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Generation of germ-line chimera zebrafish using primordial germ cells isolated from cultured blastomeres and cryopreserved embryoids

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ABSTRACT Primordial germ cells (PGCs) are the only cells in developing embryos with the potential to transmit genetic information to the next generation. In our previous study, a single PGC transplanted into a host differentiated into fertile gametes and produced germ-line chimeras of cyprinid fish, including zebrafish. In this study, we aimed to induce germ-line chimeras by transplanting donor PGCs from various sources (normal embryos at different stages, dissociated blastomeres, embryoids, or embryoids cryopreserved by vitrification) into host blastulae, and compare the migration rates of the PGCs towards the gonadal ridge. Isolated, cultured blastomeres not subject to mesodermal induction were able to differentiate into PGCs that retained their motility. Moreover, these PGCs successfully migrated towards the gonadal ridge of the host and formed viable gametes. Motility depended on developmental stage and culture duration: PGCs obtained at earlier developmental stages and with shorter cultivation periods showed an increased rate of migration to the gonadal ridge. Offspring were obtained from natural spawning between normal females and chimeric males. These results provide the basis for new methods of gene preservation in zebrafish.

KEY WORDS: *germ-line chimera, primordial germ cell, cryopreservation, dissociated blastomere*

Introduction

Germ-line chimeras are valuable tools in studies to improve our understanding of the fundamental mechanisms of germ-line development. They have also recently attracted attention in terms of the *in vivo* induction of functional gametes from donor germ cells or from cultured pluripotent cells with a different genetic background from that of the host. One potential application of germ-line chimeras is in surrogate propagation to produce seedlings of high commercial value or as valuable gene resources for poultry farming and aquaculture (Chang *et al.*, 1995; Mueller *et al.*, 1999; Takeuchi *et al.*, 2001; Horii *et al.*, 2003; Kim *et al.*, 2005; Yamaha *et al.*, 2007; Yamamoto *et al.*, 2007). Potential candidates for donors for the induction of germ-line chimeras include primordial germ cells (PGCs) and early-stage germ cells, such as spermatogonia. These germ-line cells are the only cells with the potential to transmit genetic information to the next generation, and they therefore have potential value for gene banking and cryopreservation, particularly in the production of donor gametes *via* germ-line chimeras.

PGCs are known to migrate from their emergent site to the gonadal ridge. When transplanted into an appropriate position in the host embryo, PGCs are therefore expected to migrate precisely to the host gonadal ridge. Germ-line chimeras in teleosts generated using blastoderm cells and gonadal cells, including PGCs, have successfully produced offspring and sperm (Yamaha *et al.*, 2001; Ciruna *et al.*, 2002; Takeuchi *et al.*, 2003; Yamaha *et al.*, 2003; Takeuchi *et al.*, 2004; Nagai *et al.*, 2005). In our recent

Abbreviations used in this paper: CE, cultured embryoid; DB, dissociated blastomere; GFP, green fluorescent protein; PGC, primordial germ cell.

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study (Saito *et al.*, 2008) using small freshwater fishes, such as zebrafish, we developed a method that ensured complete germ cell replacement using a single PGC transplantation (SPT) approach: namely, knockdown of endogenous PGCs in the host embryo and successive transplantation of a PGC from a donor. The SPT method provides a good model system for analyzing motility to the gonad and the potency of PGCs to differentiate into gametes. When transplanted into a host zebrafish blastula, more PGCs isolated from stages earlier than the 20-somite stage migrated to the gonadal ridge, compared with those isolated from later stages, suggesting that PGCs change autonomously during normal development (Saito *et al.*, 2008). However, the effects of stage on PGC motility require further investigation. The advantage of the SPT method is its ability to produce germ-line chimeras from a single PGC. Unlike the method used in host trout fry (about 15–20 mm) that can be transplanted'with many PGCs and spermatogonia (Kobayashi *et al.*, 2003; Takeuchi *et al.*, 2003; Yoshizaki *et al.*, 2005; Kobayashi *et al.*, 2007; Okutsu *et al.*, 2007), the SPT method is valuable for the production of germ-line chimeras in small freshwater fish, including rare species for which it is inefficient to supply many germ cells. Thus the SPT method using highly motile PGCs can provide a successful means of producing germ-line chimeras in small freshwater fish, including zebrafish.

The cryopreservation of genetic material from chickens allows its almost indefinite storage for biological conservation without deterioration, over a timescale of at least several thousands of years (Setioko *et al.*, 2007). Embryo cryopreservation, however, has not been reported for avian species, including *Gallus gallus* or quail, or for teleosts, largely because of similar limitations in their oocytes, namely their relatively large size, high lipid content, and the polar organization of the ova and early embryos (Hiemstra *et al.*, 2005). For these species, however, cryopreservation of isolated embryonic cells is an option. The production of germ-line chimeras from cryopreserved PGCs has been reported in birds (Chang *et al.*, 1998; Tajima *et al.*, 1998; Naito 2003; Tajima *et al.*, 2003; Petitte 2006), and there have been a few reports of the creation of germ-line chimeras in teleosts using cryopreserved/thawed trout PGCs (Kobayashi *et al.*, 2003; Kobayashi *et al.*, 2007). The disadvantages of the methods used, however, included the need for transplantation of many PGCs or germ cells, which is impractical, especially in small fish. Cryopreservation by vitrification has been used in the field of biology; vitrification involves the solidification of a liquid through increased viscosity during cooling (Fahy *et al.*, 1984). Previous studies have demonstrated the usefulness of this method for cryopreservation of mammalian oocytes and embryos (Atabay *et al.*, 2004, Nagano *et al.*, 2007, Fujino *et al.*, 2008). An efficient vitrification method is required for the cryopreservation of PGCs from small freshwater fish. An effective method for producing chimeras combining a means of cryopreserving PGCs with the SPT method, would help preserve the collection of zebrafish mutants, as well as preserving stocks of rare species,'such as the Japanese bitterling (Cypriniformes) and/or loach (Cypriniformes), which are among the many species designated as endangered in Japan.

In the present study, we confirmed both the differentiation of PGCs from isolated blastodiscs under dissociated or aggregated conditions, and their abilities to migrate and to differentiate into gametes in germ-line chimeras, using the SPT method. The migratory activity of PGCs derived from embryos and embryoids after a cryopreservation/thaw cycle was also determined. These results represent an improvement in the technology used for constructing germ-line chimeras and provide novel prospects for the preservation of gene stocks, such as those of zebrafish mutants, as well as presenting a method for the cryopreservation of PGCs from small fish.

TABLE 1

APPEARANCE OF GREEN FLUORESCENT PROTEIN-POSITIVE CELLS IN DISSOCIATED COLONIES OF BLASTOMERES OBTAINED USING A CULTURE ISOLATION BLASTODISC METHOD

			No. of cultured blastodiscs with GFP-positive cells		
Experiment.	Culture temp. (°C)	No. of blastodiscs	Culture day1 (%)	Culture day2 (%)	Culture day3 (%)
Exp.1	22	34	3 (12.0)	6 (24.0)	12 (48.0))
	26	25	10 (29.4)	19 (55.9)	22 (64.7)
Exp.2	22	15	1 (6.7)	1 (6.7)	2 (13.3)
	26	22	4 (18.2)	5 (22.7)	8 (36.4)
Exp.3	22	28	1 (4.4)	5 (21,7)	12 (52.2)
	24	29	3 (10.3)	9(31.0)	12 (41.4)
	26	23	5 (17.9)	13 (46.4)	17 (60.7)
Exp.4	22	31	3 (9.4)	4 (12.5)	5 (15.6)
	24	30	3 (10.0)	5 (16.7)	7 (23.3)
	26	32	5 (9.4)	10 (32.3)	10 (32.3)
Exp.5	22	31	3 (9.7)	3 (9.7)	5 (16.1)
	24	30	5 (16.7)	7 (23.3)	10 (33,3)
	26	31	12 (38.7)	13 (41,9)	17 (54.8)

GFP; green fluorescent protein.

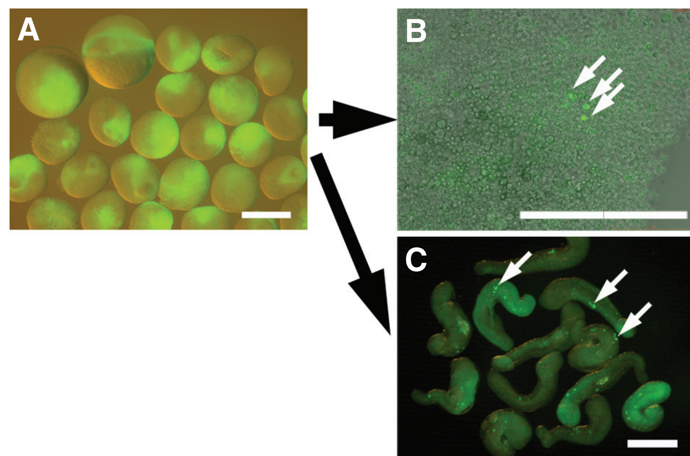

Fig. 1. Visualized green fluorescent protein (GFP)-positive cells obtained using the dissociated blastomere (DB) and cultured embryoid (CE) methods. (A) *Isolation of blastodiscs at the late-blastula stage from embryos injected with GFP-*nos1*-3'UTR strand-capped mRNA. Large embryos were blastulae before operation, whereas small ones were isolated blastodiscs.* **(B)** *DBs cultured for 1 day in a dissociated condition in sodium citrate solution.* **(C)** *Embryoid from an isolated blastodisc cultured for 1 day in Ringer's solution. Arrows indicate GFP-positive cells (primordial germ cells). Bars, 500 μm.*

Experimental Protocols

Fish embryos

Zebrafish were maintained in the Nanae Fresh Water Laboratory, Hokkaido University. Parental brood stock was maintained at 26–28°C under a 16-h light:8-h dark photoperiod. Embryos were dechorionated with 0.1% trypsin (Difco Laboratories, Detroit, MI, USA) in Ringer's solution (128 mM NaCl, 2.8 mM KCl, and 1.8 mM $CaCl_2$). Dechorionated embryos were cultured in Ringer's culture solution with 0.01% penicillin and 0.01% streptomycin. They were then cultured at 26°C in 96-well plates individually filled with Ringer's solution for 24 h, followed by culture in another separate well filled with a second culture solution (1.8 mM $CaCl_2$ and 1.8 mM $MgCl_2$) containing antibiotics, as above. The stage of embryonic development was identified according to Kimmel *et al.* (1995).

Visualization and supply of donor PGCs

Donor embryos were injected at the 2–4-cell stage with 80 pg of Green Fluorescent Protein (GFP)-*nos1*-3'UTR strand-capped mRNA, synthesized by SP6 transcription from a *Not*I-linearized plasmid (Saito *et al.*, 2008) using the mMESSAGE mMACHINE system (Ambion, Austin, TX, USA). Injected embryos were treated in one of the three following ways, after which donor PGCs with GFP fluorescence were obtained. I) Homogenization of somitogenesis-stage embryos (SE): embryos injected with GFP-*nos1*- 3'UTR were cultured at 22, 23, 24 or 26°C in 96-well plates, homogenized at the appropriate stage corresponding to 5–10, 12–17, 21–24, or 26+ somites, and Prim-25 (http://zfin.org/zf_info/zfbook/stages/) and PGCs were then obtained. II) Dissociated blastomeres (DB): the entire blastodisc of an embryo developed from eggs injected with GFP-*nos1*-3'UTR was cut off at late blastula stage (Fig. 1A), and cultured in 0.25% sodium citrate in Ringer's solution in a dissociated condition at 22, 24 or 26°C until 1 (26–27), 2 (50–51) or 3 days post-fertilization (dpf) (74–75 hours post-fertilization) of the donor fish (Fig. 1B). III) Cultured embryoids (CE): The blastodisc of donor embryos injected with artificial mRNA was removed from the yolk cell at the late blastula stage,'and then cultured in Ringer's solution in an aggregated condition at 22, 24 or 26°C until 1 or 2 dpf of the donor fish (Fig. 1C).

Cryopreservation of donor PGCs by vitrification

PGCs in embryos injected with GFP-*nos1*- 3'UTR, as described above, were cryopreserved by vitrification, according to the method of Liu *et al.* (2008) and Higaki *et al.* (2010), with slight modifications. In brief, a 12–17-somite stage embryo with its yolk sac removed, or a CE developed from an isolated blastodisc cultured at 22°C for 1 dpf was transferred into 7.5% DMSO and 7.5% ethylene glycol in Hepes-buffered TCM-199 (Invitrogen, Carlsbad, CA, USA) and 20% serum substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) at room temperature for 10–15 min. Samples were subsequently transferred into 15% DMSO and 15% ethylene glycol dissolved in TCM199 and 20% SSS. After incubation for 5–10 min, 10–15 samples were loaded into the mesh of the vitrification device and plunged into liquid nitrogen. Vitrified samples were stored in liquid nitrogen for at least 10 days (see Migishima *et al.*, 2003, Atabay *et al.*, 2004) and were then warmed by immersing the vitrification device directly in room temperature thawing solution (TCM199 and 20% SSS) for 1 min. The samples were then transferred to fresh thawing solution for 5 min twice before transfer to Ringer's solution.

PGC transplantation

Donors with visualizable PGCs, as described above, were dissociated into single cells using 0.25% sodium–citrate in Ringer's solution. PGCs from dissociated cells were transplanted into host blastulae, according to the method of Saito *et al.* (2008). Differentiation of the host PGCs was blocked by injection of a *dead end* (*dnd*) antisense morpholino oligonucleotides (Ciruna *et al.*, 2002, Weidinger *et al.*, 2003). Some of the host blastulae were injected with DsRed-*nos1*- 3'UTR strand-capped mRNA to monitor the migration of host PGCs. The transplantation procedure took around 2 h, during which one or two isolated PGCs were picked up with a glass micro-needle under a stereomicroscope and transplanted into the marginal region of the blastodisc of each zebrafish blastula. Chimeric embryos were observed and photographed using a fluorescence stereomicroscope, model MZ16F (Leica Microsystems,

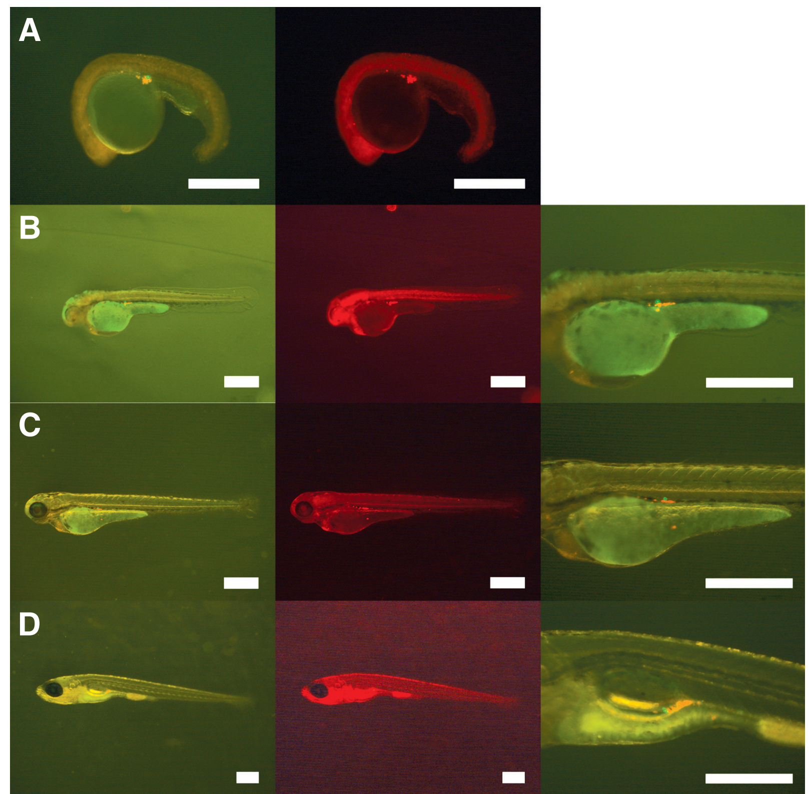

Fig. 2. Migration and proliferation of donor primordial germ cells (PGCs) derived from dissociated blastomeres cultured for 1 day at 26°C in the same germ-line chimera. *Green fluorescent protein-positive cells indicate donor PGCs and DsRed-positive cells indicate host PGCs.* **(A)** *Host at 1 day post fertilization (dpf).* **(B)** *2 dpf.* **(C)** *3 dpf.* **(D)** *11 dpf. Bars, 500 μm.*

TABLE 2

TRANSPLANTATION OF PRIMORDIAL GERM CELLS OBTAINED BY THE SOMITOGENESIS-STAGE EMBRYO, DISSOCIATED BLASTOMERE AND CULTURED EMBRYOID METHODS INTO BLASTULA-STAGE ZEBRAFISH EMBRYOS

	Culture of donor								Localization of PGCs at 2 dpf		
Experiment	Temp. (°C)	Days	Stage (donor)	No. of manipulated embryo	No. of embryo	No. hatching (%)	No. of embryos with GFP-positive cells at 1 dpf (%)	No. of fish with PGCs at 2 dpf (%)	Gonadal ridge (%)	Ectopic (%)	No. of transplantations
SE											
W to G	22	1	5-10 somites	166		149 (89.8)	90 (54.2)	71 (42.8)	9 (12.7)	62 (87.3)	4
	23	1	12-17 somites	260		172 (66.2)	122 (46.9)	76 (29.2)	14 (18.4)	62 (81.6)	8
	24	1	21-24 somites	320		205 (64.1)	119 (37.2)	52 (16.3)	4 (7.7)	49 (92.5)	8
	26	1	26+ somites	242		171 (70.1)	104 (43.0)	55 (22.7)	2 (3.6)	53 (96.4)	7
	23	2	Prim-25	117		93 (79.5)	58 (49.6)	50 (42.7)	0 (0.0)	50 (100.0)	4
G to W	23	1	12-17 somites	112		90 (80.4)	65 (58.0)	56 (50.0)	12 (21.4)	44 (78.6)	4
	24	1	21-24 somites	185		113 (71.9)	75 (40.5)	51 (27.6)	6 (11.8)	45 (88.2)	5
	26	1	26+ somites	179		119 (66.5)	78 (43.6)	51 (28.5)	4 (7.8)	47 (92.2)	4
Host control (golden)					261	208 (80.0)					
Host control (wild)					230	200 (87.0)					
DB											
W to G	26	1		202		153 (75.7)	96 (47.5)	57 (28.2)	18 (31.6)	39 (68.4)	7
	26	2		233		184 (80.0)	97 (41.6)	54 (23.2)	11 (20.4)	43 (79.6)	9
	26	3		334		255 (76.3)	127 (38.0)	52 (15.6)	3 (5.8)	49 (94.2)	15
G to W	26	1		257		185 (71.9)	112 (43.6)	50 (19.5)	16 (32.0)	34 (68.0)	8
	26	2		167		132 (79.0)	67 (40.1)	50 (29.9)	12 (24.0)	38 (76.0)	6
	26	3		200			102 (51.0)	53 (26.5)	3 (5.7)	50 (94.3)	10
Host control (golden)					263	201 (76.4)					
Host control (wild)					213	149 (70.0)					
CE											
W to G	22			238		181 (76.1)	112 (47.1)	59 (24.8)	10 (16.9)	49 (83.1)	7
	22			355		229 (64.5)	111 (31.3)	51 (14.4)	4 (7.8)	47 (92.2)	11
	24			169		105 (62.1)	79 (46.7)	53 (31.4)	7 (13.2)	46 (86.8)	6
	24			259		181 (69.9)	89 (34.4)	53 (20.5)	0 (0.0)	53 (100.0)	9
	26			244		177 (72.5)	96 (39.3)	57 (23.4)	1 (1.8)	56 (98.2)	7
	26			253		169 (66.8)	137 (54.2)	75 (29.6)	1 (1.3)	73 (98.7)	6
Host control (golden)					360	239 (66.4)					

'W to G' indicates wild-type zebrafish-to-golden-type zebrafish primordial germ cell-transplantation chimeras. 'G to W' indicates golden-type zebrafish-to-wild-type zebrafish PGC transplantation chimeras. Host control and host embryo were injected with antisense morpholino oligo of the *dead end* gene. CE; cultured embryoids, DB; dissociated blastomeres, dpf; days post-fertilization, GFP; green fluorescent protein, PGC; primordial germ cell, SE; somitogenesis-stage embryos.

Inc., Bannockburn, IL, USA).

Rearing conditions for chimeras

The chimeric fish in which migration of the transplanted donor PGCs to the germinal ridge was monitored were reared in 96-well plates in Ringer's solution at 26°C. They were transferred to a second culture solution after 1 day. Newly hatched host fish were transferred into fresh water in 24-well plates and then transferred into 6-well plates at around 10 days post-hatching (dph). The feeding scheme for the fish was as follows: rotifers, *Brachionus calyciflorus*, from 3–10 dph; and *Artemia nauplii* from 8 dph. After around 20 dph, fish were transferred from 0.5-L to 20-L tanks in series, at 27–28°C.

Results & Discussion

A blastodisc isolated at the late-blastula stage developed into an embryoid when cultured in Ringer's solution in an aggregated condition. The embryoid was an elongated cell mass with a partially developed, notochord-like structure (0–10 somites at 22°C) (N=143). GFP-positive cells were observed in the embryoid from 1 dpf after blastodisc isolation. These cells varied in number from 1–14 per blastodisc (N=87). Embryoids cultured at 22°C survived for a maximum of about 3 days under unsterile conditions (Fig. 1C).

Blastomeres from an isolated blastodisc dissociated as soon as they were immersed in Ringer's solution containing 0.25% sodium citrate, and remained dissociated during cultivation. The cells of the dissociated colonies cultured at 26°C survived for a maximum of 3 days under unsterile conditions. GFP-positive cells were observed in the dissociated colonies of blastomeres and varied in number from 1–8 per colony (Fig. 1B) (N=60). More GFP-positive cells were detected with longer cultivation periods and higher cultivation temperatures of the DB (Table 1).

A donor GFP-positive cell derived from the DB migrated from the marginal region of the host blastodisc towards the gonadal ridge in the host embryo (Fig. 2A). At 11 dpf, the donor cell was found migrating synchronously, in a burrowing fashion, on the lower side of the swim bladder, together with host PGCs (Fig. 2D). A GFP-positive cell derived from the CE also migrated towards the gonadal ridge of the host embryo (data not shown). *nos1*-3'UTR is able to direct the expression of specific genes in the

TABLE 3

TRANSPLANTATION OF ZEBRAFISH PRIMORDIAL GERM CELLS OBTAINED BY THE SOMITOGENESIS-STAGE EMBRYO AND CULTURED EMBRYOID METHODS, COUPLED WITH VITRIFICATION CRYOPRESERVATION, INTO BLASTULA-STAGE ZEBRAFISH EMBRYOS

Experiment	Culture of donor		Stage of donor (Somites)	No. of manipulated embryo	No. of embryo	No hatching (%)	No. of embryos with GFP-positive cells at 1 dpf (%)	No. of fish with PGCs at 2 dpf (%)	Localization of PGCs at 2 dpf		No. of transplantations
	Temp. (°C)	Days							Gonadal ridge (%)	Ectopic (%)	
SE											
W to G	23	1	12-17	249		189 (75.9)	91 (36.5)	50 (20.1)	4 (8.0)	46 (92.0)	8
CE											
W to G	22	1		284		232 (81.7)	110 (38.7)	54 (19.0)	4 (7.4)	50 (92.6)	8
Host control (golden)					279	242 (86.7)					

'W to G' indicates wild-type zebrafish-to-golden-type zebrafish primordial germ cell-transplantation chimeras. Host control and host embryo were injected with antisense morpholino oligonucleotides of the *dead end* gene. CE; cultured embryoids, DB; dissociated blastomeres, dpf; days post-fertilization, GFP; green fluorescent protein, PGC; primordial germ cell, SE; somitogenesis-stage embryo.

zebrafish PGCs (Köprunner *et al*., 2001). Ciruna *et al*. (2002) reported that when 50–100 GFP-expressing cells from the marginal region of a mid-blastula stage embryo injected with GFP-*nos1*- 3'UTR mRNA were transplanted into host embryos, *nos1-3'UTR* directed the specific expression of GFP in PGCs by late gastrulation. In the present study, GFP-expressing cells from the upper-part of the blastoderm at the blastula stage, which had been injected with GFP-*nos1*- 3'UTR, were transplanted into host embryos at 2 dpf, but GFP signals in chimeric fish were either absent (N=30/35) or weak (N=5/35). These results suggest that the cells from the upper part of the blastoderm differentiate into somatic cells and that GFP-*nos1*- 3'UTR mRNA is rapidly degraded in presumptive somatic cells. PGCs have been reported to be located at the marginal part of the blastoderm during the blastula stage (Yoon *et al*., 1997), and the GFP-positive cells derived from the DB or CE were thus confirmed to be PGCs.

It is well known that zebrafish PGCs are determined by the germ plasm enriched in the vicinity of the cleavage furrows. When the germ plasm was removed at the 4-cell stage, no PGCs were formed in the resultant embryo (Hashimoto *et al*., 2004), though it has not yet been confirmed whether or not direct transplantation of the germ plasm can induce PGCs. In amphibians, *goosecoid* (*gsc*), one of the dorsal signals during development, is expressed in some of the dissociated blastomeres (Lemaire and Gurdon, 1994), suggesting that a dorsal determinant autonomously induces *gsc* expression. In a similar manner, the germ plasm might autonomously differentiate PGCs in the blastomeres inherited with it. It has been reported that PGCs can differentiate in goldfish without mesoderm induction, which is the earliest induction to occur during development (Otani *et al*., 2005). However, the abilities of PGCs differentiated under conditions of mesoderm induction to migrate and develop into gametes remain unknown. These earlier findings in goldfish, together with the current results from zebrafish, namely that PGC differentiation can occur under dissociated conditions, support the idea that intercellular interactions are not required for this differentiation. Thus PGCs might be unambiguously determined by the germ plasm.

We bred some chimeric fish in which the donor PGCs migrated ectopically, and which therefore did not undergo gametogenesis (N=20). The key to the production of fertile germ-line chimeras using the current method is the ability of the donor PGCs to reach the host's gonadal ridge. The success of the germ-line chimeras could be judged on the basis of the percentage of donor PGCs reaching the gonadal ridge of the host fish at 2 dpf. When SE PGCs were used as donors, the migration rates to the gonadal ridge decreased as the stage of collection of the donor PGCs advanced, with the exception of PGCs from the SE at 22°C, corresponding to the 5–10-somite stage, which showed lower

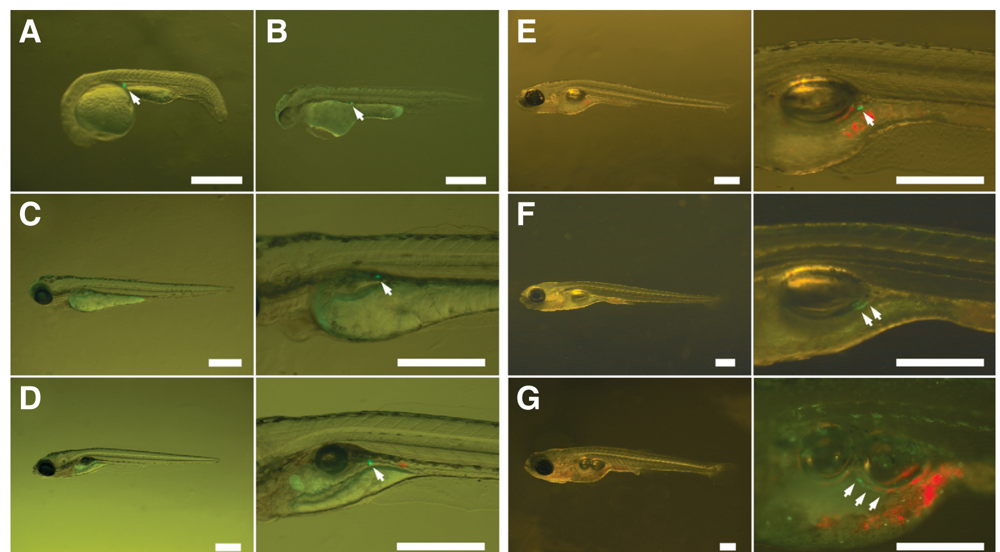

Fig. 3. Migration and proliferation of donor primordial germ cells (PGCs) derived from cryopreserved/thawed embryoids cultured for 1 day at 22ºC in the same germ-line chimera. *Arrows indicate green fluorescent protein-labeled PGCs or germ cells.* **(A)** *Host at 1 day post-fertilization (dpf).* **(B)** *2 dpf.* **(C)** *3 dpf.* **(D)** *5 dpf.* **(E)** *10 dpf.* **(F)** *13 dpf.* **(G)** *18 dpf. Bars, 500 μm.*

migration rates than those from the SE at 23°C, corresponding to the 12–17-somite stage. This tendency for PGCs from more advanced stages to migrate to the gonadal ridge at a lower rate than those from an earlier developmental stage was observed in both the transplantation of donor wild-type to host golden-type, and donor golden-type to host wild-type (Table 2). In the case of PGCs from the DB, the migration rates to the gonadal ridge also decreased with longer cultivation periods of the DB prior to the collection of donor PGCs (Table 2). The highest migration was about 32% and was observed with PGCs derived from the DB cultivated for 1 day at 26°C; this rate was even higher than that for PGCs derived from the SE. In the case of PGCs derived from the CE, higher culture temperatures were associated with lower migration rates of PGCs to the gonadal ridge. For the same cultivation temperature, 2 days of cultivation of the isolated blastodisc resulted in a lower migration rate of PGCs compared with 1 day of cultivation. A similar result was observed for PGCs derived from the DB, in that longer cultivation times led to a lower rate of PGC migration. Therefore, PGCs isolated from the DB cultured at a stage when few PGCs from normal embryos showed motility were able to migrate to the gonadal ridge, suggesting that this autonomous change in PGCs may advance more slowly under dissociated conditions compared with normal conditions.

The GFP-positive cells derived from the CE were confirmed to be PGCs capable of migrating to the gonadal ridge, as described above, and we therefore examined the effects of cryopreservation by vitrification on the PGCs from the SE and the CE. An SE cultured for 1 day at 23°C and a CE cultured for 1 day at 22°C were used as donor materials for cryopreservation by vitrification, as their PGCs demonstrated good motility towards the gonadal ridge. After defrosting, live GFP-labeled PGCs from the SE and CE were detected under a fluorescence microscope. When transplanted into a host blastula, cryopreserved/thawed PGCs from the CE migrated to the gonadal ridge (Fig. 3), and the donor PGC at the gonadal ridge underwent cell division at around 10 dpf and became localized to the bottom part of the swim bladder. The migration rates of cryopreserved/thawed PGCs from the SE and CE were 8.0 and 7.4% (Table 3), respectively, which were about 10% lower than that of PGCs without cryopreservation (Table 2). Around 10 dpf, PGCs underwent cell division (data not shown) in more than eight out of ten chimeric fish in which donor PGCs had migrated toward the gonadal ridge.

TABLE 4

MALE:FEMALE RATIO OF CHIMERAS

Experimental group		Culture of donor: Temp. (°C)	Culture of donor: Day	Stage of donor (somite)	No. of individuals	Male (%)	Female (%)
SE							
	W to G	22	1	5-10	6	6 (100.0)	0 (0.0)
	W to G	23	1	12-17	3	3 (100.0)	0 (0.0)
	G to W	23	1	12-17	3	3 (100.0)	0 (0.0)
DB							
	W to G	26	1		5	5 (100.0)	0 (0.0)
	W to G	26	2		2	2 (100.0)	0 (0.0)
	G to W	26	1		3	3 (100.0)	0 (0.0)
CE							
	W to G	22	1		3	3 (100.0)	0 (0.0)
	W to G	22	2		3	3 (100.0)	0 (0.0)
	W to G	24	1		6	6 (100.0)	0 (0.0)
	W to G	26	2		1	1 (100.0)	0 (0.0)
Vitrification cryopreservation							
Using SE							
	W to G	23	1	12-17	4	4 (100.0)	0 (0.0)
Using CE							
	W to G	22	1		2	2 (100.0)	0 (0.0)
Wild-type					16	9 (56.3)	7 (43.7)
Golden-type					18	10 (55.6)	8 (44.4)
Wild-type MO					20	20 (100.0)	0 (0.0)
Golden-type MO					24	24 (100.0)	0 (0.0)

'W to G' indicates wild-type zebrafish-to-golden-type zebrafish primordial germ cell-transplantation chimeras. Wild-type MO and Golden-type MO were injected with antisense morpholino oligonucleotides of the *dead end* gene. CE; cultured embryoids, DB; dissociated blastomeres, SE; somitogenesis-stage embryos.

Table 4 summarizes the characteristics of the germ-line chimeras induced in this study. All the chimeras were male and produced sperm. These results therefore demonstrate that transplantation of one or two PGCs isolated from SE, DB or CE, or from cryopreserved SE or CE, enabled the induction of reproductively functional chimeras.

Table 5 shows the ratio of phenotypes of the offspring produced by crosses between golden-type female zebrafish and male chimeras. The phenotypes of the wild-type and golden-type were distinguishable in the newly hatched and/or 30 dpf fish. Melanin pigmentation in the melanophores on the side of the body, which is a characteristic of wild-type, was the predominant characteristic (Fig. 4). All of the offspring generated from male chimeras in this study showed a phenotype derived from the donor, with no exceptions. Some offspring were maintained for 1 month after phenotypic discrimination at the newly hatched stage, and their phenotypes were confirmed by the presence of melanophores at 30 dpf. Melanophores were not detected in any of the fish from fry determined to be golden at the newly hatched stage, but were detected in those determined to be wild-type (Fig. 4). Therefore, all of the male chimeras induced in this study had spermatozoa derived from the donor.

In the present study, cryopreserved/thawed PGCs from the CE and SE migrated to the host gonadal ridge. Male chimeras whose germ-line cells were replaced with cryopreserved/thawed PGCs showed natural mating behavior with normal females and produced offspring. There have been a few studies of the survivability of cryopreserved/thawed whole embryos in teleosts (Chao and Liao, 2001; Chen and Tian, 2005), but the results have not been promising because of the difficulty of avoiding the formation of intracellular ice crystals (Ninhaus-Silveira *et al.*, 2008). In other words, the yolk syncytial layer plays a critical cryoprotectant role (Hagedorn *et al.*, 1997). The production of germ-line chimeras by transplantation of cryopreserved PGCs may thus offer an effective alternative method to cryopreservation of whole embryos for the maintenance of teleost species.

In conclusion, we have developed new methods for creating

TABLE 5

PHENOTYPES AT 2 AND 30 DAYS POST-FERTILIZATION OF THE F_1 GENERATION FROM A CROSS BETWEEN GOLDEN-TYPE ZEBRAFISH AND MALE CHIMERAS

		Culture of donor		Stage of donor		Phenotype at 2 dpf		Phenotype at 30 dpf	
Experimental group		Temp. (°C)	Day	(somite)	Lot. No.	Wild (%)	Golden (%)	Wild (%)	Golden (%)
SE	W to G	22	1	5-10	1	115 (100.0)	0 (0.0)		
					2	109 (100.0)	0 (0.0)		
					3	111 (100.0)	0 (0.0)		
	W to G	23	1	12-17	1	131 (100.0)	0 (0.0)		
	G to W	23	1	12-17	1	0 (0.0)	128 (100.0)		
DB	W to G	26	1		1	139 (100.0)	0 (0.0)	12 (100.0)	0 (0.0)
					2	122 (100.0)	0 (0.0)		
	W to G	26	2		1	103 (100.0)	0 (0.0)	18 (100.0)	0 (0.0)
	G to W	26	1		1	0 (0.0)	157 (100.0)	0 (0.0)	20 (100.0)
			2		2	0 (0.0)	88 (100.0)		
CE	W to G	22	1		1	113 (100.0)	0 (0.0)	20 (100.0)	0 (0.0)
					2	121 (100.0)	0 (0.0)		
	W to G	22	2		1	132 (100.0)	0 (0.0)		
					2	122 (100.0)	0 (0.0)		
	W to G	24	1		1	107 (100.0)	0 (0.0)	21 (100.0)	0 (0.0)
					2	138 (100.0)	0 (0.0)		
					3	96 (100.0)	0 (0.0)		
	W to G	26	2		1	101 (100.0)	0 (0.0)	12 (100.0)	0 (0.0)
Vitrification cryopreservation									
Using SE	W to G	23	1	12-17	1	140 (100.0)	0 (0.0)	21 (100.0)	0 (0.0)
					2	122 (100.0)	0 (0.0)	20 (100.0)	0 (0.0)
Using CE	W to G	22	1		1	111 (100.0)	0 (0.0)	12 (100.0)	0 (0.0)
					2	134 (100.0)	0 (0.0)		
Controls									
W♂ x W♀						253 (100.0)	0 (0.0)	23 (100.0)	0 (0.0)
W♂ x G♀						122 (100.0)	0 (0.0)	15 (100.0)	0 (0.0)
G♂ x G♀						0 (0.0)	139 (100.0)	0 (0.0)	23 (0.0)

'W to G' indicates wild-type zebrafish-to-golden-type zebrafish primordial germ cell-transplantation chimeras. 'G to W' indicate golden-type zebrafish-to-wild-type zebrafish PGC transplantation chimeras. CE; cultured embryoids, DB; dissociated blastomeres, dpf; days post-fertilization, SE; somitogenesis-stage embryos.

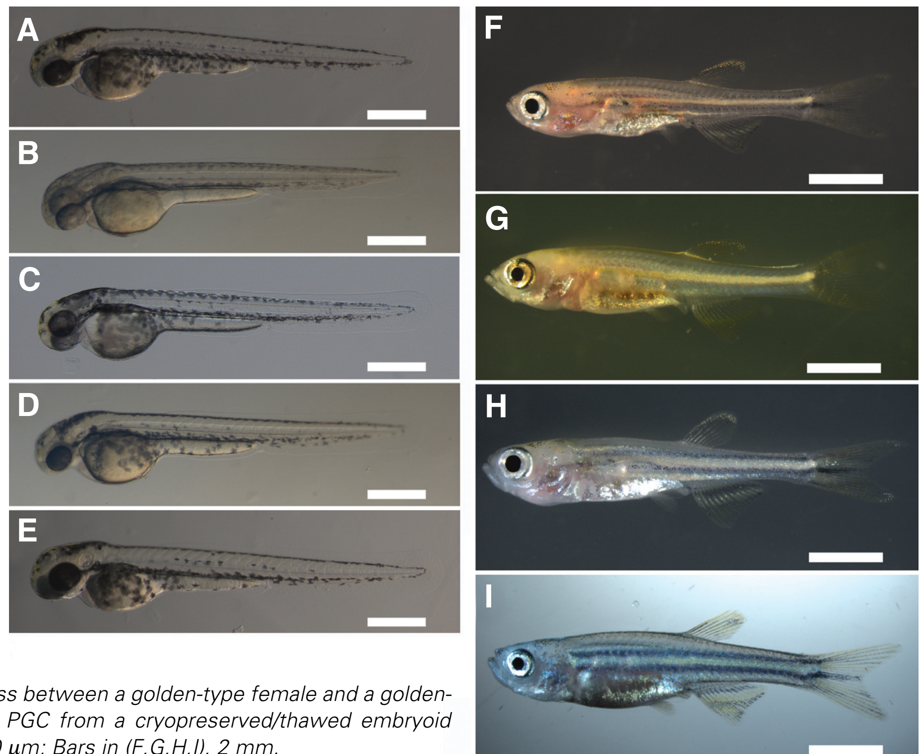

Fig. 4. Zebrafish used in this study and melanophore pigmentation of control and F_1 offspring from a cross between normal golden-type females and chimeric male fish. (A) *Control offspring of a wild-type zebrafish at 2 days post-fertilization (dpf).* **(B)** *Control offspring of a golden-type zebrafish at 2 dpf.* **(C)** *Control offspring of a cross between a golden-type female and wild-type male at 2 dpf. Phenotype is wild-type.* **(D)** *Offspring at 2 dpf of a cross between a golden-type female and a golden-type male chimera, in which a wild-type primordial germ cell (PGC) from an embryoid cultured for 2 days at 22°C was transplanted into a golden-type blastula. Phenotype is wild-type.* **(E)** *Offspring at 2 dpf of a cross between a golden-type female and a golden-type male chimera, in which a wild-type PGC from a cryopreserved/thawed embryoid cultured for 1 day at 22°C was transplanted into a golden-type blastula. Phenotype is wild-type.* **(F)** *Control offspring at 30 dpf of a cross between a golden-type female and a wild-type male.* **(G)** *Control offspring at 30 dpf of a cross between a golden-type female and a golden-type male.* **(H)** *Control offspring at 30 dpf of a cross between a wild-type male and a golden-type female.* **(I)** *Offspring at 30 dpf of a cross between a golden-type female and a golden-type male chimera transplanted with a wild-type PGC from a cryopreserved/thawed embryoid cultured for 1 day at 22°C. Bars in (A,B,C,D,E), 500 μm; Bars in (F,G,H,I), 2 mm.*

germ-line chimeras using donor PGCs from the DB or CE. Our results also highlight a novel prospect for the preservation of gene stocks. Our method focuses on the acquisition of live PGCs from cryopreserved embryos by removal of the yolk, which enhances the viability of cryopreserved/thawed PGCs (Higaki *et al.*, 2010). Moreover, the viability of PGCs from embryoids is equivalent to those from embryos after removal of the yolk. Previous studies have created germ-line chimeras in teleosts using the slow-freezing cryopreservation method, followed by transplantation of cryopreserved/thawed trout tissue fragments containing PGCs from around the gonadal ridge (Kobayashi *et al.*, 2003; Kobayashi *et al.*, 2007). One of the disadvantages of these methods is the need to transplant many PGCs, involving a large cell mass including somatic cells, and the transplantation efficiency is therefore restricted in small host fish is limited. Moreover, the supply PGCs is very limited. In contrast, the CE method has potential use in a wide range of applications. It is possible to extract PGCs effectively from recessive embryo-lethal mutants and fish with low hatching success, such as the Japanese freshwater eel (Unuma *et al.*, 2005). Further studies to investigate the use of these techniques in other fish are planned, but these methods of acquiring and transplanting PGCs, including cryo-preservation by vitrification, are likely to play important roles in future efforts to preserve genetic information.

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