ORIGINAL ARTICLE: ASSISTED REPRODUCTION

Enrichment of spermatogonial stem cells from long-term cultured human testicular cells

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Objective: To evaluate the degree of enrichment of spermatogonial stem cells (SSCs) from human testicular cell cultures by ITGA6$^+$, HLA$^-$/ITGA6$^+$, GPR125$^+$, and HLA$^-$/GPR125$^+$ magnetic-assisted cell sorting (MACS).
Design: Experimental basic science study.
Setting: Reproductive biology laboratory.
Patient(s): Multiple samples of cryopreserved human testicular cells from two prostate cancer patients with normal spermatogenesis.
Intervention(s): Cultured human testicular cells subjected to four sorting strategies based on MACS and xenotransplanted to the testes of mice to determine the enrichment for SSCs.
Main Outcome Measure(s): Enrichment for human spermatogonia and SSCs tested by expression analysis of spermatogonial markers *ITGA6*, *GPR125*, *ZBTB16*, *UCHL1*, and *ID4* using quantitative real-time polymerase chain reaction (qPCR) and by xenotransplantation into the testes of mice, respectively.
Result(s): Compared with the nonsorted cultured testicular cells, only the ITGA6$^+$ and HLA$^-$/GPR125$^+$ sorted cells showed enrichment for *ID4*. No difference in expression of *ZBTB16* and *UCHL1* was observed. Xenotransplantation of the sorted cell fractions showed a 7.1-fold enrichment of SSCs with ITGA6$^+$.
Conclusion(s): Magnetic-assisted cell sorting of cultured human testicular cells using ITGA6 allows for enrichment of SSCs, which aids in further molecular characterization of cultured human SSCs and enhances testicular colonization upon transplantation in future clinical settings. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Autotransplantation, enrichment, ITGA6, spermatogonial stem cells

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Early diagnosis of childhood cancers combined with effective treatment protocols have significantly increased the life expectancy of patients with childhood cancer (1). Given this increase in life expectancy, treatment side effects and the quality of life after treatment are of increased importance (2–4). One of the severe side effects of cancer treatment is infertility. Spermatogonia are highly sensitive to cytotoxic therapies, so sterility is common among male childhood cancer survivors (5–7). Unlike with adults, cryopreservation of semen before cancer treatment cannot be applied in prepubertal boys as spermatogenesis does not commence until puberty. For these boys, cryopreservation of a testicular biopsy before chemotherapy for later autotransplantation of spermatogonial stem cells (SSCs) has been suggested (8–11). However, SSCs are rare in the testis, so a small prepubertal testicular biopsy will only contain a limited amount of SSCs that by themselves are not enough for repopulation of an entire testis after chemo/radiotherapy. This necessitates the in vitro propagation of SSCs before SSC autotransplantation to allow for full repopulation of an adult testis. We have recently developed a testicular cell culture system that allows for in vitro propagation of both adult and prepubertal human SSCs (12, 13). However, successful culture of human SSCs requires accompanying somatic cells to provide an appropriate niche for survival and propagation of SSCs

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in vitro (12, 14). Our previously established cell culture system therefore contains a heterogeneous population of testicular cells, and SSCs represent only a minor fraction of these cells.

Before clinical application of SSC autotransplantation can take place, it is important to further analyze cultured SSCs in terms of genetic and epigenetic stability as well as functionality (11). To investigate the characteristics of human SSCs in this culture system, it is important to separate SSCs from the other cultured testicular cells. In addition, efficient autotransplantation depends on the purity of transplanted SSCs (15, 16). Thus, both from a basic science as well as a clinical point of view, enrichment of SSCs from testicular cell cultures is important.

Cell separation using either spermatogonial markers (positive selection) or somatic cell markers (negative selection) has been suggested as a way to enrich SSCs before autotransplantation (17). Indeed, several cell surface markers have been used successfully for enrichment of SSCs in rodents (16, 18, 19). Magnetic-assisted cell sorting (MACS) for ITGA6 has led to 5 to 10-fold enrichment of SSCs (16), and GPR125 sorting from mouse spermatogonial progenitor cells showed almost a ninefold enrichment of SSCs (20). Based on these rodent studies, ITGA6 sorting for noncultured human spermatogonia has also been applied, but the enrichment of SSCs by transplantation was not investigated (21, 22). Using MACS for enrichment of human spermatogonia from noncultured testicular cells has been successfully attempted using antibodies against GFRA1 (23), GPR125 (24), SSEA4 (25), and HLA/CD9 (26). However, all these studies have used freshly isolated rather than long-term cultured human testicular cells. Thus, to date, enrichment of human SSCs from long-term cultured testicular cells has not been demonstrated. The ability to enrich for SSCs from long-term testicular cell cultures might very well be different from freshly isolated noncultured cells because cultured cells are likely to alter their expression of cell surface markers in the in vitro environment.

Previously, it has been shown that ITGA6, GPR125, ZBTB16, and UCHL1 are among several spermatogonial markers that remain expressed during culture (13, 24). In this study, we investigated whether single ($ITGA6^+$ or $GPR125^+$) and double ($HLA^-/TGA6^+$ or $HLA^-/GPR125^+$) sorting allows for enrichment of SSCs from long-term cultured human testicular cells.

MATERIALS AND METHODS

Testicular Cell Isolation and Culture

Both patients (URO0034 and URO0077) provided informed consent for donating their spare tissue for research. Testis samples were donated after undergoing bilateral orchidectomy as part of prostate cancer treatment. The testicular tissues used for research were left over after clinical diagnostic and treatment procedures. According to Dutch law, these spare tissues can be used for research without approval of a medical ethical committee as none of the patients had to undergo any additional intervention to obtain the material for this research. Both patients had no history of chemotherapy or radiotherapy. Morphologic analyses of testicular sections revealed normal spermatogenesis in both cases (Supplemental Fig. 1, available online).

Testis samples were rapidly divided into small pieces and cryopreserved in minimal essential medium containing 8% dimethyl sulfoxide and 20% fetal calf serum in a slow-freezing container overnight at −80°C and then stored at −196°C. For this particular experiment, testis tissues were thawed and testicular cells isolated and cultured as previously described elsewhere (12). Briefly, testicular cells were isolated by two-step enzymatic digestion. After overnight plating, floating cells were collected and cultured in supplemented Stempro-34 (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2 in air and were passaged with Trypsin-EDTA 0.25% (25200; Invitrogen/GIBCO) every 7 to 10 days in one or several new dishes. The cultured cells were harvested around day 50 for MACS and further characterization.

Labeling and MACS of Cultured Cells

Cultured testicular cells were labeled with anti-GPR125 (AB1705; Abcam) or anti-ITGA6 (313604; Biolegend) for single positive selection and with anti-HLA I (human leukocyte antigen I [HLA I], 555555; BD Pharmingen) (Supplemental Table 1, available online) and subsequently with anti-GPR125 or ITGA6 for double selection (i.e., $HLA^-/ITGA6^+$ and $HLA^-/GPR125^+$). We performed MACS according to the manufacturer's protocol (Miltenyi Biotec Bergisch Gladbach, Germany). Briefly, after trypsinization, cells were washed in minimal essential medium and MACS buffer before incubation for 30 minutes at 4°C with one of the primary antibodies and subsequently with the appropriate conjugated microbeads (see Supplemental Table 1) for 15 minutes at 4°C. After washing in 4 mL of MACS buffer, cells were passed through the column separator. Negative selection for HLA was performed through LS columns (130-042-401; Miltenyi Biotec), and positive selection for ITGA6 or GPR125 was performed through MS columns (130-042-201; Miltenyi Biotec). The number of cells in the nonsorted as well as in the positive and negative cell fractions after sorting was counted using a Bürker counting chamber.

Quantitative Real-Time Polymerase Chain Reaction

To quantify the expression of spermatogonial markers zinc finger and BTB domain containing 16 (*ZBTB16* also known as *PLZF*) and ubiquitin carboxyl-terminal esterase l1 (*UCHL1*), RNA was extracted from the cell pellet of sorted and nonsorted human cultured testicular cells using RNeasy Mini kit (74104; Qiagen) and copy DNA (cDNA) was synthesized using the first-strand cDNA synthesis kit for real-time polymerase chain reaction AVR (11483188001; Roche). To quantify the expression of integrin A6 (*ITGA6*), G protein-coupled receptor 125 (*GPR125*), and inhibitor of DNA binding 4 (*ID4*), 2 ng of extracted RNA was amplified using the Ovation PicoSL WTA system V2 (NuGEN) and used for quantitative real-time polymerase chain reaction (qPCR).

Primer/probe sets were selected from the universal probe library of Roche Applied Science (www.roche-applied-science.com) for human *ITGA6, GPR125, ZBTB16, UCHL1*, *ID4*, and polymerase (RNA) II polypeptide A (*POLR2A*)