Production of fertile zebrafish (Danio rerio) possessing germ cells (gametes) originated from primordial germ cells recovered from vitrified embryos

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Abstract

This study aimed to produce fertile zebrafish (Danio rerio) possessing germ cells (gametes) that originated from cryopreserved primordial germ cells (PGCs). First, to improve the vitrification procedure of PGCs in segmentation stage embryos, dechorionated yolk-intact and yolk-removed embryos, the PGCs of which were labeled with green fluorescent protein, were cooled rapidly after serial exposures to equilibration solution (ES) and vitrification solution (VS), which contained ethylene glycol, DMSO, and sucrose. Yolk removal well prevented ice formation in the embryos during cooling and improved the viability of cryopreserved PGCs. The maximum recovery rate of live PGCs in the yolk-removed embryos vitrified after optimum exposure to ES and VS was estimated to be about 90%, and about 50% of the live PGCs showed pseudopodial movement. Next, to elucidate the ability of cryopreserved PGCs to differentiate into functional gametes, PGCs recovered from the yolk-removed embryos (striped-type) that were vitrified under the optimum exposure to ES and VS were transplanted individually into 218 sterilized recipient blastulae (golden-type). Two days after the transplantation, 7.5% (14/187) of morphologically normal embryos had PGC(s) in the genital ridges. Six (5 males and 1 female) of the 14 recipient embryos developed into mature fish and generated progeny with characteristics inherited from PGC donors. In conclusion, we demonstrated the successful cryopreservation of PGCs by vitrification of yolk-removed embryos and the production of fertile zebrafish possessing germ cells that originated from the PGCs in vitrified embryos.

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Introduction

The zebrafish (Danio rerio) has become an important animal model in science (Beis & Stainier 2006). However, there is an urgent need to develop efficient protocols for cryopreservation of zebrafish gametes and embryos due to their innumerable mutants (Mazur et al. 2008, Robles et al. 2009). Zebrafish sperm has been cryopreserved with a degree of success (Yang & Tiersch 2009). Although a few successful attempts to cryopreserve embryos have been reported for several fish species, including the zebrafish (Chao & Liao 2001), common carp, (Zhang et al. 1989), and flounder (Chen & Tian 2005, Robles et al. 2005), a reproducible cryopreservation method for fish oocytes and embryos has not been developed due to their large size, high sensitivity to chilling, and low membrane permeability (Zhang 2004, Robles et al. 2008).

Cryopreservation of zebrafish primordial germ cells (PGCs) may provide an alternative to cryopreservation of gametes and embryos because functional sperm and oocytes can be obtained from germline-replaced chimeras produced by the single PGC transplantation (SPT) method (Saito et al. 2008a), in which green fluorescent protein (GFP)-labeled PGCs isolated from segmentation stage (10- to 15-somite stage) embryos are transplanted individually into sterilized recipient blastulae heterochronically. Cryopreservation of isolated PGCs seems not to be an option because of the limited number of PGCs (about 20 cells/embryo) in segmentation stage zebrafish embryos (Nagai et al. 2001) and inevitable loss of PGCs during isolation. In rainbow trout, isolated genital ridges containing PGCs were frozen in order to avoid loss of PGCs, and germline chimeras were produced by transplantation of the PGCs recovered from thawed genital ridges (Kobayashi et al. 2007). Zebrafish PGCs recovered from the genital ridge cannot be used for SPT because PGCs that dissociate...
before the 20-somite stage move to the genital ridge in host embryogenesis, whereas those after that stage mostly do not (Yamaha et al. 2007).

To simplify the cryopreservation procedure and to diminish the loss of PGCs during cooling, warming, and handling of embryos, we vitrified zebrafish dechori- nated whole embryos at the 14- to 20-somite stage (Higaki et al. 2009, 2010) because before this stage, embryos are susceptible to mechanical damage (West- field 2007a). The maximum survival rates of PGCs recovered from embryos vitrified using solutions containing ethylene glycol (EG) and DMSO in combination with sucrose were estimated to be about 40 and 20% respectively (Higaki et al. 2009, 2010). However, most of the PGCs recovered from vitrified–warmed embryos did not show pseudopodial movement, and thus, we did not determine their differentiation ability by transplantation into recipient embryos. The low viability of the PGCs observed in our previous studies suggested that the PGCs suffer cryoinjuries because of the limited cryoprotectant permeation due to the presence of the yolk syncytial layer (YSL), which surrounds the yolk and prevents the permeation of the cryoprotectant into zebrafish embryos (Hagedorn et al. 1998). Toxicities of cryoprotectants at high concentrations may also have been responsible for the poor survival of PGCs in our previous studies because both EG and DMSO had high embryo toxicity (Higaki et al. 2009, 2010). Permeation of the cryoprotectant into zebrafish embryos can be enhanced by partial yolk removal (Liu et al. 2001), and toxic effects of cryoprotectant solutions can be limited by using a mixture of two or more cryoprotectants (Vajta & Kuwayama 2006). Large tissue specimens (1×10 ×10 mm) such as bovine and human ovarian tissues have been successfully vitrified using a mixture of EG, DMSO, and sucrose after stepwise exposures to equilibration solution (ES) and vitrification solution (VS) in the Cryotissue method (Kagawa et al. 2009).

This study aimed to improve the viability of zebrafish PGCs recovered from vitrified embryos and to produce fertile fish possessing germ cells (gametes) that were originated from PGCs recovered from vitrified embryos. First, we examined the effects of yolk removal and periods of embryo exposure to ES and VS in the Cryotissue method on viability of PGCs in vitrified embryos. Next, we elucidated the differentiation ability of PGCs recovered from vitrified–warmed embryos by transplanting them into sterilized recipient embryos.

Results

Experiment 1: viability of embryonic cells and PGCs recovered from cryopreserved embryos

Non-GFP-treated dechorionated embryos were used for assessing ice formation during cooling and survival of embryonic cells. For assessing survival of PGCs, GFP-labeled dechorionated embryos were cryopreserved following serial exposures to ES and VS in which embryos did not show ice formation during cooling, because few embryos that showed ice formation during cooling had live PGCs in our previous study (Higaki et al. 2010). Viability of embryonic cells and PGCs was assessed by trypan blue staining.

Yolk-intact embryos

Ice formation during cooling and the proportions of live cells/numbers of live PGCs recovered from warmed yolk-intact embryos are summarized in Table 1. Cryopreserved yolk-intact embryos were degraded after warming, regardless of the exposure protocol and ice formation during cooling. However, yolk-intact embryos that showed ice formation during cooling had lower cell survival rates (0.8–25.9%) than those without ice formation (82.4 and 83.4%; P<0.01).

Table 1 Ice formation during cooling and proportions of live cells/numbers of live primordial germ cells (PGCs) recovered from the yolk-intact zebrafish embryos cooled rapidly after serial exposure to equilibration solution (ES) and vitrification solution (VS).

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>ES</th>
<th>VS</th>
<th>Ice formation</th>
<th>Cell survival rate (%)</th>
<th>Number of live PGCs (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>+</td>
<td></td>
<td>0.8±0.7&lt;sup&gt;a,A&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td></td>
<td>18.2±5.2&lt;sup&gt;b,A&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td></td>
<td>14.6±7.4&lt;sup&gt;a,A&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>+</td>
<td></td>
<td>1.6±1.0&lt;sup&gt;a,A&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td></td>
<td>14.4±6.3&lt;sup&gt;b,A&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>±</td>
<td>+</td>
<td></td>
<td>18.6±0.9&lt;sup&gt;a,A&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>+</td>
<td></td>
<td>25.9±3.2&lt;sup&gt;b,B&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>±</td>
<td>−</td>
<td></td>
<td>83.4±11.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>±</td>
<td>−</td>
<td></td>
<td>82.4±7.5&lt;sup&gt;B&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>None (control)</td>
<td></td>
<td>100</td>
<td></td>
<td>ND</td>
<td>11.3±2.2&lt;sup&gt;c&lt;/sup&gt; (8–16)</td>
</tr>
</tbody>
</table>

<sup>a</sup>, <sup>b</sup>, <sup>c</sup>, <sup>d</sup>, <sup>e</sup>, <sup>f</sup>: ice formation was observed in all, some and none of nine embryos during cooling respectively. ND, not determined.

<sup>a</sup>–<sup>d</sup>Values (means±s.d. of three replicates) with different superscripts within the same exposure period to ES differ significantly (P<0.05).<sup>A</sup>–<sup>B</sup>Values with different superscripts within the same exposure period to VS differ significantly (P<0.05).<sup>a</sup>–<sup>e</sup>Values (means±s.d. of ten embryos) with different superscripts within the same column differ significantly (P<0.05).
Table 2 Ice formation during cooling and proportions of live cells/numbers of live primordial germ cells (PGCs) recovered from the yolk-removed zebrafish embryos cooled rapidly after serial exposure to equilibration solution (ES) and vitrification solution (VS).

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>Ice formation</th>
<th>Cell survival rate (%)</th>
<th>Number of live PGCs (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ES</td>
<td>VS</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>+</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+</td>
<td>24.0±19.3</td>
</tr>
</tbody>
</table>
|                     | 10 | −  | 59.0±0.3        | 8.3±2.5
|                     | 15 | +  | 3.6±2.4         | 10.6±3.3 | (4–16) | 5.0±1.8
|                     | 10 | −  | 51.0±12.4       | 8.5±3.4 | 5.0±1.8
|                     | 30 | −  | 59.3±7.8
|                     | 1  | +  | 19.2±10.6       | ND    | ND            |
|                     | 5  | −  | 86.9±8.2
|                     | 10 | −  | 60.5±5.1        | 7.9±3.1 | (2–14) | 2.9±2.6
| None (control)      |    |    | 100             | 11.8±2.7 | (8–15) | 10.0±2.5

+, ±, −: ice formation was observed in all, some and none of nine embryos during cooling respectively. ND, not determined. Values (means ± S.D. of three replicates) with different superscripts within the same exposure period to VS differ significantly (P<0.05). Values with different superscripts within the same exposure period to ES differ significantly (P<0.05). Values (means ± S.D. of ten embryos) with different superscripts within the same column differ significantly (P<0.05).

There was an interaction between the effects of the periods of exposure to ES and VS on the embryonic cell survival rates (P<0.01). Most embryos pretreated with ES for 10 or 15 min showed ice formation during cooling and had lower embryonic cell survival rates than the embryos pretreated with ES for 30 min, regardless of the period of exposure to VS (P<0.01). Embryos cryopreserved after serial exposure to ES for 30 min and VS for 5 or 10 min did not show ice formation during cooling, and they had higher embryonic cell survival rates than the embryos cryopreserved using other exposure protocols (P<0.01). Although all embryos in these two experimental groups had live PGCs, the mean numbers of live PGCs (3.9 and 5.5 cells/embryo) were smaller than that of fresh control embryos (11.3 cells/embryo; P<0.01), and few cryopreserved embryos had live PGCs with pseudopodial movement.

Yolk-removed embryos

Ice formation during cooling and the proportions of live cells/numbers of live PGCs recovered from warmed yolk-removed embryos are summarized in Table 2. Yolk-removed embryos that showed ice formation during cooling had lower embryonic cell survival rates (P<0.01) and more severe degradation (Fig. 1A) than the embryos cooled without ice formation. Yolk-removed embryos pretreated with ES for 10 min needed 10 min exposure to VS for preventing ice formation during cooling, whereas those pretreated with ES for 15 or 30 min did not form ice even after exposure to VS for 5 min. Yolk-removed embryos that did not show ice formation during cooling scaled off their surface and became leathery (Fig. 1B) except the embryos cryopreserved after serial exposure to ES for 15 min and VS for 5 min, which had a smooth surface, firm embryonic body, and clear somite structure (Fig. 1C).

Figure 1 Gross morphology of yolk-removed striped-type zebrafish embryos at the 15-somite stage after rapid cooling and warming. (A) An embryo that showed ice formation during cooling and has a severely degraded embryonic body. (B) An embryo that did not show ice formation during cooling and has a leathery surface embryonic body. (C) An embryo that was cooled rapidly after exposure to ES for 15 min and VS for 5 min, did not show ice formation during cooling, and has a smooth surface embryonic body and clear somite structure. Bar=250 μm (A–C).
yolk-removed embryos was estimated to be about 90% (10.6 cells/11.8 cells) based on the mean number of live PGCs in fresh embryos. Embryos cryopreserved after serial exposure to ES for 15 min and VS for 5 min had a relatively large number of live PGCs with pseudopodial movement (5.0 cells/embryo) compared with the cryopreserved embryos in other experimental groups (2.0–3.5 cells/embryo). The maximum recovery rate of live PGCs with pseudopodial movement of yolk-removed embryos was about 50% (5.0 cells/10.0 cells) based on the mean number of live PGCs with movement in fresh embryos.

Experiment 2: differentiation ability of PGCs recovered from vitrified embryos

We cryopreserved GFP-labeled striped-type zebrafish embryos after removal of the yolk and serial exposure to ES for 15 min and VS for 5 min. Fluorescent PGCs recovered from vitrified–warmed embryos were transplanted individually into sterilized golden-type zebrafish blastulae. As shown in Table 3, two days after the transplantation, fluorescent PGCs were observed in 34.8% (65/187) of morphologically normal embryos, and 21.5% (14/65) of the embryos had fluorescent PGC(s) in the genital ridges (Fig. 2A and B); one embryo had two fluorescent PGCs at the genital ridge.

Seven of the 14 recipient embryos that had live PGCs in the genital ridge grew into phenotypically golden-type mature fish (Fig. 2C and D), and the remaining recipient fish died. Six (5 males and 1 female) of the mature fish generated a large number of F1 progeny with the characteristics inherited from striped-type PGCs (Fig. 2E and F) over 3–5 cycles of natural mating with normal golden-type mature fish. These six fertile fish had one large gonad and one small gonad (Fig. 3A and C); however, the remaining one phenotypically male fish had bilateral small gonads without germ cells. Spermatogenesis was observed in large gonads (Fig. 3B) but not in small gonads of all fertile male fish. In the fertile female fish, oogenesis was observed in both the large (Fig. 3D) and small gonads.

### Discussion

The low cell survival rates obtained from ice-forming embryos during cooling, regardless of the yolk status, confirmed the necessity of rapid cooling without ice formation (i.e. vitrification) for achieving successful cryopreservation of PGCs, as intracellular ice formation causes serious damage to cells (Mazur 1984, Fowler & Toner 2005). Yolk-intact embryos that did not show ice formation during cooling had high embryonic cell survival rates (about 80%), but had relatively small numbers of live PGCs (3.9 and 5.5 cells/embryo) after warming. This might have resulted from ice formation that was not detected by visual inspection in part of the embryonic body around the PGCs during cooling and/or freezing.

### Table 3 Localization of transplanted primordial germ cells (PGCs) recovered from vitrified embryos at 2 days post transplantation.

<table>
<thead>
<tr>
<th>Trial number</th>
<th>Number of transplanted embryos</th>
<th>Number (%) of normal embryos</th>
<th>Number (%) of embryos with PGC(s) at Total</th>
<th>Ectopic</th>
<th>Genital ridges</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56</td>
<td>48 (85.7)</td>
<td>18 (37.5)</td>
<td>14 (29.2)</td>
<td>4 (8.3)</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
<td>78 (95.1)</td>
<td>24 (30.8)</td>
<td>18 (23.1)</td>
<td>6 (7.7)</td>
</tr>
<tr>
<td>3</td>
<td>41</td>
<td>36 (87.8)</td>
<td>14 (38.9)</td>
<td>12 (33.3)</td>
<td>2 (5.6)</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>25 (64.1)</td>
<td>9 (36.0)</td>
<td>7 (28.0)</td>
<td>2 (8.0)</td>
</tr>
<tr>
<td>Total</td>
<td>218</td>
<td>187 (85.8)</td>
<td>65 (34.8)</td>
<td>51 (27.3)</td>
<td>14 (7.5)</td>
</tr>
</tbody>
</table>

Percentages of embryos with PGC(s) were calculated based on the numbers of morphologically normal embryos.

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**Figure 2** Recipient golden-type zebrafish (A–D) and their offspring obtained through natural mating with normal golden-type zebrafish (E and F). (A) Gross morphology of a recipient embryo with fluorescent PGC at the genital ridge 2 days after transplantation. (B) High-magnification of a genital ridge of a recipient embryo (the delineated square in A), focusing on the GFP-labeled PGC at the genital ridge (arrow). (C and D) Recipient male and female fish with donor-derived (striped-type) characteristics respectively. (E and F) Offspring having striped-type characteristics 48 h and 1 month postspawning respectively. Bar = 500 μm (A and E), 250 μm (B), 10 mm (C and D), and 5 mm (F).
warming (i.e. devitrification). In zebrafish embryos at the 12- to 18-somite stage, PGCs form clusters in the deep cell layer close to the yolk (Braat et al. 1999). Cryoprotectants in ES and VS (EG and DMSO) might penetrate into the yolk-intact embryos from the dorsal side of the embryonic body because the YSL, which underlies the embryonic body and covers the yolk (Kimmel & Law 1985), is thought to be a major barrier to cryoprotectant permeation for the zebrafish embryo (Hagedorn et al. 1998). Concentrations of cryoprotectants in the deep cell layer close to the yolk, therefore, might not reach sufficient levels to prevent devitrification of PGCs even after prolonged (30 min) exposure to ES, whereas those in most other parts of the embryonic body were sufficient to preserve the glassy status during warming.

PGCs of yolk-removed embryos were better cryopreserved than those of yolk-intact embryos in the present and previous studies (Higaki et al. 2009, 2010). The maximum recovery rate of live PGCs in the present yolk-removed embryos (about 90%) was much higher than that of our previous studies (about 40%; Higaki et al. 2009, 2010). Partial yolk removal may well prevent ice formation in embryos during cooling and warming by reducing their total volume and enhancing permeation of cryoprotectants into them through the crack in the YSL. Reducing the embryo volume and minimizing the VS volume increase the cooling/warming rates and the probability of vitrification (Yavin & Arav 2007). In the present yolk-removed embryos, cryoprotectants could penetrate from the ventral side (yolk side) of the embryonic body into the deep cell layer in which PGCs are located.

Under the present experimental conditions, exposure of yolk-removed embryos to ES and VS for moderate periods (15 and 5 min respectively) was favorable for the cryopreservation of PGCs, though the embryonic cell survival rate was not so high (around 50%). Prolonged (30 min) embryo exposure to ES reduced the survival of PGCs. These findings indicated that the concentrations of cryoprotectants in the deep cell layer close to the yolk, but not in other parts of the embryonic body, reached sufficient levels to protect PGCs from cryoinjuries within a relatively short period (15 min) of exposure to ES. PGCs may suffer from cytotoxic effects of cryoprotectants with prolonged (30 min) exposure to ES.

In Experiment 2, we transplanted PGCs recovered from yolk-removed embryos that were cryopreserved following the exposure protocol (ES for 15 min and VS for 5 min) that resulted in the highest number of live PGCs. In the present study, the PGCs recovered from fresh embryos (fresh PGCs) were not transplanted simultaneously; however, in our separate SPT experiment that was performed under the same conditions as the present study, about 80% (273/343) of morphologically normal embryos possessed fluorescent PGC, and about 35% (99/273) of the embryos had fluorescent PGC in the genital ridges at 2 days after the transplantation of fresh PGCs recovered from embryos at the 10- to 15-somite stage (Saito et al. 2008b). These values were higher than the corresponding values in the present study using cryopreserved PGCs (34.8 and 21.5% respectively). The relative survival rate of the cryopreserved PGCs after the present SPT (43.7: 34.8/79.6%) was roughly equivalent to the proportion of live PGCs with pseudopodial movement (50%) observed in the cryopreserved embryos in Experiment 1. The apparent survival rate (about 90%) of fluorescent PGCs estimated in Experiment 1 using trypan blue staining might have been too high because determining cell viability by dye exclusion tends to result in overestimation (Freshney 2005). Pseudopodial movement of PGCs may be a more reliable indicator of PGC viability because pseudopodial activity has been proposed as a sign of survivability of teleost blastomeres (Calvi & Maisse 1998). The lower migration rate of cryopreserved PGCs to the genital ridges of recipient embryos also suggests that the PGCs recovered from vitrified embryos suffered latent cryoinjuries restricting their ability to migrate. Thus, further experiments are needed to improve the survivability of cryopreserved PGCs.
The development of gonads and germ cells in the present SPT-derived five fertile males was in agreement with the findings of a previous SPT experiment using fresh PGCs: one normally sized gonad with spermato-genesis and one small gonad without germ cells (Saito et al. 2008a). When using the SPT method with fresh PGCs, only male chimeric fish emerged without hormonal treatment, and fertile chimeric female fish were obtained by estradiol-17β treatment (Saito et al. 2008a). In the present study, however, we obtained one female chimera fish with functional large and small gonads without any hormonal treatment. Further studies are needed to clarify the sex ratio in SPT-derived chimeric fish because of the limited numbers of samples in the present study.

In conclusion, we succeeded in producing fertile zebrafish that had the germ cells originating from PGCs recovered from vitrified yolk-removed embryos. Cryopreservation of PGCs combined with the novel SPT procedure can be used not only as an alternative method for embryo cryopreservation but also for banking/re-establishment of strains with scientific value through improvement in the survival of cryopreserved PGCs after transplantation into recipient embryos.

Materials and Methods

Fish

Adult striped-type zebrafish purchased from a local fish supplier were kept in the Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido University, under a 14 h light:10 h darkness photoperiod at 27 ± 1 °C and used to produce embryos for cryopreservation and preparation of PGCs. Adult golden-type zebrafish (recessive pigment mutant: Streisinger et al. 1986) that have been maintained in a closed colony in the Nanae Fresh Water Laboratory, Hokkaido University were kept under a 14 h light:10 h darkness photoperiod at 27 ± 1 °C and used to produce embryos for the PGC recipients.

This study was conducted in accordance with the Hokkaido University guidelines for the care and use of laboratory animals and with an approved animal protocol from the university (approved protocol number 09-0098).

Preparation of embryos for cryopreservation

Striped-type zebrafish embryos at the 1- to 8-cell stage obtained through natural mating were dechorionated by gentle pipetting with a hand-drawn glass Pasteur pipette in embryo medium (EM; Westerfield 2007b) supplemented with 0.1% (w/v) trypsin (Sigma–Aldrich), 0.1% (w/v) actinase E (Kaken Pharmaceutical, Tokyo, Japan) and 0.4% (w/v) urea (Nacalai Tesque, Kyoto, Japan) as described previously (Higaki et al. 2009). To visualize the PGCs, GFP-nes1 3′UTR mRNA was injected into the yolk just under the blastodiscs of dechorionated embryos at the 1- to 8-cell stage as described previously (Saito et al. 2008a).

Dechorionated embryos with or without GFP treatment were cultured in agar-coated (1% agar in EM) plastic Petri dishes filled with EM in an incubator at 28 °C until they developed to the 14- to 18-somite stage. The stage of embryonic development was identified according to criteria described elsewhere (Kimmel et al. 1995). GFP-treated embryos were checked for GFP fluorescence under an inverted microscope (TE300, Nikon, Tokyo, Japan) equipped with a GFP filter set.

Some embryos that developed to the 14- to 18-somite stage were transferred to agar-coated plastic Petri dishes containing fresh EM, and their yolks were removed by gentle aspiration using a hand-drawn glass Pasteur pipette with a tip having a 150-μm inner diameter (Fig. 4A and B). All embryo manipulations were carried out at room temperature (20–25 °C).

Cooling and warming of embryos

Dechorionated striped-type embryos at the 14- to 18-somite stage were cooled rapidly after serial exposure to ES and VS by the Cryotissue method (Kagawa et al. 2009). The ES consisted of handling medium (HM: HEPES-buffered TCM-199 solution supplemented with 20% synthetic serum substitute) containing 7.5% (v/v) EG and 7.5% DMSO. The VS consisted of HM containing 20% EG, 20% DMSO, and 0.5 M sucrose.

After serial exposure to ES and VS, 3–5 embryos were loaded on a nylon mesh (100 μm pore size, Nytal 13XX-100, Sefar, Thal, Switzerland) that was cut into a strip (~5 × 30 mm: Fig. 4C). The embryo-loaded nylon mesh (Fig. 4D) was put on a filter paper to remove excessive VS and plunged directly into liquid nitrogen as described previously (Higaki et al. 2009). The nylon mesh was transferred into a cryogenic vial (AGC Techno Glass, Chiba, Japan) and stored in a liquid nitrogen tank for 1–25 days.

For warming, the cryopreserved nylon mesh was removed from the vial in liquid nitrogen and immersed directly into 10 ml of HM supplemented with 1 M sucrose. After 1-min

Figure 4 Preparation of striped-type zebrafish embryo for cryopreservation. (A and B) During and after yolk removal from a 15-somite stage embryo respectively. (C) A nylon mesh cut into a 5 mm × 30 mm strip. (D) A yolk-removed embryo loaded on a nylon mesh after removing excess VS. Bar = 500 μm (A, B, and D) and 10 mm (C).
immersion, embryos that detached from the mesh were transferred into 1 ml of HM supplemented with 0.5 M sucrose and kept there for 4 min. They were then washed twice in 1 ml of HM for 5 min each time. All embryo cooling and warming procedures were performed at room temperature (20–25 °C).

**Experiment 1: viability of embryonic cells and PGCs recovered from cryopreserved embryos**

To examine the ice formation in embryos during cooling and survival of embryonic cells recovered from cryopreserved embryos, non-GFP-treated embryos with or without yolk removal were cooled rapidly after serial exposure to ES for 10, 15, or 30 min and VS for 1, 5, or 10 min. During cooling of the embryos, ice formation was checked by visual inspection for the presence of a milky appearance as described elsewhere (Sheffen et al. 1986). To assess the viability of embryonic cells in cryopreserved embryos, fresh (control) and cryopreserved non-GFP-labeled embryos were digested by pipetting in a 0.5-ml microtube (Greiner Bio-One, Frickenhausen, Germany) containing 30 μl of Hank's premix solution (Westerfield 2007b) supplemented with 0.1% (w/v) collagenase (Wako Pure Chemical Industries, Osaka, Japan). The cells in the suspension were washed with Hank's premix solution by 30-s centrifugation at 2000 g (HF-120, Tomy Seiko, Tokyo, Japan) and stained with 0.5% (w/v) trypan blue (Kanto Chemical, Tokyo, Japan). The number of trypan blue-negative cells in 0.1 μl of cell suspension was counted using a hemacytometer, and the cell survival rates in cryopreserved embryos were calculated based on the number of trypan blue-negative cells in fresh embryos. The experiment was repeated three times using three embryos for each replicate.

The survival of PGCs in cryopreserved embryos was determined as described previously (Higaki et al. 2009). In brief, a single GFP-labeled fresh (control) or cryopreserved embryo placed in a 10-μl droplet of Hank's premix solution supplemented with 0.1% collagenase, and 0.5% trypan blue was partially digested by pipetting. PGCs were identified by their bright GFP fluorescence and relatively large size compared with other embryonic cells as described previously (Saito et al. 2006) under an inverted microscope equipped with a GFP filter set. The numbers of GFP-positive PGCs that were negative for trypan blue, and that showed movement with extended pseudopodia were counted. The experiment was repeated three times using three or four embryos for each replicate. All procedures for viability assessment were carried out at room temperature (20–25 °C).

**Experiment 2: differentiation ability of PGCs recovered from vitrified embryos**

GFP-labeled striped-type embryos were cooled rapidly after yolk removal and serial exposure to ES for 15 min and VS for 5 min. The cryopreserved embryos stored in liquid nitrogen were transported from Sapporo to Nanae (a distance of about 300 km).

The vitrified embryos were warmed and PGCs were isolated by pipetting in a 1.5-ml microtube (Greiner Bio-One) containing 300 μl of Ringer culture solution (128 mM NaCl, 2.8 mM KCl, and 1.8 mM CaCl₂) supplemented with 0.25% (w/v) sodium citrate. According to the procedure developed by Saito et al. (2008a), a single isolated fluorescent PGC was transplanted into the marginal region of each dechorionated golden-type blastula (recipient embryo), which was sterilized by injecting a dead end antisense morpholino oligonucleotide (Ciruna et al. 2002) in Ringer culture solution containing 2% (v/v) of FCS (Invitrogen) and antibiotics (10 IU/ml of penicillin and 10 μg/ml of streptomycin) under a fluorescence stereomicroscope (DFC300FX, Leica Microsystems, Wetzlar, Germany) equipped with a GFP filter set. We transplanted the fluorescent PGCs without viability assessments.

Following PGC transplantation, recipient embryos were individually incubated in a round-bottomed 96-well plate (Greiner Bio-One) filled with Ringer culture solution supplemented with the antibiotics for 1 day, after which they were individually incubated in a flat-bottomed 48-well plate (Sumitomo Bakelite, Tokyo, Japan) filled with culture solution (1.8 mM CaCl₂ and 1.8 mM MgCl₂) supplemented with the antibiotics. At 2 days after transplantation, the morphology of recipient embryos and location of transplanted PGCs were observed under a fluorescence stereomicroscope equipped with a GFP filter set. Recipient embryos that had a normal appearance and fluorescent PGC at the genital ridges were reared in tap water for 3–4 months to allow them to reach sexual maturity under the same conditions as for golden-type parent fish. The transplantation experiment was repeated four times using 39–82 recipient embryos for each replicate.

To obtain F1 progeny, each mature fish was placed together with a normal golden-type zebrafish of the opposite sex. The appearance of dark spots and dark stripes, striped-type characteristics, in F1 progeny was examined at 48 h and 1 month after spawning respectively. After 3–5 progeny tests (5–6 months after the PGC transplantation), the fish subjected to the test were killed by exposure to an overdose of eugenol (FA-100, Tanabe Pharma, Osaka, Japan; Borski & Hodson 2003). They were then fixed with Bouin’s fixative for 24 h. Several parts of the fish, including the gonads, were embedded in paraffin. Serial 8-μm sections were cut and stained with hematoxylin and eosin.

**Statistical analysis**

For statistical analysis, the cell survival rates were subjected to angular transformation (arcsin √%). The effects of periods of exposure to ES and VS on the cell survival rates of cryopreserved embryos were analyzed by two-way ANOVA followed by Tukey’s honestly significant difference as a post hoc test. The mean cell survival rates of embryos with and without ice formation during cooling were compared using the Mann–Whitney U test. Differences in the mean numbers of live PGCs were analyzed by Kruskal–Wallis analysis. The Bonferroni-corrected Mann–Whitney U test was used for comparisons of the mean numbers of live PGCs between groups. A computer program (SPSS for Windows, Version 12.0; SPSS Inc., Chicago, IL, USA) was used for statistical analyses.
Declarations of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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