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Original Article

Expression of SARS-CoV-2 entry genes ACE2 and TMPRSS2 at single cell resolution in the peripartum decidua

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Abstract: Angiotensin-converting enzyme 2 (ACE2) is the key receptor for severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2). However, the susceptibility of the decidua to infection during the peripartum period has not been explored, even though this may affect vertical transmission. The objective of this study was to investigate the expression of ACE2 and related genes in the decidua during delivery. Here, single-cell RNA sequencing was used to characterize the transcriptomes of decidual cells before and after the onset of labor. During the peripartum period, ACE2 expression was highly heterogeneous. ACE2 was expressed principally in decidual stromal cells, uterine smooth muscle cells, and extravillous trophoblasts. Comparison of the transcriptomes of ACE2-positive and ACE2-negative cells indicated that ACE2-positive cells exhibited integrin clusters on the cell surface interactions. ACE2-positive cells were compared before and after labor onset. After delivery, the number of ACE2-positive cells was slightly higher than before delivery. Before labor onset, ACE2-positive decidual stromal cells were in the regulation of membrane protein ectodomain proteolysis cluster. After labor onset, the upregulated genes changed to include cell junction assembly genes. The susceptibility of decidual cells to SARS-CoV-2 infection is thus heterogeneous during the peripartum period.

Keywords: ACE2, decidua, labor onset, SARS-CoV-2, single cell sequencing, vertical transmission

Introduction

The global pandemic of the novel coronavirus pneumonia has caused serious public health problems [1]. In addition to the respiratory system, multiple organs and other systems may be infected by this virus, including the cardiovascular, nervous and reproductive systems [2-4]. In particular, pregnant women are susceptible to respiratory diseases, including coronavirus disease 2019 (COVID-19). The severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) receptor angiotensin-converting enzyme 2 (ACE2) was found to be increased during pregnancy, which may contribute to increased susceptibility of infection [5] and COVID-19-mediated changes in the immune response at the maternal-fetal interface [6]. Therefore, an important issue is the vertical transmission of SARS-CoV-2. Although most pregnancy cases have been reported not to result in neonatal SARS-CoV-2 infection [7], a small number of neonates were diagnosed with SARS-CoV-2 infection [8, 9]. Therefore, vertical transmission of SARS-CoV-2 is possible [10]. It is necessary to conduct more research on this transmission route, especially with respect to the maternal-fetal interface.

SARS-CoV-2 uses the ACE2 for entry into cells, and the serine protease transmembrane protease serine 2 (TMPRSS2) for S protein priming [11]. At the same time, the heterogeneity and expression of ACE2 in lung tissue have also

been reported [12]. This expression of ACE2 in various organs is different, and ACE2 expressing organs have a higher risk of infection [4].

In addition, TMPRSS2 is one of the important therapeutic targets for prostate cancer [13], which suggests the similarity between SARS-CoV-2 and cancer. The anti-tumor drugs (especially the TMPRSS2 or ACE2 targeted drugs) may have broad treatment prospects for SARS-CoV-2 [14]. The research on TMPRSS2 and ACE2 at single cell resolution may help to evaluate the target of anti-SARS-CoV-2 drugs in decidua of maternal-fetal interface.

Due to the immunological changes during pregnancy and the existence of the placental barrier [6, 15], the study of vertical transmission of SARS-CoV-2 has brought uncertainty. Single-cell studies in early pregnancy have confirmed the heterogeneity of ACE2 expression at the maternal-fetal interface [16]. The decidual tissue at the maternal-fetal interface is important as it contains components of both maternal and fetal origin. A critical period for studying vertical transmission is the perinatal period, during which drastic changes occur in the uterus and maternal-fetal interface, increasing the risk and heterogeneity of infection.

To study the risk of vertical transmission of SARS-CoV-2 during the peripartum period (before and after labor onset), we analyzed the expression patterns of TMPRSS2 and ACE2 in the decidua at the single-cell transcriptome level.

Materials and methods

Clinical information

Six pregnant women participated in this study: three delivered by cesarean section (before onset of labor), and the other three delivered vaginally (after onset of labor). All six were singleton pregnancies, and all women gave birth between 37 and 40 weeks. No complications were found during pregnancy check-ups.

All women were diagnosed in Changsha Hospital for Maternal and Child Health Care or Xiangya Hospital Central South University (from October 1st, 2018 to Jan 1st, 2019). The informed consent was obtained from each participant prior to research start. The study protocol was approved by the Medical Ethics Committee of the Xiangya Hospital Central South University (2018081027) and Changsha Hospital for Maternal and Child Health Care Ethics Committee (2018810).

Tissue isolation and preparation

Three decidual samples were obtained from women who delivered vaginally at full-term, and three were obtained from women who underwent cesarean section. According to the general steps of making single cell suspension [17-19], decidua were obtained immediately after birth, washed in phosphate-buffered saline, dissociated, digested, and filtered. For digestion, we used collagenase (Sigma-Aldrich) in phosphate-buffered saline; a 40-μm-pore-sized cell strainer (Falcon) was used for filtration.

Single cell cDNA library preparation and sequencing

In accordance with the manufacturer's and previous instructions [20], each cell suspension was adjusted to 10^6 cells/mL and the suspensions were loaded into the Chromium Single Cell Controller of the Single Cell Library and Gel Bead Kit V2 (10 × Genomics; cat. 120237); single-cell gel beads were then generated. After reverse transcription, the beads release RNA. Single-cell RNA sequencing libraries were then prepared. Sequencing was performed by Capitalbio Technology Corporation (Beijing) using an Illumina NovaSeq 6000 sequencer (more than 100,000 reads per cell). The Cell Ranger count pipeline (ver. 2.0.1) was used to analyze the information obtained. The pipeline merges and standardizes all data to facilitate subsequent analysis.

The results were analyzed and presented using Cell Ranger (ver. 2.0.1) and Cell Browser (ver. 2.0.0; 10 × Genomics). Principal component analysis and t-distributed stochastic neighbor embedding were performed using the R t-distributed stochastic neighbor embedding package of R software. According to the guide, output and presentation files were displayed and analysed by the Cell Browser 2.0.0 (10 × Genomics).

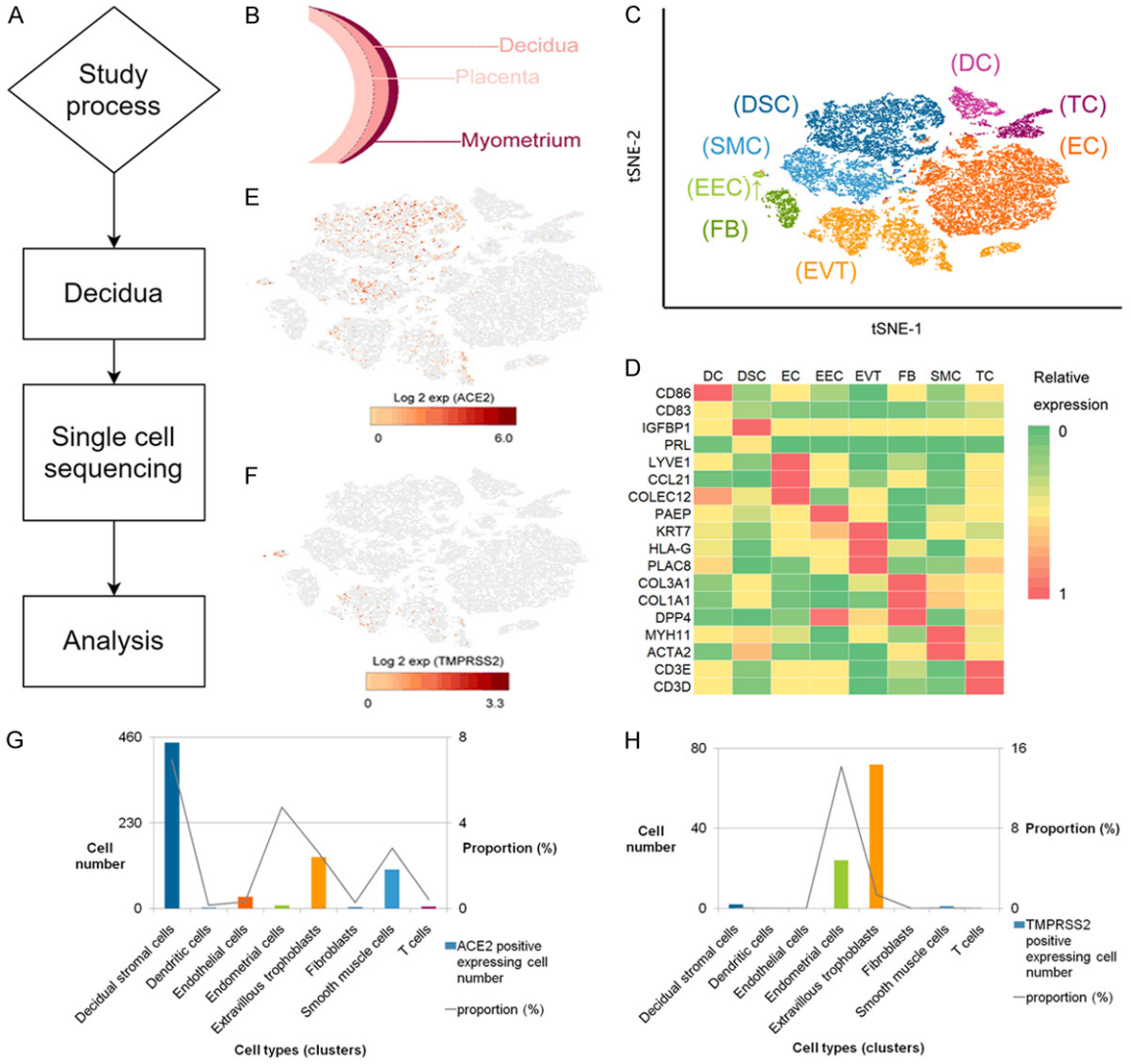

Figure 1. Heterogeneity of ACE2 and TMPRSS2 expression at single cell resolution. A. The process of the study. B. The position of decidua. C. The t-distributed stochastic neighbor embedding diagram showing the distribution of decidual cell groups. Decidual cells were divided into eight clusters (types), which are labeled with different colors. DC, dendritic cells; TC, T cells; EC, endothelial cells; EVT, extravillous trophoblasts; FB, fibroblasts; EEC, endometrial cells; SMC, (uterine) smooth muscle cells; DSC, decidual stromal cells. D. Heat map showing the relative expression of the main characteristic genes of the cell type. E. The t-distributed stochastic neighbor embedding diagram showing the relative expression of ACE2 in the peripartum decidua. F. The t-distributed stochastic neighbor embedding diagram showing the relative expression of TMPRSS2 in the peripartum decidua. G. Comparison of ACE2-positive cell numbers and proportions of each cell type. H. Comparison of TMPRSS2-positive cell numbers and proportions of each cell type.

Gene ontology analysis

After the output of gene list, Metascape was used for this step, which including pathway, gene ontology biological processes, and canonical pathways [21]. Metascape and TRRUST were used for studying transcription factors [21, 22]. The *P*-values were calculated Based on the accumulative hypergeometric distribution. Similarly, the cells with a count greater than 0 were defined as positive expressing cells [16].

Results

Heterogeneity of expression of ACE2 and TMPRSS2 in the peripartum decidua

According to the process, a total of 29231 decidual cells were analyzed (**Figure 1A**, **1B**).

Based on the differences in transcriptomes and expression of characteristic marker genes [17-19, 23, 24], we identified eight main cell groups (clusters), which included endothelial cells, decidual stromal cells, smooth muscle cells, extravillous trophoblasts, dendritic cells, fibroblasts, T cells, and endometrial cells (**Figure 1C**, **1D**).

To determine the specific cell types expressing ACE2, we investigated the expression of ACE2 in the peripartum decidua at single-cell resolution. Decidual cells were heterogeneous in terms of ACE2 expression. Specifically, ACE2 was highly expressed in a small number of cells (average of 2.52%). The t-stochastic neighbor embedding diagram showed that ACE2 was mainly expressed in decidual stromal cells, uterine smooth muscle cells, and extravillous trophoblasts, with highest expression in decidual stromal cells and smooth muscle cells, indicating that ACE2 expression was in the maternal component of the decidua. Extravillous trophoblasts (fetal component) had a lower expression level of ACE2 compared with decidual stromal cells and smooth muscle cells (**Figure 1E**).

As the TMPRSS2 protein is also involved in the entry of the virus into cells, we assessed the expression thereof. TMPRSS2 was expressed at high levels mainly by extravillous trophoblasts and endometrial cells. In contrast to ACE2, TMPRSS2 was poorly expressed in decidual stromal cells. In general, TMPRSS2 was expressed at lower levels (**Figure 1E-H**). ACE2 and TMPRSS2 were expressed in different cells in decidual tissue, and decidual cell susceptibility to SARS-CoV-2 infection is heterogeneous at the single-cell level.

Characteristics of decidual ACE2-positive cells

To determine the common characteristics of decidual ACE2-positive cells, we compared the transcriptomes of ACE2-positive and ACE2-negative cells. Transcriptome analysis showed an enrichment of integrin cell surface interactions, regulation of hormone levels, and positive regulation of secretion in ACE2-positive cells. This suggested that cell surface interactions may be related to virus entry (**Figure 2A**). ACE2-positive cells share certain common characteristics, and are mainly decidual stromal cells, uterine smooth muscle cells, and extravillous trophoblasts.

To evaluate the relevance of the data to SARS-CoV-2 infection, we compared our findings to those in the literature [25, 26]. The transcriptome of ACE2-positive cells was compared to those of cells of infected patients. The expression patterns of genes involved in regulation of growth and hormone were similar. Thus, SARS-CoV-2-susceptible and SARS-CoV-2-infected cells exhibited some common features (**Figure 2B-D**).

To explore the differences between these cells, we separately analyzed the transcriptomes of the different ACE2-positive cell types. For decidual stromal cells, compared with ACE2-negative decidual stromal cells, the genes upregulated in ACE2-positive decidual stromal cells were clustered in response to external stimulus, regulation of protein secretion, and protein activation cascade (**Figure 3A**). This may reflect a potential viral infection process. For uterine smooth muscle cells, ACE2-positive cells may be more sensitive to cell chemotaxis (**Figure 3B**). During this period, ACE2-positive extravillous trophoblasts exhibited items such as interferon α/β signaling, glycoprotein biosynthetic process (**Figure 3C**), which are characteristics of fetal components, and viral susceptibility differed among the various decidual cell types.

Since most TMPRSS2-positive cells were extravillous trophoblasts (72.73%), we also investigated the differences in the expression profiles of these cells. Compared with TMPRSS2-negative cells, TMPRSS2-positive extravillous trophoblasts were mainly represented by genes involved in response to hormone and immune system process (**Figure 4A**). ACE2-positive and TMPRSS2-positive extravillous trophoblasts expressed high levels of genes involved in extracellular matrix organization.

TMPRSS2-positive extravillous trophoblasts were next compared to infected cells [27, 28]. All cells (three groups) exhibited upregulated interferon signaling and regulated exocytosis (**Figure 4B**, **4C**). Among them, two groups were clustered on apoptotic signaling pathway. The further analysis of transcription factors showed that all three groups were regulated by STAT1 (**Figure 4D**), suggesting that STAT1 could be a transcription factor of their potential common pathway. Therefore, both susceptible and

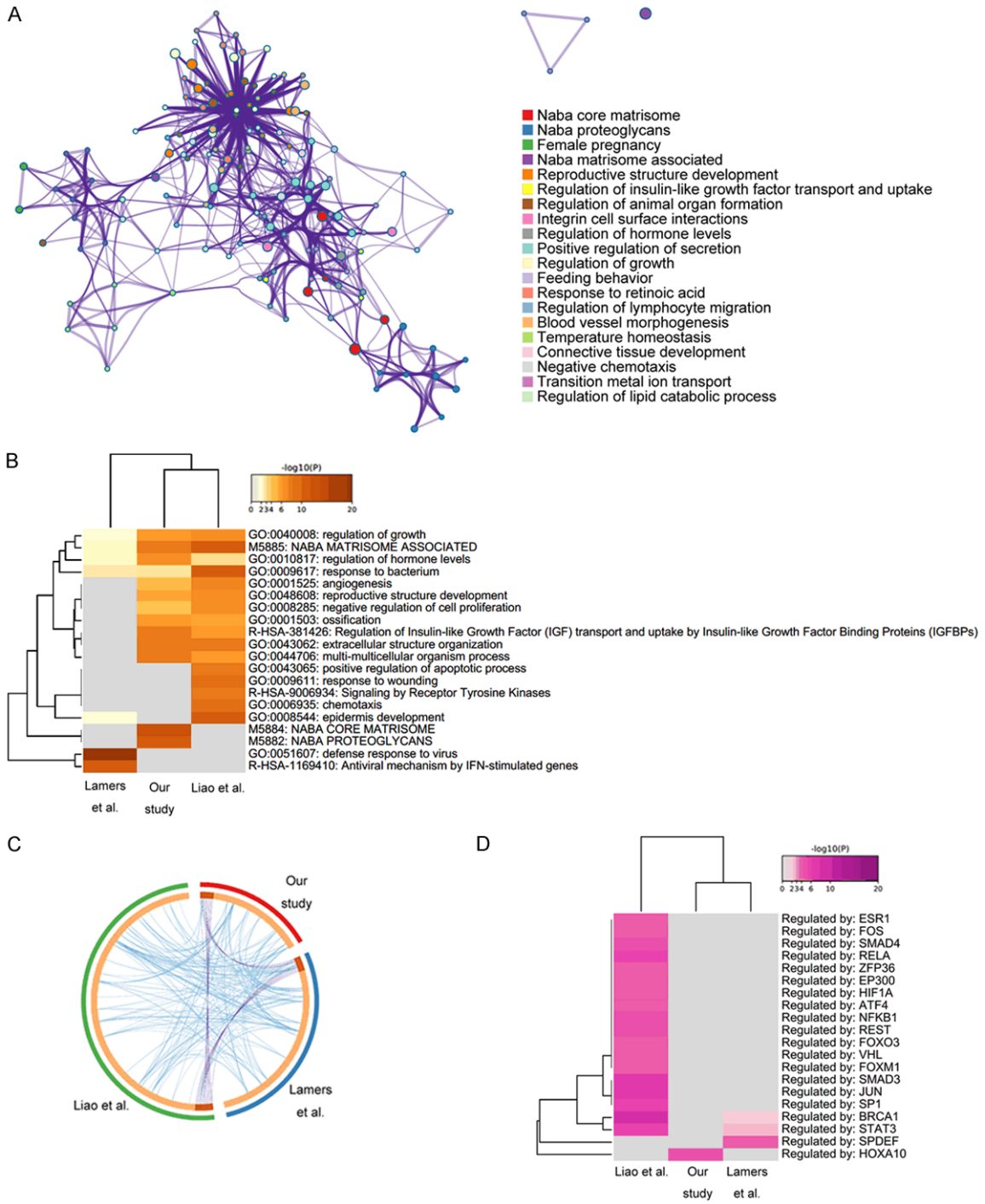

Figure 2. Characteristics of ACE2-positive cells. A. Network of enriched terms in all ACE2-positive cells (upregulated genes), colored by cluster ID. Nodes that share the same cluster ID are typically close to each other. B. Comparison of genes (up-regulated) in ACE2-positive cells to those in infected cells. The relationships of the enrichment items of different transcriptomes are shown. Each column represents a transcriptome and each row represents an enrichment item. C. Overlap among genes of ACE2-positive cells and infected cells, including the shared term level, where blue curves link genes that belong to the same enriched ontology term. The inner circle represents gene lists, where hits are arranged along the arc. Genes that hit multiple lists are coloured in dark orange, and genes unique to a list are shown in light orange. D. Summary of enrichment analysis in transcriptional regulatory interactions. Each column represents a transcriptome and each row represents an enrichment transcription factor.

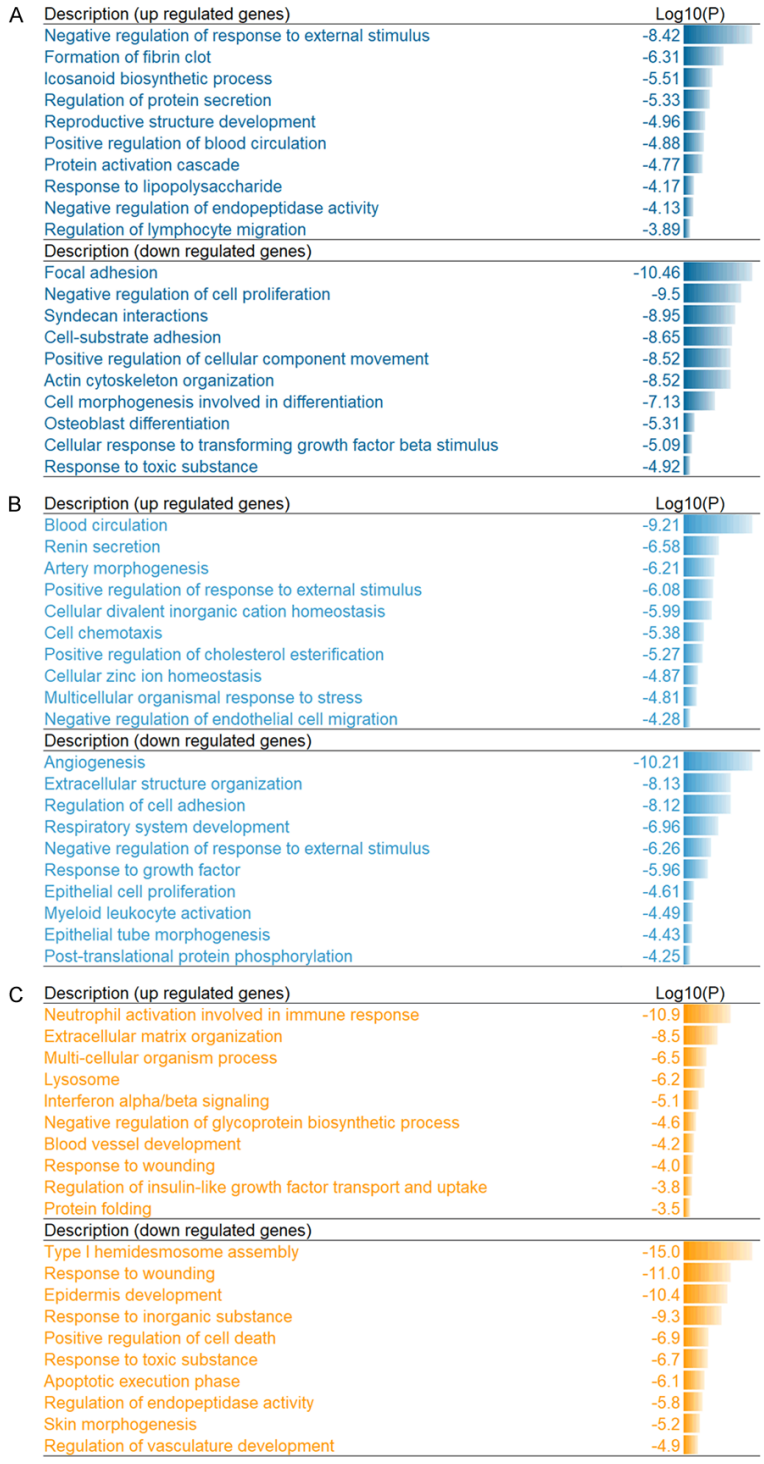

Figure 3. Characteristics of different types of ACE2-positive cells. A. Enriched terms across the upregulated and downregulated genes of ACE2-positive decidual stromal cells. Log10 (P) is the *p*-value in log base 10. B. Enriched terms across the upregulated and downregulated genes of ACE2-positive smooth muscle cells. Log10 (P) is the *p*-value in log base 10. C. Enriched terms across the upregulated and downregulated genes of ACE2-positive extravillous trophoblasts. Log10 (P) is the *p*-value in log base 10.

infected cells exhibit some features that may relate to viral invasion.

Characteristics of decidual ACE2-positive cells before and after labor

Delivery is a period associated with many cellular changes. An important question is whether the gene expression pattern of these cells changes after labor. Therefore, we investigated the changes of the main cell types after delivery. We found more ACE2-positive cells (after labor onset 55.28% vs before labor onset 44.72%), most of which were decidual stromal cells (67.40%); this was investigated further (**Figure 5A**, **5B**).

Before delivery, ACE2-positive (vs ACE2-negative) decidual stromal cells showed clusters of genes involved in regulation of membrane protein ectodomain proteolysis. This implied a possible invasion mechanism of these cells by viruses. Following labor onset, some updated terms (upregulated genes of ACE2-positive vs ACE2-negative decidual stromal cells) were found, including genes involved in regulation of cell junction assembly and G alpha signalling events (**Figure 5C**). Delivery is a complex process, and the viral susceptibility of decidual stromal cells changed over the delivery period.

Discussion

In previous studies, samples were collected from infected pregnant women as well as from throat swabs of newborns. All the samples tested

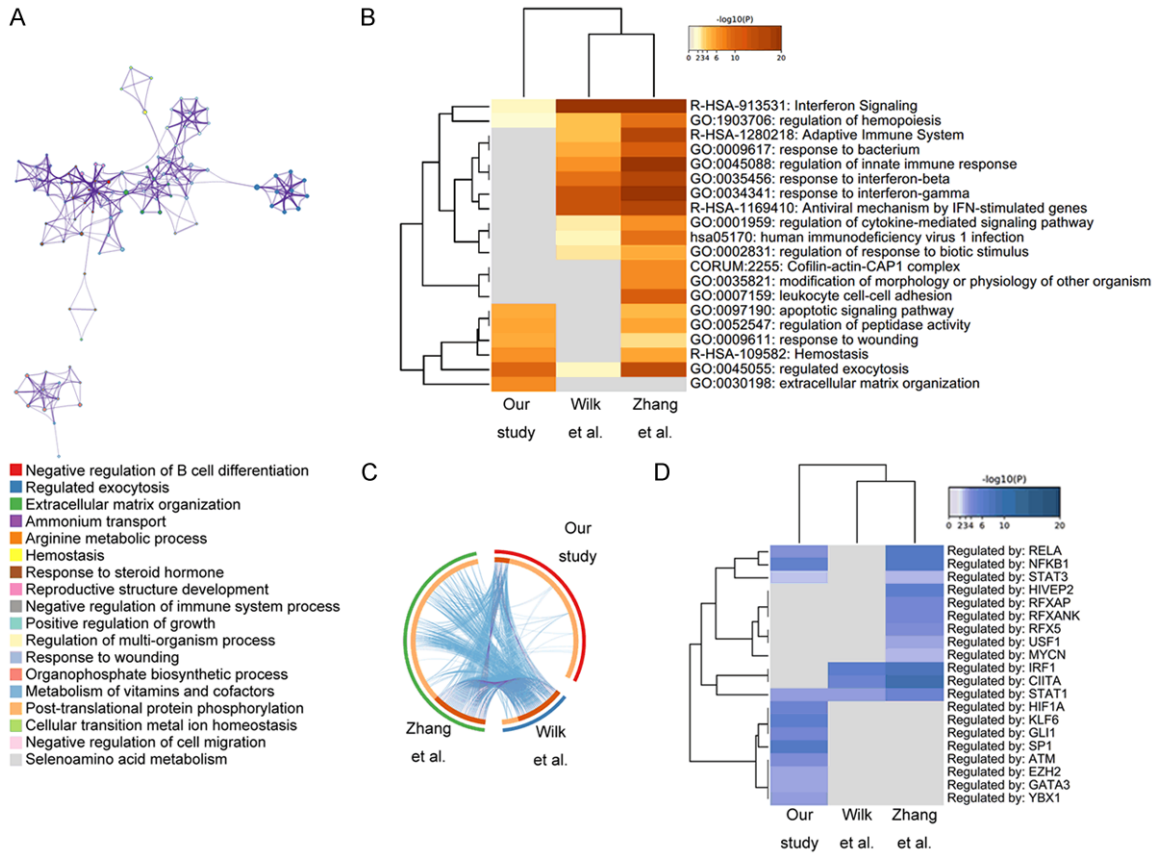

Figure 4. Characteristics of TMPRSS2-positive cells. A. Network of enriched terms in all TMPRSS2-positive extravillous trophoblasts (upregulated genes), colored by cluster ID. Nodes that share the same cluster ID are typically close to each other. B. Comparison of genes (up-regulated) in TMPRSS2-positive extravillous trophoblasts to those in infected cells. The relationships of the enrichment items of different transcriptomes are shown. Each column represents a transcriptome and each row represents an enrichment item. C. Overlap among genes of TMPRSS2-positive extravillous trophoblasts and infected cells, including the shared term level, where blue curves link genes that belong to the same enriched ontology term. The inner circle represents gene lists, where hits are arranged along the arc. Genes that hit multiple lists are coloured in dark orange, and genes unique to a list are shown in light orange. D. Summary of enrichment analysis in transcriptional regulatory interactions. Each column represents a transcriptome and each row represents an enrichment transcription factor.

negative for the virus [7, 29]. However, a few cases of infected newborns have been reported. In one neonate, elevated IgM was evident in a blood sample drawn 2 h after birth [8, 30]. Although these cases cannot completely exclude other transmission routes, this evidence raises concerns about vertical transmission.

The ACE2 receptor plays a key role in SARS-CoV-2 entry and replication [1, 31]. The differences in expression of ACE2 may indicate the susceptibility of various cells and the potential routes of SARS-CoV-2 infection. ACE2 is differentially expressed in multiple tissues and organs. In this study, we showed that this heterogeneity still existed in the decidua during the perinatal period.

Our results suggested that the expression of ACE2 in the peripartum period was highly heterogeneous, which is consistent with data from the first trimester [16, 32]. However, the possibility of increased susceptibility to placental entry of SARS-CoV-2 across pregnancy is different [33]. Therefore, it will be necessary to study the expression patterns of the related genes at the maternal-fetal interface during delivery. In particular, at single-cell resolution, we provide more accurate results for the ACE2 expression profile of decidua during this period.

During the peripartum period, decidual stromal cells, uterine smooth muscle cells, and extravillous trophoblast cells showed higher expression of ACE2, while high expression of TMPRSS2 was observed in endometrial cells and extravil-

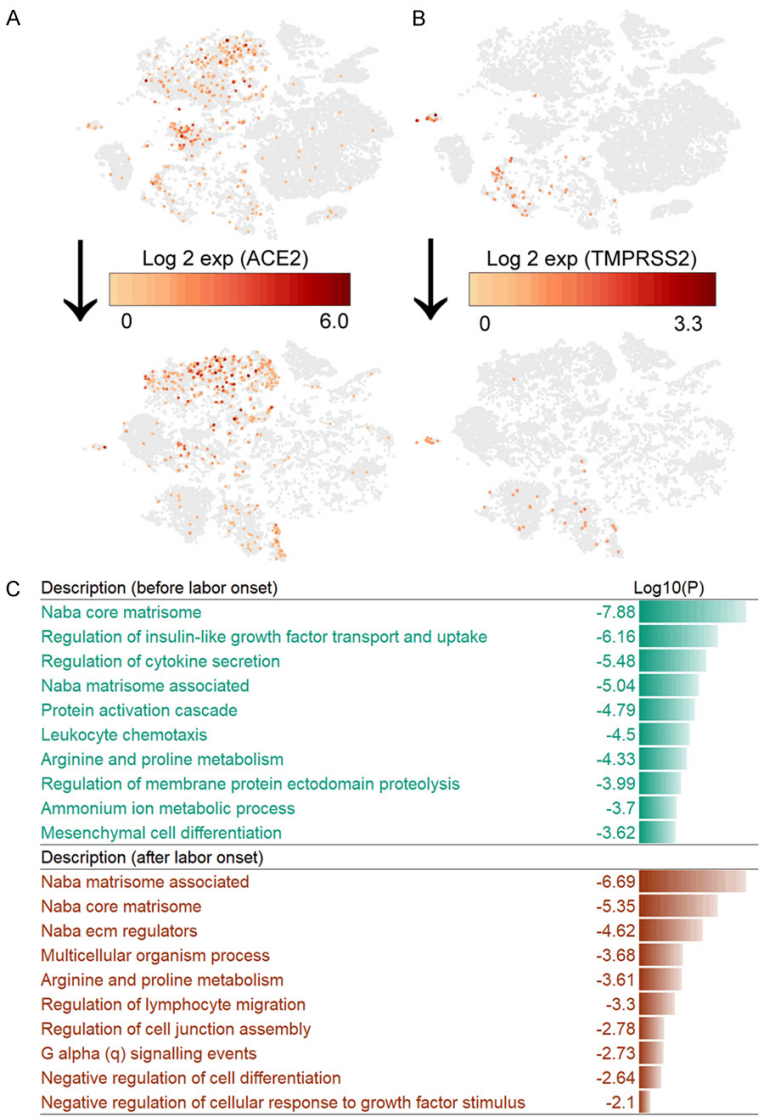

Figure 5. Comparison of cells and transcriptomes before and after delivery. A. T-distributed stochastic neighbor embedding diagram showing the relative expression of ACE2 in the decidua before (top) and after (bottom) labor onset. B. T-distributed stochastic neighbor embedding diagram showing the relative expression of TMPRSS2 in the decidua before (top) and after (bottom) labor onset. C. Enriched terms across upregulated genes of ACE2-positive decidual stromal cells before and after labor. Log10 (P) is the *p*-value in log base 10.

lous trophoblasts. This suggests the different susceptibility of these cell types within the decidua. During pregnancy, decidualization of the uterus, which consists of endometrial cells, forms decidua, which consists of decidual stromal cells, to establish pregnancy. Therefore, the two kinds of cells are closely related. Decidual extravillous trophoblasts are an invasive extravillous type of trophoblast and are important for placental development. Their high expression of ACE2 and TMPRSS2 is noteworthy. Our analysis of maternal and fetal components indicate the potential risk of vertical transmission of SARS-CoV-2 during delivery.

We next analyzed and compared the characteristics of ACE2-positive cells. We found that in ACE2-positive extravillous trophoblasts, the upregulated genes were involved in interferon signaling. A recent study showed that SARS-CoV-2 is sensitive to exogenous interferons and that type I (alpha) interferon is one candidate for the management of COVID-19 [34]. Therefore, our results are in agreement with the potential effects and mechanisms of interferon, which may explain the sensitivity to this kind of therapy. Next, the SARS-CoV-2-susceptible and SARS-CoV-2-infected cells exhibited common features including regulation of growth, apoptotic signaling pathway, and STAT1 regulation. An aberrant STAT1 pathway is central to COVID-19, and STAT1 is one key gene involved in COVID-19 [35, 36]. Combined with our results, SATA1 related pathways and the regulatory relationships may be important at the maternal-fetal interface for SARS-CoV-2 susceptibility.

We also provide a single-cell atlas of these genes before and after labor onset. After delivery, the number of ACE2-positive cells was slightly higher than before delivery. Most ACE2-positive cells after delivery were decidual stromal cells. The terms of upregulated genes after labor onset changed, which indicated the differences in the expression patterns and possible susceptibility to entry to SARS-CoV-2. After labor onset, the upregulated genes of ACE2-positive decidual stromal cells included genes involved in cell dif-

ferentiation. A recent single-cell sequencing study found altered differentiation of immune cells in the lungs of COVID-19 patients [37]. This suggests that the changes we observed after labor onset may be affected by the virus. In addition, most COVID-19 pregnant women gave birth by cesarean section (before labor onset) [7-9, 29, 38]. Considering the risks of vaginal delivery and vertical transmission, our results provide reference for vertical transmission in before and after labor, which may be of clinical relevance.

Our samples were collected during full-term deliveries; thereof, some were unavoidably affected by uterine contraction, the possible effects of which should be considered. The contractile force reflects the action potential of smooth muscle cells and varies by Ca^{2+} level [39]. In the second stage of labor, the rhythmic uterine pressure peaks, and can reach 100-150 mmHg (13.33-20.00 kpa, 1359.51-2039.26 kgf/m^2). The force applied between contractions is only 6-12 mmHg (0.80-1.60 kpa, 81.57-163.14 kgf/m^2) [40]. The average diameter of decidual cells varies [41]. The diameter of T cells is approximately 10 μm [42]. Some T-cell receptors of activated T cells are localized to structures 200-800 nm in width [43], which is close to the size of the virus (60-140 nm) [44]. At the microscopic scale, the nano-scale extracellular vesicles (100 nm-1 μm) play a role in endothelial dysfunction (endothelial cells) [45]. At the maternal-fetal interface, the diameters of extracellular vesicles were various. For example, the extracellular vesicles from endometrial stromal cells and endothelial tissue are 30-120 nm and 150-200 nm, respectively. These are involved in embryonic development and cell proliferation [46]. In general, our research found that cells in decidua have different susceptibility for SARS-CoV-2. These features could be used to identify target cells for medication. Recently, adenosine nanoparticles for targeted delivery in COVID-19 was discussed [47]. At the interface, nanoparticles may be useful for targeted medication, although further research on this is required. Hopefully, our findings could provide useful information for this kind of treatment. As this was a preliminary study, more research is needed to determine the susceptibility of the maternal-fetal interface to infection during delivery.

In summary, we found that ACE2 and related genes were differentially expressed at the single-cell level in various types of decidual cells. The susceptibility of decidual cells to SARS-CoV-2 infection is heterogeneous during the peripartum period.

Acknowledgements

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Disclosure of conflict of interest

None.

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