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***Reprogrammed iBlastoids contain amnion-like cells but not trophectoderm***

Cheng Zhao[1], Alvaro Plaza Reyes[1], John Paul Schell[1], Jere Weltner[1], Nicolás Ortega[1],Yi Zheng[2], Åsa K. Björklund[3], Janet Rossant[4], Jianping Fu[2,5,6], Sophie Petropoulos[1,7,8,9] & Fredrik Lanner[1,10#]

1. Department of Clinical Science, Intervention and Technology, Karolinska Institutet, and Division of Obstetrics and Gynecology, Karolinska. Universitetssjukhuset, Stockholm, Sweden.
2. Department of Mechanical Engineering, University of Michigan, Ann Arbor, MI 48109, USA.
3. Dept of Cell and Molecular Biology, National Bioinformatics Infrastructure Sweden, Science for Life Laboratory, Uppsala University, Husargatan 3, SE-752 37 Uppsala, Sweden
4. Program in Developmental and Stem Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada
5. Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.
6. Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA.
7. Département de Médecine, Université de Montréal, Montreal Canada
8. Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Axe Immunopathologie, H2X 19A Montreal Canada
9. Département de Biochimie et Médecine Moléculaire, Université de Montréal, Montréal Canada
10. Ming Wai Lau Center for Reparative Medicine, Stockholm Node, Karolinska Institutet, Stockholm, Sweden

# Corresponding author

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**Abstract**

Two recent papers in *Nature* show that human blastocyst-like structures (or blastoids) can be generated from human pluripotent stem cells[1] or through reprogramming of fibroblasts[2], respectively. Both papers perform extensive single cell transcriptional analysis and compare blastoid cells with the cells in preimplantation human embryos[3,4], leading to a conclusion that the blastoids contain cell lineages corresponding to the epiblast, primitive endoderm and trophectoderm in preimplantation human embryos. Transcriptional analysis is, however, critically dependent on having relevant reference samples, not only of targeted cell types but also of potential alternative cell lineages. For this reason, we have reevaluated the blastoid data with a more comprehensive cellular reference, including extended cultures of blastocysts, several stem cell-based embryo models and a gastrulation stage human specimen. From this reanalysis we resolve that reprogrammed blastoids by Liu et al. fail to generate cells with trophectoderm profiles. Instead, cells identified as trophectoderm lineages in reprogrammed blastoids possess a transcriptional profile more representative of amniotic cells in post-implantation human embryos. Our reanalysis also shows that stem cell-derived blastoids[1] did contain trophectoderm-like cells, highlighting the potential of human blastoids to model blastocyst development.

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**Results**.

Understanding human embryogenesis is critical for improving reproductive technologies, preventing pregnancy loss and supporting optimal fetal health. Studies of human embryos are, however, difficult, since donated embryos are limited in numbers, functional studies are challenging, and experiments are limited by ethical restrictions. Although extended culture of human embryos beyond preimplantation stages opens up exciting opportunities, such studies are only ethically permissible up to the 14th day post conception. So-called embryo models (or embryoids) derived *in vitro* using cultured cells offer an exciting opportunity to circumvent these challenges. However, the usefulness of such embryoids is dependent on how well the embryoids are characterized and how well they recapitulate natural human embryonic development. Human embryonic stem cells (hESCs) with primed pluripotency have been suggested to have the capacity to differentiate into the trophectoderm lineage[5]. However, more recent studies suggest that the purported trophectoderm cells derived from primed hESCs might instead be mis-annotated extraembryonic mesoderm or amnion[6,7]. Furthermore, the purported trophectoderm cells in mouse blastoids generated from extended pluripotent stem cells (EPSCs) instead represent mesoderm-like cells[8]. In light of these conflicting views and the critical need for accurate annotations of cell lineages in embryoids, we reevaluate the two recent papers suggesting that human blastocyst-like structures (or blastoids) can be generated from human pluripotent stem cells or through reprogramming of fibroblasts, respectively[1,2]. In both studies, transcriptional profiles of the blastoids were compared to those from pre-implantation human blastocysts[3,4], leading to a conclusion that the blastoids contain cell lineages corresponding to the epiblast, hypoblast and trophectoderm in the pre-implantation human blastocyst. To examine the possibility of the presence of mesoderm- or amnion-like cells in the blastoids, we generated an integrated data set, combining the blastoid transcriptome data with the

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Carnegie stage (CS) 7 human gastrula data, which contain mesoderm and amnion (also labeled as non-neural ectoderm) cells (Tyser et al 2020)[9], and data from a human amniotic sac model (Zheng et al 2019)[10] and from *in vitro* cultured human blastocysts up to day 14 (Xiang et al 2020)[11]. The integrated data clearly revealed distinct cell clusters related to the epiblast, trophectoderm, endoderm, amnion and mesoderm lineages (Fig. 1a, b). As expected, amnion- and mesoderm-like cells in the human amniotic sac model overlap with the amnion and mesoderm clusters from the CS7 human gastrula, respectively. In line with the published analysis, a significant portion of epiblast-like cells (ELC), hypoblast-like cells (HLC) and trophoblast-like cells (TLC) of the stem cell-derived blastoids overlap with their counterparts in human embryos. In contrast, cells from the reprogrammed blastoids did not contribute to the trophoblast cluster, and most cells of the annotated TLC population cluster instead with the amnion of the CS7 human gastrula and the amniotic sac model. The TLC in reprogrammed blastoids lack expression of trophectoderm markers including *GATA2/3* while instead expressing markers associated with the amnion such as *ISL1* and *GABRP* (Fig.1c)[9,12,13]. Expression of *ISL1* and *GABRP* is also clearly enriched in the TLC when exploring the reprogrammed blastoid data set in the accompanying ShinyApp (http://blastoid.ddnetbio.com). Although single cell sequencing data generated from dissociated blastocysts is not ideal for estimating the ratio of cell types present, it can still provide important information pertaining to the proportion of cell types within lineages and their ratios. Trophectoderm cells should be the dominating cell type in human blastocysts as seen in the transcriptional analysis of blastocysts of embryonic day 6-7 with 78%, 14% and 9% of trophectoderm, epiblast and hypoblast, respectively (Extended Data Fig.1.a-b). In contrast to blastocysts, both blastoids contained a much greater epiblast population and at the same time carried substantial numbers of amnion- and mesoderm-like cells (Fig.1.a and Extended Data Fig.1c), suggesting features in line with

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post-implantation human embryos. We therefore encourage the use of as complete as possible reference datasets in future transcriptome analysis of human embryoids including blastoids, and hope that our analysis can be of help in such future work.

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## Figure legends

**Fig. 1 Single-cell transcriptomic profiling for extended datasets. a,** UMAP projection of integrated datasets. Embryonic cells, cells from Zheng et al., and blastoid cells generated from stem cells (Stem-Blastoids) and reprogramming (iBlastoids) are shown in separate panels. The color of each data point represents cell annotations retrieved or restored from corresponding publications. The shape of data points for embryonic cells indicates data source. Cells belonging to blastoid datasets which were not assigned as major lineage-related cells are labeled as "Undefined" and colored in dark grey. Amnion-like cells (AMLC) and transwell amnion-like cells (Tsw-AMLC) from Zheng et al. were combined as AMLC for visualization. **b,** Expression of top 10 lineage-specific genes for Epiblast, Endoderm, Trophectoderm (TE), Mesoderm, and Amnion lineages in embryonic cells (dot plot) and blastoid cells (heat map). The size and colors of dots indicate the proportion of cells expressing the corresponding gene and averaged scaled values of log-transformed expression, respectively. The color shown in Heatmap represents scaled values of log-transformed expression in each cell. For visualization, Epiblast, Hypoblast, and Trophectoderm-like cells from both blastoid datasets with more than 150 cells are randomly down-sampled to 150. **c,** Violin plots showing log-transformed expression of key Amnion and Trophectoderm marker genes in embryonic Trophectoderm, embryonic Amnion, Amnion-like cells (AMLC), transwell Amnion-like cells (Tsw-AMLC), and previously annotated Trophectoderm-like cell of Stem-Blastoids (TLC (SB)) and iBlastoids (TLC (IB)).

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**Contributions**

C.Z. and F.L. conceived the study with advice from A.P-R., J.P.S. C.Z., A.P-R., and J.P.S., performed the data analyses with help from J.W., S. P., N.O. Y.Z., J.R., J.F., Å.B. The manuscript was written by C.Z., A.P-R, S.P., and F.L., with input from all of the authors.

**Corresponding author**

Correspondence to fredrik.lanner@ki.se

**Ethics declarations**

**Competing interests**

The authors declare no competing interests.

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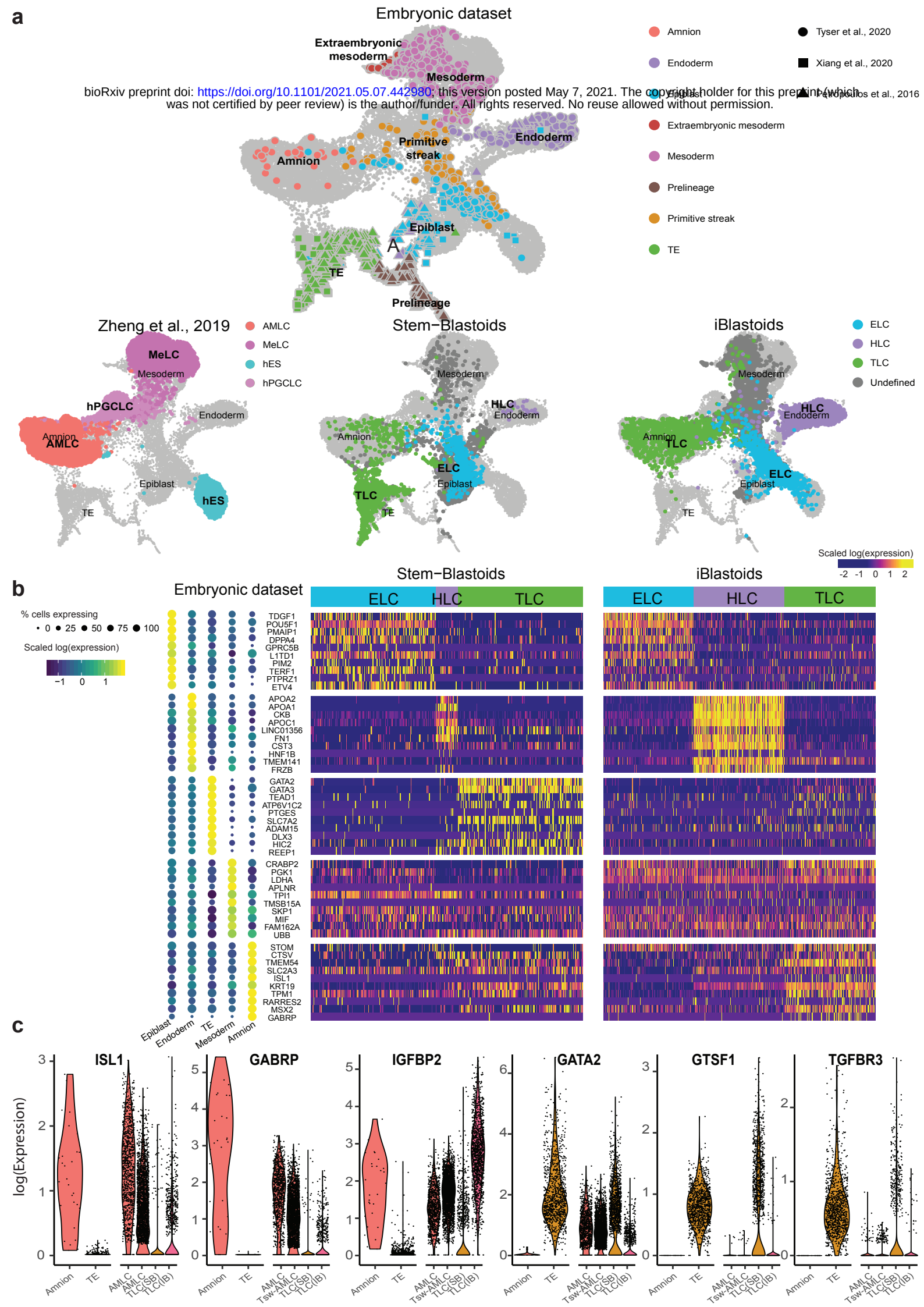
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