Stage-specific expression of mouse germ cell-less-1 (mGCL-1), and multiple deformations during *mgcl-1* deficient spermatogenesis leading to reduced fertility

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Summary. A mouse homologue of *Drosophila* germ cell-less, mouse germ cell-less-1 (*mgcl-1*), is highly expressed in the testis. Previous report revealed that the fertility of the *mgcl-1*^{-/-} male mice is reduced significantly as a result of various morphological abnormalities in the sperm (Kimura *et al.*, 2003). To elucidate the function of *mgcl-1* in spermatogenesis, the expression of mGCL-1 in the wild-type testis was examined. Immunohistochemical studies demonstrated that mGCL-1 first appeared in the nuclei of the pachytene spermatocytes at stage VI of the seminiferous epithelium, and existed in those of spermatids until step 8 during spermatogenesis. mGCL-1 was not detectable after step 9 spermatids. The testicular cells and epididymal sperm were further analyzed morphologically using *mgcl-1*^{-/-} mice. In the testis, deformed nuclei first occurred in the pachytene spermatocytes at stage VI, which is consistent with the time of the first appearance of the mGCL-1 protein in the wild-type testis. Abnormal nuclei and acrosomes were found in spermatids after step 5, and nuclei of the spermatids and epididymal sperm were frequently invaginated. In addition, variously deformed sperm such as bent-neck, multi-headed

or multi-nucleated sperm were observed in the *mgcl-1*^{-/-} cauda epididymidis. However, several key structures such as the acroplaxome marginal ring (Kierszenbaum *et al.*, 2003), postacrosomal sheath, and posterior ring apparently formed. In addition, MN7 and MN13, essential substances for fertilization that are located in sperm heads, were detectable in the *mgcl-1* null sperm. These observations provide important insights into the mechanisms regulating the nuclear architecture and causes of human infertility.

Introduction

In *Drosophila* embryos, germ cell-less (*gcl*) gene product (GCL) is a component of germ plasm and an essential factor in germ cell formation (Jongens *et al.*, 1992; 1994). Recently, a mouse homologue of *gcl*, mouse germ cell-less-1 (*mgcl-1*) was identified (de la Luna *et al.*, 1999; Kimura *et al.*, 1999; Leatherman *et al.*, 2000). De la Luna *et al.* (1999) isolated the mGCL-1 protein as a DP(E2F-dimerization partner)-interacting protein, DIP, which interacts with the DP component of E2F. E2F is a transcription factor and plays a critical role in orchestrating early cell cycle progression (Lavia and Jansen-Durr, 1999). mGCL-1 acts as a repressor of the E2F-DP transcriptional activity and is localized in the nuclear envelope, which consists of outer and inner nuclear membranes, nuclear pore complexes, and the nuclear lamina (de la Luna *et al.* 1999). Other groups identified mGCL-1 as a lamina-associated polypeptide 2 β (LAP2 β)-binding protein (Nili *et al.*, 2001). LAP2 β is an integral membrane protein of the nuclear envelope involved in chromatin and nuclear architecture (Foisner and Gerace, 1993). Nili *et al.* (2001) showed that GCL is co-localized with LAP2 β in the nuclear envelope in mammalian cells.

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Recently, it has been demonstrated that mGCL-1 binds to another integral nuclear protein, emerin (Holaska *et al.*, 2003), whose deficiency causes Emery-Dreifuss muscular dystrophy (Nagano *et al.*, 1996). These data indicate that mGCL-1 is localized in the nuclear lamina, which is involved directly or indirectly in a number of nuclear activities, including DNA replication and transcription, nuclear and chromatin organization, cell cycle regulation, and cell development and differentiation (Mattout-Drubezki and Gruenbaum, 2003).

Both the *Drosophila* GCL and mouse mGCL-1 proteins contain the BTB/POZ domain, a conserved protein-protein interaction motif (Bardwell and Treisman, 1994). It is suggested that the domain serves to organize higher order macromolecular complexes involved in nuclear events such as chromatin folding (Albagli *et al.*, 1995). The *gcl* gene is conserved in evolution, and *mgcl-1* can rescue the *Drosophila gcl*-null phenotype (Leatherman *et al.*, 2000). GCL and mGCL-1 show the same unique subcellular localization in the nuclear envelope (Jongens *et al.*, 1994; de la Luna *et al.*, 1999; Leatherman *et al.*, 2000; Nili *et al.*, 2001). However, *mgcl-1* is not highly expressed during primordial germ cell formation in the mouse as is found in *Drosophila*. *mgcl-1* is expressed ubiquitously, but the highest level of expression occurs in the testis (Kimura *et al.*, 1999; Leatherman *et al.*, 2000), suggesting a unique function of *mgcl-1* in spermatogenesis. Our previous study on *mgcl-1* gene-deficient mice (Kimura *et al.*, 2003) revealed an abnormal nuclear structure in several organs such as the liver, pancreas and testis. As expected, the most striking feature of the *mgcl-1*^{-/-} mouse phenotype was detected in the testis. Severe morphological abnormalities were found in the spermatogenic cells, and fertility was significantly impaired in *mgcl-1*^{-/-} male mice.

This study sought to elucidate the mechanisms of abnormal spermatogenesis by investigating the precise localization of the mGCL-1 protein in the testis. We revealed the stage-specific subcellular localization of mGCL-1 during spermatogenesis. In addition, an ultrastructural analysis of the testis and epididymal sperm from *mgcl-1*^{-/-} mice was performed to obtain further understanding of the role of mGCL-1 in spermatogenesis. The results demonstrate that mGCL-1 is involved in spermatogenesis at specific stages and has an influence on the morphogenesis of spermatogenic cells, especially on the nuclear formation, further leading to multiple sperm deformations.

Materials and Methods

Animals and antibodies

The generation of mice with a targeted disruption of the *mgcl-1* gene has been reported (Kimura *et al.*, 2003). *mgcl-1* gene deficient mice and control wild-type mice were supplied from the Research Institute for Microbial Diseases, Osaka University, Osaka Japan. Animal handling was approved by the Animal Research Committee of Chiba University. An anti-mGCL-1 antibody was raised in rabbits, as previously reported (Kimura *et al.*, 2003). Monoclonal antibodies mM9 and mM13 were described in Toshimori *et al.* (1992) and Toshimori *et al.* (1991), respectively.

Immunohistochemistry

Adult wild-type mice were anesthetized with pentobarbital and fixed with Bouin's fluid by perfusion through the left ventricle. Testes were removed and immersed in the same fixative for 18 h. The testes were then dehydrated with ethanol and xylene, embedded in paraffin, and cut at a thickness of 3 μ m. The sections were autoclaved in phosphate-buffered saline (PBS) at 120°C for 5 min to activate the antigen. After treatment with 0.1% Triton X-100 for 30 min, endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol for 30 min. Further, non-specific binding of the antibody was blocked in PBS containing 2% normal goat serum (NGS; GIBCO BRL, NY, USA) for 30 min at room temperature (RT). The sections were then incubated with a rabbit anti-mGCL-1 antibody at a 1:400 dilution at 4°C overnight. After washing with PBS, samples were incubated with biotin-conjugated goat anti-rabbit IgG for 1 h, followed by incubation with S-ABC (streptavidin-biotin peroxidase complex) solution (DAKO LSAB2 System; Dako Japan Company, Kyoto) for 30 min. Immunohistochemical reactions were visualized using 3, 3'-diaminobenzidine (DAB) and H₂O₂. The sections were then counterstained with periodic acid Schiff.

Confocal laser scanning microscopy

Wild-type and *mgcl-1*^{-/-} mice were perfused through the heart with 4% paraformaldehyde (PFA) in PBS. Testes were removed and immersed in the fixative for additional 2 h. Then the testes were rinsed with PBS, embedded in OCT compound, and cut at a thickness of 20 μ m on a cryostat. The sections were incubated with a blocking solution (3% BSA and 5% NGS in PBS) for 1 h at RT to block the non-specific binding of the antibody, and then incubated with the anti-mGCL-1 antibody (at a 1:100 dilution) at 4°C

Fig. 1. Immunohistochemical staining of mGCL-1 in the mouse testis. **A:** Paraffin sections of adult mouse testis were immunostained with an anti-mGCL-1-specific antibody (brown), followed by a periodic acid Schiff reaction (red). Immunopositive signals were detected in the nuclei of late pachytene spermatocytes at stage IX. An **inset** shows a higher magnification of a pachytene spermatocyte. Bar=5 μ m. **B:** An immunoperoxidase-electron micrograph of a pachytene spermatocyte, stained with an anti-mGCL-1 antibody. The arrow indicates a positive reaction. N: nucleus.

overnight. After washing with PBS, the slides were incubated with Alexa Fluor 488 goat anti-rabbit IgG (0.5 μ g/ml; Molecular Probes) and propidium iodide (1 μ g/ml) in the blocking solution for 1 h at RT. After washing with PBS, the samples were mounted with PermaFluor (Immunon, Pittsburgh, PA, USA) and observed using a confocal laser scanning microscope (LSM410; Zeiss, Germany).

Immunofluorescent labeling of epididymal sperm

Sperm were collected from the cauda epididymidis of the adult mice. Live sperm were double-stained with Mito-Tracker Red CMXRos (20 nM; Molecular Probes, OR, USA) and Hoechst 33258 (0.05 μ g/ml; Sigma) and fixed with 0.4% PFA before observation. Other sperm were attached on APS-coated slides (Matsunami Glass Ind. Ltd., Osaka) and treated with 0.1 % Triton X-100. They were incubated with mMN9 (1:100 dilution) and mMN13 (1:100 dilution) monoclonal antibodies in PBS containing 5% NGS and 3% bovine serum albumin (BSA) at 4°C overnight. The slides were washed with PBS and sequentially incubated with Alexa Fluor 546 goat anti-mouse IgG (0.2 μ g/ml) for 1 h also and with Alexa Fluor 488 goat anti-mouse IgM (0.2 μ g/ml) also for 1 h. After washing with PBS, they were observed with Olympus BX50 (Olympus Ltd., Tokyo).

Transmission electron microscopy

Animals were perfused transcardially with 3% glutaraldehyde (GA) in 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer. The testes were excised and immersed in the same fixative for 2 h at RT. After post-fixation with 1% OsO₄, the tissues were embedded in epoxy resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed in an electron microscope (JEM 1200EX, JEOL, Tokyo; Hitachi H-7100, Tokyo).

Immunoperoxidase electron microscopy was performed as described previously (Yao *et al.*, 2001). Briefly, adult male mice were fixed with periodate/lysine/2% PFA (PLP) fixative by perfusion through the left ventricle. Testes were removed and immersed in the same fixative for 72 h at 4°C, and frozen sections of 6- μ m thickness were prepared. The sections were treated with 0.3% H₂O₂ in methanol for 30 min, and blocked with 10% NGS in PBS for 30 min. Then they were incubated with an anti-mGCL-1 antibody (1:100 dilution) at 4°C overnight, followed by incubation with the horseradish peroxidase-conjugated Fab' fragment of goat anti-rabbit IgG (Protos Immunoresearch, San Francisco, LA, USA). The samples were fixed with 0.5% GA, reacted with 0.05% DAB/H₂O₂, and postfixed with 1% OsO₄. They were dehydrated and embedded in epoxy resin,

and ultrathin sections were examined with a Hitachi H-7100 transmission electron microscope.

Results

Immunohistochemical localization of the mGCL-1 in the mouse testis

Immunohistochemical localization of mGCL-1 in the wild-type mouse testis was determined by an enzyme-linked antibody method (Fig. 1A). Paraffin sections of the testes were immunostained with a rabbit anti-mGCL-1 antibody, followed by a periodic acid Schiff (PAS) reaction. The results revealed that a positive immunoreactivity was found in the nuclei—especially in the nuclear envelope—of spermatocytes with a speckled pattern (Fig. 1A). Immunoelectron microscopy was additionally employed to detect the exact localization of mGCL-1 in the testis. A positive reaction was detected in the spotted area, including the nuclear envelope of a pachytene spermatocyte (Fig. 1B).

Stage-specific localization of mGCL-1 in the mouse testis

To improve conservation of the antigen, frozen sections of the testes were prepared for an indirect immunofluorescence study. The sections were stained with an anti-mGCL-1 antibody and propidium iodide, and observed by confocal laser scanning microscopy. The results revealed the stage-specific localization of mGCL-1 in the testicular germ cells (Fig. 2). Positive immunoreactivity for mGCL-1 was first detected in the nuclei of mid pachytene spermatocytes at stage VI, and observed in those of round spermatids until step 8. Spermatogonia through early pachytene spermatocytes showed no immunoreactivity, and no staining was detectable in the step 9 spermatids thereafter. These results of the immunolocalization of mGCL-1 in the testis are summarized in Figure 3. The immunoreactivity for mGCL-1 was detected not only at the nuclear envelope but also

inside the nuclei of the pachytene spermatocytes and round spermatids by using cryosections. The reaction in the nucleus was not distributed uniformly, showing a speckled pattern. In spermatocytes at the meiotic metaphase, the immunoreactivity was not detected on the chromosomes but rather in the cytoplasm (Fig. 2D).

Control experiments using *mgcl-1*^{-/-} testes revealed no immunoreaction for mGCL-1 in the spermatogenic cells. However, a faint reaction in the Sertoli cells was observed in both *mgcl-1*^{-/-} and wild-type testes (data not shown), indicating the non-specific staining of Sertoli cells. No positive immunoreactions were observed in any other testicular cell types (data not shown).

Morphological deformation of the mgcl-1^{-/-} testis

Ultrastructural analysis of the testes from *mgcl-1*^{-/-} mice revealed morphological defects: pachytene spermatocytes with the irregularly shaped nuclei (Fig. 4A; arrows), round spermatids with deformed nuclei and acrosomes (Fig. 4B, C), and elongated spermatids with invaginated nuclei (Fig. 4D, E). We determined the precise onset of these abnormalities during spermatogenesis, and the results are summarized in Figure 5. Deformed nuclei first appeared in pachytene spermatocytes at stage VI, which coincides with the appearance of the mGCL-1 protein in wild-type testes (Fig. 3). The deformation was observed until the meiotic prophase, and after meiotic division, round spermatids at steps 1 to 4 exhibited an apparently normal morphology. Deformed nuclei then reappeared in the spermatids after step 5. The deformation of the nuclear shape usually occurred where acrosomes were attached to the nuclei in the spermatids at step 5 to 8. In step 9 spermatids and thereafter, a different type of deformation was observed: finger-like invaginations of nuclei were seen at the caudal pole. Other abnormalities, such as multi-nucleated and multi-headed spermatids at stage VIII or a widening of intercellular bridges between spermatids, were observed (data not shown) as previously reported (Kimura *et al.*, 2003). Fur-

Fig. 2. Temporal and spatial immunohistochemical localization of mGCL-1 in the mouse seminiferous epithelium. Frozen sections of the testis were immunostained with an anti-mGCL-1 antibody (green) and counterstained with propidium iodide for nuclei (red). **A:** Stage IV–V, **B:** stage VIII, **C:** stage X, **D:** stage XII. Three right columns show higher magnifications of the part of the left columns, **A** to **D**. Spermatogonia (G in **B**), leptotene spermatocytes (LS in **C**), and pachytene spermatocytes at stage IV–V (PS in **A**) show negative reactions. The nuclei of pachytene spermatocytes at stage VIII (PS in **B**) and stage X (PS in **C**) are immunoreactive for mGCL-1. In the meiotic metaphase (M in **D**), the immunostaining is not detectable in the chromosomes but rather in the cytoplasm. The immunoreactivity is evident in the nuclei of step 4–5 (S4–5 in **A**) and step 8 (S8 in **B**) spermatids, and finally disappears in step 10 (S10 in **C**) and step 12 (S12 in **D**) spermatids. Bar = 10 μ m

Fig. 2. Legend on the opposite page.

thermore, an electron microscopic analysis revealed that nuclear pores of the deformed nuclei of pachytene spermatocytes were not distributed uniformly (data not shown). Invaginated regions of the nuclei had relatively abundant nuclear pores, consistent with the observation of the immunolocalization of NPC (nuclear pore complex) by Kimura *et al.* (2003).

Morphological deformation of the $mgcl-1^{-/-}$ epididymal sperm

As previously reported (Kimura *et al.*, 2003), caudal epididymal sperm from $mgcl-1^{-/-}$ mice showed an abnormal

morphology. Bent-necked sperm (Fig. 6A) were the most frequently (nearly 40%) occurring deformation found in the cauda epididymidis of $mgcl-1^{-/-}$ mice. Decapitated sperm were also found frequently (nearly 20%) in the $mgcl-1^{-/-}$ epididymis, probably arising from the bent-neck sperm during epididymal transport. Less than 10 % of the sperm had multiple heads and flagella as shown in Figure 6. Some of them were motile (data not shown) and had an apparently normal mitochondrial sheath, visualized by the staining with MitoTracker (Fig. 6A, B). In addition, immunostaining with mMN9 and mMN13 revealed that abnormal sperm from $mgcl-1$ KO mice could form MN9 and MN13, which are responsible for sperm-oocyte fusion and for oocyte

Fig. 3. Summary of the immunolocalization of mGCL-1 in the testis. Positive immunostaining is shown in green. During spermatogenesis, immunostaining for mGCL-1 was first observed in the nuclei of pachytene spermatocytes at stage VI. mGCL-1 immunoreactivity continued through round spermatids until step 8. After elongation of the nuclei of spermatids began, the staining became undetectable. The square area in red indicates the cells expressing *mgcl-1* mRNA, according to Leatherman *et al.* (2000).

Fig. 4. Electron micrographs of the $mgcl-1^{-/-}$ testis showing morphological defects. **A:** Stage VII. Pachytene spermatocytes show deformed nuclei (arrows). A round spermatid (step 7) with an irregularly shaped acrosome is also found (arrowhead). **B** and **C:** Stage V. Pachytene spermatocytes seem to be normal, but some round spermatids (step 5) have irregularly shaped nuclei and acrosomes (arrows). **C:** Step 5 spermatid with an indented nucleus and deformed acrosome. **D** and **E:** Stage II-III. Pachytene spermatocytes and round spermatids (step 2-3) appear to be normal. However, deformed nuclei of elongate spermatids (step 14) are found (arrows). **E:** Invagination of the nucleus is obvious in the step 14 spermatid (arrow). Bars=5 μ m (A, B, C), 1 μ m (D, E)

Fig. 4. Legend on the opposite page.

Fig. 5. Summary of results of a morphological analysis of the *mgcl-1*^{-/-} testis. Deformed nuclei of the pachytene spermatocytes were first found at stage VI. After meiotic division, nuclei of the round spermatids appeared to be normal until step 4. At step 5, when the acrosome flattened over the nucleus, deformed nuclei were observed again. After step 9, invaginations of the nuclei were frequently observed. Cell nuclei are shown in pale blue.

activation, respectively (Fig. 6C).

The morphology of the *mgcl-1*^{-/-} sperm was further examined by electron microscopy (Fig. 7). Most of the sperm had nuclear invaginations (Fig. 7A–C; asterisks), and microtubules were occasionally seen in the intruding cytoplasm. The bent-neck sperm had an apparently normal basal plate, but a partial detachment between basal plate and capitulum had occurred (Fig. 7C). An abnormal nuclear envelope showing a myelin-like structure was also observed (Fig. 7D; arrowhead). Both a posterior ring (PR) and postacrosomal sheath (PAS) were formed even in abnormal sperm (Fig. 7D). However, a part of the PAS was sometimes detached from the nucleus (Fig. 7A). Other deformations such as abnormal acrosomes (Fig. 7A–C), redundant cytoplasm around the head (Fig. 7A, C), and the incomplete condensation of chromatin (Fig. 7B, C) were frequently observed.

Discussion

Several studies have reported that mGCL-1 is localized in the nuclear envelope (de la Luna *et al.*, 1999; Leatherman

et al., 2000; Nili *et al.*, 2001). However, the precise localization of the protein has not yet been revealed in the testis, where the highest expression level of *mgcl-1* occurs. In this study, we demonstrated the stage-specific expression of the mGCL-1 protein in the nuclei of the spermatogenic cells.

In a previous study, *in situ* hybridization analysis indicated the strong expression of *mgcl-1* mRNA in pachytene spermatocytes (Kimura *et al.*, 1999), and the *mgcl-1* transcripts were detected in pachytene spermatocytes at stage VI up to diplotene spermatocytes at stage XI, to disappear during stage XII (Leatherman *et al.*, 2000). We revealed that mGCL-1 first appeared in the nuclei of the mid-pachytene spermatocytes at stage VI. Thus, the first expression of the mGCL-1 protein was consistent with that of mRNA during spermatogenesis. This means that the translational repression, which has often been observed in the testis (Steger, 1999), does not occur in case of *mgcl-1*. The *mgcl-1* mRNA was not detectable after meiotic division (Leatherman *et al.*, 2000), while the mGCL-1 protein persisted until round spermatids at step 8. It is considered that the round spermatids make use of the stored *mgcl-1* transcripts for translation, as an event of post-meiotic expression.

In the present study, immunohistochemical analyses demonstrated that mGCL-1 was distributed in the nuclear envelope in spermatogenic cells at a specific stage with a speckled pattern. This result is in agreement with the previous data about the intracellular distribution of mGCL-1 (DIP) in mammalian cells (du la Luna *et al.*, 1999). In addition, analyses of stage-specific localizations of mGCL-1 in cryosections revealed that it was located inside the nuclei. As reported by Nili *et al.* (2001), mGCL-1 is not an integral membrane protein of the nuclear envelope but is rather a nuclear matrix protein, although it is specifically associated with LAP2 β at the nuclear envelope. This fact may account for the intranuclear speckled localization of mGCL-1. It is suggested that a part of mGCL-1 interacts with a partner(s) other than LAP2 β (Nili *et al.*, 2001) or emerin (Holaska *et al.*, 2003).

At the meiotic metaphase, mGCL-1 was not colocalized with chromosomes but was diffused in the cytoplasm. This pattern of mGCL-1 distribution has been reported by Kimura *et al.* (2003) in the case of mitosis, as seen in other nuclear lamina proteins. When mGCL-1 was located in the nuclei of the pachytene spermatocytes, it did not seem to be present in the condensing chromatin, suggesting that mGCL-1 may not be a component of the condensing chromatin.

A variety of abnormalities were detected in testicular germ cells and epididymal sperm when the *mgcl-1* gene was deficient (Kimura *et al.*, 2003). We determined that the abnormalities were first induced as the deformed nuclei of pachytene spermatocytes at stage VI during spermatogenesis in the *mgcl-1*^{-/-} testes. In the wild-type testes, the mGCL-1 protein and mRNA were also detectable first in the nuclei of the same cells at this stage. Thus, it is suggested that pachytene spermatocytes at stage VI carry out a key role in the nuclear architecture during spermatogenesis.

Nuclear deformation in pachytene spermatocytes was observed up to diplotene spermatocytes, and then no morphological abnormality was detectable in the round spermatids at steps 1 to 4. Apparently the nuclear fragility of spermatocytes was recovered or concealed after meiotic division. This may reflect the difference in the nuclear components between spermatocytes and round spermatids. However, an abnormal nuclear morphology emerged again in spermatids after step 5, when the acrosome becomes flattened. Both a deformed nucleus and acrosome were observed frequently after step 5 spermatids, while the acrosome appeared to be normally formed until step 4.

The acroplaxome, recently designated by Kierszenbaum *et al.* (2003), is an attachment plate linking the inner acrosomal membrane to the nuclear envelope. It is suggested that the marginal ring of the acroplaxome guides the caudal descent of the acrosome (Kierszenbaum *et al.*, 2003). In

Fig. 6. Abnormal sperm in the cauda epididymidis from the *mgcl-1*^{-/-} mice. Sperm with multiple heads and flagella are observed. **A** and **B**: Sperm stained with MitoTracker (mitochondrion: red) and Hoechst 33258 (nucleus: blue). **C**: Sperm double-immunostained with MN9 for the equatorial segment (red) and MN13 for the postacrosomal sheath (green).

mgcl-1^{-/-} testes, the marginal ring was normally found at the descending leading edge of the acrosome. Thus, during the flattening of the acrosome, the vulnerability of the nuclear envelope lacking the *mgcl-1* might cause a deformation of the nuclei, especially the site covered by the acrosome. Deformation of the acrosome seemed to be caused indirectly although the mechanism is unknown.

The formation and organization of the sperm heads are highly complicated processes, and deformed nuclei and acrosomes of spermatids have been observed in other mutant mice (Toshimori and Ito, 2003). For example, *GOPC* (Golgi-associated PDZ [PSD-95/Discs-large/ZO-1]- and coiled-coil motif-containing protein)-null mice cause infertile round-headed spermatozoa (Yao *et al.*, 2002; Ito *et al.*, 2004). They have a defect in acrosome formation which is likely to bring about abnormal nuclear shaping

Fig. 7. Legend on the opposite page.

(Ito *et al.*, 2004). In contrast, nuclear deformation seems to be the primary effect in *mgcl-1*^{-/-} mice. The difference between *GOPC*^{-/-} and *mgcl-1*^{-/-} mice may be based on the fact that GOPC is localized in the cytoplasm and involved in the Golgi-derived intracellular transport. *GOPC*^{-/-} spermatids also showed similar nuclear invaginations at the caudal side, as seen in *mgcl-1*^{-/-} elongate spermatid. Interestingly, however, an ectopic manchette is found in the intrusion into the nucleus in the *GOPC*^{-/-} spermatids, while the cytoplasmic intrusion is not usually associated with microtubules in *mgcl-1*^{-/-} elongate spermatids.

Bent-necked sperm were observed frequently in the *mgcl-1*^{-/-} epididymis. Although they had an apparently normal basal plate, *mgcl-1* null mutation might have a destructive influence on the nuclear envelope at the basal plate, and cause the separation between the basal plate and capitulum. In the case of humans, sperm with bent necks have been reported in infertile patients (Lüders, 1976; Toyama *et al.*, 1995) and even seen in the fertile men (Holstein, 1975). In addition, other various deformations were observed in *mgcl-1*^{-/-} epididymal sperm. Nearly 10 % of the *mgcl-1*^{-/-} epididymal sperm were found to have multiple heads and flagella. Some sperm had excess cytoplasm around the head and/or incomplete condensed chromatin, as has been reported by Kimura *et al.* (2003). However, elongation of the head was completed in most sperm, and an ultrastructural analysis indicated that the postacrosomal sheath and posterior ring were formed in the *mgcl-1*-null sperm. Flagella formation was also apparently normal in ultrastructure, although sperm with multiple tails were occasionally observed.

MN9 and MN13 were detected in the *mgcl-1*^{-/-} epididymal sperm by immunocytochemistry with monoclonal antibodies. MN9 localizes the equatorial segment of the sperm head (Toshimori *et al.*, 1992), and is required for successful sperm-oocyte fusion in mice (Toshimori *et al.*, 1998). MN13 exist in the postacrosomal perinuclear theca (Toshimori *et al.*, 1991), which is involved in oocyte activation during fertilization (Manandhar and Toshimori, 2003). The substances, which have important functions in fertilization, were apparently normally formed in spite of the deficiency of *mgcl-1*. This result may account for the fact that *mgcl-1*^{-/-} male mice are not completely infertile, although the

fertility is reduced compared with control mice.

As pointed out by Kimura *et al.* (2003), deformation of the nucleus is probably the primary effect of the loss of *mgcl-1* gene. However, the null mutation also causes other multiple deformations in sperm, and it is not certain whether they are direct results of an *mgcl-1* deficiency or due to the indirect effects of the abnormal nuclear structure. Since mGCL-1 is known to act as a transcriptional repressor of E2F-DP (de la Luna *et al.*, 1999; Nili *et al.*, 2001), it is possible that transcriptional activities may be altered and cause abnormal gene expression in *mgcl-1*^{-/-} mice. However, the transcriptional function of E2F-DP complex is unlikely to be involved in the testis because the expression levels of genes whose transcription was driven by E2F-DP were not altered significantly in the *mgcl-1*^{-/-} testes (Kimura *et al.*, 2003). Recently, a new function of mGCL-1 has been reported by Masuhara *et al.* (2003). mGCL-1 can modify the MDM2-p53 complex by the enhanced degradation of MDM2, although the significance in the testis is uncertain at present. Other possible functions of mGCL-1 remain to be elucidated.

It is intriguing that the functional abnormalities of *mgcl-1*^{-/-} mice are confined to spermatogenesis (Kimura *et al.*, 2003). The nuclear envelope of spermatocytes contains short meiosis-specific lamin isoforms, such as lamin C2 (Furukawa *et al.*, 1994) and B3 (Furukawa and Hotta, 1993). They are involved in the reorganization of nuclear and chromosomal structures during meiotic division. It is likely that a meiosis-specific mechanism for nuclear organization exists in the testis, and *mgcl-1*^{-/-} may be implicated in it.

Recently, Kleiman *et al.* (2003) reported that human germ cell-less (HGCL) expression was reduced in azoospermic men with severe germinal cell impairment and that defective sperm motility was associated with the absence of HGCL. Various mutant mice induce the impairment of spermatogenesis, and have served as good models for male infertility (Braun, 1998; Okabe *et al.*, 1998; Escalier, 2001; Toshimori *et al.*, 2004). Accordingly, analyses of the deformations in *mgcl-1*^{-/-} mice, together with other studies on the mutant mice showing defective spermatogenesis, will lead us to a better understanding of the mechanisms of human infertility.

Fig. 7. Electron micrographs of epididymal sperm from *mgcl-1*^{-/-} mouse. **A:** Sperm with an invaginated nucleus and deformed acrosome. The postacrosomal sheath (PAS) is detached. **B:** Multi-nucleated sperm. One of the two nuclei includes incompletely condensed chromatin. **C:** Sperm with a bent neck. The basal plate is apparently normal, but a partial detachment between the basal plate and capitulum is observed. **D:** The posterior ring (PR) and PAS are apparently normally formed. The arrowhead indicates an abnormal nuclear envelope, showing a myelin-like structure. Asterisks in **A**, **B** and **C** indicate the invagination of nuclei. Ac: acrosome, N: nucleus. Bars = 1 μ m (A, B, C), 200 nm (D)

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