Title

Artificially induced tetraploid masu salmon have the ability to form primordial germ cells

Running Title

PGCs in tetraploid masu salmon

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Abstract

Diploid gametes generated with tetraploid animals are a stepping stone to improving techniques of chromosome manipulation. But artificially induced tetraploid individuals generally die soon after hatching. Diploid gametes could have been induced by *in vivo* cultures of tetraploid primordial germ cells (PGCs) through germ-line chimera. In the present study, characteristics of PGCs were studied in inviable tetraploid masu salmon, *Oncorhynchus masou.* Histological observation of tetraploid embryos revealed that the same or smaller numbers of PGCs were observed and they migrate into the genital ridges as did diploid PGCs during gonadogenesis. By whole mount *in situ* hybridization using *vasa* mRNA, 4-35 *vasa*-positive signals were detected in a pair of genital ridges of tetraploids. By the cytological observation of genital ridge cell suspensions, several large round cells were observed, some of which extended pseudopodia. They also contained large nuclei and round granules in their cytoplasm, characteristics of PGCs. As the results suggest that inviable artificial tetraploids have PGCs, we expect to achieve diploid gamete production through surrogate propagation and tetraploid fish production.

Key Words

tetraploidy, primordial germ cells (PGCs), pseudopodium, *vasa*, surrogate propagation, germ-line chimera

Introduction

Chromosome set manipulation is a technique to control the number and combination of a haploid set of chromosomes. This technique is useful for genetic analyses and the improvement of aquaculture traits through sex manipulation, sterilization, inbreeding, cloning and so on [1-3]. A fertile tetraploid animal with four haploid sets of chromosomes is key because it produces functional diploid gametes which are able to generate progenies [4-10]. For example, mass production of triploid progeny would be possible with diploid gametes of tetraploids, when used for crosses with haploid gametes of normal diploids. With genetically-inactivated sperm or eggs, diploid gametes can develop to gynogenetic or androgenetic diploids without any treatment to duplicate chromosomes. The resultant triploids and gyno- or androgenetic diploids can be used for the production of sterile and unisex populations, respectively [1-3].

In several teleost species that tetraploid embryos have been successfully induced by first cleavage inhibition, but showed extremely low survival potential and died soon after hatching or feeding before growing to adult size [11-18]. In our previous study [18], gynogenetic diploids in masu salmon survived beyond hatching and grew, but tetraploids died just before hatching, when first cleavage was inhibited under the same conditions using single-pair mating to eliminate the difference in genetic components. Consequently, we concluded that the drastic mortality in tetraploids was caused by the elevation of ploidy itself in this species. This suggests that, at least in masu salmon, diploid gametes cannot be obtained with mature tetraploid individuals induced by first cleavage inhibition. Therefore, innovative approaches are required for the production of diploid gametes.

Recently, the use of germ-line chimera has been proposed in the aquaculture of teleost fish [19-21]. This technique allows us to obtain the gametes of a favored genotype through the gonads of another genotype. It is achieved by inducing germ-line chimera between different species or between different strains during early development. We already

reported the induction of germ-line chimera by transplanting single primordial germ cells (PGCs) into a sterile host blastula (SPT method), that functional eggs and sperm differentiated in an inter-specific host (donor; pearl danio, host; zebrafish), and that functional sperm differentiated in an inter-familial host (donor; loach, host; zebrafish) [22]. In salmonids, germ-line chimera has been induced by transplanting PGCs isolated from the genital anlage into the peritoneal cavity, and functional gametes were obtained [23-26]. If tetraploid PGCs could be isolated from tetraploid embryos and transplanted into other viable hosts, and the transplanted PGCs could be induced to differentiate into diploid gametes, tetraploid progenies would be obtained by normal mating between transplanted individuals. Thus, tetraploid PGC transplantation via germ-line chimera offers a workable strategy to by-pass the difficulties associated with the production of tetraploid individuals by first cleavage inhibition.

In this study, as a first step toward the acquisition of diploid gametes *via* surrogate propagation, we examined histological specimens and suspensions of cells from the genital ridges of artificially induced tetraploids in order to confirm whether or not they have PGCs. We also confirmed the presence of PGCs by whole mount *in situ* hybridization using a *vasa* probe specifically expressed with germ cells.

Materials and methods

Gamete collection

Eggs and sperm were collected from masu salmon reared at the Mori Research Branch, Hokkaido Fish Hatchery, in 2004. They were cooled on ice and transported to the Nanae Freshwater Laboratory, Hokkaido University. Fertilized eggs were obtained by the dry method using three females and two males (Family 1) and another two females and two males (Family 2). Each batch of fertilized eggs was kept in a water bath regulated at 10 ºC

until further treatment.

Tetraploid induction

The fertilized eggs of Family 1 and Family 2 were divided into two groups. One group was used as the intact diploid control (2N) and the other was treated to induce tetraploidy (4N). Tetraploidy was induced by inhibiting the first cleavage using hydrostatic pressure shock (PS) (700 kg/cm^2, 7 min duration) at six hours post-fertilization (hpf) as described previously [18]. Immediately after the treatments, the four groups (Family 1, 2N and 4N; Family 2, 2N and 4N) were incubated separately in a hatching tank running 10 ºC well water.

Ploidy determination

Ploidy status was determined using a flow cytometer (FCM) (Ploidy Analyzer, Partec, Germany). In Family 1, the embryos were chosen at random from each group at 22 and 32 days post-fertilization (dpf) and whole individual embryos were used for flow cytometry after removal of the chorion and yolk manually. In Family 2, after the removal of chorion and yolk sac at 30 dpf, tail tips of embryos were manually cut off and ploidy was analyzed by FCM. The remaining parts were individually incubated on 24-well plates with Ringer's solution at 4 ºC for the isolation of PGCs described below. For nuclear extraction, the whole embryo or tail tip was minced with 100 µl of A solution (High Resolution DNA Kit type T, Partec, Germany), 20 min later, filtered through a 50µm nylon mesh, added to 500 µl of B solution including DAPI (4-6-diamino- 2-phenylindole dihydrochloride) as a nuclear staining solution and left for 10 min. Then, relative DNA content was measured using control diploid embryos as a standard (2C) and ploidy status was determined as reported [18].

Histological observation of the genital ridges in the 2N and 4N groups

To confirm whether tetraploids form PGCs or not, the surviving embryos of Family 1

were fixed with Bouin's solution at 21, 26, 31 and 36 dpf after the removal of the yolk. Then, the fixed embryos were embedded in paraffin, sectioned 8 μm thick and stained with hematoxylin-eosin.

Cytological observation of living genital ridge cells in the 2N and 4N groups

After the ploidy determination, the embryos verified as diploids in the 2N group and tetraploids in the 4N group were selected from 24-well plates. The extraction and dissociation of genital ridges were performed as described previously [27]. After the embryos were anesthetized with 2-phenoxyethanol, the genital ridges were manually extracted together with the mesonephric duct which was then removed because the genital ridges were scissile at this stage. About 30 genital ridges were extracted from each group of diploids and tetraploids and samples were collected separately in different 500 μl microtubes, incubated with 0.5% trypsin (Worthington Biochemical Corp., Lakewood, USA) in PBS(+) (pH=8.2), 5% fetal bovine serum (FBS) and 150 U/ml of DNase at 20 ºC for 2 hr. One hour into the incubation, gentle pipetting was performed to aid the cell dissociation (trypsinization).

After the trypsinization, the cell suspensions of genital ridges from both diploids and tetraploids were observed by light microscope (BH-2, Olympus, Tokyo) and stereomicroscope (SZX-12, Olympus, Tokyo). Photographs were taken using a digital camera attached to each microscope and cell size was measured in at least 20 samples of PGCs and somatic cells. PGCs were distinguishable from somatic cells in their size and inner structure, being larger and having nuclei and round granules [28-30].

Whole mount *in situ* hybridization (WM-ISH) using *vasa* mRNA probe

After the ploidy determination, the embryos verified as diploids in the 2N group and tetraploids in the 4N group were selected from 24-well plates. Embryos were manually

removed from the liver and gut, and fixed with 4% paraformaldehyde in PBS overnight at 4 ºC. The fixed embryos were then stored in methanol at -20 ºC prior to use. WM-ISH was performed as described [31] with modifications to examine whether the key genes specifically expressed in germ cells were also observed in tetraploid embryos. The antisense *vasa* RNA probe transcribed from a 0.56Kb fragment of the 3' untranslated region (3'-UTR) of zebrafish *vasa* complementary DNA (cDNA) was labeled with digoxigenin (DIG; in accordance with the Roche DIG RNA labeling protocol) and detected using anti-DIG alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) as substrate.

Statistical analysis

Diameters of the PGCs and somatic cells were compared between control diploids and artificially induced tetraploids using Student's *t*-test.

Results

Ploidy status of 2N and 4N embryos

The ploidy status of surviving embryos was flow-cytometrically determined in Family 1 and 2 (Table 1). In the 2N groups of both Family 1 and 2, almost all the embryos had a DNA content in the diploid range (1.9-2.1C) except for a few hypodiploid (1.7-1.9C) embryos. In the 4N groups, high proportions of embryos developed with the PS treatment (83.2-100%) were in the tetraploid range (3.8-4.2C). The other embryos had a DNA content in the near tetraploid range; hypotetraploid (3.6-3.8C), hypertetraploid (4.2-4.4C) or mosaic containing tetraploid cells.

Histological observation of the genital ridge during early development

At 22, 26, 31 and 36 dpf, the early gonadal development of both 2N and 4N embryos was observed histologically (Fig. 1). In the 2N control group (Fig. 1A-D), PGCs were attached to a pair of mesonephric ducts at 22 dpf (eyed stage) (Fig. 1A). At 26 dpf, PGCs were gradually migrating toward the ventral side along the mesonephric duct wall, PGCs were covered with somatic cells and a pair of genital ridges had formed (Fig. 1B). At 31 dpf (Fig. 1C) and especially at 36 dpf (hatching stage) (Fig. 1D), the supporting cells covering PGCs were thickened and the genital ridges were separated from each other and hanging down from the wall of the body cavity. In the 4N group (Fig. 1E-H), PGCs were attached to a pair of mesonephric ducts at 22 dpf as in the 2N control group (Fig. 1E). The development of the genital ridge proceeded behind that in the 2N control group (Fig. 1F, G), but at 36 dpf, PGCs covered with somatic cells were hung down from the wall of the body cavity (Fig. 1H).

Cytological characteristics of dissociated cells from the genital ridge

After ploidy status was determined with tail tip cells at 30 dpf, live cell suspensions from the genital ridge were microscopically observed (Fig. 2). The cell suspension contained a few large and round cells (arrowheads) in both the 2N (Fig. 2A, C, E) and 4N (Fig. 2B, D, F) groups. They also contained large nuclei and granules in their cytoplasm, a histological feature of fish PGCs (Fig. 2A, B). Some of these cells extended pseudopodia (Fig. 2, arrow). These large cells were in fact identified as salmonid PGCs in the genital ridge according to previous reports [27, 32, 33].

The diameters of the PGCs of diploid and tetraploid embryos were 17.66±2.15 μm and 25.53±2.65 μm, respectively (Table 2), and the difference was significant ($p<0.05$). Those of somatic cells except for PGCs were 10.74±0.81μm and 14.70±1.65μm, respectively, and also significantly different ($p<0.05$) (Table 2).

Detection of PGCs by WM-ISH using the *vasa* mRNA probe

vasa mRNA was detected by WM-ISH in diploid and tetraploid embryos after ploidy status was determined with tail tip cells at 30 dpf using FCM (Fig. 3). Normal diploid embryos had a total of 25-45 *vasa*-positive signals in a pair of genital ridges in the trunk region (Fig. 3A, B). In the tetraploids, both normal (Fig. 3C, D) and abnormal embryos with a curved body axis and nanism (Fig. 3E, F) also had 4-35 *vasa*-positive signals in a pair of genital ridges, regardless of their morphology (Fig. 3, arrowheads).

Discussion

Gonadal development in tetraploid embryos

By WM-ISH using zebrafish *vasa* mRNA, 25-45 signals in diploids and 4-35 signals in tetraploids were detected in a pair of genital ridges in the trunk region. This expression pattern was well consisted with a previous finding with eyed-stage embryos of diploid rainbow trout [34]: 30-50 PGCs expressing green fluorescent protein (GFP)-positive cells were observed in living *vasa*-GFP transgenic rainbow trout [32]. So the *vasa* mRNA-positive cells in this study were considered to be PGCs. Histological observation of the tetraploid embryos revealed that the same or smaller numbers of PGCs and they migrated into the genital ridges as did diploid PGCs during gonadogenesis. The *vasa* signals in the genital ridge, however, were not found in straight lines in the tetraploid, when compared with those in the diploid (Fig. 3G). The results suggest that the genital ridge is not always constructed correctly in tetraploid embryos. Therefore, the best time for the excision of genital ridges was judged to be 26-31 dpf.

Ploidy of PGCs in tetraploid individuals

In this study, PGCs were detected by histological and WM-ISH analyses in normal and

even malformed tetraploid embryos at the hatching stage. As tetraploidy was confirmed in advance by flow cytometry of fin tip, PGCs were also expected to be tetraploid.

The cytological observation of genital ridge cell suspensions revealed two types of cells, large and small. The features of the larger cells such as a large nucleus, round granules and extended pseudopodia were well consistent with the characteristics of PGCs reported previously [27, 32, 33]. Thus, these large cells are considered to be PGCs. The diameter of tetraploid PGCs (25.53±2.65 μm) was 1.45-fold that of diploid PGCs (17.66±2.15 μm). In previous studies mainly performed with erythrocytes, tetraploidization induced a 1.20-1.45-fold [35], 1.33-fold [36], 1.33-1.68-fold [37] and up to 1.49-fold [38] enlargement of cell size and nuclear size. The values obtained in this study were consistent with those in previous reports suggesting that PGCs are tetraploid.

Tetraploid PGCs for surrogate propagation

In the present study, tetraploid PGCs were successfully induced and extracted. Tetraploids induced by first cleavage inhibition were inviable before the hatching stage in our previous report [18]. However, these tetraploid embryos, even those with serious malformations, were able to form PGCs.

For the production of diploid gametes through the surrogate propagation technique, tetraploid embryos must satisfy the following four steps; i) PGC specification can occur during embryonic development even with malformation, ii) tetraploid PGCs can be extracted from tetraploid embryos before death, iii) tetraploid PGCs have motility in the host genital ridge after their transplantation and iv) transplanted tetraploid PGCs can proliferate and differentiate into functional gametes in the viable host gonad. In this study, we examined the first and second steps. Therefore, to produce diploid gametes through surrogate propagation and tetraploid fish through the normal mating of transplanted individuals, further investigation is required to clarify whether tetraploid PGCs can migrated

toward the genital ridge and have the ability to proliferate and differentiate.

Acknowledgments

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Figure legends

Fig. 1. Histological observation of PGCs (arrows) in diploid control (A-D) and putative tetraploid (E-H) embryos. A, E; 22 days post-fertilization (dpf), B, F; 26 dpf, C, G; 31 dpf, D, H; 36 dpf. g; gut, md; mesonephric duct. Scale bar indicates 500 μm.

Fig. 2. Cell suspensions of the genital ridges of diploid control (A, C, E) and experimental tetraploid (B, D, F) embryos at 30dpf. Observations made using a light microscope (A, B) and a stereomicroscope (C-F). E and F are higher magnification views of the rectangles in C and D, respectively. Arrowheads indicate PGCs. Tetraploid PGCs also possessed large nuclei with a granular cytoplasm, typical morphological characteristics of PGCs. Note the pseudopodia (arrow) extended from PGCs (arrowheads). Scale bars indicate 50 μm.

Fig. 3. *vasa*-positive cells detected with whole mount *in situ* hybridization in diploid control (A, B) and experimental tetraploid (C-F) embryos at 30dpf. Embryos were hybridized with a digoxigenin-labeled zebrafish *vasa* antisense probe. Arrowheads indicate *vasa*-positive cells in the genital ridge region. B, D and F are higher magnification views of the abdominal areas in A, C and E, respectively. Scale bars indicate 1 mm (A, C, E) and 500 μm (B, D, F).

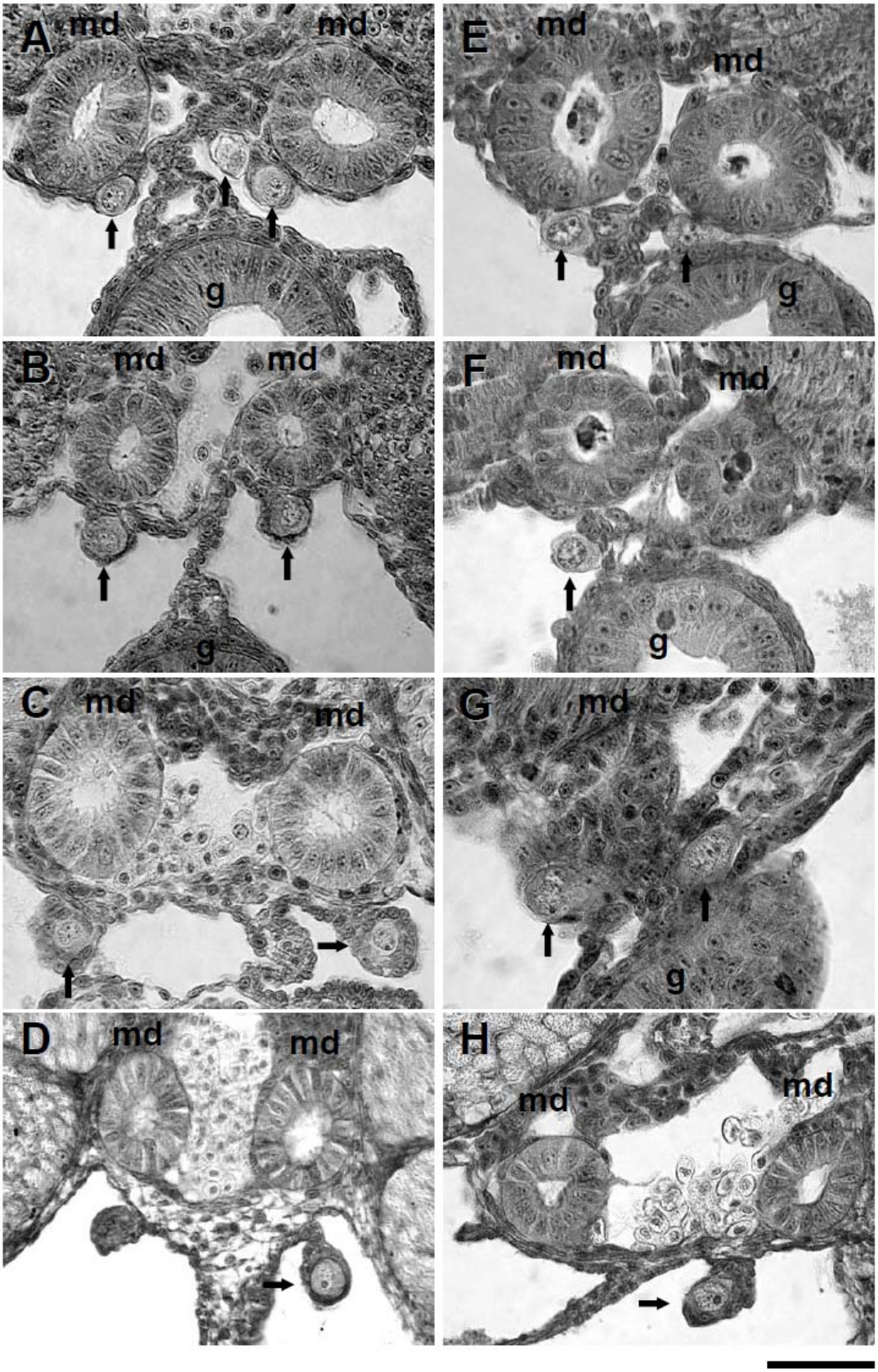
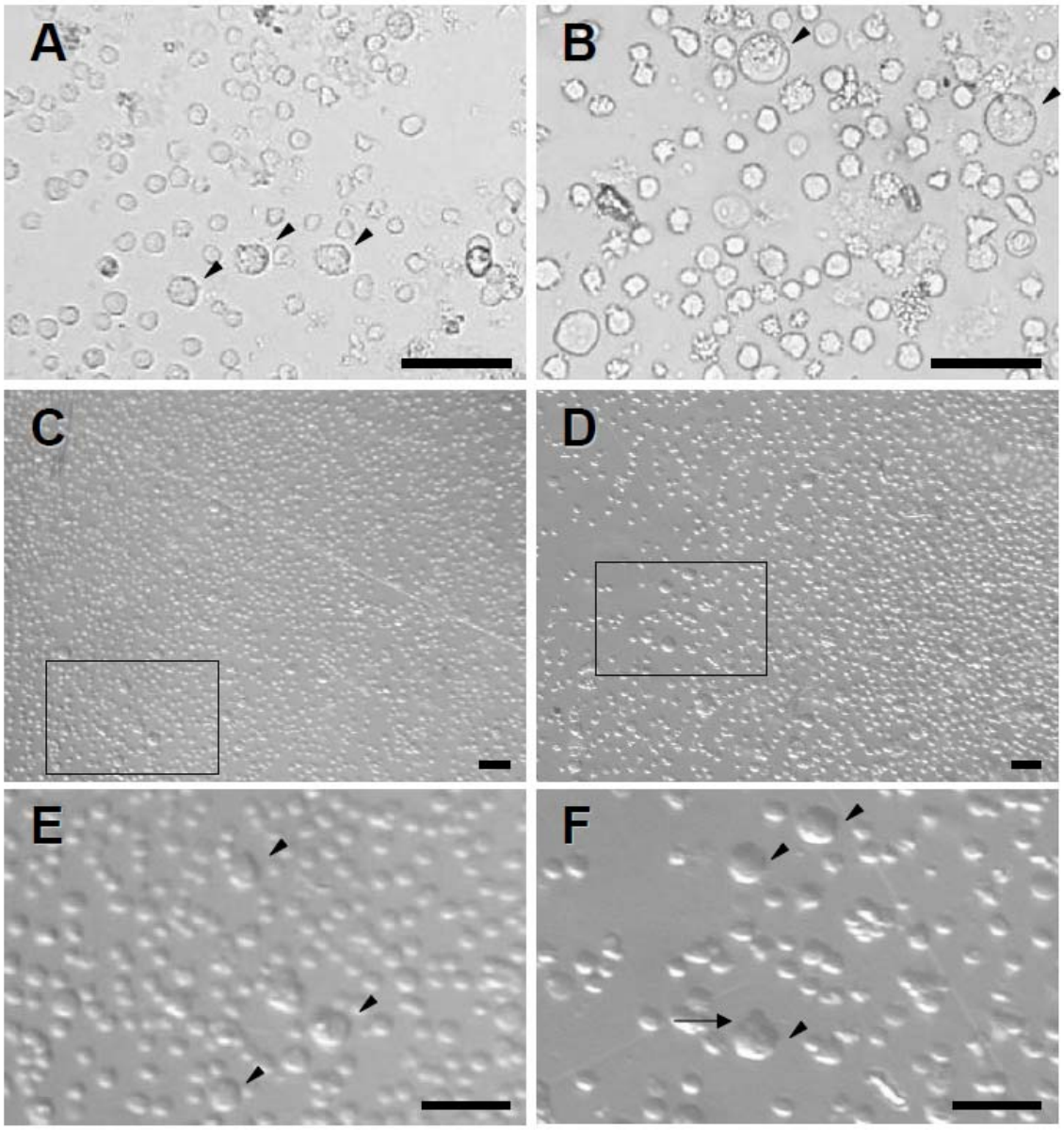


Fig. 1. (Sakao et al.)



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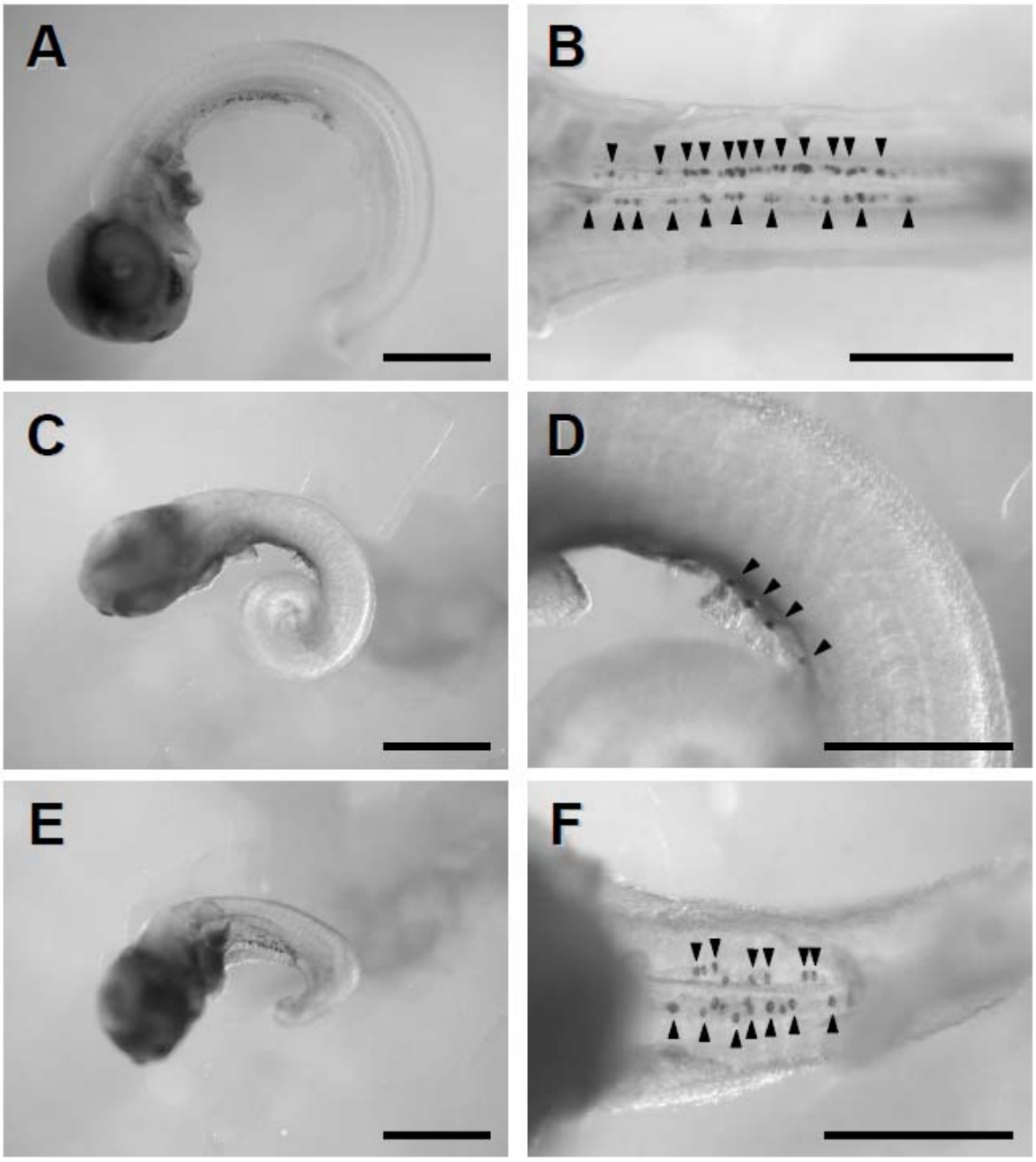

Fig. 3. (Sakao et al.)

Table 1. Ploidy status of control (2N) and tetraploid induced (4N) embryos subjected to histological observation (Family 1) and the observation of genital ridge cell suspensions and whole mount *in situ* hybridization (Family 2).

Treatment	dpf [1]	Total No.	Ploidy status						
			Hypo 2n (%) [2]	2n (%) [3]	Hypo 4n (%) [4]	4n (%) [5]	Hyper 4n (%) [6]	Mosaic (%)	ND [8] (%)
Family 1									
2N group	22	5		5 (100)					
	32	6	1 (16.7)	5 (83.3)					
4N group	22	15				15 (100)			
	32	18			2 (11.1)	16 (88.9)			
Family 2									
2N group	30	30	2 (4.0)	28 (96.0)					
4N group	30	95			11 (11.6)	79 (83.2)	3 (3.2)	1 (1.1) [7]	1 (1.1)

[1]; days post-fertilization. [2]; 1.7-1.9C. [3]; 1.9-2.1C. [4]; 3.6-3.8C. [5]; 3.8-4.2C. [6]; 4.2-4.3C. [7]; mosaic with 2n and hypo 4N. [8]; no data.
[2-6]; Each range was defined according to Sakao *et al*. (2006).

Table 2. The average sizes (μm) of PGCs and somatic cells in the suspension of genital ridges from control diploid and experimental tetraploid embryos.

Cell	2n	4n
PGCs	17.66±2.15 (n=26)	25.53±2.65 * (n=20)
(min.-max.)	(15.37-22.74)	(21.31-30.44)
Somatic cells	10.74±0.81 (n=32)	14.70±1.65 * (n=46)
(min.-max.)	(9.26-12.56)	(11.26-18.71)

* Significant difference between diploid and tetraploid embryos at each cell size ($p < 0.05$).