Spermatogonial stem cell autotransplantation: towards clinical application
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In Vitro Propagation of Human Prepubertal Spermatogonial Stem Cells

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To the editor: Treatment of pediatric cancer has continuously improved over the past decades, but fertility is often compromised in survivors of childhood cancer. Fertility preservation in prepubertal boys with cancer could theoretically be achieved by cryopreserving testicular tissue before cancer treatment, and then propagating and autotransplanting spermatogonial stem cells (SSCs) from this tissue.1 We describe in vitro propagation of human prepubertal SSCs using a culture system for adult human SSCs.2

METHODS

This study was conducted from July 2009 to September 2010. Open testicular biopsies were performed on 2 boys aged 6.5 and 8 years, diagnosed with Hodgkin lymphoma, who were referred to the Avicenna Research Institute (Tehran, Iran) for fertility preservation. The biopsy specimens were cryopreserved in 5% dimethyl sulfoxide and 5% human serum albumin.3 The main part of each biopsy specimen was stored for possible future clinical use, and with written informed consent from both parents, a small part was donated for research and transferred in liquid nitrogen to the Academic Medical Centre (Amsterdam, the Netherlands). Approval for using the material for research was obtained from the ethical committee of the Avicenna Research Institute.

After rapid thawing and washing, testicular tissues were subjected to 2-step enzymatic digestion, and single cells were cultured in supplemented StemPro medium (Invitrogen, Carlsbad, California). The medium was refreshed every 3 to 4 days; cells were passaged every 7 to 10 days; and depending on the ratio of somatic vs germ cells, differential plating was applied.2 All visible testicular-derived, embryonic stem cell–like colonies4 were removed from the culture.

To determine the presence of spermatogonia during culture, the expression of spermatogonial markers5,6 was studied by reverse transcriptase–polymerase chain reaction, immunohistochemistry, or both. To confirm the presence and propagation of SSCs during culture, cells of early and later passages were transplanted into testes of busulfan-treated immunodeficient mice.2
**RESULTS**

Two and a half weeks after initiation of the testicular cell culture, the first germline stem cell (GSC) clusters appeared. Testicular cells were cultured for 20 and 15.5 weeks from the 6.5- and 8-year-old boys, respectively. GSC clusters were subcultured on laminin for a total of 29 and 20 weeks from the 6.5- and 8-year-old boys, respectively. Expression of spermatogonial markers was detected throughout the entire culture period at the RNA (FIGURE 1) and protein levels (ZBTB16 and UCHL1). Eight weeks after xenotransplantation, human SSCs were detected on the basal membrane of seminiferous tubules of recipient mouse testes (FIGURE 2). Xenotransplantation of cultured cells from early and later passages from the 8-year-old boy showed a 9.6-fold increase in the number of SSCs in 11 days of culture. Similarly, subcultured GSCs from the 6.5-year-old boy showed a 6.2-fold increase in SSCs within 21 days and a 5.6-fold increase within 14 days from the 8-year-old boy.

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**Figure 1. Culture Characterization**

Reverse transcriptase–polymerase chain reaction showing expression of ZBTB16 (PLZF), ITGA6, ITGB1, CD9, GFRA1, GPR125, and UCHL1 (spermatogonial markers) in human adult testis (control), long-term cultured testicular cells, and several independent subcultured germline stem cell (GSC) clusters of the 6.5-year-old (clusters C0164 and C0170) and 8-year-old boys (clusters C0232 and C0230). The TBP gene was used as a positive control, and the second lane for each sample tested shows the negative control (without reverse transcriptase).
Assuming SSCs grow in an exponential way, 35 days of testicular cell culture or 58 to 83 days of GSC subculture would be necessary to achieve the 1300-fold increase in SSC number that we previously estimated as necessary for repopulation of adult human testes after autotransplantation. No intratesticular tumors were observed in any of the 11 recipient mice after xenotransplantation.

Figure 2. Detection of Human Spermatogonial Stem Cells After Transplantation to Immunodeficient Mouse Testis

Migration of human spermatogonial stem cells (cultured cells from testicular cell culture of the 8-year-old boy, passage 6 at 9 weeks) to the basal membrane of the seminiferous epithelium of immunodeficient mouse testis 8 weeks after transplantation. Cells were detected using a (A) human COT-1 fluorescence in situ hybridization probe, and detection with Cy3 (red) and cells were visualized using (B) DAPI (blue) staining. The merged image (C) indicates COT-1 staining in the nucleus of a migrated human spermatogonial stem cell.

COMMENT

We have demonstrated in vitro propagation of human prepubertal SSCs. Although these results are preliminary and need to be confirmed, they support the potential for autotransplantation of SSCs in infertile survivors of childhood cancer. Given the time between preservation of testicular tissue during childhood and potential SSC autotransplantation later in adult life, it is important to counsel prepubertal boys with cancer on the possibility of cryopreserving testicular tissue before undergoing gonadotoxic cancer treatment.
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