

Immunomagnetic Isolation and Long-Term Culture of Mouse Type A Spermatogonia

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ABSTRACT: In the mammalian testis, type A spermatogonia proliferate and differentiate into sperm under the tight control of both endocrine and paracrine factors. In order to study the complex process of spermatogenesis at the molecular level, an in vitro system must be devised in which type A spermatogonia can be cultured for a prolonged period of time. Therefore, cocultures including type A spermatogonia and Sertoli cells, which act as nurse cells to the developing germ cells, are desirable. We have developed a method for the specific isolation of type A spermatogonia using magnetic beads and antibodies that recognize the c-kit receptor or the homophilic adhesion molecule, Ep-CAM. Purified spermatogonia could survive for a period of 25 days when cocultivated on Sertoli cell monolayers.

Moreover, we recently established Sertoli cell lines that produce growth factors that are essential for the maintenance of spermatogonia in a proliferative state. Some of these Sertoli cell lines are able to reorganize into tubular structures when cultivated on a layer of Matrigel as extracellular matrix. We show here that type A spermatogonia associate specifically with the Sertoli cell tubules, and are able to replicate their DNA in this environment. Thus, these in vitro culture systems could be used for the long-term culture of primary, nonimmortalized type A spermatogonia.

Key words: Germ cells, c-kit, Ep-CAM, magnetic beads, Sertoli cell.

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Spermatogenesis is a complex, highly organized process that, in the postnatal and adult mouse testis, originates from spermatogonial stem cells. The generally accepted scheme of spermatogonial renewal and differentiation has been proposed by Huckins (1971) and Oakberg (1971). In this model, the A_s (A single) spermatogonia are considered to be the stem cells of spermatogenesis. Upon division of A_s spermatogonia, the daughter cells can either separate and become 2 independent stem cells or they can remain connected by an intercellular bridge and become A_{pr} (A pair) spermatogonia. The A_{pr} spermatogonia divide further to form chains of 4, 8, or 16 A_{al} (A aligned) spermatogonia. A_{al} spermatogonia divide into differentiating type A spermatogonia (A1-A4) that further develop into intermediate (In) and B spermatogonia. Finally, type B spermatogonia differentiate into primary spermatocytes that will enter the process of meiosis.

Spermatogonial stem cells and their progeny are contained in the germinal epithelium of the seminiferous tubules, in close association with the somatic Sertoli cells (Fritz, 1994). Regulatory mechanisms mediated by

growth factors produced by Sertoli cells induce or inhibit the proliferation, differentiation, and further development of the germ cells (Skinner, 1991; Jegou, 1993). Currently, there is a limited understanding of the molecular mechanisms that control the development of type A spermatogonia into mature sperm. In order to study the complex event of mammalian spermatogenesis, an in vitro system in which germ cells can be maintained in long-term cultures would be ideal. No such system exists to date. In fact, studies on type A spermatogonia have been severely hampered because the number of these cells is low and no unique markers for them exist. However, a number of recent findings support the presence of c-kit, the receptor for stem cell factor (SCF), at the surface of some type A spermatogonia, type B spermatogonia, and spermatocytes (Sorrentino et al, 1991; Bachvarova et al, 1993; Besmer et al, 1993; Manova et al, 1993; Dym et al, 1995; Vincent et al, 1998; Feng et al, 2000). Because in the 6-day old mouse testis the only germ cells present are type A spermatogonia, c-kit can be used as a marker to distinguish and characterize germ cells from the somatic cells in these prepubertal animals. In the present work, we took advantage of this specific expression of c-kit by type A spermatogonia to isolate these cells from 6-day-old testes using an immunomagnetic bead technique. Another molecule shown to be important in the mouse germ line through embryonic, fetal, and adult stages is the homophilic adhesion molecule, Ep-CAM (Anderson et al,

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1999). Because Ep-CAM seems to be specific for spermatogonia in the adult mouse testis, it is also an attractive candidate for use in the specific isolation of these types of germ cells from the adult.

Recently, it has been shown that stem cells such as spermatogonia need to fit within the proper tissue architecture in order to express specific genes (Meng et al, 2000). Within the seminiferous epithelium, developing germ cells are in close association with Sertoli cells. Sertoli cells provide many necessary growth factors for germ cell survival, proliferation, and differentiation. This proximity must be mimicked in vitro in order to adequately cultivate freshly isolated germ cells. In the present study we attempted to establish an in vitro culture system that recreates some in vivo characteristics of seminiferous tubules. We used immortalized Sertoli cell lines, which are able to produce SCF and other growth factors, and were also able to reconstitute tubular structures when cultured on Matrigel (van der Wee and Hofmann, 1999). When freshly isolated spermatogonia were cocultured with these Sertoli cells as feeder layers, they could survive for some time and replicate their DNA. Our studies show that a tridimensional tissue architecture coupled with the production of growth factors by Sertoli cells may be essential for maintaining spermatogonia in a proliferating state. This coculture system could be useful for studying spermatogonial proliferation and differentiation at the molecular level and elucidating the role of several newly discovered surface receptors in germ cell–Sertoli cell interaction and recognition.

Materials and Methods

Isolation of Sertoli Cells and Type A Spermatogonia

Animal investigations were conducted according to the NCR *Guide for Care and Use of Laboratory Animals*. Sequential enzymatic digestion of testicular tubules was performed essentially according to the method of Dym et al (1995). Briefly, testes of 8 Balb/c 6-day-old mice were isolated, placed into a Petri dish, and covered with sterile phosphate-buffered saline (PBS) containing 10% penicillin-streptomycin (Atlanta Biologicals, Norcross, Ga). Testes were decapsulated under a dissection microscope, and the seminiferous cords/tubules were pooled and placed into a conical tube containing a solution of 2 mg/mL collagenase (Sigma, St Louis, Mo) and 10 µg/mL DNase I (Sigma) in Dulbecco modified Eagle medium (DMEM; Atlanta Biologicals). They were incubated for 30 minutes at 37°C on a shaker with occasional gentle pipetting to dissociate the interstitial Leydig cells from the seminiferous tubules. After this incubation step, the tubules were allowed to settle down and the supernatant, containing the Leydig cells, was pipetted out. This first digestion step and settling was repeated once. The tubules were washed twice in DMEM and further digested with 2 mg/mL collagenase, 10 µg/mL DNase, and 1 mg/mL hyaluronidase

type III (Sigma) for 20–30 minutes in a shaking water bath at 37°C until the peritubular cells detached from the tubules. Digestion was monitored by phase-contrast microscopy. The tubules were allowed to settle and the supernatant, containing peritubular cells, was discarded. Finally, a fourth digestion was performed by adding to the pellet 1 mL of DMEM containing 2 mg/mL collagenase, 10 µg/mL DNase I, and 1 mg/mL hyaluronidase type III until a single cell suspension was obtained. This digestion step resulted in a cell suspension containing primarily Sertoli cells and type A spermatogonia. The dispersed cells were then washed twice with medium and filtered through 80-µm nylon mesh (Tetko, Briarcliff Manor, NY).

Immunomagnetic Isolation of Type A and Type B Spermatogonia

After sequential enzymatic digestion, type A spermatogonia were isolated from Sertoli cells using magnetic beads. Briefly, cells were incubated for 1 hour with a 1:200 dilution of a rat anti-mouse antibody that recognizes the extracellular domain of the c-kit receptor (clone 2B8; Pharmingen, San Diego, Calif). Cells were incubated for 30 minutes on an Orbitron rotator (Boeckel Scientific, Feasterville, Pa). The cell suspension was then centrifuged, and the supernatant was removed and washed once with DMEM to remove any excess antibody. The cells were resuspended in 4 mL of complete cell culture media containing 5% fetal calf serum (FCS). Then, M-450 magnetic beads, coated with a sheep anti-rat immunoglobulin G (Dynabeads; Dynal, Lake Placid, NY), were mixed with the cell suspension at a ratio of 4 beads/target cell for 1 hour at 34°C on a shaker. The c-kit-positive cells were pulled out of the suspension with a magnet (Dynal) applied to the wall of the centrifuge tube. The c-kit-positive cells (type A spermatogonia) stuck to the wall, and the cells remaining in suspension (Sertoli cells) were collected. Type A spermatogonia were resuspended in 5 mL of tissue culture medium containing 5% FCS. The isolation experiments were repeated 6 times, and we used an average of 16 testes (8 mice, 6 days post-partum [dpp]) per experiment. As negative controls, we attempted to isolate NIH-3T3 fibroblasts using the same method.

Alternatively, type A and type B spermatogonia were isolated from adult mouse testes (average of 8 mice, 2.5 months old, per experiment) using a 1:200 dilution of a rat anti-mouse antibody that recognizes the homophilic adhesion molecule, Ep-CAM (clone G8.8, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Ia; Anderson et al, 1999) and the method described above. Also, c-kit-positive germ cells were isolated from adult mice as described above.

Cell Purity

After magnetic isolation with the anti-c-kit (2B8) or the anti-Ep-CAM (G8.8) antibody, the cells were counted with a hemacytometer. Depending on the number of cells recovered, 2000 to 20 000 cells were fixed with 2% paraformaldehyde and spotted onto a microscope slide. Alternatively, the cells were cytocentrifuged at 500 × g for 5 minutes in a cytospin (Shandon, Pittsburgh, Pa). The cells were then immunostained using the procedure described below and the percentage of double-stained cells (beads and immunopositive) was calculated.

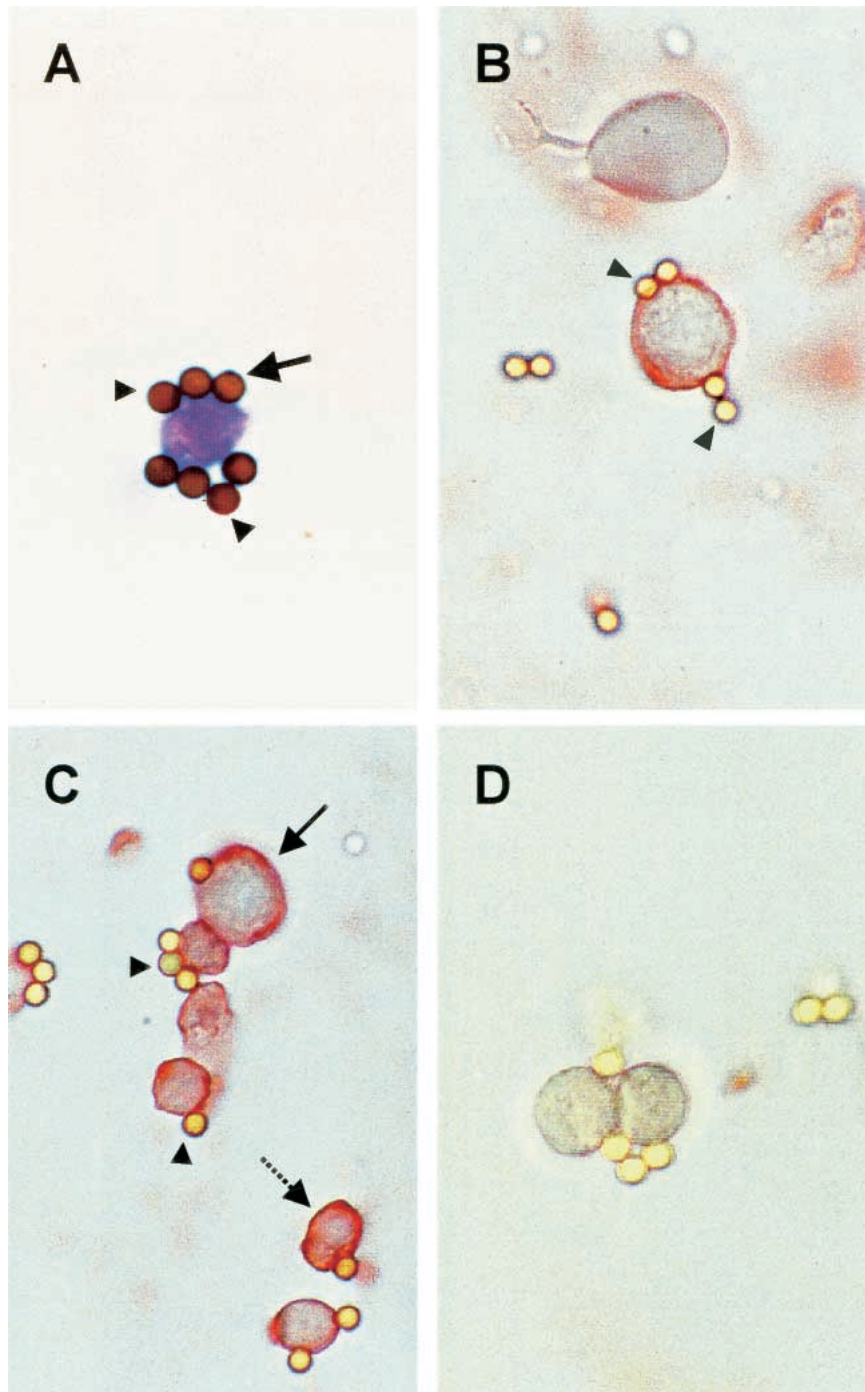


Figure 1. Mouse spermatogonia after immunomagnetic isolation using a c-kit (2B8) or an Ep-CAM (G8.8) antibody. **(A)** Type A spermatogonium isolated with the 2B8 antibody and stained with the primary rat anti-mouse c-kit antibody, ACK45, using the immuno-alkaline phosphatase reaction. The spermatogonium (arrows) is still associated with the magnetic beads (arrowheads). Brightfield microscopy, 600 \times . **(B)** Type A spermatogonium isolated with the G8.8 antibody and stained with the primary rat anti-mouse c-kit antibody, 2B8, using the immunoperoxidase reaction. The spermatogonium is still associated with the magnetic beads (arrowheads). Brightfield microscopy, 600 \times . **(C)** Type A (arrow) and type B (broken arrow) spermatogonia isolated with the G8.8 antibody and stained with the primary rat anti-mouse c-kit antibody, 2B8, using the immunoperoxidase reaction. The spermatogonia are still associated with the magnetic beads (arrowheads). Brightfield microscopy, 600 \times . **(D)** Negative control without the 2B8 primary antibody. Brightfield microscopy, 600 \times .

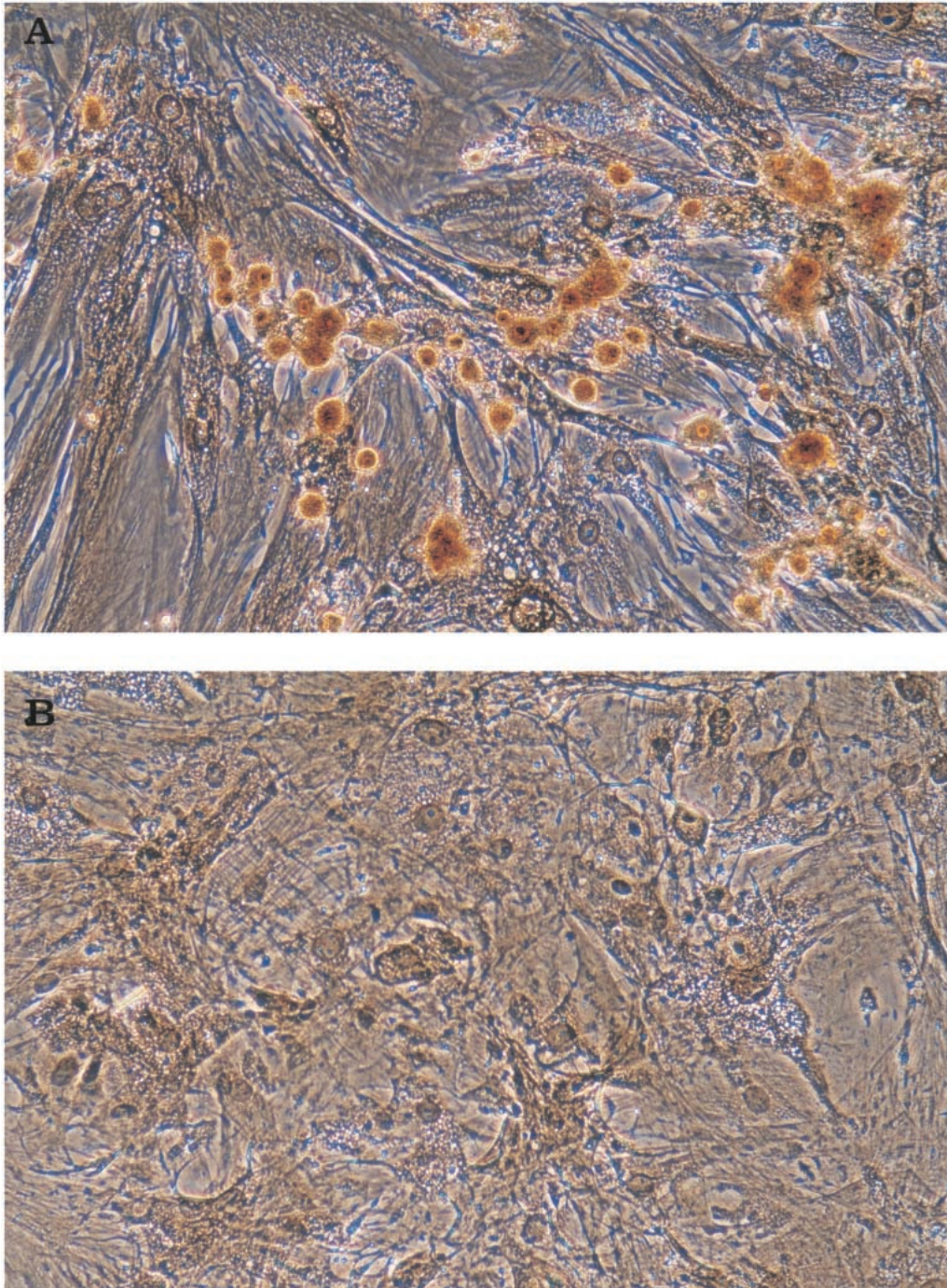


Figure 2. Mouse type A spermatogonia maintained in vitro for 25 days on a feeder layer of primary Sertoli cells. **(A)** c-Kit-positive type A spermatogonia cocultured on a monolayer of primary Sertoli cells for 25 days. The cells were then stained with the primary rat anti-mouse c-kit antibody, ACK45, and revealed with a secondary antibody coupled to peroxidase. Phase-contrast microscopy, 400 \times . **(B)** Negative control without primary antibody. Phase-contrast microscopy, 400 \times .

Tissue Culture and Cell Lines

Isolated germ cells and isolated primary Sertoli cells were maintained in DMEM supplemented with 5% FCS or 5% Nu serum type IV culture supplement (Fisher Scientific, Pittsburgh, Pa), 1 mM sodium pyruvate, 2 mM glutamine, 50 U/mL penicillin, 50 mg/mL streptomycin, and 100 mM nonessential amino acids. All

tissue culture reagents were purchased from Atlanta Biologicals. The SF7 cell line was obtained by immortalizing 10-day-old mice Sertoli cells with the large T antigen gene (Hofmann et al, 1992), and cultivating it in the medium described above. The SG5-1.13 Sertoli cell line was conditionally immortalized with the large T antigen gene under the control of an ecdysone-in-

ducible promoter (manuscript in preparation). Immortalized Sertoli cell lines were cultivated in DMEM supplemented as described above but containing 5% Nu serum (Fisher Scientific) instead of 5% FCS. Cocultures of Sertoli cells and germ cells were performed using minimal medium.

Immunocytochemistry

Staining for c-kit using an antibody that recognizes a different epitope (rat anti-mouse ACK-45, Pharmingen, San Diego, Calif) was performed on type A spermatogonia freshly isolated with the immunomagnetic beads and the 2B8 antibody. Briefly, after isolation, cells were fixed with 2% paraformaldehyde in PBS, spotted on FCS-coated slides, dried, and incubated with ACK-45 at dilutions of 1:500 to 1:10 000. After washing in PBS, the cells were incubated with a sheep anti-rat secondary antibody conjugated to biotin (Vector Laboratories, Burlingame, Calif). Finally, the cells were incubated with streptavidin-alkaline phosphatase or streptavidin-peroxidase, and the enzymes revealed with the Vector Blue Alkaline Phosphatase substrate kit or the Vector Blue Peroxidase substrate kit (Vector Laboratories, Burlingame, Calif).

Alternatively, cells isolated with the anti-Ep-CAM antibody were stained with the anti-c-kit 2B8 antibody at dilutions of 1:10 to 1:500 using the method described above. In this case, the presence of peroxidase in the final step was revealed using the AEC substrate-chromogen mixture from Zymed Laboratories, San Francisco, Calif).

Cytochemistry for Alkaline Phosphatase

Cells removed from the seminiferous tubules by sequential enzymatic digestion were evaluated for their expression of alkaline phosphatase after 3 days in culture using the method of Narisawa et al (1990). Briefly, the cells were fixed in situ with 10% formaldehyde (Sigma) in PBS for 10 minutes at 4°C, 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 salt (both from Sigma) in 0.1 M Tris buffer (pH 8.9). This solution was immediately filtered and incubated with the fixed cells for 30 minutes at 37°C. After incubation, the cells were washed with distilled water and observed under brightfield microscopy (IMT-2 microscope, Olympus Corp) for a red bright color indicating the expression of alkaline phosphatase.

Dialkylcarbocyanines I and O Staining of Type A Spermatogonia

Stocks of the dialkylcarbocyanines, DiI and DiO, were prepared at a concentration of 2 mg/mL in dimethylsulfoxide (DMSO; Molecular Probes, Eugene, Ore). The stock solutions were then added to cell suspensions in a 1:1000 final dilution in complete culture medium serum-free. The cells were incubated for 20 minutes on an Orbitron rotator. The cell suspensions were then placed on a magnet to recover the labeled cell-bead complexes and discard the supernatants. The cell-bead complexes were washed twice with serum-free medium. After washing, the cell-bead complexes were resuspended in complete culture medium with 5% FCS or 5% Nu serum and plated out with Sertoli cell lines for cocultures on plastic substratum or Matrigel (Fisher

Scientific). The labeled cells were viewed using fluorescence and phase-contrast microscopy on a Nikon Eclipse TS100 microscope, with filter blocks B-2 E/C (fluorescein-isothiocyanate [FITC]), or G-2A (rhodamine).

Cocultures of Sertoli Cells and Germ Cells

Adult Sertoli cells obtained after sequential enzymatic digestion were plated in 60-mm tissue culture dishes in serum-free culture medium (0.5% Nu serum) and incubated at 37°C and 5% CO₂ for 5 days. The Sertoli cells were then trypsinized and seeded into 24-well plates (Falcon/Fisher Scientific) at a concentration of 1×10^5 cells per well in medium containing 0.5%–5% Nu serum. Low concentrations of Nu serum, an artificial serum, were used to limit the proliferation of peritubular myoid cells. Some culture wells of the same plate were stained for alkaline phosphatase to assess contamination by peritubular myoid cells. Spermatogonia were added 3 to 5 hours after the Sertoli cells were seeded. After incubation times ranging from 5–25 days, the cultures were then fixed and immunocytochemistry was performed for the expression of c-kit using the ACK-45 antibody (Pharmingen) as described above.

Alternatively, SF7 Sertoli cells (Hofmann et al, 1992; van der Wee and Hofmann, 1999) were plated out onto Matrigel-coated wells at a concentration of 60 000 cells per well in a 24-well plate (Falcon/Fisher). Three to 5 hours after the SF7 cells were seeded, freshly isolated type A spermatogonia were added to the culture. The cocultures were maintained for 3 to 5 days in media supplemented with 5% FCS until Sertoli cell tubules formed (van der Wee and Hofmann, 1999). The cells in coculture were fixed and embedded for sectioning as described previously (van der Wee and Hofmann, 1999).

The SG5-1.13 cell line was seeded at a concentration of 180 000 cells per well onto Matrigel-coated wells in a 24-well plate. After 3 to 5 hours, freshly isolated spermatogonia, labeled with DiI, were added to the wells at a concentration of 12 000 cells per well. The cocultures were grown in media supplemented with 5% FCS for 6 days.

Tissue Sections

SF7 tubules grown on Matrigel were carefully picked up with forceps and fixed overnight at 4°C with 4% paraformaldehyde in PBS containing 20% sucrose. After 2 washes for 5 minutes each with PBS, the tubules were embedded in Tris-buffered saline (TBS) freezing medium (Fisher Scientific) and 8- μ m-thick sections were prepared with a cryotome.

Bromodeoxyuridine Staining of Sertoli Cell–Germ Cell Cocultures

After 5 days of incubation, the germ cell–Sertoli cell cocultures were incubated with 100 μ M of bromodeoxyuridine (BrdU) in medium containing 5% FCS for 16 hours. The cell culture media was removed and the cultures were washed with PBS. The cultures were fixed with 2% paraformaldehyde for 1 hour at 4°C. The tubular structures were then picked from the surface of the Matrigel and placed on poly-L-lysine (Sigma) coated slides. The tubular structures were washed 3 times with PBS containing 0.02% Tween-20 (PBST), then incubated 1 hour at 37°C with 1:500 mouse anti-BrdU (Sigma) in PBST containing 0.2% horse

serum (Vector Laboratories). Following antibody incubation, the tubules were washed 2 times with PBST and incubated for 1 hour at 37°C with a 1:1000 dilution of FITC-labeled horse anti-mouse antibody (Vector Laboratories) in PBS containing 0.2% horse serum. The tubules were then washed 2 times with PBST. The labeled cells were viewed using fluorescence or phase-contrast microscopy on a Nikon Eclipse TS100 microscope, with filter blocks B-2 E/C (FITC), or G-2A (rhodamine).

Results

Immunomagnetic Isolation of Spermatogonia

Spermatogonia were identified on the basis of their morphology in phase-contrast and brightfield microscopy. They are large cells (14–16 μm) showing a typical spherical shape and a large nucleus/cytoplasm ratio (Figure 1B). We isolated an average of 3.2×10^4 type A spermatogonia per experiment (2×10^3 cells/testis) from the 6-day-old pups using the c-kit antibody, 2B8. Further, they were identified by their expression of the c-kit receptor using the ACK-45 antibody (Figure 1A). The purity of the isolated population was greater than 95%. The experiment was repeated with NIH3T3 cells and cells from the Sertoli cell line, SF7. The magnetic beads never attached to these cells, showing that the method of isolation is specific. Using adult mice we could isolate an average of 2.5×10^4 type A and B spermatogonia per testis using beads coated with the anti Ep-CAM antibody. These cells were then stained for the c-kit receptor using the 2B8 antibody (Figure 1B through D). As shown in Figure 1C, 2 different c-kit-positive cell populations could be retrieved that show different cell size, possibly type A and type B spermatogonia. More than 95% of the cells exhibit both markers (ie, c-kit and Ep-CAM tagged with the beads).

Survival of Spermatogonia in Monolayer Cultures

The spermatogonia isolated using the immunomagnetic procedure were then cultivated on a monolayer of freshly isolated Sertoli cells. Alkaline phosphatase staining was performed in order to ensure that these feeder layers were not contaminated by alkaline phosphatase-positive peritubular myoid cells. In the germ cell–Sertoli cell cocultures, different types of serum (FCS, Nu serum, and horse serum) and serum concentrations were tested (0.5% to 10% serum). Cocultures on a feeder layer of Sertoli cells and 5% Nu serum were the most successful, because viable germ cells, still expressing the c-kit receptor, could be visualized after 25 days of culture (Figure 2A and B). In this type of culture, the cells could survive but did not proliferate.

Colonization of In Vitro Sertoli Cell Tubules by Type A Spermatogonia

In the next experiment, type A spermatogonia isolated with the immunomagnetic beads were cocultivated with the SF7 Sertoli cell line (Hofmann et al, 1992). This cell line has the ability to form hollow tubules, in vitro, when cultivated onto a layer of Matrigel in defined conditions (van der Wee and Hofmann, 1999). As seen in Figure 3A, type A spermatogonia are able to specifically attach, then colonize the in vitro tubules, whereas other cell types such as NIH3T3 fibroblasts do not enter these structures. The in vitro tubules are hollow, as shown in Figure 3B, and the spermatogonia are easily recognizable through the magnetic beads still attached to them (arrows).

Incorporation of BrdU by Type A Spermatogonia

After 1 week of culture, all type A spermatogonia were incorporated within the Sertoli cell tubules, which are readily visible in phase-contrast microscopy (Figure 4A). The spermatogonia could be localized within these structures when viewed under fluorescence microscopy because they were labeled with DiI and appeared red (Figure 4B). The incorporation of BrdU was revealed as a green fluorescence due to the FITC-labeled secondary antibody localized to the sites where BrdU was present. In cocultures of germ cells and the SF7 Sertoli cell line, no significant BrdU incorporation was observed. In cocultures of germ cells and the SG5-1.13 cell line there was a notable amount of green fluorescence (BrdU incorporation) corresponding to the location of primary spermatogonia in S phase within the tubule structures (Figure 4C).

Discussion

Rapid and effective preparation of pure populations of type A spermatogonia from prepubertal or adult animals is the basis for in vitro experiments and attempts at germ cell transplantation. In the past, isolation procedures using elutriation (Bucci et al, 1986) or velocity sedimentation (Bellve et al, 1977; Dym et al, 1995; Dirami et al, 1999) have been described. With both methods, a cell population containing up to 90% spermatogonia can be obtained. Morena et al (1996) also described a method for isolating rat spermatogonia using centrifugation in a discontinuous Percoll gradient. In this case, specific fractions of the gradient contained up to 85% spermatogonia. More recently, von Schonfeldt et al (1999) reported a fast and effective method for isolating spermatogonia from a variety of species, including adult mouse. The method uses magnetic beads coupled to the anti-c-kit antibody, which recognizes the c-kit receptor at the surface of type A spermatogonia (Sorrentino et al, 1991; Manova et al, 1993; Dym et al,

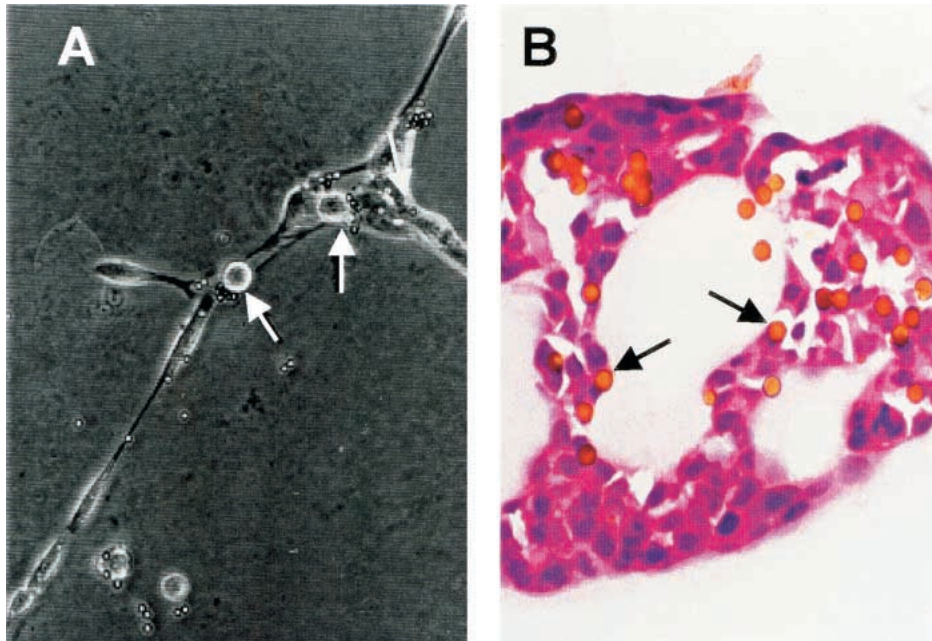


Figure 3. Integration of mouse type A spermatogonia into Sertoli cell tubules. **(A)** Adhesion of type A spermatogonia to Sertoli cells (SF7 cell line) 3 hours after seeding. The spermatogonia (arrows) are still coupled to the magnetic beads. Phase-contrast microscopy, 200 \times . **(B)** Cross-section of a Sertoli cell tubule containing type A spermatogonia (arrows) after 4 days in culture. Brightfield microscopy, 200 \times .

1995). In their study, the authors stained the isolated cells with FITC and propidium iodide, and analyzed them by flow cytometry. Thus, in this case, the cells were characterized as spermatogonia according to their morphology, their diploid DNA content, and the fact that they are in the S phase of the cell cycle. Because in the adult

mouse several different cell types (eg, Leydig cells, spermatocytes) express the c-kit receptor, type A spermatogonia were enriched only up to 54%. Moreover, the study does not account for Sertoli cells that could have been unspecifically collected and raises the question of the purity of the isolated population.

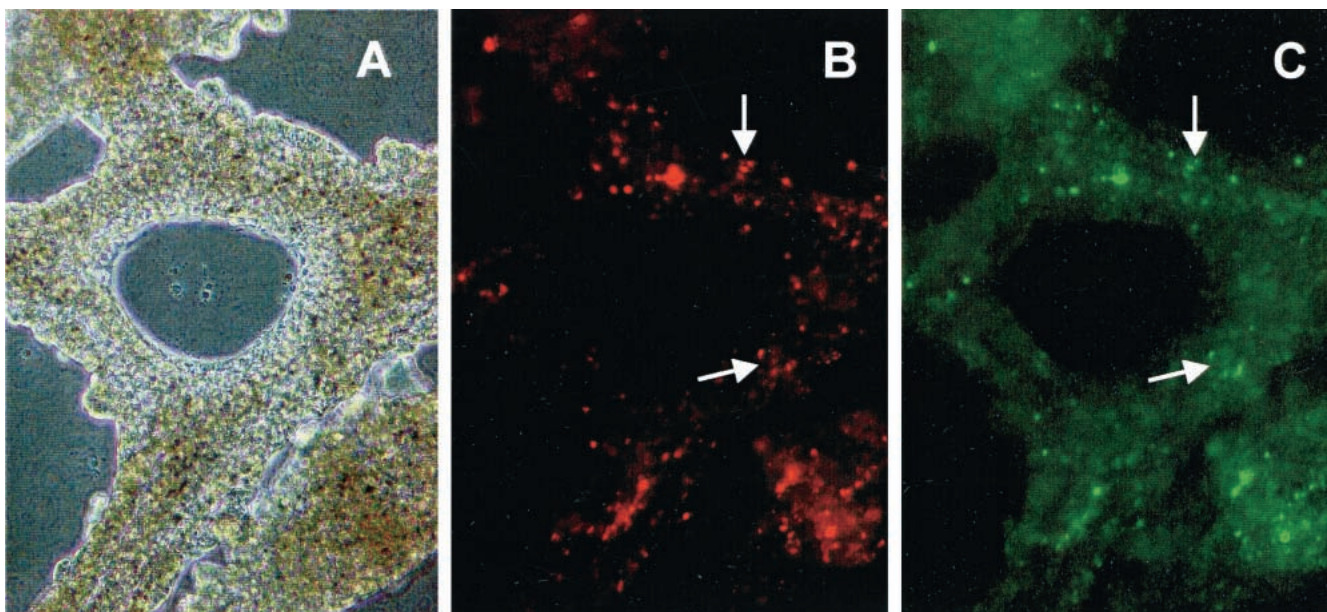


Figure 4. Incorporation of BrdU by mouse type A spermatogonia in Sertoli cell tubules. **(A)** Sertoli cell tubules produced by the cell line SG5-1.13 and containing type A spermatogonia. Phase-contrast microscopy, 100 \times . **(B)** Same tubules in fluorescence microscopy showing type A spermatogonia labeled with the red fluorescent dye, Dil (100 \times). **(C)** The same tubule in fluorescence microscopy showing type A spermatogonia expressing BrdU. BrdU was revealed with a rabbit anti-mouse primary antibody and a secondary antibody coupled to FITC (100 \times).

We described in this paper an immunomagnetic bead technique used to isolate spermatogonia from prepubertal mice with an anti-c-kit antibody. However, by using another anti-c-kit antibody that recognizes an epitope of the c-kit receptor that is different from the epitope used during the isolation procedure, we showed that >95% of the cells isolated by the immunomagnetic bead technique are c-kit-positive. Because Leydig cells have been eliminated during the sequential enzymatic digestion of the testes, and because type A spermatogonia account for all germ cells in a 6-day-old mouse testis, we are confident that this technique provides a rapid and reliable means for specifically isolating c-kit-positive type A spermatogonia in these animals. We also extended the application of this method for the isolation of spermatogonia in the adult mouse testis, using an antibody that recognizes the surface protein Ep-CAM. Ep-CAM is a calcium-independent homophilic adhesion molecule found in most vertebrate simple epithelia and carcinoma (Litvinov et al, 1994a, b). In the developing mouse gonads, Ep-CAM is expressed exclusively in the germ line throughout embryonic and fetal stages. It is down-regulated in the ovary after birth, although it remains expressed in the testis. In the adult mouse testis, Ep-CAM seems to be expressed only by type A and type B spermatogonia (Anderson et al, 1999). We could confirm these data by using the anti-EpCAM antibody to isolate adult germ cells and then staining these cells with an anti-c-kit antibody.

Isolation of spermatogonia using immunobeads is rapid and effective. Although the number of cells recovered is about 10-fold lower than with other methods using mice testes (Bellve et al, 1977), the purity of the spermatogonial cell population is very high. Moreover, we were able to isolate many more spermatogonia using the Ep-CAM antibody and adult mice than using the c-kit antibody and prepubertal animals. Whereas c-kit has been shown to be present on primordial germ cells, gonocytes, type A, In, and type B spermatogonia (Sorrentino et al, 1991; Dym et al, 1995; Orth et al, 1996), there is still controversy as to its presence on the stem cell type A spermatogonia (aka A_s). Therefore, we can conclude that we isolated at least differentiating type A spermatogonia from the 6-day-old mouse testis using this method, while we isolated type A and type B spermatogonia using the Ep-CAM antibody and adult mice. Thus, the stage of differentiation of the isolated cells, coupled with the age of the mice, likely explains the difference in the number of cells recovered by each method.

Initial studies to establish an in vitro system allowing survival and multiplication of porcine spermatogonia have been described (Dirami et al, 1999). When cultured alone or with Sertoli cells, the spermatogonia did not proliferate and remained viable only for up to 9 days. However, spermatogonial proliferation was achieved when

small fragments of seminiferous tubules were cultured in the presence of FCS or extracellular matrix, as shown by BrdU incorporation. Taken together, these results indicate that intercellular communication and extracellular matrix are important for spermatogonial multiplication and that FCS promotes the survival of porcine spermatogonia under in vitro conditions.

We have been able to maintain purified spermatogonia, which retain their expression of c-kit, in coculture with Sertoli cells for up to 25 days. We believe that the use of Nu serum to supplement the media is an important factor in these long-term cultures, as primary germ cell cocultures supplemented with either FCS or horse serum (HS) did not survive as long. There could be inhibitory substances in FCS or HS that affect murine spermatogonia that are not present in Nu serum, which explain this effect. However, germ cells did not proliferate in these types of cultures.

Recently, Nagano et al (1998) were able to maintain spermatogonial stem cells in culture for up to 4 months without the cells losing their proliferation or differentiation potential. However, these cells were part of a mixed population of testicular cells cultivated on a feeder layer of STO fibroblasts (Martin and Evans, 1975). In this study, we have been able to maintain purified spermatogonia in cocultures with a Sertoli cell line, SG5-1.13, for up to 6 days without losing their ability to replicate their DNA. In this case, the germ cells were incorporated within tubular structures formed by the Sertoli cells. The primary spermatogonia under the same conditions did not incorporate BrdU when cocultured with another Sertoli cell line, SF7. The ability of the spermatogonia to enter S phase in vitro in tubule culture with the SG5-1.13 Sertoli cell line indicates that this cell line provides a physiological structure, essential factors, or both, which are necessary for germ cell proliferation. To date, we have found no difference between the SF7 cell line and the SG5-1.13 cell line in their expression of Sertoli cell specific growth factors such as bFGF, TGF- β , LIF, SCF, or α -inhibin. Future studies will elucidate which particular genes are differentially expressed in these cell lines, and which allow germ cell proliferation in vitro.

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