Single-cell RNA expression profiling of ACE2 and TMPRSS2 in the human trophectoderm and placenta

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Contribution:

What are the novel findings of this work?

Our study has demonstrated that the angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) are expressed in human trophectoderm (TE) and placentas throughout the three trimesters of pregnancy.

What are the clinical implications of this work?

Our study revealed the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) target cells in human TE and placentas, suggesting that pregnancies complicated with novel coronavirus disease 2019 (COVID-19) are potentially at risk of

intrauterine fetal SARS-CoV-2 infection of or placental insufficiency, which might lead to fetal growth restriction and even fetal loss.

Abstract

Objectives

The objective of this study was to examine the characteristics and distributions of possible SARS-CoV-2 target cells in the human TE and placenta.

Methods

Single-cell transcriptomic datasets of the early TE as well as the first- and second-trimester placentas have been reported[1,2]. Here, we conducted the transcriptomic analysis of 4198 early TE cells, 1260 first-trimester placental cells and 189 EVTs at 24-week placentas (EVT_24W) by SMART-Seq2 method. Immunohistochemical staining of the human first-, second- and third- trimester placentas was performed to confirm bioinformatic results.

Results

Via bioinformatic analysis, we identified the existence of ACE2 and TMPRSS2 expression in human TE as well as in the first- and second- trimester placentas. In human TE data, 54.4% of TE1 cells, 9.0% of CTBs, 3.2% of EVTs and 29.5% of STBs were ACE2 positive. As for TMPRSS2, 90.7% of TE1 cells, 31.5% of CTBs, 22.1% of EVTs

and 70.8% of STBs were TMPRSS2 positive. Amongst the placental cells, 20.4% of CTBs, 44.1% of STBs, 3.4% of EVT_8W and 63% of EVT_24W were ACE2 positive. And 1.6% of CTBs, 26.5% of STBs, 1.9% of EVT_8W and 20.1% of EVT_24W were TMPRSS2 positive. Pathway analysis revealed associations to morphogenesis of branching structure, extracellular matrix interaction, oxygen binding and antioxidant activity in ACE2+TMPRSS2+ EVT_24W cells. The ACE2+TMPRSS2+ TE1 cells were correlated with an increased capacity of viral invasion, epithelial cell proliferation and cell adhesion. Based on immunohistochemical results, expression level of ACE2 and TMPRSS2 in first- and second- and third-trimester placentas was observed.

Conclusions

Our study has demonstrated the presence of ACE2 and TMPRSS2 positive cells in the human TE and placentas at different stages of pregnancy, which indicates the possibility that the SARS-CoV-2 could spread via the placenta and cause intrauterine fetal infection.

Introduction

The COVID-19, caused by SARS-CoV-2, has become a global health crisis[3]. At present, there is much controversy relating to the possibility of vertical transmission of SARS-CoV-2. In addition, there is limited data on whether SARS-CoV-2 could cause fetal infection, congenital malformation, fetal growth restriction and even fetal loss. High quality evidence is needed to address these issues.

The placenta is the interface between the mother and the fetus, mediating protection for the fetus against various infections[4]. Chorionic villi serve as functional units of the placenta and contain three types of trophoblasts: syncytiotrophoblasts (STBs), extravillous trophoblasts (EVTs) and cytotrophoblasts (CTBs). The STBs form the outer layer of the trophoblasts and establish an interface between maternal blood and embryonic extracellular fluid, facilitating exchange of materials between the mother and the fetus. The EVTs form cell columns at the base of the stem villi. The CTBs line the inside of the STBs and function as trophoblastic stem cells. When maternal infection occurs, the STBs usually serve as barrier against pathogen invasion. Unlike the STBs, the EVTs represent a possible route through which pathogens can breach the placental barrier and cause vertical transmission[5,6].

Recent studies have indicated that ACE2 serves as the putative surface receptor of sensitive cells for SARS-CoV-2, which employs downstream serine protease TMPRSS2 for S protein priming, enabling viral invasion into host cells[7–11]. Cysteine proteases, such as cathepsin B (CTSB) and cathepsin L (CTSL), have also been demonstrated to facilitate coronavirus entry into certain cell lines, such as Ebola virus, SARS- and MERS-coronaviruses[12].

In the recent years, the development of single-cell RNA-seq (scRNA-seq) technology has offered tremendous transcriptomic information at unprecedented resolution. To better understand the possibility of vertical transmission of SARS-CoV-2, with the analysis of previously published single-cell transcriptomic profiles, we examined the characteristics and distributions of possible SARS-CoV-2 target cells in human TE and placenta.

Material and Methods

Patient recruitment and sample collection

This study is composed of the scRNA-seq analysis part and the immunohistochemical staining part. Women were prospectively recruited for the component of immunohistochemical study. Three healthy pregnant women from each trimester, with a total of nine, were recruited to the study and informed consent were provided. Placentas of the following gestational age were collected: 8 weeks (n=3), 17 weeks (n=1), 18 weeks (n=1), 24 weeks (n=1), 38 weeks, (n=1) and 39 weeks (n=2). After delivery, the placentas were placed on ice and sent to the laboratory of State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. For the second- and third-trimester cases, placentas with decidua around the center (maternal side) were cut into 0.5 * 0.5 * 0.5 cm pieces and fixed with 4% paraformaldehyde overnight at 4℃ before paraffin section.

Preprocessing of the scRNA-seq data

For the bioinformatics analysis, we explored published scRNA-seq profiles of the first- and second-trimester human placentas[1] and *in vitro* cultured human embryos[2]. The datasets were acquired from the NCBI Gene Expression Omnibus (GEO) database: the

datasets of the human placentas were from GSE89497, and the datasets of *in vitro* cultured human embryos were from GSE109555. All the transcriptomic libraries of scRNA-seq data analyzed in this paper were constructed with the Smart-seq2 method[13,14]. The expression levels of genes were normalized to transcripts-per-million mapped reads (TPM). In the human placenta datasets, single-cell transcriptomes of 8 placentas were analyzed. We retained cells with at least 3 000 genes detected, and extracted 1260 cells from the 8-week placentas and 189 EVT cells from the 24-week placenta. In total 16845genes with TPMs over 5 in at least 2 cells were used for further analysis. In the *in vitro* cultured human embryo datasets, single-cell transcriptomes (5911 cells) of 48 embryos (6, 8, 10, 12, 14 days post fertilization) were used. We filtered out cells with genes detected less than 5000 and genes expressed in less three cells. Finally, 4198 trophectoderm derived cells with 22359 genes were subjected to the following analysis.

Clustering and visualization of the scRNA-seq data

Single cell data were mainly analyzed with Seurat R package. We used "ElbowPlot" function to determine appropriate number of dimensions to perform the nonlinear dimensional reduction (t-SNE or UMAP). Unsupervised clustering method was used to identify cell clusters by the "FindClusters" function of the Seurat R package. In the

datasets from the human placentas, the clusters and cell types were visualized via t-SNE (t-Distributed Stochastic Neighbor Embedding) method using Seurat V3.0 R package[15,16]. In the datasets of trophectoderm derived cells from *in vitro* cultured human embryos, the clusters and cell types were visualized via UMAP (Uniform Manifold Approximation and Projection) method using Seurat V3.0 R package[15,16]. ggplot2 V3.3.0 R package[17] was used to illustrate markers of different cell types.

ACE2 and TMPRSS2 expression analysis

'Featureplot' function of Seurat V3.0 R package[15,16] was used to illustrate the expression of ACE2, TMPRSS2, CTSB and CTSL among all cell types. To show percentages of cells that were positive for individual molecules in each cell type, we used the 'geom_bar' function of the ggplot2 V3.3.0 R package[17] to generate bar plot. Pie chart was used to show the percentages of different cell types among double positive cells (ggplot2 V3.3.0 R package)[17].

Functional analysis

We further conducted the functional analysis of the ACE2+TMPRSS2+ cells in different cell types to explore possible impact on TE or placental function after COVID-19

infection. Differentially expressed genes that were specifically highly regulated in the ACE2+TMPRSS2+ cells were used for further GO (Gene Ontology) analysis. To compare the functional differences between ACE2+TMPRSS2+ cells and the rest of the cells from EVT_24W and TE1 cells, 'FindMarkers' function of the Seurat V3.0 R package was used to acquire the upregulated genes in ACE2+TMPRSS2+ cells. Then ClusterProfiler V3.14.0 R package[18] was utilized for subsequent GO analysis.

Paraffin section

Paraffin section was performed as described by Hoffman et al[19]. Generally, tissues were collected, fixed, dehydrated and cleared before being embedded into paraffin. Embedded tissues from the first-, second- and third-trimester placentas were sliced into 5 μm per section, floated on the same glass slide and subjected to further immunohistochemical staining.

Immunohistochemical staining

Immunohistochemical staining was performed as described by Hoffman et al[19]. In brief, paraffin sections were incubated with antibodies against KRT7 (ZSGB, ZA-0573), HLA-G (Santa Cruz, SC-21799), VIM (ZSGB, ZA0511), TMPRSS2 (Abcam, ab109131) and

ACE2 (Abcam, ab246511) after deparaffinization, hydratation, endogenous peroxidase activity blocking, and antigen retrieval. DAB substrate solution (ZSGB, ZLI9018) was used for color staining after section incubation with biotinylated secondary antibody and Sav-HRP conjugates (ZSGB, SP9001). The sections were counterstained, dehydrated, cleared and coverslipped for long-term storage at room temperature. Finally, the sections were imaged with Leica Aperio VESA8 scanner (Leica, Wetzlar, Germany).

Results

ACE2+ and TMPRSS2+ cells in human trophectoderm

Using publicly available scRNA-seq datasets from human TE[2], we divided the cells into four clusters as TE1, CTBs, EVTs and STB according to their markers. Unsupervised clustering analysis presented an unbiased distribution of 4198 cells using UMAP method. The cells from TE1 subgroup exhibited a high expression level of TE related molecule CDX2, and these cells were mainly from day 6 after fertilization. Therefore, we considered TE1 cells to be TE cells before implantation. The cluster with high expression of HLA-G was EVT. The cluster with high expression of CYP19A1 was STB. The other cluster which showed high expression of PARP1 was considered to be CTB (Figure 1A-B). 54.4% of TE1 cells, 9.0% of CTBs, 3.2% of EVTs and 29.5% of STBs were ACE2 positive (Figure 1C, 1G). As for TMPRSS2, 90.7% of TE1 cells, 31.5% of CTBs, 22.1% of EVTs and 70.8% of STBs were TMPRSS2 positive (Figure 1D, 1H). CTSB was positive in 100% of TE1 cells, 95.9% of CTBs, 96.8% of EVTs and 97.4% of STBs. At the same time, CTSL was positive in 100% of TE1 cells, 99.9% of CTBs, 99.7% of EVTs and 100% of STBs(Figure 1E-F, 1I-J).

ACE2+TMPRSS2+ cells in first- and second-trimester placentas

Based on online scRNA-seq data from the first- and second-trimester placentas[1], we separated the cells into different clusters. Unsupervised clustering analysis presented the unbiased distribution of placental cells using t-SNE method. From the first-trimester placenta, the cluster that exhibited high expression of HLA-G was EVT_8W. The cluster that showed high expression of PARP1 was CTB. The cluster that showed CD68 expression was macrophage. Mesenchymal cells showed high expression of VIM, which is a classical marker for stromal cells. Cells that highly expressed CYP19A1 were identified as STBs. Blood cells showed high expression of ALAS2. EVT_24W cells showed high expression of HLA-G (Figure 2A-B). Amongst the first-trimester placental cells, 20.4% of CTBs, 44.1% of STBs and 3.4% of EVTs were ACE2 positive; 1.6% of CTBs, 26.5% of STBs and 1.9% of EVTs were TMPRSS2 positive (Figure 2G-H). Compared to trophoblasts, macrophages, blood cells, as well as mesenchymal stromal cells showed lower expression level of ACE2 and TMPRSS2 (Figure 2G-H). As for the EVT_24W, 63% and 20.1% of cells were ACE2 positive and TMPRSS2 positive, respectively (Fig 2C-D, 2G-H). All cells were CTSB positive (Figure 2E, 2I). 99.6% of CTBs, 98.1% of blood cells and 98.0% of mesenchymal cells showed CTSL positive while all cells from other cell types showed CTSL expression (Figure 2F, 2J).

Immunohistochemical analysis of ACE2 and TMPRSS2 in the human placenta

Immunohistochemical staining of ACE2 and TMPRSS2 on serial sections of human first-, second- and third-trimester placentas confirmed ACE2 and TMPRSS2 expression in human placentas. Markers including KRT7, HLA-G and VIM were stained to show distributions of trophoblasts, EVTs and stromal cells, respectively. In the first-trimester placentas, ACE2 was expressed mainly in the STBs, and TMPRSS2 was positive in STBs, EVTs as well as CTBs (Figure 3A). In the second-trimester placentas, the main sites of ACE2 expression were EVTs and STBs, and TMPRSS2 was expressed in all trophoblasts. (Figure 3B). In the third-trimester placentas, ACE2 was expressed mainly in EVTs and STBs, and TMPRSS2 was expressed in all trophoblasts (Figure 3C).

Functional analysis of ACE2+TMPRSS2+ cells

We further conducted the functional analysis of the ACE2+TMPRSS2+ cells in different cell types. At first, distributions of the ACE2+TMPRSS2^{+} cells from each cell type were described in the dataset from the first- and second-trimester placentas (Figure 4A) and the TE (Figure 5A). Among all cell types, the EVT_24W and TE1 cells contributed to the ACE2+TMPRSS2+ cells at the highest proportion. The GO analysis[20,21] identified the

functional differences between the ACE2+TMPRSS2+ cells and their counterparts from these two cell types, respectively. The ACE2+TMPRSS2+ EVT_24W cells were shown to be associated with morphogenesis of branching structure, extracellular matrix interaction, oxygen binding and antioxidant activity (Figure 4B-G). The ACE2+TMPRSS2+ TE1 cells showed a strong association with viral invasion, epithelial cell proliferation and cell adhesion molecule binding (Figure 5B-G).

Discussion

Principal findings of this study

Through scRNA-seq analysis and immunohistochemical staining, our major findings are as follows: (1) the ACE2 and TMPRSS2 are expressed in human TE and placentas throughout pregnancy; (2) the ACE2+TMPRSS2+ EVTs from the second-trimester placentas have shown association with morphogenesis of branching structure, extracellular matrix interaction, oxygen binding and antioxidant activity; and (3) the ACE2+TMPRSS2+ early TE cells have shown a strong association with viral invasion, epithelial cell proliferation and cell adhesion molecule binding.

Previous scRNA-seq analysis concerning SARS-CoV-2 in placenta

Recent development of scRNA-seq technologies have enabled us to examine the expressions of certain genes at single cell level[22]. Recently, the NIH (National Institutes of Health)-HCA (Human Cell Atlas) has released the analysis of ACE2 and CTSL expression in the first- and third-trimester placental scRNA-seq transcriptomes[23,24,25,26]. Their research has demonstrated that only a small fraction of the maternal decidua and fetal trophoblasts in the first-trimester placentas and some of the EVTs and STBs in the third-trimester placentas express both the ACE2 and CTSL. In comparison with their

findings, our study has observed a higher proportion of SARS-CoV-2 target cells in the TE and the first- and second-trimester placentas. The major difference in regarding to the sequencing methodology was that the NIH-HCA research employed 10x□ Genomics and Drop-seq platforms for all the scRNA-seq whilst in this study, we utilized the transcriptomes with SMART-Seq2 method, which has been shown to have higher sensitivity of gene detection and with lower drop-out rates[27].

Clinical implications

It is well accepted that the SARS-CoV-2 transmits via respiratory droplets and direct contact[28,29]. During pregnancy, there is the additional concern that the virus can be transmitted to the fetus, however, existing evidence on the possibility of vertical transmission remains highly controversial[30,31,32]. Studies of Chen H et al., Lei D et al. and Chen S et al. evaluated the risk of intrauterine infection by testing for SARS-CoV-2 RNA in amniotic fluid, cord blood, placental tissue and vaginal secretion samples collected from pregnant women with confirmed COVID-19 and pharyngeal swab samples collected from neonates born to these women[33,34,35]. All biological samples from these three studies tested negative[33,34,35]. Zeng et al. reported that 3 of 33 (9%) infants were diagnosed with neonatal early-onset COVID-19 based on positive SARS-CoV-2 RNA

results in two consecutive nasopharyngeal and anal swabs obtained on day 2 and day 4 of age[36]. All three infants tested negative for SARS-CoV-2 RNA on day 6 or 7 of age. Though strict infection control and prevention measures were implemented during the delivery, the possibility of postpartum neonatal infection cannot be completely excluded because of the delay in testing[36]. For some pathogens like Rubella virus, the risk of vertical transmission varies at different stages of pregnancy[37]. As for the SARS-CoV-2, we have observed a lower level of ACE2 and TMPRSS2 expression in EVTs in the second-trimester than in the first- -trimester placentas based on scRNA-seq results. Most recently, Alexandre et al reported a case of maternal and neonatal COVID-19 infection based on positive SARS-CoV-2 RNA results in the maternal nasopharyngeal swab, vaginal swab, blood, placenta, amniotic fluid, neonatal blood, neonatal nasopharyngeal swab as well as neonatal rectal swab[38].

Our findings have confirmed the presence of the target cells of SARS-CoV-2 in the human TE and placenta, highlighting the possibility of a transplacental approach for vertical transmission. A case of miscarriage at 19 weeks' gestation in a pregnant woman with COVID-19 has been reported recently[39]. In this case, reverse transcriptase-polymerase chain reaction (RT-PCR) test for SARS-CoV-2 RNA was positive in the placenta but negative in the abortus. Such findings have led us to speculate that COVID-

19 can cause miscarriage, especially when there has been a long duration of viral exposure and heavy viral load. Whether COVID-19 in pregnancy is associated with fetal growth restriction and structural malformation, more data is needed to answer this question.

Previous results have demonstrated that the ACE2+TMPRSS2+ cells are considered to be related to viral invasion. Considering the SARS-CoV-2 invasion might cause functional impairment to its target cells, we conducted functional analysis in the two cell types that showed the highest percentage of ACE2+TMPRSS2+ cells. Compared with the rest of the EVT_24W cells, ACE2+TMPRSS2+ EVT_24W cells were associated with morphogenesis of branching structure, extracellular matrix interaction, oxygen binding and antioxidant activity. Compared with the rest of the early TE cells, ACE2+TMPRSS2+ early TE cells showed correlation with increased capacity of viral invasion, epithelial cell proliferation and cell adhesion molecule binding. These findings suggest that pregnancies complicated with COVID-19 are potentially at risk of placental insufficiency, which might lead to fetal growth restriction and even fetal loss.

Strengths and limitations

This study has several strengths. First, the scRNA-seq transcriptomic analysis was used to identify SARS-CoV-2 target cells at single cell level. Second, differences of SARS-

CoV-2 target cell numbers in the placentas from the three trimesters of pregnancy were explored. Third, the SMART-Seq2 sequencing platform was used to achieve better accuracy of the analysis. Fourth, immunohistochemical method was included to confirm the bioinformatic results. Finally, functional analysis of SARS-CoV-2 target cells was conducted to illustrate potential impact on placental function after SARS-CoV-2 infection. A major limitation of the study was that we only did immunohistochemical staining of the third-trimester placentas without scRNA-seq analysis for the reason that as far as we know, no scRNA-seq transcriptome of the third-trimester placenta has been analyzed by the SMART-Seq2 platform until now. In addition, since our bioinformatic analysis and immunohistochemical experiments were based on a limited number of samples, considering the impact of individual heterogeneity, whether the expression patterns of ACE2 and TMPRSS2 can be extended to the general population requires further exploration.

Conclusions

Our study has demonstrated the presence of ACE2 and TMPRSS2 positive cells in the human placentas at the different stages of pregnancy, as well as in the human TE, which indicates the possibility that the SARS-CoV-2 could spread via the placenta and cause

intrauterine fetal infection. Functional analysis has revealed association with morphogenesis of branching structure, extracellular matrix interaction, oxygen binding and antioxidant activity in the ACE2+TMPRSS2+ EVT_24W cells and viral invasion, epithelial cell proliferation and cell adhesion in the ACE2+TMPRSS2+ early TE cells.

Disclaimer

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Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1 - Expression of SARS-CoV-2 related molecules in early trophectoderm (TE)

UMAP distributions (A) and representative markers for each cell type (B) of the single-cell RNA sequencing data from embryonic TE obtained at 6-14 days after fertilization. The expression of angiotensin-converting enzyme 2 (ACE2)(C), serine protease transmembrane protease serine 2 (TMPRSS2)(D), cathepsin B (CTSB)(E) and cathepsin L (CTSL)(F) in early TE. Percentages of ACE2 (G), TMPRSS2 (H), CTSB (I), CTSL (J) positive cells in early TE across all cell types.

Figure 2-Expression of SARS-CoV-2 related molecules in the first- and second-trimester placenta

t-SNE distributions (A) and representative markers for each cell type (B) of the single-cell RNA sequencing data from the 8-week (8W) and 24-week (24W) placentas. The expression of ACE2 (C), TMPRSS2 (D), CTSB (E), CTSL (F) in the first and second-trimester placenta. Percentages of ACE2 (G), TMPRSS2 (H), CTSB (I), CTSL (J) positive cells in the first- and second-trimester placenta across all cell types.

Figure 3-Immunohistochemistry staining of ACE2 and TMPRSS2 in different trimesters of human placenta

Immunohistochemistry staining of ACE2 and TMPRSS2 in the first- (A), second- (B), and third- (C) trimester human placenta. KRT7 (marker of trophoblasts), HLA-G (marker of EVTs) and VIM (marker of stromal cells) staining were also conducted to show different cell types (n=3).

Figure 4-ACE2+TMPRSS2+ cells in the first- and second-trimester placenta

Distributions of ACE2+TMPRSS2+ cells in 8-week (8W) and 24-week (24W) human placenta (A) across all cell types. Enriched Gene Ontology (GO) terms in ACE2+TMPRSS2+ EVTs at 24-week placentas (EVT_24W) versus the rest of the EVT_24W from human placentas, including GOBP (Biological Process) analysis (B-C), GOCC (Cellular Component) analysis (D-E), and GOMF (Molecular Function) analysis (F-G). The colors in the dot plots (B, D, F) were coded by significance of enrichment scores and sizes were on the basis of the counts of overlapping genes. In the network plots (C, E, G), the sizes of term dots

represented the counts of overlapping genes and the color of each gene dot represented the significance of enrichment scores.

Figure 5-ACE2+TMPRSS2+ cells in early TE

Distributions of ACE2+TMPRSS2+ cells in early TE (A) across all cell types. Gene Ontology (GO) analysis of the upregulated genes in ACE2+TMPRSS2+ TE1 cells versus other cells in TE1 cells from early embryo, including GOBP (Biological Process) analysis (B-C), GOCC (Cellular Component) analysis (D-E), and GOMF (Molecular Function) analysis (F-G). In the network plots (C, E, G), the sizes of term dots represented the counts of overlapping genes and the color of each gene dot represented the significance of enrichment scores. The colors in the dot plots were coded by significance of enrichment scores and sizes were on the basis of the counts of overlapping genes (F). The colors in the bar plot were coded by significance of enrichment scores and heights were on the basis of overlapping genes (B, D).

Figure 1

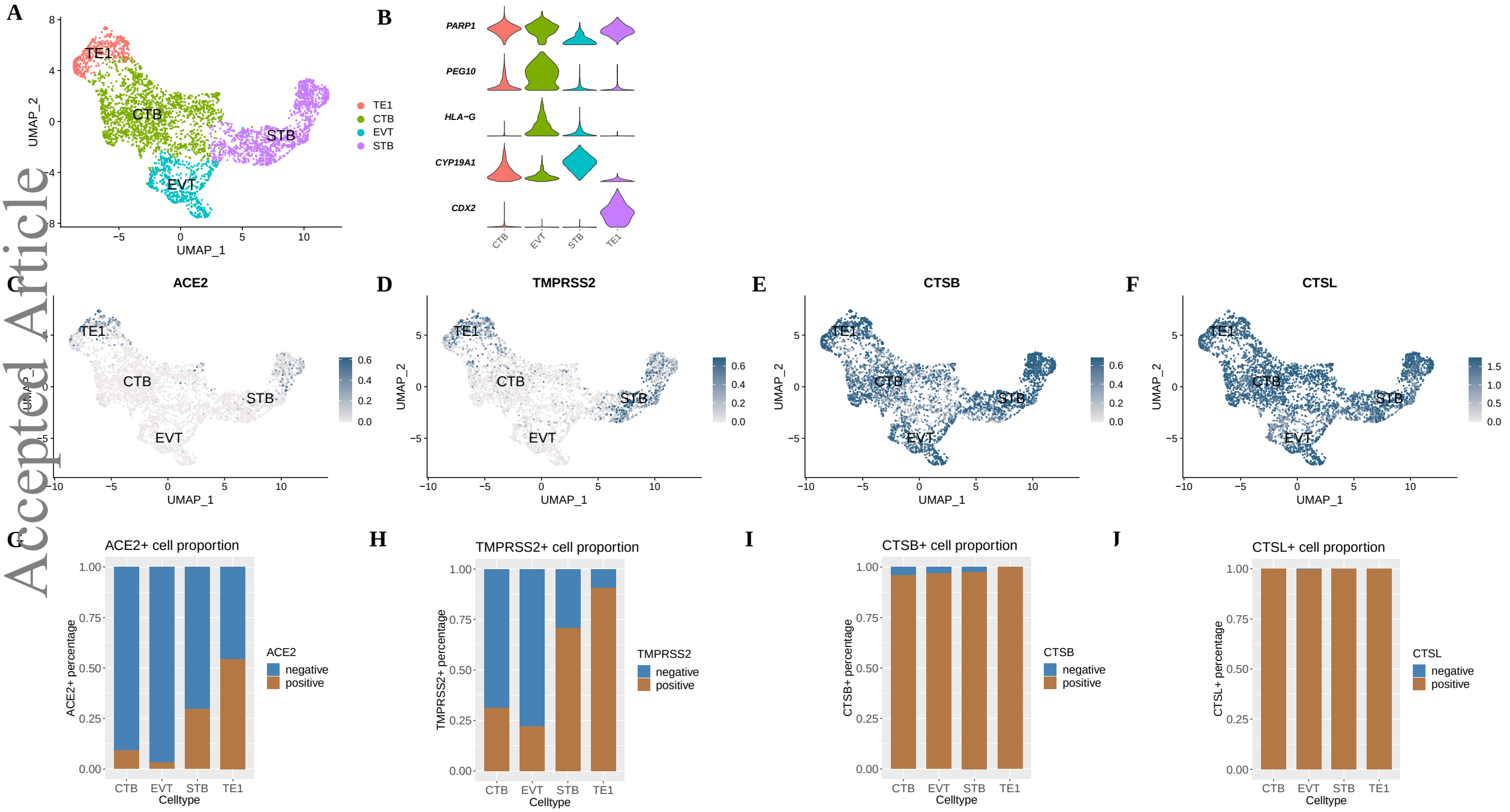
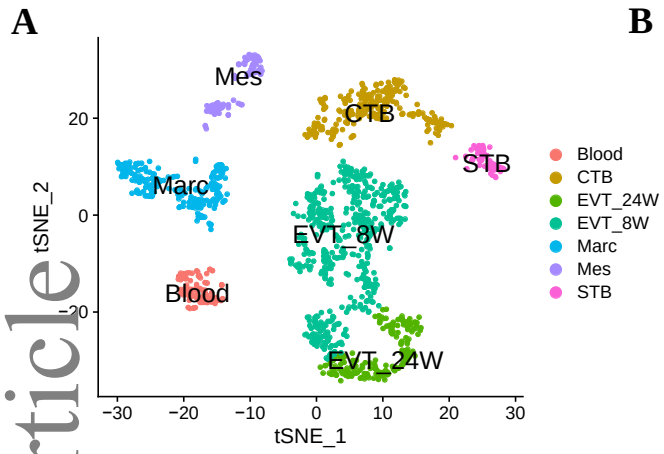
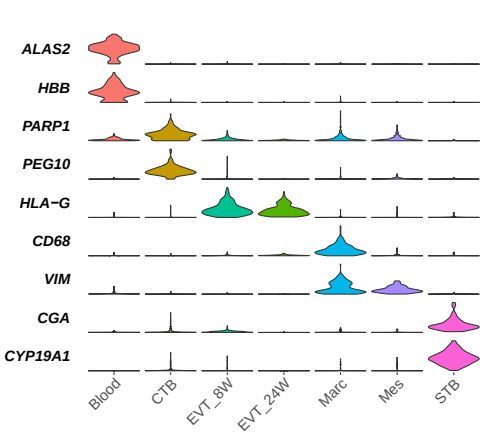
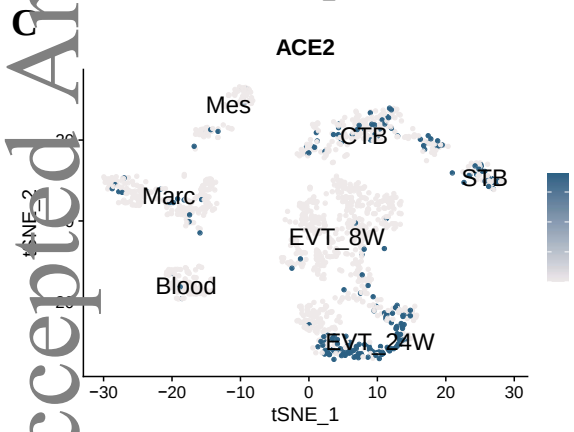
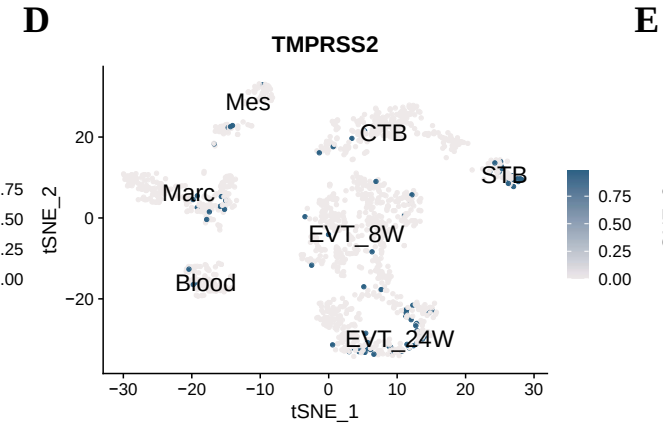


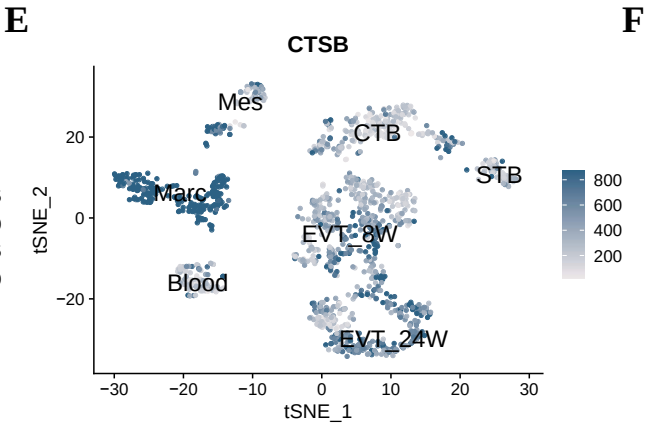
Figure 2

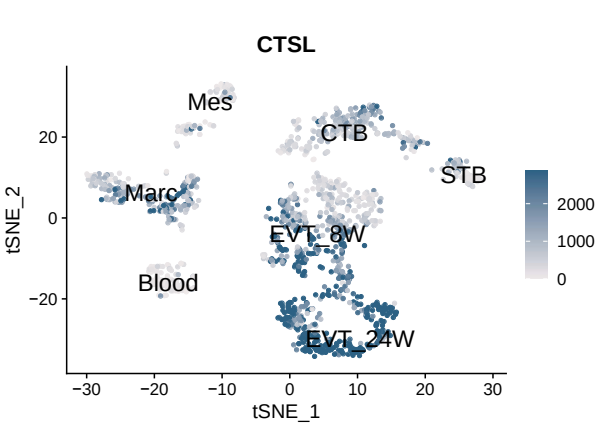


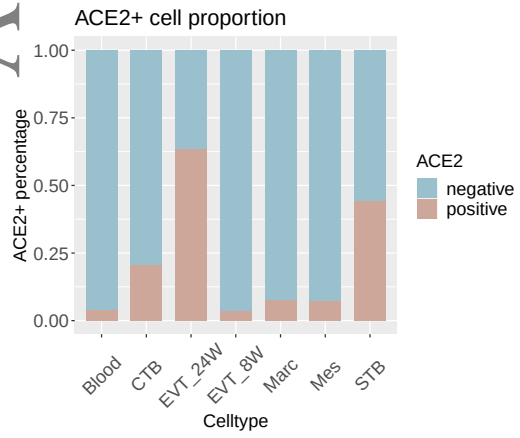


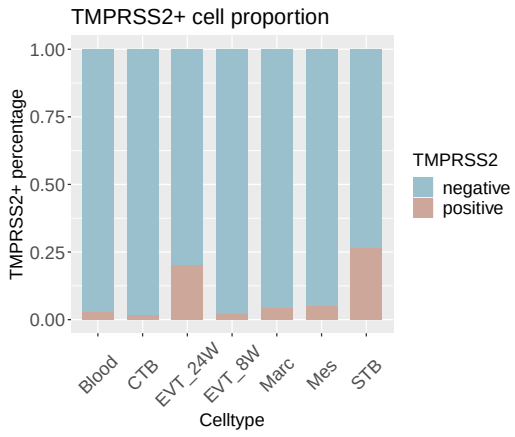


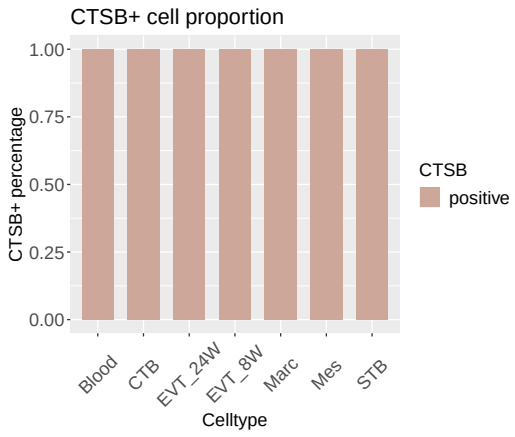












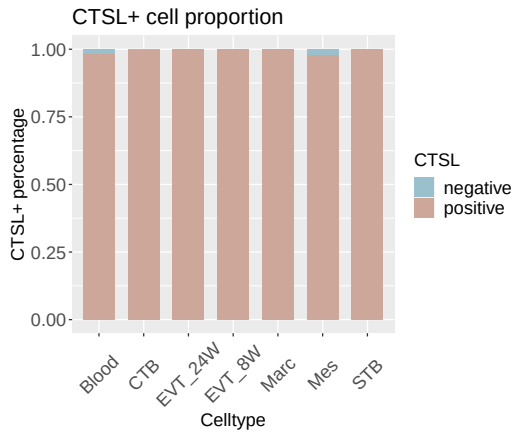


Figure 3

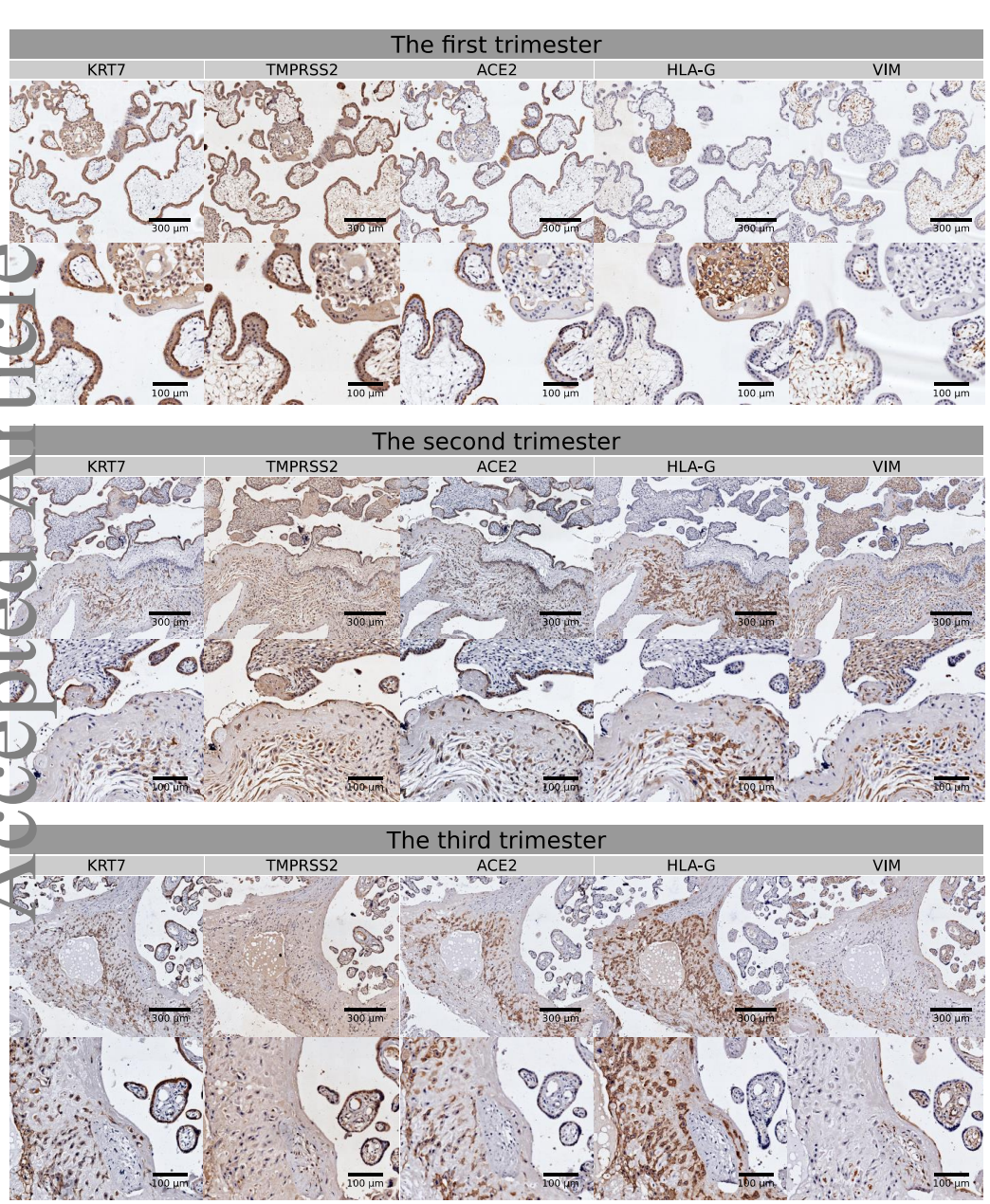


Figure 4

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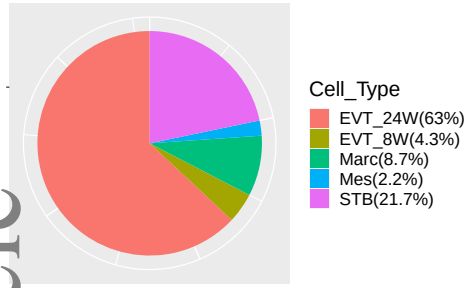

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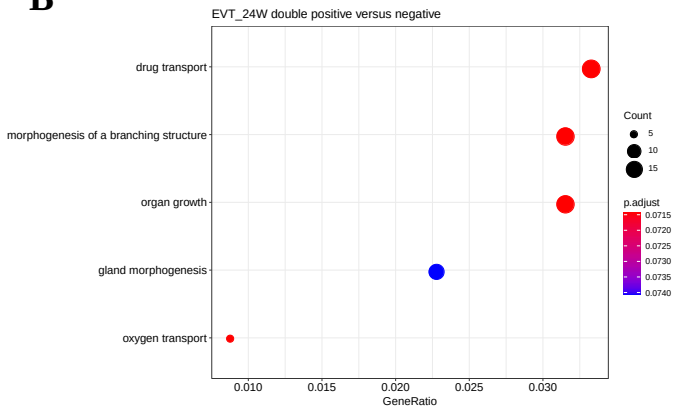

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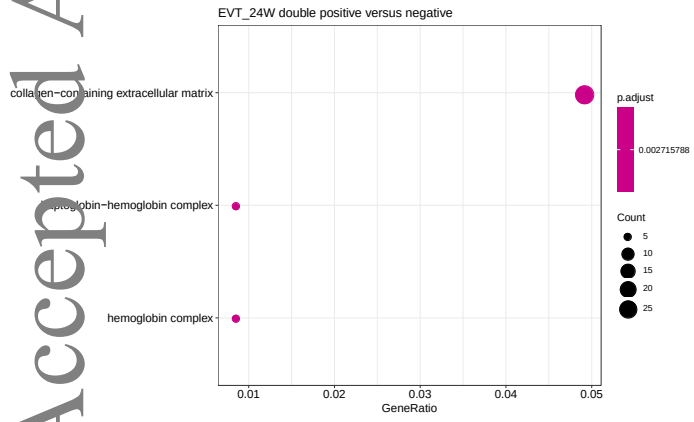

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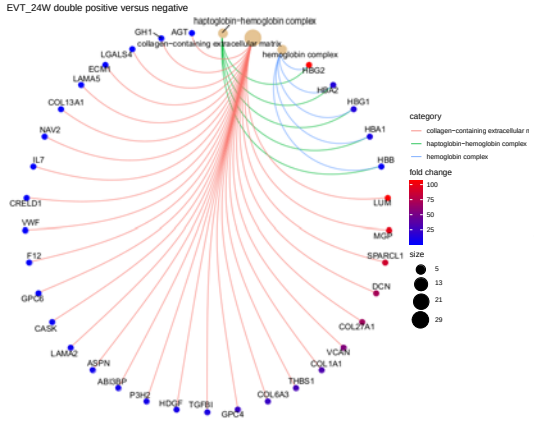

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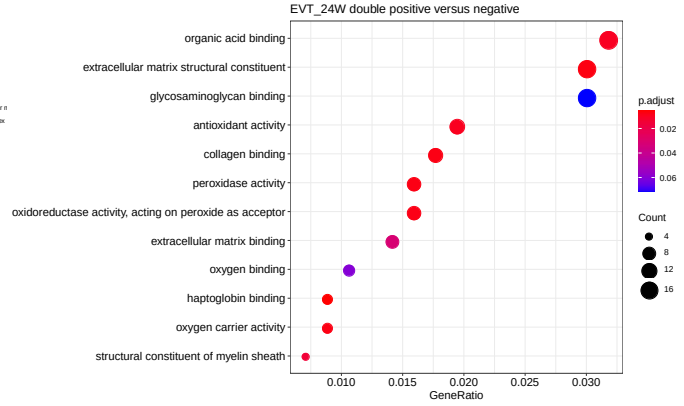

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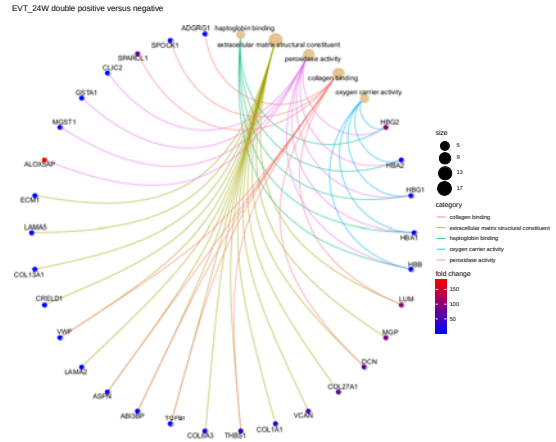
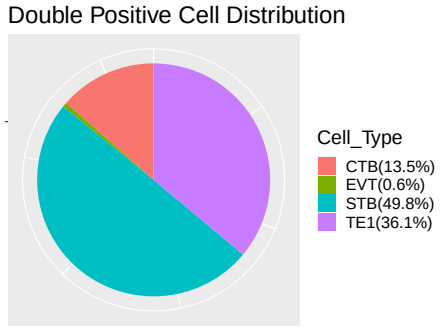


Figure 5

A



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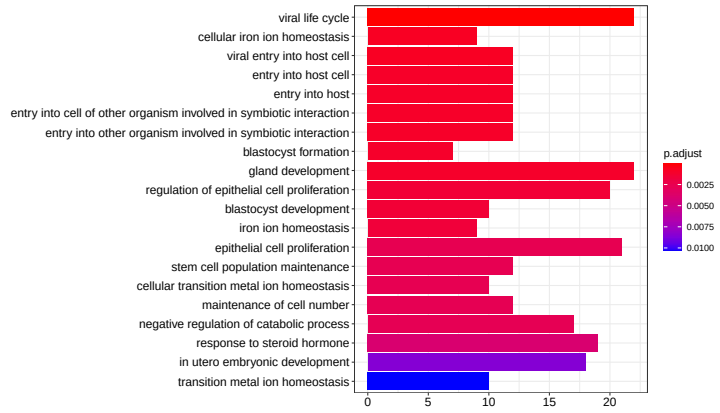

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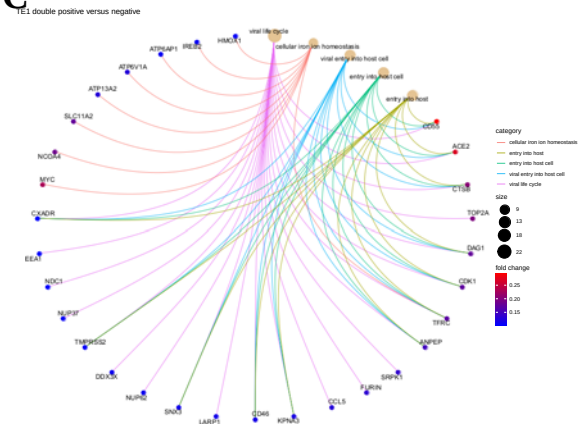

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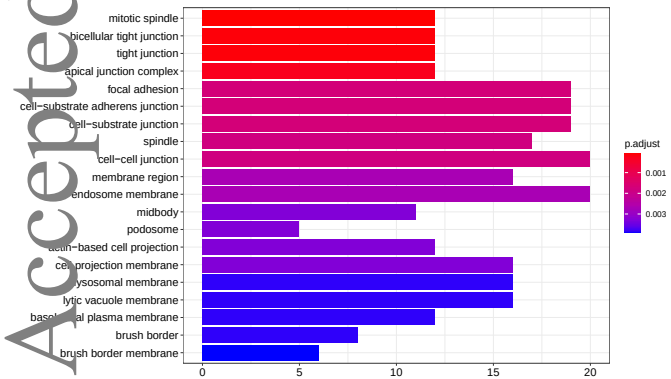

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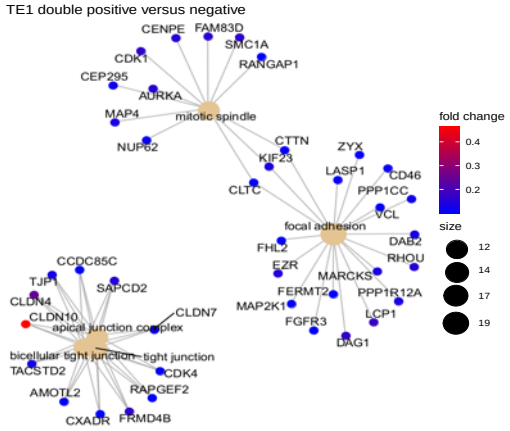

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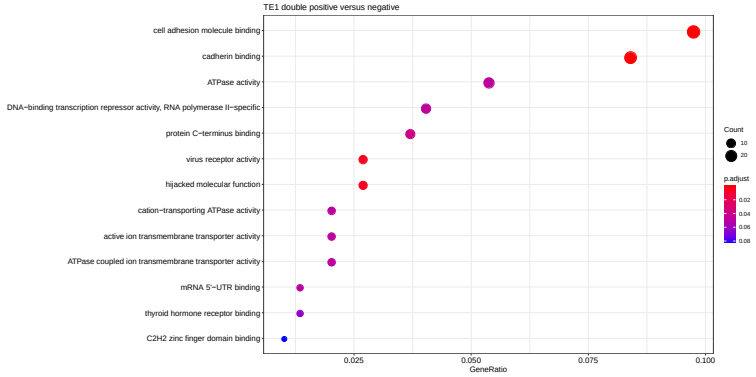

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