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The Germ Cell Lineage Identified by vas-mRNA during the Embryogenesis in Goldfish

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ABSTRACT—vas RNA has been identified in germ-line cells and its precursors in zebrafish, with the result that the germ-line lineage can be traced throughout embryogenesis. In the present study, we described vas localization and the migration of vas-positive cells in goldfish, using whole mount in situ hybridization. The signals of vas mRNA localization appeared at the marginal part of the first to third cleavage planes. The eight signals were detected during the period from the 8-cell to the 512-cell stage. At the late-blastula stage, additional numbers of vas-positive cells were observed, suggesting the proliferation of these cells. At the segmentation period, vas-positive cells showed a long extended distribution along the embryonic axis, but did not form any clusters. vas-positive cells were occasionally distributed at the head region, especially around the future otic vesicle. These signals were inherited to the primordial germ cells, suggesting that vas-positive cells were primordial germ cells (PGCs) in goldfish.

Key words: goldfish, PGCs, primordial germ cell, vas, zebrafish

INTRODUCTION

Primordial germ cells (PGCs) play a unique role in providing a continuity of life from generation to generation. In most animals, PGCs are set apart early during development and migrate to the gonadal anlage later in development (Gilbert, 2000). In the species in which the migration of PGCs has been studied, PGCs are identified during the early stages by peculiar elements, such as germ plasm (or pole plasm in Drosophila) (reviewed in Ikenishi, 1998) or the enzymatic activity of alkaline phosphatase (Mintz and Russell, 1957). Recently, a gene homologue related to Drosophila vasa has been introduced as a specific marker of the germ line cells in rats (Komiya and Tanigawa, 1995), in mice (Fujiwara et al., 1994), chick (Tsunekawa et al., 2000) and Xenopus (Komiya et al., 1994; Ikenishi and Tanaka, 1997). These results suggest that vasa homologue is useful as a germ line specific marker in vertebrates.

In teleost, recent studies on vasa (a Drosophila vasa homologue) mRNA have been successfully identified in germ cell precursors in zebrafish (Yoon et al., 1997; Olsen et al., 1997; Weindinger et al., 1999), medaka (Shinomiya et al., 2000), and rainbow trout (Yoshizaki et al., 2000). In zebrafish, PGC’s progenitors containing vasa mRNA localized at the lower border of the blastoderm at the blastula stage, migrated to the dorsal side with convergence movements, and formed two clusters at the level of the first somite during the early segmentation stage (Yoon et al., 1997; Weindinger et al., 1999). In medaka, blastomeres expressing olvas (Oryzias latipes vasa homologue) were first detected in the posterior one-third of the embryonic shield, but were not observed before the late-gastrula stage (Shinomiya et al., 2000). Blastomeres containing olvas mRNA moved in the embryonic body at the early neurula stage and were then lined along the anterior-posterior axis on both sides of the embryonic body by the 4-somite stage (Shinomiya et al., 2000). In rainbow trout, vasa expressing cells were detected only after the eyed stage (80 somite) (Yoshizaki et al., 2000). These results show that the location of vasa mRNA is not always the origin of PGCs in all fish species. They also suggest that PGCs containing vasa mRNA show different distributions and migration routes to the...
gonadal anlage during embryogenesis in each species.

In goldfish, *Carassius auratus*, PGCs have been identified only histologically (Kazama-Wakabayashi et al., 1999). Goldfish PGCs were first detected widely in the region from head to trunk along the embryonic axis at 30 h post-fertilization (hpf) (7–9 somite stage). Although PGCs were not histologically detected before 30 hpf, micro-surgical experiments suggest that potential PGCs are located at the lower part of blastoderm at the blastula stage (Kazama-Wakabayashi et al., 1999). In the present study, we identified the distribution of *vas* mRNA in goldfish by *in situ* hybridization in order to elucidate the location of PGCs during the early embryogenesis and to compare PGCs migration with other species of teleost.

**MATERIALS AND METHODS**

**Fertilization and Dechorionation**

Goldfish, *Carassius auratus*, were reared in the Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University. Ovulation was induced by hormonal injection and artificial insemination and fertilization were performed as previously described (Yamaha et al., 1998). Briefly described, inseminated eggs were fertilized in the fertilization solution, containing 0.2% urea and 0.25% NaCl in tap water. Dechorionation was carried out before blastodisc formation using a method slightly modified from an established procedure (Yamaha et al., 1986). Fertilized eggs were dechorionated with 0.1% Trypsin (DIFCO) and 0.4% urea in Ringer’s solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM CaCl₂). Embryos were cultured in Ringer’s solution containing 1.6% albumen at 20°C. Developmental stages of goldfish before 15 hpf were determined according to Yamaha et al. (1999), and after 15 hpf according to Kajishima (1960).

**Whole-mount *in situ* hybridization analyses**

Dechorionated embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for overnight at 4°C at every stage during development. Fixed embryos were stored in 100% methanol at –20°C. Two-color *in situ* hybridization was performed as described by Jowett and Lettice (1994) with slight modifications using a 0.4Kb fragment from the 3’-UTR region of *vas* cDNA in zebrafish as a template. *no tail* (*ntl*) in zebrafish and *myoD* in goldfish were used for the marker gene. After *in vitro* transcription, the RNA probe was purified using ProbeQuant G-50 Micro columns (amasham pharmacia biotech). Proteinase K treatment was performed for 10 min at 10 µg/ml for early stage embryos, for 20 min at 10 µg/ml for somite-stage embryos, and 20 min at 20 µg/ml for 4 day larvae. For double *in situ* hybridization, RNA probes were labeled with digoxigenin for *vas* and *myoD*, and labeled with Fluorescein Isothiocyanate (FITC) for *ntl*. Hybridized temperature was at 55°C.

After staining, embryos were cleared in 50% glycerol, and mounted on a dimple of an agarose plate. Photographs were taken.

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Fig. 1. Whole-mount *in situ* hybridization from the early cleavage to the late blastula stage. Embryos were hybridized with a *vas* (purple: a-h) or *vas* and *ntl* (brown: i) riboprobe. Each panel shows a side view of embryos. Arrows indicate *vas* signals. (a) 2-cell stage; a dense signal is detected on the marginal part of the cleavage plane, though the entire cytoplasm of blastodisk is stained faintly. (b) 4-cell and (c) 8-cell stage; *vas* signals are clearly visible on the marginal parts of the cleavage planes. (d) 16-cell stage; *vas* signals are localized on the 1st, 2nd, and 3rd cleavage planes, but not on the 4th cleavage planes (arrowheads). (e) 32-cell stage; (f, g) 256-cell stage; *vas* signals of the same embryo. Three signals are detected on one side (f), and single signals on the other side (g). (h) 512-cell stage; three and single signals are observed. (i) late-blastula; plural *vas* signals are detected on every cluster. Almost all signals distribute in the marginal part, expressing *ntl*.
using an Olympus CAMEDIA imaging system DS3030U equipped with a SZX-12 stereomicroscope. For histological observation, stained embryos were refixed with 4% paraformaldehyde and 0.25% glutaraldehyde in PBS, and embedded in a paraffin block. Photographs were taken using an Olympus CAMEDIA C3040 equipped with a BH-2 microscope.

RESULTS

In goldfish, localization of vas mRNA was identified during development by using a vas probe from zebrafish. The maternal vas message was not localized in freshly fertilized

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**Table 1.** The frequency of embryos with vas-expressing cells located out of blastoderm margin

<table>
<thead>
<tr>
<th>Stage</th>
<th>N</th>
<th>Normal (%) (In mesodermal region)</th>
<th>Ectopic (%) (Out of mesodermal region)</th>
<th>Undistinguishable</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hpf (mid blastula)</td>
<td>31</td>
<td>29 (94)</td>
<td>2 (6)</td>
<td>0</td>
</tr>
<tr>
<td>8 hpf (late blastula)</td>
<td>42</td>
<td>24 (57)</td>
<td>16 (38)</td>
<td>2</td>
</tr>
<tr>
<td>10 hpf</td>
<td>85</td>
<td>31 (36)</td>
<td>48 (56)</td>
<td>6</td>
</tr>
<tr>
<td>12 hpf (30% epiboly)</td>
<td>30</td>
<td>11 (37)</td>
<td>16 (53)</td>
<td>3</td>
</tr>
<tr>
<td>14 hpf (50% epiboly)</td>
<td>31</td>
<td>13 (42)</td>
<td>16 (52)</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig. 3. Location of vas-positive cells after segmentation period. Arrows indicate vas signals. (a, b) 30 hpf (8–12 somite); vas-positive cells are located in both trunk and head (red arrow) region. Higher magnification of vas signals in head region of (a). (d) 36 hpf (18-somite). (d-f) An embryo at 12-somite stage, this sample is double-stained with vas and MyoD. vas-positive cells (purple dots) are distributed from 1–2 somite (d) to the end of the tail bud (f). (g, h) Transverse sections of in situ hybridized embryos at 30 hpf. nt, neural tube; no, notochord; s, somite; me, mesendoderm; y, yolk; ysl, yolk syncytial layer. Scale bar = 20µm. (i) 48 hpf, and (j) 72 hpf; vas-positive cells are detected in the dorsal to yolk extension.
eggs and the 1-cell stage (data not shown). After the first cleavage forming the 2-cell stage, two signals resembling small dark bars were observed along the cleavage furrow at opposite sides (Fig. 1a, with these observations schematically illustrated in Fig. 4a). As the embryo developed to the 4-cell stage, a total of four signals were observed along each furrow (Figs. 1b and 4b). At the 8-cell stage, four new signals formed along the two new cleavage furrows (Figs. 1c and 4c). At the 16-cell stage, however, new vas signals were not observed along the 4th cleavage furrow (Figs. 1d and 4d). At the 32-cell stage, the shape of the eight vas signals changed from the line to the point (Fig. 1e). At the 256-cell stage, almost all the eight vas signals were located where left from the cleavage plane (Fig. 1f: arrow). At 512-cell (mid-blastula) stage, the eight vas signals were observed in each cells (Fig. 1h). The eight vas signals were detected during the period from the 8- to 512-cell stage (Figs. 1c-h, Fig. 4c-f). They were usually in four separate groups; each of two groups consisted of three points, and each of the others comprised only a single point (Figs. 1e-g and 4e-f). After 8 hpf (late-blastula), additional numbers of vas-positive cells were observed in each groups (Fig. 1h, Fig. 4g).

Almost all the vas-positive cells were detected in the lower part of the blastoderm, especially in the mesendodermal region detected by no tail (ntl) as a marker (Fig. 2a-e), but a few signals were occasionally observed at the upper or middle part of the blastoderm (Fig. 2c-f: red arrows). The frequency of embryos with vas-positive cells out of the ntl region was increased with development, from 8 hpf (late blastula stage) through 10 hpf (dome stage) (Table 1).

During gastrulation, vas-positive cells appear to move towards the dorsal side of the embryo with the convergence movement and many of these cells were seen still to lie in the ntl expressing region (Figs. 2e-g and 4j). At the 70–90% epiboly stage, vas-positive cells located on the dorsal side were observed to leave from the marginal part and migrate to the animal side along the axis (Fig. 2f). At the 100% epiboly stage, vas-positive cells showed wide distribution along the paraxial mesoderm. These cells were usually observed on the trunk region of the embryonic body (Figs. 2h and 4k), however, they were sometimes observed on the anterior side, the head region of embryonic axis (Fig. 2i: red arrow).

During the segmentation period, the vas-positive cells still showed a long extended distribution along the axis (Fig. 3a). Almost all of the cells were observed on the posterior side from the level of the first somite (Fig. 3d-f), but some of...
the cells showed anterior localization in the head region, especially on the future otic vesicle (Fig. 3a-c: red arrow). The frequency of the embryos with vas-positive cells in this position at the 8- to 18-somite stage was 18% (32/175). Transverse sections of in situ hybridized embryos at 30 hpf showed that the vas-positive cells located at the lateral to the somite (Fig. 3g), and in the mesendodermal region (Fig. 3h). By 48 hpf, the vas-positive cells were located between the embryonic body and the yolk extension (Fig. 3i). vas-positive cells still showed the wide distribution along the yolk tube at 72 hpf (Fig. 3j).

**DISCUSSION**

**vas-positive cells in goldfish**

In the present study, we used vas RNA probes made from zebrafish cDNA for PGCs marker in goldfish. The distribution of cells in goldfish detected by zebrafish vas RNA probes was similar to these of PGCs reported by histological observation after 30 hpf (Kazama-Wakabayashi et al., 1999). Both goldfish and zebrafish belong to the same carp family, Cyprinidae. Some probes, such as goosecoid and no tail, from zebrafish cDNA are also useful in the goldfish (Yamaha et al., 1999). Transverse sections showed that vas-positive cells were located at the similar positions as morphologically identified PGCs (Kazama-Wakabayashi et al., 1999). Therefore, we conclude that the vas-positive cells in this study were goldfish PGCs.

**vas signals location at the cleavage stage**

In this study, strong signals of vas mRNA were observed at the marginal part of the first to third cleavage furrows. In zebrafish, it has been reported that accumulations of maternal vas mRNA were occasionally detected at eight points of the marginal parts (Yoon et al., 1997), as in the present study. In zebrafish, occasional signals at the third furrows are weaker than those at the first and second furrows, and become undetectable at later stages (Yoon et al., 1997). Maternal vas mRNA was localized around the blastodisc at the 1-cell stage (Braat et al., 1999; Howley and Ho, 2000). The correlations between the microtubule array of cleavage furrows and vas mRNA localization have been reported in zebrafish nebel mutant and normal embryos (Peleguri et al., 1999). Therefore, the accumulation of vas mRNA might depend on the total amount and/or structure of the third cleavage furrows. In goldfish, since all the eight signals were maintained at least until the 512-cell stage and almost all signals inherited to PGCs, the amounts of vas mRNA might be necessary for maintenance of the accumulation and/or PGC differentiation. More detailed study is required to confirm the mechanism and role of vas mRNA accumulation.

**vas-positive cells proliferation**

In goldfish, the number of vas-positive cells increased at 8 hpf (late-blastula, about 4k cells). After the proliferation of vas-positive cells, these cells became easily detectable. In zebrafish, vas-positive cells proliferate at the dome stage (late-blastula stage: Yoon et al., 1997). Zygotic gene expression is thought to begin after mid-blastula transition (MBT: Newport and Kirshner, 1982ab; Kane and Kimmel, 1993). Analysis of hybrid fish with two vas genetic types demonstrates that zygotic vas expression is first detectable at the late sphere stage (Knaut et al., 2000). It has been reported that MBT occurred at approximately 3 hpf in zebrafish (Kane and Kimmel, 1993) and 6 hpf in goldfish (Yamaha et al., 1999). These results suggest that the proliferation of vas-positive cells should be influenced by zygotic factors.

vas-positive cells at 8 hpf (late-blastula) are PGCs, according to the terminology established by Nieuwoorko and Sutasurya (1979), because these cells segregate from somatic lineage and multiply vas-positive cells.

**Appearance of vas-positive cells on the upper part of the blastoderm**

In the present study, the frequency of embryos with vas-positive cells located in the outside region from the blastoderm margin increased after 8 hpf. Since the surgical elimination of the upper part of the blastoderm did not decrease the number of PGCs at gonadal anlage in goldfish (Kazama-Wakabayashi et al., 1999), new vas-positive cells hardly emerge in the upper part of the blastoderm. In the zebrafish blastula and early gastrula, PGCs are associated with the blastoderm margin and subsequently remain in the vicinity of the germ ring (Yoon et al., 1997). These differences regarding the location of vas-positive cells between the two species might owe to cell movement before epiboly. Goldfish blastomeres show active movement and wide mixing before gastrulation (Yamaha et al., 1999, 1997). In zebrafish, cell mixing was reported only in the stages after late-blastula with epiboly movement (Kimmel and Warga, 1988). Marginal blastomeres are hardly mixed after the 64-cell stage (Wilson et al., 1995). These results suggest that cell mixing in the blastoderm might mingle vas-positive cells to the upper part of the blastoderm in goldfish.

In teleost fish, mesoderm induction occurs in the marginal part of the blastoderm (Mizuno et al., 1996). The fate of PGCs might be related to their original position at the blastoderm margin (Yoon et al., 1997). But, our observation suggests that PGCs are not regulated to stay on the marginal region. Transplantation experiments have shown that presumptive PGCs transplanted to the upper part of the blastoderm migrated to the gonadal anlage and changed to germ cells (Kazama-Wakabayashi et al., 1999; Yamaha et al., 2001). Therefore, PGCs located outside of the marginal region may still have a potential to differentiate germ cells.

**vas-positive cells location during gastrulation**

In zebrafish, PGCs migrate from the blastoderm margin to dorsal side along the boundary between the trunk and head paraxial mesoderm during epiboly, and form two lat-
eral clusters at the 1st to the 3rd somite level as zebrafish (Yoon et al., 1997; Weidinger et al., 1999; Braat et al., 1999). In goldfish, vas-positive cells were frequently observed in the trunk and head paraxial mesoderm and did not form clusters. It is also peculiar that the vas expression was very frequently observed at the head region, especially around the future otic vesicle. Histological observation also reveals PGCs at this position (Kazama-Wakabayashi et al., 1999), so these vas-expressing cells might be PGCs. In zebrafish, PGCs located in such an ectopic position have been rarely observed (18% of embryos in goldfish versus 1.3% in zebrafish). Abnormal alignment of PGCs has been observed in spadetail (spt) mutants (Weidinger et al., 1999), in which the convergence-extension movement was reduced at the early stage of gastrulation (Ho and Kane, 1990). The frequency of embryos with ectopic PGCs in zebrafish spt mutant (16%; Weidinger et al., 1999) is similar to that in goldfish.

In conclusion, the migration route of goldfish PGCs before segmentation still remains unclear. Weidinger et al. (1999) proposed in zebrafish that PGCs migration are regulated by mesodermal movement during gastrulation. However, the present results about PGCs localization, such as out of the germ ring at the late-blastula stage, among the paraxial mesoderm during epiboly, and in the head during the segmentation period indicate that goldfish PGCs would prefer to migrate independently on mesodermal patterning. The detailed interaction between PGCs migration and convergence-extension movement in mesoderm during gastrulation remains to be investigated.

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