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scRNA-seq profiling of human testes reveals the presence of ACE2 receptor, a target for SARS-CoV-2 infection, in spermatogonia, Leydig and Sertoli cells

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Abstract

In December 2019, a novel coronavirus (SARS-CoV-2) was identified in patients with pneumonia (called COVID-19) in Wuhan, Hubei Province, China. SARS-CoV-2 shares high sequence similarity and uses the same cell entry receptor, angiotensin-converting enzyme 2 (ACE2), as does severe acute respiratory syndrome coronavirus (SARS-CoV). Several studies have provided bioinformatic evidence of potential routes for SARS-CoV-2 infection in respiratory, cardiovascular, digestive and urinary systems. However, whether the reproductive system is a potential target of SARS-CoV-2 infection has not been determined. Here, we investigate the expression pattern of ACE2 in adult human testis at the level of single-cell transcriptomes. The results indicate that ACE2 is predominantly enriched in spermatogonia, Leydig and Sertoli cells. Gene ontology analyses indicate that GO categories associated with viral reproduction and transmission are highly enriched in ACE2-positive spermatogonia while male gamete generation related terms are down-regulated. Cell-cell junction and immunity related GO terms are increased

in ACE2-positive Leydig and Sertoli cells, but mitochondria and reproduction related GO terms are decreased. These findings provide evidence that human testes are a potential target of SARS-CoV-2 infection which may have significant impact on our understanding of the pathophysiology of this rapidly spreading disease.

Introduction

In December 2019, a novel coronavirus designated SARS-Cov-2 was identified in patients with pneumonia in Wuhan, Hubei Province of China. It can cause acute respiratory distress syndrome and infected patients have a relatively high risk of death (1-3). It has been reported that SARS-CoV-2 shares 76% amino acid sequence identity with severe acute respiratory syndrome coronavirus (SARS-CoV) and is likely to use the same receptor, angiotensin-converting enzyme 2 (ACE2), for entry into target host cells (4-6).

scRNA-seq analysis document that ACE2 is specifically expressed in type II alveolar epithelial cells (AT2) in human lung (6), suggesting that this virus targets ACE2-positive AT2 cells to induce pneumonia. Liver function damage has been reported in SARS-infected and middle east respiratory syndrome coronavirus (MERS-CoV)-infected patients (7,8). A recent epidemiologic study indicates that some patients infected with SARS-CoV-2 have signs of severe liver damage (1). By analyzing healthy liver cells at single-cell resolution, investigators have determined that ACE2 is significantly enriched in cholangiocytes (9), suggesting that the virus might directly bind to ACE2-positive cholangiocytes to dysregulate liver function. Moreover, a recent study has explored the composition and proportion of ACE2-expressing cells in the digestive system by scRNA-seq analysis, and showed that ACE2 is not only highly expressed in lung AT2 cells, esophagus upper and stratified epithelial cells but also in absorptive enterocytes from ileum and

colon (10), implying that digestive system is a potential route for SARS-CoV-2 infection. In addition, cardiovascular and urinary systems have been reported as potential organ targets of SARS-CoV-2 infection (11).

A series of studies have provided the bioinformatics evidence of potential routes for infection of SARS-CoV-2 in respiratory, cardiovascular, digestive and urinary systems. However, whether the reproductive system is susceptible to SARS-CoV-2 infection has not been determined. In this study, we investigate the RNA expression profiles of ACE2 in adult human testis at single-cell resolution. Our study documents that ACE2 is predominantly enriched in spermatogonia, Leydig and Sertoli cells. ACE2-positive cells possess higher abundance of transcripts associated with viral reproduction and transmission and lower abundance of transcripts related with male gametogenesis. Taken together, ACE2 expression in human testis suggests that SARS-CoV-2 could infect the male gonad and risk male reproductive dysfunction.

Materials and Methods

Data Sources

Adult human testis scRNA-seq datasets were obtained from Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA) databases under accession number (GSE109037).

scRNA-seq data processing

Raw read processing was carried out using the Cell Ranger Single-Cell Software Suite (version 3.1.0, 10X Genomics Inc., CA). The primary data analyses which included alignment, filtering, barcode counting and UMI quantification for determining gene transcript counts per cell

(generated a gene-barcode matrix) and quality control, were performed using CellRanger *count* command. Gene positions were annotated using Ensembl build 93 and filtered for biotype (only protein-coding, long intergenic non-coding RNA, antisense, immunoglobulin or T-cell receptor).

Single-cell transcriptomes to identify cell types

Raw gene expression matrices generated per sample using CellRanger (version 3.1.0) were imported into R (version 3.6.2) and converted to a Seurat object using the Seurat R package (version 3.1.2). Cells which had either fewer than 300 expressed genes or over 15% UMIs derived from mitochondrial genome were discarded. For the remaining cells, gene expression matrices were normalized to total cellular read count and to mitochondrial read count using negative binomial regression method implemented in Seurat *SCTransform* function. Cell-cycle scores were also calculated using Seurat *CellCycleScoring* function since the cell-cycle phase effect was observed. The gene expression matrices were then further normalized to cell-cycle scores. The Seurat *RunPCA* functions was performed to calculate principal components (PCs). We further performed the batch effect correction using Hormany because batch effects were observed. The *RunUMAP* function with default setting was applied to visualize the first 35 Harmony aligned coordinates. The *FindClusters* function with *resolution=0.6* parameter was carried out to cluster cells into different groups. Canonical marker genes were applied to annotate cell clusters into known biological cell types.

Identification of differential expression genes (DEG)

To identify DEG between two groups, we use the Seurat *FindMarkers* function with default parameter of method "MAST" and cells ID from each defined group (e.g. ACE2 expressed AT2 vs ACE2 not-expressed AT2) as input.

Results

Identification of cell types in adult human testis

To assess the expression pattern of ACE2 in human testis, we first analyzed a published scRNA-seq dataset from three individual adult human testis samples (12). From a total of 17,520 testicular cells, 16,632 cells passed standard quality control and were retained for subsequent analyses. On average, we detected 9,398 unique molecular indices (UMIs) and 2,388 genes in each individual cell.

Uniform Manifold Approximation and Projection (UMAP) and marker gene analyses were performed for cell type identification of the total 16,632 testicular cells. Based on UMAP, we identified nine major cell clusters and none of the clusters solely derived from one individual (Figure 1A and B). Cluster identity was assigned based on expression patterns of known marker genes in human testis. We have identified five major germ cell types including spermatogonia, early spermatocytes, late spermatocytes, round spermatids and elongated spermatids that recapitulated the temporal order of spermatogenesis. We also identified somatic cell types including endothelial, Sertoli and Leydig cells as well as monocytes (Figure 1A and B).

Cell-specific expression of ACE2

To determine the specific cell type expressing ACE2, we analyzed the RNA expression profile of ACE2 at single-cell resolution in human testis. UMAP plot revealed that ACE2 was primarily enriched in two major clusters corresponding to spermatogonia, Leydig and Sertoli cells (Figure 2A and C). ACE2-positive spermatogonia represented 1.28% of all spermatogonia in human testis (Figure 3A), with similar expression level of ACE2-expressing cells (1.40% ±0.40%) in AT2 cells (6). Since we could not separate Leydig and Sertoli cells as distinct clusters, we combined these two somatic cell types together for subsequent analyses. We found that enrichment of ACE2 in Leydig and Sertoli cells had a significant higher frequency compared with ACE2-expressing cells in AT2 cells (4.25% vs 1.40%) (Figure 3A). Violin plot further demonstrated that ACE2 was highly expressed in spermatogonia, Leydig and Sertoli cells. Early spermatocytes, late spermatocytes, spermatids and other somatic cells had very low expression levels of ACE2 (Figure 2C). A recent study reported that SARS-CoV-2 uses the SARS-coronavirus receptor ACE2 and the cellular serine protease TMPRSS2 for entry into target cells (5). Feature and violin plots indicated that TMPRSS2 expression was concentrated in spermatogonia and spermatids with relatively low levels in other cell types (Figure 2B and D). Thus, ACE2 expression in spermatogonia, Leydig and Sertoli cells and TMPRSS2 expression in spermatogonia and spermatids suggest a high potential of SARS-CoV-2 infection in human testis. Furthermore, pseudotime analysis provided the trajectory of male germ cell development (Figure 3B), and further suggest that spermatogenesis would be disrupted if spermatogonia were infected and damaged by the SARS-CoV-2.

Characteristics of ACE2-positive cells in human testis

To further characterize ACE2-positive cells in human testis, gene ontology enrichment analysis was performed to determine which biological processes were enriched within either spermatogonia or Leydig and Sertoli cells by comparing ACE2-positive cells with ACE2-negative cells. We found that 24 GO terms associated with viral reproduction and transmission were positively enriched in ACE2-positive spermatogonia and included viral gene expression (e.g., *Nup133*, *Polr2a*, *Jun*, *Ranbp2*, *Rpl12*, *Eif3l*, *Rpl3*, *Rpl4*, *Rps19*, *Rps2*, *Nup85*), positive regulation of viral processes (e.g., *Top2a*, *Rsf1*, *Ppia*, *Chmp2a*, *Nucks1*, *Trim11*, *Polr2b*, *Nelfb*, *Chd1*, *Nelfcd*, *Tsg101*), viral latency, positive regulation of viral release from host cell, viral life cycle, viral translation, viral genome replication and viral budding (Figure 4A and B). In contrast, there were multiple GO terms related to male reproduction that were decreased in ACE2-positive spermatogonia and include male gamete generation (e.g., *Adcy10*, *Mettl3*, *Rnf8*, *Cdc42*, *Sycp1*, *Dazl*, *Etv5*, *Ythdc2*, *Tex14*, *Rec8*, *Morc1*, *Meioc*, *Sun1*, *Taf7l*, *Ybx2*, *Nanos3*, *Ddx4*, *Syce3*), spermatid differentiation (e.g., *Spag16*, *Cfap157*, *Spo11*, *Oca2*, *Rfx2*, *Pygo1*, *Ttc26*, *Catsper4*), fertilization (e.g., *Nectin2*, *Plb1*, *Cct7*, *Npm2*, *Rad21l1*, *Tdrkh*, *Mael*, *Izumo1*, *Spag8*, *Cd9*, *Tnp2*), sperm motility (e.g., *Hist1h1t*, *Sord*, *Anxa5*, *Slc22a16*, *Cfap44*, *Slirp*), sperm capacitation (e.g., *Pebp1*, *Catsperd*, *Slc26a6*, *Catsper3*), sperm-egg recognition, acrosome reaction, sperm chromatin condensation and male meiosis (Figure 4A and B). Therefore, SARS-CoV-2 may directly target ACE2-positive spermatogonia and disrupt spermatogenesis.

We further compared the characteristics of ACE2-positive Leydig/Sertoli cells with ACE2-negative cells. GO enrichment analysis documented that cell junction and immunity related GO terms were enriched in ACE2-positive cells including cell-cell junction organization (e.g., *Ace2*, *Flcn*, *Whrn*, *Mtdh*, *Rhoa*, *Ctnna1*), leukocyte mediated immunity (e.g., *Mlec*, *Kpnb1*, *Sptan1*,

Prss3, *Pafah1b2*, *Kcmf1*), cell surface (e.g., *Ctsv*, *Sparc*, *Ptn*, *Hspa5*, *Phb2*, *App*), cell-cell contact zone (e.g., *Cxadr*, *Ctnnb1*, *Cdh2*, *Pcdh9*, *Dlg1*, *Gja1*), secretory granule, cell activation, immune effector process, exocytosis (Figure 5A and B). Thus, SARS-CoV-2 may replicate and transfer through cell-cell junctions. Contrarily, some mitochondria and reproduction related GO terms were not enriched in ACE2-negative cells including mitochondrial matrix, mitochondrial envelope, mitochondrial gene expression (e.g., *Mrps18a*, *Mrpl21*, *Qrsl1*, *Mterf2*, *Coa3*, *Mrpl58*, *Hars*, *Mto1*, *Rcc1l*), mitochondrial translational termination, ATPase activator activity, fertilization, spermatid differentiation, sperm capacitation, sperm motility and sperm egg recognition (Figure 5A and B). These data suggest that ACE2-positive Leydig/Sertoli cells have lower potential to support spermatogenesis.

Discussion

Two known coronaviruses, SARS-CoV and MERS-CoV, are infection sources of respiratory disease in humans that caused public panic in past years (13). In December 2019, a newly identified coronavirus (SARS-CoV-2) was discovered in patients that has similar respiratory symptoms as SARS and MERS. Until now, no effective drugs are clinically approved for these etiologic agents, but it appears that they share the ACE2 receptor for entry into the host cells for reproduction and transmission. Thus, investigation of the composition and expression pattern of ACE2 may suggest potential routes for SARS-CoV-2 infection in humans. Recent studies have shown that respiratory, cardiovascular, digestive and urinary systems are affected by SARS-CoV-2 infection (10,11). Based on our current study of the scRNA-seq data in adult human testis, we suggest that the testis also is potentially vulnerable to SARS-CoV-2 infection.

Mammalian spermatogenesis is a coordinate and dynamic cell differentiation process supported by the self-renewal and differentiation of spermatogonial stem cells (SSCs). It is stringently controlled in a special niche microenvironment in testicular seminiferous tubules. Sertoli cells are the only somatic cell type in the tubules and directly interact with spermatogenic cells to control spermatogenic cell differentiation through paracrine signaling (14). The interstitial cells of Leydig are adjacent to the seminiferous tubules and produce testosterone in the presence of luteinizing hormone to support spermatogenic cell differentiation (15). Functional abnormalities in male germ cells or these supporting somatic cells cause spermatogenic failure and male infertility.

By analyzing the expression pattern of ACE2 in adult human testis at single-cell transcriptome resolution, we find that ACE2 is primarily expressed in spermatogonia, Leydig and Sertoli cells in the human testis. ACE2-positive spermatogonia express a higher number of genes associated with viral reproduction and transmission and a lower number of genes related to spermatogenesis compared to ACE2-negative spermatogonia. ACE2-positive Leydig and Sertoli cells express higher genes involved in cell-cell junction and immunity and lower genes associated with mitochondria and reproduction. These findings suggest that the testis is a high-risk organ vulnerable to SARS-CoV-2 infection that may result in spermatogenic failure.

In summary, our study provides bioinformatics evidence that the testis may be highly vulnerable to SARS-CoV-2 infection. These investigations may provide potential clues for further investigation and may have translational implications for treatment of reproductive defects caused by SARS-CoV-2 infection.

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Conflict of interest

The authors declare no conflict of interest.

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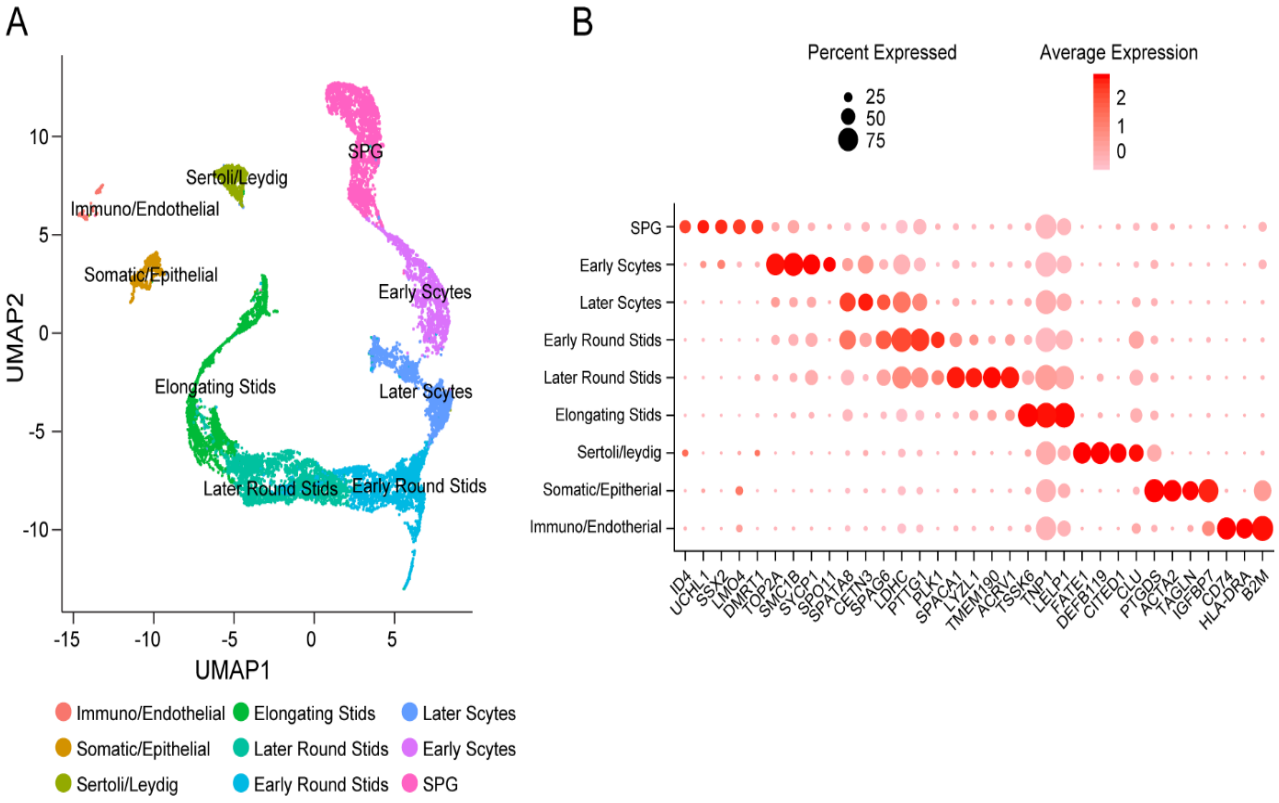

Figure 1. Single-cell transcriptome profiling from published adult human testes. (A) UMAP clustering of combined adult human testicular cells from three individual samples. Nine major cell clusters were identified across a total of 16,632 cells. (B) Dotplot for expression of selected marker genes across all identified cell types. SPG, spermatogonia; Early S'cytes, early spermatocytes; Late S'cytes, late spermatocytes; Early Round S'tids, early round spermatids; Later Round S'tids, later round spermatids; Elongating S'tids, elongating spermatids; Immuno, immune cells.

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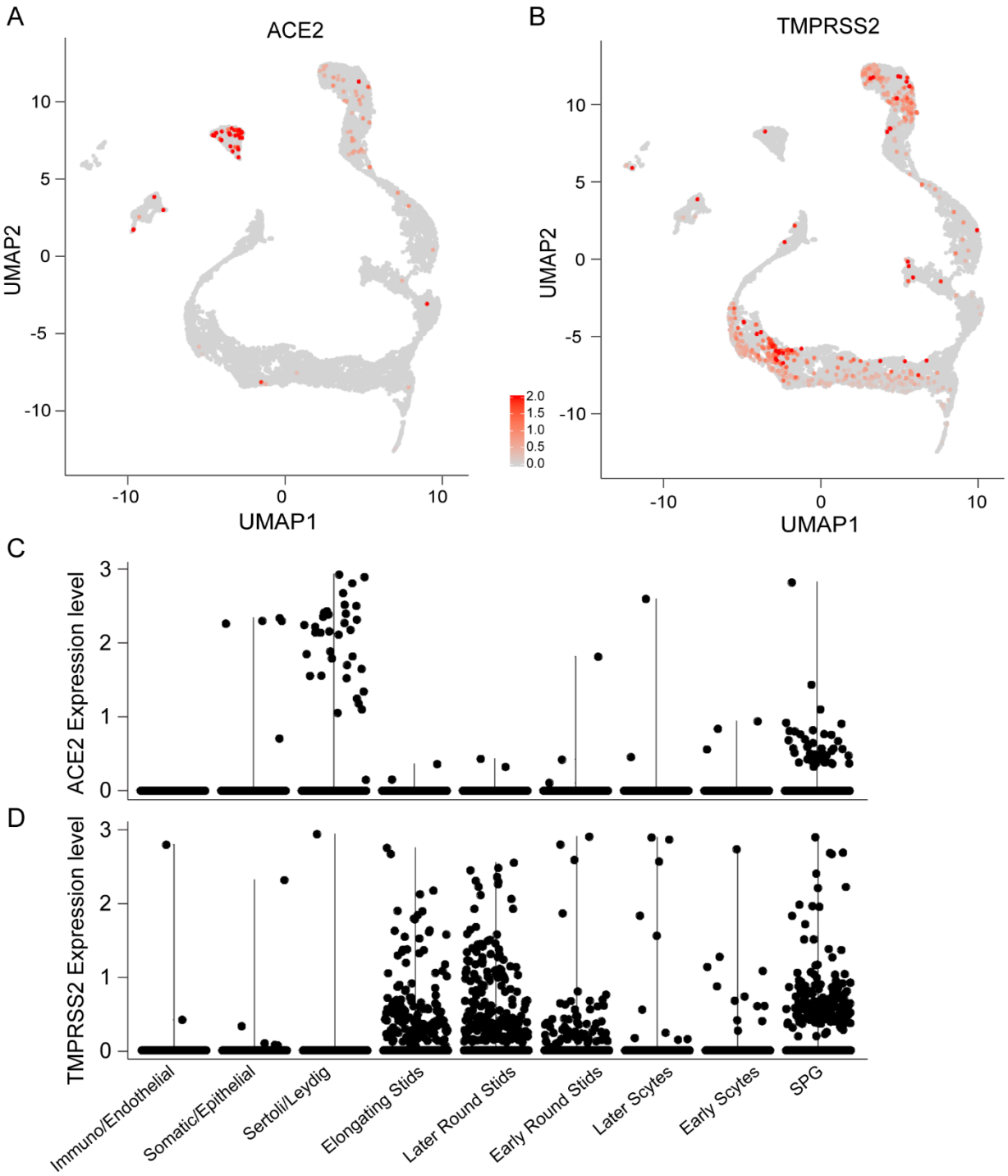

Figure 2. ACE2 expression pattern in adult human testis. (A) Per-cell expression level of ACE2 of human testicular cells visualized on the UMPA plot. (B) UMAP plot of TMPRSS2 expression across all cell clusters. (C) Violin plots of ACE2 expression in all identified cell types. (D) Violin plots for TMPRSS2 expression across all cell types.

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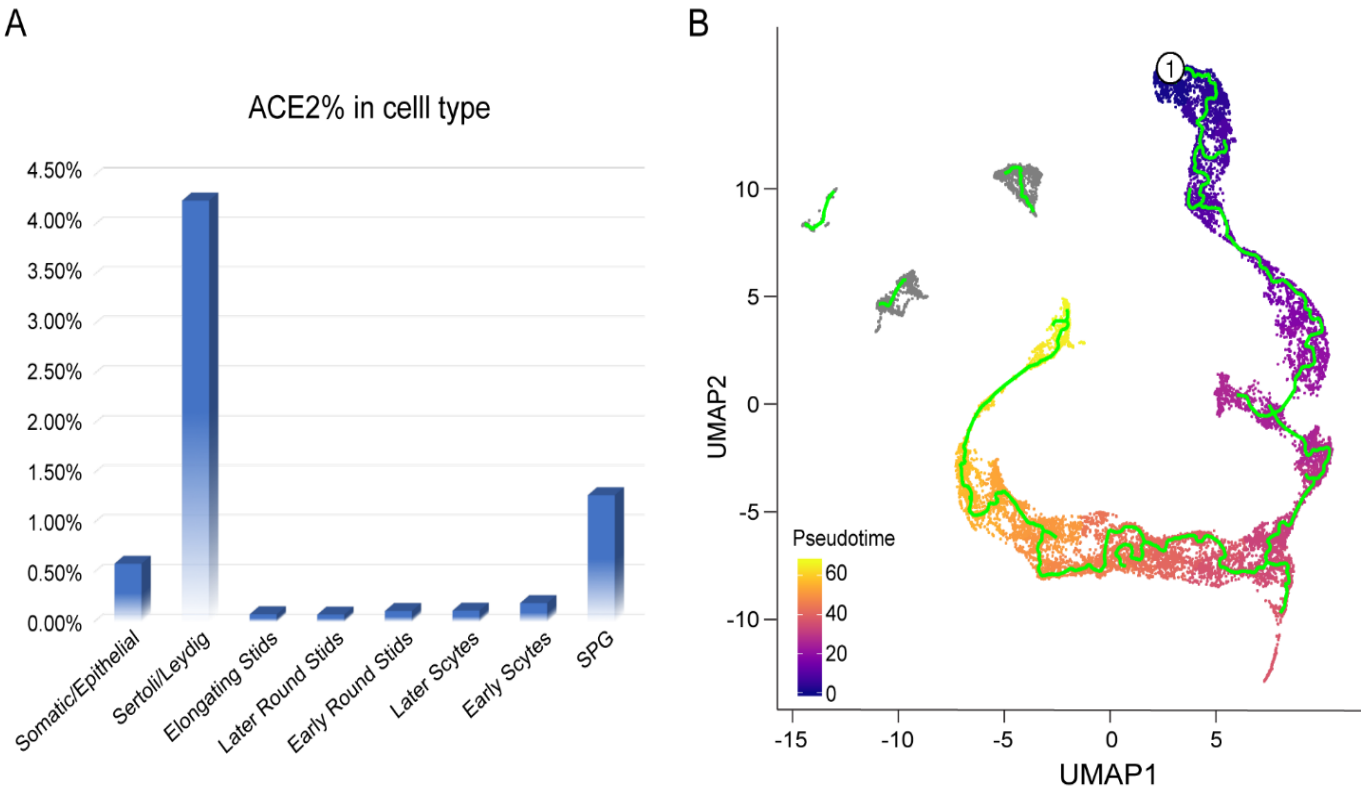

Figure 3. Composition of ACE2-positive cells and pseudotime analysis of human testicular cells. (A) ACE2-expression cells in each identified cell type. (B) Trajectory of male germ cell development by pseudotime time analysis of human testicular cells.

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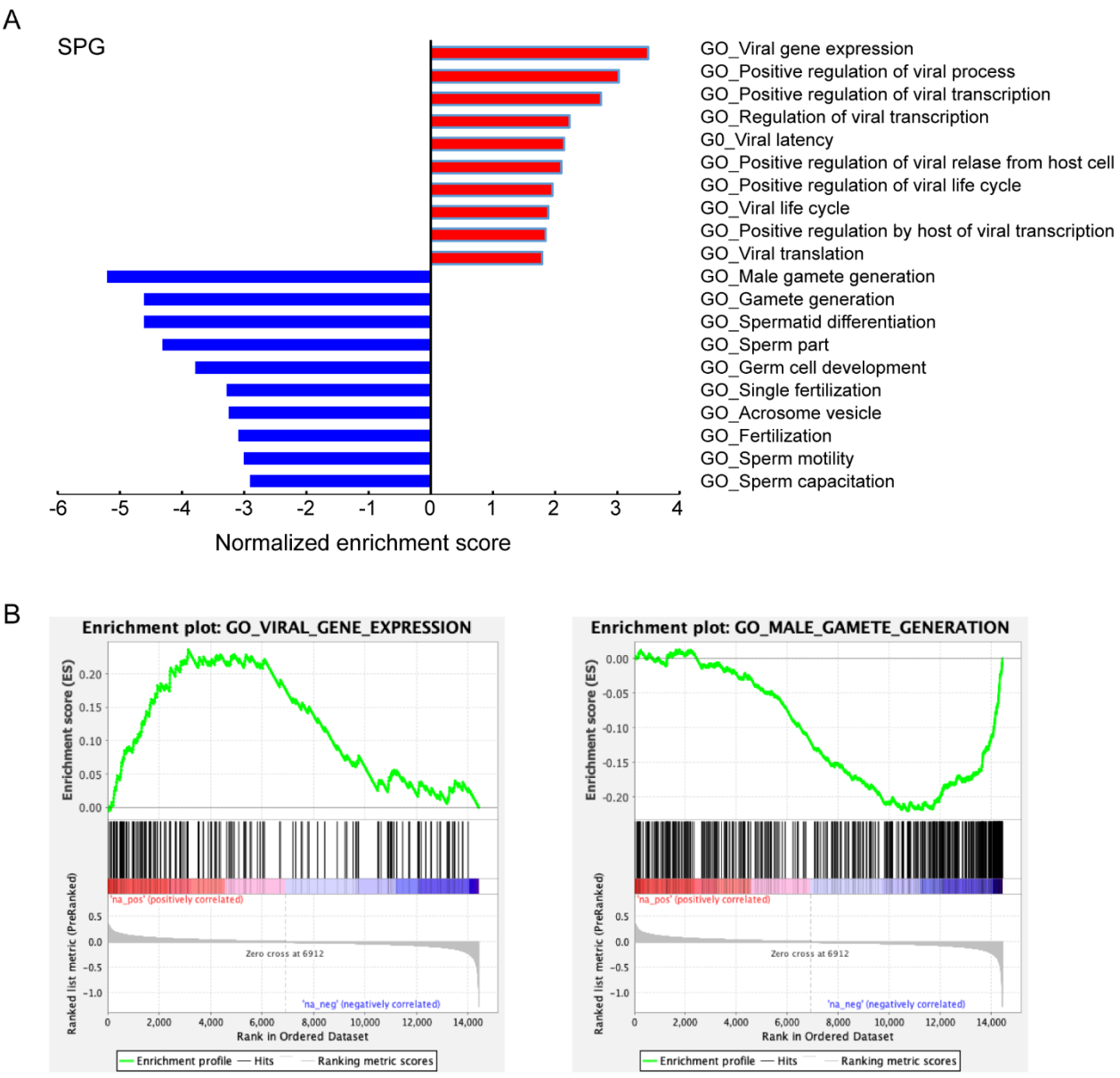

Figure 4. Characteristics of ACE2-positive spermatogonia. (A) Gene ontology enrichment analysis of biological process categories for ACE2-positive spermatogonia compared with ACE2-negative spermatogonia. (B) Examples of the enrichment plot for terms of viral gene expression and male gamete generation.

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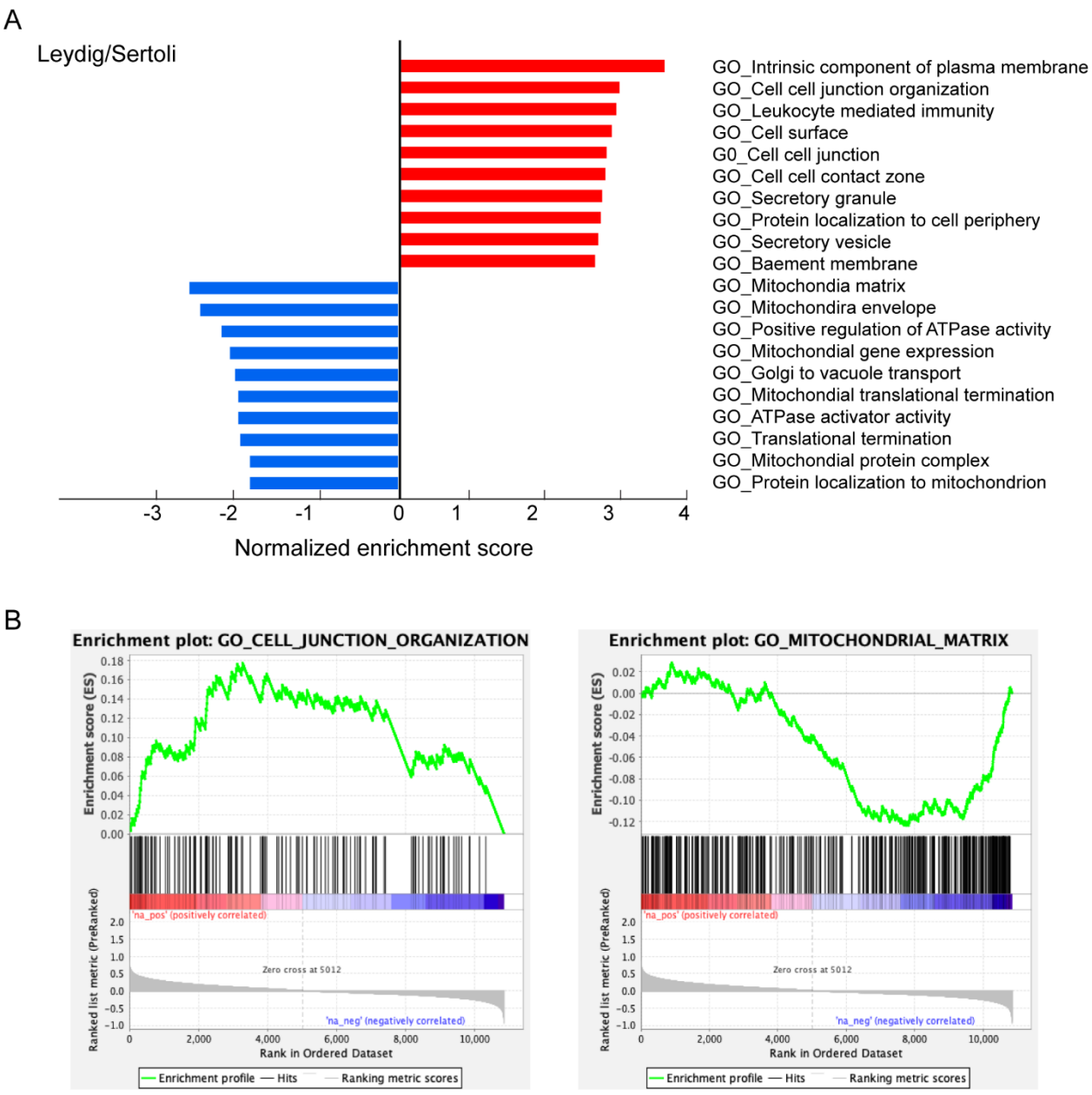

Figure 5. Characteristics of ACE2-positive Leydig and Sertoli cells. (A) Gene ontology enrichment analysis of biological process categories for ACE2-positive Leydig and Sertoli cells compared with ACE2-negative cells. (B) Examples of the enrichment plot for terms of cell-cell junction organization and mitochondrial matrix.