**Single-Cell Transcriptomic Analysis of Wnt/β-Catenin-mediated Differentiation Pathways in Mouse Trophoblast Stem Cells**

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**Background and aims.** Placental trophoblast stem cells (TSCs) differentiate into various placental trophoblast lineages, including trophoblast giant cells (TGCs), spongiotrophoblasts (SpTs), and syncytiotrophoblasts (SynTs). In mice, the placental barrier is comprised of two layers of SynTs, with SynT-II comprising the fetal side layer. Wnt/β-catenin signalling is reportedly essential for differentiation to SynT-II cells *in vivo*. This study aims to observe whether mouse TSCs can be selectively differentiated into SynT-II via Wnt/β-catenin activation using single-cell RNA sequencing (scRNA-seq).

**Methods.** The scRNA-seq was conducted on mouse TSCs that were cultured in standard differentiation medium or in differentiation medium supplemented with the Wnt/β-catenin activator CHIR99021. Data were integrated with existing single-nucleus RNA-seq datasets from mouse placental tissues (E9.5–E14.5) for comparative analysis. Cell clusters were identified based on the expression of established markers. Epithelial-mesenchymal transition (EMT) gene expression profiles were analysed using principal component analysis (PCA).

**Results.** The activation of Wnt/β-catenin signalling resulted in an increase in the proportion of SynT-II-related cell clusters from 11% to 25%. However, a more pronounced increase in junctional zone-like linage cell clusters was observed, from 8% to 32%, suggesting that Wnt activation broadly promotes differentiation into multiple trophoblast lineages. PCA indicated that SynT-II-related clusters exhibited epithelial characteristics, whereas non-SynT-II trophoblast lineages displayed mesenchymal traits. Genes upregulated in the initial differentiation step into non-SynT-II trophoblasts under Wnt activation includes transcription factors such as Ascl2 (involved in SpT and TGC differentiation), Satb1 (associated with TSC maintenance), Myc (a downstream target of Satb1 in EMT), and Irx3 (a reported Wnt target), highlighting their potential roles in trophoblast lineage specification.

**Conclusion/Discussion.** Wnt/β-catenin signal activation in mTSCs promotes differentiation towards SynT-II cells, however, it also significantly induces differentiation to other trophoblast lineages, particularly junctional zone-like lineages. EMT appears to be a key mechanism in the differentiation of non-SynT-II trophoblasts. This study provides valuable insights into the regulatory networks governing trophoblast differentiation and offers a foundation for developing in vitro models of the placental barrier.