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Establishment and Characterization of Neonatal Mouse Sertoli Cell Lines

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ABSTRACT: Sertoli cells isolated from 6-day postpartum mouse testes were conditionally immortalized with the simian virus 40 large tumor antigen gene (SV40-LTAg) under the control of a promoter inducible with ponasterone A, an analog of ecdysone. This strategy produced 2 cell lines, which exhibited mixed phenotypes. We first tested the conditional expression of the LTAg gene in the presence or absence of ponasterone A. The results showed that both cell lines expressed LTAg when the inducer was present in the culture media. When ponasterone A was removed, the majority of the cells died. After 60 generations, however, the continued expression of LTAg in the absence of the hormone indicated that unknown changes may have occurred in the genome of the cells. One of the cell lines was further subcloned, resulting in 7 new lines exhibiting a morphology resembling that of Sertoli cells in tissue culture. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on RNA collected from each cell line in order to determine which cells were phenotypically similar to Sertoli cells in vivo. All cell lines expressed the products of the Sertoli cell–specific genes stem cell factor (SCF) and sulfated glycoprotein-2 (SGP-2), in addition to α-inhibin, GATA-1, and steroidogenic factor-1. Further, the lines express growth and differentiation factors known to act upon germ cells in vivo and in vitro such as leukemia inhibitory factor (LIF), transforming growth factor beta (TGF-β), and basic fibroblast growth factor (bFGF). Moreover, when used as feeder layers in cocultures, at least 2 of these lines are able to maintain the viability of type A spermatogonia for at least 7 days and to support the first steps of spermatogonial differentiation.

Key words: Testis, immortalization, simian virus large T antigen, ecdysone, ponasterone A, growth factors.

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Within the seminiferous epithelium of the testis, developing germ cells and Sertoli cells are in close association with each other. Sertoli cells produce many soluble factors necessary for germ cell survival and proliferation, as well as basement membrane components and the tubule structure within which the germ cells differentiate. Sertoli cell–derived growth factors such as stem cell factor (SCF), basic fibroblast growth factor (bFGF), and leukemia inhibitory factor (LIF) seem to be particularly important for the survival and proliferation of primordial germ cells, gonocytes, and type A spermatogonia (Dolci et al, 1991; Resnick et al, 1992; De Felici and Pesce, 1994; Nikolova et al, 1997; Dirami et al, 1999; Feng et al, 2000), while transforming growth factor beta (TGF-β) has been implicated in germ cell differentiation at later stages (Mullaney and Skinner, 1993; Fritz, 1994; Kierszenbaum, 1994). Another Sertoli cell–secreted factor, α-inhibin, might also have a role in the regulation of spermatogonial cell number and differentiation (van Dissel-Emiliani et al, 1989; Hakovirta et al, 1993; Mather et al, 1997).

More recently, it has been shown that spermatogonial stem cells have to fit within a proper 3-dimensional tissue architecture in order to express specific genes and that a physical association with Sertoli cells is necessary (Meng et al, 2000). Thus, it seems that a close association between spermatogonia and Sertoli cells, coupled with the supply of specific growth factors, needs to be mimicked in vitro to maintain germ cells in long-term cultures. Therefore, we attempted to cultivate freshly isolated germ cells on Sertoli cell line feeder layers. We initially worked with a Sertoli cell line immortalized with a plasmid for the constitutive production of the simian virus 40 large tumor antigen (SV40-LTAg) (Hofmann et al, 1992; van der Wee and Hofmann, 1999). However, upon long-term culture, the cells became transformed and lost some of their typical characteristics, making them unsuitable for coculture with freshly isolated germ cells. Thus, a conditional immortalization system that allows the modulation of expression of the gene coding for SV40-LTAg was desirable. We conditionally immortalized freshly isolated Sertoli cells by transfecting them with 2 plasmids, pVgRXR and pIND-LTAg. The pVgRXR plasmid codes

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for the ecdysone nuclear receptor, which forms a complex with the *Drosophila* steroid hormone ecdysone (No et al, 1996). We constructed the pIND-LTAg plasmid that contains the large T-antigen gene under the control of an ecdysone-inducible promoter. When ponasterone A (an analog of ecdysone) is present in the culture medium, the complex ponasterone A receptor binds to the upstream hormone response element on the pIND-LTAg plasmid, driving the promoter and activating the transcription of the LTAg gene. Using this method, we established 7 Sertoli cell lines that we describe in the present study. These lines were characterized using light and electron microscopy, immunocytochemistry, and reverse transcriptase-polymerase chain reaction (RT-PCR). We analyzed the expression of 16 genes known to be specific for various cell types within the testis. All cell lines expressed genes specific for Sertoli cells in the testis, such as SCF and sulfated glycoprotein-2 (SGP-2). Moreover, they also expressed the genes coding for α-inhibin, GATA-1, bFGF, LIF, and TGF-β. Thus, the pattern of gene expression displayed by these Sertoli cell lines indicated that they could be used as feeder layers to support the survival of type A spermatogonia in vitro.

Materials and Methods

Construction of pIND-LTAg

The plasmid pIND-LTAg was constructed from the pIND vector (Invitrogen, Carlsbad, Calif), which contains the *neo*^{r} gene and an ecdysone-inducible promoter upstream of the multiple cloning site (No et al, 1996). Ponasterone A, an analog of the Drosophila hormone ecdysone (Invitrogen), served as the inducer. pIND-LTAg was derived from both the plasmid pSV3-neo (ATCC 37150) and pIND by excising the 3.3-kb LTAg sequence out of pSV3-neo and ligating it into the *Bam*HI site of pIND. In order to determine whether pIND-LTAg was functional, the plasmid was cotransfected into MDA-231 breast carcinoma cells with the plasmid pVgRXR (Invitrogen), which codes for the ecdysone receptor and the *zeocin*^{r} gene.

Tissue Culture

Cells were cultivated in complete cell culture media, which consisted of Dulbecco modified Eagle medium (DMEM) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, and 100 mM nonessential amino acids (Atlanta Biologicals, Norcross, Ga). Cells were plated in culture dishes (Falcon, Fisher Scientific, Pittsburgh, Pa) containing complete culture medium supplemented with 5% or 10% fetal calf serum (FCS; Atlanta Biologicals) and incubated at 37°C and 5% CO_2 in a humidified incubator. For cultures in minimal conditions, FCS was replaced by 5% synthetic NU serum (Fisher).

Cell Isolation, Transfection, and Subcloning

Animal investigations were conducted according to the National Research Council *Guide for the Care and Use of Laboratory Animals.* After the testes were removed from 6-day-old BALB/c mice, the seminiferous tubules were reduced to a single-cell suspension using a series of enzymatic digestions (Bellve et al, 1977; Hadley et al, 1985, 1990). Cotransfections with the pIND-LTAg plasmid and the pVgRXR plasmids were performed with the Lipofectin technique (GIBCO/Life Technologies, Grand Island, NY), and the transfected cells were selected with the antibiotics zeocin (Invitrogen) and G418 (GIBCO/Life Technologies). We obtained 2 cell lines, G5 and J5, that showed notably different morphologies. The G5 cell line was subcloned by 2 rounds of limiting dilutions in 96-well plates. Seven subclones—SG5-1.13, SG5-2, G5F2, G5G2, G5B1, G5B2, and G5B3—were selected on the basis of morphological resemblance to Sertoli cells in tissue culture (cytoplasmic projections, lipid inclusions, and indented nuclei) using phase-contrast microscopy. These cells were maintained in culture with complete culture medium with the addition of 5% NU serum (Fisher), ponasterone A (5 μM) (Invitrogen, La Jolla, Calif), zeocin (100 μg/mL) (Invitrogen), and G418 (100 μg/mL) (GIBCO/Life Technologies).

Inactivation/Reactivation of the Inducible Oncogene LTAg

The G5 and J5 cell lines were cultured in LabTek chambers (Fisher) with complete culture media supplemented with 10% FCS, ponasterone A (5 μM), zeocin (100 μg/mL), and G418 (100 μg/mL). Immunocytochemistry was initially performed on these cells to confirm the expression of the LTAg, the oncoprotein produced by the SV40-LTAg gene. MDA-231, a breast carcinoma cell line, was used as a negative control for LTAg expression, and MDA-231 clone B, a clone of the MDA-231 cell line transfected with pIND-LTAg and pVgRXR, served as the positive control. The cell lines were cultivated in complete culture media with 10% FCS. The cells were fixed with ice-cold methanol for 20 minutes and then incubated with a purified mouse anti-LTAg primary antibody (Calbiochem, San Diego, Calif) at a dilution of 1:500, 1:1000, or 1:2000 or no primary antibody (negative control). The secondary antibody was a biotinylated anti-mouse immunoglobulin G made in the horse (Vector Laboratories, Burlingame, Calif). The streptavidin-alkaline phosphatase technique (Vector) was used to visualize the presence of LTAg, and observations were made using brightfield microscopy (IMT microscope, Olympus Corp, Melville, NY).

Once LTAg expression was confirmed in the G5 and J5 cell lines, the cells were cultured without ponasterone A for 10 days, and immunocytochemistry was performed to determine if the LTAg gene had been inactivated. Then, ponasterone A was again added to the cultures for either 1 day or 7 days, the cells were fixed, and immunocytochemistry for LTAg was performed to determine how long it took for the oncogene to be reactivated.

Growth Curves

The SF7 (Hofmann et al, 1992) and SG5-2 cell lines were counted and seeded at 20 000 cells per dish in 18 different 60-mm tissue culture dishes with complete cell culture media and 5% FCS. For the next 6 days, 3 of the dishes for each cell line were trypsinized, and the cells were counted using trypan blue exclusion. The cell numbers are presented as mean plus or minus standard deviation.

Immunocytochemistry for c-kit, α-Actin, GATA-1, and 3β-ol-Hydroxysteroid-Dehydrogenase

Cells were grown on FCS-coated coverslips until 80% confluency and fixed with ice-cold methanol for 5 minutes. After washing with phosphate-buffered saline (PBS), the cells were stained with a mouse monoclonal α-actin antibody at a dilution of 1:100 (Roche Molecular Biochemicals, Indianapolis, Ind), a rabbit polyclonal 3β-ol-hydroxysteroid-dehydrogenase antibody at a dilution of 1:200 to 1:500 (a kind gift from J. I. Masson, University of Edinburgh, United Kingdom), a rabbit polyclonal c-kit antibody at a dilution of 1:50 to 1:200 (Santa Cruz Biotechnology, Santa Cruz, Calif), or a rabbit GATA-1 antibody at a dilution of 1:100 (Santa Cruz Biotechnology). The sites of reaction of the primary antibodies were shown by biotinylated secondary antibodies and the streptavidin-peroxidase technique (Zymed, San Francisco, Calif) or by the immunofluorescence technique.

Cytochemistry for Alkaline Phosphatase

In order to visualize the expression of alkaline phosphatase (AP), the cells were cultured in LabTek chambers (Fisher), fixed in situ with 10% formaldehyde (Sigma Chemical Co, St Louis, Mo) in PBS for 10 minutes at 4°C, and then washed twice with 0.2 M Tris buffer (pH 8.9). The substrate reagent, prepared fresh, consisted of 0.01% naphthol-AS-MX phosphate and 0.06% fast violet B salt (Sigma) in 0.1 M Tris buffer (pH 8.9). The staining solution was immediately filtered and then incubated with the fixed cells for 45 minutes at 37°C. After incubation, the cells were washed with distilled water and observed under brightfield microscopy (IMT-2 microscope, Olympus). In this assay, a red color at the plasma membrane indicates the expression of AP. NIH 3T3 fibroblasts were used as a negative control since they do not express this enzyme.

RT-PCR

In order to determine the phenotypes of the 7 sublines of G5, RT-PCR was performed using primers for genes specifically expressed by various cell types within the testis. Total RNA was collected from the subclones using TRI REAGENT according to the manufacturer's protocol (Molecular Research Center Inc, Cincinnati, Ohio). Total RNA samples were treated with 1 U/1 μg RNA of RQ1 RNase-free DNase (Promega, Madison, Wis) to degrade any genomic DNA present. Complementary DNA (cDNA) was synthesized from 5 μg of the total RNA using SuperScript II RT and oligo(dT) primers to selectively reverse transcribe messenger RNA (mRNA; GIBCO/Life Technologies).

Two microliters of the cDNA obtained from each subclone was amplified for 40 cycles (denaturation at 94°C for 30 seconds, annealing at specific temperatures for 45 seconds, and elongation at 72°C for 45 seconds) using primers specific for various genes expressed by different testicular cell types. As a positive control and to check for the presence of contaminating genomic DNA, PCR primers for cyclophilin, a housekeeping gene, were also used. The primers were designed using published sequences from mouse or other mammalian species (Stratagene, La Jolla, Calif) (Walther et al, 1996; Schrans-Stassen et al, 1999). The primers and PCR conditions used were as follows.

1) Cyclophilin, a housekeeping gene expressed by all mouse cells (5′-TGG GCC GCG TCT CCT TCG AG-3′ [sense strand] and 5′-GGG GAA TGA GGA AAA TAT GG-3′ [antisense strand], with an annealing temperature of 48°C), for a PCR product fragment of 589 bp (GenBank accession number 10090);
2) c-kit, expressed by spermatogonia in the 6-day-old mouse testis (5′-TCA ACG ACC TTC CCG AAG GCA CCA-3′ [sense strand] and 5′-CTG GTG GTT CAG AGT TCC ATA GAC-3′ [antisense strand], with an annealing temperature of 37°C), for a PCR product fragment of 385 bp (Schrans-Stassen et al, 1999);
3) SCF, produced by Sertoli cells (5′-TAA CCC TCA ACT ATG TCG CC-3′ [sense strand] and 5′-TGA AGA GAG CAC ACA GTC AC-3′ [antisense strand], with an annealing temperature of 53°C), for a PCR product fragment of 344 bp (Stratagene);
4) α-Inhibin, produced by Sertoli cells (5′-GCA ATG GAT GGG GAA GGT GG-3′ [sense strand] and 5′-GGT GGC TGC GTA TGT GTT GG-3′ [antisense strand], with an annealing temperature of 54°C), for a PCR product fragment of 237 bp (Walther et al, 1996);
5) 3β-ol-Hydroxysteroid-dehydrogenase, expressed specifically by Leydig cells (5′-GTG AC(A/C) GGA G(C/G)A GGA GG-3′ [sense strand] and 5′-AGG AAG CTC AC(A/T) (G/A)TT TCC A-3′ [antisense strand], with an annealing temperature of 33°C), for a PCR product fragment of 889 bp (Walther et al, 1996);
6) GATA-1, produced specifically by Sertoli cells (5′-TGT GTG AAC TGT GGA GCA ACG GC-3′ [sense strand] and 5′-AAA TGA AGG CCG CAG GCA TTG CA-3′ [antisense strand], with an annealing temperature of 59°C), for a PCR product fragment of 247 bp (Walther et al, 1996);
7) Androgen receptor, expressed by Sertoli cells, Leydig cells, and peritubular myoid cells (5′-GAG GAA CGA CAG CCT TCA CAG CAG C-3′ [sense strand] and 5′-GCT GCT GCT GAA GAA GTT GCA T-3′ [antisense strand], with an annealing temperature of 54°C), for a PCR product fragment of 258 bp (Walther et al, 1996);
8) Luteinizing hormone (LH) receptor, expressed specifically by Leydig cells (5′-AAT CCC ATC ACA AGC TTT CAG-3′ [sense strand] and 5′-TGC CTG TGT TAC AGA TGC-3′ [antisense strand], with an annealing temperature of 42°C), for a PCR product fragment of 214 bp (Walther et al, 1996);
9) Steroidogenic factor-1 (SF-1), expressed by Sertoli cells and Leydig cells (5′-GTG AAG TTC CTG AAC AAC CAC AGC-3′ [sense strand] and 5′-GTC TGC TTG GCC TGC AGC ATC TCG-3′ [antisense strand], with an annealing temperature of 54°C), for a PCR product fragment of 241 bp (Walther et al, 1996);
10) SGP-2, produced by Sertoli cells (5′-GAC AAT GAG CTC CA(G/A) GAA (A/C)TG-3′ [sense strand] and 5′-CAG GCA TCC TGT GGA GTT (G/A)TG-3′ [antisense strand], with an annealing temperature of 33°C), for a PCR product fragment of 806 bp (Walther et al, 1996);
11) LIF, produced by Sertoli cells, germ cells, and Leydig cells (5′-TCA CCC CTG TAA ATG CCA CC-3′ [sense strand] and 5′-CTT CTT TGT CAG AGT GGT CG-3′ [antisense

strand], with an annealing temperature of 52°C), for a PCR product fragment of 450 bp (Stratagene);

12) TGF-β, produced by Sertoli cells and peritubular myoid cells (5′-CGG GGC GAC CTG GGC ACC ATC CAT GAC-3′ [sense strand] and 5′-CTG CTC CAC CTT GGG CTT GCG ACC CAC-3′ [antisense strand], with an annealing temperature of 68°C), for a PCR product fragment of 406 bp (Stratagene);
13) bFGF, produced by Sertoli cells and peritubular myoid cells (5′-AAC TCA AAC TTC AAG CAC AAG AGA GA-3′ [sense strand] and 5′-TTA AGA TCA GCT CTT AGC AGA CAT-3′ [antisense strand], with an annealing temperature of 53°C), for a PCR product fragment of 293 bp (Stratagene); and
14) Follicle-stimulating hormone (FSH) receptor, expressed by Sertoli cells (5′-ACT GGC TGT GTC ATT GCT CT-3′ [sense strand] and 5′-CTG AGA GAT CTC TAT TTT CTC-3′ [antisense strand], with an annealing temperature of 37°C), for a PCR product fragment of 200 bp (Walther et al, 1996).

As negative controls, PCR was performed on RT reaction products obtained without the use of RT. All PCR reaction products were electrophoresed on 2% agarose gels made in 1× Tris-boric acid-EDTA buffer (TBE, pH 8.0) with 0.01% ethidium bromide in a running buffer consisting of TBE with 0.01% ethidium bromide. Ten microliters of PCR product and 2 μL of 6× loading dye were loaded into the gel and electrophoresed for 32 minutes at 100 V, 200 mA, and 600 W. Results were viewed on an ultraviolet light lamp table.

Light and Electron Microscopy

Cells were cultivated in 60-mm petri dishes until 80% confluency, washed with PBS, and treated with 0.05% trypsin-EDTA in PBS (Atlanta Biologicals). After trypsin inhibition with 1% (vol/vol) FCS in PBS, the cell samples were centrifuged at 200 × *g*. The pellets were carefully resuspended in 1 mL of 5% glutaraldehyde in 0.2 M s-collidine buffer, pH 7.4, and then transferred into microcentrifuge tubes. Glutaraldehyde and s-collidine buffer were purchased from Electron Microscopy Sciences (Fort Washington, Pa). After a 30-minute fixation with occasional inversion of the tube, the cells were centrifuged at 200 × *g* and washed 3 times with 0.2 M collidine buffer. The samples were then postfixed in a final concentration of 1% OsO_4 in 1.5% potassium ferrocyanide for 1 hour at room temperature and washed for 15 minutes with 0.2 M s-collidine buffer. After dehydration in increasing concentrations of ethanol, the samples were cleared in propylene oxide and then embedded in Epon (Electron Microscopy Sciences). One-micrometer sections were cut with an LKB microtome, stained with toluidine blue, and examined by light microscopy. Thin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy.

Cocultures of Germ Cells and Sertoli Cells

Sertoli cells of the lines SG5-1.13 and SG5-2 were seeded in 24-well plates at a concentration of 2 × 10^4 cells per well in 1 mL of culture medium. Type A spermatogonia were isolated using the STAPUT method (Dym et al, 1995), and a single-cell suspension was immediately seeded with the Sertoli cells at a concentration of 4 × 10^4 cells/well. The culture medium used was DMEM completed as described above. However, in this case, 5% synthetic NU serum (Becton-Dickinson, Sparks, Md) was used instead of FCS to provide defined culture conditions. Three days after seeding, the cocultures were fixed and stained with a rabbit polyclonal anti–c-kit antibody (Santa Cruz Biotechnology) at a dilution of 1:100 to visualize the spermatogonia. The sites of reaction of the primary antibody were shown by a biotinylated secondary antibody and the streptavidin-peroxidase technique (Zymed). For comparison, a single-cell suspension of type A spermatogonia was seeded on adherent 6-day-old primary Sertoli cells or NIH 3T3 cells using the same culture conditions.

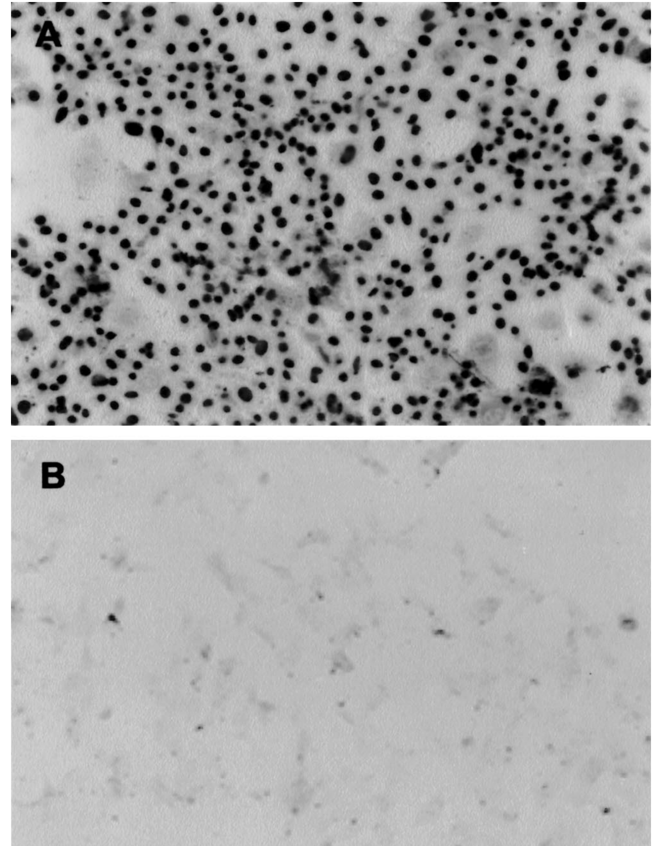

Figure 1. Expression of the large tumor antigen (LTAg) shown by immunocytochemistry. **(A)** J5 cell line positive for LTAg as seen by the dark nuclei (100×). **(B)** J5 cell line negative control without primary antibody (100×).

Results

Modulation of Expression of the Inducible Oncogene SV40-LTAg

After antibiotic selection, 2 cell lines were initially obtained, which we termed J5 and G5. Immunocytochemistry performed on these cell lines cultured in the presence of ponasterone A showed that the LTAg is expressed in the nucleus of these cells. As an example, Figure 1

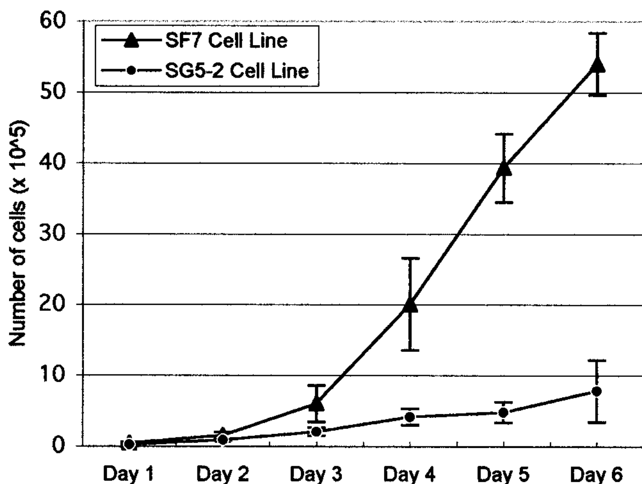

Figure 2. Rates of proliferation of the SF7 cell line vs the SG5-2 cell line.

shows LTAg expression in the J5 cell line. The positive control cell line MDA-231 clone B (which expresses LTAg) was also positive, while the negative control MDA-231 cell line was negative (data not shown).

After the 2 testicular cell lines, G5 and J5, were cultured for 10 days without ponasterone A, many of the cells entered a crisis state and died. However, 10% of the original cell populations survived, and these cells continued to express LTAg without the addition of ponasterone A in the culture medium. After 6 months of continuous culture, the ability to turn off the expression of LTAg in these cells was lost. Since both lines exhibited a mixed phenotype, the G5 line was further subcloned by limiting dilution, resulting in 7 cell lines exhibiting Sertoli cell-like morphology in tissue culture. While the expression of the LTAg remained constitutive in all of the lines, their rate of proliferation was markedly lower than the rate of proliferation of the SF7 Sertoli cell line previously established (Hofmann et al, 1992). SF7 cells were immortalized using the LTAg under the permanent control of the SV40 promoter. Figure 2 compares the rate of proliferation of the cell line SF7, which expresses a high level of LTAg, to the rate of proliferation of the SG5-2 cell line, a subclone of G5.

Characterization of the Cell Lines by Immunocytochemistry and Phase-Contrast Microscopy

The J5 and G5 cell lines were stained by immunocytochemistry for the presence of the following markers: c-kit, α-actin, and 3β-ol-hydroxysteroid-dehydrogenase. None of the lines expressed the c-kit receptor or 3β-ol-hydroxysteroid-dehydrogenase, excluding the presence of differentiating spermatogonia and Leydig cells using this method (data not shown). However, about 50% of the J5 cells expressed α-actin, indicating the presence of peritubular myoid cells. This cell line was not further characterized.

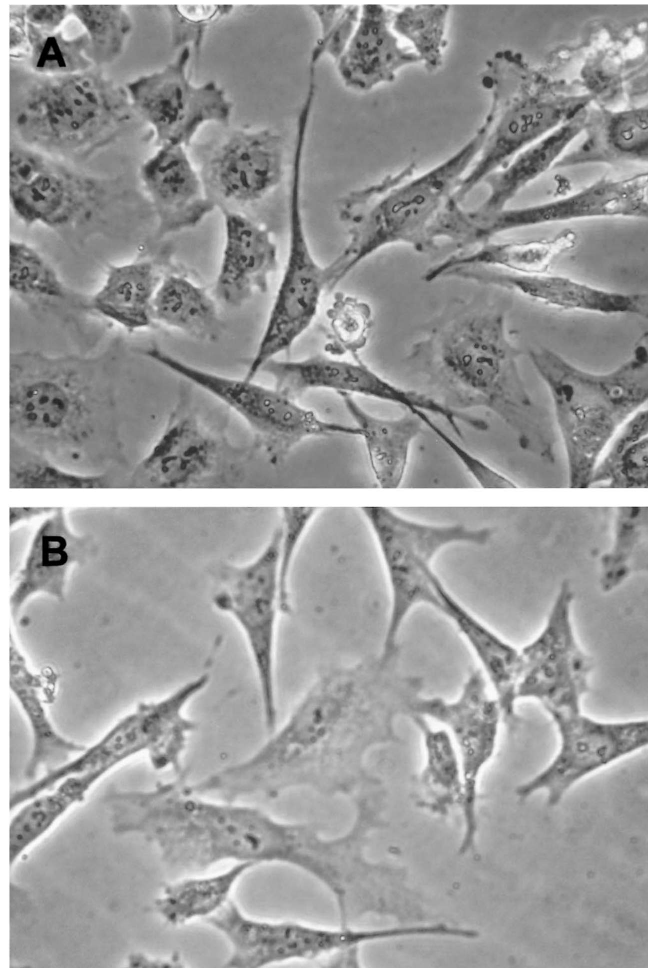

Figure 3. Phase-contrast micrographs of G5 subclones. **(A)** SG5-1.13 cell line (200×). **(B)** SG5-2 cell line (200×).

Seven cell lines resulted from the subcloning of G5 and were designated SG5-1.13, SG5-2, G5B1, G5B2, G5B3, G5F2, and G5G2. These new lines were cultivated for more than 2 years. In phase-contrast microscopy, the SG5-1.13 and SG5-2 cell lines appeared mostly spindle shaped. Cells from both lines exhibited some lipid inclusions within the cytoplasm. All cell lines showed cytoplasmic projections resembling those of primary Sertoli cells (Figure 3A and B). The SG5-1.13 and SG5-2 lines were again stained by immunocytochemistry for c-kit, α-actin, desmin, and 3-β-ol-hydroxysteroid-dehydrogenase. The cells were negative for these markers, which are specific for germ cells, peritubular cells, and Leydig cells (data not shown).

Expression of AP

Cytochemistry for AP was performed to confirm the presence or absence of peritubular myoid cells. In this assay,

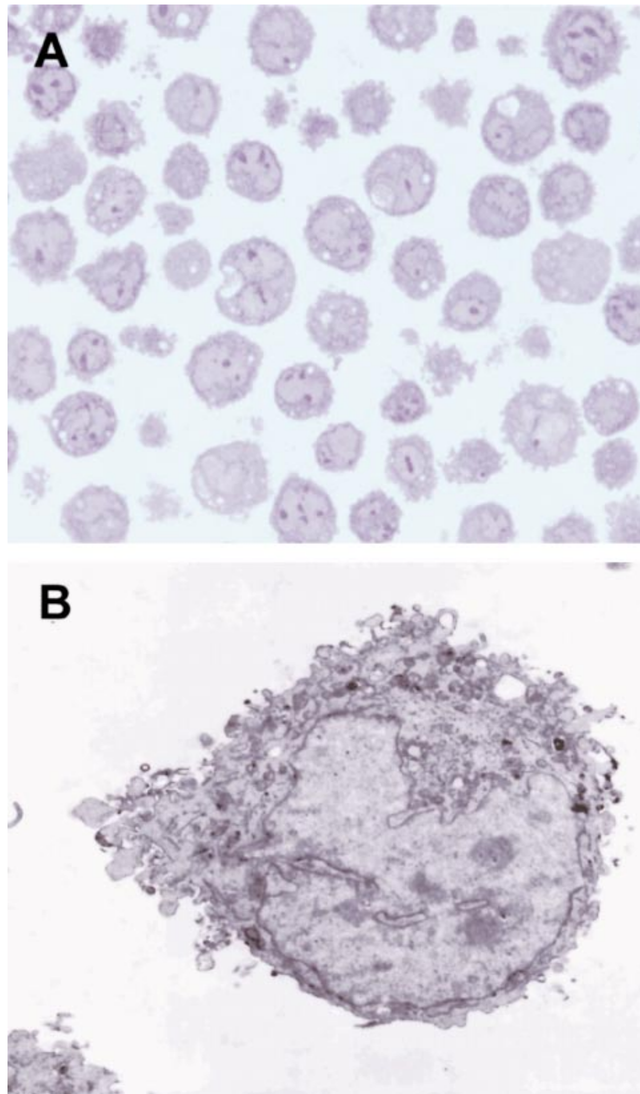

Figure 4. Morphology of the Sertoli cell line SG5-2 in light and electron microscopy. **(A)** Toluidine blue–stained 1-μm-thick section (200×). **(B)** Electron micrograph showing the nucleus indentation and numerous cytoplasmic inclusions (5000×).

AP activity is shown by the presence of a red precipitate at the cell membrane of positive cells. AP activity is a characteristic feature of peritubular myoid cells (Anthony and Skinner, 1989) and germ cells in early stages of development (Narisawa et al, 1992). All cell lines were negative for AP.

Cellular Morphology in Light and Electron Microscopy

Toluidine blue–stained 1-μm-thick sections confirmed that the SG5-1.13 and SG5-2 cell lines had many of the morphological characteristics of in vivo Sertoli cells. In particular, the SG5-2 cell line consists of a homogenous population of cells that are 8–9 μm in diameter with an irregular outline (Figure 4A). Electron microscopy analysis revealed that the nucleus is indented and contains dense nucleoli, some in association with the nuclear envelope (Figure 4B). The cytoplasm contains many spherical and elongated mitochondria, some lipid inclusions, and some phagosomes. The SG5-1.13 cells have a very similar morphology.

Characterization of the Cell Lines Using RT-PCR

The Table summarizes the results of the RT-PCR experiments. Total RNA was extracted from the cell lines and from freshly isolated Sertoli cells as a control (6-day-old testes) (Figure 5). There was a PCR product for the housekeeping gene cyclophilin in all of the cell lines and freshly isolated Sertoli cells, indicating the presence of cDNA in every sample after RT. All of the Sertoli cell–specific cDNAs tested were present in the freshly isolated Sertoli cells (positive control). The cDNAs for the androgen and FSH receptors were absent in all cell lines. The cDNA for SCF and SGP-2, genes specifically expressed by Sertoli cells in the testis, was present in all of the cell lines. TGF-β, α-inhibin, and LIF cDNAs were obtained from all of the cell lines with the exception of the G5B1 cell line. SF-1 and GATA-1 cDNAs were present in all of the cell lines. bFGF cDNA was present in all cell lines with the exception of SG5-1.13 and G5G2. There were no specific PCR bands for the LH receptor and 3β-ol-

*Gene expression of Sertoli cell ines shown by RT-PCR**

Clone Name	Cyclophilin	c-kit	SCF	SGP-2	TGF-β	α-Inhibin	LIF	SF-1	GATA-1	bFGF	Ar	FSHr	LHr	3β-HD
SG5-1.13	+	−	+	+	+	+	+	+	+	−	−	−	−	−
SG5-2	+	−	+	+	+	+	+	+	+	+	−	−	−	−
G5G2	+	−	+	+	+	+	+	+	+	−	−	−	−	−
G5F2	+	−	+	+	+	+	+	+	+	+	−	−	−	−
G5B3	+	−	+	+	+	+	+	+	+	+	−	−	−	−
G5B2	+	−	+	+	+	+	+	+	+	+	−	−	−	−
G5B1	+	−	+	+	−	−	−	+	+	+	−	−	−	−
Primary Sertoli	+	−	+	+	+	+	+	+	+	+	+	+	−	−

* Ar indicates androgen receptor; bFGF, basis fibroblast growth factor; FSHr, follicle-stimulating hormone receptor; LHr, luteinizing hormone receptor; LIF, leukemia inhibitory factor; RT-PCR, reverse transcriptase-polymerase chain reaction; SCF, stem cell factor; SF-1, steroidogenic factor-1; SGP-2, sulfated glycoprotein-2; TGF-β, transforming growth factor beta; and 3β-HD, 3β-ol-hydroxysteroid-dehydrogenase.

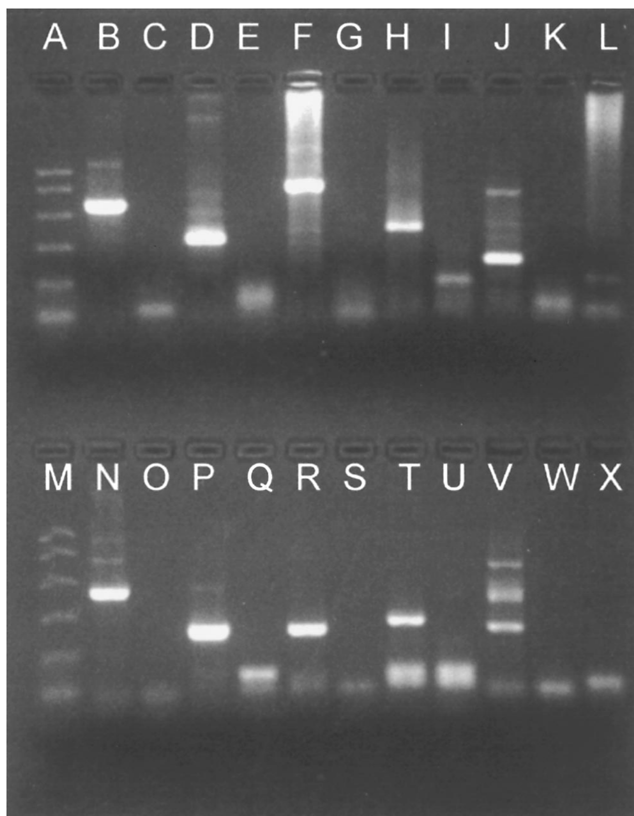

Figure 5. Gene expression of freshly isolated Sertoli cells, shown by reverse transcriptase-polymerase chain reaction (RT-PCR). **(A)** PCR size markers. **(B)** Cyclophilin PCR product, 589 bp. **(C)** Negative control cyclophilin (no RT enzyme used for RT reaction). **(D)** Stem cell factor (SCF) PCR product, 344 bp. **(E)** Negative control SCF. **(F)** Sulfated glycoprotein-2 (SGP-2) PCR product, 806 bp. **(G)** Negative control SGP-2. **(H)** Transforming growth factor beta (TGF-β) PCR product, 406 bp. **(I)** Negative control TGF-β. **(J)** α-Inhibin PCR product, 237 bp. **(K)** Negative control α-inhibin. **(L)** Follicle-stimulating hormone (FSH) receptor PCR product, 150 bp. **(M)** PCR molecular-weight markers. **(N)** Leukemia inhibitory factor (LIF) PCR product, 405 bp. **(O)** Negative control LIF. **(P)** Steroidogenic factor-1 (SF-1) PCR product, 241 bp. **(Q)** Negative control SF-1. **(R)** GATA-1 PCR product, 247 bp. **(S)** Negative control GATA-1. **(T)** Basic fibroblast growth factor (bFGF) PCR product, 293 bp. **(U)** Negative control bFGF. **(V)** Androgen receptor PCR product, 258 bp. **(W)** Negative control androgen receptor. **(X)** Negative control FSH receptor.

hydroxysteroid-dehydrogenase, genes specifically expressed by Leydig cells, in any of the cell lines. Also, there was no PCR band for the c-kit receptor, indicating that none of the lines were of germ cell origin.

Culture of Freshly Isolated Type A Spermatogonia on Sertoli Cell Monolayers

All cell lines expressed growth factors that are known to induce germ cell survival and growth in cocultures, except for SG5-1.13 and G5G2, which do not produce bFGF (Resnick et al, 1992). We thus assessed whether SG5-1.13 and another cell line, SG5-2, were able to maintain the survival of isolated type A spermatogonia in vitro. In vivo, undifferentiated spermatogonia proliferate and differentiate synchronously as A_{paired} (A_{pr}) spermatogonia in clusters of 2 germ cells and as $A_{aligned}$ (A_{al}) spermatogonia in rows of 4, 8, or 16 germ cells. At this stage, these cells remain connected by intercellular bridges (de Rooij and Russell, 2000). We observed that, in minimal culture conditions, freshly isolated type A spermatogonia could survive for at least 7 days, and they were able to differentiate into both A_{pr} and A_{al} cells in cocultures with both Sertoli cell lines (Figure 6A and B). This result is similar to what is observed when type A spermatogonia are cultivated on a feeder layer of primary Sertoli cells (Figure 6C and D). In our hands, the germ cells never attached to the NIH 3T3 cells.

Discussion

The purpose of this study was to conditionally immortalize Sertoli cells for use as feeder layers for the long-term culture of type A spermatogonia. In the absence of hormone receptors at their surface, such as FSH or LH receptors, the survival, proliferation, and differentiation of spermatogonia are likely to be regulated by paracrine factors derived from Sertoli cells (Kierszenbaum, 1994). In order to be able to modulate the rate of proliferation of these Sertoli cell lines and to allow them to revert to a normal phenotype if necessary, the immortalizing SV40-LTAg gene was placed under the control of a promoter inducible with ponasterone A. In the presence of the hormone analog in the culture medium, the expression of the oncogene was activated; upon its removal, most of the cells stopped proliferating and died within 10 days. Some cells, however, escaped the hormonal control and continued to express the LTAg, even without ponasterone A. Previous studies have shown that, after a period of time, cells that have been conditionally immortalized with the SV40 viral oncogene may still proliferate, even in nonpermissive conditions (Ewald et al, 1996). Cumulative changes occur within the genome of these cells that prohibit the reversal of the immortalization, even when the inducer is absent. However, the nature of these changes is still unknown. On the other hand, constitutive expression of the LTAg gene possibly indicates that the promoter might be driven by another type of steroid hormone present in the serum used to cultivate the cells. However, the use of hormone-free NU serum did not restore the complete inducibility of the promoter. Nonetheless, this immortalization strategy allowed us to better modulate the rate of proliferation of these cells in comparison to the Sertoli cell lines previously used (Hofmann et al, 1992). A slow growth rate of Sertoli cell lines is desirable for their use as feeder layers for isolated germ cells.

In situ, Sertoli cells exhibit a typical epithelial morphology and are highly polarized, with a tall, columnar

cytoplasm extending from the basal part of the seminiferous epithelium to the lumen. Their nuclei are usually localized close to the basement membrane. It is, however, well known that Sertoli cells lose their characteristic morphology after enzymatic digestion of the tissue. The cells become rounded once their connections to the basal membrane and the other cells have been severed. Bellve et al (1977) have described the morphology of a pure preparation of Sertoli cells from the testes of prepubertal mice by light and electron microcopy. After enzymatic digestion, the cells became rounded, with an irregular cytoplasmic contour, an indented nucleus, and heterochromatin in association with the nuclear membrane. The Sertoli cell lines that we established exhibit the same characteristics in light and electron microscopy analysis.

Freshly isolated Sertoli cells are also known to lose their typical morphology in culture when conventionally cultivated on a plastic substratum. Hadley et al (1985) reported that the Sertoli cell phenotype was best maintained when the cells were cultivated on a reconstituted basement membrane such as Matrigel. Moreover, when cultivated within the gel, Sertoli cells were also able to form cord-like structures. The cell lines that we describe in the present study behave in a similar way; they spread when cultivated directly on plastic but form cords when cultivated on Matrigel. This behavior has already been described in a previous report (van der Wee et al, 2001).

Several paracrine growth factors produced by Sertoli cells in vivo have been recently identified, and their role in germ cell survival and proliferation has been demonstrated. In particular, the transmembrane form of SCF, the ligand for c-kit, supports the survival of primordial germ cells (Dolci et al, 1991), while the soluble form stimulates the proliferation of primordial germ cells and DNA synthesis in type A spermatogonia (Rossi et al, 1993; Dym et al, 1995; Dirami et al, 1999; Feng et al, 2000). LIF is a survival and proliferation factor for primordial germ cells and gonocytes (Matsui et al, 1991; De Miguel et al, 1996; Nikolova et al, 1997). LIF has also been shown to maintain long-term cultures of embryonic stem cells in an undifferentiated stage (Smith et al, 1988; Williams et al, 1988) and might have a similar function in maintaining the stem cell potential of certain spermatogonia (Piquet-Pellorce et al, 2000). Another factor important for the survival and proliferation of primordial germ cells in culture is bFGF (Matsui et al, 1992; Resnick et al, 1992), which is produced by Sertoli cells. Sertoli cells are also the primary source of testicular inhibin, a paracrine regulator of various cell types (Mather et al, 1997). α-Inhibin might also have a role in the regulation of spermatogonial cell number and differentiation (van Dissel-Emiliani et al, 1989; Hakovirta et al, 1993; Mather et al, 1997). TGF-β produced by Sertoli cells has also been linked to germ cell differentiation (Mullaney and Skinner, 1993; Fritz, 1994; Kierszenbaum, 1994). Thus, although their function in spermatogenesis has not been clearly elucidated, any of these molecules, alone or in combination, could play a crucial role in spermatogonia proliferation and/or differentiation. Therefore, the Sertoli cell lines we established were screened specifically for the expression of these growth factors. We show here that, in addition to the morphological characteristics of Sertoli cells in light and electron microscopy analysis, these cell lines exhibit a molecular phenotype similar to that of Sertoli cells in vivo. The genes expressed include SGP-2, α-inhibin, and GATA-1, as well as genes coding for major growth and differentiation factors such as SCF, LIF, bFGF, and TGF-β. Moreover, the expression of the orphan nuclear receptor SF-1 by all of the cell lines indicates that transactivation of male-specific genes could occur in these cells, which might influence the development of germ cells in coculture. However, the lack of expression of androgen and FSH receptors suggests that these cells may not be suitable for modeling the hormonal control of spermatogenesis.

A number of Sertoli cell lines have been already established. However, none of them expresses the FSH receptor stably, except for a line reported by Walther et al (1996), where it was transcribed at very low levels. The Sertoli cell line MSC-1 has been specifically transfected with an expression vector for the FSH receptor, allowing the cells to recover this original characteristic (Peschon et al, 1992; Eskola et al, 1998). The testicular cell line TM4, derived after multiple passages in culture, has been used in many studies but does not show a very differentiated phenotype (Mather and Phillips, 1984). Sertoli cell lines have been established using the SV40 or the polyoma T antigen (Hofmann et al, 1992; Peschon et al, 1992; Paquis-Flucklinger et al, 1993; McGuinness et al, 1994; Walther et al, 1996). Some of these lines express growth factors, in particular SCF, but their ability to promote the survival and differentiation of the type A spermatogonia in vitro has not been reported. Several murine Sertoli cell lines have been established using a temperature-sensitive mutant of LTAg (Boekelheide et al, 1993; Roberts et al, 1995; Walther et al, 1996; Jiang et al, 1997). These Sertoli cells show signs of differentiation when LTAg is turned off at 40°C (nonpermissive temperature). However, this temperature is unsuitable for the culture of germ cells, which proliferate and differentiate at the optimal temperature of 34°C. The Sertoli cell lines that we established can be used in a defined medium at the critical temperature of 34°C, and they express a combination of growth factors that have not been described in other Sertoli cell lines. Although some of these factors are produced by STO fibroblasts, the latter cannot always be used to study somatic cell–germ cell interactions since

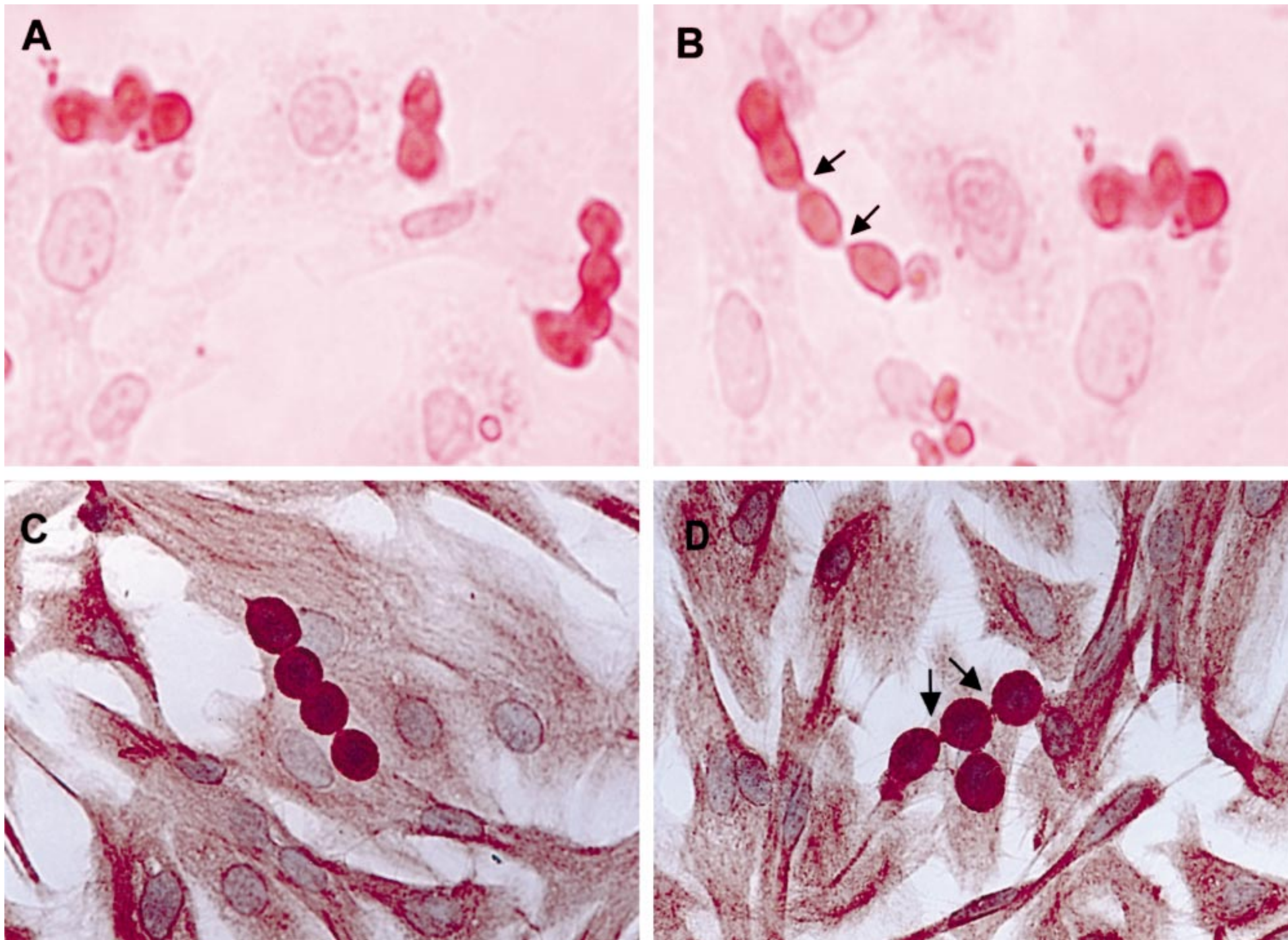

Figure 6. Culture of type A spermatogonia on Sertoli cell line feeder layers. A single-cell suspension of freshly isolated type A spermatogonia was seeded on feeder layers of SG5-1.13 and SG5-2 Sertoli cells. After 3 days, signs of spermatogonial differentiation were observed. **(A)** Paired and aligned type A spermatogonia after 3 days of culture on an SG5-2 feeder layer (400×). **(B)** Aligned type A spermatogonia after 3 days of culture on an SG5-1.13 feeder layer. Intercellular bridges are apparent (arrows) (400×). **(C, D)** Aligned type A spermatogonia after 3 days of culture on primary Sertoli cell feeder layers. Intercellular bridges are apparent (arrows) (400×). The cells were stained with an antibody to leukemia inhibitory factor (LIF) receptor, and as expected, both Sertoli cells and germ cells express the protein.

they do not express Sertoli cell–specific genes such as α-inhibin, SF-1, SGP-2, and GATA-1.

In order to study cell fate decisions in the early phases of spermatogenesis at the molecular level, an in vitro system must be devised whereby type A spermatogonia, including the testis stem cells, can be cultured for a prolonged period of time. The availability of type A spermatogonia that can be maintained in vitro while retaining their differentiation potential is highly desirable. In vitro culture of these cells is essential for the introduction of foreign genes in order to study spermatogenesis at the molecular level as well as for preliminary studies leading to germ line gene therapy. In this paper, we present the characterization of several Sertoli cell lines that could be used as feeder layers for the survival and early differentiation steps of primary type A spermatogonia in vitro. In particular, the lines SG5-2 and SG5-1.13 seem to be able to maintain the viability of spermatogonia, since these germ cells still express the c-kit receptor after several days of coculture in serum-free medium. In a previous study, we showed that the cell line SG5-1.13 was able to maintain the replication potential of freshly isolated type A spermatogonia for at least 1 week using bromodeoxyuridine incorporation (van der Wee et al, 2001). In the present study, we show that type A spermatogonia isolated in a single-cell suspension are able to form paired and aligned structures when cocultivated with SG5-1.13 and SG5-2. This indicates that these cell lines, like primary Sertoli cells, can sustain the early stages of spermatogonial differentiation in vitro. In the absence of primary Sertoli cells, or in the absence of these cell lines, the formation of A_{al} spermatogonia never occurs. Also, when we used NIH 3T3 fibroblasts as feeder layers, the germ cells never attached.

In summary, we believe that the Sertoli cell lines described in the present report represent a valuable experimental tool because they can grow in minimal serum conditions at the critical temperature of 34°C. In addition, their growth rate is slow, and they produce a combination of growth and differentiation factors that have not been reported in previously established Sertoli cell lines. Such features make these lines suitable for cocultures with undifferentiated spermatogonia and also suitable for the study of germ cell–Sertoli cell interactions.

References

Anthony CT, Skinner MK. Cytochemical and biochemical characterization of testicular peritubular myoid cells. *Biol Reprod.* 1989;40:811–823.

Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J Cell Biol.* 1977;74:68–85.

Boekelheide K, Lee JW, Hall SJ, Rhind NR, Zaret KS. A tumorigenic murine Sertoli cell line that is temperature-sensitive for differentiation. *Am J Pathol.* 1993;143:1159–1168.

De Felici M, Pesce M. Growth factors in mouse primordial germ cell migration and proliferation. *Prog Growth Factor Res.* 1994;5:135–143.

De Miguel MP, De Boer-Brouwer M, Paniagua R, van den Hurk R, de Rooij DG, van Dissel-Emiliani FM. Leukemia inhibitory factor and ciliary neurotropic factor promote the survival of Sertoli cells and gonocytes in coculture system. *Endocrinology.* 1996;137:1885–1893.

De Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. *J Androl.* 2000;21:776–798.

Dirami G, Ravindranath N, Pursel V, Dym M. Effects of stem cell factor and granulocyte macrophage-colony stimulating factor on survival of porcine type A spermatogonia cultured in KSOM. *Biol Reprod.* 1999;61:225–230.

Dolci S, Williams DE, Ernst MK, et al. Requirement for mast cell growth factor for primordial germ cell survival in culture. *Nature.* 1991;352:809–811.

Dym M, Jia MC, Dirami G, Price JM, Rabin SJ, Mocchetti I, Ravindranath N. Expression of c-kit receptor and its autophosphorylation in immature rat type A spermatogonia. *Biol Reprod.* 1995;52:8–19.

Eskola V, Ryhanen P, Savisalo M, Rannikko A, Kananen K, Sprengel R, Huhtaniemi I. Stable transfection of the rat follicle-stimulating hormone receptor complementary DNA into an immortalized murine Sertoli cell line. *Mol Cell Endocrinol.* 1998;139:143–152.

Ewald D, Li M, Efrat S, Auer G, Wall RJ, Furth PA, Hennighausen L. Time-sensitive reversal of hyperplasia in transgenic mice expressing SV40 T antigen. *Science.* 1996;273:1384–1386.

Feng LX, Ravindranath N, Dym M. Stem cell factor/c-kit up-regulates cyclin D3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia. *J Biol Chem.* 2000;275:25572–25576.

Fritz IB. Somatic cell–germ cell relationships in mammalian testes during development and spermatogenesis. *Ciba Found Symp.* 1994;182:271–274.

Hadley MA, Byers SW, Suarez-Quian CA, Kleinman HK, Dym M. Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ cell development in vitro. *J Cell Biol.* 1985;101:1511–1522.

Hadley MA, Weeks BS, Kleinman HK, Dym M. Laminin promotes formation of cord-like structures by Sertoli cells in vitro. *Dev Biol.* 1990;140:318–327.

Hakovirta H, Kaipia A, Soder O, Parvinen M. Effects of activin-A, inhibin-A, and transforming growth factor-beta 1 on stage-specific deoxyribonucleic acid synthesis during rat seminiferous epithelial cycle. *Endocrinology.* 1993;133:1664–1668.

Hofmann MC, Narisawa S, Hess RA, Millan JL. Immortalization of germ cells and somatic testicular cells using the SV40 large T antigen. *Exp Cell Res.* 1992;201:417–435.

Jiang C, Hall SJ, Boekelheide K. Development and characterization of a prepubertal rat Sertoli cell line, 93RS2. *J Androl.* 1997;18:393–399.

Kierszenbaum AL. Mammalian spermatogenesis in vivo and in vitro: a partnership of spermatogenic and somatic cell lineages. *Endocr Rev.* 1994;15:116–134.

Mather JP, Moore A, Li RH. Activins, inhibins, and follistatins: further thoughts on a growing family of regulators. *Proc Soc Exp Biol Med.* 1997;215:209–222.

Mather JP, Phillips DM. Establishment of a peritubular myoid-like cell line and interactions between established testicular cell lines in culture. *J Ultrastruct Res.* 1984;87:263–274.

Matsui Y, Toksoz D, Nishikawa S, Nishikawa S, Williams D, Zsebo K, Hogan BL. Effect of steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. *Nature.* 1991;353:750–752.

Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell.* 1992;70:841–847.

McGuinness MP, Linder CC, Morales CR, Heckert LL, Pikus J, Griswold MD. Relationship of a mouse Sertoli cell line (MSC-1) to normal Sertoli cells. *Biol Reprod.* 1994;51:116–124.

Meng X, Lindahl M, Hyvonen ME, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science.* 2000;287:1489–1493.

Mullaney BP, Skinner MK. Transforming growth factor-beta (beta 1, beta 2, and beta 3) gene expression and action during pubertal development of the seminiferous tubule: potential role at the onset of spermatogenesis. *Mol Endocrinol.* 1993;7:67–76.

Narisawa S, Hofmann MC, Ziomek CA, Millan JL. Embryonic alkaline phosphatase is expressed at M-phase in the spermatogenic lineage of the mouse. *Development.* 1992;116:159–165.

National Research Council (NRC). *Guide for the Care and Use of Laboratory Animals.* Washington, DC: National Academy Press; 1996.

Nikolova DB, Martinova YS, Seidensticker M, Bellve AR. Leukaemia inhibitory factor stimulates proliferation of prospermatogonial stem cells. *Reprod Fertil Dev.* 1997;9:717–721.

No D, Yao TP, Evans RM. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc Natl Acad Sci USA.* 1996;93:3346–3351.

Paquis-Flucklinger V, Michiels JF, Vidal F, Alquier C, Pointis G, Bourdon V, Cuzin F, Rassoulzadegan M. Expression in transgenic mice of the large T antigen of polyomavirus induces Sertoli cell tumours and allows the establishment of differentiated cell lines. *Oncogene.* 1993;8:2087–2094.

Peschon JJ, Behringer RR, Cate RL, Harwood KA, Idzerda RL, Brinster RL, Palmiter RD. Directed expression of an oncogene to Sertoli cells in transgenic mice using mullerian inhibiting substance regulatory sequences. *Mol Endocrinol.* 1992;6:1403–1411.

Piquet-Pellorce C, Dorval-Coiffec I, Pham MD, Jegou B. Leukemia inhibitory factor expression and regulation within the testis. *Endocrinology.* 2000;141:1136–1141.

Resnick JL, Bixler LS, Cheng L, Donovan PJ. Long-term proliferation of mouse primordial germ cells in culture. *Nature.* 1992;359:550–551.

Roberts KP, Banerjee PP, Tindall JW, Zirkin BR. Immortalization and characterization of a Sertoli cell line from the adult rat. *Biol Reprod.* 1995;53:1446–1453.

Rossi P, Dolci S, Albanesi C, Grimaldi P, Ricca R, Geremia R. Follicle-

stimulating hormone induction of steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. *Dev Biol.* 1993;155:68–74.

Schrans-Stassen BH, van de Kant HJ, de Rooij DG, van Pelt AM. Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. *Endocrinology.* 1999;140:5894–5900.

Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature.* 1988;336:688–690.

Van der Wee K, Hofmann MC. An in vitro tubule assay identifies HGF as a morphogen for the formation of seminiferous tubules in the postnatal mouse testis. *Exp Cell Res.* 1999;252:175–185.

Van der Wee K, Johnson E, Dirami G, Dym M, Hofmann MC. Immunomagnetic isolation and long-term culture of mouse type A spermatogonia. *J Androl.* 2001;22:696–704.

Van Dissel-Emiliani FM, Grootenhuis AJ, de Jong FH, de Rooij DG. Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. *Endocrinology.* 1989;125:1899–1903.

Walther N, Jansen M, Ergun S, Kascheike B, Ivell R. Sertoli cell lines established from H-2Kb-tsA58 transgenic mice differentially regulate the expression of cell-specific genes. *Exp Cell Res.* 1996;225:411–421.

Williams RL, Hilton DJ, Pease S, et al. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature.* 1988;336:684–687.