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Establishment of Cell Lines with Rat Spermatogonial Stem Cell Characteristics

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Spermatogonial cell lines were established by transfecting a mixed population of purified rat A_s (stem cells), A_{pr} and A_{al} spermatogonia with SV40 large T antigen. Two cell lines were characterized and found to express Hsp90α and oct-4, specific markers for germ cells and A spermatogonia, respectively. Expression of c-kit, normally expressed in A spermatogonia from late A_{al} spermatogonia onwards, could not be detected in either cell line. Furthermore, no expression of vimentin (Sertoli cell marker) and α-smooth muscle actin (peritubular cell marker) could be found. Upon transplantation of these cell lines into recipient mice, the cells were found to be able to migrate to the basement membrane and to colonize seminiferous tubules. Taken together, we conclude that our cell lines have spermatogonial stem cell characteristics. These first spermatogonial cell lines with stem cell characteristics can now be used to study spermatogonial gene expression in comparison with more advanced germ cells. (*Endocrinology* 143: 1845–1850, 2002)

SPERMATOGENESIS IS A complex well-organized process including proliferation, differentiation and apoptosis, through which differentiating daughter cells of spermatogonial stem cells develop into elongated spermatids. Spermatogonial stem cells, A_s spermatogonia, are able to renew themselves and to differentiate by dividing into A_{pr} spermatogonia, which stay connected by intercellular bridges. These cells can divide further to form chains of 4, 8, or even 16 A_{al} spermatogonia. These A_{al} spermatogonia will differentiate without mitosis into A_1 spermatogonia, which enter a strictly time-regulated process of 6 subsequent divisions, the last one giving rise to spermatocytes. Spermatocytes carry out the meiotic divisions and develop into spermatids (1). *In vivo*, in the normal testis all these steps in germ cell development take place at the same time and are therefore difficult to study. To investigate each of these steps, *in vitro* experiments using isolated germ cells have been performed (2–5). However, only a limited number of cells can be isolated, germ cells have a limited viability in culture, and it is difficult to distinguishing spermatogonial stem cells from more differentiated A spermatogonia *in vitro*, due to the lack of specific stem cell markers.

The establishment of spermatogonial cell lines would overcome most of these problems. To date, several germ cell lines have been developed, mainly having spermatocyte characteristics (6–8). Until now, no A spermatogonial cell line has been established. We now describe the establishment of rat cell lines with spermatogonial stem cell characteristics obtained by transfection of SV40 large T antigen into a population of A_s, A_{pr}, and A_{al} spermatogonia isolated from vitamin A-deficient rats, and some of the characteristics of these cell lines.

Abbreviations: ABC, Avidin/biotin complex; HRP, horseradish peroxidase; VAD, vitamin A-deficient.

Materials and Methods

Animals

Wistar rats U:WU (CPB) (Central Laboratory Animal Institute Utrecht, Utrecht University, Utrecht, The Netherlands) were used and maintained with permission of and according to regulations provided by the Animal Ethical Committee of the University Medical Center Utrecht. Vitamin A-deficient (VAD) animals were obtained by feeding pregnant Wistar rats (from 19 d postcoitum onwards) a vitamin A-deficient diet (Teklad Trucking, Madison, WI). Male offspring received the same diet until their body weight decreased at about 8–11 wk of age. At this time of vitamin A deficiency, animals were killed, and their testes were removed for cell isolations.

Isolation and culture of A spermatogonia from vitamin A-deficient rat testis

A mixed population of A_s, A_{pr}, and A_{al} spermatogonia was isolated from VAD rat testes as previously described (9). Cell fractions containing 80% or more A spermatogonia were used. Cells were used for Western blot analysis or cultured overnight on growth factor reduced Matrigel (1:10, Collaborative Biomedical Products, Bedford, MA) in MEM (Life Technologies, Inc., Paisley, Scotland, UK) supplemented with single strength nonessential amino acids, 100 IU/ml-100 μg/ml penicillin-streptomycin, 40 μg/ml gentamicin, 15 mM HEPES, 250 ng/ml Fungizone (all from Life Technologies, Inc.), 0.12% sodium bicarbonate, 4 mM L-glutamine, 10 ng/ml platelet-derived growth factor-BB, 10 ng/ml recombinant human basic fibroblast growth factor, 10 ng/ml recombinant human LIF, 20 μM forskolin (all from Sigma, St. Louis, MO), 1 μM β-E2–17-cypionate (ICN Biomedicals B.V., Zoetermeer, The Netherlands) and 2.5% FCS at 32 C and 5% CO_2. Cultures were used for transfection or fixation for immunocytochemistry.

Transfection

Isolated A spermatogonia from vitamin A-deficient rat testes were cultured overnight in Matrigel-coated 35-mm wells (six-well plate) to

improve attachment of the cells, as described above. Cells were transfected with 3.25 μg pSV3-neo, containing the intact SV40 early region (American Type Culture Collection, Manassas, VA; no. 37150) using 6.5 μl Fugene 6 transfection reagent (Roche Diagnostics, Almere, The Netherlands) per well. For selection, G418 (Life Technologies, Inc.) was added to the medium after 7 d in a concentration of 200 μg/ml. Selection medium was refreshed twice a week. Cells surviving the selection of G418 were passaged with 0.5 mM EDTA at room temperature and cultured further without Matrigel (to avoid interference of including growth factors with those used subsequently to culture the cells) in selection medium as described above.

SDS-PAGE and Western blotting

Protein lysates from adult rat testes, from isolated A spermatogonia of VAD rat testes and from cell lines A303 and A304, were prepared in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) including 1 mM phenylmethylsulfonylfluoride. Of each sample, 50 μg were separated on a 12% SDS-polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA).

Western blots were blocked using Blotto-A, containing 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in Tris-buffered saline (10 mM Tris; 150 mM NaCl, pH 7.6), including 0.05% Tween-20. Primary antibodies used were: anti-Hsp90α (Hsp86, 1:200, SPA-771, StressGen Biotechnologies Corp., Victoria, Canada), anti-Oct-4 (1:200, H-134, sc-9081, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-c-kit (1:200, C-19, sc-168 or M-14, sc-1493, both Santa Cruz Biotechnology, Inc.), anti-vimentin, nonhematopoietic (1:200, Mu 163-UC BioGenex Laboratories, Inc., San Ramon, CA) and anti-α-smooth muscle actin (1:500, Mu 128-UC, BioGenex Laboratories, Inc.). They were diluted in Blotto-A and incubated for 1 h at room temperature. Blots were washed with Tris-buffered saline including 0.05% Tween-20. After incubation with secondary antibodies, rabbit antigoat-horseradish peroxidase (HRP), rabbit antimouse-HRP (both 1:2000, DAKO Corp., Glostrup, Denmark) or goat antirabbit-HRP (1:5000, sc-2004, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in Blotto-A or goat antimouse IgM-biotin (1:2000, Zymed Laboratories, Inc., San Francisco, CA) in Blotto-A followed by avidin/biotin complex (ABC) peroxidase (Vector Laboratories, Inc., Burlingame, CA) for 1 h, blots were incubated with electrochemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK) and exposed to x-ray film (RX-omat, Kodak, Chalone/Saone, France). Western blot analysis for each antibody was performed at least three times on different passages (between passages 14 and 31) of the cell lines.

Immunocytochemistry

For immunocytochemistry, short-term cultures of the cell lines on Matrigel coated (1:10, to increase adherence of the cells) LabTek Permanox chamber slides (Nunc Life Technology, Roskilde, Denmark) were washed in PBS and fixed in 4% paraformaldehyde for 15 min. Cells were treated with 0.4% Triton X-100 for 30 min, and endogenous peroxidase was blocked with 0.35% H_20_2 for 10 min. Pretreated cells were blocked for nonspecific staining of the secondary antibody with 10% of the corresponding serum. Cells were incubated with mouse anti-SV40 T Ag (1:20, Ab-1, Oncogene Research Products, Cambridge, MA), rabbit anti-Hsp90α (1:200), or rabbit anti-Oct-4 (1:25). For negative control the first antibody was replaced with the same concentration of mouse IgG or rabbit IgG, respectively. Detection of the primary antibody was performed with an ABC peroxidase staining kit (Vector Laboratories, Inc.) and diaminobenzidine (DAKO Corp.) as a substrate. Cells were counterstained with Mayers's hematoxylin in case of Hsp90α and SV40 T Ag immunostaining.

FACScan analysis

Cells from the cell lines were collected using trypsin/EDTA (0.05% trypsin in 0.5 mM EDTA), fixed in 70% ethanol and stored at 4 C for at least 24 h. After washing, cells were incubated in 0.25 mg/ml pepsin (Sigma) in 0.1 N HCl, and stained for 15 min at RT with a solution containing 0.01 mg/ml propidium iodide (Calbiochem Corp., La Jolla, CA) and 0.1 mg/ml RNase (Sigma). DNA content was measured using a FACScan (Becton Dickinson and Co., Bedford, MA) and compared with A spermatogonia (diploid cells) and spermatocytes (tetraploid cells) isolated from testes of synchronized VAD/RA-treated rats.

Transplantation

The recipient mice used (NMRI nude (nu/nu), Harlan Netherlands, Horst, The Netherlands) were given local doses of 1.5 and 12 Gy of X-irradiation with a 24 h interval to destroy endogenous spermatogenesis (8a). At least 1 month after irradiation, for each of the cell lines 5×10^4 cells in 20 μl MEM were injected via the efferent duct and the rete testis into the seminiferous tubules in one of the testes of each recipient mouse. The other testis served as an internal control. Transplanted and control testes were examined 8 and 14 wk after transplantation. For each time point and each cell line, three to five mice were used. Testes were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. The sections were then immunostained with a primary biotinylated anti-SV40 Large T antibody (Pab 108, BD PharMingen, San Diego, CA), after blocking endogenous peroxidase with 0.35% H_20_2 for 10 min and pretreatment with 5% BSA for 1 h. The detection of the signal was performed using the ABC peroxidase staining kit and diaminobenzidine as a substrate. Sections were counterstained with Mayers's hematoxylin.

Results

Establishment of cell lines

A mixed population of A_s, A_{pr}, and A_{al} spermatogonia isolated from vitamin A-deficient rat testes with a purity of 80% was cultured overnight at a density of 3×10^5 cells/well in a 6-well dish. Seven days after transfection of the cells with pSV3-neo using Fugene 6 transfection reagent, they were given fresh medium with the addition of G418 for selection. Cells surviving the selection of G418 were passaged several times and then investigated for expression of Hsp90α using Western blot analysis. Cells expressing Hsp90α were single-cell cloned once by limiting dilution, yielding a total of 12 cell lines of which two cell lines (A303, A304) were investigated further. These cell lines have now been cultured for 3 yr and passaged more than 50 times without any morphological changes. Cell line A304 grows about twice as fast as A303 and at present they are passaged at confluence once a week; A303 diluted 1 in 4 and A304 1 in 8. The cells still are able to proliferate even after the cultures become confluent and overgrow each other, showing low contact inhibition.

Morphology

The morphology of both cell lines, A303 and A304, appeared similar. They both had a flattened and somewhat elongated shape, round/oval nuclei with relatively large nucleoli as seen by phase contrast and Hoffman images (A303 passage 36, A304 passage 56) (Nikon Eclipse TE200, Uvikon, Bunnik, The Netherlands) (Fig. 1, A–E). The nuclei showed a finely dispersed heterochromatin after fixation with Bouin's fluid and staining with hematoxylin (Fig. 1C, A303 passage 24; Fig. 1F, A304 passage 25). Although the cells from the cell lines have a more flatten image than overnight cultured primary isolated A spermatogonia, their Bouin's fluid fixed and hematoxylin stained image look very much the same (*inset* in Fig. 1C). When detached from the culture flasks, cells from both cell lines became rounded (Hoffman image, *inset* Fig. 1B, A303 passage 46, Fig. 1E, A304 passage 53) and had a morphology very similar to that of freshly isolated A spermatogonia (9).

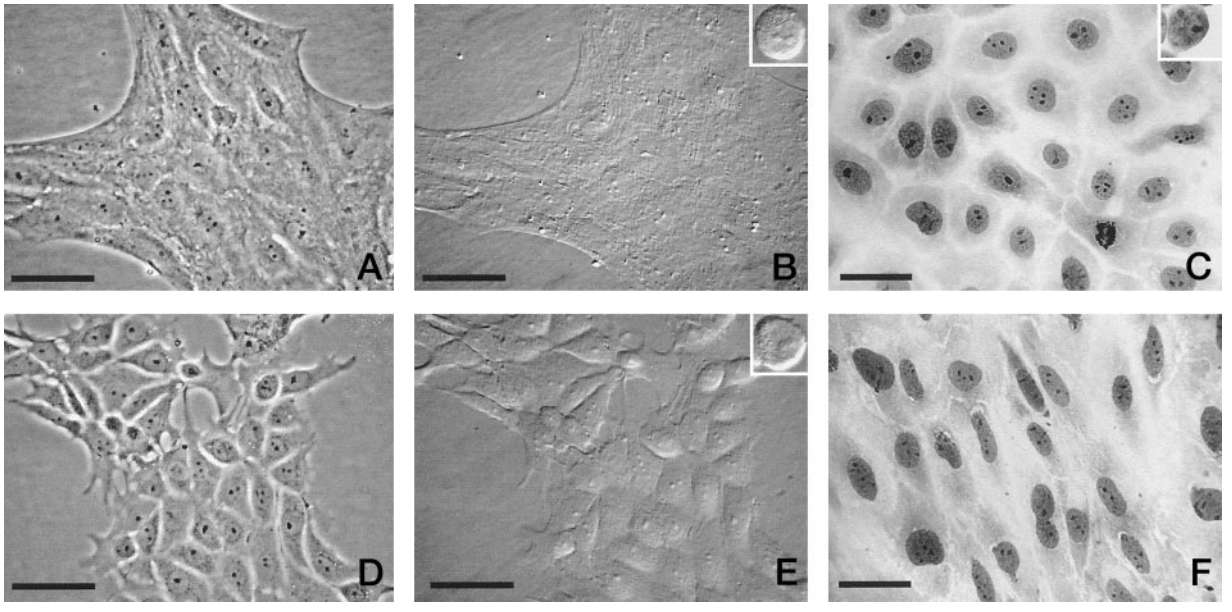

FIG. 1. Morphological characteristics of cell lines A303 (A–C) and A304 (D–F); Phase contrast (A, D), Hoffman images (B, E), Bouin's fluid fixed and hematoxylin staining (C, F) of the cells; *insets* in B and E show Hoffman images of detached cells of the respective cell line; *inset* in C shows the Bouin's fluid-fixed and hematoxylin-stained primary isolated A spermatogonia after overnight culture. *Bars,* 50 μm; *insets*, 5 μm.

Expression of testicular cell-specific markers

Cell lines were investigated for their expression of the germ cell marker Hsp90α. As shown by Western analysis (Fig. 2), Hsp90α is expressed in both cell lines A303 and A304. Furthermore, the expression of oct-4, an A spermatogonial marker, was investigated. Both cell lines showed a 42-kDa band using the oct-4 antibody (Fig. 2). As a control for this reaction, embryonal carcinoma cells F9 were used. A 42-kDa oct-4 band was also detected in protein extracts of these cells, which was down-regulated after RA treatment of the cells, as expected (10). Using two different antibodies in a Western blot analysis, hardly any signal could be detected for c-kit in both cell lines, while clear bands were found with protein extracts of primary isolated A spermatogonia from vitamin A-deficient rat testes (in Fig. 2, the analysis using the M14 antibody is shown). Furthermore, no expression could be found of vimentin and α-smooth muscle actin, markers for Sertoli cells and peritubular myoid cells, respectively. Total rat testis protein extract was used as a positive control for all markers mentioned (Fig. 2).

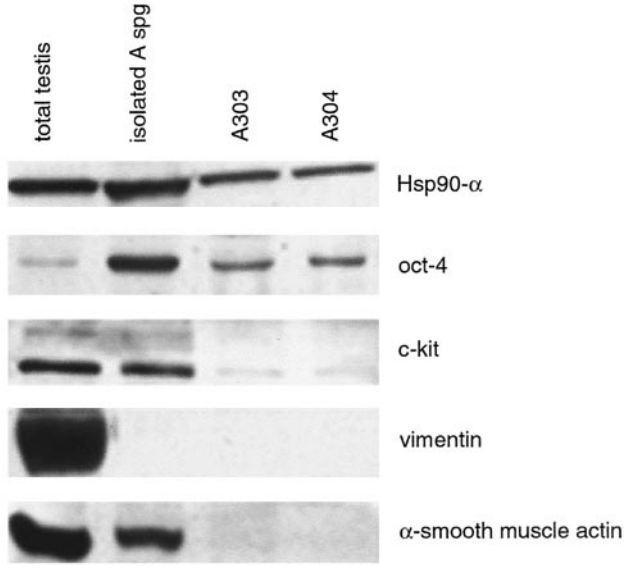

FIG. 2. Western blot analysis of the expression of several testicular cell specific markers showing a clear signal in cell lines A303 and A304 for Hsp90α and oct-4 (specific markers for germ cells and A spermatogonia, respectively). No signal could be found for vimentin and α-smooth muscle actin, markers for Sertoli cells and peritubular myoid cells, respectively. Hardly any signal for c-kit (M14) could be detected in both cell lines compared with protein extracts from isolated mixed populations of A_s, A_{pr} and A_{al} spermatogonia and an extract from total normal adult rat testes.

Immunocytochemistry

To investigate whether all individual cells within the cell lines A303 and A304 express Hsp90α and oct-4, cultured cells were immunostained for these markers. As shown in Fig. 3, a–g, all cells from both cell lines showed immunostaining for these markers, as well as immunostaining for SV40 large T antigen in the nucleus (Fig. 3, g–i).

DNA content

Using the FACScan, DNA content was measured of cell lines A303 and A304 in passage 40 and 43, respectively. Both cell lines showed DNA content profiles of proliferating diploid cells (Fig. 4, A and B). Narrow diploid peaks were found for both cell lines with a CV of 1.98 for A303 and 3.16 for A304. Furthermore, the G1 phase and G2 + M-phase peaks of both cell lines were found in the same channels compared with freshly isolated germ cells (56% A spermatogonia and 44% spermatocytes) (Fig. 4C), indicating a normal DNA content.

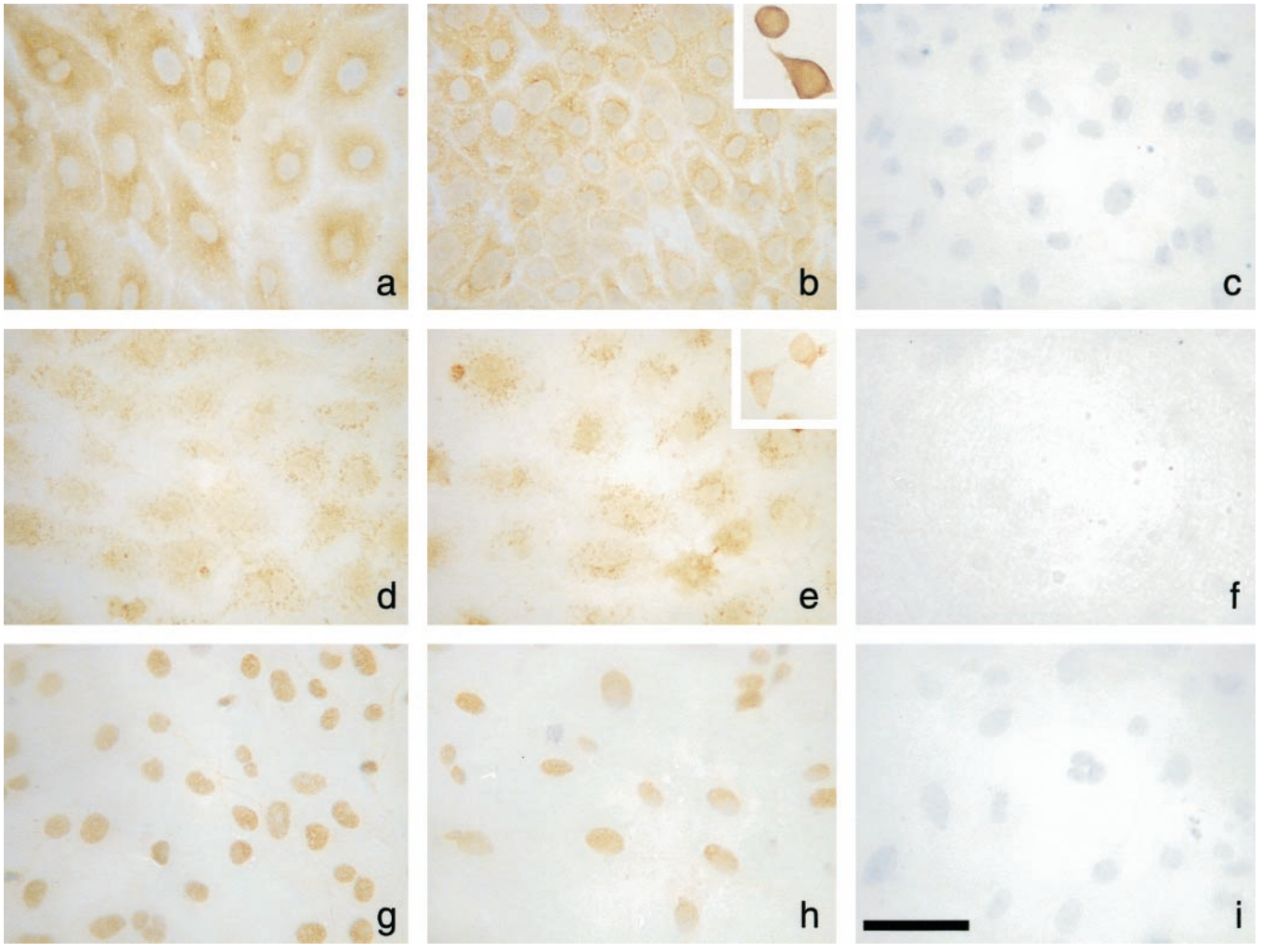

FIG. 3. Immunocytochemical staining of cell lines A303 and A304 for anti-Hsp90α (a, b), oct-4 (d, e) and SV40 large T antigen (g, h). *Insets* in b and e show immunostaining for anti-Hsp90α and oct-4 respectively of primary isolated A spermatogonia after overnight culture. Negative controls were incubated with the same concentration of normal rabbit IgGs (c, f) or normal mouse IgGs (i). Counterstaining was absent in d–f. *Bar*, 50 μm.

Transplantation

To investigate stem cell capacity of both cell lines *in vivo*, 50,000 cells were transplanted into one of the testes of recipient mice. After 8 wk, SV40 large T-positive cells from both cell lines were observed in seminiferous tubules of transplanted testes of 2 out of 3 mice transplanted with A303 and 4 out of 5 transplanted with A304 (Fig. 5, a and b). Even after 14 wk, some tubules of the transplanted mice showed SV40 large T-positive cells, 3 out of 5 transplanted with A303 and 1 out of 4 transplanted with A304. Clearly, cells from both cell lines were able to migrate to the basement membrane and colonize the tubules. The cells showed oval to elongated nuclei like normal rat A spermatogonia in stage IV-VII in paraformaldehyde fixed testes. Further developed SV40 large T-positive germ cells were not found, and no tumor formation was observed at these time points. In most tubules with large clusters of SV40 large T-positive cells, the nuclei of Sertoli cells seemed to have moved toward the lumen of the seminiferous tubules (Fig. 5a).

Discussion

In this paper, the establishment of the first cell lines with rat spermatogonial stem cell characteristics is described. It proved possible to immortalize isolated A spermatogonia by way of transfection with the SV40 large T antigen. After single cell cloning of the immortalized cells, we ended up with two pure cell lines, which have now been cultured for over 50 passages under permanent selection of G418 without noticeable changes in morphology and with a normal DNA content, indicating that these cell lines are very stable.

Morphologically, both cell lines resemble freshly isolated A spermatogonia after one night of culture. Neither cell lines expresses vimentin or α- smooth muscle actin, indicating that they are not of Sertoli cell (11) or peritubular cell (12) origin, respectively. However, both cell lines express the germ cell marker Hsp90α (Hsp86) (13) as well as oct-4, a specific marker of spermatogonia of adult male mammals (14). Furthermore, neither cell line expresses c-kit, which is expressed

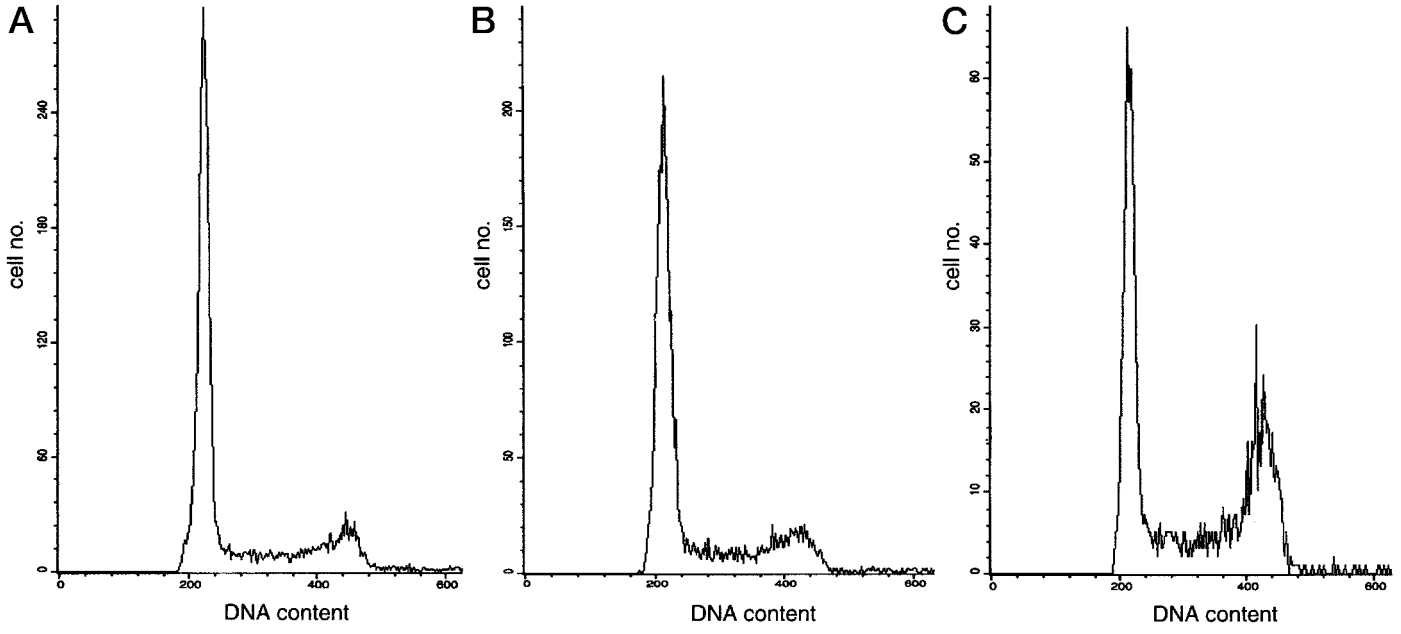

FIG. 4. FACScan analysis of A303 (40th passage) (A) and A304 (43rd passage) (B) showing narrow diploid and tetraploid peaks comparable with those of isolated A spermatogonia and spermatocytes (C).

by Leydig cells and all spermatogonia from late A_{al} to B spermatogonia, but not in A_s, A_{pr}, and early A_{al} (5). From this biochemical characterization, it is clear that the established cell lines lack somatic cell characteristics, but express A spermatogonial markers. The lack of c-kit expression suggests that both cell lines have characteristics of A_s, A_{pr}, or early A_{al} spermatogonia.

To investigate whether both rat spermatogonial cell lines displayed stem cell behavior, they were transplanted into recipient nude mice. Only stem cells are able to establish themselves on the basal membrane of a recipient seminiferous tubule and begin to replicate, subsequently providing for both renewal of the stem cell population and production of a subset of progeny that differentiate into spermatozoa (15). Although injected in a low number, the single cells of both cell lines were able to migrate to the basement membrane and colonize some tubules by forming clusters on the basal side of Sertoli cells for at least 14 wk after transplantation. This further confirmed their spermatogonial stem cell properties. However, no further differentiation of both of the rat spermatogonial cell lines could be detected. Clouthier *et al.* (16) and Russell and Brinster (17) showed complete rat spermatogenesis after transplantation of freshly isolated spermatogonia into recipient mice. Apparently, the cells from both cell lines prefer self renewal above differentiation. Within 14 wk, no germ cells tumors were formed as was found 24–130 d after transplantation of embryonic stem cells (18). Several other models have been described in which spermatogonia are not able to differentiate into more advanced germ cells. Allard *et al.* (19) described this phenomenon in rats treated with 2,5-hexanedione. In the testes of the mutant jsd/jsd mice only Sertoli cells and A spermatogonia remain (20–22) and Kangasniemi *et al.* (23) described the same problem after local irradiation of $LBNF_1$ rats. Furthermore, the atrophy in the testis of the aged Brown Norway rat is not attributed to a loss

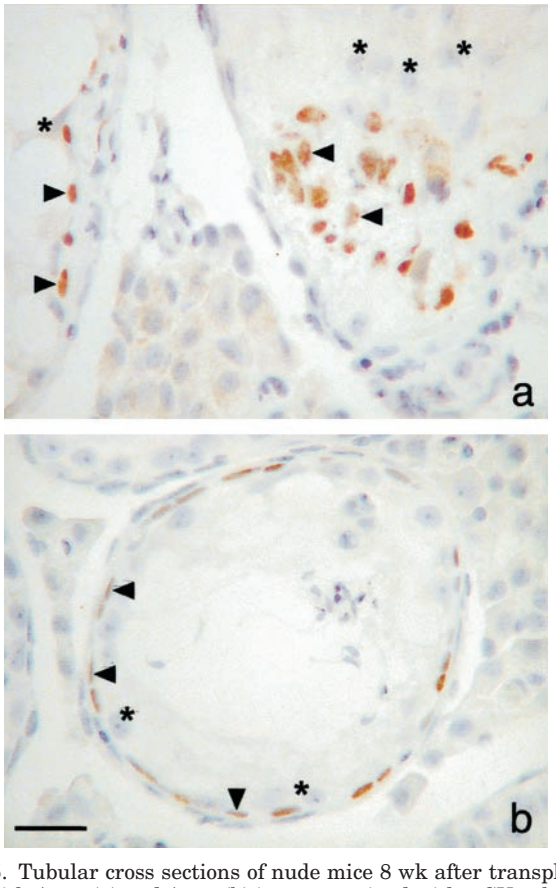

FIG. 5. Tubular cross sections of nude mice 8 wk after transplantation with A303 (a) and A304 (b) immunostained with a SV40 large T antibody showing *brown* nuclear staining in colonies of A spermatogonia from the respective cell lines (some indicated by *arrowheads*) on the basement membrane (some Sertoli cell nuclei are indicated by *asterisks*). *Bar*, 25 μm.

of spermatogonial stem cell proliferation, but is due to problems in spermatogonial stem cell differentiation (24). In all these animals, treatment with leuprolide supports the differentiation of the A spermatogonia (24–27). Leuprolide was also found to be beneficial to colonization as well as differentiation of A spermatogonia after transplantation (28, 29). Whether this could also be the case with our rat spermatogonial cell line transplanted into nude mice will be investigated. However, some endogenous (SV40 large T-negative) spermatogenesis was found in the testes of these transplanted mice, which suggests a difference in the differentiation capacity of mouse spermatogonia and rat spermatogonial cell lines or that other factors are involved.

Until now, four germ cell lines have been established, all containing the SV40 large T antigen (6–8). The first cell line established, GC-1spg, has characteristics of B spermatogonia and early spermatocytes (6). The other cell lines have characteristics of more advanced germ cell types. GC-2 spd(ts) has spermatocyte characteristics, which conditionally undergo meiosis *in vitro* (7), GC-3spc(ts) has characteristics of spermatocytes (7) and GC-4spc has characteristics between preleptotene and early pachytene spermatocytes (8). We now have established cell lines with characteristics of A spermatogonia. Moreover, our cell lines show spermatogonial stem cell characteristics. Based on the nomenclature used by others for germ cell lines, we propose to name our cell lines GC-5spg for A303 and GC-6spg for A304.

In conclusion, cell lines A303 and A304 represent the first rat spermatogonial cell lines, which express Hsp90α and oct-4, but not c-kit, and are able to colonize recipient mouse testes after transplantation. Therefore, they have characteristics of rat spermatogonial stem cells. These cell lines will greatly facilitate research on spermatogonial stem cells, enabling large scale analysis of mRNA and protein expression and will facilitate investigations into mechanisms that regulate stem cell proliferation and differentiation.

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