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Attempt at cloning high-quality goldfish breed 'Ranchu' by fin-cultured cell nuclear transplantation

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Summary

The viability of ornamental fish culture relies on the maintenance of high-quality breeds. To improve the profitability of culture operations we attempted to produce cloned fish from the somatic nucleus of the high-quality Japanese goldfish (*Carassius auratus auratus*) breed 'Ranchu'. We transplanted the nucleus of a cultured fin-cell from an adult Ranchu into the non-enucleated egg of the original goldfish breed 'Wakin'. Of the 2323 eggs we treated, 802 underwent cleavage, 321 reached the blastula stage, and 51 reached the gastrula stage. Two of the gastrulas developed until the hatching stage. A considerable number of nuclear transplants retained only the donor nucleus. Some of these had only a 2n nucleus derived from the same donor fish. Our results provide insights into the process of somatic cell nuclear transplantation in teleosts, and the cloning of Ranchu.

Keywords: *Carassius auratus auratus*, cloning, goldfish, nuclear transplantation

Introduction

It is thought that the embryonic development of medaka eggs (*Oryzias latipes*) is unaffected by transplantation of embryonic cell nuclei as some transplants survive to reach the adult stage (Niwa *et al.*, 1999; Wakamatsu *et al.*, 2001). In contrast, zebrafish (*Danio rerio*) and medaka eggs do not develop to a more advanced stage, even after reaching the larval stage, following transplantation of cultured somatic cell nuclei (Lee *et al.*, 2002; Ju *et al.*, 2003).

At present, it is not possible to predict adult phenotypes during the embryonic stage. Thus, it is not until the fish have grown sufficiently that it becomes possible to select individuals that are suitable donors, based on their expression of desirable traits. The success of somatic cell nuclear transplantation is, therefore, essential to fish cloning.

Nuclear transplantation may be used to produce individuals that have rare traits. However, it is not currently possible to mass-produce a specific breed using this technique. Our objective was to evaluate whether somatic cell nuclear transplantation may be used to clone the highly valued Ranchu breed of goldfish (*Carassius auratus auratus*).

Material and methods

Fish

We used the goldfish breeds Wakin and Ranchu as sources of recipient eggs and donor cells, respectively. The Wakin breed represents the original and most common form of goldfish, having a body shape that resembles crucian carp (*Carassius* spp.) (Fig. 1*A*). Ranchu is a high-quality breed of goldfish that has a unique body shape with a cephalocele and lacking the dorsal fin, but having triple or quadruple caudal fins (Fig. 1*B*).

Preparation of recipient eggs

We collected recipient eggs from previously unspawned Wakin breed females for each experiment. We induced ovulation by injecting each female

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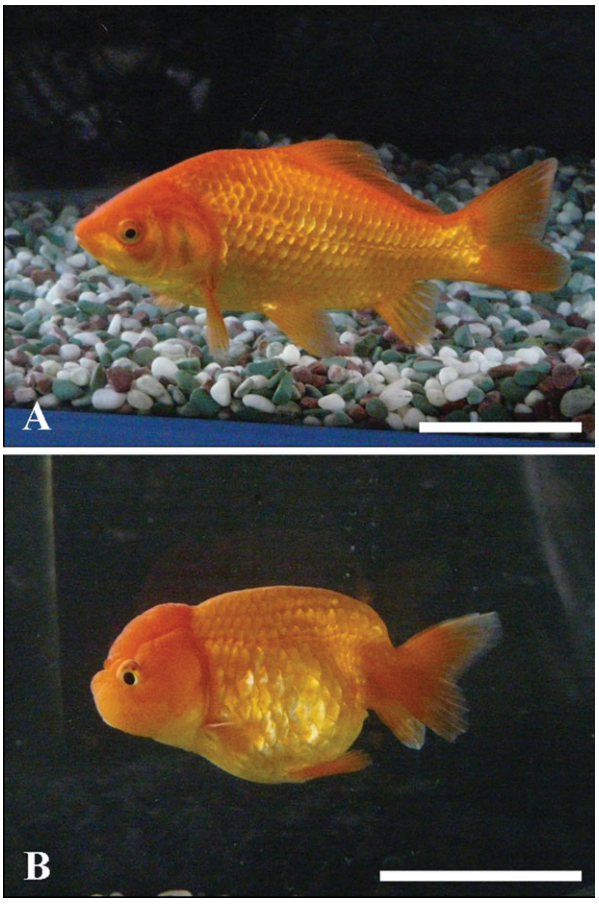

Figure 1 Recipient and donor fish. (*A*) Non-enucleated unfertilized eggs of 'Wakin' fish. (*B*) Somatic cells of 'Ranchu' fish. Bar = 50 mm.

intraperitoneally with 10 IU/g body weight human chorionic gonadotropin. The mature eggs were collected by gently massaging the abdomen. The eggs were placed in water for 3 min then treated with 0.25% trypsin solution to remove the egg membrane. Following this, the eggs were washed with Holtfreter's solution then incubated in this solution until the protoplasm accumulated at the animal pole.

Preparation of donor cells

We collected donor cells from one adult male Ranchu and one adult female Ranchu. Cultured cells from either individual were used in each nuclear transplant experiment. The primary cells were obtained by removing a section of the caudal fin. The caudal tissue was then washed in phosphate-buffered saline (PBS), sterilized with Dakin's solution, rewashed with PBS, and placed in a dish containing the Dakin's solution. A small amount of the epidermis was removed from the surface with a pair of tweezers. Each sample of caudal fin tissue was minced to yield approximately 3 mm^2 fragments then fixed to the bottom of another dish. We added Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum Cellect^{TM} and 60 mg/l kanamycin to the dish and incubated the cells for 1–2 weeks. We subcultured fibroblasts for three passages and collected the confluent state cells by centrifugation prior to nuclear transplantation.

Nuclear transplantation

The nuclear transplantation procedure is illustrated in Fig. 2. Nuclear transplantation was carried out under a stereoscopic microscope (SZX-12, Olympus) at room temperature (18°C). A 1.5 mm layer of agarose gel was laid on the bottom of a 90 mm petri dish. We excised and discarded half of the gel from the center of the plate. We then cut notches in the exposed cross section to hold the recipient eggs. We added Holtfreter's solution to the dish and placed the recipient eggs in their individual notch. The donor cells were floated in Holtfreter's solution, aspirated into a micropipette (15–20 μm internal diameter, Borosilicate Glass, Sutter Instruments) at a needle tip angle of 30°, and dispersed individually in a pipette using manipulator pressurization and depressurization to gently lyse the cell membrane. The treated eggs were then transferred to a glass dish filled with 80% Holtfreter's solution containing 0.01% kanamycin. Control eggs were obtained from matings between female fish that provided the recipient eggs and male fish that were used for the production of donor cells.

Polyploidy determination

We analysed the ploidy of the nuclear transplanted embryos, the recipient parent fish fin tissue cells, and the cultured donor cells. We used round shiner (*Gnathopogon elongates*, Cyprinidae) erythrocytes as a control. The chromosome count of this species (2n = 50) is half that of the goldfish (2n = 100). We extracted and stained the cell nuclei using a Ploidy Analyzer kit (Partec). The ploidy was determined by measuring the amount of DNA in the cell nucleus using a flow cytometer.

RAPD and microsatellite analyses

We evaluated whether the nuclei of the nuclear transplants were derived from the recipient cells, donor cells, or both using the same tissues described in the previous section. Nuclear transplantation and control DNA samples (extracted as described above) were stored in TNES-urea buffer (Asahida *et al.*, 1996). The samples were digested with proteinase K and the DNA was extracted using phenol–chloroform. We

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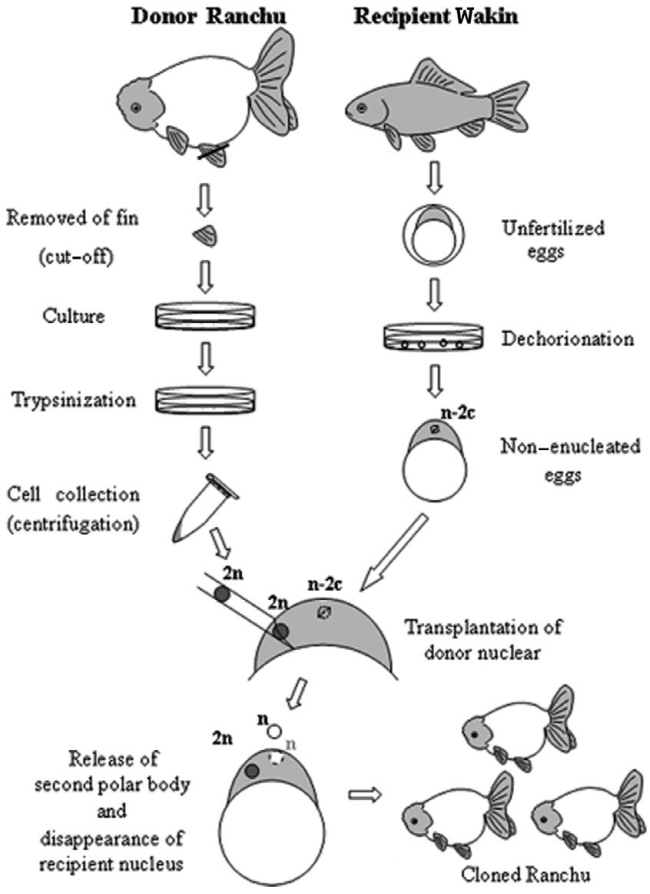

Figure 2 Schematic of the nuclear transplantation procedure for production of cloned Ranchu using cultured somatic cells.

used the random primer A-10 (5′-GTGATCGCAG-3′) from the Operon 10mer kit A (*Operon* Technologies Inc.) for RAPD analysis. RAPD PCR amplification was conducted in a total volume of 12.5 µl containing 50 ng of genomic DNA, 1.25 µl of 10× dNTP, 1.25 µl of the primer, 1.25 µl of 10× *Taq* buffer, and 0.125 µl of *Taq* DNA polymerase. We used the following thermal cycle: 5 min at 94°C, followed by 1 cycle of 1 min at 94°C and 1 min at 36°C, 45 cycles of 2 min at 94°C, and a final extension of 7 min at 72°C. The PCR amplification product was electrophoresed using a 2% agarose gel. We used Gf17 primers (forward primer, 5′-GGAACTAGAGCCCACTGACA-3′; reverse primer, 5′-TGCATTTGGGAGACGATA-3′) during the microsatellite analysis (Zheng *et al.*, 1995). Microsatellite PCR amplification was conducted in a total volume of 25 µl containing 85 ng of genomic DNA, 1.2 µl of 10× dNTP, 0.85 µl of the primer, 1.5 µl of 10× *Taq* buffer, and 0.1 µl of *Taq* DNA polymerase. The thermal conditions were: 3 min at 94°C, followed by two cycles of 30 s at 94°C, 20 s at 58°C, and 1 s at 72°C, 35 cycles of 15 s at 94°C, 20 s at 58°C, and 1 s at 72°C. There was a final extension of 30 s at 72°C. The PCR amplification product was electrophoresed in a 7.5% polyacrylamide gel.

Results

Survival rates of nuclear transplants

All eight tests using non-enucleated, unfertilized eggs transplanted with fin cell nuclei yielded viable eggs. Of the 2323 eggs that received a transplant, 802 (34.4%) underwent cleavage, 321 (13.8%) survived to the blastula stage, and 51 (2.2%) reached the gastrula stage. A considerable number of the eggs that underwent cleavage exhibited abnormal division. However, division did appear to be normal in several of the eggs. Eleven (0.5%) of the gastrula developed up to the hatching stage (Table 1) and two transplants hatched successfully.

Polyploidy of nuclear transplants

We analysed the ploidy of the four nuclear transplants that reached the segmentation and hatching stages

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Table 1 Number and percentage (in brackets) of nuclear transplants and controls surviving to each stage.

Group	No. of eggs operated	Cleavage	Blastula	Gastrula	Segmentation	Hatching
NT 1-a[a]	81	46 (56.8)	11 (13.6)	4 (4.9)	–[c]	–
NT 1-b	149	108 (72.5)	63 (42.3)	6 (4.0)	1 (0.7)	–
Control 1[b]	100	98 (98.0)	55 (55.0)	51 (51.0)	44 (44.0)	NM[d]
NT 2-a	134	41 (30.6)	20 (14.9)	3 (2.2)	2 (1.5)	–
NT 2-b	224	92 (41.1)	62 (27.7)	10 (4.5)	3 (1.3)	2 (0.9)
Control 2	100	100 (100.0)	70 (70.0)	68 (68.0)	64 (64.0)	48 (48.0)
NT 3-a	130	28 (21.5)	1 (0.8)	–	–	–
NT 3-b	220	59 (26.8)	20 (9.1)	3 (1.4)	–	–
Control 3	100	96 (96.0)	76 (76.0)	72 (72.0)	NM	NM
NT 4-a	91	35 (38.5)	–	–	–	–
NT 4-b	117	30 (36.4)	2 (1.7)	1 (0.9)	–	–
Control 4	100	96 (96.0)	1 (1.0)	1 (1.0)	NM	NM
NT 5-a	118	38 (32.2)	–	–	–	–
NT 5-b	194	77 (39.7)	–	–	–	–
Control 5	100	90 (90.0)	NM	NM	NM	NM
NT 6-a	95	29 (30.5)	–	–	–	–
NT 6-b	195	44 (22.6)	21 (10.8)	6 (3.1)	–	–
Control 6	100	100 (100.0)	47 (47.0)	46 (46.0)	NM	NM
NT 7-a	99	13 (13.1)	–	–	–	–
NT 7-b	184	67 (36.4)	57 (31.0)	7 (3.8)	1 (0.5)	–
Control 7	100	99 (99.0)	35 (35.0)	35 (35.0)	34 (34.0)	NM
NT 8-a	108	28 (25.9)	7 (6.5)	4 (3.7)	3 (2.8)	–
NT 8-b	184	67 (36.4)	57 (31.0)	7 (3.8)	1 (0.5)	–
Control 8	100	99 (99.0)	80 (80.0)	80 (80.0)	80 (80.0)	NM

[a]NT refers to individuals examined by nuclear transplantation. The numbers 1–8 denote recipient individual numbers; a, b denote donor individual numbers (a: male; b: female).
[b]Control is fertilized eggs of 'Wakin'.
[c]No nuclear transplants survived.
[d]NM, not measured.

(Fig. 3). Three of these were diploid (Fig. 3*C*, Table 2) and the remaining individual could not be analyzed because of the limited amount of DNA in the sample. The recipient egg provider (Wakin, eight parents) and the donor cell provider (Ranchu, one male parent and one female parent) were diploid (Fig. 3*A*, *B*).

Genome composition of nuclear transplants

The Wakin and Ranchu breeds were easily distinguished using both RAPD and microsatellite analyses. Both analyses yielded a band specific to Wakin and a band specific to Ranchu. Analysis of the DNA extracted from the recipient Wakin and the donor Ranchu and nuclear transplants (Figs. 4 and 5) revealed that, of the 18 nuclear transplants, 15 had only a donor nucleus, one had only the recipient nucleus, and the remaining two had both the donor and recipient nuclei (Table 2). There was no clear electrophoretic pattern for one individual in the gastrula stage.

Discussion

Generation of fin-cultured cell nuclear transplants

More than one-third of the nuclear transplanted eggs underwent cleavage. Unfortunately, the survival rate of the embryos decreased with development. However, a small number of hatched larvae were obtained in one of the experiments. Our results are consistent with those reported in zebrafish (Liu *et al.*, 2002). In another study, Ju *et al.* (2003) attempted the transplantation of caudal fin cultured cell nuclei from adult GFP transgenic medaka into the non-enucleated unfertilized eggs of orange-red medaka. The authors reported that embryonic development was generally successful, leading to the production of some larval fish. In this instance, it is thought that the nuclear division cycle of the donor nuclei was synchronized with that of unfertilized recipient eggs by endogenous factors. In general, the markedly decreased survival rate of late stage embryos is likely caused by insufficient reprogramming of the donor nuclei.

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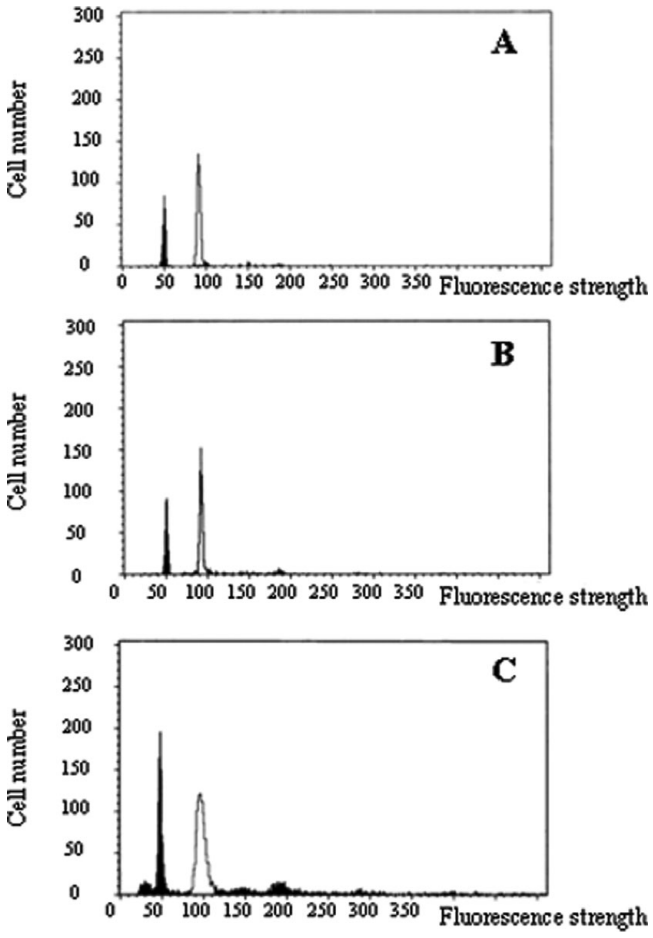

Figure 3 Histograms of the relative DNA content of somatic cells of the donor, recipient, and nuclear transplants when erythrocytes of *Gnathopogon elongatus* elongates (2n = 50) are used as control. The solid peak represents the control and the open peaks represent the donor, recipient, and nuclear transplants. (*A*) Donor diploid Ranchu (2n = 100). (*B*) Recipient diploid Wakin (2n = 100). (C) Diploid nuclear transplant.

There have been attempts to improve the developmental success of nuclear transplanted eggs in a number of animal species. Cell cycle synchronization to the G_0/G_1 phase by serum starvation culture has improved the success rate of attempts to clone mammals (Wilmut *et al.*, 1997; Kato *et al.*, 1998) and has also been trialed in fish cloning (Liu *et al.*, 2002). In mammals, nuclear transplantation after treatment of donor cells with trichostatine A® (Kishigami *et al.*, 2006), a deacetylase inhibitor, and roscovitine® (Gibbons *et al.*, 2002), a cyclin-dependent kinase, has led to an improvement in the developmental ability of nuclear transplanted eggs. A recent study also suggested that the reprogramming of somatic cell nuclei could be induced by treatment with amphibian egg cell extract (Hansis *et al.*, 2004). Attempts to reprogramme donor cells are also considered necessary for the success of nuclear transplantation using cultured somatic cells from teleosts.

Feasibility of cloning by somatic cell nuclear transplantation

The use of non-enucleated, unfertilized eggs as the recipients of nuclear material from fish embryos (Gasaryan *et al.*, 1979; Tanaka *et al.*, 2009) and somatic cells (Liu *et al.*, 2002) leads to an enhanced generation of nuclear transplants having only a 2n donor nucleus. Our results suggest that 80% of the nuclear transplants had only a donor nucleus. Among these transplants, three were diploid, two of which were produced by cultured cells from the same donor. The cytological mechanism underlying the formation of individuals having only a 2n donor nucleus is unclear. However, our results suggest that it is technically feasible to clone goldfish.

The use of non-enucleated unfertilized eggs has been associated with the formation of 100% triploids (Niwa *et al.*, 1999) or with individuals that have

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Table 2 Polyploidy and possession states of nucleus in nuclear transplants.

Nuclear transplant, no.	Polyploidy	Origin of nucleus[a]	Developmental stage
1	2n	D	Segmentation
2	NM[b]	D	Gastrula
3	–	D	Gastrula
4	–	D + R	Gastrula
5	–	D	Gastrula
6	–	R	Hatching
7	–	D + R	Gastrula
8	–	D	Gastrula
9	–	D	Gastrula
10	–	D	Gastrula
11	–	D	Gastrula
12	–	–	Gastrula
13	–	D	Gastrula
14	–	D	Gastrula
15	–	D	Gastrula
16	–	D	Gastrula
17	–	D	Gastrula
18	2n	D	Segmentation
19	2n	D	Segmentation

[a]D denotes that nuclear transplants have a donor nucleus, R denotes that they have a recipient nucleus, and D + R denotes that they have a nucleus derived from both donor and recipient.
[b]Not measured.

chromosome counts that are not compatible with the formation of a normal diploid (Ju *et al.*, 2003). Differences in the time course of recipient egg meiosis after nuclear transplantation and the phase of the cell cycle of donor cells for nuclear transplantation are thought to cause the various combinations between a recipient nucleus and a donor nucleus. Given this, successful cloning attempts will rely on optimization of the timing for nuclear transplantation and a behavioural analysis of the recipient and donor nuclei following nuclear transplantation.

Based on the current definition of cloned animals, it may be inappropriate to refer to transplants that have only a 2n donor nucleus obtained by nuclear transplantation to non-enucleated eggs as clones. However, if the recipient nucleus or chromosome is inactivated or removed following the transplantation of a donor nucleus by cytological mechanisms, the result will be the same as that of nuclear transplantation to enucleated eggs. Because the enucleation of recipient eggs requires much effort and may cause the physical impairment of fish eggs, the use of non-enucleated recipient eggs may be preferable.

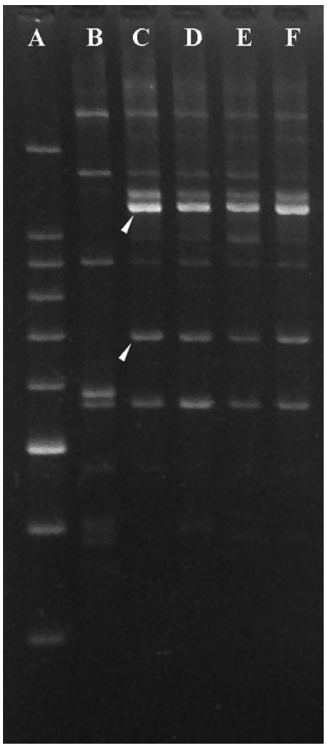

Figure 4 RAPD analysis of nuclear transplants. (*A*) 100-bp DNA ladder; (*B*) recipient Wakin; (*C*) Ranchu donor cell; (*D–F*) nuclear transplants. All of the nuclear transplants had a donor nucleus. The white arrowheads indicate a band specific for the Ranchu nucleus.

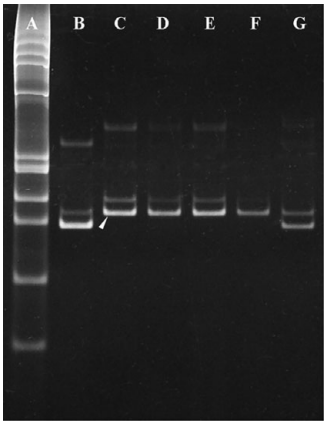

Figure 5 Microsatellite analysis of nuclear transplants. (*A*) φX174 *Hae*III digest; (*B*) recipient Wakin; (*C*) Ranchu donor cell; (*D–G*) nuclear transplants. Nuclear transplants of (*G*) had a nucleus derived from the recipient alone. The white arrowhead indicates a band specific for a donor nucleus.

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