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Production of Chimeric Loach by Cell Transplantation from Genetically Pigmented to Orange Embryos

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ABSTRACT—To establish techniques for chimera formation and to obtain further knowledge of chimerism, chimeric loach were produced using the wild strain as the donor and the orange strain as the recipient by cell transplantation. Transplantation between embryos at two different stages was performed to achieve efficient chimera formation. In the combination of the early-mid-blastula as the donor and the late-blastula as the recipient, 100–150 blastomeres were injected into the blastoderm of the recipient and the rate of chimera formation was 46.2%. On the other hand, in the combination of early-mid-blastula and early-gastrula, only 30 blastomeres were injected and the rate of chimera formation was 80.0%. These results demonstrating the combination of embryonic stages may provide a key for efficient chimera formation. We also compared the number of melanophores on chimeric larvae with that on donor cells labelled with latex beads; it was found that the number of transplanted cells has a profound effect on chimerism, whereas the site of pigmentation is not always in agreement with the site of actual transplantation of donor cells.

Key words: embryonic manipulation, blastula, melanophore, orange strain, endangered fish

INTRODUCTION

A series of studies of embryonic manipulation using several fish species models has demonstrated that until the early-gastrula stage, embryos possess pluripotency, and embryonic cells at the midblastula stage transplanted into another embryo at the same stage can differentiate not only into somatic cells but also to germ cells (Ho and Kimmel, 1993). These results indicate that germline chimeras of fish species can be produced. In fish, the electric fusion (Yamaha and Yamazaki, 1993) and the partial exchange of blastoderms (Yamaha *et al*., 1997) resulted in successful chimeric fish production and the transplantation of blastomeres at the same stage, using a micropipette; by this method, germline chimeras of zebrafish (Lin *et al*., 1992) and medaka (Wakamatsu *et al*., 1993) have been produced. However, not only is there limited information as regards techniques that can be used for chimera formation, there is also little known about chimerism in only a few model species of fish. Therefore, in the present study, we attempted to produce chimeric fish using the wild-type and the orange-type loach for the establishment of a fundamental chimera formation technique. Moreover, we obtained chimeras with embryos at two different developmental stages in order to achieve the effective production of chimeric fish. We also investigated the use of melanophores as a detective marker of chimerism.

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MATERIALS AND METHODS

Animals

Two strains of loach *Misgurnus anguillicaudatus* were obtained from commercial suppliers (Fig. 1). One strain, the wild type, had black pigmentation, and was used as the donor. The other strain, the orange strain, had no black pigmentation except in the eyes, and was used as the recipient. This combination allowed for the recognition of the resulting chimerism only in terms of apparent body color. Parent fish were maintained in an aquarium at 22°C.

Preparation of dechorionated eggs

Parent fish were intraperitoneally injected with 10 IU/g human chorionic gonadotropin 12 hr before the insemination. By pressing on the abdomen, we obtained eggs and milt. The fertilized eggs were allowed to stand for 7 min, and then were dechorionated in 0.25% trypsin/ PBS solution. The dechorionated eggs were rinsed several times with saline (NaCl, 2.8g; KCl, 0.04g; $CaCl_2$, 0.08g/ l; pH 7) and then were incubated in the same saline solution at 16, 20 and 22°C in order to regulate the developmental progress. The resulting manipulated embryos and control embryos were transferred from saline to tap water 30 hr after fertilization.

Production of chimeric fish

Donor and recipient embryos were separately placed in a petri dish filled with saline. The transplantation of blastomeres was performed under a stereoscopic microscope using a micropipette, with

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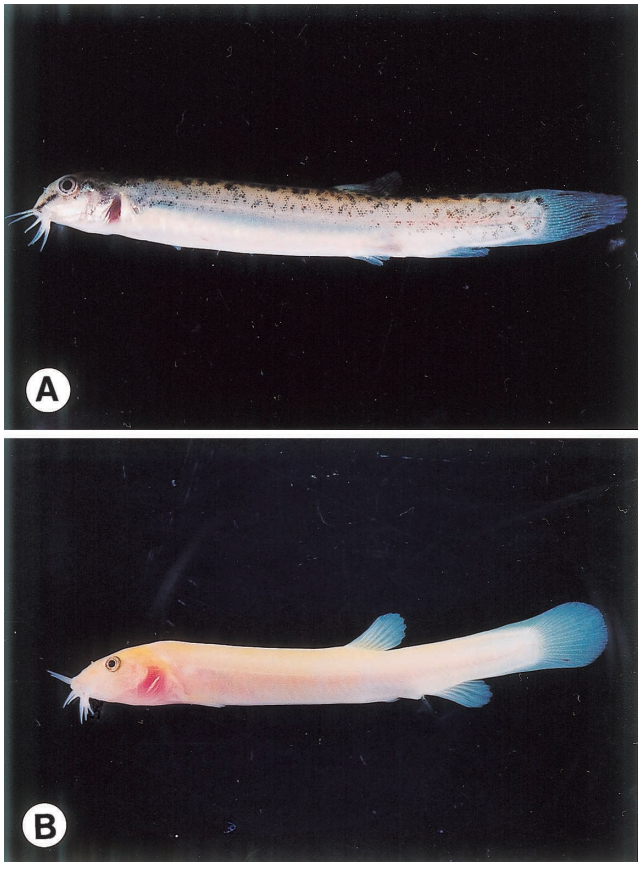


Fig. 1. Two strains of loach *Misgurnus anguillicaudatus* (A) Wild-strain, used as the donor; (B) orange-strain, used as the recipient.

a tip of 70 μm in inner diameter. We aspirated donor blastomeres from the central zone of the lower part of the blastoderm of the donor embryos, and then injected the blastomeres into the same blastodermic zone of the recipient embryos. We carefully counted the number of blastomeres transplanted through the micropipette and then altered that number for different experiments. The developmental stages used in the present experiments were the 512-cell embryo, the early-mid-blastula, the late-blastula, and the early-gastrula. The developmental stages were determined according to the classification of stages established for medaka embryos (Iwamatsu, 1994).

Observation of pigmentation

We identified an individual with at least one melanophore on its back as a chimeric fish. The first and second series of experiments were conducted in order to clarify the rates of chimera formation using 512-cell embryos, early-mid-blastulae, late-blastulae, and early-gastrulae as recipients. In these initial experiments, the pigmentation in the resulting 7-day-old larvae was observed, as normal 3-day-old larvae show distinct pigmentation, which was evident from the results of the preliminary experiments. The third series of experiments was conducted in order to demonstrate whether or not the pigmentation actually agreed with the existence of transplanted donor cells; in this series, the pigmentation in the resulting 7-day-old larvae was observed and photographed. The extent of pigmentation on the head, trunk, and tail was determined based on the following system, which was, for the purposes of practicality, based on the number of melanophores on the wild strain.

Preparation and observation of fluorescein- labelled donor blastomeres

Donor blastomeres were labelled with 0.26% solid-latex (Fluoresbrite™ Carboxylate Microspheres, 0.20 micron YG; Polysciences.) / BSS (NaCl, 7.5g; KCl, 0.1g; $CaCl_2$, 0.2g/ l; pH 7) in the second series of experiments. We used a micropipette with a tip of 20 μm in inner diameter, and injected approximately 100 pl of the solid-latex solution into the blastodisc 40 minutes after the fertilization. We then incubated the embryos at 22°C until the blastula stage. Cell manipulation using labelled cells was performed by the abovementioned method, and chimeric fish were produced. Since the loach larvae were sensitive to physical shocks, the observation of the labelled cells was performed only in 3-day-old larvae under the fluorescein channel of a stereoscopic microscope.

RESULTS

Rates of chimera formation

We transplanted donor blastomeres into a recipient of the same stage and into a recipient of a different stage. In the first series of experiments, donor blastomeres obtained from the early-mid-blastulae or the late-blastulae were injected into the recipient of the same stage (Table 1). Using the early-mid-blastulae, examinations of the transplantation of 100-150 blastomeres were performed four times. Among 192 transplanted embryos, 44 (22.9%) survived for 7 days, and among 44 larvae, 28 (63.6%) were chimeric with melanophores. Using the late-blastulae, the examination of the

Table 1. Frequency of survival and pigmented individuals in 7-day-old chimeras (Experiment 1)

Stage of embryos	Lot.	A. Transplanted embryos (%)	B. Surviving larvae (%, B/A)	C. Pigmented larvae (%, C/A)	(%, C/B)
Early-mid-blastula	1	41 (100)	9 (21.0)	7 (17.1)	(77.8)
	2	97 (100)	21 (21.6)	11 (11.3)	(52.4)
	3	28 (100)	8 (28.6)	6 (21.4)	(75.0)
	4	26 (100)	6 (23.1)	4 (15.4)	(66.7)
	Total	192 (100)	44 (22.9)	28 (14.6)	(63.6)
Late-blastula	1	12 (100)	5 (41.7)	2 (16.6)	(40.0)
	2	38 (100)	12 (31.6)	5 (13.2)	(41.7)
	Total	50 (100)	17 (34.0)	7 (14.0)	(41.2)

Embryos of the same stage were used as the donor and recipient.

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transplantation of approximately 400 blastomeres was performed twice. Among 50 transplanted embryos, 17 (34.0%) survived, and among 17 larvae, 7 (41.2%) were chimeric.

In the second series of experiments, donor blastomeres that had been obtained from the early-mid-blastulae were injected into 512-cell embryos, late-blastulae, and early-gastrulae (Table 2). In this series, the transplantation was achieved with difficulty, with the exception of the combination of early-mid-blastula and late-blastula. In the combination of early-mid-blastulae and 512-cell embryos possessing cell junctions, only 30 blastomeres were injected due to the lack of intercellular space; the rate of chimera formation was the lowest in this particular combination. On the other hand, in the case of the combination of early-mid-blastulae and late-blastulae, 100–150 blastomeres were injected without difficulty, and the rate of chimera formation (46.2%; six chimeras out of 13 larvae) was similar to that found in the combination of early-mid-blastulae. In the combination of early-mid-blastulae and early-gastrulae, which have a thin blastoderm, only 30 blastomeres were injected due to the lack of intercellular space. Therefore, it was extremely difficult to produce transplanted embryos in this case. However, the highest rate of chimera formation obtained in this study was 80.0% (four chimeras out of five larvae).

Table 2. Frequency of survival and pigmented individuals in 7-day-old chimeras (Experiment 2)

Stage of recipient embryos	Number of transplanted cells	A. Transplanted embryos (%)	B. Surviving larvae (%, B/A)	C. Pigmented larvae (%, C/A)	(%, C/B)
512-cell embryo	30	12 (100)	3 (25.0)	1 (8.3)	(33.3)
Late-blastula	100–150	35 (100)	13 (37.1)	6 (17.1)	(46.2)
Early-gastrula	30	7 (100)	5 (71.4)	4 (57.1)	(80.0)

Cells from early-mid-blastulae were used as the donor.

Table 3. Relationship between site of melanophore and fluorescein-labelled donor cells and number of cells transplanted

	Fluorescein marker									No marker		
No. of transplanted cells	30–50			100–150			200–250			200–250		
Fish no.	Head	Trunk	Tail	Head	Trunk	Tail	Head	Trunk	Tail	Head	Trunk	Tail
1	–	–	+	+++	+	–	+	+	+	++	+++	–
2	–	+	–	–	+	–	–	–	+	+	+	–
3	–	–	–	–	–	–	+	–	–	+++	+++	+++
4	–	–	–	+++	–	–	+	+	–	+	–	–
5	–	–	–	+++	–	–	+++	+	–	++	–	–
6	–	–	–	++	+	–	++	+	–	++	+	–
7	–	+	–	–	+	–	+	–	–	++	+	–
8	+	–	–	–	–	–	+++	+	–	+++	+	–
9	–	–	–	++	–	–	++	+	–	++	+	+
10	–	–	–	–	–	–	++	+	+	++	–	–
11	–	–	–	–	+	–	++	+	+	+++	+	+
12	–	–	–	++	+	–	+	+	+	+	–	–
13	++	–	–	+++	+++	+	+	–	–	++	–	–
14	–	–	–				+	+++	–			
15	++	+	–				+++	+	–			
16	–	–	–									
17	–	–	–									
18	–	–	–									
19	–	–	–									

This criterion was expediently based on the number of melanophores on the wild-strain. That is,- indicates that the fish has no pigmentation. + indicates that the fish has 1–5 melanophores on the head, 1–4 melanophores on the trunk, and/or 1–2 melanophores on the tail. ++ indicates that the fish has 6–20 melanophores on the head, 5–10 melanophores on the trunk, and/or 3–4 melanophores on the tail. +++ indicates that the fish has > 20 melanophores on the head, > 10 melanophores on the trunk, and/or > 4 melanophores on the tail. The region of cells labelled with solid latex is indicated by .

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Distribution of pigmentation on the body of chimeric fish

In order to examine the use of pigmentation as a marker of chimera formation, we produced chimeric fish species having several donor cells, and we demonstrated that the frequency of pigmented fish depended on the number of donor cells transplanted into the recipient blastoderm. In other words, when we injected 30–50 blastomeres, the frequency of pigmented fish was the lowest (number of pigmented fish/number of surviving fish: 6/19; 31.6%) and the sites of pigmentation were limited, with only a few melanophores observed. On the other hand, 76.9% of the fish resulting from the transplantation of 100–150 blastomeres had several melanophores, and all of the fish injected with 200–250 blastomeres were pigmented. Moreover, approximately one-fourth of the resulting fish had pigmentation covering the entire body (Table 3). The pigmentation had various distribution patterns in each experiment. The growth of chimeric fish was satisfactory and their pigmentation was present in the dermis and/or in the peritoneum (Fig. 2).

Distribution of labelled donor cells on the bodies of chimeric fish

We injected the labelled donor blastomeres into nonlabelled recipient embryos and examined the dynamics of the donor blastomeres after transplantation. We were able to detect labelled cells at sites in which many donor cells existed (Table 3). The labelled cells were not necessarily distributed equally, thus they were not consistently detected on the body. In the 30–50-cell transplantation, we clearly detected labelled cells in 73.7% (number of fish with labelled cells/number of surviving fish: 14/19) of the resulting chimeric fish, four of which had the labelled cells distributed on their entire bodies. In five of 19 fish, the fluorescence was too faint to detect. In the 100–150-cell and 200–250-cell transplantations, we clearly detected labelled cells in all of the resulting chimeric fish. The rate of fish possessing labelled cells on their entire body was 84.6% (number of fish with labelled cells on the entire body/number of surviving fish: 11/13) for the 100–150-cell transplantation group, and 80% (12/15) for the 200–250-cell transplantation group. In order to examine the relationship between the transplanted blastomeres and pigmentation, we compared the distribution of labelled cells with the sites of pigmentation. First, in the 200–250-cell transplantation group, we compared survival and the distribution pattern of pigmentation under conditions of labelled manipulation with that under conditions of no manipulation. It was found that the manipulation of labelled cells affected neither survival nor pigmentation (Table 3). Second, we demonstrated that at several sites, no labelled

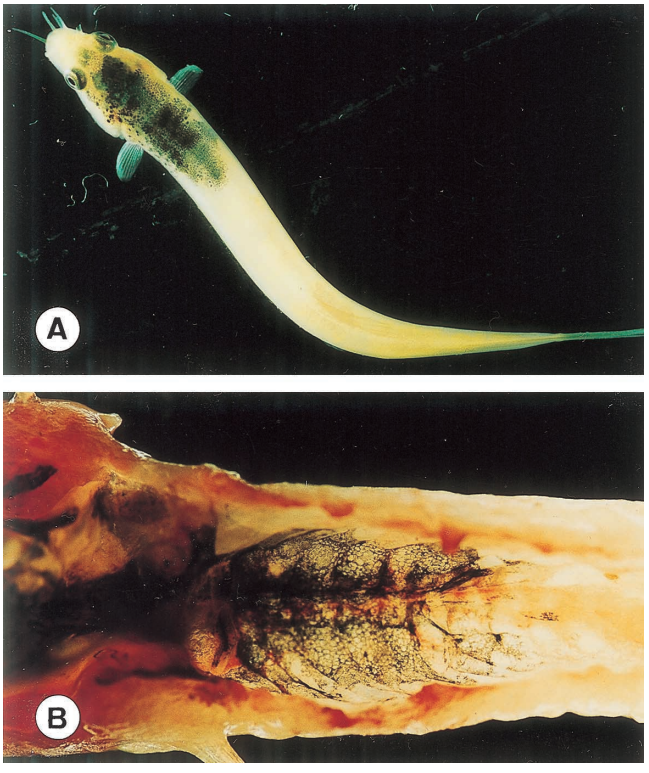


Fig. 2. Pigmentation in chimeric loach. (A) 60-day-old chimera, (B) pigmentation in the peritoneum. The fish is approximately 50 mm long.

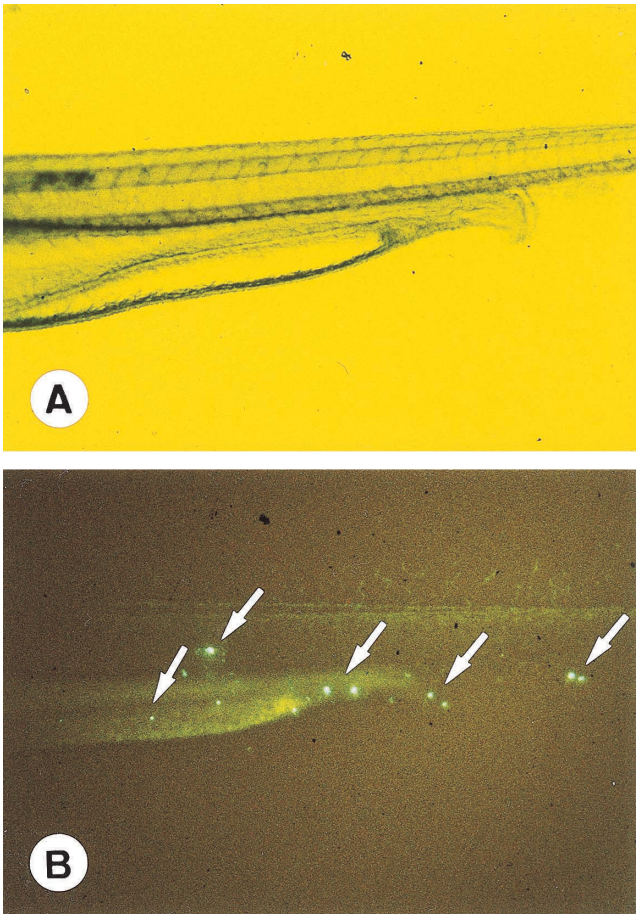


Fig. 3. Distribution of transplanted cells in chimeric loach. Donor cells were labelled with solid latex. The chimeric loach without pigmentation was photographed under a normal channel (A) and under a fluorescence channel (B). The arrows indicate labelled cells. The fish is approximately 3.5 mm long.

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cells were pigmented, and many sites in which we detected labelled cells had no pigmentation (Fig. 3). The presence of labelled cells at certain sites was not necessarily in accordance with the pigmentation distribution. In addition, the pigmentation in the resulting chimeric fish disappeared gradually as the fish developed.

DISCUSSION

We successfully produced chimeric fish with pigmentation by cell transplantation using embryos at the same stage and those at different stages. Since the donor cells of blastulae can be easily transplanted into a recipient at the same stage, and since the donor cells of blastulae can differentiate into germ cells in chimeric fish, the combination of a donor blastula and a recipient blastula is suitable for the production of germline chimeric fish (Nakagawa *et al*., 2002). This conclusion is supported by the result that a germline chimera was successfully produced using blastulae in zebrafish (Lin *et al*., 1992) and medaka (Wakamatsu *et al*., 1993). In addition, Otsuka *et al.* (1991) transplanted blastomeres into embryos at different stages and efficiently produced germline chimeras in chickens. Although in this study, it was extremely difficult to transplant blastomeres from the early-mid-blastula into the early-gastrula with an intercellular bridge (Kageyama, 1988), we were able to successfully produce pigmented fish at a high frequency. This result indicates that transplantation utilizing embryos at two different stages can be efficient for achieving successful chimera formation.

We transplanted various numbers of blastomeres and compared the resulting pigmentation in the chimera fish. In this study, the frequency of the appearance of melanophores increased with an increase in the number of transplanted blastomeres. This result is in agreement with the data from chimeric medaka (Wakamatsu *et al*., 1993). Moreover, we compared the distribution of melanophores with that of donor cells using labelled blastomeres, and we confirmed that several pigmented sites contained labelled cells. Some of these sites did not have labelled cells, but did show pigmentation. This result indicates that labelled cells could not be detected all of the sites; accounting for this result will require further study.

In the present study, it was of note that the pigmentation of the resulting chimeric fish disappeared gradually as the fish developed. The same phenomenon was observed in studies of chimeric medaka (Wakamatsu *et al*., 1993; Hyodo and Matsuhashi, 1994) and chimeric goldfish-crusian carp *Carassius auratus langsdorfii* (Yamaha *et al*., 2001); however, adult chimeric zebrafish have been shown to develop stable pigmentation (Lin *et al*., 1992). Hyodo and Matsuhashi (1994) produced germline chimeras by transplanting blastomeres of wild-type medaka into the embryo of the orangestrain and they successfully generated offspring derived from the donor-type gamete using nonpigmented chimeric fish. These reports indicate that pigmentation is not always a useful indicator of germline transmission.

Powerful techniques of producing chimera fish include embryonic cell transplantation (Nilsson and Cloud, 1992; Lin *et al*., 1992; Wakamatsu *et al*., 1993; Hyodo and Matsuhashi, 1994), electrical fusion (Yamaha and Yamazaki, 1993), and exchange of blastoderms (Yamaha *et al*., 1997; Yamaha *et al*., 2001). Among these techniques, embryonic cell transplantation using a micropipette is superior to the other techniques because it allows for the injection of a precise number of donor cells into the recipient. It is also possible to transplant isolated and/or cryopreserved blastomeres in order to regenerate endangered fish species and in order to transplant ES cells for generating transgenic fish. In this study, we successfully produced chimeric fish using two loach strains and demonstrated the use of melanophore as a pigment marker. This study revealed valuable information about chimerism, and such information will be crucial for technological developments in related areas of interest, such as the preservation of endangered fish species (Nakagawa *et al*., 2002), fish breeding (Takeuchi *et al*., 2001), and developmental biology.

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