nature portfolio

Peer Review File

Production of live monkeys and their genetically matched embryonic stem cells from single embryos

Corresponding Author: Professor Yuyu Niu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the author reports the establishment of embryonic stem cells (ESCs) and viable individuals from a single embryo using embryo splitting technology, resulting in autologous ESCs (aESCs) in non-human primates, as well as genetically matched iPSCs and ntESCs. Single-cell transcriptome analysis reveals that aESCs may offer advantages, including reduced heterogeneity, lower transcriptional noise, and enhanced genomic stability. The author refined the embryo splitting technique for rhesus and cynomolgus monkeys at the 4-cell and 8-cell stages, demonstrating that the split embryos could develop to the blastocyst stage. Split embryos also established ESCs and generated live individuals similar to control embryos, though the aESCs efficiency from equal division at the 4-cell stage was low. To improve this, the author used an unequal division method at the 8-cell stage, which more than doubled the aESCs efficiency. The author also derived iPSCs and ntESCs from monkey fibroblasts, assessing the differentiation potentials, genomic stability, and transcriptional noise among these three types of pluripotent stem cells using single-cell transcriptome sequencing. Finally, it was demonstrated that establishing human ESCs from split human embryos is feasible. The work requires a significant effort and complex data, as deriving aESCs from a single embryo and establishing three genetically consistent pluripotent stem cells (PSCs) for comparison are highly challenging. These achievements are difficult yet important, with promising applications.

Although cloning through embryo splitting was reported many years ago, the low efficiency of this process has always been a challenge. This work confirmed a live birth rate of individual split embryos (7/37, 18.9%) that was comparable to that of the control embryos in this study (11/58, 19.0%) but higher than the previous live birth rate of split embryos in rhesus monkeys (7/64, 10.9%) (Mitalipov, S.M. et al., Biol Reprod, 2002). However, the aESCs efficiency (3/26, 11.5%) remains relatively low, with room for improvement. Additionally, previous studies compared isogenic ntESCs and iPSCs but did not compare ESCs from the same individual (at least in non-human primates). This work is the first to obtain three types of PSCs in non-human primates with a consistent genetic background from a single individual, including aESCs, iPSCs, and ntESCs. This reduces the interference caused by genetic difference.

Specific points:
1. How do the authors define the blastocyst stage in Fig. 1 legend L649?
2. What does the 2/4th embryos indicate in L93? Similarly, what do 3/8th or 5/8th embryos represent?
3. What is the purpose of presenting data for 2/4th embryos that show either fewer or more Oct-4 positive cells in Fig. 1 i-k and Ext Data Fig. 1 i-j? Please clarify this in the text or figure legend.
4. What species are used in L145 and L192?
5. At what developmental stage were the split embryos transferred to surrogates in L170? Were they further cultured in vitro before this?
6. The authors should clarify their classification of cell clusters in L277-286. While the DPR designation likely refers to cells co-expressing pluripotency and lineage-specific genes, the criteria for this are unclear. Specifically, how is an ExE mech DPR cell defined, and what distinguishes Epi1 from Epi2?
7. The correlation analysis of cell lines (Fig. 2h; Fig. 7i; Extended Data Fig. 2f) shows excessive similarity among lines representing different pluripotent states (naïve, primed, EPS) and data sources. The lack of controls raises doubts about the effectiveness of the analytical methods in differentiating these cell lines. Additionally, the gene expression plots (Fig. 2g; Fig. 7h) employ a color scale that limits the visibility of differences in samples with low gene expression levels.
8. To compare primate PSC characteristics, the authors performed scRNA-seq on PSCs with identical genetic backgrounds and integrated the data with an existing dataset from a human gastrula at Carnegie stage 7. However, concerns about

differences and biases between species, like monkey and human, need to be addressed.
9. Currently, autologous ESCs cannot replace the significant advantages of iPSCs, and this point needs to be clearly articulated in discussions to ensure an accurate and comprehensive understanding of both technologies.
10. Unlike the phenomenon of poor quality and smaller volume of ICM in human split embryos observed in previous studies, this study successfully established ESCs from human split embryos. This significant achievement should be thoroughly discussed in the discussion section to explain the possible reasons for this difference.
11. The author proposed using autologous ESCs to treat maybe diseases that may occur in children born through in vitro fertilization (IVF). While this idea is intriguing, it's important to clarify its limitations, including its applicability to IVF offspring, cost-effectiveness, and safety risks related to transferring split versus intact embryos. This highlights the research's significance, especially since studying human split embryos poses ethical challenges.

Reviewer #2

(Remarks to the Author)
"Production of live monkeys and their genetically matched embryonic stem cells from single embryos" by Chenyang Si and colleagues.

Embryo and biotechnology in non-human primates (NHPs) is less developed than in mice. This is mainly due to the lower availability of NHPs, the significantly higher complexity of working with NHPs, and the fact that NHPs naturally produce only one offspring. In the present study, Chenyang Si and colleagues generated IVF embryos from macaques (rhesus and cynomolgus), split them, and analyzed the developmental potential of the split embryos through embryo transfer to surrogate mothers and ESC line derivation experiments. The authors were successful in both directions: after the transfer of the split embryos, live offspring were born and ESC lines were established. In addition, autologous iPSCs and cloned ESCs (from postnatal fibroblasts) were generated, allowing the authors to obtain three types of pluripotent stem cells (autologous ESCs from split embryos, cloned ESCs and iPSCs) from a living animal. This constellation is new and pushes the boundaries of NHP biotechnology into new directions. It provides interesting new insights into comparative pluripotent stem cell biology and theoretically opens up new perspectives for regenerative medicine with autologous ESCs. However, the medical application of this technology / resource (autologous ESCs) seems questionable to me, considering that currently in most countries where ART is available, 1-5% of all children are born with ART. However, this could change and increase in the coming decades.

The manuscript addresses and interesting and new topic, is well-written and the figures are of high quality.

Specific comments:
1. Lines 51-53: The authors say that creating genetically matched ESCs from primates poses technical and ethical challenges. Do the authors mean NHPs and humans or only NHPs? If only NHPs are meant, are there no (or even much bigger) ethical concerns with the use of human embryos for biomedical experiments, such as those conducted in this study?
2. Lines 54-59: These publication may also be considered in this context as they might be relevant also for this study: PMID: 39786543, PMID: 31504820
3. Line 67: delete “currently”.
4. Line 88: In the methods section, please describe how the sperm cells were collected. Please also include a more detailed description of the procedures in the animals, in particular the anesthetic and analgesic regimes.
5. Line 107 ff and Fig.1b: No normal embryos are shown. Therefore, it is difficult to say that the cleaved embryos do not differ from normal embryos in their development time.
6. Line 111 ff and Fig.1g: Do the authors have an explanation for the fact that the “identical pairs” have a lower blastocyst rate than the “2/4 embryos”? Is the difference in blastocyst rates between “identical couples” and the “2/4 embryos” also statistically different? If so, is there an explanation for this?
7. Fig.1i: How were the groups “few OCT4” and “more OCT4” determined? Simply by counting the number of OCT4-positive cells? If so, is this not simply a self-fulfilling prophecy?
8. Histology of the teratomas: I can easily see muscle tissue in the histological sections depicting mesoderm. I am also convinced that the histological structures you show for ectoderm and endoderm are correct. However, I would appreciate it if you could show the characteristic histological structures at a larger magnification.Line 139: I think the conclusion is a bit misleading. The number of Epi (OCT4A+) cells was significantly lower in split embryos. I would recommend to include that also in the conclusion of this part.
9. Line 178: is “surrogate monkey” the correct term for the offspring obtained from embryo transfer to surrogate mothers? I was a bit confused by this expression.
10. Line 184: I think the growth rates (increase in body weight) were different (even statistically significant) between controls and split embryos, with split embryo derived animals being - surprisingly - larger than controls. Do the authors have an explanation for this result?
11. Line 225: Please check the names of the animals in the text and in Fig. 4b.
12. Line 251 and 381: I would recommend saying “pluripotency (-related) genes” instead of “pluripotent genes”.
13. Line 333: As far as I could see from the abstracts of references 45 and 46, the cells analyzed here are mouse embryos / fetuses. Do these cells / references have any relevance to primate PSCs?
14. Fig.5c: I only see one scale bar. Therefore, I assume that all images were taken and displayed at the same magnification. However, the nuclei of the stem cells without feeders seem to have a much larger diameter than those of the feeder cells. Is this correct? If so, do you have an explanation for this finding?
15. Fig.6: what is the number of biological replicates (number of cell lines) that were used to generate all these data? This is not clear to me.

16. Fig.6d: It would be helpful if DPR would be written out once at the first mention in the figure legend.
17. Line 719: As far as I know, the nomenclature for primates suggests writing all gene and protein abbreviations in capital letters. However, proteins in normal font and only nucleic acids in italics. Please check this.

Reviewer #3

(Remarks to the Author)
In the present manuscript, Si and his colleagues have devised a valuable embryo splitting technique in monkeys. The researchers successfully obtained live monkeys from the split embryos and established genetically matched autologous embryonic stem cells (aESCs), thus validating the feasibility of the refined embryo splitting protocol. This achievement renders primates more efficacious as animal models for research.

Major Points:
The authors demonstrated that the developmental morphology, timing, and molecular characteristics of the blastocysts derived from split embryos were comparable to those of the control blastocysts. The birth of live monkeys provided robust evidence for the developmental potential of the split embryos. They also compared the autologous ESCs obtained from the split embryos with genetically matched induced pluripotent stem cells (iPSCs) and nuclear transfer embryonic stem cells (ntESCs), confirming their reduced heterogeneity.

Nonetheless, it remains uncertain whether the trophectoderm can be isolated from the blastocysts developed from split embryos, given that trophectoderm (TE) is also a crucial constituent of the blastocyst. Additionally, it is unclear whether there are disparities in the differentiation capabilities among iPSCs, ntESCs, and autologous ESCs (aESCs).

Minor Points:
The first subheading within the results section was designated as " Monkey split embryos show normal developmental potential", yet the data regarding the generation of live monkeys was presented in the second part of the results. The content of the paragraph may need some minor adjustments.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Reviewer #1 (Remarks to the Author):

In this manuscript, the author reports the establishment of embryonic stem cells (ESCs) and viable individuals from a single embryo using embryo splitting technology, resulting in autologous ESCs (aESCs) in non-human primates, as well as genetically matched iPSCs and ntESCs. Single-cell transcriptome analysis reveals that aESCs may offer advantages, including reduced heterogeneity, lower transcriptional noise, and enhanced genomic stability. The author refined the embryo splitting technique for rhesus and cynomolgus monkeys at the 4-cell and 8-cell stages, demonstrating that the split embryos could develop to the blastocyst stage. Split embryos also established ESCs and generated live individuals similar to control embryos, though the aESCs efficiency from equal division at the 4-cell stage was low. To improve this, the author used an unequal division method at the 8-cell stage, which more than doubled the aESCs efficiency. The author also derived iPSCs and ntESCs from monkey fibroblasts, assessing the differentiation potentials, genomic stability, and transcriptional noise among these three types of pluripotent stem cells using single-cell transcriptome sequencing. Finally, it was demonstrated that establishing human ESCs from split human embryos is feasible. The work requires a significant effort and complex data, as deriving aESCs from a single embryo and establishing three genetically consistent pluripotent stem cells (PSCs) for comparison are highly challenging. These achievements are difficult yet important, with promising applications.

[Response] We appreciate the recognition of the importance and relevance of our work. We agree with the reviewer on the importance of establishing aESCs from a single embryo. We believe that this advancement may contribute to the field of pluripotent stem cell research and have potential applications in regenerative medicine. Thank you very much for your valuable feedback.

Although cloning through embryo splitting was reported many years ago, the low efficiency of this process has always been a challenge. This work confirmed a live birth rate of individual split embryos (7/37, 18.9%) that was comparable to that of the control embryos in this study (11/58, 19.0%) but higher than the previous live birth rate of split embryos in rhesus monkeys (7/64, 10.9%) (Mitalipov, S.M. et al., Biol Reprod, 2002). However, the aESCs efficiency (3/26, 11.5%) remains relatively low, with room for improvement. Additionally, previous studies compared isogenic ntESCs and iPSCs but did not compare ESCs from the same individual (at least in non-human primates). This work is the first to obtain three types of PSCs in non-human primates with a consistent genetic background from a single individual, including aESCs, iPSCs, and ntESCs. This reduces the interference caused by genetic difference.

[Response] We appreciate the reviewer's insightful comments regarding our findings. We acknowledge that while our live birth rates of split embryos are comparable to controls and improved over previous reports, the aESCs efficiency is low and

needs improvement. Notably, the asymmetric embryo splitting method has significantly increased aESC efficiency, indicating that further increasing the number of EPI cells from split embryos could improve outcomes. Consequently, the stem cell chimera method may also be effective, utilizing stem cell chimerism to increase the cell numbers of split embryo for transplantation, with both stem cells and split embryos derived from the same embryo. We also appreciate the recognition of the significance of our work in obtaining three types of pluripotent stem cells-aESCs, iPSCs, and ntESCs from a single non-human primate, reducing genetic variability for more accurate comparisons.

Specific points:
1. How do the authors define the blastocyst stage in Fig. 1 legend L649?

[Response] We sincerely apologize for not clearly describing this point. In the legend for Fig. 1 (L673), we define the blastocyst stage as an embryo that has been cultured until the sixth day post-fertilization and features a well-defined cavity. Additional explanations can be found in L673-L674.

[Comment] Solved.

2. What does the 2/4th embryos indicate in L93? Similarly, what do 3/8th or 5/8th embryos represent?

[Response] We sincerely apologize for not clearly describing this point. The 2/4th embryo refers to an embryo reconstructed from any two blastomeres of a 4-cell stage embryo. Similarly, the 5/8th and 3/8th embryos denote embryos reconstructed from any five blastomeres and the remaining three blastomeres of an 8-cell stage embryo, respectively.

[Comment] Understandable, I suggest adding concrete details in the paper.

3. What is the purpose of presenting data for 2/4th embryos that show either fewer or more Oct-4 positive cells in Fig. 1 i-k and Ext Data Fig. 1 i-j? Please clarify this in the text or figure legend.

[Response] We sincerely apologize for not clearly describing this point. Epi cell number is a crucial determinant for successful implantation. Published studies have shown that fewer than four Epi cells in split mouse embryos hinder fetal development (10.1016/j.celrep.2012.08.029), while higher Epi cell counts in rhesus monkey blastocysts promote successful pregnancies (10.1016/j.cell.2011.12.007). Therefore, the low efficiency in obtaining aESCs may be due to the unintended selection of 2/4th embryos with lower Epi cell counts for transfer within genetically matched pairs. This is supported by improved aESCs efficiency through the selection of 5/8th embryos for transfer, which have characteristics similar to 2/4th embryos that contain more Epi cells. We have clarified this in L439-L450.

[Comment] This is persuasive.

4. What species are used in L145 and L192?

[Response] We sincerely apologize for not clearly describing this point. In L151, 2/4th embryos from 20 cynomolgus and 5 rhesus monkeys were used to establish ES cell lines, with detailed information provided in Table 2. In L200, 2/4th embryos were transferred to 13 cynomolgus and 1 rhesus monkey recipients, while 5/8th embryos were transferred to 4 cynomolgus and 9 rhesus monkey recipients. Specific details can be found in Supplementary Figure 3c.

[Comment] Solved.

5. At what developmental stage were the split embryos transferred to surrogates in L170? Were they further cultured in vitro before this?

[Response] We sincerely apologize for not clearly describing this point. The split embryos were cultured in vitro until they reached the morula or blastocyst stage, which typically occurs on the fifth or sixth day post-fertilization. At this developmental stage, the embryos were transferred to the recipient monkeys.

[Comment] OK.

6. The authors should clarify their classification of cell clusters in L277-286. While the DPR designation likely refers to cells co-expressing pluripotency and lineage-specific genes, the criteria for this are unclear. Specifically, how is an ExE mech DPR cell defined, and what distinguishes Epi1 from Epi2?

[Response] We thank the reviewer for the valuable comment regarding the classification of cell clusters in L277-286. We performed a systematic approach to define and annotate the cell populations as follows:
(1) Pluripotency gene expression and correlation analysis
First, we detected the expression of all known pluripotency genes and performed correlation analysis with d.p.f.10-14 Epi cells from the cynomolgus monkey to confirm the pluripotent nature of these cells. We then performed a correlation analysis of our dataset with all cells from the human Carnegie stage 7 (CS7) to define "Epi" cells. This approach allowed us to identify pluripotent stem cells.
(2) Identification of cell clusters
To further clarify the cell types, we integrated lineage marker genes (for example, endoderm (Endo), mesoderm (Meso), neural ectoderm (NE), non-neural ectoderm (NNE) , and extraembryonic mesenchyme (ExE mech),etc.) with the FateID

method, which enabled us to predict cell fate biases based on single-cell transcriptome data. This helped to confirm the identity of pluripotent and differentiating cell populations.
(3) ExE Mech DPR cells
ExE mech DPR cells were defined based on their transcriptional profile that showed differential expression of specific genes associated with the ExE mesoderm (e.g. FN1, COL1A2, COL1A1, TIMP3) as described in the methods (Figure 6d-e, Extended Data Figure 5k).
(4) Epi1 vs. Epi2
Regarding the distinction between Epi1 and Epi2, we observed a cell population (Cluster 10) that exhibited a transcriptomic profile similar to the defined Epi cells, but separated from most Epi populations. This cluster was then scored for known pluripotency genes. The stemness score of Cluster 10 is lower than that of the most Epi populations. Based on the scoring results, Cluster 10 was named "Epi2". These evidences allowed us to differentiate it from most Epi cells, which were labeled "Epi1" (Extended Data Fig. 5b-d).

[Comment] The classification of the ExE mech DPR cell population is understandable. For EPI1 and EPI2, the authors list highly expressed genes: LDHA, MT2A, CDK1, and KPNA2. Given these genes' expression profiles, which suggest a tendency for rapid proliferation and self-renewal, I consider these cells as intermediate - state cells. Unless emphasizing them is necessary, I suggest not classifying them separately as EPI2, just merging them with EPI.

7. The correlation analysis of cell lines (Fig. 2h; Fig. 7i; Extended Data Fig. 2f) shows excessive similarity among lines representing different pluripotent states (naïve, primed, EPS) and data sources. The lack of controls raises doubts about the effectiveness of the analytical methods in differentiating these cell lines. Additionally, the gene expression plots (Fig. 2g; Fig. 7h) employ a color scale that limits the visibility of differences in samples with low gene expression levels.

[Response] Thank you for your valuable comments. To emphasize the similarity at the transcriptional level between our derived spESCs and published reference ESCs, we calculated correlations using only the common genes detected in both our samples and other research samples, while excluding genes detected in one study. This strategy may attenuate differences between different stem cell states. However, our correlation analysis follows the same methodology as previous studies, which have reported correlation scores of approximately 0.97-0.99 among primed ESCs and greater than 0.95 between primed and naïve-like ESCs (10.1016/j.stem.2015.06.004). To address concerns regarding controls, we clarify that we used a primed ESC lines (CES and CES3) (10.1016/j.stem.2015.06.004), as the reference control in Fig. 2h, and human conventional ESCs (10.1016/j.cell.2014.08.029), naïve ESCs (10.1038/s41556-019-0333-2) and other PSCs (10.1016/j.biomaterials.2020.120015; 10.1016/j.cell.2014.12.01) as the reference control in Fig. 7i. Due to the correlation between H1_EPS_rep1 and H1_EPS_rep2 being below 0.94, they exhibited outlier behavior in clustering and reduced color distinction across all samples. Therefore, we removed these two samples in new Fig.7h and Fig.7i. Regarding the use of color scale, we modify it to present the results appropriately in new Fig. 2g, Fig.2h, Extended Data Fig. 2f, Fig. 7h, and Fig.7i.

[Comment] This is much more convincing.

8. To compare primate PSC characteristics, the authors performed scRNA-seq on PSCs with identical genetic backgrounds and integrated the data with an existing dataset from a human gastrula at Carnegie stage 7. However, concerns about differences and biases between species, like monkey and human, need to be addressed.

[Response] We thank the reviewer for the insightful comment regarding the potential bias due to species differences. The human CS7 data were used to assist in annotating the types and statuses of primate PSCs. The data represents the closest in vivo data available at the time for the monkey species, which includes various cell types. Comparable in vivo data for monkeys at a similar stage of development were not available at that time. Given the high degree of similarity between humans and monkeys, we used this dataset. To mitigate the effects of species differences, we first integrated human and monkey data using standard integration techniques. Subsequently, stem cell types and statuses were determined by similarity calculations based on genes co-expressed in both species. This dataset served as one of our references, and consistent results were also obtained using the marker genes and the FateID method. By combining these three methods, we were able to infer the final cell state, effectively mitigating the effects of species.

[Comment] Understandable.

9. Currently, autologous ESCs cannot replace the significant advantages of iPSCs, and this point needs to be clearly articulated in discussions to ensure an accurate and comprehensive understanding of both technologies.

[Response] Thank you for your thorough review and valuable feedback on our work, which we deeply appreciate and agree with. We have followed the reviewer’s advice and have clearly stated that aESCs are currently unable to fully replace iPSCs in L433-L434.

[Comment] Solved.

10. Unlike the phenomenon of poor quality and smaller volume of ICM in human split embryos observed in previous studies, this study successfully established ESCs from human split embryos. This significant achievement should be thoroughly discussed in the discussion section to explain the possible reasons for this difference.

[Response] Thank you for your constructive feedback. We have addressed the possible reasons for successfully

establishing human ESCs from split embryos in discussion (L468-L474), as suggested by the reviewer.

[Comment] The authors noted that the split embryos were spatially rearranged, with proper gene expression and normal developmental clock. It would be beneficial for them to further explore this successful experiment, particularly regarding the timing of splitting, the integrity of the blastomeres, and the role of cell mechanics.

11. The author proposed using autologous ESCs to treat maybe diseases that may occur in children born through in vitro fertilization (IVF). While this idea is intriguing, it's important to clarify its limitations, including its applicability to IVF offspring, cost-effectiveness, and safety risks related to transferring split versus intact embryos. This highlights the research's significance, especially since studying human split embryos poses ethical challenges.

[Response] Thank you for your valuable comments. We acknowledge the limitations of using autologous ESCs for treating potential diseases in IVF offspring. We have discussed its applicability, cost-effectiveness, and safety risks related to transferring split versus intact embryos in discussion (L466-L468).

[Comment] Thank you for the additional comments.

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my points. The biggest weakness of the manuscript is the limited number of autologous cells (n=1 for aESCs, ntESCs and iPSCs, if I understand correctly). Knowing that even cell lines produced using the same method (such as individual iPSC lines from one animal) can show significant differences from each other, the differences between PSCs produced in different ways should not be over-interpreted. In my opinion, however, this limitation was stated clearly enough in the concluding paragraph of the manuscript.

In general, the MS represents a significant and very interesting advance in the field of NHP/human stem cell research, embryology and biotechnology.

Just a few minor things:
L384: pluripotent > pluripotency (as already changed in all other places in the MS).
L485-487: I'm not a native speaker, but the syntax of this sentence seems unusual to me. Please check it.
L1175 ff: The tense of this paragraph is different from the other paragraphs in the “Materials and methods” section. Please check.

Reviewer #3

(Remarks to the Author)
I thank the authors for their detailed responses

1. Regarding the isolation and functional validation of TE from split embryos:
The data regarding the expression of trophectoderm (TE) marker genes (e.g., OCT4, GATA6, and CDX2) in split embryos, as well as the successful production of live offspring provide some evidence of TE functionality. However, these observations do not directly demonstrate the ability of TE from split embryos to differentiate into functional trophoblast stem cells (TSCs) or to support implantation and placental development. Given the low efficiency of live offspring production, it remains unclear whether the TE in split embryos is fully equivalent to that in control embryos. To strengthen their conclusions, I strongly recommend that the authors perform direct experiments to isolate and functionally validate TE-derived TSCs from split embryos. This would provide more robust evidence of the developmental potential of the TE in this context.

2. Regarding the differentiation capabilities of iPSCs, ntESCs, and aESCs:
While the teratoma assays in Figure 2 and Figure 4 demonstrate that iPSCs, ntESCs, and aESCs are capable of differentiating into all three germ layers, I believe that a more in-depth analysis and discussion of these data would significantly strengthen the manuscript.

Overall Recommendation:
If the authors can provide additional evidence (e.g., TSC derivation) or at least include a more thorough discussion of the limitations and future directions, I would be willing to recommend the manuscript for publication in Nature Communications.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the author reports the establishment of embryonic stem cells (ESCs) and viable individuals from a single embryo using embryo splitting technology, resulting in autologous ESCs (aESCs) in non-human primates, as well as genetically matched iPSCs and ntESCs. Single-cell transcriptome analysis reveals that aESCs may offer advantages, including reduced heterogeneity, lower transcriptional noise, and enhanced genomic stability. The author refined the embryo splitting technique for rhesus and cynomolgus monkeys at the 4-cell and 8-cell stages, demonstrating that the split embryos could develop to the blastocyst stage. Split embryos also established ESCs and generated live individuals similar to control embryos, though the aESCs efficiency from equal division at the 4-cell stage was low. To improve this, the author used an unequal division method at the 8-cell stage, which more than doubled the aESCs efficiency. The author also derived iPSCs and ntESCs from monkey fibroblasts, assessing the differentiation potentials, genomic stability, and transcriptional noise among these three types of pluripotent stem cells using single-cell transcriptome sequencing. Finally, it was demonstrated that establishing human ESCs from split human embryos is feasible. The work requires a significant effort and complex data, as deriving aESCs from a single embryo and establishing three genetically consistent pluripotent stem cells (PSCs) for comparison are highly challenging. These achievements are difficult yet important, with promising applications.

[Response] We appreciate the recognition of the importance and relevance of our work. We agree with the reviewer on the importance of establishing aESCs from a single embryo. We believe that this advancement may contribute to the field of pluripotent stem cell research and have potential applications in regenerative medicine. Thank you very much for your valuable feedback.

Although cloning through embryo splitting was reported many years ago, the low efficiency of this process has always been a challenge. This work confirmed a live birth rate of individual split embryos (7/37, 18.9%) that was comparable to that of the control embryos in this study (11/58, 19.0%) but higher than the previous live birth rate of split embryos in rhesus monkeys (7/64, 10.9%) (Mitalipov, S.M. et al., Biol Reprod, 2002). However, the aESCs efficiency (3/26, 11.5%) remains relatively low, with room for improvement. Additionally, previous studies compared isogenic ntESCs and iPSCs but did not compare ESCs from the same individual (at least in non-human primates). This work is the first to obtain three types of PSCs in non-human primates with a consistent genetic background from a single individual, including aESCs, iPSCs, and ntESCs. This reduces the interference caused by genetic difference.

[Response] We appreciate the reviewer’s insightful comments regarding our findings. We acknowledge that while our live birth rates of split embryos are comparable to controls and improved over previous reports, the aESCs efficiency is low and needs improvement. Notably, the asymmetric embryo splitting method has significantly increased aESC efficiency, indicating that further increasing the number of EPI cells from split embryos could improve outcomes. Consequently, the stem cell chimera method may also be effective, utilizing stem cell chimerism to increase the cell numbers of split embryo for transplantation, with both stem cells and split embryos derived from the same embryo. We also appreciate the recognition of the significance of our work in obtaining three types of pluripotent stem cells-aESCs, iPSCs, and ntESCs from a single non-human primate, reducing genetic variability for more accurate comparisons.

Specific points:
1. How do the authors define the blastocyst stage in Fig. 1 legend L649?

[Response] We sincerely apologize for not clearly describing this point. In the legend for Fig. 1 (L673), we define the blastocyst stage as an embryo that has been cultured until the sixth day post-fertilization and features a well-defined cavity. Additional explanations can be found in L673-L674.

2. What does the 2/4th embryos indicate in L93? Similarly, what do 3/8th or 5/8th embryos represent?

[Response] We sincerely apologize for not clearly describing this point. The 2/4th embryo refers to an embryo reconstructed from any two blastomeres of a 4-cell stage embryo. Similarly, the 5/8th and 3/8th embryos denote embryos reconstructed from any five blastomeres and the remaining three blastomeres of an 8-cell stage embryo, respectively.

3. What is the purpose of presenting data for 2/4th embryos that show either fewer or more Oct-4 positive cells in Fig. 1 i-k and Ext Data Fig. 1 i-j? Please clarify this in the text or figure legend.

[Response] We sincerely apologize for not clearly describing this point. Epi cell number is a crucial determinant for successful implantation. Published studies have shown that fewer than four Epi cells in split mouse embryos hinder fetal development (10.1016/j.celrep.2012.08.029), while higher Epi cell counts in rhesus monkey blastocysts promote successful pregnancies (10.1016/j.cell.2011.12.007). Therefore, the low efficiency in obtaining aESCs may be due to the unintended selection of 2/4th embryos with lower Epi cell counts for transfer within genetically matched pairs. This is supported by

improved aESCs efficiency through the selection of 5/8th embryos for transfer, which have characteristics similar to 2/4th embryos that contain more Epi cells. We have clarified this in L439-L450.

4. What species are used in L145 and L192?

[Response] We sincerely apologize for not clearly describing this point. In L151, 2/4th embryos from 20 cynomolgus and 5 rhesus monkeys were used to establish ES cell lines, with detailed information provided in Table 2. In L200, 2/4th embryos were transferred to 13 cynomolgus and 1 rhesus monkey recipients, while 5/8th embryos were transferred to 4 cynomolgus and 9 rhesus monkey recipients. Specific details can be found in Supplementary Figure 3c.

5. At what developmental stage were the split embryos transferred to surrogates in L170? Were they further cultured in vitro before this?

[Response] We sincerely apologize for not clearly describing this point. The split embryos were cultured in vitro until they reached the morula or blastocyst stage, which typically occurs on the fifth or sixth day post-fertilization. At this developmental stage, the embryos were transferred to the recipient monkeys.

6. The authors should clarify their classification of cell clusters in L277-286. While the DPR designation likely refers to cells co-expressing pluripotency and lineage-specific genes, the criteria for this are unclear. Specifically, how is an ExE mech DPR cell defined, and what distinguishes Epi1 from Epi2?

[Response] We thank the reviewer for the valuable comment regarding the classification of cell clusters in L277-286. We performed a systematic approach to define and annotate the cell populations as follows:
(1) Pluripotency gene expression and correlation analysis
First, we detected the expression of all known pluripotency genes and performed correlation analysis with d.p.f.10-14 Epi cells from the cynomolgus monkey to confirm the pluripotent nature of these cells. We then performed a correlation analysis of our dataset with all cells from the human Carnegie stage 7 (CS7) to define "Epi" cells. This approach allowed us to identify pluripotent stem cells.
(2) Identification of cell clusters
To further clarify the cell types, we integrated lineage marker genes (for example, endoderm (Endo), mesoderm (Meso), neural ectoderm (NE), non-neural ectoderm (NNE) , and extraembryonic mesenchyme (ExE mech),etc.) with the FateID method, which enabled us to predict cell fate biases based on single-cell transcriptome data. This helped to confirm the identity of pluripotent and differentiating cell populations.
(3) ExE Mech DPR cells

ExE mech DPR cells were defined based on their transcriptional profile that showed differential expression of specific genes associated with the ExE mesoderm (e.g. *FN1*, *COL1A2*, *COL1A1*, *TIMP3*) as described in the methods (Figure 6d-e, Extended Data Figure 5k).
(4) Epi1 vs. Epi2
Regarding the distinction between Epi1 and Epi2, we observed a cell population (Cluster 10) that exhibited a transcriptomic profile similar to the defined Epi cells, but separated from most Epi populations. This cluster was then scored for known pluripotency genes. The stemness score of Cluster 10 is lower than that of the most Epi populations. Based on the scoring results, Cluster 10 was named "Epi2". These evidences allowed us to differentiate it from most Epi cells, which were labeled "Epi1" (Extended Data Fig. 5b-d).

7. The correlation analysis of cell lines (Fig. 2h; Fig. 7i; Extended Data Fig. 2f) shows excessive similarity among lines representing different pluripotent states (naïve, primed, EPS) and data sources. The lack of controls raises doubts about the effectiveness of the analytical methods in differentiating these cell lines. Additionally, the gene expression plots (Fig. 2g; Fig. 7h) employ a color scale that limits the visibility of differences in samples with low gene expression levels.

[Response] Thank you for your valuable comments. To emphasize the similarity at the transcriptional level between our derived spESCs and published reference ESCs, we calculated correlations using only the common genes detected in both our samples and other research samples, while excluding genes detected in one study. This strategy may attenuate differences between different stem cell states. However, our correlation analysis follows the same methodology as previous studies, which have reported correlation scores of approximately 0.97-0.99 among primed ESCs and greater than 0.95 between primed and naïve-like ESCs (10.1016/j.stem.2015.06.004). To address concerns regarding controls, we clarify that we used a primed ESC lines (CES and CES3) (10.1016/j.stem.2015.06.004), as the reference control in Fig. 2h, and human conventional ESCs (10.1016/j.cell.2014.08.029), naïve ESCs (10.1038/s41556-019-0333-2) and other PSCs (10.1016/j.biomaterials.2020.120015; 10.1016/j.cell.2014.12.01) as the reference control in Fig. 7i. Due to the correlation between H1_EPS_rep1 and H1_EPS_rep2 being below 0.94, they exhibited outlier behavior in clustering and reduced color distinction across all samples. Therefore, we removed these two samples in new Fig.7h and Fig.7i. Regarding the use of color scale, we modify it to present the results appropriately in new Fig. 2g, Fig.2h, Extended Data Fig. 2f, Fig. 7h, and Fig.7i.

8. To compare primate PSC characteristics, the authors performed scRNA-seq on PSCs with identical genetic backgrounds and integrated the data with an existing dataset from a human gastrula at Carnegie stage 7. However, concerns

about differences and biases between species, like monkey and human, need to be addressed.

[Response] We thank the reviewer for the insightful comment regarding the potential bias due to species differences. The human CS7 data were used to assist in annotating the types and statuses of primate PSCs. The data represents the closest in vivo data available at the time for the monkey species, which includes various cell types. Comparable in vivo data for monkeys at a similar stage of development were not available at that time. Given the high degree of similarity between humans and monkeys, we used this dataset. To mitigate the effects of species differences, we first integrated human and monkey data using standard integration techniques. Subsequently, stem cell types and statuses were determined by similarity calculations based on genes co-expressed in both species. This dataset served as one of our references, and consistent results were also obtained using the marker genes and the FateID method. By combining these three methods, we were able to infer the final cell state, effectively mitigating the effects of species.

9. Currently, autologous ESCs cannot replace the significant advantages of iPSCs, and this point needs to be clearly articulated in discussions to ensure an accurate and comprehensive understanding of both technologies.

[Response] Thank you for your thorough review and valuable feedback on our work, which we deeply appreciate and agree with. We have followed the reviewer’s advice and have clearly stated that aESCs are currently unable to fully replace iPSCs in L433-L434.

10. Unlike the phenomenon of poor quality and smaller volume of ICM in human split embryos observed in previous studies, this study successfully established ESCs from human split embryos. This significant achievement should be thoroughly discussed in the discussion section to explain the possible reasons for this difference.

[Response] Thank you for your constructive feedback. We have addressed the possible reasons for successfully establishing human ESCs from split embryos in discussion (L468-L474), as suggested by the reviewer.

11. The author proposed using autologous ESCs to treat maybe diseases that may occur in children born through in vitro fertilization (IVF). While this idea is intriguing, it's important to clarify its limitations, including its applicability to IVF offspring, cost-effectiveness, and safety risks related to transferring split versus intact embryos. This highlights the research's significance, especially since studying human split embryos poses ethical challenges.

[Response] Thank you for your valuable comments. We acknowledge the limitations of using autologous ESCs for treating potential diseases in IVF offspring. We have discussed its applicability, cost-effectiveness, and safety risks related to transferring split versus intact embryos in discussion (L466-L468).

Reviewer #2 (Remarks to the Author):

"Production of live monkeys and their genetically matched embryonic stem cells from single embryos" by Chenyang Si and colleagues.

Embryo and biotechnology in non-human primates (NHPs) is less developed than in mice. This is mainly due to the lower availability of NHPs, the significantly higher complexity of working with NHPs, and the fact that NHPs naturally produce only one offspring. In the present study, Chenyang Si and colleagues generated IVF embryos from macaques (rhesus and cynomolgus), split them, and analyzed the developmental potential of the split embryos through embryo transfer to surrogate mothers and ESC line derivation experiments. The authors were successful in both directions: after the transfer of the split embryos, live offspring were born and ESC lines were established. In addition, autologous iPSCs and cloned ESCs (from postnatal fibroblasts) were generated, allowing the authors to obtain three types of pluripotent stem cells (autologous ESCs from split embryos, cloned ESCs and iPSCs) from a living animal. This constellation is new and pushes the boundaries of NHP biotechnology into new directions. It provides interesting new insights into comparative pluripotent stem cell biology and theoretically opens up new perspectives for regenerative medicine with autologous ESCs. However, the medical application of this technology / resource (autologous ESCs) seems questionable to me, considering that currently in most countries where ART is available, 1-5% of all children are born with ART. However, this could change and increase in the coming decades.

[Response] We appreciate the recognition of the importance and relevance of our work. We also agree with the reviewer on the limitations of using autologous ESCs for treating potential diseases in IVF offspring. We have discussed the applicability, cost-effectiveness, and safety risks related to transferring split versus intact embryos in discussion (L466-L468).

The manuscript addresses and interesting and new topic, is well-written and the figures are of high quality.

[Response] We sincerely appreciate your recognition of our topic's novelty and the quality of our writing and figures.

Specific comments:
1. Lines 51-53: The authors say that creating genetically matched ESCs from primates poses technical and ethical challenges. Do the authors mean NHPs and humans or only NHPs? If only NHPs are meant, are there no (or even much bigger) ethical concerns with the use of human embryos for biomedical experiments, such as those conducted in this study?

[Response] Thank you for your comments. Here, we are referring to non-human primates and humans. Although embryo splitting technology has been applied safely in animal husbandry for over 30 years, research in non-human primates is relatively limited. And the successful generation of identical twins has not yet been achieved, which may be related to their single-pregnancy nature. In contrast, humans face ethical, legal, and technical safety challenges that hinder the development of this technology, giving non-human primates a distinct advantage in this field. We have revised the text to clarify this point in L52.

2. Lines 54-59: These publication may also be considered in this context as they might be relevant also for this study: PMID: 39786543, PMID: 31504820

[Response] Thank you for your valuable comments. These publications are closely related to the heterogeneity of blastomeres, so we have included them in the discussion section (L452).

3. Line 67: delete “currently”.

[Response] We are sorry for the mistake. We have deleted the word (L68).

4. Line 88: In the methods section, please describe how the sperm cells were collected. Please also include a more detailed description of the procedures in the animals, in particular the anesthetic and analgesic regimes.

[Response] Thank you for your valuable comments. We have added a detailed description of the sperm cell collection process and outline the anesthetic regimes used in the animals in the methods section (L1003-L1011).

5. Line 107 ff and Fig.1b: No normal embryos are shown. Therefore, it is difficult to say that the cleaved embryos do not differ from normal embryos in their development time.

[Response] Thank you for your valuable comments. We have presented a clearer image showing the developmental progression of control embryos in the new Fig. 1b.

6. Line 111 ff and Fig.1g: Do the authors have an explanation for the fact that

the “identical pairs” have a lower blastocyst rate than the “2/4 embryos”? Is the difference in blastocyst rates between “identical couples” and the “2/4 embryos” also statistically different? If so, is there an explanation for this?

[Response] Thank you for your valuable comments. There is a statistically significant difference between “identical pairs” and “2/4th embryos”, as indicated by the p-value added in the new Fig. 1g. Previous studies have shown that while all blastomeres at the four-cell stage in mice are pluripotent, their developmental potentials differ due to distinctive cleavage division patterns (10.1016/j.celrep.2012.08.029). Specifically, the animal and vegetal blastomeres demonstrate varying degrees of developmental potential, with those at the vegetal pole being more restricted. In our experiments with asymmetric splitting at the 8-cell stage, the blastocyst rate of identical pairs was comparable to that of control embryos (Fig.1h). This may explain the observation, as division at the 8-cell stage avoids the selection of only developmentally restricted vegetal blastomeres. However, it remains unclear whether a similar phenomenon occurs in monkeys. Additionally, the inherent heterogeneity among blastomeres may also play a role in these observed differences (10.1242/dev.01602; 10.1038/nature05458; 10.1016/j.cell.2016.01.047; 10.1016/j.cell.2016.02.032). We have clarified the possible reasons for the significant differences in blastocyst rates in L115-L119.

7. Fig.1i: How were the groups “few OCT4” and “more OCT4” determined? Simply by counting the number of OCT4-positive cells? If so, is this not simply a self-fulfilling prophecy?

[Response] We sincerely apologize for not clearly describing this point. Regarding the classification into "few OCT4" and "more OCT4" groups, we indeed determined these by counting the number of OCT4-positive cells. However, these distinctions are relative and specifically depend on the differences observed between completely identical embryos being compared. This makes it easier to compare them with the normal control.

8. Histology of the teratomas: I can easily see muscle tissue in the histological sections depicting mesoderm. I am also convinced that the histological structures you show for ectoderm and endoderm are correct. However, I would appreciate it if you could show the characteristic histological structures at a larger magnification. Line 139: I think the conclusion is a bit misleading. The number of Epi (OCT4A+) cells was significantly lower in split embryos. I would recommend to include that also in the conclusion of this part.

[Response] Thank you for your valuable comments. We have enlarged all high-power images of the histological structures in response to the reviewers' suggestions to enhance clarity and detail in new Fig. 2e, Fig. 4d, Extended Fig.

2b and Extended Fig. 3h. In addition, following the reviewers' suggestions, we have added a conclusion that highlights the significant reduction in the cell number of split embryos, as noted in L146-L147.

9. Line 178: is “surrogate monkey” the correct term for the offspring obtained from embryo transfer to surrogate mothers? I was a bit confused by this expression.

[Response] We sincerely apologize for any lack of clarity in our description of this point. We appreciate the reviewer’ s feedback and have made the necessary corrections in L184.

10. Line 184: I think the growth rates (increase in body weight) were different (even statistically significant) between controls and split embryos, with split embryo derived animals being - surprisingly - larger than controls. Do the authors have an explanation for this result?

[Response] Thank you for your valuable comments. We have added two additional control animals (originally five control and five split-embryo animals) and re-analyzed the growth rates, as shown in the new Fig. 3c and 3d. The results indicate that the number of time points with significant differences in height and weight between split-embryo and control animals has decreased compared to previous results. This may be due to the small sample size.

11. Line 225: Please check the names of the animals in the text and in Fig. 4b.

[Response] We sincerely apologize for not clearly describing this point. We derived three live monkeys and their aESCs: sp-monkey #1, a rhesus monkey obtained through equal embryo splitting; sp-monkey #2, a rhesus monkey obtained through unequal embryo splitting; and sp-monkey #3, a cynomolgus monkey also obtained through unequal embryo splitting. The photo for sp-monkey #3 was not included. We have checked the names of the animals in the revised manuscript.

12. Line 251 and 381: I would recommend saying “pluripotency (-related) genes” instead of “pluripotent genes”.

[Response] Thank you for your valuable comments. We have revised the manuscript in accordance with the reviewers' suggestions in L258, L388 and L1266.

13. Line 333: As far as I could see from the abstracts of references 45 and 46, the cells analyzed here are mouse embryos / fetuses. Do these cells / references have any relevance to primate PSCs?

[Response] We sincerely apologize for not clearly describing this point. Reference 45 performed a single-cell analysis of the human pancreas in both young and elderly individuals, while Reference 46 examined single-cell transcriptomics and proteomics of the lungs in young and aged mice. Both studies found that transcriptional noise significantly increases across most cell types with aging, highlighting the impact of transcriptional noise on the stability of cellular phenotypes. Furthermore, Reference 47 reported that elevated transcriptional noise is associated with exit from pluripotency. For these reasons, we investigated transcriptional noise in monkey PSCs.

14. Fig.5c: I only see one scale bar. Therefore, I assume that all images were taken and displayed at the same magnification. However, the nuclei of the stem cells without feeders seem to have a much larger diameter than those of the feeder cells. Is this correct? If so, do you have an explanation for this finding?

[Response] We sincerely apologize for not clearly describing this point. In the feeder layer culture system, scale bars in the lower left corner vary in size to emphasize stem cell colony morphology. Conversely, in the feeder-free system, scale bars are uniform and generally larger, making the nuclei of stem cells appear visually larger.

15. Fig.6: what is the number of biological replicates (number of cell lines) that were used to generate all these data? This is not clear to me.

[Response] We sincerely apologize for not clearly describing this point. We generated iPSCs and ntESCs from fibroblasts in one of three monkeys with aESCs, creating the set of genetically matched aESCs, iPSCs, and ntESCs. Using these three cell lines in two culture systems, we conducted single-cell transcriptome analysis. While this provides valuable insights into the transcriptomic signatures of these cell types, individual biological variations may affect the results. We have acknowledged this limitation in our study.

16. Fig.6d: It would be helpful if DPR would be written out once at the first mention in the figure legend.

[Response] Thank you for your valuable comments. We have provided the complete annotations for DPR at the first mention in the Fig. 6d legend (L756).

17. Line 719: As far as I know, the nomenclature for primates suggests writing all gene and protein abbreviations in capital letters. However, proteins in normal font and only nucleic acids in italics. Please check this.

[Response] We appreciate the reviewer’s insightful comments, and we have

made the appropriate adjustments as suggested. Thank you for your constructive feedback.

Reviewer #3 (Remarks to the Author):

In the present manuscript, Si and his colleagues have devised a valuable embryo splitting technique in monkeys. The researchers successfully obtained live monkeys from the split embryos and established genetically matched autologous embryonic stem cells (aESCs), thus validating the feasibility of the refined embryo splitting protocol. This achievement renders primates more efficacious as animal models for research.

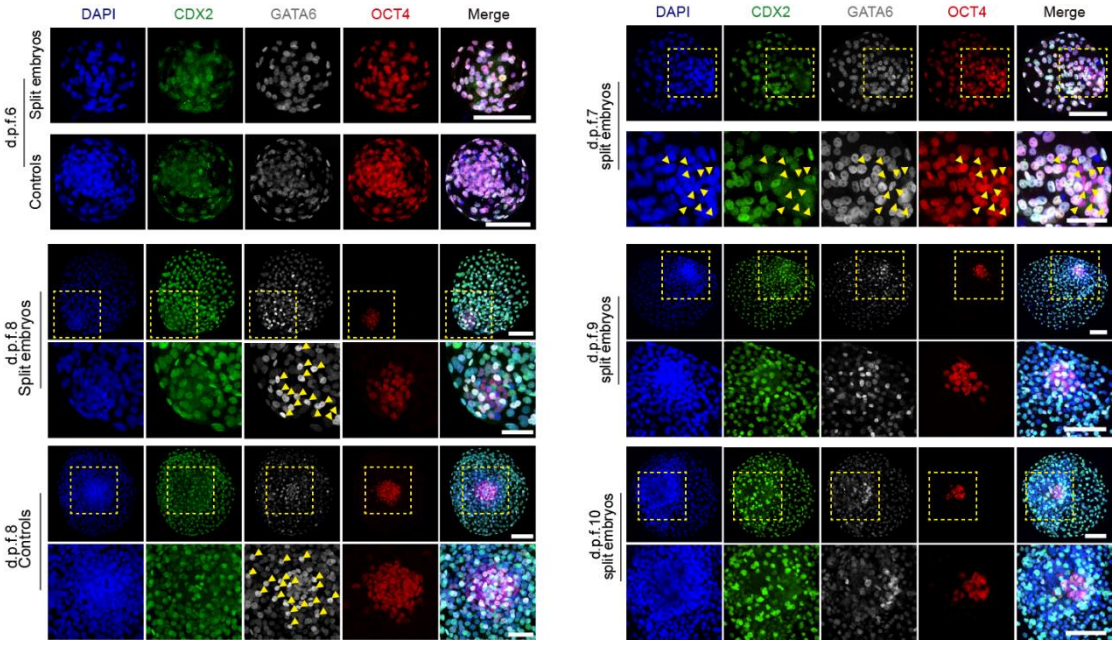
[Response] We sincerely appreciate your acknowledgment of the significance and relevance of our work.

Major Points:
The authors demonstrated that the developmental morphology, timing, and molecular characteristics of the blastocysts derived from split embryos were comparable to those of the control blastocysts. The birth of live monkeys provided robust evidence for the developmental potential of the split embryos. They also compared the autologous ESCs obtained from the split embryos with genetically matched induced pluripotent stem cells (iPSCs) and nuclear transfer embryonic stem cells (ntESCs), confirming their reduced heterogeneity.

Nonetheless, it remains uncertain whether the trophectoderm can be isolated from the blastocysts developed from split embryos, given that trophectoderm (TE) is also a crucial constituent of the blastocyst. Additionally, it is unclear whether there are disparities in the differentiation capabilities among iPSCs, ntESCs, and autologous ESCs (aESCs).

[Response] Thank you for your insightful comments.
First, we analyzed the expression of trophectoderm (TE) marker genes in split embryos from day 6 to day 10 post-fertilization. Our findings revealed that the TE lineage initially co-expressed OCT4 and GATA6. By day 8, the TE lineage predominantly expressed CDX2, with reduced GATA6 expression (as shown in the figure). This expression pattern is consistent with that observed in control embryos, indicating that normal TE lineage segregation occurs in split embryos. Supporting this, previous studies on trophoblast stem cell (TSC) derivation (10.1089/scd.2007.0020; 10.1016/j.stem.2017.11.004; 10.1016/j.cell.2023.11.008) suggest that split embryos have the potential to form TSCs.
Second, split embryos successfully produced live offspring after transplantation, with an efficiency comparable to that of control embryos, further confirming the normal developmental potential of the TE in split embryos.

Based on these findings, we hypothesize that isolating the TE from blastocysts derived from split embryos to establish TSCs is feasible, and we believe this holds significant scientific value. We plan to further investigate the possibility of deriving TSCs from split embryos in future studies.



We also greatly appreciate the reviewer's insightful comments regarding the comparative differentiation capabilities of iPSCs, ntESCs, and aESCs. Our in vivo teratoma formation assays demonstrated that all three cell types—iPSCs, ntESCs, and aESCs—can differentiate into all three germ layers. However, we did not observe significant differences in their differentiation potential. This could be due to the fact that molecular-level differences do not necessarily correlate with functional differences in differentiation capacity (10.1038/nbt.3388), or that any subtle differences may require more sensitive detection methods, such as single-cell multi-omics analysis, organoid models, or functional lineage-specific assays in both in vitro and in vivo settings. We fully agree that systematically characterizing the lineage tendencies of these pluripotent cell types is an important next step. We sincerely thank the reviewer for raising this critical point, and we have revised the manuscript to incorporate these suggestions (L423-L424).

Minor Points:
The first subheading within the results section was designated as " Monkey split embryos show normal developmental potential", yet the data regarding the generation of live monkeys was presented in the second part of the results. The content of the paragraph may need some minor adjustments.

[Response] Thank you for your valuable comments. We have added the term "in vitro" to the title of Result 1 to emphasize the in vitro developmental potential of the split embryos.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the author reports the establishment of embryonic stem cells (ESCs) and viable individuals from a single embryo using embryo splitting technology, resulting in autologous ESCs (aESCs) in non-human primates, as well as genetically matched iPSCs and ntESCs. Single-cell transcriptome analysis reveals that aESCs may offer advantages, including reduced heterogeneity, lower transcriptional noise, and enhanced genomic stability. The author refined the embryo splitting technique for rhesus and cynomolgus monkeys at the 4-cell and 8-cell stages, demonstrating that the split embryos could develop to the blastocyst stage. Split embryos also established ESCs and generated live individuals similar to control embryos, though the aESCs efficiency from equal division at the 4-cell stage was low. To improve this, the author used an unequal division method at the 8-cell stage, which more than doubled the aESCs efficiency. The author also derived iPSCs and ntESCs from monkey fibroblasts, assessing the differentiation potentials, genomic stability, and transcriptional noise among these three types of pluripotent stem cells using single-cell transcriptome sequencing. Finally, it was demonstrated that establishing human ESCs from split human embryos is feasible. The work requires a significant effort and complex data, as deriving aESCs from a single embryo and establishing three genetically consistent pluripotent stem cells (PSCs) for comparison are highly challenging. These achievements are difficult yet important, with promising applications.

[Response] We appreciate the recognition of the importance and relevance of our work. We agree with the reviewer on the importance of establishing aESCs from a single embryo. We believe that this advancement may contribute to the field of pluripotent stem cell research and have potential applications in regenerative medicine. Thank you very much for your valuable feedback.

Although cloning through embryo splitting was reported many years ago, the low efficiency of this process has always been a challenge. This work confirmed a live birth rate of individual split embryos (7/37, 18.9%) that was comparable to that of the control embryos in this study (11/58, 19.0%) but higher than the previous live birth rate of split embryos in rhesus monkeys (7/64, 10.9%) (Mitalipov, S.M. et al., Biol Reprod, 2002). However, the aESCs efficiency (3/26, 11.5%) remains relatively low, with room for improvement. Additionally, previous studies compared isogenic ntESCs and iPSCs but did not compare ESCs from the same individual (at least in non-human primates). This work is the first to obtain three types of PSCs in non-human primates with a consistent genetic background from a single individual, including aESCs, iPSCs, and ntESCs. This reduces the interference caused by genetic difference.

[Response] We appreciate the reviewer’s insightful comments regarding our findings. We acknowledge that while our live birth rates of split embryos are comparable to controls and improved over previous reports, the aESCs efficiency is low and needs improvement.

Notably, the asymmetric embryo splitting method has significantly increased aESC efficiency, indicating that further increasing the number of EPI cells from split embryos could improve outcomes. Consequently, the stem cell chimera method may also be effective, utilizing stem cell chimerism to increase the cell numbers of split embryo for transplantation, with both stem cells and split embryos derived from the same embryo. We also appreciate the recognition of the significance of our work in obtaining three types of pluripotent stem cells-aESCs, iPSCs, and ntESCs from a single non-human primate, reducing genetic variability for more accurate comparisons.

Specific points:
1. How do the authors define the blastocyst stage in Fig. 1 legend L649?

[Response] We sincerely apologize for not clearly describing this point. In the legend for Fig. 1 (L673), we define the blastocyst stage as an embryo that has been cultured until the sixth day post-fertilization and features a well-defined cavity. Additional explanations can be found in L673-L674.

[Comment] Solved.

[Response] Thanks.

2. What does the 2/4th embryos indicate in L93? Similarly, what do 3/8th or 5/8th embryos represent?

[Response] We sincerely apologize for not clearly describing this point. The 2/4th embryo refers to an embryo reconstructed from any two blastomeres of a 4-cell stage embryo. Similarly, the 5/8th and 3/8th embryos denote embryos reconstructed from any five blastomeres and the remaining three blastomeres of an 8-cell stage embryo, respectively.

[Comment] Understandable, I suggest adding concrete details in the paper.

[Response] Thank you for your valuable suggestions, we have added concrete details in L1190-1194.

3. What is the purpose of presenting data for 2/4th embryos that show either fewer or more Oct-4 positive cells in Fig. 1 i-k and Ext Data Fig. 1 i-j? Please clarify this in the text or figure legend.

[Response] We sincerely apologize for not clearly describing this point. Epi cell number is a crucial determinant for successful implantation. Published studies have shown that fewer than four Epi cells in split mouse embryos hinder fetal development (10.1016/j.celrep.2012.08.029), while higher Epi cell counts in rhesus monkey blastocysts promote successful pregnancies (10.1016/j.cell.2011.12.007). Therefore, the low efficiency in obtaining aESCs may be due to the unintended selection of 2/4th embryos with lower

Epi cell counts for transfer within genetically matched pairs. This is supported by improved aESCs efficiency through the selection of 5/8th embryos for transfer, which have characteristics similar to 2/4th embryos that contain more Epi cells. We have clarified this in L439-L450.

[Comment] This is persuasive.

[Response] We appreciate your feedback.

4. What species are used in L145 and L192?

[Response] We sincerely apologize for not clearly describing this point. In L151, 2/4th embryos from 20 cynomolgus and 5 rhesus monkeys were used to establish ES cell lines, with detailed information provided in Table 2. In L200, 2/4th embryos were transferred to 13 cynomolgus and 1 rhesus monkey recipients, while 5/8th embryos were transferred to 4 cynomolgus and 9 rhesus monkey recipients. Specific details can be found in Supplementary Figure 3c.

[Comment] Solved.

[Response] Thanks.

5. At what developmental stage were the split embryos transferred to surrogates in L170? Were they further cultured in vitro before this?

[Response] We sincerely apologize for not clearly describing this point. The split embryos were cultured in vitro until they reached the morula or blastocyst stage, which typically occurs on the fifth or sixth day post-fertilization. At this developmental stage, the embryos were transferred to the recipient monkeys.

[Comment] OK.

[Response] Thanks.

6. The authors should clarify their classification of cell clusters in L277-286. While the DPR designation likely refers to cells co-expressing pluripotency and lineage-specific genes, the criteria for this are unclear. Specifically, how is an ExE mech DPR cell defined, and what distinguishes Epi1 from Epi2?

[Response] We thank the reviewer for the valuable comment regarding the classification of cell clusters in L277-286. We performed a systematic approach to define and annotate the cell populations as follows:
(1) Pluripotency gene expression and correlation analysis
First, we detected the expression of all known pluripotency genes and performed

correlation analysis with d.p.f.10-14 Epi cells from the cynomolgus monkey to confirm the pluripotent nature of these cells. We then performed a correlation analysis of our dataset with all cells from the human Carnegie stage 7 (CS7) to define "Epi" cells. This approach allowed us to identify pluripotent stem cells.

(2) Identification of cell clusters

To further clarify the cell types, we integrated lineage marker genes (for example, endoderm (Endo), mesoderm (Meso), neural ectoderm (NE), non-neural ectoderm (NNE) , and extraembryonic mesenchyme (ExE mech),etc.) with the FateID method, which enabled us to predict cell fate biases based on single-cell transcriptome data. This helped to confirm the identity of pluripotent and differentiating cell populations.

(3) ExE Mech DPR cells

ExE mech DPR cells were defined based on their transcriptional profile that showed differential expression of specific genes associated with the ExE mesoderm (e.g. FN1, COL1A2, COL1A1, TIMP3) as described in the methods (Figure 6d-e, Extended Data Figure 5k).

(4) Epi1 vs. Epi2

Regarding the distinction between Epi1 and Epi2, we observed a cell population (Cluster 10) that exhibited a transcriptomic profile similar to the defined Epi cells, but separated from most Epi populations. This cluster was then scored for known pluripotency genes. The stemness score of Cluster 10 is lower than that of the most Epi populations. Based on the scoring results, Cluster 10 was named "Epi2". These evidences allowed us to differentiate it from most Epi cells, which were labeled "Epi1" (Extended Data Fig. 5b-d).

[Comment] The classification of the ExE mech DPR cell population is understandable. For EPI1 and EPI2, the authors list highly expressed genes: LDHA, MT2A, CDK1, and KPNA2. Given these genes' expression profiles, which suggest a tendency for rapid proliferation and self-renewal, I consider these cells as intermediate - state cells. Unless emphasizing them is necessary, I suggest not classifying them separately as EPI2, just merging them with EPI.

[Response] Thank you for your valuable suggestions. We agree with you that these 'Epi2' cells likely represent an intermediate state cell. In our study, in order to minimize the impact of these intermediate state cells on subsequent analyses, we separated them into EPI1 and EPI2.

7. The correlation analysis of cell lines (Fig. 2h; Fig. 7i; Extended Data Fig. 2f) shows excessive similarity among lines representing different pluripotent states (naïve, primed, EPS) and data sources. The lack of controls raises doubts about the effectiveness of the analytical methods in differentiating these cell lines. Additionally, the gene expression plots (Fig. 2g; Fig. 7h) employ a color scale that limits the visibility of differences in samples with low gene expression levels.

[Response] Thank you for your valuable comments. To emphasize the similarity at the transcriptional level between our derived spESCs and published reference ESCs, we

calculated correlations using only the common genes detected in both our samples and other research samples, while excluding genes detected in one study. This strategy may attenuate differences between different stem cell states. However, our correlation analysis follows the same methodology as previous studies, which have reported correlation scores of approximately 0.97-0.99 among primed ESCs and greater than 0.95 between primed and naïve-like ESCs (10.1016/j.stem.2015.06.004). To address concerns regarding controls, we clarify that we used a primed ESC lines (CES and CES3) (10.1016/j.stem.2015.06.004), as the reference control in Fig. 2h, and human conventional ESCs (10.1016/j.cell.2014.08.029), naïve ESCs (10.1038/s41556-019-0333-2) and other PSCs (10.1016/j.biomaterials.2020.120015; 10.1016/j.cell.2014.12.01) as the reference control in Fig. 7i. Due to the correlation between H1_EPS_rep1 and H1_EPS_rep2 being below 0.94, they exhibited outlier behavior in clustering and reduced color distinction across all samples. Therefore, we removed these two samples in new Fig.7h and Fig.7i. Regarding the use of color scale, we modify it to present the results appropriately in new Fig. 2g, Fig.2h, Extended Data Fig. 2f, Fig. 7h, and Fig.7i.

[Comment] This is much more convincing.

[Response] We appreciate your feedback.

8. To compare primate PSC characteristics, the authors performed scRNA-seq on PSCs with identical genetic backgrounds and integrated the data with an existing dataset from a human gastrula at Carnegie stage 7. However, concerns about differences and biases between species, like monkey and human, need to be addressed.

[Response] We thank the reviewer for the insightful comment regarding the potential bias due to species differences. The human CS7 data were used to assist in annotating the types and statuses of primate PSCs. The data represents the closest in vivo data available at the time for the monkey species, which includes various cell types. Comparable in vivo data for monkeys at a similar stage of development were not available at that time. Given the high degree of similarity between humans and monkeys, we used this dataset. To mitigate the effects of species differences, we first integrated human and monkey data using standard integration techniques. Subsequently, stem cell types and statuses were determined by similarity calculations based on genes co-expressed in both species. This dataset served as one of our references, and consistent results were also obtained using the marker genes and the FateID method. By combining these three methods, we were able to infer the final cell state, effectively mitigating the effects of species.

[Comment] Understandable.

[Response] We appreciate your feedback.

9. Currently, autologous ESCs cannot replace the significant advantages of iPSCs, and this point needs to be clearly articulated in discussions to ensure an accurate and

comprehensive understanding of both technologies.

[Response] Thank you for your thorough review and valuable feedback on our work, which we deeply appreciate and agree with. We have followed the reviewer's advice and have clearly stated that aESCs are currently unable to fully replace iPSCs in L433-L434.

[Comment] Solved.

[Response] Thanks.

10. Unlike the phenomenon of poor quality and smaller volume of ICM in human split embryos observed in previous studies, this study successfully established ESCs from human split embryos. This significant achievement should be thoroughly discussed in the discussion section to explain the possible reasons for this difference.

[Response] Thank you for your constructive feedback. We have addressed the possible reasons for successfully establishing human ESCs from split embryos in discussion (L468-L474), as suggested by the reviewer.

[Comment] The authors noted that the split embryos were spatially rearranged, with proper gene expression and normal developmental clock. It would be beneficial for them to further explore this successful experiment, particularly regarding the timing of splitting, the integrity of the blastomeres, and the role of cell mechanics.

[Response] We thank the reviewer for this constructive suggestion. Our subsequent work will employ multi-omics to compare developmental potential and regulatory mechanisms in isogenic twin embryos, while using small molecules or stem cell chimerism strategies to optimize autologous ESC efficiency. This constructive feedback significantly strengthens our research roadmap.

11. The author proposed using autologous ESCs to treat maybe diseases that may occur in children born through in vitro fertilization (IVF). While this idea is intriguing, it's important to clarify its limitations, including its applicability to IVF offspring, cost-effectiveness, and safety risks related to transferring split versus intact embryos. This highlights the research's significance, especially since studying human split embryos poses ethical challenges.

[Response] Thank you for your valuable comments. We acknowledge the limitations of using autologous ESCs for treating potential diseases in IVF offspring. We have discussed its applicability, cost-effectiveness, and safety risks related to transferring split versus intact embryos in discussion (L466-L468).

[Comment] Thank you for the additional comments.

[Response] We appreciate your feedback.

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed my points. The biggest weakness of the manuscript is the limited number of autologous cells (n=1 for aESCs, ntESCs and iPSCs, if I understand correctly). Knowing that even cell lines produced using the same method (such as individual iPSC lines from one animal) can show significant differences from each other, the differences between PSCs produced in different ways should not be over-interpreted. In my opinion, however, this limitation was stated clearly enough in the concluding paragraph of the manuscript.

In general, the MS represents a significant and very interesting advance in the field of NHP/human stem cell research, embryology and biotechnology.

Just a few minor things:
L384: pluripotent > pluripotency (as already changed in all other places in the MS).

[Response] We regret any oversight in implementing these revisions throughout the manuscript and confirm that all requested modifications have been carefully incorporated at the specified locations (Lines 242, 387).

L485-487: I'm not a native speaker, but the syntax of this sentence seems unusual to me. Please check it.

[Response] We appreciate the feedback and have revised the text in Lines 491-493 for clarity.

L1175 ff: The tense of this paragraph is different from the other paragraphs in the “Materials and methods” section. Please check.

[Response] We appreciate the feedback and confirm the verb tenses in Lines 707-714 have been standardized to ensure grammatical consistency throughout the Methods section.

Reviewer #3 (Remarks to the Author):

I thank the authors for their detailed responses

1. Regarding the isolation and functional validation of TE from split embryos:
The data regarding the expression of trophectoderm (TE) marker genes (e.g., OCT4, GATA6, and CDX2) in split embryos, as well as the successful production of live offspring provide some evidence of TE functionality. However, these observations do not directly demonstrate the ability of TE from split embryos to differentiate into functional trophoblast stem cells (TSCs) or to support implantation and placental development. Given the low

efficiency of live offspring production, it remains unclear whether the TE in split embryos is fully equivalent to that in control embryos. To strengthen their conclusions, I strongly recommend that the authors perform direct experiments to isolate and functionally validate TE-derived TSCs from split embryos. This would provide more robust evidence of the developmental potential of the TE in this context.

2. Regarding the differentiation capabilities of iPSCs, ntESCs, and aESCs:
While the teratoma assays in Figure 2 and Figure 4 demonstrate that iPSCs, ntESCs, and aESCs are capable of differentiating into all three germ layers, I believe that a more in-depth analysis and discussion of these data would significantly strengthen the manuscript.

Overall Recommendation:
If the authors can provide additional evidence (e.g., TSC derivation) or at least include a more thorough discussion of the limitations and future directions, I would be willing to recommend the manuscript for publication in Nature Communications.

[Response] We thank the reviewer for these constructive comments. In the discussion section, we have highlighted the necessity to further clarify the functional differences among the three types of stem cells (L426-427) and the establishment of TS cells from split embryos (L498-502).