

Propagation of Human Spermatogonial Stem Cells In Vitro

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TREATMENT SUCCESS IN YOUNG boys with cancer has increased tremendously over recent years, allowing most of them to survive their cancer. Currently, 1 in 250 young adults (20-29 years) is a long-term survivor of childhood cancer.¹ Given this success in pediatric oncology, long-term adverse effects of cancer treatment are of increasing importance. Infertility is a major long-term adverse effect, because there are no means to preserve fertility prior to treatment, in contrast to adult men, for whom ejaculated sperm can be cryopreserved.

The theoretical approach to overcome this problem is to store testicular tissue before chemotherapy and to propagate and autotransplant spermatogonial stem cells from this tissue after cancer survival. In 1994, spermatogonial stem cell transplantation was performed successfully for the first time in the mouse.² Since then, successful autotransplantation of spermatogonial stem cells has been achieved in a wide range of species, including bovine, goat, and

Context Young boys treated with high-dose chemotherapy are often confronted with infertility once they reach adulthood. Cryopreserving testicular tissue before chemotherapy and autotransplantation of spermatogonial stem cells at a later stage could theoretically allow for restoration of fertility.

Objective To establish in vitro propagation of human spermatogonial stem cells from small testicular biopsies to obtain an adequate number of cells for successful transplantation.

Design, Setting, and Participants Study performed from April 2007 to July 2009 using testis material donated by 6 adult men who underwent orchidectomy as part of prostate cancer treatment. Testicular cells were isolated and cultured in supplemented StemPro medium; germline stem cell clusters that arose were subcultured on human placental laminin-coated dishes in the same medium. Presence of spermatogonia was determined by reverse transcriptase polymerase chain reaction and immunofluorescence for spermatogonial markers. To test for the presence of functional spermatogonial stem cells in culture, xenotransplantation to testes of immunodeficient mice was performed, and migrated human spermatogonial stem cells after transplantation were detected by COT-1 fluorescence in situ hybridization. The number of colonized spermatogonial stem cells transplanted at early and later points during culture were counted to determine propagation.

Main Outcome Measures Propagation of spermatogonial stem cells over time.

Results Testicular cells could be cultured and propagated up to 15 weeks. Germline stem cell clusters arose in the testicular cell cultures from all 6 men and could be subcultured and propagated up to 28 weeks. Expression of spermatogonial markers on both the RNA and protein level was maintained throughout the entire culture period. In 4 of 6 men, xenotransplantation to mice demonstrated the presence of functional spermatogonial stem cells, even after prolonged in vitro culture. Spermatogonial stem cell numbers increased 53-fold within 19 days in the testicular cell culture and increased 18 450-fold within 64 days in the germline stem cell subculture.

Conclusion Long-term culture and propagation of human spermatogonial stem cells in vitro is achievable.

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monkey.³⁻⁵ In addition, it has been shown that spermatogonial stem cells from various species,⁶⁻⁹ including human,¹⁰ can home to the basal membrane of seminiferous tubules of immunodeficient mice after transplantation, making it possible to functionally test spermatogonial stem cells in experimental preclinical settings in vitro.

Because small testicular biopsies do not contain sufficient spermatogonial stem cells to fully repopulate the testis after transplantation, in vitro propagation of human spermatogonial stem cells will be

necessary to obtain an adequate amount of cells for successful transplantation. Such culture methods have been recently de-

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veloped in animal model systems¹¹⁻¹⁴ but have thus far not been reported for human spermatogonial stem cells.

We report here on an *in vitro* culture system that allows for long-term culture and propagation of human spermatogonial stem cells.

METHODS

Human Testis Material

Testis samples were donated after oral informed consent by 6 patients undergoing bilateral orchidectomy as part of prostate cancer treatment. According to Dutch law, ethics committee approval was not required, because anonymized tissue samples were used. None of these men had previously received chemotherapy or radiotherapy, and the morphology of the testes showed normal spermatogenesis in all cases.

The testes were cut into small pieces and cryopreserved in 8% dimethyl sulfoxide (Sigma-Aldrich, St Louis, Missouri) and 20% fetal calf serum (FCS) (Invitrogen, Carlsbad, California) in minimum essential medium (MEM) (Invitrogen) and stored at -196°C for later cell isolation and culture. The use of frozen-thawed material rather than fresh material most closely resembles anticipated future practice of spermatogonial stem cell autotransplantation. A small piece of tissue was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical testing.

Testicular Cell Isolation, Culture, and Cryopreservation

After thawing, testis tissue pieces weighing approximately 100 mg to 200 mg were enzymatically digested to prepare a cell suspension, as described previously.¹⁵ Testicular cells were collected and cultured overnight in uncoated dishes in supplemented MEM ($1 \times$ MEM with $1 \times$ nonessential amino acids, 40 $\mu\text{g}/\text{mL}$ gentamicin, 15 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES], 0.12% sodium bicarbonate, 4 mM L-glutamine [all from Invitrogen], penicillin (100 IU/mL)–streptomycin (100 $\mu\text{g}/\text{mL}$) [Sigma-Aldrich]) containing 10% FCS at 37°C and 5% CO_2 .

After overnight incubation, floating cells were collected and cultured at a density of 10 000 to 20 000 cells/ cm^2 in uncoated dishes with supplemented StemPro-34 (Invitrogen) as described previously,¹⁴ with minor modifications. We added 0.5% penicillin–streptomycin (Invitrogen) and replaced mouse epidermal growth factor (EGF), rat glial cell line–derived neurotrophic factor (GDNF), and mouse leukemia inhibitory factor (LIF) with recombinant human EGF (20 ng/mL) (Sigma-Aldrich), recombinant human GDNF (10 ng/mL) (Sigma-Aldrich), and recombinant human LIF (10 ng/mL) (Chemicon International Inc, Temecula, California). The cells were cultured in Costar uncoated 6-well culture plates (Cole-Parmer, Vernon Hills, Illinois) at 37°C in a humidified atmosphere with 5% CO_2 and were passaged with trypsin EDTA (0.25%) (Invitrogen) every 7 to 10 days at 80% to 90% confluency to 1 or several new dishes. Surplus cells were cryopreserved in 10% dimethyl sulfoxide and 20% FCS in MEM. In the case of overgrowth of flat cells with long extensions which were interpreted as somatic cells, only germ cells and round dividing cells were differentially passaged to fresh dishes by vigorous pipetting.

Germline stem cell clusters were subcultured on culture dishes coated with human placenta laminin (20 $\mu\text{g}/\text{mL}$) (Sigma-Aldrich) in supplemented StemPro-34. Embryonic stem cell–like colonies were subcultured as recently described.¹⁶

Immunohistochemistry and Immunofluorescence

Promyelocytic leukemia zinc finger protein (PLZF, also known as zinc finger and BTB domain containing 16 [ZBTB16]) is a well-known spermatogonial-specific marker in many species,¹⁷ although it has never been described for human spermatogonia. Specificity of PLZF for human spermatogonia was first determined by immunohistochemical testing on human testicular sections. After confirmation of the specificity of this marker, it was

used to identify spermatogonia in our culture system by immunofluorescence.

Immunohistochemical staining of PLZF was performed on 5- μm human testis sections and 4% paraformaldehyde–fixed cultured cells in Laboratory-Tek chamber slides (Nalgen Nunc International Corp, Rochester, New York). Deparaffinated testis sections and fixed cultured cells were treated in 0.2% Triton-X-100 for 10 minutes. Antigen retrieval was performed in sodium citrate, pH 6.0 at 98°C , in sections. To inhibit endogenous peroxidase, samples were treated with 0.3% hydrogen peroxide in phosphate-buffered salt for 10 minutes. Nonspecific adhesion sites were blocked in 5% bovine serum albumin for 1 hour at room temperature. Then sections and cells were incubated with anti-PLZF (sc-28319; Santa Cruz Biotechnology, Santa Cruz, California) overnight at 4°C . Signal was visualized on sections by incubation with Powervision poly horseradish peroxidase conjugated anti-mouse/rabbit/rat (Immuno Vision Technologies, Burlingame, California) for 1 hour at room temperature, followed by 3,3'-diaminobenzidine (DAB) as a substrate and hematoxylin as counterstain.

Visualization of the PLZF signal on cultured cells was performed by successive incubation with biotinylated goat anti-mouse (1 hour at room temperature) and avidin-Cy3 (1 hour at room temperature), with 4',6-diamidino-2-phenylindol (DAPI) as a nuclear counterstain. As negative controls, we used isotype mouse IgG instead of the primary antibody. Slides were examined using an Olympus BX41 bright field microscope (Olympus America Inc, Center Valley, Pennsylvania) or a Leica DMRA fluorescence microscope (Leica Microsystems Inc, Bannockburn, Illinois).

Gene Expression

To determine the presence of spermatogonia during the entire culture, the expression of spermatogonial genes^{17,18} was studied. Total RNA from cultured testicular cells, subcultured germline stem cells, embryonic stem cell–like cells,

and whole testis as a positive control was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, California). For reverse transcriptase polymerase chain reaction (PCR), first-strand cDNA was synthesized with random hexamers and the Superscript II preamplification system (Invitrogen), and PCR was carried out with specific primers for *PLZF* (*ZBTB16*) (forward: GGTCGAGCTTCCTGATAACG; reverse: CCTGTATGTGAGCGCAGGT; product size, 396 base pairs [bp]), *ITGA6* (integrin α 6) (forward: TCATGGATCTGCAAATGGAA; reverse: GCGGGGTTAGCAGTATATTCA; product size, 300 bp), *ITGB1* (integrin β 1) (forward: GTGGGTGGTGACAAATTC; reverse: GGTCAATGGGATAGTCTTCAGC; product size, 300 bp) and *TBP* (Tata box binding protein) (forward: GTGACCCAGCAGCATCACTG; reverse: GTCATGGCACCTGAGGG; product size, 224 bp) as a general marker.

PCR amplification was performed on cDNA (with reverse transcriptase) and on RNA (without reverse transcriptase) as follows: 3 minutes at 94°C followed by 35 cycles of 1 minute at 94°C, 1 minute at specific annealing temperature for each primer (*PLZF*, 55°C; *ITGA6*, 52°C; *ITGB1*, 55°C; and *TBP*, 59°C), 1 minute at 72°C, and a final elongation of 5 minutes at 72°C.

Xenotransplantation

To determine the presence of functional spermatogonial stem cells in our culture system, human testicular cells were transplanted into testes of recipient NMRI (Naval Medical Research Institute) nu/nu male mice. The procedures were approved and performed according to the regulations provided by the animal ethics committee of the Academic Medical Center of the University of Amsterdam, Amsterdam, the Netherlands. To destroy endogenous spermatogenesis, the recipient mice were given 38 to 40 mg/kg of busulfan intraperitoneally 6 weeks before donor cell transplantation. Frozen cultured human testis cells, originating from separate culture wells and from several passage numbers and several points during culture, were thawed and resuspended in MEM for transplantation, as described previously.¹⁹ Average cell viability of the cryopreserved samples was about 80%, as determined by trypan blue staining.

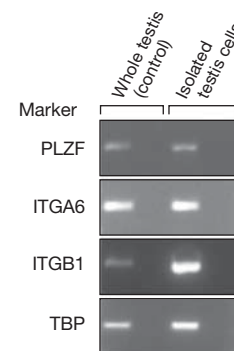
To identify colonization of human spermatogonial stem cells, recipient mouse testes were recovered 10 weeks after donor cell transplantation. Because most colonized human spermatogonial stem cells are expected to be single cells,¹⁰ we determined colonization in recipient mouse testes in serial sections, with the transplanted mouse testes fixed

in 4% paraformaldehyde and embedded in paraffin. To identify colonized human spermatogonial stem cells, every fifth section was analyzed by human COT-1 DNA (the most common human-specific repetitive DNA sequences) fluorescence in situ hybridization.²⁰

Fluorescence In Situ Hybridization

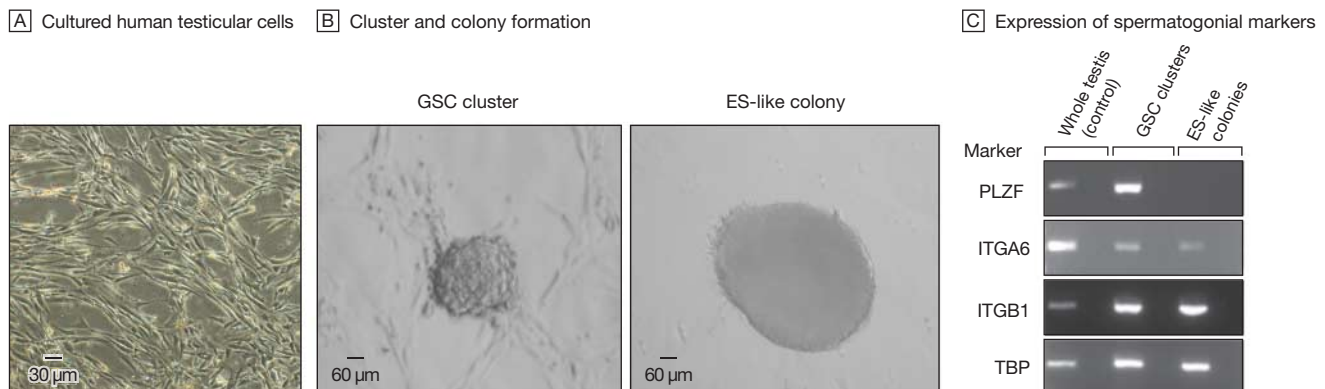
Human COT-1 DNA (Roche, Basel, Switzerland) was biotin labeled by nick

Figure 1. Expression of Spermatogonial Markers by Isolated Human Testis Cells (Study Participant UMC0001)



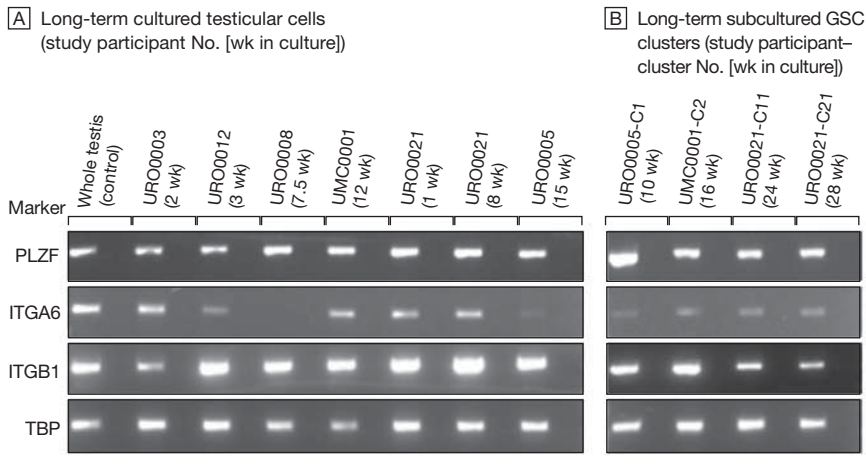
Reverse transcriptase polymerase chain reaction for spermatogonial markers *PLZF* (promyelocytic leukemia zinc finger protein), *ITGA6* (integrin α 6), and *ITGB1* (integrin β 1) on whole testis and isolated cells from human testis before culture from the same study participant. *TBP* (Tata box binding protein) was used as a reference marker. The second lane for each sample tested shows the negative (without reverse transcriptase) control.

Figure 2. Representative Germline Stem Cell (GSC) Clusters and Embryonic Stem Cell (ES)-like Colonies (Study Participant UMC0001)



A, Phase contrast image of cultured human testicular cells before formation of clusters. B, Differential interference contrast image of a GSC cluster and an ES-like colony. C, Representative reverse transcriptase polymerase chain reaction for spermatogonial markers *PLZF* (promyelocytic leukemia zinc finger protein), *ITGA6* (integrin α 6), and *ITGB1* (integrin β 1) of 1 of at least 20 subcultured GSC clusters and ES-like colonies. *TBP* (Tata box binding protein) was used as a reference marker. The second lane for each sample tested shows the negative (without reverse transcriptase) control.

Figure 3. Expression of Spermatogonial Markers in Long-term Cultured Testicular Cells and Subcultured Germline Stem Cell (GSC) Clusters

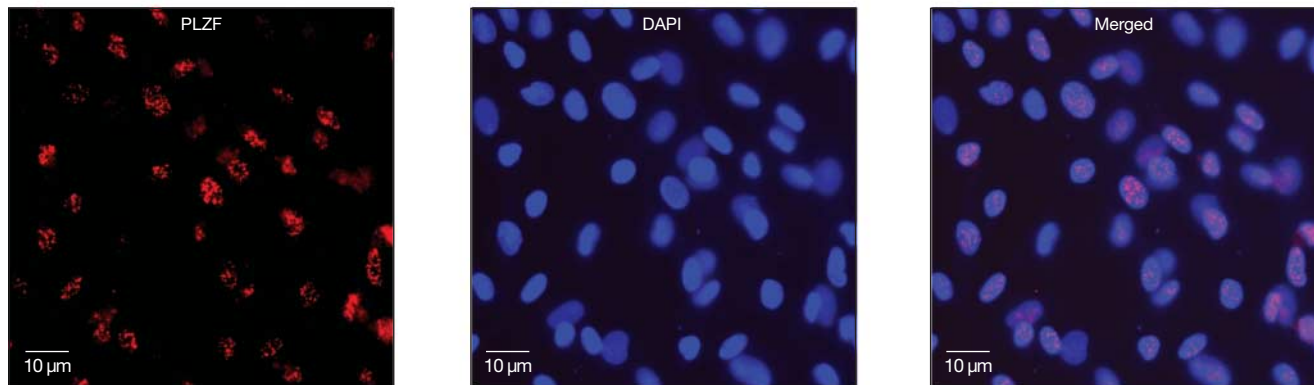


A, Reverse transcriptase polymerase chain reaction on long-term cultured testicular cells of all study participants. B, Representative reverse transcriptase polymerase chain reaction of some of the long-term subcultured GSC clusters of 3 study participants. ITGA6 indicates integrin α 6; ITGB1, integrin β 1; PLZF, promyelocytic leukemia zinc finger protein. TBP (Tata box binding protein) was used as a reference marker. For both A and B, the second lane for each sample tested shows the negative (without reverse transcriptase) control.

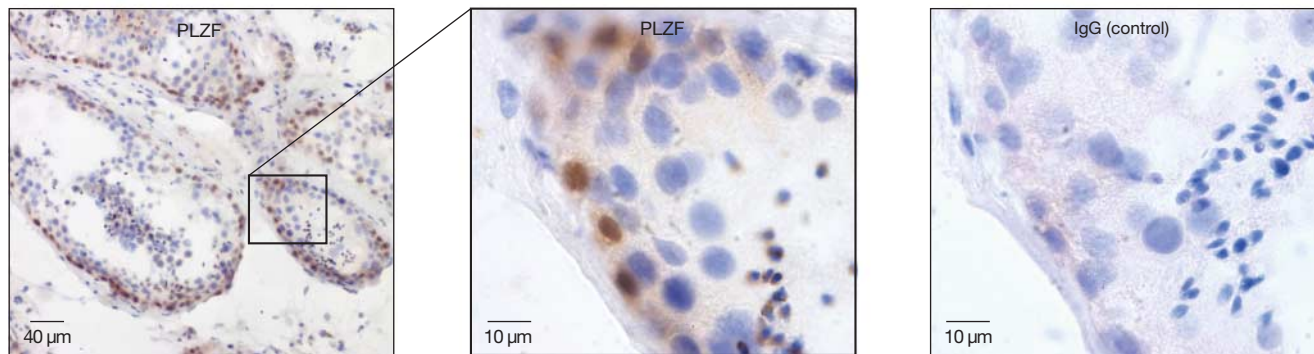
translation and used as a probe to recognize human cells in mouse testis.²⁰ Testis sections (5 μ m) mounted on 3-aminopropyltriethoxysilane (TESPA)-coated slides were deparaffinized and subsequently pretreated before hybridization with RNase A in 2 \times sodium chloride/sodium citrate solution for 1 hour at 37°C, 100 μ g/mL proteinase K in TES (50 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl) for 5 minutes at room temperature, and postfixed in 0.4% formaldehyde for 5 minutes at 4°C. After denaturation of sections at 85°C for 6 minutes, sections were hybridized at 37°C overnight with 2-ng/ μ L probe in hybridization mix (60% formamide, 2 \times sodium chloride/sodium citrate solution, 0.02M sodium phosphate buffer). Sections were washed in 50% for-

Figure 4. PLZF Expression in Human Testis and Cultured Testicular Cells

A Nuclear PLZF immunofluorescence in subcultured germline stem cells (study participant URO0021, cluster 4, passage 4)



B Immunohistochemical localization of nuclear PLZF in human testicular section (study participant UMC0001)



A, PLZF (promyelocytic leukemia zinc finger protein) immunofluorescence of Cy3 (red) staining on subcultured germline stem cells from study participant URO0021 (cluster 4, passage 4) with 4',6-diamidino-2-phenylindol (DAPI) (blue) as a nuclear stain. B, Specific immunohistochemical (3,3'-diaminobenzidine [DAB]) localization of PLZF (brown) in the nuclei of type A spermatogonia in human testicular section (study participant UMC0001) (counterstained with hematoxylin [blue]).

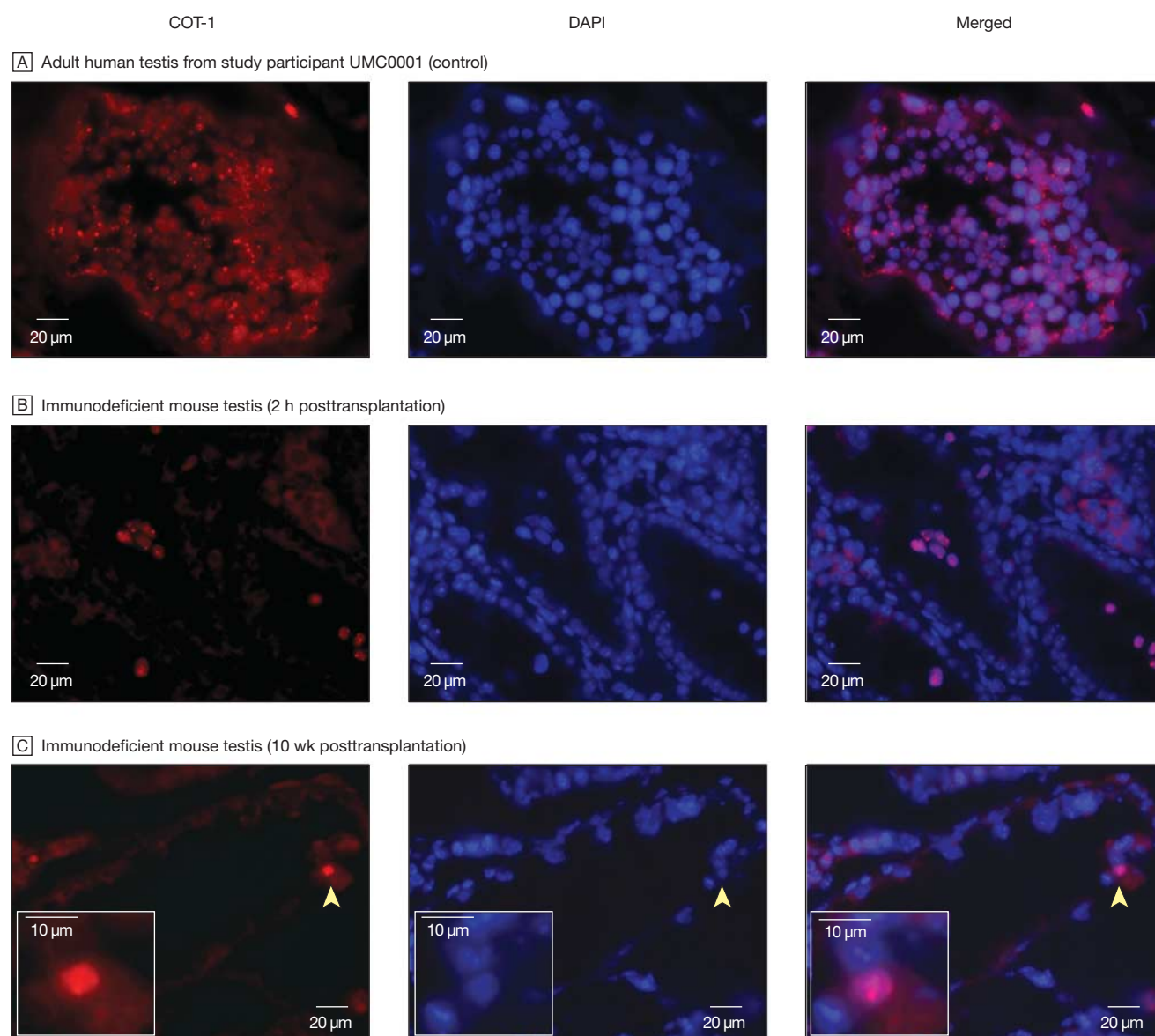
mamide in 2× sodium chloride/sodium citrate solution at 42°C, and signal was visualized by incubation in avidin–Cy3 (Jackson ImmunoResearch, Suffolk, United Kingdom) in TNB (0.1M Tris [Sigma], 0.15 M NaCl [Merck, Whitehouse Station, New Jersey], 0.02% Thimerosal

[Sigma], 0.05% blocking reagent [Roche]) for 20 minutes at 37°C and counterstained with DAPI as nuclear staining. As negative control, we only used hybridization mix.

Every fifth slide of serial-sectioned recipient testis was examined using fluorescence microscopy. The final

number of colonized spermatogonial stem cells in the whole transplanted testis was determined by multiplying the number of colonies found in every fifth section by 2.5, because the mean nuclear diameter of spermatogonial stem cells is 10 μm and the thickness of each section was 5 μm.

Figure 5. Detection of Human Spermatogonial Cells After Stem Cell Transplantation to Immunodeficient Mouse Testis



Detection of human spermatogonial stem cells after transplantation to immunodeficient mouse testis using human COT-1 fluorescence in situ hybridization (FISH) Cy3 (red, left panel), nuclear counterstaining with 4',6-diamidino-2-phenylindol (DAPI) (blue, middle panel) and merged images of COT-1 and DAPI (right panel). A, Adult testis control. COT-1 DNA FISH nuclear signal is present in all cells of adult human testis. B, Immunodeficient mouse testis 2 hours after transplantation. COT-1 FISH-positive human cells are detected in the lumen of the mouse seminiferous tubules. C, Immunodeficient mouse testis 10 weeks after transplantation. Homing of human spermatogonial stem cell from long-term subcultured germline stem cells to the basal membrane of the seminiferous epithelium of mouse testis. Insets are a higher magnification of the colonized human spermatogonial stem cell, indicated by arrowheads.

RESULTS

Isolated testicular cells contained spermatogonia as indicated by the expression of PLZF, a specific marker for undifferentiated spermatogonia, and ITGA6 and ITGB1, which are predominantly expressed by spermatogonia (FIGURE 1). When put into culture, a monolayer of flat somatic cells developed with round germ cells on top (FIGURE 2A). Cells could be passaged every 7 to 10 days. When somatic cells tended to overgrow the culture, differential passaging was performed.

In the cultures from all 6 men, we observed the appearance of clusters of germline stem cells as well as embryonic stem cell–like colonies after a mean of 22.5 (SD, 7.9) days. The germline stem cell clusters presented as clumps of individually visible cells, while the colonies of embryonic stem cell–like cells were sharply edged and compact (Figure 2B). Germline stem cell clusters expressed PLZF, while embryonic stem cell–like colonies did not (Figure 2C). On subculture of the embryonic stem cell–like colonies, these cells were able to differentiate into cells from all 3 germ layers *in vitro*.¹⁶

Testicular cells could be propagated for up to 15 weeks and 7 passages. The cells could successfully be

cryopreserved regardless of the time in culture, with a mean recovery rate of 80% (SD, 9%). Germline stem cell clusters formed between the second and eighth week in the testicular cell culture. After this period, germline stem cell clusters no longer appeared, and after 15 weeks of culture the flat, long somatic cells within the cell culture detached from the culture dish and no longer supported the germ cells.

When we observed that the culture system no longer supported spermatogonial stem cells after 15 weeks of culture in 3 men, we developed the subculture system and used it in the remaining 3 men. Under feeder cell–free conditions in laminin-coated culture dishes, we could extend the culture of germline stem cells up to 28 weeks and 15 passages, and germline stem cell clusters continued to arise up to 20 weeks of culture. The germline stem cell clusters appeared in testicular cell cultures as well as subcultures on laminin, while the embryonic stem cell–like colonies only appeared between the second and eighth week in the testicular cell culture and never in the subcultured germline stem cell clusters.

Reverse transcriptase PCR using PLZF, ITGA6, and ITGB1 (FIGURE 3) and immunofluorescence for PLZF con-

firmed the presence of spermatogonia throughout the entire culture period (FIGURE 4A). Spermatogonia-specific expression of PLZF in human testes was confirmed by immunohistochemical testing (Figure 4B).

After xenotransplantation of cultured testicular cells to the mouse testes, human spermatogonial stem cells from 4 of 6 men were found at the basal membranes of the mouse seminiferous tubules. Xenotransplantation of subcultured germline stem cell clusters occurred in 1 of 2 men (FIGURE 5 and TABLE). Because a limited number of animals were allowed for xenotransplantation, we chose to xenotransplant cells from one of the subcultures of germline stem cell clusters at several points during culture rather than transplanting cultures from all 3 men.

Transplantation of cells from an early passage (passage 2; 28 days in culture) and a later passage (passage 5; 47 days in culture) of the testicular cell culture showed a 53-fold increase in the number of human spermatogonial stem cells within this time frame of 19 days (Table). Similarly, transplantation of subcultured germline stem cells from 77 days of culture (passage 7) and from 141 days of culture (passage 12) showed an 18 450-fold increase in hu-

Table. Colonization of Cultured Human Spermatogonial Stem Cells After Transplantation in Immunodeficient Mice

Study Participant ^a	Days in Culture	Passage No.	No. of Injected Cells, ×10 ⁵	No. of Colonies/Cell, ×10 ⁵
Testicular cell culture				
UMC0001	63	4	1.3	0
URO0003	14	1	3.5	0.7
	14	1	0.2	12.5
URO0005	14	1	2.7	0.9
	42	3	0.3	0
URO0008	28	3	0.7	3.6
URO0012	21	1	0.6	0
URO0021	28	3	0.1	0
	56	7	0.4	0
	28 ^b	2	2.55	2
	47 ^b	5	3.1	0.8
Germline stem cell subculture				
URO0005	91	6	2.5	0
URO0021	77 ^c	7	2	1.25
	84	8	0.5	5
	141 ^c	12	1.9	2.6

^aTesticular cell culture was initiated once per patient. Each row represents a distinct time point, passage number, or both in culture that was used for xenotransplantation.

^bDilution factor of 133 and 53-fold increase in human spermatogonial stem cells from 28 to 47 days.

^cDilution factor of 8870 and 18 450-fold increase in human spermatogonial stem cells from 77 to 141 days.

man spermatogonial stem cells within this time frame of 64 days (Table).

COMMENT

This report outlines the first, to our knowledge, successful long-term culture and propagation of human spermatogonial stem cells. Our culture system allowed spermatogonial stem cells to increase in number by self-renewal in vitro. We used a culture medium specifically formulated to support the development of human hematopoietic stem cells but later used to culture mouse and hamster spermatogonial stem cells.^{12,14} The growth factors included in this medium, ie, GDNF, BFGF (basic fibroblast growth factor), EGF, and LIF, are able to promote animal spermatogonial stem cell survival and proliferation.²¹⁻²³

In contrast to most animal studies,¹²⁻¹⁴ we used residual testicular somatic cells in the cell suspension rather than mouse embryonic fibroblasts as feeder cells, because these somatic cells are capable of supporting mouse spermatogonial stem cells in culture.^{24,25} Importantly, the use of supporting feeder cells originating from the patient's own testicular tissue will facilitate future clinical application, because this modification avoids the use of animal products.

The presence of human spermatogonial stem cells after long-term culture was demonstrated using xenotransplantation to mouse seminiferous tubules. Migration and colonization of spermatogonial stem cells to the basal membrane of the seminiferous tubule on transplantation is so far the only functional assay to prove the presence of spermatogonial stem cells in a testicular cell population. Here we describe the first colonization of mouse seminiferous tubules with human spermatogonial stem cells from long-term cultured testicular cells, indicating the stem cell capability of these cells even after long-term culture.

Our culture system allowed for a more than 18 000-fold increase in spermatogonial stem cell number. To estimate if the propagation of the sper-

matogonial stem cells we achieved is theoretically sufficient for future clinical application, we calculated that with a biopsy size of 0.2 mL from a prepubertal testis, the number of spermatogonial stem cells within this small sample needs to be increased 65-fold to colonize an adult testis of approximately 13 mL.²⁶ From studies of transplantation of autologous animal spermatogonial stem cells, we know that the efficiency of colonization is between 5% and 12%.²⁷⁻³¹ Assuming that the efficiency with human cells is 5%, at least a 1300-fold increase in spermatogonial stem cells is needed for sufficient transplantation. This means that the more than 18 000-fold increase in human spermatogonial stem cells in our culture system would be quite sufficient.

In this study we cultured adult human testicular cells instead of prepubertal testicular cells. Although culture of testicular cells from prepubertal boys remains to be tested, culture systems similar to that described here have been used successfully to propagate spermatogonial stem cells from prepubertal animals, including mouse,^{14,32} bovine,¹¹ rat,¹³ and hamster.¹²

Some important issues have to be addressed before autotransplantation of human spermatogonial stem cells can safely be introduced into clinical practice. The most important issue is that in the case of biopsies from patients with nonsolid tumors, tumor cells must not be reintroduced when cultured spermatogonial stem cells are autotransplanted to the patient. Therefore, a reliable method of eliminating any remaining malignant cells before transplantation in the case of nonsolid tumors needs to be developed.^{33,34} In addition, the embryonic stem cell-like cells that spontaneously arise in testicular cell cultures may theoretically lead to tumor formation, but previous reports have shown that human testis-derived embryonic stem cell-like cells are unable to form extensive teratomas when injected in immunodeficient mice.^{16,35-37} Moreover, we never observed intratesticular tumors or tera-

tomias in any of the 15 recipient mice after transplantation, suggesting that propagated human spermatogonial stem cells in this long-term culture system remained completely committed to the germ line lineage.

In conclusion, these results show that long-term culture and propagation of human spermatogonial stem cells in vitro is achievable.

Author Contributions: Dr van Pelt had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Sadri-Ardekani, Mizrak, van der Veen, de Rooij, Repping, van Pelt.

Acquisition of data: Sadri-Ardekani, van Daalen, Korver, Roepers-Gajadien, Koruji, de Reijke, de la Rosette, van Pelt.

Analysis and interpretation of data: Sadri-Ardekani, Mizrak, van Daalen, Korver, Roepers-Gajadien, Hovingh, Repping, van Pelt.

Drafting of the manuscript: Sadri-Ardekani, van Daalen, Korver, Roepers-Gajadien, Koruji, Hovingh, Repping, van Pelt.

Critical revision of the manuscript for important intellectual content: Sadri-Ardekani, Mizrak, de Reijke, de la Rosette, van der Veen, de Rooij, Repping, van Pelt.

Statistical analysis: Sadri-Ardekani, Repping.

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Study supervision: Mizrak, van der Veen, de Rooij, Repping, van Pelt.

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