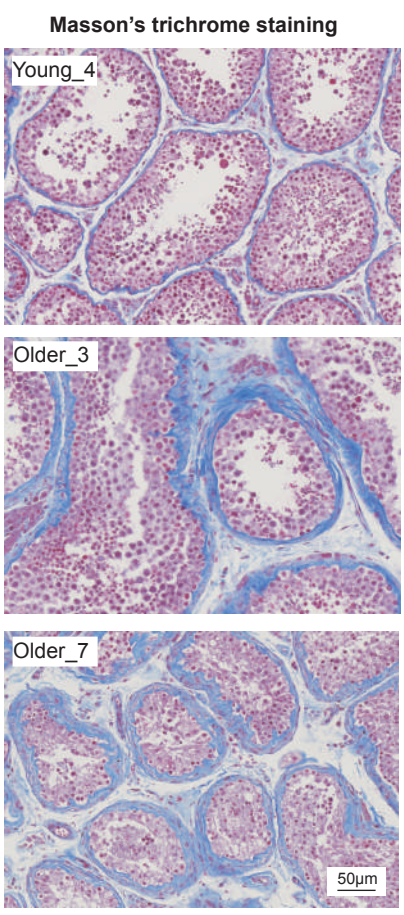
Figure S1

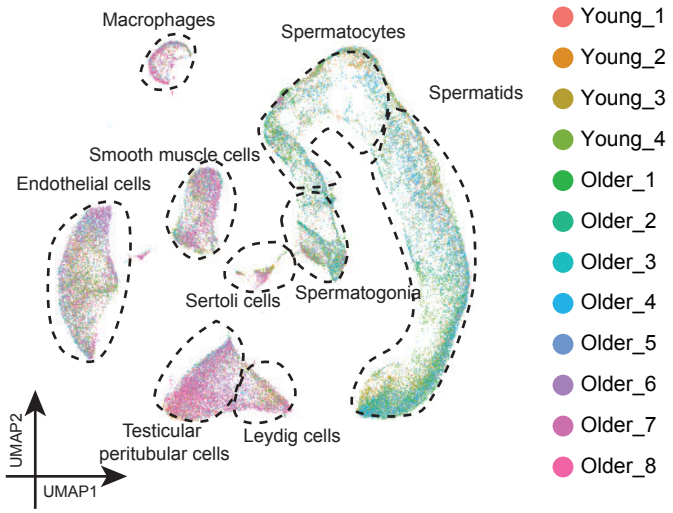
A

Donor ID	Age	Number of filtered cells	Median genes / cell
Young_1	17	2751	2519
Young_2	22	3739	2519
Young_3	22	3446	2507
Young_4	21	3220	1937
Older_1	66	3971	2441
Older_2	66	3423	3039
Older_3	66	4488	2574
Older_4	62	5058	2741
Older_5	66	3405	2769
Older_6	64	4122	2768
Older_7	76	3220	1937
Older_8	67	3814	2847

B



C



D

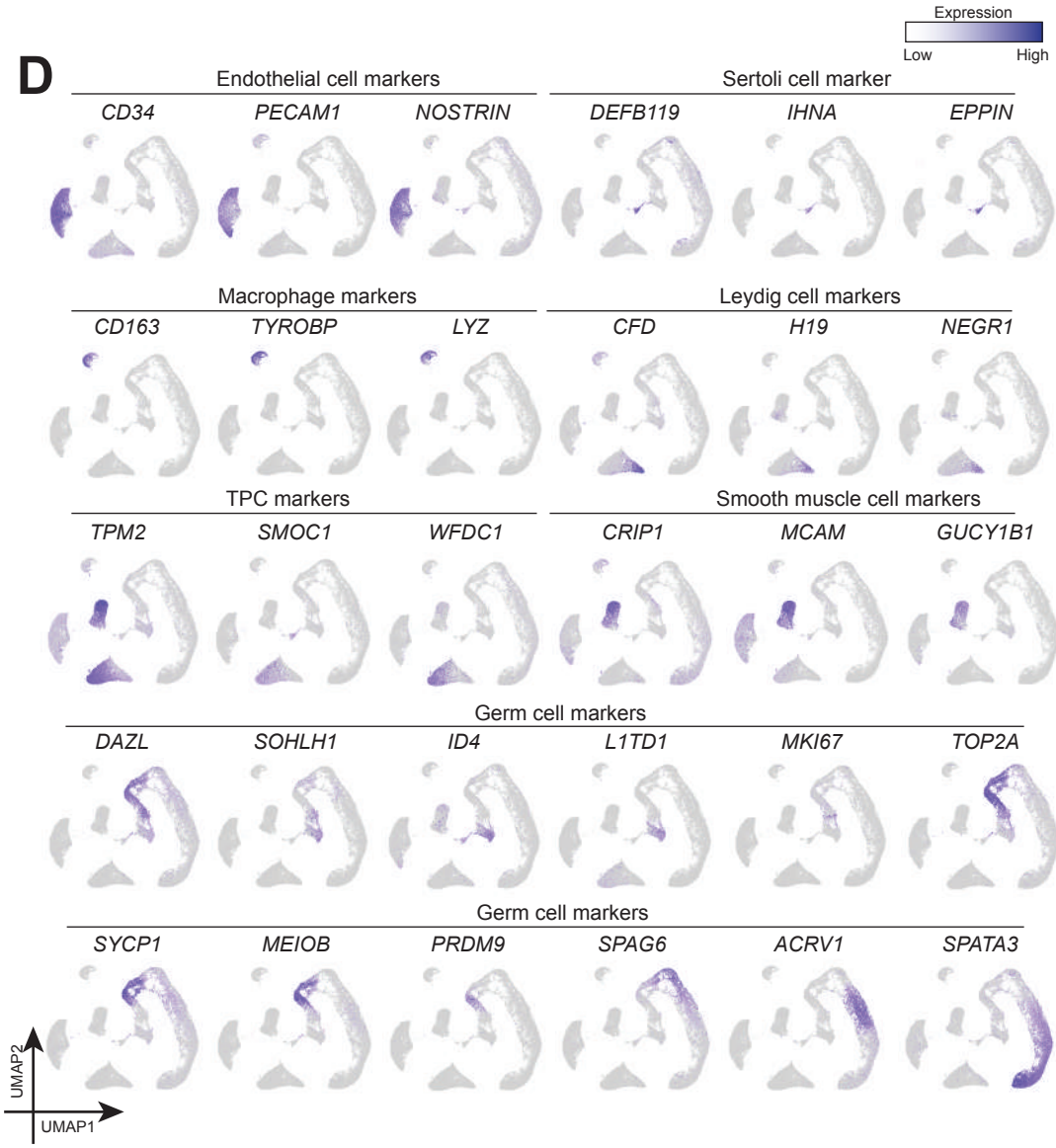


Figure S1. Information about donors and quality control of scRNA-seq

(A) Information summary of 12 donors sequenced in this study.

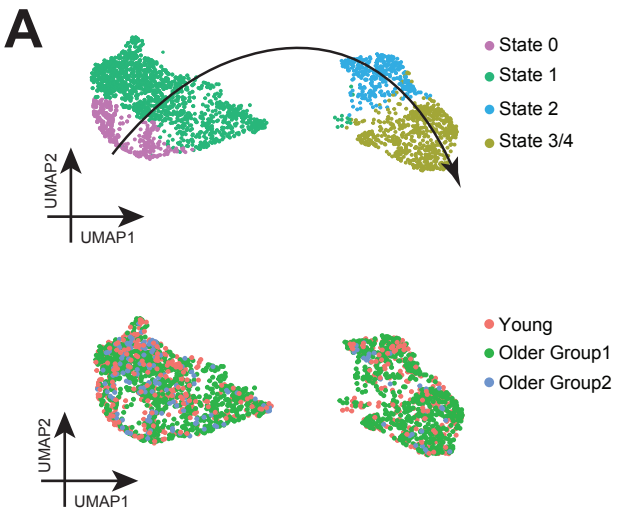
(B) UMAP plot showing major testicular cell types with color according to the donors of origin.

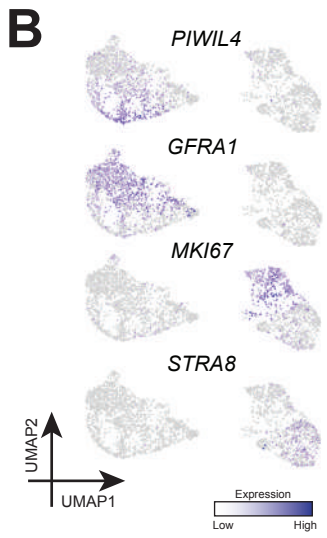
(C) Low magnification of the sections in Fig 1B, showing a thicker wall of seminiferous tubules and more ECM in interstitial tissues in older testis. The boxed regions represent the part of the area shown at the higher magnification in Fig 1B. Scale bar, 50 μm.

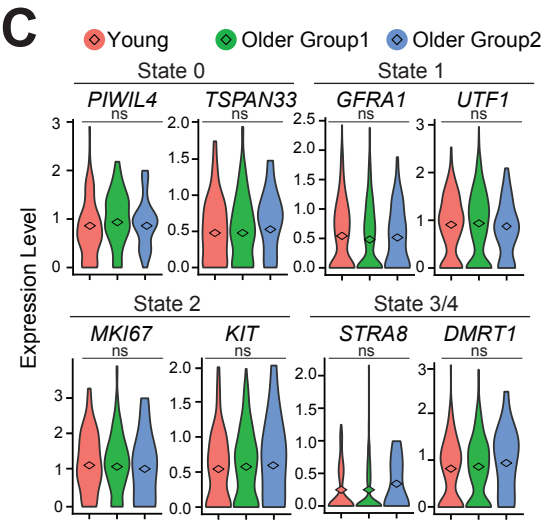
(D) Expression of additional markers identifying major testicular cell types cast on the UMAP plot. Purple (or grey) represent high (or low) expression level as shown on the color key on top right.

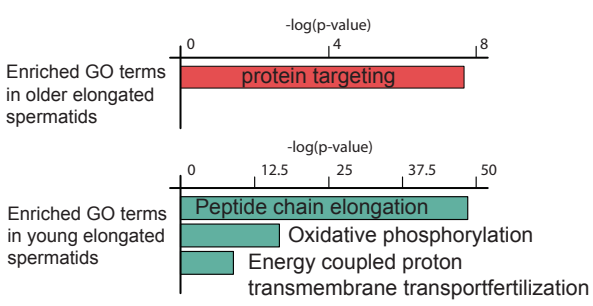
Related to Figure 1.

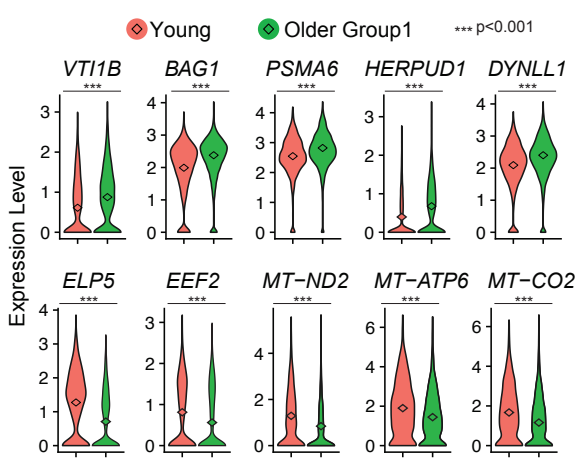
Figure S2

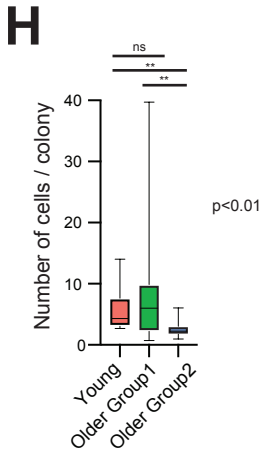

A
State 0
State 1
State 2
State 3/4
UMAP2
UMAP1
Young
Older Group1
Older Group2

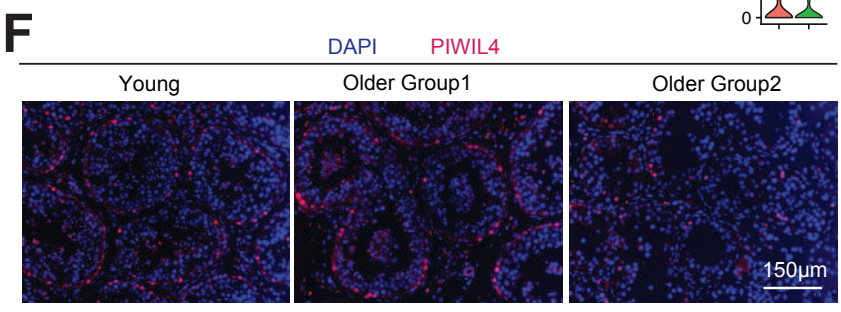

B
PIWIL4
GFRA1
MKI67
STRA8
UMAP2
UMAP1
Expression
Low
High

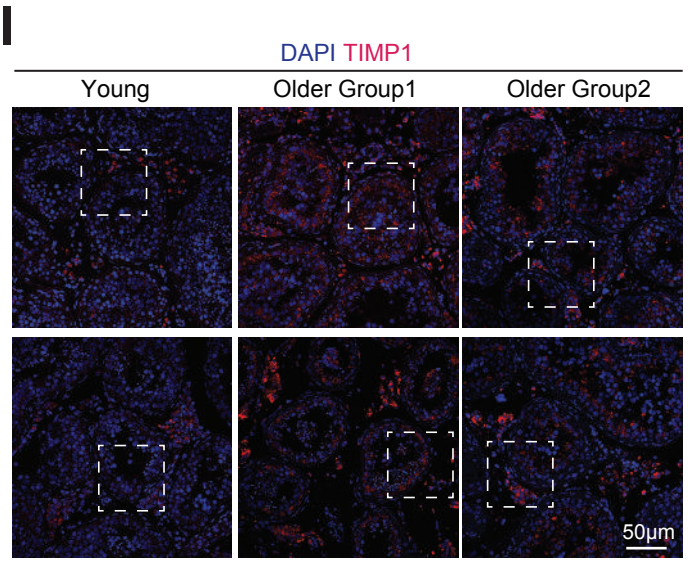

C
Young
Older Group1
Older Group2
State 0
State 1
State 2
State 3/4
PIWIL4
TSPAN33
GFRA1
UTF1
MKI67
KIT
STRA8
DMRT1
ns
Expression Level

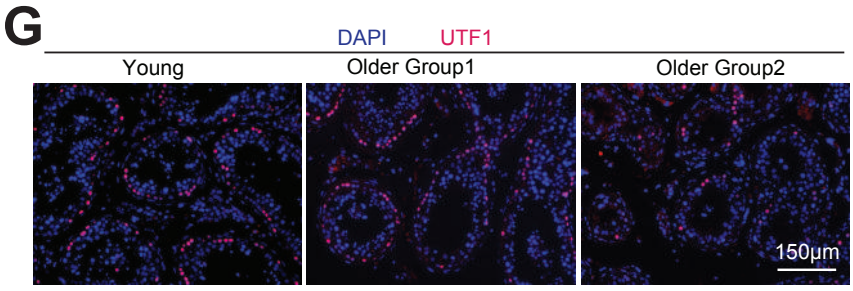

D
-log(p-value)
Enriched GO terms in older elongated spermatids
protein targeting
Enriched GO terms in young elongated spermatids
Peptide chain elongation
Oxidative phosphorylation
Energy coupled proton transmembrane transportfertilization

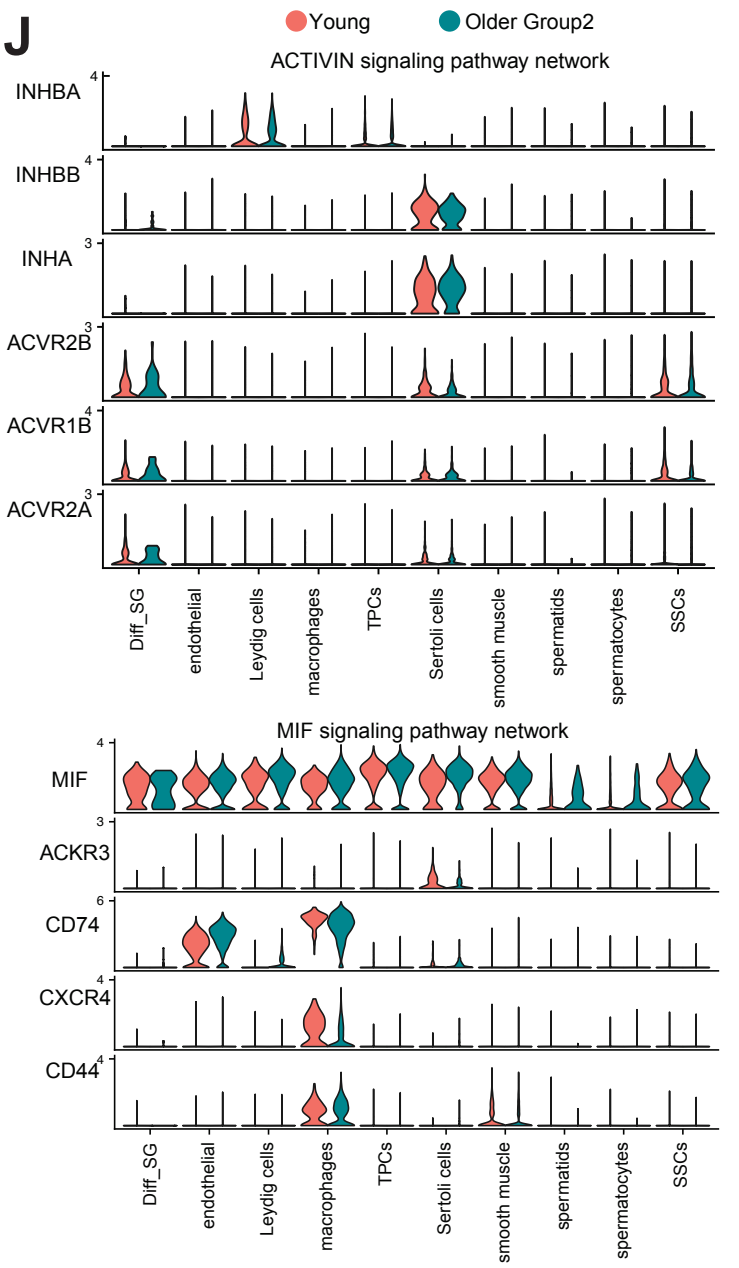

E
Young
Older Group1
*** p<0.001
VTI1B
BAG1
PSMA6
HERPUD1
DYNLL1
ELP5
EEF2
MT-ND2
MT-ATP6
MT-CO2
Expression Level


H
Number of cells / colony
p<0.01
Young
Older Group1
Older Group2


F
DAPI PIWIL4
Young
Older Group1
Older Group2
150μm


I
DAPI TIMP1
Young
Older Group1
Older Group2
50μm


G
DAPI UTF1
Young
Older Group1
Older Group2
150μm


J
Young
Older Group2
ACTIVIN signaling pathway network
INHBA
INHBB
INHA
ACVR2B
ACVR1B
ACVR2A
MIF signaling pathway network
MIF
ACKR3
CD74
CXCR4
CD44
Diff_SG
endothelial
Leydig cells
macrophages
TPCs
Sertoli cells
smooth muscle
spermatids
spermatocytes
SSCs

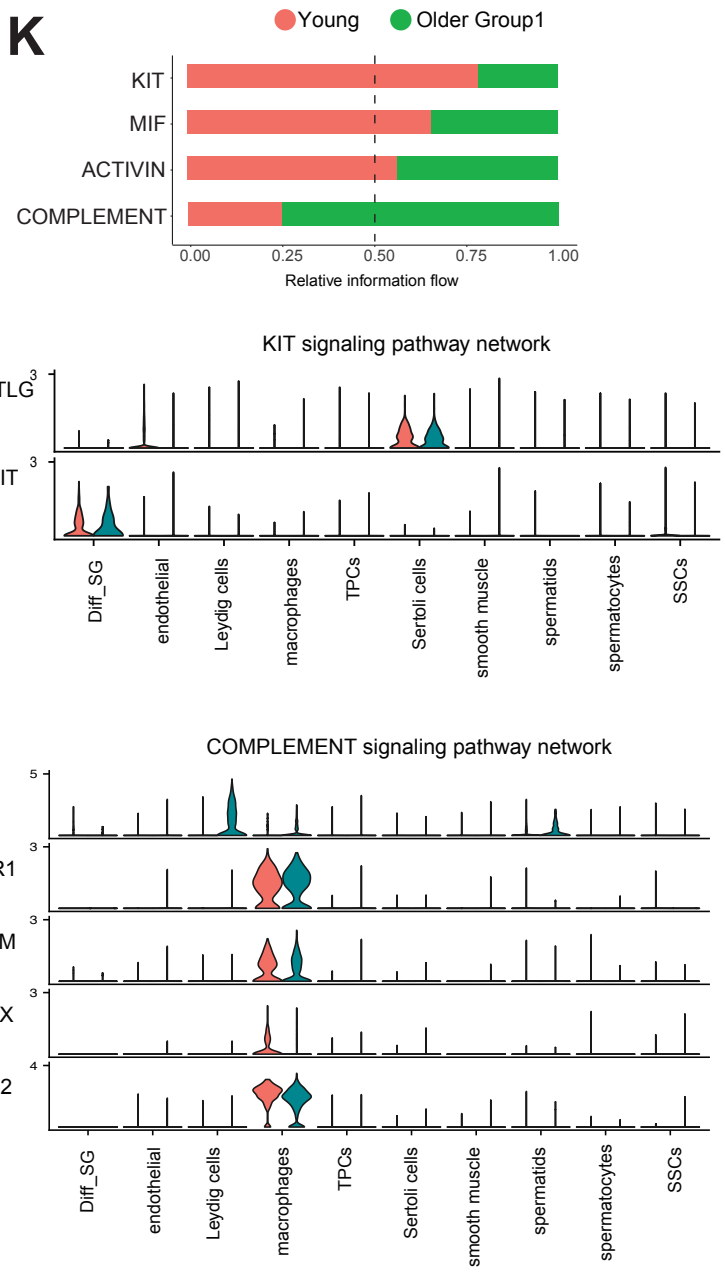

K
Young
Older Group1
KIT
MIF
ACTIVIN
COMPLEMENT
Relative information flow
KIT signaling pathway network
KITLG
KIT
COMPLEMENT signaling pathway network
C3
C3AR1
ITGAM
ITGAX
ITGB2
Diff_SG
endothelial
Leydig cells
macrophages
TPCs
Sertoli cells
smooth muscle
spermatids
spermatocytes
SSCs

Figure S2. Transcriptomic analysis of germ cells and cell-cell communication between young and older testis

(A) UMAP plots showing focused analysis of spermatogonia from Fig 2A. Top: Cells are colored according to five discrete cellular states (States 0 to 4) as described in Guo et al., 2018. The arrow points to the development direction of spermatogonia. Bottom: Cells are colored according to Young versus Older Groups.

(B) Expression of selected markers identifying major cellular states of spermatogonia cast on the UMAP plot. Purple (or grey) represents a high (or low) expression level.

(C) Violin plots showing almost no difference among different groups for each state. The diamond inside the violin plot represents the mean.

(D) Bar plots showing upregulated (top) or downregulated (bottom) GO terms enriched in the DEGs of elongated spermatids when comparing Older Group1 to Young.

(E) Violin plots showing upregulated DEGs of older elongated spermatids on the top panel and downregulated DEGs of older elongated spermatids on the bottom panel. The diamond inside the violin plot represents the mean.

(F) Low magnification of the sections in Fig 2E. Scale bar, 150 μm.

(G) Low magnification of the sections in Fig 2F. Scale bar, 150 μm.

(H) Boxplot showing the average number of cells per colony for each replicate (left/right testis) in the three different age groups. **$p < 0.01$ (two-tailed t-test).

(I) Low magnification of the sections in Fig 3C. The boxed regions represent the part of the area shown at the higher magnification in Fig 3C. Scale bar, 50 μm.

(J) Violin plots showing expression distribution of Activin / KIT / MIF / Complement signaling genes inferred by CellChat.

(K) Bar graph (by CellChat analysis) illustrating representative information flow in Older Group1 (green) and Young (red).

Related to Figure 2 and 3.

Figure S3

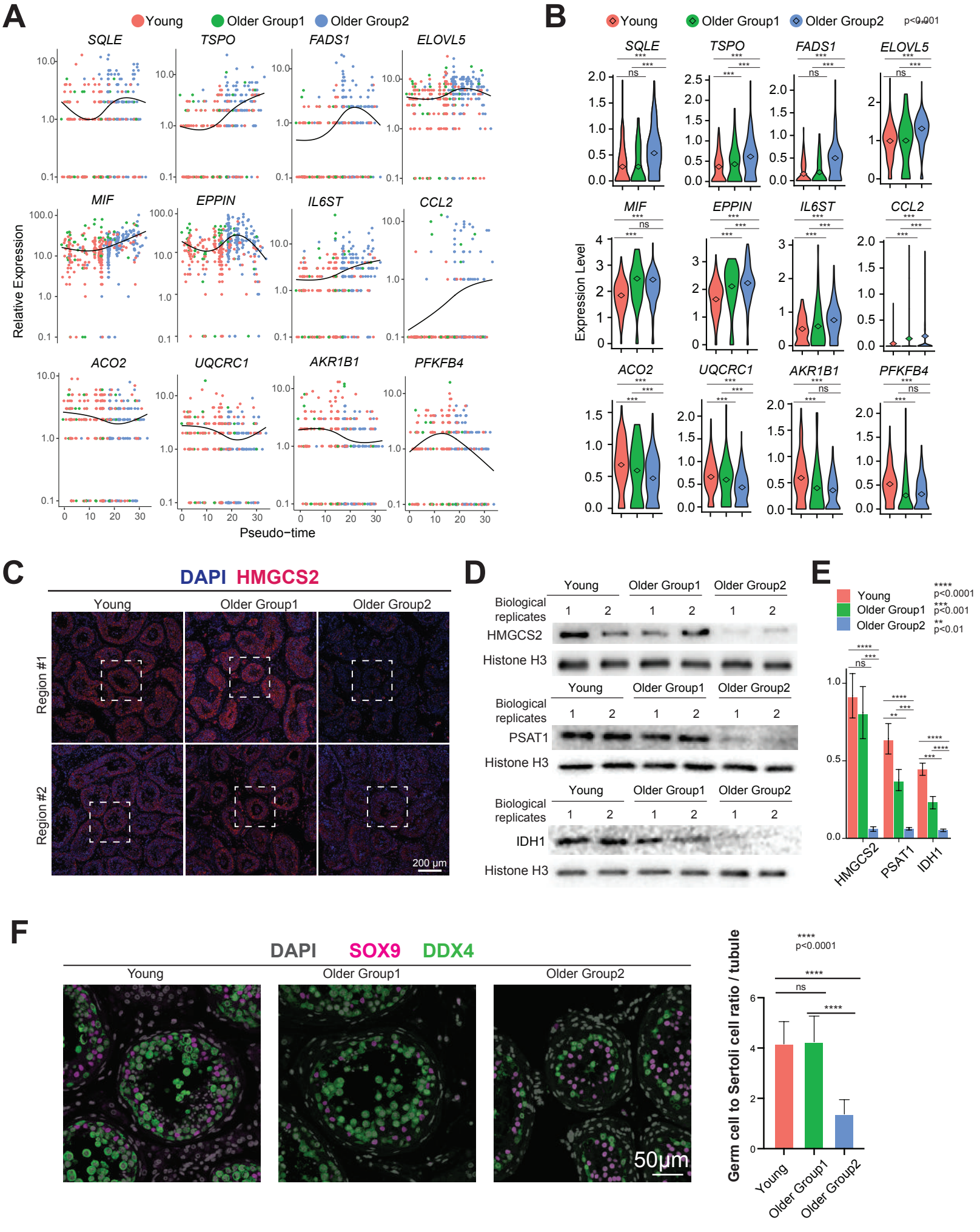

A
Young
Older Group1
Older Group2
SQLE
TSPO
FADS1
ELOVL5
MIF
EPPIN
IL6ST
CCL2
ACO2
UQCRC1
AKR1B1
PFKFB4
Relative Expression
Pseudo−time
B
Young
Older Group1
Older Group2
p<0.001
Expression Level
C
DAPI HMGCS2
Young
Older Group1
Older Group2
Region #1
Region #2
200 μm
D
Young
Older Group1
Older Group2
Biological replicates
HMGCS2
Histone H3
PSAT1
IDH1
E
Young
Older Group1
Older Group2
p<0.0001
p<0.001
p<0.01
HMGCS2
PSAT1
IDH1
F
DAPI SOX9 DDX4
Young
Older Group1
Older Group2
50μm
Germ cell to Sertoli cell ratio / tubule
p<0.0001

Figure S3. Gene expressions related to metabolism and inflammation in older Sertoli cells

(A) Expression levels of selected genes during Sertoli cell aging. The x-axis represents pseudotime as defined in Fig 4B, and the y-axis represents gene expression levels.

(B) Violin plots showing expression levels of selected genes in Fig S3A during Sertoli cell aging. The diamond inside the violin plot represents the mean.

(C) Low magnification of the sections in Fig 4F. The boxed regions represent the part of the area shown at the higher magnification in Fig 4F. Scale bar, 200 μm.

(D) Western blots of HMGCS2, PSAT1, and IDH1. Histone H3 was used for normalization. Total proteins were prepared from whole testicular tissues. Two biological replicates were used for each group.

(E) Bar plots showing the quantification in Fig S3D. Each bar includes the data derived from 2 biological replicates and 2 technical replicates.

(F) Immunofluorescence images of a Sertoli cell marker, SOX9 (magenta) and a germ cell marker, DDX4 (green) in different groups (Young, Older Group1, and Older Group2). Nuclei were counterstained with DAPI (grey). On the left is quantification of germ cell to Sertoli cell ratio per cross-section of seminiferous tubule in different groups, showing a decreased germ cell to Sertoli cell ratio in Older Group2. Bars represent the mean with SD of 20 independent tubules per group. n = 6 human samples. ****$p < 0.0001$ (two-tailed t-test). Scale bar, 50 μm.

Related to Figure 4.

Figure S4

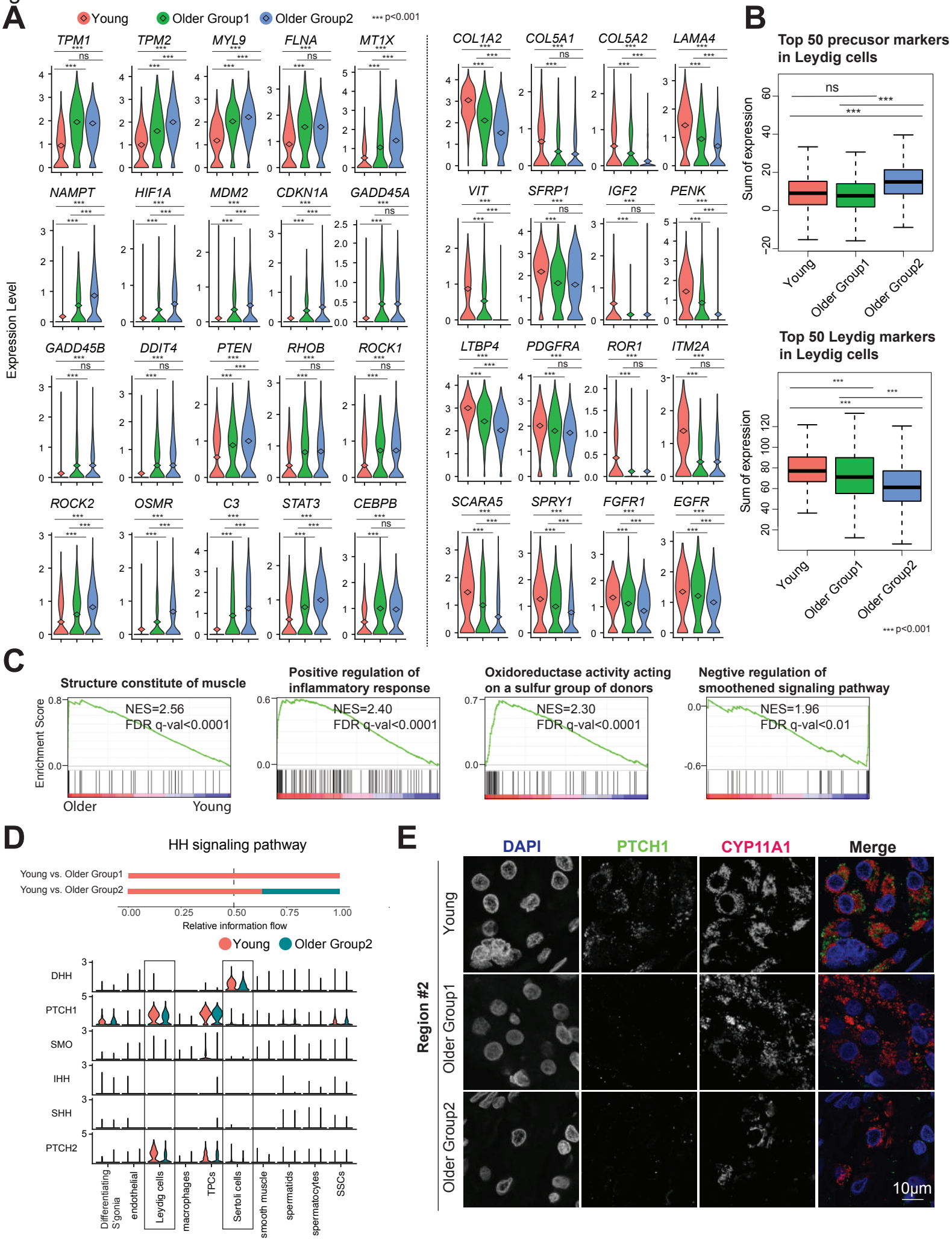

A
Young
Older Group1
Older Group2
*** p<0.001
TPM1
TPM2
MYL9
FLNA
MT1X
COL1A2
COL5A1
COL5A2
LAMA4
NAMPT
HIF1A
MDM2
CDKN1A
GADD45A
VIT
SFRP1
IGF2
PENK
GADD45B
DDIT4
PTEN
RHOB
ROCK1
LTBP4
PDGFRA
ROR1
ITM2A
ROCK2
OSMR
C3
STAT3
CEBPB
SCARA5
SPRY1
FGFR1
EGFR
Expression Level
B
Top 50 precusor markers in Leydig cells
Sum of expression
Young
Older Group1
Older Group2
Top 50 Leydig markers in Leydig cells
*** p<0.001
C
Structure constitute of muscle
NES=2.56
FDR q-val<0.0001
Positive regulation of inflammatory response
NES=2.40
FDR q-val<0.0001
Oxidoreductase activity acting on a sulfur group of donors
NES=2.30
FDR q-val<0.0001
Negtive regulation of smoothened signaling pathway
NES=1.96
FDR q-val<0.01
Enrichment Score
Older
Young
D
HH signaling pathway
Young vs. Older Group1
Young vs. Older Group2
Relative information flow
Young
Older Group2
DHH
PTCH1
SMO
IHH
SHH
PTCH2
Differentiating S'gonia
endothelial
Leydig cells
macrophages
TPCs
Sertoli cells
smooth muscle
spermatids
spermatocytes
SSCs
E
DAPI
PTCH1
CYP11A1
Merge
Region #2
Young
Older Group1
Older Group2
10μm

Figure S4. Signaling pathways are altered in older Leydig cells

(A) Violin plots showing upregulated (left) and downregulated (right) DEGs of older Leydig cells. The diamond inside the violin plot represents the mean.

(B) Overall expression levels of top 50 Leydig/TPC progenitor genes (left) or Leydig genes (right) in Leydig cells of different groups (Young, Older Group1, and Older Group2). Leydig cells of Older Group2 showed a statistically significant increase in the overall expression of Leydig/TPC progenitor genes. Leydig cells of Older Group 1 and 2 showed a progressively decreased expression of Leydig cell markers compared to Young. ***$p < 0.001$ (Wilcoxon test).

(C) Gene Set Enrichment Analysis (GSEA) of Leydig cells in young and older testis. Representative enrichment plots are shown with significant GO terms on the top that correlated with older (red, left) or young (blue, right) Leydig cells. The peak of the green curves (enrichment score curve) on the red (left) side or the blue (right) side represent a positive or negative correlation with Older Leydig cells, respectively. Normalized enrichment score (NES) and Q-value are listed within each plot.

(D) Top: Bar graph analyzed by CellChat illustrating representative information flow in Older and Young. Bottom: Violin plot showing expression distribution of HH signaling genes inferred by CellChat.

(E) Independent experiments of Fig 5D using additional 3 human samples. Scale bar, 10 μm.

Related to Figure 5.

Figure S5

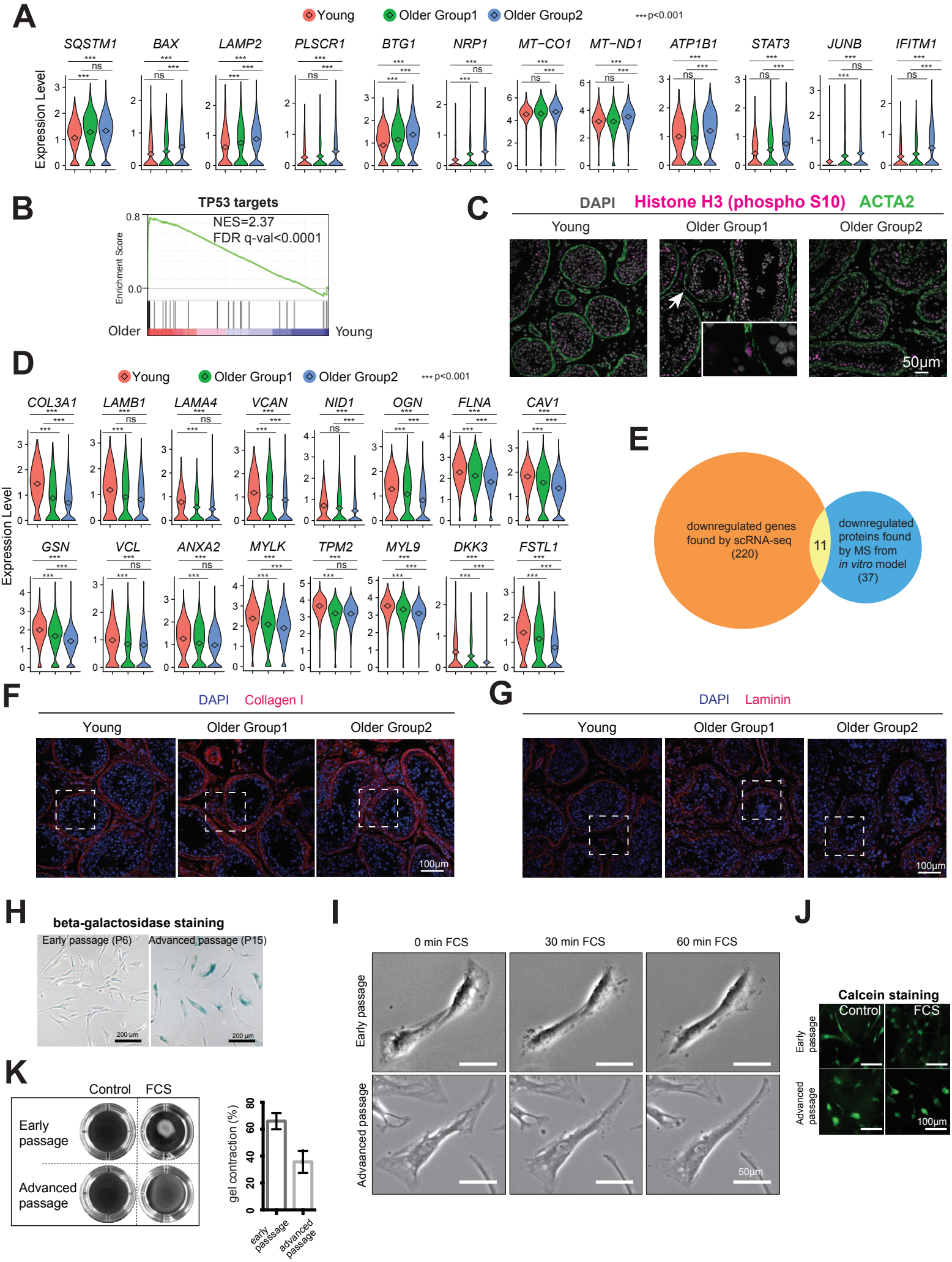

A
Young
Older Group1
Older Group2
***p<0.001
SQSTM1
BAX
LAMP2
PLSCR1
BTG1
NRP1
MT−CO1
MT−ND1
ATP1B1
STAT3
JUNB
IFITM1
Expression Level
B
TP53 targets
NES=2.37
FDR q-val<0.0001
Enrichment Score
Older
Young
C
DAPI
Histone H3 (phospho S10)
ACTA2
Young
Older Group1
Older Group2
50μm
D
Young
Older Group1
Older Group2
***p<0.001
COL3A1
LAMB1
LAMA4
VCAN
NID1
OGN
FLNA
CAV1
GSN
VCL
ANXA2
MYLK
TPM2
MYL9
DKK3
FSTL1
Expression Level
E
downregulated genes found by scRNA-seq (220)
11
downregulated proteins found by MS from in vitro model (37)
F
DAPI
Collagen I
Young
Older Group1
Older Group2
100μm
G
DAPI
Laminin
Young
Older Group1
Older Group2
100μm
H
beta-galactosidase staining
Early passage (P6)
Advanced passage (P15)
200 μm
200 μm
I
0 min FCS
30 min FCS
60 min FCS
Early passage
Advaanced passage
50μm
J
Calcein staining
Control
FCS
Early passage
Advanced passage
100μm
K
Control
FCS
Early passage
Advanced passage
gel contraction (%)
0
20
40
60
80
early passage
advanced passage

Figure S5. Disruption in ECM homeostasis in the testis of older men caused by TPCs and reduced contractile abilities

(A) Violin plots showing upregulated DEGs of older TPCs. The diamond inside the violin plot represents the mean.

(B) GSEA of TPCs in young and older testis. Representative enrichment plots are shown with significant GO terms on the top that correlated with older (red, left) or young (blue, right) TPCs. The peak of the green curves (enrichment score curve) on the red (left) side or the blue (right) side represent a positive or negative correlation with older TPCs, respectively. Normalized enrichment score (NES) and q-value are listed within each plot.

(C) Immunofluorescence images of phospho-Histone H3 (Magenta) with a TPC marker, ACTA2 (green) in different groups (Young, Older Group1, and Older Group2), revealing very few TPCs undergo proliferation. Nuclei were counterstained with DAPI (grey). Arrows indicate a double-positive cell, and a zoom-in figure of the double-positive cell is presented on the bottom right. n = 6 human samples. Scale bar, 50 µm.

(D) Violin plots showing downregulated DEGs of older TPCs. The diamond inside the violin plot represents the mean.

(E) Venn diagram illustrates 11 shared genes/proteins downregulated in scRNA-seq of this study and secreted proteomics data from *in vitro* senescence model of HPTCs via MS reported by Schmid et al.

(F) Low magnification of the sections in Fig 6G. The boxed regions represent the part of the area shown at the higher magnification in Fig 6G. Scale bar, 100 µm.

(G) Low magnification of the sections in Fig 6H. The boxed regions represent the part of the area shown at the higher magnification in Fig 6H. Scale bar, 100 µm.

(H) Staining of senescence associated beta-galactosidase of cells in early (P6) and advanced passage (P15). Note that cell size was increased in most cells in P15. Scale bar, 200 µm.

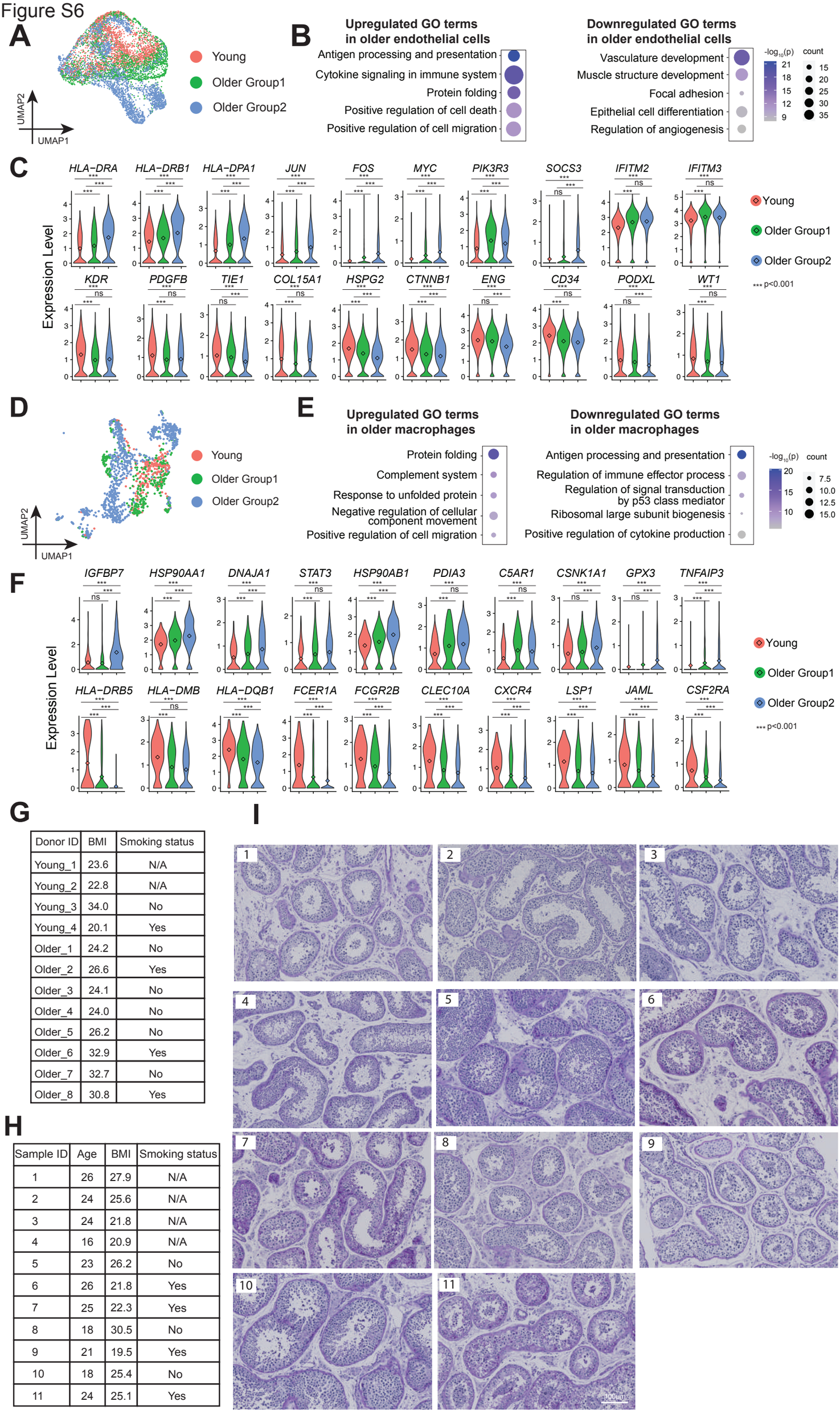
(I) Phase-contrast micrographs of TPCs in early (above, P5) and advanced (below, P15) passages. Cells were monitored for 1 h during treatment with 30% FCS. A time-dependent contraction of individual cells was observed. Considerable reductions of cell surface area are visible in cells in an early passage, in contrast to small changes in cells of an advanced passage. Scale bar, 50 µm.

(J) Example of evaluation of viability of cells in collagen gels directly after gel contraction assay using Calcein AM. Fluorescent cells were scored as alive. Scale bar, 100 µm.

(K) Example of a collagen gel contraction assay for 24 h with TPCs of an early (P5) and advanced (P16) passage. Massive reduction of gel area of early passage after treatment with 30% FCS for 24 h, in advanced passage only slight reduction of the gel area is visible. Gel area remains nearly unchanged under control conditions. One the left is quantification of collagen gel contraction with TPCs after treatment with 30% FCS for 24 h (n = 4). Note massive reduction of gel area of early passages. In contrast, the corresponding cells in an advanced passage have a significantly reduced ability to contract. Bars represent the mean with SD, statistical significance denoted with asterisks (* $p < 0.05$, t-test). Results from corresponding cells of different passages are connected with a line.

Related to Figure 6.

Figure S6



G

Donor ID	BMI	Smoking status
Young_1	23.6	N/A
Young_2	22.8	N/A
Young_3	34.0	No
Young_4	20.1	Yes
Older_1	24.2	No
Older_2	26.6	Yes
Older_3	24.1	No
Older_4	24.0	No
Older_5	26.2	No
Older_6	32.9	Yes
Older_7	32.7	No
Older_8	30.8	Yes

H

Sample ID	Age	BMI	Smoking status
1	26	27.9	N/A
2	24	25.6	N/A
3	24	21.8	N/A
4	16	20.9	N/A
5	23	26.2	No
6	26	21.8	Yes
7	25	22.3	Yes
8	18	30.5	No
9	21	19.5	Yes
10	18	25.4	No
11	24	25.1	Yes

I

Figure S6. Aging-associated changes in testicular endothelial cells and macrophages and testicular histology of donors with varying body mass index (BMI) and smoking status

(A) UMAP plot showing focused analysis of endothelial cells from Fig 1C.

(B) Dot plots showing the top 5 upregulated or downregulated GO terms enriched in the DEGs in older endothelial cells with p-value and gene numbers.

(C) Violin plots showing upregulated DEGs of older endothelial cells within the top panel and downregulated DEGs of older endothelial cells within the bottom panel. The diamond inside the violin plot represents the mean.

(D) UMAP plot showing focused analysis of macrophages from Fig 1C.

(E) Dot plots showing the top 5 upregulated or downregulated GO terms enriched in the DEGs in older macrophages with p-value and gene numbers.

(F) Violin plots showing upregulated DEGs of older macrophages within the top panel and downregulated DEGs of older macrophages within the bottom panel. The diamond inside the violin plot represents the mean.

(G) BMI and smoking status of donors who contributed to the single-cell transcriptome profile in Fig 1A.

(H) BMI and smoking status of 11 young donors other than donors contributing to the single-cell transcriptome profile in Fig 1A.

(I) Periodic acid–Schiff (PAS) staining of testis sections from donors in Fig S6H.

Related to Figure 6.