Supplementary Material For:

Isolation of Sertoli, Leydig, and spermatogenic cells from the mouse testis

Yao-Fu Chang[1], Jennifer S. Lee-Chang[1], Subbarayalu Panneerdoss[1], James A. MacLean II[2], and Manjeet K. Rao[1]
[1]Greehey Children's Cancer Research Institute, The University of Texas Health Science Center at San Antonio, San Antonio, TX, USA and [2]Department of Physiology, Southern Illinois University School of Medicine, Carbondale, IL, USA

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A thorough understanding of the events during mammalian spermatogenesis requires studying specific molecular signatures of individual testicular cell populations as well as their interaction in co-cultures. However, most purification techniques to isolate specific testicular cell populations are time-consuming, require large numbers of animals, and/or are only able to isolate a few cell types. Here we describe a cost-effective and timesaving approach that uses a single protocol to enrich multiple testicular cell populations (Sertoli, Leydig, and several spermatogenic cell populations) from as few as one mouse. Our protocol combines rigorous enzymatic digestion of seminiferous tubules with counter-current centrifugal elutriation, yielding specific testicular cell populations with >80%–95% purity.

Isolation of Leydig cells

To isolate Leydig cells, the top 35 mL Percoll was aliquoted into two 50-mL conical tubes, and 32.5 mL 1× Dulbecco's phosphate-buffered saline (DPBS) were added to each tube. Tubes were centrifuged at 500× *g* for 10 min at 4°C. The pellet was resuspended in 55% Percoll/45% 1× Hank's balanced salt solution (HBSS; vol/vol) and centrifuged at 20,000× *g* for 1.5 h at 4°C. The top 2 mL Percoll containing cellular debris were discarded, and 5-mL fractions were collected from the top of the tube. To remove the remaining Percoll, each fraction was diluted with 35 mL 1× HBSS and centrifuged at 500× *g* for 10 min at 4°C. Discard supernatant and resuspend cells in the residual 1× HBSS (approximately 400 µL). Slides for microscopic examination were prepared by adding 5 µL resuspended cells to 50 µL 4% paraformaldehyde, smearing the sample using the side of a 200 µL pipet tip, and letting the slides air dry. Centrifuge the remaining cells again at 500× *g* for 10 min at 4°C and remove all residual 1× HBSS.

Isolation of Sertoli cells

To isolate Sertoli cells, the 70-µm and 40-µm cell strainers were inverted over a 50-mL conical tube and washed with Enriched DMEM:F12. Cells were resuspended in at least 10 mL Enriched DMEM:F12. Tissue culture plates for the Sertoli fraction were prepared by coating each 100-mm plate with 5 mL 5 µg/mL DSA lectin (lectin from *Datura stramonium* that selectively binds Sertoli cells; Sigma-Aldrich, St. Louis, MO, USA) (1) in 1× HBSS, and incubating at 35°C with 5% CO_2 for 1 h. Lectin-coated plates were rinsed twice with 1× HBSS before use. The Sertoli fraction was transferred to the lectin-coated plates and incubated at 35°C for at least 1 h to achieve maximum attachment. Enriched DMEM:F12 was replaced with 10 mL Hypotonic Shock Solution (0.3× HBSS), and plates were incubated at 35°C for 3 min to lyse contaminating germ cells. Hypotonic Shock Solution was removed, and fresh Enriched DMEM:F12 was added to purified Sertoli cells.

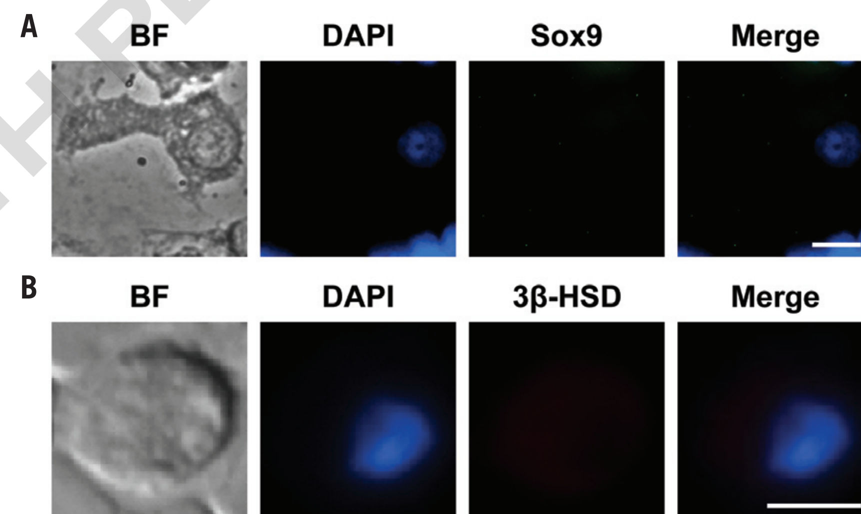

Figure S1. Immunofluorescence staining of enriched testicular fractions with pre-immune sera. (A–B) Smears of enriched testicular somatic cell fractions were air dried, fixed with Bouin's fixative, and immunofluorescence staining was performed. Sertoli and Leydig cells were incubated with pre-immune sera followed by Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 donkey anti-goat IgG, respectively. Scale bars, 10 µm. Photomicrographs were taken on the Nikon Eclipse TE2000-U. Figures shown are representative of six independent experiments.

Elutriator preparation and collection of elutriated fractions

To prepare for centrifugal elutriation, the elutriator was set to spin at 1000 rpm and 4°C and flushed with 10% bleach for 20 min, followed by sterile distilled water for 40 min at a flow rate of 10 mL/min. The elutriator was then set to spin at 2000 rpm and 4°C, and flushed with sterile 1× HBSS at 4°C and a flow rate of 10 mL/min. The single-cell suspension was fed into the elutriator using a 10-mL syringe without the plunger. The first 100 mL flow-through were collected and discarded. The flow rate was increased to 12 mL/min to collect 100 mL elongating spermatid fraction. The flow rate was further increased to 15 mL/min to collect 100 mL round spermatid fraction. The flow rate was increased to 25 mL/min, then 30 mL/min, and 50 mL were collected and discarded for each flow rate. To collect 100 mL pachytene spermatocyte fraction, the speed of the rotor was increased to 2250 rpm, and the flow rate was increased to 37 mL/min. The rotor was stopped, and 50 mL were collected at 65 mL/min, which mainly consisted of Sertoli cells and pachytene spermatocytes. These cells were transferred to datura-coated plates, and Sertoli cells were isolated as described above.

Percoll gradient preparation

Two gradients were prepared for isolating round spermatids by adding 9.5 mL 45% Percoll in 1× HBSS (vol/vol) to centrifuge tubes, then slowly overlaying with 9.5 mL 28% Percoll in 1× HBSS (vol/vol). Two gradients were prepared for isolating pachytene spermatocytes by adding 9.5 mL 38% Percoll in 1× HBSS (vol/vol) to centrifuge tubes, then slowly overlaying with 26% Percoll in 1× HBSS (vol/vol). The tops of the centrifuge tubes were sealed with Parafilm, gently tilted horizontally, and allowed to equilibrate at 4°C for at least 1 h. Divide the elutriated fractions into two equal volumes, gently tilt the centrifuge tubes back to their vertical positions, and carefully layer the elutriated fractions over their corresponding gradients.

Immunofluorescence staining

Air-dried smears were rinsed with 1× DPBS for 5 min, fixed with Bouin's fixative (RICCA Chemical, Arlington, TX, USA) for 5 min, and rinsed again with 1× DPBS for 5 min. Cells were blocked with 10% serum (goat for Sertoli cells; Vector, Burlingame, CA, USA; or donkey for Leydig cells; Abcam, Cambridge, MA, USA) in 1× DPBS (vol/vol) for 1 h in a humidified chamber, followed by rinsing with 1× DPBS for 5 min. Cells were incubated with primary antibodies diluted with 3% serum in 1× DPBS (vol/vol) at 4°C overnight in a humidified chamber (1:25 dilution of anti-Sox9 for Sertoli cells; Millipore, Billerica, MA, USA; or 1:100 dilution of anti-3β-HSD for Leydig cells; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The next day, slides were rinsed with 1× DPBS for 5 min, and cells were incubated with secondary antibodies diluted with 3% serum in 1× DPBS (vol/vol) for 1 h in a humidified chamber (1:400 dilution of Alexa Fluor 488 goat anti-rabbit IgG for Sertoli cells; Invitrogen, Carlsbad, CA, USA; or 1:400 dilution of Alexa Fluor 594 donkey anti-goat IgG for Leydig cells; Invitrogen). After the incubation, cells were rinsed with 1× DPBS for 5 min, mounted with Vectashield HardSet Mounting Medium with DAPI (Vector), and visualized on the Nikon Eclipse TE2000-U.

Supplementary Table S1. Cell number, purity, and amount of RNA obtained

Cell type	Cell number	Purity	Amount of RNA
PS	6.2×10^6	83%–90%	57.7 µg
RS	1.1×10^7	93%–95%	65.3 µg
ES	6.4×10^6	80%–85%	29.0 µg
Sertoli	7.7×10^6	92%–94%	45.6 µg
Leydig	2.1×10^6	83%–87%	21.1 µg

Values for cell numbers and amount of RNA obtained are of cells pooled from four mice and representative of six independent experiments. PS, pachytene spermatocyte; RS, round spermatid; ES, elongating spermatid.

Supplementary Table S2. qRT-PCR primers used in this study

Primer	Sequence
Acrv1	5 -TCAGCAACTTTCAAGCGAGTAT-3 5 -CTCCTGAAGAGTGCTCACCTG-3
Cyp11a1	5 -CCAGTGTCCCCATGCTCAAC-3 5 -TGCATGGTCCTTCCAGGTCT-3
Dbil5	5 -CCCAGGGCGACTGTAACATC-3 5 -GCAATGTAGATCCTCATGGCAT-3
Rhox5	5 -CACCAGGACCAAAGTGGCC-3 5 -GGTATGGAAGCTGAGGGTT-3
Rps2	5 -CTGACTCCCGACCTCTGGAAA-3 5 -GAGCCTGGGTCCTCTGAACA-3
Sycp3	5 -AGCCAGTAACCAGAAAATTGAGC-3 5 -CCACTGCTGCAACACATTCATA-3

Primer sequences were obtained from PrimerBank (2). Primers were checked for specificity and efficiency using serially diluted gene-specific cDNA constructs. qRT-PCR, quantitative RT-PCR.

Quantitative RT-PCR

One microgram RNA from purified germ cell and somatic cell fractions, as well as total testis, were reverse-transcribed using the miScript System (Qiagen, Valencia, CA, USA) and analyzed by the 7900HT Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using primer sets specific to germ cell, Sertoli cell, and Leydig cell marker genes (Supplementary Table S2). The specificity and efficiency for each primer set was determined using serially diluted gene-specific cDNA constructs. Replicate reactions were run for each cDNA sample. The relative expression of each gene was quantified using the comparative threshold cycle ($\Delta\Delta C_T$) method after normalizing against *Rps2* and primer efficiencies.

Reagents

- DMEM:F12
- L-glutamine (Mediatech, Manassas, VA, USA)
- Sodium L-lactate (Sigma-Aldrich)
- Sodium pyruvate (Invitrogen)
- MEM non-essential amino acids (Invitrogen)
- Deoxyribonuclease I (Sigma-Aldrich)
- Collagenase IA (Sigma-Aldrich)
- Percoll
- 10× HBSS
- Trypsin (Sigma-Aldrich)
- Charcoal-stripped FBS
- 10× DPBS
- Periodic Acid-Schiff (PAS) kit
- Qiazol
- miScript System
- Lectin from *Datura stramonium*
- Bouin's fixative
- Goat serum
- Donkey serum

- Anti-Sox9 antibody
- Anti-3β-HSD (P-18) antibody
- Alexa Fluor 488 goat anti-rabbit IgG
- Alexa Fluor 594 donkey anti-goat IgG
- Vectashield HardSet Mounting Medium with DAPI
- Enriched DMEM:F12 (DMEM:F12, 1 mM L-glutamine, 5 mM sodium L-lactate, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids)
- Enzymatic Solution 1 (Enriched DMEM:F12, 200 μg/mL deoxyribonuclease I, 0.5 mg/mL collagenase IA)
- Enzymatic Solution 2 (Enriched DMEM:F12, 200 μg/mL deoxyribonuclease I, 1 mg/mL trypsin)
- Enzymatic Solution 3 (Enriched DMEM:F12, 200 μg/mL deoxyribonuclease I)
- Hypotonic Shock Solution (0.3× HBSS)

Ethics statement

All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Approval of animal use for this study was granted by The Institutional Animal Care and Use Committee of University of Texas Health Science Center at San Antonio (UTHSCSA; Animal Welfare Assurance no. A3345-01; Protocol no. 07057-34-02-A).

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