# Establishment of a Simple and Effective Method for Isolating Male Germline Stem Cells (GSCs) from Testicular Cells of Neonatal and Adult Mice

KIM, KYE-SEONG[1], JUNG JIN LIM[1,2], YUN HEE YANG[2], SOO-KYOUNG KIM[1], TAE KI YOON[2], KWANG YUL CHA[2], AND DONG RYUL LEE[2]*

[1]*Department of Anatomy and Cell Biology, Hanyang University College of Medicine, Seoul, Korea*
[2]*Fertility Center of CHA General Hospital, CHA Research Institute, Pochon CHA University, Seoul, Korea*

**Abstract**  The aims of this study were to establish a simple and effective method for isolating male germline stem cells (GSCs), and to test the possibility of using these cells as a new approach for male infertility treatment. Testes obtained from neonatal and adult mice were manually decapsulated. GSCs were collected from seminiferous tubules by a two-step enzyme digestion method and plated on gelatin-coated dishes. Over 5–7 days of culture, GSCs obtained from neonates and adults gave rise to large multicellular colonies that were subsequently grown for 10 passages. During *in vitro* proliferation, oct-4 and two immunological markers (Integrin β1, α6) for GSCs were highly expressed in the cell colonies. During another culture period of 6 weeks to differentiate to later stage germ cells, the expression of oct-4 mRNA decreased in GSCs and Sertoli cells encapsulated with calcium alginate, but the expression of c-kit and testis-specific histone protein 2B (TH2B) mRNA as well as the localization of c-kit protein was increased. Expression of transition protein (TP-1) and localization of peanut agglutinin were not seen until 3 weeks after culturing, and appeared by 6 weeks of culture. The putative spermatids derived from GSCs supported embryonic development up to the blastocyst stage with normal chromosomal ploidy after chemical activation. Thus, GSCs isolated from neonatal and adult mouse testes were able to be maintained and proliferated in our simple culture conditions. These GSCs have the potential to differentiate into haploid germ cells during another long-term culture.

**Key words:** Male germline stem cells (GSCs), *in vitro* proliferation, *in vitro* differentiation, haploid germ cells, mouse testis

*Corresponding author
Phone: 82-2-3468-3421; Fax: 82-2-501-8704;
E-mail: drleedr@cha.ac.kr

Spermatogenesis is the process of germ cell proliferation and differentiation within the seminiferous tubules of the testis, leading to haploid free-swimming spermatozoa. As precursors of the spermatogonial lineage, male germline stem cells (GSCs) must maintain a balance between production of mature sperm and the self-renewal of stem cells.

Orchestrated in large measure by Sertoli cells *in vivo*, spermatogenesis requires complex endocrine [17, 30] and auto/paracrine regulation as well as direct cell-to-cell interactions [7].

Male GSCs exhibit many similarities to other stem cells. GSCs are a rare, relatively quiescent population that lies in a protected region in the testis among support cells, which may regulate their behavior. As with hematopoietic stem cells, GSCs in mammals are transplantable, with the ability to both expand the stem cell pool and regenerate an entire depleted spermatogenic lineage [2].

An effective procedure for recapitulating spermatogenesis *in vitro* would greatly facilitate mechanistic studies of the *in vivo* process while providing a biological basis for treating male infertility and genetically modifying the male germline. Attempts to achieve mammalian spermatogenesis *in vitro* using immature germ cells have been reported in the literature since the early 1960s [26], but difficulties in establishing proper conditions for germ cells to proceed to and through meiosis and subsequently to spermiogenesis have limited the success of *in vitro* spermatogenesis systems [12]. Several other reports in recent years have demonstrated that germ cells cultured in association with Sertoli cells can progress through discrete steps in the sequence of spermatogenesis during short-term culture, including initiation or completion of meiosis and initiation of spermiogenesis [8, 10, 13, 16, 24, 25]. Recently, GSCs were generated by genomic modification and differentiated into male germ

cells, round spermatids, by culturing *in vitro* [5]. By coculturing with STO cells, Kanatsu-Shinohara *et al.* [11] also achieved long-term proliferation without genetic modification *in vitro* and haploid male germ cells by transplantation of these stem cells. In addition, male haploid germ cells in the round spermatid stage have been derived from embryonic stem cells by spontaneous differentiation *in vitro*, opening up the possibility of investigating germ cell development, epigenetic reprogramming, and germline gene modification [6]. However, completion of spermatogenesis *in vitro* from GSCs through spermatocytogenesis, meiosis, and spermiogenesis has not been reported previously for any mammalian species. Furthermore, proliferation of GSCs by genomic modification or co-culture using feeder cells obtained from foreign species would be a major drawback to human applications. Isolation and proliferation of GSCs from the adult testis has not been established until now. Therefore, in order to apply *in vitro* spermatogenesis to treatment of male infertility, we developed a novel two-step culture system in which GSCs obtained from neonatal and adult testes are first isolated and allowed to proliferate by coculture with their own testicular somatic cells, and then differentiated into haploid germ cells by encapsulating them in calcium alginate during long-term culture.

## MATERIALS AND METHODS

### Isolation and Proliferation of GSCs

Testes were obtained from 50 3-day-old neonatal mice and 2 adult mice (ICR strain), and the tunicae albuginea of collected testes were manually decapsulated. The decapsulated testes were dissociated in 10 ml of an enzyme solution A containing 0.5 mg/ml collagenase (Type I, Sigma), 10 μg/ml DNAse I, 1 μg/ml soybean trypsin inhibitor (Gibco), and 1 mg/ml hyaluronidase (Sigma) in $Ca^{++}$, $Mg^{++}$-free PBS, and incubated for 30 min at ambient temperature (~25°C). After the peritubular cells were removed, seminiferous tubules were redissociated in 10 ml of enzyme solution B containing 5 mg/ml collagenase (Gibco), 10 μg/ml DNAse I (Sigma), 1 μg/ml soybean trypsin inhibitor (Gibco), and 1 mg/ml hyaluronidase (Sigma) in $Ca^{++}$, $Mg^{++}$-free PBS, and incubated for 30 min at 37°C. For isolation of GSCs from the adult testis, dissociated testicular cells were loaded onto 3 layers of 2 ml each Percoll gradient (45–70–90%, Sigma) and centrifuged at 500 ×*g* for 20 min. Each fraction was washed and collected. Dissociated cells from neonatal and adult testes were plated and grown on gelatin-coated dishes in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 15% fetal bovine serum (Gibco), 10 μM 2-mercaptoethanol (Gibco), 1% nonessential amino acids (Gibco), 1 ng/ml basic fibroblast growth factor (bFGF, Invitrogen), 10 μM forskolin (Sigma), and 1,500 U/ml human leukemia inhibitory factor (Pepro Tech Inc.).

Over a period of 5–7 days, small colonies of GSCs gave rise to large multicellular colonies that were subsequently grown for 10 passages. After the 5th passage, cells within the colonies were divided into two groups; one was fixed for characterization and the other was encapsulated and cultured for *in vitro* differentiation.

### Characterization of GSCs Isolated from *In Vitro* Culture

Colonies were fixed in 4% paraformaldehyde in Dulbecco's PBS (Gibco) for nucleus counting using 1 μg/ml 4,6-diamidino 2-phenyindiol (DAPI; Sigma). For immunocytochemical analysis of oct-4, integrin α6, integrin β1, and c-kit expression, other fixed colonies were incubated overnight with anti-oct-4 antibody (Santa Cruz), anti-integrin α6 antibody (BD/Pharmingen), anti-integrin β1 antibody (BD/Pharmingen), and anti-c-kit antibody (Santa Cruz) at 1:100–500 dilutions. The primary antibody was visualized with fluoroscein isothiocyanate-conjugated secondary antibody (Zymed).

### *In Vitro* Differentiation of GSCs

After 5 passages, over 500 GSC colonies and their underlying Sertoli cells were trypsinized into single cells. Dissociated cells were resuspended and then encapsulated with sodium alginate [16]. Alginate-encapsulated cells were then transferred into culture medium. The culture medium was HEPES-buffered DMEM/F12 medium (Gibco) supplemented with 10 μg/ml insulin-transferring-selenium solution (Gibco), $10^{-4}$ M vitamin C (Sigma), 10 μg/ml vitamin E (Sigma), $3.3 \times 10^{-7}$ M retinoic acid (Sigma), $3.3 \times 10^{-7}$ M retinol (Sigma), 1 mM pyruvate (Sigma), $2.5 \times 10^{-5}$ U recombinant human FSH (Gonal-F, Serono), $10^{-7}$ M testosterone (Sigma), 1× antibiotic-antimycotic (ABAM, containing penicillin, streptomycin, and amphotericin B, Gibco), and 10% bovine calf serum (Hyclone) [29]. Alginate-encapsulated cell aggregates were transferred to 1.0 ml of culture medium in a 24-well dish and cultured for up to 6 weeks at 32°C in a humidified atmosphere of 5% $CO_2$ in air. The medium was replaced on alternate days.

### Characterization of Haploid Male Germ Cells Derived from *In Vitro* Differentiation

*In vitro* cultured cells were decapsulated mechanically and incubated in trypsin-EDTA for 30 min. The dispersed cells were rinsed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After 3 washes, the cells were attached to precoated glass slides (Probe On Plus, Fisher, PA, U.S.A.) by cytospin (Cyto-TEK, Miles Inc., Elkhart, IN, U.S.A.) for 15 min at 1,500 rpm and permeabilized with absolute methanol for 30 min. A reaction with 10 μg/ml tetramethylrhodamine isothiocyanate (TRITC)-*Arachis hypogea* (peanut agglutinin, PNA, Sigma) was carried out for 1 h at room temperature. After cells were washed, they were mounted on glass slides with PBS and cover slipped,

and the cover slips sealed with nail varnish. The presence of acrosome vesicles on round spermatids was verified by microscopic observation.

## Immunohistochemical Localization of c-kit

Alginate-encapsulated cell aggregates were collected at 0, 3, and 6 weeks and fixed overnight in 10% formaldehyde in PBS. Each sample was washed overnight in PBS, dehydrated through graded ethanol (50%, 70%, 85%, 95%, 100%; 2×20 min each), cleared in xylene (2×30 min each), infiltrated with paraffin (60°C; 2×30 min), and embedded in paraffin wax.

Serial sections (5 μm) were made, dried overnight onto glass slides at 37°C, and stored at room temperature until the immunohistochemistry was performed.

Prior to immunohistochemistry, sections were deparaffinized in xylene and rehydrated through graded ethanol. Sections were reacted with c-kit primary antibody (1:500 dilution) overnight at 4°C on glass slides and then rinsed in reaction buffer 3 times.

The primary monoclonal antibody was localized using a biotinylated secondary antibody, followed by a streptavidin and horseradish peroxidase complex. The presence of peroxidase was revealed by addition of a DAB substrate-chromogen solution (Histostain-Plus DAB kit, Zymed, CA, U.S.A.).

## RNA Preparation and Analysis Using Reverse Transcription-Polymerase Chain Reaction

The mRNA expression of oct-4, c-kit, testis-specific histone protein 2B (TH2B), and transition protein (TP-1) genes was assessed in cells cultured from GSCs using reverse transcription-polymerase chain reaction (RT-PCR). First, testes were collected from 5-, 10-, 15-, and 20-day-old and adult mice in order to check the expression patterns of these genes. At 0 (5-day-old testicular cells), 3, and 6 weeks of culture, the cultured cells were incubated with trypsin-EDTA for 30 min, washed three times with $Ca^{++}$, $Mg^{++}$-free PBS, and then sampled.

Total RNA was extracted by the TRIzol method (Gibco) from the testicular tissues (about 100 mg) and cultured cells ($10^8$ cells).

Reverse transcription was carried out using 1 μg of total RNA, 5 mM $MgCl_2$, and 1 U of DNAse I at 37°C for 30 min, and then at 42°C for 1 h after adding 1 mM dNTP, 2.5 μM oligo-dT, and 2.5 U reverse transcriptase (Superscript, Gibco) [9].

PCR was performed in a 20-μl reaction containing 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 0.25 mM dNTP, 3–5 pmol of each primer, and 1.25 U Taq polymerase (Gibco). The following genes were amplified using the primers indicated in parenthesis. oct-4 (5'-acc atg ttt ctg aag tgc cc-3', 5'-gct cct gat caa cag cat ca-3'; 155 bp; GenBank Accession Number X52437), c-kit (5'-ctg gtg gtt

cag agt tcc ata gac-3', 5'-tca acg acc ttc ccg aag gca cca-3'; 389 bp; GenBank No. Y00864), TH2B (5'-ctc tct aga aag gtt act tga gcc atg-3', 5'-ctc tat aga tgc cgg tgt cgg ggt g-3'; 199 bp; Gen Bank X90778), TP-1 (5'-tgg cat gag gag agg caa ga-3', 5'-gct cat tgc cgc atc aca ag-3'; 155 bp; GenBank No. NM009407), and 18S ribosomal protein (5'-aga tga tcg agc cgc gc-3', gct acc agg gcc ttt gag atg ga-3'; 163 bp, [28]. The PCR was initiated with denaturation at 94°C for 5 min followed by 25–35 cycles (within linear ranges) of 30 sec at 94°C, 30 sec at 55–60°C, and 30 sec at 72°C. A final extension for 10 min at 72°C completed the PCR; then, PCR products were separated by 2% agarose gel electrophoresis and verified by automated nucleotide sequencing. Negative controls included mock transcription without RNA or PCR with distilled deionized water.

## Intracytoplasmic Spermatid Injection and Embryo Production

After 6 weeks of in vitro differentiation, presumptive round spermatids (7–10 μm) were obtained from cultured testis parenchyma and then injected into mature oocytes. Expanded cumulus cells were removed by repeated pipetting with a small-bore glass pipette followed by incubation in 0.1 mg/ml hyaluronidase for 5 min. Matured oocytes were activated by treatment with 5 μM of calcium ionophore (Sigma) and incubated for 3 h. A single round spermatid selected for injection was aspirated into an injection pipette, and injected into a denuded, matured oocyte at 90° from the first polar body. Sham injections with medium only and chemical activation were performed as controls. Injected oocytes were then transferred into KSOM [15] containing 0.3% BSA under mineral oil. The culture medium was changed every other day, and embryonic cleavage, blastocyst formation, and hatching were evaluated. For karyotyping, presumptive embryos were transferred to fresh KSOM containing 0.5 μg/ml Colcemid (Gibco) at 48–50 h post-activation and cultured for an additional 24 h.

Following Colcemid treatment, the zona pellucida was removed with 0.5% pronase E (P-5147, Sigma) and an individual blastomere was transferred to hypotonic (1%) sodium citrate for 4 min at ~24°C. The blastomere was transferred in ~0.5 μl of sodium citrate to 1.5 μl methanol: acetic acid (1:1 v/v) on a precleaned microscope slide. The chromosomes were spread by gently blowing on the preparation. After air-drying, slides were fixed in methanol: acetic acid (3:1 v/v) for 30 min. Fixed preparations were then stained with 4% Giemsa solution for 10 min and observed under a light microscope. Embryos were classified as being of normal ploidy (diploid), or of abnormal ploidy (haploid, polyploid, or mixoploid).

## Statistical Analysis

Differences in rates of embryonic development and ploidy between the round spermatid injection and sham-operation

groups were analyzed with the Chi-square test. $P<0.05$ was defined as statistically significant.

## RESULTS AND DISCUSSION

Over a period of 5–7 days of *in vitro* culture, testicular cells from neonatal and adult mice gave rise to large multicellular colonies ($\geq$100 cells in a colony). After dissociation into small pieces by trypsinization, the cell suspension was transferred to new culture dishes every week.

The number of presumptive GSCs colonies was increased by 3–5 times after every passage (Figs. 1A, 1B). Adult

testicular cells obtained from the 45% Percoll fraction could only form similar patterns of colonies to those of neonatal cells. However, the number of colonies formed from adult cells was much lower than the number derived from neonatal cells, although without comparative analysis (data not shown).

During *in vitro* proliferation, cells within colonies derived from neonatal and adult testicular cells showed positive signals for oct-4 and two immunological markers (Integrin $\beta$1 and Integrin $\alpha$6) that have been recognized for general characterization of stem cells and GSCs (Fig. 1). Despite these stem cell characteristics, presumptive GSCs colonies were detached and disappeared after about 10 passages.
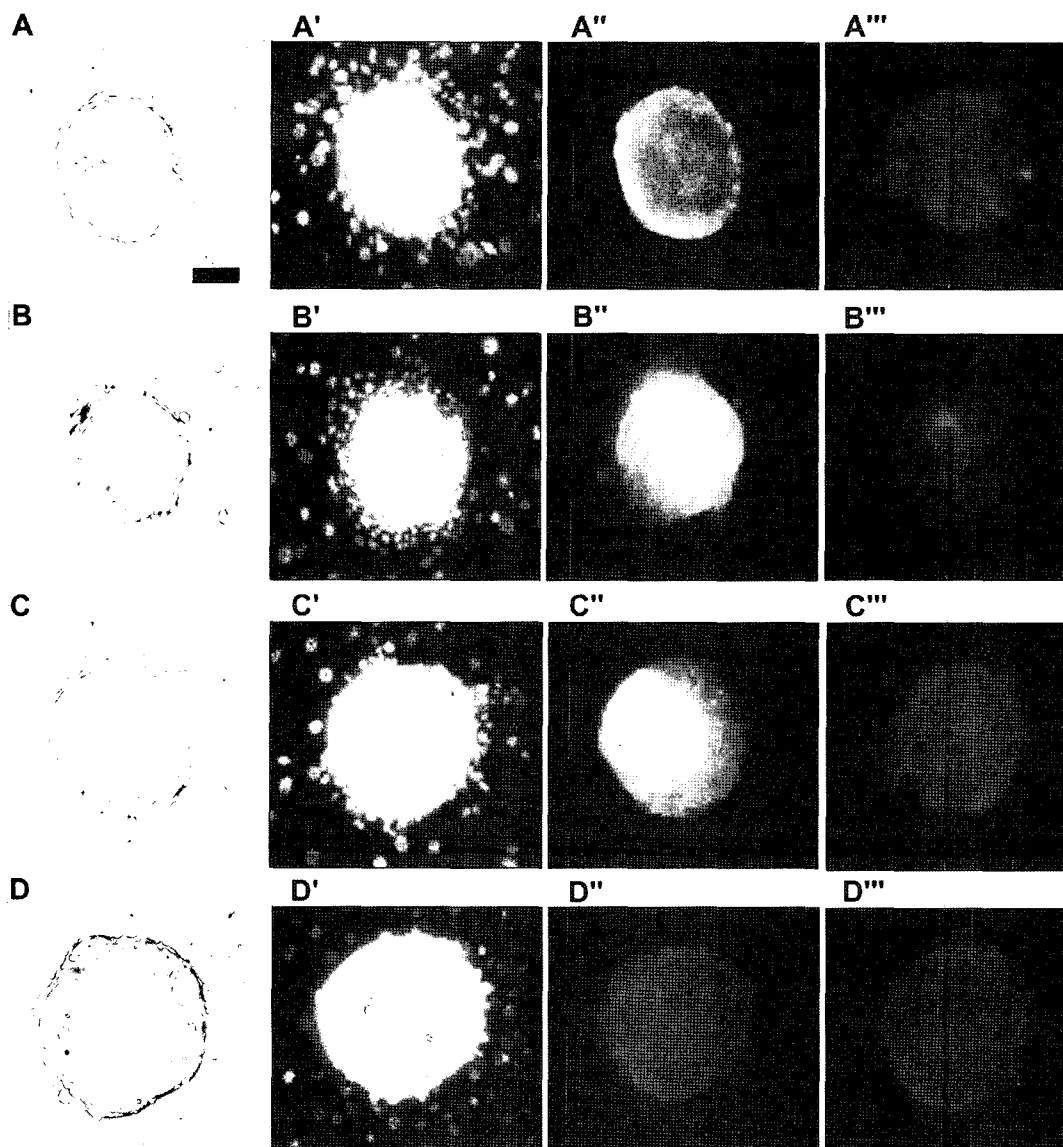


**Fig. 1.** Morphology and characterization of mouse GSCs. **A–D.** GSC colony formation after 2-weeks of cultivation. **A'–D'.** Staining with DAPI. **A"–D".** Immunocytochemistry revealing oct-4, Integrin $\alpha$6, Integrin $\beta$1, and c-kit expression in colony cells. **A'''–D'''.** Negative controls not treated with primary antibodies.
Bar=100 μm.
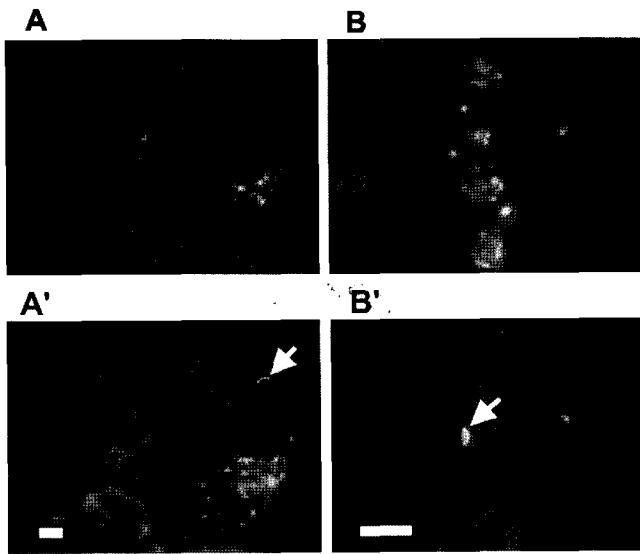
**Fig. 2.** Identification of sperm obtained from the epididymis of an adult mouse (**A** and **A'**) and round spermatids from *in vitro* culture of GSCs (**B** and **B'**) by Hoechst 33258 (**A** and **B**) and TRITC-peanut agglutinin (PNA) (**A'** and **B'**) staining.
The fluorescence was more intense in the acrosomal regions of the sperm and spermatids (arrow). Scale bar=10 μm.

To determine the function of these cultured GSCs, we performed *in vitro* differentiation by encapsulation during long-term culture. After 5 passages, GSCs colonies and their underlying Sertoli cells were detached from the plates by trypsinization, reaggregated encapsulated, and cultured further for 6–7 weeks. As reported previously, there was no change in the integrity or dimension of the extruded calcium alginate-containing GSCs during *in vitro* culture. However, when encapsulated cells were dissociated, spherical cells with sizes ranging from 7 to 20 μm were observed after 3 or 6 weeks of *in vitro* culture. Dissociated cells cultured for 6 weeks were fixed, permeabilized, mounted on glass slides, and stained with TRITC-PNA, which specifically stains acrosome granules of round spermatids. The acrosomes of spermatozoa were used as a positive control. TRITC-PNA gave a distinct signal in *in vitro* cultured testicular cells of neonates and adults, with an approximate diameter of 7–10 μm (Fig. 2).

To further characterize these spherical cells, we used RT-PCR to check for the presence of mRNAs from genes known to be expressed in male germ cells at specific stages, from spermatogonia to round spermatids. First, we confirmed the expression patterns of these marker genes (oct-4, c-kit, TH2B, and TP-1) in neonatal and adult mouse testis (5-day-old for GSCs, 10-day-old for spermatogonia, 15-day-old for spermatocytes, 20-day-old for spermatids, and adult cells as a positive control, Fig. 3A), and then analyzed gene expression in cultured GSCs using the same method. During *in vitro* culture for 6 weeks, expression of oct-4 mRNA decreased in encapsulated cells, but

expression of c-kit and TH2B mRNA increased. The spatial extent of c-kit expression was seen by 3 weeks of culture (Fig. 3B and Fig. 3c4). Expression of c-kit protein in various cells was examined for negative and positive controls; as expected, c-kit was not expressed in the GSCs and Sertoli cells of 5-day-old mice testes or the colonies from *in vitro* culture (Fig. 3c1, 3c3), and was well localized in spermatogonia and Pachytene spermatocytes (Fig. 3c2). Expression of TP-1 was not seen until 3 weeks after culturing, and then appeared by 6 weeks (Fig. 3B).

To test the capacity of the differentiated haploid germ cells to fertilize eggs, we carried out embryo production using these cells. We injected spermatid-like cells into mature mouse oocytes and chemically activated injected eggs by exposure to a $Ca^{++}$ ionophore and DMAP. Ninety-five MII oocytes were used for intracytoplasmic injection of putative round spermatids obtained from cultured GSCs, and 75 MII oocytes were used for sham operations.

Twenty-five (26.3%) and 6 (12.5%) oocytes, respectively, were damaged by the microinjection and activation procedures. Embryonic cleavage rates did not differ between injected (injection and activation) and sham-operated (just activation) control groups (80.0% [56/70] vs. 72.5% [50/69]), but rates of blastocyst formation and hatching in the injected group using cells cultured *in vitro* (42.9% [24/56]; 50.0% [12/24]) were higher than in the activated controls (24.0% [12/50]; 25.0% [3/12]), respectively ($P<0.05$). The cytogenetic composition of some 2- to 4-cell embryos was examined. In the injected group, 50% (16/32) of embryos had normal chromosomal diploidy, whereas most embryos in the control (activation) group were abnormal (96.6% [29/30]) ($P< 0.05$). Most of chromosomal aneuploidy was haploidy and mixoploidy.

Spermatogenesis is a complex, well-organized process that includes proliferation and differentiation. During fetal development, primordial germ cells migrate to the genital ridge and are enclosed by somatic Sertoli cells; they then become prospermatogonia or gonocytes [3]. The gonocytes proliferate for a few days and then arrest in the $G_0/G_1$ phase until birth.

Within a few days after birth, gonocytes resume proliferation to initiate spermatogenesis. Gonocytes migrate to the basement membrane of seminiferous tubules and become undifferentiated type A spermatogonia, the male GSCs [18]. GSCs either renew themselves to maintain the pool of stem cells or undergo differentiation to produce spermatozoa [4, 5].

The establishment of male GSCs has broad implications for the study of development, disease, and evolution. Moreover, tools to isolate, proliferate, and differentiate GSCs will provide powerful new approaches to creating transgenic animals and performing human gene therapy relating to infertility and diseases. Even though several approaches using transgenesis or long-term culture of
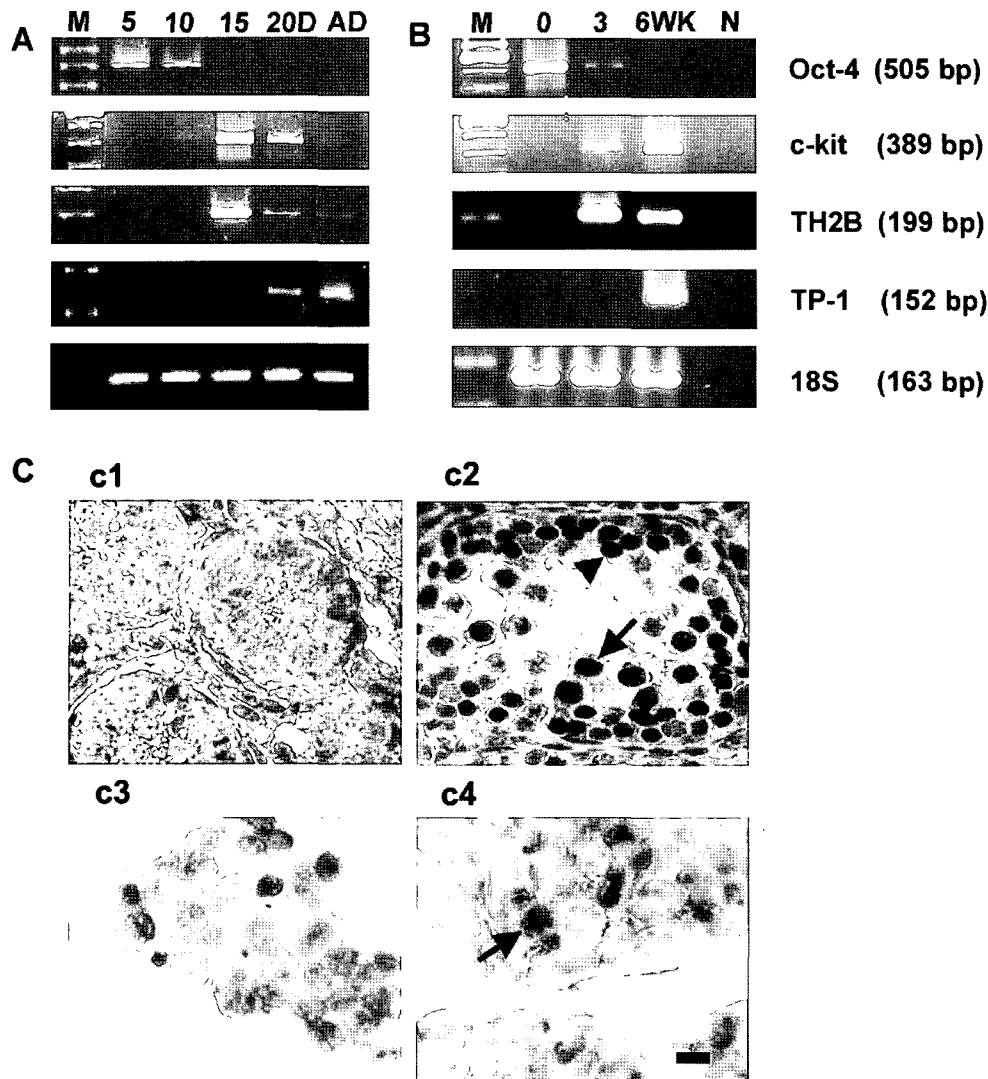
**Fig. 3.** RT-PCR expression pattern of stage-specific genes in testis (**A**) from 5-, 10-, 15-, and 20-day-old and adult mice, and in vitro diffrention of testicular cells (**B**) from neonatal mice following culture GSCs for 3 and 6 weeks.

Immunolocalization of c-kit protein in neonatal mice testes and testicular cells before and after culture *in vitro*. c1 and c3, c-kit is not expressed in the testis or the GSC colonies from a 5-day-old mouse. c2, c-kit is expressed in spermatogonia (arrowheads) and Pachytene spermatocytes (arrows) of a 15-day-old mouse testis. c4, c-kit is expressed in germ cells derived from the GSCs after 3 weeks of culture *in vitro* (arrow). M, molecular weight marker. Scale bar=10 μm.

GSCs have been successful [5, 11], an efficient method for proliferation and differentiation *in vitro* has been limited. Our present method easily established GSCs without genetic modification and additional feeder cells, and its success was independent of the maturity of the male mouse. We were able to establish GSCs from adults, which may provide applicable possibilities for treatment of human male infertility. In addition, the isolation and proliferation of GSCs by coculture with their own testicular somatic cells avoids the risk of transmitting pathogens from feeder layers. Furthermore, in our study, these GSCs were proliferated and maintained in colonies. During maintenance, similarly to the results of other researchers [14, 21, 22], a high level of oct-4 and specific integrins ($\beta 1$ and $\alpha 6$) were expressed in GSCs derived from our culture, but the c-kit receptor was not expressed.

However, proliferation of GSCs was limited to 10 passages under our culture conditions; we believe that this might be caused by aging of the self-feeder cells, mainly Sertoli cells, in extended culture. Development of a better feeder-free system may therefore extend the number of passages of GSCs. Long-term survival and proliferation of GSCs were achieved by replacement of fresh mouse embryonic feeder cells [11].

In a previous study, neonatal bovine testis cells from dissociated seminiferous tubules were reaggregated and

encapsulated in calcium alginate to promote and sustain interactions between germ cells and Sertoli cells without limiting the permeability of media components.

Results indicated that these conditions promoted *in vitro* spermatogenesis from gonocytes to round spermatids [16]. However, the number of testis cells (round spermatids and spematocytes) was low in long-term cultures, and we believed that this culture system would only support the progression of gonocytes to round spermatids, and not allow proliferation. In order to reconcile this problem, we employed a new approach for isolating and proliferating male GSCs in our culture system and finally developed a novel technique for proliferation of male GSCs and their differentiation into male haploid germ cells. However, comparative analysis with other culture methods for precise analysis of proliferating efficiency is needed before using our process for human applications, although the number of colonies increased by 3–5 times after each passage in this study.

Many aspects of differentiation occur before the cell enters the second meiotic division from secondary spermatocyte to round spermatid. These changes include reductions in size, DNA and chromosomal content, and the formation of acrosome granules. Acrosomal granules of round spermatids originate from proacrosomic granules in the Golgi regions of primary spermatocytes. This has been confirmed by electron microscopy as well as clear staining with *Arachis hypogea* agglutinin (PNA) lectin, which binds to the acrosomal outer membrane of spermatids and spermatozoa [1]. In the present study, acrosome granules were clearly detected by TRITC-PNA staining in putative round spermatids derived from *in vitro* cultured testicular cells of neonates and adults.

The nuclei of round spermatids are able to successfully support development to term in some mammals, including mice, humans, and rabbits [19, 23, 27]. However, unlike sperm with condensed nuclei, round spermatids have uncondensed nuclei with chromatin. Furthermore, round spermatids and oocytes are in different phases of the cell cycles: G2 and M, respectively. Higher levels of metaphase promoting factor in MII oocytes induce premature chromosome condensation of injected chromatin and asynchronization of pronuclei in fertilized eggs, resulting in low fertilization rates or poor development [13]. In our experiments, the round spermatids derived from GSCs supported embryonic development up to the blastocyst stage, with maintenance of normal chromosomal ploidy after chemical activation for synchronizing the cell cycle between each gamete. However, we believe that production of offspring though the transfer of embryos is needed to confirm the developmental competence of round spermatids derived from *in vitro* spermatogenesis.

These results demonstrate that our simple long-term culture method allows for the isolation and proliferation of mouse GSCs from neonatal and adult testicular cells *in*

*vitro* and may confirm a potential method for differentiating these cells into functionally normal haploid germ cells, even though the method did not produce spermatozoa from long-term culture and the experiment was not pursued to produce viable offspring. This novel approach represents a major advance for developing and characterizing improved procedures for *in vitro* spermatogenesis and has important implications for clarifying the mechanistic details of spermatogenesis, for the treatment of male-factor infertility, and possibly for germline modification.

## Acknowledgments

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