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Identification of Nuage-like Structures in the Panoistic Ovarioles of the German Cockroach Blattella germanica (Linnaeus) 【Research report】

德國蜚蠊無營養細胞型微卵管中似生殖雲構造之偵測鑑定【研究報告】

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Abstract

In some insects with meriostic ovarioles, maternal germ plasm is assembled during oogenesis and contains germline determinants that originated from the perinuclear nuage in the nurse cells. However, in insects with panoistic ovarioles containing no nurse cells, it is uncertain whether or not they contain maternal germ plasm or nuage. In order to approach this question, we used a cross-reacting antibody against the most conserved germline marker Vasa to stain the panoistic ovarioles dissected from the adult German cockroach Blattella germanica. This antibody cross-reacted with the Vasa signals in the perinuclear nuage-like structures of young oocytes and some oocytes bearing early and mid-vitellogenesis. However, staining signals in the nuclear periphery broke down in late oocytes. In the mature eggs subjected to oviposition, Vasa signals were identified in the cortex of the yolk, but a subcellular localization of Vasa signals was undetectable. This suggests that nuage exists in oocytes by mid vitellogenesis but it may not be involved in the assembly of a maternal germ plasm localized in oocytes bearing late vitellogenesis in B. germanica.

摘要

在某些具有營養細胞型微卵管的昆蟲當中，母系生殖漿在卵發育的過程形成，而且生殖漿內所含生殖細胞決定物質係來自於營養細胞的生殖雲。然而，在不具有營養細胞的無營養細胞型微卵管中，母系生殖漿或生殖雲是否存在仍屬未定。為了研究這個問題，我們用一個可辨識最保守之生殖細胞標記分子Vasa的高度交叉反應性抗體對德國蜚蠊 (Blattella germanica) 的無營養細胞型微卵管進行免疫染色。實驗結果顯示在剛分化的卵母細胞以及在卵黃生成早、中期的卵母細胞，較集中的Vasa訊號出現在核膜周圍。然而，免疫染色訊號在一些卵黃生成中期的卵母細胞之核膜外緣呈現不連續之點狀分佈，而接著在較晚期的卵母細胞，染色訊號便無法偵測得到。在即將被排出體外的生熟卵中，Vasa訊號出現在充滿卵黃的卵外緣；然而Vasa訊號並無聚集在卵腔內部任何地方的跡象。由上述結果顯示，在德國蜚蠊的微卵管中，生殖雲存在於卵黃發育中期以前的卵母細胞；然而在卵黃發育後期，生殖雲並不參與母系生殖漿的組成。

Key words: Blattella germanica, germ cell, germ plasm, nuage, Vasa
關鍵詞: 德國蜚蠊、生殖細胞、生殖漿、生殖雲、Vasa。

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Identification of Nuage-like Structures in the Panoistic Ovarioles of the German Cockroach *Blattella germanica* (Linnaeus)

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ABSTRACT

In some insects with meriostic ovarioles, maternal germ plasm is assembled during oogenesis and contains germline determinants that originated from the perinuclear nuage in the nurse cells. However, in insects with panoistic ovarioles containing no nurse cells, it is uncertain whether or not they contain maternal germ plasm or nuage. In order to approach this question, we used a cross-reacting antibody against the most conserved germline marker Vasa to stain the panoistic ovarioles dissected from the adult German cockroach *Blattella germanica*. This antibody cross-reacted with the Vasa signals in the perinuclear nuage-like structures of young oocytes and some oocytes bearing early and mid-vitellogenesis. However, staining signals in the nuclear periphery broke down in late oocytes. In the mature eggs subjected to oviposition, Vasa signals were identified in the cortex of the yolk, but a subcellular localization of Vasa signals was undetectable. This suggests that nuage exists in oocytes by mid vitellogenesis but it may not be involved in the assembly of a maternal germ plasm localized in oocytes bearing late vitellogenesis in *B. germanica*.

Key words: *Blattella germanica*, germ cell, germ plasm, nuage, Vasa

Introduction

In insects germ cells are either specified by a preformed germ plasm during early embryogenesis or they are segregated later, usually during gastrulation, by induction

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signals released by adjacent somatic cells (Hegner, 1917; Anderson, 1972; Extavour and Akam, 2003). After the germ cells are determined, they start proliferating and migrate toward the somatic gonads. In the gonads, usually after embryogenesis, germ cells differentiate into cyst founder cells (cystoblasts), and after several rounds of incomplete cytokinesis they become germline cysts. Through meiosis, germline cysts differentiate into gametes (Pepling *et al*., 1999).

The model insect *Drosophila melanogaster* is the most-studied example of the formation of germ plasm in the (meriostic) polytrophic ovarioles, where each developing oocyte is accompanied by a group of nurse cells (trophocytes). The assembly of the maternal germ plasm is driven by the localization of *oskar* mRNA at the posterior pole of the oocyte during stage 8 of oogenesis (Ephrussi *et al*., 1991). After the *oskar* mRNA is localized it acts as a molecular anchor to localize other germline determinants transported from the nurse cells to the oocyte (Ephrussi and Lehmann, 1992). In the nurse cells, germline determinants are first aggregated to a perinuclear organelle – nuage, and thereafter germline determinants are detached from the nuage and move into the oocyte during mid oogenesis (Snee and Macdonald, 2004). Among all germline determinants localized in the preformed germ (pole) plasm, the DEAD-box protein Vasa is the most conserved because it has been specifically identified in germ cells across many metazoan species (Raz, 2000; Extavour and Akam, 2003).

Homologues of the *Drosophila oskar* gene have not been identified in insects with (meriostic) telotrophic or panoistic ovarioles outside Diptera, suggesting that the formation of a maternal germ plasm, if there is any, in these two types of ovariole does not depend on the localization of *oskar*. Nevertheless, in the telotrophic ovarioles of the parthenogenetic pea aphid *Acyrthosiphon pisum*, Vasa is synthesized in the nurse cells of the germarium at the distal end of the ovariole and afterwards Vasa is transported to the posterior region of the syncytium, a potential location of the forming germ plasm (Chang *et al*., 2006). In the panoistic ovarioles of the grasshopper *Schistocerca gregaria*, expression of Vasa occurs in young oocytes, but Vasa signals are almost undetectable in late oocytes and mature eggs, suggesting that a localized germ plasm expressing Vasa may not exist (Chang *et al*., 2002).

In order to determine whether the expression of Vasa in panoistic ovarioles of other insects follows a pattern similar to that in *S. gregaria*, we stained the ovarioles dissected from the German cockroach *Blattella germanica* using a cross-reacting antibody against *Drosophila* Vasa. Combining the results of Vasa staining and previous studies on germline segregation in *B. germanica*, we propose a model of how germ cells are specified in this species.

Materials and Methods

Insects

Drosophila melanogaster were maintained at 23°C under a 12:12 LD cycle and *Blattella germanica* were reared at 28°C under a 16:8 LD cycle. The staging of *Drosophila* oogenesis was based on the scheme of Spradling (1993). We did not find any reports in the literature on a detailed staging table of oogenesis in cockroaches. In this study we characterize morphological features of *Blattella* ovarioles according to a description of oogenesis in the American cockroach *Periplaneta americana* by Anderson (1964).

Immunochemistry and microscopy

We stained ovarioles dissected from adult *D. melanogaster* and *B. germanica* using identical steps. Ovarioles were dissected in pre-chilled 1X phosphate buffered saline (PBS: 137 mM NaCl; 2.68 mM KCl; 1.47 mM KH_2PO_4; 8.10 mM Na_2HPO_4; pH 7.0), followed by fixation in

3.8% formaldehyde in 1X PBS for 20 minutes at room temperature. Dissected ovarioles were washed three times for 10 minutes with 0.2% Triton-X 100 (Sigma) in 1X PBS. After that, we used fine forceps and needles to peel off the outer layer of each ovariole follicle in order to increase the penetration of antibodies. The steps of blocking, antibody staining, and washing followed those of Chang *et al*. (2006). The primary antibody was an antibody against the *Drosophila* Vasa protein (1:200; resource: rabbits), a gift from the Paul Lasko Laboratory (Lasko and Ashburner, 1990; Liang *et al*., 1994). We used Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (1:500; Invitrogen) as the secondary antibody to generate fluorescent signals. Counter staining was performed using DAPI (Sigma; 2 ng/μL) for nuclear staining and Phalloidin TRITC (Sigma; 100 nM) for F-actin staining. As for light microscopy, we used biotinylated anti-rabbit IgG (1:200; Vector Laboratories). Signals were developed using a VECTASTAIN® Elite ABC Kit (Vector Laboratories) where the Nitroblue tetrazolium (NBT) (18.75 mg/mL)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (9.4 mg/mL) is the substrate for the alkaline phosphatase conjugated to biotin.

We took confocal images using a Zeiss LSM510 META and DIC (differential interference contrast) images of whole-mount samples using a Leica DMR connected to a Fuji FinePix S2 Pro digital camera.

Results

We used an antibody against the *Drosophila* Vasa protein to stain ovarioles dissected from adult *Blattella germanica* and *Drosophila melanogaster* respectively. Staining the *Drosophila* ovarioles serves as a positive control to test the antibody activity. In *D. melanogaster*, this antibody marked Vasa preferentially expressed in the perinuclear region of germline stem cells in the germaria and that of nurse cells in the egg chambers by stage 12 of oogenesis (Fig. 1) (Hay, *et al*. 1988b; Spradling, 1993). In the stage-10 oocyte, Vasa was first identified in the germ plasm localized at the posterior pole (Fig. 1). The above staining results correspond to previous studies concerning the expression of Vasa protein in *D. melanogaster* (Hay *et al*., 1988a, b; Lasko and Ashburner, 1990). It shows that this antibody is Vasa-specific and that it may have the potential to cross-react homologous protein of Vasa in *B. germanica.*

The ovarioles of *B. germanica* are panoistic, a type of ovarian tubules containing no specialized nurse cells (Büning, 1994). Morphological structures of the ovariole, from anterior to posterior, are described as follows (Fig. 2A): (1) terminal filament: a thin tubule located at the anterior-most end of the ovariole, serving as a suspensory ligament; (2) germarium: a region containing oogonia (precursors of oocytes) and young oocytes; (3) vitellarium: a zone containing growing oocytes where the uptake of yolk takes place. In the ovarioles of *B. germanica*, staining signals were almost undetectable in the oogonia (Fig. 2A), whereas they were preferentially localized to the periphery of the nuclear envelope in the young oocytes (Fig. 2B). During early vitellogenesis (length of an egg chamber: about 10 to 30 μm), the expression of Vasa signals, like that in young oocytes, was also localized to the nuclear periphery of the oocyte (Fig. 2C). Weak staining signals were detected within the nuclei of young oocytes (Fig. 2B) and those in the oocytes bearing early and mid vitellogenesis (length of an egg chamber: about 50 to 80 μm) (Fig. 2C). In ovarioles without the staining of the Vasa antibody, Vasa activity was almost unidentifiable (Fig. 2D).

The development of egg chambers in each ovariole is asynchronous. However, during the transition period from mid to late vitellogenesis, we could identify significant changes of Vasa signals in most ovarioles. In some mid vitellogenic oocytes

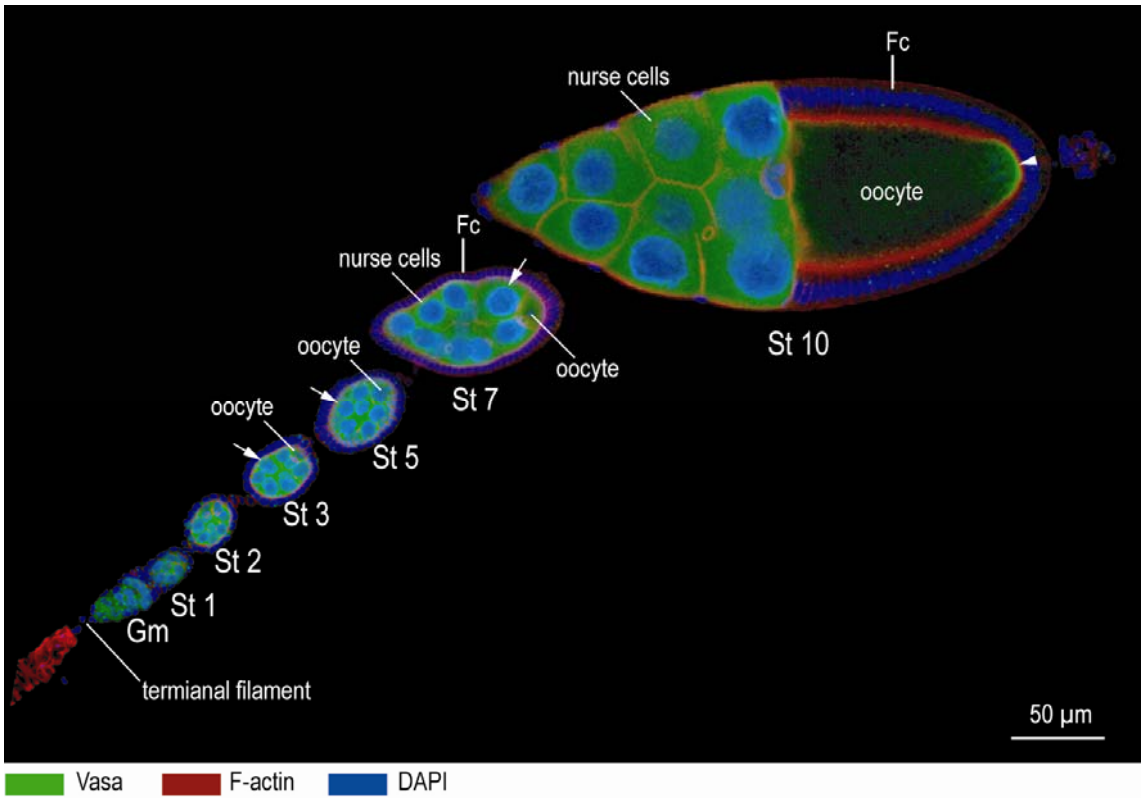

Fig. 1. Expression of Vasa protein during oogenesis in *D. melanogaster*. Ovarioles were stained with the anti-*Drosophila* Vasa antibody. Germarium and Stage (St) 1-5 egg chambers: the anterior is toward the location of the terminal filament; St 7 and St 10 egg chambers: the anterior is to the left. Staining signals representing the distribution of Vasa, F-actin adjacent to the inner surface of the cell membrane, and the nuclei are indicated with color keys. The migration of oocytes toward the posterior of the egg chamber is identified in the St 3 egg chamber. Preferential expression of Vasa in the nuclear periphery of nurse cells is indicated with arrows in the egg chambers from stages 3 to 10 of oogenesis. In the germarium and in the stages 1-2 egg chambers the localization of Vasa to nuclear envelope is not clear in the focal plane shown. In the st-10 egg chamber, Vasa is localized to the posterior pole (arrowhead) of the oocyte. Fc, follicle cells. For the staging of *Drosophila* oogenesis please refer to Spradling (1993).

Vasa signals were localized as discontinuous dots in the nuclear periphery (Fig. 3A). They were followed with late vitellogenic oocytes (length of an egg chamber: about 100 µm or longer) where the preferential expression of Vasa signals in the perinuclear region was almost undetectable. Instead, staining signals were randomly distributed within the nuclei (Fig. 3B). In some late vitellogenic oocytes, we did not identify Vasa signals either in the nuclear periphery or within the nuclei (Fig. 3C). In the mature eggs subjected to oviposition, Vasa signals appeared in the cortex of the yolk, but localized signals within a subcellular area were undetectable (Fig. 4).

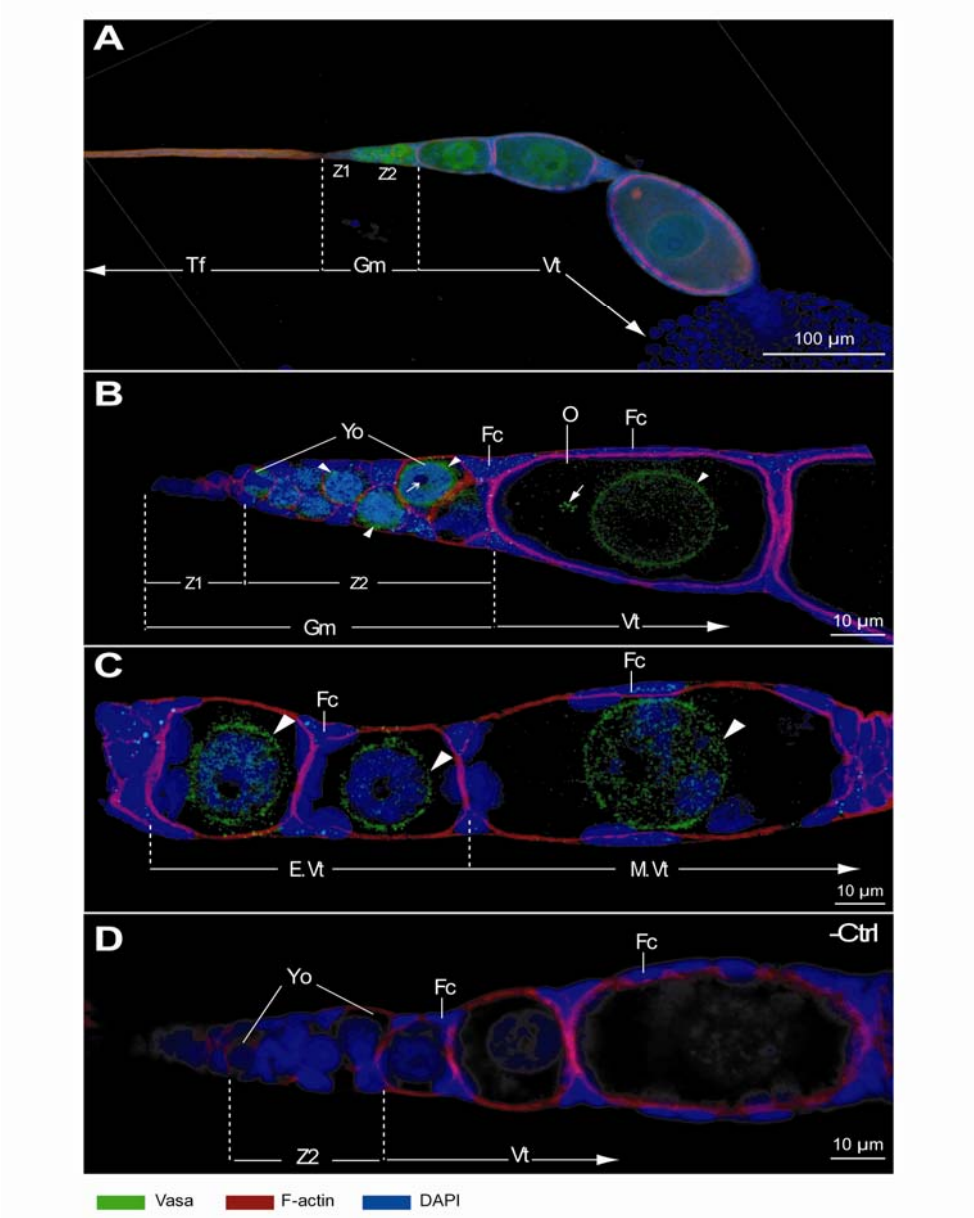

Fig. 2. Distribution of Vasa signals during early and mid vitellogenesis in *B. germanica*. Ovarioles dissected from the adult females were stained with the anti-*Drosophila* Vasa antibody. Staining signals representing the distribution of Vasa signals, F-actin adjacent to the inner surface of the cell membrane, and the nuclei are indicated with color keys. **(A)** An overview showing the morphology of an ovariole. From left to right: Tf, terminal filament; Gm, germarium (including Zone1 (Z1) and Z2); Vt, vitellarium. **(B)** A magnification of the germarium (Gm) and the oocyte (O) adjacent to the Z2, which undergoes mid vitellogenesis. Oogonia in Z1 are not clear in the focal plane shown. Young oocytes (Yo) are located within Z2. Vasa signals in the nuclear periphery of the young oocytes (Yo) and the vitellarium oocyte are indicated with arrowheads. The nucleoli visible in this focal plane are marked with double arrowheads. Cytoplasmic granules labelled with the Vasa antibody are indicated with an arrow. Fc, follicle cells. **(C)** Distribution of Vasa signals in oocytes undergoing early vitellogenesis (E. Vt) and mid vitellogenesis (M. Vt). Vasa signals (arrowhead) are preferentially detectable in the nuclear periphery. **(D)** Negative control (-Ctrl) without primary antibody.

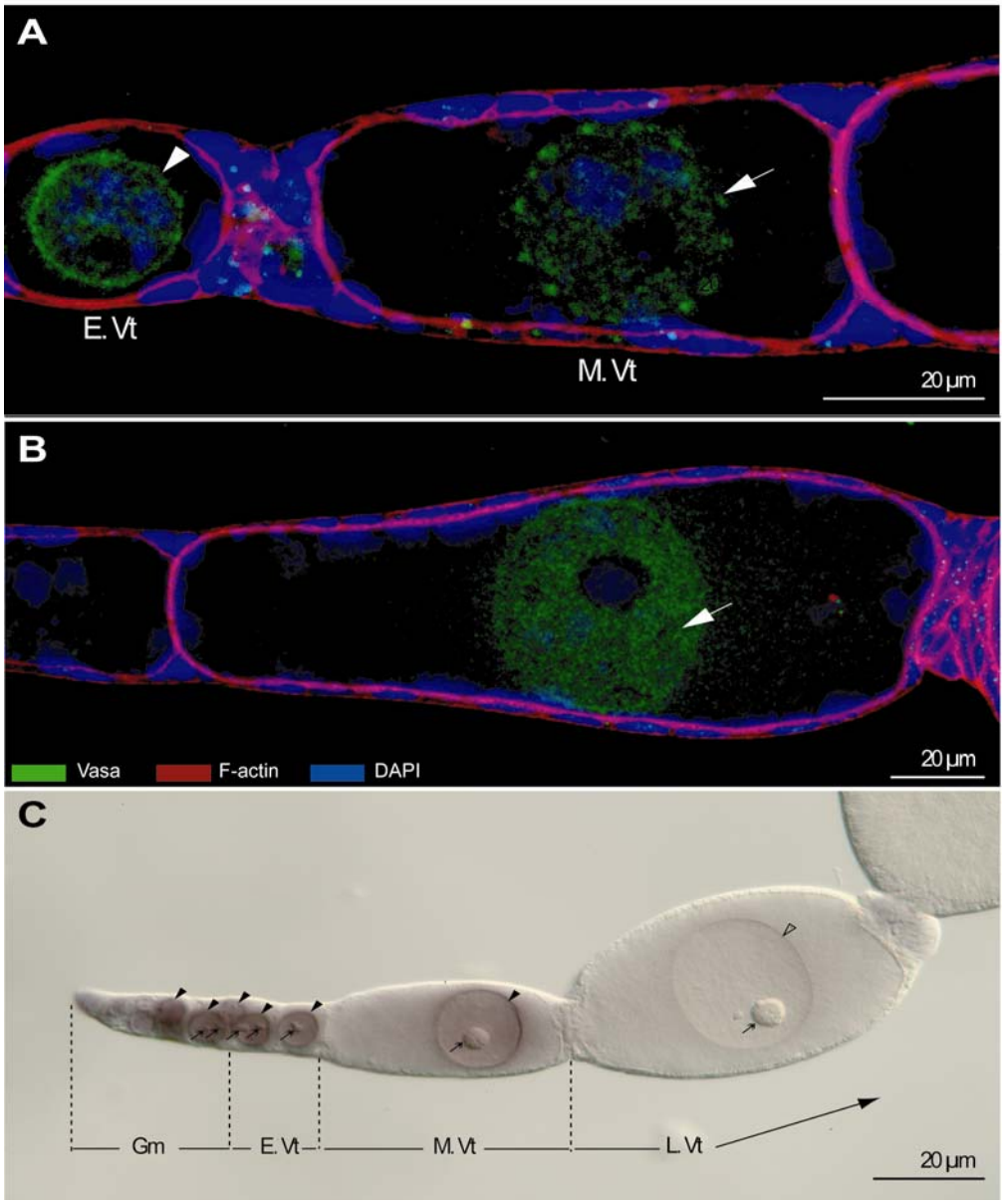

Fig. 3. Distribution of Vasa signals from mid to late vitellogenesis in *B. germanica*. Ovarioles dissected from the adult females were stained with the anti-*Drosophila* Vasa antibody. **(A)** Change of the distribution pattern of Vasa signals from early vitellogenesis (E. Vt) to mid vitellogenesis (M. Vt). Staining signals representing the distribution of Vasa, F-actin adjacent to the inner surface of the cell membrane, and the nuclei are indicated with color keys in panel B. Vasa is identified in the peripheral region (arrowhead) in an early vitellarium (E. Vt) oocyte, whereas in an oocyte undergoing mid vitellogenesis (M. Vt) Vasa signals in the nuclear periphery appear as dots (arrow). **(B)** Distribution of Vasa signals in the egg chamber posteriorly adjacent to the mid vitellarium oocyte shown in panel A. Localized signals do not preferentially appear in the nuclear periphery and are distributed within the nucleus (arrow). **(C)** Vasa signals developed with the NBT/BCIP substrate are preferentially identified in the nuclear periphery (arrowhead) in young oocytes in the germarium (Gm) and in oocytes undergoing early vitellogenesis (E. Vt) and mid vitellogenesis (M. Vt). In the late vitellarium (L. Vt) oocytes, Vasa signals are not detectable (open arrowhead). Nucleoli are indicated with double arrowheads.

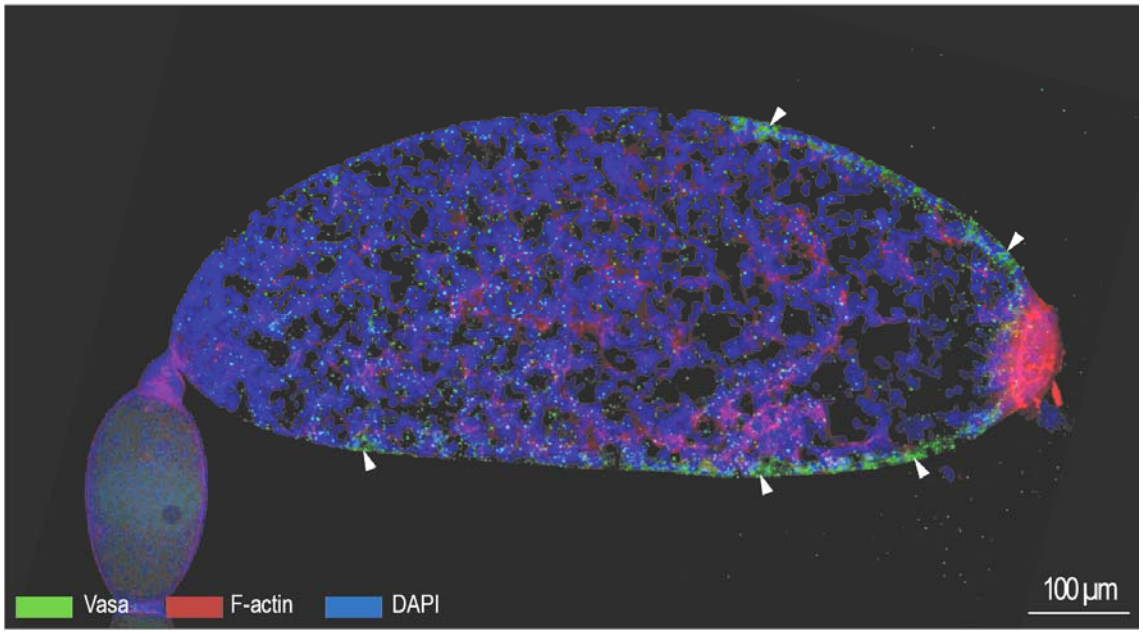

Fig. 4. Distribution of Vasa signals in the mature eggs of *B. germanica*. Ovarioles dissected from the adult females were stained with the anti-*Drosophila* Vasa antibody. Staining signals representing the distribution of Vasa, F-actin adjacent to the inner surface of the cell membrane, and the nuclei are indicated with color keys. Vasa signals are identified in the cortex (arrowhead) of the mature egg full of yolk. In the focal plane shown the Vasa signals are not clear in the oocyte linked perpendicularly to the mature egg.

Discussion

In many animal species the nuage is an electron-dense perinuclear organelle and has been regarded as a hallmark of germline cells (Eddy, 1975). In *Drosophila melanogaster* (fly), *Caenorhabditis elegans* (nematode) (Kuznicki *et al*., 2000), *Dugesia japonica* (planarian) (Shibata, *et al*. 1999), *Danio rerio* (zebrafish) (Knaut *et al*., 2000), and *Mus musculus* (mouse) (Toyooka *et al*., 2000), the most conserved germline marker *vasa*/Vasa is a component of nuage in the germ cells, suggesting that nuage is associated with the maintenance of germline identity.

We did not find any reports on the study of nuage in the panoistic ovarioles of *Blattella germanica*. However, in *Periplaneta americana* electron-dense particles were identified in the perinuclear region of young oocytes and in some oocytes before or during mid vitellogenesis (Anderson, 1964). Because of the electron-dense and perinuclear features, Eddy (1975) categorized these particles as nuage-like structures. In *Blattella* ovarioles, our staining results show that Vasa signals are preferentially localized to the nuclear periphery of oocytes within the equivalent periods of oogenesis (Fig. 2B, C). This strongly suggests that the Vasa-positive perinuclear structures are nuage in *B. germanica*. Given that nuage is an electron-dense organelle in germ cells of most phyla (Eddy, 1975), further examination of the presence of electron-dense particles in the Vasa-positive nuclear periphery by electron microscopy is required to confirm the nuage identity.

Apart from the continuous nuage-like structures labelled with the *Drosophila* Vasa antibody in the germarium and early vitellarium, we observed the discontinuous distribution of the perinuclear signals of

Vasa in some mid vitellogenic oocytes (Fig. 3A). Furthermore, in oocytes bearing late vitellogenesis the perinuclear signals were not visible (Fig. 3B, C). We hypothesize that the breakdown of nuage occurs between the transition from mid to late vitellogenesis (Fig. 3A, B). In the mature eggs, we did not identify any localized Vasa signals except in the egg cortex (Fig. 4), implying that a localized maternal germ plasm expressing Vasa does not exist. In effect, segregated germ cells in *B. germanica* are not detectable in the newly-laid eggs, and they are first identified at the posterior region of the germ band after the mesoderm is formed (Heymons, 1901). Together with the lack of a detectable germ plasm localized in the mature egg (Fig. 4), it suggests that: (1) Vasa signals in nuage-like structures are not localized to a preformed germ plasm; (2) the specification of germ cells in *B. germanica* does not depend on a preformed germ plasm in the ovarioles, and the segregation of germ cells may be induced later by epigenetic signals from neighboring somatic cells during gastrulation. Nevertheless, the nuage-like structures may be associated with the development and survival of young oocytes and oocytes by mid vitellogenesis.

We find that the distribution of Vasa signals are not localized to a subcellular region in late oocytes of both *S. gregaria* (Chang *et al*., 2002) and those in *B. germanica* (Fig. 3B, C; Fig. 4). This implies that insects with panoistic ovarioles may not have a preformed germ plasm expressing Vasa, or that Vasa is not a component of the preformed germ plasm in panoistic ovarioles. Nevertheless, nuage-like structures expressing Sgv have not been identified in the ovarioles of *S. gregaria* (Chang *et al*., 2002), which is different from our staining results showing nuage-like structures in the oocytes by mid vitellogenesis (Figs 2 and 3). At present we are not sure whether nuage-like structures cross-reacted by the *Drosophila* Vasa antibody in *B. germanica* express the Vasa orthologue(s) or other non-Vasa DEAD-box proteins. We therefore cannot conclude that the expression pattern of Vasa by mid vitellogenesis is different in these two species embodying panoistic ovarioles. Cloning a *vasa* orthologue for further studies is essential to clarify the relationship between nuage and germ plasm in *B. germanica*.

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德國蜚蠊無營養細胞型微卵管中似生殖雲構造之偵測鑑定

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摘　要

在某些具有營養細胞型微卵管的昆蟲當中，母系生殖漿在卵發育的過程形成，而且生殖漿內所含生殖細胞決定物質係來自於營養細胞的生殖雲。然而，在不具有營養細胞的無營養細胞型微卵管中，母系生殖漿或生殖雲是否存在仍屬未定。爲了研究這個問題，我們用一個可辨識最保守之生殖細胞標記分子 Vasa 的高度交叉反應性抗體對德國蜚蠊 (*Blattella germanica*) 的無營養細胞型微卵管進行免疫染色。實驗結果顯示在剛分化的卵母細胞以及在卵黃生成早、中期的卵母細胞，較集中的 Vasa 訊號出現在核膜周圍。然而，免疫染色訊號在一些卵黃生成中期的卵母細胞之核膜外緣呈現不連續之點狀分佈，而接著在較晚期的卵母細胞，染色訊號便無法偵測得到。在即將被排出體外的生熟卵中，Vasa 訊號出現在充滿卵黃的卵外緣；然而 Vasa 訊號並無聚集在卵腔內部任何地方的跡象。由上述結果顯示，在德國蜚蠊的微卵管中，生殖雲存在於卵黃發育中期以前的卵母細胞；然而在卵黃發育後期，生殖雲並不參與母系生殖漿的組成。

關鍵詞：德國蜚蠊、生殖細胞、生殖漿、生殖雲、Vasa。

+這兩位作者對本論文具有等量貢獻。
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