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There was an error published in *Development* **137**, 1227-1230 in the version that appeared on ePress on March 10, 2010.

The name of author Yoshiko Iwasaki was spelled incorrectly. The corrected author list, as above, appears in the full online and print versions.

The authors apologise to readers for this mistake.

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Sexual plasticity of ovarian germ cells in rainbow trout

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SUMMARY

The sexual plasticity of fish gonads declines after the sex differentiation period; however, information about the plasticity of the germ cells themselves after sex differentiation is limited. Using rainbow trout (*Oncorhynchus mykiss*), we recently established a novel germ cell transplantation system that provides a unique platform with which to dissect the developmental and cellular mechanisms underlying gametogenesis. Using this technique, we show here that transplanted ovarian germ cells isolated from 6- to 9-month-old donors can colonize sexually undifferentiated embryonic gonads and resume gametogenesis. Ovarian germ cells containing oogonia and early oocytes isolated from female rainbow trout were transplanted into the peritoneal cavities of hatching-stage fry of both sexes and the behavior of the donor cells was observed. The transplanted ovarian germ cells migrated towards the recipient gonads, interacted with embryonic gonadal somatic cells, proliferated rapidly, and eventually differentiated into eggs in female recipients and sperm in male recipients. Furthermore, the donor-derived eggs and sperm obtained from the recipient fish were functional and were able to produce normal offspring. These findings indicate that mitotic germ cells, the oogonia, possess a high level of sexual plasticity.

KEY WORDS: Oogonia, Spermatogonial stem cell, Plasticity, Sex, Germ cell, Fish, Rainbow trout

INTRODUCTION

In fish, sexually undifferentiated gonads are plastic: their sex can be manipulated easily by exogenous sex steroid administration regardless of their genetic sex (Devlin and Nagahama, 2002). After the sex differentiation period, however, exogenous steroids are no longer effective and only aromatase inhibitor can induce masculinization (Nakamura et al., 2003; Ogawa et al., 2008). Thus, the plasticity of fish gonads declines after the sex differentiation period; however, information about the plasticity of the germ cells themselves after sex differentiation is limited.

In this study, we transplanted germ cells isolated from the meiotic ovaries of 6- to 9-month-old rainbow trout that had already completed sex differentiation into hatching-stage fry in order to characterize the sexual plasticity of oogonia. Rainbow trout are a gonochoristic species – that is, they never change their sex throughout their lifetime. They also have a rigid XY sex-determining system (Brunelli et al., 2008). Sex differentiation in this species is completed ~70 days after fertilization at 10°C and, at almost the same time, some of the female germ cells enter meiosis (Takashima et al., 1982). They reach sexual maturity at 2 years of age.

MATERIALS AND METHODS

Germ cell transplantation

Ovarian cell suspensions were prepared from 6- to 9-month-old p*vasa-Gfp* transgenic trout (Yoshizaki et al., 2000; Takeuchi et al., 2002). Freshly isolated ovaries were minced and incubated with 1 ml of L-15 containing 2 mg/ml collagenase H (Roche, Basel, Switzerland) and 500 IU/ml dispase (Godo-Shuzo, Tokyo, Japan) for 7-9 hours at 10°C. The resultant cell suspension was filtered through a 20-μm pore-size nylon screen to eliminate cell clumps and vitellogenic oocytes. The cell pellets were resuspended in 200 μl of L-15 medium. Approximately 20-30 nl of the cell suspension, containing ~15,000 cells, were intraperitoneally transplanted into 25-day-old fry.

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Cell sorting by flow cytometry

Ovaries isolated from 9-month-old p*vasa-Gfp* transgenic trout were subjected to GFP-dependent cell sorting to purify ovarian somatic cells and germ cells in a flow cytometer (Epics Altra, Beckman Coulter, Miami, FL, USA) according to the method of Kobayashi et al. (Kobayashi et al., 2004). To dissociate testes, 0.5% trypsin (Worthington Biochemical, Lakewood, NJ, USA) was used instead of collagenase and dispase. Approximately 100 GFP-positive germ cells, GFP-negative somatic cells, and non-sorted cells were transplanted intraperitoneally into the 25-day-old recipients. Colonization efficiency was obtained by the following formula: colonization efficiency (%) = (number of fry incorporating GFP-labeled cells in their gonadal anlagen at 22 days post-transplantation/number of fry used for cell transplantation) × 100.

Measurement of the cross-sectional area of germ cells

In order to compare the sizes of donor ovary-derived germ cells labeled with GFP and recipient primordial germ cells (PGCs) labeled with DsRed, we observed the recipient gonads using an Olympus FV1000 confocal microscope. Samples were optically sectioned at consecutive intervals of 2 μm and the images reconstructed to one projection image. For quantitative assays, we made 6-μm paraffin sections, measured the cross-sectional areas of donor-derived germ cells that were positive for GFP immunostaining, and compared them with those of oogonia in the donor ovary and those of PGCs in undifferentiated gonads of hatching-stage fry. Only cells showing a clear view of the nucleus were selected from serial sections and used to measure the maximum cross-sectional area of each cell.

Progeny tests

Triploids were produced as described previously (Okutsu et al., 2007). All recipients were reared until maturity. To determine the production of offspring from gametes derived from donor ovarian cells, eggs and sperm obtained from triploid recipients were fertilized with the sperm and eggs obtained from wild-type trout. The number of donor-derived spermatozoa was calculated by the method of Okutsu et al. (Okutsu et al., 2006a).

RESULTS AND DISCUSSION

In this study, we used a transgenic trout strain carrying the *Gfp* gene driven by cis-regulatory elements derived from the trout *vasa* gene (Yoshizaki et al., 2000; Takeuchi et al., 2002) as a donor. Since the precise expression pattern of GFP in the ovaries of this

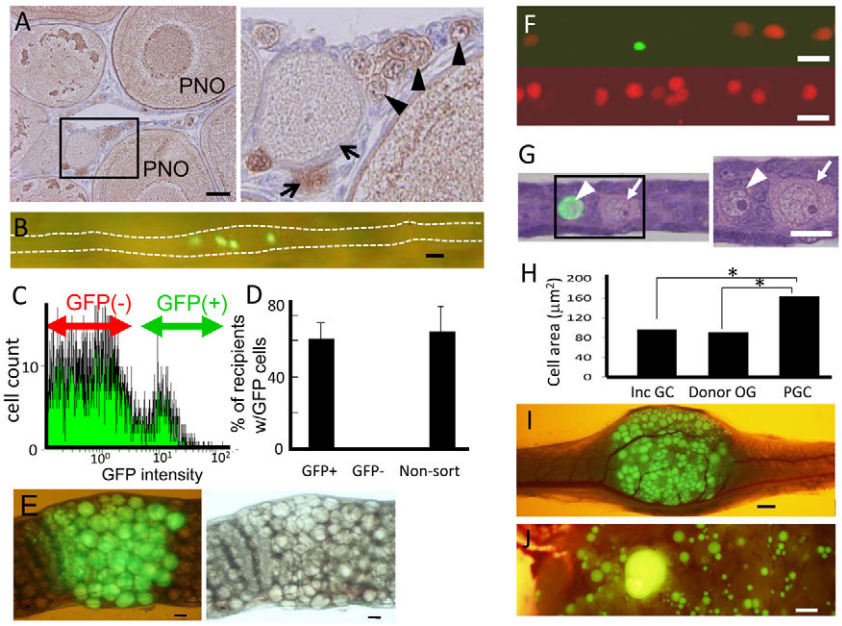

Fig. 1. Ovarian germ cells can colonize female recipient gonads and resume oogenesis. (**A**) Oogonia (arrowheads) and oocytes (arrow) specifically expressed GFP in a p*vasa-Gfp* transgenic trout ovary as examined by immunohistochemistry with a GFP-specific antibody. The boxed area is shown at high magnification on the right. PNO, peri-nucleolus stage oocyte. (**B**) Incorporation of GFP-labeled donor germ cells into a recipient gonad 20 days post-transplantation. Dashed lines outline the undifferentiated recipient gonad. (**C**) Fluorescence intensity patterns of donor ovarian cells. The *x*-axis represents the GFP intensity (logarithmic scale) and the *y*-axis indicates the cell count (linear scale). (**D**) GFP-positive germ cells, but not GFP-negative cells, were incorporated into the recipient gonads after the transplantation of GFP-positive or GFP-negative populations into allogeneic recipients. Data are the mean ± s.e.m. (*n*=3). (**E**) GFP-labeled donor germ cells proliferated and differentiated into oocytes in the recipient ovary. Left, fluorescent view; Right, bright-field view. (**F**) Donor ovary-derived germ cells (showing green fluorescence) were clearly distinct from the sexually undifferentiated PGCs (showing red fluorescence) of recipients (45 days old; upper panel) and those of hatching-stage fry (30 days old; lower panel) by their small size. (**G**,**H**) The cross-sectional areas of donor ovary-derived germ cells that were incorporated into the recipient gonad, those of oogonia of 9-month-old fish, and those of PGCs of hatching-stage fry were measured and compared. In G, the arrowheads indicate donor-derived germ cells stained with GFP antibody, and arrows indicate the endogenous PGCs of the recipient fish. The boxed area is shown at high magnification on the right. Donor-derived germ cells were distinguished from PGCs by their small size and stronger staining with Hematoxylin. *, $P<0.01$. Data are the mean ± s.e.m. (*n*=12). (**I**) GFP-labeled donor germ cells proliferated and differentiated into oocytes in the sterile ovary of a triploid recipient. (**J**) GFP-labeled donor-derived germ cells remained in the post-ovulatory follicles of a triploid recipient. Scale bars: 10 μm in G; 30 μm in A,B,E,F; 1 mm in I,J.

transgenic trout has not been examined, we first performed immunohistochemistry using an antibody against GFP. We found that GFP was expressed specifically in ovarian germ cells, including oogonia and early stage oocytes (Fig. 1A).

In the next experiment, we transplanted ~15,000 ovarian cells [11.9±7.4% (± s.e.m.) GFP-positive germ cells] into the peritoneal cavities of non-transgenic allogeneic rainbow trout fry that possessed sexually undifferentiated gonadal anlagen containing primordial germ cells (PGCs). We performed intraperitoneal transplantation using hatching-stage fry as their immune systems are immature (Manning and Nakanishi, 1996) and because we previously proved that they are incapable of rejecting allogeneic cells (Takeuchi et al., 2003; Takeuchi et al., 2004). By 20 days after the transplantation of ovarian germ cells, 55 out of 133 recipient fish had donor-derived germ cells in their sexually undifferentiated gonads (Fig. 1B). The mean number of GFP-positive germ cells incorporated into the recipient gonads was 3.3±2.6.

In order to clarify which type of ovarian cell repopulated the embryonic gonads, we performed GFP-dependent cell sorting using ovarian cell suspensions prepared from p*vasa-Gfp* transgenic rainbow trout (Fig. 1C). Transplantation experiments with sorted cell populations (GFP-positive germ cells and GFP-negative somatic cells) revealed that only GFP-positive germ cells, which were identified as oogonia or small oocytes, possessed the ability to migrate towards, and be incorporated into, the recipient gonads (Fig. 1D). This suggests that somatic donor cells without green fluorescence did not transdifferentiate into GFP-positive germ cells after transplantation.

By fluorescence observation of female recipients at 5 months post-transplantation, we found that 24 out of 51 recipients carried donor-derived germ cells in their ovaries (Fig. 1E). The mean number was 133±110. Taken together with the fact that the mean number of germ cells incorporated into the recipient gonads was 3.3, these data indicate that the donor-derived germ cells proliferated more than 40-fold (133/3.3) over the 130-day period after being incorporated into the recipient gonads. This strongly suggests that the ovarian germ cells incorporated into the recipient gonads were oogonia, having mitotic activity.

In order to rule out the possibility that PGCs, a few of which might remain in the donor ovary, were incorporated into the recipient gonad, we performed morphological characterization of donor-derived germ cells in the recipient gonads. We compared the cross-sectional area of each donor-derived germ cell incorporated into the recipient gonads with that of oogonia in the donor ovary and PGCs in hatching-stage fry. The donor-derived germ cells incorporated into the recipient gonads were nearly identical in size to the oogonia

in the donor ovary and were significantly smaller ($P<0.05$) than the PGCs (Fig. 1F-H). This indicates that the donor-derived germ cells incorporated into the recipient gonads were oogonia and not PGCs.

In order to confirm whether donor-derived germ cells have the ability to differentiate into functional eggs in the allogeneic recipient ovary, we performed progeny tests using mature female recipients. For this experiment, we transplanted diploid ovarian germ cells from pvasa-*Gfp* hemizygous (Gfp/–) fish and dominant orange-colored mutant (Boonanuntanasarn et al., 2004) heterozygous [OR/wild type (WT)] fish into wild-type (black-pigmented, WT/WT/WT) non-transgenic (–/–/–) triploid recipients. The triploid recipients were produced by suppression of the second polar body extrusion by heat-shock treatment. It is widely known that triploid female trout are completely sterile and do not proceed to vitellogenesis (Carrasco et al., 1998). Twenty-month-old triploid recipients that received diploid ovarian germ cells had ovaries that possessed a large colony of vitellogenic oocytes (Fig. 1I). Furthermore, these vitellogenic oocytes showed clear green fluorescence, demonstrating that they were derived from donor ovarian germ cells. Two years after the transplantation, we obtained one mature female trout out of 20 female recipients. The mature recipient showed normal fecundity and the resulting eggs showed a normal fertilization rate (76.0%). Furthermore, the ratios of orange-colored to wild-type trout and of p*vasa*-*Gfp*-positive to p*vasa*-*Gfp*-negative trout were both nearly 1:1 (Fig. 2A,B; Table 1). By contrast, none of the control triploid trout without germ cell transplantation matured. Taken together with the fact that nearly 50% of offspring showed donor-trout-derived phenotypes (GFP-positive and orange-colored), this indicates that the offspring obtained from the triploid recipients were all donor derived. Furthermore, the post-ovulatory ovary contained many oogonia and early-stage oocytes, indicating that the donor-derived oogonia can support oogenesis for multiple spawning seasons (Fig. 1J). Thus, oogonia obtained from meiotic ovaries can interact with the microenvironment produced by hatching-stage fry, resume oogenesis, and eventually differentiate into functional eggs in the allogeneic recipient ovary.

To further confirm the sexual plasticity of oogonia, we examined the behavior of donor-derived germ cells transplanted into allogeneic male recipients. The recipients were evaluated at 5 months of age. Out of 52 male recipients, 26 carried donor-derived germ cells in their testes. In most of these, the donor-derived germ cells formed a large colony comprising 200-400 GFP-positive cells (Fig. 3A). In fact, we could not find any significant differences in the rates of recipients containing donor germ cells in their gonads and those having colonies with proliferated donor germ cells between female and male recipients (Fig. 3B). Similar results have also been obtained in PGC and spermatogonia transplantation in rainbow trout (Takeuchi et al., 2003; Okutsu et al., 2006a).

Two years after the transplantation of ovarian germ cells isolated from transgenic orange-colored trout (Gfp/–, OR/WT), 11 out of 20 male triploid recipients (–/–/–, WT/WT/WT) matured. Although we could obtain milt from the 11 male recipients, only three recipients produced normal white-colored milt; the rest produced transparent milt that contained extremely small amounts of sperm. By contrast, control triploid males never produced white-colored milt. It has been reported that triploid male rainbow trout produce small amounts of aneuploid sperm, which cannot fertilize (Carrasco et al., 1998). Therefore, we concluded that the three recipients that produced white-colored milt showed donor-derived spermatogenesis, and these recipients were used for further progeny tests. The milts obtained from the recipients were used to inseminate eggs obtained from non-transgenic (–/–) wild-type (WT/WT) rainbow trout. The resulting fertilized eggs developed and hatched normally (Fig. 2C). Furthermore, the ratios of orange-colored trout to wild-type trout and of p*vasa*-*Gfp*-positive to p*vasa*-*Gfp*-negative were both nearly 1:1 (Fig. 2C,D). These results indicated that the male recipients receiving ovarian germ cells produced donor oogonia-derived sperm but not recipient-derived sperm. Furthermore, the resulting sperm were fully functional.

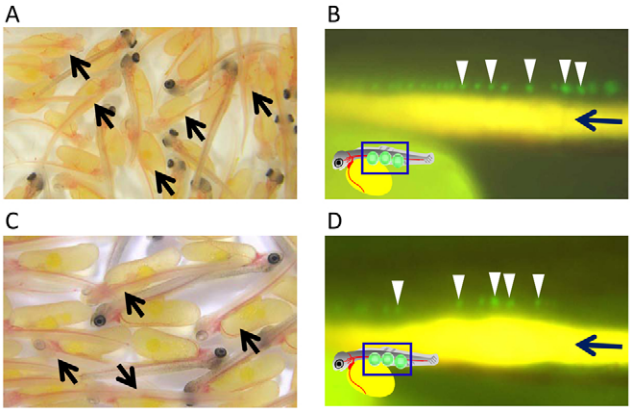

Fig. 2. Donor-derived offspring obtained from progeny tests. (**A**,**C**) Approximately 50% of newly hatched F1 offspring derived from the female (A) and male (C) recipients showed the orange-colored phenotype (arrow), suggesting that all F1 offspring were donor (OR/WT) derived. (**B**,**D**) Approximately 50% of F1 offspring derived from the female (B) and male (D) recipients possessed GFP-positive germ cells, suggesting that all F1 offspring were donor (GFP/–) derived. The boxed areas in the schematics indicate the regions imaged in B,D. Arrows indicate intestines showing autofluorescence, and arrowheads indicate germ cells.

If oogonia carrying XX sex chromosomes produced sperm in the recipient males, then the F1 fish produced by the milt obtained from the recipient fish and eggs obtained from wild-type females (XX) should all be female. To confirm this, we examined the sex ratio of the resulting F1 fish. All 45, 100 and 51 F1 offspring derived from the above-mentioned three recipients were female, demonstrating that the sperm produced by the recipient males was derived from oogonia carrying XX sex chromosomes.

In this study, donor-derived oogonia, transplanted into male recipients, could produce fully functional sperm. Furthermore, during these studies we always obtained recipients showing a nearly 1:1 sex ratio and did not find any ovo-testis-like morphology, suggesting that oogonial transplantation did not induce any sex reversal of the recipient fish at either the gonadal or cellular levels.

Table 1. Hatching date and appearance rate of donor-derived offspring among the F1 generation

Recipient	No. of eggs used	Hatchlings (%)	GFP positive (%)	Orange colored (%)
Female 1	2115	69.3	54.2	52.2
Control 1	906	95.8	0	0
Male 1	472	99.2	50.4	48.1
Male 2	452	96.9	41.1	44.8
Male 3	356	64.3	52.4	50.7
Control 2	493	98.4	0	0

Milt obtained from one male was divided into two groups and used to inseminate eggs obtained from female 1 and control 1. Eggs obtained from one female were divided into four groups and used for insemination with milt from males 1-3 and control 2.

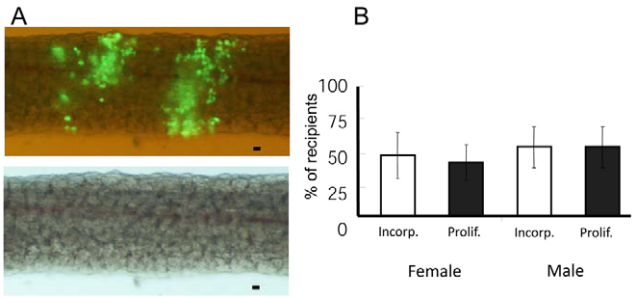

Fig. 3. Ovarian germ cells can colonize male recipient gonads and resume spermatogenesis. (**A**) GFP-labeled donor germ cells were incorporated into, and started to proliferate in, recipient testis at 5 months post-transplantation. Upper panel, fluorescent view; lower panel, bright-field view. Scale bars: 20 μm. (**B**) The percentage of recipients that contained donor germ cells in their gonads (white bars) and those having colonies with proliferated donor germ cells (black bars) did not differ significantly between female recipients and male recipients. Data are the mean ± s.e.m. (*n*=3).

Several previous reports have described natural sex reversal (Devlin and Nagahama, 2002) and artificial sex reversal induced by an aromatase inhibitor (Nakamura et al., 2003; Ogawa et al., 2008) in fish, but none has clarified whether this was due to differentiated germ cells changing their sexual characteristics or undetermined cells within the gonad being recruited to initiate a new course of differentiation. Our study has demonstrated that oogonia isolated from sexually differentiated ovaries contain a cell population that can differentiate into fully functional sperm. This clearly indicates that oogonia are sexually plastic and that the sexual differentiation of germ cells is controlled solely by the somatic microenvironment, rather than being cell-autonomous.

Once fish spermatogonia are committed to differentiate, they lose their self-renewal activity and possess only a limited ability to proliferate (Schultz and Miura, 2002). In rainbow trout, differentiated spermatogonia are reported to undergo mitosis no more than seven times, followed by two consecutive cycles of meiosis (Loir, 1999); thus, one founder spermatogonium can produce up to 512 spermatozoa. In this study, the average number of donor-derived spermatozoa produced in the recipient testes was 3.2×10^9 (range, $1.58\text{-}5.85\times10^9$). The average number of donor cells incorporated into the gonads of a recipient fish was 3.3±2.6. These values imply that the donor-derived germ cells underwent mitosis 30 times on average, which is far greater than the predicted proliferation rate of differentiated spermatogonia. Furthermore, these recipients also produced donor oogonia-derived sperm during the following spawning season. These data suggest that the oogonia, incorporated into the recipient gonads, differentiated into spermatogonial stem cells that could differentiate into spermatozoa and had a high, or even unlimited, capacity for self-renewal.

PGCs are well known to migrate towards the gonadal anlagen by chemotaxis during embryogenesis (Raz and Reichman-Fried, 2006), but it has been believed that their migration abilities are lost after they differentiate into either spermatogonia or oogonia. Since, for technical reasons, we failed to microinject donor germ cells directly into gonadal anlagen in hatching-stage trout fry, we delivered oogonia into peritoneal cavities instead. We clearly showed that oogonia transplanted into the peritoneal cavities of recipient fry migrated towards, and were eventually incorporated into, the gonadal anlagen, suggesting that even oogonia isolated from the meiotic ovaries of 6- to 9-month-old fish might still possess, or reacquire, the ability to respond to chemoattractants secreted from the embryonic gonadal anlagen.

In addition to the transplantation of PGCs (Takeuchi et al., 2003) and spermatogonia (Okutsu et al., 2006a), in this study we developed a method to transplant oogonia in rainbow trout. From an applicational point of view, this novel technique expands the versatility of fish germ cell transplantation, especially for surrogate broodstock technology (Okutsu et al., 2006b).

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Competing interests statement
The authors declare no competing financial interests.

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