Appearance of an oocyte activation-related substance during spermatogenesis in mice and humans

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BACKGROUND: Recently we reported that an oocyte activation ability in human and mouse sperm is associated with head flatness or the presence of perinuclear theca (PT) substance, MN13, which is an oocyte activation-related protein localized on the post-acrosomal sheath (PAS). As such, we hypothesize that the appearance of oocyte activation ability is stage-specifically regulated and depends on the formation of the acrosome or PAS/PT in spermatids.

METHODS: We monitored the appearance and movement of MN13 as a PT-specific molecule during spermatogenesis and analysed how the MN13 localization is affected in mouse and human globozoospermic acrosomeless sperm.

RESULTS: MN13 was first detected on the surface of acrosomic vesicles, i.e. on the nascent outer acrosomal membrane of step 5–6 round spermatids (Sb1 spermatids in human), and it was then translocated via the outer acrosomal membrane surface to the most distal region of the acrosome in step 7 round spermatids (Sb2 spermatids). As spermatids elongated, MN13 was translocated via the cytoplasmic space between the nuclear envelope and the overlying plasma membrane towards the post-acrosomal region, and it was organized on the top of the nascent PAS that was typically found in step 14 elongated spermatids (Sd1 spermatids). In contrast, MN13 was not found in any GOPC-deficient spermatids, which completely lack the acrosome but have manchettes (microtubule bundles), nor in mouse and human acrosomeless sperm.

CONCLUSIONS: The MN13 appearance or the MN13-related PAS/PT formation is highly dependant on acrosome formation; the MN13-related oocyte activation factor/ability is stage-specifically acquired in elongating/elongated spermatids.

Key words: acrosome / perinuclear theca / MN13 / spermatogenesis / testis

Introduction

The perinuclear theca (PT) is formed by the cytoskeletal substance surrounding the nucleus at the perinuclear region (Fig.1 and Ito et al., 2009). PT is subdivided into two major regions: the subacrosomal layer and the post-acrosomal sheath (PAS) at the post-acrosomal region (PAR). The subacrosomal layer, which forms the acroplaxome (Kierszenbaum et al., 2003), is located between the inner acrosomal membrane and the nuclear envelope, while the PAS is a bottomless, funnel-like structure that is located at the PAR, except for the implantation fossa region that connects the head to the neck (Oko and Maravei, 1994; Mountjoy et al., 2008; Toshimori, 2009). Biochemically, the PT is formed mostly by non-ionic detergent-resistant substance(s). Functionally, the subacrosomal layer binds the acrosome to the nuclear envelope to maintain the anterior head morphology, while the PAS maintains the posterior head morphology, which forms characteristic periodic, ladder-like structures that connect the overlying plasma membrane to the perinuclear substance or envelope in mature sperm (Toshimori et al., 1991; Toshimori, 2009). PAS is formed after the acrosome is fully formed, during the elongating spermatid phase, starting at step 8 elongating spermatids in mice (equivalent to Sc spermatids in human), which is towards the end of spermatid nuclear elongation and condensation; this event is concurrent with manchette (microtubule) formation and descent. Functionally, it is thought that the PAS, where MN13 is specifically located, is closely related with oocyte activation (Manandhar and Toshimori, 2003).

Proteins known to make up the PT are actin (Fouquet et al., 1990), Arp-T1 and Arp-T2 (Heid et al., 2002), calcin (Longo et al., 1987;
Lécuyer et al., 2000), calmodulin (Kann et al., 1991; Moriya et al., 1993), CPX3 and CPB3 (von Bülow et al., 1997), calcin I and II (Hess et al., 1993, 1995), CYPT1 (Kitamura et al., 2004), MN13 (Toshimori et al., 1991; Manandhar and Toshimori, 2003; Ito et al., 2009), PERF 15 (Korley et al., 1997), PAWP (Wu et al., 2007), PT polypeptides (Oko, 1995), RAB2A (Mountjoy et al., 2008) and SubH2Bv (Aul and Oko, 2002). Proteins specifically localized to the PAR are Arp-T1, Arp-T2, calcin, calmodulin (also at the subacrosomal region), CPX3, CPB3, calcin I and II, MN13, CYPT1 and PAWP, whereas proteins within the subacrosomal region are calmodulin, PERF 15, RAB2A and SubH2Bv. Translocation of SubH2Bv and RAB2A to the PT is dependent on acrosome formation (Aul and Oko, 2002; Mountjoy et al., 2008), although translocation of PAWP is independent of acrosome formation but dependent on microtubular manchette development (Wu et al., 2007).

Recently we presented data that the oocyte activation ability is associated with the head flatness or the PT substance MN13, an oocyte activation-related molecule, localized on the PAS in human and mouse sperm. This was shown by comparing normal flat-headed sperm and round-headed sperm from a human globozoospermic patient and GOPC−/− mice, which are animal models of human globozoospermia (Ito et al., 2009); Golgi-associated PDZ- and coiled-coil protein encoding (GOPC) deficient (GOPC−/−) male mice are infertile due to fusion failure of transport vesicles to acrosome (Yao et al., 2002). The human round-headed acrosomes sperm have neither MN13, PAS/PT, nor oocyte activation ability, while GOPC−/− flat-headed sperm with acrosome have MN13 and can activate oocytes (Yao et al., 2002; Ito et al., 2004, 2009). As such, we hypothesize that the appearance of oocyte activation ability is stage-specifically regulated during spermatogenesis and depends on acrosome formation or PAS/PT formation in spermatids. Since developing spermatids are used for infertility treatment by intracytoplasmic sperm injection (ICSI) such as round spermatid injection (ROSI) and elongated spermatid injection (ELSI), it is important to examine when the oocyte activation substance appears during spermatogenesis and how its appearance relates with structure (acrosome or PAS/PT) formation. To address these issues, experiments were designed to monitor the appearance and movement of MN13 during spermatogenesis and to analyse how MN13 localization is affected in mouse and human acrosomeless (globozoospermic patient) sperm.

Materials and Methods

Animals

Adult male ICR mice (12–14 weeks old) were purchased from Takasugi Experimental Animal Supply Company (Saitama, Japan). GOPC deficient (GOPC−/−) and wild-type (GOPC+/+) male mice were supplied from the Department of Cell Biology of the Japanese Foundation Cancer Research (JFCR) Cancer Institute (Yao et al., 2002).

Human testis

Human testis used in this study was obtained from surgical specimens at the Chiba University hospital. The donor had not been treated with medicines detrimental to the testis such as carcinostatic agents and estrogens. The testis was immediately fixed in 10% neutral buffered formaldehyde and then post-fixed with Bouin’s solution for 1 h. The testis was dehydrated through graded ethanol and xylene, embedded in paraffin and sliced in thin sections. After deparaffinizing routinely, the testis was subjected to immunohistochemistry with anti-MN13 or anti-α tubulin antibody.

Sample preparation and general characteristics of mouse sperm

Normal (GOPC+/+) mouse sperm (control)

Sperm were collected from the cauda epididymides of male ICR mice and GOPC+/+ mice.

Round-headed, acrosomeless (GOPC−/−) sperm

Round-headed sperm, which accounted for >95% of the population, were collected from the cauda epididymides of GOPC−/− male mice. In GOPC−/− spermatids, the acrosome does not develop due to the failure of Golgi-derived vesicles to fuse (Yao et al., 2002). The oocyte activation ability rate (i.e. pronuclear formation rate) was low (mean ± SD: 0.7% ± 1.15) in GOPC−/− round-headed sperm but significantly higher (mean ± SD: 63% ± 20.8) in GOPC−/−/flat-headed sperm (Ito et al., 2009). Other characteristics were previously reported (Yao et al., 2002; Ito et al., 2004, 2009; Suzuki-Toyota et al., 2004, 2007).

All mice used were sacrificed by deep anesthesia with pentobarbital (Nembutal, Dainippon Sumitomo Pharma, Osaka, Japan) or by neck dislocation. Live sperm were collected from the cauda epididymides and stored in R18S3 medium [180 mg of Raffinose/ml (Wako Pure Chemical Industries Ltd, Osaka, Japan) and 30 mg of skimmed milk/ml] at −80°C until use.

Sample preparation and general characteristics of human sperm

Normal, flat-headed sperm (control)

Sperm from a healthy male volunteer (50 years old with two children) were used as controls for normal sperm. Sperm were collected by masturbation after 3 days of abstinence. The semen volume was 2.5 ml with a concentration of 80 × 10^6 sperm/ml. The proportion of normal sperm

Figure 1

Schematic drawing showing the localization and spatial relationship of the membrane system and cytoplasmic layers in developing spermatids at around step 7. A, acrosome; AG, acrosomic granule; G, Golgi apparatus; IAM, inner acrosomal membrane; N, nucleus; NE, nuclear envelope; OAM, outer acrosomal membrane; PAR, post-acrosomal region; PAS, postacrosomal sheath.
with flat heads was >80%, in agreement with the World Health Organization (WHO) criteria (>60%, according to Kruger's strict criteria). The sperm motility was >70%. The sperm were stored in R1853 medium at −80°C until use.

Round-headed, acrosomeless (globozoospermic patient) sperm
Round-headed acrosomeless sperm from an infertile globozoospermic patient were used for the analysis of sperm without oocyte activation ability. Sperm were collected as described above. The semen volume was 2 ml with a concentration of 38 × 10⁶ sperm/ml, and the percent of round-headed sperm (acrosomeless sperm) was 100%. The sperm motility was ~39%. The oocyte activation ability rate of the round-headed sperm from the patient was 0% (0 oocytes/19 injected oocytes) when injected mouse oocytes were not artificially activated; meanwhile, the rate was 68.4% (13 oocytes/19 injected oocytes) when the injected mouse oocytes were artificially activated with SrCl₂ (Kyono et al., 2008).

The treatment history of this patient and the partial characterization of the round-headed sperm have been previously reported (Kyono et al., 2008). The collected sperm not used for treatment were stored in Sperm-Freeze (FertPro N.V., Beemen, Belgium) at −80°C.

In this study, round-headed, acrosomeless sperm from the GOPC−/− mice and a globozoospermic patient were examined to determine whether acrosomeless sperm without oocyte activation ability had MN13 and formed PAS/PT by immunocytochemistry with MN13 antibody. These two characteristics were compared with those of normal sperm with acrosome from wild-type mice and a healthy human volunteer.

Informed consent, animal handling and ethical approval
Written informed consent was provided by all donors before the experiments, in accordance with the ethical approval of our institutes. The individual names of the donors were encoded to hide personal information. Animal handling was performed in accordance with Chiba University guidelines for the care and use of laboratory animals and with the approval of the Animal Research Committee of the JFCR Cancer Institute and Chiba University.

Antibodies
Primary antibodies
The monoclonal MN13 mouse IgM antibody made by us (Toshimori et al., 1992) was used in this study at a 1/5000 to 1/10 000 dilution of stock solution, which was ~0.1–0.2 μg/ml final concentration for immunohistochemistry (IHC) and indirect immunofluorescence (IF) and at a dilution of 1/1000 for immunogold electron microscopy (IEM). The MN13 antibody specifically recognizes the antigenic molecule MN13 that is present on the PAS of the PT. Since MN13 is an oocyte activation-related molecule (Manandhar and Toshimori, 2003; Ito et al., 2009), the presence of MN13 is considered representative of PT substance with the oocyte activation function (Fujimoto et al., 2004; Kitamura et al., 2004; Maekawa et al., 2004; Ito et al., 2009). The monoclonal MN9 mouse IgG₂a antibody specifically recognizes equatorin in the acrosome, particularly on the equatorial segment. MN9 antibody, which has been previously characterized (Toshimori et al., 1992, 1998; Manandhar and Toshimori, 2001; Yoshinaga et al., 2001, Yamatoya et al., 2009), was used to detect the existence of acrosome and to identify the stage of developing germ cells. MN9 antibody was used at a 1/20 000 dilution of stock solution, with a final concentration of ~0.02 μg/ml for IF. The rabbit polyclonal anti-α tubulin antibody (Thermo Fisher Scientific, Anatomical Pathology, Fremont, CA, USA) was used at 1/100 dilution, with a final concentration of 1 μg/ml for IHC.

Secondary antibodies
Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM antibody (Jackson Immuno Research, West Grove, PA, USA) and HRP-conjugated donkey anti-rabbit IgG antibody (GE healthcare Ltd, Buckinghamshire, UK) were used for IHC with MN13 antibody and anti-α tubulin antibody, respectively. Alexa Fluor 488 goat anti-mouse IgM and Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR, USA) were used for IIF with anti-MN13 and anti-MN9 antibodies, respectively. Aliquots of 5 or 10 nm colloidal gold-conjugated anti-mouse IgM (BBInternational, Cardiff, UK) were used for IEM with the MN13 antibody.

Other chemicals
Hoechst 33258, Hoechst 33342 (Sigma Aldrich, St. Louis, MO, USA) and Mayer's hematoxylin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) were used for nuclei staining. Schiff's reagent (Wako Pure Chemical Industries) was used for periodic acid Schiff (PaS) staining for the acrosome.

Preparation of cryosections of mouse testes for IHC and immunogold electron microscopy (IEM) with MN13 antibody
Testes were fixed by perfusion through the left ventricle with Zamboni's solution, immersed in the same fixative for 2 h, and dehydrated through a sucrose gradient [10, 20, 40% in phosphate-buffered saline (PBS)]. The tissues were embedded in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek; Sakura, Tokyo, Japan), quick-frozen in liquid nitrogen, sectioned on a cryostat (Leica CM1850, Leica Microsystems, Wetzlar, Germany) and mounted on polylysine-coated glass slides.

Preparation of paraffin sections of mouse testes for IHC with anti-α tubulin antibody
Mouse testes were fixed by perfusion through the left ventricle with Bouin's solution, immersed in the same fixative for 1 h and dehydrated through graded ethanol and xylene. The testes were routinely embedded in paraffin, sliced in thin sections, deparaffinized and then used for immunohistochemistry with anti-α tubulin antibody.

IHC of mouse and human testes with MN13 antibody; MN13-IHC
IHC was performed to analyse how MN13 is translocated and organized into the PAS/PT as described below. The sections of testes were treated with PBS containing 0.1% Triton X-100 and were then treated with 0.3% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. The samples were washed with PBS, incubated with 5% normal goat serum (NGS) and 3% bovine serum albumin (BSA) and treated with anti-MN13 antibody at 4°C overnight. After washing with PBS, the samples were incubated for 1 h with HRP-conjugated goat anti-mouse IgM antibody (1/400). After washing with PBS, the samples were visualized with 0.05% diaminobenzidine as a chromogen. The mouse samples were incubated with 1% periodic acid for 10 min, washed in running water for 10 min, stained with Schiff’s reagent for 20 min and rinsed in running water for 5 min for staining acrosome, or incubated with methyl green (DAKO Japan Company, Kyoto, Japan) for staining nuclei. The samples were then analysed using images captured by a charge-coupled device (CCD) camera (RETIGA Exi FAST 1394; Qimaging, Surrey, Canada).

IHC of human and mouse testes with anti-α tubulin antibody; α tubulin-IHC
α tubulin-IHC was performed to show when and how manchettes (microtubules) are formed. The samples were treated with PBS containing 0.1%
Triton X-100 and then with 0.3% H$_2$O$_2$ in methanol for 30 min to block endogenous peroxidase activity. After being blocked with 5% NGS and 3% BSA for 30 min, the samples were treated with anti-$\alpha$ tubulin antibody at 4°C overnight. After washing with PBS, the samples were incubated for 1 h with HRP-conjugated donkey anti-rabbit IgG antibody (1/400). After washing with PBS, the samples were visualized with 0.05% diaminobenzidine as a chromogen. The samples were then counterstained with Mayer’s hematoxylin.

IIF of wild-type and GOPC$^{-/-}$ mouse testicular germ cells with MN13 antibody and MN9 antibody; MN13-IIF and MN9-IIF

IIF was performed to observe the distribution of MN13 in individual testicular germ cells isolated from the testes. MN9 co-staining was performed to stain the developing acrosome and to determine the germ cell stage. Fresh testes were decapsulized and treated in TYH medium (a modified Kreb’s-Ringer bicarbonate solution made by Toyoda, Yokoyama and Hoshi medium; Toyoda et al., 1971) containing 0.01% collagenase (Wako Pure Chemical Industries). After washing in TYH medium, the testes were minced with scissors to collect germ cells, fixed with Zamboni’s solution and then washed in PBS. The germ cells were then treated in PBS containing 0.1% Triton X-100 and attached to pollysine-coated slides. After incubation with MN13 and MN9 antibodies for 1 h, cells were washed with PBS several times and sequentially incubated with Alexa Fluor 488 goat anti-mouse IgM (0.5 μg/ml) for MN13, Alexa Fluor 546 goat anti-mouse IgG (0.5 μg/ml) for MN9 (equatorion) and Hoechst 33258 (1 μg/ml) for nuclei staining at room temperature for 1 h. For a negative control, germ cells were incubated without MN13 antibody but with MN9 antibody, and treated with the same secondary antibodies described above. After washing with PBS, the germ cells were covered with a cover glass in a drop of PBS and visualized with an Olympus BX50 epifluorescence microscope (Olympus Corporation, Tokyo, Japan).

Conventional transmission electron microscope

Transmission electron microscope (TEM) was employed to show the sub-acrosomal layer (marginal ring region). Testes of an adult mouse were fixed with 10% NGS and incubated with MN13 antibody at 4°C overnight. The sperm were rinsed with PBS several times and sequentially incubated with 5 or 10 nm colloidal gold-conjugated goat anti-mouse IgM at 4°C overnight. After washing with PBS, the sperm were fixed with 2.5% glutaraldehyde and post-fixed with 1% OsO$_4$. After washing with distilled water, the sperm were embedded in 2% agar, dehydrated through an ethanol gradient, and then the sections were fixed with 2.5% glutaraldehyde and post-fixed with 1% OsO$_4$. The sections were dehydrated through an ethanol gradient, embedded in Epon 812 and cut into ultrathin sections using an ultramicrotome (Ultracut E; Reichert-Jung, Vienna, Austria). After staining with uranyl acetate and lead citrate, the sections were observed with JEOL 1200 EX TEM (JEOL, Tokyo, Japan).

IIF for normospermic and globozoospermic sperm

IIF was performed to demonstrate that sperm with acrosome (normal sperm) had the MN13 protein, but sperm without normal developing acrosome (globozoospermic sperm) did not have the MN13 protein. MN9 co-staining was performed to check for the presence or absence of the acrosome. Frozen spermatozoa were thawed in a water bath at 37°C and washed with PBS. The sperm were treated with MN9 containing 0.1% Triton X-100 and then incubated in PBS containing MN13 antibody and MN9 antibody at 4°C overnight. The sperm were then rinsed twice with PBS and sequentially incubated in PBS containing Alexa Fluor 488 goat anti-mouse IgM, Alexa Fluor 546 goat anti-mouse IgG and Hoechst 33258 at room temperature for 1 h. The sperm were then rinsed twice with PBS, covered with a cover glass in a drop of PBS or mineral oil (MP Biomedicals, LLC, Solon, OH, USA) and observed with an Olympus BX50 epifluorescence microscope, as described above. Normal wild-type mouse sperm with acrosome were added to the samples of interest as an internal control.

Immunogold electron microscopy with MN13 antibody (MN13-IEM) for mouse developing spermatids and mature sperm from mouse and human

For developing spermatids

IEM was performed to monitor the MN13 in developing spermatids or to monitor the appearance and translocation of MN13 to the PAS/PT during mouse spermatogenesis. Cryopreserved testicular sections were treated with PBS containing 0.1% Triton X-100 for 30 min, blocked with 10% NGS and incubated with MN13 antibody at 4°C overnight. The sections were rinsed with PBS several times and sequentially incubated with 5 nm colloidal gold-conjugated goat anti-mouse IgM at 4°C overnight. After washing in PBS, the sections were fixed with 2.5% glutaraldehyde and post-fixed with 1% OsO$_4$. The sections were dehydrated through an ethanol gradient, embedded in Epon 812 and cut into ultrathin sections using an ultramicrotome (Ultraht E; Reichert-Jung, Vienna, Austria). After staining with uranyl acetate and lead citrate, the sections were observed with JEOL 1200 EX TEM (JEOL, Tokyo, Japan).

For mature sperm from mouse and human

Cryopreserved sperm were blocked with 10% NGS and incubated with MN13 antibody at 4°C overnight. The sperm were rinsed with PBS several times and sequentially incubated with 5 or 10 nm colloidal gold-conjugated goat anti-mouse IgM at 4°C overnight. After washing with PBS, the sperm were fixed with 2.5% glutaraldehyde and post-fixed with 1% OsO$_4$. After washing with distilled water, the sperm were embedded in 2% agar, dehydrated through an ethanol gradient, embedded in Epon 812 and cut into ultrathin sections using an ultramicrotome. After staining with uranyl acetate and lead citrate, the sections were observed with JEOL 1200 EX TEM.

Image acquisition of differential interference contrast and fluorescence

Observation with a differential interference contrast (DIC) and fluorescence system was performed with an Olympus BX50 microscope with a UPlanApo 100 × NA 1.35 oil objective lens, which was equipped with an imaging system with the appropriate filters for fluorescence and a CCD camera. SlideBook 4 software (Intelligent Imaging Innovations, Denver, CO, USA) was used for acquisition and storage of the data.

Results

Appearance and translocation of MN13 to the PAR in mouse spermatids

First, we looked at the appearance or translocation of the MN13 protein during wild-type (GOPC$^{+/+}$) and GOPC$^{-/-}$ spermatogenesis by MN13-IHC and MN13-IIF. In wild-type spermatogenesis, MN13 was first detected faintly on the nascent acrosome (acrosome vesicles) in step 5–6 round spermatids except for the acrosomal granule region (Fig. 2A and F), and then the staining intensity increased and was

Scope (Olympus Corporation, Tokyo, Japan).
detected at the acrosomal region of step 7 round spermatids (Fig. 2B). MN13-immunofluorescence was detected in the middle region between the acrosomal granule and the marginal ring (Fig. 2F and G), and then the intensity became strong at the marginal ring region in step 7 spermatids (Fig. 2G). In step 10–12 elongating spermatids, MN13 was found at the more distal region near the PAR (inset of Fig. 2C and H). In the control lacking MN13 antibody (inset of Fig. 2H), immunostaining for MN13 was negative, while that for MN9 was positive. During late spermiogenesis, in step 14 and 16 elongated spermatids, MN13 was more strongly positive at the PAR, when compared with the MN9 immunostained region (acrosome). Step 14 spermatids were immunostained with MN13, counterstained with methyl green for nuclei, showing strong dark immunostaining due to the close contact of MN13 with nuclei, not with the manchette (inset in D). Note the lack of immunostaining by MN13 and MN9, except for degraded remnants (detached acrosome fragments (red)) near the nucleus (blue) in both insets of I and J. Scale bar = 1 μm in A–J, and insets in C, D, H, I and J.

**Figure 2** Appearance of MN13 or MN13 translocation route. MN13 immunostaining change in developing germ cells. (A–E) MN13-IHC with PaS staining (red for acrosome). Inset in D: MN13-IHC with methyl green staining (green for nuclei). (F–J) MN13/MN9-IIF image. MN9 (acrosome): red. MN13 (oocyte activation-related protein): green. A and F, B and G, C and H, D and I, and E and J: step 5–6, step 7, step 11, step 14 and step 16 spermatids, respectively. Inset in C: a head of a step 10 spermatid. Inset in H: an around step 12 spermatid (control lacking MN13 antibody), in which immunostaining is negative to MN13 (green), but positive to MN9 (red). Insets in I and J: MN13/MN9-IIF image with DIC image. GOPC−/− spermatids at step 14 and step 16, respectively (Hoechst staining for nucleus). In step 5–6 round spermatids, MN13 (arrows) is faintly detected at the acrosomal region except for the acrosomal granule region (small arrows in A and F). Immunofluorescence is found in the middle region between the acrosomal granule and the marginal ring (compare the immunofluorescence intensity indicated by arrowheads in F and G). The staining intensity increases in step 7 round spermatids, as PAS positive substance is accumulated (B and G). In step 10 and 11 spermatids, the MN13-staining (arrows) is extended towards the post-acrosomal region (arrows in C and H), as compared with the MN9 immunostained region (acrosome). During late spermiogenesis, at step 14 (D and I) and 16 (E and J) spermatids, MN13 (arrows) is more strongly positive at the PAR, when compared with the MN9 immunostained region (acrosome). Step 14 spermatids were immunostained with MN13, counterstained with methyl green for nuclei, showing strong dark immunostaining due to the close contact of MN13 with nuclei, not with the manchette (inset in D). Note the lack of immunostaining by MN13 and MN9, except for degraded remnants (detached acrosome fragments (red)) near the nucleus (blue) in both insets of I and J. Scale bar = 1 μm in A–J, and insets in C, D, H, I and J.

**Translocation of MN13 to the PAR in mouse spermatids**

MN13-IEM confirmed the results from MN13-IHC, as demonstrated by the following data. MN13 was first detected on the outer surface of the outer acrosomal membrane in step 5–6 round spermatids (Fig. 3A), and then at the marginal region, i.e. at the marginal ring region formed at the most peripheral region of the acrosome in step 7 round spermatids (Fig. 3B). MN13 started to move towards the PAR in the space between the nuclear envelope and overlying plasma membrane (Fig. 3C in step 11 spermatids). Thereafter, MN13 was found at the amorphous substance on the nuclear envelope just beneath the plasma membrane (Fig. 3C in step 11 spermatids). Step 14 spermatids were immunostained with MN13, counterstained with methyl green for nuclei, showing strong dark immunostaining due to the close contact of MN13 with nuclei. MN13 was not found in any developing spermatids, as shown in Fig. 2 (insets of 2I and J for step 14 and 16 spermatids, respectively).

**Manchette formation in mouse spermatids**

By IHC with anti-α tubulin antibody, manchette (microtubules) were found near the nuclei of both GOPC−/− and GOPC+/+ spermatids typically at step 9 spermatids (Fig. 4A and B). All of the above findings (Figs. 2–4) are summarized in Fig. 5.

**MN13 appearance and manchette formation in human testis**

We investigated MN13 appearance and manchette (microtubule) formation in human spermatogenesis by IHC with anti-MN13 and anti-α...
Figure 3 Location change of MN13 to the PAR. MN13-immunogold staining in developing germ cells revealed by MN13-IEM. (A–E) Step 5–6 round, step 7 round, step 11 elongating, step 14 elongated and step 16 elongated spermatids, respectively. In round spermatid phases, immunogold particles (arrows) are detected on the surface of acrosomal membranes (A) and are then detected at the distal edge of the acrosome, i.e. at the marginal ring area (boxed area in B), which is also shown by routine transmission electron microscopy (inset in B). In elongating or elongated spermatid phases, immunogold particles (asterisks) move towards the PAR in the perinuclear space, that is the widely expanded (C) or narrowed (D) space between the nuclear envelope and plasma membrane, and it is finally organized on the top of the nascent PAS at the PAR (E). Scale bar = 100 nm in A–E. A, acrosome; IAM, inner acrosomal membrane; N, nucleus; NE, nuclear envelope; OAM, outer acrosomal membrane; PAR, post-acrosomal region; PAS, post-acrosomal sheath; PM, plasma membrane.

Figure 4 Manchette (microtubule) formation in GOPC−/− and GOPC+/+ spermatids. IHC with anti-α tubulin antibody. Hematoxylin co-staining. (A and B) Stage 9. Note that manchettes (microtubule bundles; arrows) are formed in both GOPC−/− (A) and GOPC+/− (B) spermatids. Scale bar = 5 μm (shown in A). L, leptotene spermatocytes; P, pachytene spermatocytes.

Expression levels of MN13 in normal and round-headed acrosomeless sperm from mouse and human

Since it is very difficult to get human globozoospermic tests, we investigated the expression level of MN13 in normospermic and round-headed acrosomeless sperm to confirm that MN13 translocation is associated with acrosome formation by MN13-IIF and MN9-IIF. Normal sperm with acrosome from mouse and human were immunopositive to both MN13 and MN9 antibodies (Fig. 7A and B). In contrast, round-headed acrosomeless sperm from GOPC2/2 mice and a human globozoosperm patient were completely negative to both MN13 and MN9 antibodies (Fig. 7A).

Localization of MN13 in mouse and human mature sperm

MN13-IEM for mature sperm from mouse cauda epididymides and human ejaculate also confirmed the results of MN13-IIF, as demonstrated by the following data. Immunogold particles (MN13) were localized on the PAS, which was present just beneath the plasma membrane at the PAR in both mouse and human sperm (Fig. 8A and B). Although not shown here, the ladder-like structure can be found just beneath the plasma membrane when this region was perpendicularly cut as we had shown previously (Toshimori et al., 1991).

Discussion

Initially we hypothesized that the appearance of an oocyte activation ability is stage-specifically regulated and depends on acrosome formation or PAS/PT formation in spermatids. The overall results of this study showing the appearance and movement (organization) of MN13 during spermiogenesis propose that as the acrosome system expands, with underlying PT expansion, oocyte activation-related protein MN13 first appears in early round spermatids and is subsequently translocated towards the PAR, eventually forming the PAS. This scheme is shown in Fig. 9. The presence and absence of factors that affect the PAS formation are summarized in Table I.
An important fact is that MN13 is faintly detected at the peri-acrosomal region in round (step 5–7) spermatids, but the MN13 moves towards the PAR in elongating (step 11) spermatids and becomes clearly detected at the PAR in elongated (step 14 and 16) spermatids. These results would indicate that the oocyte activation ability increases as spermatids develop, and that early round spermatids do not have a sufficient capacity to activate oocytes. Furthermore, acrosome formation appears to be prerequisite for MN13 to be accumulated on the PAS in elongating/elongated spermatids.

The appearance and translocation route for MN13 could be called the acrosome formation-dependent route or the acrosome route. Consistent with previous work, PAS formation occurs after the acrosome is fully formed, which occurs towards the end of spermatid nuclear elongation and condensation (Oko and Maravei, 1994). Interestingly, however, oocyte activation-related or PAS-related MN13 was initially detected on the surface of the acrosomal membrane in step 5–6 round spermatids (equivalent to Sb1 spermatids in human), and then MN13 was localized to the most peripheral and marginal area of the acrosome in step 7 round spermatids (Sb2 spermatids in human). These data indicate that MN13 is not translocated via the subacrosomal layer route, but translocated via the outer acrosomal membrane route. The subacrosomal layer route was previously reported for the translocation of RAB2A, which is within the subacrosomal layer component of the PT and remains in mature sperm (Mountjoy et al., 2008). The subacrosomal layer SubH2Bv is also translocated via the subacrosomal layer route and involved in the acrosome formation, since the subacrosomal layer protein SubH2Bv attaches to and coats the expanding acrosomic vesicles and gets trapped between the inner acrosomal membrane and nuclear envelope, which attaches the acrosome to the nucleus to form the anterior head shape (Aul and Oko, 2002).

In contrast, our data show that after appearance on the acrosome external surface MN13 moves towards the PAR in the space between the nuclear envelope and overlying plasma membrane, as spermatids start to elongate. As such, MN13 is translocated to the PAS at PAR via the cytoplasm that is just above the nuclear envelope; MN13 translocation occurs in conjunction with the elongation of the nuclear envelope. This conclusion is supported by the data demonstrating that MN13 was absent in round-headed acrosomeless human sperm and in sperm from \( \text{GOPC}^{-/-} \) mice, a model mice for globozoospermia (this report and Yao et al., 2002; Ito et al., 2004). Thus,
since the acrosome is not formed or misformed during the acrosome phase of round spermatids but rather forms small acrosome fragments that are destined to be detached from the nuclei (Yao et al., 2002; Ito et al., 2004), MN13 would fail to be translocated to the acrosomal membrane. Therefore, it is plausible that the lack of acrosome results in the absence of some scaffold proteins that are necessary to anchor MN13 to the acrosome surface. These events will be similar in the human globozoospermic patient testes. Thus, oocyte activation ability or the relating protein MN13 is lost in round-headed acrosomeless (globozoospermic patient) sperm.

Another interesting result is that MN13 is not found at regions apart from the nuclear envelope or at the manchette (microtubule bundle) area. Furthermore, we did not detect MN13 immunostaining at the manchette region in $G_{O_{PC}}^{-/-}$ elongating spermatids, in which microtubules were formed. These data indicate that MN13 does not associate with manchette (microtubule) formation, and that MN13 is not translocated to the PAS via the manchette route which was previously reported for another PT protein, PAWP (Fig. 9K–O; Wu et al., 2007).

Thus, our data obtained in this study indicate that there is an acrosome-dependent route (outer acrosomal membrane-marginal ring route) to the PAR for the PAS/PT-associated protein in spermatogenesis, as typically described in this study for MN13 translocation, which begins from the early stage of acrosome formation; possibly termed as ‘early translocation’. This continues throughout spermiogenesis from round to elongated spermatids. In contrast, there is another route, a manchette- (or microtubule-) dependent route, as shown typically for PAWP translocation, which starts from elongating spermatids.

Figure 7 Expression level and localization of MN13 in mature sperm of mouse and human. MN13-IIF and MN9-IIF. MN9 (EQT); red. MN13; green. (A) MN13 immunostaining shown in comparison with MN9 (acrosomal protein equatorin/EQT) in normal mouse sperm and human acrosomeless (globozoospermic patient) sperm. Inset; globozoospermic sperm from a $G_{O_{PC}}^{-/-}$ mouse. Note that control (normal-shaped) mouse sperm are stained with both anti-MN13 and anti-MN9 antibodies (arrow), but staining of round-headed acrosomeless sperm of globozoospermic semen from both human patients (*) and $G_{O_{PC}}^{-/-}$ mouse (inset) was completely negative for these antibodies. (B) Control human sperm that have both acrosomes and PAS/PT were stained positive with both anti-MN9 and anti-MN13 antibodies. Scale bar = 5 μm in A and B.

Figure 8 Localization of MN13 in mature sperm from mouse and human. These typical figures (of control sperm) show the localization of MN13 on PAS. MN13-IEM after removal of plasma membrane by 0.1% Triton X-100 treatment before MN13-IEM. (A) Mouse (cauda epididymal) sperm. (B) Human (ejaculate) sperm. Inset in A and B; higher magnification of boxed area in each figure. MN13 is present on the PAS at the PAR (when typically shown, the ladder-like structure can be found just beneath the plasma membrane, which was removed in this study, as we have shown previously (Toshimori et al., 1991). Note that immunogold particles were found neither on the plasma membrane, nor on the acrosome membrane, nor on the nuclear envelope. Asterisk indicates the broken area of PAS. Scale bar = 500 nm for A and B, and 50 nm for the insets in A and B. AA, anterior acrosome; ES, equatorial segment (posterior acrosome); IAM, inner acrosomal membrane; N, nucleus; NE, nuclear envelope; OAM, outer acrosomal membrane; PAR, post-acrosomal region; PAS, postacrosomal sheath.
spermatids and occurs at the same time as the manchette develops; possibly termed as ‘late translocation’.

In summary, the appearance of MN13 is tightly related to acrosome formation. In round spermatids only a small amount of oocyte activation-related substance (MN13) is found, but in elongating/elongated spermatids enough substance (not only of MN13 but also of PAWP) is accumulated. These lines of fundamental information will be valuable to design the ICSI treatment cycles utilizing testicular

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**Table 1** Summary of the presence and absence of various factors that affect PAS/PT formation.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Human</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild (fertile)</strong></td>
<td><strong>Healthy (fertile)</strong></td>
<td></td>
</tr>
<tr>
<td>Spermatid</td>
<td>Spermatid</td>
<td>Spermatid</td>
</tr>
<tr>
<td>Acrosome</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MN9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Manchette</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>MN13</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PAS/PT</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Presence of MN9 or acrosome formation is thought to be prerequisite to translocate MN13 (oocyte activation-related molecule) to form PAS/PT at PAR, when considering the results of this study together with the summarized schematic drawing shown in Fig. 9.

1 The mouse globozoospermic (round-headed) acrosomeless sperm were from GOPC−/− mice (Yao et al., 2002; Ito et al., 2004).
2,3 The human globozoospermic (round-headed) acrosomeless sperm were reported in our previous studies (Kyono et al., 2008; Ito et al., 2009), but in this study the spermatids from the patient’s testis could not be examined due to the difficulty to obtain the sample; thus described as ‘?’ in Table 1.

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**Figure 9** Schematic drawing as a summary to show the MN13 appearance and translocation route in developing germ cells in mouse and human. Original results are from MN13-IHC and MN13-IIF (Fig. 2) and MN13-IEM (Fig. 3). Red particles indicate MN13. (A–E) MN13 found in step 5–6 round, step 7 round, step 11 elongating, step 14 elongated and step 16 elongated spermatids, respectively. (F–H) Theoretical images in early round spermatids (no photos due to technical difficulty). (I and J) Sc elongating spermatid (I) and 5d elongated spermatid (J). Boxed area shown in B (step 7 spermatid) indicates the marginal ring area of the acrosome, as shown in Fig. 3B. (K–O) These results are from Wu et al. (2007) for PAWP, which showed ‘Manchette-dependent translocation route’. In elongating spermatids, enough amount of MN13 and PAWP is accumulated in PAS (asterisks in D, I and N). A, acrosome; AA, anterior acrosome; AG, acrosomic granule; ES, equatorial segment (posterior acrosome); G, Golgi apparatus; N, nucleus; NE, nuclear envelope; PAR, post-acrosomal region; PAS, post-acrosomal sheath; PM, plasma membrane; PS, posterior acrosome.
germ cells such as ROSI and ELSI, since it has been reported that the oocyte activation rate of ROSI is lower than that of ELSI, which is much lower than conventional ICSI with mature sperm (Tachibana et al., 2009).

**Authors’ roles**

C.I.: conception and design, immunogold staining electron microscopy, immunofluorescence assay, collection and/or assembly of data, data analysis and interpretation, writing and final approval of the manuscript. K.Y.: immunofluorescence assay, data analysis, critical review and final approval of the manuscript. K.K.: collection of clinical data, clinical review and final approval of the manuscript. R.Y.: establishment and maintenance of GOPC-/- mouse and final approval of the manuscript. T.N.: establishment and maintenance of GOPC-/- mouse and final approval of the manuscript. K.T.: conception and design, data analysis and interpretation, writing and final approval of the manuscript.

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**References**


