EMBO reports

Temporal Activation of LRH-1 and RAR-γ in Human Pluripotent Stem Cells Induces a Functional Naïve-Like State

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1st Editorial Decision 10th Jan 2019

Dear Prof. Baharvand,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports that are copied below.

As you will see, all three referees have major concerns regarding the publication of your manuscript in EMBO reports. They point out that the experimental evidence does not support in many cases the conclusions, and also note several technical shortcomings. As the reports are below, I will not further detail them here.

Given these comments, the amount of work required to address them, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from the referees upon initial assessment, we cannot offer to publish your manuscript.

I am sorry to have to disappoint you this time. I nevertheless hope, that the referee comments will be helpful in your continued work in this area, and I thank you once more for your interest in our journal.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

The manuscript from Taei and colleagues described a new method for the generation of human naive PSCs from different sources, based on the use of two agonists of nuclear receptors. The findings are potentially interesting. but despite the thorough characterisation of the cells generated, some key points need to be clarified.

1) Both in figure 4, where naive ES cells are derived from human embryos, or in figure 7, where naive cells are obtained from primed PSCs, the authors said that cells were expanded in 2iL medium. This seems to be also what is described in the Supplementary experimental material.
If this is indeed the case, such findings are extremely surprising, given that in all papers where naive conditions have been defined (Gafni et al. 2013; Takashima et al. 2014; Theunissen at al. 2014) 2iL was consistently found NOT sufficient for the maintenance of naive PSCs.
So the author should be very clear about what conditions were used for induction of naive pluripotency and for maintenance of naive pluripotency, by indicating it on figure panels and figure legends.
Moreover, if the authors really expanded their naive cells in just 2iL they should comment on why the cells they obtained do not need additional inhibitors, as reported by all other groups in the field.

2) In the case of naive iPS cells directly generated from fibroblasts, or from conversion of primed iPS cells, the authors should make sure that the cells obtained are indeed transgene-free. Episomal vectors could integrate at low frequency, as reported by the Yamanaka group.. So a PCR or Southern blot on genomic DNA from naïve iPS cells should be performed.

3) How robustly are expressed naive markers in the cells obtained by the authors?
KLF4 and TFCP2L1 are two functional regulators of human naive pluripotency (Takashima et al).
Are they detectable by immunostaining or Western Blot?
Alternatively, what is their ABSOLUTE expression measured by RNAseq?
is it comparable to the one reported for cells generated by the Smith and Jaenisch groups?
Of note, some protocols are known to generate naive PSCs with a mild activation of naive markers, as reviewed by Hanna and colleagues in Weinberger et al. Nat Rev Mol Cel Biol 2016.
Do naive cells described in this study also display mild activation?

4) The results about human-mouse chimeras are extremely interesting, yet the authors failed to show convincingly that naive cells functionally participated in embryo formation. In other worlds, they might have just survived inside the host embryo without differentiating and contributing to formation of tissues.
The authors should perform immunostaining for markers of pluripotency, to show that naive PSCs down regulated them and also markers of early differentiation to show differentiation and integration into the host embryo.

Referee #2:

In this work the authors claim that transient treatment of human PSCs with 2 agonists of 2 nuclear receptors together with 2i yields human naive PSCs. then the authors claim that similar effect can be achieved by treatment of TGFB instead together with 2i. Unfortunately, by carefully observing the characteristics of cell described, one can easily conclude that the cells are not in a naive pluripotent state and i even question that they are pluripotent at all.

1) By looking at RT-PC r (Fig. 2) and gene array data one can easily see that many crucial naive markers like KLF17, ARGFX and KHDC2L! are not induced at all. Remaining naive markers are barely up-regulated (2 fold increase from their levels in primed cells which are close to zero expression is not impressive at all). These markers are expected to be up-regulated by thousands of folds.

2) X chromosome analysis provided is insufficient. FISH analysis on multiple line should be provided. The authors show that XIST is down-regulated upon transition from primed to naive cells, however we now know that this is actually an indicator for priming. Naive human cells, unlike their mouse counterparts, up-regulate XIST in naive conditions. (Theunissen et al. Cell Stem Cell 2016)

3) Knock-in reporters for Oct4 distal enhancer activation are the only reliable reporter for naive pluripotency and they should be used in this line of research (Theunissen et al. Cell Stem Cell 2014)

4)Cross-species chimera assay is not a specific marker of naive pluripotency either, as also primed human and monkey cells can make chimeric contribution (https://www.ncbi.nlm.nih.gov/pubmed/30485820)

5) Single cell cloning efficiency is not a marker of naive vs. primed pluripotency either, particularly given that the authors also supplement their naive conditions with Y27632 ROCK inhibitor.

6) 2iL conditions with and without TGFb growth conditions were previously described in the highly controversial study by Ware et al. PNAS 2010, that many find their cells not to be in a naive state as

i indicate above (Takashima et al. Cell 2014).

The conditions described above are overall another variation of primed conditions, were described before by Ware et al. , and also do not represent naive human cells.

Referee #3:

Within this manuscript, Taei et al, show that in combination with 2i/LIF the chemical agonists (2a) of LRH1 and RARγ induce a naïve-like state in human embryonic stem cells and induced pluripotent stem cells. Furthermore, they show that the effects of 2a are achieved via the transient activation of TGF-β. The generated naïve-like cells show morphological characteristics and some transcriptional characteristics of the naïve phenotype. An advantage of their protocol is the use of episomal vectors and chemical agents negating the need for gene transfer and viruses. However, in contrast to what they claim; neither "all criteria" of naïve pluripotency nor the "functionality naïve state" is clearly demonstrated. This problem arises as most of the molecular and functional criteria used here to judge naïve pluripotency are those used for primed pluripotent stem cells.

Major comments:
The major improvements which should be made mainly relate to a more definitive characterisation of naïve-specific features of the naïve-like cells created here, as outlined below:
1. Naivety is claimed as early as the first figure, however there is little within this figure to demonstrate this beyond morphology. The dome shaped colonies observed after 2a conversion clearly represent a naïve-like morphology, however the markers stained for are not naïve specific (e.g. Figure1C OCT4/NANOG/TRA-1-81/SOX2), the authors should therefore endeavour to stain with markers more characteristic of the naïve state (e.g. KLF17/CD75). It is noted that the resultant cells are negative for SSEA1, but this is not sufficient to imply naivety.

2. qRT-PCR analysis (Figure 2) of markers does show the expression of several naïve-associated markers, however there is no comment on the lack of expression of expected markers, such as KLF17 and DPPA5. Furthermore, the relative increase in expression is generally small and not demonstrated at the protein level. The only convincing protein marker shown here nuclear TFE3. As mentioned above the authors should endeavour to stain with more markers of a definitive naïve phenotype.
3. We would expect the Oct4 DE-Luciferase activity to be higher within naïve cells than what is shown in Figure2E. The relatively low increase is not commented on. We would suggest repeating this experiment with the Oct4 DE-GFP reporter line from Theunissen et al. (Jaenisch lab).

4. It is stated in the text that "X-chromosome inactivation is a hallmark of naïve PSCs", this is a mistake, as the X-chromosome should reactive in naïve cells. The experiments performed to test for X-reactivation do not sufficiently demonstrate this. The loss of XIST and H3K27me3 is more representative of erosion of X chromosome inactivation. To definitively demonstrate X-chromosome reactivation the authors should perform RNA-FISH for XIST to demonstrate that a cloud of XIST is still present on one or two of the active X-chromosomes (demonstrated in Sahakyan et al, Cell Stem Cell 2017) and RNA-FISH for X-linked genes to demonstrate bi-allelic expression of the two X chromosomes.

5. Figure 3E, to definitively show integration of cells into the ICM they should be stained for markers of the ICM, such as NANOG.

6. The presence of 2a2iL-induced nRH6-eGFP cells in chimeras is not sufficient to demonstrate contribution. Expression of molecular markers should be assessed to demonstrate that these cells are expressing the markers of the respected lineages in which they reside.

7. Single pictures of a karyotype are not sufficient to demonstrate genomic stability, more karyotypes should be analysed at various passages and the quantification clearly displayed.

Minor comments:
1. Page 7: Tone down the use of language "all characteristic of naivety" this is neither definitively shown nor definitively known
2. Page 11: Tone down language "these data clearly indicated that treatment by 2a2iL induced the naïve state"
3. The authors interchange "naïve" and "naïve-like", we suggest using "naïve-like" throughout to describe their cells
4. Figure S1 show/describe what happens between Day 20 and P18
5. Figure S1B - show 2a control (i.e. without 2iL)
6. Figure S1F: H&E staining is not sufficient to confirm lineages
7. Figure 4H: The increase of efficiency of 70-82% shown for the generation of mESCs does not seem noteworthy or significant for inclusion, furthermore there is no functional test or characterization of these cells.
8. Figure 5E: The numbers on individual points are too small to see, hence we cannot review this figure.
9. Figure 5D and 5E: should display the percentage of explained variance for each PC on the axis legend
10. The first two result sections use the term naïve, however show no definitive characteristics of naivety (Figures 1 and S1) apart from perhaps the dome shaped morphology (which is a low stringency criteria). Consider demonstrating the features of naivety demonstrated in Figure 2 earlier.
11. Key references missing/overlooked: Di Stefano et al. Reduced MEK inhibition preserves genomic stability in naive human embryonic stem cells. Nat Methods 2018; Liu et al. Comprehensive characterization of distinct states of human naive pluripotency generated by reprogramming. Nat Methods 2017.

We hope that these comments help the authors to improve the manuscript.

EMBO reports

Editor-in-chief

Dear Dr Esther Schnapp,

I submitted a manuscript entitled "**Temporal Activation of LRH-1 and RAR-γ in Human Pluripotent Stem Cells Induces a Functional Naïve-Like State**" before for publication in *EMBO Reports*. It was rejected but we tried to reply to the questions of referees and revised the manuscript.

Therefore, I highly appreciate if you please reconsider our manuscript as Original Article for publication in EMBO Reports.

To reply the comments of referees, we reviewed the published papers (nine studies) on human naïve derivation and analyzed. This led to a recent comprehensive review entitled "**Signal regulators of human naïve pluripotency**" (Taei et al., Exp Cell Res. 2020 Feb 26, PMID: 32112799).

In this analysis, we found that despite specific requirements of distinct signaling events, all human naïve-like cells share certain cellular phenotypes such as high growth rate, dome-shape morphology, responsiveness to 2iL medium, and to some extent resistance to single cell dissociation. However, human naïve cells exhibit a variety of naïve pluripotency in expression of specific genes and differentiation potency which can be depended on the protocol used. Therefore, we compared the expression profile of naïve, primed, and lineage specific-associated marker genes. We identified two separated groups using unsupervised hierarchical clustering of genes related to naïve, primed, and differentiation lineages within generated naïve-like pluripotent cells using different protocols. According to this clustering, naïve-like cells derived from studies including Chan et al 2013, Duggal et al 2015, Chen et al 2015, Qin et al 2016, Zimmerlin et al 2017, Gafni et al 2013, and Ware et al 2014 are grouped together and 5i/LAF- and t2iLGö -derived cells associated with two other studies of Thuenissen et al 2014 and Takashima et al 2014 are clustered in one group. Since numerous naïve gene markers are upregulated and many primed markers are downregulated in 5i/LAF- and t2iLGö -derived cells, we therefore labeled this cluster "bona fide naïve pluripotent cells" and other group as "intermediate naïve cells". The latter seems to exhibit an incomplete conversion to full naivety due to the lack of significant expression in conserved and human specific naïve- associated marker genes. 2a2iL cells that we derived are similar to "intermediate naïve cells" in gene expression pattern, thus we named them naïve-like cells. Interestingly, these cells have shown extreme participation in three embryonic germ layers in interspecies mouse chimera. Of note, most protocols for derivation of human naïve cells are not able to generate authentic naïve cell while all of them called their produced cells "naïve cells" not naïve-like cells.

Additionally, at the moment, the gold standard for testing pluripotency of mouse ESCs (mESCs) is the creation of allogeneic chimeras, which is not possible in humans, requiring the need to develop hPSCs in a xenogeneic environment for generation of interspecies chimeras (Mascetti et al, 2016). Thus far, most studies relied on mice as hosts for evaluating the competency of hPSCs to form chimeras. However, only a few of these experiments have been successful (Theunissen et al, 2016; Wu et al, 2017). Numerous efforts were made for improving the efficiency of hPSCs to form interspecies chimeras with mice or other mammals (Wu et al, 2016). However, the lack of an appropriate animal test system has made it difficult to assess potential differences for chimera formation. We submitted a manuscript that indicates the developing chicken embryo is a permissive host for hPSCs, allowing analysis of

the pluripotency potential of hPSCs. Transplantation of our naïve (current study) at blastodisc resulted in robust chimerism as indicated by fluorescence imaging and PCR analysis.

Please find a point-by-point answers to the questions and comments of the respected reviewers in the following lines.

We respectfully await your kind response.

Sincerely yours,

Hossein Baharvand, Ph.D.

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Referee #1:

Comment #1: Both in figure 4, where naive ES cells are derived from human embryos, or in figure 7, where naive cells are obtained from primed PSCs, the authors said that cells were expanded in 2iL medium.
This seems to be also what is described in the Supplementary experimental material.
If this is indeed the case, such findings are extremely surprising, given that in all papers where naive conditions have been defined (Gafni et al. 2013; Takashima et al. 2014; Theunissen at al. 2014) 2iL was consistently found NOT sufficient for the maintenance of naive PSCs.
So the author should be very clear about what conditions were used for induction of naive pluripotency and for maintenance of naive pluripotency, by indicating it on figure panels and figure legends.
Moreover, if the authors really expanded their naive cells in just 2iL they should comment on why the cells they obtained do not need additional inhibitors, as reported by all other groups in the field.

Response: Our derived naïve-like cells have been produced using 2a2iL medium in a short time (5 days for conversion of primed into naïve hPSCs and 7-10 days for derivation of naïve hESCs) and have been maintained in 2iL culture medium as has been clearly shown in schematic overview of protocols in Figure 1B and Figure 4A. Our experiments showed that this 5-day period of 2a2iL induction can produce a genetic pattern (TGF-beta signaling pathway upregulation) which can further support maintaining these cells in 2iL media.

Comment #2: In the case of naive iPS cells directly generated from fibroblasts, or from conversion of primed iPS cells, the authors should make sure that the cells obtained are indeed transgene-free. Episomal vectors could integrate at low frequency, as reported by the Yamanaka group. So, a PCR or Southern blot on genomic DNA from naïve iPS cells should be performed.

Response: According to the comment about integration of episomal vectors, we have performed RT-PCR for three episomal vectors, PCXLE-hul, PCXLE-hsk and PCXLE-OCT4, and the results showed that the first two vectors had no expression whereas the third one is still expressing but to a lesser extent comparing to the positive control sample (Figure S1-A).

Comment #3: How robustly are expressed naive markers in the cells obtained by the authors?
KLF4 and TFCP2L1 are two functional regulators of human naive pluripotency (Takashima et al). Are they detectable by immunostaining or Western Blot?
Alternatively, what is their ABSOLUTE expression measured by RNAseq?
is it comparable to the one reported for cells generated by the Smith and Jaenisch groups?
Of note, some protocols are known to generate naive PSCs with a mild activation of naive markers, as reviewed by Hanna and colleagues in Weinberger et al. Nat Rev Mol Cel Biol 2016.
Do naive cells described in this study also display mild activation?

Response: We have analyzed some of the naïve markers by immunostaining and western blot, as suggested by the reviewer. The results showed that, induced naïve cells by our protocol, can express TFCP2L1, KLF4, KLF17, REX1 in addition to TFE3 and STAT3 which have been analyzed in the previous version of this manuscript, by immunostaining. Moreover, expression of NANOG, TFCP2L1 and KLF17 have been confirmed by Western blot in these cells. These data have been shown in Fig.2 E & F. According to our results, and our recent

review entitled "**Signal regulators of human naïve pluripotency**" (Taei et al., Exp Cell Res. 2020 Feb 26, PMID: 32112799), we called our cells, naïve-like cells.

Comment #4: The results about human-mouse chimeras are extremely interesting, yet the authors failed to show convincingly that naive cells functionally participated in embryo formation. In other words, they might have just survived inside the host embryo without differentiating and contributing to formation of tissues.
The authors should perform immunostaining for markers of pluripotency, to show that naive PSCs down regulated them and also markers of early differentiation to show differentiation and integration into the host embryo.

Response: We have examined functionality of the naïve-like cells in mouse chimeras, as the referee had suggested. To this, we performed immunostaining for TUJ1, BRACHYURY (BRA) and SOX17 in mouse chimera sections. Results showed that naïve-like cells had contributed in differentiation of three embryonic lineages (Endoderm, Ectoderm and Mesoderm). We have also showed that these sections have not expressed NANOG pluripotency marker indicating that 2a2iL naïve-like cells have lost pluripotency features and differentiated to progenitors of three embryonic germ layers. The results have been shown in Figure 3-H and 3-I.

Referee #2:

Comment #1: By looking at RT-PCR (Fig. 2) and gene array data one can easily see that many crucial naive markers like KLF17, ARGFX and KHDC2L! are not induced at all. Remaining naive markers are barely up-regulated (2-fold increase from their levels in primed cells which are close to zero expression is not impressive at all). These markers are expected to be up-regulated by thousands of folds.

Response: We analyzed the expression of naïve markers ARGFX and KHDC1L in 2a2iL- and TGFβ2iL-hPSCs. The results showed the expression of *KHDC1L* in 2a2iL-hPSCs and *ARGFX* and *KHDC1* in TGFβ2iL-hPSCs. Although there is no significant differential expression in *KLF17* between primed and naïve hPSCs, we could clearly detect this naïve marker in protein level using immunostaining and western blot analysis. We also named 2a2iL-hPSCs, naïve-like cells due to the lack of significant expression in naïve specific markers.

Comment #2: X chromosome analysis provided is insufficient. FISH analysis on multiple line should be provided. The authors show that XIST is down-regulated upon transition from primed to naive cells, however we now know that this is actually an indicator for priming. Naive human cells, unlike their mouse counterparts, up-regulate XIST in naive conditions. (Theunissen et al. Cell Stem Cell 2016)

Response: I am sorry, there is no the possibility of RNA-FISH analysis in our laboratory. We have many restrictions to perform experiments due to USA sanctions on my country.

Comment #3: Knock-in reporters for Oct4 distal enhancer activation are the only reliable reporter for naive pluripotency and they should be used in this line of research (Theunissen et al. Cell Stem Cell 2014).

Response: Thank you for the comment, but there is no feasibility to performed this experiment in our lab due to USA sanctions on my country.

Comment #4: Cross-species chimera assay is not a specific marker of naive pluripotency either, as also primed human and monkey cells can make chimeric contribution (https://www.ncbi.nlm.nih.gov/pubmed/30485820).

Response: According to stage matching theory, chimera formation can be considered as a marker for identification of different state of pluripotency. In other words, primed pluripotent cells can contribute to host embryo when are injected into gastrula stage and naïve cells can generate chimera when the cells are injected into blastocyst embryo. However, there are challenges in generation of human interspecies chimera. Recently, using anti-apoptosis agents or selection of suitable host embryo (our submitting manuscript) could overcome to this barrier.

at the moment, the gold standard for testing pluripotency of mouse ESCs (mESCs) is the creation of allogeneic chimeras, which is not possible in humans, requiring the need to develop hPSCs in a xenogeneic environment for generation of interspecies chimeras (Mascetti et al, 2016). Thus far, most studies relied on mice as hosts for evaluating the competency of hPSCs to form chimeras. However, only a few of these experiments have been successful (Theunissen et al, 2016; Wu et al, 2017). Numerous efforts were made for improving the efficiency of hPSCs to form interspecies chimeras with mice or other mammals (Wu et al, 2016). However, the lack of an appropriate animal test system has made it difficult to assess potential differences for chimera formation. We submitted a manuscript that indicates the developing chicken embryo is a permissive host for hPSCs, allowing analysis of the pluripotency potential of hPSCs. Transplantation of our naïve (current study) at blastodisc resulted in robust chimerism as indicated by fluorescence imaging and PCR analysis.

In the following please find some of the related data that prepared for you.

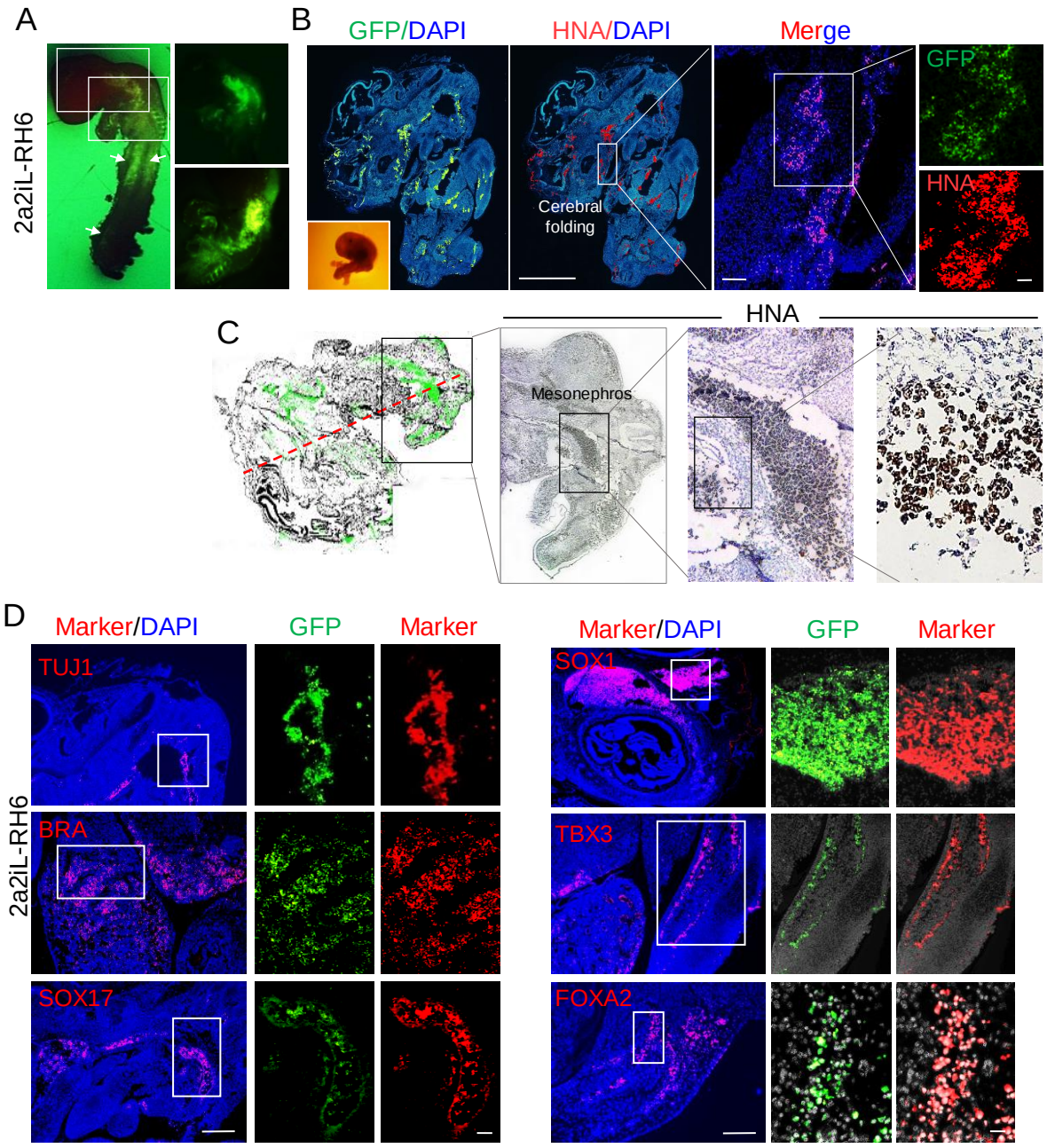

Figure legend. Efficient interspecies chimera formation with our naïve-like hPSCs injected into the blastodisc stage chicken embryos.

(A) Live image of D6 chicken chimeras derived from injections of 2a2iL-RH6 cells into BLD stage embryos. Higher magnifications of the white boxes are shown in the right panels.

(B) Representative images of a whole-mount collage from sagittal sections of GFP expressing 2a2iL-RH6-derived chick chimera (left panel). The bright field image in the lower left corner shows the morphology of one of the 2a2iL-RH6 derived chimeras. All sagittal sections that form the collage were stained with HNA (middle panel; red). Sequential higher magnifications of a piece of the collage are shown in a box (right panels). The insets (right lower panel) are the zoom-in pictures of the areas of the white boxes. Nuclei are stained with DAPI (blue).

Scale bars: 500 μm (left and middle panel), 200 μm (right panel: larger image), 100 μm (right panel: smaller images).

(C) Immunofluorescence staining for HNA in 2a2iL-RH6-derived chimera embryos. The insets are zoomed-in images of the white boxes.

(D) Contribution of 2a2iL-RH6 to different tissues of chick chimeras. Immunofluorescence staining with human antibodies against TUJ1, BRA, SOX17, SOX1, TBX3, and FOXA2 lineage markers (red) of day 6 (D6) chimeras formed by 2a2iL-RH6. Nuclei were stained with DAPI (blue) and GFP. The insets represent zoomed-in images of areas in the white box. Scale bars: 500 μm (merge images), 100 μm (GFP and markers).

Comment #5: Single cell cloning efficiency is not a marker of naive vs. primed pluripotency either, particularly given that the authors also supplement their naive conditions with Y27632 ROCK inhibitor.

Response: Primed cells are dependent on ROCK inhibitor after single cell dissociation and we expect naïve cells to be independent of ROCK inhibitor. The majority of protocols related to naïve cells production use ROCK inhibitor in their chemical cocktail but our cells, are passagable without this small molecule and we have shown high clonogenicity with and without the mentioned chemical.

Comment #6: 2iL conditions with and without TGFb growth conditions were previously described in the highly controversial study by Ware et al. PNAS 2010, that many find their cells not to be in a naive state as I indicate above (Takashima et al. Cell 2014).

Response: One of the most significant issues in pluripotency is the role of TGF-β signaling pathway. It is accepted that the maintenance of primed cells is depended on TGF-beta. However recently, some of the experiments have shown that this signaling pathway plays a role in human naïve cells. Usage of TGF-β or Activin A in maintaining human naïve cells has been reported by Gafni et al 2013, and Qin et al 2016 (intermediate naïve state) and Theunissen et al 2014 (bona fide naïve state). Besides, it has been shown that some of the ligands (like Nodal, LEFTY1/2) belonging to TGF-β family are upregulated in human naïve cells. However, the exact role of TGF-β signaling pathway in human naïve cells still remains arguable. In our study, KEGG analysis showed that TGF-beta signaling pathway has been upregulated in 2a2iL cells. Indeed, 2a lead to activation of TGF-β signaling pathway in our naïve-like cells via increase in expression of NODAL and LEFTY1/2. Our experiments showed that TGFβ2iL can induced naïve-like cells even better than 2a2iL cells regarding the expression of naïve specific genes. Thus, we can conclude that TGF-β has a positive effect on naivety.

Referee #3:

Major comments:

The major improvements which should be made mainly relate to a more definitive characterisation of naïve-specific features of the naïve-like cells created here, as outlined below:

Comment #1: Naivety is claimed as early as the first figure, however there is little within this figure to demonstrate this beyond morphology. The dome shaped colonies observed after 2a conversion clearly represent a naïve-like morphology, however the markers stained for are

not naïve specific (e.g. Figure1C OCT4/NANOG/TRA-1-81/SOX2), the authors should therefore endeavour to stain with markers more characteristic of the naïve state (e.g. KLF17/CD75). It is noted that the resultant cells are negative for SSEA1, but this is not sufficient to imply naivety.

Response: In the first figure, we intended to show 2a2iL combination could change the morphology and have no effect on pluripotency. So, common pluripotency markers such as OCT4, NANOG, SOX2, TRA-1-81, and SSEA1 were analyzed. Next, we assayed naïve specific markers in the second figure.

Comment #2: qRT-PCR analysis (Figure 2) of markers does show the expression of several naïve-associated markers, however there is no comment on the lack of expression of expected markers, such as KLF17 and DPPA5. Furthermore, the relative increase in expression is generally small and not demonstrated at the protein level. The only convincing protein marker shown here nuclear TFE3. As mentioned above the authors should endeavour to stain with more markers of a definitive naïve phenotype.

Response: We have analyzed some of the naïve markers by immunostaining and western blot, as suggested by the referee. The results showed that, induced naïve cells by our protocol, can express TFCP2L1, KLF4, KLF17, REX1 in addition to TFE3 and STAT3 which have been analyzed in the previous version of this manuscript, by immunostaining. Moreover, expression of NANOG, TFCP2L1 and KLF17 have been confirmed by Western blot in these cells. These data have been shown in Fig.2 E & F. According to our results, we called our cells, naïve-like cells.

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Response: There is no feasibility to do this experiment in our lab due to USA sanctions on my country.

Comment #4: It is stated in the text that "X-chromosome inactivation is a hallmark of naïve PSCs", this is a mistake, as the X-chromosome should reactive in naïve cells. The experiments performed to test for X-reactivation do not sufficiently demonstrate this. The loss of XIST and H3K27me3 is more representative of erosion of X chromosome inactivation. To definitively demonstrate X-chromosome reactivation the authors should perform RNA-FISH for XIST to demonstrate that a cloud of XIST is still present on one or two of the active X-chromosomes (demonstrated in Sahakyan et al, Cell Stem Cell 2017) and RNA-FISH for X-linked genes to demonstrate bi-allelic expression of the two X chromosomes.

Response: Thank you for the comment. Reactivation of X-chromosome is a naïve marker as we mentioned in introduction. This is just an error in writing, although we have changed the text of our manuscript in this new version. Moreover, we tried to prepare RNA-FISH of X-linked genes for demonstrating of X-chromosome reactivation. Unfortunately, we could not success to access them due to USA sanctions on my country.

Comment #5: Figure 3E, to definitively show integration of cells into the ICM they should be stained for markers of the ICM, such as NANOG.

Response: Due to our imaging system we could not take a good picture to show GFP and NANOG together. Then We add Fig 5H and I to show the integration of transplanted cells and their fate in vivo at day 10.5. If the respected referee does not satisfy with this, we can remove the claim of ICM integration and the related fig in the manuscript.

Comment #6: The presence of 2a2iL-induced nRH6-eGFP cells in chimeras is not sufficient to demonstrate contribution. Expression of molecular markers should be assessed to demonstrate that these cells are expressing the markers of the respected lineages in which they reside.

Response: We examined the molecular markers of the naïve-like cells that differentiated into three embryonic lineages in mouse chimeras at E10.5. To this, we performed immunostaining for TUJ1, BRACHYURY (BRA) and SOX17 in mouse chimera sections. Results showed that naïve-like cells had contributed in differentiation of three embryonic lineages (Endoderm, Ectoderm and Mesoderm). We have also showed that these sections have not expressed NANOG pluripotency marker indicating that 2a2iL naïve-like cells have lost pluripotency features and differentiated to progenitors of three embryonic germ layers. The results have been shown in Figure 3-H and 3-I.

Comment #7: Single pictures of a karyotype are not sufficient to demonstrate genomic stability, more karyotypes should be analysed at various passages and the quantification clearly displayed.

Response: Indeed, we did not intend to demonstrate genomic stability in this manuscript. We wanted to show karyotype of these cells under mentioned condition is normal. Therefore, we removed the word of “stability” in the text.

Minor comments:
1. Page 7: Tone down the use of language "all characteristic of naivety" this is neither definitively shown nor definitively known

Response: We removed this phrase.

2. Page 11: Tone down language "these data clearly indicated that treatment by 2a2iL induced the naïve state"
Response: We named 2a2iL-hPSCs naïve-like cells due to the lack of significant expression in some naïve markers.

3. The authors interchange "naïve" and "naïve-like", we suggest using "naïve-like" throughout to describe their cells

Response: We have revised naïve into naïve-like as you suggested.

4. Figure S1 show/describe what happens between Day 20 and P18

Response: Figure S1 show what happens between day 0 until day 20 of

5. Figure S1B - show 2a control (i.e. without 2iL)

Response: Figure 1B does not show control group. It shows naïve-like cells produced during reprogramming process using episomal vectors and 2a2iL.

6. Figure S1F: H&E staining is not sufficient to confirm lineages

Response: H&E staining is a routine analysis for studying different lineages in teratomas.

7. Figure 4H: The increase of efficiency of 70-82% shown for the generation of mESCs does not seem noteworthy or significant for inclusion, furthermore there is no functional test or characterization of these cells.

Response: We removed this result from this new version of our manuscript.

8. Figure 5E: The numbers on individual points are too small to see, hence we cannot review this figure.

Response: It was redesign to be clearer.

9. Figure 5D and 5E: should display the percentage of explained variance for each PC on the axis legend

Response: They were added. Now X and Y labels show the percentage of variance explained by each principal component.

10. The first two result sections use the term naïve, however show no definitive characteristics of naivety (Figures 1 and S1) apart from perhaps the dome shaped morphology (which is a low stringency criteria). Consider demonstrating the features of naivety demonstrated in Figure 2 earlier.

Response: As mentioned earlier, we have used naïve-like in this version of manuscript. In addition, we first assayed common pluripotency markers such as morphology, karyotype and the expression of OCT4, NANOG, SOX2, TRA1-81, SSEA1, ALP. Next, we analyzed naïve specific markers in Figure 2.

11. Key references missing/overlooked: Di Stefano et al. Reduced MEK inhibition preserves genomic stability in naive human embryonic stem cells. Nat Methods 2018; Liu et al. Comprehensive characterization of distinct states of human naive pluripotency generated by reprogramming. Nat Methods 2017.

Response: These two references were added in the text as the referee suggested.

Dear Hossein,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports on it.

As you will see, while referee 2 is more critical, both referees 1 and 3 support the publication of your findings here, if the last concerns can be successfully addressed. I would therefore like to invite you to do so and submit a final manuscript as soon as possible.

A few other changes are also required:

Please add a direct link to your deposited data at the NCBI Gene Expression Omnibus. This information needs to be moved to a "Data Availability Section" at the end of the materials and methods.

Please remove "data not shown" on pages 7, 8 and 9, or add data. You may use the term "personal communication".

Please send us a completed author checklist that can be found here: <https://www.embopress.org/page/journal/14693178/authorguide>. The completed checklist will be part of our Review Process File (RPF).

Please upload all figures as separate files and move the figure legends to the end of the main manuscript file.

Please add a callout for figure 4D.

For the supplementary information, you can show all figures and tables together in an Appendix file that also needs a table of content with page numbers and that can have supplementary methods. Alternatively, you can upload 5 individual expanded view (EV) figures that will be integrated into the manuscript text online. If the tables are not part of the Appendix, they can be uploaded as EV tables or as Datasets; in this case the legends need to be part of the table files. For example, supplementary table 2 should be uploaded as Dataset EV1 with a legend and/or title in the first tab of the excel sheet. Please see our guide to authors for more information.

The figure quality should be improved for the final manuscript submission.

The reference style needs to be changed to the numbered EMBO reports style, if the manuscript will be submitted before the end of June. From the 1st of July, the EMBO reports reference style will change to the Harvard style. If the manuscript will be accepted after the 1st of July, the reference style will need to be changed to Harvard style.

I would like to suggest a few changes to the abstract that needs to be written in present tense. Do you agree with the following:

Naïve pluripotency can be established in human pluripotent stem cells (hPSCs) by manipulation of transcription factors, signaling pathways or a combination thereof. However, differences exist in the molecular and functional properties of naïve hPSCs generated by different protocols, which include

varying similarities with pre-implantation human embryos, differentiation potential, and maintenance of genomic integrity. We show here that short treatment with two chemical agonists (2a) of nuclear receptors, liver receptor homologue-1 (LRH-1) and retinoic acid receptor gamma (RAR-γ), along with 2i/LIF (2a2iL) induces naïve-like pluripotency in human cells during reprograming of fibroblasts, conversion of pre-established hPSCs, and generation of new cell lines from blastocysts. 2a2iL-hPSCs match several defined criteria of naïve-like pluripotency and contribute to human-mouse interspecies chimeras. Activation of TGF-β signaling is instrumental for acquisition of naïve-like pluripotency by the 2a2iL induction procedure, and transient activation of TGF-β signaling substitutes for 2a to generate naïve-like hPSCs. We reason that 2a2iL-hPSCs are an easily attainable system to evaluate properties of naïve-like hPSCs and for various applications.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript file.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550 pixels x 200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I am looking forward to receiving the final files. Please let me know if you have any questions or comments.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors addressed the majority of points raised by Referees and now they define their cells as naive-like. Overall the manuscript improved significantly therefore I support its publication.

Referee #2:

The authors provide a revised manuscript in response to comments by 3 different reviewers. Unfortunately, the revision is far from being complete and the conclusions of the manuscript are not solid.

For example, the authors did not use Oct4-GFP reporters and did not add FISH experiments on X chromosome status. Data is not solid as exemplified by lack of change in KLF17 makers by RNA but only by protein. Many of the conclusions are based only after short term passaging the cells in claimed conditions, which is unsatisfactory to reach conclusions about any pluripotent state stability.

Very few of the concerns were addressed, while most of the major points raised by all reviewers remain unaddressed.

Referee #3:

Within this manuscript, Taei et al, show that in combination with 2i/Lif, the chemical agonist (2a) of LRH1 and RARγ induce a naïve-like state in human ESC/iPSCs. Furthermore, they show that TGF-β is integral for the induction of a naïve-like state within 2a2iL conditions and that TGF-β supplementation can replace 2a to achieve a similar conversion. The generated naïve-like cells show morphological characteristics and some transcriptional characteristics of the naïve phenotype. Importantly, it is shown that 2a2iL cells can contribute to mouse-human chimeras and that cells take on, at least some, functional characteristics of several cell types in the developing embryos. An advantage of their protocol is the use of chemical agents to achieve a naïve-like state, negating the need for gene transfer and viruses - however this is not the only approach described which can do this.

Minor comments:

1. As the KLF17 qPCR in Figure 2D is not significantly higher in naïve vs primed I think this should be clearly stated in the text and commented on, as this is not what we would expect in comparison to other naïve studies. Furthermore, the immuno-staining for KLF17 in figure 2E is not convincing - it looks rather non-specific and certainly not nuclear. Particularly as it looks like KLF17 staining has worked well in Figure 4E and 7G. To overcome this - I would suggest repeating this staining and use proper controls to rule out false results - if better pictures cannot be obtained then I would suggest saying that protein expression using immuno-fluorescent was not able to adequately detect KLF17 protein expression.

2. Within the rebuttal letter it is mentioned that "We identified two separated groups using unsupervised hierarchical clustering of genes related to naïve, primed, and differentiation lineages within generated naïve-like pluripotent cells using different protocols. According to this clustering, naïve-like cells derived from studies including Chan et al 2013, Duggal et al 2015, Chen et al 2015, Qin et al 2016, Zimmerlin et al 2017, Gafni et al 2013, and Ware et al 2014 are grouped together and 5i/LAF- and t2iLGö -derived cells associated with two other studies of Thuenissen et al 2014 and Takashima et al 2014 are clustered in one group." However, this does not actually seem to be discussed in the manuscript, nor is the clustering shown. I believe this would be beneficial to include.

3. Regarding this statement: "We found that all sectioned embryos exhibited GFP-positive cells in the head, trunk, and other parts of the body; however, the contribution of GFP-positive cells in some embryos was very low." It would be interesting to know what 'very low' means here (even if semi-quantitatively).

4. The embryo stainings appear very good within Figure 3I, as noted all antibodies used here are human specific, therefore we only see overlapping GFP and protein (RED). One issue I have, however, is that all green cells are also always red. Which makes me sceptical - whole embryo staining can be difficult as there are innate differences in cellular auto-fluorescence. Can appropriate controls be shown in the supplement (e.g. non-injected embryos which are stained with the same human antibodies (showing no green or red fluorescence) and/or the same injected embryos stained with an antibody for the mouse protein - so that you see non-overlapping red (protein) and GFP cells. (There is also a box missing from the TUJ1/DAPI staining in figure 3I).

5. This statement: "2a2iL-hPSCs clustered closely to early human pre-implantation embryos (Yan, Yang et al., 2013) together with other naïve hPSCs in the PCA analysis" should be toned down. Firstly, 'clustered' implies you have performed some level of clustering (which I do not believe has been done here). Secondly, the 2a2iL cells actually lie closer to several primed (dark blue) points than anything else. The text could be a bit more detailed in its description of this figure (5E).

6. I think it would be good to reference/mention recent studies, which have used the mentioned TGF-β signalling inhibitors (SB431542 and A83-01) within protocols to induce TSC cells from naïve PSCs (Dong/Theunissen, eLife, 2020 and Guo/Smith, bioRxiv, 2020). In this regard, it is not very surprising that cells do not remain naïve with the addition of these chemicals and it would be a nice link to the wider literature.

7. Within the discussion you mention many studies which have used different approaches to make naïve cells. One notable study missing is that by Guo/Smith (Development, 2017), in which no transgenes are used and naïve cell are created which faithfully resemble those made in T2iLGo (transgene induced) settings.

8. There are several grammatical mistakes/typos which can easily be fixed by careful proof-reading, some I noticed
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i. page 11: "develop" ->developped

Cross-comments from referee 3:

I agree with referee 2 that the revision is not complete, but also do not think that every little request needs to be done. I disagree with referee 2 that the main conclusion is not solid: there is enough evidence to suggest a naive-like state. Clearly the naive cells are not as naive as other naive media

but still is distinct from the primed state. Related to the use of the Oct4-GFP reporter, the authors have used an alternative strategy to address this point. The FISH experiment was not done but its outcome would not change the conclusion that cells are naive-like (other naive-like media have cells that still show X inactivation: X reactivation is only robustly found in t2iLGo and 5iLAF naive media). I agree with referee 2 that the KLF17 data is not solid: as indicated in our previous review, there are likely issues with the KLF17 IF. Hope this helps.

Cross-comments from referee 1:

I agree with referee 3.
Clearly the authors have not addressed all concerns that were raised, but is some cases they had good reasons not to.
I think it is pretty clear that their cells are not fully naive, like t2iLGö or 4/5iLAF, but this will be also clear to experts in the field.
This is why I think the paper is suitable for publication, after the KLF17 staining issue is sorted out.

Referee #3:

Minor comments:

Comment #1: As the KLF17 qPCR in Figure 2D is not significantly higher in naïve vs primed I think this should be clearly stated in the text and commented on, as this is not what we would expect in comparison to other naïve studies. Furthermore, the immuno-staining for KLF17 in figure 2E is not convincing - it looks rather non-specific and certainly not nuclear. Particularly as it looks like KLF17 staining has worked well in Figure 4E and 7G. To overcome this - I would suggest repeating this staining and use proper controls to rule out false results - if better pictures cannot be obtained then I would suggest saying that protein expression using immuno-fluorescent was not able to adequately detect KLF17 protein expression.

Response #1: Thank you for pointing this out. We checked our data again and found that in the first set of our experiments we had used a culture medium with "Advanced DMEM/F12" basal medium. But during the continuing of the procedure, we used "DMEM/F12" as basal medium. When we compared the results of qRT-PCR from the studies of converting the primed to naïve pluripotency with these two different basal medium, we found that the expression of KLF17 is not significant between naïve and primed cells in "Advanced DMEM/F12". Although the reason for this is not clear to us, we replaced the data of KLF17 with data of cells cultivated in DMEM/F12. It is noteworthy that the results of KLF17 expression in human embryo and TGFβ condition is also the result of cells cultured in DMEM/F12 (Fig.2D and 2E).

Comment #2: Within the rebuttal letter it is mentioned that "We identified two separated groups using unsupervised hierarchical clustering of genes related to naïve, primed, and differentiation lineages within generated naïve-like pluripotent cells using different protocols. According to this clustering, naïve-like cells derived from studies including Chan et al 2013, Duggal et al 2015, Chen et al 2015, Qin et al 2016, Zimmerlin et al 2017, Gafni et al 2013, and Ware et al 2014 are grouped together and 5i/LAF- and t2iLGö -derived cells associated with two other studies of Thuenissen et al 2014 and Takashima et al 2014 are clustered in one group." However, this does not actually seem to be discussed in the manuscript, nor is the clustering shown. I believe this would be beneficial to include.

Response #2: Thank you for your suggestion. We inserted the following text in the first paragraph of discussion. We also added a supplementary figure showing cluster dendrogram (Figure S5).

"The main motivation for development of an efficient protocol to establish human naïve pluripotency is the production of cells that have a high growth rate, are resistant to single cell dissociation, and show the capability to differentiate into three germ layers while maintaining genome integrity. Various approaches have been tried, including forced expression of naïve-related transcription factors (Buecker et al, 2010; Chen et al, 2015; Hanna et al, 2010; Li et al, 2009; Takashima et al, 2014), manipulation of different signaling pathways with small

molecules (Chan et al, 2013; Duggal et al, 2015; Qin et al, 2016; Ware et al, 2014) or targeting of numerous protein kinases such as PKC, p38, JNK, BRAF, SRC, CDK, and ROCK (Gafni et al, 2013; Guo et al, 2016; Szczerbinska et al, 2019; Theunissen et al, 2014; Zimmerlin et al, 2016) to induce the naïve pluripotent state in human cells. These different culture conditions induce different levels of naivety in pluripotent stem cells. Although most of these protocols lead to the generation of similar cellular phenotype, their gene profiling shows a spectrum of naïve pluripotency levels that can be typically clustered into two separate groups; bona fide and intermediate naïve pluripotency (Taei et al, 2020). According to this clustering, naïve-like cells derived from studies including Chan et al 2013, Duggal et al 2015, Chen et al 2015, Qin et al 2016, Zimmerlin et al 2017, Gafni et al 2013, and Ware et al 2014 are in intermediate state, while 5i/LAF- and t2iLGö -derived cells related to studies of Thuenissen et al 2014 and Takashima et al 2014 are in bona fide state of naïve pluripotency (Taei et al, 2020).

Here, we report that synthetic small molecule ligands specific to the nuclear receptors LRH-1 and RAR-γ in combination with 2i and LIF (2a2iL) induce naïve-like pluripotency in human cells during (i) reprogramming of fibroblasts, (ii) conversion of existing primed hPSCs, and (iii) derivation of hESCs from human blastocysts. We found that 2a2iL-hPSCs own the majority of key criteria of naïve pluripotency: (i) dome-shape morphology of the colonies, (ii) single cell passaging and high cloning efficiency without the need for ROCKi treatment, (iii) short doubling time that resembled mouse PSCs, (iv) activation of luciferase reporter constructs under the DE of *OCT4*, (v) nuclear localization of naïve-related markers such as STAT3 and TFE3, and (vi) normal karyotype after long-term passaging. The expression of a panel of naïve-related markers including REX1, KLF17, and TFCP2L1 indicated upregulation in 2a2i-PSCs versus primed cells at the transcript and protein levels. Our

transcriptome analysis also indicated that 2a2iL-hPSCs are molecularly in the intermediate state of naïve pluripotency (Figure S5) (Taei et al, 2020).”

Comment #3: Regarding this statement: "We found that all sectioned embryos exhibited GFP-positive cells in the head, trunk, and other parts of the body; however, the contribution of GFP-positive cells in some embryos was very low." It would be interesting to know what 'very low' means here (even if semi-quantitatively).

Response #3: Thank you for your comment. We obtained this result via fluorescent microscopy imaging. Indeed, we cannot quantify this data in hPSC-mouse embryo chimera. Instead, we have quantified the percentage of contribution of our naïve pluripotent cells in the chick chimera that the result is currently under review as a separate paper. Since there is no feasibility to quantification of this experiment, we removed this sentence from the text of the manuscript.

Comment #4: The embryo staining appears very good within Figure 3I, as noted all antibodies used here are human specific, therefore we only see overlapping GFP and protein (RED). One issue I have, however, is that all green cells are also always red. Which makes me sceptical - whole embryo staining can be difficult as there are innate differences in cellular auto-fluorescence. Can appropriate controls be shown in the supplement (e.g. non-injected embryos which are stained with the same human antibodies (showing no green or red fluorescence) and/or the same injected embryos stained with an antibody for the mouse protein - so that you see non-overlapping red (protein) and GFP cells. (There is also a box missing from the TUJ1/DAPI staining in figure 3I).

Response #4: Thank you for your valuable comment. To exclude auto-fluorescence staining we have presented a section from negative control group with no GFP or RED staining signal. You can find this data in Figure S3C. We also added the following sentence in the text at page 11. The box was also added to the TUJ1/DAPI staining in figure 3I.

“We also checked green and red signals in non-injected mouse (E10.5, negative control) that had been stained with HNA (negative control) to exclude cellular auto-fluorescents (Figure S3C).”

Comment #5: This statement: "2a2iL-hPSCs clustered closely to early human pre-implantation embryos (Yan, Yang et al., 2013) together with other naïve hPSCs in the PCA analysis" should be toned down. Firstly, 'clustered' implies you have performed some level of clustering (which I do not believe has been done here). Secondly, the 2a2iL cells actually lie closer to several primed (dark blue) points than anything else. The text could be a bit more detailed in its description of this figure (5E).

Response #5: Thank you for your comment. We replaced “clustered” with “are” in the text of the manuscript. We also added the following sentence in the text of our manuscript.

"While PC1 showed that 2a2iL-hPSCs are in line with morula and closer to primed cells, they have been entirely separated from their parental primed cells."

Comment #6: I think it would be good to reference/mention recent studies, which have used the mentioned TGF-β signalling inhibitors (SB431542 and A83-01) within protocols to induce TSC cells from naïve PSCs (Dong/Theunissen, eLife, 2020 and Guo/Smith, bioRxiv, 2020). In this regard, it is not very surprising that cells do not remain naïve with the addition of these chemicals and it would be a nice link to the wider literature.

Response #6: Thank you for your suggestion. We added the following sentence in the last paragraph of discussion. We also added three references related to this concept as reviewer suggested.

"Several reports also used TGF-β/Activin A to establish naïve pluripotency in hPSCs (Qin et al, 2016). Consistence with this, it has been reported that inhibition of TGF-β signaling in bona fide naïve pluripotent cells can generate extraembryonic trophectoderm indicating the dependency of human naïve pluripotency to TGF-β signaling (Dong *et al*, 2020; Guo *et al*, 2020). Compared to primed hPSCs in which continuous supplementation of TGF-β/Activin A is necessary to protect pluripotency, we found that transient stimulation of TGF-β signaling is sufficient to induce and maintain the naïve-like state in hPSCs. Further experiments need to determine the molecular mechanisms of TGF-β signaling pathway in human naïve pluripotency regulation."

Comment #7: Within the discussion you mention many studies which have used different approaches to make naïve cells. One notable study missing is that by Guo/Smith (Development, 2017), in which no transgenes are used and naïve cell are created which faithfully resemble those made in T2iLGo (transgene induced) settings.

Response #7: I agree with you. We inserted this reference in the manuscript.

Comment #8: There are several grammatical mistakes/typos which can easily be fixed by careful proof-reading, some I noticed

a. "In the naïve pluripotency, these cells can participate in embryo" -> Naïve pluripotent stem cells can participate....

Response: This sentence was changed according to the reviewer suggestion.

b. "hPSCs into naïve ones" -> hPSCs into naïve cells

Response: This correction was done.

c. "OCT4 transcription dependents on" -> OCT4 transcription depends on

Response: This mistake was corrected.

d. "In obtain a more detailed" -> To obtain a more detailed

Response: "To" was replaced with "in".

e. "regulated pluripotency" -> pluripotency regulation

Response: Pluripotency regulation was written in the text of manuscript.

f. "LEFTTY1/2" -> LEFTY1/2

Response: This mistake as corrected.

g. "2a2iL-hPSCs might dependent on" -> 2a2iL-hPSCs might be dependent on

Response: This sentence was corrected.

i. page 11: "develop" ->developed

Response: This sentence was corrected.

Cross-comments from referee 3:

I agree with referee 2 that the revision is not complete, but also do not think that every little request needs to be done. I disagree with referee 2 that the main conclusion is not solid: there is enough evidence to suggest a naive-like state. Clearly the naive cells are not as naive as other naive media but still is distinct from the primed state. Related to the use of the Oct4-GFP reporter, the authors have used an alternative strategy to address this point. The FISH experiment was not done but its outcome would not change the conclusion that cells are naive-like (other naive-like media have cells that still show X inactivation: X reactivation is only robustly found in t2iLGo and 5iLAF naive media). I agree with referee 2 that the KLF17 data is not solid: as indicated in our previous review, there are likely issues with the KLF17 IF. Hope this helps.

Response: Thank you for pointing this out. We corrected the result from KLF17 expression as mentioned before.

Cross-comments from referee 1:

I agree with referee 3. Clearly the authors have not addressed all concerns that were raised, but is some cases they had good reasons not to. I think it is pretty clear that their cells are not fully naive, like t2iLGö or 4/5iLAF, but this will be also clear to experts in the field.
This is why I think the paper is suitable for publication, after the KLF17 staining issue is sorted out.

Response: Thank you for pointing this out. We corrected the result from KLF17 expression as mentioned before.

The senior editor comments:

Comment #1: Please add a direct link to your deposited data at the NCBI Gene Expression Omnibus. This information needs to be moved to a "Data Availability Section" at the end of the materials and methods.

Response: Your comments considered in our manuscript.

Comment #2: Please remove "data not shown" on pages 7, 8 and 9, or add data. You may use the term "personal communication".

Response: “Data not shown” were removed.

Comment #3: Please upload all figures as separate files and move the figure legends to the end of the main manuscript file.

Response: It was done.

Comment #4: Please add a callout for figure 4D.

Response: Added.

Comment #5: For the supplementary information, you can show all figures and tables together in an Appendix file that also needs a table of content with page numbers and that can have supplementary methods. Alternatively, you can upload 5 individual expanded view (EV) figures that will be integrated into the manuscript text online. If the tables are not part of the Appendix, they can be uploaded as EV tables or as Datasets; in this case the legends need to be part of the table files. For example, supplementary table 2 should be uploaded as Dataset EV1 with a legend and/or title in the first tab of the excel sheet. Please see our guide to authors for more information.

Response: We showed all figures and tables together in an Appendix file with a table of content. Supplementary methods have been also included in Appendix file.

Comment #6: The figure quality should be improved for the final manuscript submission.

Response: Thank you. We performed it.

Comment #7: The reference style needs to be changed to the numbered EMBO reports style, if the manuscript will be submitted before the end of June. From the 1st of July, the EMBO

reports reference style will change to the Harvard style. If the manuscript will be accepted after the 1st of July, the reference style will need to be changed to Harvard style.

Response: Thank you. But in your site, we found "**Please note: for all manuscripts submitted from 1 May 2020, and for all articles published beginning 1 July 2020, the EMBO Reports reference style will change to the Harvard style for all article types. This updated EndNote style is available for download here"**. Then we redownload it and prepared as it not numbered. If we should change please let us know.

Comment #8: I would like to suggest a few changes to the abstract that needs to be written in present tense.

Response: Thank you. We changed the abstract according your suggestion as you can see in the following:

"Naïve pluripotency can be established in human pluripotent stem cells (hPSCs) by manipulation of transcription factors, signaling pathways or a combination thereof. However, differences exist in the molecular and functional properties of naïve hPSCs generated by different protocols, which include varying similarities with pre-implantation human embryos, differentiation potential, and maintenance of genomic integrity. We show here that short treatment with two chemical agonists (2a) of nuclear receptors, liver receptor homologue-1 (LRH-1) and retinoic acid receptor gamma (RAR-γ), along with 2i/LIF (2a2iL) induces naïve-like pluripotency in human cells during reprograming of fibroblasts, conversion of pre-established hPSCs, and generation of new cell lines from blastocysts. 2a2iL-hPSCs match several defined criteria of naïve-like pluripotency and contribute to human-mouse interspecies chimeras. Activation of TGF-β signaling is instrumental for acquisition of naïve-like pluripotency by the 2a2iL induction procedure, and transient activation of TGF-β signaling substitutes for 2a to generate naïve-like hPSCs. We reason that 2a2iL-hPSCs are an easily attainable system to evaluate properties of naïve-like hPSCs and for various applications."

Comment #9: I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript file.

Response: All of your comments were considered.

Comment #10: EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550 pixels x 200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Response: We have prepared a separated file as the synopsis including one sentences and four highlights and a synopsis image.

Comment #11: We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

Comment #12: As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts.

This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Response: It is our pleasure to published RPF Review Process File along with the accepted manuscripts.

3rd Revision - Editorial Decision 17th Jul 2020

Prof. Hossein Baharvand
Royan Institute for Stem Cell Biology and Technology
Banihashem St
Tehran, Tehran 1665659911
Iran

Dear Hossein,

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.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-47533V3

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was choosen based on standard protocols in the field. All experiments were repeated at least three independent times. RNA-Seq experiments were conducted in two technical replicates for naïve hESC samples and one for primed hESC samples in two biological replicates (two different hESC lines).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical test was performed to estimate sample size for. Animal studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No amples were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We did not use parametric tests.
Is there an estimate of variation within each group of data?	Yes.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

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http://www.ncbi.nlm.nih.gov/gap

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Not applicable.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used in this study are listed in Table 2 in "Materials and Methods" section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Source of cell lines are spcified in the "Materials and Methods" section. All cells were regularly tested for mycoplasma and were found to be negative.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	MEF was derived from 12.5-day mouse embryos. For human-mouse interspecies chimera, we used 6-8 weeks NMRI strain for obtaining blastocysts and BALB/C/C57BL/6 mixed background as foster mother (2.5 days post coitum). All animals had free access to chow and water and were housed in 12 hours dark and 12 hours light cycles.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed in accordance with Ethical Committee of Royan institute.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Human embryo experiments were conducted in accordance with Ethical Committee of Royan institute.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	We used surplus and poor quality embryos.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not Applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not Applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	A "Data Availibility" section has been included at the ed of the Materials and Methods section.RNA-Seq data has been deposited in Gene Expression Omnibus database repository with accession number: GSE116501.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	The RNA-Seq data is deposited in the Gene Expression Omnibus database, accession number.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not Applicable.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not Applicable.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable.