Supplementary Materials

Supplementary Materials and Methods

All animal experiments were approved by the Ethics Committee of Northwest Agriculture and Forestry University and were conducted at the Shaanxi Centre of Stem Cells Engineering & Technology. Every effort was made to minimize the suffering of animals.

Sample collection

Testis samples were collected from a 100-day-old healthy male Guanzhong dairy goat fed at Northwest A&F University Institutional Animal Care and Use Committee. After tissue collection, the testes (56.67 g) were transported to the laboratory within 1 h, and cells were collected from the testicular parenchyma using two-step enzymatic digestion (Setthawong et al., 2019).

Preparation of single testicular cell suspension for single-cell RNA sequencing (scRNA-seq)

The testes were washed 5–8 times in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, after which the testicular white membrane tissue was removed. The testicular tissue (3–5) were cut into several small pieces in a glass Petri dish. The tissue was digested in an enzyme cocktail containing 2 mg/ml collagenase type IV (Invitrogen, Carlsbad, CA, USA), 20 μg/ml DNase (Sigma-Aldrich, St. Louis, MO, USA), and 2 mg/ml dispase (Invitrogen) for 20 min at 37 °C in a shaker (250 rpm). The fragments were centrifuged at 1 200 rpm for 5 min at 37 °C, then digested in 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and

DNase I, shaken vigorously, and incubated at 37 °C for 20 min. Digestion was stopped by adding Dulbecco's modified Eagles medium (DMEM)/F12 (Invitrogen) with 10% fetal bovine serum. The cells were obtained by filtering through 74 μm strainers and then resuspended in PBS after centrifugation at 1 200 rpm for 5 min at 37 °C. Cell viability was determined by trypan blue staining, and cell number was measured using a hemocytometer.

scRNA-seq, cDNA library construction, and sequencing

scRNA-seq was performed with the 10x Genomics system. The single-cell library was constructed using the Chromium™ Controller and Chromium™ Single Cell 3' Reagent v2 Kit (10x Genomics, Pleasanton, CA, USA). Briefly, single cells, reagents, and gel beads containing barcoded oligonucleotides were encapsulated in nanoliter-sized gel beads in emulsion (GEMs) using GemCode Technology. Lysis and barcoded reverse transcription of polyadenylated mRNA from single cells were performed inside each GEM, and cleaned it up. cDNA was amplified, then fragmented, and fragment end-repaired. We used an Agilent 2100 bioanalyzer to check the size distribution of the fragments, and used quantitative real-time PCR (qRT-PCR) (TaqMan Probe) to quantify the library. The final product was sequenced using Illumina Hiseq 4000 (BGI Shenzhen, China).

Processing scRNA-seq data

After removing low-quality reads, Cell Ranger software was used for gene expression quantification and cell-type identification based on the cell barcode and UMI information of reads in every single cell. We obtained the matrix data files generated

from the 10x Genomics Cell Ranger alignment software. The files were then used as input in the R analysis package Seurat for subsequent analysis, including cell type identification, principal component analysis (PCA), and t-Stochastic-Neighbor Embedding (t-SNE) dimensionality reduction to visualize single-cell clustering. We used Seurat 2.3.0 to import the matrix data files, and then modify it into the object of Seurat 3.1.4, and then carried out downstream analysis.

Cell trajectory analysis

Germ cells from the t-SNE plot were used for pseudotime analysis with Monocle 2. Through data filtering, normalization, cell classification and clustering, key gene selection, dimensionality reduction, and sorting, the pseudotime trajectory of germ cells in spermatogenesis was obtained. Functional enrichment of marker genes within each cluster was then investigated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses.

Hematoxylin-eosin (HE) staining and immunofluorescence

Goat testis tissue (1–3 cm^3) was collected and fixed in 4% paraformaldehyde for 48 h, dehydrated with gradient alcohol, made transparent with xylene, immersed in tissue block in melted paraffin, and embedded. The embedded tissues were sectioned (5 μm thick) and stained with HE. The expression location of several candidate genes was detected by immunofluorescence analysis. The immunofluorescence staining methods have been described previously (Wei et al., 2018; Li et al., 2019; Liu et al., 2020; Yang et al., 2021). The antibody information used for immunofluorescence staining is as follows: Alexa Fluor 488 (1:500; Chemicon, USA) and Alexa Fluor 594 (1:500;

Chemicon, USA), anti-SYCP3 (1:400, Abcam, Cambridge, UK), anti-DDX4 (1:200; Abcam, Cambridge, UK), anti-SOX9 antibody (1:200, Abcam, Cambridge, UK), and Hoechst 33342 (Sigma-Aldrich, USA).

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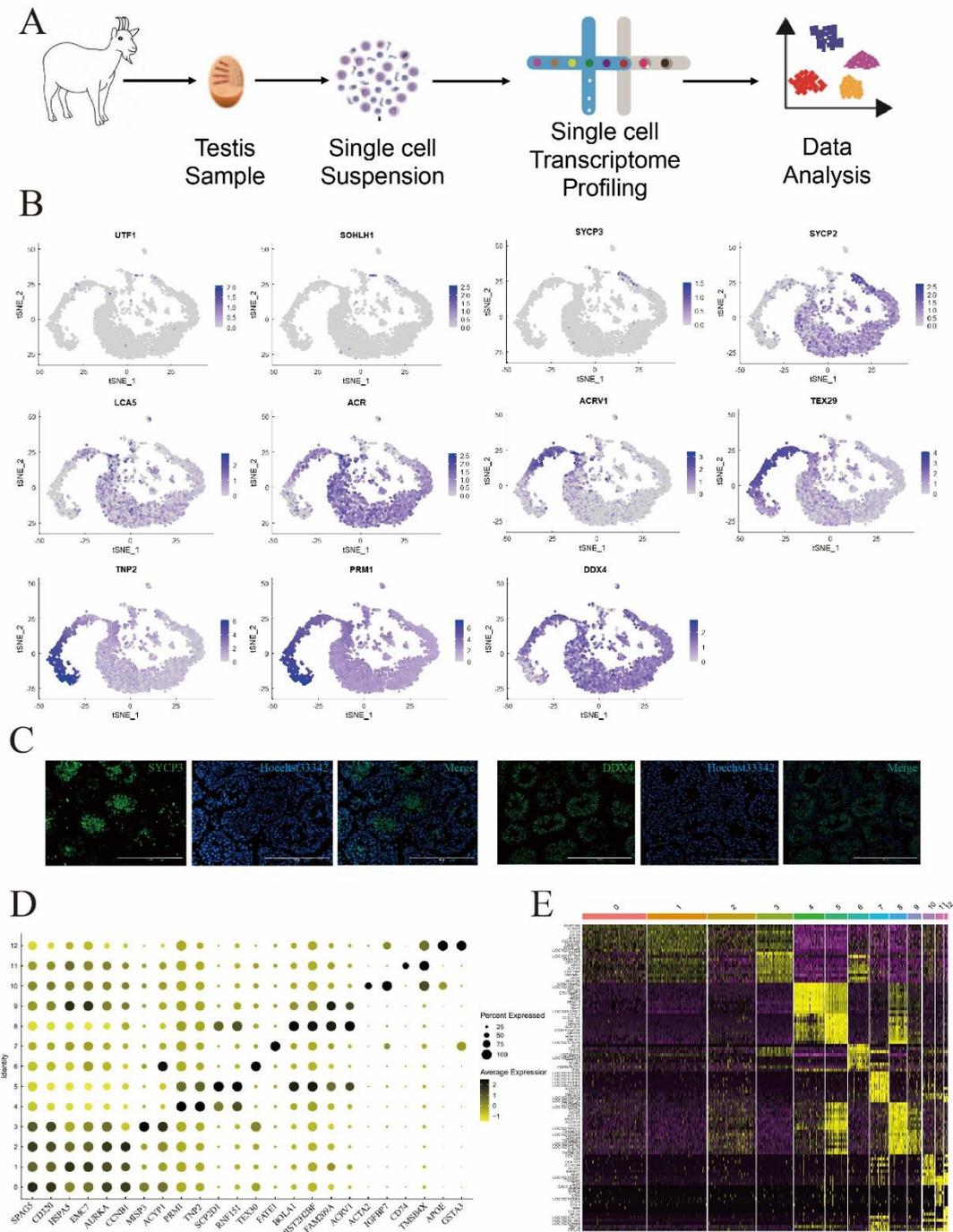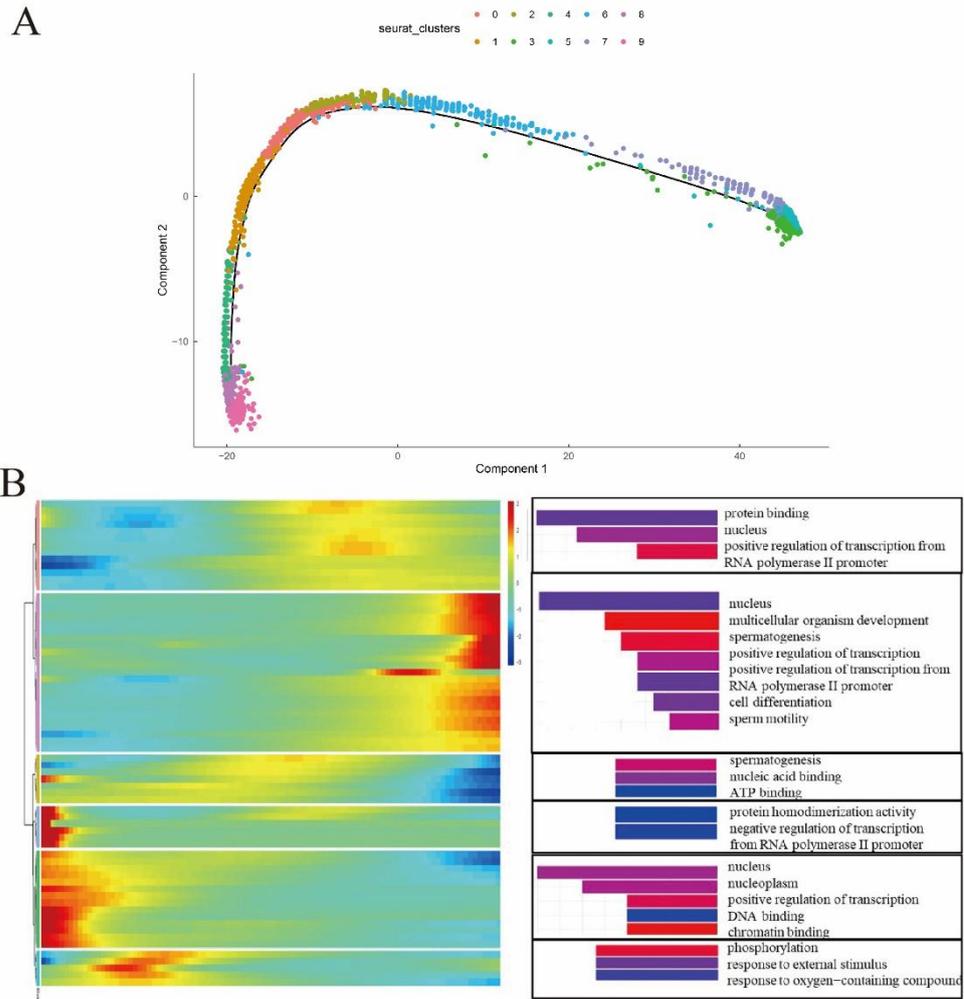
Supplementary Figure S1 Cell map of dairy goat testis by single-cell RNA sequencing. (A) Overview of data collection and experimental workflow. (B) Expression patterns of known conservative markers at different stages projected on t-SNE map. Colors from blue to gray indicate level of expression from high to low. (C)

Section immunofluorescence staining of expression position of SYCP3 and DDX4 (in green) in testis of 100-day-old Guanzhong dairy goat, blue is Hoechst 33342 signal, scale bar = 200 μm. (D) Specific highly expressed genes in each taxon. Colors from black to yellow indicate level of expression from high to low. Size of dot indicates average expression proportion in group. (E) Heatmap of marker genes for each cell type. Different colors at top of heatmap indicate different cell cluster types. Colors from yellow to pink indicate level of expression from high to low.
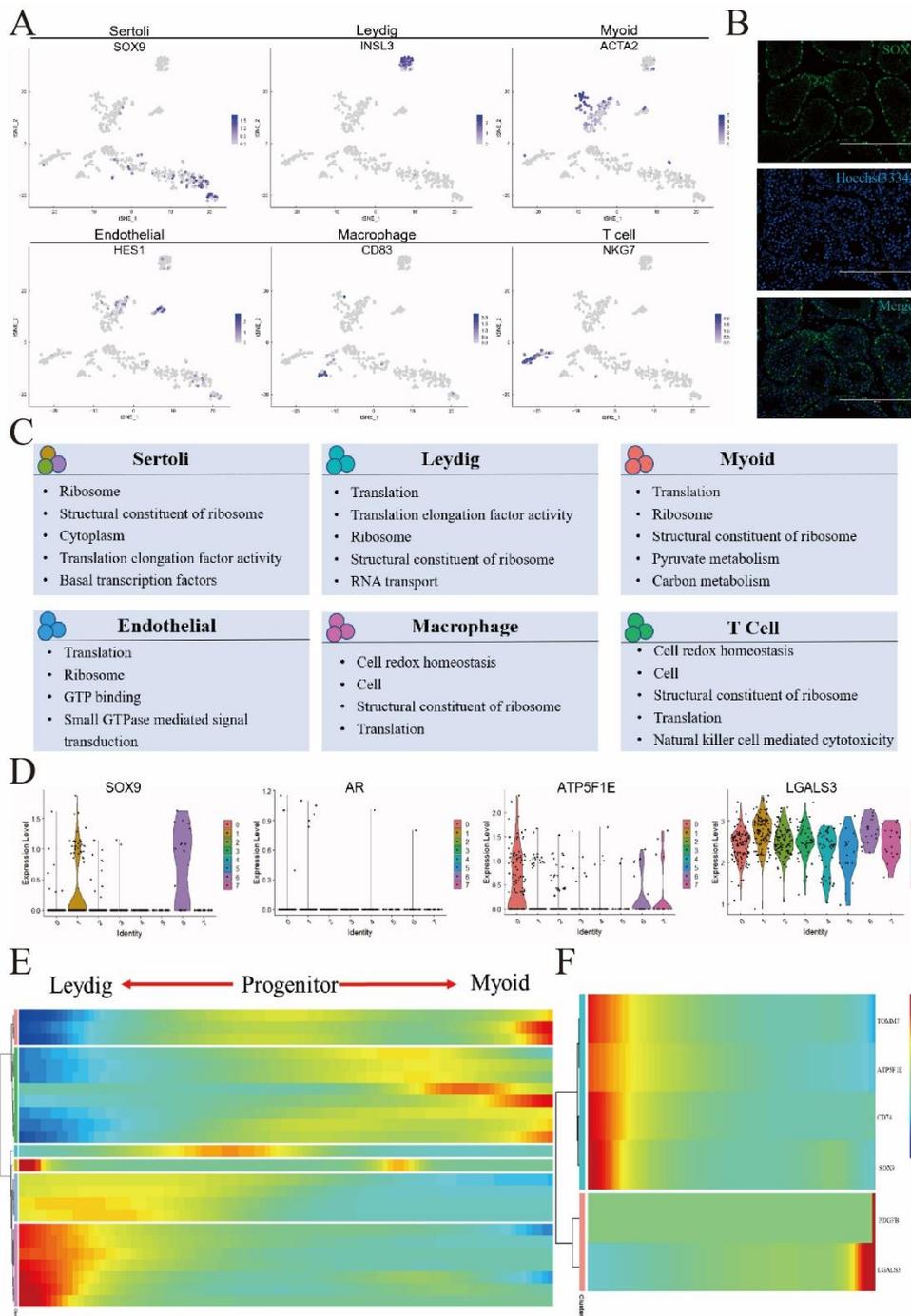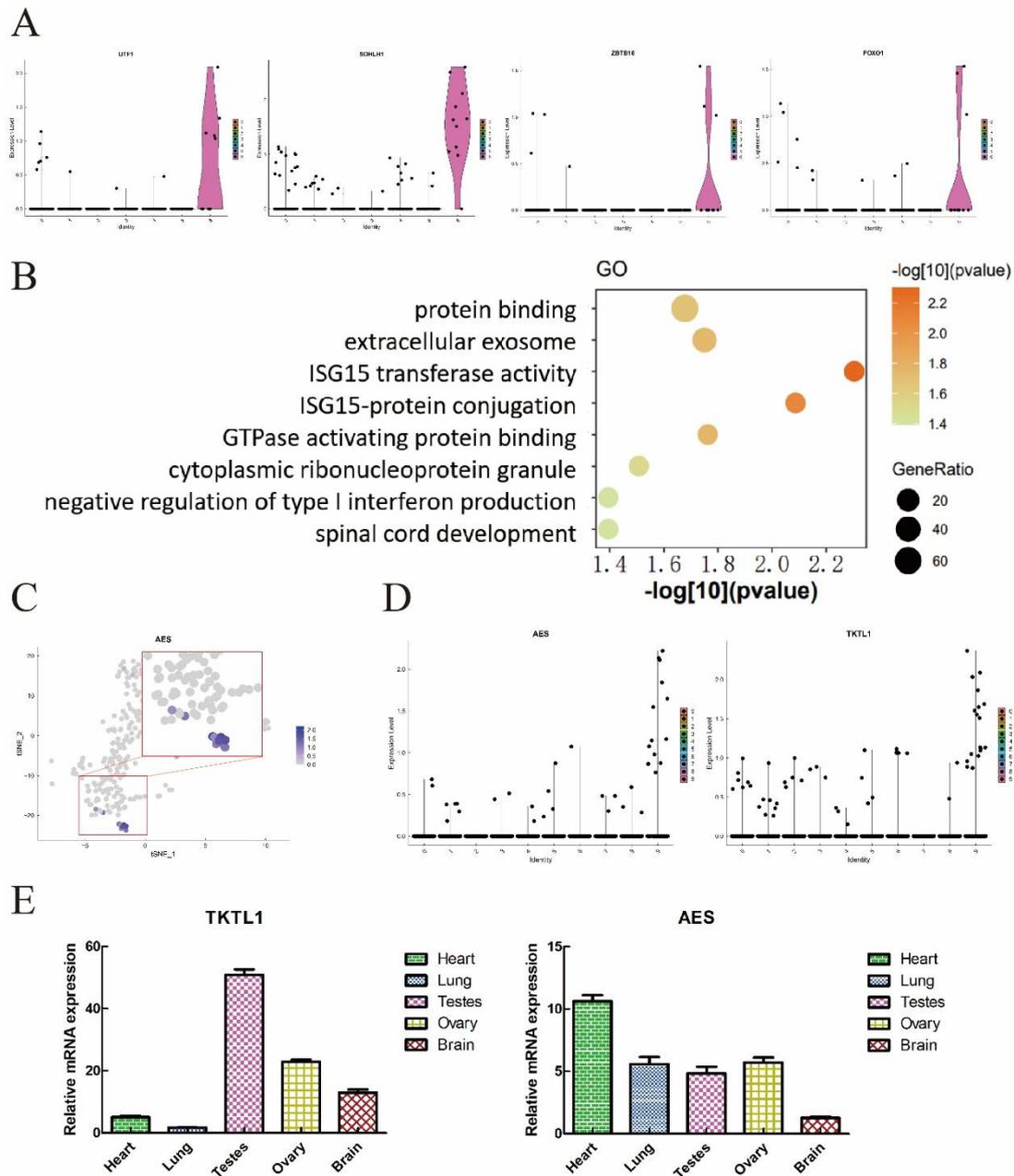
Supplementary Figure S2 Identification and cell trajectory analysis of testicular germ cell clusters in dairy goat. (A) Representative markers of six somatic cell populations. Colors from blue to gray indicate level of expression from high to low. (B) Section immunofluorescence staining of expression position of SOX9 (in green) in testis of 100-day Guanzhong dairy goat, blue is Hoechst 33342 signal, scale bar =

200 μm. (C) Gene Ontology (GO) terms for differentially expressed genes (DEGs) in eight somatic cell clusters. (D) Expression of genes related to Sertoli cells (*SOX9*, *AR*, *ATP5F1E*, and *LGALS3*) after somatic cell re-clustering. Clusters 1, 2, and 6 are Sertoli cell clusters. (E) Heatmap of DEGs corresponding to pseudotime trajectory of Sertoli cells. (F) Heatmap of DEGs corresponding to pseudotime trajectory of Leydig and Myoid cells.

Supplementary Figure S3 Identification and cell trajectory analysis of testicular germ cell clusters in dairy goat. (A) Pseudotime trajectory analysis of germ cell cluster, color of cell cluster corresponds to cell type in Figure 1Ci. (B) Heatmap of DEGs during spermatogenesis according to pseudotime trajectory shown in Figure 1Cii. Left: Representative enrichment genes of six processes. Right: GO terms of representative enriched genes for each cluster.

Supplementary Figure S4 Identification of spermatogonia clusters in dairy goat testis. (A) Distribution and expression of known spermatogonia marker genes (*UTF1*, *SOHLH1*, *ZBTB16* and *FOXO1*) in seven re-clustering cell clusters. Numbers correspond to cell cluster numbers in Figure 1E. Cluster 6 is spermatogonia cluster. (B) GO terms for specific genes expressed in spermatogonia (Cluster 6 in Figure 1E). (C) Expression distribution of *AES* in spermatogonia clusters. (D) *AES* and *TKTL1* gene

distribution and expression levels in all germ cell clusters. (E) Tissue expression

profiles of *AES* and *TKTL1* in dairy goat heart, lung, testis, ovary, and brain.
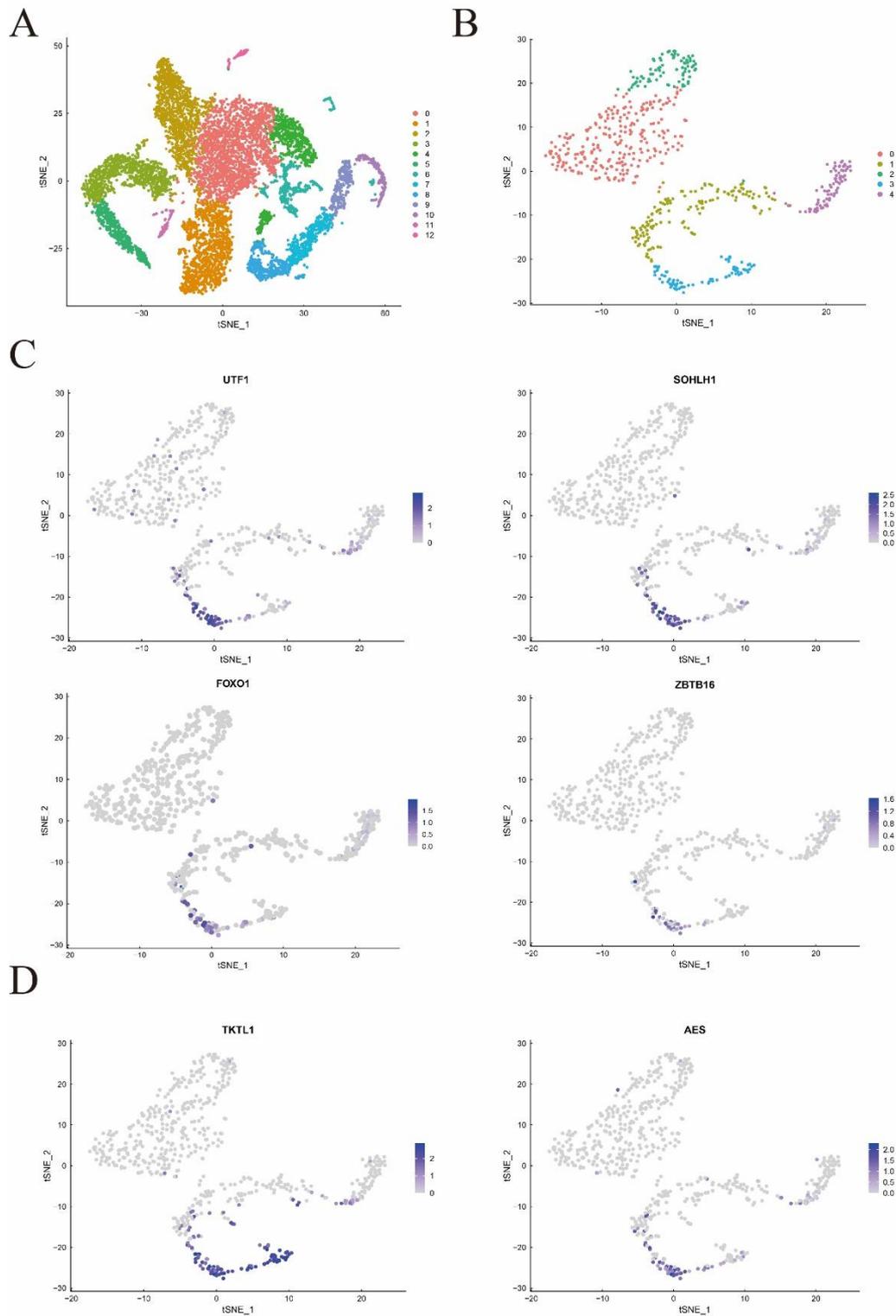
Supplementary Figure S5 Expression of genes in sheep spermatogonia. (A) t-SNE

diagram of somatic cells in testis of dairy goat (cells are colored by 13 broad cell

types). (B) t-SNE diagram of re-clustering of Cluster 6 in (A) (cells are colored by

five broad cell types). (C) Distribution and expression of known spermatogonia

marker genes (*UTF1*, *SOHLH1*, *FOXO1* and *ZBTB16*) in five re-clustering cell clusters. Numbers correspond to cell cluster numbers in (B). Clusters 3 and 4 are spermatogonia. (D) Distribution and expression of *TKTL1* and *AES* in five re-clustering cell clusters.