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Supplemental information

AAV-mediated gene therapy produces fertile offspring in the *Lhcgr*-deficient mouse model of Leydig cell failure

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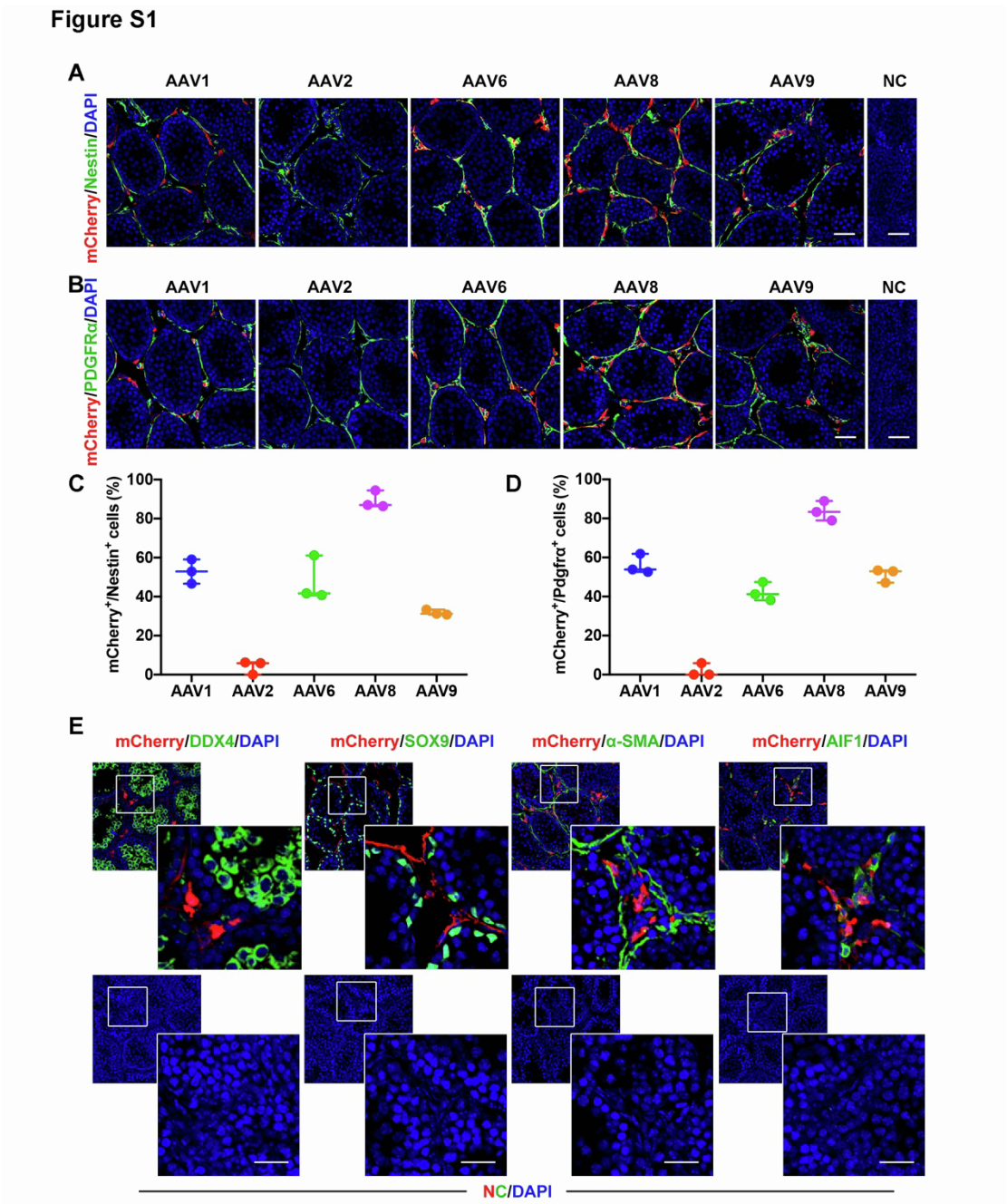

Figure S1. Testicularly injected AAV8 targets progenitor Leydig cells. Related to Figure 1.
(A and B) Representative confocal images of the testicular sections of *Lhcgr*$^{-/-}$ mice injected with AAV-CAG-mCherry (n=3) of the indicated capsid serotypes. The testis tissues were collected and immunostained with progenitor Leydig cells markers (Nestin, PDGFRα) at 7 days after AAV injection. NC indicates negative control. Scale bars: 50 μm. (C and D) Viral transduction rates were determined from the number of mCherry^{+} cells divided by the number of Nestin^{+} or PDGFRα^{+} progenitor Leydig cells. Data are represented by plots, and whiskers are minimum to maximum values. (E) Immunostaining of AAV8-mCherry-injected testes for the germ cell marker DDX4, or the Sertoli cell marker SOX9, peritubular myoid cells marker α-SMA, and macrophage markers AIF1 at 7 days after injection. Nuclei were counterstained with DAPI. NC indicates negative control. Scale bar: 25 μm.

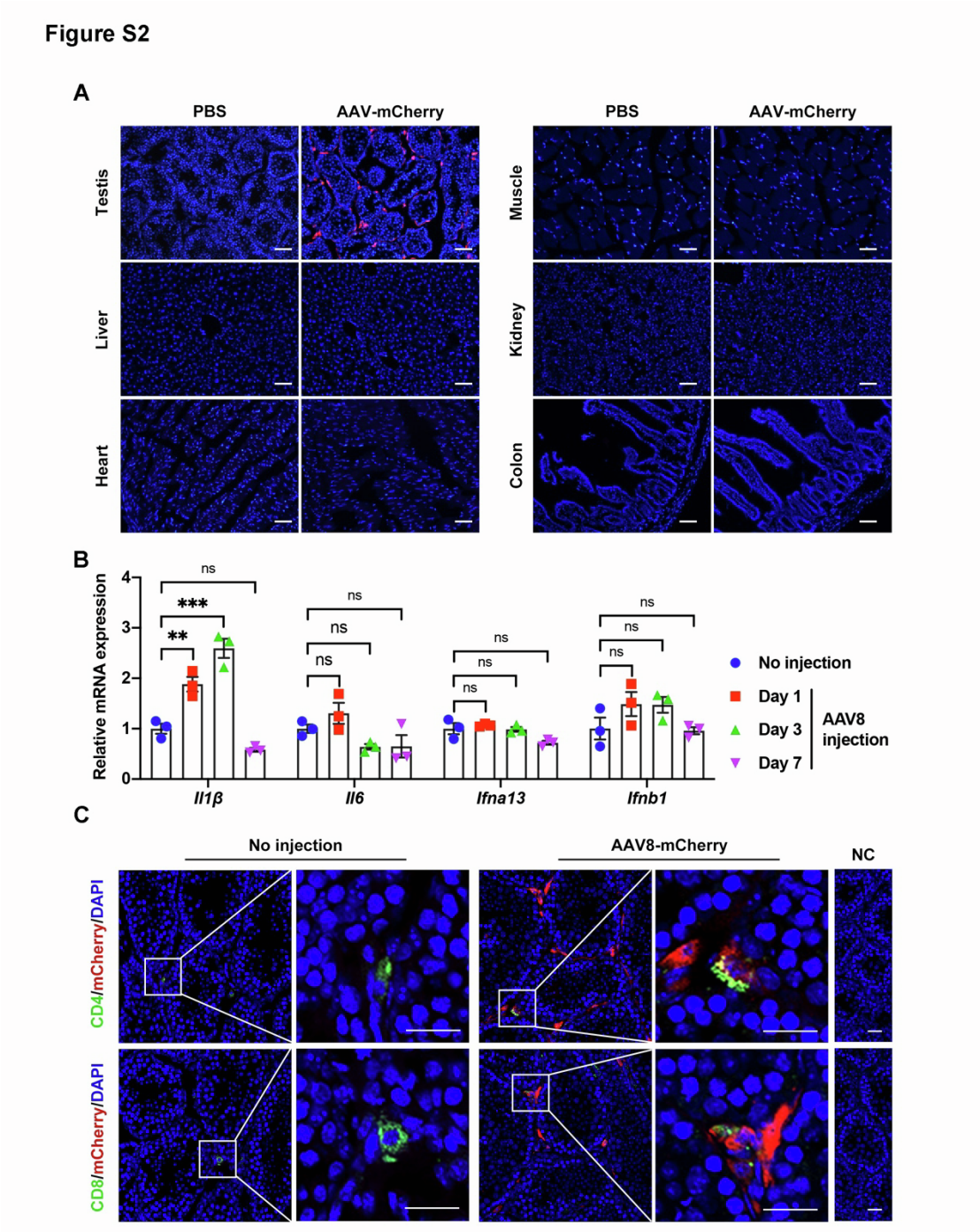

Figure S2. AAV8 showed a clear testis tropism and safety profile with no apparent inflammatory reaction. Related to Figure 1.

(A) Representative fluorescent photographs of mCherry expression (red) in indicated organs of AAV8-mCherry treated mice for 4 weeks. Scale bar: 50 μm. (B) Quantitative RT-PCR was used to analyze key inflammatory markers expression in testes samples collected from *Lhcgr*$^{-/-}$ mice on days 1, 3, and 7 following AAV-mCherry injection, respectively. β-actin was used for normalization. Data are expressed as mean ± sem. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant. (C) Immunostaining of *Lhcgr*$^{-/-}$ mouse testes by CD4 and CD8 antibodies 7 days after microinjection AAV8-mCherry into the interstitium. NC indicates negative control. Scale bar: 50 μm.

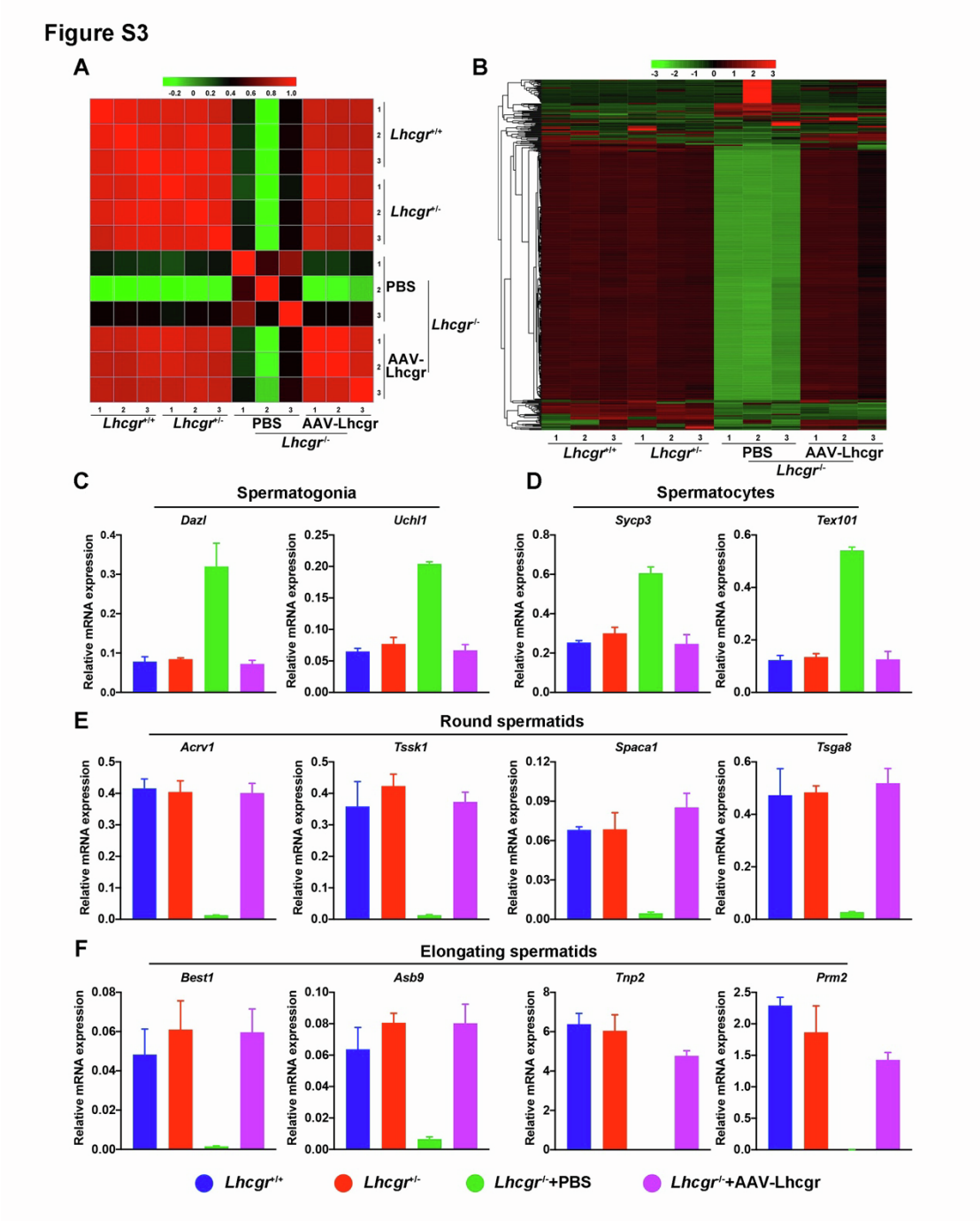

Figure S3. Testicular transcriptome profiles and mRNA expression of specific germ cell marker genes 4 weeks after treatment in pubertal cohort. Related to Figure 4.

(A) Pearson correlation coefficient (PCC) values were calculated to identify the correlation among *Lhcgr*$^{+/+}$ mice, *Lhcgr*$^{+/-}$ mice, and *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=3). Each symbol represents an individual sample. (B) Heat map of top 500 gene expression profiles in the testes of *Lhcgr*$^{+/+}$ mice, *Lhcgr*$^{+/-}$ mice, and *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=3). The color code indicates z-score-normalized expression values. (C-F) Quantitative RT-PCR analysis was performed in testes samples collected from *Lhcgr*$^{+/+}$ mice, *Lhcgr*$^{+/-}$ mice, and *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=3) at 4 weeks after treatment. The expression levels of marker genes for spermatogonia (*Dazl*, *Uchl1*), spermatocytes (*Sycp3*, *Tex101*), round spermatids (*Acrv1*, *Tssk1*, *Spaca1*,

and *Tsga8*), and elongating spermatids (*Best1*, *Asb9*, *Tnp2*, and *Prm2*) were detected from each group. Data are expressed as mean ± sem.

Figure S4

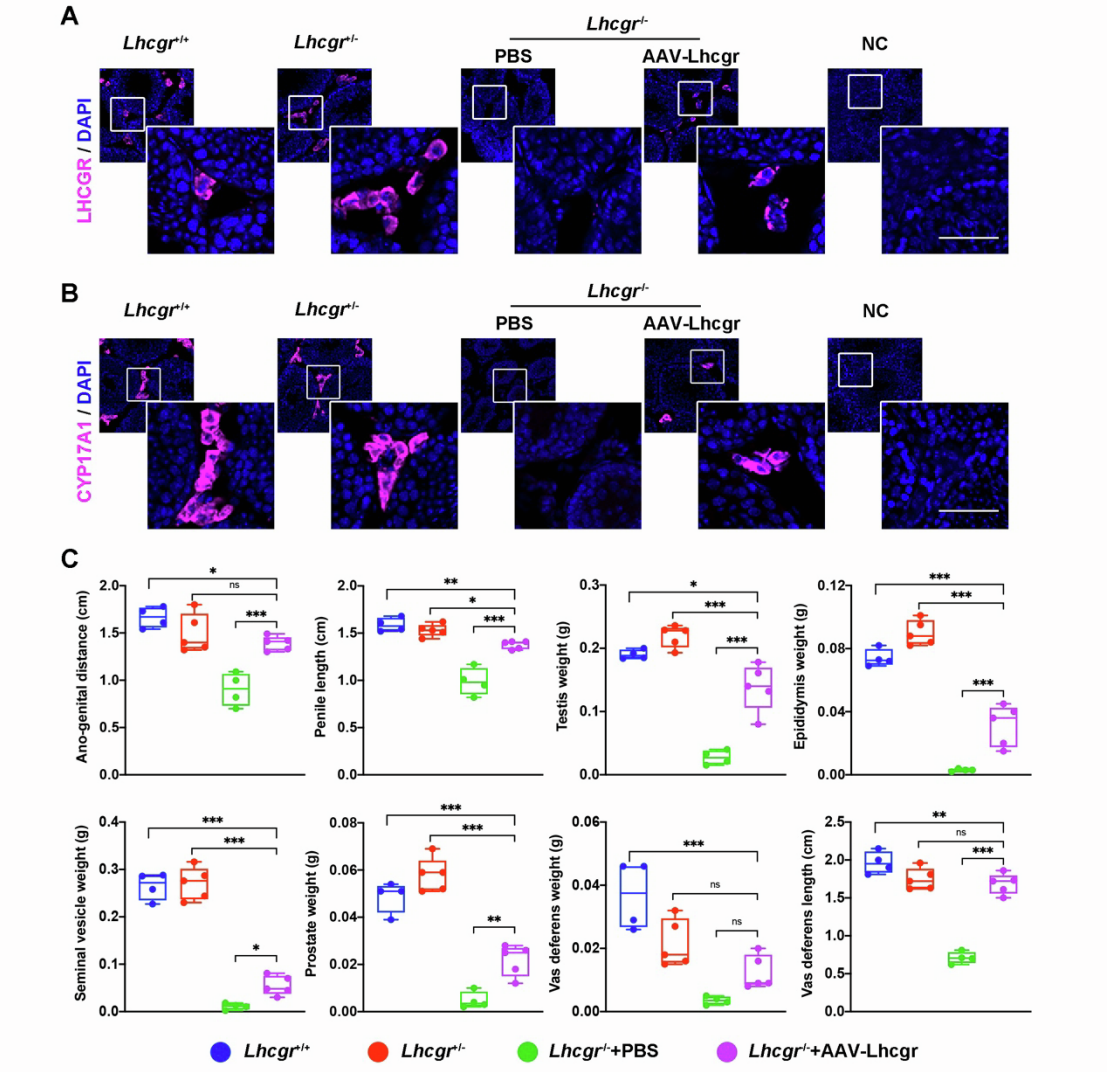

Figure S4. Testicular injection of AAV8-Lhcgr rescues Leydig cell function and restarts sexual development in 8-week-old adult *Lhcgr*$^{-/-}$ mice. Related to Figure 7.

(A) Representative images of LHCGR expression in the testicular interstitium at 4 weeks after treatment in 8 weeks *Lhcgr*$^{+/+}$ mice, *Lhcgr*$^{+/-}$ mice, and *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=4). Nuclei were counterstained with DAPI. NC indicates negative control. Scale bar: 50 μm. (B) The Leydig cells marker, CYP17A1, was evaluated in the testicular interstitium by immunofluorescence assay at 4 weeks after treatment in *Lhcgr*$^{+/+}$ mice, *Lhcgr*$^{+/-}$ mice, and *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=4). NC indicates negative control. Scale bar: 50 μm. (C) The ano-genital distance, penile length, testis weight, epididymis weight, seminal vesicle weight, prostate weight, vas deferens weight, and vas deferens length of *Lhcgr*$^{+/+}$ mice (n=4), *Lhcgr*$^{+/-}$ mice (n=5), *Lhcgr*$^{-/-}$ mice injected with PBS (n=4) or AAV8-Lhcgr (n=5) at 4 weeks after treatment. Data are represented by box plots, and whiskers are minimum to maximum values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

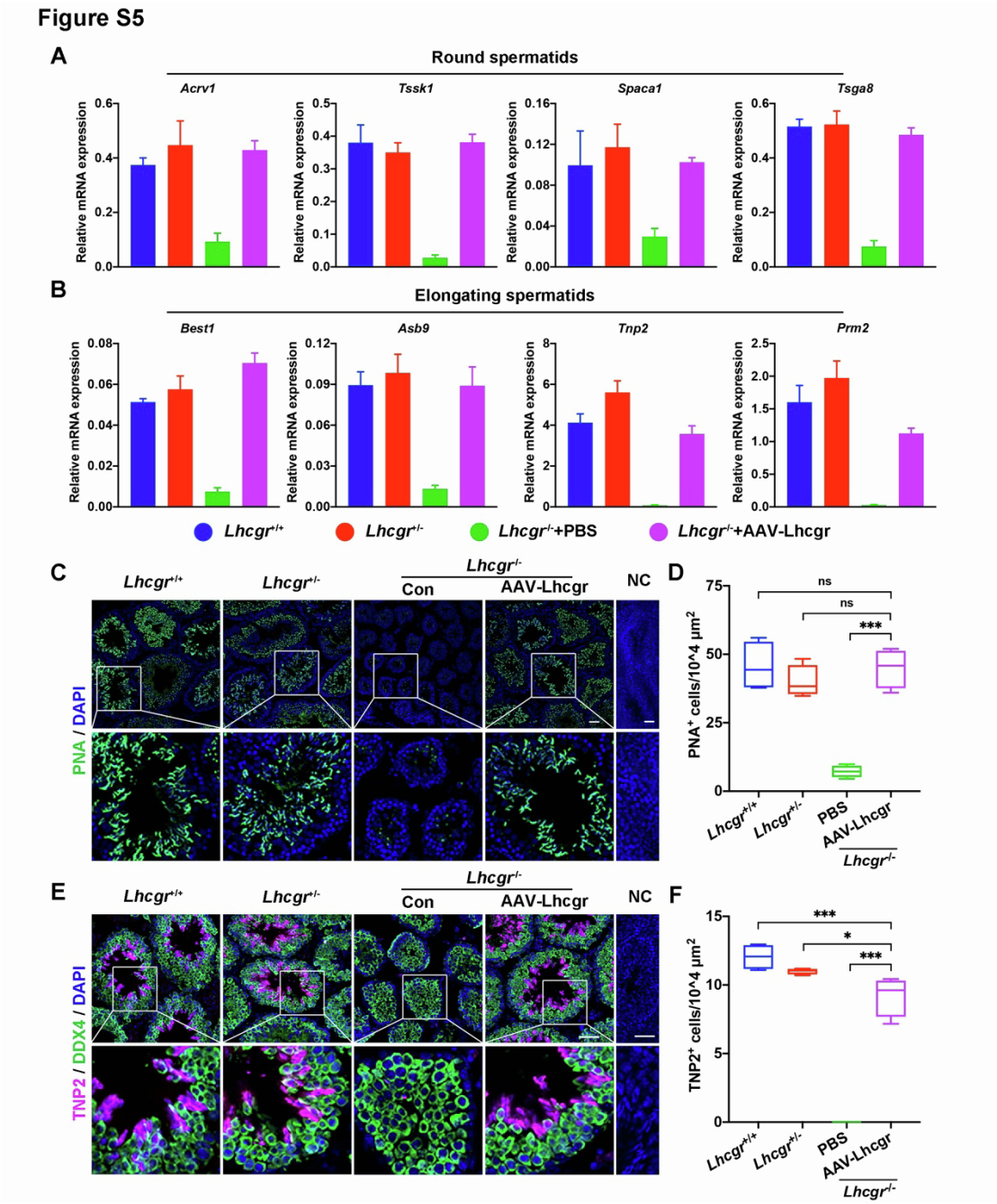

Figure S5. AAV8-Lhcgr promotes the formation of elongating spermatids in 8-week-old adult *Lhcgr*$^{-/-}$ mice. Related to Figure 7.

(A and B) Quantitative RT-PCR was used to analyze marker expression in testes samples collected from *Lhcgr*$^{+/+}$ mice, *Lhcgr*$^{+/-}$ mice, and *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr 4 weeks after treatment (n=3). The expression levels of marker genes for round spermatids (*Acrv1*, *Tssk1*, *Spaca1*, and *Tsga8*) and elongating spermatids (*Best1*, *Asb9*, *Tnp2*, and *Prm2*) were detected for each group. Data are expressed as mean ± sem. (C-F) Representative images of testis sections from *Lhcgr*$^{+/+}$ mice, *Lhcgr*$^{+/-}$ mice, and *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=4). Sections were immunostained for PNA I, DDX4 and TNP2 (E), and counterstained with DAPI. Quantitative analysis showing the percentages of PNA^{+} (D) and TNP2^{+} (F) germ cells in the seminiferous tubules of the testes. NC indicates negative control. Scale bars: 50 μm. * $p < 0.05$, *** $p < 0.001$, ns = not significant.

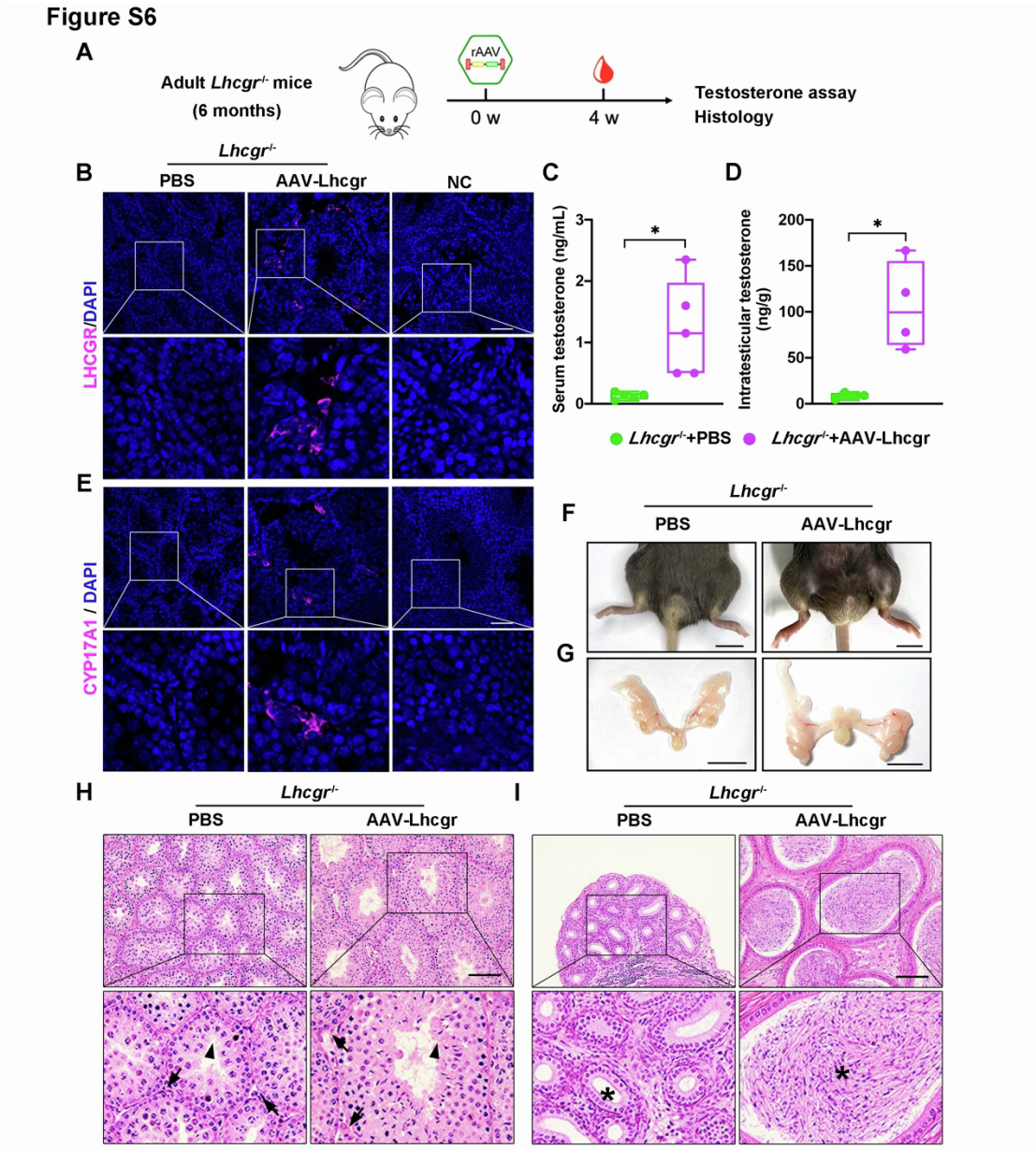

Figure S6. AAV8-Lhcgr recovers testosterone production and rescues spermatogenesis in 6-month-old adult *Lhcgr*$^{-/-}$ mice. Related to Figure 7.

(A) Experimental overview for the *in vivo* studies. (B) Representative images of LHCGR expression in the testicular interstitium at 4 weeks after treatment in 6 months *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=4). Nuclei were counterstained with DAPI. NC indicates negative control. Scale bar: 50 µm. (C and D) The concentrations of serum (C; n=5) and intratesticular (D; n=4) testosterone were analyzed 4 weeks after treatment in 6 months old *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=5). (E) Representative images of CYP17A1 expression in the testicular interstitium at 4 weeks after treatment in 6 months *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=4). Nuclei were counterstained with DAPI. NC indicates negative control. Scale bar: 50 µm. (F and G) Representative photographs of external (F) and internal genitalia (G) of 6 months *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=4) at 4 weeks after treatment. Scale bar: 1 cm. (H and I) Representative light micrographs of testes sections (H) and cauda epididymis (I) from *Lhcgr*$^{-/-}$ mice injected with PBS or AAV8-Lhcgr (n=4). Samples were taken 4 weeks after treatment. Arrows indicates Leydig cells and

arrowheads indicates full spermatogenesis in testis (H). Stars indicates spermatozoa in the cauda epididymis (I). Scale bars: 100 μm. Data are represented by box plots, and whiskers are minimum to maximum values. * $p < 0.05$.

Figure S7

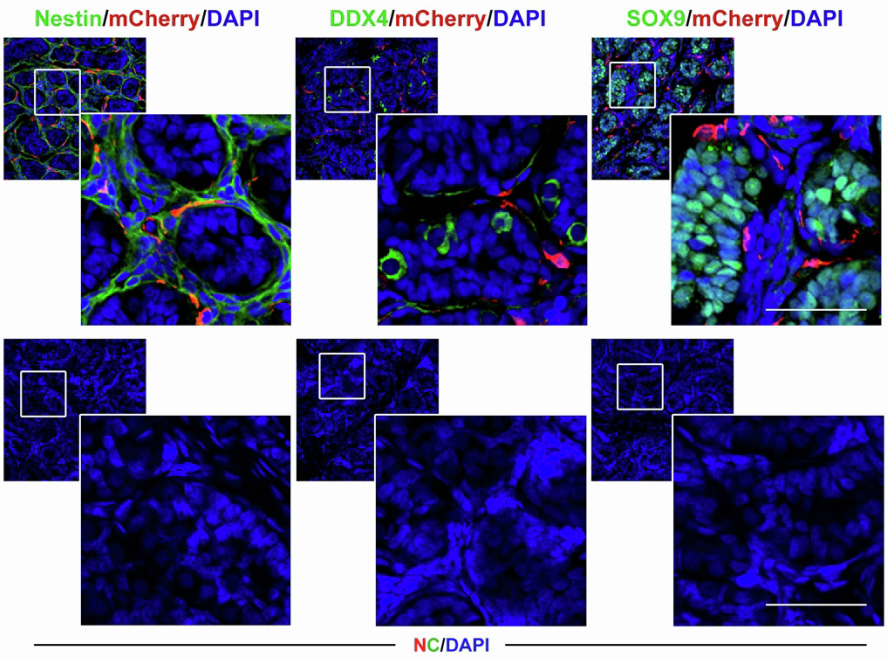

Figure S7. Testicularly injected AAV8 targets progenitor Leydig cells in non-human primates. Related to Figure 7.

Representative confocal images of the testicular sections of pubertal male cynomolgus monkeys injected with AAV8-CAG-mCherry (n=2). The testicular tissues were collected and immunostained with progenitor Leydig cells markers (Nestin), germ cell marker (DDX4), and Sertoli cell marker (SOX9) at 7 days after injection. Nuclei were counterstained with DAPI. NC indicates negative control. Scale bar: 50 μm.

Table S1. Development of embryos generated via IVF with sperm from AAV-Lhcgr-treated *Lhcgr*-/- mice in pubertal cohort. Related to Figure 5.

Lhcgr$^{-/-}$ male mice	No. of oocytes	No. of 2-cell (%)	No. of embryos transferred	No. of pseudo-pregnant mice	No. of pups (%)
M03	139	59 (42.4%)	40	2	20 (50%)
	79	31 (39.2%)	31	2	3 (9.6%)
M18	144	18 (12.5%)	18	1	10 (55.6%)
	361	70 (19.4%)	60	3	25 (41.7%)

Table S2. Primers used to amplify the transcripts in PCR analysis. Related to Star methods.

Primers for Quantitative RT-PCR

Gene	Forward Primer	Reverse Primer
Lhcgr	CACTCTCCAGAGTTGTCAGGG	GAGGTTTGTAAAAGCACCGGG
Dazl	CTTCATCAGCAACCACAA	TTCATCCATCCTAACATCAAT
Uchl1	TGGAATTTGAGGATGGAT	AACACTTGGCTCTATCTT
Sycp3	CGCTGAGCAAACATCTAAAGA	CAACCAAAGGTGGCTTCC
Tex101	TACCTTTAACTGGACTTCA	CCATCTGCTTTAATCAACA
Acrv1	CAGGTGAACAGGTGTCTA	CAGATGTGCTTGGAAGTG
Tssk1	CAAGGACTTCAACATCAA	GGTCTTGCTTAATATCAGT
Spaca1	ATTCACCGTCTATACAAC	CAGATAATGACTCCTATGG
Tsga8	GTGAAGCCTATAATGCCAAT	ACCCTTTCCACAAAGAATG
Asb9	ACTATAACATCAGCCATC	CCTTGATTCACAGATACT
Best1	AACTTGAACATTCCAGAG	TCATTAGAGCCTGTATATTG
Prm2	GGACTATGGGAGGACACA	ATCCTATGTAGCCTCTTACGA

Tnp2	AAAGTGAGCAAGAGAAAGG	TTGTATCTTCGCCCTGAG
Il1β	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
Il6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Ifnb1	CAGCTCCAAGAAAGGACGAAC	GGCAGTGTAACTCTTCTGCAT
Ifna13	AGGATGTGACCTGCCTCAGACT	CACCTTCTCCTGTGGGAATCCA
Primers for Genotyping		
Lhcgr wild type	TGACCTGTTCCTGGGGCTGCTG	AAATGGCCTCAACGGGTGTGCA
Lhcgr mutant type	ATGGGATCGGCCATTGAACAAG	TCAGAAGAACTCGTCAAGAAGGC
Primers for Integration Assay		
Lhcgr	AGCTAATGCCTTTGACAACCTC	CGAGATTAGCGTCGTCCCAT
CAG	TTCGGCTTCTGGCGTGTGA	GGTGAGAGATAGTCGGGCG
Primers for Probe Synthesis		
AAV vector	TCCTGTCCCCTCAGTTCA	AATTGCATTCATTTTATGTTTC