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Research Article

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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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Abstract: The mechanisms underlying pregnancy complications caused by advanced maternal age (AMA) remain unclear. We analyzed the cellular signature and transcriptomes of human placentas in AMA women to elucidate these mechanisms. Placental tissues from two AMA women and two controls were used for single-cell RNA-sequencing (scRNA-seq). Controls consisted of AMA women who did not experience any pregnancy complications and pregnant women below the age of 35 years without pregnancy complications. Trophoblast cells were obtained from the placentas of another six pregnant women (three AMA women and three controls), and in-vitro transwell assays were conducted to observe the cell invasion ability. Thirty additional samples (from 15 AMA women and 15 controls) were analyzed to verify the specific expression of serine protease inhibitor clade E member 1 (SERPINE1). Preliminary study of the role of SERPINE1 in cell invasion was carried out with HTR8-S/Vneo cells. High-quality transcriptomes of 27 607 cells were detected. Three types of trophoblast cells were detected, which were further classified into eight subtypes according to differences in gene expression and Gene Ontology (GO) function. We identified 110 differentially expressed genes (DEGs) in trophoblast cells between the AMA and control groups, and the DEGs were enriched in multiple pathways related to cell invasion. In-vitro transwell assays suggested that the invading trophoblast cells in AMA women were reduced. SERPINE1 was specifically expressed in the trophoblast, and its expression was higher in AMA women (P<0.05). Transfection of human SERPINE1 (*hSERPINE1*) into HTR8-S/Vneo trophoblast cells showed fewer invading cells in the hSERPINE1 group. Impaired cell invasion may underlie the increased risk of adverse pregnancy outcomes in AMA women. Abnormal expression of SERPINE1 in extravillous trophoblast (EVT) cells appears to play an important role.

Key words: Advanced maternal age (AMA); Pregnancy complications; Placenta; Trophoblast; Cell invasion; Serine protease inhibitor clade E member 1 (SERPINE1)

1 Introduction

A pregnant woman is said to have advanced maternal age (AMA) if she is 35 years or older at the time of delivery. With a continuous increase in the number of women in this category worldwide, more attention is being given to reproductive health problems due to AMA. Previously, birth defects in children of AMA mothers were of primary concern, as the risk of chromosomal abnormalities is higher in these children (Heffner, 2004). Additionally, AMA women are at an increased risk of conditions that result in poor maternal and neonatal outcomes, including pre-eclampsia, gestational diabetes mellitus (GDM), and premature rupture of membranes (Heffner, 2004; Laopaiboon et al., 2014), which may lead to premature delivery. It has been reported that the risk of preterm birth, especially very preterm birth, increases with advancing age. In one study, risks of very preterm birth, expressed as adjusted odds ratios, were 1.18–1.28 at 30–34 years, 1.59–1.70 at 35–39 years, and 1.97–2.40 at ⩾40 years (Waldenström et al., 2017). Therefore, the risk of poor pregnancy outcomes in AMA women is of great concern.

The mechanisms underlying the poor pregnancy outcomes caused by AMA remain unclear. Notably, these complications are closely related to placental dysfunction (Norwitz, 2006). There appear to be many factors involved, including the developmental potential of oocytes (Nelson et al., 2013), microstructure of

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the uterus (Sweeney et al., 2014), and placental structure and function (Napso et al., 2019). Increasing data have shown that placental defects might play an important role in the occurrence and development of adverse pregnancy outcomes in AMA women. Woods et al. (2017) reported a dramatic increase in developmental variability, such as developmental delay and growth retardation, in old female mice, but the abnormal variability was no longer observed when embryos from old mice were transferred to young recipients. They speculated that an age-associated placental defect in decidualization was the major problem, not the oocyte. However, the exact underlying molecular mechanism remains elusive. Being a complex, dynamic, and heterogeneous organ, the placenta performs gas exchange, nutrient absorption, metabolite excretion, and immune functions between the mother and fetus. Due to its complexity and technical limitations, the full picture of placental function is yet to be clearly described.

Trophoblast cells serve as a functional unit at the maternal–fetal interface during pregnancy. They are not only one of the main cells in the human placenta, but also the only cells in contact with the decidua and immune cells. Their differentiation and development are a complex and changeable process, involving many factors. Interaction between the environment and genes is important for the development of trophoblasts at the maternal–fetal interface (Io et al., 2020). There are three types of placental trophoblasts: villous cytotrophoblasts (VCTs), syncytiotrophoblasts (SCTs), and extravillous trophoblasts (EVTs). They are heterogeneous cell populations with different morphologies which perform distinct processes essential for placental function and maintenance of pregnancy. However, there have been few studies of the heterogeneity of these cells. Recently, it was proven that single-cell RNA-sequencing (scRNA-seq) can contribute to specific profiling of cell populations and understanding the nature of disease (Lambrechts et al., 2018; Park et al., 2018; Aizarani et al., 2019; Park et al., 2020). Sporadic studies with scRNA-seq have also revealed the heterogeneity of human placental cells (Nelson et al., 2016; Pavličev et al., 2017). Tsang et al. (2017) defined individual cell-type-specific gene signatures by analyzing more than 24 000 cells, which enabled reconstruction of the trophoblast differentiation trajectory. Vento-Tormo et al. (2018) described the cellular characteristics and immune interaction network at the maternal–fetal interface. Liu et al. (2018) discovered new cell subtypes of VCT and EVT. However, recent studies were conducted on a small scale and were not extensive enough. The microenvironment of the placenta and the interaction network among its different cells remain unknown. It is also unknown whether there are characteristic placental defects in AMA women.

To profile and compare the cellular signatures and transcriptomes of human placentas in AMA women and reveal the molecular mechanism underlying their poor pregnancy outcomes, we built a comprehensive cell atlas of the human placenta in AMA women using scRNA-seq. This enabled us to explore new subtypes of trophoblast cells and their differentiation tracks, and revealed that reduced cell invasion ability might be an important mechanism of placental defect in AMA women. Overall, our data provide a rich resource for elucidating the molecular mechanism leading to poor pregnancy outcomes among AMA women.

2 Materials and methods

2.1 Study design and participants

All study participants were recruited from the Changzhou Maternal and Child Health Care Hospital. AMA women (aged ≥35 years) who did not have any adverse pregnancy outcomes and normal pregnant women (aged <35 years without pregnancy complications) were selected as controls. The clinical characteristics of the participants are shown in Table S1.

2.2 Sample collection

Placental tissues were collected during delivery. Tissues from two AMA women and two controls were used for scRNA-seq; six placental tissue samples (from three AMA women and three controls) were used for cell culture; and 15 AMA women and 15 controls participated in validated experiments. For scRNA-seq, within 5 min of delivery, we immediately obtained a small piece of placental tissue (about 1.5 cm^3) from a region 1–2 cm deep and 2–5 cm away from the umbilical cord insertion. For other tests, whole placental tissues were sent to the Department of Pathology, Changzhou Maternal and Child Health Care Hospital, Changzhou Medical Center, Nanjing Medical University, Changzhou, China, within 30 min, and a

professional pathologist dissected and extracted specimens from the same region.

2.3 Experimental method

The key resources of the present study are shown in Table S2.

2.3.1 Single-cell RNA-sequencing

The method used was described in our previous study (Yang et al., 2021), and included the following main experimental steps: first, single-cell suspensions were prepared from the placental samples. Then, scRNA-seq libraries were prepared using chromium single cell 3' Reagent v3 Kits (10x Genomics, Pleasanton, USA). Single-cell suspensions were loaded on a chromium single cell controller instrument (10x Genomics) to generate single-cell gel beads in emulsions (Burl et al., 2018). The scRNA-seq data were analyzed with the Cell Ranger software pipeline (Version 3.0, 10x Genomics). The unique molecular identifier count matrix was analyzed with the R package Seurat (Version 2.3.4, Paul Hoffman, New York, USA). The top variable genes across single cells were identified using the method described by Macosko et al. (2015). Differentially expressed genes (DEGs) (P<0.05 and fold change>2) were identified using the FindMarkers function (test.use=MAST) in Seurat (Butler et al., 2018). Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEGs were performed using R, based on the hypergeometric distribution. Meanwhile, we determined the developmental pseudotime with the Monocle2 package (Trapnell et al., 2014), and RNA velocity with the R package velocyto. R v 0.6 (la Manno et al., 2018). Further experimental details of scRNA-seq are highlighted in Material S1.

2.3.2 Other methods

The other validation methods and in vitro research methods are shown in Material S2, including immunofluorometric assay, immunohistochemistry, western blots, transwell assay, and construction and transfection of lentiviral vector of human serine protease inhibitor clade E member 1 (*hSERPINE1*).

2.4 Statistical analyses

All analyses were performed using R version 3.4.3 (http://www.R-project.org). Student's t-test was used to analyze differences in age, height, weight, gestational age at delivery, and the expression of *SERPINE1* between the AMA and control groups. P-values of ⩽0.05 were considered statistically significant.

3 Results

3.1 Single-cell transcriptome profiling of human placental cells

To discover the effects of maternal aging on the human placenta, we used high-throughput scRNA-seq to examine the transcriptional profiles of the placentas of controls and AMA women. Four placental tissues freshly delivered via cesarean section (two from AMA women and two from controls) were collected and dissociated into single-cell suspensions (Table S3). The transcriptomes of single cells were sequenced and analyzed using microfluidic technology (based on 10x Genomics platform) (Fig. 1a). After quantitative quality control, high-quality transcriptomes of 27 607 cells (14 978 from AMA women and 12 629 from controls) were detected. The number of genes per cell ranged from 1054 to 1786, and mean reads per cell ranged from 67 825 to 130 068 (Figs. S1a and S1b, Table S3). First, we transcriptionally aggregated similar cells using principal component analysis. Cells were clustered using a graph-based clustering approach, and were visualized in two dimensions using t-distributed stochastic neighbor embedding (t-SNE). A total of 18 clusters were detected (Fig. S1c). There were no significant differences between the AMA and control groups. Next, we used the SingleR (Version 0.2.2, Dvir Aran, San Francisco, USA) to infer the origin of every single cell independently and identify the cell types. We further identified these cell clusters with cell-type-specific marker genes in the t-SNE plot, and finally annotated cell clusters into nine main cell types (Fig. 1b).

Three types of trophoblasts were clearly identified with cell-type-specific marker genes. Consistent with previous reports (Pavličev et al., 2017; Tsang et al., 2017; Liu et al., 2018), clusters 2, 3, 12, and 15, corresponding to VCTs, were identified by the expression of classic markers c-MET proto-oncogene (*MET*) (Vento-Tormo et al., 2018), cadherin 1 (*CDH1*) (Liu et al., 2018), and poly(ADP-ribose) polymerase 1 (*PARP1*) (Suryawanshi et al., 2018). The markers of

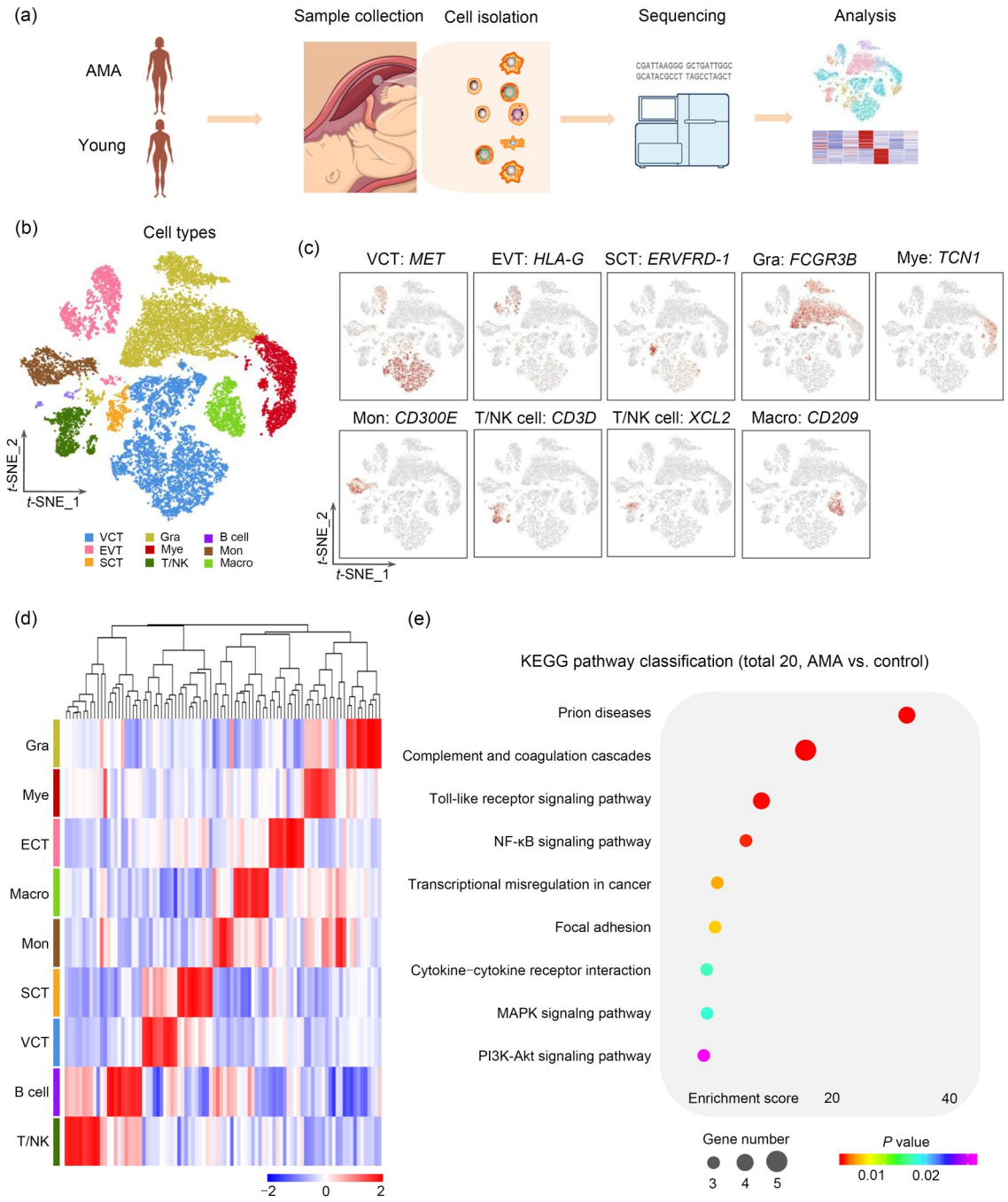

Fig. 1 Overview of cell signatures from human placentas determined by scRNA-seq analysis. (a) Flowchart overview of scRNA-seq analysis of human placentas in the present study; (b) Cell-type-specific marker genes in the *t*-SNE plot, in which nine cell types were transcriptomically identified in the human placenta; (c) Expression of marker genes used to identify the nine cell types; (d) Heatmap point plot of the top 10 marker genes among different cell types; (e) KEGG analysis based on DEGs (AMA vs. control). scRNA-seq: single-cell RNA-sequencing; *t*-SNE: *t*-distributed stochastic neighbor embedding; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEG: differentially expressed gene; AMA: advanced maternal age; VCT: villous cytotrophoblast; EVT: extravillous trophoblast; SCT: syncytiotrophoblast; Gra: granulocytes; Mye: myelocytes; Mon: monocytes; Macro: macrophages; *MET*: c-MET proto-oncogene; *HLA-G*: human leukocyte antigen G; *ERVFRD-1*: endogenous retrovirus group FRD member 1; *FCGR3B*: Fcγ receptor type IIIAB; *TCN1*: transcobalamin 1; *CD300E*: cluster of differentiation 300E; *XCL2*: X-C motif chemokine ligand 2; NF-κB: nuclear factor-κB; MAPK: mitogen-activated protein kinases; PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B.

SCTs (endogenous retrovirus group FRD member 1 (*ERVFRD-1*) (Suryawanshi et al., 2018), cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*) (Lv et al., 2019), and chromogranin A (*CGA*)) were specifically expressed in cluster 14. Strong expression of human leukocyte antigen G (*HLA-G*) (Suryawanshi et al., 2018), pregnancy-associated plasma protein A2 (*PAPPA2*) (Vento-Tormo et al., 2018), and matrix metallopeptidase 11 (*MMP11*) was detected in clusters 7 and 11, likely representing EVTs. We also detected many immune cells. Clusters 10 and 17 seemed to be similar to T/natural killer (NK) cells, with T cell markers (cluster of differentiation 3D (*CD3D*) (Han et al., 2020), *CD3G*, and granzyme A (*GZMA*)) and NK cell markers (X-C motif chemokine ligand 2 (*XCL2*), C-C motif chemokine ligand 5 (*CCL5*) (Vento-Tormo et al., 2018), and granzyme K (*GZMK*) (Reyes et al., 2020)). Preliminary analysis showed that the upper part was more similar to NK cells, while the lower part was similar to T cells. Monocytes (*CD300E*, *CD244*, and HLA-DR α chain (*HLA-DRA*) (Reyes et al., 2020)) were identified in cluster 5, and macrophages (*CD209*, *CD163* (Tsang et al., 2017), and allograft inflammatory factor 1 (*AIF1*) (Yao et al., 2019)) in cluster 6. A few B cells were found in cluster 18, and they strongly expressed *CD79A* (Han et al., 2020), *CD79B*, and *CD19*. Finally, a large group of cells from clusters 1, 8, 9, and 16 specifically expressed some marker genes (Fcγ receptor type IIIAB (*FCGR3B*), C-X-C motif chemokine ligand 8 (*CXCL8*), myeloid nuclear differentiation antigen (*MNDA*), and selectin L (*SELL*)). They were defined as granulocytes. Clusters 4 and 13 corresponded transcriptionally to myelocytes with strong expression of transcobalamin 1 (*TCN1*), carcinoembryonic antigen-related cell adhesion molecule 8 (*CEACAM8*), *MMP8*, defensin α4 (*DEFA4*), and cyclic adenosine monophosphate (*CAMP*). The expression of marker genes for the different cell types is shown in Figs. 1c and S1d.

There were no differences in placental cell type between AMA women and controls. However, the proportions of the various cell types differed (Figs. S1b and S1c). Fig. 1d shows the heatmap point plot of the top ten marker genes among the different cell types. To explore the biological function of each cell type, the DEGs were compared between AMA women and controls, using bioinformatics analysis by GO and KEGG (Figs. 1e and S1e). For example, KEGG pathway classification suggested that the DEGs of AMA were significantly enriched in some important pathways, such as "complement and coagulation cascades," "Toll-like receptor signaling pathway," "NF-κB signaling pathway," and "transcriptional misregulation in cancer." GO terms specific to AMA women included "synapse pruning" and "platelet degranulation" in the biological process, and "protease binding" and "cytokine activity" in the molecular function. The genes significantly up-regulated in AMA women were involved mainly in the immune system and were similar to the up-regulated genes observed in cancers.

In summary, using scRNA-seq we identified nine cell subtypes in the human placenta based on their transcriptomes.

3.2 Deep excavation of subtypes of trophoblast cells

We detected a total of 10 326 trophoblast cells, about 41.6% of the total placental cells. We verified the expression of cell-type-specific markers of the trophoblast cells. *HLA-A* was specifically expressed in non-trophoblast cells (Tsang et al., 2017), while keratin 7 (*KRT7*) and p53 effector related to PMP-22 (*PERP*) were expressed in almost all the trophoblast cells (Suryawanshi et al., 2018) (Fig. S2a). With the canonical marker genes (Liu et al., 2018; Suryawanshi et al., 2018; Vento-Tormo et al., 2018; Lv et al., 2019), all three types of trophoblast cells were detected, namely VCT (7258, 70.3%), EVT (2333, 22.6%), and SCT (735, 7.1%) cells (Fig. 2a). We also found a panel of novel markers for distinguishing the three kinds of trophoblast cells (Fig. S2b). The expression of these new marker genes (homeodomain-only protein homeobox (*HOPX*), neuronal regeneration-related protein (*NREP*), and potassium two pore domain channel subfamily K member 12 (*KCNK12*)) was verified by immunofluorescence (Fig. S2c).

Furthermore, we revealed the trophoblast cell subtypes on the *t*-SNE plot. Due to a lack of unequivocal marker genes, we classified the subtypes by differences in gene expression and GO function. The 2333 EVT cells could be roughly divided into two subtypes: EVT1 and EVT2 (Fig. 2a). Some classic marker genes (such as *HLA-G* and *PAPPA2*) were strongly expressed in EVT1 cells and only slightly expressed in EVT2 cells. Some potential markers,

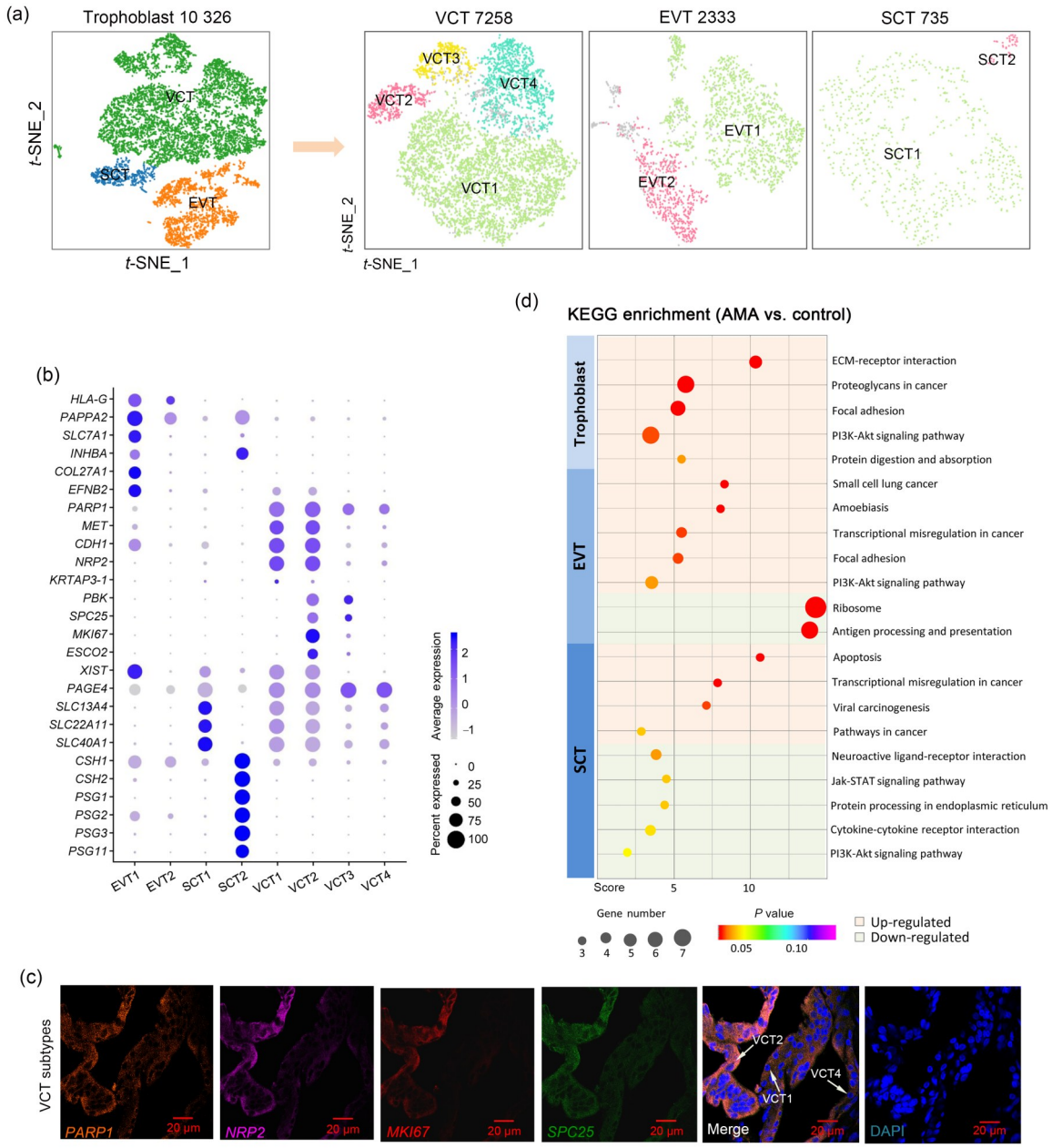

Fig. 2 Transcriptome profiling of trophoblast cells. (a) *t*-SNE plot grouping of 10 326 trophoblast cells into three types and re-clustering into eight subtypes; (b) Heatmap of potential markers that contributed to distinguishing the eight trophoblast cell subtypes; (c) New subtypes of VCT confirmed by expression of *PARP1*, *NRP2*, *MKI67*, and *SPC5* with immunofluorescence tests (five placental samples); (d) KEGG pathway analysis based on 110 DEGs that were identified in trophoblast cells between the AMA and control groups (P<0.05 and fold change>2). *t*-SNE: *t*-distributed stochastic neighbor embedding; VCT: villous cytotrophoblast; EVT: extravillous trophoblast; SCT: syncytiotrophoblast; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEG: differentially expressed gene; AMA: advanced maternal age; *HLA-G*: human leukocyte antigen G; *PAPPA2*: pregnancy-associated plasma protein A2; *SLC7A1*: solute carrier family 7 member 1; *INHBA*: inhibin subunit βA; *COL27A1*: collagen type XXVII α1 gene; *EFNB2*: ephrin B2; *PARP1*: poly(ADP-ribose) polymerase 1; *MET*: c-MET proto-oncogene; *CDH1*: cadherin 1; *NRP2*: neuropilin 2; *KRTAP3-1*: keratin-associated protein 3-1; *PBK*: PDZ-binding kinase; *SPC25*: spindle pole body component 25 homolog; *MKI67*: marker of proliferation Ki-67; *ESCO2*: establishment of cohesion 1 homolog 2; *XIST*: X inactive-specific transcript; *PAGE4*: prostate-associated gene 4; *CSH*: chorionic somatomammotropin hormone; *PSG*: pregnancy-specific β-1-glycoprotein; ECM: extracellular matrix; PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B; Jak-STAT: Janus kinase-signal transducer and activator of transcription; DAPI: 4',6-diamidino-2'-phenylindole.

such as solute carrier family 7 member 1 (*SLC7A1*), inhibin subunit βA (*INHBA*), collagen type XXVII α1 (*COL27A1*), and ephrin B2 (*EFNB2*), contributed to distinguishing the two subtypes (Fig. 2b). Most of these genes were specifically expressed in EVT1 cells. VCT cells could also be divided into four subtypes (Fig. 2a). Among them, VCT1 and VCT2 cells had stronger expression of the classical marker genes (*PARP1*, *MET*, and *CDH1*), while VCT3 and VCT4 cells had reduced expression of those genes. The four subtypes of VCT cells were distinguished based on markers such as keratin-associated protein 3-1 (*KRTAP3-1*), PDZ-binding kinase (*PBK*), spindle pole body component 25 homolog (*SPC25*), marker of proliferation Ki-67 (*MKI67*), and establishment of cohesion 1 homolog 2 (*ESCO2*) (Fig. 2b). Almost all the marker genes related to VCT were strongly expressed in VCT2 cells, while their expression was inferior in VCT4 cells. Similarly, the 735 SCT cells could be divided into SCT1 and SCT2 cells (Fig. 2a). There were significant differences in expression of gene family (chorionic somatomammotropin hormone (*CSH*), pregnancy-specific β-1-glycoprotein (*PSG*), etc.) between the two subtypes (Fig. 2b).

The new subtypes of VCT cells were also initially confirmed by immunofluorescence tests for *PARP1*, neuropilin 2 (*NRP2*), *MKI67*, and *SPC5*. These new subtypes of VCT cells were roughly identified, and more detailed information on their identification is required (Fig. 2c).

Compared with those of normal pregnant women, the subtypes of trophoblast cells in AMA women showed characteristic changes. In particular, among EVT cells, EVT1 in AMA women was absolutely dominant, both in cell number and proportion. Similarly, SCT1 in AMA women increased significantly (Fig. S2d).

A total of 110 DEGs were identified in trophoblast cells between the AMA and control groups, including 97 up-regulated genes and 13 down-regulated genes. KEGG pathway analysis showed that the up-regulated DEGs were significantly enriched in extracellular matrix (ECM)–receptor interaction, and included five genes (heparan sulfate proteoglycan 2 (*HSPG2*), fibronectin 1 (*FN1*), integrin α5 (*ITGA5*), collagen α-1 (IV) chain (*COL4A1*), and laminin subunit α3 (*LAMA3*)) (Fig. 2d). Further analysis showed that they were strongly expressed in EVT cells, especially EVT1 cells (Fig. S2e). The down-regulated genes in EVT cells were genes involved in "ribosome biogenesis" and "antigen processing and presentation."

Finally, we compared the biological processes of different trophoblast cell subtypes by GO analysis and proved some of the hypotheses stated above (Fig. S2f). For example, GO enrichment analysis comparing EVT1 with EVT2 cells revealed that the top enrichment process was ECM organization, further confirming the important role of EVT1 cells in ECM–receptor interaction. Among the four subtypes of VCT cells, the biological processes of VCT2 and VCT3 cells were very similar. VCT1 cells had a variety of biological processes (including transcriptional regulation and apoptotic regulation), while VCT4 cells were responsible mainly for cell division.

Thus, we classified, for the first time, the subtypes of the three trophoblast cell types (VCT, EVT, and SCT) and described their roles in placental defects in AMA women.

3.3 Pseudotemporal ordering of the subtypes of trophoblast cells

As the progenitor, VCT cells can differentiate into EVT or SCT cells under different conditions during placental development. To further explore trophoblast cell differentiation, we ordered 10 326 trophoblast cells (VCT, EVT, and SCT) computationally in a 2D "pseudotime" trajectory and by RNA velocity. Both tests revealed that EVT and SCT cells were originated from VCT cells (Fig. 3a). Concurrently, there were certain differentiation tracks among the subtypes of trophoblast cells. For example, RNA velocity predicted a well-ordered differentiation pattern first from VCT to EVT2 cells, then to EVT1 cells. Among the four subtypes of VCT cells, VCT3 cells seemed to be the primitive cells that gradually differentiated into VCT2, and eventually into VCT1 cells. Fig. 3b shows our proposed trophoblast subtype differentiation trajectory.

SLC7A1 was selected for preliminary experiments. It was strongly expressed in EVT1 and weakly expressed in EVT2 cells. Theoretically, its expression may gradually increase with increasing gestational age. This was verified with three specimens, each of villous tissue in the first trimester and each of placental tissue in the third trimester. Although the sample size was small, we found that the expression of *SLC7A1* was higher in the third-trimester placentas (Fig. 3c).

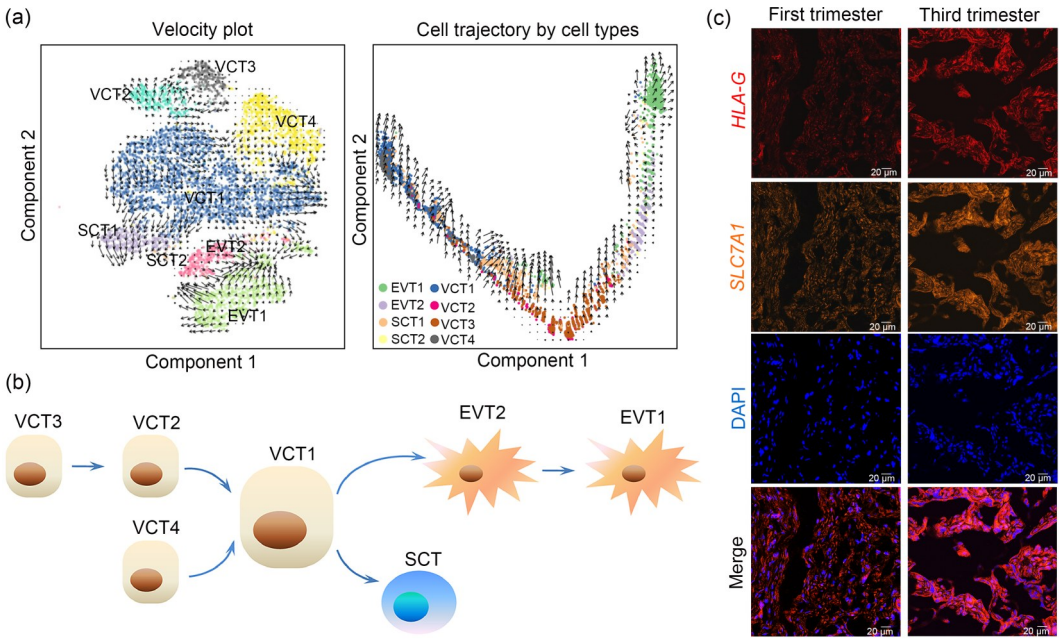

Fig. 3 Pseudotemporal ordering of subtypes of trophoblast cells. (a) Pseudotime analysis and RNA velocity of trophoblast cells; the cells were ordered computationally in terms of RNA velocity (left) and 2D "pseudotime" trajectory (right). (b) Proposed trophoblast subtype differentiation trajectory. (c) Comparison of *SLC7A1* expression in villous and placental tissues (three specimens, each in first trimester and in third trimester). VCT: villous cytotrophoblast; EVT: extravillous trophoblast; SCT: syncytiotrophoblast; *HLA-G*: human leukocyte antigen G; *SLC7A1*: solute carrier family 7 member 1; DAPI: 4',6-diamidino-2'-phenylindole.

Therefore, we proposed a new differentiation trajectory of subsets of trophoblast cells.

3.4 Placental defects in AMA women characterized by decreased cell invasion

Referring to the method of Wang et al. (2020), we performed a comparative analysis of the DEGs in the trophoblast with the aging-associated genes annotated in the GenAge database, and eight genes were found to overlap (Fig. 4a). *SERPINE1*, an inhibitor of serine protease, which is involved in cellular and replicative senescence, was one of the eight genes. The results of scRNA-seq showed that *SERPINE1* was specifically expressed in the trophoblast, especially in EVT cells (Figs. 4b and 4c). The results were verified by immunohistochemistry (Fig. 4d). Next, 30 samples (from 15 AMA women and 15 controls) were used to further verify the specific expression of *SERPINE1* in AMA women by western blot. Compared with the controls, the expression of *SERPINE1* was significantly increased in the placental tissue of AMA women ($P<0.01$; Fig. 4e).

To investigate the biological function of *SERPINE1*, a lentiviral vector of *hSERPINE1* was constructed and transfected into HTR8-S/Vneo trophoblast cells. Transfection with *hSERPINE1* resulted in significantly increased expression of SERPINE1 (Fig. 4f). Meanwhile, the results of the transwell assay demonstrated that there were fewer invading cells in the hSERPINE1 expression group than in the negative control group 24 h after transfection (Fig. 4g).

To verify the scRNA-seq finding and investigate whether AMA women also have a decline in trophoblast cell invasion ability, trophoblast cells were obtained from the placentas of six pregnant women (three AMA women and three controls). After primary cell culture, in-vitro transwell assays were conducted to observe the degree of cell invasion. Compared with the controls, there were fewer invading cells in AMA women, and the difference between the two groups was statistically significant ($P<0.0001$; Fig. 5). This suggests that the cell invasion capability of trophoblast cells in AMA women might be reduced.

Together, these preliminary studies confirmed a decrease in the cell invasion capability of placental trophoblast cells in AMA women. The abnormal expression of *SERPINE1* appears to play an important role.

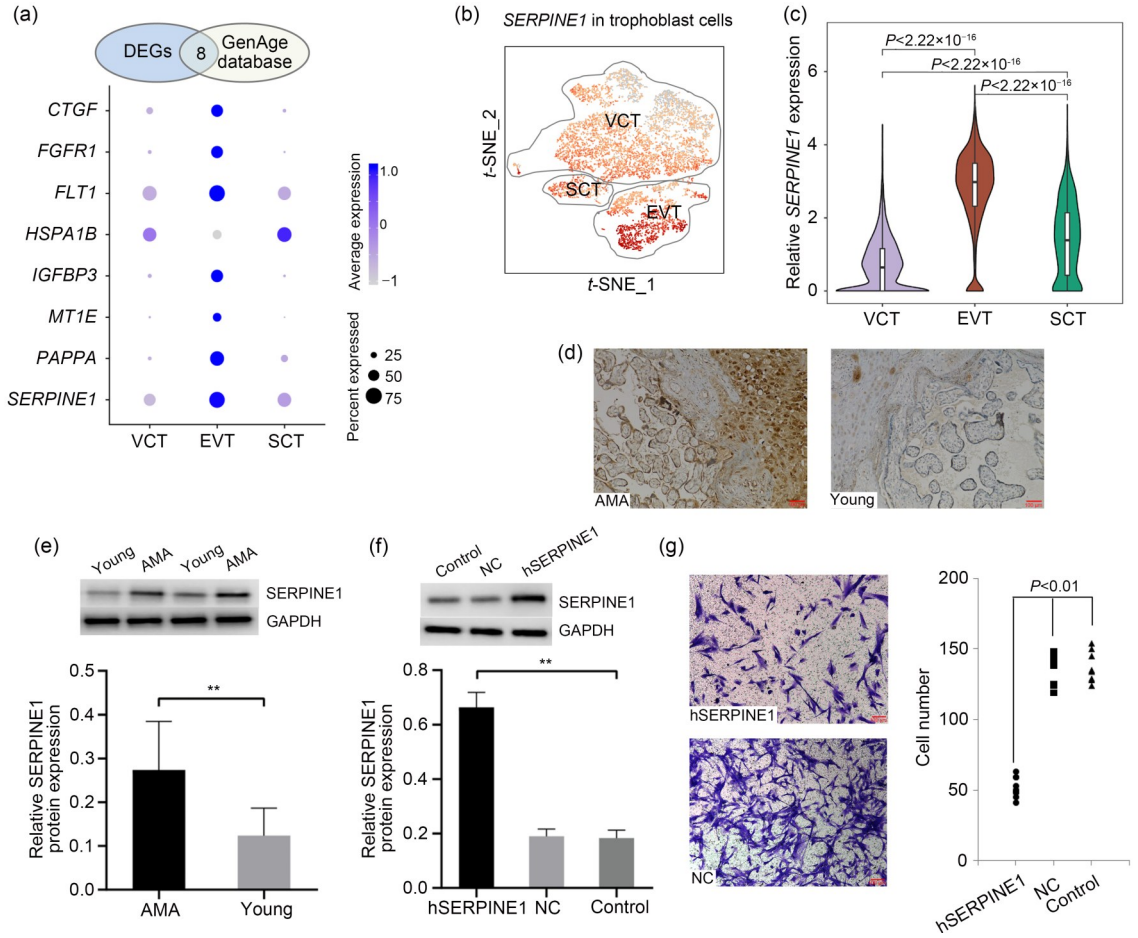

Fig. 4 Expression and role of *SERPINE1* in trophoblast cells of AMA women. (a) A comparison between DEGs (P<0.05 and fold change>2) in the trophoblasts and aging-associated genes annotated in the GenAge database. Eight genes were pooled. (b) *SERPINE1* was specifically expressed in trophoblasts, as shown by scRNA-seq. (c) Violin plots of *SERPINE1* expression in three trophoblasts. (d) Specific expression of SERPINE1 in AMA women by immunohistochemistry (15 AMA women vs. 15 controls), scale bar=100 μm. (e) Specific expression of *SERPINE1* in AMA women by western blot (15 AMA women vs. 15 controls): compared with the controls, *SERPINE1* expression significantly increased in the placental tissue of the AMA women. Data are expressed as mean±SD, n=15; $^{**}P$<0.01. (f) Lentiviral vector of *hSERPINE1* was constructed and transfected into HTR8-S/Vneo cells. Data are expressed as mean±SD, n=3; $^{**}P$<0.01. (g) There were fewer invading cells in the hSERPINE1 expression group than in the NC group 24 h after transfection (scale bar=100 μm). *SERPINE1*: serine protease inhibitor clade E member 1; *hSERPINE1*: human *SERPINE1*; AMA: advanced maternal age; DEG: differentially expressed gene; scRNA-seq: single-cell RNA-sequencing; SD: standard deviation; NC: negative control; *t*-SNE: *t*-distributed stochastic neighbor embedding; *CTGF*: connective tissue growth factor; *FGFR1*: fibroblast growth factor receptor 1; *FLT1*: fms-related tyrosine kinase 1; *HSPA1B*: heat shock 70-kDa protein 1B; *IGFBP3*: insulin-like growth factor binding protein 3; *MT1E*: metallothionein 1E; *PAPPA*: pregnancy-associated plasma protein A; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

4 Discussion

This is the first study to describe a comprehensive cell atlas of the placenta in AMA women, using scRNA-seq. We focused mainly on the characteristic changes in the trophoblasts and their cell subtypes in AMA pregnant women, including cell type composition, number, proportion, development, differentiation, and biological function.

Initially, nine transcriptomically identified cell types and many DEGs were identified in the placentas of AMA women. Although there were no significant differences in placental cell type distribution, there were significant changes in the biological function of

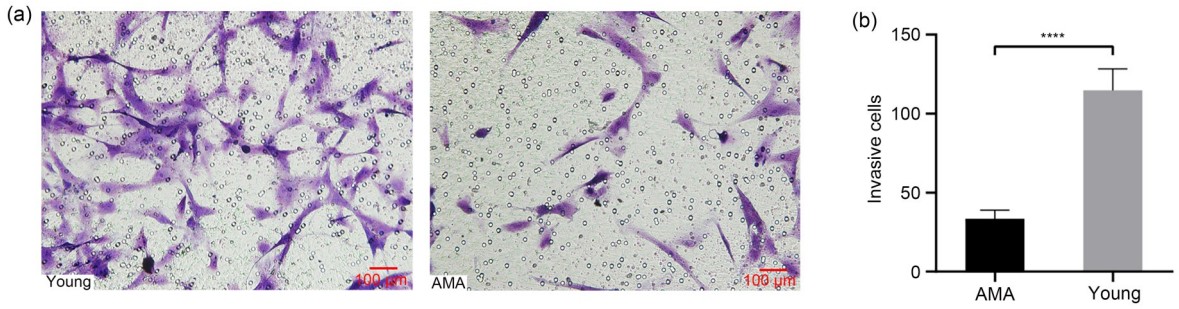

Fig. 5 Cell invasion capability of trophoblast cells in AMA women. (a) After primary placental cell cultures were created from six pregnant women (three AMA women and three controls), we compared cell invasiveness by in-vitro transwell assay. Compared with the young pregnant women, there were fewer invading cells in the AMA women. (b) Reduced cell invasion capability of trophoblast cells in AMA women. Data are expressed as mean±SD, *n*=3; ** *P*<0.0001. AMA: advanced maternal age; SD: standard deviation.**

placental cells in AMA women. As previously reported (Sood et al., 2006; Tsang et al., 2017), we also noted significant spatial heterogeneity in placental cells. This confirms that human placental cells have high heterogeneity, and it is necessary to elucidate the placental cell atlas at the cellular level. This study focused mainly on the characteristic changes in trophoblast cells. Remarkably, eight new trophoblast cell subtypes were identified, and some potential markers for distinguishing them were also found. This is an important new discovery in advancing the understanding of the human placenta. Liu et al. (2018) isolated three subtypes each of VCT and EVT cells from first-trimester placentas (8 weeks), and two subtypes of EVT cells from second-trimester placentas (24 weeks). Among the three subtypes of VCT cells, VCT-8W-3 cells were highly proliferative, while VCT-8W-1 and VCT-8W-2 cells had already exited the cell cycle. In the present study, VCT, EVT, and SCT cells were divided into four, two, and two subtypes, respectively, based on differences in gene expression and biological function. Compared with EVT2 cells, EVT1 cells may play an important role in cell invasion and metastasis. VCT1 cells perform a variety of biological functions, including transcriptional regulation and apoptotic regulation. VCT4 cells are mainly responsible for cell division, while VCT3 cells are relatively naive. Pique-Regi et al. (2019) had reported one new cluster named non-proliferative interstitial cytotrophoblasts, which was similar to VCT4. Reduced expression of X inactive-specific transcript (*XIST*) and increased expression of prostate-associated gene 4 (*PAGE4*) were detected in VCT4 cells. Members of the solute carrier family (*SLC13A4*, *SLC22A11*, and *SLC40A1*) were specifically expressed in SCT1, but showed negligible expression in SCT2 cells. They may be involved in the transfer of substances between maternal and fetal circulations (Markovich et al., 2005). In contrast, *CSH1* and *CSH2* were highly expressed in SCT2 cells, which are produced only during pregnancy and are involved in stimulating lactation, fetal growth, and metabolism (Walsh and Kossiakoff, 2006). *PSG1*, *PSG2*, *PSG3*, and *PSG11* (Khan et al., 1992; Teglund et al., 1995), which are produced in high quantities during pregnancy, were predominantly expressed in SCT2 cells. Meanwhile, there were certain differentiation tracks among these subtypes of trophoblast cells. Fig. 3b shows our proposed trophoblast subtype differentiation trajectory, providing an interesting possibility for future research. A clear understanding of the biological functions of the new trophoblast cell subtypes will help to reveal more comprehensively and accurately the mechanisms underlying many adverse pregnancy outcomes.

The up-regulated DEGs in AMA women, which were significantly enriched in ECM–receptor interaction, were strongly expressed in EVT cells, especially in EVT1 cells. This suggests that EVT1 cells may play a major role in abnormal ECM–receptor interaction in the placentas of AMA pregnant women. EVT seems to be the main cause of the cancer characteristics of trophoblastic cells. The up-regulated genes in EVT cells are enriched by some tumor pathways, such as small cell lung cancer, transcriptional misregulation in cancer, and focal adhesion. Likewise, the up-regulated genes in SCT cells may follow the pathway of apoptosis. Recent studies have found that abnormal proliferation and apoptosis of trophoblast cells lead to placental dysfunction, which is the pathological basis of pregnancy complications (Huppertz et al., 2006;

Fisher, 2015; Burton and Jauniaux, 2017). However, whether it is also the cause of placental defects in AMA women, thus increasing the risk of adverse pregnancy outcomes, is worthy of further study.

Currently, the characteristic placental defects in AMA women are unknown. The KEGG analysis performed in this study suggested that the DEGs of trophoblast cells in AMA women were enriched in multiple pathways related to cell invasion, such as ECM–receptor interaction and focal adhesion. Meanwhile, AMA women have a high risk of developing pre-eclampsia (Laopaiboon et al., 2014). It is well known that the cell invasion ability of the trophoblast is reduced in pre-eclampsia (Zhou et al., 2019; Sun et al., 2021). Based on the results of this experiment, we considered that, in AMA pregnant women, the cell invasion ability of trophoblasts may be impaired, and that *SERPINE1* may play an important role in placental dysfunction. Although it was only a small-scale in-vitro experiment, it was obvious that the invasion ability of trophoblast cells isolated from the placentas of AMA women was decreased. Clinical studies have also confirmed the strong expression of *SERPINE1* in the placental tissue of AMA women. Meanwhile, cell experiments confirmed that the cell invasion ability reduced with the over-expression of *SERPINE1*. Although it is only a preliminary study, we were able to derive the results highlighted above. As previously described, *SERPINE1* is involved in cellular and replicative senescence (Kortlever et al., 2006). However, its role in placental development and pregnancy maintenance remains controversial. One report suggested that tumor necrosis factor-α (TNF-α) restricts trophoblast invasion mainly by increasing the expression of plasminogen activator inhibitor-1 (PAI-1), which is encoded by *SERPINE1* (Huber et al., 2006). Other studies confirmed that transforming growth factor-β (TGF-β) enhanced PAI-1 expression in HTR-8/SVneo cells, and was associated with pregnancy complications such as pre-eclampsia and intrauterine growth restriction (Ma et al., 2002; Bauer et al., 2004). However, Zhang et al. (2005) reported that adrenomedullin could stimulate cell proliferation and the invasion capabilities of trophoblasts, and was associated with increased gelatinolytic activity and reduced *SERPINE1* expression. However, it is consistently maintained that *SERPINE1* is closely related to the occurrence and development of pre-eclampsia. It is well known that AMA pregnant women have a higher risk of developing pre-eclampsia. Therefore, we speculate that *SERPINE1* may mediate the impairment of cell invasion in AMA pregnant women.

This study had a few limitations. First, the small sample size used for scRNA-seq might have been affected by variation due to the heterogeneity of the placenta. Also, no corrections were made for multiple testing in the scRNA-seq experiment. Although we found eight new subtypes of trophoblast cells and their trajectories of development, the verification experiments were insufficient. It is not clear whether there are characteristic changes in these subtypes and trajectories in AMA women. We also hypothesized that impaired cell invasion may be one of the characteristics of placental defects in AMA pregnant women, and that it may lead to an increased risk of adverse pregnancy outcomes. Second, although an initial experiment on *SERPINE1* was carried out, more clinical evidence and experiments are required to prove our findings and elucidate the mode of action of *SERPINE1*. Moreover, scRNA-seq suggested that *SERPINE1* is specifically expressed in EVT cells, but it is also expressed in VCT and SCT cells. Therefore, another limitation of the study is that we used only HTR8-S/Vneo as a model for a pure trophoblast. Furthermore, some reports suggested that the placental phenotype of AMA women is altered in a sex-specific fashion (Napso et al., 2019). Fetal sex might contribute to placental transcriptomic signatures. However, in the present study we did not carry out an analysis according to sex due to the limited sample size. This is an important direction for further studies.

5 Conclusions

This study is the first to reveal the specific changes in the placenta at the cellular level and find new trophoblast cell subtypes and development trajectories. The results suggest that placental dysfunction, especially reduced cell invasion ability, may be the reason for the increased risk of adverse pregnancy outcomes in AMA women, and that abnormal expression of *SERPINE1* in EVT cells plays an important role. Our findings provide new insights for prenatal healthcare in AMA pregnant women and may contribute to the prevention of poor pregnancy outcomes.

Availability of data and materials

The data presented in the study (next generation sequencing (NGS) raw data) were uploaded to GEO repository, accession number GSE173193 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE173193).

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Author contributions

Bin YU and Bin ZHANG carried out the assays and participated in the study design. Bin ZHANG, Feng ZHANG, Fengying LU, Jing WANG, Wenbai ZHOU, and Huihui WANG performed clinical consultations, laboratory tests, and statistical analysis. Bin YU conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Bin ZHANG, Feng ZHANG, Fengying LU, Jing WANG, Wenbai ZHOU, Huihui WANG, and Bin YU declare that they have no competing interests.

The study design and protocol were reviewed and approved by the Ethics Committee of Changzhou Maternal and Child Health Care Hospital, Changzhou, China (No. 2020160). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

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Supplementary information

Tables S1–S3; Figs. S1 and S2; Materials S1 and S2