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Germ Cell Lineage from a Single Blastomere at 8-Cell Stage in Shiro-uo (ice goby)

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ABSTRUCT—Shiro-uo (ice goby; teleost fish), *Leucopsarion petersii,* shows a unique cleavage pattern characterized by two tires of blastomeres at 8-cell stage, like that of echinoderm and amphibian embryo. Such a pattern is suitable to isolation and cell lineage experiments. In this study, cell lineage of germ-line was traced by histological observation and cell labelling experiment at the 8-cell stage. Primordial germ cells (PGCs) were first detected histologically at the 10-somite stage, and migrated to gonadal anlage at 10 days post-fertilization, through usual way described in other teleost species. When a single blastomere was labelled with tracer dye at 8-cell stage, both upper and lower tires generated labelled PGCs at gonadal anlage although upper tires occasionally. This result suggests that all blastomeres at the 8-cell stage have potential to produce PGCs in shiro-uo.

Key words: primordial germ cells (PGCs), cell lineage, shiro-uo

INTRODUCTION

Cell lineage study reveals developmental system about degrees of regulation and mosaicism in embryogenesis of a given species. In general, the more mosaicism of development increases, the more invariable cell lineage is, because the given cytoplasmic factors distribute a fixed blastomere (Davidson, 1990).

In teleost, cell lineage seems to be variable before early gastrulation (Kimmel *et al*., 1990), suggesting that the development is comparatively more regulative than mosaic in embryogenesis. Actually, extirpation and transplantation treatments of blastoderm before the blastula stage do not affect following development in several species (*Fundulus*, Oppenhemer, 1936; goldfish, Tung *et al*., 1944; Yamaha *et al*., 1997; zebrafish, Lin *et al*., 1992; medaka, Wakamatsu *et al*., 1994; rainbow trout, Takeuchi *et al*., 2001). On the other hand, germ cell lineages in teleost fish are not regulative, because extirpation of lower blastoderm at the blastula stage affects the number of PGCs at gonadal anlage (Kazama-Wakabayashi *et al*., 1999). Germ cell lineage inherits maternal factors during early cleavage stage in zebrafish, including *vasa* and *nanos* mRNA (Yoon *et al*., 1997; Köprunner *et al*., 2001). The maternal factors aggregated at marginal parts of early cleavage planes, are inherit to single cells located marginal part of blastoderm at the mid-blastula stage, and thereafter establish germ cell lineage in zebrafish (Yoon *et al*., 1997; Knaut *et al*., 2000) and goldfish (Otani *et al*., 2002). In marginal part of blastoderm, cytoplasm of marginal blastomeres join through yolk cell during early cleavage stage and collapse into yolk cell to form yolk syncytial layer after MBT (Kimmel and Law, 1985; Gevers *et al*., 1992; Yamaha *et al*., 1999). Therefore, it is difficult to trace germ cell line by cell labelling with tracer dye.

Shiro-uo (ice goby), *Leucopsarion petersii*, shows a unique cleavage pattern among fish embryos (Nakatsuji *et al*., 1997; Arakawa *et al*., 1999). During the initial steps of embryogenesis, the first two vertical cleavages produce 4 blastomeres on the yolk surface at the 4-cell stage. The third cleavage is horizontal in all blastomeres, and produces two tiers of blastomeres at the 8-cell stage. This cleavage pattern of blastodisc is resemble that of echinoderm and amphibian embryo. Therefore, the blastomeres at the 8-cell

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stage are suitable for cell lineage study, using tracer dye, because of the complete division of blastomeres without connection at this stage. The mechanical extirpation of upper tiers does not affect following development, suggesting the presence of regulative developmental system in shiro-uo (Saito *et al.*, 2001). Germ cell lineage, however, has not been studied in this species.

In the present study, we first describe the positioning of PGCs during embryonic development by histological observation, and next trace blastomere lineage at 8-cell embryo by cell labelling, in order to elucidate establishment of germ cell lineage.

MATERIALS AND METHODS

Animals and gametes

Adult ice goby (shiro-uo), *Leucopsarion petersii*, used in this study, were purchased from local dealers. Maturation, fertilization, dechorionation and cultivation procedure were according to the method of Saito *et al.* (2001). Staging of embryo was according to Arakawa *et al.* (1999).

Injection of tracer dye

In order to reveal cell lineage from upper or lower blastomeres at the 8-cell stage, we used fluorescent dye as a tracer. Injected solution was a 5% Biotin-Dextran-fixable (Sigma) and 5% FITC-Dextran (Sigma) in 0.2M KCl. The embryos in which the tracer dye leaked from labelled blastomere to adjacent ones by checking under fluorescence microscope were discarded. Blastomeres with Biotin-Dextran-fixable was histologically detectable. Injected embryos were incubated in the culture medium at 19°C according to Saito *et al.* (2001), and fixed with Bouin's fixative for 2 hr, and used for histological analysis (described below).

Optical analysis

Fluorescence photomicrographs of embryos were taken by using an inverted microscope (Zaiss Axiovert 135) with transmission optics and an automatic camera. FITC fluorescence images were observed by Zaiss filter set.

Histology

For histological observation, embryos were fixed with Bouin's fixative for 2 hr, dehydrated in a butyl alcohol series and embedded in paraffin. Serial sections were cut at a thickness of 8 μm, and stained with hematoxylin-eosin, using standard procedures. PGCs were confirmed based on location and characteristics similar to those observed in other teleosts; round shape, relatively large size, large nuclei, and clear nuclear membrane (Hamaguchi, 1982; Timmermans and Taverne, 1989; Braat *et al.*, 1999; Kazama-Wakabayashi *et al.*, 1999; Nagai *et al.*, 2001).

For cell fate analysis, sections of biotin-labelled embryos were attached to slide glasses by 0.005% poly-L-lysine (WAKO). Biotin-labelled cells were stained with diaminobenzidine (DAB) using Histofine SAB-PO (M) kit (Nichirei Co. Ltd.).

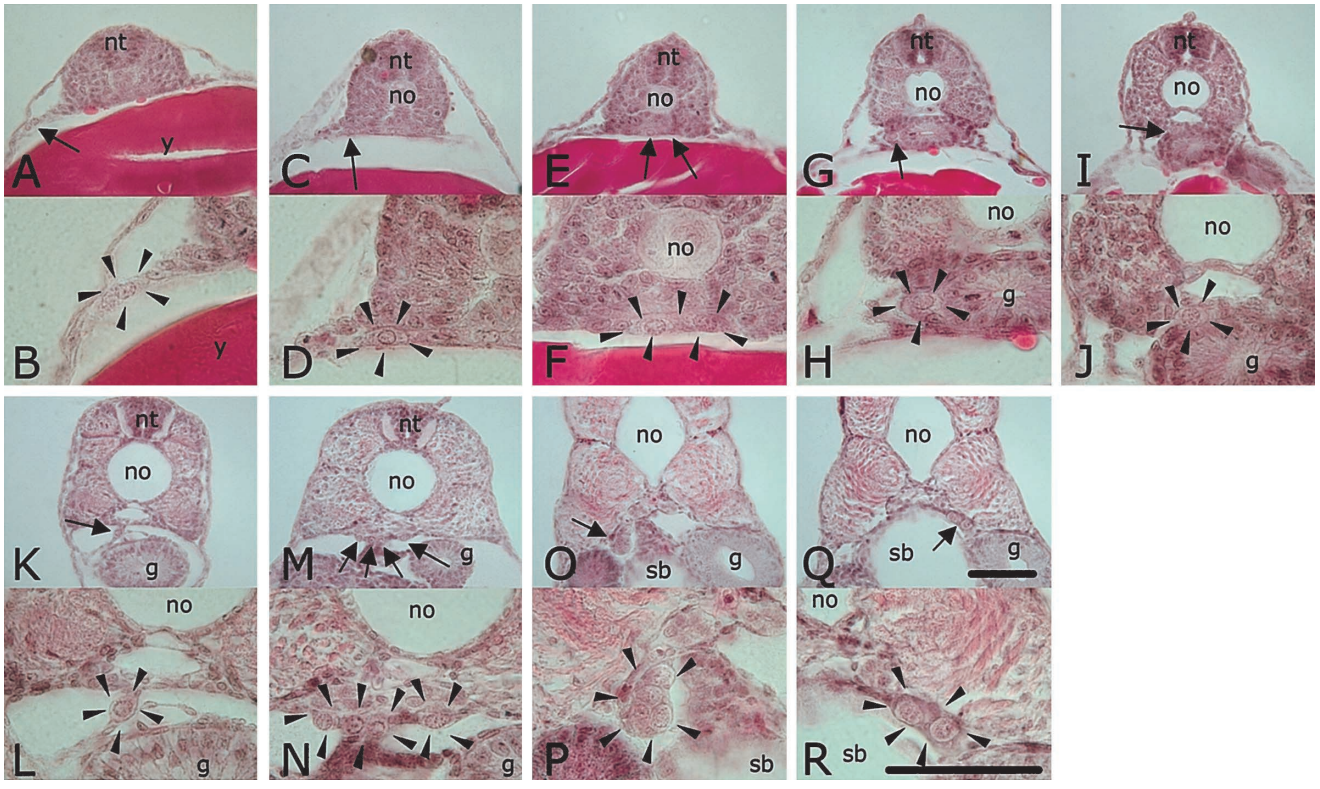

Fig. 1. Distribution of PGCs in transverse sections of shiro-uo embryos. (A-B) 10-somite. (C–D) 20-somite. (E–F) 30-somite. (G–H) 5 dpf. (I–J) 6 dpf (K, L) 8 dpf. (M, N) 10 dpf, (O–R) 13 dpf. B, D, F, H, J, L, N, P, and R, are higher magnification of A, C, E, G, I, K, M, O, and Q., respectively. Arrows and arrowheads indicate PGCs. g, gut; no, notochord; nt, neural tube; sb, swim bladder; y, yolk. Scale bars indicate 50 μm.

RESULTS

Migration of the PGCs in shiro-uo embryo

To identify the localization of the PGCs, we analyzed paraffin sections of shiro-uo embryos from 50%-epiboly (24 hr postfertilization [hpf]) stage until hatch out stage (13 days postfertilization [dpf]) of development. Initially, we observed the morphology of PGCs at 10 dpf, and then traced back the location of PGCs using the characteristics, described above. PGCs were found around the upper part of body cavities, the future gonadal region. Their sizes varied between 10 and 20 μm in major diameter, and PGCs were apparently larger than other surrounding cells. The cytoplasm was stained faintly with eosin.

PGCs were first detectable histologically at the 10-somite stage (50 hpf). Before 10-somite stage embryo, we couldn't distinguish PGCs from somatic cells, because of

Table 1. Number of PGCs in embryos at the 10-somite stage and 10 dpf

Stage (hpf)	No. of PGCs	Average
10-somite (50)	18, 18, 19, 21, 24	20.0
10 dpf (240)	9, 19, 20, 21, 23	18.4

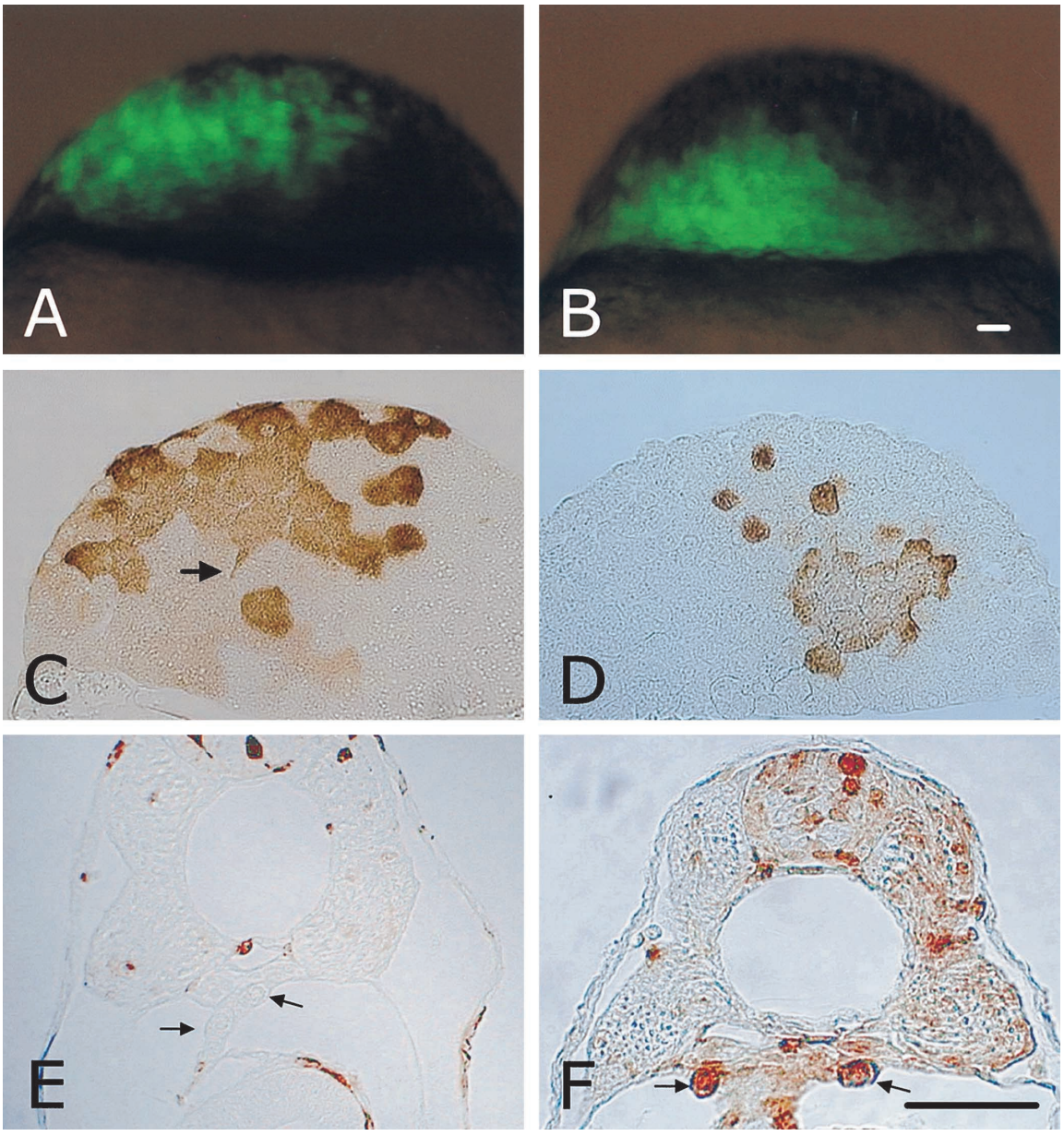

Fig. 2. FITC-biotin-labelled descendants from upper or lower blastomere of 8-cell stage. (A) Fluorescent view of an upper-blastomere-labelled embryo at the blastula. (B) Fluorescent view of a lower-blastomere-labelled embryo at the blastula. (C, D) Histological detection of descendants of labelled upper-blastomere at 15 hpf and 16 hpf. Arrow indicates cytoplasmic protrusion. (E, F) Histological detection of descendants of labelled blastomere at 10-dpf. Single upper (E) or lower (F) blastomere of 8-cell stage embryo was labelled. Arrows indicate unlabelled (E) and labelled (F) PGCs. Scale bars indicate 50 μm.

yolk granules in blastomeres. Yolk granules disturbed histological detection of PGCs. At the 10-somite stage, PGCs were located at mesendodermal region lateral to embryonic body and close contact to the YSL (Fig. 1A, B). At the 20-somite stage, the location of PGCs were shifted medially, and located in mesendodermal region (Fig. 1C, D). At the 30-somite stage, the locations of PGCs were more medially, and some PGCs were in contact with the gut primordium (Fig. 1E, F). At the 5 dpf, PGCs were positioned at the inside of lateral plate mesoderm (Fig. 1G, H). At the 6 dpf, the locations of PGCs were shifted dorsally along inside of lateral plate mesoderm and some PGCs are located at the upper part of gut (Fig. 1I, J). At the 7-9 dpf, when the body cavity was expanded, PGCs were located on the peritoneum dorsal to the intestine (Fig. 1K, L). At the 10 dpf, PGCs were located at the upper part of the body cavity. PGCs were situated at both sides of the upper part of body cavity (Fig. 1M, N). At the 13 dpf, the pair of PGCs clusters were observed at the dorsal part of body cavity. The clustered PGCs were covered with the somatic cells (Fig. 1O-R).

The numbers of the PGCs were different in each individual even at the same stage, but the average number of the PGCs was almost conserved in embryos from 10-somite to 10-dpf stages (Table 1).

Clonal analyses by tracer injection into 8-cell blastomere

When the tracer dye was injected into single blastomere, resultant embryos continued to develop, and tracer dye was transmitted only to the clonal progeny of a founder blastomere. The clonal descendants of the blastomere were dispersed among other blastomeres in the blastoderm. The dispersion of labelled cells gradually initiated after 15 hpf, the blastula stage (Fig. 2A, B). During early epiboly (20 hpf), labelled cells were located around almost entire blastoderm. Histological detection of biotin-labelled cells revealed active shape after the 15 hpf. They showed protrusions from their main bodies (Fig. 2C). After 16 hpf, labelled cells had distance each other (Fig. 2D). At 10 dpf, labelled cells were histologically observed at almost all organs. Thus, both upper and lower blastomeres at the 8-cell stage differentiated to various kinds of cells belonging to all the three germ layers.

When labelled PGCs were counted at the gonadal anlagen of 10 dpf in labelled embryos, labelled PGCs were identified in all embryos developed from labelling of lower blastomere. The number of labelled PGCs varied from 3 to 8 in 10 embryos. In the case of labelling of upper blastomere, only 2 out of 11 resultant embryos had labelled PGCs with 4 or 5 in number (Fig 2E, F. Table 2).

Table 2. Number of biotin-labelled PGCs in 10 dpf embryos after labelling of a single upper or lower blastomere at the 8-cell stage

Labelled blastomere	No. of labelled PGCs	Average
Upper	0, 0, 0, 0, 0, 0, 0, 0, 4, 5	0.8
Lower	3, 4, 4, 4, 6, 6, 7, 7, 7, 8	5.6

DISCUSSION

In shiro-uo, PGCs were first detected at the 10-somite stage. Because yolk granules were distributed in all cells of embryonic body until early segmentation period, like in goldfish (Kazama-Wakabayashi *et al*., 1999), it was difficult to distinguish PGCs from surrounding somatic cells. In both zebrafish and goldfish, histological detection of PGCs is later in development than those of identification of *vasa* transcripts, germ line maker (Nagai *et al*., 2001; Otani *et al*., 2002). Therefore, it must be difficult to identify PGCs by histological characters in teleost at early developmental stage.

In final step of PGCs migration to the gonadal anlage, it is shown that PGCs are related to the inner layer of lateral plate mesoderm in other teleost fish (Richards and Thompson, 1921; Hann, 1927; Jhonston, 1951). But in medaka embryo, when PGCs migrate dorsalward, they are related to the outer layer of lateral plate mesoderm (Gamo, 1961; Hamaguchi, 1982). In the shiro-uo, the present study reveals that PGCs migrated through former route. Finally, the PGCs were covered with somatic cells at 13 dpf, suggesting gonadal formation thereafter.

In shiro-uo, the clonal cells of labelled blastomere, showing pseudopodia, scattered among unlabelled cells at blastula stage, finally differentiated almost all tissues from the three germ layers. The mixing pattern, described here, clearly shows that developmental fate of shiro-uo blastoderm is undetermined at the blastula stage. Namely, blastomeres have pluripotency at this stage. Moreover, our previous report shows that shiro-uo embryos without yolk cell at the 8-cell stage develop abnormally, although without upper tire of blastomeres develop normally (Saito *et al*., 2001). These results suggest that differentiation of blastoderm is integrated by inductions from yolk cell after blastula stage like shown in zebrafish and goldfish (Mizuno *et al*., 1996; Mizuno *et al*., 1997; Mizuno *et al*., 1999; Yamaha *et al*., 1998).

Origins of PGCs were investigated by cell linage experiments and indicated that PGCs were mainly originated from lower blastomeres at the 8-cell stage. In zebrafish embryo, molecular marker *vasa* gene has identified PGCs origins (Yoon *et al*., 1997; Knaut *et al*., 2000). Maternal *vasa* transcripts are localized around the blastodisc at the one-cell stage (Braat *et al*., 1999; Howley and Ho., 2000). At the two- to four-cell stage, *vasa* transcripts are localized along the first and second cleavage plane, and eventually these clumps localize into four cells at the 32-cell stage (Yoon *et al*., 1997). The present results are generally consistent with the zebrafish pattern. But PGCs sporadically rose from upper blastomeres at the 8-cell stage in shiro-uo. Recently, *vasa* gene was isolated in shiro-uo. The expression pattern of *vasa* by whole mount *in situ* hybridization reveals that germ cell lineage is detected from early cleavage stage (Miyake *et al*., 2001). This suggests existence of germ-line determinants from early cleavage stage in shiro-uo. In shiro-uo, *vasa* transcripts are observed at the one- and two-cleavage furrows. Moreover, at the 16-cell stage, new four dots of *vasa* transcripts are frequently observed just under the upper blastomeres, although not confirmed at the 8-cell stage. In the goldfish, *vasa* gene transcripts are always

identified at the third-cleavage furrows, in addition to one- and two- cleavage furrows (Otani *et al*., 2002). In zebrafish, occasional signals are observed at third-cleavage plane, but are weaker than those at the first and second furrows (Yoon *et al*., 1997). The frequency of labelled PGCs originated from upper blastomere in 10 dpf embryos is low (2/11 embryos). This possibly suggests that the descendants of lower blastomeres receive the germinal factors include *vasa* transcripts with high frequency more than upper blastomeres one.

We previously showed pluripotency of blastomere by extirpation experiment in shiro-uo embryo at 8-cell stage (Saito *et al*., 2001). Moreover, in this study, we revealed that all the blastomeres had a potential to produce the PGCs. This indicates that shiro-uo could be useful model to study PGCs because their blastomeres are suitable for isolation and transplantation experiments at early cleavage stage.

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