizing it in subsequent divisions, though this is not the intended question our system is designed to answer. We now state our explicit intent to study the pre-induction state as it effects reprogramming outcome (page 6 and page 10) and highlight the limitations of live imaging over the 14 day reprogramming experiment itself.

4. In fig 3.F; the basis for the experiment is that reprogramming results in decrease cell size (as mentioned in the Ref 15.). However, the authors choose to monitor the nucleus area (instead of the cell size). Is the nucleus area really correlated to cell size? In Ref 15 paper, the authors don't seem to study or mention nucleus size. A better experiment might want to study the correlation between OSKM intensity and cell size.

The reviewer is indeed correct in that we had not shown the relation between nucleus size and lineage fate as we have for cell size in Smith *et. al.*. We have now measured the nucleus size distributions on days 0, 2 and 3 for cells in tracked lineages, verifying both a connection between nucleus size and response (NR vs. FD/iPS), and a decrease over time in nucleus size only in responding (FD/iPS) cells, which we include as a new SI Figure S8. For technical image analysis reasons, the estimate of nucleus size is more accurate than cell size, since cell body segmentation in fibroblasts is noisier due to variable sizes, shapes and cell contact. Alternatively, nucleus segmentation is much clearer and consistent.

5. Figure 3: Replating CFP-labelled cells onto another plate does not remove effect of the lineage niche; after replating, subsequent division might recreate the self-supporting lineage niche or be embedded in similar niche. The experiment setups do not exclude signalling from neighbouring lineages do not play a significant role in determining lineage fate. Signalling could also be paracrine in nature. Replating onto another YFP-labelled plate of parallel reprogramming still provide signalling from neighbouring lineages. The conclusion drawn from this experiment is too far-fetch and the evidence are not evident.

At the moment of replating, lineage niche effect is completely lost and effectively randomized. The reviewer is correct in that it could be hypothetically regained with time after some divisions, but it is cut off for at least a short period immediately following replating. We now stress this point in the text (page 7).

The purpose of the replating experiment is to break potential signaling from the original niche, including from within the lineage and from neighboring lineages, to allow signaling from random neighboring lineages in the new plate. We then use statistical analysis to show that the signaling in the new environment has no significant effect on a lineage's final fate by showing that the distance distribution to different lineage types is not informative about a lineage's subsequent fate. It is true that there could be an autocrine effect, but this does not deviate substantially from our interpretation of the data, that reprogramming outcome is largely cell intrinsic.

6. Subtitle 'Perturbing chromatin pre-induction alters future lineage fates' should be changed for 'Reducing H3K27me3 epigenetic mark alters future lineage fates' as to make the title more accurate. The authors only studied 1 specific chromatin post-translational modification.

Agreed. We have modified the section heading, as well as heading of Figure 4, and have also included data for a modifier of H3K4 methylation (next point).

7. To further improve on Fig4., the authors should look at other epigenetic marks, more specifically activating epigenetic marks; or alternatively look at the effect of increasing the basal level of H3K27me3 mark (using Ezh2 overexpression for example).

We have now added a test for an activating mark, using an Lsd1 inhibitor pre-treatment that increases H3K4 methylation before induction of reprogramming. This treatment, as seen in Figure 4B, indeed increases the efficiency of reprogramming. We have further repeated the pre-treatment experiment for 7 of the treatments with more replicates over an expanded imaging area, now reported in an improved Figure 4B. As a result, the previous Figure S5 became redundant, as it only adds information on different working concentrations tried, and we have removed it from the SI in favor of our more systematic follow-ups. The rest of Figure 4 (C,D) is focused on Ezh2 inhibition to provide a deeper analysis into one potential perturbation. The new results are described on page 9 of the revised text.

8. One could argue that the effect observed on reprogramming increase following Ezh2 inhibition might be attributable to simply a higher expression of OSKM. It would be good if the author show the mRNA or protein level of OSKM in the different conditions.

We thank the reviewer for this suggestion. We have measured OSKM protein levels in Ezh2 inhibitor pre-treated cells by immunostaining on days 2 and 4 following dox induction. Compared to non-treated control, there is no change in the distribution of protein levels for any of those factors. This result is described in SI Figure S12 and referred to in the Discussion on page 11.

9. Figure S3: Replating the cells at later stage of reprogramming allows each reprogrammed colonies to be typsinized to single cell and each of these cell can potentially form a new colony after replating. This results in the higher number of iPSC colonies form after replating, but there is no evidence to suggest that the iPSCs cells are restricted in their route. It is also possible that some of these cells did not form iPSC colonies after replating, the authors did not have any experimental data to suggest otherwise.

We agree that the increased number of iPSC colonies generated by splitting reprogramming populations at later time points does not mean that all cells within a fated lineage are restricted to their route. In fact, given previous results by Buganim *et. al.* (2012) showing that split progeny of a single lineage can adopt different fates, it is very likely. We apologize for this overstatement. We now use more careful language to clarify this point in the text on page 8 and acknowledge that we do not explicitly mean that all cells from an iPSC-forming lineage will reprogram, but may just do so at a higher per cell efficiency than the overall population after a certain point in the reprogramming timeline.

10. Although the secondary system was derived from a single clone, heterogeneity in the starting secondary MEF was demonstrated in term of proliferation and other properties mentioned in the paper. This heterogeneity could be due to genetic variability acquired through differentiate of the iPSC clone, and could also contribute to the phenotypic variability.

The secondary system employed in this work has been extensively characterized by us and others, including through the generation of viable, germline competent chimeras, indicating a low likelihood of deleterious mutation stemming from the original clone. Moreover, batches of secondary MEFs are routinely regenerated via blastocyst injection and isolation of fibroblasts from E13.5 embryos. As such, the likelihood of a single clone at the injection stage containing a reprogramming promoting mutation is unlikely, and would be evident from abnormal reprogramming efficiency compared to prior batches. The high reproducibility of isolated MEFs from this secondary line is, in fact, a major benefit of its utility for studying reprogramming to pluripotency. We also feel that the acquisition of heterogeneous mutations following injection of the clone would be unlikely, as it would again contribute to abnormal reprogramming efficiency and kinetics, which we did not observe during these experiments. Moreover, it would occur at an exceedingly low efficiency, as such mutations would not be advantaged until doxycycline was induced. As cited, the efficiency estimates from all experiments fell within the expectation for this system as it was initially described in Wernig *et. al.* 2008 and again in Smith *et. al.* 2010.

Referee #3:

Comments to the authors:

Nachman and colleagues propose a pre-determined per-lineage model to explain somatic cell reprogramming, which contrasts with the prevalent view nowadays that the initial phase is stochastic. This is interesting work but I have the following comments:

We thank the reviewer for the comments and suggestions, particularly in reference to the Polo *et. al.* work. Below we address the comments and describe the relevant experiments and their results.

1-The manuscript is very well written but in occasions is not clear enough.

We have now added further clarifications throughout and expanded description in various points in the main text and SI.

2-Is it possible that a small Thy1- fibroblast population accounts for the pre-determined susceptibility described by the authors? This could be easily assessed by FACS. If this population exists then the authors should test their model with them and with the remaining Thy1+ population.

We thank the reviewer for this suggestion. The identification of a distinguishing marker of reprogramming fate in pre-induction MEFs is indeed a natural next step. We have now tried to use Thy1 in this context in two different experiments: (1) Live-staining MEFs for Thy1 on day 0 of reprogramming, followed by imaging and tracking lineages until final fate is determined. (2) Sorting of Thy1 high vs. Thy1 low MEFs, followed by reprogramming and efficiency estimation in comparison to unsorted cells. We provide the detailed results of these two experiments in SI Figure S11 and refer to them in the Discussion on page 10. Briefly, the live tracking experiment shows no significant difference in the distribution of pre-induction Thy1 levels for different terminal lineage fates. The Thy1+ vs. Thy1- efficiency estimation shows that Thy1+ cells have the same efficiency as unsorted cells, while Thy1- cells produce far less reprogramming colonies, which is not normally evident as they represent a minority of the cells in the unsorted population. This is consistent with the results described in Polo *et. al.*, which suggest factor-responsive MEFs initially express Thy1, and only lose it after a few days.

3-The results by the authors contrast with the Cell roadmap paper by Polo and Hochedlinger. In that paper they show that additional expression of the reprogramming factors can turn refractory cells into iPSCs. I wonder whether the current findings are related to the specific threshold of expression of the reprogramming factors. By this I mean that with low/moderate expression (as it is common in some secondary systems) only a fraction of cells are sensitive/predetermined to become reprogrammed, but with higher expression the chances increase for other cells as well. It would be useful if the authors could try to prove this experimentally, if possible.

The secondary system used in our work (originally described in Wernig et. al. 2008) produces high OKSM expression levels (as shown in Figure S1 of Smith *et. al.* 2010 and Figure S1 in Brambrink *et. al.* 2008.). We have now verified that the expression levels in our system are stronger than in the polycistronic OKSM system used by Polo *et. al.* , as shown in SI Figure S7. We further verified that the addition of OK or OKSM expression to this system does not improve reprogramming efficiency in the same figure. We conclude that our main results are not due to low factor expression. Consistently, the Ezh2 pre-perturbation that we test does improve reprogramming efficiency, but does so without elevating OKSM levels (Figure S12). All these results are now referred to in the main text on page 8 and page 11.

4-I think that with their current model the authors can not exclude that among the privileged population the chances to become reprogrammed still depend on stochastic events. This should be explained.

Our results suggest that the potential to become iPS is set before induction, that no new potential is "born" along the way, and that this potential is maintained over the first two observed divisions. As

we now clarify in the text, there is indeed loss of potential (or lack of realization) later in parts of the sub-lineages. Which parts of an iPS lineage tree eventually lose or realize this potential may still depend on stochastic events, but proceed downstream of an overall predisposition. The percentage of cells realizing the potential is high enough such that every sub-lineage tracked within an iPS lineage tree eventually contributed to iPS colonies within the prescribed timeline. We now state this explicitly in the Results section on page 6, and elaborate this point in the Discussion on page 10 to ensure it is clearly understood.

2nd Editorial Decision 08 December 2014

Many thanks for submitting your revised manuscript to our editorial office and for your patience while we were waiting to hear back from the original reviewers who were asked to assess the revised version.

As you will see, while referees 1 and 3 now support publication of the study in our journal, referee 2 still raises concerns about some of the data and the conclusions based on them. Upon further discussions of these criticisms with reviewer #3, we agreed that it would be necessary to further discuss these issues and to tone down your conclusions accordingly. This refers mainly to referee 2's point 5 (on the lineage niche/neighboring niche issue) and point 10 (on the possibility of mutations having been acquired during reprogramming). I would therefore kindly ask you to change the text of the manuscript accordingly before we can accept it for publication here. Please do not worry about length restrictions too much as I think an insightful discussion of these points is more important than publishing a short paper in this case. Please also highlight these changes in the text, so that it will be easier for us to identify them.

Formally, please expand the materials and methods section, as it currently is completely shifted to the supplementary section. Also, please indicate for each experiment for which you have provided statistical analysis, how many independent times it has been performed. Finally, please complete the author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision)

You can submit the final version of your manuscript through our website.

Thank you very much for your cooperation and I look forward to receiving the revised manuscript as soon as it is ready, as we do not want to unnecessarily delay its publication, which, in the end, the reviewers support.

REFEREE REPORTS:

Referee #1:

Revised manuscript addressed all previous comments and is ready to get published.

Referee #2:

Major Concern:

1. In fig 1. Does the decrease in reprogramming efficiency of the '+dox count' is attributable to other factors? At 5 days post-seeding, one could expect the cells to be packed and more confluent than for the 'initial count' samples; this increase in confluence might decrease cell surface available for Dox to enter cells and this might lead to a reduced reprogramming efficiency.

Answer:
To assess whether local density effects could either reduce dox availability, or hinder

reprogramming efficiency in some other unforeseen way, we have repeated the Luria-Delbruck type experiment with a 5 days delay before dox, adding one change: just before addition of dox, cells were trypsinized and allowed to resettle in the same wells, dissociating any possible clumps that could have affected dox availability and eliminating any local relationship. The experiment is fully detailed in SI Figure S4, and referenced in the main text on page 5 as a critical control. The resulting reprogramming efficiency from this experiment is similar to the original experiment that proceeded without dissociation.

We further stress that the paired-lineages experiment alone suffices to conclude statistically that potential acquisition occurs before the first division. To test the effect of cell density in this context, we repeated our observations from Figure 2 at increased cell density by mixing YFP labeled cells with the dilute CFP population, and again only obtained same-fate pairs. We conclude that the predetermined effect we observe is not a result of low density reprogramming. The experiment is fully detailed in SI Figure S5, and referred from the main text on page 6.

Reply:
The authors reseeding experiment addressed the density issues sufficiently by replating the cells and letting them resettle in the same well as demonstrated in figure S4.

2. Figure 1A: Condition of individual fibroblast differs significantly after 5 day of culture, cell division results in a cluster of cells around the same small area, the formation of these cluster affect the capacity to be reprogrammed, therefore the decrease in efficiency could be due to the overcrowding of some cluster (5 000 cells reprogrammed in a small cluster would give a different efficiency when 5000 cells are evenly spread). Since the starting cell number and the number on the day of induction are trivially the same, after 5 days of division, the same well could be split into another well for dox induction and the efficiency assayed.

Answer:
We thank the reviewer for this suggestion. We have implemented it in our resuspension+resettling experiment described for Reviewer 1 in point 3 above, with the slight variation that cells are resettled in the same wells to reduce variability and cell loss. See Figure S4 for full description of the experiment and results, which demonstrate that the reprogramming efficiency is similar to the original experiment where cells were not dissociated prior to factor induction. Figure S1 additionally shows that delaying induction by 5 days does not affect efficiency due to altered internal state of the cells during a conventional reprogramming experiment with high cell numbers.

Reply:
By letting it resettle in the same well, does not change the cell density, the cells may be spread out, but cell/volume is still the same and reprogramming conditions are not the same as those before 5 days of expansion.

3. Figure 2: It is established that there is a pre-disposition towards different reprogramming fate before the first cell division; the authors have to emphasize the context of prior to first cell division in the subheading as well as discussion as the fate of the cells can still be changed in subsequent division. Cells are capable of acquiring different fate after the first cell division and are evident in figure 2b where part of the circled colony are not positive for Nanog.

Answer:
By adding analysis of second division pairs (point 4 of Reviewer 1), we have expanded the context of this section. We now emphasize in the section and its heading (page 5), as well as in the discussion, that our analysis is explicitly limited to the existence and maintenance of potential during the early observed divisions, mostly prior to factor induction. Cells can indeed acquire different fates by losing potential or partially realizing it in subsequent divisions, though this is not the intended question our system is designed to answer. We now state our explicit intent to study the pre-induction state as it effects reprogramming outcome (page 6 and page 10) and highlight the limitations of live imaging over the 14 day reprogramming experiment itself
Reply: Authors have taken the steps to limits its claims.

4. In fig 3.F; the basis for the experiment is that reprogramming results in decrease cell size (as mentioned in the Ref 15.). However, the authors choose to monitor the nucleus area (instead of the

cell size). Is the nucleus area really correlated to cell size? In Ref 15 paper, the authors don't seem to study or mention nucleus size. A better experiment might want to study the correlation between OSKM intensity and cell size.

Answer:
The reviewer is indeed correct in that we had not shown the relation between nucleus size and lineage fate as we have for cell size in Smith et. al.. We have now measured the nucleus size distributions on days 0, 2 and 3 for cells in tracked lineages, verifying both a connection between nucleus size and response (NR vs. FD/iPS), and a decrease over time in nucleus size only in responding (FD/iPS) cells, which we include as a new SI Figure S8. For technical image analysis reasons, the estimate of nucleus size is more accurate than cell size, since cell body segmentation in fibroblasts is noisier due to variable sizes, shapes and cell contact. Alternatively, nucleus segmentation is much clearer and consistent.

Reply: The authors have provided the relation between nucleus size and lineage fate as shown in SI Figure S8.

5. Figure 3: Replating CFP-labelled cells onto another plate does not remove effect of the lineage niche; after replating, subsequent division might recreate the self-supporting lineage niche or be embedded in similar niche. The experiment setups do not exclude signalling from neighbouring lineages do not play a significant role in determining lineage fate. Signalling could also be paracrine in nature. Replating onto another YFP-labelled plate of parallel reprogramming still provide signalling from neighbouring lineages. The conclusion drawn from this experiment is too far-fetch and the evidence are not evident.

Answer:
At the moment of replating, lineage niche effect is completely lost and effectively randomized. The reviewer is correct in that it could be hypothetically regained with time after some divisions, but it is cut off for at least a short period immediately following replating. We now stress this point in the text (page 7).

The purpose of the replating experiment is to break potential signaling from the original niche, including from within the lineage and from neighboring lineages, to allow signaling from random neighboring lineages in the new plate. We then use statistical analysis to show that the signaling in the new environment has no significant effect on a lineage's final fate by showing that the distance distribution to different lineage types is not informative about a lineage's subsequent fate. It is true that there could be an autocrine effect, but this does not deviate substantially from our interpretation of the data, that reprogramming outcome is largely cell intrinsic.

Reply:
The authors did not address the issue sufficiently, the same signalling from neighbouring lineages and the new environment is not entirely new, and it was plated to another plate undergoing parallel reprogramming. Although randomized, most of the replated cells could still be embedded in the same neighbour lineages, due to the high confluency of the experiment. Reprogramming is also highly dependent on extrinsic signals, the failure to provide the right cellular environment or culture medium will dramatically affect reprogramming efficiency. This series of experiment does not allow the author to conclude that signalling from neighbouring lineages affect the determination of lineage fate.

6. Subtitle 'Perturbing chromatin pre-induction alters future lineage fates' should be changed for 'Reducing H3K27me3 epigenetic mark alters future lineage fates' as to make the title more accurate. The authors only studied 1 specific chromatin post-translational modification.

Answer:
Agreed. We have modified the section heading, as well as heading of Figure 4, and have also included data for a modifier of H3K4 methylation (next point).
Reply: Authors have taken appropriate steps to accurately describe the achievement.
7. To further improve on Fig4., the authors should look at other epigenetic marks, more specifically

activating epigenetic marks; or alternatively look at the effect of increasing the basal level of H3K27me3 mark (using Ezh2 overexpression for example).

Answer:

We have now added a test for an activating mark, using an Lsd1 inhibitor pre-treatment that increases H3K4 methylation before induction of reprogramming. This treatment, as seen in Figure 4B, indeed increases the efficiency of reprogramming. We have further repeated the pre-treatment experiment for 7 of the treatments with more replicates over an expanded imaging area, now reported in an improved Figure 4B. As a result, the previous Figure S5 became redundant, as it only adds information on different working concentrations tried, and we have removed it from the SI in favor of our more systematic follow-ups. The rest of Figure 4 (C,D) is focused on Ezh2 inhibition to provide a deeper analysis into one potential perturbation. The new results are described on page 9 of the revised text.
Reply: Authors have included data for H3K4 methylation and restrict the subheading to a more appropriate claim.

8. One could argue that the effect observed on reprogramming increase following Ezh2 inhibition might be attributable to simply a higher expression of OSKM. It would be good if the author show the mRNA or protein level of OSKM in the different conditions.

Answer:
We thank the reviewer for this suggestion. We have measured OSKM protein levels in Ezh2 inhibitor pre-treated cells by immunostaining on days 2 and 4 following dox induction. Compared to non-treated control, there is no change in the distribution of protein levels for any of those factors. This result is described in SI Figure S12 and referred to in the Discussion on page 11.
Reply: Authors have clearly demonstrate in SI Figure S12, the inhibition of Ezh2 does not affect OSKM protein level

9. Figure S3: Replating the cells at later stage of reprogramming allows each reprogrammed colonies to be typsinized to single cell and each of these cell can potentially form a new colony after replating. This results in the higher number of iPSC colonies form after replating, but there is no evidence to suggest that the iPSCs cells are restricted in their route. It is also possible that some of these cells did not form iPSC colonies after replating, the authors did not have any experimental data to suggest otherwise.
Answer:
We agree that the increased number of iPSC colonies generated by splitting reprogramming populations at later time points does not mean that all cells within a fated lineage are restricted to their route. In fact, given previous results by Buganim et. al. (2012) showing that split progeny of a single lineage can adopt different fates, it is very likely. We apologize for this overstatement. We now use more careful language to clarify this point in the text on page 8 and acknowledge that we do not explicitly mean that all cells from an iPSC-forming lineage will reprogram, but may just do so at a higher per cell efficiency than the overall population after a certain point in the reprogramming timeline.
Reply: Authors have made the recommended edits in Figure S3 and limited their claim in text on page 8.

10. Although the secondary system was derived from a single clone, heterogeneity in the starting secondary MEF was demonstrated in term of proliferation and other properties mentioned in the paper. This heterogeneity could be due to genetic variability acquired through differentiate of the iPSC clone, and could also contribute to the phenotypic variability.

Answer:
The secondary system employed in this work has been extensively characterized by us and others, including through the generation of viable, germline competent chimeras, indicating a low likelihood of deleterious mutation stemming from the original clone. Moreover, batches of secondary MEFs are routinely regenerated via blastocyst injection and isolation of fibroblasts from E13.5 embryos. As such, the likelihood of a single clone at the injection stage containing a reprogramming promoting mutation is unlikely, and would be evident from abnormal reprogramming efficiency compared to prior batches. The high reproducibility of isolated MEFs

from this secondary line is, in fact, a major benefit of its utility for studying reprogramming to pluripotency. We also feel that the acquisition of heterogeneous mutations following injection of the clone would be unlikely, as it would again contribute to abnormal reprogramming efficiency and kinetics, which we did not observe during these experiments. Moreover, it would occur at an exceedingly low efficiency, as such mutations would not be advantaged until doxycycline was induced. As cited, the efficiency estimates from all experiments fell within the expectation for this system as it was initially described in Wernig et. al. 2008 and again in Smith et. al. 2010.
Reply: The authors did not offer an adequate explanation on what was reported in this current manuscript where cells could varies drastically in terms of proliferation that the authors mentioned themselves.

Referee #3:

The revision is satisfactory. It is now ready for publication.

2nd Revision - authors' response 11 December 2014

We are pleased to submit our revision to manuscript 2014-39264. Below we provide detailed answers to each of the three remaining concerns of Reviewer #2, including references to where these issues have been addressed in the main text. We have also expanded the Materials and Methods section as requested, and indicated experiment replicate numbers where appropriate.

Response to Reviewer #2:

(2) The different potential effects of the 5 days delayed reprogramming in Figure 1 were tested in three separate experiments: the effect of cell "age" was addressed in the experiment described in Figure S1, by comparing day 0 and day 5 fibroblasts under the same density, showing the same reprogramming efficiency; We have also shown that the parameters of reprogramming efficiency are unaffected on a per cell basis when uniquely CFP labeled cells are reprogrammed at either isolated low densities or within far higher densities of YFP labeled cells, establishing an experimental range where density does not influence reprogramming efficiency; finally, the resuspension/resettling experiment (**Figure S4**) deals with potential confounding effects of local density, again showing no effect on efficiency. Together, we believe these three experiments test each of the parameters that differ between day 0 and day 5 cells on Figure 1, and conclude that heterogeneity in local or global density within the range of macroscopic densities used in this study, nor the cell "age", confers substantial influence on reprogramming outcome.

(5) We agree that some average paracrine signaling may support the cell's progress through reprogramming. However the spatial statistics show this would have to be a strictly non-localized effect that is dispersed evenly in the well. We observed no effect on spatial statistics of distances between different lineage types (e.g. iPS lineages are not more likely than FD lineage to land next to another FD colony) but agree that we cannot rule out longer range communication between lineages as an effect of signaling. We now phrase our conclusions from this result more carefully to include these additional possibilities (page 8).

(10) In mammalian somatic cells, proliferation can be exceedingly heterogeneous even for genetically identical populations (see e.g. (Tzur et al., 2009)). The common explanation includes heterogeneity in terms of expression and status of growth factor sensing pathways as well as epigenetic state, similar to models that describe decreased proliferation rate within ES cells as they begin to differentiate (Li and Kirschner, 2014). Though one cannot entirely rule out genetic differences that may have accrued in MEFs prior to induction of

reprogramming (for example during embryo development) evidence by other works have shown that this variation in proliferation and response can be effected by augmenting epigenetic modifications (Rais et al.), exogenous growth factor conditions, or by p53 inhibition (Hanna et al., 2009). These data suggest that the source of the observed variability in initial replication timing is epigenetic, rather than genetic, in origin. Previous descriptions of biased genetic variation in iPSCs generated from clonal MEF populations have found minimal over-representation of mutations that contribute to this process, with those that were identified associated with rare variants predicted to be found in less than 1 in 500 cells at the onset of reprogramming (Young et al., 2012). As such, somatic mosaicism is unlikely to account for the 1% efficiency observed in our secondary system. Nevertheless, we agree that we cannot fully exclude this possibility and now refer to these considerations both in the results section (page 7) and in the Discussion (page 10).

We note that mutations that may have arisen during the reprogramming process we assay are not relevant to the pre-existing differences between the MEFs. Such possible mutations or abberations, previously a hotly debated topic, have been shown to be a minor effect (as recently reviewed in (Liang and Zhang, 2013)), and observed genetic variation in final iPS cells (including CNVs and SNVs) were shown to mostly represent pre-existing genetic variability in the starting cell population.

References

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3rd Editorial Decision 12 December 2014

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