Subcellular localization of MS4A13 isoform 2 in mouse spermatozoa

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Abstract

To identify upregulated genes during the development of spermatozoa, we performed PCR-selected subtraction analysis of testes RNA samples from 10-day-old and 12-week-old shrews. A transcript, highly homologous to two mouse transcripts, *Ms4a13-1* and *Ms4a13-2*, was differentially regulated. *Ms4a13-2*, but not *Ms4a13-1*, was shown to be primarily expressed in mouse testes in an age-dependent manner. *Ms4a13-2* cDNA contains an open-reading frame of 522 nucleotides, encoding a protein of 174 amino acids, with predicted molecular mass, 19,345 Da. MS4A13-2 protein was expressed along the periphery of nuclei of round and elongated spermatids (steps 3–16) in adult mouse testes, and in the equatorial region of the heads of fresh mature mouse spermatozoa. In addition, MS4A13-2 was found to localize to the outer acrosomal membrane in the equatorial region of heads in fresh spermatozoa. In acrosome-reacted spermatozoa, the MS4A13-2 expression extended to the entire sperm head including the postacrosomal region and acrosomal cap. MS4A family proteins are known to facilitate intracellular protein–protein interactions as ion channel/adaptor proteins by oligomerization, and have important regulatory roles in cellular growth, survival and activation. We report that the MS4A family member, MS4A13-2, may form oligomers in sperm membranes, which may be involved in an interaction with the zona pellucida or cumulus during fertilization.

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Introduction

In many mammals, the sperm–oocyte interaction involves a sequence of processes, including passage of sperm through cumulus cells, binding of sperm to the zona pellucida, penetration of sperm through the zona pellucida and adhesion and fusion of the sperm and oocyte plasma membranes.

In contrast to the diffuse hyaluronic acid matrix-rich structure in many mammals, in the musk shrew, *Suncus murinus*, the cumulus appears as a compact hyaluronidase-insensitive ball of cells, devoid of intercellular matrix (Bedford *et al.* 1994). *S. murinus* spermatozoa consistently bind to the surface of an outer cumulus cell through the face of the intact sperm head, followed by fusion of the sperm plasma and outer acrosomal membranes at this point. Thereafter, individual spermatozoa without an acrosome have been observed within a cumulus cell displaying signs of necrosis. Loss of the acrosome exposes an apical body/perforatorium covered with barbs, which appears to assist in binding of the fertilizing spermatozoa to the zona pellucida. Finally, a spermatozoon fuses with the oocyte plasma membrane (Kaneko *et al.* 2001).

Although the cellular events of mammalian fertilization are not fully understood at the molecular level despite numerous studies, they depend on the numerous different binding patterns of the molecules on the surface of the sperm head (Stein *et al.* 2004, Okabe 2014).

There are more than 16 members of the human and mouse membrane-spanning 4-domains, subfamily A (MS4A) gene family, which map to clusters on human chromosome 11q12–13 and mouse chromosome 19, respectively (Ishibashi *et al.* 2001, Liang & Tedder 2001, Liang *et al.* 2001). The MS4A gene family encodes various cell membrane proteins, the majority of which characteristically contain four membrane-spanning regions and N- and C-terminal cytoplasmic domains (Ishibashi *et al.* 2001, Zuccolo *et al.* 2010, Eon Kuek *et al.* 2016).

MS4A1 (CD20) is involved in an increase in Ca²⁺ conductance, possibly by acting as an ion channel (Bubien *et al.* 1993); this protein can homo-oligomerize into tetramers and physically associate with the antigen-binding component of B cell receptors to positively regulate B cell receptor-induced cytoplasmic Ca²⁺ mobilization (Polyak *et al.* 2008). MS4A2 (FCRB) is

a component of the high-affinity IgE receptor and associates with FcεRIα and FcγR to form a tetrameric receptor complex (αβγ2) expressed at high density on mast cells and basophils (Dombrowicz *et al.* 1998), resulting in amplification of IgE-mediated signals (Lin *et al.* 1996). MS4A1 and MS4A2 are associated with the activation and proliferation of B lymphocytes (Tedder & Engel 1994) and mast cells and basophils (Cruse *et al.* 2010), respectively. Many MS4A family members, including MS4A3 (HTm4; Donato *et al.* 2002, Chinami *et al.* 2005, Kutok *et al.* 2011) and MS4A4B (Xu *et al.* 2010), regulate the cell cycle and act as chemosensors expressed in necklace sensory neurons (Greer *et al.* 2016). Abnormal or absent MS4A protein expression has been observed in various cancers (Bangur *et al.* 2004, Koslowski *et al.* 2008, Kutok *et al.* 2011), while MS4A4 and MS4A6 are associated with the development of late-onset Alzheimer's disease (Hollingsworth *et al.* 2011, Naj *et al.* 2011), and polymorphisms of MS4A2 are commonly associated with atopy (Shirakawa *et al.* 1994).

MS4A5 (TETM4; Hulett *et al.* 2001), MS4A13 (NYD-SP21; Turner *et al.* 2008) and MS4A14 (SP3111; Xu *et al.* 2014) are testis-specific proteins, and MS4A14 is thought to be expressed in the acrosome and the midpiece of the flagella in mature spermatozoa, and to be involved in fertilization. MS4A13 is specifically expressed in the testis (Turner *et al.* 2008); however, its precise localization and function in spermatozoa are yet to be reported.

In this study, we focused on the mouse, because of the ease of obtaining samples for experiments, rather than *S. murinus*, for which various interesting features have been observed in the process of fertilization, and may help to elucidate the diversity of mammalian fertilization. We aimed to identify genes differentially regulated in sperm development to determine the precise expression pattern of MS4A13-2 in mouse testes and spermatozoa before and after the acrosome reaction and to reveal the role of MS4A13-2 in fertilization.

Materials and methods

Animals

Investigations were conducted in accordance with the National Research Council Publication, Guide for Care and Use of Laboratory Animals. The Animal Experiment Committee of Kyushu University investigated our research plan and granted permission for the animal experiments (document numbers A27-058-1 and A29-022-0). Male shrews (*S. murinus*) of three months or more in age, originating from stocks caught in Katmandu, were raised at Kyushu University or at the Laboratory of Animal Management and Resources, Department of Zoology, Faculty of Science, Okayama University of Science. ddY mice were obtained from Kyudo Company (Fukuoka, JP) or bred at Kyushu University.

RNA isolation and PCR-selected subtraction analysis

Total RNA samples were isolated from testes of 10-day-old and 12-week-old isoflurane (5%)-anesthetized shrews using a QuickPrep Total RNA Extraction Kit (GE Healthcare UK Ltd). Poly(A)+ mRNA was purified using an mRNA Purification Kit (GE Healthcare UK Ltd.) and used for PCR-selected subtraction analysis to identify differentially expressed cDNAs, performed with the Clontech PCR-Select cDNA Subtraction Kit (Clontech); cDNAs from the testis of 12-week-old and 10-day-old shrews were used as 'tester' and 'driver' samples, respectively. Subtracted PCR products were cloned into the pGEM-T easy vector (Promega Corp) and sequenced using an ABI PRISM 377 DNA Sequencer (Applied Biosystems), following the manufacturer's instructions. Generated sequences were submitted to BLAST searches of the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>)-NR database and unpublished *Suncus* genome resource (currently undergoing preparation for publication) to determine the identity of clones.

RNA isolation, cDNA synthesis and reverse transcription (RT)-PCR

Total RNA samples from the testis, epididymis, intestine, kidney, spleen, liver and heart of 12-week-old isoflurane-anesthetized ddY mice were isolated using the QuickPrep Total RNA Extraction Kit (GE Healthcare UK Ltd.). Total RNA was also isolated from the testes of 1-, 2-, 3- and 12-week-old mice. First-strand cDNA was synthesized from 2 µg of total RNA, using a PrimeScript first-strand cDNA synthesis Kit (Takara Biotech.) with oligo-(dT) primers. The reverse-transcribed cDNA was used as a PCR template to amplify gene sequences. PCR was performed using GoTaq DNA polymerase (Promega). Primers to amplify the glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*) gene were 5'-TGAAGGTCGGTGTCAACGGATTGGC-3' (forward) and 5'-CATGTAGGCCATGAGGTCCACCAC-3' (reverse). The primers used to amplify the mouse open-reading frames (ORFs), *Ms4a13-1* and *Ms4a13-2*, were 5'-ATGGAATGTAGAAATCCAAAAGTATC-3' (forward) and 5'-AGGAGTTGCCTTCCACTACTTC-3' (reverse). PCR products were analyzed by agarose gel electrophoresis.

Antibody production

Two peptides, VSYRNYGQAKLGR and AGNQEETLESVTEVVEGNS, derived from the hydrophilic regions of the putative extracellular region and C-terminus, respectively, of mouse MS4A13-2 were used to raise antibodies. The peptides were coupled to keyhole limpet hemocyanin (Pierce), dissolved in sterilized water, emulsified with Freund's adjuvant and injected into a rabbit. Antisera were collected within 2 weeks after the five injections. Affinity purification of the antibodies was carried out over matrix-immobilized peptide coupled to 2-fluoro-1-methylpyridinium toluene-4-sulfonate-activated Sephadex (Seikagaku Kogyo, Chiyoda-ku, Tokyo, Japan). The concentrations of two purified antibodies were estimated using a protein assay.

Specificity of the anti-MS4A13-2 antibodies

Mouse *Ms4a13-2* mRNA contains an ORF of 522 nucleotides encoding 174 amino acids (AAs). Nucleotides encoding the carboxy-terminus (AAs 75–174) of MS4A13-2, with a predicted molecular weight of approximately 11.2 kDa, were PCR-amplified using the primers 5'-GCCAAGCTTGTAATGGCAGCAAAATATCAAAGTAGAC-3' (forward) and 5'-GAGGGATCCGAGTTGCCTTCCACTACTTC-3' (reverse). The PCR product was then digested with HindIII and BamHI and cloned in-frame into the pTagRFP-N expression vector (Evrogen JSC, Moscow, Russia). The PCR product sequence was confirmed by Sanger sequencing using an ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Plasmids were transfected into COS7 cells, using Lipofectamine 2000 reagent (Invitrogen), following the manufacturer's instructions. After 24 h of culture in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), transfected cells were either lysed directly in SDS-PAGE buffer (62.5 mM Tris-HCl; 2% (w/v) SDS; 10% (v/v) glycerol; 0.005% (w/v) bromophenol blue; 5% (v/v) 2-Mercaptoethanol) for electrophoresis and immunoblot analysis or fixed and examined under a fluorescence microscope (Leica DM RXA; Leica Microsystems). Non-transfected COS7 cells were used as controls.

For immunoblot analysis, control and transfected COS7 cells prepared for SDS-PAGE were separated by electrophoresis on 15% (w/v) acrylamide gels; separated proteins were either stained with Coomassie brilliant blue (40% (v/v) methanol; 10% (v/v) acetic acid; 0.1% (w/v) Coomassie brilliant blue R250) or transferred to PVDF membranes (Hybond-P; GE Healthcare UK). The membranes were incubated for 2 h with either a polyclonal anti-RFP-Tag antibody (Invitrogen) diluted 1:5000, or each anti-MS4A13-2 antibody diluted 1:5000, in blocking buffer (phosphate-buffered saline (PBS) containing 5% (w/v) non-fat milk and 0.05% (v/v) Tween-20), followed by incubation with anti-rabbit IgG conjugated with HRP (BioRad) diluted 1:2000 in the same buffer. Antigen-antibody complexes were visualized using an ECL-Plus detection Kit (BioRad).

For immunofluorescence microscopy, control and transfected COS7 cells were fixed using 4% (w/v) paraformaldehyde in PBS at 4°C, washed in PBS and treated with 0.1% (v/v) Triton X-100 for 5 min. Cells were then stained with each anti-MS4A13-2 antibody diluted 1:200, followed by incubation with FITC-conjugated goat anti-rabbit IgG (GE Healthcare UK). For nuclear staining, immunostained samples were incubated for 30 min with PBS containing Hoechst 33342. Samples were then washed with PBS before examination under a fluorescence microscope (Olympus BX-50; Olympus Optical, or Leica DM RXA). As a control, the two anti-MS4A13-2 antibodies were substituted with pre-immune rabbit sera.

Immunoblot analyses of mouse testis

Testes of isoflurane-anesthetized adult mice were immersed in PBS on ice, and capsules (tunica albuginea) torn away using tweezers. Seminiferous tubules released from testes were

untangled and washed several times in PBS at 4°C with gentle agitation to remove interstitial cells. Seminiferous tubules were homogenized and extracted in RIPA buffer (50 mM Tris, pH 7.2; 1 mM EDTA; 0.1% (w/v) SDS; 0.1% (w/v) Na deoxycholate; 1% (v/v) Nonidet P-40) plus protease inhibitor cocktail (Roche Diagnostics GmbH), followed by centrifugation at 20,000 g at 4°C for 20 min. Clarified supernatants in RIPA buffer were used as samples for immunoblot analysis with the anti-MS4A13-2 antibody (extracellular region) and immunoprecipitation with the anti-MS4A13-2 antibody (C-terminus). Samples for immunoprecipitation were incubated with either 0.003% (w/v) anti-MS4A13-2 antibody (C-terminus) or pre-immune serum for 12 h at 4°C with mild agitation. Protein A-agarose (0.05% (w/v); Roche Diagnostics GmbH) was then added, and incubation continued for 4 h at 4°C. The resultant protein A-agarose-antibody-antigen complexes were washed five times with RIPA buffer, and twice with 50 mM Tris-Cl (pH 6.9). After washing, antigen was eluted from the beads using SDS-PAGE sample buffer.

Clarified supernatant in RIPA buffer and immunoprecipitated proteins were separated by SDS-PAGE on a 15% (w/v) acrylamide gel and either stained with Coomassie brilliant blue or transferred to PVDF membranes. The membranes from clarified supernatant in RIPA buffer and immunoprecipitated proteins were incubated for 2 h with anti-MS4A13-2 antibody (extracellular region), and for 3 h with anti-MS4A13-2 antibody (C-terminus), respectively, diluted 1:5000 with blocking buffer, followed by incubation with HRP-conjugated goat anti-rabbit IgG (BioRad), and HRP-conjugated protein G (BioRad), respectively, diluted 1:2000 in the same buffer. Antigen-antibody complexes were visualized using an ECL-Plus detection Kit.

Immunoblot analyses of mouse spermatozoa from epididymides

Spermatozoa collected from the caput and cauda epididymides of adult mice were purified by Percoll density gradient centrifugation, as reported previously (Awano *et al.* 1993). Samples were then dissolved in SDS-PAGE buffer for electrophoresis.

Samples prepared for SDS-PAGE were separated on 15% (w/v) acrylamide gels and either stained with Coomassie brilliant blue or transferred to PVDF membranes. The membranes were incubated for 2 h with anti-MS4A13-2 antibody (extracellular region or C-terminus) diluted 1:5000 in blocking buffer, followed by incubation with HRP-conjugated goat anti-rabbit IgG diluted 1:2000 in the same buffer. Antigen-antibody complexes were visualized using an ECL-Plus detection Kit.

Solubilization of sperm flagella, heads and membranes

Purified spermatozoa were diluted in PBS and probe-sonicated (TOMY UR-150P; Tominaga Works, Tokyo, Japan) at maximum power on ice for three 10 s bursts to separate sperm heads from flagella. The extent of cell breakage was monitored microscopically. Sonicated samples suspended in PBS containing 0.9 M sucrose were layered onto 2.05 M

sucrose in 10 mM Tris-Cl (pH 7.0), and onto 2.2 M sucrose in 10 mM Tris-Cl. The samples were centrifuged at 100,000 *g* for 60 min at 4°C. The band at the interface of 0.9/2.05 M sucrose was collected as the flagella fraction and pellets at the bottom of tubes were collected as the sperm head fraction. Samples were washed once in PBS and subjected to a second round of sucrose-density centrifugation for further purification. Highly purified flagella and sperm heads were extracted for 1 h in RIPA buffer, after which samples were centrifuged for 15 min at 20,000 *g* to separate extracts from undissolved pellets. Supernatants were dissolved in SDS-PAGE buffer for electrophoresis. An aliquot of probe-sonicated spermatozoa was centrifuged for 10 min at 1000 *g*, followed by 15 min at 5000 *g* twice. Resultant supernatants, in which neither nuclei nor flagella contamination were found, were further centrifuged at 100,000 *g* for 60 min at 4°C to obtain the membrane fraction (mainly containing plasma membrane), which was dissolved in SDS-PAGE buffer for electrophoresis. Samples were processed for immunoblot analysis, as described earlier.

Immunohistochemistry

Caput and cauda epididymides and testes of adult mice were fixed in 4% (w/v) paraformaldehyde in PBS at 4°C for 4 h, washed thrice in PBS, incubated in PBS containing 50 mM NH₄Cl for 30 min and then rinsed in PBS. After infiltration with 20% (w/v) sucrose in PBS, they were immersed in OCT compound (Tissue-Tek; Miles, Inc., Elkhart, IN, USA) and immediately frozen in liquid nitrogen. Frozen sections (10 µm) were cut using a cryostat (CM1850; Leica Biosystems), and then attached to poly-L-lysine (Sigma-Aldrich)-coated glass slides for immunolabeling.

Fresh spermatozoa collected from caput and cauda epididymides of adult mice were fixed in 4% (w/v) paraformaldehyde in PBS at 4°C for 1 h, washed in PBS and then mounted on poly-L-lysine-coated glass slides for immunolabeling.

The sections and spermatozoa on glass slides were washed in PBS, exposed to 0.1% Triton X-100 in PBS and then immunostained with the anti-MS4A13-2 antibody (extracellular region or C-terminus) diluted 1:200 with blocking buffer, followed by incubation with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Cy3) diluted 1:1000 in the same buffer. For double immunostaining, sections and spermatozoa were stained with the anti-MS4A13-2 antibody (C-terminus) and the monoclonal anti-MN7 antibody diluted 1:200 in blocking buffer, followed by incubation for with goat anti-rabbit IgG conjugated with Cy3 and anti-mouse IgG conjugated with FITC. The 90-kDa acrosome protein recognized by the MN7 antibody localizes to the acrosomal vesicle, including the acrosomal granule in early round spermatids, and at the anterior acrosome, but not at the equatorial region in elongated spermatids and mature spermatozoa (Tanii *et al.* 1994, Ito & Toshimori 2016). MN7 antigen can therefore be used as a marker protein for the acrosome, but not for the equatorial region. Alternatively, pure *Arachis hypogaea* lectin conjugated with FITC (FITC-PNA; 1 mg/mL, EY Lab, San Mateo, CA, USA) was used to label acrosomes of spermatozoa instead of the monoclonal anti-MN7 antibody.

For nuclear staining, immunostained samples were incubated for 30 min with PBS containing Hoechst 33342. The samples were washed with PBS before examination by fluorescence microscopy. For controls, primary antibodies were substituted with pre-immune sera.

Immunoelectron microscopy

For pre-embedding immunoelectron microscopy, purified mouse spermatozoa collected from the caput and cauda epididymides of adult mice were probe-sonicated (TOMY UR-150P) at maximum power in 50 mM Tris-HCl (pH 8.0) on ice for 10 s bursts, and then centrifuged at 5000 *g* for 10 min to generate pellets. The pellets were fixed in PBS containing 6% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde at 4°C for 3 h, washed in PBS and incubated for 30 min in PBS containing 0.5% (w/v) bovine serum albumin and 0.5% (w/v) gelatin. Samples were then immunostained for 3 h with the anti-MS4A13-2 antibody (C-terminus), followed by incubation for 2 h with 10 nm gold particle-conjugated goat anti-rabbit IgG (GE Healthcare UK). For controls, the primary antibody was replaced by pre-immune serum. After immunostaining, samples were washed in PBS and fixed in 3% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% (v/v) osmium tetroxide, dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections (80 nm) cut with a diamond knife were mounted on copper grids and examined in a Hitachi H-600 electron microscope (Tokyo, Japan) at 75 kV, after staining with 2% (w/v) uranyl acetate and lead citrate (Sigma-Aldrich).

Triton X-100 treatment of spermatozoa collected from the caput and cauda epididymides

Spermatozoa collected from the caput and cauda epididymides of adult mice were purified by Percoll density gradient centrifugation and extracted for 1 h in PBS containing 1% (v/v) Triton X-100 and protease inhibitor cocktail, after which the samples were centrifuged for 3 min at 3300 *g* to separate extracts from undissolved pellets. To observe the ultrastructure of spermatozoa treated with Triton X-100, 1% Triton X-100-treated spermatozoa were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed in 1.3% osmium tetroxide, dehydrated in a graded ethanol series and embedded in epoxy resin. Samples were then processed for electron microscopy, as described earlier.

Both the supernatant and the pellet obtained after treatment with 1% Triton X-100 were dissolved in SDS-PAGE buffer for electrophoresis and processed for immunoblot analyses. Membranes were incubated for 2 h with either polyclonal anti-MS4A13-2 antibody (C-terminus) diluted 1:5000, monoclonal anti-MN7 antibody diluted 1:2000, or monoclonal anti- α -tubulin antibody (clone No. 10G10) (Wako Pure Chemical) diluted 1:1000, in blocking buffer, followed by incubation with anti-rabbit or anti-mouse IgG conjugated with HRP (BioRad) diluted 1:2000 in the same buffer. Antigen-antibody complexes were visualized using an ECL-Plus detection Kit (BioRad).

1% Triton X-100-treated spermatozoa collected from caput and cauda epididymides of adult mice were fixed in 4% (w/v) paraformaldehyde in PBS at 4°C for 1 h, washed in PBS and then mounted on poly-L-lysine-coated glass slides for immunolabeling. Samples were processed for immunostaining and examination by fluorescence microscopy, as described earlier.

Acrosome reaction in vitro

Spermatozoa isolated from cauda epididymides of mature male mice were capacitated for 1 h at 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air in TYH capacitation medium (modified Krebs-Ringer bicarbonate; 119.37 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂·2H₂O, 1.19 mM MgSO₄·7H₂O, 1.19 mM KH₂PO₄, 25.07 mM NaHCO₃, 5.56 mM glucose, 0.5 mM sodium pyruvate, 75 µg/mL potassium penicillin G, 50 µg/mL streptomycin sulfate, 2 µg/mL phenol red; pH 7.2; Toyoda *et al.* 1971) supplemented with BSA (4 mg/mL). To undergo the acrosome reaction, capacitated spermatozoa were treated at 37°C with or without (control) 10 µM calcium ionophore A23187 (Sigma-Aldrich). Spermatozoa treated for 0, 5, 10, 15, 20, 30 and 60 min with calcium ionophore and for 30 min without calcium ionophore were fixed with 3% (w/v) paraformaldehyde for 15 min, washed in PBS and mounted on poly-L-lysine-coated glass slides. Spermatozoa immunostained for MS4A13-2 (C-terminus), MN7 (or PNA) and nuclei were examined by fluorescence microscopy, as described earlier. At least 200 spermatozoa treated for each time with calcium ionophore A23187 were examined for the presence or absence of PNA and change in localization of MS4A13.

Spermatozoa treated for 30 min with or without calcium ionophore A23187 were dissolved in SDS-PAGE buffer for electrophoresis. Samples were processed for immunoblot analysis, as described earlier.

Treatment with BS3 crosslinker in acrosome-reacted spermatozoa

BS3 (Bis (sulfosuccinimidyl) suberate, Thermo Scientific) was used to investigate whether MS4A13-2 forms hetero/homo-oligomers in sperm membranes. Spermatozoa treated for 30 min with calcium ionophore A23187, as described earlier, were exposed for 1 h at 4°C to PBS in the presence or absence (control) of BS3 crosslinker at a final concentration of 2 mM. The reaction was quenched by the addition of Tris-HCl (pH 7.0) to a final concentration 20 mM and incubated at 4°C for 30 min. Spermatozoa were then centrifuged at 5000g for 10 min to generate pellets. The pellets were dissolved in SDS-PAGE buffer for electrophoresis and processed for immunoblot analysis, as described earlier.

IVF investigation

Mature female mice were induced to ovulate by consecutive injections of 5 IU eCG (SEROTROPIN; ASKA Animal Health Tokyo, Japan) followed 48 h later by 5 IU hCG (GONATROPIN; ASKA Animal Health).

Oocytes with cumulus were collected 13–15 h after the injection of hCG by rupturing the ampulla of oviducts under oil in an IVF dish (Falcon Plastics, Brookings, SD, USA). For the zona pellucida-intact experiment, in which capacitated spermatozoa inseminated zona pellucida-intact oocytes with cumulus, the oocytes were transferred to another IVF dish containing TYH medium (pH 7.2) equilibrated with 5% CO₂ in air at 37°C. For the zona pellucida-free experiment, in which spontaneous acrosome-reacted spermatozoa inseminated zona pellucida-free oocytes, oocytes were treated with 0.05% hyaluronidase in TYH medium for 10 min to remove cumulus cells, and then the cumulus-free oocytes were washed twice in TYH medium. The zona pellucida was removed from these oocytes by brief treatment with low-pH (pH 2.5) TYH medium. Zona pellucida-free oocytes were transferred to an IVF dish that contained TYH medium (pH 7.2) equilibrated with 5% CO₂ in air, and then incubated for 1 h at 37°C for recovery before use (Toshimori *et al.* 1998).

Spermatozoa collected from the cauda epididymis of mature male mice, as described earlier, were allowed to disperse into 400 µL TYH medium and capacitated for 1 h at 37°C. The capacitated spermatozoa were diluted to a final concentration of 4 × 10⁴ sperm/mL in TYH medium with or without antiserum (100 µg/mL), anti-MS4A13-2 antibody (C-terminus; 100 µg/mL) or anti-MS4A13-2 antibody (extracellular region; 25, 50, 100 µg/mL), and preincubated for 30 min before insemination.

The zona pellucida-intact and -free oocytes were then added to the spermatozoa in TYH medium containing antibodies. The formation of two cells was determined by phase-contrast microscopy (Leica DM RXA) 24 h after insemination.

Results

Isolation of Ms4a13-2 by PCR-selected subtraction analysis

To identify genes developmentally upregulated in the shrew testis, transcripts derived from the testes of 10-day-old and 12-week-old animals were examined by PCR-selected subtraction analysis, which resulted in isolation of 174 DNA fragments (data not shown, currently undergoing analysis). Sequence data from these clones were subjected to a homology searching of the NCBI database and an unpublished *Suncus* genome resource (currently undergoing preparation for publication) using the program, BLAST. One of the genes was highly homologous to two mouse transcripts: membrane-spanning 4-domains, subfamily A, member 13, transcript variant 1 (*Ms4a13-1*; NCBI Reference Sequence, NM_198224) and *Ms4a13* transcript variant 2 (*Ms4a13-2*; NCBI Reference Sequence, NM_001347435), and to a *Suncus* transcript: *Ms4A13* (*Suncus* genome resource, reference number R57005, currently under analysis). A BLAST search against the NCBI mouse genomic database revealed that *Ms4a13* was composed of 8 exons (Fig. 1A) and located on mouse chromosome 19A, and that exon 7 of *Ms4a13-2* mRNA was removed by splicing (Fig. 1B). The cDNAs of

Figure 1 (A) Schematic representation of the mouse *Ms4a13* gene. Numbered boxes indicate exons and lines connecting the exons represent introns. The *Ms4a13* gene is composed of 8 exons. (B) Schematic representation of partial *Ms4a13-1* and *Ms4a13-2* mRNAs. The *Ms4a13-1* and *Ms4a13-2* mRNAs are composed of 8 and 7 exons, respectively, because exon 7 of *Ms4a13-2* is removed by splicing. (C) The cDNA coding sequences of mouse *Ms4a13-1* (accession number NM_198224) and *Ms4a13-2* (accession no. NM_001347435). The cDNAs of mouse *Ms4a13-1* and *Ms4a13-2* contain open-reading frames (ORFs) of 609 and 522 nucleotides, encoding proteins of 203 (accession no. NP_937867) and 174 (accession no. NP_001334364) amino acid residues, respectively. Deletions are indicated by dashes. Asterisks denote shared identical nucleotides between the two cDNAs. The DNA fragment located between the two white arrows, which is shared by *Ms4a13-1* and *Ms4a13-2*, was amplified by RT-PCR in Fig. 1D. (D) Left panel, RT-PCR analysis to examine the expression levels of *Ms4a13-1* and *Ms4a13-2* in various organs of adult mouse. *Ms4a13-2* (523 bp) is highly expressed in testis and epididymis, and detected at much lower levels in kidney, spleen, liver and heart; however, *Ms4a13-1* (610 bp) was not detected in any organ examined. Right panel, RT-PCR analysis of the expression of *Ms4a13-2* in testis of 1-, 2-, 3- and 12-week-old mice. A PCR product of 523 bp was first detected at 2-week postpartum and continued to be observed into adulthood. *G3pdh* was amplified as a control. (E) The cDNA coding sequence and predicted amino acid sequence of mouse *MS4A13-2*. The termination codon, TGA, is indicated by an asterisk. *MS4A13-2* contains transmembrane regions at AAs 14–36, 56–77, 87–109 and 131–153 (underlined). Two polyclonal antibodies were raised against the synthetic peptides, VSRYNYGQAKLGR and AGNQEETLESVTEVEGNS, corresponding to AAs 115–127 (putative extracellular region) and 156–174 (C-terminus) of *MS4A13-2* (boldface type), respectively. An *Ms4a13-2* DNA fragment of 300 bp in length, located between the two arrows, was amplified by PCR and used for transfection into COS7 cells.

Ms4a13-1 and *Ms4a13-2* contain ORFs of 609 and 522 nucleotides, encoding proteins of 203 (NCBI Reference Sequence, NP_937867) and 174 (NCBI Reference Sequence, NP_001334364) AA residues, respectively. The deduced nucleotide sequences of the mouse *Ms4a13-1* and *Ms4a13-2* ORFs are shown in Fig. 1C. The predicted molecular masses and isoelectric points of mouse MS4A13-1 and MS4A13-2 are 22,783 Da and 19,345 Da and 9.11 and 8.26, respectively. A BLAST search against the *Suncus* genome resource revealed that the *Suncus Ms4a13* cDNA contains an ORF of 519 nucleotides, encoding proteins of 173 AA residues (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

We examined the expression of *Ms4a13-1* and *Ms4a13-2* mRNAs in various organs of adult mice by RT-PCR. A 523-bp *Ms4a13-2* RT-PCR product was strongly expressed in the testis and epididymis, and was detected at much lower levels in the kidney, spleen, liver and heart. A 610-bp *Ms4a13-1* RT-PCR product was undetectable in all organs examined (Fig. 1D, left panel). We next performed RT-PCR to study the developmental expression of *Ms4a13-2* mRNA in testis of 1-, 2-, 3- and 12-week-old mice and found that it was first detected at 2 weeks postnatally and continued to be expressed into adulthood (Fig. 1D, right panel).

SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) predicted that AA residues 14–36, 56–77, 87–109 and 131–153 of mouse MS4A13-2 form transmembrane regions (Fig. 1E), indicating that MS4A13-2 has four potential membrane-spanning domains, with N- and C-terminal cytoplasmic domains. In contrast, analyses with SMART (<http://smart.embl-heidelberg.de>), TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) and Ensembl (<http://www.ensembl.org/index.html>) suggested that mouse MS4A13-2 has three potential transmembrane regions with an N-terminal cytoplasmic domain and an extracellular C-terminus, and that AAs 15–117 of mouse MS4A13-2 comprise a CD20 domain.

Specificity of the anti-MS4A13-2 antibodies

To examine the expression and localization of MS4A13-2 protein in the mouse testis, epididymis and spermatozoa, two polyclonal antibodies were raised against two synthetic peptides (VSRYNYGQAKLGR and AGNQEETLESVTEVVEGNS) corresponding to AAs 115–127 (putative extracellular region) and 156–174 (C-terminus) of mouse MS4A13-2, respectively (Fig. 1E).

Specificity of the affinity-purified anti-MS4A13-2 antibodies was first examined by immunoblot of RFP-tagged MS4A13-2 (AAs 75–174) expressed in COS7 cells. As shown in Fig. 2A, both the anti-MS4A13-2 (extracellular region, Fig. 2A, third panel from the right; and C-terminus, Fig. 2A, second panel from the right) antibodies and the anti-RFP antibody (Fig. 2A, right

panel) recognized a protein migrating at approximately 38 kDa, which was not detected in control samples (untransfected cells).

Next, COS7 cells transfected with RFP-tagged MS4A13-2 were examined by fluorescence microscopy after labeling with each of the two anti-MS4A13-2 antibodies (green color). The RFP-tagged MS4A13-2 (AAs 75–174) detected by the anti-MS4A13-2 antibodies (extracellular region, Fig. 2B, upper panel; and C-terminus, Fig. 2B, lower panel) appeared as small aggregates in the cytoplasm. The fluorescence representing the anti-MS4A13-2 antibodies overlapped with that representing RFP. Replacement of the anti-MS4A13-2 antibodies with pre-immune sera produced little or no green labeling in the cells (Fig. 2B, right panel). These results demonstrate that both the anti-MS4A13-2 antibodies specifically recognize RFP-tagged MS4A13-2 expressed in COS7 cells. The two anti-MS4A13-2 antibodies were used for subsequent experiments.

Detection of MS4A13-2 in mouse testis

We next performed immunoblot analysis to investigate the expression of MS4A13-2 in mouse testis. On blots containing proteins extracted in RIPA buffer and immunoprecipitated with the anti-MS4A13-2 antibody (C-terminus) from mouse seminiferous tubule, the two anti-MS4A13-2 antibodies (extracellular region, Fig. 3A, lane 1 in right panel; and C-terminus, Fig. 3A, lane 2 in right panel) recognized a protein migrating at approximately 19 kDa, close to the theoretical molecular weight (19.3 kDa) calculated from the MS4A13-2 AA sequence, deduced from the cDNA sequence. The 19-kDa protein was not detected when control pre-immune serum was used instead of the anti-MS4A13-2 antibody (C-terminus; Fig. 3A, lane 3 in right panel). These data indicate that the antibodies, which were raised against two synthetic MS4A13-2 peptides, specifically recognize MS4A13-2 protein expressed in the mouse testis.

Immunolocalization of MS4A13-2 protein in the mouse testis

We used fluorescence microscopy of frozen sections of adult mouse testis stained with anti-MS4A13-2 antibodies (extracellular region and C-terminus) to determine the cell types expressing MS4A13-2 protein. In cross sections of stages I–XII of the cycle of the mouse seminiferous epithelium, MS4A13-2 was detected along the periphery of nuclei of round (Fig. 3B, upper panel) and elongating (Fig. 3B, lower left panel) spermatids, while spermatogonia, spermatocytes, Sertoli cells and interstitial cells were devoid of the signal (Fig. 3B, upper panel). No specific staining was visible in controls stained with pre-immune serum (Fig. 3B, lower right panel). No distinct difference in immunolabeling was discernible

Figure 2 (A) Immunoblot analysis demonstrating the specificity of the anti-MS4A13-2 antibodies. Proteins extracted from COS7 cells expressing RFP-tagged truncated MS4A13-2 (transfected), as well as non-transfected COS7 cells (control), were separated by SDS-PAGE and either stained with Coomassie brilliant blue (left panel) or subjected to immunoblot analysis using anti-MS4A13-2 (extracellular region, Fig. 2A, third panel from the right), anti-MS4A13-2 (C-terminus, Fig. 2A, second panel from the right) or anti-RFP (right panel) antibodies. A protein migrating at approximately 38 kDa was detected by the three antibodies in transfected cells, and not in non-transfected cells. The molecular masses (kDa) of the standard proteins are shown on the left. (B) Fluorescence microscopy demonstrating the specificity of the anti-MS4A13-2 antibodies. COS7 cells transfected with the C-terminus of MS4A13-2-RFP were examined by immunofluorescence microscopy. The green signals from the two anti-MS4A13-2 antibodies (E, extracellular region, upper panel; and C, C-terminus, lower panel; each leftmost panel) and the red signal from RFP (panels second from the left) were merged (panels third from the left, yellow color). Nuclear DNA was stained using Hoechst 33342 (blue color). In transfected COS7 cells, truncated MS4A13-2-RFP appears as small aggregates in the cytoplasm. Little or no green labeling was visible in control samples stained with pre-immune sera (rightmost panels). Scale bars, 10 μ m.

between the two anti-MS4A13-2 antibodies against the extracellular region (not shown) and C-terminus.

To determine the subcellular distribution of MS4A13-2 protein in mouse spermatids, fluorescence microscopy was carried out by double-staining mouse testis with anti-MS4A13-2 antibody (C-terminus) and MN7 monoclonal antibody. At higher magnification, MS4A13-2 was initially observed at acrosomal vesicles labeled by MN7 antibody (a marker protein for the acrosome, but not for the equatorial region) in step 3 round spermatid at stages II–III (Fig. 3C, step 3). As the acrosomal vesicle extended, MS4A13-2 appeared to cover the outer surface of the acrosomal vesicle (Fig. 3C, step 5), and to gradually extend backward (Fig. 3C, steps 7 and 8). In the elongating spermatid, MS4A13-2 was detected mainly along the posterior part of nuclei, separate from the MN7 signal (Fig. 3C, elongating spermatid).

Detection of MS4A13-2 in mouse spermatozoa

We next examined whether MS4A13-2 was expressed in mouse spermatozoa. Spermatozoa derived from the caput and cauda of mouse epididymides were purified by Percoll density gradient centrifugation and subjected

to SDS-PAGE and immunoblotting. We found that the two anti-MS4A13-2 antibodies (extracellular region, Fig. 4A; and C-terminus, not shown) recognized a single protein migrating at 19 kDa in spermatozoa derived from both caput and cauda of epididymides, indicating that MS4A13-2 is expressed in spermatozoa.

To investigate the subcellular distribution of MS4A13-2 in mouse spermatozoa in the epididymis, membrane (mainly containing plasma membrane), head (without membrane fraction) and flagella (without membrane fraction) fractions of spermatozoa were subjected to immunoblot analysis using the anti-MS4A13-2 antibody (C-terminus). MS4A13-2 was detected strongly in the head fraction, suggesting that MS4A13-2 is primarily expressed in the sperm head without the plasma membrane (Fig. 4B).

To examine the localization of MS4A13-2 in mouse spermatozoa, we performed fluorescence immunocytochemistry. In both the caput (not shown) and the cauda of adult mouse epididymides, MS4A13-2 was detected in sperm, but not in epididymal epithelial cells (Fig. 4C, upper panel). At high magnification of sperm from both caput (not shown) and cauda epididymides, MS4A13-2 was visible along the periphery of nuclei in the sperm heads (Fig. 4C, lower left panel). No distinct

Figure 3 (A) Immunoblot analyses of proteins extracted in RIPA buffer and immunoprecipitated from seminiferous tubules using the two anti-MS4A13-2 antibodies. Clarified supernatants in RIPA buffer from seminiferous tubules of adult mouse testes were immunoprecipitated using either the anti-MS4A13-2 antibody (C-terminus) or pre-immune serum. Proteins extracted in RIPA buffer (lane 1) and immunoprecipitated (lane 2 and 3) were separated by SDS-PAGE, and stained with Coomassie brilliant blue (left panel) or subjected to immunoblot analyses using the anti-MS4A13-2 antibodies (extracellular region, lane 1 in right panel; and C-terminus, lanes 2 and 3 in right panel). The anti-MS4A13-2 antibodies recognized a protein migrating at approximately 19 kDa (black arrow) in samples extracted in RIPA buffer (lane 1 in right panel) and immunoprecipitated (lane 2 in right panel). No band was detected when control pre-immune serum was used instead of the anti-MS4A13-2 antibody (lane 3 in right panel). White arrow, heavy chains (HC) of IgG. Molecular masses (kDa) of standard proteins are shown on the left. (B) Immunohistochemical localization of MS4A13-2 in the seminiferous tubules of adult mouse testis. Frozen sections of adult mouse testis stained with the anti-MS4A13-2 antibody (C-terminus; red) were counter-stained with Hoechst 33342 to visualize nuclear DNA (blue). Immunofluorescence representing MS4A13-2 was detected along the periphery of round (step 8 spermatid in stage VIII, upper panel) and elongating (lower left panel) spermatid nuclei (arrows), and was not detected in spermatogonia, spermatocytes, somatic Sertoli cells or testis interstitial cells (upper panel). No specific staining was observed using the pre-immune serum control (lower right panel). Lu, lumen of the seminiferous tubules. Scale bars, 10 µm. (C) Subcellular distribution of MS4A13-2 protein in mouse spermatids. Frozen sections of adult mouse testis were double-stained with anti-MS4A13-2 antibody (C-terminus; red) and anti-MN7 monoclonal antibody (green; marker of the acrosome). Nuclear DNA was stained using Hoechst 33342 (blue). Three color images are superimposed in right panels (merge). MS4A13-2 is visible as small round area of immunofluorescence in the MN7 positive acrosomal vesicle in a step 3 round spermatid at stage II–III (step 3). As the acrosomal vesicle extends, MS4A13-2 immunolabeling covers the outer surface of the acrosomal vesicle (step 5), gradually extending backward (step 7 and step 8). In the elongating spermatid, MS4A13-2 is detected primarily along the posterior part of nuclei, separate from the MN7 signal (elongating spermatid). Scale bars, 3 µm in steps 3–8, and 1 µm in elongating spermatid.

immunosignal was observed in controls labeled with pre-immune serum (Fig. 4C, lower right panel). When spermatozoa isolated from caput and cauda mouse epididymides were immunostained, MS4A13-2 was strongly detected in the equatorial region of sperm heads marked by FITC-PNA (Fig. 4D), and weakly in the principal piece of sperm flagella (not shown).

To determine the localization of MS4A13-2 in sperm heads in detail, mouse caput and cauda epididymis spermatozoa treated with sonication to almost remove plasma membranes were evaluated by pre-embedding immunoelectron microscopy using the anti-MS4A13-2

antibody (C-terminus) and 10-nm gold-conjugated secondary antibody. The majority of MS4A13-2 was associated with naked outer acrosomal membrane at the equatorial region (Fig. 4E, left panel). The outer acrosomal membrane at the acrosomal cap exhibited very little labeling, even when the outer acrosomal membrane was exposed by removal of the majority of the plasma membrane by sonication. Little or no labeling was observed in control samples stained with pre-immune serum (Fig. 4E, right panel). No distinct difference in immunolabeling was discernible between caput (not shown) and cauda epididymis spermatozoa.

Figure 4 (A) Immunoblot analysis of MS4A13-2 in spermatozoa. Proteins from spermatozoa purified from caput and cauda epididymides were separated by SDS-PAGE, and either stained with Coomassie brilliant blue (left panel) or transferred to a filter for immunoblot analysis with anti-MS4A13-2 antibody. Molecular masses (kDa) of standard proteins are shown on the left. (B) Subcellular distribution of MS4A13-2 in mouse spermatozoa from epididymides. The membrane (mainly containing plasma membrane), head (except membrane fraction) and flagella (except membrane fraction) fractions of spermatozoa were separated by SDS-PAGE, and either stained with Coomassie brilliant blue (left panel) or subjected to immunoblot analysis using the anti-MS4A13-2 antibody (C-terminus; right panel). MS4A13-2 is visible strongly in the head fraction of spermatozoa. Molecular masses (kDa) of standard proteins are shown to the left. (C) Immunohistochemical localization of MS4A13-2 in mouse epididymides. Frozen sections of cauda of adult mouse epididymides stained with anti-MS4A13-2 antibody (C-terminus; red) were counter-stained with Hoechst 33342 to visualize nuclear DNA (blue). MS4A13-2 is associated with sperm and not found in epididymal epithelial cells (arrowheads in upper panel). At high magnification in sperm from cauda epididymis, MS4A13-2 was visible along the periphery of nuclei in the sperm heads (lower left panel). Staining with pre-immune serum produced negative results (lower right panel). Scale bars, 10 μ m. (D) Spermatozoa stained with the anti-MS4A13-2 antibody (C-terminus; red), followed by incubation with PNA-FITC (green) and Hoechst counterstaining (blue). Merged images are shown in the bottom panel. At high magnification, MS4A13-2 immunolabeling is visible in the equatorial region of sperm heads. Scale bar, 3 μ m. (E) Pre-embedding immunoelectron microscopy of MS4A13-2 in mouse spermatozoa. Purified spermatozoa from cauda epididymides were sonicated, fixed and processed for immunolabeling using the anti-MS4A13-2 antibody (C-terminus) and 10-nm gold-conjugated goat anti-rabbit IgG. Plasma membrane was mostly removed by sonication. MS4A13-2 is primarily visible at the naked outer acrosomal membrane (arrows) at the equatorial region (Eq), with no distinct immunolabeling on the exposed outer acrosomal membrane at the acrosomal cap (AC) (left panel). Little or no labeling is visible in control samples stained with pre-immune serum (right panel). M, middle piece. N, nucleus. Scale bars, 0.5 μ m.

Biochemical examination of caput and cauda epididymis spermatozoa

Morphologically, there was no difference in the subcellular localization of MS4A13-2 between caput and cauda epididymis spermatozoa. In order to biochemically examine differences between spermatozoa from caput and cauda epididymides, we performed 1% Triton X-100 extraction for 1 h to remove some of the sperm membranes. By electron microscopy, the removal of the plasma membranes, some of the inner and outer acrosomal membranes, and a small proportion of other organelles from both caput and cauda epididymis spermatozoa were observed, as a result of

treatment with Triton X-100 (Fig. 5A). The inner and outer acrosomal membranes of caput epididymis spermatozoa (Fig. 5A, left panel) were more extensively removed after Triton X-100 treatment than those of cauda epididymis spermatozoa (Fig. 5A, right panel). By immunoblotting and immunolabeling analyses, we determined that a substantial fraction of MS4A13-2 was released from caput epididymis spermatozoa by 1% Triton X-100 treatment for 1 h, while only half of the protein was extracted from cauda epididymis spermatozoa by the same treatment (Fig. 5B, third panel from the bottom, and 5C). Detection of an MN7 (a component of the acrosome) in supernatants of both caput and cauda

Figure 5 (A) Electron microscopy of mouse spermatozoa extracted with Triton X-100. Caput (left panel) and cauda (right panel) epididymis spermatozoa were treated for 1 h with 1% Triton X-100, fixed, embedded in epoxy resin and ultrathin sections examined by electron microscopy. Triton X-100 treatment removed the plasma membranes, some of the inner and outer acrosomal membranes, and a small proportion of other organelles from both caput and cauda epididymis spermatozoa. The inner and outer acrosomal membranes of caput epididymis spermatozoa were more extensively removed by Triton X-100 treatment than those of the cauda epididymis spermatozoa. M, middle piece. N, nucleus. Scale bars, 0.3 µm. (B) Immunoblot analyses of MS4A13-2 in mouse spermatozoa extracted using Triton X-100. Proteins in both extracts and pellets were separated by SDS-PAGE, and either stained with Coomassie brilliant blue (upper panel) or transferred to a membrane for immunoblot analysis with anti-MS4A13-2 antibody (C-terminus; third panel from the bottom) or monoclonal anti-MN7 antibody (second panel from the bottom), or monoclonal anti- α tubulin antibody (bottom panel). MS4A13-2 protein migrating at 19 kDa was visible primarily in the supernatant (S) from caput epididymis spermatozoa, while protein is distributed equally in both the supernatant and the pellet (P) from cauda epididymis spermatozoa. MN7 protein, migrating at 90 kDa, was detected primarily in the supernatants of caput and cauda epididymis spermatozoa, and α tubulin protein, migrating at 50 kDa, was detected in the pellets of caput and cauda epididymis spermatozoa. Molecular masses (kDa) of standard proteins are shown on the left. (C) Immunocytochemical localization of MS4A13-2 in mouse spermatozoa extracted with Triton X-100. Caput (left panels) and cauda (right panels) epididymis spermatozoa were treated for 1 h with 1% Triton X-100, fixed and immunostained with anti-MS4A13-2 antibody (C-terminus), followed by incubation with Cy3-conjugated goat anti-rabbit IgG (red). Immunostained samples were counterstained with Hoechst 33342 to visualize nuclear DNA (blue). Merged immunofluorescence images for MS4A13-2 and Hoechst 33342 are shown in upper panels. The MS4A13-2 was detected in cauda epididymis spermatozoa, whereas it was not detected in caput epididymis spermatozoa. Scale bars, 3 µm.

epididymis spermatozoa (Fig. 5B, second panel from the bottom) and of tubulins (components of the axoneme) in pellets of both caput and cauda epididymis spermatozoa

by the same treatment (Fig. 5B, bottom panel) indicated accurate separation of supernatants and pellets from caput and cauda epididymis spermatozoa. These data suggest that the membranes of sperm heads become resistant to biochemical processing during epididymal maturation, resulting in more MS4A13-2 remaining in the outer acrosome membrane of spermatozoa in cauda epididymis after 1% Triton X-100 treatment.

Alterations in MS4A13-2 after the acrosome reaction

We examined whether localization of MS4A13-2 altered after the acrosome reaction. Capacitated spermatozoa were incubated for 30 min in the presence or absence (control) of the calcium ionophore A23187 (10 µM). The acrosome reaction of spermatozoa was monitored by staining with the monoclonal anti-MN7 antibody and anti-mouse IgG conjugated with FITC. We observed that immunolabeling for MS4A13-2 extended to the postacrosomal region and acrosomal cap of sperm heads after the acrosome reaction (Fig. 6A, lower panel), whereas MS4A13-2 was limited to the equatorial region in fresh (Fig. 6A, upper panel) or capacitated (Fig. 6A, middle panel) spermatozoa.

We next examined when MS4A13-2 extended to the postacrosomal region and acrosomal cap of sperm heads during the acrosome reaction. Capacitated spermatozoa were incubated for 0, 5, 10, 15, 20, 30 and 60 min in the presence of the calcium ionophore A23187 (10 µM) (Supplementary Fig. 2). The acrosome reaction of spermatozoa was monitored by FITC-PNA. We found that the proportion of spermatozoa, in which PNA disappeared ($P < 0.05$) and localization of MS4A13-2 was diffused ($P < 0.05$), increased significantly between 15 and 20 min after the addition of calcium ionophore, and that for all the spermatozoa in which the localization of MS4A13-2 was diffused, PNA had disappeared. These data suggest that MS4A13-2 extended to the postacrosomal region and acrosomal cap of sperm heads within a maximum of 5 min of the disappearance of PNA.

To investigate whether the molecular size of MS4A13-2 changed before and after the acrosome reaction, spermatozoa treated for 30 min with or without calcium ionophore A23187 were processed for immunoblot analysis (Fig. 6B). We found that the anti-MS4A13-2 antibody (extracellular region) recognized a single protein migrating at 19 kDa in both capacitated and acrosome-reacted spermatozoa.

BS3 crosslinker analysis of acrosome-reacted spermatozoa

BS3 is a cell-surface crosslinker used to investigate the formation of protein complexes, such as dimers and tetramers. We investigated the effect of BS3 treatment on the migration of MS4A13-2 by immunoblotting.

Figure 6 (A) Fluorescence microscopy of MS4A13-2 in acrosome-reacted spermatozoa. The acrosome reaction of spermatozoa was monitored by staining with the monoclonal anti-MN7 antibody and anti-mouse IgG conjugated with FITC (green). Immunostained samples were counter-stained with Hoechst 33342 to visualize nuclear DNA (blue). Immunolabeling of MS4A13-2 (red) extended to the postacrosomal region and acrosomal cap of sperm heads after the acrosome reaction (lower panel), whereas in fresh (upper panel) and capacitated (middle panel) spermatozoa, MS4A13-2 was limited to the equatorial region. Scale bars, 3 μ m. (B) Immunoblot analysis of MS4A13-2 in spermatozoa before (capacitated) and after (AR) the acrosome reaction. Spermatozoa treated with or without calcium ionophore were separated by SDS-PAGE, and either stained with Coomassie brilliant blue (left panel) or transferred to a filter for immunoblot analysis with the anti-MS4A13-2 antibody (extracellular region; right panel). A band migrating at 19 kDa was detected in both samples. Molecular masses (kDa) of standard proteins are shown on the left. (C) Immunoblot analysis of MS4A13-2 in non-treated (control) or BS3-treated (BS3) acrosome-reacted spermatozoa. MS4A13-2 was detected at 19 kDa in the absence of BS3, whereas it migrates at 19 kDa, 40 kDa and the bands larger than 40 kDa in the presence of BS3. Molecular masses (kDa) of standard proteins are shown on the left. (D) Schematic diagrams showing the estimated translocation of MS4A13-2 (red dots) during the acrosome reaction. MS4A13-2 is initially localized on the outer acrosomal membrane of the equatorial region (Eq) (left diagram). After the acrosome reaction, it translocated to the exposed to the postacrosomal region (PA) and the acrosomal cap (AC) (right diagram).

Spermatozoa with outer acrosomal membranes exposed by treatment with calcium ionophore were treated with or without (control) BS3 crosslinker, and examined by immunoblot analysis. As shown in Fig. 6C, in the absence of BS3, MS4A13-2 was detected at 19 kDa (monomer); however, in the presence of BS3, it migrated at 19 kDa (monomer), 40 kDa and the bands larger than 40 kDa. These data indicate effective crosslinking

of MS4A13-2 molecules by BS3 and suggest that BS3 generates homophilic or heterophilic crosslinks with MS4A13-2 in acrosome-reacted spermatozoa.

Effect of MS4A13-2 on fertilization

As shown in Table 1, in an experiment using zona pellucida-intact oocytes, the rate of two-cell formation decreased in a dose-dependent manner on treatment with anti-MS4A13-2 antibody (extracellular region). The difference between experimental (treated with anti-MS4A13-2 antibody (extracellular region)) and control groups (treated with TYH capacitation medium, pre-immune serum or anti-MS4A13-2 antibody (C-terminus)) was significant ($P < 0.05$). As shown in Table 2, in the experiment using zona pellucida-free oocytes, the rate of two-cell formation was not decreased by treatment with anti-MS4A13-2 antibody (extracellular region).

Discussion

In this study, we isolated 174 DNA fragments (under analysis) upregulated during the development of sperm heads in *S. murinus* testes. We found that one of the genes had high homology with one *Suncus* transcript, *Ms4a13* (under analysis), and two mouse transcripts, *Ms4a13-1* and *Ms4a13-2*. Mouse *Ms4a13-2* mRNA was strongly expressed in the mouse testis and epididymis, and at much lower levels in the kidney, spleen, liver and heart; however, mouse *Ms4a13-1* was undetectable in all organs examined. The expression of *Ms4a13-2* mRNA in mouse testis was first detected at 2 weeks postpartum and continued to be expressed into adulthood.

Recent studies have reported that, among MS4A family members, MS4A5 (Hulett *et al.* 2001), MS4A13 (Turner *et al.* 2008) and MS4A14 (Xu *et al.* 2014) are specifically expressed in the testis; however, details of the localization and functions of MS4A5 and MS4A13 have not previously been revealed. In the rat, MS4A14 locates in the acrosome region of the sperm head and at the midpiece of the flagellum (Xu *et al.* 2014). Pretreatment of mature sperm with MS4A14 antibody during the IVF procedure caused a clear reduction in the normal fertilization rate (Jia *et al.* 2010) and *in vivo* fertilization using transgenic males in which levels of testicular *Ms4a14* mRNA were reduced by 30%, with WT females, resulted in a reduced number of litters; the fertility rate declined to 34.48% using transgenic males, compared with 70.95% in the control group (Shi *et al.* 2010). We found that pretreatment of mature sperm with the anti-MS4A13-2 antibody during the IVF procedure caused a clear reduction in the normal fertilization rate of zona pellucida-intact oocytes with cumulus, whereas it had no significant effect on the fertilization rate of the zona pellucida-free oocytes without cumulus. These data suggest that MS4A13-2 may be involved in an

Table 1 Effect of anti-MS4A13-2 antibodies on fertilization of mouse zona pellucida-intact oocytes.

Treatment	Concentration (µg/mL)	Two-cell formation* (n=3)	
		Total number of oocytes observed	% (mean ± S.E.M.) [†]
TYH (control)	—	115	70.3 ± 6.5 ^{a1}
Pre-immune (control)	100	108	74.4 ± 2.8 ^{a2}
mMS4A13-2 C-terminus (control)	100	108	72.1 ± 5.8 ^{a3}
mMS4A13-2 extracellular region	25	74	43.1 ± 10.8 ^{a4}
	50	70	31.7 ± 1.3 ^{a5}
	100	112	7.5 ± 3.4 ^{a6}

*Two-cell formation was checked at 24 h after insemination. †, a1, a2, a3 vs a4, a5, a6: $P < 0.05$.

interaction with the zona pellucida or cumulus, but not oocytes, during fertilization.

MS4A family members share the topology of their transmembrane domains; however, they exhibit low sequence similarity. The regions demonstrating the greatest variation among MS4A proteins are those in the N- and C-terminal cytoplasmic domains and the putative second extracellular loop (Ishibashi *et al.* 2001), suggesting that these regions may impart unique functional characteristics. Although MS4A13-2 and MS4A14 also share the same basic structure, there are large differences between their N- and C-terminal domains and putative second extracellular loops; for example, MS4A13-2 has a very short N-terminus and MS4A14 has a much longer C-terminal tail, including phosphorylation and glycosylation sites (Xu *et al.* 2014). Additionally, in molecular phylogenetic analysis of mouse MS4A family members using CLUSTALW (<http://www.genome.jp/tools/clustalw/>), MS4A13-2 was found to have higher homology to MS4A1, MS4A3, MS4A4c, MS4A4d and MS4A9 than to MS4A5 and MS4A14. These data suggest that MS4A13-2 and MS4A14 may have diverse binding targets and different roles in fertilization.

In this study, we found that the localization of MS4A13-2 extended to the postacrosomal region and acrosomal cap of sperm heads within a maximum of 5 min after the acrosome reaction, whereas in spermatozoa freshly isolated from cauda epididymides and capacitated, it was limited to equatorial region. Furthermore, we found that the molecular size of MS4A13-2 did not alter before and after the acrosome reaction, indicating that MS4A13-2 was not cleaved on relocalization. The localization of several membrane proteins changes in spermatozoa after the acrosome reaction. Izumo1, the only candidate in sperm-egg fusion protein, is a type I membrane glycoprotein with one immunoglobulin-like domain and a putative N-glycoside link motif, detectable on the inner and outer acrosomal membranes

of the anterior acrosome before the acrosome reaction, and on the entire surface of the sperm head, including the equatorial region (Inoue *et al.* 2005), within a maximum of 30 s after the acrosome reaction (Satouh *et al.* 2012). Moreover, recent studies have reported that the molecular size and amount of unphosphorylated Izumo1 remained unchanged by the acrosome reaction, whereas that of phosphorylated Izumo1 was greatly diminished after the acrosome reaction (Ellerman *et al.* 2009). The specific loss of phosphorylated Izumo1 may be due to its localization on the outer acrosomal membrane, which is lost as vesicles after the acrosome reaction, or to the dephosphorylation of Izumo1. SPACA1, an important protein that functions to shape the sperm head and in sperm-egg fusion, localizes to the inner acrosomal membrane of the entire acrosome and on the outer acrosomal membrane of the equatorial region before the acrosome reaction, and remains on the inner acrosomal membrane in capacitated and acrosome-reacted spermatozoa (Fujihara *et al.* 2012). Since MS4A13-2 shows somewhat similar localization changes to SPACA1 during spermatogenesis, and is detectable on the entire surface of the sperm head, including the equatorial region, immediately after the acrosome reaction, it may have a similar role to those of SPACA1 and Izumo1 in the testis and during fertilization. However, how MS4A13-2 relocates to the entire surface of the sperm head and how it behaves during fertilization remain to be elucidated.

In conclusion, we demonstrate that mouse MS4A13-2, but not MS4A13-1, is a constituent molecule of the outer acrosomal membrane of the equatorial region of fresh sperm heads. In addition, the expression of MS4A13-2 extends to whole sperm heads, including the postacrosomal region and acrosomal cap, after the acrosome reaction. Current evidence suggests that some MS4A family members may form hetero/homo-oligomers and function as ion channel/adaptor proteins

Table 2 Effect of anti-MS4A13-2 antibody on fertilization of mouse zona pellucida-free oocytes.

Treatment	Antibody concentration (µg/mL)	Two-cell formation* (n=3)	
		Total number of oocytes observed	% (mean ± S.E.M.)
TYH (control)	—	102	76.8 ± 3.1
mMS4A13-2 extracellular region	100	126	77.4 ± 1.8

*Two-cell formation was checked at 24 h after insemination.

to facilitate intracellular protein–protein interactions, with important regulatory roles in cellular growth, survival and activation (Eon Kuek *et al.* 2016). As MS4A13-2 was detected at 19 kDa (monomer), 40 kDa and the bands larger than 40 kDa in the presence of BS3, MS4A13-2 may also form hetero/homo-oligomers, such as dimers and multimer, and have an important role in fertilization. Further studies of MS4A13-2 will provide additional information about, and understanding of, the molecular complexity involved in the mechanism of fertilization. Furthermore, gene knockout models could facilitate elucidation of the function of MS4A13-2.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0477>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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