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Supplemental Information

Lovastatin promotes the self-renewal of murine and primate spermatogonial stem cells

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Supplementary Information

Figure S1

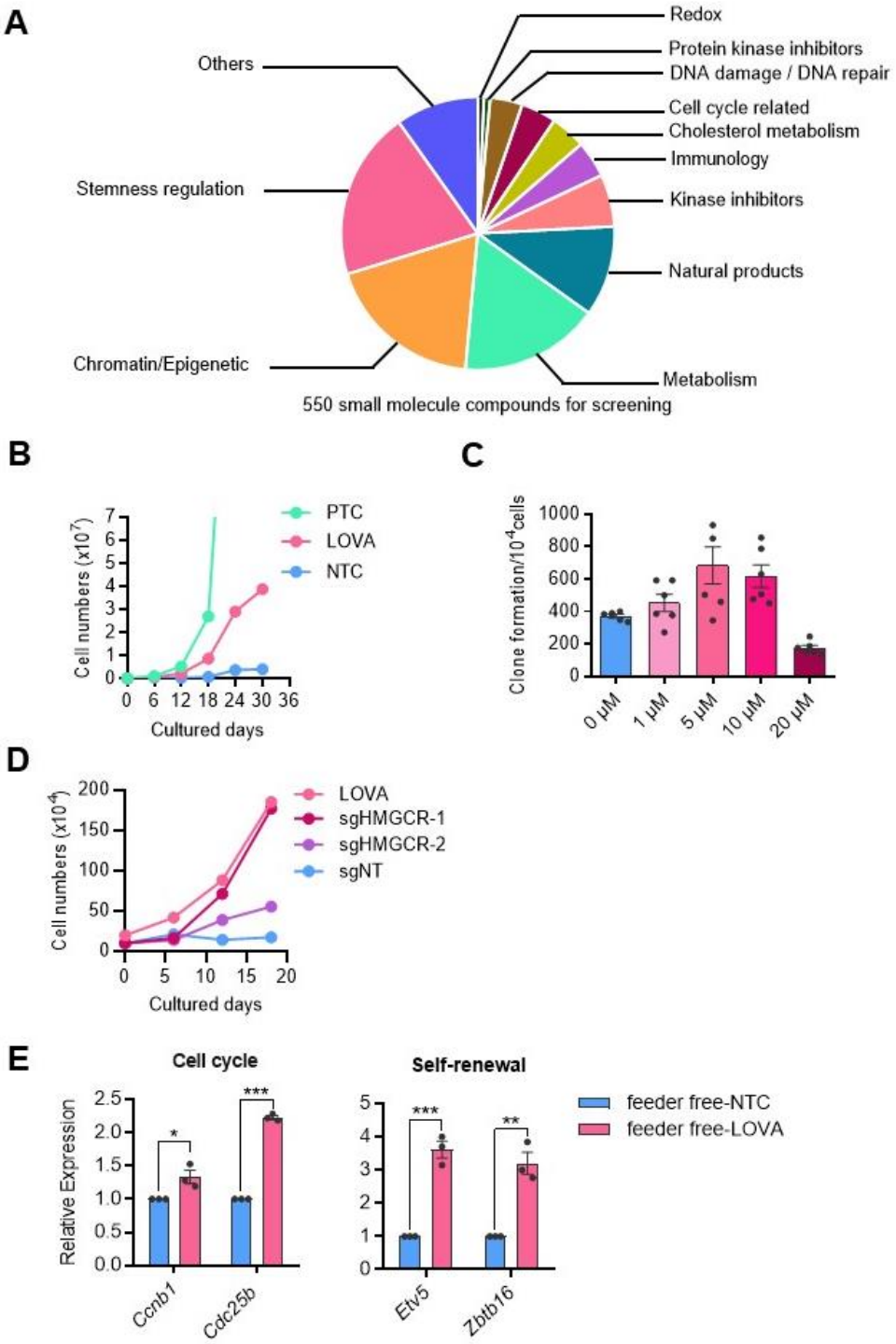


Figure S1. Small molecule screening for mSSCs self-renewal, related to Figure 1.

A) Classes of small molecule compounds tested.

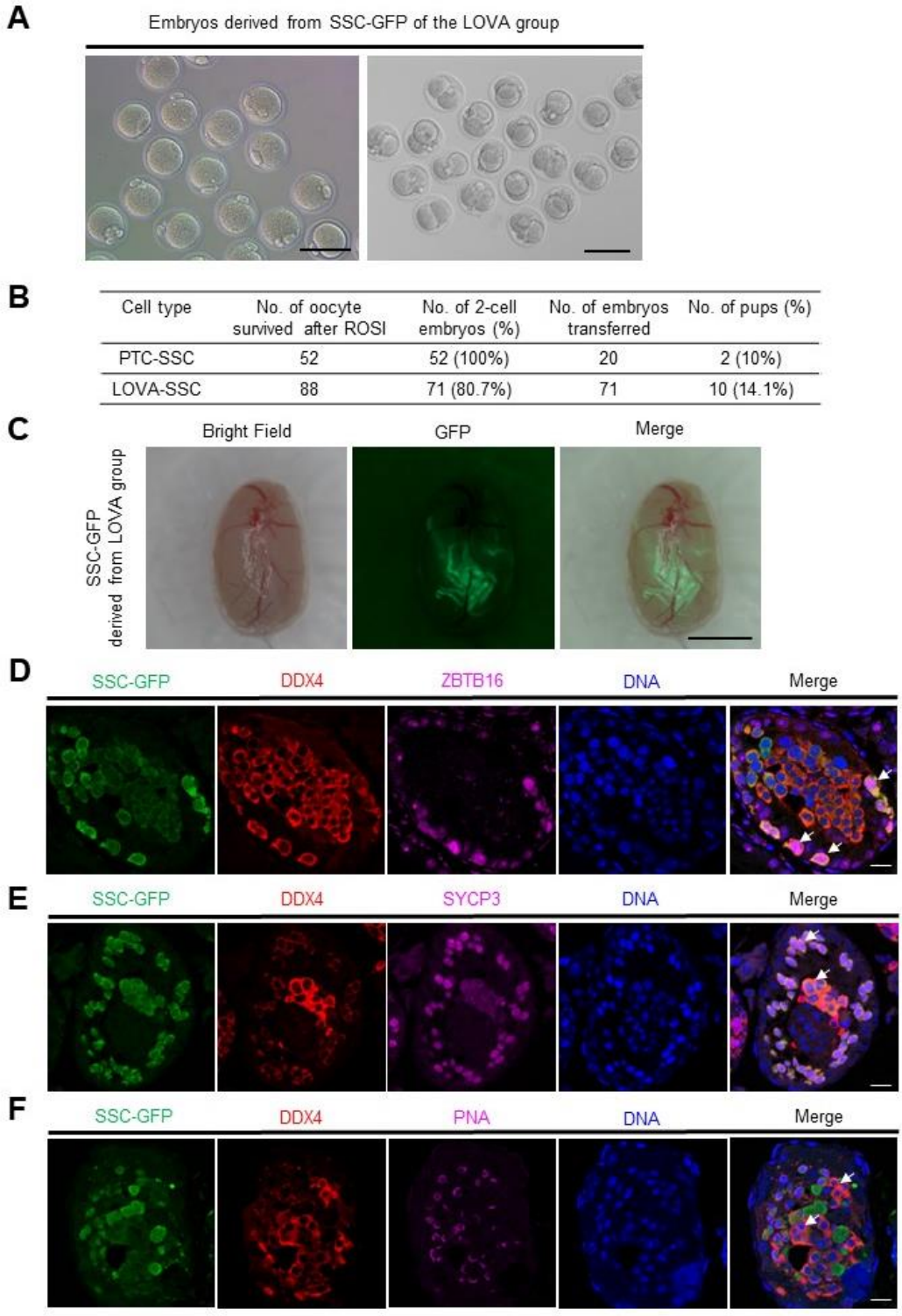
B) Cell growth curve of *in vitro* cultured mSSCs in 3 different conditions.

C) Histogram of Lovastatin concentration gradient test. Error bars indicate mean ± SEM from five independent experiments.

D) Cell growth curve of *in vitro* cultured HMGCR knockdown of mSSCs cell lines and mSSCs from LOVA group (LOVA) was taken as the control.

E) qPCR analysis of mSSCs and undifferentiated spermatogonia cultured in feeder-free mSSCs culture system of NTC (feeder free-NTC) and LOVA (feeder free-LOVA) group. The relative expression levels of representative genes regarding cell cycle and self-renewal were assessed. Error bars indicate mean ± SEM from three independent experiments, **$p \leq 0.01$.

Figure S2



Cell type	No. of oocyte survived after ROSI	No. of 2-cell embryos (%)	No. of embryos transferred	No. of pups (%)
PTC-SSC	52	52 (100%)	20	2 (10%)
LOVA-SSC	88	71 (80.7%)	71	10 (14.1%)

Figure S2. Functional analysis of mSSCs from the LOVA group, related to Figure 2.

A) Photomicrographs of embryos at the pronuclear (left) or 2-cell (right) stage were injected with differentiated spermatids from LOVA derived SSC-GFP cells. Scale bars, 100 μm.

B) Statistics for embryo development and the generation of offspring from spermatids derived from SSC-GFP from the PTC (PTC-SSC) or LOVA (LOVA-SSC) group, respectively.

C) Macroscopic appearance of busulfan-treated adult mouse recipient testes that were transplanted with SSC-GFP derived from LOVA condition. Scale bar, 2 mm.

D, E, F) Immunofluorescent staining of busulfan-treated adult mouse recipient testes that were transplanted with SSC-GFP derived from LOVA condition. ZBTB16 (D), SYCP3 (E), and PNA (F) were labeled as markers of undifferentiated spermatogonia, spermatocyte, and spermatid, respectively. DDX4 was used as the germ cell marker. Scale bars, 20 μm.

Figure S3

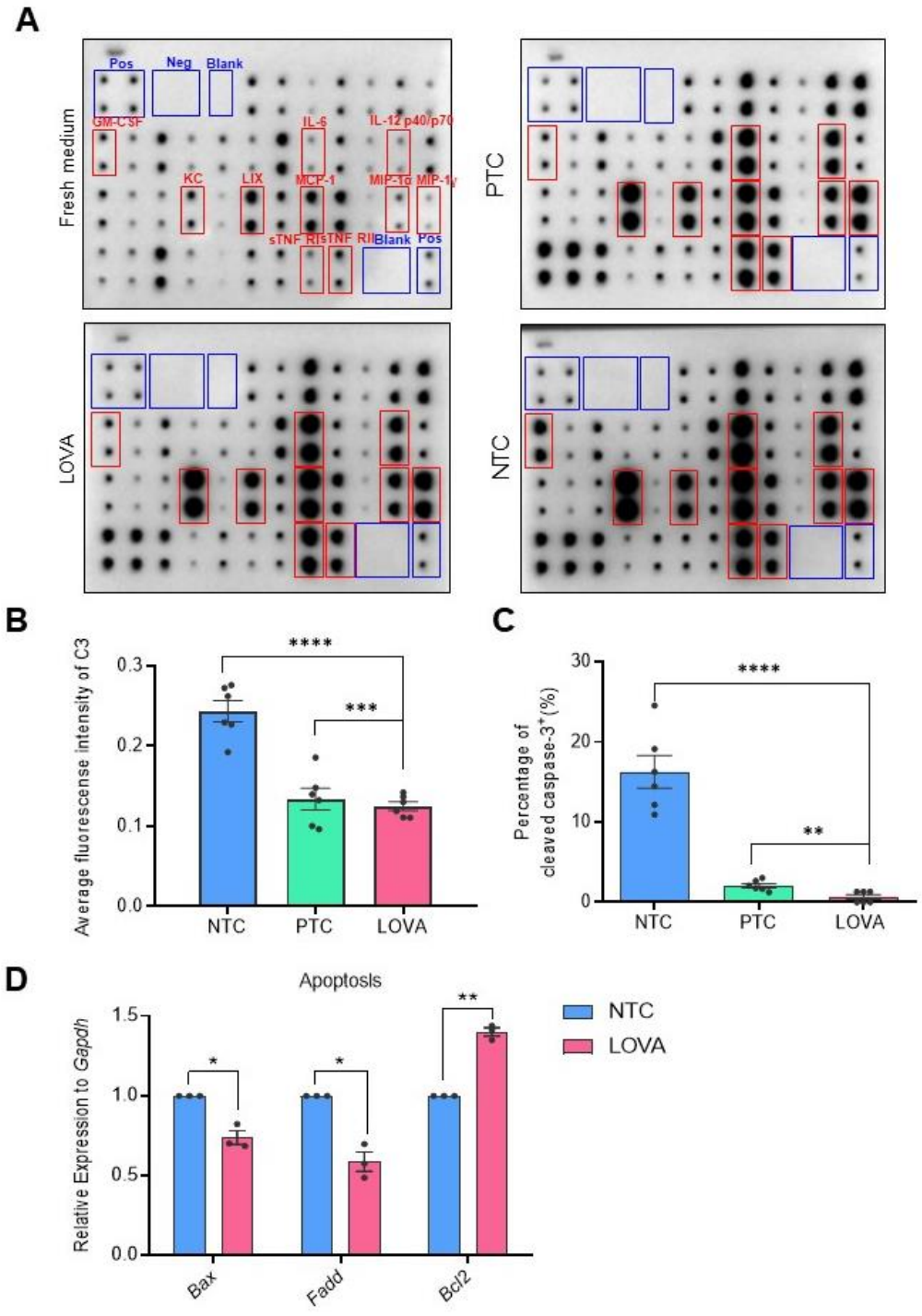


Figure S3. Levels of cytokines and apoptosis were reduced in LOVA, related to Figure 3.

A) Cytokine array analysis. Blue box labeled control of this assay. Red box labeled inflammatory factors which significantly upregulated in LOVA groups compared to the fresh medium group.

B) Statistics of average fluorescence intensity of complement 3 in cell clones cultured in 3 different conditions. Error bars indicate mean ± SEM from six independent experiments, ***p≤0.001, ****p≤0.0001.

C) Statistics of cleaved caspase-3^{+} cells in cell clones cultured in 3 different conditions. Error bars indicate mean ± SEM from six independent experiments, **p≤0.01, ****p≤0.0001.

D) qPCR analysis of the relative expression level of representative genes regarding apoptosis. Error bars indicate mean ± SEM from three independent experiments, *p≤0.05, **p≤0.01.

Figure S4

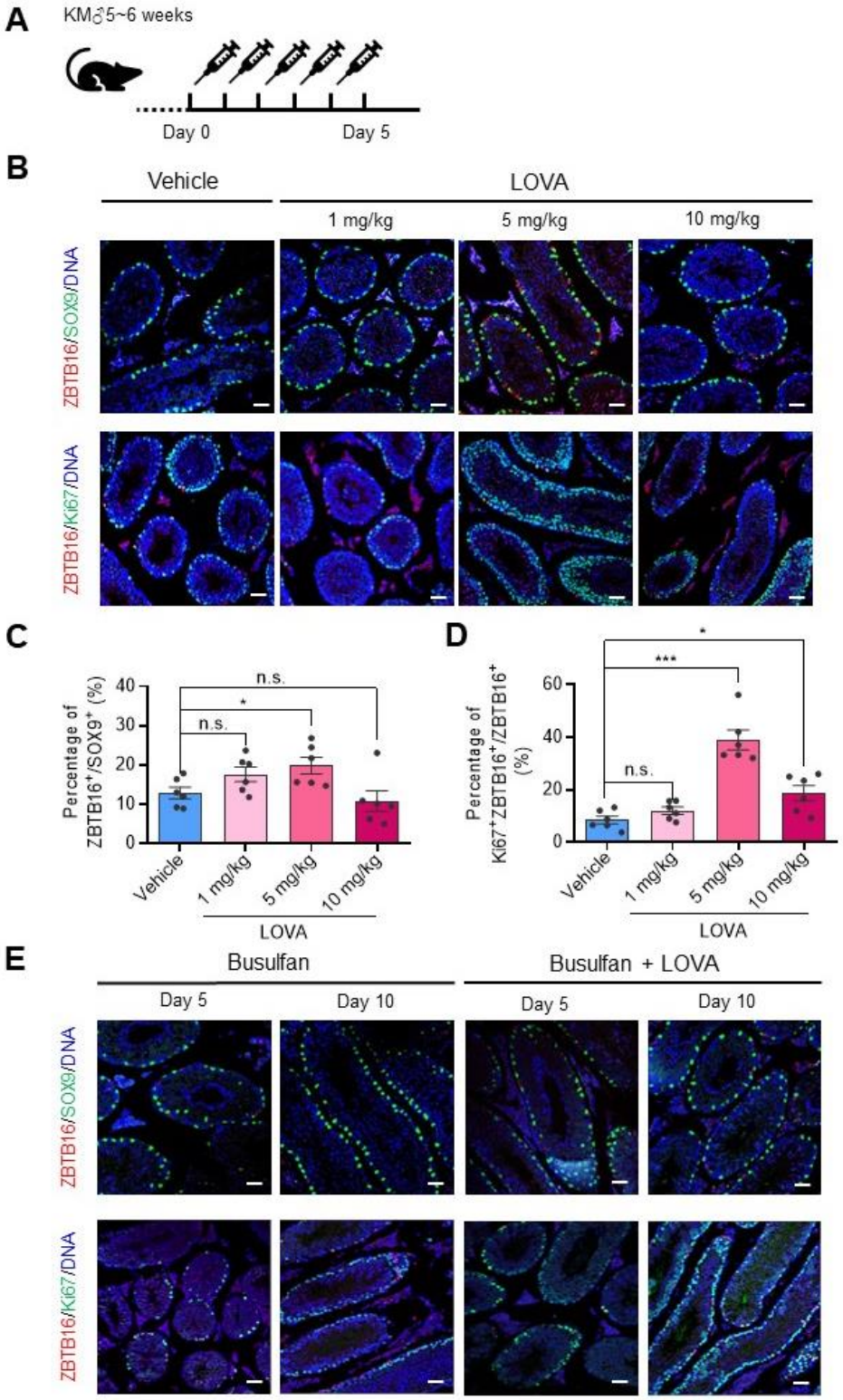


Figure S4. Lovastatin could promote the regeneration of undifferentiated spermatogonia *in vivo*, related to Figure 5.

A) Schematic outline of LOVA injection under physiological conditions.

B) Immunofluorescent staining of undifferentiated spermatogonia marker (ZBTB16) and Sertoli cell marker (SOX9) or cell proliferation marker Ki67 expression in mouse testis treated with Lovastatin under physiological conditions. Scale bars, 50 μm.

C) Histogram calculation of the proportion of undifferentiated spermatogonia ($ZBTB16^+$ cells) compared to Sertoli cells ($SOX9^+$ cells) in B) from six independent experiments. Error bars indicate mean ± SEM, *p≤0.05, ns, non-significant.

D) Histogram calculation of the proportion of proliferative undifferentiated spermatogonia ($Ki67^+ZBTB16^+$ cells) compared to total undifferentiated spermatogonia ($ZBTB16^+$ cells) in B) from six independent experiments. Error bars indicate mean ± SEM, *p≤0.05, ***p≤0.001, ns, non-significant.

E) Immunofluorescent staining of spermatogonia marker (ZBTB16) and Sertoli cell marker (SOX9) or cell proliferation marker (Ki67) in mouse testis under chemotherapy condition. Scale bars, 50 μm.

Table S1: List of primers used in RT-qPCR, related to Figure 3 and Figure4.

Gene name	Forward primer sequence	Reverse primer sequence
Gapdh	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGA
Zbtb16	CTGGGACTTTGTGCGATGTG	CGGTGGAAGAGGATCTCAAACA
Etv5	TCAGTCTGATAACTTGGTGCTTC	GGCTTCCTATCGTAGGCACAA
C-kit	GCCACGTCTCAGCCATCTG	GTCGCCAGCTTCAACTATTAACT
Cd68	TGTCTGATCTTGCTAGGACCG	AGGAGAGTAACGGCCTTTTTG
Apoe	GACCCAGCAAATACGCCTG	CATGTCTTCCACTATTGGCTCG
Ccnb1	GCGTGTGCCTGTGACAGTTA	CCTAGCGTTTTTGCTTCCCTT
Cdc25b	TCCGATCCTTACCAGTGAGG	GGGCAGAGCTGGAATGAGG
Bax	CCGGCGAATTGGAGATGAACT	CCAGCCCATGATGGTTCTGAT
Bcl2	ATGCCTTTGTGGAACTATATGGC	GGTATGCACCCAGAGTGATGC
Fadd	GCGCCGACACGATCTACTG	TTACCCGCTCACTCAGACTTC

Supplemental Experimental Procedures

Animal and human tissues

Mouse experiments were performed in the Animal Experimental Center of Southern Medical University under the approval for animal studies by the competent institution. Cynomolgus monkeys (*Macaca fascicularis*) were purchased from Guangdong Institute of Biological Resources Application which has been approval for animal studies by the competent institution.

All human samples related experiments were carried out in accordance with The Code of Ethics of the World Medical Association. The medical ethics committee of Southern medical university is No. NFEC-2019-219. The materials used in the experiment were from 2 patients with obstructive azoospermia, and the patients were informed of the purpose of the experiment.

mSSC cell line establishment

Testicular cells were derived from CAG-GFP transgenic C57/DBA mice on day 3 after birth as previously described (Kanatsu-Shinohara et al., 2003). In brief, testicular tissue was cut into small pieces then digested with collagenase IV (1 mg/ml; Invitrogen) for 15 min, followed by 0.25% trypsin/1 mM EDTA digestion (Invitrogen) for 10 min. Cells were resuspended in αMEM with 10% FBS then inoculated in 0.1% gelatin-coated culture dishes for 24 hours to separate the germ cells from the somatic cells. Purified germ cells were cultured on Mitomycin-treated mouse embryonic fibroblasts (MEFs) for long-term propagation. For the basic culture medium, StemPro-34 SFM (Invitrogen) supplemented with 1% FBS, 0.1 mM NEAA, 1 mM sodium pyruvate, 2 mM GlutaMAX, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 50 μM β-mercaptoethanol, 1 μL/mL DL-lactic acid, 1 × minimal essential medium (MEM) Vitamin Solution, 10 μg/mL biotin, 100 μM ascorbic acid, 60 ng/mL progesterone, 30 ng/mL β-estradiol, 6 mg/mL Glucose, 5 mg/mL Bovine

Serum Albumin (BSA), and 1% N2 supplement. PTC group culture medium contains 10 ng/mL recombinant rat GDNF (R&D Systems, 512-GF), 10 ng/mL recombinant mouse EGF (R&D Systems, 2028-EG), 5000 U recombinant mouse LIF (Millipore, ESG1107), and 10 ng/mL recombinant mouse FGF2 (R&D Systems, 3139-FB). The difference between NTC and PTC group culture medium is the presence of GDNF. The LOVA group culture medium replaces GDNF with 5 μM Lovastatin (TargetMol, T1207). For feeder-free culture system, long-term propagated mSSCs were cultured on dishes coated with 20 mg/ml laminin (BD Biosciences) as previously described (Kanatsu-Shinohara et al., 2011). The feeder-free culture medium was supplemented with B27 (Invitrogen) with basic culture medium described above. The culture medium was changed every other day and the subcultivation ratio of 1:3-5 per week was used.

mSSCs *in vivo* transplantation and Intracytoplasmic Sperm Injection

The day12.5 pregnant ICR mouse was used to generate the recipient male pups. The pregnant mice were treated with 40 mg/kg busulfan by intraperitoneal injection. Testicular transplantation of mSSCs was performed on the newborn male pups at 5-10 days postpartum. Anesthesia the recipient and split the hypogastrium to pull out testis. Prepare a glass needle with a 40 μm inner diameter. Fill the needle with 2×10^5 cell/μL mSSC cell suspension and add a small amount of trypan blue at the tip of the needle. Found the output tubules, which along the parallel direction of the testicular artery under the microscope, then poked the needle into the output tubules and inserted into the direction of the testicular reticulum along the output tubules, then blown the cells into the seminiferous tubules of the testis. After injection, Place the testicles and fat pads back into the abdominal cavity carefully and avoid torsion of the testicles. Sutured abdominal wound layer by layer. Finally, alcohol cotton was applied to the abdomen of the mice to prevent infection. The mice were placed in the worm table at 37°C. Then put them back into

the feeding room while woke up. 2 months after SSC transplantation, the testicular tissue from recipient mice was digested into single cells suspension and stained with 10 mg/mL Hoechst 33342 for 20 min and washed three times with PBS. The haploid spermatids with GFP fluorescence from the CAG-EGFP transgenes were collected by loading on a MoFlo XDP (Beckman Coulter). The cell suspension was kept at 4°C until ROSI. The ROSI was performed essentially as described previously (Kishigami and Wakayama, 2007).

RNA isolation and library preparation

SSC cells were collected after 18 days cultured in different conditions. Total RNA isolation and Illumina transcriptome sequencing library preparation were performed according to the manufacturer's instructions with the NEB Next Ultra II Directional RNA Library Prep Kit (NEB, E7760L). The constructed NGS libraries were sequenced with Illumina Hiseq XTEN platform at Novogene and sequencing strategy was paired-end 150 bp (PE150).

Transcriptome data analysis

The total reads were quality- and adapter-trimmed using BBDuk and Trimmomatic (v0.38) (Bolger et al., 2014). The mouse transcriptome index was generated using the reference genome mm10 with Ensembl version 95 and the clean reads were aligned to mm10 transcriptome using RSEM (Li and Dewey, 2011) integrated Bowtie2 (v 2.4.1) (Langmead and Salzberg, 2012). Gene counts were calculated using RSEM. To ensure data quality, the low expressed genes which counts did not >=50 in at least two samples were eliminated for following analysis. R-Bioconductor package DESeq2 (Love et al., 2014) was used to normalized count data and calculated differentially expressed genes across different groups ($P< 0.05$).

Testicular tissue paraffin-embedded section

The testicular tissue was washed twice with PBS then fixed in 4% paraformaldehyde overnight at 4°C after. The fixed tissue was transferred to the embedding frame and dehydrated with 70%, 80% and 90% ethanol solution successively, followed by 100% alcohol and xylene transparent sample successively, and then paraffin penetration. FFPE sample was sectioned into 5-7 μm pieces and pasted on slide for further use.

Statistics and Reproducibility

Microsoft Excel, GraphPad Prism (version 8.0.1) and R (version 3.5.1) software were used for statistical calculations. Specific statistical tests, sample number and other information are indicated in the main text or figure legends. The t-test and two-sided statistical analysis approach was used to determine the significance of the difference between the different sets of data if there was no specific indication. All experiments were conducted at least two times independently, and similar results were adopted for further analysis to guarantee reproducibility.

Immunostaining

mSSCs cultured on the glass slides were fixed with 4% paraformaldehyde after clone formation. Then the sample was permeated with PBS containing 0.5% TritonX-100 for 20 min and blocked with 2% BSA for 1 hour at room temperature.

The testicular tissue sections were re-hydrated gradually in Xylene and serial concentrations (100%, 90%, 80%, and 70%) of ethanol. Then the tissue sections were boiled at 95°C for 10 min in EDTA antigen retrieval solution (pH 8.0) and followed by 30 min incubation at room temperature then block the slides with 2% BSA for 1 hour. After finished the blocking, the primary antibody was added to the sample and incubation at 4°C overnight. Wash the sample with PBS 3 times, and then the corresponding secondary Immunofluorescence

antibody and Hoechst33342 were added for incubation at room temperature for 1 hour. Wash the samples in PBS 3 times. Finally, the section was sealed using anti fluorescence quenching agent and the confocal microscope (Carl Zeiss) was used for further detection. At least 3 fields were calculated in each experiment.

Cas13d-mediated HMGCR knockdown

The knockdown of HMGCR was conducted by using CRISPR/Cas13d system. Two sgRNAs designed to target the coding sequence of HMGCR mRNAS were prepared：

sgHMGCR-1F	AAACAACACAAAGTAGTTGGCCAACAC
sgHMGCR-1R	AAAAGTGTTGGCCAACTACTTTGTGTT
sgHMGCR-2F	AAACACACAAAGTAGTTGGCCAACACT
sgHMGCR-2R	AAAAAGTGTTGGCCAACTACTTTGTGT

Non-targeting sgRNA (sgNT) for control was used as previously described (He et al., 2020). According to previous reports (He et al., 2020; Konermann et al., 2018), lentivirus packaging and transfection were performed.

Western blot

Cells were lysed in RIPA Lysis buffer (R0010, Solarbio) to collect the cellular proteins, which were resolved by SDS-PAGE and transferred to PVDF membrane (RPN303F, GE), blocked, probed with the primary antibody target HMGCR (ab174830, abcam) and the secondary antibody (KM9007, Sungene). Immuno-reactive bands were visualized using the ECL Western Blotting Substrate Kit (YEASON, 36208ES60) before exposure. The intensities of bands were quantified with ImageJ software (http://imagej.nih.gov/ij/).

Quantitative real-time PCR analysis

SSC samples were collected after 18 days cultured in different conditions and total RNA was extracted using Trizol reagent (TIANGEN, DP424) according to the manufacturer's instructions. RNA was subsequently reversely transcribed

to cDNA using the HiScript Q RT SuperMix Kit (Vazyme, R123-01). Then cDNA was diluted and used for RT-qPCR with RealStar Green Fast Mixture reagent (GenStar, A301-101) and designed primers on a LightCycler®96 (Roche, 900012C). Every sample has triplications and the relative mRNA expression levels of targeted genes were calculated through the $2^{-\Delta\Delta CT}$ method after normalized by housekeeping gene *Gapdh*. The detailed sequence of primers was listed in Table S1.

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