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Dissociated Blastomeres at the Mid-blastula Stage Differentiate Autonomously to Ectoderm Lineage under the Cultured Condition in Goldfish (*Carassius auratus*)

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Abstract

In bony fish, somatic-lineage blastomeres in the blastula are thought to possess pluripotency and differentiate into meso-endoderm lineages through induction from the yolk cells. This is supported by their ability to alter their developmental fate when transplanted from their original location to a different area around the blastoderm during the blastula stage. However, whether pluripotency can be sustained under dissociated conditions without cytokines remains unclear. Here, we conducted experiments to clarify the differentiation potential of blastomeres dissociated at the mid-blastula stage in goldfish (*Carassius auratus*). When dissociated blastomeres were cultured under conditions chelated with 0.25% trisodium citrate at 20°C, they began to aggregate and formed cell clusters after 6 h, whereas control embryos developed to the germ-ring stage. When dissociated blastomeres were cultured for 12 h and then transplanted into host blastulae, most donor cells were incorporated as clusters into the host organs and differentiated exclusively into the ectoderm lineage. These results suggest that, in the absence of cytokines, blastomeres at the blastula stage are destined to differentiate into the ectoderm lineage.

Key words : Additional Organ, Blastomere, Ectoderm, Goldfish, Blastula, Pluripotency, Heterochronic transplantation.

Abbreviations used in this paper :

hpf, hours post-fertilization.

Introduction

In zebrafish, the cell fates of three germ layers are committed at the early gastrula stage, depending on their localization within the blastoderm (Kimmel et al., 1990). Before this embryonic stage, their fates remain changeable by experimental transplantation of single cells among the blastoderm (Ho and Kimmel, 1993), suggesting that blastomeres possess pluripotency. Fate mapping in zebrafish has been conducted through cell lineage tracing of single or small groups of labeled blastomeres (Kimmel and Law, 1985abc ; Kimmel and Warga, 1988 ; Warga and Kimmel, 1990). In this fate map, meso- and endoderm lineages localize around the marginal region of the blastoderm (Kimmel et al., 1990). These lineages are induced by morphogens from the yolk syncytial layer (YSL), located beneath the blastoderm, in a process known as mesoderm induction (Mizuno et al., 1996). Zygotic transcription is reported to initiate at the mid-blastula stage (Kane and Kimmel, 1993). Therefore, the commitment process is thought to occur after the mid-blastula transition to the late-blastula stage.

In goldfish, which belong to the same subfamily as zebrafish, similar but slightly different results were observed regarding the differentiation of blastomeres during early development. When the upper or lower part of the blastoderm was extirpated at the blastula stage, the resulting embryos developed almost normally, suggesting that blastomeres showed pluripotency similar to zebrafish (Kazama-Wakabayashi et al., 1999). When the blastoderm was excised just above the yolk cell and transplanted back onto the same yolk cell after a 180° horizontal rotation, an additional embryonic axis formed in a small part of the resultant embryos (Yamaha et al., 2003). When the entire blastoderm was transplanted onto another blastoderm, *no tail* (*ntl*), a pan-mesodermal marker (Schulte-Merker et al., 1994), and *goosecoid* (*gsc*), a dorsal mesodermal marker, were expressed in a marginal part of the donor graft (Yamaha et al., 1997).

In zebrafish, the zygotic expression of mesendoderm mark-

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ers in many blastomeres of the blastoderm disappeared following RNase injection into the YSL, although a small number of cells retained the expression of dorsal mesoderm markers (Chen and Kimelman, 2000). These findings suggest that, in both goldfish and zebrafish, a small portion of the blastoderm is destined for dorsal specification as a group of cells. However, as mentioned above, individual blastomeres of blastoderm maintained pluripotency until the late blastula stage (Ho and Kimmel, 1993). Thus, during early developmental stages, individual blastomeres seem to have different differentiation potentials than those in groups. The differentiation of embryonic cells as a population has been described by Gurdon (1988) as a "community effect." However, the mechanism by which individual cells differentiate under culture conditions remains unclear.

Individual blastomeres differentiate through interactions with neighboring cells, including the yolk cell, within a single embryo during the early stages of development. Their differentiation is expected to depend on the maternal cytoplasm with spatial distribution in the fertilized egg. If blastomeres are dissociated and cultured without cell interactions under artificial conditions during the mid-blastula transition (MBT), a period characterized by minimal zygotic gene expression (Kane and Kimmel, 1993), and subsequently transplanted into the blastula, what fate can be anticipated for these cells *in vivo*? In such a condition without induction from the yolk cell *in vitro*, it is expected to be destined for an ectoderm fate. There are many studies on the cultivation of dissociated blastomeres under artificial conditions containing leukemia inhibitory factor (LIF), which maintains the pluripotency of early blastomeres, in order to induce embryonic stem cells (Wakamatsu et al., 1994 ; Hong et al., 1996 ; Xiao et al., 2016). There are few reports about blastomere culture under a simple medium, which includes no nutrients and inhibiting factors for differentiation.

Primordial germ cells (PGCs) differentiate from blastomeres that inherit maternal germ plasm in zebrafish (Yoon et al., 1997). PGCs in zebrafish can also differentiate from dissociated blastomeres at the blastula stage (Kawakami et al., 2010). When the PGCs from dissociated blastomeres are cultured for three days under artificial conditions and heterochronically transplanted into the blastula, they can differentiate into functional gametes through surrogate hosts (Kawakami et al., 2010). In addition, established embryonic stem (ES) cells from zebrafish blastulae contribute to organogenesis in the host body when transplanted (Xiao et al., 2016). Therefore, heterochronic transplantation can be used to reveal the pluripotency of donor cells.

We studied the early embryonic developmental speed under different temperature conditions in goldfish and zebrafish (Urushibata et al., 2019, 2021), enabling us to perform heterochronic transplantation in these species. In this article, we reveal the developmental potential of dissociated blastomeres, when they are cultured for several hours at an advanced developmental stage and then transplanted into the blastula stage. Our results indicate that the developmental potential of dissociated blastomeres becomes restricted to the ectoderm lineage after long-term cultivation, suggesting that blastomeres dissociated at the blastula stage lose the ability to be induced into mesodermal fate.

Materials and methods

Ethics

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University and Field Science Center for Northern Biosphere, Hokkaido University, Japan (#22-1).

Eggs and sperm

Parental goldfish were housed at the Nanae Freshwater Laboratory, Hokkaido University, and maintained at a temperature of 10-14°C. Artificial fertilization of goldfish eggs was carried out as described by Yamaha et al. (2001).

Dechorionation and incubation conditions

Fertilized embryos were dechorionated by treatment with 0.1% trypsin and 0.4% urea in freshwater fish Ringer's solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$) for approximately 10 min. Dechorionated embryos were then incubated in 1% agar-coated petri dishes filled with the first culture solution (Ringer's solution supplemented with 0.01% penicillin, 0.01% streptomycin, and 1.6% egg white). The dechorionated embryos were kept at 20°C in an incubator until the embryos reached the blastula stage.

Dissociation of donor cells

To detect transplanted graft-cells histologically in developing embryos, the cytoplasm of the donor embryos was labeled with biotin. Biotin-dextran-fixable (5%) (Sigma) and 5% Fluorescein isothiocyanate-dextran (Sigma : FITC) solution in 0.2 M KCl was injected just beneath the blastoderm at the 1- to 2-cell stage, following Ho and Kane (1990).

At the MBT stage, when embryos reached the 1,000-cell stage (6 hpf at 20°C), the central part of the donor blastoderms was dissected horizontally with a fine glass needle under a dissecting microscope (Mizuno et al., 1996 ; Yamaha et al., 1997). The excised blastoderms were then dissociated into single cells by treatment with 0.25% trisodium citrate in Ringer's solution in a 96-well plate (Greiner Bio-One : Falcon 650180).

Transplantation of donor cell

Donor cells were cultured at 20°C for 0 to 15 h (Fig. 1). The developmental speed of host embryos was adjusted by modifying temperature conditions, following the method

described by Urushibata et al. (2019), to ensure they reached the MBT stage at the time of transplantation. After cultivation, donor cells were transferred from the 96-well plate to 1% agar-coated petri dishes filled with the first culture solution. The donor cells were then transplanted into the host blastula at the MBT stage using an injector (Eppendorf : Vario or CellTram) and a micromanipulator (Narishige : M-152). The injection site was the central part of the blastoderm. Host embryos with donor cells were cultured at 20°C for 24 h before being transferred to a second culture solution (1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$) (Yamaha and Yamazaki, 1993) and cultured at 20°C for 48 h in a 96-well plate.

Photographing the external appearance of developing embryos

The external appearance of each embryo was photographed using a stereoscopic microscope (Leica : MZ16F-RCFL equipped with DFC300FX).

Histochemical staining

Embryos were fixed with Bouin's fixative for 3 h, dehydrated through a series of butyl alcohols, and embedded in a paraffin block. Serial sections were cut at a thickness of 8 μm and mounted on slide glasses. The paraffin was removed from the sections using prewarmed xylene, followed by rehydration through an alcohol series and immersion in 0.1 M phosphate-buffered saline (PBS) (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4). Biotin-labeled cells were stained following the protocol of the Histofine SAB-PO(M) kit (Nichirei). Specifically, the sections were treated with peroxidase-conjugated streptavidin (Nichirei) for 30 min, rinsed with PBS and developed with diaminobenzidine (DAB) (Nichirei). The stained sections were observed and photographed using a biological microscope (Leica : DM2500L).

Results

External appearances of dissociated blastomeres after cultivation

Dissociated blastula cells were cultured for different durations (0, 3, 6, 9, 12 and 15 h) in Ringer's solution containing 0.25% trisodium citrate (Fig. 1). The size of donor cells decreased as the culture time increased (Fig. 2). Additionally, donor cells began to aggregate after 6 h of culture (Fig. 2).

Developmental potency of dissociated blastomeres after cultivation

When blastomeres were transplanted into the host blastula shortly after dissociation (0 h), FITC imaging revealed that donor cells incorporated into various organs and tissues, including ectodermal and mesodermal lineages in the resultant embryos (Fig. 3A, B). They were also histochemically detected in various organs in ectoderm (epidermis, olfactory placode, forebrain, midbrain, hindbrain, spinal cord, optic cup and lens), mesoderm (myotome) and triploblastic organ (visceral pouch) (Table 1 ; Fig. 3C-H). A large number of donor cells were mainly distributed in the head region. Donor cells were mixed with host cells in these organs and tissues. These differentiation trends of donor cells in the resulting embryos were detected in the transplantation after 3 h of cultivation of donor cells.

When blastomeres after dissociation were cultured for 6 h and transplanted into a host blastula, external observations of the resultant embryos revealed that a small number of donor cells were incorporated into the brain and spinal cord (Fig. 4A2 and 4A3 : ectoderm) and muscle fibers (Fig. 4A2 : mesoderm). Additionally, large aggregates of donor cells were distributed around the head region (Fig. 4A, 4B). Histochemical analysis showed that donor cells formed larger aggregates in the resultant embryos (Table 1 ; Fig. 4C-G) compared to donor cells transplanted immediately after dissociation (Fig. 3). Furthermore, some additional organs composed mostly of donor cells were observed, such as the optic cup with lens placode (Fig. 4F) and the auditory vesicle (Fig. 4G). Histologically, these organs appeared to be at a less advanced stage of development than their host counterparts. Similar distribution patterns of donor cells were also observed in embryos following transplantation after 9 h of cultivation of donor cells.

When blastomeres were dissociated and cultured for 12 h before transplantation into the host blastula, donor cells appeared as small and large aggregates around the head region (Fig. 5A). Histochemical analysis revealed that a small number of scattered donor cells were incorporated into the telencephalon without any morphological abnormalities (Table 1 ; Fig. 5B, C, E). Donor cells were also observed in the olfactory placode and neural retina (Fig. 5C). Additionally, donor cells formed an abnormal aggregate within the optic cup (Fig. 5E) and contributed to the formation of an additional optic cup with a pigmented retina and lens (Fig. 5F).

Discussion

The present study shows that blastomeres cultured under dissociated conditions change their developmental characteristics. When dissociated blastomeres at the mid-blastula stage were cultured under chelating conditions, they initially remained separate, but gradually began to form cell-aggregates after 6 h of cultivation. In the results of the blastomere transplantation into blastula embryos, the blastomeres also began to form aggregated distributions after longer cultivation and contributed to the ectoderm lineage. In these

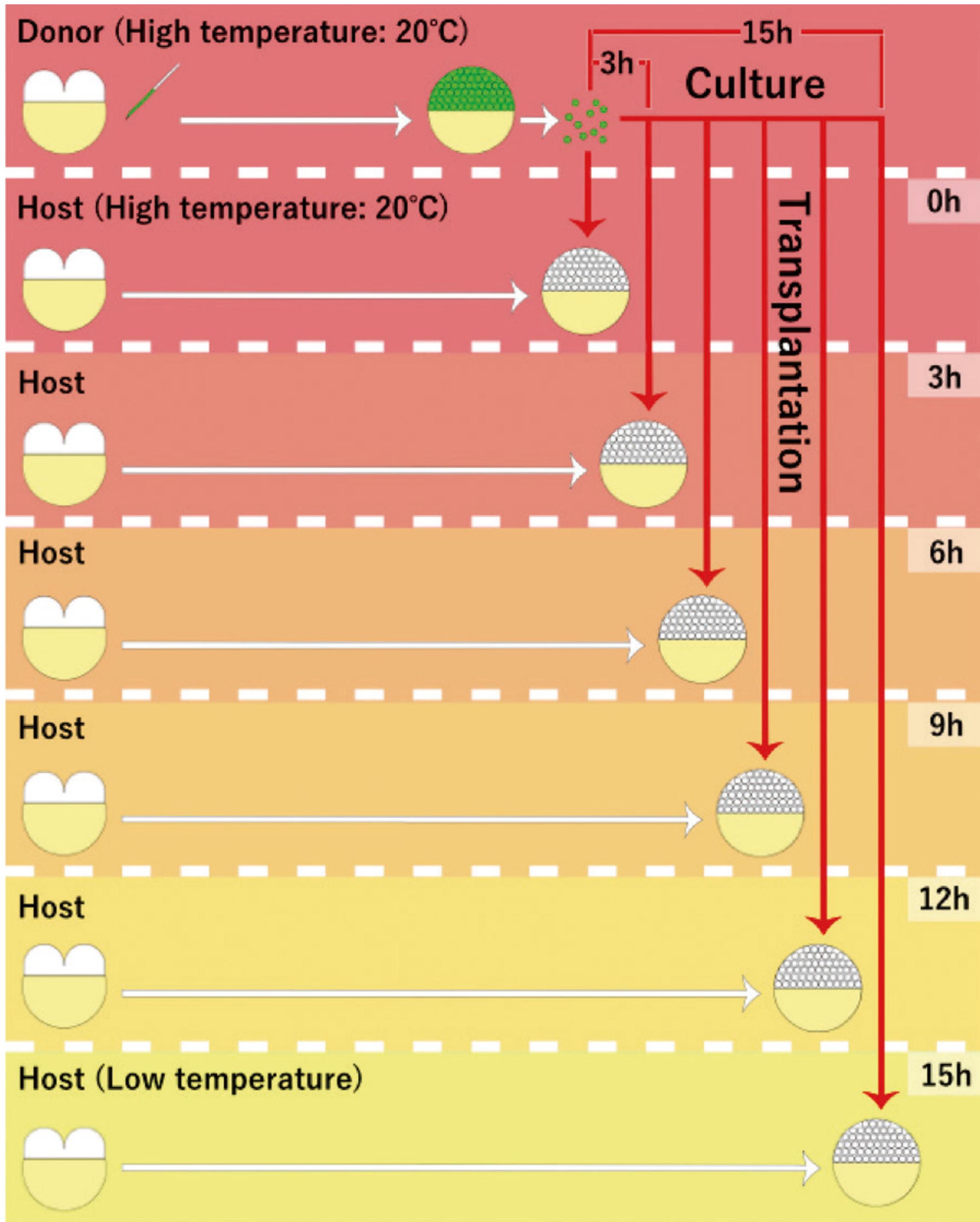

Fig. 1. Schematic illustration of transplantation of dissociated and cultured donor blastomeres into host blastulae. Donor blastomeres are collected at the blastula stage from the blastoderm by treatment with 0.25% trisodium citrate in Ringer's solution, cultured for several hours under this solution at 20˚C, and transplanted into host blastulae which developmental speeds are controlled under different temperature conditions according to Urushibata et al. (2019).

cases, scattered donor cells were incorporated into the host organ, whereas aggregated donor cells maintained their aggregated properties and/or differentiated into further additional organs as separated cell aggregates. These observations indicate that dissociated blastomeres change their adhesive cellular properties and lose their differentiation potential towards mesendodermal lineages. Furthermore, the integration of donor cells into the host organ appears to depend on the state of the cells at the time of transplantation —that is, whether they remain dispersed or have begun to aggregate.

During gastrulation, ectodermal cells attach to the enveloping layer and form the epiblast as a sheet-like structure

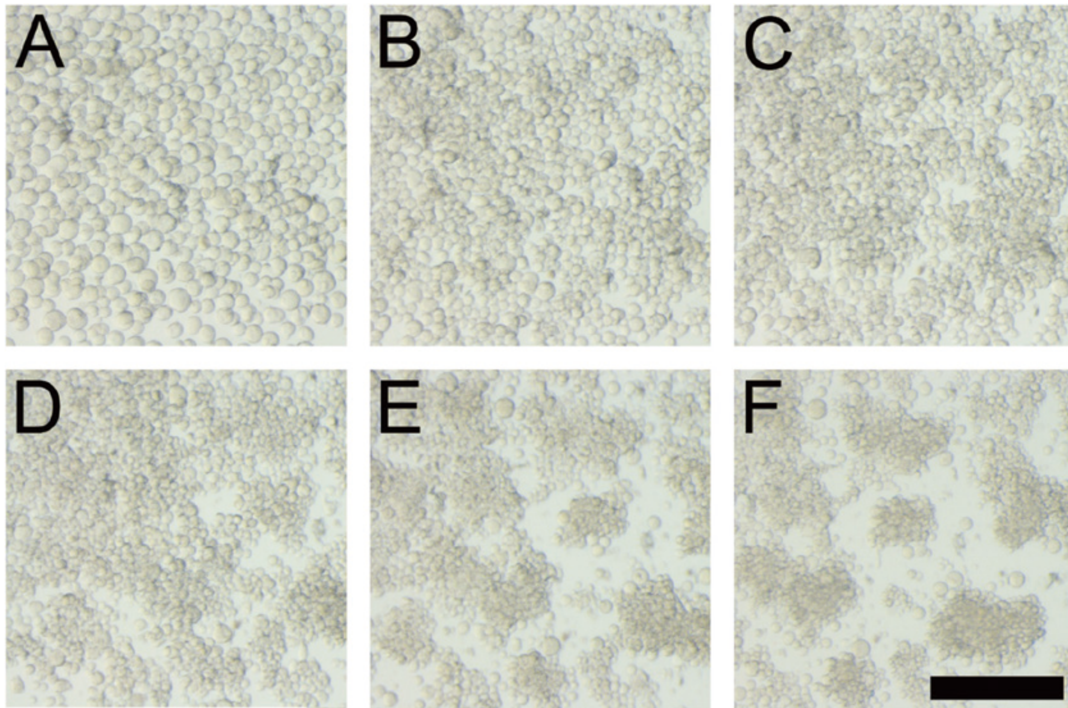

Fig. 2. Dissociated donor cells. A : Without cultivation (0 h). B : Cultivated for 3 h. C : Cultivated for 6 h. D : Cultivated for 9 h. E : Cultivated for 12 h. F : Cultivated for 15 h. The scale bar indicates 0.5 mm.

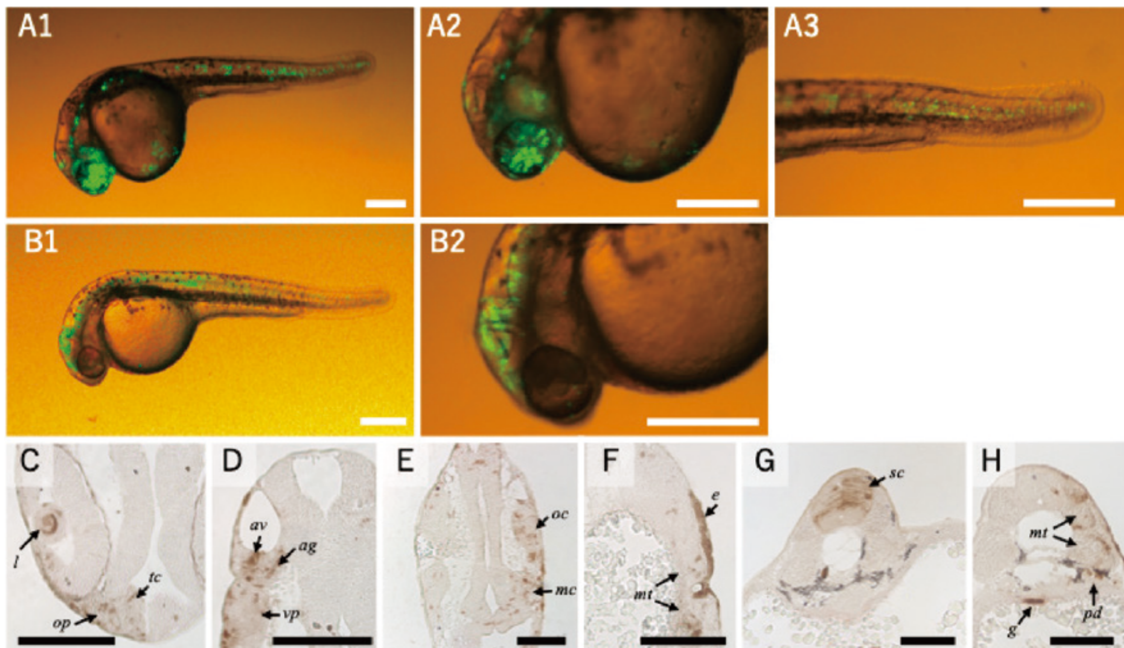

Fig. 3. External (A and B) and histological (C–H) appearances of chimeric embryos developed from blastulae transplanted with labeled dissociated donor cells without cultivation. A1 : Embryo with donor cells in the optic cup, the lenses and the notochord. A2 and A3 : The higher magnification around the head and the tail of A1, respectively. B1 : Embryo with donor cells distributed mainly in the brain. B2 : The higher magnification of B1 around the head region. C–H : Distribution of donor cells in histological sections. Sections were cut horizontally around the head region and vertically after the trunk region. C : Donor cells are distributed in the lens (*l*) and olfactory placode (*op*) and telencephalon (*tc*). D : Donor cells in the auditory vesicle (*av*), auditory ganglion (*ag*), visceral pouch (*vp*). E : Donor cells in optic cup (*oc*) and mesencephalon (*mc*). F : Donor cells in epidermis (*e*), and myotome (*mt*). G : Donor cells in spinal cord (*sc*). H : Donor cells in the myotome, pronephric duct (*pd*) and gut (*g*). Note the scattered distribution of donor cells around many organs. Scale bars indicate 0.5 mm.

Table 1. Chimeric distribution of donor cells, except for additional organs, in two-days-old resultant embryos transplanted with cultivated dissociated blastomeres.

Organ	0 h cultivation			6 h cultivation			12 h cultivation		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
Epidermis	+	-	+	+	-	+	+	+	*
Olfactory placode	-	+	+	-	+	+	-	+	-
Fore brain	++	+	+	-	+	++	+*	+*	+
Midbrain	-	+	+	-	-	-	+	-	-
Hindbrain	-	-	+	-	-	-	-	-	-
Spinal cord	-	+	-	-	-	+	-	-	-
Optic cup	+	+	+	-	-	-	+	-	+
Lens	++	+	++	++	-	-	-	-	-
Auditory vesicle	-	-	++	-	-	-	-	-	-
Myotome	+	-	++	-	-	-	-	-	-
Pronephric duct	-	-	-	-	-	-	-	-	-
Gut	-	-	-	-	-	-	-	-	-
Visceral pouch	-	+	+	+	-	*	-	-	-

++ About half of the cells in the organ were labeled.
\+ Plural single cells in the organ were labeled.
\- No labeled cells in the organ.
* Cell clusters in the organ were labeled.

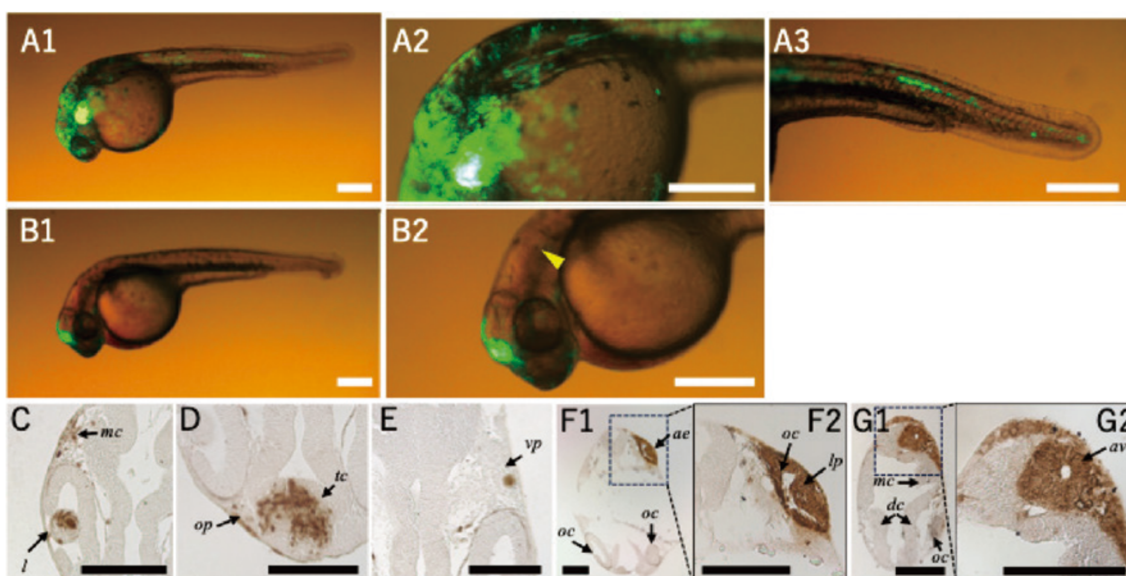

Fig. 4. External (A and B) and histological (C–G) appearances of chimeric embryos developed from blastulae transplanted with labeled dissociated donor cells cultivated for 6 h. A and B : Donor cells labeled with FITC distribute around several parts of the embryonic body. A1 : Many donor cells are distributed in the head region and the spinal cord as some clusters. A2 and A3 : The higher magnification of A1. B1 : Most donor cells are distributed in the head region, especially the diencephalon, as a cluster. B2 : The higher magnification of B1. C–G : Histological distribution of donor cells. Sections were cut horizontally around the head region. C : Donor cells distributed in the lens (*l*) and mesenchymal cells (*mc*) in the mid-brain region. D : Donor cells are in the olfactory placode (*op*) and the telencephalon (*tc*). E : Small aggregate of donor cells in the visceral pouch region (*vp*). F1 : Donor cells in the additional eye (*ae*) with an optic cup (*oc*) and lens placode (*lp*). F2 : The higher magnification of F1. Note a pair of more differentiated optic cups (*oc*) without labeling in the lower part of histological section in F1. G1–2 : Donor cells distributed in the additional auditory vesicle (*av*) and its surrounding area. G2 : The higher magnification of G1. *dc* : diencephalon, *mc* : mesencephalon. At this stage of the host embryo, the auditory vesicles differentiate into a thinner membranous vesicle containing otoliths, as seen in B2 (yellow arrowhead). Scale bars indicate 0.5 mm.

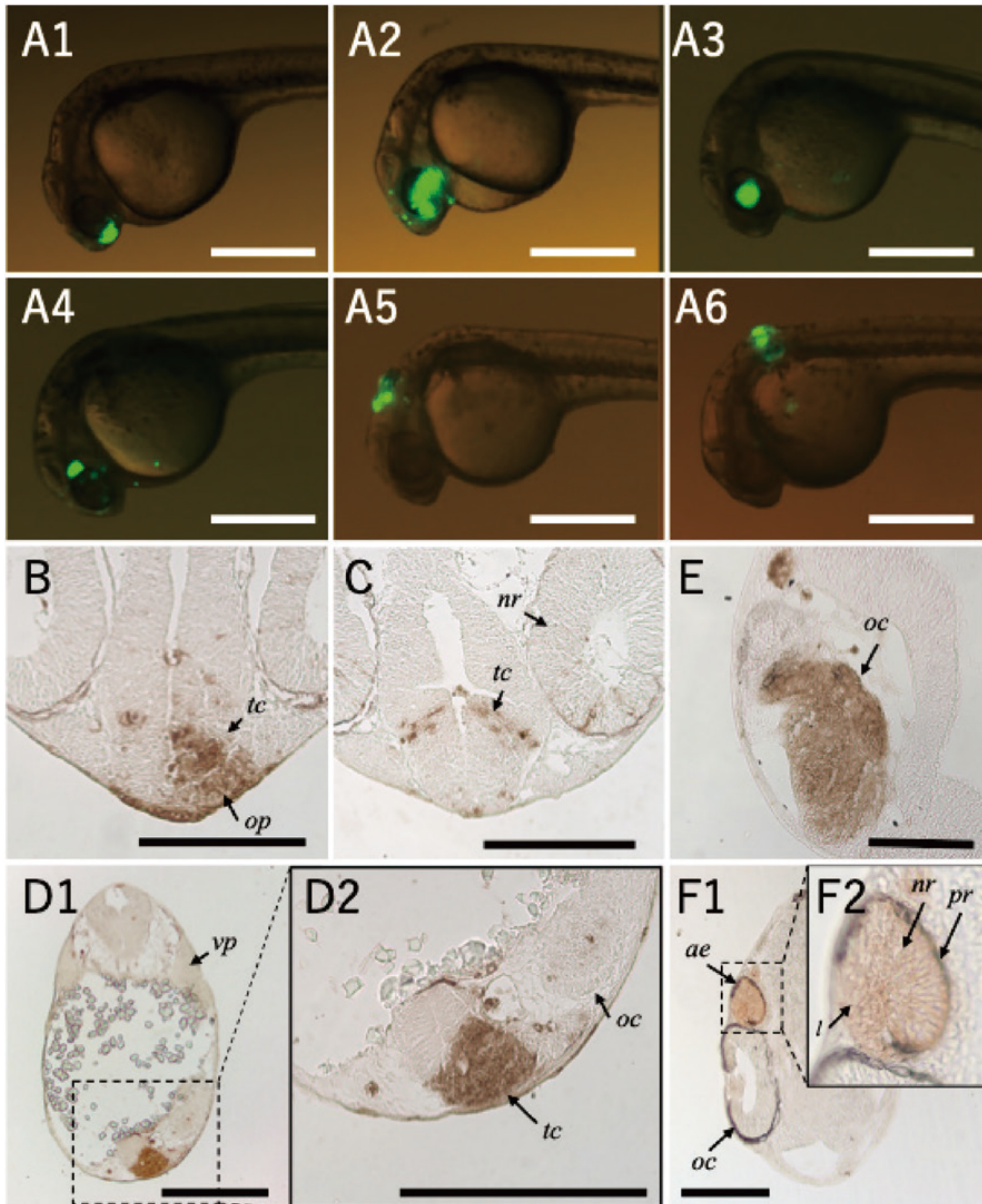

Fig. 5. External (A) and histological (B–F) appearance of chimeric embryos with transplanted donor cells cultured for 12 h. A1–6 : Distribution of donor cells with FITC labeling in the host embryos. Note that the donor cells are mainly distributed as cell clusters in host embryos. Scale bars in A1–6 indicate 1 mm. Sections were cut horizontally around the head region and vertically after the trunk region. B : Donor cells stained with DAB are integrated into the telencephalon (*tc*) and olfactory placode (*op*). C : Donor cells are integrated into the telencephalon (*tc*) and neural retina (*nr*). D : Donor cells are differentiated in the dorsal part of the telencephalon (*tc*). D2 is the higher magnification of the rectangles in D1. E : Donor cells distribute to an optic cup (*oc*) at the normal position but are less differentiated than the host *oc*. F : Donor cells form additional eye (*ae*) with pigmental (*pr*) at the adjacent portion of the host eye, neural retina (*nr*), and lens (*l*). F2 is the higher magnification of the rectangles in F1. Scale bars in B–F indicate 0.5 mm.

(Beams et al., 1985 ; Kimmel et al., 1995). In contrast, mesodermal and endodermal cells break apart and contribute to the hypoblast layer ; the former moves below the ectodermal sheet, and the latter moves above the yolk cells (Beams et al., 1985 ; Warga and Kimmel, 1990 ; Warga and Nüsslein-Volhard, 1999). The aggregation of dissociated blastomeres

observed in this study suggests their differentiation into the ectodermal lineage. Blastomeres distributing the marginal region of the blastoderm are induced into the mesendoderm lineage, expressing *gsc* and *ntl*, by the underlying yolk cell in zebrafish, during normal development (Mizuno, 1996 ; Ober and Schulte-Merker, 1999 ; Rodaway et al., 1999). In goldfish, *gsc* and *ntl* begin to be expressed around the marginal region of the blastoderm at 8 hpf (Yamaha et al., 1999). The timing of 6 and 12 h post-dissociation in this study corresponds to the 50% and 90% epiboly stages in normal development, respectively (Urushibata et al., 2019). The timing of cell aggregation coincides with the 50%-epiboly stage, namely the establishment of three germ layers, suggesting that the normal developmental program proceeds in the dissociated cells.

In this study, longer culture periods for the dissociated blastomeres led to reduced differentiation of the donor cells toward the mesendoderm lineage under chimeric conditions in the host. Similarly, in zebrafish at the 90%-epiboly stage, ectodermal cells lose their responsiveness to mesendodermal differentiation when the mesendoderm inducer Nodal is ectopically injected into the animal cap region (Vopalensky et al., 2018). Both findings indicate that blastomeres gradually lose their pluripotency for the three germ layers as embryogenesis progresses, suggesting that the developmental program also proceeds in the dissociated cells.

In this study, the isolated blastomeres lost their ability to differentiate into mesendodermal lineages after long-term culture and did not differentiate into mesendodermal-lineage organs. On the other hand, a small number of donor cells successfully integrated into ectodermal organs, such as the telencephalon, even after 12 h of culture under isolation conditions. This result suggests that donor cells may adopt the differentiation program of the host ectoderm. However, further experiments are required to determine whether they can differentiate into all ectodermal organs. On the other hand, donor cell masses were observed in the resulting embryos without integration into host organs. Donor cells transplantation into the host blastula was performed without resuspension after cultivation under chelating conditions. Therefore, the donor cell masses observed after transplantation likely originate from aggregation during culture. It has been reported that cell masses respond differently to isolated cells during development, a phenomenon known as the community effect (Gurdon, 1988). Therefore, it is not surprising that scattered and aggregated donor cells exhibited different responses.

In the resultant embryos transplanted with donor cells after long-term cultivation, additional optic cups or auditory vesicles were observed. These organs were comprised entirely of donor cells and were at an earlier developmental stage than those in the host. In the additional eye, both the optic cup and lens were formed from donor cells (Figs. 4F and 5F). The lens is induced from the embryonic epithelium by signals from the optic vesicle (Heavner and Pevny, 2012). Previous studies have shown that a graft cell mass from a donor optic vesicle can develop into an additional optic cup when transplanted into an adjacent portion of the host optic vesicle (Dragomirow, 1933 ; cited in Balinsky, 1965). However, in the present study, the donor cells differentiated into optic vesicles in the host embryo and induced the formation of a lens. It remains unclear whether these eyes will develop further. Since the differentiation process of donor cells was not tracked over time, future studies should focus on examining the dynamic behavior of transplanted cells throughout development.

Chimeras are organisms that contain cells from two different zygotes, effectively having more than one set of parents. Recently, germline chimeras have been investigated as a technology of surrogate reproduction in livestock breeding (Ledesma et al., 2023) and fisheries science (Goto and Saito, 2019). In somatic-line chimera, chimeric heterosis has been reported (Mikami and Onishi, 1985 ; Onishi and Mikami, 1987). Blastula-stage embryonic cells of bony fishes have pluripotency, and are suitable to produce embryonic chimera by micromanipulation. If these cells can be systematically integrated into specific organs of another individual, they could potentially be utilized to modify individuals using somatic chimeras. To achieve this, it is necessary to clarify the characteristics of blastula-stage embryonic cells. In the present study, it was revealed that when cells at the mid-blastula stage are isolated and cultured, they maintain pluripotency for up to 3 h, and when cultured for up to 12 h and transplanted in a separated state into a host blastula, they maintain the ability to differentiate into ectodermal organs. Such cultured blastomeres may be useful for incorporating donor cells only into ectodermal organs.

However, in embryonic chimeras induced by transplantation between individuals, the donor cells are often rejected by the host's immune system during development. It has been suggested that, when foreign cells are incorporated into the thymus, genetically similar cells may be tolerated within the chimera (Ohki et al., 1987). Consequently, if an experimental framework can be established in which donor cells are introduced into the thymus and differentiate exclusively into mesodermal or endodermal lineages, it may be feasible to induce the formation of chimeric individuals wherein donor cells are integrated into specific organs. In the next paper, we will report on experiments to integrate donor blastomeres into the endoderm.

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