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Chemical conversion of human conventional PSCs to TSCs following transient naive genes activation

Irene Zorzan, Riccardo Betto, Giada Rossignoli, Mattia Arboit, Andrea Drusin, Clelia Corridori, Paolo Martini, and Graziano Martello
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Corresponding author(s): Graziano Martello (graziano.martello@unipd.it) , Irene Zorzan (irene.zorzan@babraham.ac.uk)

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1st Editorial Decision **4th May 2022**

Dear Prof. Martello,

Thank you for the submission of your research manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, all referees have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be carefully addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Figure legends should be compiled at the end of the manuscript text.

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4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

In the past two years, a number of groups have successfully converted specially cultured "naïve" human pluripotent stem cells (hPSC) to trophoblast stem cells (hTSC). There have also been reports of conversion of cells from conventionally cultured primed hESC to hTSC, though there is controversy as to how efficient this process is, and whether the resulting cells are actually amnion or are contaminated with amnion-like cells.

In this paper, the authors demonstrate that relatively short-term treatments designed to promote naïve reversion (periods of 7 or even 3 days) are sufficient to allow conversion to trophoblast. The research is reasonably well conducted, but a few important comparisons are missing (see major comments below). From a technical standpoint, the research is useful to researchers who want to generate hTSC lines from hPSC lines as quickly as possible, but the biological value of the data is limited and the paper may be better suited to a more specialized journal.

Major comments:
1. The question of whether primed hPSCs generate trophoblast or amnion upon differentiation is still not settled (yet another possibility is that they may form trophoblast-like cells via an amnion-like intermediate). The introduction section strikes too certain a tone that primed hESC cannot generate trophoblast.
2. Relevant to the comment above, the authors demonstrate a lack of amnion markers in their chemically converted trophoblast cells in Figure 3e. However, they omit these markers in Figure 4b, in which they show differentiation of chemically converted TSCs over a time course. It is possible that the d3-reverted cells are generating amnion as well as trophoblast upon culture in hTSC media or passing through an amnion-like intermediate. Furthermore, the authors should include markers in these figures (e.g. ADAP2, HAVCR3, SLC28A3) specific to trophoblast as opposed to amnion. Many classical trophoblast genes are also expressed in amnion.
3. The authors draw attention to a population of non-naïve cells formed by d7 of reversion (Figures 1b,c, Figure2f) and seem to suggest that these could be trophoblast-like. However, they never demonstrate this. Either they should withdraw the claim or perform an immunofluorescence staining for trophoblast markers to show that they are trophoblast-like. Physical resemblance is insufficient proof, a lot of epithelial cells look vaguely similar.
4. Ideally, a publication claiming to have generated hTSCs would use, as a comparison point, authentic hTSCs generated from placenta or blastocyst. The authors here compare their ccTSC lines to naïve hPSC-derived hTSCs. This is perhaps tolerable given that there is pretty good literature establishing the similarity of naïve-derived hTSCs to authentic hTSCs, but at very least the authors should directly compare differential gene expression between naïve-derived hTSC and cchTSC and see if there are any statistically significant differences. Showing that they are found in similar places in a PCA plot is not sufficient.
5. Is there any concept as to the molecular identity of the d3-reverted cells, compared with classically primed or naïve hESCs. Does their ostensibly superior ability to form trophoblast arise from an earlier developmental state than conventional primed cells?

Minor comments:
- Page 5: "We asked ourselves what these cells might be". Too colloquial.
- A table detailing the passage number, sample descriptions, mapping statistics etc. of the RNA-seq samples is necessary.
- There are hCG ELISA kits which will deliver data far more quantitative than a home pregnancy test. These would be appropriate for Figure 2d.

Referee #2:

This manuscript from Zorzan et al. reports an efficient and rapid method for generating trophoblast stem cells (TSCs) from human pluripotent stem cells (PSCs) using a modified naïve resetting protocol. This work joins a number of recent studies that report methods to derive TSCs from naïve PSCs, all of which take advantage of the culture conditions for isolation of TSCs that were first described by Okae et al. in 2018. The difference with these prior studies is that Zorzan et al. show that 3 days of chemical resetting using a MEK inhibitor, HDAC inhibitor and LIF is sufficient to induce competence for TSC derivation, which removes the need for prolonged expansion under naïve conditions. Establishing more efficient methods to generate TSCs is important for modeling placental disorders. However, the authors need to clarify a number of points; in particular, the nature of the intermediate cells from which they derive TSCs after 3 days of chemical resetting remains unclear. This issue needs to be clarified since it is currently unclear whether TSCs are directly converted from conventional PSCs into TSCs (as the title suggests) or emerge from a partially reprogrammed intermediate state.

Major comments:

1. The current title of the manuscript gives the impression that the authors have developed a method for directly converting human conventional (i.e. primed) PSCs into TSCs without passing through naïve pluripotency. It's not clear that this is actually the case since this chemical resetting protocol was developed to induce naïve pluripotency and many naïve markers are already

upregulated within 3 days (see Fig. 4b). A more appropriate title might be: "Chemical conversion of human pluripotent stem cells to trophoblast stem cells following transient naïve reversion".

2. The nature of the intermediate cells that emerge within 3 days of chemical resetting is only investigated by bulk RNA-seq, which obscures likely heterogeneity within the population. It is unclear whether the TSCs arise from partially reprogrammed naïve cells, early trophoblast precursors (the "flat polygonal cells"?), or a true intermediate state in which naïve and trophoblast genes are co-expressed at the single cell level. It's also possible that some fully naïve cells are already present within 3 days. Since this issue is critical for the authors' interpretation, single cell analyses and/or prospective sorting based on distinctive cell-surface-markers is needed to understand the origin of TSCs.

3. According to the bulk RNA-seq data in Fig. 4b, the majority of naïve and TSC specific genes are already induced within two days of chemical resetting. Is the extra day of chemical resetting really necessary to induce competence for TSC derivation?

4. In Fig. 1 the authors compare gene expression in KiPS primed cells, KiPS cells undergoing chemical resetting, and HPD06 naïve iPSCs. At what timepoint were the KiPS undergoing chemical resetting analyzed? The text mentions 7 days, but the figure legend mentions 14 days. I'm also confused why KiPS cells undergoing chemical reprogramming are compared to HPD06 naïve iPS cells, which have a different genetic background. Fully reprogrammed KiPS naïve cells following prolonged expansion under naïve conditions would provide a better or at least complementary control.

5. In Fig. 2 and 3 gene expression in chemically converted (cc) TSCs is compared to TSCs generated from H9 and HPD06 naïve PSCs. These data need to be compared to previously published RNA-seq data from TSCs isolated from placental tissues or naïve PSCs by other labs. Also, can the authors comment on whether there are any transcriptional differences between naive TSCs and ccTSCs?

6. In Fig. 2 and 3 STB differentiation is assessed, but the authors do not investigate whether ccTSC can complete differentiation into the extravillous trophoblast (EVT) lineage, which is the other major specialized trophoblast lineage generated from TSCs. Upregulation of EVT markers should be assessed at the RNA and protein level relative to EVT generated from naïve TSC controls.

Minor points:

1. Please define the term "GETMS" in the Introduction.

2. Fig. 3d shows an image of ccTSC at passage 5 and KiPS treated with BAP media for 3 days. Given the significant difference in time, it would be useful to include phase contrast images of ccTSC at multiple timepoints during the derivation process.

Referee #3:

In this manuscript, Zoran and colleagues propose an alternative way to generate hTSCs from primed hPSCs. The manuscript is overall well written and nicely presented.

While of potential interest, some important controls are missing to clearly assess their findings.

Major remarks:
- An important omission is the study by Wei et al., Sci. Adv. 2021; 7 : eabf4416 11 August 2021. The approach is different, but should be at least discussed. It is possible than BMP signaling is a better "priming toward TSC reprogramming" strategy than inducing a naïve conversion. What would be the pros and the cons of each approach?
- When mentioning lack of amniotic signature, author should use the results of the recently published study by Rostovskaya and colleagues, Cell Stem Cell 2022.
- Authors might consider using placental or embryonic derived TSC, as reference.
- It is necessary to check if ccTSCs obtained after 3 days in MEKi/LIF/HDACi and directly switched to TSC medium are able to form bona fide syncytiotrophoblasts, looking at syncytialization, and also extravillous trophoblasts.
- How many passages where the ccTSCs cultured? Did extended passaging impaired differentiation potential?
- « We conclude that a modified chemical resetting protocol allowed the efficient conversion of conventional PSCs to TSCs. » Going back to my first comment, how efficient is the conversion? Did the author assess the homogeneity of the population?

Minor:
- Fig4B, it would be interesting to include ccTSC in the Heatmap.

Point-by-point response to Reviewers' comments

Referee #1:

In the past two years, a number of groups have successfully converted specially cultured "naïve" human pluripotent stem cells (hPSC) to trophoblast stem cells (hTSC). There have also been reports of conversion of cells from conventionally cultured primed hESC to hTSC, though there is controversy as to how efficient this process is, and whether the resulting cells are actually amnion or are contaminated with amnion-like cells.

In this paper, the authors demonstrate that relatively short-term treatments designed to promote naïve reversion (periods of 7 or even 3 days) are sufficient to allow conversion to trophoblast. The research is reasonably well conducted, but a few important comparisons are missing (see major comments below). From a technical standpoint, the research is useful to researchers who want to generate hTSC lines from hPSC lines as quickly as possible, but the biological value of the data is limited and the paper may be better suited to a more specialized journal.

We thank the Referee for the useful comments, which helped us improve the manuscript.
In particular, we made two observations that we deem biologically valuable:

1. Single-cell analysis of the conversion revealed rapid co-expression of naive pluripotency and TSC markers at day 3 (Fig 6). This phenomenon has not been observed in any of the recent TSC conversion protocols (Viukov *et al*, 2022; Wei *et al*, 2021; Jang *et al*, 2022; Soncin *et al*, 2018). Interestingly, also human naive cells co-express pluripotency and extraembryonic markers, a feature linked to their broad differentiation potential (Dong *et al*, 2020; Guo *et al*, 2021).
2. We noticed that our protocol leads to more robust activation of TSC markers than those based on BMP stimulation (Fig 3E-F), indicating that different conversion trajectories might have an impact on the quality of TSCs.
3. Concerning the controversy about amnion/TSC identity, we clarified that neither our protocol, nor those based on BMP stimulation (Wei *et al*, 2021; Jang *et al*, 2022) generates TSCs stably expressing amnion markers at a

significant level, by analysing a large set of amnion markers recently identified by Rostovskaya and colleagues (Rostovskaya *et al*, 2022).. Only some amnion markers are transiently upregulated during the conversion, but expression is lost upon expansion.

Major comments:

1. The question of whether primed hPSCs generate trophoblast or amnion upon differentiation is still not settled (yet another possibility is that they may form trophoblast-like cells via an amnion-like intermediate). The introduction section strikes too certain a tone that primed hESC cannot generate trophoblast.

We agree with the reviewer, considering very recent publications (Seetharam et al, 2022; Soncin et al, 2018; Rostovskaya et al, 2022), it is still not clear whether primed hPSCs generate amnion, trophoblast or a combination of the two. Also, it is hard to find markers that unambiguously distinguish between the two cell types.
For this reason, we included additional analyses (see below) about the expression of amnion markers in TSCs obtained from different sources.
We have edited the Introduction and Results sections accordingly (line 81-97 and 248-267).

2. Relevant to the comment above, the authors demonstrate a lack of amnion markers in their chemically converted trophoblast cells in Figure 3e. However, they omit these markers in Figure 4b, in which they show differentiation of chemically converted TSCs over a time course. It is possible that the d3-reverted cells are generating amnion as well as trophoblast upon culture in hTSC media or passing through an amnion-like intermediate.

This is a very good suggestion, we analysed the amnion markers previously shown in Figure 3E (i.e. POSTN, SPARCL1, ITGB6, ISL1 and SEMA3C).
We observed a mild and transient upregulation of BAMBI, ISL1, POSTN and SEMA3C. However, their expression dropped in ccTSC (Fig EV3A-B).

As a reference, we analysed transcriptomes of a set of TSCs obtained from different sources (from embryos, from naive PSCs or from primed PSCs, see also Point 4), and found that the amnion markers ITGB6, ISL1 and SEMA3C showed low expression in several TSC lines derived from primed PSCs via different protocols

(Wei et al, 2021; Jang et al, 2022) and this study) and in embryo-derived TSCs (Fig EV2E). BAMBI was detected in heterogeneous fashion in TSCs - including embryo-derived ones - and in all PSCs (Fig EV2E-F).
We conclude that the low levels of expression BAMBI, ITGB6, ISL1 and SEMA3C observed during conversion and in established TSCs might not indicate acquisition of amnion identity, but rather background/spurious expression.

To further investigate the potential acquisition of amnion identity we analysed additional markers identified by Rostovskaya and colleagues (Rostovskaya et al, 2022), in a recent study reporting that amnion forms from two waves of epiblast differentiation (early and late amniogenesis).

We observed that early amnion markers (e.g. PGF, TIMP3, S100P) are highly expressed in all TSCs, regardless of their origin (Fig EV2E). Indeed, Rostovskaya and colleagues reported that early amniogenesis occurs via a trophectoderm-like route, thus indicating a shared transcriptional programme between the two cell fates. Late amnion markers (IGFBP3, PRKD1, KCNMA1, GABRP) were barely detectable in all TSCs analysed (Fig EV2D-F). Also these markers showed a similar low/background expression in primed/conventional PSCs.

During conversion, we measured the expression of unambiguous amnion markers (Fig EV2E) and detected rapid upregulation of only ISL1 and POSTN during the first 3 days. We conclude that cells do not acquire a transient amnion identity during conversion (Fig EV3A-B).
At day 9 in TSC medium the late amnion markers KCNMA1 and GABRP were upregulated (Fig EV3A-B). In established ccTSCs all these amnion markers were expressed at negligible levels. These results might suggest that either a fraction of amnion is generated during the conversion, but these cells do not expand in TSC conditions or, alternatively, that some amnion markers are erroneously activated by transcription factors shared by TE and amnion identity (e.g. TFAP2A).

Formally, we cannot rule out a transition through an amnion-like intermediate. Future studies, based on genetic inactivation of key amnion regulators and/or lineage tracing will be needed to distinguish between the two hypotheses, but these experiments go beyond the scope of the current study.

Furthermore, the authors should include markers in these figures (e.g. ADAP2, HAVCR3, SLC28A3) specific to trophoblast as opposed to amnion. Many classical trophoblast genes are also expressed in amnion.

We added the suggested markers and observed that only ADAP2 is rapidly induced, from day 2 onwards (Fig 5D and EV3A).

We then checked the expression of these 3 markers in a panel of TSCs, including established ccTSCs.
ADAP2 is robustly expressed by ccTSCs to levels found in TSC lines obtained from embryos and from naive PSCs (Fig EV2E and 3F).
HAVCR1 (we think the Referee wrote 'HAVCR3' but meant 'HAVCR1', as only the latter is used as a TSC marker) is upregulated in established ccTSC and naive-TSC, relative to primed PSCs, as detected by qPCR and RNAseq (Fig 3F and EV2D-E).
SLC28A3 is also highly expressed by ccTSCs to levels comparable to embryo-derived TSCs (Fig 3F and EV2E). Of note, HAVCR1 and SLC28A3 were not expressed in several TSCs derived from primed PSCs via BMP induction (Wei et al, 2021; Jang et al, 2022)(Fig 3F).

We conclude that ADAP2 is rapidly induced during conversion of primed PSCs to ccTSCs, while HAVCR1 and SLC28A3 expression increases during expansion of ccTSCs.

3. The authors draw attention to a population of non-naïve cells formed by d7 of reversion (Figures 1b,c, Figure2f) and seem to suggest that these could be trophoblast-like. However, they never demonstrate this. Either they should withdraw the claim or perform an immunofluorescence staining for trophoblast markers to show that they are trophoblast-like. Physical resemblance is insufficient proof, a lot of epithelial cells look vaguely similar.

We withdrew the claim as suggested and simply reported the activation of TSC and TE markers. We also clarified, both in the text and in the figures/legends, that the analyses performed in Figure 1 refer to day 14, rather than day 7.

4. Ideally, a publication claiming to have generated hTSCs would use, as a comparison point, authentic hTSCs generated from placenta or blastocyst. The authors here compare

their ccTSC lines to naïve hPSC-derived hTSCs. This is perhaps tolerable given that there is pretty good literature establishing the similarity of naïve-derived hTSCs to authentic hTSCs, but at very least the authors should directly compare differential gene expression between naïve-derived hTSC and cchTSC and see if there are any statistically significant differences. Showing that they are found in similar places in a PCA plot is not sufficient.

We completely agree on the importance of comparing our data against embryo-derived TSCs. To do so, we analysed RNA-seq data from 3 studies (Okae et al, 2018; Wei et al, 2021; Jang et al, 2022) in which TSCs were obtained from embryos or placental tissues, as well as from PSCs.
We found that ccTSCs and TSC derived from our naive PSCs expressed a large set of TSC markers at levels comparable to, or higher than, embryo-derived TSCs (Fig 3E).

Importantly, functional TSC regulators have been recently identified (e.g. SKP2, TEAD1, ARID5B, TFAP2C, GATA3)(Guo et al, 2021; Dong et al, 2022), and all those genes are highly expressed in ccTSCs.

To further characterise the transcriptome of ccTSCs we used the PlacentaCellEnrich program (Jain & Tuteja, 2021), which find specific expression of cell types found in first-trimester human placenta (Vento-Tormo et al, 2018).
The genes upregulated in ccTSCs, compared to isogenic primed kiPS cells, were highly enriched for Syncytiotrophoblast, the in vivo couterpart of TSCs (Fig 3D).
A similarly strong enrichment was found comparing H9 naive-TSCs against their isogenic primed PSCs (H9 ESCs). These analyses indicate correct trophoblast identity acquisition in ccTSCs.

To compare the global transcriptome of ccTSC against those TSC obtained from naive PSCs, we also performed a correlation analysis (Fig EV2B). We found that ccTSCc and naive-TSC are highly correlated, with coefficients ranging between 0.85 and 0.92. In contrast, comparing ccTSC vs naive PSC gave coefficients below 0.72, and comparing ccTSC vs primed PSC gave coefficients between 0.69 and 0.72. These results indicate that the global transcriptome of ccTSC and naive-TSC are highly correlated.

We then focussed on differences between ccTSCs and naive-TSCs.

None of the TSC markers were found differentially expressed between ccTSCs and TSC obtained from naive PSCs (see Reviewer Figure 1, at the end of this document). We performed Gene Ontology and KEGG pathways enrichment analyses on the 279 genes (Cluster B) distinguishing ccTSCs from naive TSCs, and failed to find any significant term.

However, manual inspection revealed aberrant expression of 7 imprinted genes in TSC derived from naive PSCs (Reviewer Figure 1).
Prolonged expansion of human naive PSCs has been reported to induce imprinting abnormalities (Perrera & Martello, 2019), thus it is interesting to observe aberrant expression of imprinted genes in TSC derived from naive PSCs.
However, we believe that a thorough investigation of this aspect might be suited for a future publication comparing a larger set of TSC obtained with different protocols.

Thus, based on the comparable expression of several functional TSC markers, PlacentaCellEnrich analysis, the PCA and the global correlation analysis, we conclude that the ccTSCs, naive TSCs and embryo-derived TSCs are highly similar.

5. Is there any concept as to the molecular identity of the d3-reverted cells, compared with classically primed or naïve hESCs. Does their ostensibly superior ability to form trophoblast arise from an earlier developmental state than conventional primed cells?

To address this crucial question we performed two experiments:

1. *We analysed the expression of developmental stages earlier than conventional primed PSCs, namely naive pluripotency and 8-cell like cells (8CLCs - Taubenschmid-Stowers et al, 2022; Mazid et al, 2022). We could not detect expression of 8-cell stage markers (Fig EV3A), while several naive markers were rapidly upregulated, together with TSC markers like GATA3.*

2. *We performed quantitative immunostaining for markers of TSC (GATA3 and KRT7), general pluripotency (OCT4) and naive pluripotency (SUSD2) (Fig 6B-D).*
Remarkably, after 3 days of resetting we found co-expression of OCT4, SUSD2, GATA3 and KRT7 in 54% of cells, and of OCT4, SUSD2, GATA3 in 21%.
This is in line with rapid mRNA increase of GATA3 and SUSD2 measured by RNAseq (Fig 5B and 5D).

We conclude that 3 days of resetting activate both naive and TSC markers in the same cells. The subsequent signalling environment steers cells towards one of the two alternative fates.
This is interesting, as a recent study proposed that the co-expression of embryonic and extraembryonic genes observed in human naive pluripotent cells confers them the competence for somatic and extraembryonic differentiation (Dong et al, 2020).

Minor comments:
- Page 5: "We asked ourselves what these cells might be". Too colloquial.

We rephrased the sentence.

- A table detailing the passage number, sample descriptions, mapping statistics etc. of the RNA-seq samples is necessary.

We generated the requested table (see Appendix Table S1).

- There are hCG ELISA kits which will deliver data far more quantitative than a home pregnancy test. These would be appropriate for Figure 2d.

We agree on the fact that an ELISA test would be more sensitive than a home pregnancy test, but we think that a qualitative result (i.e. present/absent) is sufficient in this context, as also found in several high profile studies (Turco et al, 2018; Liu et al, 2020).

Referee #2:

This manuscript from Zorzan et al. reports an efficient and rapid method for generating trophoblast stem cells (TSCs) from human pluripotent stem cells (PSCs) using a modified naïve resetting protocol. This work joins a number of recent studies that report methods to derive TSCs from naïve PSCs, all of which take advantage of the culture conditions for isolation of TSCs that were first described by Okae et al. in 2018. The difference with these prior studies is that Zorzan et al. show that 3 days of chemical resetting using a MEK inhibitor, HDAC inhibitor and LIF is sufficient to induce competence for TSC derivation, which removes the need for prolonged expansion under naïve conditions. Establishing more efficient methods to generate TSCs is important for modelling placental disorders. However, the authors need to clarify a number of points; in particular, the nature of the intermediate cells from which they derive TSCs after 3 days of chemical resetting remains unclear. This issue needs to be clarified since it is currently unclear whether TSCs are directly converted from conventional PSCs into TSCs (as the title suggests) or emerge from a partially reprogrammed intermediate state.

Major comments:

1. The current title of the manuscript gives the impression that the authors have developed a method for directly converting human conventional (i.e. primed) PSCs into TSCs without passing through naïve pluripotency. It's not clear that this is actually the case since this chemical resetting protocol was developed to induce naïve pluripotency and many naïve markers are already upregulated within 3 days (see Fig. 4b). A more appropriate title might be: "Chemical conversion of human pluripotent stem cells to trophoblast stem cells following transient naïve reversion".

> *We thank the reviewer for the suggestion, but we think there must have been some technical issues or confusion.*
> *The title of our submitted manuscript is “Chemical conversion of human conventional Pluripotent Stem Cells to Trophoblast Stem Cells”.*
> *We actually never implied that the conversion occurs without passing through the naive state. The Reviewer might maybe refer to the title of an older version of our bioRxiv preprint, which we anyway amended.*
> *We agree that we should mention the activation of naive genes, especially in light of the novel results showing widespread expression of the naive marker SUSD2 during*

conversion (Fig 6B-D). Also, no activation of naive genes have been reported in other recent studies (Viukov et al, 2022; Soncin et al, 2018).
However we think that "Chemical conversion of human pluripotent stem cells to trophoblast stem cells following transient naïve genes activation", as "naive reversion" implies functional reversion to the naive state, which was never tested.

2. The nature of the intermediate cells that emerge within 3 days of chemical resetting is only investigated by bulk RNA-seq, which obscures likely heterogeneity within the population. It is unclear whether the TSCs arise from partially reprogrammed naïve cells, early trophoblast precursors (the "flat polygonal cells"?), or a true intermediate state in which naïve and trophoblast genes are co-expressed at the single cell level. It's also possible that some fully naïve cells are already present within 3 days. Since this issue is critical for the authors' interpretation, single cell analyses and/or prospective sorting based on distinctive cell-surface-markers is needed to understand the origin of TSCs.

We completely agree on the importance of resolving at the single cell resolution the potential heterogeneity of day 3 cells.
We thus performed quantitative immunostaining for two markers of TSC (GATA3 and KRT7), a general pluripotency marker (OCT4) and a naive pluripotency marker (SUSD2).
GATA3 and OCT4 have been demonstrated to be functionally required for TSC and PSC identity, respectively (Dong et al, 2022; Guo et al, 2021; Takashima et al, 2014; Hay et al, 2004; Matin et al, 2004; Zaehres et al, 2005; Wang et al, 2012).
Remarkably, after 3 days of resetting we found co-expression of OCT4, SUSD2, GATA3 and KRT7 in 54% of cells, and of OCT4, SUSD2, GATA3 in 21%.

Interestingly, the population of cells expressing only OCT4+SUSD2, or only GATA3+KRT7 were negligible (1% and 0%, respectively).
See also Results (line 315-344).
We conclude that at day 3 of conversion the majority of cell co-express markers of naive PSC and TSC. The subsequent exposure to different signalling environments steer the cells towards two alternative cell fates.
This is interesting, as a recent study proposed that the co-expression of embryonic and extraembryonic genes observed in human naive pluripotent cells confers them the competence for somatic and extraembryonic differentiation (Dong et al, 2020).

3. According to the bulk RNA-seq data in Fig. 4b, the majority of naïve and TSC specific genes are already induced within two days of chemical resetting. Is the extra day of chemical resetting really necessary to induce competence for TSC derivation?

> *We respectfully disagree with this comment. Between day 2 and day 3 there is a significant increase in the expression of GATA3 and SUSD2 (Fig 5D), so it is reasonable to perform chemical resetting for 3 days.*

4. In Fig. 1 the authors compare gene expression in KiPS primed cells, KiPS cells undergoing chemical resetting, and HPD06 naïve iPSCs. At what time point were the KiPS undergoing chemical resetting analyzed? The text mentions 7 days, but the figure legend mentions 14 days.

> *We apologise for the confusion. The figure legends are correct, as those images refer to day 14 of the conversion. In the text we stated that 'After 7 days, several dome shaped, compact colonies were present, that we could readily expand for multiple passages in PXGL medium'. We simply meant that naive-like colonies appeared after 7 days, as reported by other authors (Guo et al, 2017; Bredenkamp et al, 2019).*
> *We amended the text, figures and figure legends to avoid misunderstandings (line 133-136).*

I'm also confused why KiPS cells undergoing chemical reprogramming are compared to HPD06 naïve iPS cells, which have a different genetic background. Fully reprogrammed KiPS naïve cells following prolonged expansion under naïve conditions would provide a better or at least complementary control.

> *We have reset KiPS cells, sorted them for SUSD2 positivity (Bredenkamp et al, 2019) and negativity for the primed marker CD24 (Collier et al, 2017) expanded and analysed, as suggested. Fig 1D shows that KiPS undergoing chemical resetting for 14 days upregulated naive markers and downregulated the primed state marker ZIC2.*

5. In Fig. 2 and 3 gene expression in chemically converted (cc) TSCs is compared to TSCs generated from H9 and HPD06 naïve PSCs. These data need to be compared to previously published RNA-seq data from TSCs isolated from placental tissues or naïve PSCs by other labs. Also, can the authors comment on whether there are any transcriptional differences between naive TSCs and ccTSCs?

We compared the transcriptome of ccTSC, H9-TSC and HPD06-TSC to those of TSC generated in other laboratories, either from placenta/blastocysts or from naive PSCs (Wei et al, 2021; Jang et al, 2022; Okae et al, 2018).

We found that ccTSCs and TSC derived from our naive PSCs expressed a large set of TSC markers at levels comparable to, or higher than, embryo-derived TSCs (Fig 3D). Importantly, we analysed a set of crucial TSC regulators (e.g. SKP2, TEAD1, ARID3A, ARID5B, TFAP2C) identified by via CRISPR/Cas9 inactivation (Dong et al, 2022; Guo et al, 2021) and all those genes are highly expressed in TSCs generated in our study (Fig 3D, in bold).

To further characterise the transcriptome of ccTSCs we used the PlacentaCellEnrich program (Jain & Tuteja, 2021), which finds specific expression of cell types found in first-trimester human placenta (Vento-Tormo et al, 2018).
The genes upregulated in ccTSCs, compared to isogenic primed KiPS cells, were highly enriched for Syncytiotrophoblast, the in vivo couterpart of TSCs. A similarly strong enrichment was found comparing H9 naive-TSCs against their isogenic primed PSCs. These analyses indicate correct trophoblast identity acquisition in ccTSCs.

To compare the transcriptome of ccTSC against those TSC obtained from naive PSCs, we also performed a correlation analysis (Fig EV2B). We found that ccTSCc and naive-TSC are highly correlated, with coefficients ranging between 0.85 and 0.92. In contrast, comparing ccTSC vs naive PSC gave coefficients below 0.72, and comparing ccTSC vs primed PSC gave coefficients between 0.69 and 0.72. These results indicate that the global transcriptome of ccTSC and naive-TSC are highly correlated.

We then focussed on differences between ccTSCs and naive-TSCs.
None of the TSC markers were found differentially expressed between ccTSCs and TSC obtained from naive PSCs (see Reviewer Fig. 1, at the end of this document).

We performed Gene Ontology and KEGG pathways enrichment analyses on the 279 genes (Cluster B) distinguishing ccTSCs from naive TSCs, and failed to find any significant term.

However, manual inspection revealed aberrant expression of 7 imprinted genes in TSC derived from naive PSCs (Reviewer Fig. 1).
Prolonged expansion of human naive PSCs has been reported to induce imprinting abnormalities (Perrera & Martello, 2019), thus it is interesting to observe aberrant expression of imprinted genes in TSC derived from naive PSCs.
However, we believe that a thorough investigation of this aspect might be suited for a future publication comparing a larger set of TSC obtained with different protocols.

Thus, based on the comparable expression of several functional TSC markers, PlacentaCellEnrich analysis, the PCA and the global correlation analysis, we conclude that the ccTSCs, naive TSCs and embryo-derived TSCs are highly similar, but future studies will be needed to investigate the impact of aberrantly expressed imprinted genes found in naive TSCs.

6. In Fig. 2 and 3 STB differentiation is assessed, but the authors do not investigate whether ccTSC can complete differentiation into the extravillous trophoblast (EVT) lineage, which is the other major specialized trophoblast lineage generated from TSCs. Upregulation of EVT markers should be assessed at the RNA and protein level relative to EVT generated from naïve TSC controls.

We first performed EVT differentiation from naive-TSCs and observed robust induction of HLA-G surface protein and of VGLL3, NOTUM, SNAI1 and ITGA5 mRNAs, accompanied by an elongated, mesenchyme-like morphology (Fig 2C-D and 4).

We then repeated the differentiation protocols for STB and EVT on naive-TSCs and on ccTSCs. We used ccTSCs2, obtained after 3 days in MEKi/LIF/HDACi and directly switched to TSC medium (Fig 3), as requested by Reviewer #3.
Analysis of a large set of STB and EVT markers, both at the mRNA and protein levels, confirmed successful differentiation (Fig 4).

Minor points:

1. Please define the term "GETMS" in the Introduction.

We rephrased the sentence and listed the 5 transcription factors.

2. Fig. 3d shows an image of ccTSC at passage 5 and KiPS treated with BAP media for 3 days. Given the significant difference in time, it would be useful to include phase contrast images of ccTSC at multiple timepoints during the derivation process.

We have included in the revised manuscript phase contrast images of the derivation process *(Fig 6A). Cells at day 3 of the conversion are morphologically distinct from BAP cells.*

Referee #3:

In this manuscript, Zorzan and colleagues propose an alternative way to generate hTSCs from primed hPSCs. The manuscript is overall well written and nicely presented.

While of potential interest, some important controls are missing to clearly assess their findings.

Major remarks:
- An important omission is the study by Wei et al., Sci. Adv. 2021; 7 : eabf4416 11 August 2021. The approach is different, but should be at least discussed. It is possible than BMP signaling is a better "priming toward TSC reprogramming" strategy than inducing a naïve conversion. What would be the pros and the cons of each approach?

We apologise for not citing and discussing this important study.
We analysed transcriptomes from cells generated by Wei and colleagues (Wei et al, 2021) and found that some TSC markers were not expressed (HAVCR1 and SLC28A3, Fig 3F), or expressed at levels lower than ccTSCs (e.g. TEAD1, TCAF1 and ARID5B - Fig 3E-F). We also analysed a large set of amniotic markers (Rostovskaya et al, 2022; Guo et al, 2021; Cinkornpumin et al, 2020; Jang et al, 2022) and found no significant differences among TSCs of different origin.
We discussed that the trajectories followed during conversion appear different. In our protocol we detected robust induction of naive markers, together with TSC markers. This aspect was not investigated by Wei and colleagues. However, two recent studies (Soncin et al, 2018; Viukov et al, 2022) reported the conversion of conventional PSC to TSC without induction of naive markers, indicating that different protocols induce distinct trajectories.
A recent study also proposed the co-expression of embryonic and extraembryonic genes as a functional attribute of naive pluripotency (Dong et al, 2020). In agreement with this idea, we detected co-expression of naive PSCs and TSCs markers at the single-cell levels after 3 days of chemical conversion, indicating an intermediate cellular state responsive to different external stimuli.

Given the robust expression of a large set of TSC markers in ccTSCs (Fig 3E) and the co-expression of KRT7 and GATA3 in 97% of cells at day 14 of conversion (Fig 6B-D) we do not think that inducing a naive conversion is worse than using BMP

signalling (for which Wei and colleagues reported less than 25% of KRT-positive cells by day 10, while GATA positivity ranged from ~25 to ~90%), although additional studies, comparing side by side different protocols would be needed to draw conclusions about efficiency.

- When mentioning lack of amniotic signature, authors should use the results of the recently published study by Rostovskaya and colleagues, Cell Stem Cell 2022.

This is a very good suggestion, we analysed the amnion markers previously shown in Figure 3E of the submitted manuscript (i.e. POSTN, SPARCL1, ITGB6, ISL1 and SEMA3C), together with additional markers identified by Rostosvskaya and colleagues (Rostovskaya et al, 2022), in a recent study reporting that amnion forms from two waves of epiblast differentiation (early and late amniogenesis).

First, we analysed a panel of embryo-derived TSCs, naive-TSCs and TSCs derived from primed/conventional PSCs with different protocols (Fig EV2E).
We observed that early amnion markers (e.g. PGF, TIMP3, S100P) are highly expressed in all TSCs, regardless of their origin. Indeed, Rostovskaya and colleagues reported that early amniogenesis occurs via a trophectoderm-like route, thus indicating the presence of transcriptional programme shared by the two cell fates.
In contrast, the late amnion markers (PRKD1, KCNMA1, GABRP) were barely detectable in all TSCs, either embryo-derived or obtained from PSCs (Fig EV2E-F). These late amnion markers showed equally low expression levels in primed/conventional PSCs. Given that conventional PSCs are not amnion cells, we conclude that the low expression of late amnion markers observed in TSCs does not indicate acquisition of amnion identity in TSCs.

- Authors might consider using placental or embryonic derived TSC, as reference.

We included the suggested samples from 3 different studies (Okae et al, 2018; Wei et al, 2021; Jang et al, 2022) and included them in our analyses (Fig 3E-F, EV2E-F). A large number of TSC markers were expressed at comparable levels in ccTSCs and embryo-derived TSCs.

- It is necessary to check if ccTSCs obtained after 3 days in MEKi/LIF/HDACi and directly switched to TSC medium are able to form bona fide syncytiotrophoblasts, looking at syncytialization, and also extravillous trophoblasts.

We first performed EVT differentiation from naive-TSCs and observed robust induction of HLA-G surface protein and of VGLL3, NOTUM, SNAI1 and ITGA5 mRNAs, accompanied by an elongated, mesenchyme-like morphology (Fig 2C-D and 4).

We then repeated the differentiation protocols for STB and EVT on naive-TSCs and on ccTSCs. We used ccTSCs2, obtained after 3 days in MEKi/LIF/HDACi and directly switched to TSC medium (Fig 3), as requested by Reviewer #3.
Analysis of a large set of STB and EVT markers, both at the mRNA and protein levels, confirmed successful differentiation (Fig 4).

- How many passages where the ccTSCs cultured? Did extended passaging impaired differentiation potential?

The ccTSC line we generated expanded robustly for >20 passages.
All differentiation experiments were performed between passage 7 and 10, so we do not know whether extended passaging could affect the differentiation potential, but such studies would require several months of expansion and differentiation.

- « We conclude that a modified chemical resetting protocol allowed the efficient conversion of conventional PSCs to TSCs. » Going back to my first comment, how efficient is the conversion? Did the author assess the homogeneity of the population?

We have measured the protein expression levels of TSC and PSC markers throughout the conversion (Fig 6B-D). On day 3 we observed coexpression of PSC (OCT4) and TSC (GATA3) markers in >75% of cells. At day 7, after the switch to TSC medium, OCT4 expression was retained, together with GATA3 in 37% of cells. 40% of cells expressed OCT4, GATA3 and KRT7. A similar co-expression of PSC (OCT4 and SUSD2) and TSC markers (GATA3 and KRT7) was recently reported by Zijlmans et al. (Zijlmans et al, 2022), when naive PSC were converted to TSCs.

On day 14 we found that 97% of cells were GATA3 and KRT7 double positive, indicating high homogeneity of the population.
As a comparison, the most efficient protocol from Wei and colleagues, based on BMP stimulation, gave rise to less than 25% of KRT-positive cells by day 10, while GATA positivity ranged from ~25 to ~90%. Although a direct side-by-side comparison would be needed, our protocol seems at least equally efficient.

Minor:

- Fig4B, it would be interesting to include ccTSC in the Heatmap

Thanks for the good suggestion, we added ccTSCs to the heatmap.

References

Bredenkamp N, Stirparo GG, Nichols J, Smith A & Guo G (2019) The Cell-Surface Marker Sushi Containing Domain 2 Facilitates Establishment of Human Naive Pluripotent Stem Cells. *Stem Cell Rep* 12: 1212–1222

Cinkornpumin JK, Kwon SY, Guo Y, Hossain I, Sirois J, Russett CS, Tseng H-W, Okae H, Arima T, Duchaine TF, *et al* (2020) Naive Human Embryonic Stem Cells Can Give Rise to Cells with a Trophoblast-like Transcriptome and Methylome. *Stem Cell Rep* 15: 198–213

Collier AJ, Panula SP, Schell JP, Chovanec P, Plaza Reyes A, Petropoulos S, Corcoran AE, Walker R, Douagi I, Lanner F, *et al* (2017) Comprehensive Cell Surface Protein Profiling Identifies Specific Markers of Human Naive and Primed Pluripotent States. *Cell Stem Cell* 20: 874-890.e7

Dong C, Beltcheva M, Gontarz P, Zhang B, Popli P, Fischer LA, Khan SA, Park K, Yoon E-J, Xing X, *et al* (2020) Derivation of trophoblast stem cells from naïve human pluripotent stem cells. *eLife* 9: e52504

Dong C, Fu S, Karvas RM, Chew B, Fischer LA, Xing X, Harrison JK, Popli P, Kommagani R, Wang T, *et al* (2022) A genome-wide CRISPR-Cas9 knockout screen identifies essential and growth-restricting genes in human trophoblast stem cells. *Nat Commun* 13: 2548

Guo G, von Meyenn F, Rostovskaya M, Clarke J, Dietmann S, Baker D, Sahakyan A, Myers S, Bertone P, Reik W, *et al* (2017) Epigenetic resetting of human pluripotency. *Dev Camb Engl* 144: 2748–2763

Guo G, Stirparo GG, Strawbridge SE, Spindlow D, Yang J, Clarke J, Dattani A, Yanagida A, Li MA, Myers S, *et al* (2021) Human naive epiblast cells possess unrestricted lineage potential. *Cell Stem Cell* 28: 1040-1056.e6

Hay DC, Sutherland L, Clark J & Burdon T (2004) Oct-4 Knockdown Induces Similar Patterns of Endoderm and Trophoblast Differentiation Markers in Human and Mouse Embryonic Stem Cells. *Stem Cells* 22: 225–235

Jain A & Tuteja G (2021) PlacentaCellEnrich: A tool to characterize gene sets using placenta cell-specific gene enrichment analysis. *Placenta* 103: 164–171

Jang YJ, Kim M, Lee B-K & Kim J (2022) Induction of human trophoblast stem-like cells from primed pluripotent stem cells. *Proc Natl Acad Sci* 119: e2115709119

Liu X, Ouyang JF, Rossello FJ, Tan JP, Davidson KC, Valdes DS, Schröder J, Sun YBY, Chen J, Knaupp AS, *et al* (2020) Reprogramming roadmap reveals route to human induced trophoblast stem cells. *Nature* 586: 101–107

Matin MM, Walsh JR, Gokhale PJ, Draper JS, Bahrami AR, Morton I, Moore HD & Andrews PW (2004) Specific knockdown of Oct4 and β2-microglobulin expression by RNA interference in human embryonic stem cells and embryonic carcinoma cells. *Stem Cells* 22: 659–668

Mazid MA, Ward C, Luo Z, Liu C, Li Y, Lai Y, Wu L, Li J, Jia W, Jiang Y, *et al* (2022) Rolling back human pluripotent stem cells to an eight-cell embryo-like stage. *Nature* 605: 315–324

Okae H, Toh H, Sato T, Hiura H, Takahashi S, Shirane K, Kabayama Y, Suyama M, Sasaki H & Arima T (2018) Derivation of Human Trophoblast Stem Cells. *Cell Stem Cell* 22: 50-63.e6

Perrera V & Martello G (2019) How Does Reprogramming to Pluripotency Affect Genomic Imprinting? *Front Cell Dev Biol* 7

Rostovskaya M, Andrews S, Reik W & Rugg-Gunn PJ (2022) Amniogenesis occurs in two independent waves in primates. *Cell Stem Cell* 0

Seetharam AS, Vu HTH, Choi S, Khan T, Sheridan MA, Ezashi T, Roberts RM & Tuteja G (2022) The product of BMP-directed differentiation protocols for human primed pluripotent stem cells is placental trophoblast and not amnion. *Stem Cell Rep* 17: 1289–1302

Soncin F, Khater M, To C, Pizzo D, Farah O, Wakeland A, Arul Nambi Rajan K, Nelson KK, Chang C-W, Moretto-Zita M, *et al* (2018) Comparative analysis of mouse and human placentae across gestation reveals species-specific regulators of placental development. *Dev Camb Engl* 145: dev156273

Takashima Y, Guo G, Loos R, Nichols J, Ficz G, Krueger F, Oxley D, Santos F, Clarke J, Mansfield W, *et al* (2014) Resetting Transcription Factor Control Circuitry toward Ground-State Pluripotency in Human. *Cell* 158: 1254–1269

Taubenschmid-Stowers J, Rostovskaya M, Santos F, Ljung S, Argelaguet R, Krueger F, Nichols J & Reik W (2022) 8C-like cells capture the human zygotic genome activation program in vitro. *Cell Stem Cell* 29: 449-459.e6

Turco MY, Gardner L, Kay RG, Hamilton RS, Prater M, Hollinshead MS, McWhinnie A, Esposito L, Fernando R, Skelton H, *et al* (2018) Trophoblast organoids as a model for maternal–fetal interactions during human placentation. *Nature* 564: 263–267

Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Tormo M, Meyer KB, Park J-E, Stephenson E, Polański K, Goncalves A, *et al* (2018) Single-cell reconstruction of the early maternal–fetal interface in humans. *Nature* 563: 347–353

Viukov S, Shani T, Bayerl J, Aguilera-Castrejon A, Oldak B, Sheban D, Tarazi S, Stelzer Y, Hanna JH & Novershtern N (2022) Human primed and naïve PSCs are both able to differentiate into trophoblast stem cells. *Stem Cell Rep* 0

Wang Z, Oron E, Nelson B, Razis S & Ivanova N (2012) Distinct Lineage Specification Roles for NANOG, OCT4, and SOX2 in Human Embryonic Stem Cells. *Cell Stem Cell* 10: 440–454

Wei Y, Wang T, Ma L, Zhang Y, Zhao Y, Lye K, Xiao L, Chen C, Wang Z, Ma Y, *et al* (2021) Efficient derivation of human trophoblast stem cells from primed pluripotent stem cells. *Sci Adv* 7: eabf4416

Zaehres H, Lensch MW, Daheron L, Stewart SA, Itskovitz-Eldor J & Daley GQ (2005) High-efficiency RNA interference in human embryonic stem cells. *Stem Cells* 23: 299–305

Zijlmans DW, Talon I, Verhelst S, Bendall A, Van Nerum K, Javali A, Malcolm AA, van Knippenberg SSFA, Biggins L, To SK, *et al* (2022) Integrated multi-omics reveal polycomb repressive complex 2 restricts human trophoblast induction. *Nat Cell Biol* 24: 858–871

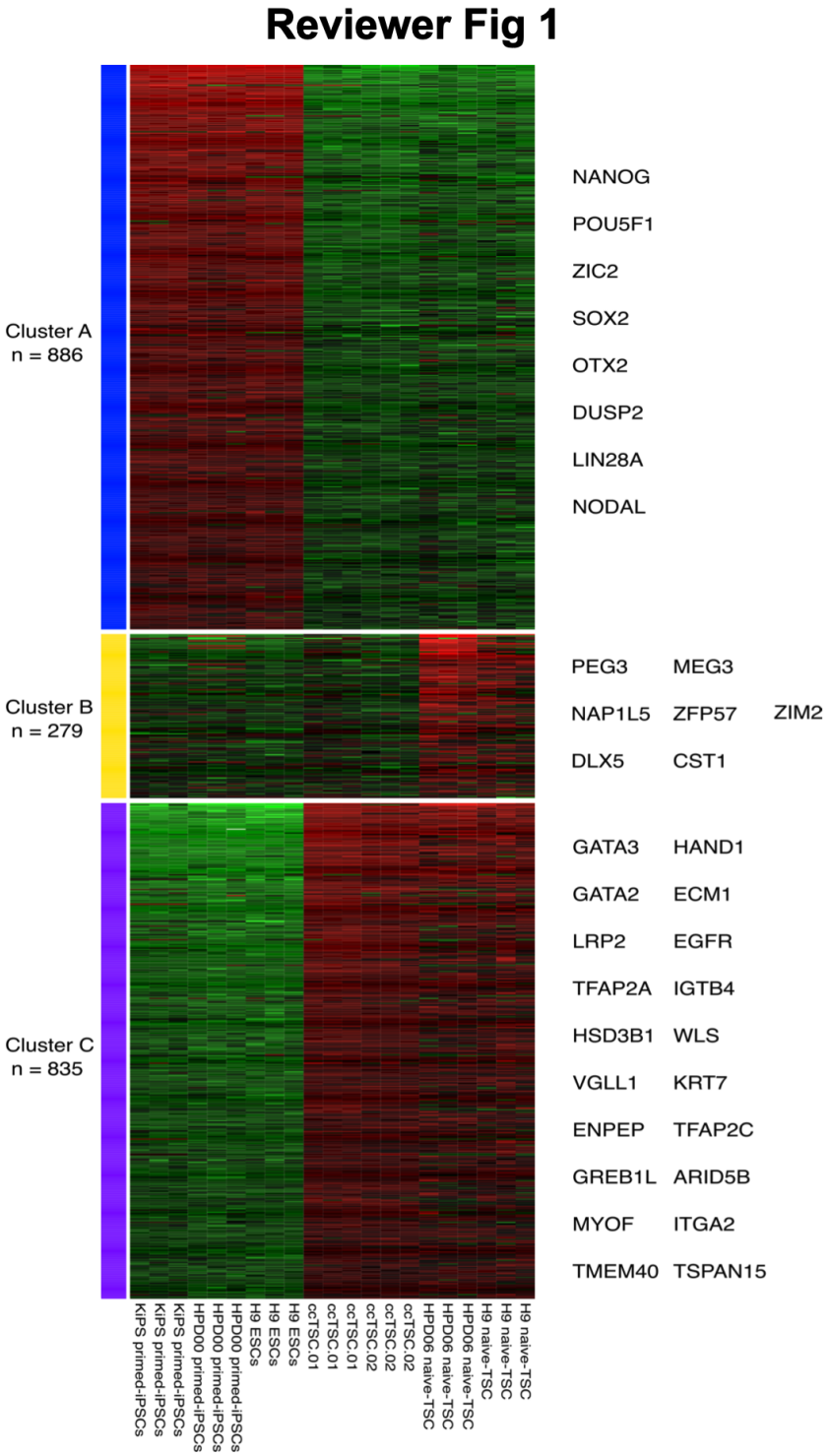

Figure legend: K-means clustering of the 2000 most variable genes in Primed-iPSCs (KiPS primed-iPSCs, HPD00 primed-iPSCs and H9 ESCs), ccTSCs (ccTSC.01 and ccTSC.02) and Naive-TSC (HPD06 naive-TSC and H9 naive-TSC. Red and green indicate high and low expression, respectively. Representative genes of each cluster are shown on the right.

Dear Prof. Martello,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports. Nevertheless, referees #1 and #2 have remaining concerns and suggestions to improve the manuscript, I ask you to address in a final revise manuscript.

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Presently, most diagrams seem to lack statistical testing!

- The labelling of the x-axes in Fig. 5D is hardly readable. Please provide the diagrams enlarged and in 2-3 rows with bigger fonts.

- Please add scale bars of similar style and thickness to the microscopic images (main and EV), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, many scale bars are rather thin.

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and

similar to the one in the acknowledgement section of the manuscript text file.

- Please make sure that all figure panels are called out separately and sequentially (main and EV figures). Presently, Fig. 6D is called out before 6A,
Fig. EV2C is called out before EV2A and there seems to be no callout for Fig. EV2F.
Moreover, there are callouts to panels EV3E-F, which don't seem to exist. Please check.

- Please add a title to the Appendix file (Appendix for ...), page numbers and also add the page numbers to the table of contents.

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Yours sincerely,

Achim Breiling
Senior Editor
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Referee #1:

The authors have substantially addressed most of my concerns, and I do not object to the eventual publication of this paper. However, I still have one significant concern about this submission. To the extent that there is anything approaching a consensus in the field about hTSC derived from primed hPSC, it is that most trophoblast genes can be reactivated relatively easily but that certain heavily methylated imprinted require extended naïve culture. This point was best demonstrated by a paper published since the initial submission of Zorzan et al: Kobayashi et al. "The microRNA cluster C19MC confers differentiation potential into trophoblast lineages upon human pluripotent stem cells" Nature Communications 2022. Kobayashi and colleagues find that activation of the imprinted C19MC locus occurs when naïve hPSCs are converted to trophoblast but not when primed cells are.

It is logical to worry therefore, that certain imprinted genes including but not limited to C19MC will not reactivate in three days of chemical conversion. In response to previous comments, the authors compare expression of naïve and cc-derived hTSCs, and note failure to activate imprints including PEG3. C19MC is presumably not analyzed, not being a coding transcript. The authors should include Reviewer Figure 1 in the extended data and discuss it briefly. Also they should analyze reactivation of C19MC or at least mention the Kobayashi 2022 finding in the Discussion and say that the C19MC reactivation status of the ccTSC is unknown.

Minor comments:
1. Reviewer Figure 1: ZFN57 is not imprinted, it is a regulatory of imprinting. Did the authors mean ZNF597?
2. Line 167/Figure 1I "We also analysed a wide range of markers expressed by TSCs in vitro and observed strong upregulation of some trophoblast markers (Figure 1I)."
EOMES is not expressed in conventional hTSCs or for that matter first trimester placenta (see RNA-seq data in Okae 2018). It is really only a trophoblast marker for mouse. The authors should change their phrasing.
3. Figure 3E, EV2E: Legend makes extensive reference to RNA-seq data from Okae 2018 that appears to be from Dong 2020. There are no H9 ESCs in Okae 2018.
4. Line 297: "APAD2" is presumably ADAP2
5. Figure 5D labels are too small.

Referee #2:

Zorzan and colleagues have carefully addressed the reviewers' comments and their manuscript has been improved as a result. In particular, their newly incorporated single cell analysis of primed cells undergoing chemical resetting reveals the co-expression of naïve and trophoblast markers on day 3, which suggests the emergence of a transient intermediate state that is amenable for either naïve reversion or trophoblast differentiation. In addition, the revised their title better conveys the main message of the manuscript. This study will be of interest to the readers of EMBO Reports. I only have a few minor suggestions remaining:

- On lines 230-231 the authors conclude that "a transient inhibition of histone deacetylases is sufficient to allow efficient and rapid conversion of conventional PSCs to ccTSCs, without need of an intermediate step in a naïve supporting medium". However, MEK inhibition and LIF are also included in the chemical resetting recipe and likely contribute to the observed gene expression changes. It would be better to write "transient histone deacetylase and MEK inhibition with LIF stimulation", as is indicated in the abstract.

- Line 284: same issue as above.

- Line 330: please change "SUD2" to "SUSD2"

- In Fig. 3E and Fig. EV2E it looks like the samples from Dong et al. 2020 are mislabeled as "Okae et al., 2018". Okae et al. 2018 did not generate naïve TSC.

Referee #3:

The authors have improved their manuscript and correctly addressed my points.

Point-by-point Response to Reviewers' Comments

Comments from Referee #1:

The authors have substantially addressed most of my concerns, and I do not object to the eventual publication of this paper. However, I still have one significant concern about this submission. To the extent that there is anything approaching a consensus in the field about hTSC derived from primed hPSC, it is that most trophoblast genes can be reactivated relatively easily but that certain heavily methylated imprinted require extended naïve culture. This point was best demonstrated by a paper published since the initial submission of Zorzan et al: Kobayashi et al. "The microRNA cluster C19MC confers differentiation potential into trophoblast lineages upon human pluripotent stem cells" Nature Communications 2022. Kobayashi and colleagues find that activation of the imprinted C19MC locus occurs when naïve hPSCs are converted to trophoblast but not when primed cells are.

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> We thank the reviewer for the insightful comment. We included the Figure as suggested. We discussed the importance of analysing imprinted genes expression in TSCs obtained via different methods.
> We also mentioned the findings of Kobayashi and colleagues, but we have been unable to analyze the expression of C19MC as small-RNA sequencing would be needed.

Minor comments:
1. Reviewer Figure 1: ZFN57 is not imprinted, it is a regulatory of imprinting. Did the authors mean ZNF597?

> We deleted ZNF57 from the figure.

2. Line 167/Figure 1I "We also analysed a wide range of markers expressed by TSCs in vitro and observed strong upregulation of some trophoblast markers (Figure 1I)."
EOMES is not expressed in conventional hTSCs or for that matter first trimester placenta (see RNA-seq data in Okae 2018). It is really only a trophoblast marker for mouse. The authors should change their phrasing.

> We deleted the EOMES panel from the figure.

3. Figure 3E, EV2E: Legend makes extensive reference to RNA-seq data from Okae 2018 that appears to be from Dong 2020. There are no H9 ESCs in Okae 2018.

We amended the figure and the legend; we should have indeed referred to Dong 2020.

4. Line 297: "APAD2" is presumably ADAP2

We apologize for the typo.

5. Figure 5D labels are too small.

We made sure all labels are easy to read.

Comments from Referee #2:

Zorzan and colleagues have carefully addressed the reviewers' comments and their manuscript has been improved as a result. In particular, their newly incorporated single cell analysis of primed cells undergoing chemical resetting reveals the co-expression of naïve and trophoblast markers on day 3, which suggests the emergence of a transient intermediate state that is amenable for either naïve reversion or trophoblast differentiation. In addition, the revised their title better conveys the main message of the manuscript. This study will be of interest to the readers of EMBO Reports. I only have a few minor suggestions remaining:

We are glad the Reviewer appreciates the improvements in our revised manuscript.

- On lines 230-231 the authors conclude that "a transient inhibition of histone deacetylases is sufficient to allow efficient and rapid conversion of conventional PSCs to ccTSCs, without need of an intermediate step in a naïve supporting medium". However, MEK inhibition and LIF are also included in the chemical resetting recipe and likely contribute to the observed gene expression changes. It would be better to write "transient histone deacetylase and MEK inhibition with LIF stimulation", as is indicated in the abstract.

- Line 284: same issue as above.

We have rephrased the text on line 230-231, and other instances, clearly stating that it is "transient histone deacetylase and MEK inhibition with LIF stimulation".

- Line 330: please change "SUD2" to "SUSD2"

We apologise for the typo.

- In Fig. 3E and Fig. EV2E it looks like the samples from Dong et al. 2020 are mislabeled as "Okae et al., 2018". Okae et al. 2018 did not generate naïve TSC.

We amended the figure and the legend; we should have indeed referred to Dong 2020.

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We are glad the Reviewer appreciates the improvements in our revised manuscript.

Prof. Graziano Martello
University of Padova
Department of Biology
Viale G. Colombo, 3
Padova 35131
Italy

Dear Prof. Martello,

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The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
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  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Material and Methods

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Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
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