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ORIGINAL ARTICLE

Sperm Biology

Characterization, isolation, and culture of spermatogonial stem cells in *Macaca fascicularis*

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Spermatogonial stem cells (SSCs) have great applications in both reproductive and regenerative medicine. Primates including monkeys are very similar to humans with regard to physiology and pathology. Nevertheless, little is known about the isolation, the characteristics, and the culture of primate SSCs. This study was designed to identify, isolate, and culture monkey SSCs. Immunocytochemistry was used to identify markers for monkey SSCs. Glial cell line-derived neurotrophic factor family receptor alpha-1 (GFRA1)-enriched spermatogonia were isolated from monkeys, namely *Macaca fascicularis* (*M. fascicularis*), by two-step enzymatic digestion and magnetic-activated cell sorting, and they were cultured on precoated plates in the conditioned medium. Reverse transcription-polymerase chain reaction (RT-PCR), immunocytochemistry, and RNA sequencing were used to compare phenotype and transcriptomes in GFRA1-enriched spermatogonia between 0 day and 14 days of culture, and xenotransplantation was performed to evaluate the function of GFRA1-enriched spermatogonia. SSCs shared some phenotypes with rodent and human SSCs. GFRA1-enriched spermatogonia with high purity and viability were isolated from *M. fascicularis* testes. The freshly isolated cells expressed numerous markers for rodent SSCs, and they were cultured for 14 days. The expression of numerous SSC markers was maintained during the cultivation of GFRA1-enriched spermatogonia. RNA sequencing reflected a 97.3% similarity in global gene profiles between 0 day and 14 days of culture. The xenotransplantation assay indicated that the GFRA1-enriched spermatogonia formed colonies and proliferated *in vivo* in the recipient c-Kit^{W/W} (W) mutant mice. Collectively, GFRA1-enriched spermatogonia are monkey SSCs phenotypically both *in vitro* and *in vivo*. This study suggests that monkey might provide an alternative to human SSCs for basic research and application in human diseases.

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INTRODUCTION

According to the World Health Organization (WHO), about 15% of couples at child-bearing age suffer from infertility. Male factors account for about 50% of fertility,¹ and 15% of male infertility cases result from azoospermia. Spermatogenesis is a complex process in which spermatogonial stem cells (SSCs) undergo self-renewal and differentiation to become haploid spermatozoa.² SSCs are a subpopulation of type A spermatogonia in mammalian testes, and as the initial cells of spermatogenesis, they are responsible for self-renewal to maintain the stem cell pool and differentiation to male germ cells of different stages. SSCs are the unique adult stem cells that transmit genetic information to subsequent generations. Increasing evidence has indicated that SSCs from rodents and humans can develop pluripotency to become embryonic stem (ES)-like cells that differentiate to all cell types of three germ layers.^{3–7} In addition, SSCs can transdifferentiate to cells of other lineages, including hepatocytes and neurons,^{8–10} which could provide sufficient cells for treating various kinds of human diseases and

have important implications in the field of regenerative medicine. Nevertheless, the severe shortage of human testicular tissues, the limited number of primary SSCs, and the lack of a long-term culture system have hampered basic studies and the potential application of human SSCs.

Nonhuman primates are the most advanced models in life science research. They are close to humans in testicular anatomy, spermatogenic cell composition and types, physiology, and biochemistry. Therefore, nonhuman primate SSCs have been regarded as an ideal source of stem cells for moral and ethical reasons as well as for legality and safety. Moreover, genome sequencing shows that 97.5% of the genes in *Macaca mulatta* are similar to those of *Homo sapiens*.¹¹ Compared with rodents, very little is known about the characteristics of nonhuman primate SSCs owing to the high cost of specimen and the long time required for sexual maturity of primates. *Macaca fascicularis* (*M. fascicularis*), also known as the long-tailed macaque, is a nonhuman primate that has recently received much attention in medical research, and it is a widely used

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experimental animal. In 2018, Chinese scientists have successfully cloned two *M. fascicularis*, namely “Zhongzhong” and “Huahua,” which were the first primates cloned through somatic cell cloning technology.¹² The cloning *M. fascicularis* will make it feasible to solve many issues related to human medicine, including efficacy, safety, and toxicology studies for drug development, especially for human immunodeficiency virus (HIV) vaccine development.¹³ Additionally, *M. fascicularis* has been utilized as a model to examine the progression and regression of atherosclerotic lesions.¹⁴

SSCs can be isolated, cultured, and cryopreserved in bulls,¹⁵ goats,¹⁶ pigs,¹⁷ sheep,¹⁸ and humans,^{19,20} and the ability to manipulate them in different species is feasible. Accumulating evidence reveals that there is a conserved expression of numerous genes across species, including mice, monkeys, and human, and Glial cell line-derived neurotrophic factor family receptor alpha-1 (GFRA1) has been shown to be a marker for SSCs in primates.^{21,22} Furthermore, monkey testicular cells can be enriched by differential plating on an extracellular matrix, laminin, and gelatin, and they can be purified by fluorescence-activated cell sorting (FACS) with high efficiency.²³ Studies on *M. fascicularis* for the isolation, purification, and culture of SSCs would lay the foundation to uncover molecular mechanisms underlying human spermatogenesis and the pathology of male infertility and could provide novel insights into the protection of endangered species, high-quality animal breeding, and preparation of transgenic animals.²⁴ In this study, immunohistochemistry of numerous hallmarks of germ cells and SSCs was used to identify SSCs as a distinct population in *M. fascicularis* testes. Subsequently, GFRA1-enriched spermatogonia with high purity and viability were isolated from *M. fascicularis* testicular tissues by utilizing a two-step enzymatic digestion and magnetic-activated cell sorting (MACS) with an antibody against GFRA1. We also established a short-term culture system for SSCs and compared the differences in phenotypes between the SSCs after culture for 0 day and 14 days. Transcriptomic analysis revealed that only 2.8% of global genes were affected after 14 days' culturing. GFRA1-enriched spermatogonia from *M. fascicularis* were transplanted into recipient c-Kit^{w/w} (W mutant) mice to determine the identity of these cells. In summary, we have for the first time characterized, identified, isolated, and cultured monkey SSCs, which could provide important alternative cells to human SSCs for molecular research and application in both reproductive medicine and regenerative medicine.

MATERIALS AND METHODS

Procurement and processing of the testes from M. fascicularis

The testes tissues of four 6-year-old *M. fascicularis* were obtained from Guangdong Landao Biological Technology Limited Company (Guangzhou, China), and they were transported in ice-cold saline to the laboratory within 12 h of extirpation. After the tunica vaginalis was removed, testicular tissues were washed three times in phosphate-buffered saline (PBS) with 1% (*w/v*) penicillin/streptomycin (Gibco, Waltham, MA, USA) and cut into pieces about 1 cm³. These tissues were used for characterization, isolation, and culture of SSCs, or placed in freezing medium including 10% (*v/v*) dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA), 80% (*v/v*) fetal bovine serum (FBS; Gibco), and 10% (*v/v*) Dulbecco's Modified Eagle's Medium (DMEM; Gibco) and then stored in liquid nitrogen. Monkey testicular tissues were used only for research, and all experiments were performed in accordance with relevant guidelines and regulation of animals from The Biomedical Research Ethical Committee of Hunan Normal University (Changsha, China).

Histological examination

Testicular tissues from four 6-year-old *M. fascicularis* were cut and then fixed with 4% (*w/v*) paraformaldehyde (PFA; BBI Life Sciences Corporation, Shanghai, China) for 24 h, dehydrated, and embedded in paraffin. Serial sections were cut at 5- μ m thickness, stained with hematoxylin and eosin (H and E; Abcam, Cambridge, UK), and assessed for spermatogenic status.

Isolation of GFRA1-enriched spermatogonia from M. fascicularis

Testicular tissues were placed in a 10-cm dish containing DMEM and mechanically sliced into small pieces. A single cell suspension was prepared by a two-step enzymatic digestion with minor modification.^{25,26} The first enzymatic digestion involved the incubation of testicular tissues in 2 mg ml⁻¹ collagenase IV (Gibco) and 1 μ g μ l⁻¹ DNase I (Gibco) at 37°C for 15 min with gentle agitation every 5 min. The digestion was set for 5 min, and the seminiferous tubules were allowed to sediment. After removing the supernatant, seminiferous tubules were incubated with 4 mg ml⁻¹ collagenase IV, 2.5 mg ml⁻¹ hyaluronidase (Sigma), 2 mg ml⁻¹ trypsin (Sigma), and 1 μ g μ l⁻¹ DNase I at 37°C for 10 min until single cells were obtained. Enzymatic digestion was terminated by adding 10% (*v/v*) FBS, and the cell suspension was centrifuged at 300g (5804R, Eppendorf, Hamburg, Germany) for 5 min. The cell pellet was resuspended in DMEM, and cell clumps were removed by a 40- μ m nylon cell strainer (Corning, New York, NY, USA). Male germ cells were obtained by differential plating. The dishes were precoated with 0.1% (*w/v*) gelatin (Sigma) in ultrapure water at 37°C in 5% (*v/v*) CO₂ for at least 30 min before differential plating, and cells were seeded onto plates in DMEM/F-12 supplemented with 10% (*v/v*) FBS and incubated at 34°C in 5% CO₂ for 3 h. When the somatic cells attached to the dishes, male germ cells remained in suspension and were collected by centrifuging at 300g for 5 min. GFRA1-enriched spermatogonia were separated by magnetic-activated cell sorting (MACS) by using an antibody against GFRA1 (Bio-Techne, Minneapolis, MN, USA) at a concentration of 0.1 μ g μ l⁻¹. Finally, the cells were seeded on specifically prepared plates as described below. The viability of the freshly isolated cells was examined by trypan blue exclusion. The conditioned medium mentioned below was changed every other day, and cell morphology was observed in a phase-contrast microscope (DMI8, Leica, Buffalo Grove, IL, USA).

Preparation of poly-lysine and laminin-coated plates

GFRA1-enriched spermatogonia were grown in 6-well plates precoated with poly-lysine (Shanghai Weiao Ltd., Shanghai, China) and laminin (Sigma).²⁷ To prepare the culture plates, poly-lysine was diluted to the working concentration of 50 μ g ml⁻¹ and incubated 4 h in 5% CO₂ at 37°C. After being washed three times for 5 min each in distilled water and drying at room temperature, the laminin product was slowly thawed at 2°C–8°C to avoid the formation of gel, and the product was diluted in sterile PBS to 25 μ g ml⁻¹. The plates were incubated at 37°C for 1–2 h and then treated three times for 5 min each with DMEM before GFRA1-enriched spermatogonia were placed on the plates.

Culture of GFRA1-enriched spermatogonia

The conditioned-culture medium for GFRA1-enriched spermatogonia comprised DMEM/F-12 supplemented with 1 \times B27 (Invitrogen, Carlsbad, CA, USA), 1% (*v/v*) FBS, 0.5% (*w/v*) bovine serum albumin (BSA; Invitrogen), 6 g l⁻¹ D-glucose (Sigma), 1 \times 10⁻⁴ mol l⁻¹ vitamin C (Invitrogen), 30 mg ml⁻¹ sodium pyruvate (Shanghai Weiao Ltd.), 2 mmol l⁻¹ glutamine (Invitrogen), 50 mmol l⁻¹ β -mercaptoethanol (Invitrogen), 1-X nonessential amino acid (Invitrogen), 1% (*v/v*) D-L-lactate (Sigma), 15 ng ml⁻¹ recombinant human glial cell line-derived

neurotrophic factor (GDNF; Sigma), 10 ng ml⁻¹ basic fibroblast growth factor (bFGF; Sigma), 20 ng ml⁻¹ epidermal growth factor (EGF; Sigma), 10 ng ml⁻¹ leukemia inhibitory factor (LIF; Sigma), and 1% penicillin/streptomycin (Gibco). The conditioned culture medium was changed every other day, and GFRA1-enriched spermatogonia were manually collected and passaged every 7 days at a 1:3–5 dilution. The culture was maintained at 34°C in 5% CO₂ atmosphere.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the freshly separated cells, the GFRA1-enriched spermatogonia after culture, and the *M. fascicularis* testicular cells separately, with Trizol (Invitrogen), and the quality and concentrations of total RNA were assessed by Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The cDNA was synthesized by the First Strand cDNA Synthesis Kit (Thermo Scientific), and PCR was performed according to a protocol described previously.²⁸ The primer sequences of the chosen genes, including DEAD-box helicase 4 (*VASA*), Deleted in azoospermia like (*DAZL*), Thy-1 cell surface antigen (*THY1*), *GFRA1*, ubiquitin C-terminal hydrolase L1 (*UCHL1*), POU class 5 homeobox 1 (*OCT4*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were designed as listed in **Supplementary Table 1**. The PCR reactions started at 94°C for 2 min and were performed under the following conditions: 30 s at 95°C, annealing temperature (54°C–60°C) for 30 s, 72°C for 30 s, 35 cycles, and extended at 72°C for 10 min. The PCR products were separated by electrophoresis on 2.0% (*w/v*) agarose, and the gels were exposed to chemiluminescence (Chemi-Doc XRS, Bio-Rad, Hercules, CA, USA). The integrated density values (IDV) of target gene products were quantified in relation to the expression of the housekeeping gene *GAPDH*.

Immunocytochemistry of the freshly separated and cultured GFRA1-enriched spermatogonia

Immunocytochemistry was used to identify the phenotypic characteristics of the freshly separated cells and the cultured GFRA1-enriched spermatogonia as described previously.²⁸ The cells were placed on the glass slides by cytospin (Thermo Scientific), and were fixed with 4% (*w/v*) PFA for 15 min and permeabilised in 0.4% (*v/v*) Triton-X-100 (Sigma) for 15 min at room temperature, followed by three washes in PBS for 5 min. After being blocked in 1% (*w/v*) BSA for 1 h, the cells were incubated with primary antibodies, including UCHL1 (MCA4750, 1:200, Bio-Rad), zinc finger and BTB domain containing 16 (PLZF; ab104854, 1:200, Abcam), GFRA1 (ab8026, 1:200, Abcam), and THY1 (ab133350, 1:200, Abcam) overnight at 4°C. After three 5-min washes in PBS, the cells were incubated with appropriate fluorescein isothiocyanate (FITC)-conjugated or rhodamine-conjugated IgG secondary antibodies (Sigma) at a 1:200 dilution for 1 h at room temperature. The nuclei of cells were stained by 4',6-diamidino-2-phenylindole (DAPI, Sigma), and the cells were observed under fluorescence microscope (Leica, Wetzlar, Germany).

Immunohistochemistry of *M. fascicularis* testes and recipient W mutant mouse testes with transplanted monkey SSCs

Tissues from five *M. fascicularis* testes and ten recipient W mutant mouse testes with transplanted GFRA1-enriched *M. fascicularis* spermatogonia were fixed in 4% (*w/v*) PFA for 24 h, dehydrated, and embedded in paraffin. Serial testicular sections (5 µm thickness) were dewaxed in xylene and rehydrated through a series of the graded alcohol from 100% to 50%. Antigens were retrieved with citrate buffer solution (0.01 mol l⁻¹, pH 6.0; Guangzhou Dingguo Biology

Inc., Guangzhou, China) for 20 min at 99°C and allowed to cool at room temperature for 30 min. The sections were then treated with 3% (*v/v*) H₂O₂ for 10 min in order to block the endogenous peroxidase activity and then rinsed with PBS for 5 min. The tissue sections were incubated with a blocking buffer (5% [*w/v*] BSA in PBS) for 30 min at room temperature to prevent nonspecific binding and 0.4% (*v/v*) Triton-X-100 (Sigma) in PBS for 5 min for tissue permeation. The sections were incubated with primary antibodies, including GFRA1 (ab8026, 1:100, Abcam), proliferating cell nuclear antigen (PCNA; 2586, 1:100, Cell Signaling Technology, Danvers, MA, USA), PLZF (ab104854, 1:200, Abcam), THY1 (ab133350, 1:100, Abcam), and UCHL1 (MCA4750, 1:100, Bio-Rad), overnight at 4°C. Replacement of primary antibodies with their respective isotype IgGs, including normal goat IgG and rabbit IgG, served as negative controls. After three washes with PBS, testicular sections were incubated with secondary antibodies to rhodamine-conjugated IgG or FITC-conjugated IgG at a 1:200 dilution for 1 h at room temperature and washed three times with PBS. DAPI was used to stain cell nuclei, and the sections were observed under the fluorescence microscope (Nikon Eclipse Ti-S, Nikon Corporation, Tokyo, Japan).

Xenotransplantation of GFRA1-enriched spermatogonia from *M. fascicularis* into recipient W mutant mouse testes

The xenotransplantation of freshly separated GFRA1-enriched spermatogonia from *M. fascicularis* was performed according to a procedure previously described²⁹ with minor modification. Briefly, ten W mutant mice (Kit mutation) at 6–8 weeks of age were used because there was no male germ cell in the testis.³⁰ Animals were maintained and experiments were carried out strictly in accordance with the care and use of laboratory animals and the related ethical regulation of Ren Ji Hospital, Shanghai Jiao Tong University (Shanghai, China). Freshly separated GFRA1-enriched spermatogonia from *M. fascicularis* were collected and resuspended in DMEM/F-12 medium at a concentration of 10⁷ cells per ml, and 0.04% (*w/v*) trypan blue was added to the cell suspension for monitoring the injection and agitated before cell transplantation. Recipient W mutant mice were anaesthetized with avertin (2, 2, 2-tribromoethanol; Sigma) and tert-amyl alcohol (2-methyl-2-butanol; Sigma). The efferent duct that connects the testis to the epididymis was identified, and GFRA1-enriched monkey spermatogonia were transplanted into the seminiferous tubules of the testis through the efferent duct by the capillary glass tube. Approximately 15 µl of cell suspension was transplanted into the seminiferous tubules of one testis via the efferent ducts, whereas the other testis without cell injection but with medium and trypan blue (Batch lot No. 711742, Saint-Bio, Shanghai, China) served as negative controls. The injection was stopped when 90% of seminiferous tubules had been filled as indicated by the trypan blue dye. One month after cell transplantation, the testes of the recipient mice were collected for preparing the sections and for immunohistochemistry as described above.

RNA sequencing

Total RNA was extracted from the freshly separated GFRA1-enriched spermatogonia from four *M. fascicularis* and the cultured GFRA1-enriched spermatogonia by Trizol (Invitrogen). RNA purity and quantification were evaluated in a Nanodrop 2000 spectrophotometer (Thermo Scientific). RNA integrity was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with RNA integrity number (RIN) >7.0. The process of RNA-Seq analysis was performed according to the standard procedure of the Illumina HiSeqTM 2500 instrument (Oebiotec, Shanghai, China). Raw data

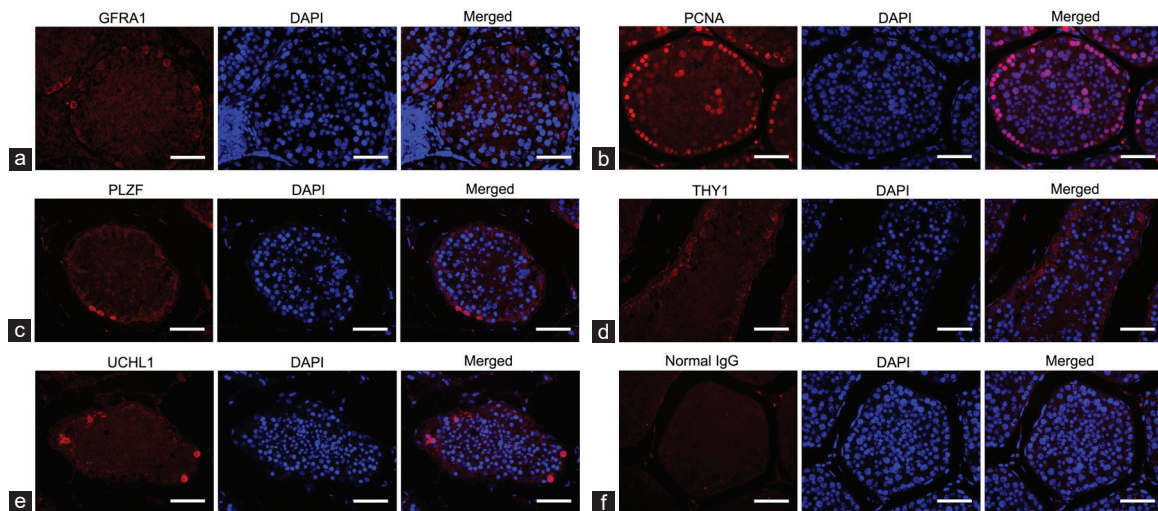


Figure 1: The cellular localization of numerous markers for SSCs in the testes of *M. fascicularis*. (a–e) Immunohistochemistry displayed the expression of (a) GFRA1, (b) PCNA, (c) PLZF, (d) THY1, and (e) UCHL1 in the *M. fascicularis* testes. (f) Replacement of primary antibodies with isotype rabbit or goat IgGs was used as negative controls. DAPI was used to label cell nuclei. Scale bars = 50 μ m. SSC: spermatogonial stem cell; GFRA1: glial cell line-derived neurotrophic factor family receptor alpha-1; PCNA: proliferating cell nuclear antigen; PLZF: zinc finger and BTB domain containing 16; THY1: Thy-1 cell surface antigen; UCHL1: ubiquitin C-terminal hydrolase L1; DAPI: 4',6-diamidino-2-phenylindole; *M. fascicularis*: *Macaca fascicularis*.

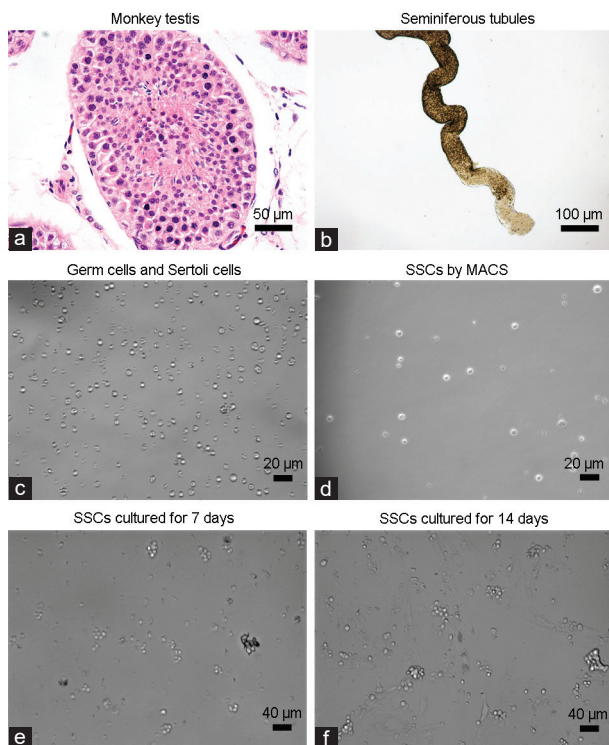


Figure 2: Morphological and phenotypic characterization of the freshly isolated and cultured GFRA1-enriched spermatogonia of *M. fascicularis*. (a) H and E staining revealed the normal spermatogenesis of testicular tissues. (b) Seminiferous tubules were obtained after the first enzymatic digestion with collagenase IV and DNase I. (c) Cell mixture comprising male germ cells and Sertoli cells was isolated from seminiferous tubules by the second enzymatic digestion. (d) GFRA1-enriched spermatogonia were separated by MACS. (e) GFRA1-enriched spermatogonia formed obvious colonies after culturing for 7 days. (f) After two weeks of culture, the GFRA1-enriched spermatogonia grew into larger clones with dozens of cells. H and E: hematoxylin and eosin; GFRA1: glial cell line-derived neurotrophic factor family receptor alpha-1; MACS: magnetic-activated cell sorting; SSC: spermatogonial stem cell; *M. fascicularis*: *Macaca fascicularis*.

(raw reads) of fastq format were first processed by using trimmomatic.³¹ After removing low-quality reads and reads with adapter or poly-N (nucleobases), from the raw data, the clean reads were mapped to *M. fascicularis* by using HISAT2.³² HISAT2 is a fast and sensitive alignment program for mapping next-generation sequencing reads (both RNA and DNA) to a population of human genomes and a single reference genome. The fragments per kilobase of exon model per million mapped fragments (FPKM) value of each gene was calculated from Cufflinks,³³ and the read counts of each gene were obtained by HTSeq-count.³⁴ Differential gene expression analysis was performed with the DESeq (2012) R package (<http://bioinfo.au.tsinghua.edu.cn/software/degseq/>). $P < 0.05$ and fold changes ≥ 2 were set as the threshold for significantly differential expression of genes.

RESULTS

Identification of SSC markers for *M. fascicularis*

We first made efforts to identify hallmarks for monkey SSCs in the testis of the *M. fascicularis*. Immunohistochemical staining revealed that there were subpopulations of spermatogonia along the basement membrane of the testes which were positive for GFRA1 (about 6 positive cells in each seminiferous tubule cross section, **Figure 1a**), PCNA (around 42 positive cells, **Figure 1b**), PLZF (about 3 positive cells, **Figure 1c**), THY1 (also known as CD90, about 5 positive cells, **Figure 1d**), and UCHL1 (around 4 positive cells, **Figure 1e**). No immunostaining was seen when primary antibodies were replaced with isotype rabbit or goat IgGs (**Figure 1f**).

Isolation of GFRA1-enriched spermatogonia from *M. fascicularis* testes

We first assessed the monkey spermatogenic status by histology that revealed all types of male germ cells, including several subpopulations of spermatogonia, various kinds of primary and secondary spermatocytes, as well as round and elongated spermatids (**Figure 2a**). Male germ cells were separated from the testicular tissue by two enzymatic digestions followed by differential plating. Firstly, seminiferous tubules (**Figure 2b**) were obtained from the first enzymatic digestion to remove potential contamination of myoid and Leydig cells. Secondly, mixtures

of male germ cells and Sertoli cells (**Figure 2c**) were acquired from the second enzymatic digestion. Somatic cells attached to the plates, and cell suspension containing male germ cells, were collected by centrifugation. GFRA1-enriched spermatogonia (**Figure 2d**) were obtained by MACS with an antibody against GFRA1, and they began to divide and propagate at day 7 after plating (**Figure 2e**). Small clones comprising numerous cells emerged after culturing and they enlarged to contain dozens of cells after 14 days of culture (**Figure 2f**).

Phenotype of GFRA1-enriched spermatogonia from the *M. fascicularis* testis

RT-PCR was used to determine the identity of the isolated GFRA1-enriched spermatogonia. This assay showed that *GFRA1*, *THY1*, *UCHL1*, *OCT4*, *VASA*, and *DAZL* were transcribed in the isolated spermatogonia (**Figure 3a**, left panel). RT-PCR showed that the transcripts of *GFRA1*, *THY1*, *UCHL1*, and *OCT4*, as well as *VASA* and *DAZL*, were detected in testicular tissue (**Figure 3a**, right panel), whereas the mRNA of these genes was undetectable in the attached somatic cells (data not shown). To check whether the GFRA1-enriched monkey spermatogonia could proliferate and maintain phenotypic characteristics of SSCs, we carried out RT-PCR and immunocytochemical analysis after 14 days of culturing. Semi-quantitative RT-PCR demonstrated that the expression levels of *GFRA1*, *THY1*, *UCHL1*, *VASA*, and *DAZL* after 14 days of culture were comparable to that of genes on day 0 of culturing (**Figure 3b**). The expression of the housekeeping gene *GAPDH* in these cells was used as the loading control of RNA. Immunocytochemistry revealed that almost all the isolated GFRA1-enriched spermatogonia were positive for GFRA1 (**Figure 4a**), THY1 (**Figure 4b**), UCHL1 (**Figure 4c**), and PLZF (**Figure 4d**). No staining was observed when primary antibodies were replaced with isotype rabbit or goat IgGs (**Figure 4e**), reflecting the specific immunostaining of these antibodies in the cells.

Proliferation and maintenance of stemness of GFRA1-enriched spermatogonia from *M. fascicularis* in vitro

Immunocytochemistry further showed that GFRA1-enriched spermatogonia after culturing for 14 days were positive for GFRA1 (**Figure 5a**), THY1 (**Figure 5b**), and UCHL1 (**Figure 5c**). Double immunostaining revealed that the cultured GFRA1-enriched spermatogonia co-expressed UCHL1 and PLZF (**Figure 5d**) as well as GFRA1 and PLZF (**Figure 5e**). No immunostaining was seen when primary antibodies were replaced with isotype rabbit or goat IgGs (data not shown), verifying the specific staining of these antibodies.

Transcriptomes of freshly separated SSCs and GFRA1-enriched spermatogonia from *M. fascicularis* after culture for 14 days

In order to test the effectiveness and stability of the conditioned-culture medium for GFRA1-enriched spermatogonia, RNA was sequenced and comparison was made for the global gene profiles in freshly isolated and cultured GFRA1-enriched spermatogonia. Total RNA was extracted from these two kinds of cells, and the quality of these cells was evaluated by RNA sequencing using electropherogram (**Figure 6a** and **6b**) to ensure high quality. The total reads and gene numbers in freshly isolated and cultured GFRA1-enriched spermatogonia are shown in **Supplementary Table 2**. In total, there were 20 886 genes in the freshly isolated and cultured GFRA1-enriched spermatogonia. Compared with freshly separated GFRA1-enriched spermatogonia, 414 genes were upregulated and 161 genes were downregulated after culture for 14 days (**Figure 6c**), reflecting a similarity of 97.3% for global gene profiles in GFRA1-enriched spermatogonia between 0 day and 14 days. There were

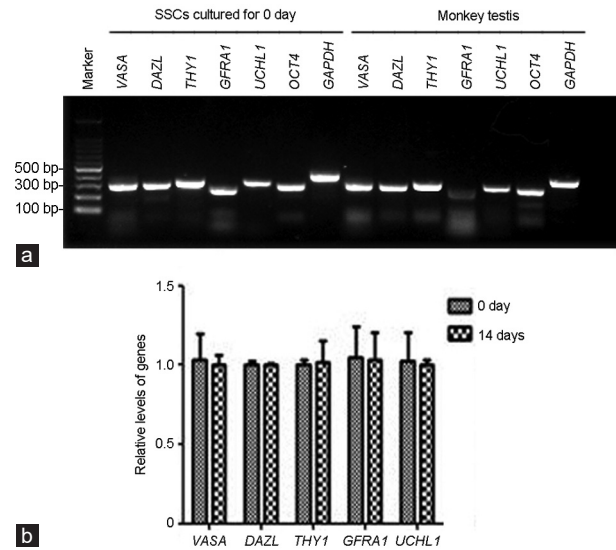


Figure 3: The expression of a number of genes for male germ cells and SSCs in the testicular tissues of *M. fascicularis* and GFRA1-enriched spermatogonia. RT-PCR showed that the transcription of *GFRA1*, *THY1*, *UCHL1*, *OCT4*, *VASA*, and *DAZL* in (a) the freshly isolated GFRA1-enriched spermatogonia (left panel) and the testicular tissues (right panel), as well as (b) the expression of *GFRA1*, *THY1*, *UCHL1*, *VASA*, and *DAZL* in GFRA1-enriched spermatogonia after culture for 0 day and 14 days. *GAPDH* was used as a loading control of total RNA. SSC: spermatogonial stem cell; RT-PCR: reverse transcription-polymerase chain reaction; *GFRA1*: glial cell line-derived neurotrophic factor family receptor alpha-1; *THY1*: Thy-1 cell surface antigen; *UCHL1*: ubiquitin C-terminal hydrolase L1; *OCT4*: POU class 5 homeobox 1; *DAZL*: deleted in azoospermia like; *VASA*: DEAD-box helicase 4; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; *M. fascicularis*: *Macaca fascicularis*.

575 differentially expressed genes (DEGs) in GFRA1-enriched spermatogonia between culture for 0 day and 14 days (data not shown), while the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of these DEGs was illustrated in **Supplementary Figure 1**.

Colonization, proliferation, and maintenance of stemness of GFRA1-enriched spermatogonia from *M. fascicularis* in vivo

In total, 1.5×10^5 GFRA1-enriched spermatogonia were transplanted into each testis of W mutant mice, which are sterile owing to the lack of male germ cells. One month after xenotransplantation, immunohistochemistry revealed that GFRA1 (**Supplementary Figure 2a**), PLZF (**Supplementary Figure 2b**), and UCHL1 (**Supplementary Figure 2c**) were expressed in the cells along the basement membrane of seminiferous tubules of recipient W mutant mice. Immunohistochemistry displayed PCNA expression in these cells (**Supplementary Figure 2d**). Negative control using isotype IgG but not primary antibody showed that no immunostaining was detected within the cells of the seminiferous tubules (**Supplementary Figure 3**), confirming the specificity of our immunostaining results.

DISCUSSION

Rodents and primates have many differences in age, brain size and complexity, and reproductive physiology. Nonhuman primates have emerged as one of the better animal models because the *rhesus macaque* (*Macaca mulatta*) genome has 93% similarity to that of humans.³⁵ Moreover, centrosomal inheritance and fertilization in rhesus monkeys are similar to those in humans but different from those of rodents.³⁶ The SSC is one of the most important adult stem cells, as judged

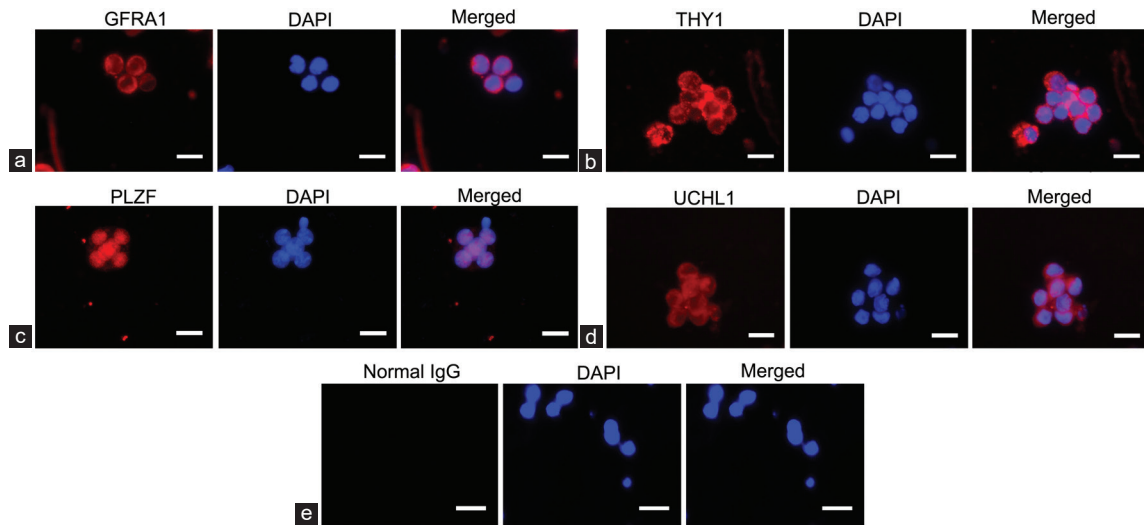


Figure 4: Identification of the freshly isolated GFRA1-enriched spermatogonia from *M. fascicularis* by MACS. (a–d) Immunocytochemistry revealed the expression of (a) GFRA1, (b) THY1, (c) PLZF, (d) UCHL1, and (e) normal IgG in the isolated GFRA1-enriched spermatogonia. Scale bars in a–e = 10 μ m. GFRA1: glial cell line-derived neurotrophic factor family receptor alpha-1; MACS: magnetic-activated cell sorting; PLZF: zinc finger and BTB domain containing 16; THY1: Thy-1 cell surface antigen; UCHL1: ubiquitin C-terminal hydrolase L1; DAPI: 4',6-diamidino-2-phenylindole; *M. fascicularis*: *Macaca fascicularis*.

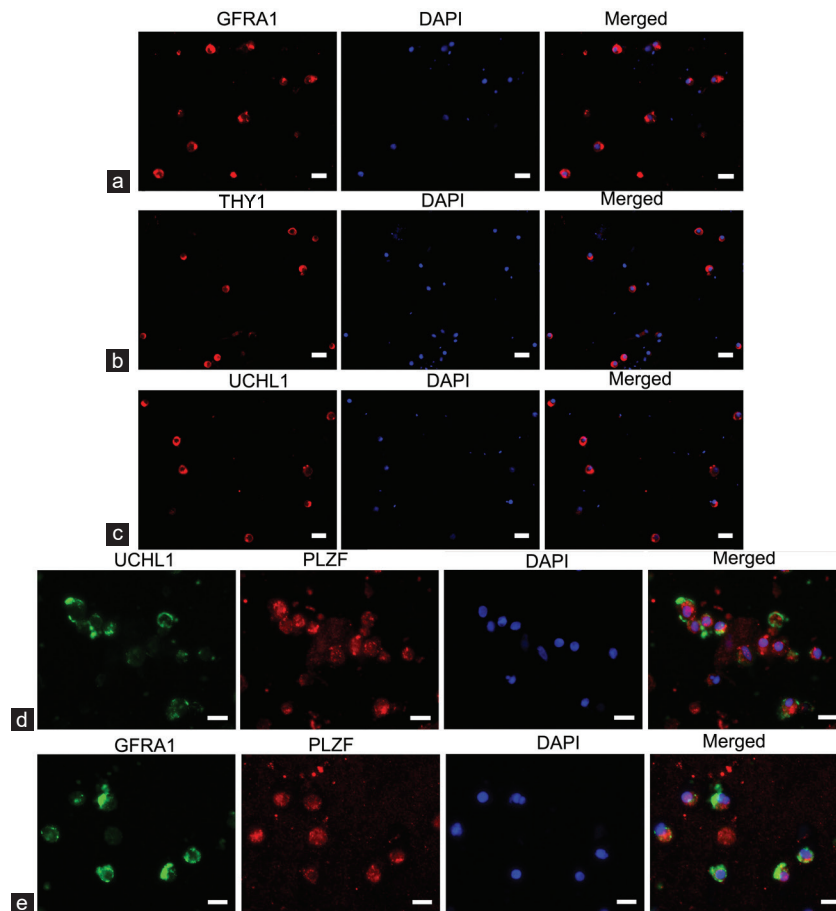


Figure 5: Phenotypic features of GFRA1-enriched spermatogonia from *M. fascicularis* after 14 days of culture. Immunocytochemistry revealed the expression of (a) GFRA1, (b) THY1, and (c) UCHL1, as well as the co-expression of (d) UCHL1 and PLZF and (e) GFRA1 and PLZF in GFRA1-enriched spermatogonia of *M. fascicularis* after culture of 14 days. Scale bars = 10 μ m. GFRA1: glial cell line-derived neurotrophic factor family receptor alpha-1; THY1: Thy-1 cell surface antigen; UCHL1: ubiquitin C-terminal hydrolase L1; PLZF: zinc finger and BTB domain containing 16; DAPI: 4',6-diamidino-2-phenylindole; *M. fascicularis*: *Macaca fascicularis*.

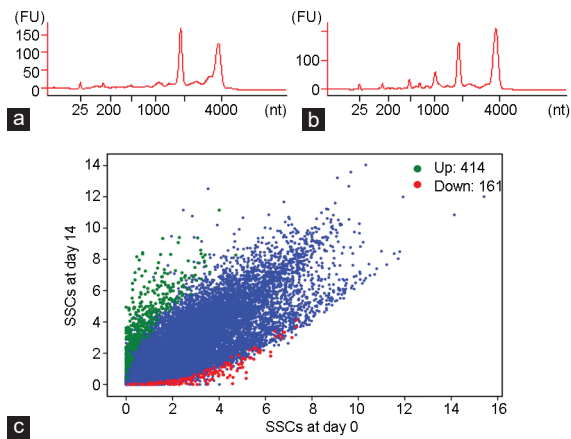


Figure 6: Global gene profiles in the freshly isolated and cultured GFRA1-enriched spermatogonia from *M. fascicularis*. (a and b) Electropherogram by Agilent bioanalyzer displayed the concentrations and nucleotide (nt) sizes of RNAs for (a) freshly separated spermatogonia and (b) the cultured GFRA1-enriched spermatogonia. (c) Scatter plots revealed large scale of gene expression levels in the freshly separated spermatogonia and the cultured GFRA1-enriched spermatogonia. GFRA1: glial cell line-derived neurotrophic factor family receptor alpha-1; SSC: spermatogonial stem cell; *M. fascicularis*: *Macaca fascicularis*; FU: fluorescence unit.

by not only having hereditary genetic functions but also becoming pluripotent stem cells through dedifferentiation.³⁷ SSC manipulation techniques are common in life science, agricultural science, and clinical applications.^{38,39} Long-term culture systems for mouse and rat SSCs have been established.^{40,41} In order to use SSCs *in vitro*, they should be isolated, purified, and cultured. The number of primary SSCs in the testis is limited, and thus the isolation and proliferation of SSCs are essential for basic research and application of SSCs. In this study, we found that GFRA1, UCHL1, PLZF, and THY1 were potential markers for SSCs in *M. fascicularis*, and GFRA1, a surface marker, is suitable for separating SSCs from the testis of this species.

The method for obtaining male germ cells from the testis should be effective. The timing of SSC separation in different animals is particularly important. If animals are too young, male germ cells within the testis are gonocytes rather than SSCs. By contrast, the percentages of SSCs are very low if the animals are relatively old. Generally, the age of animals is selected after birth or before puberty. In this study, we chose *M. fascicularis* at 6 years of age because this is a period of enriched SSCs within the testis.⁴² We used collagenase IV, trypsin, and hyaluronidase to digest testicular tissue to single cells. The addition of DNase I removes the DNA from the lysed cells and prevents adhesion between cells.⁴³ The two-step enzymatic digestion method was used to obtain a cell suspension containing male germ cells and Sertoli cells. Seminiferous tubules were isolated from testicular tissue by the first enzymatic digestion in collagenase IV and DNase I. Single cells were acquired from the seminiferous tubules by the second digestion in collagenase IV, hyaluronidase, trypsin, and DNase I. The method can fully digest the testicular tissue of *M. fascicularis*, which resulted in high single-cell yield and cell viability. Male germ cells can be separated from Sertoli cells by differential plating.⁴⁴ In the specific culture system containing gelatin, Sertoli cells and the plastic culture dish are preferentially adherent, whereas male germ cells remain in the suspension. Gelatin is a derivative of matrix constituency, and it assists in attachment of cells to a surface and allows them to proliferate.⁴⁵ Here, we used multiple differential adherence methods to remove Sertoli cells, which achieved a high purification of male germ cells.

GFRA1 is specifically expressed at the surface of SSCs along the basement membrane of the seminiferous tubules. GFRA1 is the receptor for GDNF, which is an important regulator of self-renewal of SSCs, and it has been shown to be expressed in different species including mice, rats, bulls, and primates.^{46–50} In this study, we isolated GFRA1-enriched spermatogonia with a high viability from *M. fascicularis* testis by MACS with an antibody against GFRA1. MACS is a combination technology using a cell surface antigen and a specific antibody linked to a magnetic bead, and a magnetic field can be applied to achieve cell sorting. The freshly isolated GFRA1-enriched spermatogonia were stained positively for numerous markers of SSCs and germ cells, including *GFRA1*, *THY1*, *PCNA*, *PLZF*, *OCT4*, *UCHL1*, *VASA*, and *DAZL*. *VASA* and *DAZL* have been recognized as hallmarks for male germ cells,^{51–54} and *PCNA* is a marker for proliferating cells including spermatogonia.⁵⁵ In addition, *OCT4* has been shown to be a marker for rodent SSCs.^{56,57} *PLZF* has been characterized as a specific transcriptional factor for rodent SSCs.^{58,59} Considered together, our data implicate that GFRA1-enriched spermatogonia are phenotypically SSCs, and we demonstrate that the isolation of SSCs from the *M. fascicularis* testis by MACS is effective.

Establishing an *in vitro* culture system of SSCs while maintaining their stemness and proliferation is required for uncovering mechanisms underlying the fate determinations of SSCs. The maintenance of stem cell status is closely related to their microenvironment. In the current study, we simulated the *in vivo* microenvironment of SSC division and differentiation, with plates precoated with poly-lysine and laminin. Poly-lysine-precoated plates were used because they were necessary for cell adhesion, while laminin has been known to stimulate cell attachment and outgrowth.⁶⁰ A conditioned-culture medium containing DMEM/F-12, GDNF, bFGF, EGF, and LIF was employed to maintain *M. fascicularis* SSCs in an undifferentiated status, which promoted the proliferation and division of these cells. GDNF is an important factor in maintaining the self-renewal and survival of rodent SSCs.⁶¹ GDNF is secreted by testicular support cells and bound by SSC receptor GFRA1 to activate downstream signaling pathway,⁶² while bFGF, bEGF, and LIF enhance the proliferation of SSCs. To assess the effectiveness and stability of the culture system further, RNA sequencing revealed high similarities in global gene profiles between 0 day and 14 days culturing cells. These results indicate that *M. fascicularis* SSCs can be cultured for a short period while maintaining stemness. Our modification of digestion and purification methods accelerated the preparation of testicular cell suspension; shortened the purification time of SSCs; and accelerated the process of production, formation, and stabilization of SSCs clones in culture. Therefore, SSC clones appearing in culture can be observed at the initial culture for 7–14 days. These clones have SSC activity and exhibit clonal growth behavior.

Spermatogonial transplantation is a unique functional assay *in vivo* for SSCs,^{38,63} and SSC xenotransplantation including nonhuman primates has been achieved. Honaramooz *et al.*⁶⁴ transplanted SSCs from juvenile macaques into immunodeficient mouse testes, and they found sperm production. Sadri-Ardekani *et al.*⁶⁵ transplanted human SSCs into immunodeficient mice. SSCs are the only cell that can migrate to the basement membrane of seminiferous tubules.⁶⁶ For xenotransplantation of primate SSCs, it remains unclear whether these cells can survive for a long time in seminiferous tubules of recipient rodents. In the present study, *M. fascicularis* SSCs could settle down and colonize the seminiferous tubules of recipient W mutant mouse testes for one month, and they were able to migrate to the basement membrane of seminiferous tubules and proliferate. The

expression of UCHL1, GFRA1, PLZF, and PCNA confirmed their stem cell properties. These results demonstrate that GFRA1-enriched spermatogonia of *M. fascicularis* possess SSC cell characteristics *in vivo*.

CONCLUSIONS

We have effectively isolated SSCs from *M. fascicularis* with high viability and purity by MACS. These cells could colonize and proliferate in recipient W mutant mice after xenotransplantation. We developed a feeder-free culture system using poly-lysine and laminin and certain growth factors to culture and expand *M. fascicularis* SSCs while maintaining their stem cell characteristics. Studies on *M. fascicularis* SSCs, including *in vitro* culture, cryopreservation, differentiation to mature spermatozoa *in vitro*, and transgenic animals, would provide theoretical reference and technical basis for the use of human SSCs, and they may shed light on stem cell therapy of male reproductive disorders, genetic diseases, regenerative medicine, and transgenic animal research.

AUTHOR CONTRIBUTIONS

GPM performed the experiments, analyzed the data, and wrote the paper. MHN, WC, and RLT performed the experiments and analyzed the data. YHC and BL performed the experiments. ZPH designed the study, analyzed the data, and wrote the paper. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests

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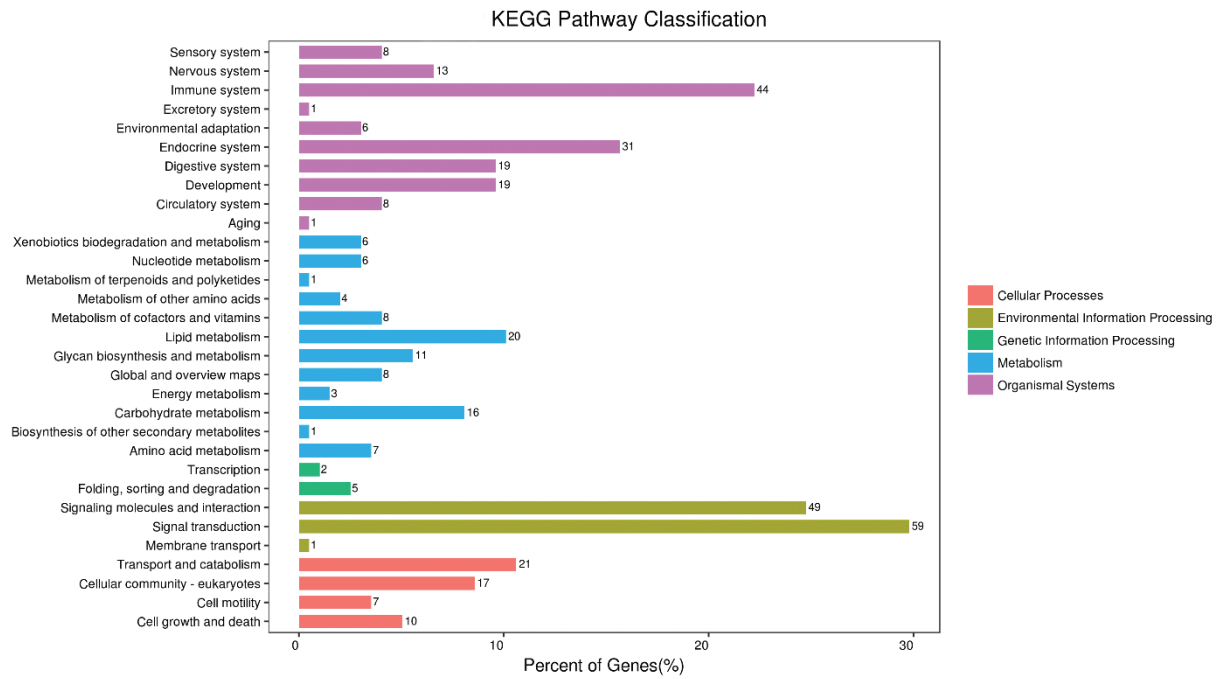
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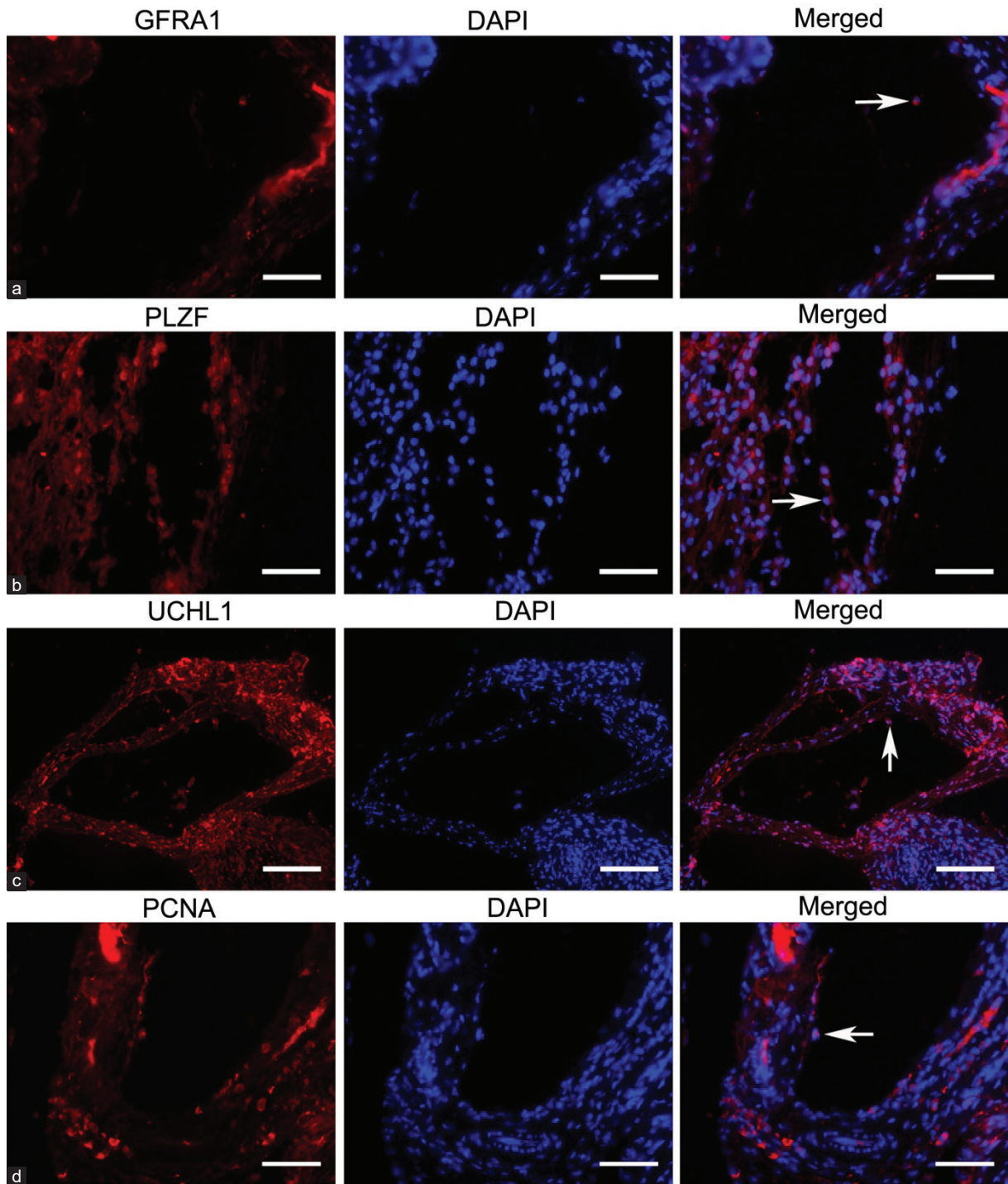
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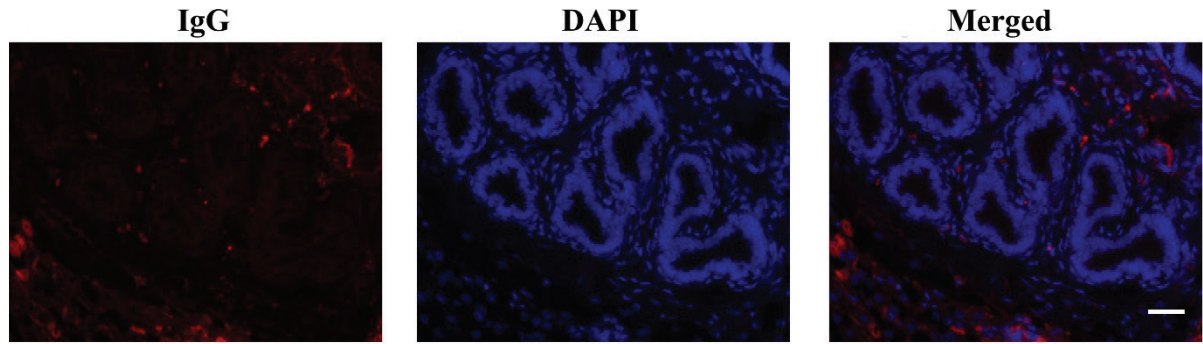
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Supplementary Figure 1: KEGG pathway classification of the DEGs in GFRA1-enriched spermatogonia between culture for 0 day and 14 days. In total, 59 DEGs were involved in signal transduction, while 49 DEGs were signaling molecules and interaction. DEGs: differentially expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes.



Supplementary Figure 2: Phenotypic characteristics of GFRA1-enriched spermatogonia of *M. fascicularis* after xenotransplantation into the recipient W mutant mice. Immunohistochemistry illustrated the expression of GFRA1 (a), PLZF (b), UCHL1 (c), and PCNA (d) in the cells of seminiferous tubules of recipient W mutant mice with xenotransplantation of the GFRA1-enriched spermatogonia. Arrows within the seminiferous tubules in a-d indicated the positive cells. Scale bars in a-d = 100 μ m.



Supplementary Figure 3: Phenotypic characteristics of GFRA1-enriched spermatogonia of *Macaca fascicularis* after xenotransplantation into the recipient W mutant mice. Immunohistochemistry illustrated the expression of isotype IgG in the cells of seminiferous tubules of recipient W mutant mice with xenotransplantation of the GFRA1-enriched spermatogonia from *Macaca fascicularis*. Scale bar = 50 μ m.

Supplementary Table 1: The sequences of gene primers used for reverse transcription-polymerase chain reaction

Genes		Primer sequences	Product size (bp)	Tm ($^{\circ}$ C)
VASA	Forward	TGGAAACAGAGATGCTGGTG	300	55
	Reverse	GGGCTTCCTAGACCAAATCC		
DAZL	Forward	ATCATCCTCCTCCACCACAG	292	55
	Reverse	TCATTTGGCACAACCTCAGC		
THY1	Forward	CTAGTGGACCAGAGCCTTGG	312	54
	Reverse	GCCCTCACACTTGACCAGTT		
GFRA1	Forward	TCGGTTCTCCTACTCCTTAGC	240	60
	Reverse	CCGCTTTTAGGGGTTCAAGT		
UCHL1	Forward	TGCTGAACAAAGTGCTGTCC	299	56
	Reverse	TCCATCCTCAAATCCCAGTT		
OCT4	Forward	G TTCAGCCAAACGACCATCT	296	58
	Reverse	ACACTCGGACCACATCCTTC		
GAPDH	Forward	ATCACTGCCACCCAGAAGAC	302	55
	Reverse	ACCTGGTGCTCAGTGAGCC		

DAZL, DEAD-box helicase 4; THY1, thy-1 cell surface antigen; GFRA1, glial cell line-derived neurotrophic factor family receptor alpha-1; UCHL1, ubiquitin C-terminal hydrolase L1; OCT4, POU class 5 homeobox 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Supplementary Table 2: The reads and gene numbers in glial cell line-derived neurotrophic factor family receptor alpha-1-enriched spermatogonia stem cells of *Macaca fascicularis*

Cell types	Total reads	Gene numbers
Freshly separated GFRA1-enriched spermatogonia	47,027,940	20,886
GFRA1-enriched spermatogonia after culture for 14 days	47,440,288	20,886

GFRA1, glial cell line-derived neurotrophic factor family receptor alpha-1