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In mouse oocytes the mitochondrion-originated germinal body-like structures accumulate mouse *Vasa* homologue (MVH) protein

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Summary

Mouse *Vasa* homologue (MVH) antibodies were applied to mouse Graafian oocytes to clarify if mitochondrion-originated germinal body-like structures, described previously by conventional electron microscopy, were associated with the germ plasm. It was found that both the mitochondrion-like structures with cristae and the germinal body-like structures that lacked any signs of cristae were labelled specifically by the anti-MVH antibody. Moreover, some granules were MVH-positive ultrastructural hybrids of the mitochondria and germinal body-like structures, the presence of which clearly supported the idea of a mitochondrial origin for the germinal body-like structures. This finding is the first evidence that mitochondrion-originated germinal body-like granules represent mouse germ plasm.

Keywords: Germ plasm, Germinal body-like structures, Mouse, MVH protein, Oocytes

Introduction

During the life cycle of metazoan animals the germ plasm, the cytoplasmic substance that determines the segregation of primordial germ cells (PGCs), undergoes similar events of formation and transformation. An initial step in germ plasm development is its appearance during oogenesis (Saffman & Lasko, 1999; Kloc *et al.*, 2002; Cox & Spradling, 2003; Chang *et al.*, 2004; Wilk *et al.*, 2004; King *et al.*, 2005). Newly formed germ plasm is known as polar granules in *Drosophila* (Mahowald, 1962), as germinal granules in *Xenopus* (Kloc *et al.*, 2000, 2002; Chang *et al.*, 2004; Cuykendall & Houston, 2010), and as P granules in *Caenorhabditis* (Strome & Wood, 1983; Wolf *et al.*, 1983). After formation and incorporation into PGCs, the germ plasm is transferred during embryogenesis and is present throughout in the next generation of germ-line cells (Eddy, 1975; Mahowald, 1977; Strome & Wood, 1982; Williamson & Lehmann, 1996; Ikenishi, 1998).

It has been assumed that mice are an exception to this situation, as germ plasm-forming structures had not been found in oocytes and early embryos, although germ plasm had been observed in PGCs and in differentiating gametes (see for review Eddy, 1975; Eddy & Hahnel, 1983; Snow & Monk, 1983; McLaren, 2003). It was accepted that germ plasm was absent in mouse early development and that PGCs arise *de novo* during embryogenesis due to signals from the extra-embryonic ectoderm (for review see Matova & Cooley, 2001; Noce *et al.*, 2001; Yoshimizu *et al.*, 2001; Extavour & Akam, 2003; McLaren, 2003; Matsui & Okamura, 2005; Hayashi *et al.*, 2007; Ewen-Campen *et al.*, 2010). However, the exclusiveness of mammalian germ plasm and germ line has been always doubted by some researchers and a re-examination of this dogma has been discussed as being desirable (for review see Edwards & Beard, 1999). Hübner *et al.* (2003) have shown that mouse embryonic stem cells are capable of differentiating into gametes; their research gave support to the self-sufficiency of mouse stem cells that presumably have all the necessary equipment to exhibit the germ cell

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pattern. Moreover, it has been stressed that, although mammals have no maternally deposited structures for germ cell differentiation, many mRNAs and proteins characteristic for *Drosophila* germ plasm are conserved in mouse and other mammalian species (for review see Thomson & Lasko, 2005). Furthermore, the repressive mechanisms in animals characteristic of the preformation pattern of germ-line development have been found surprisingly in animals thought to have an epigenetic pattern of germ-line differentiation (for review see Seydoux & Braun, 2006). Another argument in support of conserved mechanisms of development in mice was the finding of the Balbiani body. Despite the fact that mouse oocytes were thought initially to lack a Balbiani body (McLaren, 2003), the presence of this structure was demonstrated by Pepling *et al.* (2007). In some metazoan animals the Balbiani body is thought to be involved in localization of the germ line (for review see Kloc *et al.*, 2004), therefore its presence in mouse oocytes may indicate the presence of germ plasm and therefore increase the effort to find it.

Initial controversial data, which assumed that mouse oocytes and early embryos actually contained tiny, mitochondrially originated, germinal body-like structures that were comparable with germinal granules of metazoan animals, were obtained as a result of focused electron microscopy study (Reunov, 2004, 2006). However, molecular-based investigations were still required to clarify if these 'mitochondrion-germinal body-like' structures were really related to the germ plasm. As the germ plasm structures of some animal oocytes were found to be *Vasa* immuno-positive (for review see Williamson & Lehmann, 1996; Saffman & Lasko, 1999), it seemed likely that the best way to perform this sort of experiment was an immuno-test for MVH (mouse *Vasa* homologue), which had been found by electron microscopy to be specific for germ plasm structures in mouse spermatogenesis (Toyooka *et al.*, 2000; Noce *et al.*, 2001; Onohara *et al.*, 2010).

The purpose of this study was to use immunoelectron microscopy for mouse Graafian oocytes and the germinal body-like structures described early (Reunov, 2004), with the application of anti-MVH antibodies labelled with colloidal gold. This research could extend our knowledge of mouse germ-line cell localization.

Materials and methods

Western blot analysis

The testes were removed surgically, then were immediately cut on ice, pieces were fast frozen on ice with ethanol. Samples were kept at –80°C. To prepare protein extracts for electrophoresis, 0.5 g of testes in an equal volume of RIPA buffer were homogenized with the 1× cOmplete, Mini, Protease Inhibitor (Roche Applied Science, cat. no. 11836153001) in a homogenizer. The homogenate was prepared for electrophoresis by boiling the sample in Laemmli sample buffer (Bio-Rad, cat. no. 161-0737) at a ratio of 1:1. Samples (≥35 μl total volume) were separated on Bio-Rad 10% Tris–HCl Precast gels in a Bio-Rad vertical mini slab gel system.

Proteins were transferred to Bio-Rad Immun-Blot polyvinylidene difluoride (PVDF) membranes and blots were incubated in Bio-Rad 1× TBE/casein blocker for 1 h at room temperature and incubated overnight at 4°C in the same buffer that contained 1:1000 rabbit polyclonal antibody generated to a DDX4/MVH primordial germ cell marker (Abcam, cat. no. ab13840). Blots were washed three times for 5 min in Bio-Rad 1× Tris-buffered saline with 0.05% Tween-20 (washing buffer), incubated for 2 h at room temperature at a 1:2000 dilution of goat anti-rabbit IgG–horseradish peroxidase (HRP) (Santa Cruz Biotechnology, cat. no. sc-2030), and then washed four times for 15 min in washing buffer. Immunoreactive bands were visualized with Promega 3,3′,5,5′-tetramethylbenzidine (TMB) Stabilizer Substrate for HRP. Molecular mass was estimated by comparing the bands with Bio-Rad Kaleidoscope pre-stained standards (cat. no. 161–0324).

Immunoelectron microscopy

Ovaries were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer and washed three times for 15 min each in phosphate-buffered saline (PBS) with 0.05% Tween 20. Materials were then blocked in casein buffer (Bio-Rad 1% casein in Tris-buffered saline (TBS)) overnight at room temperature, followed by overnight incubation with shaking in rabbit polyclonal antibody generated to a DDX4/MVH primordial germ cell marker (Abcam, cat. no. ab13840) and diluted in casein buffer (1:200). Materials were washed a further three times for 60 min each in PBS + 0.05% Tween 20, incubated with anti-rabbit Nanogold® (Nanoprobes, cat. no. 2003) in casein buffer (1:50), and washed again three times for 60 min each in PBS + 0.05% Tween 20. The negative control omitted the primary antibodies. Materials were postfixed in the same fixative for 20 min, washed twice for 5 min each in PBS + 0.05% Tween 20, then washed three times for 10 min each in molecular grade water and enhanced with gold for 30 min (Nanoprobes, GoldEnhance™ EM, cat. no. 2131). The enhancement reaction was terminated by washing three times in molecular grade water for 10 min, and materials were postfixed in the same fixative. Fixed ovaries were rinsed in cacodylate buffer and distilled water,

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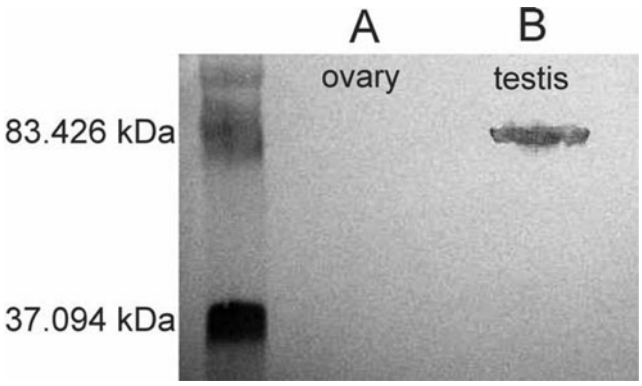

Figure 1 Western blots using anti-MVH antibodies in material extracted from the ovary (*A*) and testis (*B*) of mouse. No band is present in the ovary, but the testis has a band of 83.426 kDa in weight.

dehydrated in an ethanol series and acetone, and then infiltrated and embedded in Spurr's resin. Ultra-thin sections were stained with 2% alcohol uranyl acetate and aqueous lead citrate before examination using a transmission electron microscope (Zeiss Libra 120).

Quantification of MVH-positive granules

For transmission electron microscopy three ovary pieces were taken from each of three individuals and these pieces were embedded in resin blocks. From each of these nine blocks, three sections were taken from different levels, thus 27 sections were investigated. The presence and ultrastructure of MVH-positive granules were investigated. Percentages were calculated and results were analyzed statistically using Student's *t*-test.

Results

Antibody control by western blotting and immunoelectron microscopy

Binding of rabbit polyclonal antibody to MVH was visualized by western blot for the ovaries and testes of mice (Fig. 1*A*,*B*). The assay did not show any signal for the ovary, although the signal on the blots was quite pronounced for the testes. The band on the blot had an equivalent weight of 83.426 kDa, by comparison with the molecular weight standard. The ultrastructure section stained with both primary and secondary antibodies showed labelling of the cytoplasm and some cell structures. However, the negative control that omitted the primary antibodies showed total absence of any staining (data not shown).

An investigation of mitochondrially originated germinal body-like structures by immunoelectron microscopy

We investigated the localization of anti-MVH antibody in the periphery of mouse Graafian oocytes that, based on a previous study, contained about 15% cortical granules and about 85% mitochondria derivatives that transform into electron-dense bodies (Reunov, 2004). It was found that the anti-MVH antibody was located randomly in the cytoplasm. Staining of the cortical granules followed a typical pattern in that some anti-MVH antibodies were visualized along their surface, but there was no internal labelling (Fig. 2*A*). As seen in the same micrograph, the mitochondria that were located in close proximity to the cortical granules were recognizable by the presence of cristae and were labelled by anti-MVH antibody both internally and externally (Fig. 2*A*). Our attention was focused on reinvestigation of the groups of granules that represented continuous stages of mitochondrial condensation that occurred during the formation of germinal body-like structures (Reunov, 2004; Fig. 1*C*). To be consistent with our previous study, we examined the localization of anti-MVH antibodies in the same granule groups and found that the typical mitochondrion-like granules that had discernable mitochondrial cristae were MVH positive (Fig. 2*B*). The same result was characteristic of the granules or germinal body-like structures that lacked any cristae (Fig. 3*B*). In addition, some novel types of granules were discovered. These were 'hybrid' structures that shared ultrastructural characteristics of both the mitochondria with cristae and the granules that lacked cristae (Fig. 2*C*). Our calculation showed that 39% of MVH-positive structures were mitochondrion-like granules, whereas 16% and 45% were hybrid granules and germinal body-like granules, respectively (Fig. 3).

Discussion

Using western blot assays we did not detect any MVH signal in mouse ovaries, although the signal was quite strong in the testes. An absence of signal could be explained by the presence of only small numbers of oocytes in the ovaries of adult animals, as was suggested by Castrillon *et al.* (2000) who registered the same result during a *Vasa* localization study in female and male human gonads. Toyooka *et al.* (2000) demonstrated by confocal scanning microscopy that MVH is distributed uniformly in mouse oocytes, followed by a decrease in the anti-MVH signal and then total disappearance of signal in mature oocytes and absence at any sites of localization. Using immunoelectron microscopy,

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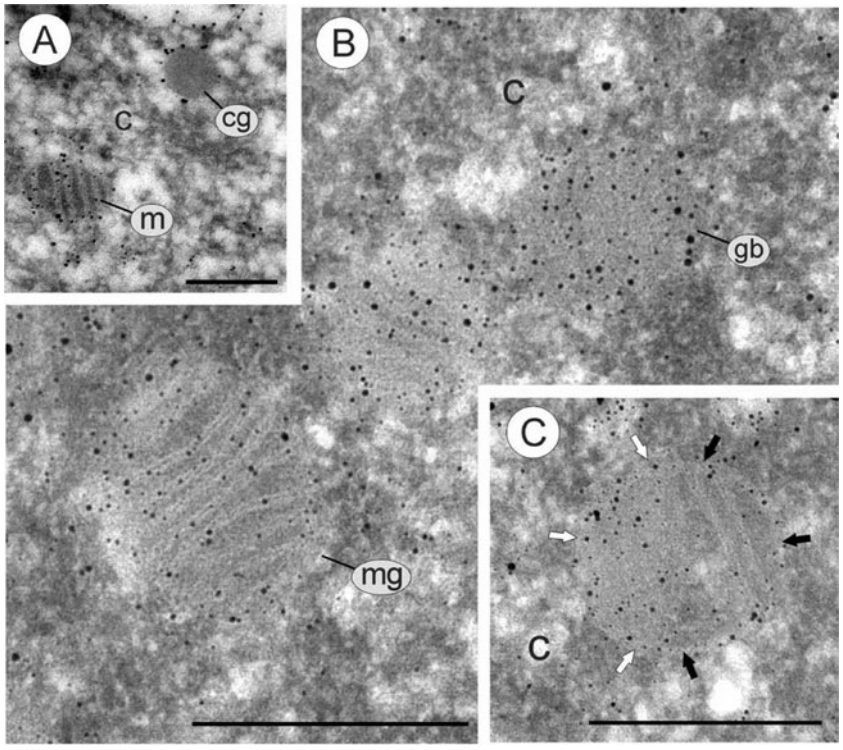

Figure 2 Mouse oocyte periphery visualized by immunoelectron microscopy. (*A*) The anti-MVH antibodies are seen in the cytoplasm adjacent to the cortical granule, but not inside this structure. Note the localization of anti-MVH antibodies in the mitochondrion. (*B*) Both the mitochondrion-like granule that still contains cristae and germinal body-like structures that lacked any cristae show marked affinity to anti-MVH antibodies. Note that the scarce gold particles are distributed randomly in the cytoplasm around these structures. (*C*) An MVH-positive hybrid structure that has characteristics of both a mitochondrion-like granule that still has cristae (right part shown by black arrows) and a germinal body-like structure that lack cristae (left part shown by white arrows). Some gold particles are seen in the surrounding cytoplasm. c, cytoplasm; cg, cortical granule; gb, germinal body-like structure; m, mitochondrion; mg, mitochondrion-like granule. Scale bar = 0.5 μm.

which clearly provided much higher magnification, we also observed the random cytoplasmic distribution of anti-MVH antibodies. Although the examination of dynamic changes in the presence of MVH was not the object of our work, we nevertheless believe that the anti-MVH signal normally occurs in the late stage oocyte cytoplasm, although the number of gold particles present at this stage might be not very high. Our analysis showed that, in late stage mouse oocytes, the cortical granules that were present in the oocyte periphery were negative for MVH because a signal was never found internally for the cortical granules. However the germinal body-like structures, originating from mitochondria and present in the oocyte periphery together with the cortical granules, were clearly stained with anti-MVH antibodies. This finding proves the germ plasm nature of these structures as was suggested previously by Reunov (2004). Hence, it is more likely that mice have universal mechanisms of germ-line cell differentiation rather than experience any unique mechanisms that are suggested in a number of reports (for review see Matova & Cooley, 2001; Noce *et al.*, 2001; Yoshimizu *et al.*, 2001; Extavour & Akam, 2003; McLaren, 2003; Matsui & Okamura, 2005; Hayashi *et al.*, 2007; Ewen-Campen *et al.*, 2010).

The results obtained here clearly support the hypothesis for a mitochondrion origin of mouse germ plasm (see Reunov, 2004). It seems likely that any internal anti-MVH antibody penetration is possible because mitochondria lose their membranes, as was shown by Reunov (2004). The possibility of mitochondrial membrane disappearance was seconded by Ninomiya & Ichinose (2007), who suggested that this phenomenon may explain why DIG-labelled probes applied in their study labelled mtrRNA so easily. It should be emphasized that some MVH-positive granules were at an intermediate stage and shared structural signs both of mitochondria and germinal granules; the discovery of these combined structures also strengthens the idea of mitochondrial transformation. It should be stressed that the levels of hybrid structures were comparatively low compared with mitochondrion-like granules and germinal

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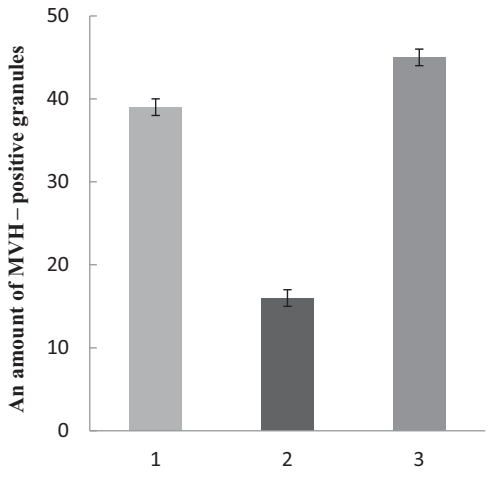

Figure 3 Frequency of MVH-positive granules in mouse oocytes. Mitochondrion-like granules (1); hybrid granules (2); and germinal body-like granules (3).

body-like granules. Presumably, this finding may indicate that the life of the intermediate granules is short or, alternatively, the transformation of mitochondria to germinal granules occurs relatively quickly.

The hypothesis of mitochondrion involvement in germ plasm formation has been voiced in previous studies of *Drosophila* and *Xenopus*. It has been found that the germinal granules in *Xenopus* and polar granules in *Drosophila* are unusual due to the presence of mitochondrial ribosomal RNAs (Kobayashi *et al.*, 1993, 1994, 1998; Iida & Kobayashi, 1998; Ogawa *et al.*, 1999; Kloc *et al.*, 2000). Some evidence has shown that in *Drosophila* embryos the mitochondrion-type ribosomes, which included both mitochondrial rRNAs and some mitochondrial ribosomal proteins, are able to integrate into polysomes on the germ-plasm polar granules and presumably provide germ-line directed translation (Amikura *et al.*, 2001, 2005). As was found by immunoelectron microscopy, both the germinal granules and the matrices of the mitochondria in *Xenopus* embryo germ-line cells accumulate anti-*Vasa* antibodies (Watanabe *et al.*, 1992). It seems likely that, in *Xenopus*, the germinal granules and the mitochondria may share the same antigen as a result of structural continuity. It seems quite possible that, in different taxa, mitochondria could be a universal platform for germ plasm as it was suggested by Reunov (2004); further research in more animal species is necessary to determine if this hypothesis is true.

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