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E-cadherin is crucial for ESC pluripotency and can replace Oct4 during somatic cell reprogramming

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

1st Editorial Decision 18 October 2010

Thank you for the submission of your manuscript to our editorial office. I am sorry for the slight delay in getting back to you, but we have only now received the full set of referee reports that are copied below.

As you will see, while the referees agree on the potential interest of the finding that E-cadherin can replace Oct4 in iPS cell production, they also indicate that this part of the manuscript, as it stands, is somewhat preliminary and would have to be strengthened before the manuscript can be considered for publication in EMBO reports.

Both referees 2 and 3 point out that E-cadherin is still present in the knockout cells after recombination and that iPS cells can still be produced from these cells, albeit at lower frequency. The iPS cells generated from E-cadherin knockout cells should be examined for E-cadherin expression in order to clarify whether E-cadherin is indeed essential for reprogramming or whether it rather enhances reprogramming efficiency. Both referees also request that the reprogramming process with E-cadherin replacing Oct4 needs to be characterized in more detail (for example by analyzing ESKM virus integration and germline contribution of ESKM cells to chimeras) and referee 3 adds that in general better images need to be provided throughout the figures and that the experimental design needs to be explained in more detail. Both referees also remark that the citations need to be corrected.

From the analysis of these comments it is clear that, as it stands, the experimental evidence provided is insufficient to support the conclusion that E-cadherin can replace Oct4 during reprogramming, and publication of the manuscript in our journal can therefore not be considered at this stage. On the other hand, given the potential interest of your study, I would like to give you the opportunity to address the reviewers concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS

Referee #1 (Revision Comments):

This manuscript reports the roles of E-cadherin in the maintenance and acquisition of pluripotency by mouse embryonic cells. The authors found that E-cadherin is required for ES cells to maintain their undifferentiated states, and that this cadherin is even required for the reprogramming of MEFs into iPS cells. Considering the established importance of E-cadherin in cell-cell contacts and communications between embryonic stem cells, these findings are not surprising. However, another finding that E-cadherin can be substituted for Oct4 in the Yamanaka factors-mediated transformation of MEFs into iPS cells was unexpected and quite intriguing. These studies were carefully done; and the conclusions were corroborated by multiple approaches. I have only minor suggestions to this manuscript:

The authors propose that E-cadherin acts upstream of Oct4; e.g., E-cadherin-mediated signals enhance Oct4 expression. However, the present observations equally suggest the opposite possibility that Oct4 is required for inducing E-cadherin expression in iPS cells; in other words, E-cadherin is not properly re-expressed when Oct4 is omitted from the Yamanaka factors, leading to the failure of pluripotency acquisition. The authors should comment this possibility, too.

In Fig. 2A and 4A, "MET" should be read as "MEF".

Referee #2 (Revision Comments):

Only the last part seems novel and interesting. If confirmed and expanded upon it could be in fact very interesting. But as pointed out in the report it would need more relevant support on how and why E-Cad would be able to replace Oct4.

Referee #2 (Remarks to the Author):

The manuscript by Redmer et al. investigates the role of E-Cadherin in pluripotency and reprogramming by transcription factors. The results largely confirm previously established facts about the expression and role of E-Cad in ES cells, but claim a novel finding: E-Cad does not

increase the efficiency in combination with the four factors (OSKM)(different from previously published data), but can replace Oct4.

The manuscript is well written and organized, but most of the data are not entirely novel and while many of the characterizations of pluripotent cells are valid they are quite expected.

Specific comments:
Much of the first part of the paper is only confirming published data and while some previous studies are cited other are left out (Soncin et al 2009 Stem Cells; Larue et al 1996 Development) or cited in a different context at the end (Chou et al 2008 Cell).
Most of the data are nice, but provide little new insights.

Figure 2F needs more convincing chimeric contributions of the E-Cad high.

The first interesting section begins at conditional deletion of E-Cad for the reprogramming experiments. This is nice because it's a genetic approach rather than knockdown.
However, it raises two main concerns:
1) The blot in Figure 3A still shows that E-Cad is still present after adding Cre. This needs to be explained or addressed. Incomplete recombination?
2) If E-Cad is essential for reprogramming as the authors suggest, then Figure 3B makes no sense. The authors still report more than 20% of colonies after deletion. To clarify the colonies need to be picked and characterized. No deletion? Heterozygous? Residual levels seen in 3A are sufficient for a few clones to reprogram? Related to that the authors say in the discussion that iPS cells could not be obtained after Cre, but again Figure 3B still shows plenty of colonies.

The last section of the manuscript turns to an interesting observation. E-Cad can replace Oct4. This would be novel, but also seems very surprising. Therefore, rather than finishing with the characterization of the ESKM cells the authors should put significantly more focus (in fact most of the paper) around this finding and support it with more data.
In order to make such a claim it would be important to better characterize the effect the E-Cad overexpression has on MEFs and more details about the process need to be established. Certain cell types express E-Cad. Could these be reprogrammed with just SK (since Myc is also not really required for reprogramming)?

In summary the authors touch upon something quite interesting here, but it seems premature to claim than E-Cad can really replace Oct4 without additional supporting and mechanistic data.

Referee #3 (Revision Comments):

In its present form this manuscript cannot be published in EMBO Reports. However, if substantiated, the results are of great interest and following the successful implementation of the points outlined above, I would strongly support publication.

Referee #3 (Remarks to the Author):

The manuscript by Redmer et al. claims that E-cadherin plays a crucial role in the pluripotency of ESCs and that the substitution of ectopic expression of Oct4 by E-cadherin leads to the generation of iPS cells.

In Figure 1, the authors present E-cadherin shRNA KD data, thereby claiming that E-cadherin is required for the maintenance of pluripotency. In Figure 1B, the authors should consider including phase-contrast images for infection with the empty vector and with the shRNA-containing vector. In general, all images on immunocytochemical analysis as well as cell morphology have to be improved. The authors should also provide information on the timeframe between infection of the shRNA and the real-time PCR analysis demonstrated in Figure 1C. In general, in this first section of the manuscript where the authors discuss the requirement for E-cadherin in the maintenance of mESC pluripotency, qRT-PCR and immunostainings for lineage-specific markers should be

included to show the direction of ESCs differentiation. In Figure S1C, the authors claim to show that Oct4 expression is downregulated; however, Oct4 expression looks like it is not downregulated. Although one can expect that Oct4 is downregulated, evidence for this needs to be provided.

With Figure 2, the authors claim that SSEA-1-positive/E-cad-high cells derived from MEFs with OSKM comprise the fully reprogrammed population. The SSEA-1 data are not really clear, however (Figures 2B, S2A). Are E-cad-negative cells also SSEA-1-positive? It is also not clear when the authors performed the immunostaining-Was it on day 8 of viral infection? The Oct4 levels in the E-cadLOW population shown in Figure 2D look contradictory to the Oct4 levels presented in Figures 2C and S2C; in the latter figures, Oct4 levels in "E-cadLOW" cells appear to be comparable to those in "E-cad-high" cells. To demonstrate pluripotency, the authors present chimera formation in Figure 2F. Image #2 is not very convincing. The authors should consider including a table summarizing their chimera work. As shown in Figure 2C, the authors performed a typical "RT-PCR," and not a "qRT-PCR."

In Figure 3, the authors demonstrate the requirement for E-cadherin in iPS cell generation. Cre-inducible E-cadherin excision reduces iPS cell colony numbers. Although the level of E-cad has been clearly reduced, E-cadherin has not been completely excised. Therefore all statements describing these data need to be modified to be more cautious with their conclusions. In regard to Figure 3C, the authors should indicate the primers used for the qRT-PCR analysis (endogenous-specific, viral-specific, or coding sequence-specific) and how long after infection the analysis was performed.

With Figure 4, the authors draw their main claim that E-cadherin is capable of replacing Oct4 in iPS cell generation. With Figure 4B, the authors claim to generate iPS cell colonies that exhibit a typical ESC morphology with ESKM. However, the images provided are of poor quality, making it nearly impossible to see whether the colonies have a typical ESC shape or a partially reprogrammed shape.

In addition to the RT-PCR in Figure 4D, the integration of either ESKM or OSKM virus should be confirmed. To claim that ESKM iPS cells are authentic iPS cells, with pluripotency comparable to ESCs or OSKM iPS cells, the authors should consider showing global gene expression profiling by microarray. This certainly would make this a stronger paper.

For all teratoma data, such as that presented in Figure 4C, the authors should show the entire section images and provide more detailed information, such as the number of teratomas and the average size. As the authors`major claim is that Oct4 can be replaced by E-cadherin in iPS cell generation, the contribution of ESKM iPS cells to different organs of the chimera and hopefully the germline should be presented as in Figure 2F. Considering that E-cad is central to pluripotency, do the authors see an increase in reprogramming efficiency when coexpressing E-cad with OSKM?

In addition to the experimental changes described above, I would like to suggest some additional changes to the manuscript. At the end of the first paragraph in the Introduction, the authors mention that the "concept of cellular reprogramming was initiated by iPSC." I think it is widely accepted that it was initiated with somatic cell nuclear transfer. In the manuscript, the author attempt to associate E-cad with both reprogramming and EMT. However, evidence showing an association with EMT is unclear and should be deleted from the manuscript. In addition, the authors provide other statements regarding previous findings without the supporting citations, and in some cases, the citations provided are incorrect. For example, the authors mention that Chou et al. (2008) could revert epiSCs to ESCs with E-cad. Actually those authors were able to revert FAB-SCs to ESC-like cells but not to epiSCs. In addition, the reference list is rather short and should be lengthened. The authors should cite and discuss the recent PLoS One report by Mohamet et al. (PLoS One. 2010;5(9):e12921) demonstrating improved propagation of ES cells in suspension culture after E-cadherin abrogation as well as the supporting report by Chen et al. (Stem Cells. 2010; 28:1315-1325).

1st Revision - authors' response 28 February 2011

Detailed point by point response to the referees:
We like to thank all three referees for the time they took to review our manuscript and the constructive suggestions, which we have used to improve our study.

Referee #1:
This manuscript reports the roles of E-cadherin in the maintenance and acquisition of pluripotency by mouse embryonic cells. The authors found that E-cadherin is required for ES cells to maintain their undifferentiated states, and that this cadherin is even required for the reprogramming of MEFs into iPS cells. Considering the established importance of E-cadherin in cell-cell contacts and communications between embryonic stem cells, these findings are not surprising. However, another finding that E-cadherin can be substituted for Oct4 in the Yamanaka factors-mediated transformation of MEFs into iPS cells was unexpected and quite intriguing. These studies were carefully done; and the conclusions were corroborated by multiple approaches. I have only minor suggestions to this manuscript:

The authors propose that E-cadherin acts upstream of Oct4; e.g., E-cadherin-mediated signals enhance Oct4 expression. However, the present observations equally suggest the opposite possibility that Oct4 is required for inducing E-cadherin expression in iPS cells; in other words, E-cadherin is not properly re-expressed when Oct4 is omitted from the Yamanaka factors, leading to the failure of pluripotency acquisition. The authors should comment this possibility, too.

We agree with the reviewer that Oct4 could also be upstream of the E-cadherin expression. Nevertheless, from our experiment that viral expression of E-cadherin leads to the expression of endogenous Oct4 we have to conclude that E-cadherin is in this scenario upstream of Oct4. Since Oct4 has a major role in establishing the pluripotent state we cannot speculate that just the expression of E-cadherin can overcome the requirement of all of the Oct4 functions in the nuclear reprogramming of somatic cells. Due to space limitations in the manuscript we cannot discuss these possibilities further.

In Fig. 2A and 4A, "MET" should be read as "MEF".
MET refers to mesenchymal to epithelial transition in this context.

Referee #2 (Revision Comments):

Only the last part seems novel and interesting. If confirmed and expanded upon it could be in fact very interesting. But as pointed out in the report it would need more relevant support on how and why E-Cad would be able to replace Oct4.

We have added more data towards the reprogramming of somatic cells by E-cadherin in the absence of Oct4 and hope that we have satisfied some of the questions of the referee. However, given the limitations in time we are unable to provide the molecular mechanisms that E-cadherin evokes to trigger the

reprogramming in the absence of Oct4. Based on this publication we will analyze the molecular mechanisms further and hope that it will promote other investigation into this interesting topic.

Referee #2 (Remarks to the Author):

The manuscript by Redmer et al. investigates the role of E-Cadherin in pluripotency and reprogramming by transcription factors. The results largely confirm previously established facts about the expression and role of E-Cad in ES cells, but claim a novel finding: E-Cad does not increase the efficiency in combination with the four factors (OSKM)(different from previously published data), but can replace Oct4.
The manuscript is well written and organized, but most of the data are not entirely novel and while many of the characterizations of pluripotent cells are valid they are quite expected.

While we agree that some of our data confirm previously established facts, we still believe that we have for the first time combined these analyses here in one comprehensive publication on this topic. Our data also prove that E-cadherin is in a central position to control pluripotency via spatial cues. This has not been shown this clear in previous publications. We have also found in our settings, as opposed to the publication by Chen et al. (Stem Cells, 28 (8), 1315-1325), that E-cadherin cannot in general increase reprogramming efficiency. We believe that this is an important finding for the field. We have now tried to improve on our central finding that E-cadherin can replace Oct4 in the reprogramming of MEFs.

Specific comments:
Much of the first part of the paper is only confirming published data and while some previous studies are cited other are left out (Soncin et al 2009 Stem Cells; Larue et al 1996 Development) or cited in a different context at the end (Chou et al 2008 Cell).
Most of the data are nice, but provide little new insights.
As mentioned above, we believe that our data significantly combine previous findings and point out for the first time the central role E-cadherin plays in the maintenance and establishment of pluripotency. We have added the suggested literature in the introduction: "It has also been shown that E-cadherin controls processes of early differentiation (Larue et al, 1996) and plays an important role in regulating pluripotency of different stem cell compartments (Chou et al, 2008; Soncin et al, 2009). ". Especially, our findings here require a different discussion of the data by Soncin et al. It appears that in this study not the maintenance of mESC-like cells has been analyzed but rather of mFABSC-like cells that appear to be closer to EpiSCs and do not require E-cadherin for stemness (Chou et al. (2008), Cell 135, 449-461). We believe that also in light of these controversial findings regarding the function of E-cadherin in pluripotency our report is of significant importance.

Figure 2F needs more convincing chimeric contributions of the E-Cad high.

We have now transferred these results to the supplemental material and instead are showing similar data for our ESKM cell clones in Figure 4D. The same criticism can be raised towards our data shown for the ESKM clone. We agree with the referee that we do not observe the expected chimerism, especially we cannot found off-spring with germline transmission. We believe that this is due to the limited capacities we have to inject different iPS lines into a large number of blastocysts. Nevertheless, the main point we are trying to prove with this experiment is that these cells can contribute to organogenesis while Ecad^{low} cells never showed any contribution. Further blastocyst injections with larger numbers will most likely generate germline transmission but will be not achievable in the time frame and with the current resources. Together with the other data (teratoma formation, gene expression, bisulfite sequencing, cell morphology and protein staining, embryoid body formation) we provided, we believe that we have shown sufficient evidence that our iPS cells are indeed pluripotent.

The first interesting section begins at conditional deletion of E-Cad for the reprogramming experiments. This is nice because it's a genetic approach rather than knockdown.

However, it raises two main concerns:
1) The blot in Figure 3A still shows that E-Cad is still present after adding Cre. This needs to be explained or addressed. Incomplete recombination?

Yes, we do not observe ablation of E-cadherin in all cells, i.e. that soluble Cre fails to recombine the locus in around 20% of cells. We can show that iPS cells that resulted from this experiment and show expression of Nanog still express E-cadherin.

2) If E-Cad is essential for reprogramming as the authors suggest, then Figure 3B makes no sense. The authors still report more than 20% of colonies after deletion. To clarify the colonies need to be picked and characterized. No deletion? Heterozygous? Residual levels seen in 3A are sufficient for a few clones to reprogram? Related to that the authors say in the discussion that iPS cells could not be obtained after Cre, but again Figure 3B still shows plenty of colonies.

We agree with the referee that this is a crucial point. We have now provided evidence that all iPS-like colonies that formed after Cre-treatment of Ecad$^{flox/flox}$ MEFs are still E-cadherin positive, thus, failed to recombine the E-cadherin locus. Co-staining with Nanog and E-cadherin showed an overlap of the expression of these proteins in all cells with an ESC-like morphology (now Fig. 2D). We have added this to the result section and changed the sentence in the discussion: "Second, in reprogramming of mouse embryonic fibroblasts by the four Yamanaka factors (OSKM), we found that iPS cells were exclusively E-cadherin positive, and could not be obtained from Ecadflox/flox cells after E-cadherin has been deleted by Cre.".

The last section of the manuscript turns to an interesting observation. E-Cad can replace Oct4. This would be novel, but also seems very surprising. Therefore, rather than finishing with the characterization of the ESKM cells the authors should put significantly more focus (in fact most of the paper) around this finding and support it with more data.

We have now added a significant amount of data describing the ESKM iPS cells. We now show data that determine the methylation state of pluripotency gene promoters Oct4 and Nanog by bisulfite sequencing and have added a global expression profiling. Both experiments show that ESKM iPS are indeed pluripotent cells comparable to mESCs. We can also show that there are multiple viral Oct4 integration sites in the case of OSKM cells while in ESKM cells only the endogenous Oct4 locus is detected. We would like to perform a more detailed molecular analysis of the interaction between E-cadherin and Oct4. However, given the time frame for this publication we believe that this should be done in future experiments. We agree that the replacement of Oct4 by E-cadherin is an interesting finding and we would like to publish these finding as soon as possible.

In order to make such a claim it would be important to better characterize the effect the E-Cad overexpression has on MEFs and more details about the process need to be established. Certain cell types express E-Cad. Could these be reprogrammed with just SK (since Myc is also not really required for reprogramming)?

These are indeed interesting experiments and we have tried to reprogram keratinocytes in the course of this study. However, due to technical difficulties these results were not conclusive. Since reprogramming experiments are very time consuming (we generally need at least five weeks before we can establish stable cell clones in great numbers for further analysis) we cannot screen different cell types for this study.

In summary the authors touch upon something quite interesting here, but it seems premature to claim than E-Cad can really replace Oct4 without additional supporting and mechanistic data.

We can understand the criticism of this referee and we would like to be able to provide more mechanistic data but this will require significant new sets of experiments. However, we believe that our initial findings regarding the role of E-cadherin in maintenance and induction of pluripotency and its ability to replace Oct4 during reprogramming is of significant interest for this fast evolving scientific field.

Referee #3 (Revision Comments):

In its present form this manuscript cannot be published in EMBO Reports.

However, if substantiated, the results are of great interest and following the successful implementation of the points outlined above, I would strongly support publication.

We have tried to substantiate the results in regard to the characterization of the E-cad$^{lox/flox}$ MEFs and the ESKM reprogrammed cells. We hope the referee can based on this additional data support the publication of our manuscript.

Referee #3 (Remarks to the Author):

The manuscript by Redmer et al. claims that E-cadherin plays a crucial role in the pluripotency of ESCs and that the substitution of ectopic expression of Oct4 by E-cadherin leads to the generation of iPS cells.

In Figure 1, the authors present E-cadherin shRNA KD data, thereby claiming that E-cadherin is required for the maintenance of pluripotency. In Figure 1B, the authors should consider including phase-contrast images for infection with the empty vector and with the shRNA-containing vector.

This Figure is now supplemental Fig. S1. We show the phase contrast pictures in Fig. S1E (PH).

In general, all images on immunocytochemical analysis as well as cell morphology have to be improved.

We have improved the images for the immunocytochemical data. However, the data generated by the submission system were not quite as strong as our original data, which are very large. We hope that in the new version of our manuscript the problem does not occur.

The authors should also provide information on the timeframe between infection of the shRNA and the real-time PCR analysis demonstrated in Figure 1C.

The time frame in this experiment is four days and this was mentioned in the figure legend, now Fig. S1C. This time frame applies to the data in Fig. S1D and E as well.

In general, in this first section of the manuscript where the authors discuss the requirement for E-cadherin in the maintenance of mESC pluripotency, qRT-PCR and immunostainings for lineage-specific markers should be included to show the direction of ESCs differentiation.

While we agree with the referee that this is an important line of investigation, our focus in this manuscript was on establishment and maintenance of pluripotency.

We cannot with the restriction of space proceed to analyze the differentiation of the E-cadherin low cells. We believe that several cell lineages are no longer observed after E-cadherin knock-down and we have investigated this by global expression profiling. A throughout analysis of the different cell fates will require further experiments and we would like to perform these in further projects.

In Figure S1C, the authors claim to show that Oct4 expression is downregulated; however, Oct4 expression looks like it is not downregulated. Although one can expect that Oct4 is downregulated, evidence for this needs to be provided.

We agree with the referee that this was not clearly visible. In the recent version of this figure (Fig. S1C) we believe that the downregulation of Oct4 is more evident. Oct4 decrease is generally observed quite late after differentiation of cells, due to its maintained expression even if cells are differentiating (compare Fig. S1 A and B).

With Figure 2, the authors claim that SSEA-1-positive/E-cad-high cells derived from MEFs with OSKM comprise the fully reprogrammed population. The SSEA-1 data are not really clear, however (Figures 2B, S2A). Are E-cad-negative cells also SSEA-1-positive?

The referee is correct in assuming that not all SSEA-1 positive cells are E-cadherin positive. In fact, only around 20% of the SSEA-1 positive cells 10 days after the infection are also E-cadherin high. Nevertheless, all stable colonies that convert to mESC-like morphology are double positive. Thus, sorting for SSEA-1 only allows for enrichment for reprogrammed cells. We have not shown all our data in this regard in the interest of space.

It is also not clear when the authors performed the immunostaining-Was it on day 8 of viral infection?

This staining has been performed on established cell clones as stated in the figure legend. The cells have been passaged at least five times before staining. It is important, that only SSEA-1 and E-cadherin high clones show full reprogramming.

The Oct4 levels in the E-cadLOW population shown in Figure 2D look contradictory to the Oct4 levels presented in Figures 2C and S2C; in the latter figures, Oct4 levels in "E-cadLOW" cells appear to be comparable to those in "E-cad-high" cells.

The referee is correct in pointing out this discrepancy. Fig. 2D (now Fig.1D) has been carried out with the Oct4 endo primer against the endogenous Oct4 mRNA. The primer used in Fig. 2C (now Fig. 1C) is the total Oct4 primer that does not distinguish between viral and endogenous Oct4. This primer as the referee has pointed out has also been used in Fig. S2C. Nevertheless, here the data are shown in logarithmic scale. The level of Oct4 endo is lower in Ecad^{low} cells

although only around 2-fold and not as low as shown in Fig. 1D around 10-fold. This discrepancy is most likely due to the residual activation of the endogenous Oct4 by the viral Oct4, that is present in Ecad^{low} cells, at the earlier passages of the cells.

To demonstrate pluripotency, the authors present chimera formation in Figure 2F. Image #2 is not very convincing. The authors should consider including a table summarizing their chimera work.

We agree with the statement of the referee, which is also pointed put by referee #2: We have now transferred these results to the supplemental material and instead are showing similar data for our ESKM cell clones in Figure 4D. (see below)
We have included tables summarizing the chimera work with SSEA-1 positive cells and ESKM clones (Table 4a and 4b, respectively) in the supplemental material.

As shown in Figure 2C, the authors performed a typical "RT-PCR," and not a "qRT-PCR."

Agreed and corrected. "In line with the IF, RT-PCR analysis revealed that SSEA-1 positive..."

In Figure 3, the authors demonstrate the requirement for E-cadherin in iPS cell generation. Cre-inducible E-cadherin excision reduces iPS cell colony numbers. Although the level of E-cad has been clearly reduced, E-cadherin has not been completely excised. Therefore all statements describing these data need to be modified to be more cautious with their conclusions.

Referee #2 raised the same criticism:
Yes, we do not observe ablation of E-cadherin in all cells, i.e. that the soluble Cre fails to recombine the locus in around 20% of cells. We can show that iPS cells that resulted from this experiment and show expression of Nanog still express E-cadherin. We have now provided evidence that all iPS-like colonies that formed after Cre-treatment of Ecad$^{flox/flox}$ MEFs are still E-cadherin positive, thus, failed to recombine the E-cadherin locus. Co-staining with Nanog and E-cadherin showed an overlap of the expression of these proteins in all cells with an ESC-like morphology (now Fig. 2D). We have added this to the result section and change the sentence in the discussion: "Second, in reprogramming of mouse embryonic fibroblasts by the four Yamanaka factors (OSKM), we found that iPS cells were exclusively E-cadherin positive, and could not be obtained from Ecadflox/flox cells after E-cadherin has been deleted by Cre.".

In regard to Figure 3C, the authors should indicate the primers used for the qRT-PCR analysis (endogenous-specific, viral-specific, or coding sequence-specific) and how long after infection the analysis was performed.

The referee is correct. This figure has been changed and does not include Oct4 but only endogenous genes that were not virally transduced. The analysis was done 10 days after infection as stated in the figure legend now.

With Figure 4, the authors draw their main claim that E-cadherin is capable of replacing Oct4 in iPS cell generation. With Figure 4B, the authors claim to generate iPS cell colonies that exhibit a typical ESC morphology with ESKM. However, the images provided are of poor quality, making it nearly impossible to see whether the colonies have a typical ESC shape or a partially reprogrammed shape.

We don't agree with this statement. Although the quality of these phase contrast images are not perfect, the morphology of the cell clones can be appreciated and it is clear that ESKM cells show sub clones while in SKM cells only single cells are observed 4 days after passaging of the cells.

In addition to the RT-PCR in Figure 4D, the integration of either ESKM or OSKM virus should be confirmed.

We agree with the referee and have included a Southern blot analysis for Oct4 in Fig. S3E showing that only OSKM clones have multiple integrations of viral Oct4 while ESKM clone only show the endogenous Oct4 loci.

To claim that ESKM iPS cells are authentic iPS cells, with pluripotency comparable to ESCs or OSKM iPS cells, the authors should consider showing global gene expression profiling by microarray. This certainly would make this a stronger paper.

Agreed. This has been included in Fig. 4 B.

For all teratoma data, such as that presented in Figure 4C, the authors should show the entire section images and provide more detailed information, such as the number of teratomas and the average size.

Tables describing the teratoma experiments have been included in supplemental material (Table 2 and 3, for E-cad^{high} clones and ESKM clones, respectively).

As the authors` major claim is that Oct4 can be replaced by E-cadherin in iPS cell generation, the contribution of ESKM iPS cells to different organs of the chimera and hopefully the germline should be presented as in Figure 2F.

We agree with this statement of the referee as has been discussed for referee #2 and for this referee under Fig. 2F:
We agree with the referee that we do not observe the expected chimerism, especially we cannot found off-spring with germline transmission. We believe that this is due to the limited capacities we have to inject different iPS lines into a large

number of blastocysts. Nevertheless, the main point we are trying to prove with this experiment is that these cells can contribute to organogenesis while Ecad^{low} cells never showed any contribution. Further blastocyst injections with larger numbers will most likely generate germline transmission but will be not achievable in the time frame and with the current resources. Together with the other data (teratoma formation, gene expression, bisulfite sequencing, cell morphoplogy and protein staining, embryoid body formation) we provided, we believe that we have shown sufficient evidence that our iPS cells are indeed pluripotent

Considering that E-cad is central to pluripotency, do the authors see an increase in reprogramming efficiency when coexpressing E-cad with OSKM?

This was suggested by the publication Chen et al. (2010), Stem Cells, 28, 1315-1325. We have tested whether expression of E-cadherin can enhance reprogramming efficiency in general (Fig. S3 C and D) and we were not able to see any increase in efficiency. This finding triggered the analysis of single clones that lead to the discovery that E-cadherin was able to partially replace Oct4.

In addition to the experimental changes described above, I would like to suggest some additional changes to the manuscript. At the end of the first paragraph in the Introduction, the authors mention that the "concept of cellular reprogramming was initiated by iPSC." I think it is widely accepted that it was initiated with somatic cell nuclear transfer.

Agreed, we have changed the sentence: "The concept of somatic cell reprogramming was initiated with somatic nuclear transfer (Wilmut et al, 1997) and further developed by the seminal finding that mouse embryonic fibroblasts (MEFs)...".

In the manuscript, the author attempt to associate E-cad with both reprogramming and EMT. However, evidence showing an association with EMT is unclear and should be deleted from the manuscript.

Agreed. We have omitted EMT in the text describing Fig. 1 (now Suppl. Fig. 1).

In addition, the authors provide other statements regarding previous findings without the supporting citations, and in some cases, the citations provided are incorrect. For example, the authors mention that Chou et al. (2008) could revert epiSCs to ESCs with E-cad. Actually those authors were able to revert FAB-SCs to ESC-like cells but not to epiSCs.

Agreed, we have changed this statement accordingly: "Another study also shows that E-cadherin is a critical determinant in the conversion of Fgf2-, Activin-, BIO-derived mouse stem cells (FAB-SCs) to pluripotent mESCs (Chou et al, 2008). FAB-SCs appear to be closely related to EpiSCs.".

In addition, the reference list is rather short and should be lengthened. The authors shouldcite and discuss the recent PLoS One report by Mohamet et al. (PLoS One. 2010;5(9):e12921) demonstrating improved propagation of ES cells in suspension culture after E-cadherin abrogation as well as the supporting report by Chen et al. (Stem Cells. 2010; 28:1315-1325).

*We have now introduce the publication by Mohamet et al.: "It has been reported that the loss of E-cadherin allows the maintenance of mESCs in suspension cultures (Mohamet et al, 2010), suggesting that under these conditions they may convert to a intermediated cell state like mEpiSCs." The publication by Chen et al. has been discussed: "*For instance, shRNA viruses directed against E-cadherin could prevent iPS cell reprogramming and chemical intervention leading to upregulation of E-cadherin promoted somatic cell reprogramming (Chen et al, 2010)." *And: "In our reprogramming system, overexpression of E-cadherin alone could not enhance the efficiency of iPS cell production; such an effect was recently reported (Chen et al, 2010)." We agree that there are a great number of other publications that could and should be discussed. However, the space for a EMBO Report publication is rather limited and an scientific field like pluripotency and nuclear reprogramming with its large novel literature data can not be covered completely.*

2nd Editorial Decision 18 March 2011

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. Although referee 2 still does not support publication of the manuscript in its present state, the main concern of this referee is the missing mechanistic insight, which is not required for publication in EMBO report. Both referees acknowledge that overall the manuscript has been much improved, however, referee 3 still thinks that the images in figure 3B should be improved in order to better visualize the morphology of the cells. I would like you to address this remaining concern, and please also add a scale bar to this figure, so that we can proceed with the official acceptance of your manuscript.

I noticed that error bars are not defined in both the main and supplementary figures. Please do so in the figure legends, and also indicate the number of experiments (n=x) the graphs and bars represent for all the figures this applies to. I further noticed that you have included results in the supplementary information, which EMBO reports does not allow. Please either delete the results from the supplementary information or include them in the main text, but in this case the text will have to be somewhat shortened, as the character count is already at our limit. You can move some of the materials and methods to supplementary information, but only the most commonly used ones, like immunofluorescence, western blots, FACS, etc. Methods essential for the understanding of the experiments described in the main body of the manuscript must remain in the materials and methods section of the main manuscript file.

I hope you find my suggestions of how to revise the manuscript acceptable, and I look forward to seeing a new revised version of your work as soon as possible.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS

Referee #2 (Revision Comments):

The revised version is somewhat improved, but many of the previously raised points have not been addressed. Surprisingly the authors often cite space and time limitations as an excuse for not performing the suggested experiments.

As stated before the E-Cad replacement of Oct4 seems the most novel and interesting finding. The authors certainly provide now much better data on the characterization of the ESKM lines, but have not added a single experiment that helps explain how this somewhat surprising result can be explained.

I don't think it makes sense to reiterate all the previous points (many of which overlapped with reviewer 3) that have not been addressed. Overall I still think there is something interesting in this story, but don't feel comfortable proceeding with publication based on the limited number of revisions.

Referee #3 (Remarks to the Author):

The manuscript by Redmer et al. claims a crucial role of E-cadherin for ESC pluripotency and the generation of iPS cells substituting ectopic Oct4 expression by E-cadherin.

The authors have addressed most points raised during the first review round.

- The images for the immunocytochemical data have been improved.

- The authors have responded to the point concerning SSEA-1 positivity and E-cadHIGH and E-cadLOW populations.

- The chimera data are presented much better.

- The Cre-inducible E-cadherin excision section has been modified.

- The qRT-PCR has been improved and the authors now include a Southern Blot analysis to demonstrate Oct4 (non) integration.

- The authors have now added a microarray analysis to compare ESKM iPS cells with ESCs.

- The teratoma analysis has been improved.

- Albeit germline transmission has not been achieved due to technical limits of the group, the current pluripotency characterization seems sufficient.

- The minor points in changing phrases and adding citations have been mostly addressed.

- I still would like to suggest that the authors provide an improved figure demonstrating the morphology of ESKM iPS cells in Figure 3B.

The manuscript has been greatly improved and is now a nice piece of work.

2nd Revision - authors' response 23 March 2011

Please, find the newly revised version of our manuscript ìE-cadherin is crucial for ESC pluripotency and can replace Oct4 during somatic cell reprogrammingî attached. We are very happy that you consider our manuscript now ready for publication in EMBO Reports based on some minor changes.
In the revised version we have improved the quality of Fig. 3B as suggested by referee #3 and by you. Furthermore, throughout the figure legends we have defined the error bars and indicated the number of experiments. We have deleted the result texts from the supplementary information, as you suggested. Some of the material and methods have been moved to the supplementary information. We believe that the methods essential for the understanding of the manuscript are still available in the main manuscript. The character count is reduced to 27,337, we hope that this is acceptable for publication.
We are looking forward to your response and hope that you will find the newly revised version of our manuscript now suitable for publication. Please, let us know if you require further information or documents.

3rd Editorial Decision 08 April 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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

Best,

Editor
EMBO Reports

