Proliferative Activity In Vitro and DNA Repair Indicate that Adult Mouse and Human Sertoli Cells Are Not Terminally Differentiated, Quiescent Cells


Published in:
Biology of Reproduction

DOI:
10.1095/biolreprod.108.071662

Citation for published version (APA):
Proliferative Activity In Vitro and DNA Repair Indicate that Adult Mouse and Human Sertoli Cells Are Not Terminally Differentiated, Quiescent Cells

Emad A. Ahmed, Angelique D. Barten-van Rijbroek, Henk B. Kal, Hooman Sadri-Ardekani, S. Canan Mizrak, Ans M.M. van Pelt, and Dirk G. de Rooij

Department of Endocrinology and Metabolism, Faculty of Science, Utrecht University, Utrecht, The Netherlands
Department of Radiotherapy, University Medical Center Utrecht, Utrecht, The Netherlands
Department for Reproductive Medicine, Academic Medical Center, Amsterdam, The Netherlands
Reproductive Biotechnology Research Centre, Avicenna Research Institute (ARI), Tehran, Iran

ABSTRACT

Sertoli cells isolated from the adult mouse and human testis resume proliferation in culture. After 20 days of culture in Dulbecco modified Eagle medium/Ham F12 (DMEM/F12) medium containing 5% fetal calf serum, about 36% of the mouse Sertoli cells, identified by their immunohistochemical staining for the Sertoli cell marker vimentin, incorporated bromodeoxyuridine (BrdU). The renewed proliferation was associated with a 70% decrease in expression of the cell cycle inhibitor CDKN1B (P27kip1) and a 2-fold increase in the levels of the proliferation inducer ID2. In vivo, the balance between cell cycle inhibitors and inducers probably is such that the cells remain quiescent, whereas in culture the balance is disturbed such that Sertoli cells start to proliferate again. The renewed proliferative activity of Sertoli cells in culture was further confirmed by double staining for BrdU and the Sertoli cell marker clusterin (CLU), showing about 25% of the CLU-positive Sertoli cells to be also positive for BrdU after 13 days of culture. Radiobiologically, Sertoli cells are also different from other quiescent somatic cells in the testis because they express several DNA repair proteins (XRCC1, PARP1, and others). Indeed, a comet assay on irradiated Sertoli cells revealed a 70% reduction in tail length and tail moment at 20 h after irradiation. Hence, Sertoli cells repair DNA damage, whereas other quiescent somatic testicular cells do not. This repair may be accomplished by nonhomologous end joining via XRCC1 and PARP1. In conclusion, cell kinetic and radiobiological data indicate that Sertoli cells more resemble arrested proliferating cells than the classic postmitotic and terminally differentiated somatic cells that have always been assumed to be.

cell cycle genes, culture, DNA repair, Sertoli cells, testis

INTRODUCTION

Cells that do not divide, no longer show changes in phenotype, and perform a specific function are known as terminally differentiated cells. Many cell types are considered to be terminally differentiated in the adult animal, including neurons, myocytes, auditory hair cells, epidermal cells, and Sertoli cells [1, 2]. In mammals, Sertoli cells are formed before puberty, and after puberty these cells are considered to be unable to proliferate except in seasonal breeders, in which season-dependent variations in Sertoli cell numbers per testis occur [3–5]. The latter implicates a residual capacity of adult Sertoli cells to proliferate or the long-term presence of some Sertoli cell precursors in the testis of adult seasonal breeders. The terminal differentiation of Sertoli cells involves loss of proliferative activity, formation of inter-Sertoli cell tight junctions, and establishment of the Sertoli cell barrier, as well as acquisition of the ability to fully sustain the spermatogenic process [1]. Sertoli cell differentiation is also accompanied by expression of many gene products that are not present in immature cells [6, 7].

In the mouse, Sertoli cells proliferate until Day 16 after birth [8]; thereafter, they terminally differentiate. However, the period of Sertoli cell differentiation can be postponed in the event of hypothyroidism [9] or deficiency of the testicular gap junction protein GIA1 [10]. Some growth factors and follicle-stimulating hormone increase proliferation of Sertoli cells in neonatal and prepubertal testes, but adult Sertoli cells fail to respond [11–13]. Intriguingly, although adult Sertoli cells are quiescent, they express genes that are involved in the regulation of the cell cycle (e.g., CDKN1B [P27kip1], a cell cycle inhibitor that arrests cells in G1 phase of the cell cycle [14]). Furthermore, after infliction of DNA damage by irradiation, Sertoli cells express TP53, which causes cell cycle arrest [15]. Besides cell cycle inhibitors, adult Sertoli cells also express ID proteins, which are helix-loop-helix proteins that inhibit differentiation and promote proliferation, which is in contrast to other quiescent and differentiated cells in which ID proteins are expressed at low levels or not at all [1, 16, 17]. Taken together, unlike other terminally differentiated cells, Sertoli cells express a puzzling mixture of proliferation inducers and inhibitors.

In addition to the presence of cell cycle regulatory proteins, Sertoli cells differ from most other quiescent somatic cells in that they express a number of DNA damage repair proteins. Adult Sertoli cells express XRCC6 (KU70), XRCC5 (KU86), and TP53 before irradiation and phosphorylated ATM in response to irradiation [15, 18, 19]. Furthermore, after irradiation, TP53BP1 foci are formed in Sertoli cells that decrease in number over time, suggesting that, in contrast to other quiescent somatic cells in the testis, Sertoli cells may repair DNA double-strand breaks (DSBs) [20].

Hence, with respect to expression of cell cycle genes and to radiobiological aspects, Sertoli cells differ from other supposedly terminally differentiated cells that perform a specific task in an adult tissue. We show herein that adult mouse and human Sertoli cells resume proliferation in culture and that adult
Sertoli cells repair DNA DSBs. Our results indicate that Sertoli cells should no longer be regarded as an example of terminally differentiated quiescent cells.

**MATERIALS AND METHODS**

**Animals, Irradiation, and Fixation**

Testes of male FVB mice, 8- to 12-wk-old, were given a 4-Gy dose of x-rays (local irradiation, 200 kV, 20 mA, 0.5-mm Cu filter; Philips, Eindhoven, The Netherlands). Mice were killed by cervical dislocation, and testes were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C and embedded in paraffin (Stemcowax; Adamas Instruments, Amerongen, The Netherlands). The animals were used and maintained according to regulations provided by the animal ethics committee of Utrecht University, which also approved the experiments.

**Immunohistochemistry**

Sections (5 μm) of testes of control and irradiated mice were mounted together on 3-aminoproyl-tri-ethoxysilane-coated glass slides and dried overnight at 37°C. Sections were dewaxed in xylene and hydrated in a graded series of alcohols. The sections were boiled once or twice (each for 10 min) in 0.01 M sodium citrate using a microwave oven (H2500; BioRad, Hercules, CA) and incubated in 0.35% H2O2 in PBS for 10 min. Blocking was done in 5% bovine serum albumin (Sigma) in 1% Hanks fluid (pH 7.4) containing 0.04% DNase I, 0.05% hyaluronidase, and 0.5% trypsin (all from Sigma) for at least 10 min at 34°C with agitation. The fragmented tubules were allowed to settle and then were washed two times in PBS. After repeated pipetting in Dulbecco modified Eagle medium/Ham F12 (DMEM/F12) medium, cells were centrifuged at 900 rpm and at 700 rpm (each for 3 min). Cells in the supernatant were collected and cultured in DMEM/Ham F12 medium containing 5% FCS; after 3 h in culture, the medium was carefully removed, the culture was washed, and fresh medium was added. During overnight culturing, Sertoli cells attached to the bottom and acquired an irregular shape, whereas the germ cells did not attach and could easily be removed by repeated washing. Hypotonic shock (with 0.02 M Tris-HCl [pH 7.2] for 2 min) was used when needed. Following this protocol, the purity of Sertoli cells in culture exceeded 90% and could reach 99% after 20 days. Human testicular cells were enzymatically (trypsin, hyaluronidase, and collagenase) isolated from testes donated by patients undergoing bilateral castration as part of prostate cancer treatment. Cells were cultured in Stempro medium (Invitrogen, Carlsbad, CA) according to the technique described by Kanatsu-Shinohara et al. [23].

**Cell Identification**

Sertoli cells in culture were identified immunocytochemically by mouse monoclonal anti-vimentin or by goat polyclonal anti-CLU. Peritubular cells were identified using α-smooth muscle actin antibody (Biogenex).

**Immunocytochemistry and Cell Counts**

After fixation in methanol, cells were incubated in 0.05% Triton X-100 in PBS for 15 min. For vimentin detection, after blocking with goat serum (0.5%), cells were incubated for 60 min with mouse monoclonal anti-vimentin antibody, followed by incubation with secondary biotinylated goat anti-mouse IgMs (Vector Laboratories) in a humidified chamber for 30 min at room temperature. The horseradish peroxidase (HRP) avidin-biotin complex reaction was performed according to the manufacturer’s protocol (Vector Laboratories). Bound antibodies were visualized in red by 3-amin0-9-ethyl carbazole (AEC) in AEC buffer activated with 0.3% H2O2, or in brown using 0.3 μg/ml of DAB in PBS to which 0.03% H2O2 was added. When the avidin-biotin-peroxidase (ABC)-alkaline phosphatase standard kit (Vector Laboratories) was used according to the manufacturer’s protocol, vimentin-labeled cytoplasm was blue after incubation with an alkaline phosphatase substrate.

For peritubular cell staining, the same protocol was used. Exceptions were that the primary antibody was mouse monoclonal IgG anti-α-smooth muscle actin (Biogenex) and the secondary antibody was biotinylated horse anti-mouse (Vector Laboratories).

**Table 1.** Sources, concentrations, and dilutions of the primary and secondary antibodies used in immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Raised in</th>
<th>Concentration (mg/ml)</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal anti-XRCC1</td>
<td>Mouse</td>
<td>0.2</td>
<td>1:10</td>
<td>Abcam</td>
</tr>
<tr>
<td>Polyclonal anti-phospho-XRCC1</td>
<td>Rabbit</td>
<td>0.2</td>
<td>1:200</td>
<td>Bethyl Labs</td>
</tr>
<tr>
<td>Polyclonal anti-PARP1</td>
<td>Rabbit</td>
<td>0.1</td>
<td>1:200</td>
<td>Abcam</td>
</tr>
<tr>
<td>Monoclonal IgM anti-vimentin</td>
<td>Mouse</td>
<td>0.1</td>
<td>1:100</td>
<td>Biogenex</td>
</tr>
<tr>
<td>Monoclonal anti-BrdU</td>
<td>Mouse</td>
<td>0.25</td>
<td>1:80</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Monoclonal anti-DKN1B</td>
<td>Mouse</td>
<td>0.25</td>
<td>1:100</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Polyclonal anti-id2 (C-20)</td>
<td>Rabbit</td>
<td>0.2</td>
<td>1:100</td>
<td>Santa Cruz Biotech</td>
</tr>
<tr>
<td>Polyclonal anti-CLU</td>
<td>Goat</td>
<td>0.2</td>
<td>1:100</td>
<td>Santa Cruz Biotech</td>
</tr>
<tr>
<td>Monoclonal anti-smooth muscle actin</td>
<td>Goat</td>
<td>0.1</td>
<td>1:200</td>
<td>Biogenex</td>
</tr>
<tr>
<td>Monoclonal anti-alpha tubulin</td>
<td>Mouse</td>
<td>0.1</td>
<td>1:100</td>
<td>Biogenex</td>
</tr>
<tr>
<td>Poly-HRP anti-mouse</td>
<td>Goat</td>
<td>Ready to use</td>
<td></td>
<td>ImmunoVision Techn</td>
</tr>
<tr>
<td>Poly-HRP anti-mouse/rabbit/rat</td>
<td>Goat</td>
<td>Ready to use</td>
<td></td>
<td>ImmunoVision Techn</td>
</tr>
<tr>
<td>Biotinylated anti-mouse IgMs</td>
<td>Goat</td>
<td>0.5</td>
<td>1:1000</td>
<td>Vector Labs</td>
</tr>
<tr>
<td>HRP anti-mouse</td>
<td>Rabbit</td>
<td>0.2</td>
<td>1:1000</td>
<td>DAKO A/S</td>
</tr>
<tr>
<td>Biotinylated anti-mouse</td>
<td>Goat</td>
<td>0.5</td>
<td>1:1000</td>
<td>Vector Labs</td>
</tr>
<tr>
<td>Biotinylated anti-mouse</td>
<td>Horse</td>
<td>0.5</td>
<td>1:1000</td>
<td>Vector Labs</td>
</tr>
<tr>
<td>Biotinylated anti-goat</td>
<td>Rabbit</td>
<td>0.5</td>
<td>1:1000</td>
<td>Vector Labs</td>
</tr>
</tbody>
</table>

**Sertoli Cell Isolation and Culture**

Testes of male FVB mice, 8- to 12-wk-old, were decapsulated, and Sertoli cells were isolated as described [21, 22] with some modifications. Briefly, only two testes per isolation were used, and the isolation was done in a 15-ml centrifuge tube. Testes were decapsulated with forceps in 1× Hanks fluid washed twice in PBS (calcium/magnesium free [pH 7.4]), and dispersed (but not fragmented) in 0.1% collagenase (type IV) and 0.04% DNase I (all from Sigma) in 1× Hanks fluid (pH 7.4) at 34°C for 10–15 min, shaking at 100 oscillations/min. The tubules were allowed to settle, and after being washed two times with sedimentation with PBS, they were incubated in 1× Hanks fluid (pH 7.4) containing 0.04% DNase I, 0.05% hyaluronidase, and 0.5% trypsin (all from Sigma) for at least 10 min at 34°C with agitation. The fragmented tubules were allowed to settle and then were washed two times in PBS. After repeated pipetting in Dulbecco modified Eagle medium/Ham F12 (DMEM/F12) medium, cells were centrifuged at 900 rpm and at 700 rpm (each for 3 min). Cells in the supernatant were collected and cultured (in DMEM/F12 medium containing 5% FCS); after 3 h in culture, the medium was carefully removed, the culture was washed, and fresh medium was added. During overnight culturing, Sertoli cells attached to the bottom and acquired an irregular shape, whereas the germ cells did not attach and could easily be removed by repeated washing. Hypotonic shock (with 0.02 M Tris-HCl [pH 7.2] for 2 min) was used when needed. Following this protocol, the purity of Sertoli cells in culture exceeded 90% and could reach 99% after 20 days. Human testicular cells were enzymatically (trypsin, hyaluronidase, and collagenase) isolated from testes donated by patients undergoing bilateral castration as part of prostate cancer treatment. Cells were cultured in Stempro medium (Invitrogen, Carlsbad, CA) according to the technique described by Kanatsu-Shinohara et al. [23].
periodic acid for 30 min at 60°C and blocked in goat serum (0.5%) or horse serum (0.5%). Cells were then incubated with secondary antibody-conjugated HRP (PowerVision Poly Hrp-anti-mouse) or with biotinylated horse anti-mouse in the case of double labeling with CLU. After incubation with the biotinylated secondary antibody, the HRP avidin-biotin complex reaction was performed according to the manufacturer’s protocol (Vector Laboratories). BrdU-positive nuclei were visualized (in dark blue color) by using 0.3 µg/ml of DAB in PBS, to which cobalt (2.5%), nickel (2%), and H2O2 (0.03%) were added. Cells that incorporated BrdU were stained in red or brown as already described.

To quantify the proliferative activity of Sertoli cells, the numbers of anti-BrdU/anti-vimentin double-stained Sertoli cells relative to the numbers of vimentin-positive Sertoli cells were determined from four different isolations. These were calculated after 1, 3, 7, 13, and 20 days of culture.

To study the ability of adult human Sertoli cells to proliferate in vitro, BrdU (11 µg/ml for 3 h) was added to 3-wk-old cultures of cells from a human testicular biopsy specimen before fixation in methacarn (60% methanol, 30% chloroform, and 10% acetic acid) for 10 min. For double staining of adult human Sertoli cells, BrdU/vimentin, or BrdU/CLU, we used the same protocol as that for mouse Sertoli cells.

To determine whether increasing proliferative activity of Sertoli cells over time in culture was associated with changes in expression of CDKN1B or ID2, expression of CDKN1B and ID2 was studied by immunocytochemistry and Western blot analysis. For immunocytochemistry of CDKN1B, we used the same double-staining protocol as that for vimentin/BrdU staining except that the primary antibody rabbit anti-ID2 was used instead of mouse monoclonal anti-CDKN1B. The secondary antibody was used except that the primary antibody rabbit anti-ID2 was used instead of mouse monoclonal anti-CDKN1B and rabbit anti-ID2, were diluted 1:100 in Blotto A. After incubation with secondary antibody-conjugated HRP rabbit anti-mouse (DAKO) or goat anti-rabbit (Santa Cruz Biotechnology), the antigens were visualized using chemiluminescence (ECL; Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) and exposed to x-ray film (RX; Fuji Photo Film Co., Tokyo, Japan). SDS-PAGE was performed as described by Laemmli [26]. Proteins were blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Western blots were blocked using Blotto A containing 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline (10 mM Tris- HCl [pH 8.0] and 150 mM NaCl) plus 0.05% Tween-20 (TBST) and were washed in TBST between each step. The first antibodies, mouse monocolonal anti-CDKN1B and rabbit anti-ID2, were diluted 1:1000 in Blotto A. After incubation with secondary antibody-conjugated HRP rabbit anti-mouse (DAKO) or goat anti-rabbit (Santa Cruz Biotechnology), the antigens were visualized using chemiluminescence (ECL; Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) and exposed to x-ray film (RX; Fuji Photo Film Co., Tokyo, Japan). The blots were analyzed using a mouse polyclonal antibody against α-tubulin (InnoGenex, San Ramon, CA) after incubation with secondary antibody-conjugated HRP rabbit anti-mouse (DAKO). The experiments were performed four times. Quantification of the signal was performed using ImageJ software (http://rsb.info.nih.gov/ij/index.html).

**Statistical Analysis**

Statistical analysis between groups from four different experiments was performed by one-way ANOVA (Dunnett multiple comparisons test). GraphPad software (GraphPad Software, Inc., San Diego, CA) was used.

**Western Blot Analysis**

Total protein lysates from cultured Sertoli cells at 1, 3, 13, and 20 days were scraped using a Teflon scraper after the addition of ristocetin-induced platelet agglutination buffer. Lysates were sonicated on ice and then washed three times with 0.3 M Tris-HCl buffer (pH 7.5) and dehydrated in ethanol. For analysis, slides were stained with ethidium bromide (2 µg/ml in distilled water), scoring was done in a blinded manner. Image analysis was performed using the software program Comet Assay IV (Perceptive Instruments Ltd., Haverhill, Suffolk, England) on 70 randomly selected cells (three experiments). The DNA lesions were quantified by measuring the increase in tail length and tail moment. Tail length is the distance of DNA migration from the body of the nuclear core, with longer tails correlating with more DNA damage inflicted. Tail moment is the product of the tail length and the fraction of total DNA in the tail. Both are used to evaluate the extent of DNA damage.

**Statistical Analysis**

Statistical analysis between groups from four different experiments was performed by one-way ANOVA (Dunnett multiple comparisons test). GraphPad software (GraphPad Software, Inc., San Diego, CA) was used.
RESULTS

Sertoli Cells Can Proliferate In Vitro

After 16 h in culture followed by repeated washing and hypotonic shock, at least 89% of the cells were positive for the Sertoli cell marker vimentin (Fig. 1A). The specificity of the vimentin antibody was confirmed by using a mouse testis section as a positive control. In this control section, only the cytoplasm of Sertoli cells became stained, whereas all other cell types in the testis were negative (Supplemental Fig. S1A, all Supplemental Data are available at www.biolreprod.org). In culture, Sertoli cells formed a monolayer, had an irregular shape (Fig. 1, B–F), and stretched out at early time points (Fig. 1F). After a few days in culture and repeated washings, the purity of the vimentin-positive cells in culture increased to more than 98% (Fig. 1A). The few other cells were round-shaped germ cells or irregular-shaped cells with less cytoplasm than Sertoli cells that were negative for vimentin and possibly were peritubular cells [27]. Over time in culture, the irregular-shaped vimentin-positive Sertoli cells enlarged in size (Fig. 1, B and C). Besides these large cells, there were groups of smaller cells that were positive for vimentin after 20 days in culture (Fig. 1D).

To determine whether Sertoli cells were able to proliferate in vitro, BrdU was added to the culture for 15 h, and double staining was performed for BrdU and vimentin. The specificity of the anti-BrdU antibody was confirmed by studying staining in testes of mice that received a dose of BrdU. Only cells capable of proliferation, spermatogonia and preleptotene spermatocytes, were found to be stained (data not shown). After 3 days in culture, 10% of the vimentin-positive Sertoli cells were positive for BrdU, and this number increased to 15% after 6 days. The BrdU-labeled Sertoli cells were dispersed over the culture (Fig. 1B), suggesting that increasing numbers of Sertoli cells resumed proliferation and that the labeled Sertoli cells did not derive from colonies formed by rare Sertoli cells that had retained their proliferative capacity. After 1 wk in culture, Sertoli cells were flattened and larger in size, and the number of BrdU/vimentin double-positive cells reached 36% after 20 days (Fig. 1A). Sertoli cells remained positive for vimentin during the full culture period. After 16–20 days of culture, the enlarged and the smaller Sertoli cells were capable of incorporating BrdU (Fig. 1, C and D).
It has been reported that serum can induce ID2 in cells, which stimulates proliferation [28, 29]. To evaluate whether, after the initiation of the culture during which 5% serum is needed, Sertoli cells were induced to proliferate by the serum in the culture medium, Sertoli cells were isolated and cultured for 24 h in 5% serum containing medium and then left in serum-free medium for 5 days. At the end of the culture, 15% of Sertoli cells were positive for BrdU, similar to Sertoli cells that are continuously exposed to 5% serum during culture (Supplemental Fig. S1B).

To investigate whether mouse Sertoli cells behaved differently from those in other species not showing season-dependent spermatogenesis, BrdU was added to cultures of cells from a human testicular biopsy specimen. In 3-wk-old cultures, double staining for vimentin and BrdU revealed many double-positive cells (Fig. 1E), indicating that human Sertoli cells also resume proliferation in vitro.

To further confirm the identity and purity of the cultured Sertoli cells, cultures were stained for the additional Sertoli cell marker CLU and for the peritubular cell marker α-smooth muscle actin (Fig. 2A). After 1 wk, approximately 91% and 6.3% of cells in culture were positive for CLU and α-smooth muscle actin, respectively (Fig. 2B). After 20 days, 92.4% of the cells were positive for CLU and 7% for α-smooth muscle actin, showing that the purity of Sertoli cells did not change much during culture.

When triple staining was performed (Fig. 2, C–G) for CLU, anti-BrdU, and α-smooth muscle actin after 20 h (A), 4 days (B), and 16 days (C) of culture, CDKN1B expression in the adult mouse testis. Arrows indicate Sertoli cells; arrowheads, Leydig cells. Bar = 20 μm. E) Western blot analysis of CDKN1B levels after various times in culture (1, 3, 13, and 20 days). Expression levels significantly decrease over time in culture. The error bar represents the SEM of results from three experiments (***P < 0.01).

Expression of CDKN1B Decreases over Time in Culture

To investigate whether the proliferative activity of Sertoli cells in culture was accompanied by changes in expression of the cell cycle inhibitor CDKN1B, we studied expression of this protein in normal testis sections and after culture for 1–20 days. Immunohistochemistry revealed that the numbers of vimentin-positive Sertoli cells that stained for CDKN1B decreased over time in culture (Fig. 3, A–C). Most Sertoli cell nuclei were positive for CDKN1B after 20 h in culture (Fig. 2A). After 2 wk, most of these nuclei were negative or only lightly stained for CDKN1B. Western blot analysis confirmed decreasing expression levels of CDKN1B protein (Fig. 3E). After 3 days in culture, CDKN1B protein levels were reduced to 30% of those found after 20 h. Western blots showed that the antibody recognized only one band at 27 kDa. In testis sections, CDKN1B stained the nuclei of Sertoli cells, whereas weak staining was seen in Leydig cells (Fig. 3D).

Expression of ID2 Increases over Time in Culture

To find out whether the renewed proliferative activity of Sertoli cells was associated with increased levels of the proliferation inducer ID2, Western blot analysis was carried out on testis lysates and on Sertoli cell protein extracts from 1-, 3-, 13-, and 20-day cultures. The antibody recognized two bands, ID2 at 15 kDa and ID2 fusion proteins at 75 kDa. Increased expression of ID2 (15 kDa) was found over time in culture. After 3 days in culture, expression of ID2 showed a 2-fold increase compared with that after 20 h and more than a 3-fold increase after 13 days (Fig. 4). Immunohistochemistry showed clear ID2 staining of Sertoli cell nuclei and weak cytoplasmic staining in 1-day and 1-wk cultures (Supplemental Fig. S2, A and B).
Adult Sertoli Cells Express DNA Repair Proteins

In testis sections before and after irradiation, Sertoli cells were found to express XRCC1, phosphorylated XRCC1, and PARP1 proteins (Fig. 5, A–C). This expression did not clearly vary among the stages of the cycle of the seminiferous epithelium. Other somatic cells in the testis were negative for these proteins (Fig. 5). In Supplemental Table S1, we summarize expression of proteins involved in DNA DSB repair in Sertoli cells in vivo before and after ionizing radiation, as reported by us and by other groups. None of these proteins were found in Leydig cells or other somatic cells.

Adult Sertoli Cells Are Able to Repair Ionizing Radiation-Induced DNA Damage

In the comet assay during electrophoresis, damaged DNA migrates from the nucleus toward the anode, forming the shape of a “comet” with a head (cell nucleus with intact DNA) and a tail (relaxed and broken DNA). The DNA damage and repair were quantified by measuring changes in the length of the nuclear tail and tail moment. Shortly after irradiation, the changes in the shape of Sertoli cell nuclear comets (Fig. 6, A–D) indicated a 5-fold increase in tail length and tail moment compared with the control. Thereafter, these parameters decreased by 50% and 70% at 5 h and 20 h, respectively, compared with 30 min after irradiation. At 20 h, a less than 1-fold increase remained compared with the control (Fig. 6E). These results clearly show that Sertoli cells are capable of repair of DNA DSBs.

DISCUSSION

Our results show that mouse and human Sertoli cells reenter the cell cycle in vitro. Unlike other somatic cells in the testis, Sertoli cells express DNA repair genes, suggesting their ability to repair DNA DSBs. The latter was proven using the comet assay. These findings completely change the traditional view about the nature of Sertoli cells in adult nonseasonal breeding mammals.

Adult mouse Sertoli cells were isolated and put into culture. The purity of the isolated Sertoli cells exceeded 90%, and this remained so throughout the culture as determined by staining for the Sertoli cell markers vimentin and CLU. After 3 days in culture and as characterized by double immunohistochemical staining for the Sertoli cell marker vimentin and for incorporated BrdU, 10% of Sertoli cells were positive for BrdU, and this percentage increased over time. After 20 days in culture, 36% of Sertoli cells incorporated BrdU when exposed to precursor for 15 h. After the transition of Sertoli cells from their in vivo situation in the seminiferous tubules to the culture conditions, these cells were apparently gradually released from their cell cycle arrest and started to proliferate. These cell kinetic results were confirmed by similar culture experiments using CLU as a Sertoli cell marker in which comparable data were obtained. Immunohistochemical experiments with the peritubular cell marker α-smooth muscle actin revealed that these cells remained rare (<7%) throughout the cultures and did not overgrow the Sertoli cells. Additional experiments were carried out in cultures of a cell suspension of human testis to which BrdU was added. Here too, vimentin/BrdU and CLU/BrdU double-positive cells were found, indicating that adult human Sertoli cells can also resume proliferation in vitro. Apparently, the potential proliferative capacity of adult Sertoli cells is not specific to mice.

The increased BrdU incorporation in mouse Sertoli cells was accompanied by decreasing numbers of Sertoli cells expressing the cell cycle inhibitor CDKN1B and by decreasing
levels of CDKN1B protein over time in culture. CDKN1B has an important role in the regulation of Sertoli cell proliferation during development, and Cdkn1b−/− mice demonstrate increased testis size [14, 30], testis weight (42%), and Sertoli cell numbers (26%) [31, 32]. The concomitant increase in the proliferative activity of Sertoli cells and their decreased expression of CDKN1B suggest that this protein is actively suppressing Sertoli cell proliferation in vivo. Nevertheless, CDKN1B cannot be the only factor involved in regulating Sertoli cell proliferation in the adult mouse testis, as Sertoli cells in Cdkn1b−/− mice do not continue proliferation beyond a certain age [14].

Our results also show that the decrease in CDKN1B expression in culture and the onset of Sertoli cell proliferation are associated with increased expression of ID2. The ID proteins are considered dominant-negative regulators of cellular differentiation pathways and act as positive regulators of cellular proliferation [31]. Adult Sertoli cells express the four ID proteins, but only ID2 is present in Sertoli cell nuclei [16, 17]. In adult mice, Sertoli cell nuclei at all stages exhibit immunoreactivity for ID2, indicating that ID2 protein is present throughout the cycle, irrespective of the germ cell types with which Sertoli cells are associated [17]. ID2-null mice display a late-$\gamma$H-2AFX incorporation [36]. In this organ culture, the continued presence of Sertoli cell proliferation in serum-free cultures is similar to that in cultures containing 5% serum.

Steinberger and Steinberger [36] previously found that Sertoli cells repair DNA DSBs, but we were not able to find any published results on the proliferative activity of these cells. Recent studies have shown that Sertoli cells are capable of proliferating in response to certain stimuli, such as the presence of germ cells [37]. Furthermore, Sertoli cells have been shown to express DNA repair proteins, including PARP1 and XRCC1 [40]. These proteins are involved in the base excision repair pathway [40]. Sertoli cells express PARP1 and XRCC1 and may use these proteins to repair DNA DSBs.

In conclusion, our results indicate that postpubertal Sertoli cells cannot be regarded as terminally differentiated cells that have lost the capacity to proliferate. In vivo, the testis barrier and the formation of tight junctions after maturation may prevent Sertoli cells from reentering the cell cycle. A balance seems to exist between cell cycle inhibitors and inducers that in the normal in vivo situation keeps the cells quiescent. The residual proliferation capacity of Sertoli cells in vivo is probably the underlying reason for Sertoli cell tumors seen rarely in humans [41] and more often in dogs [42]. Mitotically active Sertoli cells have been found in testis nodules of infertile humans [43]. Placement of Sertoli cells in culture clearly disturbs the in vivo balance in such a way that these cells gradually start to proliferate again. Sertoli cells also do not behave as terminally differentiated somatic cells in that they are still able to repair DSBs. When not repaired, DSBs will be fatal and cause cell death. Our results show that these cells still have the capacity to proliferate, rendering it necessary to remove DSBs.

These results in nonseasonal breeding mammals completely change the traditional views about the nature of Sertoli cells, and it will be enlightening to study the regulatory mechanisms governing Sertoli cell proliferation in further detail. In this respect, seasonal breeders may provide a clue to investigate these regulatory mechanisms, as the same mechanisms may likely trigger Sertoli cell proliferation in these animals at the start of each breeding season.
REFERENCES


25. Bouquet F, Muller C, Salies B. The loss of gammaH2AX signal is a marker of DNA double strand breaks repair only at low levels of DNA damage. Cell Cycle 2006; 5:1116–1122.


