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*Original Article*

# Cloning and pattern of expression of the shiro-uo *vasa* gene during embryogenesis and its roles in PGC development

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**ABSTRACT The *vasa* genes are expressed in the germ cell lineage in many organisms, but their expression patterns show large variations. Recent studies suggest that *vasa* transcripts are involved in germ cell lineage development. In this paper, we isolated the *vasa* cDNA clone from a teleost, shiro-uo, *Leucopsarion petersii* and examined its expression pattern during embryogenesis. Then, we examined the functional significance of *vasa* mRNA during the formation of primordial germ cells (PGCs). The amino acid sequence of shiro-uo VASA is 61.1% identical to that of zebrafish. In whole-mount *in situ* hybridization, *vasa* transcripts appeared at the 4- and 8-cell stages as four spots at both ends of two cleavage planes between the lower tier of blastomeres and the yolk cell mass. At the 16-cell stage, eight spots were observed. After the blastula stage, shiro-uo *vasa* transcripts showed similar localization as in the zeblafish. Ultrastructural analysis of 4-cell stage embryos revealed the presence of a subcellular organelle that resembled "nuage" in the germ cell lineage observed in the embryos of various organisms. We carried out micromanipulation of 4- or 8-cell stage embryos to remove the *vasa* mRNA-containig spots and then measured the number of the *vasa*-expressing PGCs in the genital ridge of the manipulated embryos. The numbers decreased when all of the four spots were removed, indicating that the *vasa*-containing spots at early cleavage stages have important functions in the development of PGCs.**

KEY WORDS: *Shiro-uo, vasa, primordial germ cell (PGC), mRNA localization, teleost*

## Introduction

The germ cell lineage is an important and specialized group of cells that possesses the ability to produce gametes and offspring. The morphology of PGCs in various fish is similar to that in other organisms (Braat *et al*., 1999). PGCs are morphologically distinct from somatic cells because of their large cell size, large nuclei and the presence of "nuage" in their cytoplasm (Mohowald, 1962; Czolowska, 1969; Wolf *et al*., 1983).

Molecular markers used for identification of PGCs have become useful research tools. The *Drosophila vasa* homolog gene has been identified in many other organisms, but its expression patterns during early embryogenesis shows large variations (Start-Gaiano and Lehmann, 2001). During embryonic development in zebrafish, *vasa* transcrips are localized to the distal parts of the first two cleavage furrows as condensed granules during cleavage and are eventually localized into four cells in the blastula. The number of *vasa* mRNA-positive cells remains at four until the late blastula stage. Then, zygotic *vasa* transcription is initiated in PGCs and these *vasa* mRNA-positive cells migrate to the gonads (Olsen *et al*., 1997: Yoon *et al*., 1997: Weidinger *et al*., 1999; Knaut *et al*., 2000). In medaka, however, *vasa* mRNA can be detected uniformly among the blastomeres until late gastrulation. Blastomeres with *vasa* transcripts move in the embryonic body at the early neurula stage and line up along the anterior-posterior axis on both sides of the embryonic body until the 4-somite stage (Shinomiya *et al*., 2000). For rainbow trout, *vasa* mRNA-positive cells are detectable later at the 80-somite stage (Yoshizaki *et al*., 2000).

It was also reported that *vasa* RNA is localized to subcellular

*Abbreviations used in this paper:* PGC, primordial germ cell.

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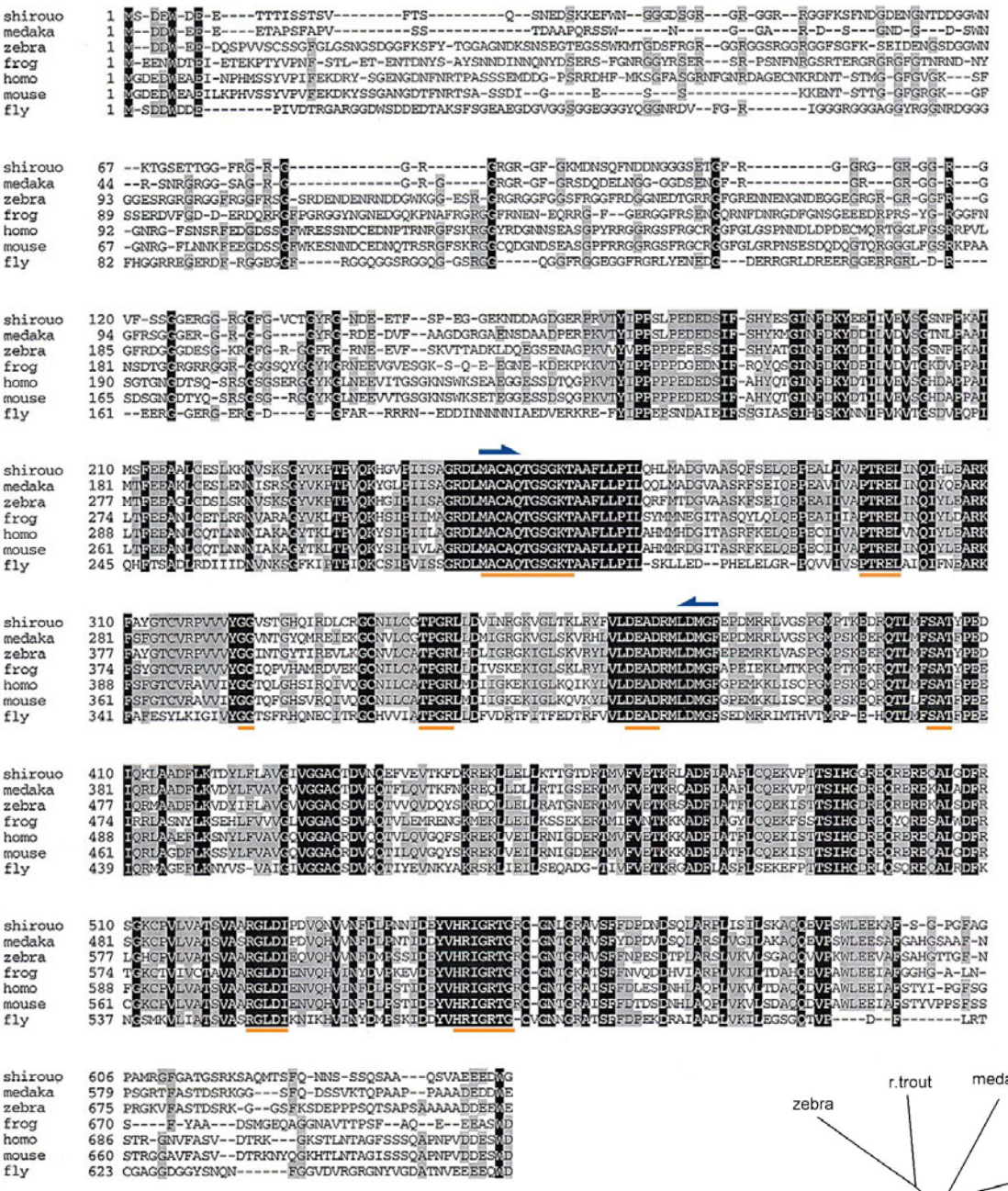
**A**



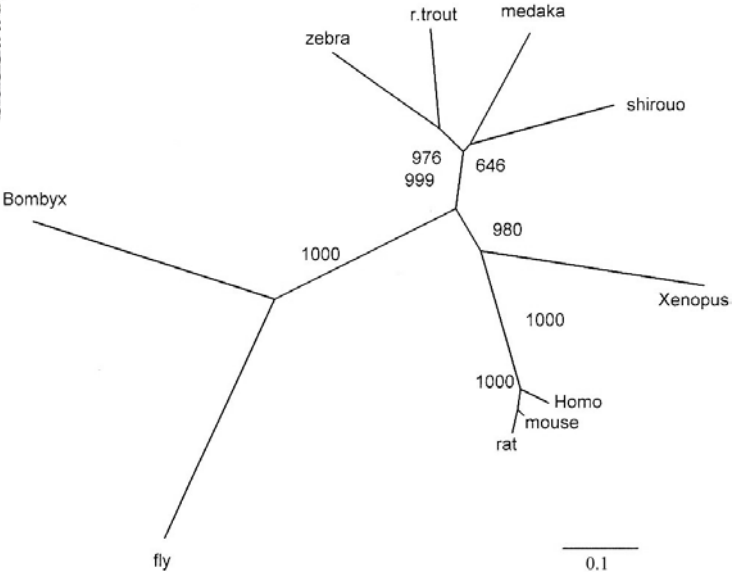




**Fig. 1. Sequence and phylogeny of the shiro-uo VASA protein. (A)** *The aligment of the shiro-uo VASA protein and the VASA proteins of other animals. The amino acid sequence is 69.7, 61.4, 52.1, 54.3, 55.9 and 45.2% identical to that of the medaka, zebrafish,* Xenopus *(frog),* Homo sapiens *(Homo), mouse and* Drosophila *(fly) VASA proteins, respectively. Orange lines indicate conserved regions. Arrows indicate the probe region used for whole-mount* in situ *hybridization.* **(B)** *Phylogeny of the VASA proteins. The numbers after the internal branches indicate the bootsrap value (value/1000) for each group.*

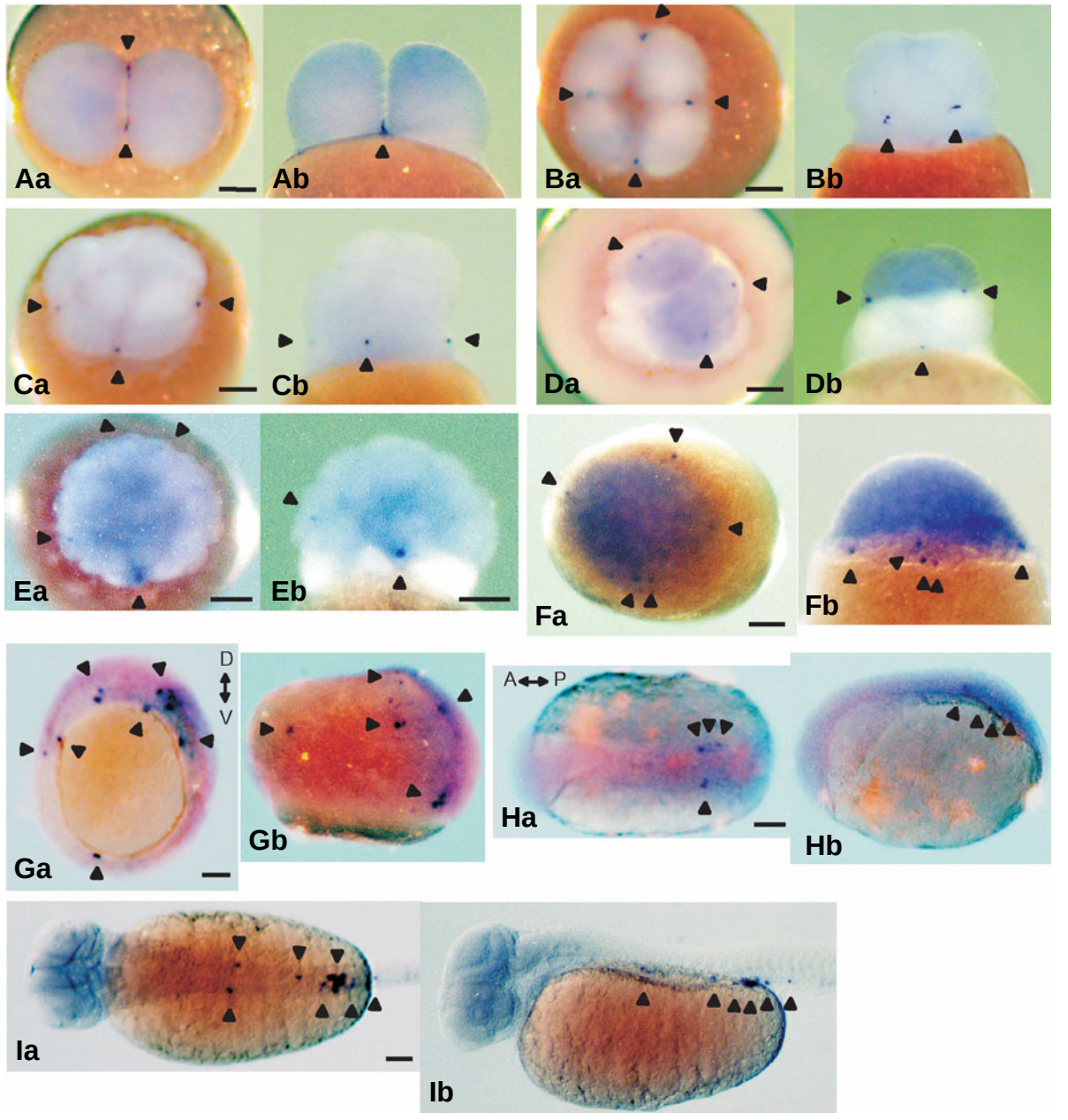



**Fig. 2. Whole-mount *in situ* hybridization using *vasa* probe. (A)** *2-cell stage,* **(B)** *4-cell stage,* **(C)** *8-cell stage,* **(D)** *16-cell stage,* **(E)** *32-cell stage,* **(F)** *blastula stage,* **(G)** *90%-epiboly stage,* **(H)** *bud stage and* **(I)** *32-somite stage. (Aa, Ba, Ca, Da, Ea, Fa) view from the animal pole. (Ga) view from the vegetal pole. (Ha, Ia) dorsal view. (Ab, Bb, Cb, Db, Eb, Fb, Gb, Hb, Ib) lateral view. D↔V, dorsal↔ventral; A↔P, anterior↔posterior. Arrowheads indicate* vasa *spots (A, B, C, D, E) and* vasa *positive cells (F, G, H, I). Scale bars, 100 μm.*

structures resembling the germ plasm organelle, “nuage”. These expression patterns of *vasa* RNA are suggestive of its function in PGC development (Knaut *et al.*, 2000). Furthermore, removal of the cytoplasm at the ends of the cleavage planes in zebrafish with the aim of removing *vasa* and other mRNA components results in a severe reduction in the number of germ cells (Hashimoto *et al.*, 2004).

In this paper, we isolated the *vasa* cDNA from a teleost, shiro-uo (Family Gobiidae) and studied its expression pattern during embryogenesis while investigating the development of the shiro-uo germ cell lineage. We reported that shiro-uo embryos show a unique cleavage pattern among teleosts (Nakatsuji *et al.*, 1997) and that they are highly transparent and suitable for micromanipulation and other experiments. Furthermore, we examined the functional significance of the *vasa* mRNA-containing spots in the formation of PGCs using micromanipulation. We carried out micromanipulation of 4- or 8-cell stage embryos to remove the *vasa* mRNA-containig spots and then counted the number of *vasa*-expressing PGCs in the genital ridges of the manipulated embryos. The numbers decreased significantly when the four

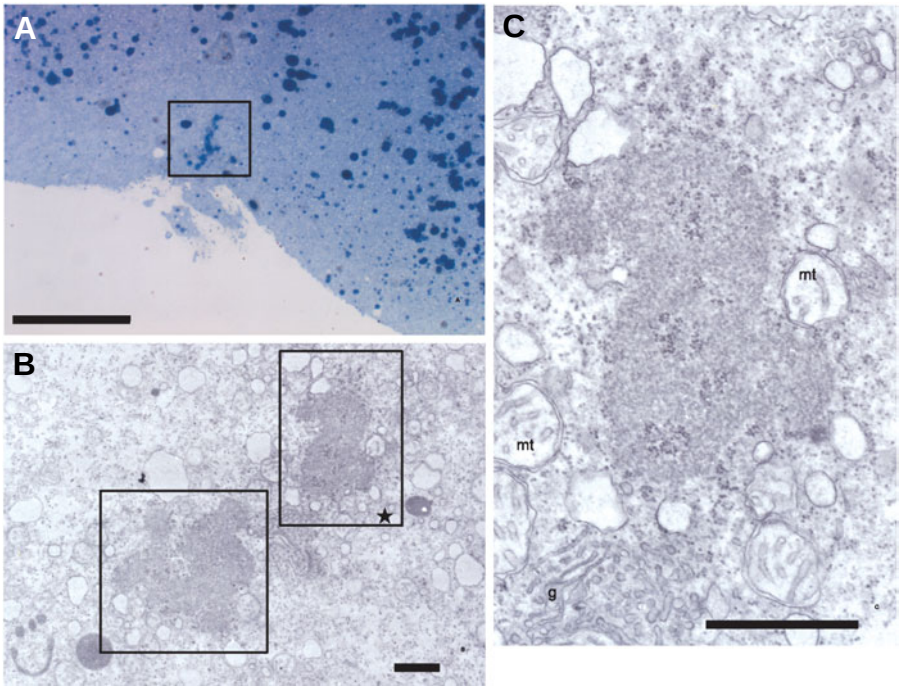



**Fig. 3. A semi-thin (2 μm) vertical section (A) and electron micrograph (B,C) of the 4-cell stage of the shiro-uo embryo. (A)** *Distinct aggregated granules, presumable* vasa*-containing areas, are indicated in the square.* **(B)** *A low magnification view of the electron micrograph of the granules indicated in (A).* **(C)** *A higher magnification view of the presumptive germ plasm, "nuage", indicated by the square labeled with a star in (B). mt, mitochondria; g, Golgi. Scale bars; 50 μm (A); 1 μm (B,C).*

spots were removed, indicating that the *vasa*-containing spots at early cleavage stages have important functions in the development of PGCs.

## Results

### *Sequence analysis*

We isolated a full-length *vasa* cDNA clone from shiro-uo, which had an open reading frame of 1938bp and encoded 646 amino acids (Fig. 1A). The amino acid sequence contained eight consensus sequences for the DEAD protein family (Fig.1, underlined). FASTA analysis indicated that the shiro-uo VASA protein is 69.7% and 61.4% identical to the medaka (Shinomiya *et al.*, 2000) and zebrafish (Olsen *et al.*, 1997; Yoon *et al.*, 1997), respectively. Additionally, the sequence showed 52.1% identity to *Xenopus* (Komiya *et al.*, 1994), 54.3% identity to *Homo sapiens* (Castrillon *et al.*, 2000), 55.9% identity to mouse (Fujiwara *et al.*, 1994) and 45.2% identity to *Drosophila* (Hay *et al.*, 1988; Lasko and Ashburner, 1988). A radial phylogenic tree of VASA proteins produced by the Neighbor-joining method indicated that the deduced shiro-uo VASA protein falls into the fish cluster (Fig. 1B).

### *Expression pattern during embryogenesis*

Whole-mount *in situ* hybridization was carried out using shiro-uo probes prepared from the 383bp *vasa* cDNA fragment containing the DEAD box region (Fig. 1A, arrowed region) and the localization of *vasa* transcripts was investigated from the 2-cell stage to the 32-somite stage (5 day post-fertilization). At the 2-cell stage, *vasa* transcripts formed two spots at both ends of the first cleavage plane (Fig. 2A) and at the 4-cell stage, four spots were observed at both ends of the first and second cleavage planes (Fig. 2B). As we have shown previously (Nakatsuji *et al.*, 1997) during shiro-uo embryogenesis, the third cleavage is horizontal in all blastomeres and produces two tires of blastomeres at the 8-cell stage. The forth cleavage plane is formed vertically in all blastomeres at the 16-cell stage (Arakawa *et al.*, 1999). At the 8-cell stage, *vasa* transcripts were still observed as four spots at both ends of two cleavage planes between the lower tier of the blastomeres and the yolk cell mass (Fig. 2C). For the 16-cell stage, new *vasa* spots appeared and we observed eight spots, four of which were still between the lower tier of the blastomeres and the yolk cell mass. The other four spots were present between the upper and lower tiers of the blastomeres (Fig. 2D). At the 32-cell stage, eight *vasa* transcripts were taken, respectively, into each blastomere located in the lower half of embryos (Fig. 2E). At the blastula stage, the number of *vasa*-positive cells increased and they were clustered (Fig. 2F). By the 50% epiboly stage, four clusters of *vasa*-positive cells were observed in the marginal region of the blastodisc. At the 90% epiboly stage, the clusters were observed in the dorsal area of the embryonic body (Fig. 2G). By the bud stage, *vasa*-positive cells were aligned along both sides of the embryonic body in the trunk region (Fig. 2H) and for the 12-somite stage, the tailbud detached and *vasa*-positive cells migrated toward the posterior region along the body axis (data not shown). At the 32-somite stage, *vasa*-positive cells were observed as aggregates in the presumptive genital ridge region, although some *vasa*-positive cells were still localized along both sides of the body axis (Fig. 2I).

### *Ultrastructural analysis*

Semi-thin sections of 4-cell stage embryos were examined by light microscopy. Distinct aggregated granules were observed at the distal ends of the cleavage furrows, presumptive *vasa*-containing areas (Fig. 3A, square). These granules were distinct from the dark stained round yolk granules. Furthermore, ultrastructural analysis of such granule revealed distinct subcellular structures that resembled nuage, a germ plasm organelle (Fig. 3B, squares). A higher magnification view (square in Fig. 3B) indicated that these nuages were made of fine electron-dense amorphous bodies and were present in close association with mitochondria and the Golgi apparatus (Fig. 3C).

### *Functional analysis of* vasa *transcripts*

Because of clear morphological criteria, we can easily identify the region in which *vasa* mRNA may be present. In 4- or 8-stage shiro-uo embryos, the presumed location of the *vasa* mRNA-containing four spots can be easily identified. To examine the functional significance of the *vasa* mRNA-containing spots during

the formation of PGCs, we carried out micromanipulation of 4- or 8-cell stage embryos to remove the cytoplasm at the presumed four sites of the *vasa* mRNA-containing spots and examined the manipulated embryos at later stages. By whole-mount *in situ* hybridization, the *vasa*-positive cells in manipulated and staged embryos were counted. When the four spots were removed, the number of PGCs at the bud stage decreased to 11.9±6.4 (mean±SD, n=36). When those manipulated embryos were examined at the 32-somite stage, the number was 13.1±7.3 (n= 28). As a control experiment, we removed the cytoplasm around the animal pole and the number of PGCs in those control embryos were 21.5±3.6 (n=13) at the bud stage and 20.7±5.2 (n=22) at the 32-somite stage. In the untreated embryos, the number of PGCs were 21.6±4.0 (n=11) at the bud stage and 21.4±7.0 (n=23) at the 32-somite stage. Thus, the number of *vasa*-positive cells significantly decreased when all of the four sites, which correspond to the *vasa* mRNA-containing spots, were removed from the cleavage stage embryos (t-test: $p<0.01$) (Fig.4).

## Discussion

In the present study, we cloned and examined the shiro-uo (ice goby), *Leucopsarion petersii*, *vasa* gene and identified the eight conserved homology boxes present in all DEAD box proteins. The shiro-uo VASA protein also shares with other VASA-related proteins an N terminus rich in glycine, with multiple repeats of an RGG motif (Fig. 1).

The expression patterns of *vasa* transcripts during cleavage stages show significant variations among fish. In shiro-uo, upon initiation of the first cleavage, *vasa* transcript-containing spots were not detectable yet in the whole-mount samples, although there were partial aggregates at the center of the blastomere detectable in histlogical sections of whole- mount samples after *in situ* hybridization (data not shown). The four *vasa* spots became visible at the 4-cell stage. In ukigori and goldfish embryos at the 8-cell stage, *vasa* transcript accumulation results in eight *vasa* spots (Otani *et al*., 2002; Saito *et al*., 2004). In the shiro-uo 8-cell stage embryos, however, only four *vasa* spots were observed at both ends of two cleavage planes between the lower tier of the blastomeres and the yolk cell mass, as same as at the 4-cell stage (Fig. 2C). In our previous study on the shiro-uo, involving cell-lineage examination via the injection of a tracer dye, we found that the PGCs mainly originated from lower blastomeres at the 8-cell stage (Saito *et al*., 2002) and this could be explained by the localization of *vasa* transcripts at the 8-cell stage. At the 16-cell stage, four new *vasa* spots appeared between the upper and lower tires of the blastomeres and thus resulted in eight *vasa* spots (Fig. 2D). These four new spots may be formed by division of the four original *vasa* spots, or by newly concentrated *vasa* mRNA. Similarly, in ukigori and goldfish embryos at the16-cell stage, eight *vasa* spots are present. In contrast, for zebrafish the *vasa* spots only total four from the 4-cell stage until the 32-cell stage (Olsen *et al*., 1997; Yoon *et al*., 1997). After the blastula stages, the expression patterns of *vasa* transcripts show more similarity among teleost species.

Detection of "nuage-like" subcellular structures using electron microscopy revealed the presence of amorphous fine granules and mitochondria, indicating similarity between the *vasa*-containing complex and the germ plasm of various groups of organisms.

Removal of *vasa*-containing spots resulted in a significant decrease in PGCs. The number of PGCs was, however, not always lower than that of the controls. This could be due to incomplete removal of *vasa*-containing spots. Also, the *vasa*-containing complex might not be confined to the spots but also be more diffusely distributed, which might be the origin of the four new spots at the 16-cell stage. In such a case, complete removal

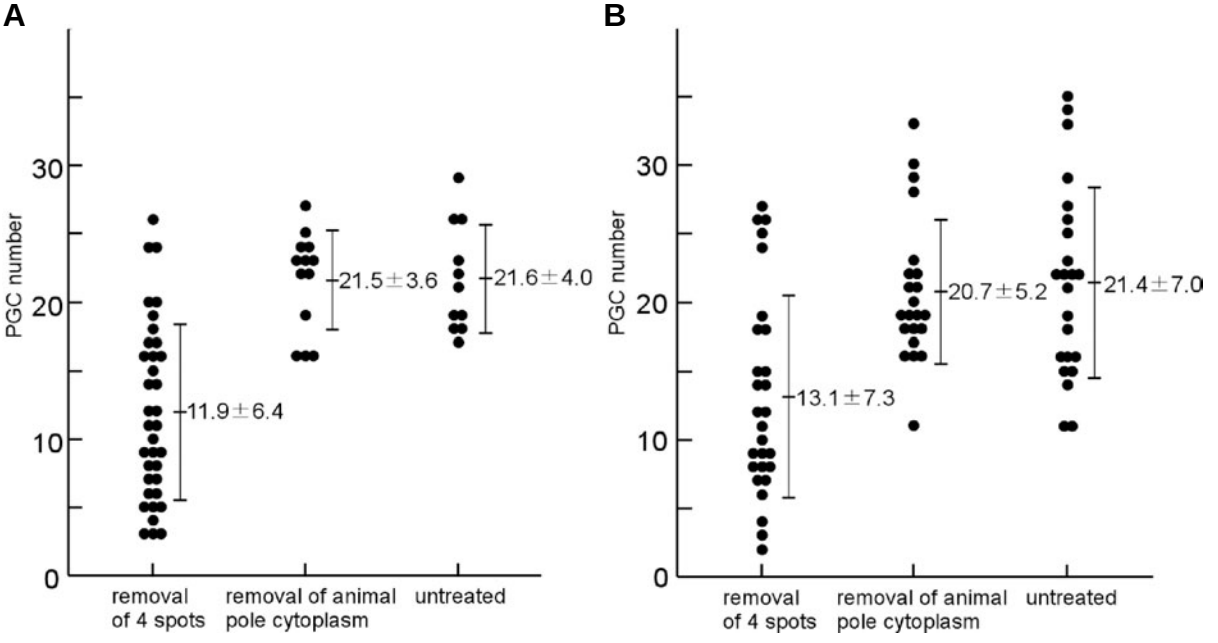



**Fig. 4. Removal of the *vasa*-containg cytoplasm resulted in a decrease in the number of primordial germ cells. (A)** *The number of PGCs counted at the bud stage.* **(B)** *The number of PGCs counted at the 32-somite stage.*

would be difficult and it may be the reason of decreased but remaining number of PGCs. Recent studies have shown that *nanos1, dead end, zDazl* and *bru* mRNAs are also localized to the ends of the cleavage furrows at the 4-cell stage and that their distribution is very similar to that of *vasa* during early zebrafish development (Köprunner *et al*., 2001; Weidinger *et al*., 2003; Hashimoto *et al*., 2004; Theusch *et al*., 2006). Our micromanipulation probably removed these complexes at the same time. Taken together, we conclude that the *vasa*-containing spots at early cleavage stages are functionally important in the development of PGCs in shiro-uo.

## Materials and Methods

### *Embryos*

Shiro-uo embryos were obtained by artificial insemination as described previously (Arakawa *et al*., 1999). Fertilized eggs and embryos were cultured at 19°C in diluted (10%) sterile Hank's salt solution. The chorion was removed manually using forceps in diluted (50%) sterile Hank's salt solution supplemented with 5 mM CaCl2.

### *cDNA Cloning of Shiro-uo* vasa

mRNAs were extracted from 30 shiro-uo embryos at the 4- to 5-somite using a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). First strand cDNAs were synthesized using an oligo (dT) primer and 2nd strand cDNAs were generated using a TimeSever cDNA Synthesis Kit (Amersham Pharmacia Biotech). The following PCR primers were designed using a highly conserved region of *vasa* homologous to that found in zebrafish (Olsen *et al*., 1997; Yoon *et al*., 1997), *Xenopus* (Komiya *et al*., 1994), mouse (Fujiwara *et al*., 1994), rat (Komiya and Tanigawa, 1995) and *Drosophila* (Hay *et al*., 1988; Lasko and Ashburner, 1988) to amplify a 383bp cDNA fragment of the *vasa* gene including the DEAD-box RNA helicase domain;
5'-ATGC(ATGC)TG(CT)GC(ATGC)CA(AG)AC(ATGC)G-3'(upper) and
5'-(AG)AA(ATGC)CCCAT(AG)TC(ATGC)AGCAT-3'(lower). To isolate a full-length coding sequence, 5'- and 3'-RACE were performed after determining the DNA sequence of the 383bp cDNA fragment of the *vasa* gene. For 5'-RACE, mRNA was extracted from shiro-uo ovaries and first-strand cDNA was synthesized using a SMART cDNA Library Construction Kit (Clontech). For 3'-RACE, a shiro-uo embryonic cDNA library in the λ zapII vector (Stratagene) was used. The primer sequences used for 5'- and 3'-RACE were as follows.
5'-RACE; 5'-CCTAGCCTCAAGGTGAATCTGGTTG-3' and
5'-TGCTACGCCGTCTGCCATAAGATGC-3'.
3'-RACE; 5'-GTAAGCCACAGGACACCAGATCAGAG-3' and
5'-GTATTGGACGAGGCTGACCGAATGC-3'.

The sequence data of the full-length *vasa* gene are available from DDBJ under accession number (AB098252).

### *Whole-mount* in situ *hybridization*

Staged embryos were fixed overnight at 4°C with 4% paraformaldehyde dissolved in 50% PBS. *In situ* hybridization was performed as previously described (Jowett and Lettice, 1994) using Digoxigenin (Dig)-labeled riboprobes, which were prepared from the 383 bp cDNA fragment of shiro-uo *vasa* containing the DEAD-box region. Antisense RNA probes were generated using T7 RNA polymerase (Roche).

### *Electron microscopy*

Embryos were fixed with a mixture of 4% paraformaldehyde and 5% glutaraldehyde in 0.1M PBS, rinsed with 0.1M PBS and post-fixed with 1% osmic acid in 0.1M cacodylate buffer. After dehydration through an ethanol series and propylene-oxide, embryos were embedded in Epon, separated from the yolk mass and cut along the animal–vegetal axis. Semi-thin (2 μm) sections were examined by light microscopy and were then cut to 80 nm for electron microscopy examination.

### *Micromanipulation*

Dechorionated embryos were horizontally laid along the side of slide glass in a 60mm plastic dish containing diluted (50%) culture medium supplemented with 2% albumen and 5mM $CaCl_2$. Approximately 50 pl of cytoplasm, at the ends of the cleavage furrows in which *vasa* transcripts are localized at the 4- or 8-cell stage, was removed from each site with sterile Femtotips II (Eppendorf) and injection equipment (IM-6-2 Microinjector, NARISHIGE). The tip of FemtotipsII was cut to 6 μm diameter for use. We removed all the cytoplasm of the four sites at the ends of the cleavage furrows in which *vasa* transcripts were presumably contained. We removed only a limited amount of cytoplasm, ca. 50 pl, from each of the presumed *vasa*-containing spots for a maximum total of 200 pl from each embryo to avoid interfering with embryonic development and limiting survival. Thirty minutes was allowed to pass to permit the ablated embryos to recover and they were then transferred to dilute (50%) fresh sterile media supplemented with 5 mM $CaCl_2$, antibiotics (100 units/ml penicillin and streptomycin) and methylene blue. The manipulated embryos were maintained in culture until the bud or 32-somite stage and then fixed with 4% paraformaldehyde. *In situ* hybridization was then performed as described and the number of *vasa*-positive cells was counted.

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