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# Cryopreservation of Mouse Spermatogonial Stem Cells in Dimethylsulfoxide and Polyethylene Glycol<sup>1</sup>

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## ABSTRACT

Assisted reproductive techniques involving isolation, culture, and transplantation of spermatogonial stem cells (SSCs) have the potential to create transgenic livestock and to treat male infertility caused by cancer treatments such as chemotherapy or radiation. Because stem cells may need to be preserved for several years before reintroduction to the patients' testes, efficient SSC cryopreservation techniques need to be developed. SSCs can reinitiate spermatogenesis in recipient testes after freezing; however, optimal cryopreservation protocols have not been identified. The objective of this study was to develop an efficient cryopreservation method for SSCs using permeable cryoprotectant agents (PCAs) or additive cryoprotectant agents (ACAs). To identify an efficient cryopreservation method, populations of mouse testis cells enriched for SSCs were cultured in vitro and frozen using conventional freezing media containing various PCAs or ACAs for 1 wk or 1, 3, 6, 12, or 24 mo. Additionally, various molecular weights and concentrations of polyethylene glycol (PEG) were evaluated. Recovery rate, culture potential, and stem cell activity were significantly greater for cells frozen in 2.5% PEG with a molecular weight of 1000 compared to other treatment groups. These cells also retained the ability to colonize recipient testes, generate normal spermatogenesis, and contribute to viable offspring. The systematic analysis of many cryoprotectant agents indicates that 2.5% PEG (molecular weight 1000) is the most effective agent for efficient SSC cryopreservation.

*cryopreservation, polyethylene glycol, spermatogonial stem cells, transplantation*

## INTRODUCTION

Spermatogonial stem cells (SSCs) are the foundation of adult spermatogenesis because they have the capacity to undergo both self-renewal and differentiation into spermatozoa throughout the lifetime of a male [1, 2]. The ability to self-renew and differentiate into spermatozoa results in the ability of donor SSCs to produce a nearly limitless number of mature spermatozoa after transplantation to a recipient testis [3, 4]. The ability to isolate, culture, and transplant SSCs has facilitated the development of practical and clinical applications such as producing transgenic animals and preserving the germ lines of valuable livestock, endangered species, and prepubertal human cancer patients [5–8].

To maximize the efficiency of SSC manipulation, cryopreservation of the SSC must be feasible. Initial studies exploring SSC transplantation demonstrated that mouse SSCs could reinitiate spermatogenesis in recipient testes following cryopreservation [9]. Subsequent research has demonstrated that SSCs from a variety of mammals, including livestock and humans, have the capacity to survive long-term cryopreservation [10–18]. Furthermore, cryopreservation provides for the long-term preservation of SSCs from species for which SSC culture and transplantation techniques have yet to be developed. Although these initial studies have demonstrated a potential for cryopreservation of SSCs, techniques used have been very similar to those used for somatic cells. Additionally, few attempts have been conducted to optimize germ cell cryopreservation, or to directly evaluate the effects of cryopreservation on the SSC. To maximize the efficiency of future applications, novel SSC cryopreservation protocols must be developed and optimized.

Development of cryopreservation techniques for SSCs has utilized uncontrolled slow-freezing methods coupled with rapid thawing. These methods have proven to be convenient methods for the long-term preservation of SSCs capable of restoring fertility after thawing and transplantation into infertile recipient mice [19–21]. Furthermore, it has been demonstrated that cryopreservation does not lead to the development of genetic or epigenetic errors [19]. A common consequence of cryopreservation using uncontrolled slow freezing is the development of cryoinjury caused by osmotic stress. However, the osmotic stress can be overcome by including permeable cryoprotectant agents (PCAs), which reduce intracellular ice formation, in cryopreservation media. Furthermore, the addition of additive cryoprotectant agents (ACAs) can further improve cellular viability after thawing [22–24].

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The objective of this study was to develop an efficient cryopreservation method for SSCs using PCAs and/or ACAs. To complete this objective, mouse testis cells enriched for SSCs were cultured *in vitro* and preserved using various cryopreservation media. After thawing, testis cells enriched for SSCs were evaluated for recovery rate, culture potential, and functional capacity to restore fertility after transplantation into recipient testes.

## MATERIALS AND METHODS

### *Isolation and Culture of Testis Cells Enriched for SSCs*

Animal procedures were approved by the Animal Care and Use Committee of Chung-Ang University (permit number 11-0038) in accordance with the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Donor testis cells for all experiments except progeny generation transplantation experiments were isolated from 6- to 8-day-old F1 progeny of C57BL/6-TgEGFP X DBA/2 crosses (C57GFP X DBA). Donor testis cells for progeny generation transplantation experiments were isolated from 6- to 8-day-old C57BL/6-TgEGFP (C57GFP) males. All donor cells expressed the enhanced green fluorescent protein (EGFP). Isolation and culture of testis cells enriched for SSCs was conducted as previously reported [25] with slight modification. After collection, fresh testes were washed in Dulbecco PBS (DPBS; Invitrogen, Grand Island, NY) and decapsulated. To create a single-cell suspension, decapsulated testis tissue was incubated at 37°C for 5 min in a 2:1 solution of 0.25% trypsin-EDTA (Invitrogen) and 7 mg/ml DNase I (Roche, Basel, Switzerland) in DPBS. After the initial 5-min digestion, tubules were dispersed and allowed to incubate an additional 5 min. Incubation was followed by addition of fetal bovine serum (FBS; Hyclone; Thermo Scientific, Logan, UT) equivalent to 10% of the reaction volume. To remove undigested tissue and debris, testis cell suspensions were filtered through a nylon mesh with 40-μm pores (BD Biosciences, San Jose, CA). After digestion and filtration, cell viability was determined to be greater than 95% by trypan blue exclusion. Single-cell suspensions were centrifuged at  $600 \times g$  at 4°C for 7 min and cells were resuspended to  $5 \times 10^6$  cells/ml in Dulbecco modified Eagle medium (Invitrogen) containing 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. To remove erythrocytes and debris from the single-cell suspension,  $10 \times 10^6$  cells were overlaid on 2 ml of 30% Percoll and centrifuged. The percentage of undifferentiated germ cells (based on PLZF expression) in the resulting Percoll-enriched cell fraction was  $1.8\% \pm 0.5\%$  ( $n = 3$ ). The cell pellet isolated after Percoll centrifugation was resuspended for magnetic-activated cell sorting (MACS) to isolate THY-1-positive testis cells enriched for SSCs using anti-THY-1 microbeads (Miltenyi Biotech, Auburn, CA) as previously described [25]. The percentage of undifferentiated germ cells (based on PLZF expression) in the resulting THY-1-positive cell fraction was  $39.5\% \pm 3.2\%$  ( $n = 3$ ). To initiate testis cell cultures enriched for SSCs,  $0.1 \times 10^6$  THY-1<sup>+</sup> cells/well were placed in 12-well culture plates containing mitotically inactivated STO (SIM mouse embryo-derived thioguanine- and ouabain-resistant) feeder cells. Germ cell cultures were maintained in mouse serum-free medium (mSFM) as previously described [26] containing 1 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN), 10 ng/ml GDNF (R&D Systems) and 75 ng/ml GFRα1 (R&D Systems). The serum-free nature of the SSC culture media prevents overgrowth of the SSCs by testis somatic cells. Culture medium was replaced every 2–3 days and wells were passaged weekly 1:2 or 1:3. In all experiments, passage numbers used were between passage 6 and 12. Within an experiment, the same passage number cells ( $\pm 1$  passage) were used. The percentage of undifferentiated germ cells (based on PLZF expression) in culture after 6 passages was  $93.8\% \pm 0.8\%$  ( $n = 3$ ).

### *Cryopreservation*

Cultured testis cells enriched for SSCs were recovered 6 wk after initial testis cell isolation by digestion in 0.25% trypsin. After digestion, cells were suspended at  $5 \times 10^5$  cells/ml of Minimum Essential Medium (MEM) alpha (Invitrogen). Freezing medium containing FBS, PBS, and ACA/PCA (in a 1:3:1 ratio) was added to 0.5 ml of cell suspension in a drop-wise manner to a final volume of 1 ml and placed in 1.8 ml cryovials (Corning, Midland, MI). Cryovials were frozen in a Nalgene freezing container (Nalgene, Rochester, NY) that provided a 1°C/min cooling rate to  $-80^\circ\text{C}$ . After overnight storage at  $-80^\circ\text{C}$ , cryovials were placed in liquid nitrogen for long-term storage.

Several different freezing media were evaluated to determine their efficacy for cryopreservation of testis cells enriched for SSCs. In our initial experiment,

we evaluated four PCAs: dimethylsulfoxide (DMSO), ethylene glycol (1,2-ethane diol; EG), propylene glycol (1,2-propanediol; PG), and glycerol. The PCAs were added at a concentration of 10% to MEM alpha containing 10% FBS (v/v). Of the PCAs evaluated, DMSO was the most effective cryoprotectant. In our second experiment, we evaluated six ACAs: hydroxyethyl starch (HES), collagen, laminin, catalase, Y-27362, and polyethylene glycol (PEG). After initial ACA analysis, we examined two different concentrations (2.5% and 5% w/v) and three different molecular weights (MWs) of PEG (1000, 3000, and 10000). PEG was also compared to the cryoprotective sugar trehalose. To mix the various ACA cryopreservation media, the cryoprotectants were added to MEM alpha containing 10% FBS and 10% DMSO (v/v).

### *Postthaw Analyses of Recovery Rate and Culture Potential*

Frozen cells were thawed after freezing for 1 wk–24 mo by incubation in a 37°C water bath for 2.5 min and diluted 1:10 with MEM alpha containing 10% FBS in a drop-wise manner. Analysis of cell viability was determined after thawing by trypan blue exclusion. The recovery rate of viable cells was calculated as follows:

Recovery rate (%)

$$= \frac{\text{number of recovered viable cells after freeze, thaw, and washing} \times 100}{\text{number of frozen cells} (5 \times 10^5 \text{ cells})} \quad (1)$$

To determine the culture ability of thawed testis cells enriched for SSCs, all cells recovered from a vial after thawing were cultured in mSFM for 1 wk as described above. The 1-wk culture period was used because the doubling rate for nonfrozen cells in our culture system is approximately 1 wk. Noncryopreserved cell cultures of equivalent culture age and seeding number ( $5 \times 10^5$ ) were used for normalization. One week after thawing, cells were removed from culture wells by trypsin digestion and EGFP-positive cells were counted. The number of EGFP cells in cultures established from cryopreserved cells was normalized to the number of noncryopreserved cells using the following equation:

Culture potential (%)

$$= \frac{\text{number of recovered cells after freeze, thaw, and culture} \times 100}{\text{number of recovered noncryopreserved cells after culture}} \quad (2)$$

### *Transplantation of Cryopreserved Testis Cells Enriched for SSCs*

The only way to unequivocally quantify SSCs in a cell population is to transplant them into a recipient testis. Thus, germ cell transplantation was conducted to determine the effects of different cryopreservation techniques on the SSCs. BALB/c immunodeficient nude mice (Nara Biotech., Seoul, Republic of Korea) were used for recipients in SSC quantification experiments. Because of their lack of endogenous spermatogenesis, W/Wv mice were used as recipients for progeny generation transplantation experiments. All animal lines, except nude mice, were purchased from The Jackson Laboratory (Bar Harbor, ME).

To quantify C57GFP X DBA SSCs, thawed testis cells enriched for SSCs were transplanted into nude mice whose endogenous spermatogenesis had been depleted by treatment with 40–44 mg/kg busulfan at 6 wk of age. To determine if transplanted SSCs maintained the ability to generate complete spermatogenesis and contribute to offspring, thawed C57GFP donor cells were transplanted into 12- to 14- or 24-day-old W/Wv mice. Different strains of donor cells were transplanted for the quantification and progeny generation experiments because of the background strains of recipients. After recipient preparation, testis cell populations enriched for SSCs that were frozen for 3 mo were thawed, cultured for 1 wk, concentrated, and stained with trypan blue to visualize injection. Before injection, recipient animals were anesthetized i.p. with 75 mg/kg ketamine and 0.5 mg/kg medetomidine. Approximately  $8 \mu\text{l}$  ( $5 \times 10^6$  cells/ml) and  $2\text{--}4 \mu\text{l}$  ( $50 \times 10^6$  cells/ml) of donor cells, resulting in filling of 80% of surface seminiferous tubules, were injected into the testes of nude and W/Wv mice respectively through efferent ducts as previously described [27].

Two months after transplantation, recipients were euthanized and testes were recovered and decapsulated. To quantify SSCs, the tubules were gently dispersed and colonies were quantified using fluorescence microscopy. A colony of donor-derived germ cells was defined by size ( $\geq 1$  mm) as previously described [28]. Colony numbers were expressed as the number of colonies per  $10^5$  cells transplanted or normalized as the number of colonies per total cells recovered after culture because of the potential effects of cryopreservation on

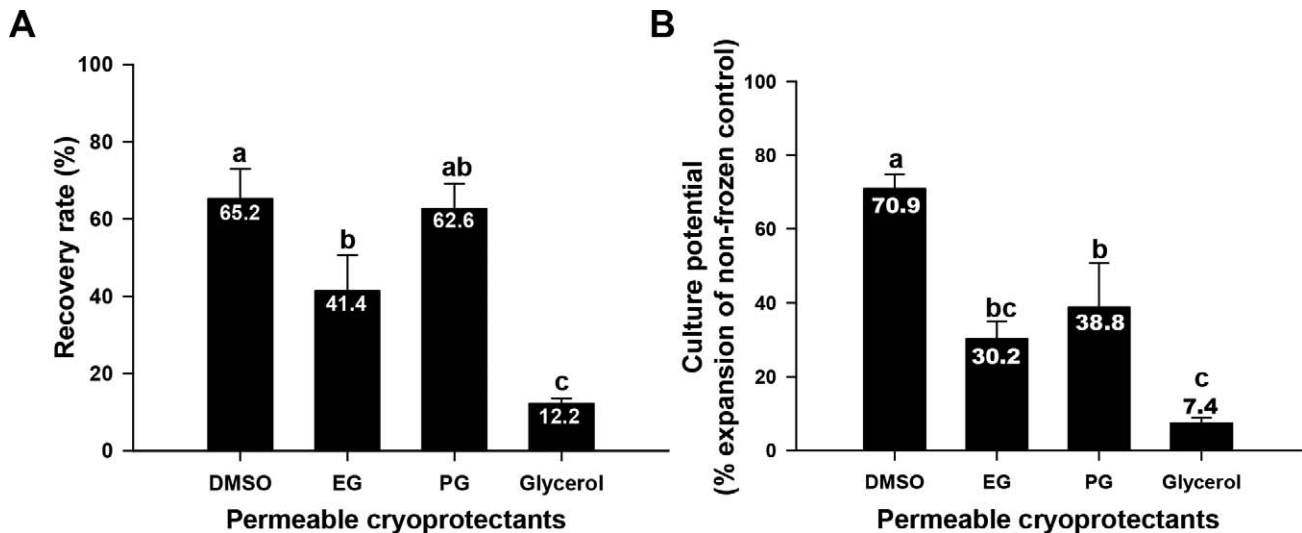


FIG. 1. Effects of PCAs on recovery rate and culture potential of testis cells enriched for SSCs. **A)** Percentage of viable cells after thawing. **B)** Culture (proliferation) potential of frozen cells in SSC culture for 1 wk after thawing. Values are means  $\pm$  SEM ( $n = 3$  independently established cultures for each treatment). Bars with different letters are significantly different ( $P < 0.05$ ).

SSC culture potential, by using the following equations:

$$\text{Colonies}/10^5 \text{ cells transplanted} = \frac{\text{number of colonies} \times 10^5}{\text{number of cells transplanted}} \quad (3)$$

Normalized colonies/total number of cells cultured

$$= \frac{\text{number of colonies} \times \text{total number of cells cultured}}{\text{number of cells transplanted}} \quad (4)$$

To confirm the extent of donor spermatogenesis in recipients, some testes were recovered 3 mo after transplantation, cryosectioned, and histologically visualized using fluorescent microscopy.

#### Analysis of Apoptosis in Thawed Testis Cells Enriched for SSCs

To determine if observed differences in viability were due to apoptosis, the percentage of apoptotic cells from different freezing protocols was determined immediately after thawing and after a 12-h postthaw culture period using an annexin V-phycoerythrin (PE) apoptosis kit (BD Biosciences) with a modified protocol. Thawed cells were washed twice with DPBS and resuspended at  $2 \times 10^5$  cells/200  $\mu$ l in  $1 \times$  binding buffer followed by the addition of 10  $\mu$ l of annexin V-PE. Cells were then incubated for 15 min in the dark at room temperature. After incubation, propidium iodide (PI) buffer was added to a final concentration of 5  $\mu$ g/ml and cells were placed on ice. The percentages of apoptotic cells were determined with BD FACS Diva software analysis of data obtained using a FACS Aria flow cytometer (BD Biosciences) equipped with Cell Quest software.

#### Statistical Analysis

Analysis of variance and Tukey honestly significant difference test were used to determine differences between treatment groups for viability, culture potential, apoptosis, and colony number. All analysis was conducted using SPSS version 18 software (SPSS Inc., Mechanicsburg, PA) and differences were considered significant if  $P < 0.05$ . Unless stated, each experiment was conducted in triplicate.

## RESULTS

#### Evaluation of PCAs on the Cryopreservation of Testis Cells Enriched for SSCs

Four different PCAs, DMSO, EG, PG, and glycerol, were evaluated for their effects on cell recovery rate and culture potential after thawing of cryopreserved testis cells enriched for

SSCs. One week after freezing, frozen cells were thawed and recovery rate was determined (Fig. 1A). The recovery rate of cells frozen in medium containing DMSO ( $65.2\% \pm 7.7\%$ ), EG ( $41.4\% \pm 4.1\%$ ), and PG ( $62.6\% \pm 6.5\%$ ) was significantly greater than the recovery rate of cells frozen in glycerol ( $12.2\% \pm 1.4\%$ ). Furthermore, freezing in DMSO resulted in a significantly greater recovery rate than freezing in EG. No significant difference was observed between DMSO and PG or PG and EG.

Thawed cells were also evaluated for their culture potential relative to nonfrozen controls (Fig. 1B). The culture potential of cells frozen in medium containing DMSO ( $70.9\% \pm 3.9\%$ ) was significantly greater than that of cells frozen in medium containing EG ( $30.2\% \pm 4.8\%$ ), PG ( $38.8\% \pm 11.8\%$ ), and glycerol ( $7.4\% \pm 1.5\%$ ). Additionally, cells frozen in medium containing PG had significantly greater culture potential than cells frozen in glycerol. Collectively, the culture potential and recovery rate data indicated that DMSO was the most effective PCA for cryopreservation of testis cells enriched for SSCs; thus, DMSO was included as a comparative cryoprotectant for further studies.

#### Evaluation of PEG on the Cryopreservation of Testis Cells Enriched for SSCs

Preliminary evaluations of ACAs, including HES, collagen, laminin, catalase, Y-27362, and PEG, indicated that most ACAs were not effective for the cryopreservation of testis cells enriched for SSCs (Supplemental Table S1, available online at [www.biolreprod.org](http://www.biolreprod.org)). In contrast to other ACAs, PEG markedly increased recovery and culture potential of cryopreserved testis cells enriched for SSCs. To further evaluate the effects of PEG on cryopreservation of testis cells enriched for SSCs, PEG was added to freezing medium at various MWs (1000, 3000, and 10000) and concentrations (0%, 2.5%, and 5% w/v). Cells were thawed 1 wk or 1 or 3 mo after freezing. After thawing, recovery rates of viable EGFP-positive cells were compared to control cells frozen in DMSO. Compared to DMSO alone, testis cells enriched for SSCs frozen in PEG, regardless of concentration and MW, had significantly greater recovery rates after 1 wk of freezing. After 1 mo of freezing, testis cells enriched for SSCs frozen in 2.5% and 5% PEG

TABLE 1. Effects of PEG on the recovery of thawed testis cells enriched for SSCs.

Final concentration (%) of PEG	Molecular weight of PEG	Recovery rate (%) after thawing		
		1 wk*	1 mo*	3 mo*
0 <sup>†</sup>	—	71.4 ± 3.3 <sup>b</sup>	74.0 ± 5.0 <sup>cd</sup>	63.8 ± 5.0 <sup>bc</sup>
2.5	1000	87.4 ± 0.5 <sup>a</sup>	88.3 ± 1.9 <sup>ab</sup>	89.6 ± 2.8 <sup>a</sup>
	3000	93.9 ± 3.0 <sup>a</sup>	88.2 ± 5.5 <sup>ab</sup>	73.2 ± 4.7 <sup>ab</sup>
	10 000	87.3 ± 0.8 <sup>a</sup>	86.2 ± 3.8 <sup>abc</sup>	78.0 ± 6.0 <sup>ab</sup>
5	1000	91.2 ± 2.3 <sup>a</sup>	90.1 ± 1.8 <sup>ab</sup>	91.8 ± 3.8 <sup>a</sup>
	3000	88.5 ± 1.3 <sup>a</sup>	90.7 ± 0.8 <sup>a</sup>	86.0 ± 9.5 <sup>ab</sup>
	10 000	88.3 ± 2.6 <sup>a</sup>	76.9 ± 2.9 <sup>bcd</sup>	64.4 ± 4.7 <sup>bc</sup>

\* Period of freezing.

<sup>†</sup> Freezing control. (Freezing medium is MEM alpha supplemented with 10% FBS and 10% DMSO.)<sup>a,b,c,d</sup> Different letters within a column indicate significant differences ( $P < 0.05$ );  $n = 3$  independently established experiments.

(MW 1000 and 3000) had significantly greater recovery rates than control. However, after 3 mo of freezing, only the cells frozen in 2.5% and 5% PEG (MW 1000) had significantly greater recovery rates than controls (Table 1).

Thawed cells were also evaluated for culture potential relative to nonfrozen control cells. Compared to DMSO control, culture potential was significantly greater when testis cells enriched for SSCs were frozen in 2.5% PEG (MW 1000) at all freezing periods (Table 2). Because of the consistent improvement in recovery and culture potential, the percentages of apoptotic cells were evaluated for freezing media containing PEG (MW 1000; Fig. 2). Compared to control, 2.5% PEG had a significantly lower percentage of apoptotic cells after 1 wk of freezing ( $3.6\% \pm 0.2\%$  vs.  $1.7\% \pm 0.2\%$ ); however, no difference was observed after 1 or 3 mo of freezing ( $8.9\% \pm 0.5\%$  vs.  $9.7\% \pm 1.3\%$  and  $8.5\% \pm 0.4\%$  vs.  $7.8\% \pm 0.2\%$  respectively). Compared to control and 2.5% PEG, 5% PEG had significantly more apoptotic cells after freezing for 1 wk ( $5.2\% \pm 0.4\%$ ), 1 mo ( $13.7\% \pm 0.3\%$ ), and 3 mo ( $12.4\% \pm 0.3\%$ ; Fig. 2).

To determine if the short-term effects of cryopreservation of testis cells enriched for SSCs with 2.5% PEG (MW 1000) could be maintained for longer periods, cells were evaluated for culture potential after freezing for 6, 12, and 24 mo. As seen after freezing for 1 wk and 1 and 3 mo, testis cells enriched for SSCs frozen in 2.5% PEG (MW 1000) had significantly greater culture potential compared to DMSO (as a percentage of noncryopreserved controls) after 6, 12, and 24 mo of freezing ( $53.1\% \pm 1.2\%$  vs.  $34.6\% \pm 2.7\%$ ,  $45.8\% \pm 1.3\%$  vs.  $30.8\% \pm 2.4\%$ , and  $50.7\% \pm 2.9\%$  vs.  $34.1\% \pm 2.6\%$ ; Fig. 3).

Previously, we demonstrated that the addition of 200 mM trehalose to freezing medium significantly improved the cryopreservation of SSCs. In accordance with these results, we compared the effect of 2.5% PEG (MW 1000) and 200 mM trehalose on cryopreservation of SSCs for an extended freezing period. No significant differences were observed between the culture potential of SSCs cryopreserved for 6, 12, or 24 mo in PEG (MW 1000) or trehalose ( $50.5 \pm 0.9$ ,  $50.3 \pm 2.3$ , and  $52.6 \pm 2.9$  for trehalose treatment respectively; Fig. 3). Collectively, these data indicated that 2.5% PEG (MW 1000) was the most effective freezing medium examined for cryopreservation of testis cells enriched for SSCs, and the effect of 2.5% PEG (MW 1000) was comparable to that of 200 mM trehalose on culture potential of germ cells after freeze-thawing.

TABLE 2. Effects of cryopreservation in PEG on the culture potential of thawed testis cells enriched for SSCs.

Final concentration (%) of PEG	Molecular weight of PEG	Expansion (% of control) after freezing and thawing and 1 wk of culture		
		1 wk*	1 mo*	3 mo*
0 <sup>†</sup>	—	69.3 ± 8.3 <sup>b</sup>	43.8 ± 6.9 <sup>b</sup>	36.6 ± 0.4 <sup>b</sup>
2.5	1000	91.2 ± 4.4 <sup>a</sup>	67.0 ± 3.5 <sup>a</sup>	49.8 ± 3.8 <sup>a</sup>
	3000	74.7 ± 2.9 <sup>ab</sup>	48.2 ± 1.8 <sup>ab</sup>	42.4 ± 3.4 <sup>ab</sup>
	10 000	77.5 ± 11.0 <sup>ab</sup>	49.2 ± 5.4 <sup>ab</sup>	45.6 ± 1.5 <sup>ab</sup>
5	1000	77.0 ± 4.5 <sup>ab</sup>	53.8 ± 2.2 <sup>ab</sup>	41.7 ± 3.5 <sup>ab</sup>
	3000	75.1 ± 3.7 <sup>ab</sup>	44.6 ± 3.2 <sup>ab</sup>	41.6 ± 1.8 <sup>ab</sup>
	10 000	65.7 ± 10.0 <sup>b</sup>	44.1 ± 6.3 <sup>b</sup>	41.1 ± 2.3 <sup>ab</sup>

\* Period of freezing.

<sup>†</sup> Freezing control. (Freezing medium is MEM alpha supplemented with 10% FBS and 10% DMSO.)<sup>a,b</sup> Different letters within a column indicate significant differences ( $P < 0.05$ );  $n = 3$  independently established cultures for each treatment.

### Evaluation of SSC Activity Following Cryopreservation in 2.5% PEG (MW 1000)

Direct evaluation of the effects of different cryopreservation techniques on the SSC can be determined only by transplantation of thawed SSCs into recipient testes. Based on the preliminary experiments, we chose to evaluate the ability of 2.5% PEG (MW 1000) on SSC preservation using transplantation experiments. Donor EGFP-positive cells that had been frozen for 3 mo with 2.5% PEG (MW 1000) were thawed, cultured for 7 days (Fig. 4, A and B), and transplanted into recipient testes. Noncryopreserved, cultured testis cells enriched for SSCs and testis cells enriched for SSCs and frozen in DMSO were transplanted as controls. Two months after transplantation, recipient testes were recovered and visualized for donor colonies (Fig. 4C). Histological analysis demonstrated that complete spermatogenesis was present in colonies of donor cells, including those that were frozen in 2.5% PEG (MW 1000; Fig. 4D). Furthermore, testis cells enriched for SSCs that were frozen in 2.5% PEG (MW 1000) or DMSO and

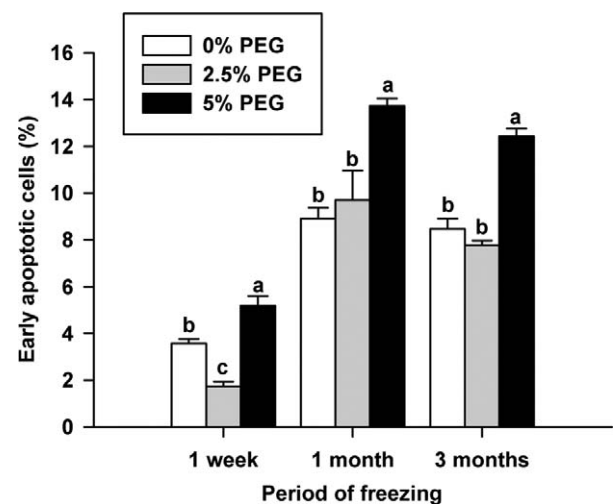


FIG. 2. Effects of PEG (MW 1000) on apoptosis of frozen testis cells enriched for SSCs. Percentage of annexin V-binding, PI-excluding, apoptosis-positive, EGFP-positive cells 12 h after thawing. As expected, very low percentages of apoptotic cells (0.2%–0.5%) were observed among the concentrations of PEG immediately after thawing regardless of freezing period. Values are means  $\pm$  SEM ( $n = 3$  independently established cultures for each treatment). Bars within a freezing period with different letters are significantly different ( $P < 0.05$ ).

cultured for 1 wk generated the same number of donor colonies per  $10^5$  cells transplanted as nonfrozen cells ( $127.0 \pm 14.7$ ,  $87.1 \pm 13.0$ , and  $93.7 \pm 12.1$  for 2.5% PEG [MW 1000] cryopreserved, DMSO cryopreserved, and noncryopreserved cells respectively; Fig. 4E), indicating that freezing did not diminish viable SSC function. However, this analysis did not account for differences in recovery rate, viability, and apoptosis between cryopreservation treatments. To account for cell loss during freezing, thawing, and culture, colony numbers were also evaluated normalized to the total number of viable recovered cells after freezing, thawing, and 1 wk of culture. No significant difference was observed in normalized colony number between noncryopreserved testis cells ( $814.1 \pm 104.6$ ) and testis cells cryopreserved in 2.5% PEG (MW 1000;  $761.0 \pm 90.1$ ); however, cryopreservation in DMSO resulted in significantly fewer normalized colonies ( $305.0 \pm 44.0$ ) than noncryopreserved or 2.5% PEG (MW 1000) cryopreserved testis cells enriched for SSCs (Fig. 4F).

To confirm that complete SSC function was retained in cryopreserved SSCs, EGFP-positive testis cells enriched for SSCs that were frozen in 2.5% PEG (MW 1000) for 3 mo were thawed and transplanted into testes of W/W<sup>v</sup> mice after 1 wk of culture. Several months after transplantation, recipient males were mated with wild-type females and donor-derived, EGFP-positive offspring were produced (Fig. 5, A and B). Additionally, offspring were phenotypically normal and fertile (Fig. 5C).

## DISCUSSION

The objective of this study was to develop an efficient cryopreservation method for SSCs using various cryoprotectant agents. To complete this objective, populations of testis cells enriched for SSCs were developed using standard isolation and culture techniques [25]. After selection of THY-1-positive cells using MACS selection, about 40% of cells were positive for PLZF (a marker of undifferentiated germ cells); however, the percentage of PLZF-positive cells after six passages in culture was greater than 90%. The utilization of cultured SSCs in this work provided two advantages over using freshly isolated cells. First, culturing of populations of testis cells enriched for SSCs allows for additional enrichment of undifferentiated PLZF-positive germ cells. Second, it is likely that any practical utilization of nonhuman mammalian SSCs will require a period of culture to amplify the numbers of SSCs transplanted into recipient males. After freezing in various cryoprotectants, thawed cells were evaluated for recovery rate, culture potential, and capacity to generate donor colonies of spermatogenesis after transplantation to recipient testes, an SSC-specific function. The data indicated that DMSO and 2.5% PEG (MW 1000) were the most effective PCA and ACA evaluated, respectively.

Previous research has shown that DMSO, EG, PG, and glycerol are effective PCAs for the cryopreservation of cells and tissues; however, their effectiveness is highly variable for different testis cell populations. DMSO has been regularly used for the cryopreservation of murine and human germ cells and has been shown to provide better preservation than cryopreservation with EG, PG, and glycerol. Previous research has evaluated the efficiency of germ cell and testis tissue cryopreservation using PCAs [19, 29, 30]; however, these experiments did not evaluate cryopreservation of populations of germ cells enriched for SSCs. In the current study we confirmed the effectiveness of DMSO compared to other PCAs. Our results showed that postthaw recovery was best when cells were preserved in DMSO or PG; however, postthaw

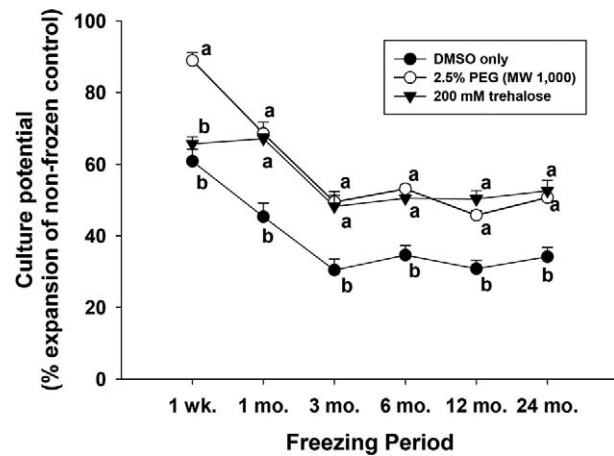


FIG. 3. Effects of 2.5% PEG (MW 1000), trehalose, and DMSO on culture potential of testis cells enriched for SSCs after extended freezing times. Culture (proliferation) potential of frozen cells in culture for 1 wk after thawing. Cells were frozen for up to 24 mo prior to thawing. Values are means  $\pm$  SEM ( $n = 3$  independently established cultures for each treatment). Different letters indicate significant difference ( $P < 0.05$ ) among groups within each freezing period.

culture potential was nearly two times greater when cells were cryopreserved in DMSO compared to PG, likely because of differential postthaw apoptosis levels between the two treatments. These data confirm the effectiveness of cryopreservation of testis cells enriched for SSCs in DMSO and demonstrate that evaluation of postthaw recovery rate by itself is not an efficient evaluation of the effects of cryopreservation agents on testis cells.

In addition to PCAs, ACAs have also been demonstrated as effective for cryopreservation for various cell types [31–35]. Preliminary evaluations of ACAs, including HES, collagen, laminin, catalase, Y-27362, and PEG, indicated that most ACAs were not effective for the cryopreservation of testis cells enriched for SSCs (Supplemental Table S1). In contrast to other ACAs, PEG markedly increased recovery rate and culture potential of cryopreserved testis cells enriched for SSCs compared to DMSO. Thus, we chose to further evaluate different concentrations (2.5% and 5%) and MWs (1000, 3000, and 10 000) of PEG on cryopreservation of testis cells enriched for SSCs.

Evaluation of postthawed testis cells enriched for SSCs demonstrated that cryopreservation in 2.5% and 5% PEG (MW 1000) improved recovery rate compared to cryopreservation in DMSO at all freezing periods. However, significant improvements in culture potential were observed only when cells were frozen in 2.5% PEG (MW 1000). The loss of significant functional differences is most likely caused by cell injury due to high osmotic pressure and cell fusion caused by PEG [36], as evidenced by the excellent recovery rates coupled with the relatively poor proliferation potential of cultured cells frozen in higher concentrations of PEG (5%). When evaluating the percentage of apoptotic cells postthawing, 2.5% PEG (MW 1000) was unable to limit the percentage of apoptosis compared to control at freezing times beyond 1 wk. Because PEG does not cross the cell membrane, it is likely that PEG promotes cell survival by decreasing the likelihood of extracellular ice formation. The mechanism of action of extracellular PEG has not been definitively identified, but the affinity of PEG for water molecules has been demonstrated in studies examining cell fusion [37]. Although PEG cannot prevent intracellular ice formation directly, it is possible that



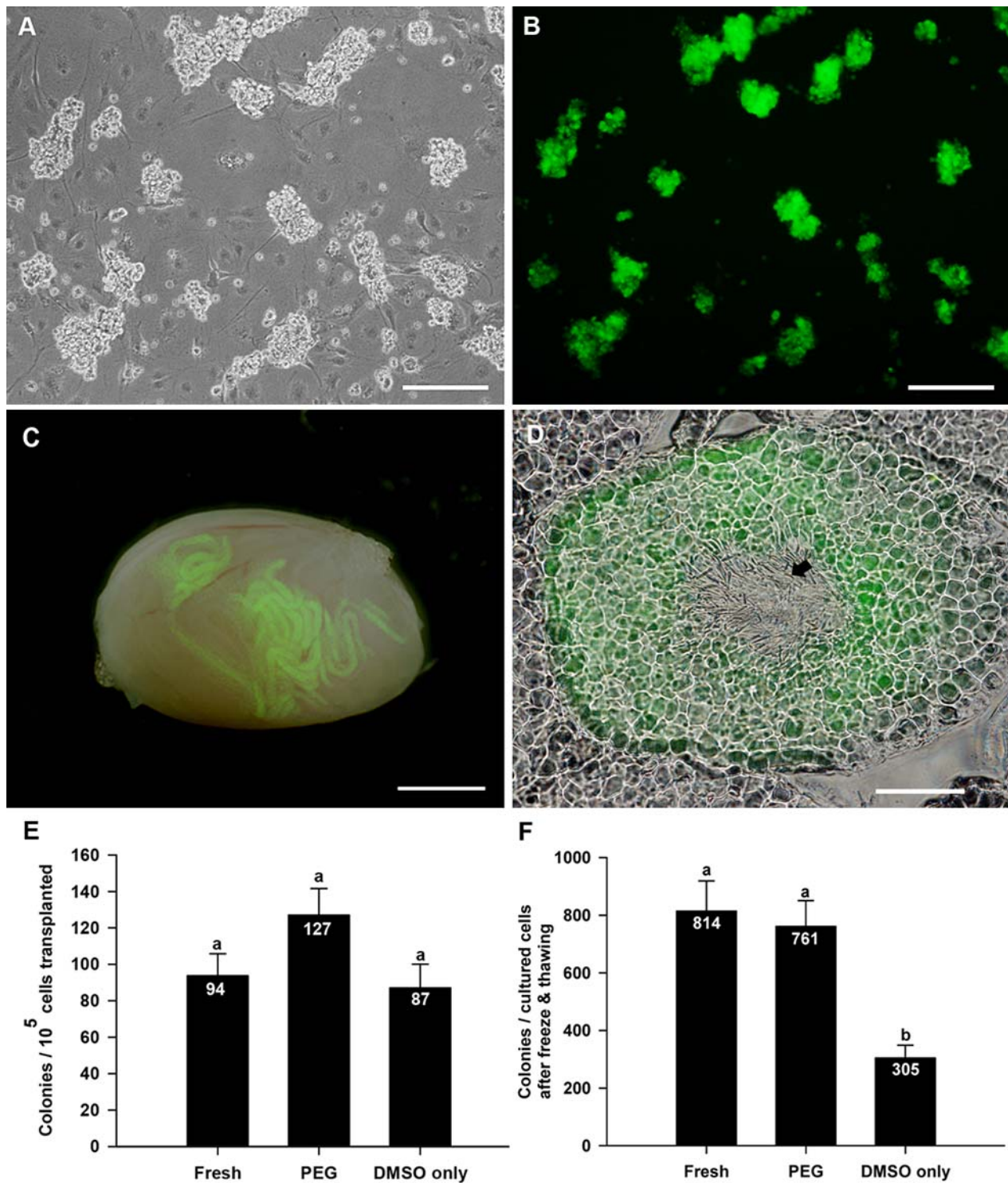


FIG. 4. Effects of 2.5% PEG (MW 1000) on SSC activity after thawing. **A, B** EGFP-positive testis cells enriched for SSCs that were frozen in 2.5% PEG (MW 1000) and thawed after 3 mo were able to form clumps after 1 wk in culture; bright-field (**A**), dark-field fluorescence (**B**). **C** EGFP-positive germ cells, presumably SSCs, that were frozen in 2.5% PEG (MW 1000) thawed after 3 mo, and cultured for 1 wk were able to form colonies in recipient testes; dark-field fluorescence images are overlaid on the bright-field image of the same section of the recipient testis. **D** Cryosection of donor-derived germ cell colony. Donor colonies contained complete spermatogenesis, indicating that SSC function was retained after freezing; Green image showing multiple layers of green donor germ cells is overlaid on the bright-field image of the same section. Complete spermatogenesis is evidenced by the presence of sperm (black arrow) in the lumen of the seminiferous tubules. **E** The number of colonies per  $10^5$  transplanted cells after freezing, thawing, and 1 wk of culture; colonies/ $10^5$  cells transplanted = number of colonies  $\times$   $10^5$ /number of cells transplanted. **F** The number of colonies normalized to total number of viable cells recovered after freezing, thawing, and 1 wk of culture; normalized colonies/total number of cells cultured = number of colonies  $\times$  total number of cells cultured/number of cells transplanted. Bars = 200  $\mu$ m (**A, B**), 2 mm (**C**), and 25  $\mu$ m (**D**). Values are means  $\pm$  SEM ( $n = 3$  experiment per treatment; total numbers of mice/testes analyzed were 8/14, 9/16, and 8/16 for fresh, 2.5% PEG (MW 1000), and DMSO respectively). Different letters indicate significant difference ( $P < 0.05$ ) between treatments.

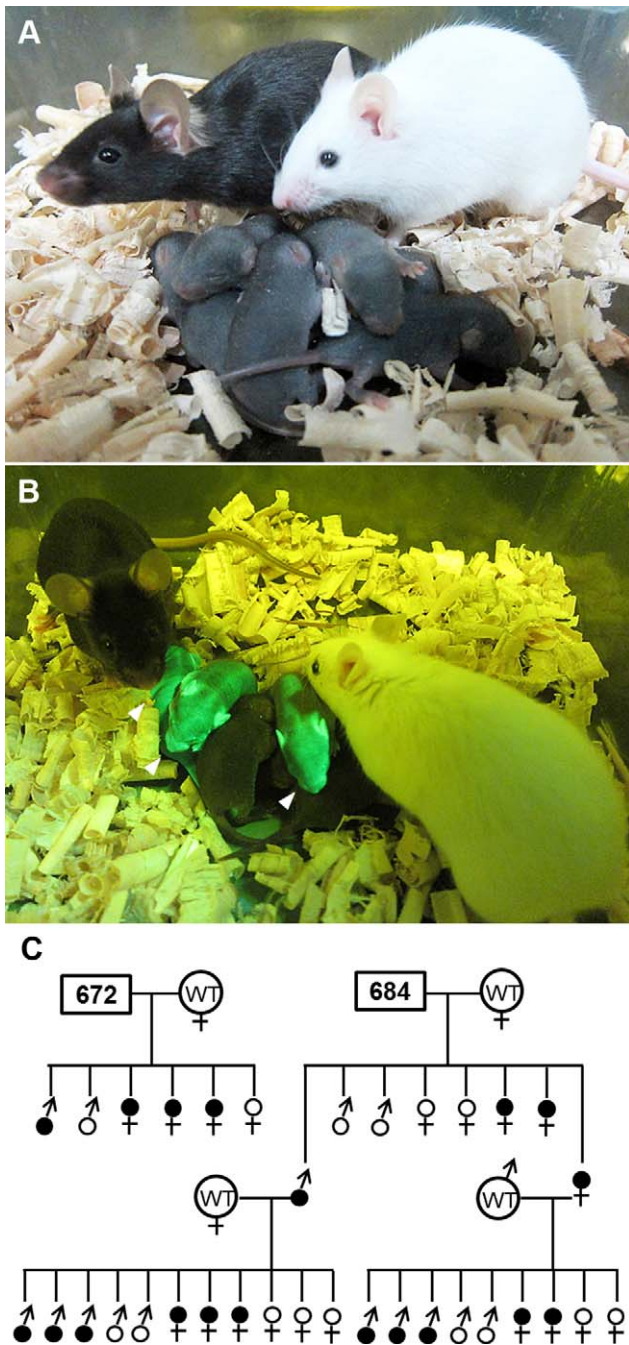


FIG. 5. Generation of offspring after transplantation of SSCs frozen in 2.5% PEG (MW 1000). **A**) Offspring from a C57BL/6 female crossed with a W/Wv recipient male that was transplanted with SSCs frozen in 2.5% PEG (MW 1000). **B**) The same individuals under UV exposure. EGFP+ pups (arrow heads) indicate complete donor spermatogenesis in recipient males. **C**) Pedigree for W/Wv recipient mice number 672 and 684 that were transplanted with germ cells frozen in 2.5% PEG (MW 1000) demonstrates that the F2 generation retained normal fertility. Solid symbols indicate progeny expressing EGFP.

the sequestration of water molecules by PEG may help protect the plasma membrane during freeze-thawing. Interestingly, it may be possible to improve the effectiveness of cryopreservation of testis cells enriched for SSCs in PEG by including cryoprotectant agents such as caspase inhibitor or Y-27632 that prevent apoptosis [34, 38–40].

Although recovery rates remained constant for testis cells enriched for SSCs frozen for 1 wk–3 mo, culture potential

gradually decreased over the same period. Although culture potential (determined as proliferation rate during 1 wk of culture) does not definitively evaluate SSC proliferation, given that >90% of cultured cells were germ cells and that long-term perpetuation of a culture is dependent on SSCs, it is likely that germ cell amplification is due to proliferation of SSCs. Theoretical models have argued that cryostability exists at  $-196^{\circ}\text{C}$  as long as photophysical events such as free radical formation due to background radiation and cosmic rays are minimized. Thus, living cells should be stable at  $-196^{\circ}\text{C}$  for thousands of years [41]. To determine if culture potential continued to decrease after 3 mo freezing time, in vitro proliferation rates were determined in thawed cells that were frozen for 6, 12, or 24 mo. Interestingly, testis cells enriched for SSCs that were frozen in 2.5% PEG (MW 1000) for 3–24 mo did not have different culture potentials, indicating that deleterious effects of cryopreservation are established during the first 3 mo of freezing. This result is corroborated by recent work indicating that mouse SSCs can retain functional fertility for at least 14 yr [19]. However, these experiments did not systematically evaluate different cryopreservation methods. Collectively, the current data demonstrate that freezing media containing 2.5% PEG (MW 1000) can maintain testis cells enriched for SSC for at least 24 mo.

To preserve an individual male's germ line, cryopreservation of germ cells must result in specific survival of SSCs. Because of the improved cryopreservation capacity observed in preliminary experiments, we chose to evaluate the cryoprotective capacity for SSCs using 2.5% PEG (MW 1000). Noncryopreserved testis cells enriched for SSCs and testis cells enriched for SSCs that were frozen in DMSO or 2.5% PEG (MW 1000) were thawed after 3 mo, cultured for 1 wk, and transplanted into recipient testes. No difference in colony number per  $10^5$  transplanted cells was observed, indicating that SSCs are not deleteriously affected by cryopreservation. When normalized to number of viable cells recovered, SSCs frozen in DMSO generated significantly fewer colonies than noncryopreserved SSCs or SSCs frozen in 2.5% PEG (MW 1000). Lack of a difference in germ cell colonization between noncryopreserved cells and those frozen in 2.5% PEG (MW 1000) was unexpected because thawed cells had approximately 50% of the in vitro culture (proliferative) potential of noncryopreserved cells. It is possible that the observed improvement of PEG versus DMSO is due to improved culture potential of SSCs frozen in PEG. However, given the fact that greater than 90% of all cultured cells were PLZF-positive germ cells, this difference may be due to a difference in the ratio of survival of SSCs to other testis cells, including differentiating spermatogonia in the germ cell populations between the noncryopreserved and cryopreserved cells. These data indicate the SSC may be more resistant to the negative effects of cryopreservation compared to other germ cells, which is consistent with the resistance of the SSC to irradiation or chemical insult [42–43]. Nevertheless, even if observed colony differences are due to different culture proliferation capacities, an increased culture potential indicates a more robust undifferentiated germ cell population.

Formation of colonies in recipient testes does not definitively evaluate the function of donor SSCs. Testis cells enriched for SSCs that were cryopreserved in 2.5% PEG (MW 1000) for 3 mo were thawed, cultured for 1 wk, and transplanted into testes of W/Wv recipient mice. After mating, recipient mice generated donor-derived offspring. Additionally, donor-derived offspring were able to generate viable offspring. These data indicate that the SSC is not adversely affected by cryopreservation in 2.5% PEG (MW 1000).



Previous work has shown that inclusion of DMSO in cryopreservation media allows for the long-term preservation of mouse SSCs [9]. It has recently been demonstrated that cryopreservation in media containing DMSO and 200 mM trehalose serves as a more effective cryoprotectant for murine SSCs than DMSO alone [21]. In the current study we directly compared the effect of 2.5% PEG (MW 1000) and 200 mM trehalose on the culture potential of cryopreserved cells. No significant difference was observed between these methods for cells cryopreserved up to 24 mo. However, when comparing the transplantation data between this study and previous work [21] with identical transplantation methods, it appears that the numbers of donor colonies of spermatogenesis were higher for cells frozen in 2.5% PEG (MW 1000) compared to 200 mM trehalose. Because of the similar data between 2.5% PEG (MW 1000) and trehalose, it would be of interest to systematically evaluate the efficacy of sugar molecules such as dextran and sucrose, which have been used previously to cryopreserve SSCs [16, 44], on SSC cryopreservation.

In conclusion, the data presented here indicate that cryopreservation of murine SSCs is significantly improved when a combination of 2.5% PEG (MW 1000), DMSO, and FBS is used as cryoprotectant. The methods developed here will serve as foundations for the long-term preservation of mammalian SSCs.

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