nature portfolio

Peer Review File

Crosstalk between H3K4me3 and oxidative stress is potential target for the improvement of ART-derived embryos

Corresponding Author: Professor Keliang Wu

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 vitro fertilization (IVF) is one important type of assisted reproductive technology. The different outcome between IVF and in vivo fertilization (IVO) has been a long-known problem associated with IVF. In this study, the authors find that global reduction of H3K4me3 is seen in IVF blastocysts. This phenomenon is associated with increased oxidative stress and increased expression of Kdm5a. Inhibition of Kdm5 by CPI-455 treatment significantly rescued the differential gene expression. In addition, the authors find that in human clinics day5 IVF blastocysts has higher H3K4me3 and better clinical outcome compared to day6 IVF blastocysts, which is somewhat similar to that seen in IVO embryos compared to IVF embryos. Overall, the findings are very interesting and has the potential to improve IVF clinics. I think it is a very good candidate for Nature communications, pending some suggestions for further improvement of the manuscript.

1. For oxidative stress, could anti-oxidant be more effective or feasible than Kdm5 inhibitor treatment? Why NMN was selected as the anti-oxidant tested? Have the authors tried other small molecules? A better comparison between the treatment of CPI-455 and anti-oxidants may strengthen our understanding of this process and potential clinical application of the small molecules.

2. The IVO embryos performed better than IVF embryos in terms of birth rate, which is expected. The key conclusion of this study includes that CPI-455 treatment alleviates defects associated with in vitro culture. Therefore, whether CPI-455 treatment improves birth rate of IVF embryos is interesting to test. To be clear, it is a suggestion that this reviewer is interested to ask, but not a requirement.

3. The decrease of H3K4me3 is very big in IVF blastocysts. It will be great to compared the current data to the earlier reports of H3K4me3 in blastocysts cultured in vitro (DOI: 10.1038/nature19362) and IVO blastocysts (DOI: 10.1038/nature19361). This comparison may strengthen the finding.

4. Similarly, DNA methylation from published in IVF blastocysts (DOI: 10.1016/j.stem.2018.07.017;) and IVO blastocysts (DOI: 10.1016/j.cell.2014.04.017; 10.1038/cr.2017.82) could be a good reference for the data in the current study.

5. The language of the manuscript make it difficult to read at many places. Therefore, it is recommended to be improved by a native speaker or professional editing service. For example, Page 3 Line 22 "At last, human data based on day5- and day6-blastocysts obtained via ART also revealed a correlation between H3K4me3 level (at blastocyst stage) and clinical outcomes given that day5-blastocysts harbored globally higher H3K4me3 levels as well as better clinical outcomes." is better to be "Finally, analysis of human ART-derived day5 and day6 blastocysts revealed a correlation between H3K4me3 levels at the blastocyst stage and clinical outcomes, with day5 blastocysts exhibiting globally higher H3K4me3 levels and better clinical outcomes compared to day6 blastocysts." Page 5 Line 7, "At blastocyst stage, both ICM (inner cell mass) and TE (trophectoderm) markers genes became dysregulated following IVF." is better to be "At the blastocyst stage, expression of marker genes for both the inner cell mass (ICM) and the trophectoderm (TE) was dysregulated in IVF-derived embryos."; Page 8, Line 20, "all" is not appropriate here which I'd suggest to be removed.

6. In Figure 1b, the labeling of developmental stages should follow their chronological order.

7. The number of DEGs in L2C stage is a lot. The conclusions related the L2C shall be carefully made. For example, Page

5, Line 27, "Moderate differences between IVO and IVF at gene transcription level during early embryo development from PN5 to morula" might not be appropriate.

8. The H3K27me3 intensity in Extended Data Fig. 3c does not match the description in Page 9. Since based on the Figure, H3K27me3 is almost non-detectable in IVO blastocysts.

9. It will be good to describe the samples collected for scRNA-seq more clearly because IVC embryos are known to vary from each other.

10. Did gene analyzed in Fig. 5d include all the genes shown in Extended Data Fig. 2b? It is recommended to do so to give a whole picture.

11. The H2O2 treatment needs to be described more clearly. Were IVO or IVF embryos used in the treatment?

12. Day5 embryos performs significantly better than Day6 blastocyst in human clinics. This finding is fantastic. It would be better to discuss more to give the readers a clear connection between Day5 vs Day6 and IVF vs IVM. In addition, mouse IVF embryos developed a bit slower compared to IVO embryos, does CPI-455 treatment improve this? This might be another link.

Reviewer #2

(Remarks to the Author)
It is crucial to understand the underlying mechanisms that lead to adverse outcomes in ART. In this manuscript, the authors report a significant reduction in H3K4me3 levels and an increase in oxidative stress in IVF-derived embryos. Further analysis demonstrate that treatment with CPI-455 can rescue these adverse effects and promote embryo development in vitro. Importantly, their findings in human embryos indicate that CPI-455 may have potential for future clinical application.
I have following questions and suggestions that may help strengthen their conclusions:
Major:
1.In Figure 5, the authors show that CPI-455 treatment facilitates blastocyst formation in IVF-embryos. Could the authors further examine whether this treatment improves the live-birth rate of IVF-embryos, similar to the analysis in Figure 6a?
2.Could the authors explore inter-individual variability in H3K4me3 levels among human day-5 blastocysts, for example across different morphological grades? As day-5 blastocysts are generally preferred for embryo transfer, such analysis may help enhance the clinical significance of their findings.
3.The authors may consider revise the statements on page 16, lines 1-4. Their current Chip-seq data are informative, but not directly linked to clinical outcomes. Whereas previous studies have reported direct associations between DNA methylation and ART clinical outcome (e.g. https://doi.org/10.1038/s41422-023-00809-z).
Minor:
1.The authors state that IVF-embryo have lower level of Carm1 expression, which would promote the formation of TE (page 8, lines 12-14). This appears inconsistent with the findings that the number of TE cells is decreased in IVF embryos, as shown Figure 2a. Some clarification would be helpful.
2.In mouse IVF embryos, it appears that the increased ROS (Figure 2d) and Tet3 level (Figure 2g) are the potential contributors of decreased global DNA methylation level (Figure 3h). Moreover, CPI-455 does not seem to reduce ROS levels in IVF embryos to those seen in IVO embryos (Figure 5g vs. Figure 2d). These raise my question that, instead of inhibiting a single histone demethylase use CPI-455, would decrease the oxidative stress offer a broader improvement in embryo quality? (e.g. using NMN, as the authors mentioned)
3.The authors forgot to cite Figure 6d in the main text.

Reviewer #3

(Remarks to the Author)
The work titled “The crosstalk between H3K4me3 and oxidative stress could be potential
target for the improvement of ART-derived embryos ” examined transcriptomic and epigenetic marks of mouse and human pre-implantation embryos generated by IVF or in vivo
The authors found that:
1. Moderate differences between IVO and IVF at gene transcription level at each stage of preimplantation development
2. Enrichment of NRF2 and Bach1 sequences (TF involved in dealing with Oxidative stress) in promoter of upregulated in vivo generated blastocysts.
3. ICM marker genes, such as Pou5f1 and Nanog had lower expression, and TE marker gene Eomes, exhibited higher expression levels in mouse IVF-conceived blastocysts, suggesting a failure of activation of Carm1 during ZGA process following IVF.
4. Tet3 and Kdm5a (responsible for demethylation of H3k4me3, activating) are increased and kdm6b (responsible for demethylation of H3k27me3, repressive) are decreased in mouse IVF blastocysts.
5. Massive decreased H3K4me3 in IVF blastocysts (by IF and ULI-NChiP) in more than 14,000 regions which could be ascribed to the higher oxidative stress in IVF-derived blastocysts. On the other hand there was minimal higher H4K27me3 (54 regions) in IVF blastocysts.
6. Increasing oxidative stress, by culturing embryos in H2O2 resulted in decreased in H3k4me3
7. There is also decreased DNA methylation in IVF embryos (Figure 3h), particularly in area where tet3 binds the promoter.
8. IVF generated embryos cultured for 96 hours show normal trilaminar structure but reduced size and had higher proportions of trophoblast progenitor cells (TPC), trophoblast giant cells (TGC) and Spongiotrophoblast (SpT) which are in a

more differentiated status compared to ExE ectoderm. Also the proportion of epiblast cells was lower.
9. The use CPI-455 (5 microM for 96 hours, started at the zygote stage), the inhibitor of KDM5 (which catalyzes the demethylation of H3K4), successfully rescued the H3K4me3 as well as transcription levels of genes related to ATP, mitochondrion, oxidative stress and signal transduction.
10. Day 5 human blastocyst had higher H3K4me3 levels than CD6 human blastocysts; day 5 blastocysts had better clinical outcomes
11. The use of CPI-455 supplement enhanced the formation of high-quality human embryos

The authors conclude that there is crosstalk between increased oxidative stress and decreased levels of H3K4me3, which may underlie the negative clinical outcomes associated with ART. They propose that disruption of the epigenetic landscape in IVF-conceived blastocysts is secondary to impaired bivalency at promoter regions, particularly due to reduced H3K4me3, which in turn may alter gene expression. They further suggest that the use of CPI-455 could improve developmental outcomes in ART-derived embryos.
The overall topic is of high significance, given the large and growing number of children conceived through IVF. The authors should be commended for performing extensive studies and applying multiple omics techniques to explore how IVF-derived embryos differ from their in vivo counterparts. The observation of reduced H3K4me3 in mouse IVF embryos is important and supports a mechanistic link between oxidative damage and altered chromatin states.
However, several major concerns remain, especially regarding the interpretation and use of human embryo data:

Major Concerns
1. CD5 vs CD6 Human Embryos:
The comparison between CD5 and CD6 human embryos is not convincing. The authors claim that CD6 embryos show an epigenomic profile resembling IVF mouse embryos, whereas CD5 embryos resemble in vivo mouse embryos. However, this interpretation appears inconsistent with their hypothesis. If CD6 embryos show lower H3K4me3, does this imply they have experienced more oxidative stress? This seems unlikely, and alternative explanations should be considered. The authors are encouraged to conduct additional experiments using mouse embryos to determine whether delayed blastocyst development (e.g., 113+24h) further alters H3K4me3 levels and/or DNA methylation.
2. Human Embryo Ethics and Consent:
The generation and use of human embryos require clearer ethical documentation. Human embryos were generated from immature oocytes and donor sperm. A full description of the informed consent process should be provided, including whether patients were aware that their oocytes would be fertilized with donor sperm and used for research. A translated version of the consent form in English should be included.
3. Embryo Numbers and Metadata:
A detailed summary table should be added in the supplementary materials, specifying:
o The total number of human embryos generated, donated, and used in each experiment
o Whether they were treated with CPI
o Gardner morphological scores
o Egg and sperm donor age and diagnosis
o Whether embryos were generated by IVF or ICSI
o If preimplantation genetic testing (PGT) was performed
4. Morphology Matching and Infertility Cause:
Were CD5 and CD6 embryos matched for morphology grade? Were causes of infertility similar between the two groups?
5. DNA Methylation Literature:
The introduction and discussion should better address the contrasting reports on DNA methylation differences between IVF- and naturally-conceived children. Studies have shown only minimal differences in some tissues. Relevant mouse studies (e.g., Doherty, 2004; Ruggeri, 2020) should also be cited.
6. NRF2/Bach1 Binding in IVO Embryos:
The authors note increased NRF2 and Bach1 binding in promoters of genes upregulated in in vivo embryos. Please clarify the significance of this observation—how does it support or contradict the proposed model?
7. Discussion Clarity:
The discussion is overly superficial and should be strengthened, particularly regarding the observed decline in H3K4me3 (Figure 3G). A deeper synthesis of the findings is necessary.
8. TET3 Expression in Figure 3K:
The figure shows two populations of IVF embryos with high and low TET3 expression. What accounts for this heterogeneity? Could it reflect embryo subtypes with differential epigenetic responses?
9. Marker Expression vs Cell Types (Figure 4i):
The authors conclude that IVF embryos contain more trophoblast progenitor cells (TPC), trophoblast giant cells (TGC), and spongiotrophoblast (SpT) cells. However, this is based solely on marker gene expression. In situ hybridization or lineage tracing would be necessary to validate differences in actual cell populations.
10. Replicates in Omics Experiments:
The number of biological replicates used for each omics approach should be clearly stated. A minimum of three replicates is expected.
11. Causality in Embryo Transfer Findings:
On page 14, the authors suggest that reduced H3K4me3 accounts for lower live birth rates after IVF. However, this is correlative. They should test whether CPI-455 treatment improves both H3K4me3 and live birth rates to support causality.
12. Embryo Developmental Speed:
On the same page, the authors assert that IVO embryos develop faster. Please provide evidence for this claim.
13. Clinical Relevance of CD5 vs CD6 Transfers:
The discussion should include literature on outcomes of CD5 vs CD6 embryo transfers. Additionally, the reported dataset

(n=2584 per group) is only relevant if all patients underwent single embryo transfer of a CD5 or CD6 embryo. Otherwise, the data interpretation is flawed.

Minor Points
14. Replace "delayed embryo culture" with "extended culture."
15. Define P5 stage and stage 4 blastocyst in the text.
16. In Figure 1C, show IVF and IVO results side-by-side for each stage to enable easier comparison.
17. In Figure 3E, provide a more detailed description of the ChromHMM method.
18. In Figure 3F, clarify how to interpret overlaps in bivalent regions between IVF and IVO embryos.
19. The increase in Eomes expression in IVF embryos contradicts prior findings (e.g., Giritharan 2012), which showed downregulation. This discrepancy should be addressed.
20. In Figure 5B, explain what 4× and 10× represent (e.g., microscopy magnification?).
21. In Figure 6G, human day 5 embryos appear to have both higher and lower DNA methylation than day 6 embryos. Could this indicate multiple subpopulations?
22. Figure 6J requires more detailed explanation of the findings and their biological significance.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done a great job in revising the manuscript. My comments have been fully addressed.

Reviewer #2

(Remarks to the Author)
I have reviewed the revised manuscript. The authors have fully addressed all of my previous comments. The paper is now suitable for publication.

Reviewer #3

(Remarks to the Author)
The authors have responded satisfactorily to the majority of queries

Few comments persist:

1. Ethics of Human Embryos:
The consent states: “Research is limited to abandoned sperm, seminal plasma, follicular fluid, immature eggs, abnormal fertilized eggs or embryos of poor quality that cannot be transferred, and will not be used for reproductive purpose”. More explanations are needed for the following points:
o A large number of embryos (n=256) were generated from M1 oocytes, underwent ICSI (with sperm donor: Were egg donors and sperm donors aware that embryos would have been generated for research purposes?) and were developed to the blastocyst stage. Is this covered by the consent?
o Same excellent embryos 4AA were tested
2. Table R3-1: infertility causes by non ovarian factors is not a clinical diagnosis. This should be well explained

Reviewer #4

(Remarks to the Author)
This research triggers ethical concerns about source of reproductive materials, adequacy of consent from 'donor' parties, Oversight by a research ethics committee and manner and length of manipulation of donated embryonic materials.

I am satisfied that the research reported has followed appropriate ethical standards in all respects. The consent form for donors seems sound, IRB oversight and approval is documented. Manipulation of reproductive materials is ethically sound both intervention and time of interventions.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In vitro fertilization (IVF) is one important type of assisted reproductive technology. The different outcome between IVF and in vivo fertilization (IVO) has been a long-known problem associated with IVF. In this study, the authors find that global reduction of H3K4me3 is seen in IVF blastocysts. This phenomenon is associated with increased oxidative stress and increased expression of Kdm5a. Inhibition of Kdm5 by CPI-455 treatment significantly rescued the differential gene expression. In addition, the authors find that in human clinics day5 IVF blastocysts has higher H3K4me3 and better clinical outcome compared to day6 IVF blastocysts, which is somewhat similar to that seen in IVO embryos compared to IVF embryos. Overall, the findings are very interesting and has the potential to improve IVF clinics. I think it is a very good candidate for Nature communications, pending some suggestions for further improvement of the manuscript.

1. For oxidative stress, could anti-oxidant be more effective or feasible than Kdm5 inhibitor treatment? Why NMN was selected as the anti-oxidant tested? Have the authors tried other small molecules? A better comparison between the treatment of CPI-455 and anti-oxidants may strengthen our understanding of this process and potential clinical application of the small molecules.

We thank the reviewer for these helpful comments. In this work, we found that mouse IVF-conceived blastocysts exhibit aberrant H3K4me3 and ROS/ATP/NADH/GSH levels. The profound H3K4me3 differences between IVF- and IVO-conceived blastocysts thus inspired us to further apply CPI-455 to early embryo development *in vitro*.

In fact, on the other hand, as mentioned by the reviewer, the abnormal ROS levels in IVF-conceived blastocysts may indicate anti-oxidant could be effective towards the improvements of the quality of IVF-conceived embryos. In this study, we selected NMN, an effective precursor of NAD^{+}[1], for several reasons: Firstly, NMN has already been suggested to possess antioxidant property[2]. More importantly, previous studies have revealed that female reproductive aging was accompanied by mitochondrial dysfunction[3] (including the increase of ROS and decrease of ATP, NADH and NAD^{+}) and the reduction of H3K4me3[4,5], reminiscent of the properties of IVF-conceived blastocysts reported in our work. Of note, a recent review[3] emphasized NMN was a potential therapeutic strategy to alleviate aspects of female reproductive aging (i.e., improving pregnancy rate and blastocyst development via restoring NAD^{+}, NADH and mitochondrial activity). Hence, we speculated NMN may also be capable of improving the quality of IVF-conceived embryos. At last, unlike astaxanthin and vitamin E, another two anti-oxidants, the supplementation of NMN could indeed facilitate the formation of blastocyst (Figure R1-1A). We thus further performed Smart-seq2 and measured ROS/NAD^{+}/ATP levels to clarify the potential mechanisms behind such a phenomenon and compared the effects of NMN to that

upon CPI-455 treatment.

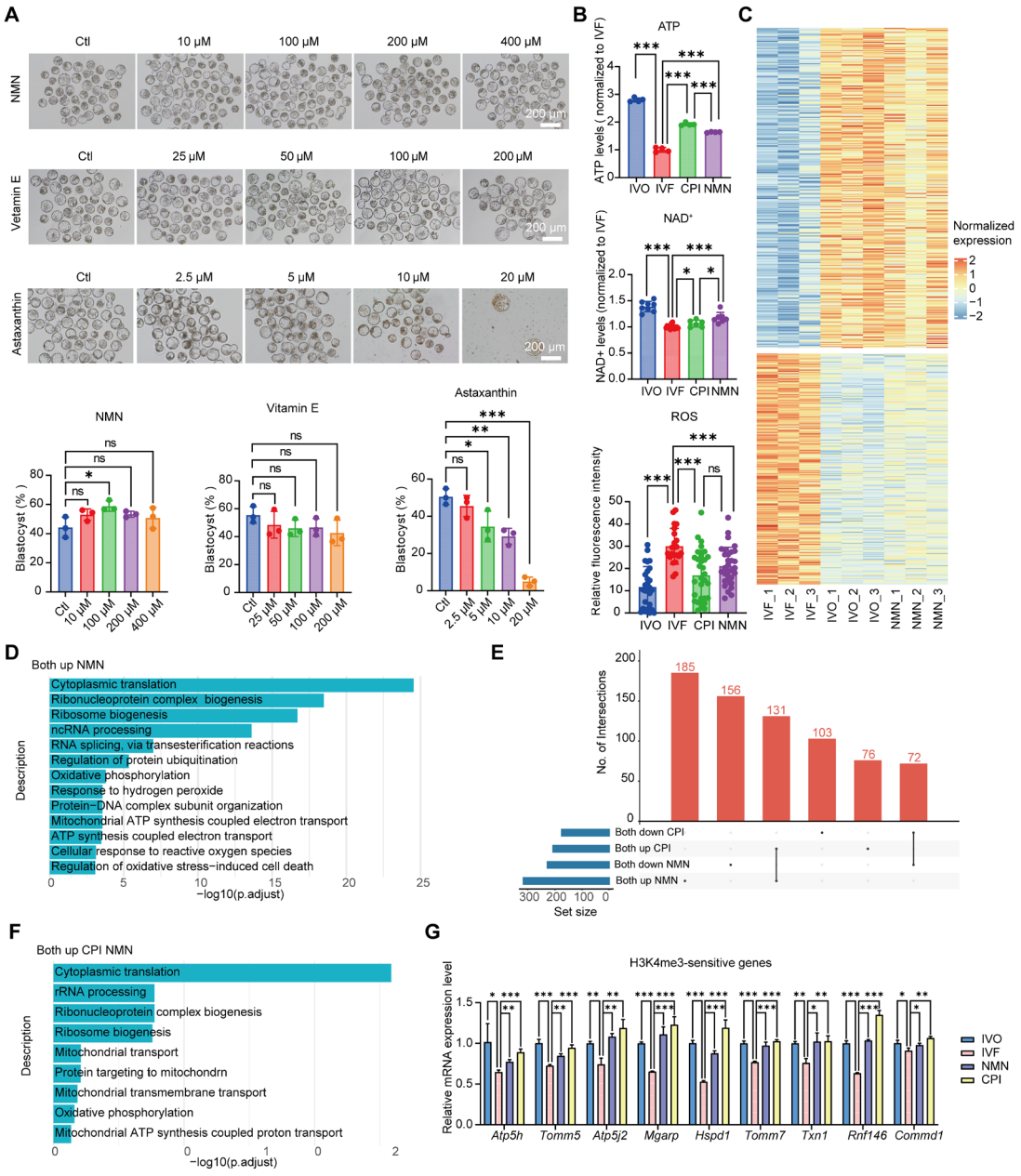
Akin to CPI-455, NMN treatment mitigated oxidative stress and promoted ATP/NAD^+ formation (Figure R1-1B). In addition, as expected, NMN seems to be more effective towards the restoration of NAD^+ level (Figure R1-1B). Nevertheless, with respect to oxidative stress, NMN treatment alleviated oxidative stress to a level similar to that upon CPI-455 treatment (Figure R1-1B). Taken together, these results indicated that NMN supplementation could restore mitochondrial activity, which further contributed to the formation of blastocyst.

On the other hand, based on Smart-seq2 data, we further identified successfully rescued genes (SRGs) upon NMN treatment (Figure R1-1C). Expectedly, SRGs are associated with "oxidative phosphorylation", "mitochondrial ATP synthesis coupled electron transport", "cellular response to reactive oxygen species" (Figure R1-1D). Surprisingly, we further found many SRGs are conserved between CPI-455 and NMN treatment (Figure R1-1E), functions of which included "cytoplasmic translation", "mitochondrial transport", "protein targeting to mitochondrion", "mitochondrial transmembrane transport", "oxidative phosphorylation" and "mitochondrial ATP synthesis coupled proton transport" (Figure R1-1F). The transcription variation of genes with interest and involved in oxidative stress, mitochondrion and ATP were further validated by RT-qPCR (Figure R1-1G).

At last, we performed embryo transfer and examined whether CPI-455 and NMN could improve pregnancy outcome. In fact, CPI-455 and NMN improved the rates of birth and live-birth to similar levels (see reply to comment 2 of reviewer 1 for detailed information).

In summary, although the effects of CPI-455 and NMN treatment on embryo development are comparable in many ways, including the alleviation of oxidative damage, the restoration of mitochondrial activity and eventually the enhancement of blastocyst formation, birth rate and live-birth rate, the potential mechanisms behind such phenomena were distinctly different with NMN treatment directly providing NAD^+, while the addition of CPI-455 firstly rescued the H3K4me3 levels at promoter regions for genes related to mitochondrion, ATP and oxidative stress, and then their expression levels, which further contributed to the restoration of mitochondrial activity.

The above results have been added in the revised manuscript.



FigureR1-1. (A) The effects of NMN, vitamin E and astaxanthin on blastocyst formation. (B) The levels of ATP, NAD+ and ROS in IVO, IVF, CPI-455- and NMN-blastocysts. (C) Expression heatmap showing the SRGs identified upon NMN treatment. (D) Functions of SRGs upon NMN treatment. (E) Overlap between SRGs upon CPI-455 and NMN-treatment. (F) Functions of SRGs conserved between CPI-455 and NMN-treatment. (G) Relative mRNA expression level of SRGs with interest and conserved between CPI-455 and NMN treatment.

2. The IVO embryos performed better than IVF embryos in terms of birth rate, which is expected. The key conclusion of this study includes that CPI-455 treatment alleviates defects associated with in vitro culture. Therefore, whether CPI-455 treatment improves birth rate of IVF embryos is interesting to test. To be clear, it is a suggestion that this reviewer is interested to ask, but not a requirement.

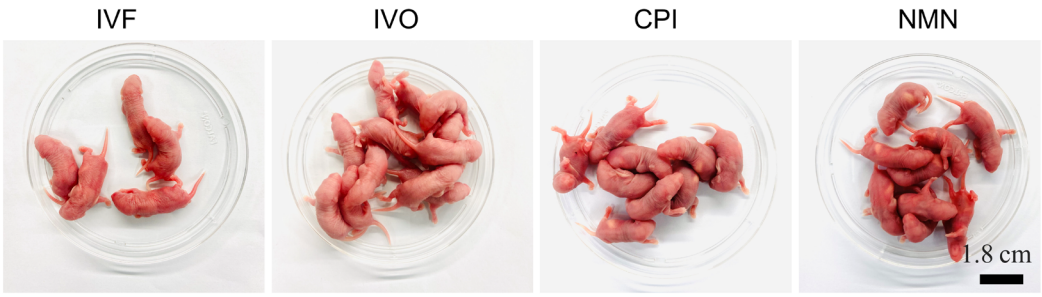
We thank the reviewer for this kind suggestion. We performed the transfer of blastocyst and calculated the birth-rate, live birth-rate under several conditions including IVO, IVF, CPI-455 and NMN. As expected, in contrast to IVO, IVF possessed lower birth rate as well as live birth rate, indicating poorer clinical outcomes (Figure R1-2). Intriguingly, the supplementation of both CPI-455 and NMN could significantly enhance the rate of both birth and live-birth (Figure R1-2), thus improving the clinical outcomes.

The above contents have been added in the revised manuscript.

Pregnancy outcomes following the transplantation of mouse blastocysts subjected to different treatments.

Treatment	Number of Embryos Transferred	Number of Recipients	Rate of Pregnancies (%)	Rate of Births (%)	Rate of Live Births (%)	Rate of Stillbirths (%)
IVF	198	11	10 (90.90%)	65 (36.11%)	63 (35.00%)	2 (1.11%)
IVO	126	7	7 (100%)	79*** (62.70%)	79*** (62.70%)	0 (0)
CPI	108	6	6 (100%)	55* (50.93%)	55* (50.93%)	0 (0)
NMN	90	5	5 (100%)	46* (51.11%)	44* (48.89%)	2 (2.22%)

Notes: The pregnancy rate is calculated as the number of pregnant mice divided by the number of recipient mice. The birth rate is calculated as the number of births divided by the total number of embryos transferred. The live birth rate is calculated as the number of live births divided by the total number of embryos transferred. The stillbirth rate is calculated as the number of stillbirths divided by the total number of embryos transferred. *p<0.05, ***p<0.001



FigureR1-2. The pregnancy outcomes under several conditions including IVF, IVO, CPI and NMN.

3. The decrease of H3K4me3 is very big in IVF blastocysts. It will be great to compared the current data to the earlier reports of H3K4me3 in blastocysts cultured in vitro (DOI: 10.1038/nature19362) and IVO blastocysts (DOI: 10.1038/nature19361). This comparison may strengthen the finding.

We thank the reviewer for this helpful suggestion. We noticed that one study[6] also performed H3K4me3 ULI-NChIP-seq experiments on NM (natural mating) and IVF (in vitro fertilization and in vitro culture), similar to our design. Therefore, we downloaded the raw data and reanalyzed it. We firstly projected H3K4me3 peaks

identified in our study to their data to further ensure the fidelity of H3K4me3 peaks (Figure R1-3A). Then we calculated the H3K4me3 signals within these peaks and found that compared to IVF, NM samples harbored significantly higher H3K4me3 signals, consistent with our observations (Figures R1-3A, B).

Furthermore, we also wondered why our data exhibited more striking H3K4me3 differences. We speculated that longer in vitro culture time may lead to more severe oxidative stress and thus lower H3K4me3 signal given that in our original manuscript we showed the negative correlation between H3K4me3 intensity and H_2O_2 concentration and discussed the potential mechanism behind such a correlation: The timepoint of NM and IVF data reported in Bai et al. was E3.5[6], however, in our work, to better link our study to clinical practice, in which blastocyst at stage four was practically transferred to the uterus, we specifically selected this kind of blastocyst (timepoint: ~E4.5 for IVF-conceived stage four blastocyst) for further investigation. Therefore, it is possible that IVF-conceived embryos in our work suffered more oxidative stress due to the longer in vitro culture time, resulting in more significant H3K4me3 differences. To test this hypothesis, we measured the H3K4me3 intensities and ROS level of IVF-conceived E4.5 and E5.5 (mouse) blastocysts with the latter indeed bearing significantly lower H3K4me3 signals and higher ROS levels (Figure R1-3C). Results derived from human embryos further corroborated our hypothesis as human day6-blastocysts were characterized by lower H3K4me3 and higher ROS levels, relative to day5-blastocysts (Figures R1-3D).

<u>As suggested, we also downloaded H3K4me3 data mentioned by reviewer and reanalyzed it.</u> Once again, this data showed IVO-conceived embryos possessed higher H3K4me3 signals (Figure R1-3E).

Taken together, both our and previously published data revealed in vitro culture procedure could influence H3K4me3 signals. Furthermore, our latest data also uncovered a correlation among in vitro culture time, oxidative stress and H3K4me3 intensity.

The above results have been added in the revised manuscript to better support our discoveries and strengthen the depth of discussion.

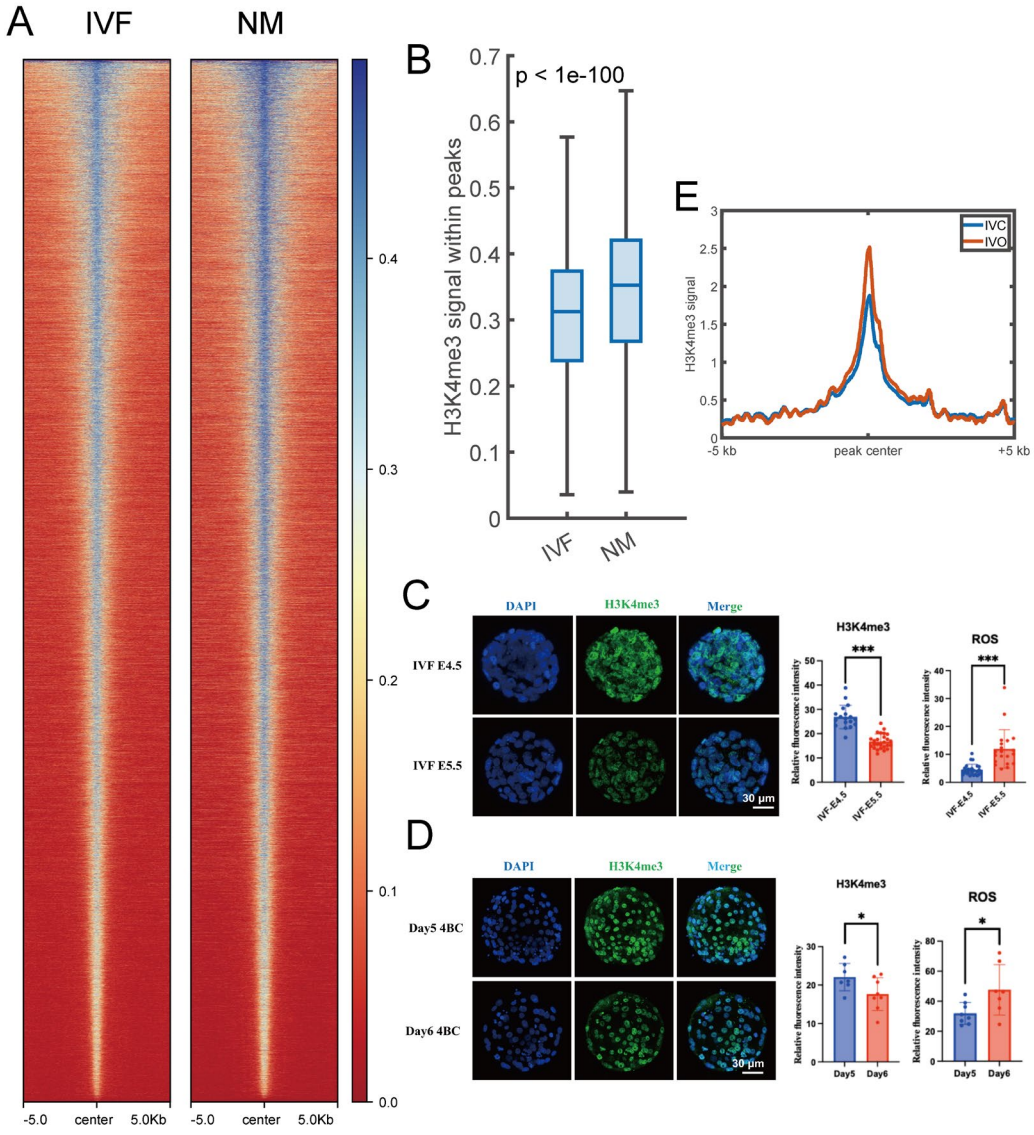


Figure R1-3. (A) Heatmap showing the distribution of H3K4me3 signal around H3K4me3 peaks in IVF and NM[6]. (B) Boxplot showing the H3K4me3 signal within H3K4me3 peaks. (C)-(D) The levels of H3K4me3 and ROS in mouse E4.5 and E5.5 IVF-conceived blastocysts (C) and human day5- and day6-(4BC) blastocysts (D). Of note, in our original manuscript, we performed H3K4me3 ULI-NChIP-seq on human day5- and day6-(4BB) blastocysts and found day5-blastocysts were characterized by globally higher H3K4me3 signal. Here, we collected human day5- and day6-(4BC) blastocysts and measured their H3K4me3 levels for further validation. (E) The distribution of H3K4me3 signal around H3K4me3 peaks in IVC[7] (in vivo fertilization and in vitro culture) and IVO[8].

4. Similarly, DNA methylation from published in IVF blastocysts (DOI: 10.1016/j.stem.2018.07.017;) and IVO blastocysts (DOI: 10.1016/j.cell.2014.04.017; 10.1038/cr.2017.82) could be a good reference for the data in the current study.

We thank the reviewer for this kind suggestion. As suggested, we downloaded and reanalyzed the raw DNA methylation data generated in these researches, and

found the results indeed resembled that reported in our work, that is, in vitro culture-conceived blastocysts were characterized by lower DNA methylation levels (Figure R1-4), in other words, in vitro culture could affect the DNA methylation landscape of early embryos.

DNA methylation results derived from published work have been added in the revised manuscript to better support our conclusions.

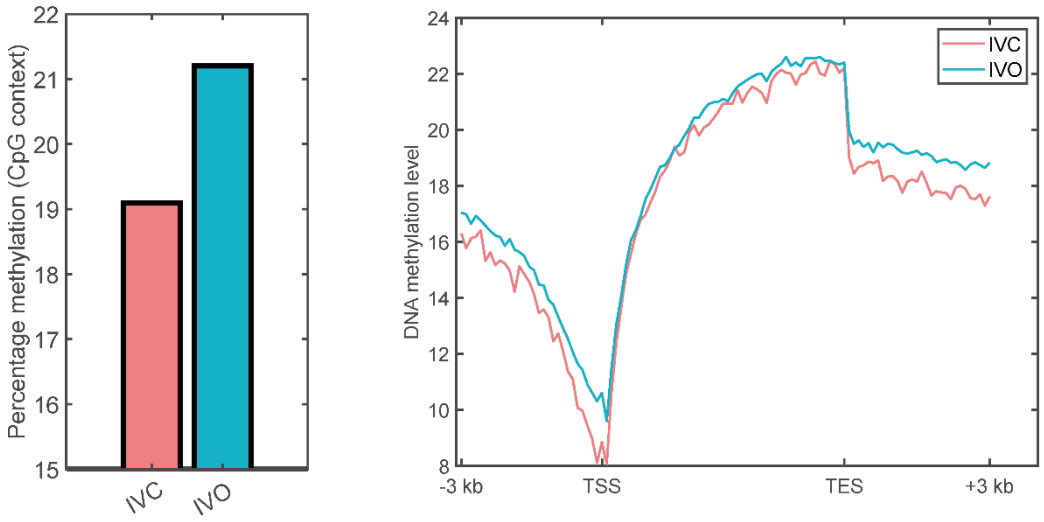


Figure R1-4. Global DNA methylation level (left) and methylation level around TSS (transcription start site) and TES (transcription end site, right). These results were calculated from two previously published datasets[9,10].

5. The language of the manuscript make it difficult to read at many places. Therefore, it is recommended to be improved by a native speaker or professional editing service. For example, Page 3 Line 22 "At last, human data based on day5- and day6- blastocysts obtained via ART also revealed a correlation between H3K4me3 level (at blastocyst stage) and clinical outcomes given that day5-blastocysts harbored globally higher H3K4me3 levels as well as better clinical outcomes." is better to be "Finally, analysis of human ART-derived day5 and day6 blastocysts revealed a correlation between H3K4me3 levels at the blastocyst stage and clinical outcomes, with day5 blastocysts exhibiting globally higher H3K4me3 levels and better clinical outcomes compared to day6 blastocysts." Page 5 Line 7, "At blastocyst stage, both ICM (inner cell mass) and TE (trophectoderm) markers genes became dysregulated following IVF." is better to be "At the blastocyst stage, expression of marker genes for both the inner cell mass (ICM) and the trophectoderm (TE) was dysregulated in IVF-derived embryos."; Page 8, Line 20, "all" is not appropriate here which I'd suggest to be removed.

We thank the reviewer for these helpful comments.

① **Regarding page3 line 22, the original sentence has been replaced by "Finally, analysis of human ART-derived day5 and day6 blastocysts revealed a correlation between H3K4me3 levels at the blastocyst stage and clinical**

outcomes, with day5 blastocysts exhibiting globally higher H3K4me3 levels and better clinical outcomes compared to day6 blastocysts."

② **For page 5 line 7, the original sentence has been replaced by "At the blastocyst stage, expression of marker genes for both the inner cell mass (ICM) and the trophectoderm (TE) was dysregulated in IVF-derived embryos."**

③ **For page 8 line 20, "all" has been removed.**

④ **As suggested, the manuscript has been carefully checked by a native speaker and then revised to present our conclusions more clearly.**

6. In Figure 1b, the labeling of developmental stages should follow their chronological order.

We thank the reviewer for this helpful suggestion and the labeling of developmental stages has been modified.

7. The number of DEGs in L2C stage is a lot. The conclusions related the L2C shall be carefully made. For example, Page 5, Line 27, "Moderate differences between IVO and IVF at gene transcription level during early embryo development from PN5 to morula" might not be appropriate.

We thank the reviewer for this helpful comment and agreed it. The conclusion related to the L2C has been modified as "Moderate transcriptional differences between IVO and IVF during early embryo development from PN5 to morula, except late 2-cell" in the revised manuscript.

8. The H3K27me3 intensity in Extended Data Fig. 3c does not match the description in Page 9. Since based on the Figure, H3K27me3 is almost non-detectable in IVO blastocysts.

We thank the reviewer for this comment. We newly performed immunofluorescence on H3K27me3 (Figure R1-5), the conclusions from which match the description in manuscript: IVF-conceived blastocyst harbored higher H3K27me3 signals.

The original figure has been replaced in the revised manuscript.

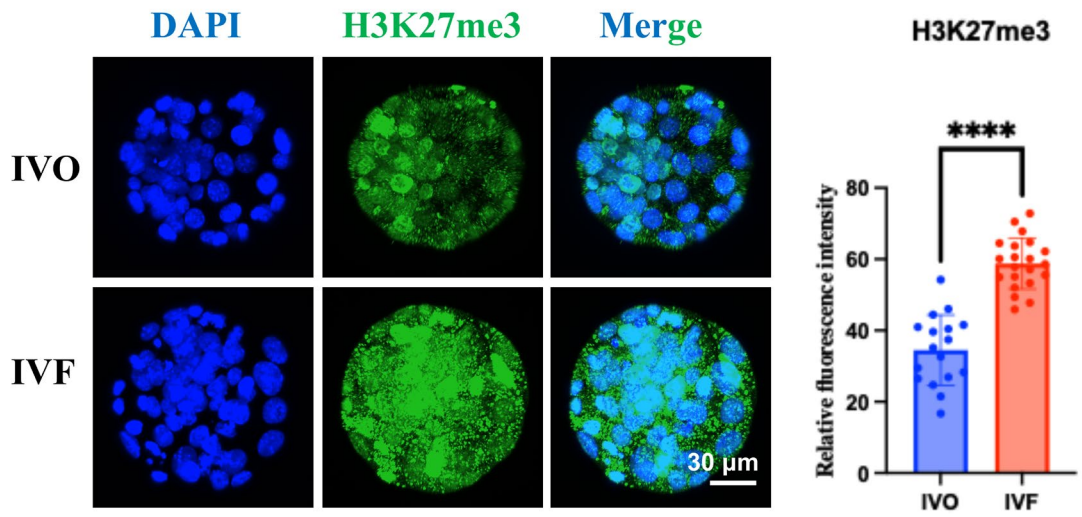


Figure R1-5. The H3K27me3 level in IVF- and IVO-conceived blastocysts.

9. It will be good to describe the samples collected for scRNA-seq more clearly because IVC embryos are known to vary from each other.

We thank the reviewer for this helpful comment. As suggested, methods we used to collect embryos for scRNA-seq were described more clearly in the revised manuscript: IVO- and IVF- blastocysts were collected for extended mouse embryo culture as described above. After cultured in IVC1 for 48h and then cultured in IVC2 for 48h (the ingredients of IVC1 and IVC2 mediums were shown in supplemental tables S1 and S2, respectively), 45 embryos were collected in each group and digested by TrypLETM Express Enzyme (1×), no phenol red (Thermo Fisher, 12604013) at 37°C for 10 minutes. The scRNA-seq libraries were constructed following the protocol provided in the Singleron GEXSCOPE single-cell sequencing kit (Singleron Biotechnologies). Each library was individually diluted to a concentration of 4 nM and then pooled for combined sequencing on the Illumina novaseq 6000.

10. Did gene analyzed in Fig. 5d include all the genes shown in Extended Data Fig. 2b? It is recommended to do so to give a whole picture.

No, Fig. 5d in the original manuscript only exhibited the expression levels of candidate successfully rescued genes (SRGs) among IVF-, IVO- and CPI-blastocysts.

As suggested, we also give a whole picture (Figure R1-6) summarizing the expression levels of differentially expressed genes (DEGs) between IVF- and IVO-conceived blastocysts among IVF-, IVO- and CPI-blastocysts in the revised manuscript. Of note, to seek candidate SRGs, we firstly used a less stringent criterion ($p < 0.05$) to identify the DEGs of two processes (IVF VS CPI-455 and IVF VS IVO) and the expression variation of genes with interest was further validated by RT-qPCR.

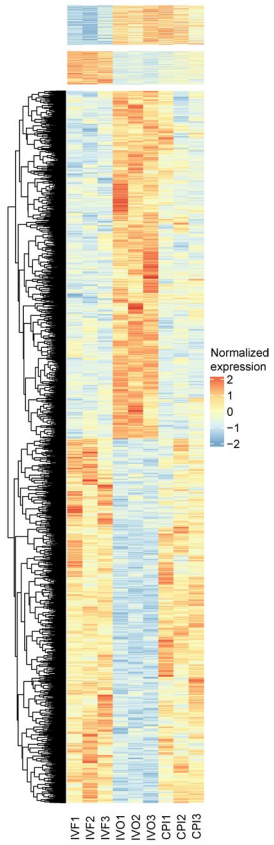


Figure R1-6. The expression level of DEGs (p < 0.05) between IVF- and IVO-conceived blastocysts in IVF-, IVO- and CPI-treated blastocysts.

11. The H2O2 treatment needs to be described more clearly. Were IVO or IVF embryos used in the treatment?

We thank the reviewer for this helpful suggestion. In the revised manuscript, method related to H_2O_2 treatment was added: IVO-blastocysts were collected as described above. Embryos were incubated in G-1 PLUS medium supplemented with 0 μM, 200 μM, 400 μM, or 1000 μM H_2O_2 under dark conditions at 37°C with 6% CO_2 and 5% O_2 for 3 hours. After washing at least five times in M2 medium, the embryos were imaged using a confocal microscope to measure their H3K4me3 levels.

12. Day5 embryos performs significantly better than Day6 blastocyst in human clinics. This finding is fantastic. It would be better to discuss more to give the readers a clear connection between Day5 vs Day6 and IVF vs IVM. In addition, mouse IVF embryos developed a bit slower compared to IVO embryos, does CPI-455 treatment improve this? This might be another link.

We thank the reviewer for these helpful suggestions.

In our work, we found mouse IVF-conceived blastocysts indeed resembled human day6-blastocysts in many ways. Both of them suffered more oxidative stress

and possessed lower H3K4me3 signals. On the other hand, in contrast to mouse IVO- and human day5-blastocysts, mouse IVF- and human day6-blastocysts developed a bit slower with poor clinical outcomes.

In fact, the potential mechanisms behind the above phenomena between human and mouse were also similar: relative to mouse IVO- and human day-5 blastocysts, mouse IVF- and human day6-blastocysts underwent longer in vitro culture time and thus accumulated more oxidative stress, which consequently influenced their H3K4me3 signal. Intriguingly, the correlation between H3K4me3 and clinical outcomes exist in both mouse and human given that the supplementation of CPI-455 could facilitate blastocyst formation and improve clinical outcomes, further investigation revealed the mechanism related to such improvements upon CPI-455 treatment may also be conserved between human and mouse.

As suggested, the above contents have been added in the revised manuscript to strengthen the depth of discussion.

Regarding developmental speed, yes, CPI-455 treatment indeed facilitated embryo development (Figure R1-7), which was also added in the revised manuscript.

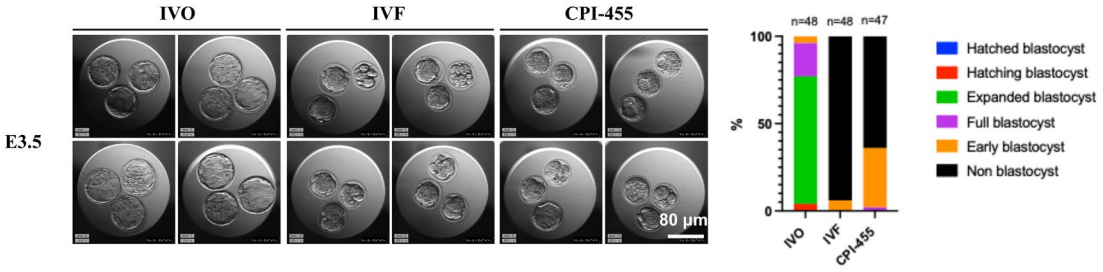


Figure R1-7. The proportion of blastocyst in E3.5 under several conditions including IVO, IVF and CPI-455.

Reviewer #2 (Remarks to the Author):

It is crucial to understand the underlying mechanisms that lead to adverse outcomes in ART. In this manuscript, the authors report a significant reduction in H3K4me3 levels and an increase in oxidative stress in IVF-derived embryos. Further analysis demonstrate that treatment with CPI-455 can rescue these adverse effects and promote embryo development in vitro. Importantly, their findings in human embryos indicate that CPI-455 may have potential for future clinical application.

I have following questions and suggestions that may help strengthen their conclusions:

Major:

1.In Figure 5, the authors show that CPI-455 treatment facilitates blastocyst formation in IVF-embryos. Could the authors further examine whether this treatment improves the live-birth rate of IVF-embryos, similar to the analysis in Figure 6a?

We thank the reviewer for this helpful comment. As mentioned in the reply to the comment 2 of reviewer 1, CPI-455 treatment indeed improves both birth rate and live-birth rate of IVF embryos (FigureR1-2).

2.Could the authors explore inter-individual variability in H3K4me3 levels among human day-5 blastocysts, for example across different morphological grades? As day-5 blastocysts are generally preferred for embryo transfer, such analysis may help enhance the clinical significance of their findings.

As suggested, we measured H3K4me3 levels among day5-blastocysts with different morphological grades including 4AA, 4BB and 4BC. Nevertheless, no significant difference was observed among them (Figure R2-1A). We next sought to understand the underlying mechanism behind such a phenomenon. In the original manuscript, we revealed that day5- and day6-blastocysts with the same morphological grade (4BB) exhibited distinct H3K4me3 levels with day5-blastocysts bearing significantly higher H3K4me3 signals. Based on these discoveries, we surmised in vitro culture time may be related to H3K4me3 levels as longer in vitro culture time may be accompanied by more severe oxidative stress and consequently lower H3K4me3 signal (a negative correlation between oxidative stress level and H3K4me3 intensity has already been shown in the original manuscript). In fact, both mouse and human studies confirmed this hypothesis (Figures R2-1B, C): relative to human day5-blastocyst (4BC) and mouse IVF-conceived E4.5 blastocyst, human day6-blastocyst (4BC) and mouse E5.5 blastocyst were characterized by lower H3K4me3 and higher ROS levels, hinting the association among in vitro culture time, oxidative stress and H3K4me3. Together, similar H3K4me3 levels among day5-blastocysts with different morphological grades may be due to the similar in vitro culture time.

On the other hand, in the original manuscript, we showed that in contrast to mouse IVO-conceived blastocyst and human day5-blastocyst, mouse IVF-conceived blastocyst and human day6-blastocyst harborer significantly lower H3K4me3 and poor clinical outcomes, indicating a possible connection between epigenetic modifications and clinical outcome. Such a correlation further inspired us to apply CPI-455 to the in vitro culture system of both human and mouse embryos and we found the supplementation of CPI-455 indeed facilitated blastocyst formation and improved clinical outcomes. The mechanisms behind it were investigated in detail in mouse and may be conserved between human and mouse.

Taken together, we emphasized in the revised manuscript the association among in vitro culture time, oxidative stress and H3K4me3. We also emphasized H3K4me3 is related to clinical outcomes, at least between mouse IVF- and IVO-conceived blastocyst, and between human day5- and day6-blastocysts, and consistently, CPI-455 may be a potential selection towards the improvements of embryo quality as well as clinical outcomes. Of note, the similar H3K4me3 levels among day5-blastocysts with different morphological grades may indicate other factors (such as DNA methylation[11]) other than H3K4me3 could also be related to clinical outcomes.

The above contents have been added in the revised manuscript to strengthen our discussion part.

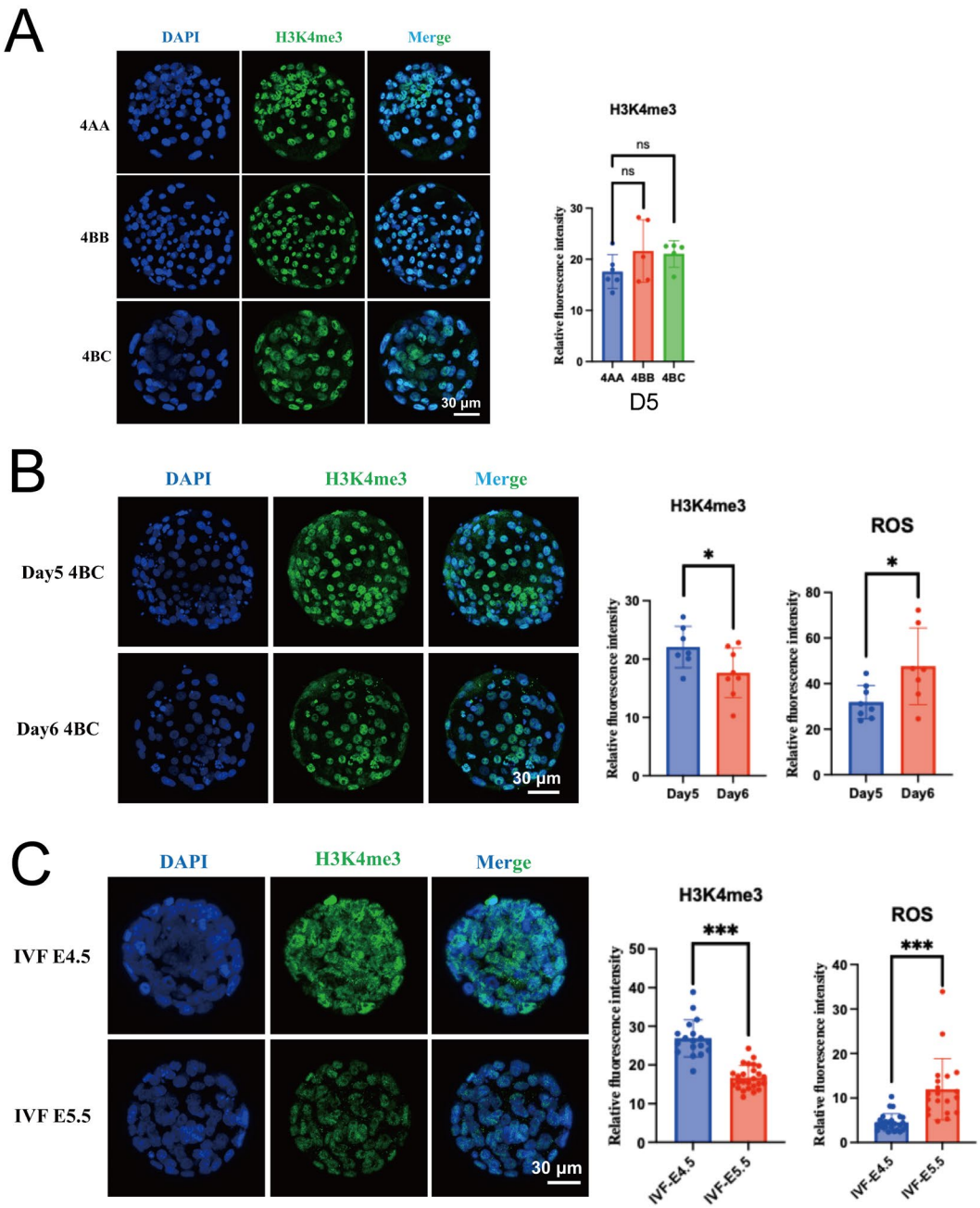


Figure R2-1. (A) The H3K4me3 levels among human day5-blastocysts with different morphological grades. (B)-(C) The levels of H3K4me3 and ROS in human day5- and day6-(4BC) blastocysts (B) and mouse E4.5 and E5.5 IVF-conceived blastocysts (C).

3.The authors may consider revise the statements on page 16, lines 1-4. Their current Chip-seq data are informative, but not directly linked to clinical outcomes. Whereas previous studies have reported direct associations between DNA methylation and ART clinical outcome (e.g. https://doi.org/10.1038/s41422-023-00809-z).

We thank the reviewer for this kind comment. In this work, regarding human studies, we focused on day5- and day6-blastocysts due to the inaccessibility of human preimplantation in vivo embryos. Through the analysis of collected clinical

data, we firstly found that relative to day6-blastocyst, day5-blastocyst was characterized by better clinical outcomes. Further inspection revealed that unlike DNA methylation, H3K4me3 signal was significantly higher in day5-blastocysts and CPI-455 treatment could indeed improve clinical outcomes, thus indicating a possible connection between H3K4me3 and clinical outcomes.

After carefully reading the paper referred to by the reviewer, we recognized that firstly, the sample size in our work was limited, furthermore, epigenetic modification data (including H3K4me3 ULI-NChIP-seq and WGBS) and clinical data were not from the same embryo. Therefore, we cited this paper and revised the statements as "On the other hand, we also compared the DNA methylation levels of day5- and day6-blastocysts, which seemed to be irregular. However, compared to previous studies[11], the sample size in our work was limited, more data taking into account both epigenetic information and clinical outcomes from the same embryo are needed to better compare the correlation of clinical outcomes to H3K4me3/DNA methylation."

Minor:

1.The authors state that IVF-embryo have lower level of Carm1 expression, which would promote the formation of TE (page 8, lines 12-14). This appears inconsistent with the findings that the number of TE cells is decreased in IVF embryos, as shown Figure 2a. Some clarification would be helpful.

We thank the reviewer for this insightful comment. In fact, the number of both ICM and TE cells was smaller in IVF-conceived blastocyst. We then further calculated the proportion of ICM and TE cells, intriguingly, relative to IVO, IVF-conceived blastocysts were characterized by significantly higher and lower proportion of TE and ICM cells, respectively (Figure R2-2), hinting the aberrant cell compositions upon IVF, which may be ascribed to the activation failure of *Carm1* during ZGA process.

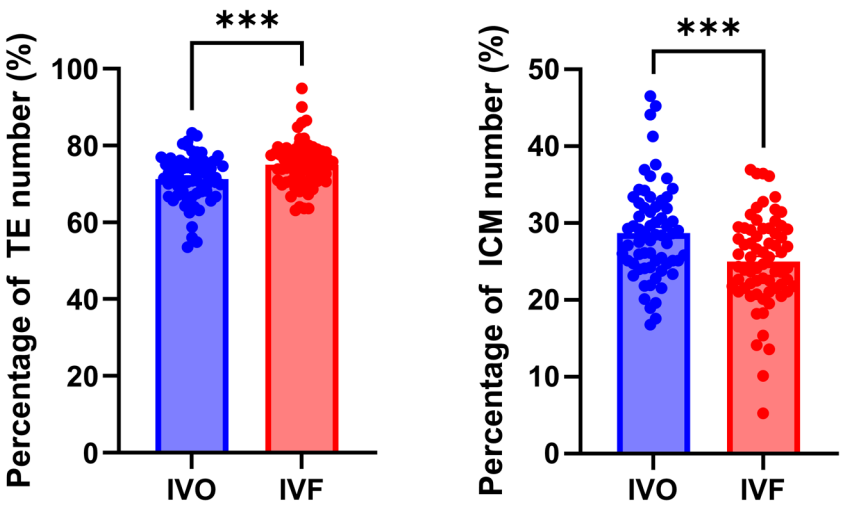


Figure R2-2. The proportion of ICM and TE cells in IVF- and IVO-conceived blastocysts.

2.In mouse IVF embryos, it appears that the increased ROS (Figure 2d) and Tet3 level (Figure 2g) are the potential contributors of decreased global DNA methylation level (Figure 3h). Moreover, CPI-455 does not seem to reduce ROS levels in IVF embryos to those seen in IVO embryos (Figure 5g vs. Figure 2d). These raise my question that, instead of inhibiting a single histone demethylase use CPI-455, would decfrease the oxidative stress offer a broader improvement in embryo quality? (e.g. using NMN, as the authors mentioned)

We thank the reviewer for this helpful suggestion. <u>As suggested, we firstly examined whether the supplementation of NMN could facilitate blastocyst formation and improve pregnancy outcome.</u> In fact, the effects of NMN- and CPI-455 treatment on blastocyst formation, birth rate and live-birth rate are highly comparable: both of which enhanced the formation of blastocyst (FigureR1-1), and improved the rates of birth and live-birth to similar levels (FigureR1-2).

We further performed Smart-seq2 and measured the levels of ROS, ATP and NAD^+ to investigate the potential mechanism behind NMN supplementation. <u>Detailed information could be seen in reply to comment 1 of reviewer 1. With respect to oxidative stress, NMN treatment alleviated oxidative stress to a level similar to that upon CPI-455 treatment.</u>

In summary, although the effects of CPI-455 and NMN treatment on embryo development are comparable in many ways, including the alleviation of oxidative damage, the restoration of mitochondrial activity and eventually the enhancement of blastocyst formation, birth rate and live-birth rate, the potential mechanisms behind such phenomena were distinctly different with NMN treatment directly providing NAD^+, while the addition of CPI-455 firstly rescued the H3K4me3 levels at promoter regions for genes related to mitochondrion, ATP and oxidative stress, and then their expression levels, which further contributed to the restoration of mitochondrial activity.

The above contents have been added in the revised manuscript.

3.The authors forgot to cite Figure 6d in the main text.

This error has been corrected in the revised manuscript.

Reviewer #3 (Remarks to the Author):

The work titled “The crosstalk between H3K4me3 and oxidative stress could be potential target for the improvement of ART-derived embryos ” examined transcriptomic and epigenetic marks of mouse and human pre-implantation embryos generated by IVF or in vivo

The authors found that:

1. Moderate differences between IVO and IVF at gene transcription level at each stage of preimplantation development
2. Enrichment of NRF2 and Bach1 sequences (TF involved in dealing with Oxidative stress) in promoter of upregulated in vivo generated blastocysts.
3. ICM marker genes, such as Pou5f1 and Nanog had lower expression, and TE marker gene Eomes, exhibited higher expression levels in mouse IVF-conceived blastocysts, suggesting a failure of activation of Carm1 during ZGA process following IVF.
4. Tet3 and Kdm5a (responsible for demethylation of H3k4me3, activating) are increased and kdm6b (responsible for demethylation of H3k27me3, repressive) are decreased in mouse IVF blastocysts.
5. Massive decreased H3K4me3 in IVF blastocysts (by IF and ULI-NChiP) in more than 14,000 regions which could be ascribed to the higher oxidative stress in IVF-derived blastocysts. On the other hand there was minimal higher H4K27me3 (54 regions) in IVF blastocysts.
6. Increasing oxidative stress, by culturing embryos in H2O2 resulted in decreased in H3k4me3
7. There is also decreased DNA methylation in IVF embryos (Figure 3h), particularly in area where tet3 binds the promoter.
8. IVF generated embryos cultured for 96 hours show normal trilaminar structure but reduced size and had higher proportions of trophoblast progenitor cells (TPC), trophoblast giant cells (TGC) and Spongiotrophoblast (SpT) which are in a more differentiated status compared to ExE ectoderm. Also the proportion of epiblast cells was lower.
9. The use CPI-455 (5 microM for 96 hours, started at the zygote stage), the inhibitor of KDM5 (which catalyzes the demethylation of H3K4), successfully rescued the H3K4me3 as well as transcription levels of genes related to ATP, mitochondrion, oxidative stress and signal transduction.
10. Day 5 human blastocyst had higher H3K4me3 levels than CD6 human blastocysts; day 5 blastocysts had better clinical outcomes
11. The use of CPI-455 supplement enhanced the formation of high-quality human embryos

The authors conclude that there is crosstalk between increased oxidative stress and decreased levels of H3K4me3, which may underlie the negative clinical outcomes associated with ART. They propose that disruption of the epigenetic landscape in IVF-conceived blastocysts is secondary to impaired bivalency at promoter regions, particularly due to reduced H3K4me3, which in turn may alter gene expression. They further suggest

that the use of CPI-455 could improve developmental outcomes in ART-derived embryos. The overall topic is of high significance, given the large and growing number of children conceived through IVF. The authors should be commended for performing extensive studies and applying multiple omics techniques to explore how IVF-derived embryos differ from their in vivo counterparts. The observation of reduced H3K4me3 in mouse IVF embryos is important and supports a mechanistic link between oxidative damage and altered chromatin states.

However, several major concerns remain, especially regarding the interpretation and use of human embryo data:

Major Concerns

1. CD5 vs CD6 Human Embryos:

The comparison between CD5 and CD6 human embryos is not convincing. The authors claim that CD6 embryos show an epigenomic profile resembling IVF mouse embryos, whereas CD5 embryos resemble in vivo mouse embryos. However, this interpretation appears inconsistent with their hypothesis. If CD6 embryos show lower H3K4me3, does this imply they have experienced more oxidative stress? This seems unlikely, and alternative explanations should be considered. The authors are encouraged to conduct additional experiments using mouse embryos to determine whether delayed blastocyst development (e.g., 113+24h) further alters H3K4me3 levels and/or DNA methylation.

We thank the reviewer for these comments.

① **Regarding human embryos, we measured their oxidative stress level and found that relative to day5-blastocysts, day6-blastocysts harbored higher ROS level (Figure R3-1A), which may consequently lead to the lower H3K4me3 signal (a negative correlation between oxidative stress level and H3K4me3 signal has already been shown in the original manuscript and the potential mechanism behind such a correlation have also been discussed).**

② **As suggested, we also measured the levels of H3K4me3 and ROS using mouse IVF embryos including E4.5 and delayed (E5.5) blastocysts. Akin to observations from human embryos, mouse E5.5 blastocysts were characterized by lower H3K4me3 and higher ROS levels, compared to E4.5 blastocysts (Figure R3-1B).**

Taken together, our newly performed experiments in both human and mouse revealed the association among in vitro culture time, oxidative stress and H3K4me3.

The above contents have been added in the revised manuscript.

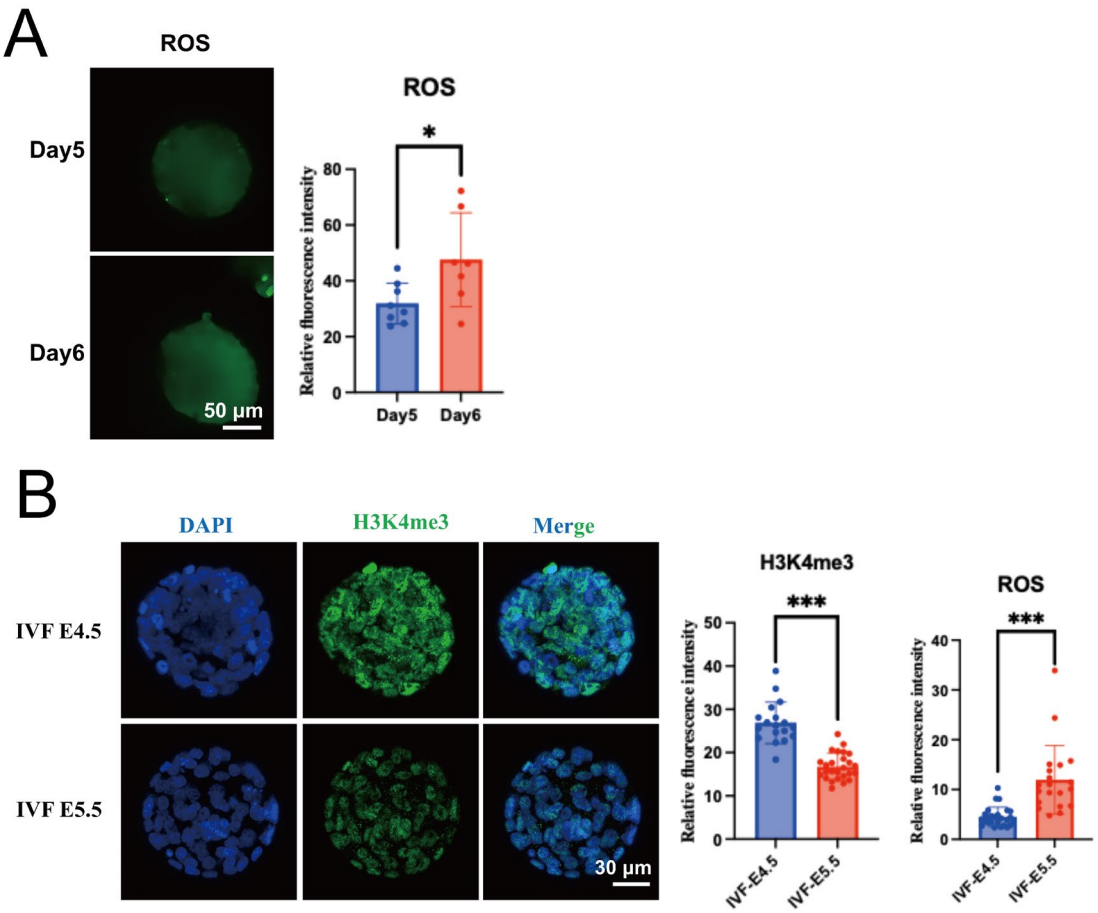


Figure R3-1. (A) The ROS level in human day5- and day6-blastocysts. (B) The levels of H3K4me3 and ROS in mouse E4.5 and E5.5 blastocysts.

2. Human Embryo Ethics and Consent:

The generation and use of human embryos require clearer ethical documentation. Human embryos were generated from immature oocytes and donor sperm. A full description of the informed consent process should be provided, including whether patients were aware that their oocytes would be fertilized with donor sperm and used for research. A translated version of the consent form in English should be included.

We thank the reviewer for these kind suggestions. The informed consent and ethical documentation in both Chinese and English versions have all been provided along with our revised manuscript.

3. Embryo Numbers and Metadata:

A detailed summary table should be added in the supplementary materials, specifying:

o The total number of human embryos generated, donated, and used in each experiment

o Whether they were treated with CPI

o Gardner morphological scores

o Egg and sperm donor age and diagnosis

o Whether embryos were generated by IVF or ICSI

o If preimplantation genetic testing (PGT) was performed

We thank the reviewer for this helpful suggestion. A detailed table summarizing the information of human embryos we used has been added in the supplementary materials, which could also be seen below (Table R3-1).

Type	Stage	Morphological scores	Number	Age and diagnosis	IVF or ICSI	PGT
ULI-NChIP-seq	Blastocyst	4BB	18	<38，Infertility caused by non-ovarian factors	ICSI	No
WGBS	Blastocyst	4BB	24	<38，Infertility caused by non-ovarian factors	ICSI	No
Smart-seq2 of Day5 and Day6 embryos	Blastocyst	4BB	18	<38，Infertility caused by non-ovarian factors	ICSI	No
CPI-455 Treatment and Smart-seq2	MI	-	256	<38，Infertility caused by non-ovarian factors	ICSI	No
Determination of ROS	Blastocyst	4BB	15	<38，Infertility caused by non-ovarian factors	ICSI	No
Immunofluorescence staining of Day5 and Day6	Blastocyst	4BC	15	<38，Infertility caused by non-ovarian factors	ICSI	No
Immunofluorescence staining in Day5 embryos of different grades	Blastocyst	4AA、4BB、4BC	16	<38，Infertility caused by non-ovarian factors	ICSI	No

Table R3-1. The information of human embryos used in this work.

4. Morphology Matching and Infertility Cause:
Were CD5 and CD6 embryos matched for morphology grade? Were causes of infertility similar between the two groups?

We appreciate the reviewer's insightful question. In this study, day5 and day6-blastocysts were categorized into two quality groups—high quality and general quality—based on morphological grading criteria. Specifically, blastocysts with a grade of ≥ 4BB were classified as high quality, while those with lower grades were categorized as general quality. In our initial analysis, embryo quality was designated as an outcome variable associated with embryonic indicators for statistical evaluation. A significant difference was observed between the day 5 and day 6 groups. Notably, the proportion of high-quality embryos in the day5 group was

94.25%, which was significantly higher than the 66.23% observed in the day6 group ($p<0.001$). Based on these findings, we concluded that day5 blastocysts exhibit superior quality compared to day6 blastocysts.

During the process of addressing the reviewers' inquiries, we recognized that propensity score matching (PSM) of embryo quality as baseline characteristics could further enhance the rigor of our analysis. Given that embryo quality represents a critical determinant of clinical outcomes, this approach would allow clinical outcomes to be attributed primarily to embryo developmental factors, rather than being confounded by variations in embryo quality. Accordingly, PSM was performed on embryo quality between the two groups. Additionally, the causes of infertility were analyzed and categorized into four subtypes: tubal factor, male factor, factors, and unexplained infertility. Significant differences were observed in the distribution of these infertility subtypes between the day 5 and day 6 groups. Specifically, the proportions in the day5 group were 69.9%, 16.7%, 1.4%, and 11.9%, respectively, compared to 61.5%, 19.7%, 1.4%, and 17.4% in the day6 group ($p < 0.001$). To control for potential confounding variables, propensity score matching (PSM) was also conducted based on infertility causes. After PSM, no significant differences were found between the day 5 and day 6 groups in terms of blastocyst quality (75.47% vs. 75.15%, $p = 0.794$) or the distribution of infertility causes (tubal factor: 65.21% vs. 63.26%; male factor: 16.90% vs. 19.25%; combined: 1.75% vs. 1.43%; unexplained: 16.14% vs. 16.06%, $p = 0.146$). These findings suggest that the observed clinical outcomes were primarily attributable to the developmental duration of the blastocysts, rather than to other differences.

5. DNA Methylation Literature:

The introduction and discussion should better address the contrasting reports on DNA methylation differences between IVF- and naturally-conceived children. Studies have shown only minimal differences in some tissues. Relevant mouse studies (e.g., Doherty, 2004; Ruggeri, 2020) should also be cited.

We thank the reviewer for these helpful suggestions.

We noticed that in our original introduction part, the DNA methylation differences between IVF- and naturally-conceived children was introduced inadequately <u>with only differences in cord blood discussed.</u> <u>We thus included several human studies regarding placenta in the revised manuscript:</u> In contrast to natural conceptions, altered global DNA methylation levels have been observed in IVF-conceived placenta[12], a recent work[13] with a total of 80 ART and 77 control placentas further identified 6814 significantly differentially methylated CpG sites and 822 DMRs (differentially methylated regions). Functions of genes related to ART-associated DMRs involved in cell-cell adhesion and tissue development. Of note, previous studies[14,15] also proved that the observed DNA methylation differences between ART and natural conceptions could be attributed to ART protocols instead of underlying infertility. Taken together, these studies indeed revealed the potential effects of ART on human DNA methylation landscape.

In fact, different studies sometimes exhibited distinctly different and even contradictory results related to how and to what extent ART influenced DNA methylation levels, which may be due to, at least partially, the different sample sizes, different methods to measure and calculate DNA methylation level, etc.

The above contents have been added in the introduction part of revised manuscript.

With respect to mouse studies, firstly, as suggested by reviewer 1 (please see the reply to comment 4 of reviewer 1), we downloaded and reanalyzed published DNA methylation data related to in vitro culture- and IVO-conceived blastocysts, the conclusions from which were indeed similar to that in our work, thus supporting our observations.

Furthermore, we also cited some researches focusing on DNA methylation differences between IVF- and IVO-conceived mouse embryos at preimplantation stage and discussed them. For instance, the proper expression of imprinted genes, such as *H19*, relies on accurate epigenetic landscape, such as DNA methylation, and is essential for normal embryo development. Doherty et al. [16]found that in vitro culture could perturb the methylation level, resulting in the loss of *H19* imprinting. Another related work[17] performed WGBS experiments on IVF- and IVO-embryos and identified 1624 DMRs. Intriguingly, regions undergoing DNA methylation changes were generally distal from TSS (transcription start sites), indicating enhancer activity may change. Furthermore, given that CTCF has been suggested to play pivotal roles in establishing enhancer-promoter interactions[18] through loop-extrusion mechanism[19,20] and its binding was methylation-sensitive[21], therefore, in future, studies carefully checking the variation of enhancer activity, CTCF binding and 3D chromatin structure in IVF-conceived embryos, and their implications for gene transcription, are needed.

The above contents regarding mouse studies have been added in the revised manuscript to strengthen our discussion parts.

6. NRF2/Bach1 Binding in IVO Embryos:
The authors note increased NRF2 and Bach1 binding in promoters of genes upregulated in in vivo embryos. Please clarify the significance of this observation—how does it support or contradict the proposed model?

We thank the reviewer for this comment. When analyzing the differentially expressed genes (DEGs) between IVF- and IVO-conceived blastocysts, we found that IVO-expressed genes were related to oxidative stress. Intriguingly, sequence motif analysis on these genes revealed the potential binding of Bach1 and Nrf2 to their promoter regions. Bach1 and Nrf2 have been suggested to play essential roles in tackling oxidative stress[22], thus consistent with our gene function analysis.

7. Discussion Clarity:

The discussion is overly superficial and should be strengthened, particularly regarding the observed decline in H3K4me3 (Figure 3G). A deeper synthesis of the findings is necessary.

We thank the reviewer for these helpful comments.

① **As shown in the reply to comment 5 of reviewer 3, literatures related to DNA methylation differences between IVF- and IVO-conceived embryos have been cited and discussed.**

② **Regarding H3K4me3 decline, we firstly downloaded and reanalyzed previously published datasets, the conclusion from which were similar to that in our work, that is, IVF-conceived embryos possessed lower H3K4me3 signal (please see the reply to comment 3 of reviewer 1 for details). The potential mechanisms behind such an observation may be that relative to IVO, IVF-conceived embryos bear higher oxidative stress due to in vitro culture/exposure as well as in vitro operation. In fact, based on previous work[23], higher oxidative stress could disturb the activity of enzyme responsible for lysine-directed histone methylation. Consistently, we also found a negative correlation between H_2O_2 concentration and H3K4me3 intensity.**

Furthermore, our newly performed experiments on both human and mouse embryos revealed a non-negligible association among in vitro culture time, oxidative stress and H3K4me3 signals, given that in contrast to mouse E4.5- and human day5-blastocysts, mouse E5.5 and human day6-blastocysts were characterized by higher ROS and lower H3K4me3 signals.

③ **Besides, during embryo development, the aberrant cell compositions in IVF-conceived embryos and the possible cause were also discussed to strengthen our discussion part (please see the reply to comment 9 of reviewer 3 for details).**

Taken together, the discussion part was revised according to the above contents.

8. TET3 Expression in Figure 3K:
The figure shows two populations of IVF embryos with high and low TET3 expression. What accounts for this heterogeneity? Could it reflect embryo subtypes with differential epigenetic responses?

We thank the reviewer for these comments. The heterogeneity of TET3 expression may result from the insensitivity of the antibody we used. We newly collected IVF- and IVO-conceived blastocysts and performed immunofluorescence staining with a new antibody. Such a heterogeneity disappeared while the conclusion remained the same: IVF-conceived blastocysts bear higher Tet3 expression level (Figure R3-2). The original figure has been replaced in the revised manuscript.

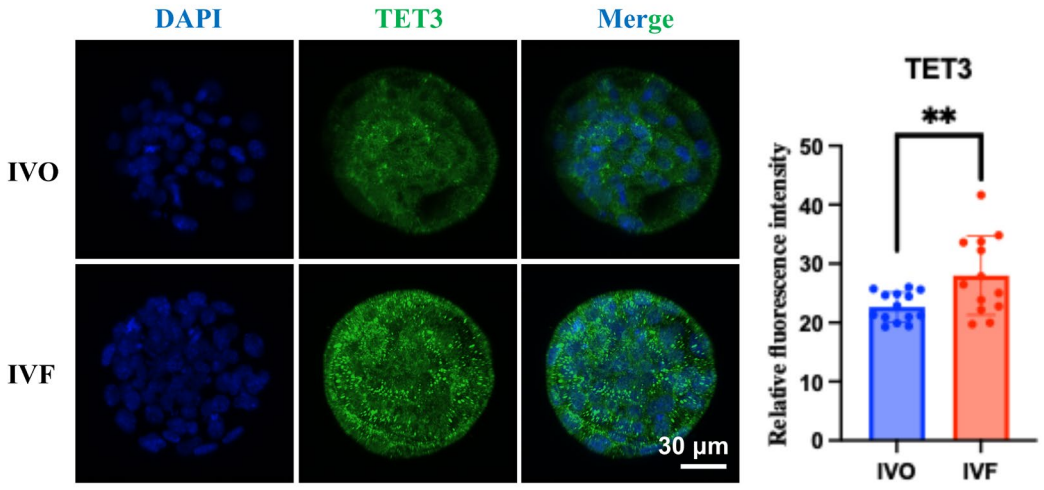


Figure R3-2. The TET3 expression level in IVF- and IVO-conceived blastocysts.

9. Marker Expression vs Cell Types (Figure 4i):
The authors conclude that IVF embryos contain more trophoblast progenitor cells (TPC), trophoblast giant cells (TGC), and spongiotrophoblast (SpT) cells. However, this is based solely on marker gene expression. In situ hybridization or lineage tracing would be necessary to validate differences in actual cell populations.

We thank the reviewer for this helpful suggestion. Aside from ~E6.5 embryos (at this time-point, we performed scRNA-seq to investigate the differences between IVF- and IVO-conceived embryos), we also performed lineage tracing at two time-points, pre-implantation (blastocyst stage) and E18.5:

At blastocyst stage, we found compared to IVO, IVF-conceived blastocysts harbored higher and lower proportion of TE and ICM cells, respectively (Figure R3-3A), which was indeed consistent with our observation at ~E6.5 stage that the proportions of TPC, TGC and SpT were higher in IVF-conceived embryos.

At E18.5 stage, we collected both mouse placenta and fetus. Intriguingly, in contrast to IVO, IVF was characterized by higher and lower percentage of placenta weight (defined as placental weight divided by the total weight of both placenta and fetus) and efficiency of placenta (defined as the ratio between the weights of fetus and placenta), respectively (Figure R3-3B), in accordance with the cell proportion differences between IVF- and IVO-conceived embryos at both pre-implantation blastocyst and ~E6.5 stages.

Of note, the above phenomena may be ascribed to, at least partially, the activation failure of *Carm1* during ZGA process, reported in our original manuscript, since previous studies[24] have revealed that the heterogeneity of *Carm1* expression plays pivotal roles in mediating the first lineage segregation with lower *Carm1* expression contributing to the formation of TE.

The above contents have been added in the manuscript.

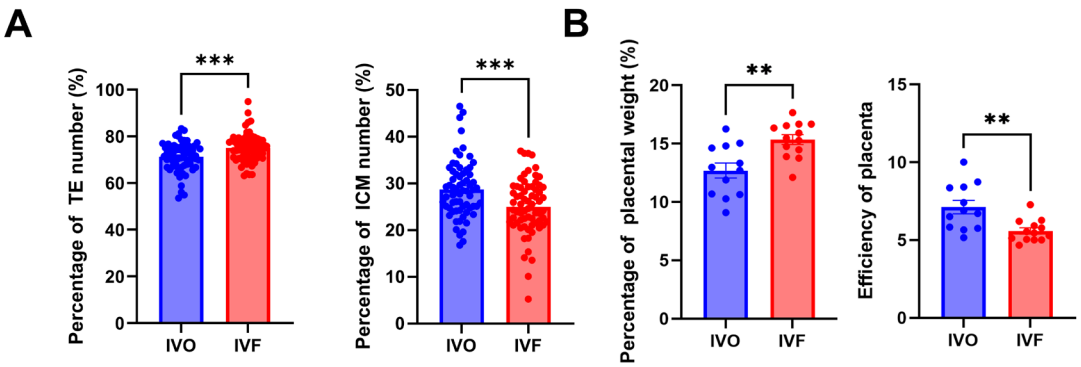


Figure R3-3. (A) The proportion of ICM and TE cells in IVF- and IVO-conceived blastocysts. (B) The percentage of placental and the efficiency of placenta at E18.5 stage.

10. Replicates in Omics Experiments:
The number of biological replicates used for each omics approach should be clearly stated. A minimum of three replicates is expected.

We thank the reviewer for this comment. For Smart-seq2 data of human (including D5-, D6-, CPI-treated-D5- and CPI-treated-D6-blastocysts) and mouse (including PN5, early 2-cell, late 2-cell, 4-cell, 8-cell, morula, blastocyst and CPI-treated blastocyst), three replicates were consistently used. For human epigenetic data including H3K4me3 and DNA methylation of D5- and D6-blastocysts, we also used three replicates. For mouse epigenetic data including H3K4me3, H3K27me3 and DNA methylation, we used two replicates, and conclusions derived from them were further validated by immunofluorescence and reanalyzing previously published datasets.

As suggested, the number of biological replicates for each omics has been added clearly in the revised manuscript.

11. Causality in Embryo Transfer Findings:
On page 14, the authors suggest that reduced H3K4me3 accounts for lower live birth rates after IVF. However, this is correlative. They should test whether CPI-455 treatment improves both H3K4me3 and live birth rates to support causality.

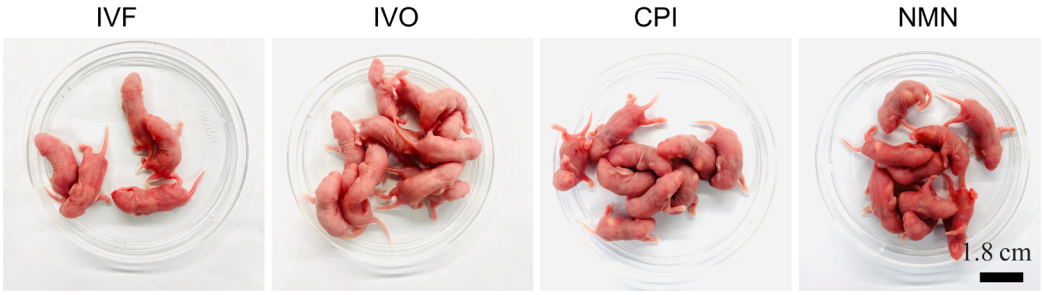
We thank the reviewer for these suggestions. In the original manuscript, we performed ChIP-seq and immunofluorescence experiments, both of which revealed the increase of H3K4me3 upon CPI-455 treatment.

Regarding live-birth rates, we performed embryo transfer and found that the supplementation of CPI-455 could indeed improve both birth rate and live-birth rate (Figure R3-4), which has been added in the revised manuscript.

Pregnancy outcomes following the transplantation of mouse blastocysts subjected to different treatments.

Treatment	Number of Embryos Transferred	Number of Recipients	Rate of Pregnancies (%)	Rate of Births (%)	Rate of Live Births (%)	Rate of Stillbirths (%)
IVF	198	11	10 (90.90%)	65 (36.11%)	63 (35.00%)	2 (1.11%)
IVO	126	7	7 (100%)	79*** (62.70%)	79*** (62.70%)	0 (0)
CPI	108	6	6 (100%)	55* (50.93%)	55* (50.93%)	0 (0)
NMN	90	5	5 (100%)	46* (51.11%)	44* (48.89%)	2 (2.22%)

Notes: The pregnancy rate is calculated as the number of pregnant mice divided by the number of recipient mice. The birth rate is calculated as the number of births divided by the total number of embryos transferred. The live birth rate is calculated as the number of live births divided by the total number of embryos transferred. The stillbirth rate is calculated as the number of stillbirths divided by the total number of embryos transferred. *p<0.05, ***p<0.001



FigureR3-4. The pregnancy outcomes under several conditions including IVF, IVO, CPI and NMN.

12. Embryo Developmental Speed:

On the same page, the authors assert that IVO embryos develop faster. Please provide evidence for this claim.

We thank the reviewer for this helpful suggestion. As suggested, we analyzed the percentage of blastocyst at E3.5 under different conditions including IVO, IVF and CPI-455 treatment. Consistent with previous study[25], we also found relative to IVF, IVO-embryos developed faster (Figure R3-5). Furthermore, the supplementation of CPI-455 could facilitate embryo development (Figure R3-5).

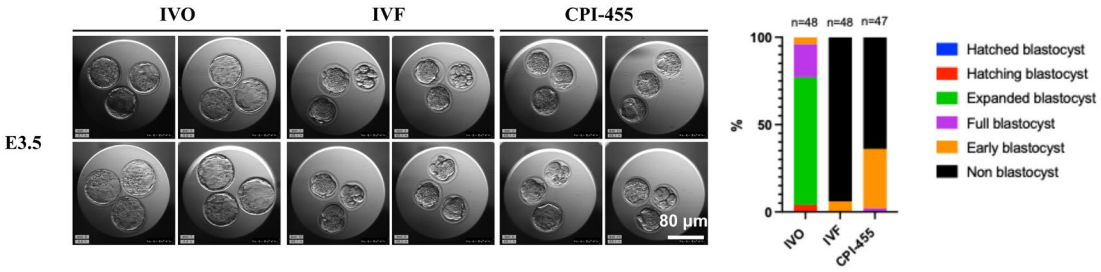


Figure R3-5. The proportion of blastocyst in E3.5 under several conditions including IVO, IVF and CPI-455.

13. Clinical Relevance of CD5 vs CD6 Transfers:
The discussion should include literature on outcomes of CD5 vs CD6 embryo transfers. Additionally, the reported dataset (n=2584 per group) is only relevant if all patients underwent single embryo transfer of a CD5 or CD6 embryo. Otherwise, the data interpretation is flawed.

We sincerely appreciate the reviewer's valuable suggestion. Relevant literature concerning the outcomes of day 5 versus day 6 embryo transfers has been integrated into the Discussion section. Regarding the post-PSM (propensity score matching) dataset, all patients included in the study underwent single blastocyst transfer during their first treatment cycle, as clearly outlined in the "Clinical Study Design and Statistical Analysis" subsection of the Materials and Methods section.

Minor Points
14. Replace "delayed embryo culture" with "extended culture."

We have replaced it.

15. Define P5 stage and stage 4 blastocyst in the text.

We infer that the P5 stage referred to by the reviewer may be PN5 stage, which is defined as follows: In mouse zygotes, post-fertilization development can be categorized into distinct pronuclear (PN) stages, specifically PN1 to PN5[26,27]. A PN5 zygote represents the terminal pronuclear stage of mouse embryonic development, occurring approximately 16-18 hours after fertilization. At this pre-mitotic G2 phase, the zygote exhibits enlarged pronuclei that are closely aligned near the embryonic center, following the completion of DNA replication. This developmental stage directly precedes pronuclear membrane breakdown and the initiation of the first mitotic division.

According to the Gardner and Schoolcraft scoring systems, blastocysts are given a numerical score from 1 to 6 based upon their degree of expansion and hatching status. A stage 4 blastocyst, also known as an expanded blastocyst, is characterized by a blastocoel volume that exceeds that of an early blastocyst (corresponding to stage 1), accompanied by thinning of the zona pellucida. This stage indicates progressive embryonic development prior to potential hatching.

The definition of both PN5 and stage 4 blastocyst have been added in the revised manuscript.

16. In Figure 1C, show IVF and IVO results side-by-side for each stage to enable easier comparison.

We thank the reviewer for this comment. In fact, in our work, we evaluated the transcriptional differences between IVF- and IVO-conceived embryos from two aspects: the one is transcriptional differences at each stage (e.g., IVF-morula VS IVO-morula, which was shown in Figure 1e), and the other is the difference of gene transcription dynamics during early embryo development (Figure 1c), for instance, a group of genes was specifically activated in IVO-morula, Figure 1c would further tell us whether these genes were also activated in IVF-morula. Both heatmap (Figure 1c) and quantitative analysis (Figure 1d) exhibited high correlation between IVF- and IVO-conceived embryos, indicating moderate transcriptional differences from this aspect.

17. In Figure 3E, provide a more detailed description of the ChromHMM method.

We thank the reviewer for this comment. ChromHMM, leveraging histone modification as well as protein binding data and based on multivariate hidden Markov model, was widely used to automatically annotate chromatin state[28]. In this work, we applied chromHMM (binsize = 500bp), using H3K4me3 and H3K27me3 ChIP-seq data as input, to divide whole genome into 4 states: none (decorated by neither H3K4me3 nor H3K27me3), active (decorated by only H3K4me3), repressive (decorate by only H3K27me3) and bivalent regions (decorated by both H3K4me3 and H3K27me3).

The above content has been added in the methods section of the revised manuscript to provide a more detailed description.

18. In Figure 3F, clarify how to interpret overlaps in bivalent regions between IVF and IVO embryos.

As mentioned above (reply to comment 17 of reviewer 3), we used chromHMM model and identified bivalent regions. Given that epigenetic landscape, especially H3K4me3, underwent reorganization in IVF-conceived blastocysts, therefore, Figure 3F revealed that most bivalent regions are unique to IVF- or IVO-conceived blastocysts (the first two columns) with only a small proportion of bivalent regions conserved between them (the last column). Overlapping bivalent regions represent bivalent genome regions remain stable (conserved) between IVF and IVO embryos.

In fact, bivalent regions could render genes in a poised state, letting them be activated rapidly by the removal of H3K27me3 in subsequent certain stage. We thus speculated the perturbation of bivalent regions in IVF-conceived blastocysts may influence specific gene regulation in subsequent developmental process and used *Pnpt1* as a typical example to illustrate it.

19. The increase in Eomes expression in IVF embryos contradicts prior findings (e.g., Giritharan 2012), which showed downregulation. This discrepancy should be addressed.

We thank the reviewer for this helpful comment. We firstly further measured the protein level of Eomes in IVF- and IVO-conceived blastocysts and found akin to transcription level, IVF-conceived blastocyst harbored significantly higher protein level for Eomes (Figure R3-6), hence supporting our discovery.

We next carefully checked the "Materials and Methods" section of the prior findings[25] (Giritharan et al., 2012), where both mouse strain and medium used for in vitro culture are different from our work: In Giritharan et al., they used CF-1 female and B6D2F1/J male mice and Whitten medium (WM), whereas in our work, we used ICR (Institute of Cancer Research) mice, G-IVF PLUS medium (for fertilization) and G1 PLUS medium (for in vitro culture). Such differences may result in the discrepancy of *Eomes* expression.

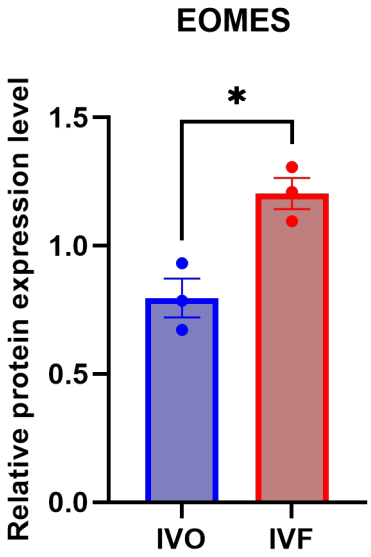


Figure R3-6. The protein expression level of Eomes in IVF- and IVO-conceived blastocysts.

20. In Figure 5B, explain what 4× and 10× represent (e.g., microscopy magnification?).

Yes, they represent the microscopy magnification and we have emphasized it in the revised figure legends.

21. In Figure 6G, human day 5 embryos appear to have both higher and lower DNA methylation than day 6 embryos. Could this indicate multiple subpopulations?

At the beginning of human embryo studies, we found compared to human day6-blastocysts, human day5-blastocysts possessed better clinical outcomes. We thus wondered which epigenetic modification was correlated to this phenomenon and found H3K4me3, instead of DNA methylation, may be related to clinical outcomes given that human day5-blastocyst harbored globally higher H3K4me3 levels and CPI-455 treatment also improved clinical outcomes, whereas the DNA methylation levels between day5- and day6-blastocysts seem to be irregular. Nevertheless, as mentioned in reply to (major) comment 3 of reviewer 2, we recognized the sample

size in our work is limited and more data are needed to infer the multiple subpopulations. In addition, we revised the statement as "On the other hand, we also compared the DNA methylation levels of day5- and day6-blastocysts, which seemed to be irregular. However, compared to previous studies[11], the sample size in our work was limited, more data taking into account both epigenetic information and clinical outcomes from the same embryo are needed to better compare the correlation of clinical outcomes to H3K4me3/DNA methylation."

22. Figure 6J requires more detailed explanation of the findings and their biological significance.

We thank the reviewer for this comment. Figure 6J exhibited the functions of CPI-455 sensitive human genes, which are defined as genes became up- (down-) regulated in both CPI-D5 and CPI-D6 blastocysts, compared to D5 and D6 blastocysts, respectively. Intriguingly, we found that the expression level of genes related to mitochondrion changed upon CPI-455 treatment, which may restore mitochondrial function and ultimately facilitate human blastocyst formation, with the latter already observed in our work. Of note, in mouse, the supplementation of CPI-455 indeed restored the expression level of genes related to mitochondrion, ATP and oxidative stress. Therefore, mitochondrial function was restored, which at last contribute to the formation of blastocyst and the improvement of clinical outcomes.

Taken together, this figure emphasized that the mechanism behind the phenomenon that CPI-455 treatment could facilitate blastocyst formation may be conserved between human and mouse.

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have done a great job in revising the manuscript. My comments have been fully addressed.

We appreciate the reviewer's helpful and supportive comments.

Reviewer #2 (Remarks to the Author):

I have reviewed the revised manuscript. The authors have fully addressed all of my previous comments. The paper is now suitable for publication.

We appreciate the reviewer's helpful and supportive comments.

Reviewer #3 (Remarks to the Author):

The authors have responded satisfactorily to the majority of queries
Few comments persist:
1. Ethics of Human Embryos:
The consent states: "Research is limited to abandoned sperm, seminal plasma, follicular fluid, immature eggs, abnormal fertilized eggs or embryos of poor quality that cannot be transferred, and will not be used for reproductive purpose". More explanations are needed for the following points:
o A large number of embryos (n=256) were generated from M1 oocytes, underwent ICSI (with sperm donor: Were egg donors and sperm donors aware that embryos would have been generated for research purposes?) and were developed to the blastocyst stage. Is this covered by the consent?
o Same excellent embryos 4AA were tested

We thank the reviewer for these helpful comments. We confirm that the generation and use of all human embryos in this research were fully compliant with the informed consent and ethical approvals.

<u>Regarding the 256 embryos generated from M1 oocytes:</u>
The use of these embryos is explicitly covered by the informed consent form for two primary reasons. First, the consent form explicitly authorizes the use of "immature eggs". In clinical embryology, M1 oocytes are definitively classified as immature oocytes as they have not completed meiosis I and are therefore not suitable for standard clinical transfer. Thus, their use falls directly within the scope of the consented materials. Second, all donors were fully informed that their biological materials would be used exclusively for non-reproductive research. Both the female

patients and dedicated sperm donor understand their discarded “immature eggs” and “abandoned sperm” would be used solely for the creation of research embryos with no reproductive intent.

<u>Regarding the use of good-quality 4AA blastocysts:</u>
The use of these blastocysts was governed by a separate, signed "Informed Consent for Disposition of Cryopreserved Embryos and Oocytes upon Expiration of the Storage Period". This document explicitly states that upon the expiration of the cryopreservation period, patients can consent to their embryos (irrespective of quality) being de-identified and donated for medical research purposes. Therefore, the application of these 4AA blastocysts in our research was conducted in strict accordance with the donors' prior authorization for research disposition.

<u>The informed consent form has been submitted together with the original ethical documentation.</u>

2. Table R3-1: infertility causes by non ovarian factors is not a clinical diagnosis. This should be well explained

We thank the reviewer for pointing this out. We have now corrected the term "clinical diagnosis" to "cause of infertility" in the revised table.

Reviewer #4 (Remarks to the Author):

This research triggers ethical concerns about source of reproductive materials, adequacy of consent from 'donor' parties, Oversight by a research ethics committee and manner and length of manipulation of donated embryonic materials.

I am satisfied that the research reported has followed appropriate ethical standards in all respects. The consent form for donors seems sound, IRB oversight and approval is documented. Manipulation of reproductive materials is ethically sound both intervention and time of interventions.

We appreciate the reviewer’s helpful and supportive comments.