Single Cell RNA Sequencing Identifies CXADR as a Fate Determinant of the Placental Exchange Surface

Dafina M. Angelova[1,2]; Malwina Prater[2,3]; Aleksandra Tsolova[4,5]; Wendi Bacon[2,6]; Russell S. Hamilton[2]; Danielle Blackwell[4,5]; Noura Ballasy[4,5]; Ziyi Yu[7]; Xin Li[8]; Xin Liu[8]; Myriam Hemberger[4,5]; D. Stephen Charnock-Jones[1,2]

[1] Department of Obstetrics and Gynaecology, University of Cambridge, NIHR Cambridge Biomedical Research Centre, Cambridge, United Kingdom

[2] Centre for Trophoblast Research, Department of Physiology, Development, and Neuroscience, University of Cambridge, Cambridge, United Kingdom

[3] Functional Genomics Centre, Cancer Research Horizons, Milner Therapeutics Institute, Jeffrey Cheah Biomedical Centre, University of Cambridge, Cambridge, United Kingdom

[4] Department of Biochemistry and Molecular Biology, Cumming School of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada

[5] Alberta Children's Hospital Research Institute, University of Calgary, Calgary, Canada

[6] School of Life, Health & Chemical Sciences, The Open University, Milton Keynes, United Kingdom

[7] State Key Laboratory of Materials-Oriented Chemical Engineering, College of Chemical Engineering, Nanjing Tech University, Nanjing, P. R. China

[8] Sphere Fluidics Ltd., Building One Granta Centre, Granta Park, Great Abington, Cambridge, CB21 6AL, United Kingdom

Early placentation is a crucial period in fetal development. Abnormalities in the formation of the placenta underly major pregnancy complications. The differentiation of the trophectoderm into the different trophoblast lineages is critical for the remodeling of the maternal spiral arteries and the establishment of placental blood supply and to generate the syncytial surface which is essential for gaseous and nutrient exchange between the mother and the fetus.

Studying the early differentiation events *in-vivo* is difficult and mouse trophoblast stem (TS) cells are one useful *in-vitro* model to achieve this goal. Here we used single cell RNA sequencing analysis of differentiating mouse trophoblast stem cells to uncover the gene expression patterns that define the entry into the trophoblast lineages. We induced mouse trophoblast stem cell differentiation by using a Mitogen-activated protein kinase kinase inhibitor (PD0325901) or by withdrawing conditioned medium and sampled cells at 0h, 1h, 4h, 24h, 36h and 48h (n=3-4). We fabricated polydimethylsiloxane microfluidic devices and encapsulated single cells with barcoded beads in microdroplets to construct single cell transcriptomic Drop-seq libraries. Our dataset comprised a total of 136,291 cells with an average of 1,740 reads and 1,059 genes per cell.

We explored the heterogeneity of mouse trophoblast stem cells before differentiation through clustering with Seurat. We identified a sub-population of mouse TS cells with a gene expression profile similar to differentiating cells suggesting they were primed for differentiation. Differential gene expression analysis identified NELL2 Interacting Cell

Ontogeny Regulator 1 (*Nicol1*) as a potential marker for the predominant non-primed stem cell population.
Further bioinformatic analysis of the full timecourse in Seurat identified candidate clusters for the junctional zone and placental labyrinth progenitor cells. Gene regulatory network analysis in SCENIC revealed that the labyrinth progenitor cell cluster, characterized by activity of the glial cells missing transcription factor 1 (*Gcm1*) regulon, also exhibited increased activity of the PHD Finger Protein 8 (*Phf8*) and E2F Transcription Factor 8 (*E2f8*) regulons. Pseudotime analysis in Monocle and marker gene expression revealed that the junctional zone and syncytiotrophoblast layer I lineage precursor cells initially shared a similar differentiation trajectory, while the syncytiotrophoblast layer II lineage was already transcriptionally distinct by 48h.
Pseudotime analysis showed that the Coxsackievirus and adenovirus receptor (*Cxadr*) transcript is retained in the syncytiotrophoblast layer I and junctional zone trajectory but is not expressed in the early syncytiotrophoblast layer II progenitors. Immunofluorescent staining of mouse trophoblast stem cells revealed that loss of CXADR preceded syncytialisation. We then determined the effect of CRISPR-Cas9 mediated knockout of *Cxadr* in TS cells. Upon differentiation this lead to increased cell fusion but with upregulation of syncytiotrophoblast layer I markers and reduction of syncytiotrophoblast layer II markers as measured by RT-qPCR. This suggests that this gap junction protein is involved in maintaining the correct balance between the two layers of syncytiotrophoblast in the mouse labyrinth by slowing or suppressing the syncytiotrophoblast layer I differentiation.
Correct trophoblast differentiation is essential to the establishment of the placental exchange interface. These findings enhance our understanding of the early events during mouse trophoblast differentiation and if a similar mechanism is present in human trophoblast this may effect human pregnancy outcome.
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