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Authors: Otani, Satoshi, Maegawa, Shingo, Inoue, Kunio, Arai, Katsutoshi, and Yamaha, Etsuro

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Satoshi Otani[1, 3]*, Shingo Maegawa[2], Kunio Inoue[2], Katsutoshi Arai[1]
and Etsuro Yamaha[3]

[1]*Laboratory of Breeding Science, Graduate School of Fisheries Sciences, Hokkaido University,
Hakodate, 041-8611, Japan*
[2]*Molecular and Developmental Biology, Graduate School of Biological Sciences,
Nara Institute of Science and Technology, Ikoma 630-0101, Japan*
[3]*Nanae Fresh Water Laboratory, Field Science Center for Northern Biosphere,
Hokkaido University, Nanae, 041-1105, Japan*

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- cells 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 *vas* (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 *vas* 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, *vas* expressing cells were detected only after the eyed stage (80 somite) (Yoshizaki *et al*., 2000). These results show that the location of *vas* mRNA is not always the origin of PGCs in all fish species. They also suggest that PGCs containing *vas* mRNA show different distributions and migration routes to the

* Corresponding author: Tel.   +81-138-65-2344;
FAX. +81-138-65-2237.
E-mail: otani@fish.hokudai.ac.jp

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 car-

ried 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 CaCl$_2$). 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
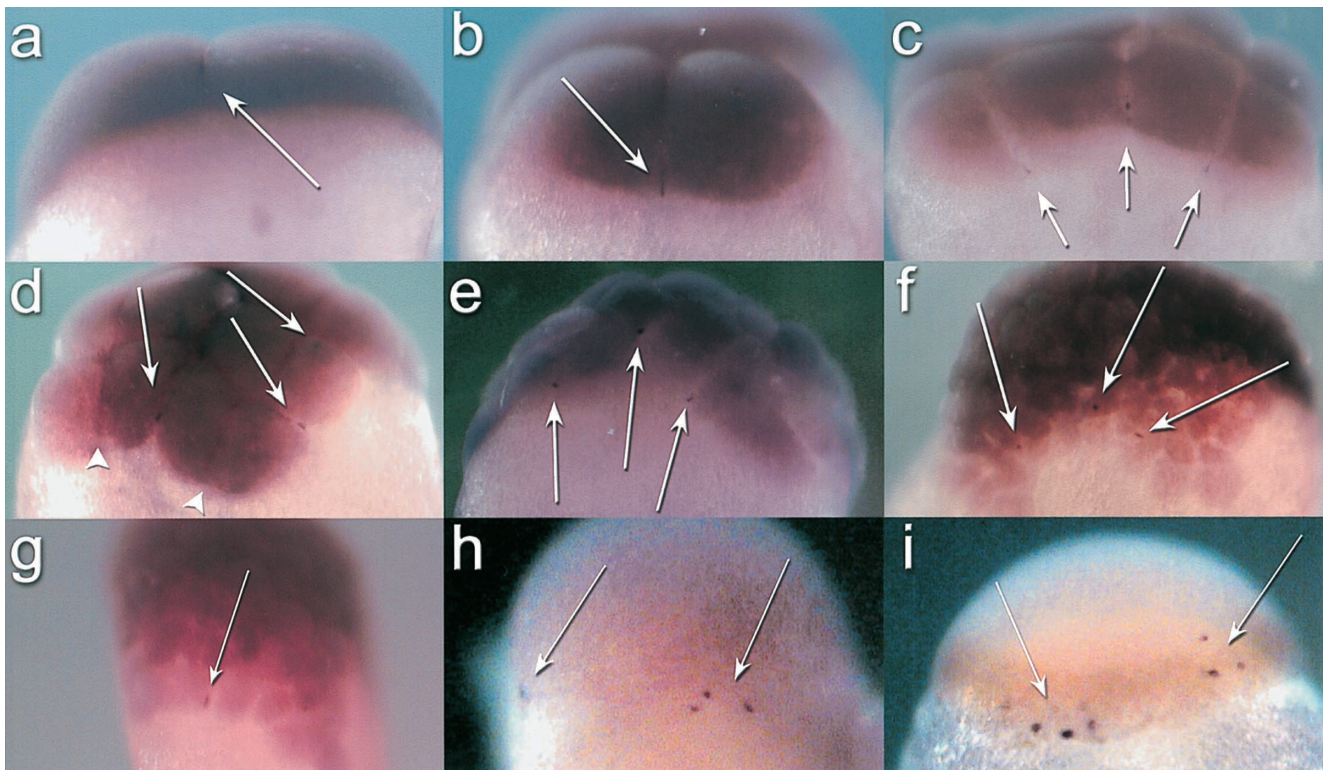

Fig. 1. Whole-mount *in situ* hybridization from the early cleavage to the late blastula stage. Embryos were hybridized with a *vas* (purple: a-h) or *vas* and *ntl* (brown: i) riboprobe. Each panel shows a side view of embryos. Arrows indicate *vas* signals. (a) 2-cell stage; a dense signal is detected 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
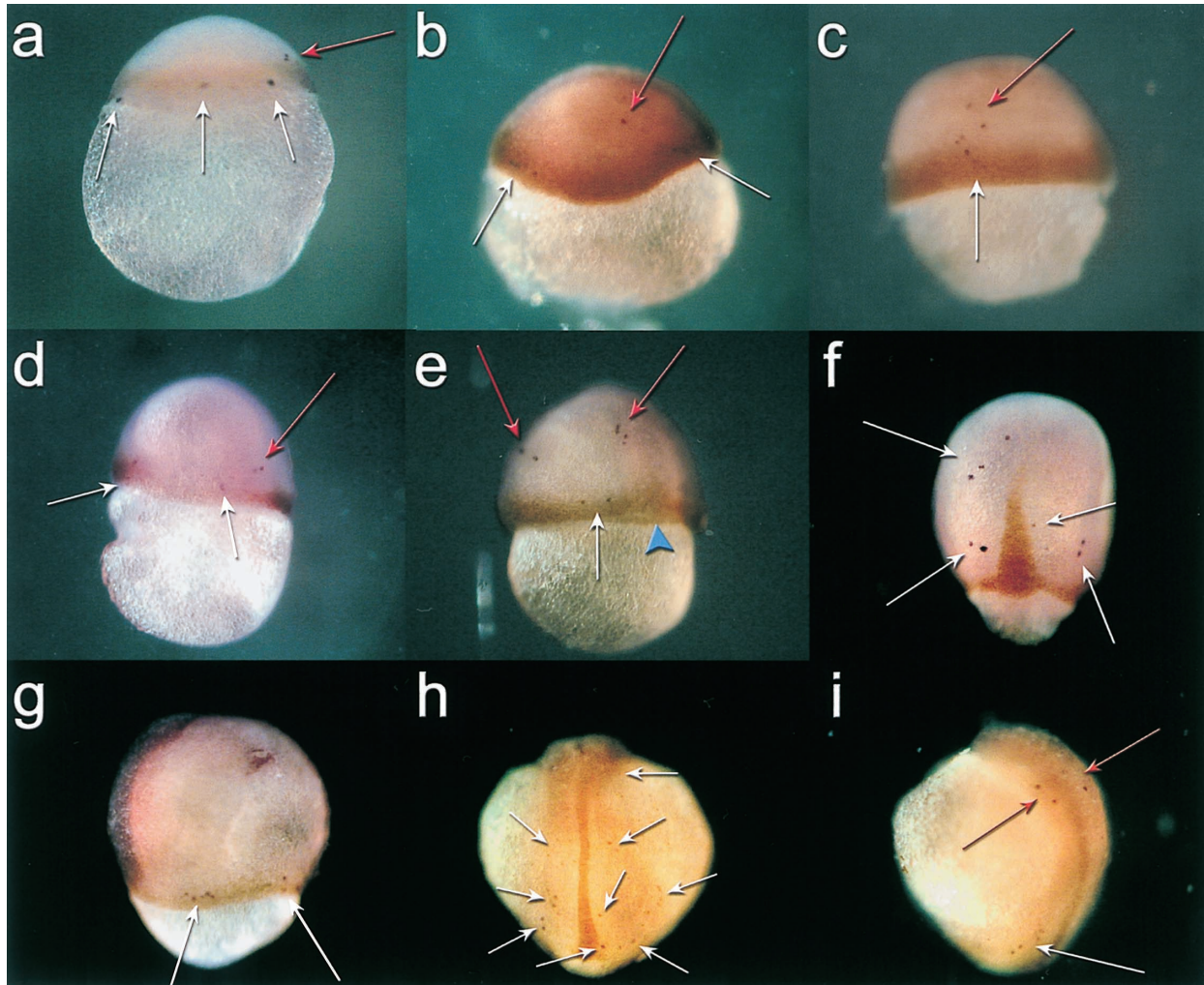

Fig. 2. Whole-mount *in situ* hybridization on embryos during gastrulation. The *vas*-positive cells are stained with dark purple (indicated with arrows) and *ntl*-expressing domain are labeled in light brown. (a) late-blastula. (b) dome. (c) 30% epiboly. (d) 50% epiboly. Many *vas*-positive cells are located in the *ntl*-expressing region (white arrow), but some cells are located outside of this region (red arrow). (e) shield; *vas*-positive cells are located on the upper part of the blastoderm (red arrow). Some cells are located above the embryonic shield (blue arrowhead). (f) 70% epiboly (dorsal view); *vas*-positive cells show wide distribution along the axis. (g) 70% epiboly (lateral view, different embryo from f); *vas*-positive cells are observed in the *ntl*-expressing region. (h) 100% epiboly (dorsal view). (i) 100% epiboly (lateral view; the same embryo with (h)); almost all *vas*-positive cells are located on the trunk region (h) but some cells are located at the head region (red arrow).

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

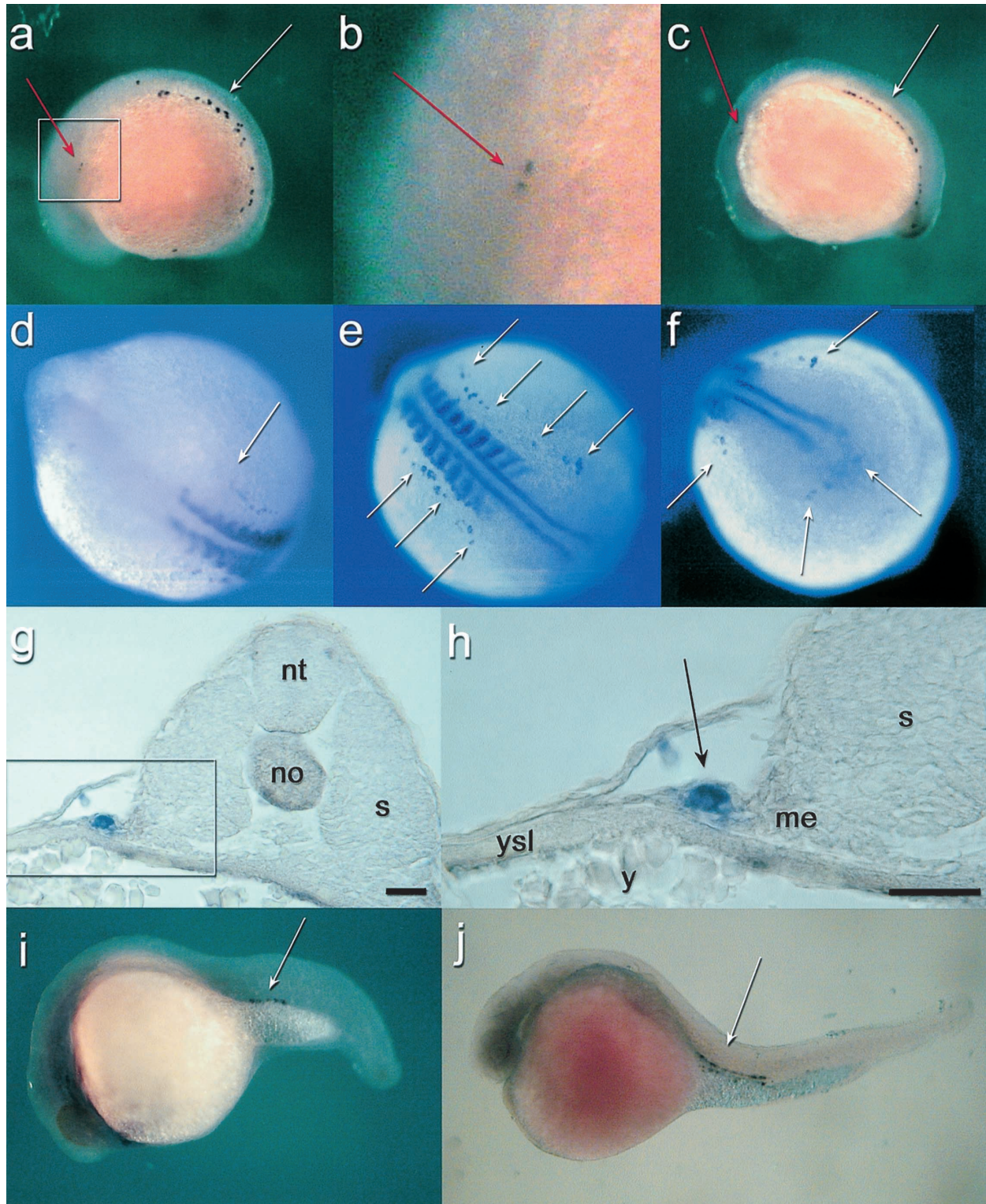| stage	N	Normal (%) (In mesodermal region)	Ectopic(%) (Out of mesodermal region)	Undistinguishable
6 hpf (mid blastula)	31	29 (94)	2 (6)	0
8 hpf (late blastula)	42	24 (57)	16 (38)	2
10 hpf	85	31 (36)	48 (56)	6
12 hpf (30% epiboly)	30	11 (37)	16 (53)	3
14 hpf (50% epiboly)	31	13 (42)	16 (52)	2

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 siganals 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
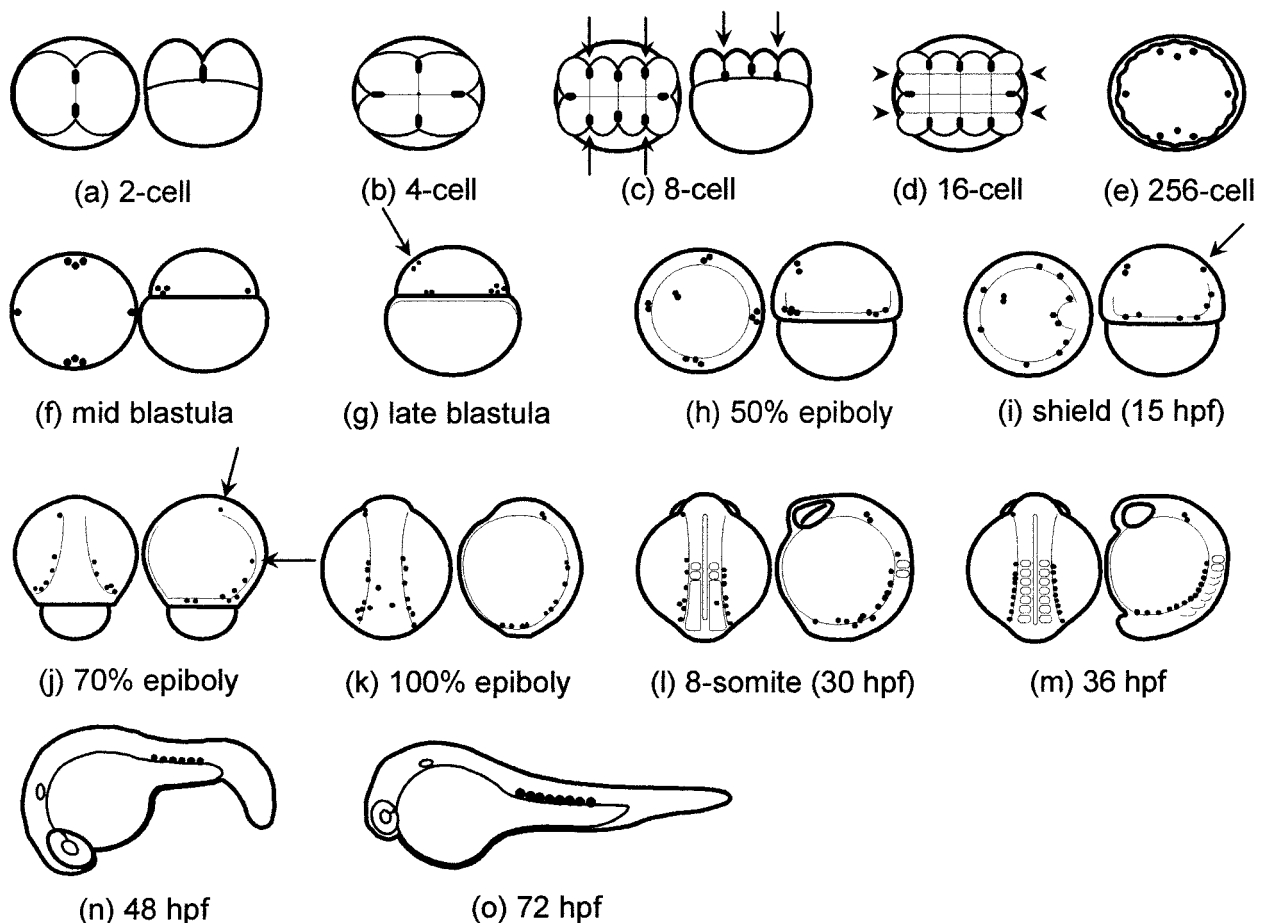

(a) 2-cell   (b) 4-cell   (c) 8-cell   (d) 16-cell   (e) 256-cell

(f) mid blastula   (g) late blastula   (h) 50% epiboly   (i) shield (15 hpf)

(j) 70% epiboly   (k) 100% epiboly   (l) 8-somite (30 hpf)   (m) 36 hpf

(n) 48 hpf   (o) 72 hpf

Fig. 4. Schematic illustration of vas signal distribution during embryogenesis in goldfish. Black points indicate vas signals. Arrows indicate the unique vas location in goldfish. vas-expressions are not detectable at the fourth cleavage plane (arrowheads).

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). Transeverse 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 Nieuwkoop 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.*, 1997, 1999). 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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