

Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (*Brachymystax lenok*)

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1 **Successful cryopreservation of spermatogonia in critically endangered Manchurian trout**
2 **(*Brachymystax lenok*)**

3
4 **Seungki Lee^{a,†}, Goro Yoshizaki^b**

5 ^aBiological and Genetic Resources Assessment Division, National Institute of Biological
6 Resources, Incheon 404-708, Korea

7 ^bDepartment of Marine Biosciences, Tokyo University of Marine Science and Technology,
8 Tokyo 108-8477, Japan

9
10 [†]Corresponding author:

11 Seungki Lee

12 Tel: +82-32-590-7120

13 Fax: +82-32-590-7472

14 Email: skmetany@gmail.com

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16 **Keywords:** Manchurian trout; Cryopreservation; Slow freezing; Spermatogonial transplantation;
17 Spermatogonial stem cells

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19 ¹

¹ **Abbreviations:** GSI, gonadosomatic index; NIBR, National Institute of Biological Resources; EG, ethylene glycol; PG, propylene glycol; Me₂SO, dimethyl sulfoxide; LN₂, liquid nitrogen; BSA, bovine serum albumin; GVC, Guava ViaCount; TB, trypan blue; dpt, days post-transplantation; SEM, standard error of the mean

Abstract

20
21
22 Because of the lack of cryopreservation techniques for fish eggs and embryos,
23 cryopreservation of fish spermatogonia and subsequent generation of eggs and sperm would be
24 an exit strategy for the long-term preservation of genetic resources. This study aimed to optimize
25 cryoprotectants, cooling rates, and thawing temperatures for slow freezing of spermatogonia from
26 endangered Manchurian trout (*Brachymystax lenok*). Whole testes were frozen with a
27 cryomedium containing 1.3 M methanol, 0.2 M trehalose, and 10% egg yolk at a cooling rate of
28 $-1^{\circ}\text{C}/\text{min}$ and then stored in liquid nitrogen for 2 days. After thawing at 30°C in a water bath,
29 testicular cells from thawed testes were intraperitoneally transplanted into allogeneic triploid
30 hatchlings. Transplanted spermatogonia migrated toward and were incorporated into recipient
31 gonads, where they underwent gametogenesis. Transplantation efficiency did not significantly
32 differ between frozen and fresh testes, demonstrating that Manchurian trout spermatogonia can be
33 successfully cryopreserved in liquid nitrogen.

34

35 Manchurian trout, *Brachymystax lenok* Li 1996, is a landlocked salmonid fish that
36 inhabits the upstream regions of East Asia and is listed as an endangered species in both Korea
37 and China [8,9]. A subspecies of the Manchurian trout, *B. lenok tsinlingensis* has an extremely
38 restricted distribution, for which the Nakdong River (Korea) defines the southernmost limit,
39 where it has a small population size and suffers from habitat fragmentation because of the effects
40 of climate change and habitat destruction [9]. This species and the regional population of
41 Manchurian trout are seriously facing extinction; thus, measures for preserving genetic resources
42 are urgently required.

43 Gamete or embryo cryopreservation could be an effective solution for the long-term
44 preservation of genetic resources. However, fish eggs and embryos are too large to be
45 successfully cryopreserved using current techniques [1,2,10]. Therefore, maternally inherited
46 materials, including mitochondrial DNA, cannot be preserved using these techniques. We
47 recently described a new method for deriving functional eggs and sperm from type A
48 spermatogonia isolated from cryopreserved whole testes of rainbow trout [6]. This study aimed to
49 establish a reliable and simple cryopreservation protocol for spermatogonia of the Manchurian
50 trout.

51 Experiments were conducted according to the Guidelines for the Care and Use of
52 Laboratory Animals by the National Institute of Biological Resources (NIBR; Incheon, Korea).
53 Manchurian trout (*B. lenok*) were obtained from a local trout hatchery (Yangyang-gun, Korea) in
54 May 2012 and maintained at NIBR to use as donor and recipient fish. Immature testes [testis
55 weight, 0.019 ± 0.002 g; gonadosomatic index (GSI), $0.040\% \pm 0.003\%$] isolated from 10-month-
56 old Manchurian trout donors (standard length, 12.3 ± 1.9 cm) were prepared for equilibrium slow
57 freezing. Whole testes were transferred into 2 mL cryovials (Corning, Sigma-Aldrich) containing

58 1 mL cryomedium (pH 7.8) that comprised permeating cryoprotectants: methanol, ethylene
59 glycol (EG), propylene glycol (PG), dimethyl sulfoxide (Me₂SO), or glycerol with 1.0, 1.3, or 1.6
60 M concentrations. We investigated four nonpermeating cryoprotectants: D-glucose, D-lactose
61 monohydrate, D-(+)-trehalose dehydrate, or D-(+)-raffinose pentahydrate with 0.1, 0.2, or 0.3 M
62 concentrations. We also tested 10% (v/v) fresh hen egg yolk vs. 1.5% (w/v) bovine serum
63 albumin (BSA). The basal recipe of the cryomedium was reported previously [6]. The samples
64 were equilibrated on ice for 60 min, then cooled at a rate of $-0.5^{\circ}\text{C}/\text{min}$, $-1^{\circ}\text{C}/\text{min}$, $-10^{\circ}\text{C}/\text{min}$,
65 or $-20^{\circ}\text{C}/\text{min}$ to -80°C using a computer-controlled rate freezer (IceCube 14S; SY-LAB). After
66 cooling, the samples were plunged into liquid nitrogen (LN₂) and were stored for 1 day, then
67 thawed at 10, 20, 30, or 40°C in a water bath. Thawed testes were rehydrated in three changes of
68 L-15 medium (Life Technologies, pH 7.8) containing 10% (v/v) FBS (Invitrogen). Extender was
69 formulated as previously described [6]; all reagents for cryopreservation were purchased from
70 Sigma-Aldrich unless otherwise stated.

71 To assess testicular cell viability, testes were trypsinized as previously described [7]. The
72 cell suspension was filtered through a 42- μm nylon screen (N-No. 330T; Tokyo Screen Company,
73 Japan) and re-suspended in Guava ViaCount (GVC) reagent (Guava Technologies, USA) to
74 count viable cells using CytoSoft software (Guava Technologies, USA). Viable cells were also
75 identified with the trypan blue (TB) exclusion test. On establishing that the total numbers of
76 testicular cells did not significantly differ between both testes of a Manchurian trout ($39.8 \pm 3.0 \times$
77 10^5 vs. $41.0 \pm 2.2 \times 10^5$, $n = 4$, $P < 0.05$), the numbers of testicular cells in frozen and fresh testes
78 were compared to determine cryopreserved testicular cell viability ($n = 4-5$). Viability was
79 calculated as follows: viability (%) = [(GVC (+) + TB (-) cells in frozen testis)/(GVC (+) cells in
80 fresh testis)] $\times 100$.

81 To determine whether spermatogonia were recoverable from thawed testes,
82 transplantation assays were performed as previously described [7]. Whole testes were
83 equilibrated with cryomedium optimized in the preceding experiment and cooled at a rate of
84 $-1^{\circ}\text{C}/\text{min}$ for 90 min using a slow-freezing container (CoolCell FTS30, USA) located in a -80°C
85 freezer before being plunged into LN_2 . After storage in LN_2 for 2 days, the cryovials were thawed
86 at 30°C in a water bath. Testicular cells obtained from thawed testes were labeled with a
87 fluorescent dye (PKH26 Cell Linker Kit, Sigma-Aldrich) to detect the donor cells in recipient
88 gonads [5]; sterile triploid recipients were produced by heat shock of fertilized eggs at 28°C for
89 10 min subsequent to 15-min postfertilization and were then allowed to develop in environmental
90 water at 10°C . Intraperitoneal transplantation was performed by microinjecting approximately 5
91 $\times 10^4$ PKH26-labeled cells (Fig. 2A and B) into hatchlings of triploid Manchurian trout (41–42
92 dpf). As control, cells harvested from fresh testes were also microinjected. At 25, 40, 151, and
93 558 days post-transplantation (dpt), the recipients were dissected; their gonads were examined
94 with fluorescence microscopy (BX-53, Olympus). Because the transplantation efficiency [ratios
95 at 21 dpt, $79.2\% \pm 4.0\%$: $77.8\% \pm 3.5\%$, $n = 33$, $P < 0.05$] did not significantly differ between the
96 testes of a given Manchurian trout, the transplantation efficiencies of testicular cells from frozen
97 and fresh testes were compared to determine the transplantability of cryopreserved testicular cells
98 ($n = 15\text{--}37$). Ratios of recipients that possessed PKH26-labeled cells within their gonads at 25
99 and 40 dpt and the number of incorporated PKH26-labeled cells at 25 dpt were recorded. Ratios
100 of recipients that possessed differentiating cells within their gonads were also examined at 151
101 and 558 dpt. The colonization, proliferation, and differentiation efficiencies of donor-derived
102 spermatogonia in the recipient gonads were calculated by the formulae: colonization rate
103 $(\%) = [(\text{number of fish incorporating PKH26-labeled cells at 25 dpt})/(\text{number of fish$

104 observed)] \times 100; proliferation rate (%) = [(number of fish proliferating PKH26-labeled cells at 40
105 dpt)/(number of fish observed)] \times 100; differentiation rate (%) = [(number of fish having mature
106 gonads at 558 dpt)/(number of fish observed)] \times 100.

107 To determine the maturational stage of each gonad at 151 dpt, the middle portions of the
108 gonadal fragments were fixed in Bouin's solution, embedded in paraffin, sectioned at 5- μ m
109 thickness, and stained with hematoxylin and eosin (H&E). Furthermore, to determine the ploidy
110 level of recipients, blood cells were fixed in 70% (v/v) ethanol and incubated for 8 h in PBS (pH
111 7.8) that contained RNase A (10 μ g/ml; Sigma) and propidium iodide (200 μ g/ml; Sigma). DNA
112 contents were analyzed using a Guava PCA-96 flow cytometry system (Millipore).

113 Data are presented as mean values \pm standard error of the mean (SEM) derived from three
114 independent experiments. Statistical significance was determined using the Student's *t*-test for
115 comparisons between groups. For comparisons among more than two groups, statistical
116 significance was determined using one-way ANOVA, followed by a Tukey test.

117 When whole testes were frozen at a cooling rate of $-1^{\circ}\text{C}/\text{min}$ and thawed at 10°C in a
118 water bath, the viability of testicular cells frozen with cryomedium containing 1.3 M methanol
119 was significantly higher than that frozen with cryomedium containing 1.3 M EG, 1.3 M PG, or
120 1.3 M glycerol (Fig. 1A). Of the testes frozen with cryomedium containing methanol or Me_2SO
121 at 1.0, 1.3, or 1.6 M concentrations, the highest survival rate of testicular cells was obtained for
122 those frozen with 1.3 M methanol (Fig. 1B). Nonpermeating cryoprotectants dissolved in
123 cryomedium containing 1.3 M methanol were also assessed. The highest survival rate was
124 observed for cells obtained from testes cryopreserved in cryomedium containing 0.1 M trehalose
125 and 10% egg yolk (Fig. 1C). Of the testes frozen with cryomedium containing lactose or
126 trehalose at 0.1, 0.2, or 0.3 M concentrations, cells obtained from those frozen with 0.2 M

127 trehalose demonstrated the highest survival rate (Fig. 1D). Next, the effects of cooling rates on
128 cell viability were examined. Cooling rates of $-0.5^{\circ}\text{C}/\text{min}$ and $-1^{\circ}\text{C}/\text{min}$ produced significant
129 increases in cell viability relative to other groups; the highest survival rate occurred with a
130 cooling rate of $-1^{\circ}\text{C}/\text{min}$ (Fig. 1E). Cell viability of whole testes frozen at a cooling rate of
131 $-1^{\circ}\text{C}/\text{min}$ with a cryomedium containing 1.3 M methanol, 0.2 M trehalose, and 10% egg yolk
132 was assessed with thawing temperatures of 10, 20, 30, or 40°C . The highest survival of testicular
133 cells ($81.0\% \pm 1.3\%$) was obtained by thawing at 30°C in a water bath (Fig. 1F).

134 To determine whether spermatogonia possessing the ability to transdifferentiate into
135 oocytes [6,7] were recovered from thawed testes, the transplantation efficiency was compared
136 between frozen and fresh groups. Recipients were dissected at 25, 40, 151, and 558 dpt; PKH26-
137 labeled donor cells were examined (Fig. 2B). Although red fluorescence was never observed in
138 the gonads of 50 non-transplanted fish (Fig. 2C), frozen/thawed cells labeled with PKH26 were
139 detected in the gonads of 88/104 recipients at 25 dpt (Fig. 2D); the cells rapidly proliferated in
140 the gonads of 71/98 recipients at 40 dpt (Fig. 2F). Moreover, similar transplantation efficiencies
141 were observed using freshly prepared PKH26-labeled cells (Fig. 2E and G). However, continued
142 proliferation of PKH26-labeled cells resulted in a loss of fluorescence in gonads of all recipients
143 at 151 dpt (0/97). Therefore, we performed histological analysis of each gonad at 151 dpt. In the
144 non-transplantation group, the gonads of the triploid fish (6/6) contained only immature germ
145 cells without advanced germ cells (Fig. 2H), whereas the ovaries of the female triploid recipients
146 that received frozen spermatogonia (4/6) contained peri-nucleolus-stage oocytes and oogonia (Fig.
147 2I). Next, we examined gonads of recipients at 558 dpt, which were reared to the pre-spawning
148 stage. As shown in Fig 2J, gonads in all 45 triploid fish that did not receive spermatogonia
149 remained immature (gonad weight, 1.531 ± 0.240 g; GSI, $0.339 \pm 0.062\%$); however, Fig. 2K

150 demonstrates that gonads in 38/61 triploid recipients that received frozen spermatogonia had
151 maturing gonads (testis weight, 11.327 ± 2.312 g; GSI, $2.658\% \pm 0.570\%$; ovary weight, $9.708 \pm$
152 3.175 g; GSI, $2.355\% \pm 0.780\%$). Efficiencies of colonization ($84.1\% \pm 7.4\%$), proliferation
153 ($72.3\% \pm 5.7\%$), and differentiation ($60.7\% \pm 7.1\%$) and the numbers of incorporated
154 spermatogonia (3.6 ± 2.0) did not significantly differ between the frozen and fresh groups (Table
155 1). All recipients were identified as triploids using flow cytometry (Fig. 2L), with the exception
156 of five recipients in which triploidy induction failed; these five were not used in this study.

157 To save Manchurian trout from extinction, it is urgent to develop cryopreservation
158 methods for long-term preservation of genetic resources. We successfully established a
159 cryopreservation methodology for spermatogonia using the Manchurian trout, as evidenced by
160 81.0% viability of frozen testicular cells with the ability to derive vitellogenic oocytes and with
161 transplantation efficiencies that did not significantly differ from the efficiencies for cells derived
162 from fresh testes. To our knowledge, this is the first study to report cryopreservation methods for
163 the Manchurian trout germ cells.

164 Previous studies distinguish live germ cells from somatic cells using *vasa*-GFP transgenic
165 constructs [6,7] and GFP-*nos1* 3'UTR chimeric RNAs [3,4]; visualized germ cells were viability
166 indicators in those studies. However, these techniques have not been developed for most
167 endangered fish species, including the Manchurian trout. We determined viability by comparing
168 results from frozen and fresh testes. Both sides of testes within a Manchurian trout were used
169 because the total numbers of testicular cells and transplantation efficiencies did not significantly
170 differ between the two testes. The method used can be directly applied to determine testicular cell
171 viability within endangered fish species, although further investigation is required for different
172 fish species.

173 Here, we optimized a protocol for the slow freezing of whole testes from Manchurian
174 trout using cryoprotectants comprising 1.3 M methanol, 0.2 M trehalose, and 10% egg yolk. This
175 protocol originated from a protocol used for testis cryopreservation in rainbow trout [6]. There
176 may be large differences in how testes from different species respond to permeating
177 cryoprotectants; namely, 1.3 M methanol and 1.3 M Me₂SO were the best permeating
178 cryoprotectants for testicular cells of Manchurian trout and rainbow trout, respectively. Although
179 it is well known that the optimal type of cryoprotectant is species specific [10], these results
180 might be because the testes used for cryopreservation in this study (testis weight, 19 ± 2 mg)
181 were larger than those in rainbow trout (testis weight, 14 ± 1 mg) [6]. Owing to the lower
182 molecular weight of methanol, it penetrates Manchurian trout testes more rapidly than Me₂SO,
183 and thus, the cryoprotectant molecules may reduce the intracellular ice formation leading to cell
184 death. These results could have practical implications for the selection of optimal cryoprotectants
185 for spermatogonial cryopreservation.

186 We recently initiated a project to cryopreserve whole testes of Manchurian trout trapped
187 in the Nakdong River and believe this effort will significantly contribute to conservation and
188 restoration of the endangered Manchurian trout.

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Acknowledgments

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Figure Legends

227 **Figure 1. Optimization of freezing conditions for testicular cells from the Manchurian trout.**

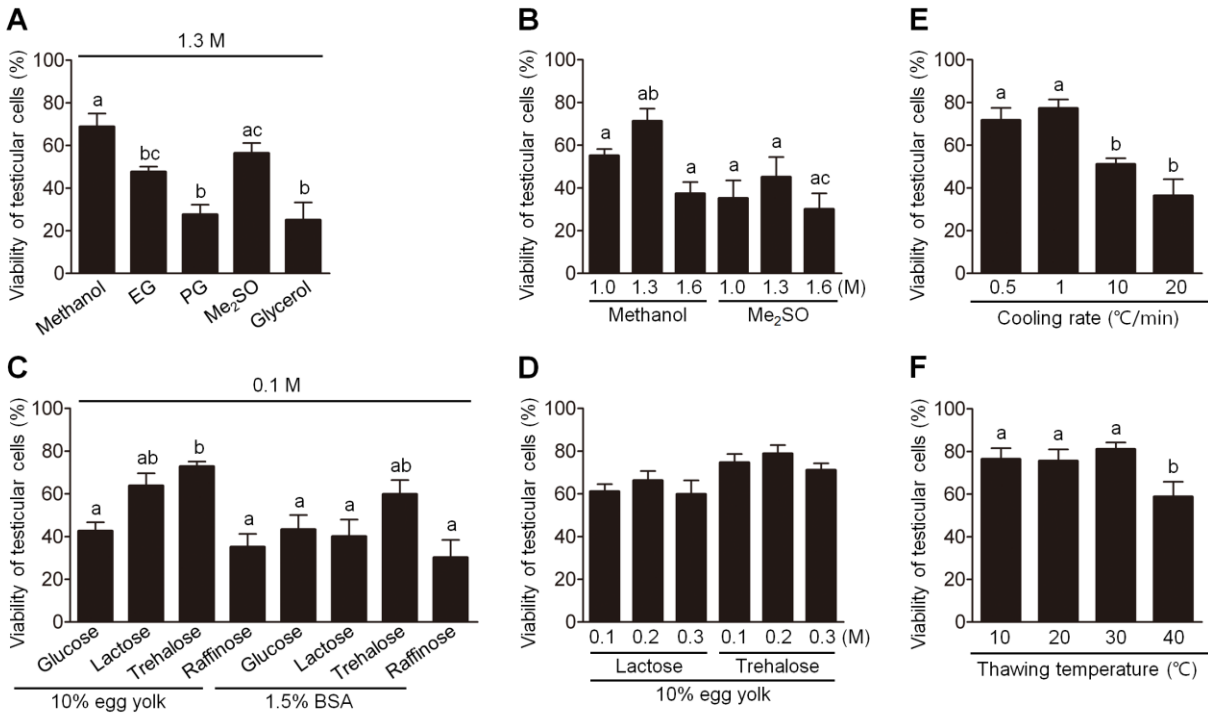
228 **(A)** Viability of testicular cells with cryomedium containing 1.3 M methanol, ethylene glycol
229 (EG), propylene glycol (PG), dimethyl sulfoxide (Me₂SO), or glycerol. **(B)** Viability of testicular
230 cells with cryomedium containing methanol or Me₂SO at 1.0, 1.3, or 1.6 M concentrations. **(C)**
231 Viability of testicular cells with cryomedium containing 0.1 M glucose, 0.1 M lactose, 0.1 M
232 trehalose, or 0.1 M raffinose with egg yolk or BSA. **(D)** Viability of testicular cells with
233 cryomedium containing lactose or trehalose at 1.0, 1.3, or 1.6 M concentrations with egg yolk. **(E)**
234 Viability of testicular cells at cooling rates of $-0.5^{\circ}\text{C}/\text{min}$, $-1^{\circ}\text{C}/\text{min}$, $-10^{\circ}\text{C}/\text{min}$, or $-20^{\circ}\text{C}/\text{min}$.
235 **(F)** Viability of testicular cells after thawing at 10, 20, 30, or 40°C. Columns represent mean \pm
236 SEM ($n = 4-5$). Columns with different letters are significantly different with $P < 0.05$.

237

238 **Figure 2. Transplantation of thawed testicular cells. (A,B)** Thawed testicular cells labeled with

239 red fluorescent dye (PKH26) in the bright-field **(A)** and fluorescent view **(B)**. **(C)** Gonad of a
240 non-transplanted triploid fish as a control of D and E. **(D-G)** Frozen/thawed and freshly prepared
241 PKH26-labeled donor cells were incorporated into the recipient gonads **(D,E)** and rapidly
242 proliferated **(F,G)**. **(H,I)** H&E-stained histological section of gonads from a non-transplanted
243 triploid fish **(H)** and ovaries from a female triploid recipient that received frozen spermatogonia
244 **(I)**. **(J)** Immature ovary of a non-transplanted triploid fish as a control of K. **(K)** Triploid fish,
245 which received frozen spermatogonia, had ovaries that possessed a large colony of differentiating
246 oocytes. **(L)** DNA contents of a diploid Manchurian trout (upper panel) and triploid recipient
247 (lower panel). Arrows indicate the gonads **(C-G,J,K)**. Scale bars, 20 μm **(A-H)**, 50 μm **(I)**, 2
248 mm **(J,K)**.

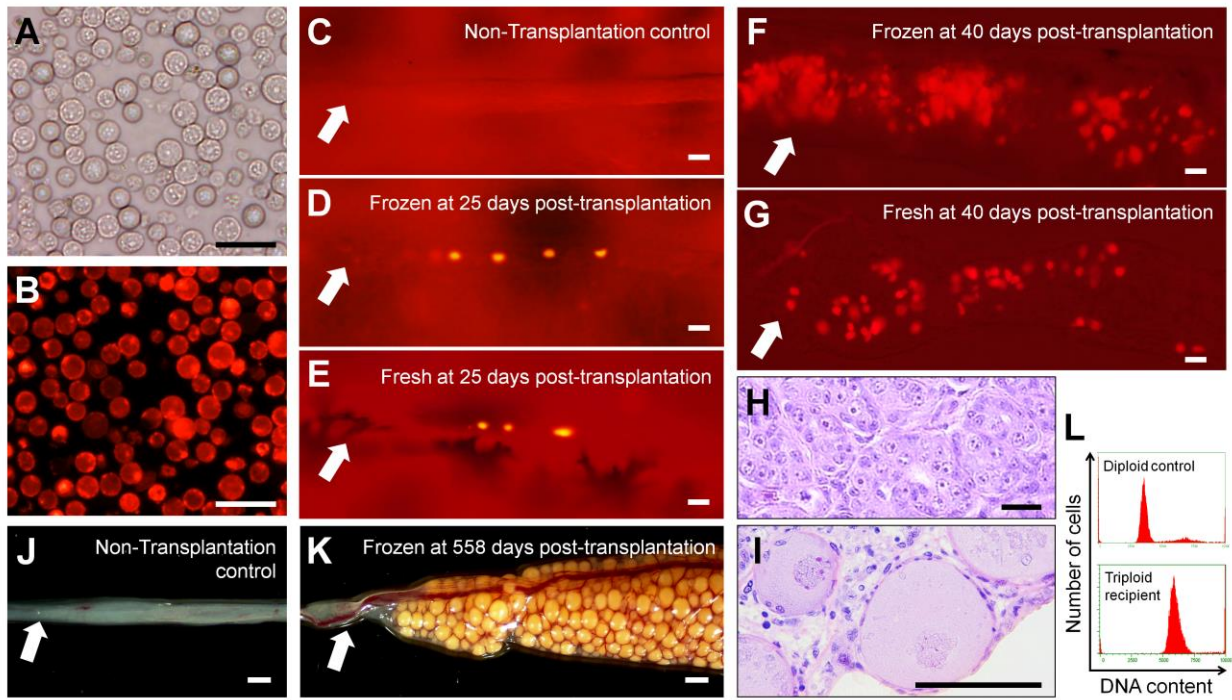
250 Fig. 1



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252

253 Fig. 2



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255

256 **Table 1. Colonization, proliferation, and differentiation of Manchurian trout spermatogonia in**
 257 **recipient gonads.**

Group	No. of fish transplanted	No. of fish survived ^d	Colonization rate (%)	No. of colonized cells	Proliferation rate (%)	Differentiation rate (%)
Frozen ^a	105	104	84.1 ± 7.4 ^e	3.6 ± 2.0 ^e	72.3 ± 5.7 ^e	60.7 ± 7.1 ^e
Fresh ^b	100	98	89.0 ± 5.5 ^e	4.5 ± 2.1 ^e	75.0 ± 4.2 ^e	53.2 ± 7.0 ^e
Control ^c	50	50	0 ^f	0 ^f	0 ^f	0 ^f

^a Triploid Manchurian trout recipients received spermatogonia cryopreserved for 2 days.

^b Triploid Manchurian trout recipients received freshly prepared spermatogonia.

^c Triploid Manchurian trout that did not receive spermatogonia.

^d Number of viable recipients at 25 days post-transplantation.

^{e,f} Values in a column with different superscripts are significantly different ($P < 0.05$).

Values are shown as mean ± SEM derived from three independent experiments.

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