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Histological Differentiation of Primordial Germ Cells in Zebrafish

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ABSTRACT—In this study, primordial germ cells (PGCs) in zebrafish were described histologically using eosinophilic granules as a marker. PGC-like cells (PL-cells) with eosinophilic granules were identified initially at the sphere stage (4-hr postfertilization), and were observed until the bud stage, the earliest stage to which PGCs with proper morphology could be traced. The morphology and distribution of eosinophilic granules in PL-cells changed during epiboly. Mitoses of PL-cells were observed only from the shield to bud stage, after eosinophilic granules aggregated to the perinuclear region in PGCs. These shifts of eosinophilic granules corresponded histologically to those of germ plasm described in *Xenopus*. These results suggest that eosinophilic granules represent germ plasm in fish and that PL-cells with these granules correspond to the PGCs or presumptive PGCs (pPGCs).

INTRODUCTION

The bodies of multicellular organisms consist of various kinds of cells which are broadly classified into somatic and germ lines. In many animals, the germ line can be identified as cells which are morphologically different from somatic cells in the early developmental period. Generally, the germ line is formed through the mechanisms of predetermination or epigenesis (Nieuwkoop and Sutasurya, 1979; Timmermans, 1996; Wakahara, 1996). In worms, flies and frogs, a special cytoplasm called germ plasm or pole plasm (in flies) is located asymmetrically within eggs and is inherited into some cells, which differentiate into germ cells (Nieuwkoop and Sutasurya, 1979). The cytoplasm is believed to predetermine the fate of germ cell differentiation, because transplantation and UV irradiation experiments have led to perturbation of germ-cell formation in *Drosophila* (Okada *et al*., 1974; Illemensee and Mahowald, 1974; Kobayashi and Okada, 1989) and in anurans (Smith, 1966; Ikenishi *et al*., 1986). The cytoplasm contains specific RNA and protein, such as *vasa* (Hay *et al*., 1988; Bardsley *et al*., 1993; Komiya *et al*., 1994; Ikenishi *et al*., 1996; Ikenishi and Tanaka, 1997). In *Drosophila*, females homozygous for a mutation of the *vasa* gene give rise to progeny that are sterile due to their lack of pole cells, the future germ cells (Williamson and Lehmann, 1996).

On the other hand, primordial germ cells (PGCs), which differentiate to germ cells, may arise epigenetically in urodeles (Kotani, 1957, 1958; Nieuwkoop and Stasurya, 1979; Michael, 1984), mammals (Lawson and Hage, 1994), and birds (Ginsburg, 1994). In these animals, germ line cells can be identified only after the expression of morphological features of germ cells. In teleost, PGCs have been investigated exclusively by both light and electron microscopy (Johnston, 1951; Gamo, 1961; Hamaguchi, 1982; Timmermans and Taverne, 1989; Gevers *et al*., 1992b; Kazama-Wakabayashi *et al*., 1999; Braat *et al*., 1999). In these studies, PGCs have been identified by their morphological characteristics, namely roundish shape, relatively large size, large nucleus, and a clear nuclear membrane. Eosinophilic granules have been reported as histological characteristics common to PGCs in some species, such as medaka (Gamo, 1961), rosy barb (Timmermans and Taverne, 1989), and goldfish (Kazama-Wakabayashi *et al*., 1999). However, fish PGCs have not been traced back by these histological markers to before the gastrula period.

In zebrafish, germ line lineage can be traced throughout embryogenesis by means of *vasa* transcripts. *Vasa* mRNA distinguishes germ cell precursors from other somatic cells in cleaving embryos, and is inherited by the future germ cells (Yoon *et al*., 1997; Braat *et al*., 1999). Electron microscopic observation has also revealed germ-plasm-like structures containing *vasa* mRNA (Knaut *et al*., 2000). These results suggest that the formation of PGCs is predetermined by maternal factors. Like as the PGCs of other teleost species, however, zebrafish PGCs are not distinguished from the somatic cells before the gastrula stage under light microscope (Braat *et al*.,1999). In the present study, to describe the histological

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characteristics of PGCs, we made detailed observations of PGCs in zebrafish, especially before the gastrula period.

MATERIALS AND METHODS

Fish embryos

Zebrafish, *Danio rerio*, were kept in the Aquarium Center of the Faculty of Fisheries, Hokkaido University. Parent fish were maintained at 29.0°C. Embryos were obtained through natural matings, and dechorionated with 0.1% trypsin (DIFCO) and 0.4% urea in Ringer's culture solution containing actinase E (KAKEN). Dechorionated embryos were rinsed with Ringer's culture solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$) containing 0.01% penicillin, 0.01% streptomycin and 1.6% albumen. Dechorionated embryos were cultured at 29±1°C in separate wells (Greiner) filled with Ringer's culture solution for 24 hr, and afterward in another separate well filled with culture solution (1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$) containing the same antibiotics as above. The stage of development was identified according to Kimmel *et al.* (1995).

Histology

For histological observation of PGCs, embryos were fixed at appropriate stages from the 2-cell stage to 10-days postfertilization (dpf) with Bouin's fixative for 2 hr and transferred to 80% ethanol. Fixed samples were dehydrated using a butyl alcohol series, and subsequently embedded in a paraffin block. Serial transverse sections of 8 μm thickness were stained with hematoxylin and eosin according to the standard procedures.

RESULTS

Histological changes of zebrafish PGCs

We observed the morphology of PGCs in zebrafish embryos after fertilization (Figs. 1, 2, 3). PGCs were found at 6 dpf around the upper part of the body cavity, the future gonadal region (Fig. 1F). These cells were confirmed as PGCs based on characteristics similar to those observed in other teleosts: i.e., roundish shape, relatively large size, large nucleus, and clear nuclear membrane. Their sizes varied between 10 and 20 μm in major diameter, and the PGCs were apparently larger than other surrounding cells. The PGC cytoplasm, nuclear membrane and granular structures in the nucleus were all stained faintly with eosin.

PGCs were detectable at 10-hr postfertilization (10 hpf; bud stage), based on the histological features described at 6 dpf (Fig. 1A). Almost all PGCs were located among hypoblast cells around the para-axial part of an embryonic body. Compared with surrounding cells, PGCs contained large eosinophilic granules adjacent to their nuclear membranes. It was difficult to distinguish PGCs from other somatic cells before the bud stage using morphological features, especially their size and shape.

When PGCs were investigated at earlier stages, cells that exhibited eosinophilic granules, but not other characteristics of PGCs, were noticeable at 4 hpf (sphere stage) (Fig. 2A). We named these cells PGC-like cells (PL-cells). The eosinophilic granules in PL-cells, as well as those in PGCs, differed from yolk in their ability to be stained with eosin. From the sphere stage to the beginning of gastrulation, eosinophilic granules in PL-cells were scattered in the cytoplasm rather than around the nuclear membrane (Fig. 2A, B). From gastrulation to early segmentation, these scattered granules came to attach to a discrete section or the entire surface of the nuclear membrane (Fig. 1A, B, C, Fig. 2C, D), forming a cap- or ring-like structure similar to those described in *Xenopus* (Whitington and Dixon, 1975). The nuclei of PL-cells differed from those of other somatic cells in their ability to be stained with hematoxylin; the former was stained more faintly than the latter. From the late segmentation period to 6 dpf, the morphology of eosinophilic granules changed to disperse as small particles attached to the nuclear membrane, and was not conspicuous in PGCs entering the gonadal primordium (Fig. 1D, E, F). In this period, PGC nuclei exhibited fainter and more homogeneous eosin staining than those at previous stages. Cell divisions of PL-cells and PGCs were observed from the shield stage to the 2-somite stage period (Fig. 3). Eosinophilic granules were distributed almost equally to daughter cells as a few large granules, which were located between two chromosome clusters in the dividing cells (Fig. 3A2, B2). The number of PL-cells increased nearly two or three times from the 30% epiboly stage to the bud stage period (Table 1).

The sizes of PL-cells and PGCs changed during development. PL-cells were about 30 μm in major diameter at the sphere stage. They became smaller, but were still larger than surrounding cells after the epiboly movement. The size of PGCs did not change following gastrulation, and was constant until at least 6 dpf.

The distribution of PL-cells and PGCs

The distribution of PL-cells and PGCs shifted with the advance of development (Fig. 4). In embryos at the sphere stage, PL-cells were found mainly in the lower part of the blastoderm, but did not cluster obviously in this region. From the beginning of epiboly to the shield stage, PL-cells were located in the deep cell layer close to the yolk syncytial layer (YSL). From the shield stage to the beginning of somitogenesis, all PGCs were observed in the hypoblast, and were often located side by side (Fig. 2C). The PGCs at the 1-to 6-somite stage (10.3-12 hpf) were located between the lateral mesoderm and the YSL, and were gradually approaching both sides of the embryonic body at the same distance from the notochord (Fig. 1B). At the 8-to 14-somite stage (13–16 hpf), when the somites were elevated from the yolk with morphogenesis of the neural primordium, PGCs were still located on the YSL. At the 21-somite stage (19.5 hpf), the location of PGCs shifted medially, near the extended pronephric duct (Fig. 1D). At the hatching stage (48 hpf), they shifted toward a more medial position around the gut (Fig. 1E). Thereafter, up to 6 dpf, they were located on the peritoneum, dorsal to the intestine during the expansion of the body cavity (Fig. 1F).

The distribution of PGCs also shifted along the anteroposterior axis with embryonic development after epiboly (Fig. 4). PGCs were located in various positions in embryos throughout the epiboly movement, gradually moved to the trunk region, and formed clusters at the level of the third to sixth somite along the embryonic body at 12 hpf (the 6-somite stage). At

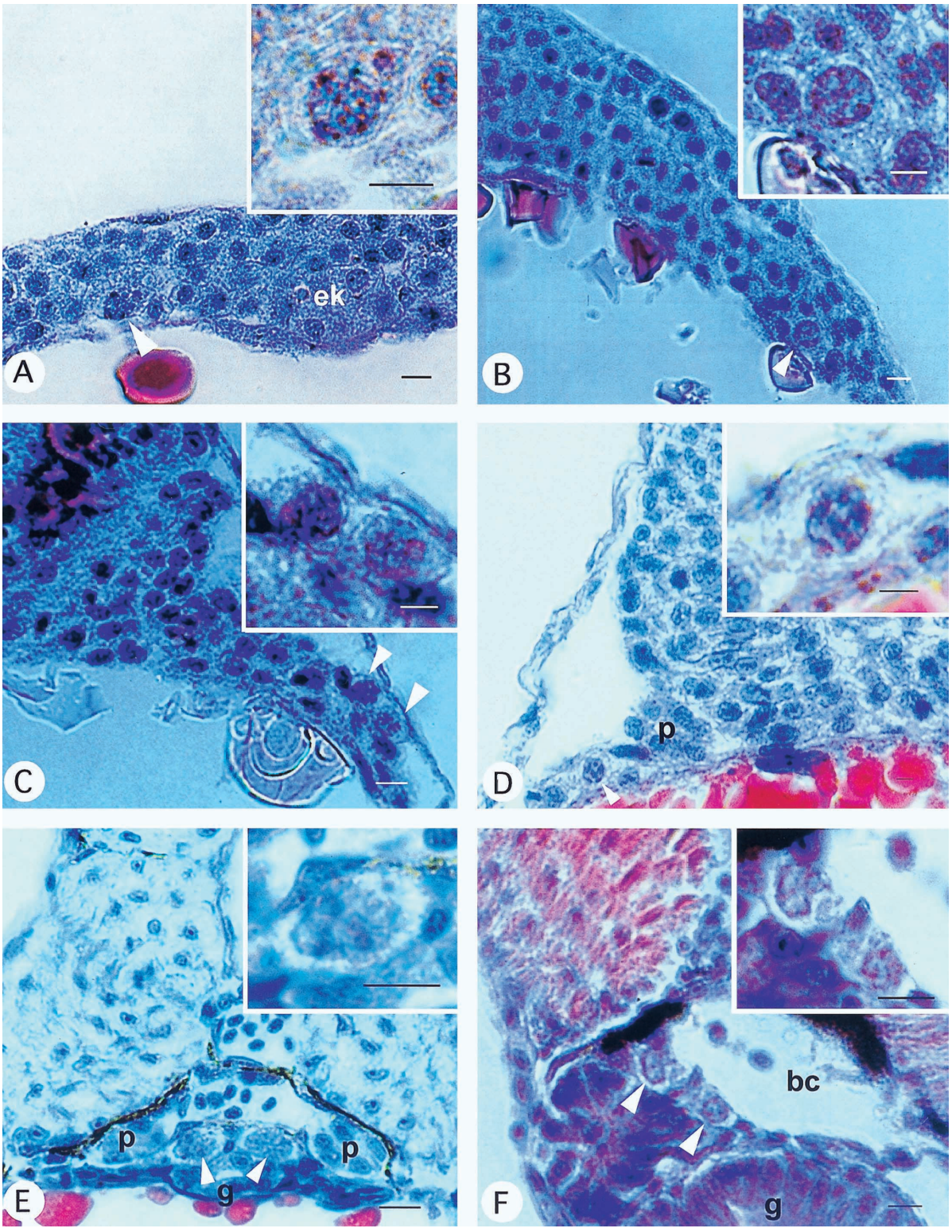


Fig. 1. Morphology of PGCs in zebrafish embryos at the bud stage (10 hpf) to 6 dpf. All figures are transverse sections. Insets are a higher magnification of PGCs in each section. (A) PGC at the bud stage (10 hpf). (B) PGC at the 4-somite stage (11.3 hpf). The PGC was located between the mesoderm and YSL. Eosinophilic granules were located around the nucleus, forming a ring. The size of the PGC was larger than that of surrounding cells. (C) PGCs at the 12-somite stage (15 hpf). Eosinophilic granules were located in a juxtanuclear region, forming a cap. (D) PGCs at the 21-somite stage (19.5 hpf). PGCs were located on the YSL ventral to the pronephric duct. Eosinophilic granules, which came to particles, were located in a juxtanuclear region and inner part of the nucleus. (E) PGCs at the hatching period (48 hpf). PGCs were located in a more medial position, in the upper part of the gut. (F) PGCs were located on the peritoneum dorsal to the gut at 6 dpf. Eosinophilic granules were in a juxtanuclear region similar to those at the hatching period, but not conspicuous. Arrowheads, PGCs; bc, body cavity; ek, embryonic keel; g, gut; p, pronephric duct. Scale bars indicate 10 μm.

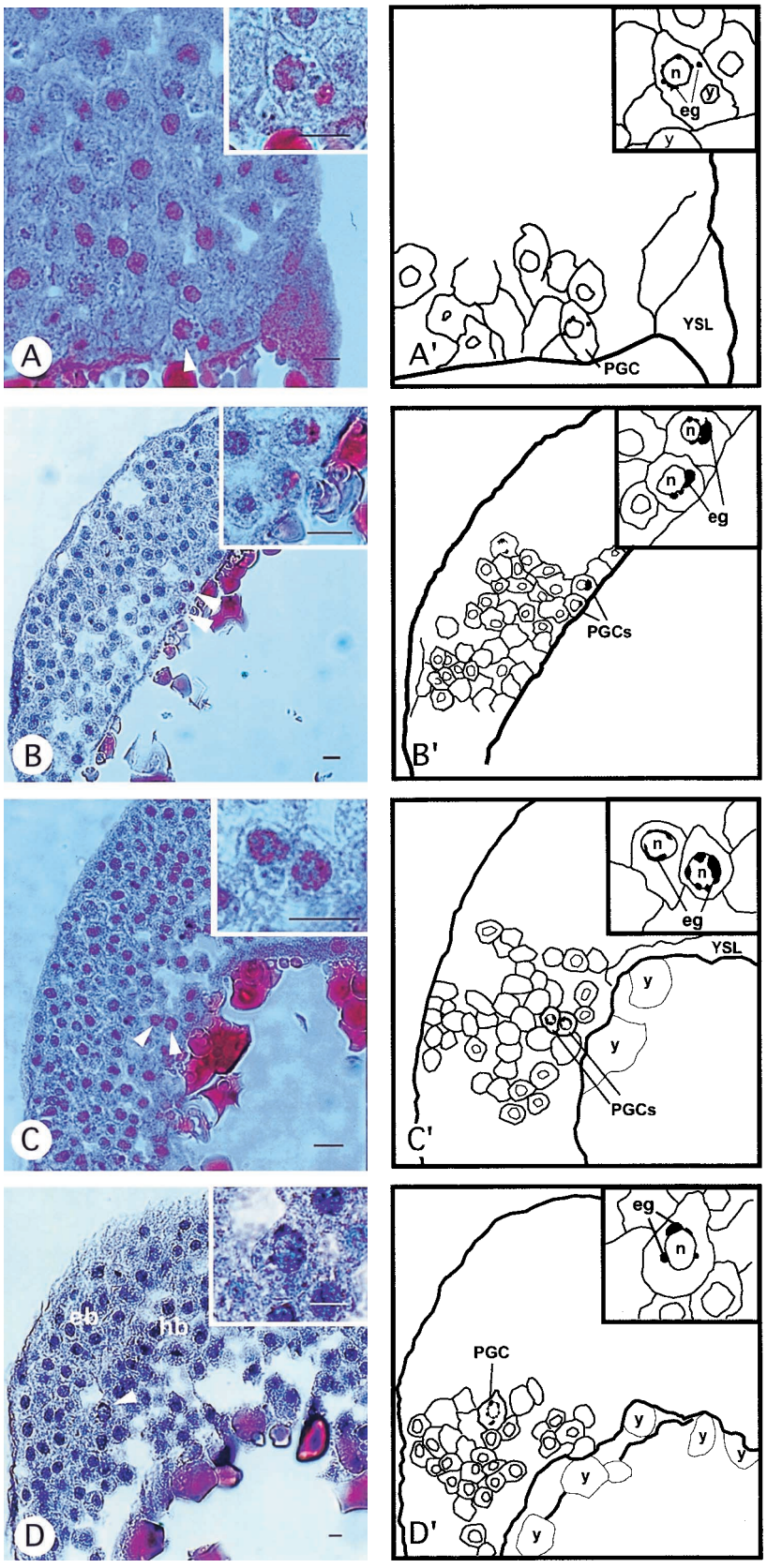


Fig. 2. Morphology of PL-cells in zebrafish embryos at the sphere stage (4 hpf) to the 90% epiboly stage (9 hpf). All figures are transverse sections. Insets are a higher magnification of PL-cells in each section. The drawings in A', B', C', and D' correspond to the sections in A, B, C, and D. (A) PL-cell at the sphere stage (4 hpf). The PL-cell was located in the lower part of the blastoderm. Eosinophilic granules, not the yolk spherule (y), were located in the cytoplasm and perinuclear region. The size of the PL- cell was equal to that of surrounding cells. (B) PL-cells at the 30% epiboly stage (4.7 hpf). Eosinophilic granules aggregated gradually to the perinuclear region in PGCs. (C) PL-cells at the shield stage (8 hpf). Eosinophilic granules were located in the perinuclear region. (D) PL-cell at the 90% epiboly stage (9 hpf). Arrowheads, PGCs; eb, epiblast; eg, eosinophilic granules; hb, hypoblast; n, nucleus; y, yolk spherule. Scale bars indicate 10 μm.

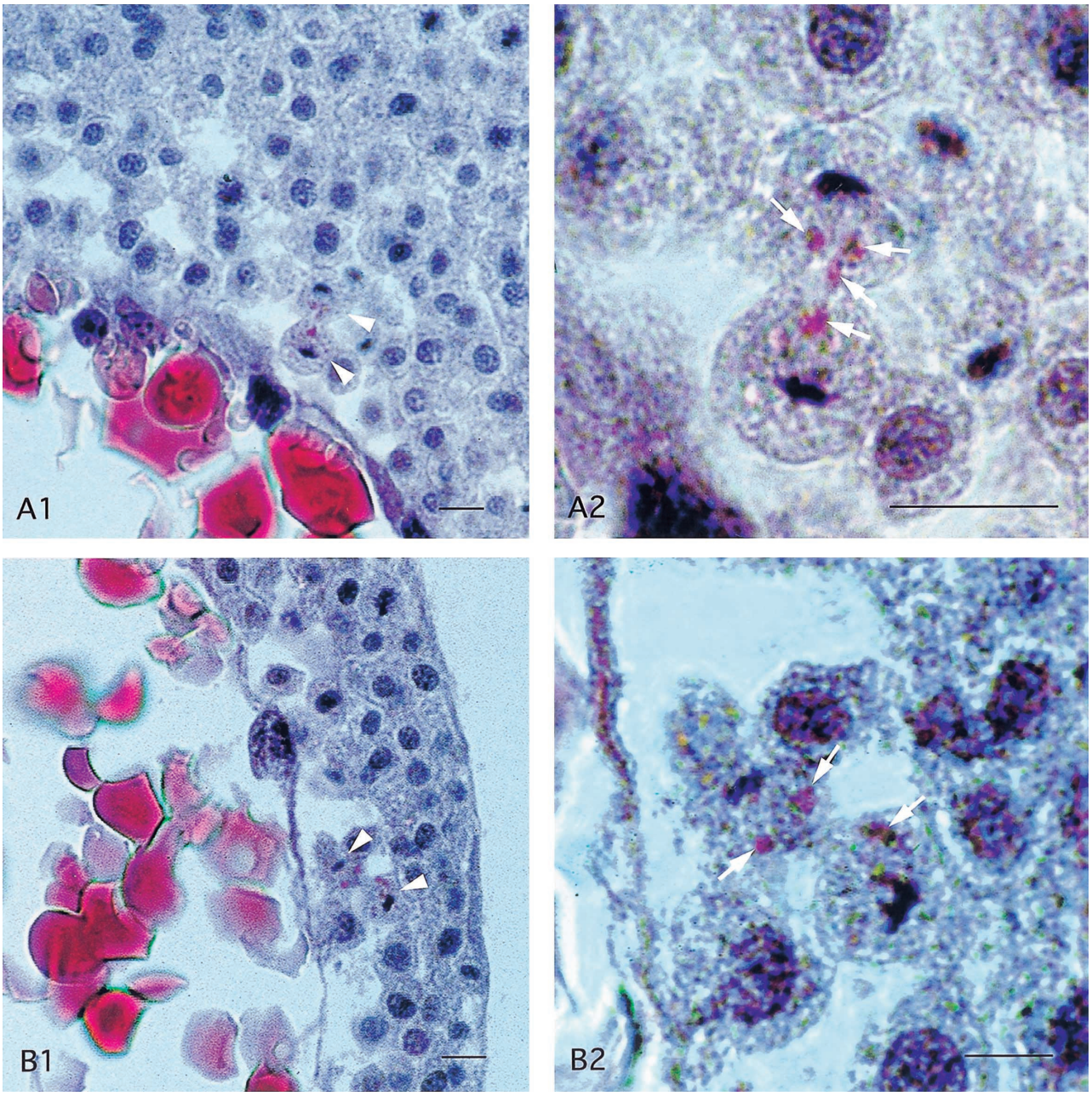


Fig. 3. Mitotic divisions of PL-cells with eosinophilic granules. (A) PL-cells at the shield stage (6 hpf) and (B) at the 75% epiboly stage (8 hpf). A2 and B2 are a higher magnification of A1 and B1, respectively. The figures show that eosinophilic granules are inherited by both daughter cells in PGCs. Arrowheads indicate PGCs, and arrows indicate eosinophilic granules. Scale bars indicate 10 μm.

15 hpf (the 12-somite stage), these clusters were located at the level of the first somite to the third somite. Subsequently, PGC clusters scattered to the posterior along the embryonic body.

DISCUSSION

PGCs could be recognized in zebrafish embryos from 10 hpf onward under light microscope, being morphologically similar to those described in other teleost species, such as blackbass (Johnston, 1951), medaka (Gamo, 1961; Hamaguchi, 1982), rosy barb (Timmermans and Taverne, 1989; Gevers *et al*., 1992b) and goldfish (Kazama-Wakabayashi *et al*., 1999). Fish PGCs have not been traced back histologically to before the gastrula stage. In the present study in zebrafish, some blastomeres, referred to as PL-cells, contained eosinophilic granules and were identified after the sphere stage (4 hpf; late-blastula). The morphological identification of PGCs at this stage has not been reported in any teleost species.

Recent studies on *vasa* mRNA, a germ line-specific marker, indicated that germ cell precursors were segregated early from somatic cell lineage and that the germ cell lineage

Table 1. Number of PL-cells and PGCs in embryos during normal development

Stage (h)		No. of PGCs	(average)
sphere	(4h)	0, 1, 3, 5, 5, 7, 7	(4)
dome	(4.3h)	0, 1, 2, 2, 6, 6, 7	(3.4)
30% epiboly	(4.7h)	5, 7, 9, 11	(8)
shield*	(6h)	11, 11, 15, 17, 17, 18, 22	(15.9)
75% epiboly*	(8h)	9, 10, 13, 14, 14, 15, 18, 21, 23	(15.2)
90% epiboly	(9h)	10, 11, 12, 15	(12)
bud	(10h)	12, 14, 20, 22, 22, 22, 30, 31	(21.6)
2-somite*	(10.7h)	19, 23, 29, 31, 36, 38	(29.3)
4-somite	(11.3h)	11, 14, 16, 21	(15.5)
6-somite	(12h)	21, 24, 26, 37, 53	(32.2)
8-somite	(13h)	13, 16, 18, 25, 32	(20.8)
10-somite	(14h)	13, 19, 22, 24, 24	(20.4)
12-somite	(15h)	11, 12, 14, 22, 25, 34	(19.7)
14-somite	(16h)	11, 13, 13, 14, 15, 19	(14.2)
18-somite	(18h)	15, 24, 28, 37	(26)
21-somite	(19.5h)	13, 14, 16, 19, 26, 26	(19)
hatch	(48h)	31, 42, 53, 25, 29, 35	(35.83)
6 days	(144h)	21, 34, 37, 39, 103, 33, 29, 106, 41	(49.22)

* The stages at which mitotic divisions were observed.

could be traced throughout embryogenesis in zebrafish (Yoon *et al.*,1997; Knaut *et al.*, 2000). Maternal *vasa* mRNA segregation changes from asymmetric to symmetric at the sphere stage (4 hpf), and zygotic *vasa* transcription is initiated shortly after the loss of unequal *vasa* mRNA segregation (Knaut *et al.*, 2000). Thereafter, proliferation of PGCs and perinuclear localization of Vasa protein are observed. The PGCs described by Knaut *et al.* (2000) had a location and proliferation corresponding to those of the presently observed PL-cells containing eosinophilic granules. This suggests that these PL-cells corresponded to PGCs or presumptive PGCs (pPGCs).

We observed granules that stained deeply with eosin in zebrafish PGCs. In medaka, rosy barb and goldfish, a few eosinophilic granules have been observed in the cytoplasm adjacent to nuclei of PGCs (Gamo, 1961; Timmermans and Taverne, 1989; Kazama-Wakabayashi *et al.*, 1999). Especially in rosy barb, perinuclear dense bodies in PGCs corresponded with “nuage”, germ line-specific materials that were detected ultrastructually, and were clearly visible after staining with eosin and gallocyanin, indicating the presence of protein and RNA

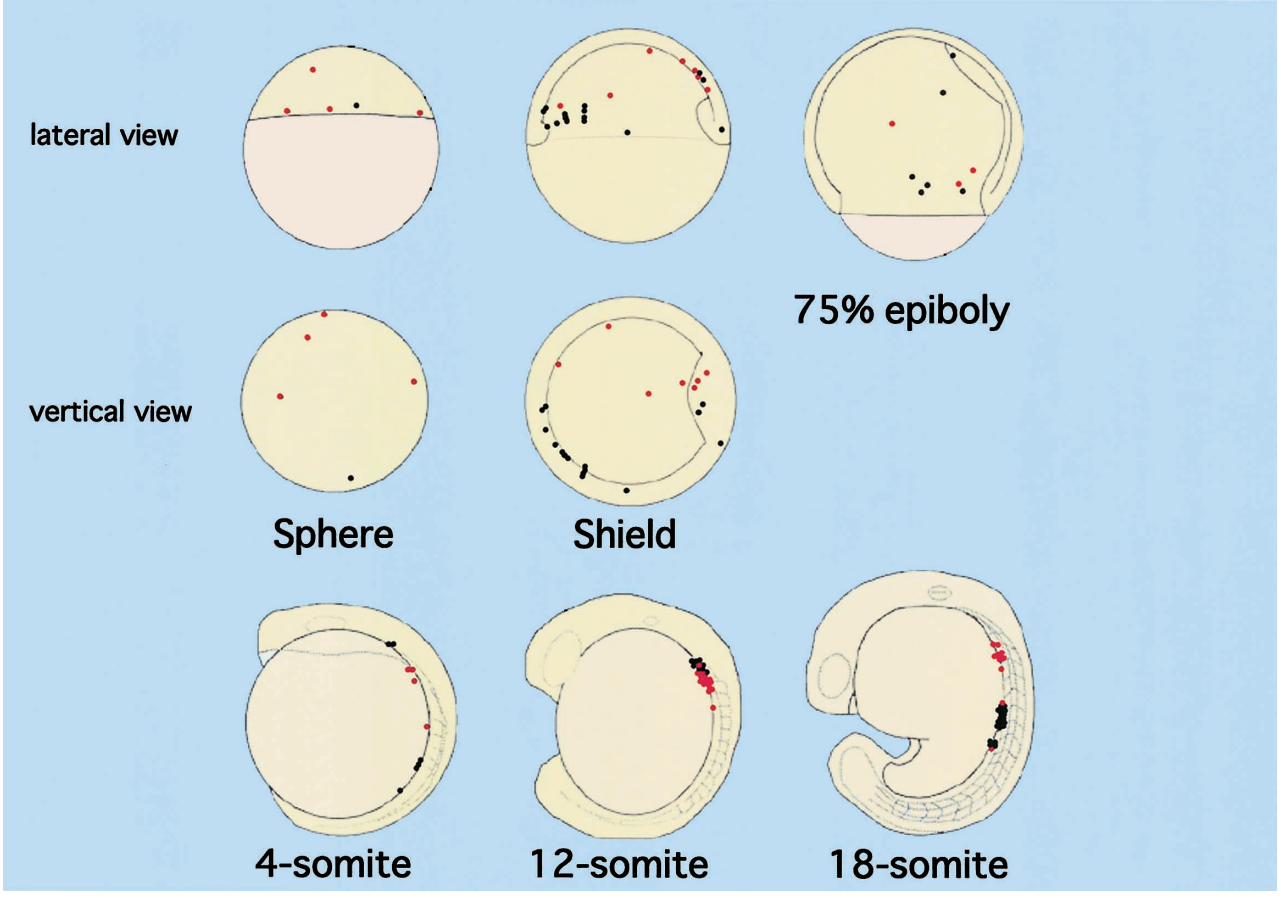


Fig. 4. Schematic distribution of PGCs with eosinophilic granules in zebrafish embryos as presumed from histological observations. The stages and views are indicated below each drawing. In the side views, red dots and black dots indicate the right and left of the notochord, respectively. In sphere-stage (4 hpf) embryos, almost all PGCs were located in the lower part of the blastoderm, and very few were located in the upper part of the blastoderm. In the top views, each PGC is distributed to some degree of distance from the others. The number of PGCs ranged from 0 to 7. In shield-stage embryos, the number of PGCs clearly increased. Many PGCs were located adjacent to others. Mitosis continued twice or three times during gastrulation, and the number of PGCs increased to 20–30. In 4-somite embryos, PGCs were located along the embryonic body. PGCs clustered completely at the level of the first- to third-somite at the 12-somite stage, and thereafter migrated to the posterior at the 18-somite stage.

(Timmermans and Taverne, 1989; Gevers *et al*., 1992b). Therefore, eosinophilic granules should be one of the characteristics used to identify PGCs in teleosts.

Eosinophilic granules changed their morphology and location in PL-cells and PGCs during development, which was roughly classified into three phases (Fig. 5) as follows: (1) large and small patches were distributed in the cytoplasm of PL-cells at the late-blastula stage; (2) smaller particles formed a cap or ring in the cytoplasm or perinuclear region of PGCs from the gastrula stage to the early segmentation period; and (3) small particles were observed in a juxtanuclear and inner part of the nuclei position in PGCs from the late segmentation period to the hatching period. These morphological profiles of eosinophilic granules were similar to the confocal profiles of Vasa protein that were determined by Knaut *et al*. (2000) using whole-mount staining. In particular, Vasa protein was detected at the sphere stage when eosinophilic granules were identified for the first time. The perinuclear distribution of eosinophilic granules corresponded to that of Vasa protein (Knaut *et al*., 2000). In *Xenopus*, germ plasm also shows similar morphological profiles: moving to a perinuclear region, forming a cap or ring in the late blastula or early gastrula, and inheritance by both daughter cells in 2–3 mitotic divisions (Whitington and Dixon, 1975; Dziadek and Dixon, 1975, 1977). It is tempting to speculate that eosinophilic granules might be fish germ plasm and contain Vasa protein. However, Braat *et al*. (2000) reported that Vasa protein localizes in many blastomeres at the early cleavage stage. In the future, it will be necessary to analyze the histochemical characters of eosinophilic granules.

During gastrulation, three kinds of alterations occurred in PL-cells; namely, the shift of eosinophilic granules to a perinuclear location, affinity for hemetoxylin in the nucleus, and clonal divisions, in which eosinophilic granules were inherited by each daughter cell. Knaut *et al*. (2000) reported that zygotic *vasa* RNA transcription is initiated after the sphere stage and that proliferation of PGCs occurs during gastrulation. In rosy barb, proliferation of pPGCs was observed at a similar stage (Timmermans and Taverne, 1989). These results suggest that segregation of PGCs in zebrafish occurs during gastrulation and that the shift of eosinophilic granules plays an important role in differentiation from pPGCs to PGCs. In *Xenopus*, Whitington and Dixon (1975) suggested that the shift of germ plasm to the perinuclear region triggered both an equal distribution of germ plasm at mitosis and an increase in the number of pPGCs. Dixon (1994) proposed that germ plasm play a role in protecting germ cells during the processes specializing the adult body form. In zebrafish, specific cell lineages arise at the gastrula stage (Kimmel and Warga, 1986; Kimmel *et al*., 1990; Warga and Kimmel, 1990). In our histological observations, the hematoxylin-stainability of the nuclei of PL-cells and other somatic cells was equivalent at the sphere stage, but the nuclei of PL-cells exhibited fainter staining during gastrulation. This would indicated the PGCs would be segregated from somatic cells during gastrulation. According to Dixon's proposal, the eosinophilic granules in zebrafish might play a role in protecting the totipotency or pluripotency of PGCs. In *Xenopus*, on the other hand, without a proper environment, cells containing germ plasm differentiate to somatic cells even if they are germ line cells (Wylie *et al*., 1985; Ikenishi and Tsuzaki, 1988). However, the role or function of eosinophilic granules in zebrafish remains to be determined.

The present results suggest that the mode of germ cell formation in zebrafish is close to those in *Drosophila* and *Xenopus*; that is, it occurs predeterminally. Gevers *et al*. (1992a)

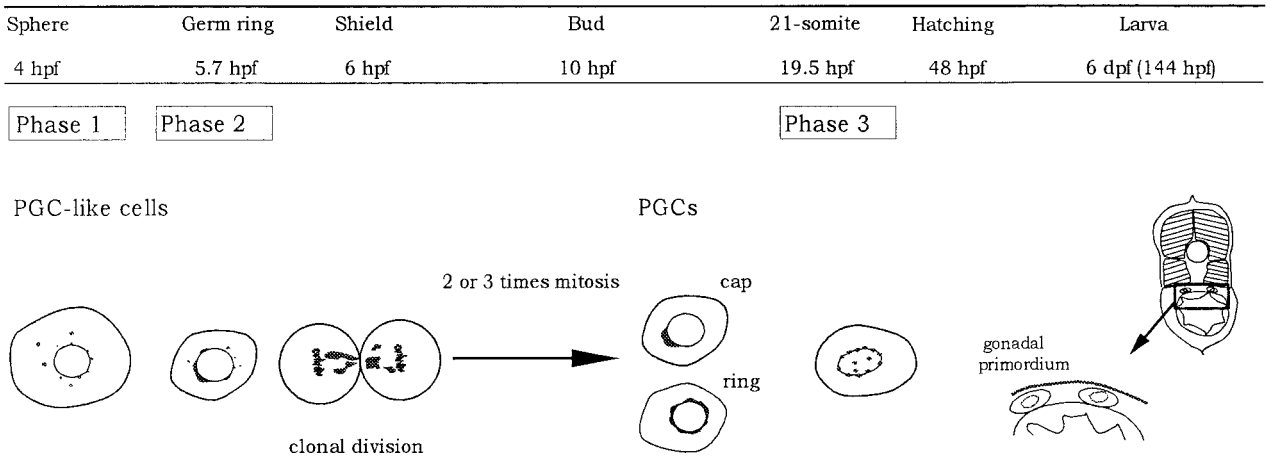


Fig. 5. Schematic illustration of morphological changes in eosinophilic granules in PGCs.
Phase 1. Eosinophilic granules distributed in the cytoplasm as large and small patches.
Phase 2. Eosinophilic granules distributed in the cytoplasm or perinuclear region as a smaller patch forming a cap or ring.
Phase 3. Eosinophilic granules distributed to a juxtanuclear position and inner part of the nuclei of PGCs as small particles.

used cell lineage analysis to demonstrate that PGCs in rosy barb originate from lower tiers of blastomeres at the 64-cell stage. In goldfish, microsurgical analysis showed that PGC-bearing cells were predetermined by cytoplasmic factors and already distributed in the lower part of the blastoderm at the mid-blastula stage, after which they could not be regenerated (Kazama-Wakabayashi *et al*., 1999). These results would also agree with the hypothesized mechanism in zebrafish, and would imply that fish species might share a common mechanism of germ cell formation. However, conflicting data have been reported in medaka: *vasa*-positive cells could not be traced to before the late-gastrula stage (Shinomiya *et al*., 2000). Therefore, it is not clear whether the germ line is predeterminant. With respect to germ plasm, the question might be resolved by means of transplantation techniques and so forth.

In conclusion, zebrafish PGCs were light-microscopically identified by the presence of eosinophilic granules as in other teleost species, and then were traced back to the sphere stage. It seemed that blastomeres containing eosinophilic granules could be set aside from specification of somatic cell lineage, because of their faint staining with hematoxylin. The germ plasm of *Drosophila* must contain a combination of factors controlling both the cellular behavior of the early germ cells as well as the activation of germ cell-specific gene expression. Eosinophilic granules are one of the candidates for controlling germ cell specification in fish. Detailed biochemical analysis of the granules in relation to *vasa* RNA and Vasa protein is needed to confirm our conclusions.

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