

Single-Cell RNA-seq Identifies Cell Subsets in Human Placenta That Highly Expresses Factors to Drive Pathogenesis of SARS-CoV-2

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Abstract

Infection by the Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) results in the novel coronavirus disease COVID-19, which has posed a serious threat globally. Infection of SARS-CoV-2 during pregnancy is associated with complications like preterm labor and premature rupture of membranes; a proportion of neonates born to the infected mothers are also positive for the virus. During pregnancy, the placental barrier protects the fetus from pathogens and ensures healthy development. However, whether or not SARS-CoV-2 can infect the placenta is unknown. Herein, utilizing single-cell RNA-seq data, we report that the SARS-CoV-2 binding receptor *ACE2* and the S protein priming protease *TMPRSS2* are co-expressed by a subset of syncytiotrophoblasts (STB) in the first trimester and extra villous trophoblasts (EVT) in the second trimester human placenta. The *ACE2*- and *TMPRSS2*-positive (*ACE2*+*TMPRSS2*+) placental subsets express mRNA for proteins involved in viral budding and replication. These cells also express mRNA for proteins that interact with SARS-CoV-2 structural and non-structural proteins in the host cells. We also discovered unique signatures of genes in *ACE2*+*TMPRSS2*+ STBs and EVTs. The *ACE2*+*TMPRSS2*+ STBs are highly differentiated cells and express genes involved mitochondrial metabolism and glucose transport. The second trimester *ACE2*+*TMPRSS2*+ EVTs are enriched for markers of endovascular trophoblasts. Further, both these subtypes abundantly expressed genes in Toll like receptor pathway, the second trimester EVTs (but not first trimester STBs) are also enriched for component of the JAK-STAT pathway that drive inflammation. To conclude, herein we uncovered the cellular targets for SARS-CoV-2 entry and show that these cells can potentially drive viremia in the developing human placenta. Our results provide a basic framework towards understanding the paraphernalia involved in SARS-CoV-2 infections in pregnancy.

Keywords: Placenta, trophoblast, SARS-CoV-2, Coronaviruses, COVID-19, Single cell RNAseq, scRNA-seq, *ACE2*, *TMPRSS2*, CD147, CTSL, inflammation

Introduction

Epidemiologic evidence indicates that pregnant women are at higher risk of severe illness and mortality from viral infections such as influenza, Ebola and Lassa fever (Silasi et al., 2015). Certain viral infections during pregnancy can lead to several adverse pregnancy outcomes like spontaneous abortion, mother-to-child transmission resulting in congenital viral syndromes, still-births and intrauterine fetal deaths (Silasi et al., 2015; Arora et al., 2018). Furthermore, viral infection also predisposes the pregnancy towards preterm birth, which has major long-term health implications for the newborn. Thus, understanding the health risks of viral infections during pregnancy is vital for designing the appropriate approaches for clinical management of these conditions. The importance of understanding the role of viral infection during pregnancy gains further relevance as we are confronted with newer pandemics, which may affect the pregnant mother and the fetus.

Coronaviruses (CoV) are positive-sense RNA viruses that, upon zoonotic transmission, lead to respiratory disease in humans and some animals. Previous outbreaks of zoonotic coronaviruses (CoV), including the severe acute respiratory syndrome (SARS)-CoV and the Middle East respiratory syndrome (MERS)-CoV, have proven to be of great public health concern. Another outbreak of severe acute respiratory syndrome called coronavirus disease-2019 (COVID-19) has been recently reported, which is due to infection by a novel coronavirus termed as SARS-CoV-2 (Zhu et al., 2020). The infection has spread rapidly worldwide due to high human-to-human transmission resulting in a public health emergency of international concern (Gupta et al., 2020; Li et al., 2020b). Presently, there are no treatments available for COVID-19 and there is an urgent need to identify the drugs and vaccines targeted against this virus (Prajapat et al., 2020).

Since SARS-CoV-2 has not been detected in humans before, limited information is available about its health effects; negligible information is available for pregnant women. In pregnant women, COVID-19 is associated with severe pregnancy complications like preterm labor and premature rupture of membranes (Gajbhiye et al., 2020). Furthermore, a proportion of neonates born to mothers with COVID-19 are positive for the virus, suggesting vertical transmission through the placental barrier (Gajbhiye et al., 2020).

The placenta is a highly specialized organ that maintains the equilibrium between immunological and biochemical factors required for fetal development. It also acts as a barrier for vertical transmission of pathogens (Maltepe and Fisher, 2015; Burton et al., 2016). However, some viruses such as Zika can infect the placental cells via receptors on trophoblasts, leading to fetal malformation and pregnancy complications (Hirsch et al., 2018; Tabata et al., 2018). Placental and amniotic fluid infection with SARS-CoV-2 has also been reported (Baud et al., 2020; Zamaniyan et al., 2020). For SARS-CoV-2 to be able to infect the placenta, the cells must harbour the necessary receptors and virus-processing machinery. It has been shown that SARS-CoV-2 binds and infects host cells by utilizing the membrane-bound Angiotensin-Converting Enzyme II (ACE2) (Jagtap et al., 2020; Letko et al., 2020; Shang et al., 2020). In addition, SARS-CoV-2 also binds to CD147/Basigin (BSG) on the cell surface that may act as an alternate receptor (Wang et al., 2020). Upon receptor binding to host cells, the viral-encoded S protein aids in membrane fusion, a process typically primed for activation by proteolytic cleavage. The main host protease that mediates S protein priming on primary target cells and initiates viral entry is the Type II transmembrane serine protease TMPRSS2 (Hoffmann et al.,

2020). The endosomal protease cathepsin L (CTSL) can also enhance viral entry (Hoffmann et al., 2020). Thus, the presence of such receptors and S protein primer proteases in host cells is a key determinant of SARS-CoV-2 infection. Indeed, expression of *ACE2* and *TMPRSS2* have been detected in lung airway cells and the upper respiratory epithelium, the primary site of SARS-CoV-2 action (Sungnak et al., 2020; Ziegler et al., 2020). Beyond respiratory distress, some patients with SARS-CoV-2 viremia develop multiple organ injuries, and cells of these tissues also express *ACE2* and *TMPRSS2* (Qi et al., 2020; Seow et al., 2020; Zou et al., 2020).

The binding of enveloped viruses like SARS-CoV-2 to its receptors results in events related to membrane fusion and/or endocytosis and establishment of the primary infection. Following its entry and uncoating, the coronavirus replicative cycle is initiated by translation of its non-structural proteins including the replicases that allow viral RNA synthesis and capping. This course requires a network of host factors to create an optimal environment for facilitating viral entry, gene expression and, RNA synthesis or virus release (de Wilde et al., 2018). Further, most enveloped viruses bud at the plasma membrane by recruiting the host endosomal sorting complex required for transport (ESCRT) machinery (Ahmed et al., 2019; Gatta and Carlton, 2019). While the precise host proteins in SARS-CoV-2 entry and replication are not yet understood, the host SARS-CoV-2 interactome has been characterized and shown to overlap with host proteins involved in endocytosis and replication of SARS-CoV-2 (Gordon et al., 2020). Thus, elucidating tissue and cell-type-specific host machinery that not only mediate viral entry but also its replication and eventually budding from the host cell is essential to understand the pathogenesis of SARS-CoV-2 infection.

Single-cell RNA-sequencing (scRNA-seq) of different tissues has transformed our ability to map the types, subsets and states of cells in healthy and diseased conditions in an unprecedented manner (Sharma et al., 2018; Szabo et al., 2019; Iyer et al., 2020). Recently, scRNA-seq has been applied to expand our understanding of the cellular landscape during viral infection including that of SARS-CoV-2 (Russell et al., 2018; Galinato et al., 2019; Liao et al., 2020), and also to identify various tissues and cells that are potential targets of SARS-CoV-2 infection (Colaco et al., 2020; Lukassen et al., 2020; Qi et al., 2020; Seow et al., 2020; Sungnak et al., 2020; Zhang et al., 2020). These studies have immensely contributed to the identification of host tissues and cellular targets for SARS-CoV-2 infection, expanding our understanding of the molecular characteristics of the host cells that are targets of viral infection.

To gain an insight into the pathogenesis of SARS-CoV-2 infection during pregnancy, it is essential to identify and characterize the placental cell types that express the host receptors *ACE2* and protease enzyme *TMPRSS2*. Previous studies have shown that *BSG/CD147* is expressed in first trimester human trophoblasts and is essential for trophoblast invasion (Lee et al., 2013). Recent studies have reported *ACE2* positive cells in early embryonic trophoblasts as well as first trimester human placenta (Colaco et al., 2020; Li et al., 2020a). Expression of *ACE2* protein is observed in term human placenta (<https://www.proteinatlas.org/ENSG00000130234-ACE2/tissue/placenta>) and also in mouse placenta on day 18 of gestation (Bharadwaj et al., 2011). However, the detailed characteristics of the *ACE2* and *TMPRSS2* positive cells in the placenta are unknown. Herein, we surveyed the single-cell RNA-seq data of human placenta for the expression of *ACE2*, *TMPRSS2*, alternate receptor *BSG/CD147* and the endosomal protease *CTSL*. The present study aimed to characterize the *ACE2* and *TMPRSS2* positive placental cells for their ability in viral

endocytosis, replication, SARS-COV-2 interactions and viral budding. The results reveal that the syncytiotrophoblasts (STB) of the first trimester and the extra villous trophoblasts (EVT) of the second trimester placenta express the receptors and S protein processing enzyme and also possess viral endocytosis, replication and viral budding machinery indicating that these cells are the potential targets for SARS-CoV-2 infection. These results provide a basic framework towards the understanding of the fundamental mechanisms of SARS-CoV-2 infections in pregnancy.

Methods

To identify the population of human placental cells that express *ACE2*, *BSG*, *TMPRSS2* and *CTSL* at single cell resolution, we analysed scRNA-seq data of first and second trimester human placenta (Liu et al., 2018) [Accession number GSE89497]. The available data was deconvoluted for extravillous trophoblast (EVT), cytotrophoblast (CTB), syncytiotrophoblast (STB) and villous stromal cells (STR) for the first trimester placenta; only EVT data was available for second trimester human placenta. Expression was determined in terms of Transcripts Per Million (TPM) per cell. In addition, pseudo-bulk scRNA-seq data was analyzed to understand the distribution of *ACE2*, *BSG*, *TMPRSS2* and *CTSL* in different maternal cell populations in the feto-maternal interface (Suryawanshi et al., 2018).

We profiled the mRNA levels of 27 host proteins involved in human ESCRT for viruses (Ahmed et al., 2019) and mRNA levels of 30 proteins involved in viral replication (de Wilde et al., 2018) in placental cells that co-express *ACE2* and *TMPRSS2* (**Supplementary Table 1**). We also analyzed the transcript profiles of 332 human proteins that physically interact with SARS-CoV-2 in placental cells that co-express *ACE2* and *TMPRSS2* (Gordon et al., 2020). To guard against viral infection, the host cells express a plethora of genes to sense the presence of the virus on the cell surface, cytosol and the endosomes. This in turn, activates the host defense program to limit or eliminate virus infection and restore tissue homeostasis. We profiled the expression of 487 host viral response genes in placental cells that co-express *ACE2* and *TMPRSS2* (**Supplementary Table 1**).

To characterize the trophoblast cells that co-express *ACE2* and *TMPRSS2* from their counterparts that do not express both of these genes, we carried out pseudo-bulk analysis of *ACE2*- and *TMPRSS2*-positive (*ACE2*+*TMPRSS2*+) cells and *ACE2*- and *TMPRSS2*-negative (*ACE2*-*TMPRSS2*-) trophoblast cells. Single-cell data for *ACE*+*TMPRSS2*+ and *ACE2*-*TMPRSS2*- cells was independently aggregated and the mean TPM values were computed. The data was filtered for genes whose mean values were ≥ 0.1 TPM and the ratio of the mean value in *ACE2*+ *TMPRSS2*+ cells over *ACE2*-*TMPRSS2*- cells was calculated. The genes that had a ratio of ≥ 1.5 or ≤ 0.5 and p value of < 0.05 were filtered and the data was deconvoluted for single cells. Gene ontology (GO) analysis was performed using the DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/home.jsp>) and PANTHER database and over-representation test was performed using reference genes of PANTHER pathways (<http://www.pantherdb.org/>).

All the data was processed using R Studio version 3.6.2. All heatmaps were plotted using heatmap.2 function from gplots R package. Statistical analysis was done using the Welch's t-test and the graphs plotted in GraphPad prism version 8.0.

Results:

Trophoblast cells express mRNA for SARS-CoV-2 receptors and its spike protein processing enzymes

The human placenta is characterized by four distinct cell lineages: extravillous trophoblast cells (EVT), cytotrophoblast (CTB), syncytiotrophoblast (STB) and villous stromal cells (STR). To understand the distribution of the SARS-CoV-2 receptors in these cell types, we analysed single-cell transcriptome data from publicly available datasets of human placenta to comprehensively resolve the status of placental cell susceptibility to SARS-CoV-2 infection during pregnancy. The result revealed that *ACE2*, *BSG*, *TMPRSS2* and *CTSL* were expressed in all the cell types of first trimester placenta; however, not every cell of each type expressed these genes (**Figure 1A**). As evident, the highest proportion of STBs in first trimester placenta (39%) expressed *ACE2*, whereas only 2% of EVTs had *ACE2* expression. *BSG* was abundantly expressed in almost all the cells (98-100%) of each cell type of the first trimester (**Supplementary Table 2**). Very few cells of the human placenta expressed *TMPRSS2*. The highest *TMPRSS2* expression was detected in STBs of first trimester placenta (23%), whereas only 1% of CTBs had *TMPRSS2* expression (**Supplementary Table 2**). *CTSL* was expressed in nearly all STBs, CTBs, EVTs and STRs of first trimester placenta (**Figure 1A**). The numbers of cells that express these genes individually are given in **Supplementary Table 2**.

We then compared the expression of these genes in EVTs of the first trimester and second trimester human placenta (**Figure 1B**). In the first trimester, 2% of EVTs expressed *ACE2*, while 62% of second trimester EVT cells expressed *ACE2*. Similarly, the numbers of EVTs expressing *TMPRSS2* also increased in the second trimester as compared to first trimester (2% vs 19%) (**Supplementary Table 2**). In both cases, the increase was statistically significant ($p\text{-value} \leq 0.001$). Similar to the first trimester, nearly 100% of EVT cells of the second trimester expressed *BSG* (195/200) and *CTSL* (199/200) (**Supplementary Table 2**). *BSG* expression was significantly reduced in second trimester EVTs as compared to first trimester ($p\text{-value} \leq 0.001$); the expression of *CTSL* was significantly ($p\text{-value} \leq 0.001$) higher in second trimester EVTs as compared to first trimester EVTs (**Figure 1B**).

We also studied the expression of other SARS-CoV receptors DPP4 and ANPEP, which are utilized by Middle East Respiratory Syndrome coronavirus (MERS-CoV) and CoV-229E respectively. *ANPEP* was detected in the EVTs, CTBs and STRs but not the STBs of the first trimester placenta. *DPP4* on the other hand was expressed by all the CTBs, STBs, EVTs and STRs (**Supplementary Figure 1A**). As compared to first trimester EVTs, the levels of *ANPEP* significantly decreased ($p\text{-value} \leq 0.001$), while that of *DPP4* significantly increased ($p\text{-value} \leq 0.001$) in the second trimester EVTs (**Supplementary Figure 1B**).

To determine whether or not endometrial cells express these genes, we analysed pseudo-bulk data of the first trimester feto-maternal interface (**Supplementary Figure 2**). Expression of *ACE2* was detected in smooth muscle cells, and a low abundance of *ACE2* transcripts was also detected in decidual stromal cells and fibroblasts, vascular endothelial cells and NK cells. *TMPRSS2* transcripts were detected in endometrial epithelial cells and lymphatic endothelial cells. *BSG* and *CTSL* were detected in all the maternal cells of the first trimester feto-maternal interface (**Supplementary Figure 2**).

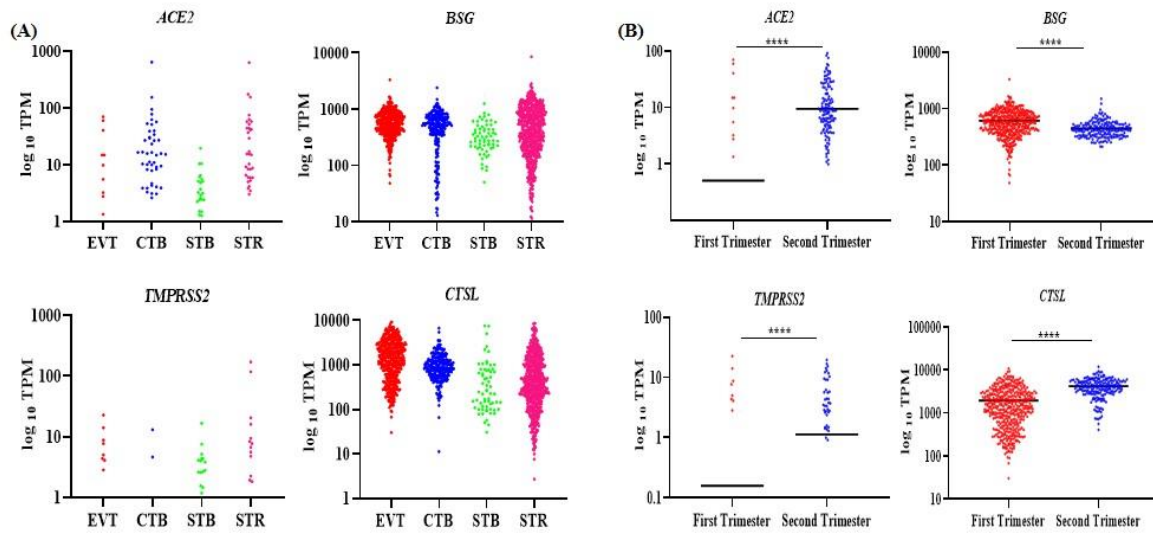


Figure 1: Trophoblast cells express mRNA for SARS-CoV-2 receptors and spike protein processing enzymes

A) mRNA level of SARS-CoV-2 receptors (*ACE2* and *BSG*) and spike protein primer enzymes (*TMPRSS2* and *CTSL*) in different cell types of first trimester human placenta [EVT (n=440), CTB (n=248), STB (n=64), STR (n= 615)]. **B)** Comparison of the mRNA levels of *ACE2*, *BSG*, *TMPRSS2* and *CTSL* in EVT of first (n=440 cells) and second (n=200 cells) trimester human placenta. Each dot represents data of a single cell, Y axis represents log₁₀ Transcripts Per Million (TPM). Bar within the cluster denote mean. Horizontal black bars denote significantly different values (**** indicates p-value ≤ 0.0001). Data was extracted from single-cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497]. EVT = Extravillous Trophoblast, CTB = Cytotrophoblast, STB = Syncytiotrophoblast, STR= Villous Stromal cell.

Co-expression of mRNA of SARS-CoV-2 receptors and spike protein processing enzymes in human placental cells

SARS-CoV-2 infection in host cells requires coordinated expression of the entry receptor and S protein primer. We aimed to identify cells co-expressing *ACE2*, *TMPRSS2*, *BSG* and *CTSL* in different combinations (**Figure 2**). The results revealed that only a subset of STBs (14%) in the first trimester placenta co-expressed *ACE2* and *TMPRSS2* (**Supplementary Table 3**). No other cell types in first trimester placenta expressed this receptor and S protein primer protease pair; although, there were a number of cells expressing *ACE2* (**Figure 2**). However, 15% of EVTs in the second trimester placenta co-expressed *ACE2* and *TMPRSS2* (**Supplementary Table 3**).

In the first trimester placenta, all the *ACE2*-positive trophoblast subtypes co-expressed *BSG* and *CTSL*, and all the *BSG*-positive cells co-expressed *CTSL*. All the *ACE2*-positive second trimester EVT co-expressed *BSG* and *CTSL*, and all the *BSG*-positive cells co-expressed *CTSL* (Figure 2). The absolute numbers and the percentage of the co-expressing cells are given in Supplementary Table 3.

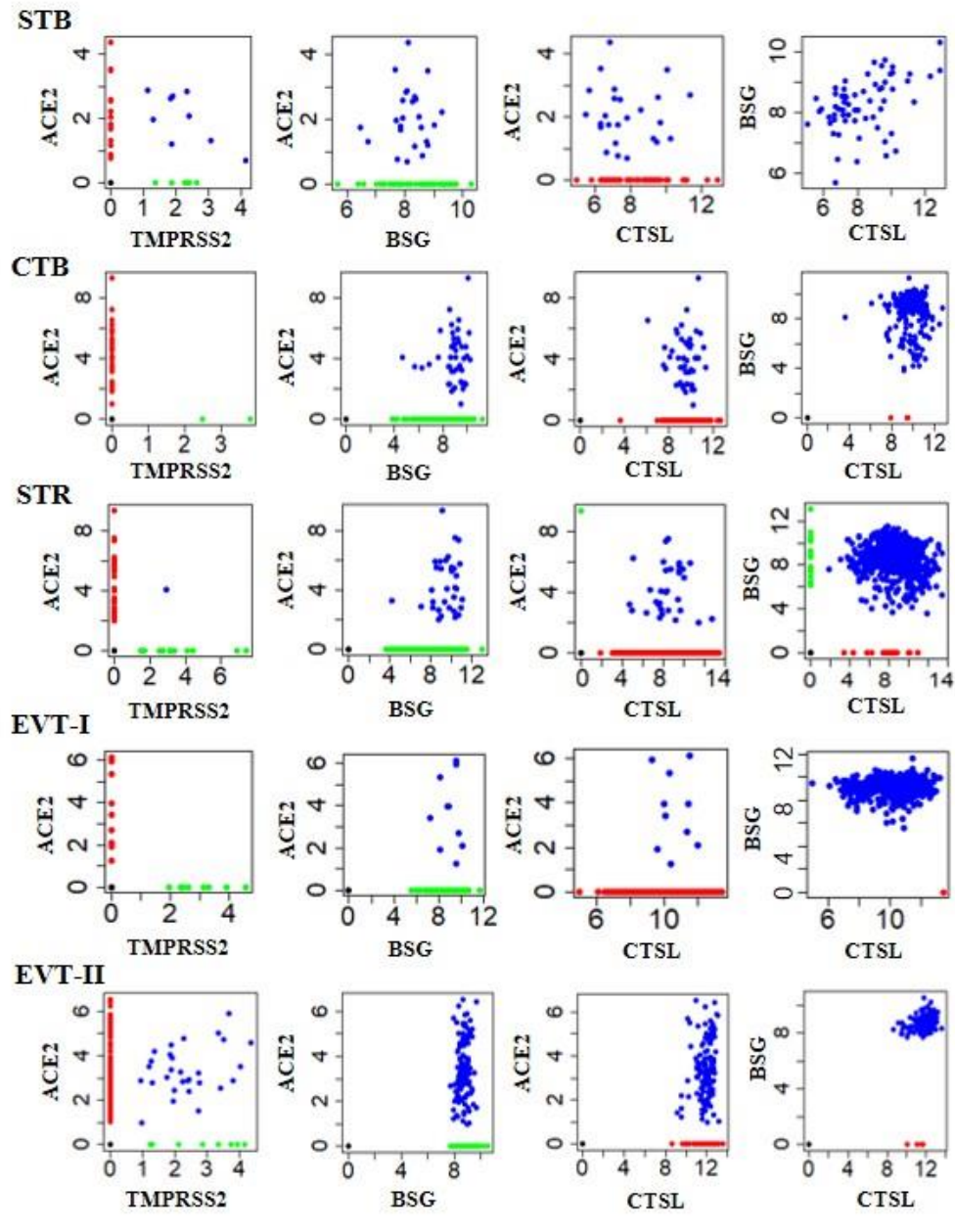


Figure 2: Co-expression of mRNA of SARS-CoV-2 receptors and spike protein processing enzymes in human placental cells

Co-expression of *ACE2* and *TMPRSS2*, *ACE2* and *BSG*, *ACE2* and *CTSL*, and *BSG* and *CTSL* in STB (n=64), CTB (n=248), STR (n= 615), first trimester EVT (EVT-I, n=440) and second trimester EVT (EVT-II, n=200). Each dot represents data of a single cell. Co-expressing cells are blue, single-positive cells are red and green. X and Y axes represent log₂ Transcripts Per Million (TPM) values for that gene. Data was extracted from single cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497]. EVT = Extravillous Trophoblast, CTB = Cytotrophoblast, STB = Syncytiotrophoblast, STR = Villous Stromal cell.

***ACE2+TMPRSS2+* first trimester syncytiotrophoblast cells are highly differentiated and express the machinery for viral endocytosis, replication and budding**

Since only STBs co-expressed *ACE2* and *TMPRSS2* in the first trimester, we carried out an in-depth characterization of these cells. 14% of the total STB population of the first trimester placenta expressed both *ACE2* and *TMPRSS2*, 52% did not express either, and the rest of the cells expressed either *ACE2* or *TMPRSS2* (**Supplementary Table 3**). We compared the expression profiles of classical STB genes between the *ACE2* and *TMPRSS2* co-expressing cells and *ACE2*- and *TMPRSS2*-negative STBs. We observed that both cell types abundantly expressed the transcripts for human chorionic gonadotropin beta 5 (*CGB5*) and somatomammotropin [placental lactogen (*CSH1*)], as well as steroid hormone biosynthesis enzymes (*HSD17B1* and *CYP19A1*). Further, both these subsets of STBs also abundantly expressed the other putative SARS-CoV-2 S protein primers *FURIN* and Cathepsin B (*CTSB*) (**Supplementary Figure 3**).

We next characterized the transcriptome differences between the *ACE2+TMPRSS2+* versus the *ACE2-TMPRSS2-* STB cells. Pseudo-bulk analysis identified 817 genes (including *ACE2* and *TMPRSS2*) between these two cell types (**Supplementary Table 4**). Of these, 444 were over represented while the others were under represented in the *ACE2+TMPRSS2+* cells as compared to *ACE2-TMPRSS2-* cells. These genes were very heterogeneously expressed in the *ACE2-TMPRSS2-* STBs, while most *ACE2+TMPRSS2+* cells uniformly expressed these genes (**Figure 3A**). The biological processes enriched by these genes included regulation of G1/S checkpoints, actin polymerization/depolymerisation, regulation of mitochondrial membrane permeability and electron-transport-coupled ATP synthesis, monosaccharide transport and unfolded protein response (**Figure 3B**). Most of the *ACE2+TMPRSS2+* cells significantly ($p\text{-value}\leq 0.05$) overexpressed the transcription factor Ovo like Transcriptional Repressor 1 (*OVOL1*) (terminal STB differentiation marker), the glucose transport regulators glypican 3 (*GPC3*) and the glucose transporter 9 (*SLC2A9*). The expression of Actinin Alpha 1 (*ACTN1*) was significantly ($p\text{-value}\leq 0.05$) downregulated in the *ACE2+TMPRSS2+* as compared to *ACE2-TMPRSS2-* STBs (**Figure 3C**).

We next analysed the mRNA levels of 27 genes involved in human ESCRT of viruses and 30 host genes involved in SARS-CoV replication in *ACE2*- and *TMPRSS2*-positive cells in STBs. All the *ACE2+TMPRSS2+* STBs uniformly expressed most of these genes (**Figure 3D**); however, the other cells had a very heterogeneous expression across the different subtypes (**Supplementary Figure 4**). We also analysed the mRNA levels of 332 host proteins that are known to interact with SARS-CoV-2 and found that there was minimal heterogeneity in expression of these genes in the first trimester STBs (**Figure 3D**) as compared to other cell types (**Supplementary Figure 4**).

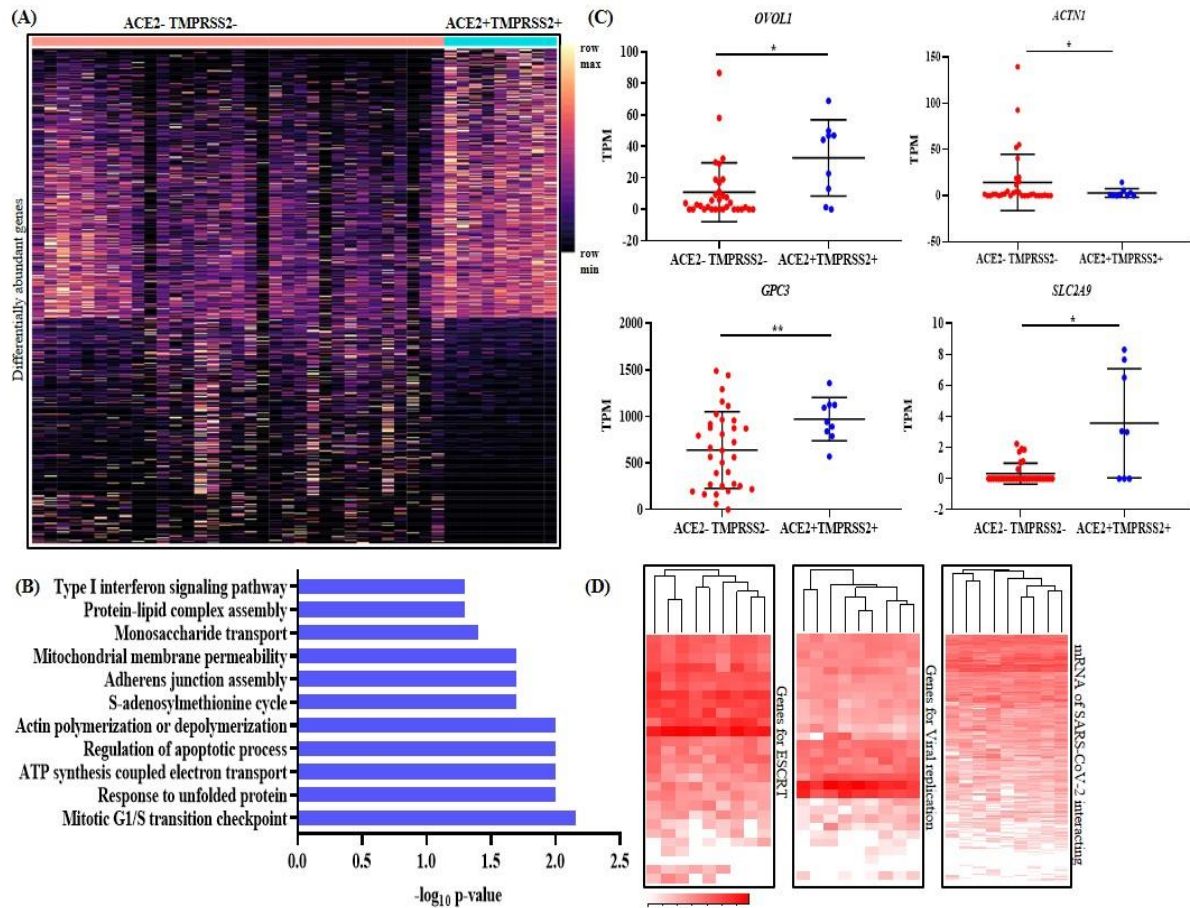


Figure 3: *ACE2*+*TMPRSS2*+ first trimester syncytiotrophoblast cells are terminally differentiated and express the machinery for viral endocytosis, replication and budding

A) Distribution of 817 differentially expressed genes in *ACE2*- and *TMPRSS2*-positive (*ACE2*+*TMPRSS2*+) and *ACE2*- and *TMPRSS2*-negative (*ACE2*- *TMPRSS2*-) cells ($n=33$) of the first trimester syncytiotrophoblast. Rows represent genes and columns represent individual cells, presented on a relative colour scale. **B)** Biological processes enriched in the differentially expressed genes of the first trimester syncytiotrophoblast. The Y axis indicates the enriched biological processes and X axis is the $-\log_{10}$ of the raw p values. **C)** mRNA levels of *OVOL1*, *GPC3*, *SLC2A9* and *ACTN1* in *ACE2*-*TMPRSS2*- and *ACE2*+*TMPRSS2*+ cells. Each dot represents data of a single cell, Y axis represents Transcripts Per Million (TPM). Bars denote mean \pm SD. Horizontal black bars denote significantly different values (* indicates p -value ≤ 0.05 , ** indicates p -value ≤ 0.001). **D)** Heat map showing the expression of genes involved in endosomal sorting complexes required for transport (ESCRT), replication and host genes involved in SARS-CoV-2 interaction. In all heat maps, each row depicts a gene and each column depicts a single *ACE2*- and *TMPRSS2*-positive ($n=9$) (*ACE2*+*TMPRSS2*+) cell in the first trimester syncytiotrophoblast. The data is presented on a relative colour scale in which the minimum and maximum values in each row are used to convert values to colours. Data was extracted from single cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497].

Second trimester *ACE2*+*TMPRSS2*+ cells are invasive extravillous trophoblasts and express markers of endovascular trophoblasts

Amongst the second trimester EVT, 15% of cells were *ACE2*+*TMPRSS2*+ while 33% did not express either of the transcripts (**Supplementary Table 3**). Two populations of second trimester EVT are reported and characterized by the expression of TAC3. Type 1 EVTs are TAC3-high and express genes involved in migration and invasion; type 2 TAC3-low cells

express genes involved in cell proliferation (Liu et al., 2018). In addition to *TAC3*, the type 1 EVTs also express *JAM2*, *SERPENIN1* and *PRG2* (Liu et al., 2018). We observed that the levels of *TAC3* were marginally but not significantly higher in *ACE2+TMPRSS2+* EVTs (**Supplementary Figure 5A**), and the mRNA levels of other genes were identical in *ACE2+TMPRSS2+* EVTs as compared to cells not expressing either of the two genes (*ACE2-TMPRSS2-*) (**Supplementary Figure 5A**). Principal component analysis did not reveal major differences in the transcriptome of the *ACE2+TMPRSS2+* and *ACE2-TMPRSS2-* cells (**Supplementary Figure 5B**). We compared the expression profiles of classical EVT genes between the *ACE2+TMPRSS2+* and *ACE2-TMPRSS2-* cells and observed that both the cell types abundantly expressed the transcripts for *HLA-G* and *ITGB1* (**Supplementary Figure 5C**). Both these subsets of EVTs also abundantly expressed other SARS-CoV-2 S protein primer proteins *FURIN* and *CTSB* of which the levels of *FURIN* were significantly higher ($p\text{-value}\leq 0.05$) in *ACE2+TMPRSS2+* EVTs as compared to *ACE2-TMPRSS2-* EVTs (**Supplementary Figure 5C**).

To characterize if there are any specific classes of genes differentially abundant between the *ACE2+TMPRSS2+* versus the *ACE2-TMPRSS2-* EVT cells, pseudo-bulk analysis was carried out. There were 983 differentially abundant genes (including *ACE2* and *TMPRSS2*) between these two cell types (**Supplementary Table 5**) of which 931 were overrepresented and 52 were underrepresented in the *ACE2+TMPRSS2+* cells as compared to *ACE2-TMPRSS2-* cells. Further, these genes were very heterogeneously expressed in the *ACE2-TMPRSS2-* EVTs while most *ACE2+TMPRSS2+* cells uniformly expressed these genes (**Figure 4A**). Most of these differentially abundant genes enriched GO biological processes including viral entry, release and intracellular transport (**Figure 4B**), and other enriched GO biological processes were nucleic acid replication, epithelial morphogenesis and cell migration (**Figure 4B**).

Of note, the *ACE2+TMPRSS2+* cells significantly ($p\text{-value}\leq 0.05$) overexpressed the markers of endovascular trophoblasts *CDH5*, *VCAM*, *CCR1* and *CD59* (**Figure 4C**). These cells also overexpressed *OVOL2* and *ICAM*, the markers of terminally differentiated EVTs, as well as the invasion-related marker *AKT1* (**Figure 4C**). The increase in the expression of *OVOL2* and *AKT1* was statistically significant ($p\text{-value}\leq 0.05$).

Analysis of the mRNA levels of 27 genes involved in human ESCRT of viruses and 30 host genes involved in SARS-CoV replication in *ACE2-* and *TMPRSS2*-positive cells at single-cell resolution revealed that all the *ACE2+TMPRSS2+* EVTs uniformly expressed most of these genes (**Figure 4D**), while the first trimester EVTs that had no *ACE2+TMPRSS2+* cells had a very heterogeneous expression of these genes (**Supplementary Figure 4**). We also analysed the mRNA levels of 332 host proteins that interact with SARS-CoV-2 and observed that almost all these genes were expressed in most *ACE2+* second trimester EVTs (**Figure 4D**), while the first trimester EVTs had a very heterogeneous expression (**Supplementary Figure 4**).

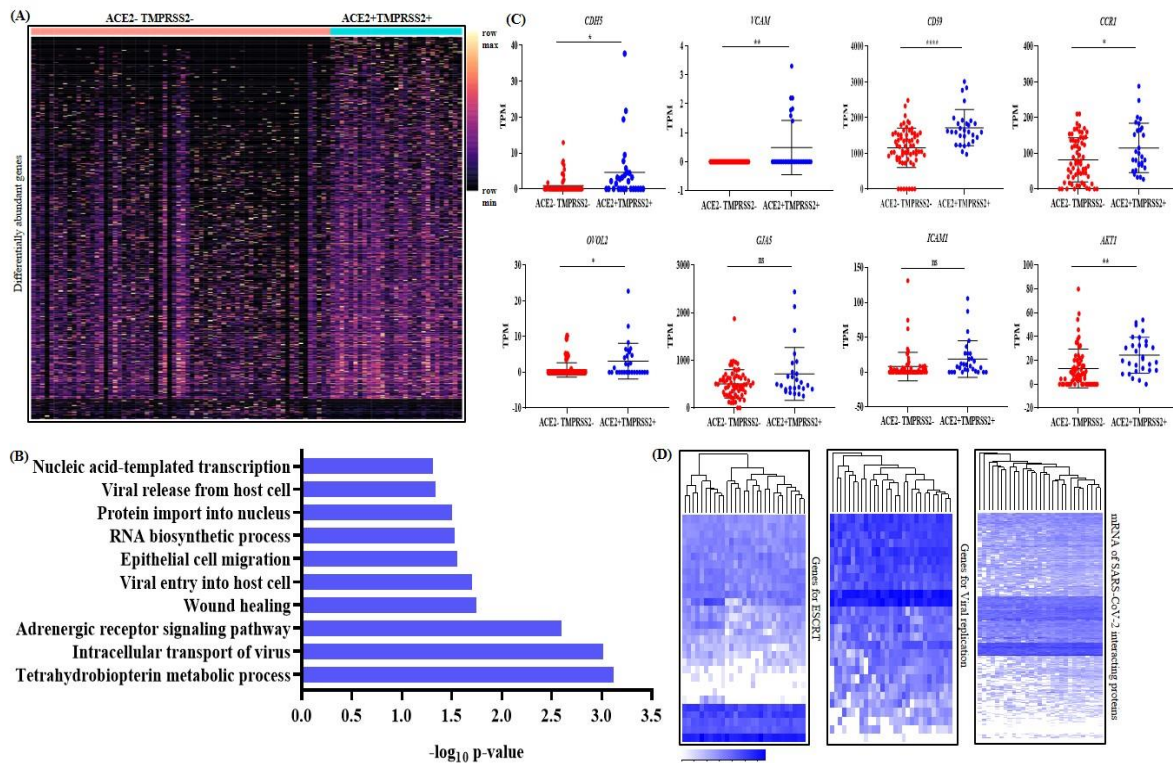


Figure 4: Second trimester *ACE2+TMPRSS2+* cells are invasive extravillous trophoblasts and express markers of endovascular trophoblasts

A) Distribution of 983 differentially expressed genes in *ACE2-* and *TMPRSS2-* positive (n=29) (*ACE2+TMPRSS2+*) and *ACE2-* and *TMPRSS2-* negative (n=66) (*ACE2-TMPRSS2-*) cells of the second trimester extravillous trophoblast. Rows represent genes and columns represent individual cells, presented on a relative colour scale. **B)** Biological processes enriched in the *ACE2+TMPRSS2+* cells (n=29) of the second trimester extravillous trophoblast. The Y axis indicates the enriched biological processes and X axis is the enrichment score. **C)** Comparison of mRNA level of *CDH5*, *VCAM*, *CCR1*, *CD59*, *OVOL2*, *ICAM* and *AKT1* in *ACE2-TMPRSS2-* (n=66) and *ACE2+TMPRSS2+* (n=29) cells. Each dot represents data of a single cell, the Y axis represents Transcripts Per Million (TPM). Bars denote mean \pm SD. Horizontal black bars denote significantly different values (* indicates $p\text{-value} \leq 0.05$, ** indicates $p\text{-value} \leq 0.001$). **D)** Heat map showing the expression of genes involved in endosomal sorting complexes required for transport (ESCRT), replication and host genes involved in SARS-CoV-2 interaction. In all heat maps, each row depicts a gene and each column depicts a single *ACE2+TMPRSS2+* cell of the second trimester extravillous trophoblast. The data is presented on a relative colour scale in which the minimum and maximum values in each row are used to convert values to colours. Data was extracted from single cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497].

Unique signatures of genes involved in viral response in first trimester STB and second trimester EVT

We studied the baseline expression of 487 genes involved in viral response in both first trimester STBs and second trimester EVTs (**Supplementary Table 1**). Only a subset of viral response genes was expressed in both EVTs and STBs irrespective of *ACE2* and *TMPRSS2* status (**Figure 5A**). The heatmaps showed minimal heterogeneity across cells but high variability in expression across genes involved in the viral response. To characterize these genes in EVT and STB cells, genes were clustered by a hierarchical clustering method using the “hclust” function available in R. Genes were grouped based on the most optimum threshold,

which resulted in four different gene clusters (**Figure 5B**). Further, the mean expression of each gene cluster was estimated and checked across EVT and STB cells of which the mean expression of genes in Clusters 1 and 2 were identical in both EVT and STBs. However, genes in Clusters 3 and 4 were more abundant in EVTs as compared to STBs (**Figure 5C**).

Next, GO analysis was performed using the PANTHER database for all the four gene clusters. For each cluster, an over-representation test was performed using reference genes of PANTHER pathways, and a pathway with the highest fold-enrichment value was selected as the enriched pathway for a given gene cluster. GO classification of these revealed that most of the genes in Cluster 1 had a role in the Toll-like receptor (TLR) signalling response and the genes in Cluster 2 had a role in apoptosis. Additionally, genes of the JAK-STAT pathway and axon guidance mediated by semaphorins were enriched in EVTs as compared to STBs ($p\text{-value} \leq 0.05$) (**Figure 5D**).

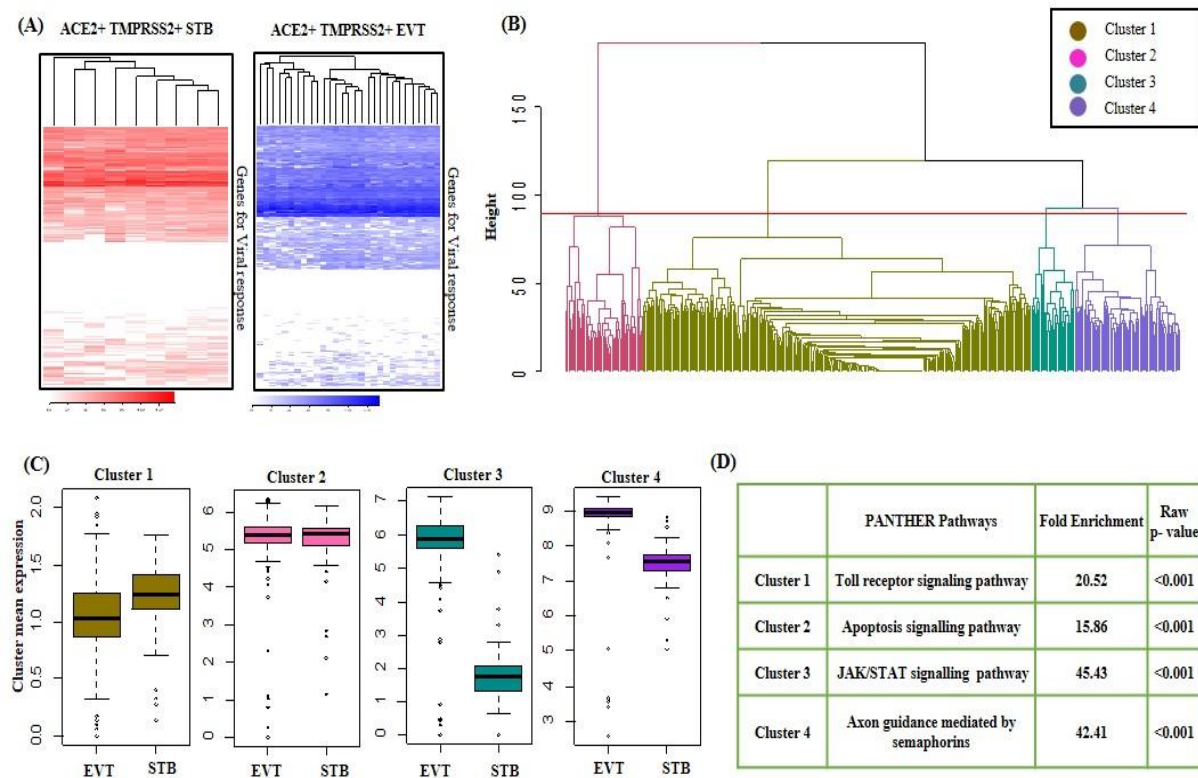


Figure 5: Viral response genes in first trimester syncytiotrophoblasts and second trimester extravillous trophoblasts

A) Heat map showing the expression of genes involved in viral response in *ACE2*- and *TMPRSS2*-positive (*ACE2*+*TMPRSS2*+) cells of first trimester STB (n=9) and second trimester EVT (n=29). Each row is a gene and each column represents data for a single *ACE2* and *TMPRSS2* co-expressing cell. **B)** Cluster dendrogram of first trimester STB and second trimester EVT cells. The horizontal axis of the dendrogram represents the distance or dissimilarity between clusters. The vertical axis represents the clusters. **C)** Mean of four clusters of upregulated genes in first trimester STB and second trimester EVT cells. Y axis represents cluster mean expression and X axis represents different cell types. **D)** Pathways enriched in the in four clusters of first trimester STBs and second trimester EVTs, with their enrichment score and p-values. EVT =, STB = Syncytiotrophoblast. Data was extracted from single cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497].

Discussion

Herein, we utilized scRNA-seq to identify and characterize the potential cellular targets of SARS-CoV-2 infection in human placenta. To review the data presented: 1) The SARS-CoV-2 binding receptor *ACE2* and the S protein priming protease *TMPRSS2* are co-expressed by a subset of syncytiotrophoblasts (STB) in the first trimester and extravillous trophoblasts (EVT) in the second trimester human placenta 2) These *ACE2*+*TMPRSS2*+ subsets are highly differentiated STBs and endovascular EVTs 3) The *ACE2*+*TMPRSS2*+ placental subsets readily express mRNA for proteins involved in ESCRT of viruses and replication as well as transcripts for proteins that are known to interact with SARS-CoV-2 structural and non-structural proteins 4) STBs and EVTs differentially express genes involved in the host response to viral infection

Using a scRNA-seq dataset of first trimester human feto-maternal interface (Vento-Tormo et al., 2018), a recent study reported the presence of *ACE2*-positive villous cytotrophoblasts and syncytiotrophoblasts but not the extravillous trophoblasts of first trimester human placenta (Li et al., 2020a). Herein using a different dataset (Liu et al., 2018), we corroborated these findings and show the presence of *ACE2* in cytotrophoblast and syncytiotrophoblast cells of first trimester placenta. However, a low-abundance subset of *ACE2*+ extravillous trophoblasts was additionally identified in the present study. Differences in methods of tissue sampling, cell isolation and inefficiencies in detection of low-abundance transcripts in scRNA-seq can underestimate the actual frequencies of *ACE2*+ cells in a given tissue. In addition to *ACE2* (the SARS CoV and SARS-COV-2 receptor) we also detected expression of *DPP4* (the receptor for MERS-CoV) and *ANPEP* (the receptor for CoV-229E) in the cells of the placenta. Like *ACE2*, *DPP4* was detected in all the cell types of the first trimester placenta and also in EVTs of second trimester, very few STBs expressed *ANPEP* suggesting different cell types could be targets of different corona viruses in the developing placenta.

While *ACE2* is the primary receptor for SARS-CoV-2 entry, the S protein of SARS-CoV-2 undergoes cleavage by the cell surface protease *TMPRSS2* (Hoffmann et al., 2020). Whether *ACE2* and *TMPRSS2* are required on the same cell to activate SARS-CoV-2 S-protein to invade *ACE2* single-positive cells is a matter of investigation. However, as active S protein has a finite lifetime (Shulla et al., 2011), its processing at the plasma membrane will make it most effective for viral entry. Thus, we assumed that for SARS-CoV-2, the *ACE2* and *TMPRSS2* co-expressing cells would have the highest infectivity. Our analysis revealed a proportion of STBs (14%) in the first trimester and a subset of EVTs (15%) in second trimester human placenta co-express *ACE2* and *TMPRSS2*. While these numbers may appear insignificant considering the total placental volume, it must be borne in mind that only 3-6% of lung airway epithelial cell subtypes (the primary site of SARS-CoV-2 action) express both *ACE2* and *TMPRSS2* (Ziegler et al., 2020). Therefore, we propose that only the first trimester STBs and second trimester EVTs are likely to be potential common entry routes for SARS-CoV-2 in the placenta.

In addition to *ACE2*-expressing STBs and EVTs, our study revealed that *BSG/CD147*, the alternate receptor for SARS-CoV-2 (Wang et al., 2020), is expressed by almost all the placental cells. Presently, the mechanism by which *BSG/CD147* mediates viral entry in host cells is

unknown. In other cells, BSG/CD147 promotes entry of viruses by endocytosis (Pushkarsky et al., 2001). It is possible that the same mechanism may be operative in the case of SARS-CoV-2. In this context, it is especially interesting that all the *BSG/CD147*-positive cells co-express the endosomal protease *CTSL*, indicating that both *ACE2*- and *BSG/CD147*- mediated viral entry may be operative in placental cells. Further, we observed that almost all the *ACE2*+ STBs and EVT_s co-expressed *BSG/CD147*, suggesting that more than one mechanism may operate for viral entry in these cells of the human placenta. Beyond *TMPRSS2*, studies have identified that SARS-CoV-2 may have a *FURIN* cleavage site, leading to a broader set of host proteases that could mediate S-protein priming (Coutard et al., 2020). The *ACE2*+*TMPRSS2*+ STBs and EVT_s abundantly express *FURIN* as well as another endosomal protease, *CTSB*. Together our data conclusively show that multiple cells of human placenta are targets for SARS-CoV-2 binding and entry by S protein priming.

We next characterized the placental cells that are potential targets for SARS-CoV-2 infection. As the *ACE2*-mediated viral entry is a well-established mechanism, we focused only on *ACE2*+*TMPRSS2*+ cells in the placenta. In the developing placenta, the trophoblast stem cells differentiate into cytotrophoblasts, which undergo further differentiation to form the non-self-renewing cytotrophoblasts, extravillous trophoblasts and syncytiotrophoblasts (Turco and Moffett, 2019; Hemberger et al., 2020). The syncytiotrophoblasts covering the villi are major hormone secreting cells and function as a protective immunological barrier (Maltepe and Fisher, 2015; Gupta et al., 2016; Liu et al., 2018; Vento-Tormo et al., 2018; Turco and Moffett, 2019). We observed that the STBs that co-express both *ACE2* and *TMPRSS2* also express the mRNA for the peptide hormones and enzymes for steroid hormone biosynthesis. However, their levels are not significantly different from the *ACE2*-*TMPRSS2*- counterparts, suggesting that both these cell types retain the basic functions of STBs. However, pseudo-bulk analysis revealed that the *ACE2*+*TMPRSS2*+ cells are enriched for genes involved in cell cycle checkpoints, actin filament remodelling, mitochondrial functions, hexose transport and type I interferon signalling. Indeed, the terminally differentiated STBs have replicative senescence and require extensive cytoskeletal remodelling for syncytialization; the mitochondria of STBs play a key role in progesterone synthesis by providing cholesterol (Martinez et al., 2015). Additionally, these cells are enriched in *OVOL1*, the transcription factor required for STB specification, as well as proteins involved in glucose transport across the feto-maternal barrier, a key function of well differentiated STBs (Jansson and Ylve, 2002; Renaud et al., 2015; Vento-Tormo et al., 2018; Turco and Moffett, 2019). These results imply that the *ACE2*+*TMPRSS2*+ cells are a subset of highly differentiated STBs and these cells are potential target for viral entry. What could be the consequence of viral infection on STBs is yet to be demonstrated, increased syncytiotrophoblastic knots are observed in placenta from pregnant women with COVID-19 (Chen et al., 2020), which is suggestive of injury to the STBs in the placenta.

We next probed the second trimester EVT_s, 15% of which co-express *ACE2* and *TMPRSS2*. The EVT_s differentiate from cytotrophoblast stem cells and populate the tips of the placental villi to form the anchoring villi, thus defining the boundary between mother and fetus. The EVT_s are central to placentation as they invade into maternal decidua and are involved in remodelling of maternal spiral arteries, veins and lymphatics (Sharma et al., 2016; Pollheimer

et al., 2018). We observed that the *ACE2+TMPRSS2+* cells abundantly express the classical EVT marker *ITGB1* and also *HLA-G* that induces tolerogenic immune responses leading to acceptance of the semi-allogeneic. Two kinds of EVTs are reported in the second trimester human placenta: the proliferative EVTs in the cell columns and the invasive endovascular or interstitial EVTs (Pollheimer et al., 2018; Turco and Moffett, 2019) and both these have a unique transcript signature (Liu et al., 2018). Herein, we observed that while the levels of *TAC3* and other molecules associated with columnar versus invasive EVTs are not significantly different between *ACE2+TMPRSS2+* and *ACE2-TMPRSS2-* second trimester EVTs, the double-positive cells overexpressed key invasive EVT markers *OVOL2*, *GJA5*, *ICAM* and *AKT1* (Sharma et al., 2016; Bai et al., 2018; Liu et al., 2018; Jeyarajah et al., 2020), suggesting that these cells are invasive trophoblasts. We further observed that many of the *ACE2+TMPRSS2+* cells were enriched in genes having a role in cell migration. The EVTs can either invade in to the decidua (called as interstitial EVTs) or remodel the spiral arteries (termed as endovascular EVTs). While both these EVTs are invasive in nature, they have differential expression of certain marker genes. For e.g. the endovascular EVTs overexpress the vascular endothelial cadherin (*CDH5*) and vascular cell adhesion molecule-1 (*VCAM-1*), they also have higher expression of *CCR1* and *CD59* (Bulla et al., 2005; Cartwright and Balarajah, 2005; Liu et al., 2018; Ueda et al., 2019; Sato, 2020). Intriguingly, the *ACE2+TMPRSS2+* cells also overexpressed several of the key endovascular EVT markers indicating that the *ACE2+TMPRSS2+* population of second trimester EVTs are potentially endovascular trophoblasts and are targets of SARS-CoV-2 infection. Indeed, pseudo-bulk analysis of the *ACE2+TMPRSS2+* cells and *ACE2-TMPRSS2-* cells revealed significant enrichment of genes with GO terms involving regulation of viral release from host cells. Most of the *ACE2+TMPRSS2+* endovascular EVTs abundantly expressed most genes whose protein products in the host are known to be involved in human endocytosis and budding of viruses and replication. Together, this data shows that SARS-CoV-2 may affect the invading EVTs at the fetomaternal interface in the second trimester and can result in damaged vasculature. In this context, it is important to note that the maternal endothelial cells in the decidua also express *ACE2* and *BSG*, making the maternal endothelium another entry point of SARS-CoV-2 infection at the fetomaternal interface; any damage to these cells can cause placental damage and vertical transmission of the virus. Indeed, increased fibrin deposition, chorionic haemangioma and massive placental infarction indicative of vasculopathy are observed in term placenta of women infected with SARS-CoV. An increase in intervillous and subchorionic fibrin and fetal thrombotic vasculopathy with zones of avascular fibrotic villi are also reported in placenta of women infected with coronaviruses including SARS CoV-2 (Ng et al., 2006; Baud et al., 2020; Mulvey et al., 2020). Together, these results indicate that the integrity of endovascular trophoblasts and the endothelial compartment of the fetomaternal interface may be compromised in women with SARS-CoV-2 infection.

Once the virus binds to their receptors on host cells and gains an entry it utilizes a plethora of host genes for its replication. Post replication, the enveloped viruses complete their cycle by forming vesicles that bud from plasma membrane via the cellular ESCRT (endosomal sorting complexes required for transport) machinery. Interestingly, we observed that the *ACE2+TMPRSS2+* STBs and EVTs were enriched for the key genes that encode for proteins

involved in ESCRT and viral replication. Using affinity-purification mass spectrometry 332 human proteins that interact with SARS-CoV-2 have been identified and many of these play a role in ESCRT and viral replication (Gordon et al., 2020). We observed that most *ACE2+TMPRSS2+* STBs and EVTs abundantly expressed most of these genes. Thus SARS-CoV-2 would hijack proteins in the EVTs and STBs thereby interfering with normal placental functions. In this context it is important to highlight that a significant proportion of the human–SARS-CoV-2 interacting proteins also interact with proteins of other viruses including Zika and HCV which replicate in the trophoblast cells (Giugliano et al., 2015; Tabata et al., 2016, 2018; Gordon et al., 2020). Together our data strongly imply the first trimester STBs and second trimester EVTs are not just targets for SARS-CoV-2 entry but the virus would also be potentially pathogenic to these cells.

A proportion of SARS-CoV-2 proteins target the members of the innate immune signalling pathway including the NF-kappa-B (Gordon et al., 2020). We decided to probe this in further details by profiling the STBs and EVTs for 487 genes whose protein products are involved in viral response in host cells. We observe that only a proportion of the genes are expressed in most EVTs and STBs. Based on their expression levels in the host cells, they could be classified in four clusters and interestingly, the first trimester STBs and the second trimester EVTs expressed genes in the TLR signalling pathway, the primary response to viral infection. Previous studies in SARS-CoV have identified involvement of TLR pathways in protection against viral response (Dosch et al., 2009; Totura et al., 2015). However, we found that the genes in the JAK-STAT pathway were overexpressed in the EVTs but not STBs. This is however not surprising as the JAK-STAT pathway is required for physiological function of EVTs mainly invasion (Fitzgerald et al., 2010; Suman et al., 2013; Sharma et al., 2016; Godbole et al., 2017). However, the JAK-STAT pathway is also central for mounting a host response to viral infection and treatment with interferon gamma induces the expression of interferon-stimulated genes in EVT cells (Verma et al., 2020). Thus, EVTs are not just the entry sites for SARS-CoV-2 infection, but they also have the cellular machinery to mount an inflammatory response towards an infection. However, with regards to coronaviruses including SARS-CoV-2 there is an overexuberant inflammatory response even at lower viral loads which contributes to the viral pathogenicity in the lungs (Liao et al., 2020). Further, the ACE2 receptors are induced by the interferon signalling at least in the lung cells (Ziegler et al., 2020) thereby amplifying the infection cycle in the host tissues. Whether a similar mechanism is operative in the placental cells is under investigation, the heightened baseline expression of the JAK-STAT pathway genes in the EVTs itself could readily lead to placental inflammation which may be detrimental to pregnancy. Infiltration of leucocytes and chorioamnionitis is observed in placenta from women with COVID-19 (Ng et al., 2006; Baud et al., 2020). Since inflammation of the fetomaternal interface causes preterm births (Silasi et al., 2015; Surve et al., 2016), it is plausible that the increased incidence of preterm delivery in women with COVID-19 could be linked to this process.

Beyond preterm births, the demonstration that the *ACE+TMPRSS2+* subpopulation of EVTs consists of invasive endovascular trophoblasts is clinically relevant in conditions like preeclampsia. The invasion of the trophoblast cells and remodelling of the spiral arteries deep into the myometrium is essential for normal fetal growth and development (Norwitz, 2006;

Soares et al., 2015). If the arteries are not sufficiently remodelled, there is disordered perfusion of blood and an inadequate supply of nutrients and oxygen, resulting in fetal growth restriction, stillbirth, preeclampsia, placental abruption and preterm labor (Brosens et al., 2019). Since SARS-CoV-2 and other coronaviruses may target the endovascular trophoblasts, it is plausible that the infection could lead to other adverse pregnancy outcomes. Indeed, preeclampsia, preterm labor, fetal distress and premature rupture of membranes are reported in pregnant women infected with SARS-CoV-2 in the third trimester (Gajbhiye et al., 2020). For other human coronavirus infections also high rates of miscarriages, preterm birth and premature rupture of membranes have been reported (Alfaraj et al., 2019; Mullins et al., 2020). These observations imply that SARS-CoV-2 infection is detrimental to pregnancy due to the possible infection of placenta cells such as EVT's.

Although our studies demonstrate the presence of SARS-CoV-2 receptors in the placenta, viral mRNA is not reported in placenta of most babies born to mothers with COVID-19 in the third trimester (Gajbhiye et al., 2020; Mulvey et al., 2020). While inadequate sampling of the cells during sample collection, site of sampling and sensitivity of the methods used can underestimate the presence of virus in tissues, it must also be borne in mind that most of the infections have occurred in the late third trimester, often just before delivery and when the viral loads are unknown. Nevertheless, vertical transmission of the virus from mother to child is observed in 8% of cases (Gajbhiye et al., 2020), presence of viral RNA in amniotic fluid and placenta of a mother infected with SARS-CoV-2 are reported (Baud et al., 2020; Zamaniyan et al., 2020). These observations point towards the possibility of placental damage resulting in the breakthrough of SARS-CoV-2 infections. However, more cases should be investigated before reaching a final conclusion.

To summarise, herein we exploited the power of scRNA-seq and identified the cellular basis of SARS-CoV-2 infection in the human placenta. This data provides crucial insight regarding where the genes required for viral endocytosis, replication, budding and response were expressed amongst specific subsets of placental cells. It will now be essential to determine how SARS-CoV-2 infection alters the temporal dynamics of host responses at the single-cell resolution. Given the complexities of host-pathogen interactions in SARS-CoV-2 infections, a deep understanding of the full spectrum of mechanisms operative in different tissues will be required to combat this pandemic. Our results provide a basic framework in understanding of the paraphernalia involved in SARS-CoV-2 infections in pregnancy. We believe that this work will also aid in developing rational strategies for management of COVID-19 and other coronavirus infections in pregnancy.

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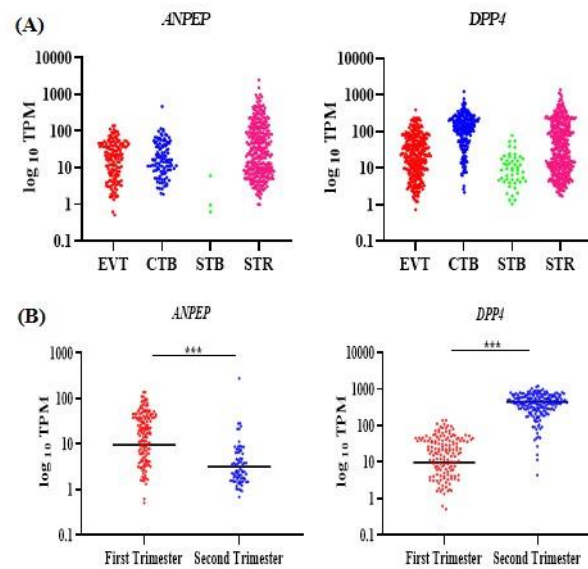
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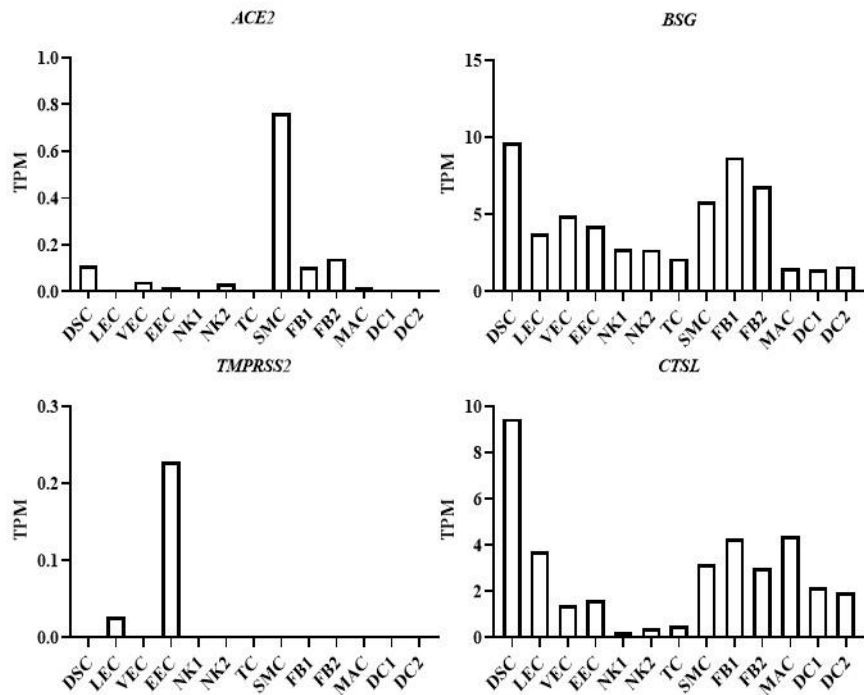
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Supplementary data



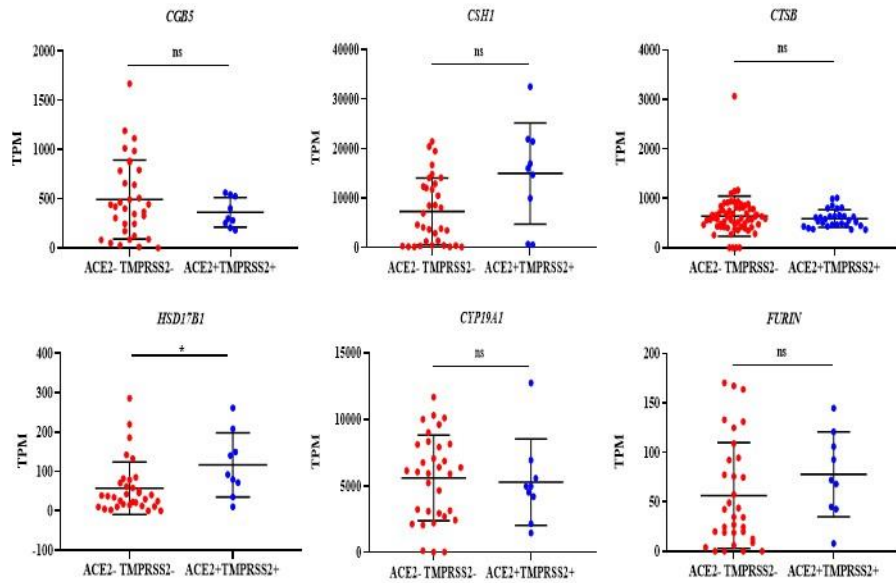
Supplementary Figure 1: Expression of the receptors for the Middle East respiratory syndrome coronavirus (MERS-CoV) (*DPP4*) and CoV-229E receptor (*ANPEP*) in human placenta

A) mRNA level of *DPP4* and *ANPEP* in different cell types of first trimester human placental cells [EVT (n=440), CTB (n=248), STB (n=64), STR (n= 615)]. **B)** Comparison of the levels of *DPP4* and *ANPEP* mRNA in EVT of first (n=440 cells) and second (n=200 cells) trimester human placenta. Each dot represents data of a single cell, Y axis represents \log_{10} Transcripts Per Million (TPM). Bars within the data points denote the mean. Horizontal black bars denote significantly different values (**** indicates p-value ≤ 0.001). Data was extracted from single cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497]. EVT = Extravillous Trophoblast, CTB = Cytotrophoblast, STB = Syncytiotrophoblast, STR = Villous Stromal cell.



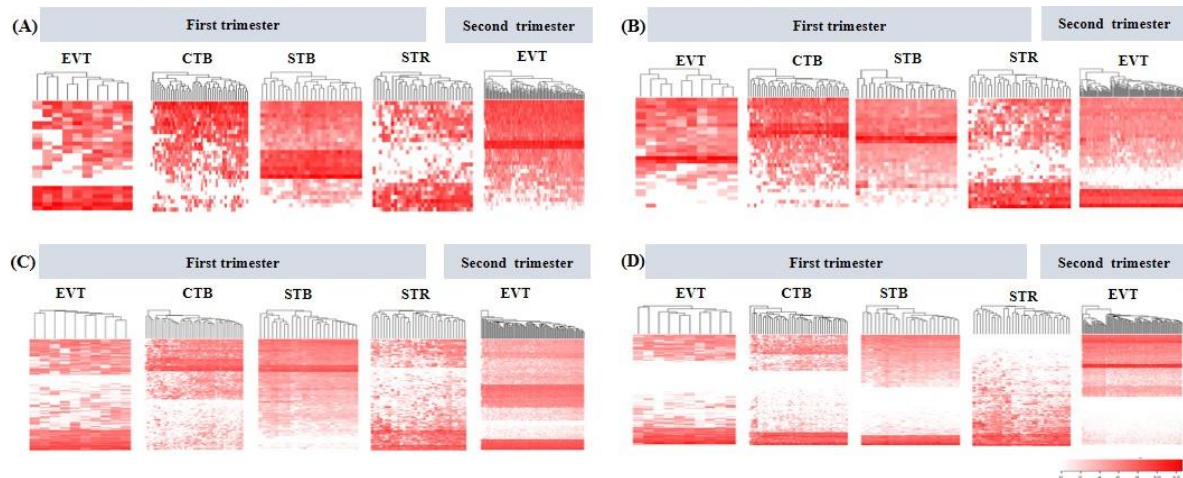
Supplementary Figure 2: Expression of SARS-CoV-2 receptors and spike protein primers in different cell types of maternal endometrium of first trimester

mRNA level of SARS-CoV-2 receptors (*ACE2* and *BSG*) and spike protein primer enzymes (*TMPRSS2* and *CTSL*) in different cell types of first trimester maternal endometrium. X axis represents the different endometrial cell types. Y axis represents average gene expression profiles in Transcripts Per Kilobase Million (TPM) for individual cell types of endometrium. Each bar represents the average TPM value of SARS-CoV-2 receptor (*ACE2* and *BSG*) and spike protein processing enzymes (*TMPRSS2* and *CTSL*) in endometrial cell types. All data was extracted from single-cell RNA-seq of endometrium cell types (Suryawanshi et al 2018). DSC = decidualized stromal cells, LEC = lymphatic endothelial cells, VEC = vascular endothelial cells, EEC = endometrial epithelial cells, NK1 & NK2 = natural killer cells, TC = T cell, SMC = smooth muscle cells, FB1 & FB2 = decidual fibroblast populations, MAC = macrophages, DC1 & DC2 = dendritic cells.



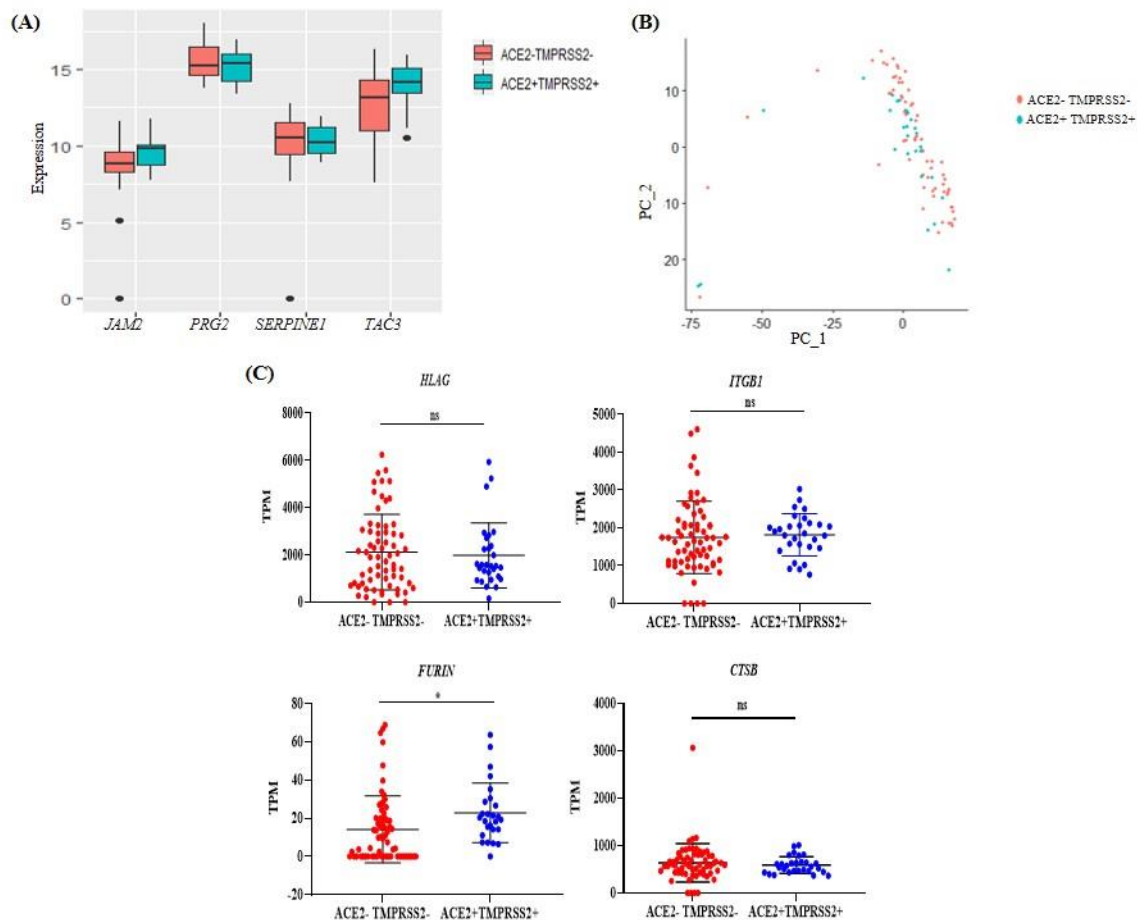
Supplementary Figure 3: *ACE2*+*TMPRSS2*+ STBs are terminally differentiated and express SARS-CoV-2 S protein primers *FURIN* and *CTSB*

Comparison of mRNA level of *CGB5*, *CSH1*, *HSD17B1*, *CYP19A1*, *FURIN* and *CTSB* in *ACE2*- and *TMPRSS2*-negative (n=33) (*ACE2*-*TMPRSS2*-) and *ACE2*- and *TMPRSS2*-positive (n=9) (*ACE2*+*TMPRSS2*+) cells. Each dot represents data of a single cell, Y axis represents Transcripts Per Million (TPM). Bars denote mean ± SD. Horizontal black bars denote significantly different values (* indicates p value ≤ 0.05). Data was extracted from single cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497].



Supplementary Figure 4: Expression of genes involved in life cycle of virus in *ACE2* and *TMPRSS2* co-expressing cells of human placenta.

A) Heat map showing the expression of genes involved in endosomal sorting complexes required for transport (ESCRT) in first trimester EVT, CTB, STB, STR and second trimester EVT. **B)** Heat map showing the expression of viral replication genes in first trimester EVT, CTB, STB, STR and second trimester EVT. **C)** Heat map showing the expression of host genes involved in SARS-CoV-2 interaction in first trimester EVT, CTB, STB, STR and second trimester EVT. **D)** Heat map showing the expression of genes involved in viral response during first trimester in EVT, CTB, STB, STR and second trimester EVT. In all heat maps each row is a gene and each column represent a single cell. The data is presented on a relative colour scale in which the minimum and maximum values in each row are used to convert values to colours. Data was extracted from single-cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497]. EVT = Extravillous Trophoblast, CTB = Cytotrophoblast, STB = Syncytiotrophoblast, STR = Villous Stromal cell.



Supplementary Fig 5: *ACE2+TMPRSS2+* cells in the second trimester placenta are well differentiated EVTs and express *FURIN* and *CTSB*.

A) Box-and-whisker plot shows comparison of mRNA levels of *TAC3*, *JAM2*, *PRG2* and *SERPINE1* genes in *ACE2*- and *TMPRSS2*-positive (*ACE2+TMPRSS2+*) and *ACE2*- and *TMPRSS2*-negative (*ACE2-TMPRSS2-*) second trimester EVTs. Y-axis represents expression values and X-axis represents different genes. Bars denote mean \pm SD. **B)** Principle component analysis (PCA) of transcriptome of *ACE2*- and *TMPRSS2*-positive (*ACE2+TMPRSS2+*) and *ACE2*- and *TMPRSS2*-negative (*ACE2-TMPRSS2-*) cells of EVT. **C)** Comparison of mRNA levels of *HLAG*, *ITGB1*, *FURIN* and *CTSB* in *ACE2*- and *TMPRSS2*-negative (n=66) (*ACE2-TMPRSS2-*) and *ACE2*- and *TMPRSS2*-positive (n=29) (*ACE2+TMPRSS2+*) cells. Each dot represents data of a single cell, the Y axis represents Transcripts Per Million (TPM). Bars denote mean \pm SD. Horizontal black bars denote significantly different values (* indicates p-value ≤ 0.05). Data was extracted from single-cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497]. EVT = Extravillous Trophoblast.

Supplementary Table 1 Excel sheet for the list of genes in viral endocytosis, ESCRT, viral replication, SARS-CoV-2 interactions and viral response.

Cell type	Total cells	ACE2	BSG	TMPRSS2	CTSL
First trimester EVT	440	10 (2%)	439 (99%)	8 (2%)	440 (100%)
Second trimester EVT	200	125 (62%)	195 (97%)	38(19%)	199 (99%)
STB	64	25 (39%)	64 (100%)	15(23%)	64 (100%)
CTB	248	45 (18 %)	244 (98%)	2 (1%)	247 (99%)
STR	615	33 (5%)	593 (96%)	13 (2%)	591 (96%)

Supplementary Table 2: Percentage of cells expressing *ACE2*, *BSG*, *TMPRSS2*, *CTSL* mRNA in human placenta. EVT = Extravillous Trophoblast, CTB = Cytotrophoblast, STB = Syncytiotrophoblast, STR= Villous Stromal Cell. Data was extracted from single-cell RNA-seq of human placenta (Liu et al 2018) [Accession number GSE89497].

Cell type	Total cells	ACE2+ BSG+	ACE2+ BSG-	ACE2+ TMPRSS2 +	ACE2+ TMPRSS2-	ACE2+ CTSL+	CTSL+ BSG+	ACE2- TMPRSS2-
First trimester EVT	440	10 (2%)	0	0	10 (2%)	10 (2%)	439 (99%)	422 (96%)
Second trimester EVT	200	125 (62%)	0	29 (15%)	96 (48%)	125 (62%)	195 (98%)	66 (33%)
STB	64	25 (39%)	0	9 (14%)	16 (25%)	25 (39%)	64 (100%)	33 (52%)
CTB	248	45 (18%)	0	0	45 (18%)	45 (18%)	244 (98%)	201 (81%)
STR	615	33 (5%)	0	1 (0.1%)	32 (5%)	33 (5%)	576 (94%)	570 (93%)

Supplementary Table 3: Percentage of cells co-expressing SARS-CoV-2 receptors (*ACE2* and *BSG2*) and its spike protein processing enzymes (*TMPRSS2* and *CTSL*). EVT = Extravillous Trophoblast, CTB = Cytotrophoblast, STB = Syncytiotrophoblast, STR= Villous Stromal cell.

Supplementary Table 4: Excel sheet for the list of differentially abundant genes in *ACE2+ TMPRSS2+* and *ACE2+ TMPRSS2-* cells of first trimester STB. Data is from Liu et al 2018 (Accession number GSE89497).

Supplementary Table 5: Excel sheet for the list of differentially abundant genes in *ACE2+ TMPRSS2+* and *ACE2+ TMPRSS2-* cells of second trimester EVT. Data is from Liu et al 2018 (Accession number GSE89497).