

## **Reduced cell invasion may be a characteristic of placental defects in pregnant women of advanced maternal age at single-cell level**

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### **Material S1**

#### **The methods of single-cell RNAsequencing (scRNA-seq)**

The method was as described in our previous study (Yang et al., 2021), mainly included the following experimental steps.

#### ***Preparation of single-cell suspensions***

After delivery, we cut small tissue (about 1.5 cm<sup>3</sup>) in the region of 2 cm~5 cm away from the umbilical cord insertion immediately (within 5 min). Placental samples were minced on ice into <1-mm<sup>3</sup> pieces, followed by enzymatic digestion using trypsin. Subsequently, the solution was centrifuged at 300 rcf for 30 s at room temperature and the supernatant was removed. 1× PBS (calcium- and magnesium-free) containing 0.04% weight/volume BSA (400 µg/mL) was added to the supernatant, followed by centrifugation at 300 rcf for 5 min. The cell pellet was then resuspended in 1 mL red blood cell lysis buffer and incubated for 10 min at 4 °C. After red blood cell lysis, samples were resuspended in 1 mL PBS containing 0.04% BSA and filtered over SciencewareFlowmi 40-µm cell strainers (VWR). Finally, cell concentration and viability were determined by haemocytometers and Trypan Blue staining.

#### ***Single-cell RNA-seq library construction and sequencing***

The scRNA-seq libraries were prepared using Chromium Single Cell 3' Reagent v3 Kits. Single-cell suspensions were loaded on a Chromium Single Cell Controller Instrument (10x Genomics) to generate single-cell gel beads in emulsions (GEMs). Briefly, about 16,000–20,000 cells were added to each channel, with a targeted cell recovery estimate of 5,000–8,000 cells. After generation of GEMs, single-cell RNA-seq libraries were prepared using the Chromium Single Cell 3'Library& Cell Bead Kit (10x Genomics) according to the manufacturer's protocol. Libraries

were sequenced with an Illumina Novaseq 6000 using high-output 75-cycle kits with previously reported read length configuration (Yang et al., 2021).

### ***Single-cell RNA sequencing data analysis***

The Cell Ranger software pipeline (version 3.0) provided by 10x Genomics was used to demultiplex cellular barcodes, map reads to the genome and transcriptome using the STAR aligner, and down-sample reads as required to generate normalized aggregate data across samples, producing a matrix of gene counts versus cells. We processed the unique molecular identifier (UMI) count matrix using the R package Seurat (version 2.3.4). Low-quality cells (UMI/gene numbers out of the limit of mean value  $\pm 2$  fold of standard and  $>10\%$  mitochondrial genes) were excluded.

The top variable genes across single cells were identified using the method described in Macosko et al. (2015). Briefly, the average expression and dispersion were calculated for each gene, and genes were subsequently placed into 22 bins based on expression. Principal component analysis (PCA) was performed to reduce the dimensionality in the log-transformed gene-barcode matrices of the top variable genes. Cells were clustered using a graph-based clustering approach and visualized in two dimensions using tSNE. We used likelihood ratio tests that simultaneously assessed changes in mean expression and in the percentage of expressed cells to identify significantly DEGs between clusters. We used the R package SingleR, a novel computational method for unbiased cell type recognition of scRNA-seq, with two reference transcriptomic datasets from the 'Human Primary Cell Atlas' (Mabbott et al., 2013) to infer the cell of origin of each of the single cells independently, and to identify cell types.

### ***Differentially expressed genes (DEGs) analysis and enrichment analysis***

Differentially expressed genes (DEGs) were identified using the FindMarkers function (test.use=MAST) in Seurat (Trapnell et al., 2014).  $P$  value  $<0.05$  and  $\log_2(\text{fold change}) > 0.58$  was set as the threshold for significant differential expression. GO enrichment and KEGG pathway enrichment analysis of DEGs were respectively performed using R based on the hypergeometric distribution.

### ***Pseudotime analysis***

We determined the developmental pseudotime with the Monocle2 package (Trapnell et al., 2014). The raw count was first converted from Seurat object into CellDataSet object with the import CDS function in Monocle. We used the differential GeneTest function of the Monocle2 package to select ordering genes ( $q\text{-val} < 0.01$ ) which were likely to be informative in the ordering of cells along the pseudotime trajectory. The dimensional reduction clustering analysis was performed with the reduce Dimension function, followed by trajectory inference with the order Cells function

using default parameters. Gene expression was plotted with the plot gene in pseudotime function to track changes over pseudo-time.

### ***RNA velocity analysis***

We performed RNA velocity analysis using the R package *velocyto*. R v0.6 (La Manno et al., 2018). The RNA velocity was calculated based on spliced and unspliced transcript reads and estimated using a gene-relative model. The resulting velocity estimates were projected onto the t-SNE embedding obtained in Seurat and the pseudotime space produced by Monocle 2.

## **Material S2**

### ***Immunofluorometric Assay***

Frozen placental tissue sections were cut into 4–5  $\mu\text{m}$  pieces and fixed with 1% acetone. After inactivation and sealing, 50–100  $\mu\text{L}$  of fluorescently labelled diluted primary antibodies were separately added and incubated at 37 °C. After rinsing with phosphate-buffered saline (PBS), 50–100  $\mu\text{L}$  diaminidino-2-phenylindole solution was added to each plate, and the plates were placed in a dark environment at room temperature. They were sealed with anti-extraction seals, and the expression of proteins in the cells was observed by confocal laser scanning (Olympus FV3000, Japan).

### ***Immunohistochemistry***

As previously reported (Yang et al., 2021), slices for immunohistochemistry were prepared according to the conventional method. After inactivation, each slice was sealed with 50–100  $\mu\text{L}$  of 1% bovine serum albumin for 20 min. Antibody was dripped and stored at 4 °C overnight. After 20 min of incubation with the second antibody, diaminobenzidine was used for colorization. After dehydration and sealing, protein expression in the cells was observed using a microscope.

### ***Western Blots***

The method used was described in a previous study (Zhou et al., 2019). Proteins were extracted from the placenta (Kaiji, China. KGP250) and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electro-transferred to polyvinylidene difluoride membranes. Blots were blocked for 2 h at room temperature with 5% nonfat milk in PBS. Membranes were incubated overnight at 4 °C with polyclonal antibodies against SERPINE1 (Lot: ab222754, Abcam, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Blots were developed using the enhanced chemiluminescence western blotting detection system (Kaiji, China. KGP1201). The blots were scanned, and the images were analyzed using Gel-Pro32 software (BioRad Laboratories, USA). Protein levels are expressed relative to GAPDH expression.

### ***Transwell Assay***

We used a method identical to that used in a previous study (Zhou et al., 2019). The upper chamber of the transwell chamber was coated with Matrigel (BD Biosciences, San Jose, CA, USA) or left uncoated. HTR-8/SVneo cells were cultured in serum-free medium for 24 h. The cell density was adjusted to  $1 \times 10^5$  cells/mL. Then, 100  $\mu$ L of cell suspension was added to the top chamber, and 500  $\mu$ L of culture medium containing 20% FBS was added to the lower chamber. After incubation at 37 °C with 5% CO<sub>2</sub> for 48 h for the invasion assay, the cells on the top surface of the transwell membrane were removed with a cotton swab. The cells on the bottom surface were fixed with cold paraformaldehyde and stained with 500  $\mu$ L of 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Five fields on each membrane were randomly selected, and the invaded cells were counted.

### ***Construction and Transfection of Lentiviral Vector of hSERPINE1***

To generate cell lines that stably overexpressed SERPINE1, HTR8/SVneo cells were infected with lentiviral particles carrying 3\*FLAG-tagged SERPINE1 or control vector (Keygen Bio, Nanjing, China), yielding cells overexpressing SERPINE1 (hSERPINE1) and control cells (NC), respectively. Polybrene (enhanced transfection reagent, 10 mg/mL) was added to a final concentration of 5  $\mu$ g/mL in each well, and properly mixed. After 24 h incubation, the culture medium was replaced with fresh medium, and incubation was continued for 72 h for later detection.

### **References**

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