Spermatogonial stem cell autotransplantation: towards clinical application
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Eliminating acute lymphoblastic leukemia cells from human testicular cells cultures

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Submitted
Abstract
Male sterility is a relatively common consequence of cancer treatment. For prepu-
bertal boys with cancer, fertility preservation can theoretically be achieved by
freezing a small testis biopsy prior to cancer treatment, followed by in vitro
propagation of spermatogonial stem cells (SSCs) from this biopsy and autotrans-
planting these SSCs after cure of cancer. Avoiding the reintroduction of any
malignant cells present in this biopsy is of critical importance before clinical
application of SSCs autotransplantation can be further explored. Here, we show
that ALL cells cultured separately in medium used to propagate human SSCs did
not survive beyond 14 days of culture. We also demonstrate that irrespective of
their initial concentrations (0.04%, 0.4%, 4% and 40%), ALL cells mixed with
testicular cells were undetectable beyond 18-26 days of culture as measured by
highly-sensitive (1:10,000 to 1:100,000 cells) antigen-receptor PCR (MRD-PCR).
These results prove that our culture system not only allows for efficient propa-
gation of SSCs but also eliminates contaminating malignant ALL cells.
INTRODUCTION

The survival rate of children with cancer has continuously improved over the past few decades and the 5-year event-free survival rates are currently around 80%\textsuperscript{1-3}. Gonadal damage is a relatively common consequence of the treatments used to cure pediatric cancer and many cancer survivors are sterile\textsuperscript{4,5}. In contrast to adult men and prepubertal or adult women with cancer\textsuperscript{6}, there are currently no methods to preserve fertility in prepubertal boys with cancer. Important advances in spermatogonial stem cell (SSC) biology have now opened an avenue to preserve fertility in prepubertal boys with cancer\textsuperscript{7} by cryopreserving a testicular biopsy before onset of cancer treatment\textsuperscript{8-11} and, when infertility has manifested itself, thawing of this tissue followed by in vitro propagating of SSCs\textsuperscript{12,13} and autotransplanting of these cultured cells.

Although the transplantation of fresh or cultured SSCs has led to live healthy offspring in mice\textsuperscript{13-15}, all aspects of this technique should be carefully evaluated on human material and proven to be safe before introducing SSC autotransplantation into clinical practice. One of the most important safety issues is avoiding the reintroduction of remaining malignant cells in case of non-solid tumors. The most common non solid cancer at prepubertal age is Acute Lymphoblastic Leukemia (ALL)\textsuperscript{16} and in up to 30% of boys with ALL, infiltration of the testis is observed\textsuperscript{14}.

Although there have been a few studies that attempted to eliminate malignant cells from mouse, non-human primate and human testicular cells\textsuperscript{15-18}, no study has attempted to eliminate malignant cells in the context of a culture system for human SSCs. We recently described a culture system that enables propagation of adult and prepubertal human SSCs necessary for efficient transplantation\textsuperscript{12,13}.

To investigate the elimination of malignant cells in this culture system, we cultured ALL cells separately or in combination with testicular cells and used highly sensitive methods to detect the presence of ALL cells at various time points in culture.

RESULTS AND DISCUSSION

When cultured separately in testicular cell culture conditions, the percentage of live ALL cells decreased dramatically in all three patients during the first 3 to 6 days of culture. After 14 days of culture all ALL cells were dead as measured by flow
cytometry analysis for live and dead cells (Figure 1), while testicular cells cultured in parallel proliferated well after 8 weeks of culture.

**Figure 1.** Viability of Acute lymphoblastic Leukemia (ALL) cells and testicular cells in human testicular cell culture conditions during 16 days of culture.

![Viability graph](image)

ALL cells from three different patients (SQ8512, SQ9850, and SQ11485) were cultured in parallel to adult human testicular cells (URO0126) as a positive control. Data are from flow cytometric analysis by using dead-alive assay kit (Mean±SEM from n=3).

Survival of ALL cells in the presence of testicular cells was subsequently investigated using real-time quantitative PCR analysis of ‘leukemia-specific’ junctional regions of rearranged antigen receptor genes (MRD-PCR). For each patient it was shown that the MRD-PCR was able to detect 1 ALL cell in the presence of $10^4$ or $10^5$ testicular cells and that the level of contamination could be accurately quantified until a concentration of $10^{-4}$ in all three patients (Figure 2). We used varying initial concentrations of ALL cells (0%, 0.04%, 0.4%, 4% and 40%) with human testicular cells and cultured them together for a period of at least 47 days. The MRD-PCR was also capable to reliably quantify the percentage of leukemic cells at the start of the culture (Table 1). At the time point of the first passage, after 10-16 days of culture, ALL cells were only detected in the condition with an initial concentration of 40% ALL cells, but the level of contamination with ALL cells was outside the quantitative range of the assay in all patients. Based on these PCR results, at least a 2 log reduction is obtained during the first 10-16 days of culture. At the second passage after 18-26 days ALL cells were completely undetectable (Table 1). At each passage cells in culture are diluted 1:6. If the leukemic cells would have survived but would not have proliferated, the PCR is sensitive enough to detect the diluted leukemic cells until after the 4th (day 35-47),
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3rd (day 18-26) or 2nd passage (day 10-16) in cultures initiated with 40%, 4% or 0.4% ALL cells, respectively. This indicated a reduction in the number of ALL cells during culture.

Reverse transcriptase PCR using PLZF, UCHL1, GFRA1 (markers specific for spermatogonia) and CD19 (a marker for lymphoblasts) confirmed the presence of spermatogonia and the absence of ALL cells at the end of the culture period (data not shown).

This study demonstrates that our culture system not only allows for propagation of SSCs\textsuperscript{11,12}, but also efficiently eliminates contaminating malignant ALL cells.

Figure 2. Standard curve constructed from the RQ-PCR (MRD-PCR).

MRD-PCR was able to detect 1 ALL cell in the presence of $10^5$ testicular cells and that the level of contamination could be accurately quantified until a concentration of $10^{-4}$. Red crosses and black boxes represent cultured cells (SQ9850-URO0126) and standards, respectively.

The ability to remove ALL cells in the context of human testicular cell cultures has never been studied, simply because this testicular cell culture system for propagation of human SSCs was only recently established\textsuperscript{12,13}. Two studies assessed the ability to remove leukemic or lymphoma cells from uncultured human spermatogonia/testicular cells\textsuperscript{15,16}. The first study, in which eight leukemia and lymphoma cell lines were used, showed that negative selection for both MHC class I and CD45 markers by FACS was effective for all cell lines except the erythroleukemic K562, while successful elimination of this cell line required incubation.
Table 1. Overview of the MRD-PCR results in different passages of cells culture

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PNQ= positive not quantifiable (= a positive signal outside the quantitative range), number on top of bracket represents the percentage of detected ALL cells based on MRD-PCR signal (Red color= positive signal). Symbol of – represents absence of any specific MRD-PCR signal. Day of culture showed in the bracket.
with IFN-y prior to FACS sorting. The second study showed ineffective elimination of human malignant cells by negative selection only for MHC class I by FACS.

One study assessed the ability to remove ALL cells from a mixture of ALL and testicular cells cultured for a period of four days prior to MACS sorting. This study demonstrated that this period of culture was inefficient in eliminating the leukemic cells. This is in line with our study since we demonstrate in three ALL patients that ALL cells mixed with testicular cells are still detectable after a few days of culture, but are completely eliminated after 18-26 days of culture. In the same study, a combination of positive selection for CD49f and negative selection for MHC class-I markers and subsequently cell selection by matrix adhesions (collagen I and laminin) was still insufficient to eliminate the malignant cells.

What is the minimal number of malignant cells capable of reintroducing cancer in the context of testicular autotransplantation? Only one study has investigated this, showing that the intraluminal injection of as few as 20 rat leukemia cells into the testes of recipient rats could induce disease relapse in three out of five animals. In all three patients in the current study, we observed at least a 2 log reduction during the first culture passage, while SSCs have to be cultured for five passages to obtain enough cells for efficient autotransplantation. If the log reduction is in the same order of magnitude during subsequent passages, there will be a 10 log reduction at the end of the culture. Based on these calculations the risk of transplanting a single leukemic cell is highly unlikely.

The MRD-PCR employed in the current study was highly sensitive in detecting ALL cells (1 ALL cells out of 10,000 to 100,000 other cells). Obviously, no test can guarantee that all ALL cells are truly gone, but the PCR assays we have applied in this study are at present the most sensitive assays for this purpose. Since a PCR reaction cannot discriminate between viable and dead cells, the absence of any positive signal makes the presence of viable leukemic cells even more unlikely. Nevertheless, since ALL cells were incapable of surviving for more than 14 days of culture and no ALL cells could be detected beyond 18-26 days of co-culture, there is in our view sufficient certainty that no live ALL cells will be present if SSCs are propagated for the required 35 days in our culture system. Because results could theoretically vary between patients and between cultures, we do suggest that when SSC autotransplantation will be applied clinically in the future, testing for the presence of ALL cells before transplantation should be an integral part of the procedure.
Our results are a crucial step towards clinical application of autotransplantation of in vitro propagated SSCs derived from a small testis biopsy of prepubertal boys with non-solid cancers. All boys with cancer, including those with non-solid tumors, should now be offered the possibility to store testicular tissue for possible future clinical use.

**METHODS**

**Cells preparation and culture**

Malignant cells were obtained by bone marrow aspirations from three patients (SQ8512, SQ9850, and SQ11485) with B-cell ALL during their diagnostic process and informed consent for study participation was obtained from each patient or legal guardian. The percentages of ALL cells (blast cells) before and after cryopreservation in these cell suspensions were evaluated based on their phenotypes determined by flow cytometry with a panel of multiple antibodies (CD 1, 2, 3, 5, 10, 19, 20 and 34)\textsuperscript{20,21}. This percentage, which varied between 77 to 94\%, was used to correct for the number of ALL cells at the start point of any culture. The number of ALL and testicular cells were also corrected for viability using trypan blue staining at the start of every culture.

Testicular cells were isolated from donated testis tissue from an adult man who underwent castration as part of their prostate cancer treatment (URO0126) and a prepubertal boy with Hodgkin Lymphoma who stored his testis biopsy prior to chemotherapy (URO0113)\textsuperscript{13}. Samples were used for research after receiving oral informed consent from adult man and written informed consent from parents of the prepubertal boy\textsuperscript{13}. Testicular cells were cultured for 3-5 weeks prior to the experiments described in this paper in our recently established testicular cell culture condition based on supplemented StemPro medium in non-coated dishes by using the remaining testicular somatic cells from the same patient as feeder cells layer for SSCs\textsuperscript{12,13}.

Testicular cells were then co-cultured with ALL cells (Figure 3) at various ratios (ALL/Testicular cells: 0\%, 0.04\%, 0.4\%, 4\% and 40\%) in the same culture condition. The medium was refreshed every 3 to 4 days. When cells became 90-100\% confluent, cells were passaged (1:6) using trypsin EDTA (0.25\%) (Invitrogen) and transferred to new dishes in a concentration of 10,000-15,000 cells per cm\textsuperscript{2}. During each passage, surplus cells were used for DNA and RNA isolation. We
previously calculated that at least 35 days of culture would be required to propagate enough SSCs for autotransplantation\(^{13}\). We therefore cultured the ALL/testicular cell mixes of all three patients for at least 47 days (duration of culture was 47-52 days).

**Figure 3.** ALL and testicular cells in culture (Start point)

Acute lymphoblastic Leukemia (ALL) cells (a) and prepubertal testicular cells (b) were seeded separately and together (c) for culturing in human testicular cell culture condition in medium used to propagate human SSCs. Scale bars: 50 µm.

**Viability of ALL and testicular cells during culture**

To examine the viability of ALL cells from three patients cultured separately in human testicular cell culture condition\(^{12,13}\) for a period of 14-16 days, the same amount of cells was plated in each well of a 48 well plate (30000 cells per well). During this 14-16 days culture period, every 24 hours cells from three wells were harvested by cell scraping (#3010, Corning Inc) and evaluated by flow cytometry with a live-dead kit based on Calcein AM and Ethidium H1 staining according to manufacturer’s instructions (MP 3224, Invitrogen). As a control, parallel cultured human testicular cells (URO0126) were used.

**Minimal residual disease (MRD) PCR to trace ALL cells**

To detect the leukemic cells in the co-cultures with testicular cells, for each patient a leukemia-specific PCR was developed based on the unique antigen receptor rearrangements present in the leukemic cells, as previously described\(^{22-24}\). Sensitivity and quantitative range of the respective PCR assays was determined according to the guidelines of European Study Group on MRD detection in ALL\(^{25}\). The sensitivity of the PCR ranged from \(10^{-4}\) (SQ11485) to \(10^{-5}\) (SQ9850 and SQ8512), and the amount of leukemic cells could be reliably quantified up to \(10^{-4}\) (SQ8512, SQ9850 and SQ11485)\(^{26,27}\). DNA from ALL cells mixed with testicular cells before and during culture were extracted using the QIAamp DNA Mini Kit (51306, QIAGEN)\(^{28}\) with an elution volume of 55 µl instead of 200 µl to increase the
concentration of DNA. Extracted DNA was measured by fluorometer Quibit (Invitrogen) using the DNA HS assay kit (Q32854, Invitrogen). PCR was performed as previously described\(^2\) using the ABI PRISM 7700 Sequence Detection System containing a 96- well thermal cycler (PE Biosystems). Reaction mixtures of 50 µl contained the TaqMan buffer A, 5 mM MgCl\(_2\), 200 mM each dNTPs, 900 nM primers, 100 nM probe, 1.25 U AmpliTaq Gold (PE Biosystems), 10% glycerol and 500 ng DNA. The reaction conditions were 10 min 95°C followed by 50 cycles of 15 sec 95°C and 1 min 60–69°C. All RQ-PCR experiments were performed in triplicate.

To verify that in a mixture of ALL cells and testicular cells the same quantitative range and sensitivity of the MRD-PCR is reached as when ALL cells are mixed with mononuclear blood cells (MNC), 10-fold dilution series of ALL cells in testicular cells and in MNCs were compared.

**Gene expression**

To determine the presence of spermatogonia and ALL cells, total RNA from cultured mixed cells, ALL cells and whole testis (positive control) was isolated using the RNeasy Mini Kit (74104, QIAGEN). Extracted RNA was measured by Quibit analysis (Invitrogen) using the RNA assay kit (Q32855, Invitrogen). For reverse transcriptase polymerase chain reaction (PCR), first-strand cDNA was synthesized with random hexamers and the Superscript II premplification system (Invitrogen). PCR was carried out with specific primers for spermatogonial markers (30, 31) ZBTB16 (PLZF) (forward: GGTCGAGCTTCTCTGATAACG; reverse: CCTGTATGTGAGCGCAGGT; product size, 396 base pairs (bp)), UCHL1 (PGP9.5) (forward: ATGCCGTTGGCACAGGAAGGC; reverse: GCCAGCGTCTGTCGTCAGCAGGG; product size, 164 bp), and GFRA1 (forward: GTTCCTGGCGACCCTGTACT; reverse: TGGTTGATATGGGGAATCCT; product size, 367 bp), and for the lymphoblasts marker CD19 (http://biogps.gnf.org)\(^3\) (forward: CCGG GCCACAGCTCAAGACG; reverse: CGTAGCCGCTGCCATCCTTG; product size, 572 bp), and housekeeping gene TBP (Tata box binding protein) (forward: GTGACCCAGCCACAGCATCT; reverse: GTGTTGATATGGGGAATCCT; 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 set (PLZF, 55°C; UCHL1, 65°C; GFRA1, 54°C; CD19, 60°C and TBP, 59°C), 1 minute at 72°C, and a final elongation of 5 minutes at 72°C.
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