----- Original Message -----
件名: Decision on Nature manuscript [redacted]
送信者: [redacted]
送信日時: 2013年04月04日(木) 22:44:04
宛先: [redacted]

4th April 2013

> *Please ensure you delete the link to your author home page in this e-mail if you wish to forward it to your co-authors.
>
> Dear Dr Obokata
>
> I hope you are well. Your manuscript entitled "Stimulus-Triggered Fate Conversion of Somatic Cells into Pluripotency" has now been seen by 3 referees, whose comments are attached below. While they find your work of great potential interest, as do we, they have raised important concerns that in our view need to be addressed before we can consider publication in Nature.
>
> Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript (unless something similar has been accepted at Nature or appeared elsewhere in the meantime).
>
> Any revised manuscript should conform to our format instructions and publication policies, which can be found at www.nature.com/nature/authors/. The revised paper should be submitted using the link below:
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>
> In the meantime we hope that you will find our referees' comments helpful. Please do not hesitate to contact me if there is anything you would like to discuss.
>
> Yours sincerely
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> [redacted]
>
> Nature
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> Referees' comments:
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> Referee #1 (Remarks to the Author):
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> In the present manuscript the authors evaluate the role of cellular stress during potential nuclear reprogramming towards pluripotency. This is a very interesting manuscript and potentially groundbreaking. However, the presentation and data supporting the conclusions are somewhat speculative and, in some cases, preliminary.
>
> The work and conclusions are purposely focus into a particular field but

obviate other, and perhaps even more feasible, explanations for the experimental results presented. From the text, the reader immediately assumes stress induces some kind of reprogramming towards pluripotency. This is best demonstrated by teratoma and chimera formation. However, reprogramming could be only one possibility. For instance, cell transformation could stand for an alternative explanation. Self-renewal, de-differentiation and capacity to populate and differentiate into different tissues is also a feature of cancer stem cell, including teratocarcinomas. I would recommend the authors to be extremely cautious in their claims regarding their discovery of generation of stem-like cells in the absence of genetic manipulation. The authors should look into the actual effect that the treatment elicits in the genome and they should assess genomic instability.
>
> The authors lead the reader to believe that somatic cell identity can only be forcefully changed by leveraging in genetic modifications and that this process does not occur naturally. I am afraid I have to disagree. This is not the case since partial dedifferentiation of cardiomyocytes is responsible for the healing of the injured heart (see Senyo et al., 2012 Nature for example). Interestingly a dynamic equilibrium in cell identity has also been shown for the maintenance of cancer stem cell populations (Gupta et al., 2011 Cell for example). In my opinion, these examples demonstrate that, contrary to the statements made by the authors, mammalian somatic cells have the capacity to dedifferentiate in the absence of genetic modifications.
>
> In addition to the possibility that the cell treatment performed may induce genetic aberrancies, it is evident that it induces massive apoptosis. The authors concentrate on survivor cells for their downstream experiments and present "cell counting" estimations in Figure 1, which is perhaps not the best assay. The authors should demonstrate and show the levels of cell death and perform this by doing co-stainings with CD45 as well as monitoring OCT4-GFP. This is a simple experiment that can be done with PI/Annexin-V.
>
> The authors should make an effort in trying to define whether the process observed is actually resembling cellular transformation and tumorigenicity. A comparison is shown between Oct4-GFP+ cells and the non-treated CD45+ cells, but the best internal control is the population of Oct4-GFP- cells that arise during the treatment.
>
> Figure 1b is not properly presented. A proper gating strategy including all different controls should be shown. Similarly, the panels should be clearly labeled. Readers will wonder why non-treated CD45+ cells will demonstrate a reduction of CD45 (d3) and even a fully negative independent population by day 7. It would also be desirable that the authors characterize their starting populations as CD45+ cells include a plethora of hematopoietic lineages and progenitor cells.
>
> What do the authors want to claim by saying that STAPs are smaller? Is this relevant? Why? And the "lamellipodia"? The authors should elaborate on what was the question/hypotheses behind these experiments and what conclusions can be drawn? This reviewer fails to see the relevance of these observations as presented. Instead, it would certainly be of more interest to provide some information of chromatin remodeling and methylation changes, though it does not necessarily need to be genome-wide profiling, simple histone marks and/or methylation arrays would very much enhance the quality of the manuscript.
>
> FACS data should be properly analyzed and presented with statistical tests throughout the manuscript. So-called "representative plots" is a poor use of a semi-quantitative single-cell methodology suitable for proper analysis. How many experiments were done for each analysis? Some graphs such as those comparing different Oct4-gfp % per cell type don't even have error bars. How is the error fluctuation? Are the results statistically significant?
>
> Soft-agar assays could serve to evaluate potential transformation of the cells.
>
> RNA microarrays shoul be performed so to highlight the new "metastable pluripotent state" that STAP cells represent and the differences with ESC/iPSCs.
>
> Several figures lack error bars. Please provide information on the number of

experiments, technical replicates and proper statistics.
>
> There is a lack of the more proper internal controls, that is CD45-GFP- stress induced cells (not all cells become Oct4-GFP+ as shown in fig 1 so this populations could be used as an additional internal control for all experiments as they have been treated yet not reprogrammed).
>
> If the cells are stem cells, why do they fail to self-renew and demonstrate a decline in their numbers? I believe this observation deserves further discussion.
>
> Figure 3c shows a clearly over-saturated GFP channel.
>
> Figure 4e demonstrates a small fraction of cells expressing ZO-1. The so-called differences between ESC, EpiSCs and STAPs could be simply due to limited and differential antibody accessibility due to the presence of cell clamps/ colonies. No statement or major conclusion based on this data should be made and /or quantitative approaches (e.g.: flow cytometry employed).
>
> Figures 1a, 1e, 1f, 1g, 1h, figure 4a should be removed. These figures are either non-informative, a mere method with no actual biological implications discussed or, as in the case of the multiple panels in figure 1, simply the same information provided by multiple means. One panel suffices in figure 1 to say that the cells are OCT4-GFP and lose CD45 expression.
>
> The authors mention that "CD45+/CD34- population is the major source for producing oct3/4::GFP+ cells", but they should discuss what might be the reason for it, particularly considering that CD34+ cells represent a more dedifferentiated state. In line with the current belief in the reprogramming field, they should be more amenable for reprogramming (please see any of the reports dealing with the reprogramming of adult stem cell populations such as Neural Stem Cells).
>
> Immunostaining in Fig. 2a shows that pluripotency factors are detected in STAP cells but some population looks negative for them. Is it correct? If so, the authors should discuss this point, whether or not this negative population was not reprogrammed at all or arises from cells that loss pluripotent marker expression (and so implying that the reprogramming of at least some cells is not stable).
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> Referee #2 (Remarks to the Author):
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> In this and the accompanying manuscript Obokata and colleagues describe an unanticipated and highly provocative finding. They report that somatic cells exposed to low pH change phenotype and acquire broad developmental potency, including the capacity to colonise host embryos and generate gametes. The authors term this process "Stimulation triggered acquirement (should be 'acquisition') of pluripotency" or STAP. Provided the results are correct and reproducible this promises to be a transformational discovery in developmental and stem cell biology with major implications for regenerative medicine.
> The phenomenology described leaves open many questions for future investigation. However, the authors set their work fairly in the context of previous studies and acknowledge the outstanding issues. Accordingly I confine this critique to the substance and description of the data presented. There are several issues that I consider should be clarified beyond doubt because of the potential revolutionary nature of the observations.
> The most challenging issue for the authors is to provide irrefutable evidence of somatic cell origin. Genomic analysis of TCR rearrangement is a good approach but the data presented are not compelling because they indicate a mixture of T cell and non-T cell derived DNA. This result could reflect presence of residual T cells within the STAP clusters. In the absence of ability to clone or rigorously purify the STAP cells, an alternative route would be prospective FACS purification of T cells then conversion to STAP cells and TCR analysis. The described results suggest this should be feasible. Most convincing, however, would be to demonstrate visually by time lapse tracking of single cells conversion of CD45 immunofluorescent cells into CD45 negative/Oct4 GFP positive cells that can also be stained with Ecadherin and/or Nanog. This approach has been exemplified several years ago by Schroeder and colleagues and should be

technically rather straightforward for these authors.
> It is also important that the authors buttress their observations with more quantitative information. How many independent trials of STAP cell generation have been performed with the final protocol and what is the variability? Does the GFP reporter faithfully reproduce Oct4 expression - please provide a time course of Oct4 immunostaining during STAP cell generation. It is of particular interest to know when Oct4 protein is first detectable. Do all GFP positive cells go on to form clusters or do a significant number perish - it is not evident that fluorescent clusters in the movies are necessarily living cells. How were the cell aggregates analysed in Figure 2a obtained - they appear much larger than the cell clusters in Figure 1 or in the movies. Are all cells in each cluster GFP positive? From the immunostaining (also in Fig 3c) they are not uniformly Oct4 positive. This is important to clarify. The authors suggest that the clusters form through accretion rather than
> proliferation. The text is uncertain on whether the STAP cells proliferate after day 7 and whether they are viable after day 14. Please provide clear information on these points, including BrdU labelling. Data should be provided on the frequency and reproducibility of in vitro differentiation. How many cells were grafted for the teratoma assay? Why were NOD/Scid rather than syngeneic mice used as recipients? The frequency and sizes (weights) of tumours should be presented. Are they teratomas or teratocarcinomas (easily monitored via Oct4-GFP)?
> Of paramount importance for the legitimacy of this paper is that the authors provide a full step by step account of their method such that the community can rapidly validate the reproducibility of the findings. The present method description is minimal and key elements are not properly defined. Notably the authors say that conversion is achieved in B27 medium, but B27 is a medium supplement, not a medium itself. Is serum present and if so have different serum batches been tested. Furthermore B27 is a complex supplement with considerable batch to batch variation. Is B27 essential for the conversion and have the authors tested several batches? How exactly were cells transferred from low pH to "B27 medium" and were any measures taken to control the final pH. It is not mentioned in the text, but the methods say that samples were taken from 1 week old mice. If conversion has only been demonstrated with cells from pre-pubertal mice, this should be clearly stated in the main results
> section. How exactly are STAP cells identified and isolated without use of the Oct4-GFP reporter?
> The cell shrinkage data are not entirely convincing. How can the authors be sure this is not attributable to selective survival of small cells, e.g. lymphocytes? Unless they can exclude this possibility these data may be better omitted. Furthermore, the two fluorescent cells at the end of the time lapse movie do not look healthy, indeed one looks to be disintegrating.
> The description of STAP cell generation from other tissues is rather rudimentary and the data are not presented in the most helpful way. To be able to gauge the frequency of conversion it is essential to know the input number in each experiment and the number surviving at day 7. If the authors want to assert a non-blood cell origin, they must do a negative sort for CD45 and/or positive sort for non-haematopoietic marker(s).
> A troublesome part of the paper is the account of chimaera production. The details are very confusing and in part contradictory. Most glaringly we are told in the legend for Figure 5b that the Oct4-GFP transgene is used to show wide tissue contribution. I assume this is an error and a constitutive GFP reporter was used, but as pointed out above, at no point in the manuscript is there a description of how STAP cells are isolated without use of the Oct4 reporter. Figure 5c supposedly shows chimaeras made with STAP cells derived from BL/6 mice, but the starred high contribution chimaera is not black and nor are the offspring in panel e? Please explain. Important data on chimaera production are presented in Supplementary Tables, but these also are not clear or comprehensive. Table 1 reports offspring, but some of the chimaeras are analysed as foetuses. Please discriminate. GOF mice are not defined. Table 2 reveals that chimaeras were inter-crossed. Why? This is very unusual. The data
> in the table cannot be fully interpreted without information on the strain, genotype and gender of the STAP cells.
> The authors conclude based on in vitro differentiation, teratoma formation and chimaera colonisation that STAP cells are pluripotent. The assays are valid, but as performed they test pluripotency at the population level, not single cell level. In the absence of clonal analysis the authors should be more circumspect. Indeed it would be relevant to examine expression of early lineage markers in

STAP cell clusters and evaluate the possibility that they comprise a mix of lineage progenitors rather than a unitary cell state.
> It is not clear from the description if LIF is actually necessary for STAP cell generation? This is significant in light of recent reports that LIF signalling is required for other reprogramming modalities. Addressing this point, even in preliminary fashion, would appropriately herald future mechanistic investigations.
> Finally, in the abstract and discussion the authors refer to "initialization" of the somatic cell state -what is meant by this?
>
> Minor points:
> Label or legend does not describe the difference between upper and lower panels in Figure 1b.
> Although commonly asserted, foetuses generated by tetraploid complementation are not in fact "derived only from donor cells" (Eakin et al., Developmental Biology, 2005).
> What is the basis for describing the histological section in the supplementary figure as pancreas?
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> Referee #3 (Remarks to the Author):
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> This manuscript and the accompanying study report some truly remarkable findings. The authors show somatic cells can readily be converted to a pluripotent state through short-term exposure to moderately stressful conditions in vitro. In the most widely studied paradigm, exposure of splenic CD45+ cells from the spleens of mice transgenic for an Oct-4 reporter to low pH for a brief period converts them into Oct-4 positive cells (STAP cells) which express other markers of pluripotency, can differentiate into multiple lineages in vitro, and give rise to teratomas and contribute to chimera formation under appropriate assay conditions. The STAP cells cannot multiply extensively in vitro, unlike embryonic stem (ES) cells. Exposure to other forms of stress including cell membrane damage, trituration through a narrow pipet, and hypoxia, also induce measurable numbers of Oct-4 positive cells.
>
> It is difficult to fault the data in the study, so far as it goes; the observations reported are relatively straightforward and could readily be reproduced by any laboratory familiar with pluripotent stem cell culture. The authors offer no insight into the mechanism whereby low pH or other forms of stress convert cells to the STAP pluripotent state. This is a concern because it is not clear why or how these relatively simple manipulations should destabilize the differentiated state of somatic cells so profoundly and at such high frequency. There are circumstances in which cells would be exposed to the reprogramming conditions used in this study. For example, exposure of cells to pH6 (which induces significant reprogramming) would not be unusual in cell culture if cells are growing rapidly and medium is not replenished frequently, or in the urinary tract or stomach in vivo, and what the authors refer to as hypoxia (5% O2, which seems reproducibly to induce 1-5% of cells from
> various tissues to express Oct-4) approximates the oxygen tension present in most tissues in vivo and is used as standard practice by many workers to culture mammalian cells in vitro (including some labs studying reprogramming). There is some indication that LIF or other factors required for mouse ES cell culture are necessary to observe the emergence of reprogrammed cells, but whether these factors play an active role in the reprogramming or are simply permissive for survival of reprogrammed cells is not fully clear from the data presented.
>
> Because the STAP cells cannot be expanded in vitro, it is impossible to derive clonal lines, and therefore the claim that these cells are pluripotent is not fully validated. The possibility remains that the tissues formed in vitro or in teratomas or chimeras are derived from multiple (perhaps partially) reprogrammed cells, each with limited differentiation capacity. The authors might take advantage of the fact that some of the reprogrammed cells have T-cell receptor rearrangements to elucidate whether the differentiated cells in teratomas or mice are clonally derived. The data on conversion of STAP to ES-like cells in the accompanying manuscript do not really speak to this point (pluripotency of

STAP cells per se) directly.
> The populations in Figure 2a and 3c look reasonably convincing.
>
> More extensive data on gene expression in the Oct-4 positive cells at various stages of the reprogramming process would shed light on the mechanism of the conversion and the similarities and differences between STAP cells and other pluripotent cell types.
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>
> *Please ensure you delete the link to your author home page in this e-mail if you wish to forward it to your co-authors.
>
> Dear Dr Obokata
>
> I hope this email finds you well. Your manuscript entitled "Developmental potential for embryonic and placental lineages in reprogrammed cells with acquired pluripotency" has now been seen by 3 referees, whose comments are attached below. While they find your work of great potential interest, as do we, they have raised important concerns that in our view need to be addressed before we can consider publication in Nature.
>
> Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript (unless something similar has been accepted at Nature or appeared elsewhere in the meantime). We agree with the reviewers that as it stands this Letter relies too much on the other paper and at Nature each manuscript needs to stand on its own merit, therefore you may consider merging the two papers. However, we would offer you more space than our usual 5 pages in case of publication.
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>
> In the meantime we hope that you will find our referees' comments helpful. Please do not hesitate to contact me if there is anything you would like to discuss.
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> Yours sincerely
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> Referees' comments:
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> Referee #1 (Remarks to the Author):
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> In the present manuscript the authors leverage on an accompanying manuscript

in which they described reprogramming induced by stress. In here the authors try to expand their findings to the observation that STAP cells (the acronym used to define these cells) have the ability to contribute to extra-embryonic tissues.
>
> Both manuscript present very interesting observations and are potentially groundbreaking. Yet two major issues apply:
> 1) The two manuscripts should not be considered independently, as this particular one seems rather suitable to be a simple figure inside the main manuscript describing STAP conversion.
> 2) There are multiple experimental concerns in the accompanying manuscript that hamper this Letter as a stand-alone manuscript because by leveraging in the paper describing STAP formation, and with no additional characterization, all issues related to the accompanying manuscript apply here as well.
>
> This is of special importance because whereas the main Article describing STAP formation was more descriptive, this manuscript claims "STAP stem cell technology may offer a versatile, powerful resource for new-generation regenerative medicine." This might be the case, or not, but it is important to realize no single experiment in any of the two manuscript evaluates the "quality" of the cells, performs comparative genome-wide analysis or precise quantifiable assays side-by-side with ESCs/iPSCs.
>
> I am afraid I cannot recommend the current manuscript for publication until a comprehensive study on the identity, quality, mutational load, and epigenetic remodeling occurring in the cells is shown. As it stands, and whereas this reviewer does not doubt the data presented, the process can be summarized as a "magical" approach and none of the conclusions related to "next-generation" or applications in regenerative medicine is supported experimentally.
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> Referee #2 (Remarks to the Author):
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> In this study, Obokata and colleagues extend their description of stimulus triggered acquired pluripotent (STAP) stem cells introduced in the accompanying paper. They characterise the developmental potency of STAP cells and generate proliferative derivatives that resemble trophoblast stem (TS) cells and embryonic stem (ES) cells. From these observations they conclude that STAP cells represent a new type of pluripotent cell with both extraembryonic and embryonic lineage potential. These findings significantly extend the observations in the accompanying paper, though they might be judged insufficient to justify a stand-alone publication because they do not decisively illuminate the identity of STAP cells.
> The experiments are generally well performed and the authors demonstrate the major findings convincingly. However, in terms of broader understanding the study does not decisively distinguish between two possibilities: (i) that single STAP cells can generate both trophectoderm and embryonic germ layers; (ii) that STAP cells are heterogeneous comprising separate trophectoderm and germ layer progenitors. In fact the authors do not discuss the second possibility despite evidence that STAP cells may be heterogeneous, for example variable levels of Oct4 and Nanog evident in the accompanying paper. It would be particularly informative to double stain STAP cells for Oct4 and Cdx2, and perhaps other markers.
> Quantitative data should be provided on the frequencies and reproducibility of conversion into TS-like and ES cells. The authors should clarify whether the different ES cell derivatives used to produce chimaeras were all generated in one experiment or came from different STAP cells. If all originated from the same STAP cells, the experiment should be repeated.
> The authors suggest that STAP cell-derived TS-like cells are distinct from embryo-derived TS cells, but the data for this are not decisive and could be attributed to persistent contamination with STAP cells or ES cells (which could explain the occasional Nanog positive cells). Presence of ES cells could be excluded by culture in the presence of JAK inhibitor, and monitored by Oct4 and Nanog immunostaining. A global transcriptome analysis could then be performed for comparison with TS cells. Data should also be provided on Fgf4 withdrawal from the TS-like cells - does this induce the expected differentiation into trophoblast giant cells?
> It is surprising that among the conditions tested for ES cell generation the authors do not refer to 2i plus LIF which appears to be the combination favoured

by others for primary ES cell derivation and for reprogramming.
> The authors describe the STAP cells as having "metastable" pluripotency but do not explain why or what they mean. More clarity on an embryonic counterpart of these cells would really strengthen the paper. Given the availability of several recent datasets, transcriptome profiling should be highly informative, provided measures are taken to allow for potential heterogeneity among STAP cells.
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> Referee #3 (Remarks to the Author):
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> This study extends the remarkable findings on cell reprogramming reported in the accompanying manuscript. In this second report, the authors report that STAP cells can contribute to extraembryonic tissues, unlike embryonic stem (ES) cells, that STAP cells can be converted to cells with the potential to give rise to trophoblast, and that they can also give rise to cell lines with the properties of ES cells.
>
> Clonal derivation of the trophoblast like cell lines would indicate whether there are cells with both trophoblast and pluripotent developmental potential or whether a single cell possesses both capacities.
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> It is important to report the properties of clonally derived STAP ES like stem cells, otherwise, it is not clear whether one cell population gives rise to all the lineages in teratomas or in chimeras.
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> The use of ACTH to establish STAP stem cell lines appears to be critical. It would be important to establish why this is the case and why LIF or feeders or 3i failed to achieve this effect.
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> More extensive transcriptome and epigenomics data would shed light on the nature of the STAP-derived trophoblast like and ES like cell lines.
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