Group III secreted phospholipase A₂ regulates epididymal sperm maturation and fertility in mice

Hiroyasu Sato,1,2 Yoshitaka Taketomi,1,3 Yuki Isogai,1,4 Yoshimi Miki,1,2 Kei Yamamoto,1 Seiko Masuda,1,2 Tomohiko Hosono,3 Satoru Arata,3 Yukio Ishikawa,5 Toshiharu Ishii,5 Tetsuyuki Kobayashi,4,6 Hiroki Nakanishi,6,7 Kazutaka Ikeda,6,7 Ryo Taguchi,6,7 Shuntaro Hara,2 Ichiro Kudo,2 and Makoto Murakami1,8

1Biomembrane Signaling Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. 2Department of Health Chemistry, School of Pharmaceutical Sciences, and 3Center for Biotechnology, Showa University, Tokyo, Japan. 4Department of Biology, Faculty of Science, Ochanomizu University, Tokyo, Japan. 5Department of Pathology, Toho University School of Medicine, Tokyo, Japan. 6Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Kawaguchi, Japan. 7Department of Metabolome, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan. 8Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Kawaguchi, Japan.

Although lipid metabolism is thought to be important for the proper maturation and function of spermatooza, the molecular mechanisms that underlie this dynamic process in the gonads remains incompletely understood. Here, we show that group III phospholipase A₂ (sPLA₂-III), a member of the secreted phospholipase A₂ (sPLA₂) family, is expressed in the mouse proximal epididymal epithelium and that targeted disruption of the gene encoding this protein (Pla2g3) leads to defects in sperm maturation and fertility. Although testicular spermatogenesis in Pla2g3−/− mice was grossly normal, spermatooza isolated from the cauda epididymidis displayed hypomotility, and their ability to fertilize intact eggs was markedly impaired. Transmission EM further revealed that epididymal spermatooza in Pla2g3−/− mice had both flagella with abnormal axonemes and aberrant acrosomal structures. During epididymal transit, phosphatidylcholine in the membrane of Pla2g3+/+ sperm underwent a dramatic shift in its acyl groups from oleic, linoleic, and arachidonic acids to docosapentaenoic and docosahexaenoic acids, whereas this membrane lipid remodeling event was compromised in sperm from Pla2g3−/− mice. Moreover, the gonads of Pla2g3−/− mice contained less 12/15-lipoxygenase metabolites than did those of Pla2g3+/+ mice. Together, our results reveal a role for the atypical sPLA₂ family member sPLA₂-III in epididymal lipid homeostasis and indicate that its perturbation may lead to sperm dysfunction.

Introduction

The male reproductive system consists of a series of organs that act together to produce and deliver functional spermatooza into the female reproductive tract. After the complex differentiation process of male germ cells, spermatooza exit the seminiferous tubules of the testis through the efferent ducts toward the epididymis. During their transit from the caput to the cauda epididymidis, spermatooza undergo significant morphological and biochemical modifications, which lead to acquisition of their forward motility and ability to recognize and fertilize oocytes (1). Since 1 out of 6 couples of reproductive age present with infertility, understanding of the molecular mechanisms underlying the proper development of gametes in the gonadal niche is of considerable biomedical and clinical importance.

A number of knockout mouse models have been reported to display reproduction disorders, among which, those harboring abnormalities of lipid metabolism are often accompanied by fertility defects. For instance, disruption of several genes regulated for the biosynthesis or receptors for arachidonic acid–derived eicosanoids perturbs the reproduction process in females rather than in males (2–5). In contrast, knockout of genes affecting lipid signaling (6, 7) and transport (8–11) often profoundly affects the maturation and function of spermatooza. In addition, accumulating evidence suggests that composition changes of sperm lipids can affect sperm quality and functions (12). Rodents fed an essential fatty acid–deficient diet or those unable to synthesize polyunsaturated fatty acids (PUFAs) show degeneration of germ cells (13). Inactivation of genes involved in the synthesis or transport of PUFAs hampers fertilization, even in invertebrates such as Drosophila and Caenorhabditis elegans (14, 15).

Amounts of cholesterol and phospholipids are lower in mature and motile sperm than in immature and immotile sperm of both human and mouse species (16, 17). A quality unique to mammalian sperm cells is the abundance of phospholipid species with C22-PUFAs, particularly docosahexaenoic acid (DHA; an ω-3 fatty acid), whose proportion in membrane phospholipids appears to correlate with sperm maturity, motility, and fertility (17–20). In humans, the percentage of DHA relative to total fatty acids is correlated with the normal morphology of sperm cells, whereas an inverse relationship between the percentage of atypical sperm cells and that of DHA is seen (19). Sperm cells from subfertile men with low sperm motility or counts contain a percentage of DHA lower than that from normal men (21). Sperm maturation involves the remodeling of membrane phospholipids toward the acquisition of motility and fertility during sperm migration through the epididymis. Indeed, the increase in C22-PUFAs, such as DHA and docosapentaenoic acid (DPA), and the reciprocal decrease in ω-6 arachidonic acid favor an increase in the unsaturation degree of fatty acids in mouse sperm membrane during epididymal transit (17), which could consequently contribute to increasing the mouse sperm membranous fluidity, as it was
Phospholipase A\(_2\) (PLA\(_2\)) is a group of enzymes that hydrolyze the sn-2 position of glycerophospholipids to yield fatty acids (typically unsaturated) and lysophospholipids, which are further converted to a variety of lipid mediators. Of more than 30 mammalian PLA\(_2\)-related enzymes identified so far, targeting of 3 genes has been shown to affect reproduction. Ablation of \(\text{Pla2g4a}\) (group IVA cytosolic PLA\(_2\)\(\alpha\), also known as cPLA\(_2\)\(\alpha\)), impairs multiple steps of female reproduction, because of reduced synthesis of PGs (2, 3), whereas that of \(\text{Pla2g6}\) (group VIA Ca\(^{2+}\)-independent PLA\(_2\), also known as iPLA\(_2\)\(\beta\)) and \(\text{Pafah1b2}\) (type I platelet-activating factor acetylhydrolase or group VIIIB PLA\(_2\)) results in defective sperm motility and severe impairment of spermatogenesis, respectively, through unknown mechanisms (24, 25). Secreted PLA\(_2\) (sPLA\(_2\)) enzymes represent a group of structurally related enzymes, with strict Ca\(^{2+}\)-dependence and a His-Asp catalytic dyad, and 10 catalytically active sPLA\(_2\) enzymes have been identified in mammals (26, 27). Recent studies employing transgenic (gain-of-function) and knockout (loss-of-function) mice have revealed that individual sPLA\(_2\)s are involved in distinct biological events, such as dietary phospholipid remodeling during epididymal transit still remain unknown.

Figure 1
Targeted disruption of the \(\text{Pla2g3}\) gene. (A) Expression of transcripts for \(\text{Pla2g3}\) in various tissues of male C57BL/6 mice was assessed using RT-PCR. After electrophoresis of the PCR products in agarose gels with ethidium bromide (top panel), the intensities of the band for \(\text{Gapdh}\), were quantified using a densitometer (CS analyzer 2.0; mean ± SD; \(n = 3\)) (bottom panel). (B) RT-PCR of \(\text{Pla2g3}\) mRNA expression in male and female reproductive organs from C57BL/6 mice. (C) Immunoblotting of sPLA\(_{\text{III}}\) protein in sperm and fluid from the cauda epididymis of C57BL/6 mice. Arrow indicates a fully processed sPLA\(_{\text{III}}\). (D) Schematic representation of the \(\text{Pla2g3}\) gene, targeting vector, and mutated \(\text{Pla2g3}\) allele. Details of the construction of the targeting vector are given in Methods. Arrows indicate positions of primers for PCR genotyping. Boxes indicate exons of the \(\text{Pla2g3}\) gene. Large arrows indicate the 5.5-kb long-arm and 1.7-kb short-arm genomic fragments of the \(\text{Pla2g3}\) gene. Small arrows indicate the positions of primers used for PCR genotyping (III-F, WT-R, and MT-R indicate \(\text{Pla2g3 WT}\) forward primer, WT reverse primer, and mutant reverse primer, respectively). “X” indicates the regions where homologous recombination occurs. A bold bar shows a region used as a probe for Southern blotting. (E) Genotyping using PCR of samples from mouse tails. The WT and mutant alleles gave 375-bp and 627-bp amplified fragments, respectively. (F) Confirmation by RT-PCR of the absence of \(\text{Pla2g3}\) mRNA in the testis and epididymis of nullizygous mice. (G) PLA\(_2\) enzymatic activity in cauda epididymal fluid of \(\text{Pla2g3}\)\(-/-\) mice and their littermates. (H) PLA\(_2\) enzymatic activity in cauda epididymal fluid of \(\text{Pla2g10}\)\(-/-\) mice and their littermates. (G and H) Values are mean ± SD; \(n = 4\). *\(P < 0.05\).
digestion (28), host defense (29, 30), inflammation (31, 32), tissue injury (33, 34), and atherosclerosis (35). However, the physiological roles of individual sPLA₂s still remain largely obscure.

Group III sPLA₂ (sPLA₂-III) is distinctive among mammalian sPLA₂s, consisting of a central sPLA₂ (S) domain flanked by unique N-terminal and C-terminal domains, the S domain being homologous to bee venom PLA₂, rather than to other mammalian sPLA₂s (36–38). Transgenic overexpression of human sPLA₂-III in mice, in which the enzyme is processed to the S domain–only form, results in accelerated atherosclerosis, following a high-cholesterol diet, through increased hydrolysis of phospholipids in plasma lipoproteins (39) and in systemic inflammation through increased lipid mediator levels (40). In the present study, we show that targeting disruption of the Pla2g3 gene in mice results in male hypofertility due to impaired sperm maturation in the epididymis. This is the first demonstration to our knowledge that a particular sPLA₂ enzyme regulates the process of sperm maturation, likely through control of local lipid homeostasis in the genital tract.

**Results**

Expression of sPLA₂-III in reproductive organs. RT-PCR showed broad expression of Pla2g3 mRNA in various tissues of C57BL/6 mice, among which the highest level of expression was found in the brain and testis (Figure 1A). Of the male genital organs, Pla2g3 mRNA was expressed at high levels in the testis and epididymis and to a lesser extent in the seminal vesicles and prostate (Figure 1B, left). In females, more Pla2g3 transcript was detected in the ovary and oviduct than in the uterus (Figure 1B, right). Immunoblotting of cauda epididymal exudates with anti–sPLA₂–III antibody detected an 18-kDa immuno-reactive band in epididymal fluid but not in sperm cells (Figure 1C), suggesting that sPLA₂–III is secreted into the epididymal duct and is fully processed (38, 39) but is not present in mature sperm.

Generation of Pla2g3 knockout mice. The Pla2g3-directed targeting vector, in which the exons encompassing the catalytic domain of the Pla2g3 gene were replaced with a neomycin resistance cassette (Neo⁺), was constructed (Figure 1D) and transfected into mouse ES cells of 129SvEv origin. Appropriately targeted ES cell clones, as evaluated by
epididymis, whereas it was barely detectable in the cauda of the testis (Supplemental Figure 1D). In comparison, expression of Pla2g3 in Sertoli cells was confirmed using quantitative RT-PCR analysis, in which 527-bp and 375-bp amplified fragments represented the mutant and WT alleles, respectively. Successful gene ablation of Pla2g3 mice was confirmed by the absence of its transcript in the testis and epididymis of Pla2g3−/− mice (Figure 1F). Also, the Pla2g3 mRNA expression was reduced partially in heart, kidney, and liver from Pla2g3+/− mice compared with Pla2g3+/+ mice and was undetectable in Pla2g3−/− mice (Supplemental Figure 1B). Two independent ES clones gave rise to mutant mouse lines with virtually identical phenotypes (see below).

PLA2 enzymatic activity in the cauda epididymal fluid was decreased to approximately 40% in Pla2g3−/− mice compared with the activity in Pla2g3+/+ mice (Figure 1G), indicating that PLA2-III accounts for nearly half of the total PLA2 activity in epididymal fluid and that the remaining activity detected in Pla2g3−/− mice may be due to the presence of other PLA2s (s) present in the male reproductive organs (41). As shown in Figure 1H, the sPLA2 activity in the fluid of Pla2g3−/− mice was reduced by approximately half compared with that of Pla2g3+/+ mice. These results suggest that sPLA2-III and sPLA2-X are 2 major sPLA2s present in the mouse epididymal fluid. Considering that sPLA2-X is a major sPLA2 isoform residing in sperm acrosomes (41), the sPLA2-X activity in the fluid may reflect a fraction of the enzyme that had been leaked from capacitated spermatozoa during sample preparation.

Immunohistochemical staining of testis sections using anti–sPLA2-III antibody revealed distribution of sPLA2-III in a net-like structure in spermatogenic cells and cilia in the caput to corpus of the epididymis (Figure 2, A and B). The staining was also associated with spermatozoa, luminal space, and luminal cilia on the epithelium in the caput and corpus but not in the cauda (Figure 2B). No such positive staining was detected in the epididymis of Pla2g3+/− mice stained with control serum (Figure 2A) or that of Pla2g3−/− mice stained with anti-sPLA2-III antibody (Figure 2C). These results, together with the apical border locations within principal and apical cells, suggest that PLA2-III is secreted apically from the epithelium and associates with luminal sperm cells and cilia in the caput to corpus epididymidis. The faint staining of connective tissue in all regions of the epididymis appeared to be nonspecific, since similar

Table 1
Fecundity of Pla2g3+/+, Pla2g3−/−, and Pla2g3+/− mice

<table>
<thead>
<tr>
<th>Male genotype</th>
<th>Female genotype</th>
<th>Delivery</th>
<th>Litter size (mean ± SEM)</th>
<th>Total pups (breakdown of pup genotypes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>s/+</td>
<td>s/+</td>
<td>33</td>
<td>8.2 ± 0.4</td>
<td>270 (270 s/+ s/+; 20 s/+ s/−)</td>
</tr>
<tr>
<td>s/+</td>
<td>s/−</td>
<td>60</td>
<td>7.4 ± 0.3</td>
<td>464 (217 s/+ s/−; 229 s/+ s/−)</td>
</tr>
<tr>
<td>s/+</td>
<td>−/−</td>
<td>6</td>
<td>6.7 ± 0.7</td>
<td>43 (43 s/− s/−)</td>
</tr>
<tr>
<td>s/−</td>
<td>s/+</td>
<td>20</td>
<td>4.4 ± 0.5t</td>
<td>87 (45 s/+ s/+; 42 s/+ s/−)</td>
</tr>
<tr>
<td>s/−</td>
<td>s/−</td>
<td>47</td>
<td>2.5 ± 0.5t</td>
<td>128 (34 s/− s/−; 65 s/− s/−; 29 s/− s/−)</td>
</tr>
<tr>
<td>s/−</td>
<td>−/−</td>
<td>7</td>
<td>3.7 ± 0.8t</td>
<td>26 (13 s/− s/−; 13 s/− s/−)</td>
</tr>
<tr>
<td>−/−</td>
<td>s/+</td>
<td>13</td>
<td>2.9 ± 1.2t</td>
<td>29 (29 s/+ s/−)</td>
</tr>
<tr>
<td>−/−</td>
<td>s/−</td>
<td>20</td>
<td>4.2 ± 0.6t</td>
<td>80 (37 s/− s/−; 43 s/− s/−)</td>
</tr>
<tr>
<td>−/−</td>
<td>−/−</td>
<td>22</td>
<td>2.2 ± 0.4t</td>
<td>49 (49 s/− s/−)</td>
</tr>
</tbody>
</table>

*P < 0.01 versus +/+ × s/+.

Reproduction abnormality in male Pla2g3-deficient mice. During the course of backcrossing (to C57BL/6J mice) and test mating, we found that the litter size after intercrossing heterozygous mutant male and female mice (2.5 ± 0.5 pups per litter) was significantly smaller than that after intercrossing the WT male and female mice (8.2 ± 0.4 pups per litter) (Table 1). Despite this reproductive problem, the ratio of the genotypes of heterozygous male and female offspring exhibited roughly mendelian proportions (Pla2g3−/−/Pla2g3+/−: Pla2g3+/−/Pla2g3−/−: 34:65:29, among 128 pups), and the homozygous-null mice were indistinguishable from their WT littermates in terms of survival rates, appearance, and gross behavior, suggesting that the lack of sPLA2-III did not affect embryonic and postnatal development under normal housing conditions. When mutant males were mated with WT females, the litter sizes were reduced in a genotype-related manner, with only 4.4 ± 0.5 and 2.9 ± 1.2 pups per litter after breeding of Pla2g3+/− and Pla2g3−/− males with Pla2g3−/− females (Table 1), respectively, regardless of normal mounting behavior and production of copulatory plugs. In contrast, reduction in the litter size was not evident when mutant females were mated with WT males (Table 1). These results suggest that the loss of sPLA2-III results in male hypofertility. It was noteworthy that, after backcrossing to a C57BL/6J background for several generations, mutant males did not produce any pups, suggesting a genetic influence on the sterile phenotype, as has been demonstrated for several other targeted mutations in sperm development. Therefore, subsequent experiments were conducted using mice with a 129Sv × C57BL/6J mixed background.

Body, testis, and epididymis weights of the mutant mice did not differ from those of their WT counterparts (Supplemental Table 1). Serum testosterone levels were comparable between Pla2g3−/− and Pla2g3+/− mice (Supplemental Table 1), implying that the recombination abnormality of Pla2g3−/− mice was not caused by genomic Southern blotting (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI40493DS1), were used to obtain chimeric mice that transmitted the disrupted locus through the germ line. Genotyping was performed on genomic DNA isolated from tail biopsy samples using PCR (Figure 1E), in which 627-bp and 375-bp amplified fragments represented the mutant and WT alleles, respectively. Successful gene ablation of Pla2g3 was confirmed by the absence of its transcript in the testis and epididymis of Pla2g3−/− mice (Figure 1F). Also, the Pla2g3 mRNA expression was reduced partially in heart, kidney, and liver from Pla2g3+/− mice compared with Pla2g3+/+ mice and was undetectable in Pla2g3−/− mice (Supplemental Figure 1B). Two independent ES clones gave rise to mutant mouse lines with virtually identical phenotypes (see below).

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imbalance of the gonadal hormone. Although defects in testicular spermatogenesis are often accompanied by the reduction of sperm counts, the numbers of cauda epididymal spermatozoa from both genotypes were similar (Supplemental Table 1), suggesting that germ cell development in the testis was not deeply affected by the lack of sPLA2-III. The testicular (data not shown) and epididymal (Supplemental Figure 3) expressions of other PLA2 enzymes, including Pla2g4a (cPLA2α), Pla2g5 (sPLA2-V), Pla2g10 (sPLA2-X), Pla2g6 (iPLA2β), and Pnpla8 (iPLA2γ), were unaffected by Pla2g3 deficiency, confirming that the male infertility in Pla2g3–/– mice was not due to altered expression of other PLA2s.

Impaired motility and fertility of spermatozoa from Pla2g3-deficient mice. As shown in Figure 3A, sperm from mutant animals showed severely reduced ability to fertilize zona pellucida–intact (ZP-intact) eggs in an in vitro fertilization (IVF) assay. Indeed, epididymal spermatozoa from WT animals fertilized more than 50% of WT eggs at 24 hours after insemination, whereas those from null animals fertilized only approximately 10% of the eggs (Figure 3B). In this experimental setting, spermatozoa from heterozygous mice showed an intermediate reduction of IVF. Accordingly, unfertilized eggs and aborted embryos were increased by the Pla2g3 deficiency in the IVF assay (Supplemental Figure 4). However, spermatozoa from the mutant mice were able to fertilize eggs normally once the ZP had been removed (Figure 3C). The average numbers of sperm associated with the egg surface was greatly reduced in Pla2g3–/– mice, indicating impaired binding of the mutant sperm to the egg ZP (Figure 3, D and E). In contrast, eggs from Pla2g3–/– mice underwent normal cell division after incubation with WT sperm (Figure 3A), in line with the normal breeding score of Pla2g3–/– females after crossing with Pla2g3+/+ males (Table 1).
We noted that spermatozoa from mutant mice showed impaired motility. The flagellar motion of sperm from Pla2g3–/– animals was sluggish and rarely resulted in forward movement. Computer-assisted sperm analysis demonstrated that the sperm tracks differed clearly between the genotypes (Figure 3F and Table 2). Various parameters of sperm motility, including straight-line and curvilinear velocities and amplitude, were markedly reduced in spermatozoa from Pla2g3–/– mice (Supplemental Video 1) as compared with those from Pla2g3+/+ mice (Supplemental Video 2). The motility parameters of spermatozoa from heterozygous mice showed values that were intermediate between those from the WT and null animals (Table 2).

To observe the effect of Pla2g3 disruption on sperm migration within the female reproductive tract, cross sections of the uterus and oviduct 0.5 days after coitus were prepared. Spermatozoa from Pla2g3+/+ mice were readily found throughout the uterine tract, and a few sperm cells were present in the oviduct. Although spermatozoa from Pla2g3–/– mice accumulated in the lower part of the uterus, they did not reach the upper part of the uterus and the oviduct (Supplemental Figure 5). These results suggest that, because of their impaired forward progression, the mutant sperm failed to swim up through the female genital tract, thereby displaying reduced fertilization efficiency.

We further evaluated the effect of sPLA2-III deficiency on morula development following sperm-egg fusion in vitro. To this end, after WT eggs had been inseminated with Pla2g3+/+ or Pla2g3–/– sperm, those that developed to the 2-cell stage were selected on day 1 and cultured for additional 2 days. In this setting, as expected, morulae were normally recovered from eggs that had fused with the WT sperm, whereas fewer eggs that had fused with the knockout sperm progressed beyond the 2- or 4-cell stage (Figure 3G), thus revealing poor morula development (Figure 3H). Consistently, the reimplanted embryos obtained from Pla2g3–/– sperm gave rise to smaller litter size than those from WT sperm (data not shown).

In addition, spermatozoa from Pla2g3–/– mice contained a markedly lower level of ATP than those from Pla2g3+/+ mice, while those from Pla2g3+/+ mice had an intermediate level (Figure 4A). Probably due to the low level of ATP as well as to defects in other signals, inductive tyrosine phosphorylation of several proteins in spermatozoa that were placed in a capacitating medium, a prerequisite step leading to hyperactivation of flagellar beating (46), was partially, if not completely, reduced in Pla2g3–/– mice compared with Pla2g3+/+ mice (Figure 4, B and C). During sperm maturation, fertilin β, in concert with the molecular chaperone calmegin, is processed from a 100-kDa precursor form present in the testis to a 45-kDa mature form in the epididymis (Figure 4D) (47). An additional approximately 30-kDa immunoreactive band was markedly increased in the epididymis of Pla2g3–/– mice compared with that of WT mice (Figure 4D), suggesting unusual fertilin β processing in the knockout epididymis. Since the processing of fertilin β to the 30-kDa species usually takes place after the acrosome reaction (48), the result shown in Figure 4D suggests that the acrosomes of Pla2g3-deficient sperm are unstable. Indeed, light microscopic examination showed that, in contrast to the normal appearance (hook-shaped head and straight tail) of Pla2g3+/+ sperm, Pla2g3–/– sperm frequently possessed round-shaped heads (indicative of defective acrosome biogenesis) as well as coiled, bent, or duplicate tails (Figure 4E). When counted, nearly half of the cauda epididymal spermatozoa had abnormal heads in Pla2g3–/– mice (Figure 4F) (also see below).

Ultrastructural abnormalities in spermatozoa from Pla2g3-deficient mice. Transmission EM analysis further revealed structural defects in cauda epididymal spermatozoa of Pla2g3–/– mice. In the axoneme of WT sperm, the outer and inner dynein arms attached to the 9 microtubule doublets that are connected to each other via nexin links (Figure 5A). The axonemal central apparatus consists of 2 microtubules, bridges between the 2, and the central sheath. The radial spokes extend from each doublet, and the radial spoke heads are adjacent to the central sheath. Each axoneme doublet is associated with a dense fiber, and the fibrous sheath has 2 longitudinal columns and regularly spaced ribs. In contrast, Pla2g3–/– mice had abnormal sperm with an inappropriate 9 plus 2 configuration of doublets in the flagellar axoneme, in which the most prevalent structural abnormality involved incomplete axonemal doublets, due to loss of one doublet or half of the axonemal complex (Figure 5A). Many of these sperm also lacked the central pair of microtubules and possessed disorganized outer dense fibers. Furthermore, some Pla2g3–/– sperm had developed double axonemes in the principal piece and even in the midpiece of the flagellum, where outer dense fibers were mislocalized outside the mitochondrial lines (Figure 5B). Such alterations have often been observed in mutant mice harboring (even haploinsufficient) defects in structural proteins for flagellar axonemes (49–53). In addition, the acrosomes of some spermatozoa from Pla2g3–/– mice were unusually expanded (Figure 5C), and vacuoles were often found beneath the acrosome cap (Figure 5C). Furthermore, the middle piece of Pla2g3–/– spermatozoa appeared disorganized, with unequally sized and swollen mitochondria that had failed to establish the usual regular helical pattern (Figure 5D), which may have some relation to the reduced ATP synthesis, resulting from Pla2g3 deficiency (Figure 4A). In contrast to the marked ultrastructural abnormalities in cauda spermatozoa, sperm cells with abnormal morphology were scarcely found in the testis and even in the caput epididymidis of Pla2g3–/– mice (Figure 5E). Thus, the sperm flagellar malformation in Pla2g3–/– mice occurred mainly during epididymal transit from the caput to the cauda (Figure 5F). Although the ultrastructural morphology of epithelial principal cells in the epididymis of Pla2g3–/– mice displayed few overt abnormalities, there was a trend toward accumulation of larger cytoplasmic vesicles in the caput epithelium in Pla2g3–/– mice when compared with Pla2g3+/+ mice (Supplemental Figure 6).
Altered lipid profiles in the gonads of Pla2g3−/− mice. Considering that sPLA2-III is a lipolytic enzyme, it was anticipated that abnormalities in spermatozoa of Pla2g3−/− mice would be caused by alteration of lipid metabolism in the gonads. To address this issue, we performed electrospray ion source/mass spectrometry (ESI-MS) profiling of phosphatidylcholine (PC), a major phospholipid, in spermatozoa from the caput (Figure 6A) and cauda (Figure 6B) epididymidis. Relative abundances of individual PC molecular species, in comparison with the signal intensity of the internal standard PC28:0 (14:0–14:0; m/z = 678), are summarized in Figure 6C. In sperm of Pla2g3+/+ mice, peaks of PC species with sn-2 oleic, linoleic, and arachidonic acids (e.g., PC34:1 [16:0–18:1; m/z = 760], PC36:1 [18:0–18:1; m/z = 788], PC36:2 [18:0–18:2; m/z = 784], PC36:4 [16:0–20:4; m/z = 810]) were less abundant in the cauda epididymidis than in the caput epididymidis, whereas those with sn-2 DPA and DHA (e.g., PC40:5 [18:0–22:5] and PC40:6 [18:0–22:6]) were higher in the cauda than in the caput (Figure 6C). These results indicate a significant increase in the degree of unsaturation of the fatty acyl components of PC during epididymal transit of sperm, which has been proposed to confer greater flexibility on the sperm membrane and thus higher motility (17–21).

We found that the fatty acyl composition of PC in cauda but not caput epididymal spermatozoa of Pla2g3−/− mice differed markedly from that of Pla2g3+/+ mice (Figure 6, A–C). Although PC species with oleic acid (PC34:1 and PC36:1) in sperm cells were decreased following their transit from the caput or cauda epididymidis in Pla2g3+/+ mice, this reduction was noticeably compromised in Pla2g3−/− mice (Figure 6C). Thus, the ratios of oleate-containing PC to total PC in cauda epididymal spermatozoa from Pla2g3+/+ and Pla2g3−/− mice were 10.1% and 21.3%, respectively, revealing 11% more oleate-containing PC in Pla2g3−/− sperm than in Pla2g3+/+ sperm. Furthermore, although the epididymal transit of sperm from the caput to the cauda was accompanied by marked decreases in PC species with arachidonic acid (PC36:4 and PC38:4) in both genotypes, cauda spermatozoa from Pla2g3−/− mice still retained significantly more arachidonate-bearing PC than those from Pla2g3+/+ mice (Figure 6C).
PC with linoleic acid (PC36:2) also showed a similar trend. Conversely, increases of PC species with DHA and DPA (PC40:6 and PC40:5) in the cauda epididymidis were less obvious in \textit{Pla2g3}$^-/-$ mice than in \textit{Pla2g3}$$^+/+$ mice (Figure 6C). Accordingly, the ratios of DHA/DPA-containing PC to total PC in cauda epididymal spermatozoa of \textit{Pla2g3}$$^+/+$ and \textit{Pla2g3}$^-/-$ mice were 31.2% and 23.4% (for DHA) and 35.2% and 26.4% (for DPA), respectively, revealing an 8%–9% reduction in DHA/DPA in \textit{Pla2g3}$^-/-$ mice relative to \textit{Pla2g3}$$^+/+$ mice. ESI-MS profiles of PC composition in the sperm-depleted epididymis of the two genotypes were nearly identical (Supplemental Figure 7, A and B).

These results suggest that sPLA$_2$-III secreted from the epididymal epithelium may act on maturing spermatozoa in the luminal duct in a paracrine manner, thereby facilitating the replacement of fatty acyl chains in PC from $\omega$-6/$\omega$-9 to $\omega$-3 PUFAs in sperm membrane.

Given the aforementioned results, we next investigated whether sPLA$_2$-III would affect the production of some oxygenated metabolites from the arachidonic acid pool released from membrane PC. As assessed by enzyme immunoassay, the levels of PGE$_2$ and PGF$_{2\alpha}$, which are arachidonate-derived products of the cyclooxygenase pathway, in the epididymal fluid from \textit{Pla2g3}$^-/-$ mice were approximately the same as those from \textit{Pla2g3}$$^+/+$ mice (Supplemental Figure 8A), suggesting that \textit{Pla2g3} deficiency does not affect the production of gonadal prostanoids. To search for other arachidonate metabolites, we performed liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) that was optimized for semiquantitative detection of various PUFA-oxygenated products (54, 55). Relative quantities of PUFA metabolites detected thus far in the epididymis of \textit{Pla2g3}$^-/-$ mice in comparison
with Pla2g3–/– mice are summarized in Figure 6D. Consistent with the results of enzyme immunoassay (Supplemental Figure 8A), the levels of PGE2, as well as other prostanoids, PGD2 and PGL2, in both genotypes were indistinguishable (Figure 6D), thus validating the results of LC-ESI-MS/MS. Notably, we found significantly lower levels of several lipoxynegenase metabolites, namely 8-hydroxyeicosatetraenoic acid (8-HETE), 12-HETE, and 15-HETE, in the epididymis of Pla2g3–/– mice than in that of Pla2g3+/+ mice (Figure 6D). Moreover, the levels of 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE, which are produced from linoleic acid by 12/15-lipoxygenase, were also markedly lower in Pla2g3+/+ mice than in Pla2g3–/– mice (Figure 6D). The levels of 5-HETE and 11,12-eicosysexatrienoic acid (11,12-EET), which are arachidonate metabolites of the 5-lipoxygenase and cytochrome P-450 pathways, respectively, did not differ appreciably between the genotypes (Figure 6D). It is notable that a reduction of the 12/15-lipoxygenase products 12- and 15-HETE and 13-HODE, rather than the cyclooxygenase products, was also found in the testis of Pla2g3–/– mice compared with Pla2g3+/+ mice (Figure 6E), even though the compositions of PC molecular species in the testis of the 2 genotypes were similar (Supplemental Figure 7C). The expression level of 12/15-lipoxygenase in the gonads was unaffected by Pla2g3 deficiency (data not shown). These results collectively suggest that the arachidonic and linoleic acids released by the action of sPLA2-III are coupled with the 12/15-lipoxygenase pathway, in preference to the cyclooxygenase, 5-lipoxygenase, and cytochrome P-450 pathways, in the male reproductive tract of mice. It is unlikely that the selective reduction of 12/15-lipoxygenase products in the gonads of Pla2g3–/– mice was due to altered innate immune inflammatory equilibrium, since expression profiles of classical inflammatory cytokines were not altered in the epididymis and testis of the knockout mice (data not shown).

In addition, we found that the epididymal fluid contained substantial levels of PC species with oleic acid (PC34:1 [16:0–18:1] and PC36:1 [18:0–18:1]), which were significantly more abundant in Pla2g3–/– mice than in Pla2g3+/+ mice (Figure 6F). This suggests that the lipid-associated components of epididymal luminal fluid, which appears to include lipoproteins or epididymosomes (microvesicles secreted from epididymal epithelial cells; ref. 17), may be altered in Pla2g3–/– mice. Since it has been proposed that PUFAs in the sperm membrane play a protective role against oxidative stress by scavenging reactive oxygen species (18), we assessed the state of lipid peroxidation in the epididymis of Pla2g3+/+ and Pla2g3–/– mice. Although the relative degree of unsaturation of fatty acids in cauda epididymal spermatozoa was lower in Pla2g3–/– mice than in Pla2g3+/+ mice (see above), the level of epididymal lipid peroxidation, as evaluated by accumulation of malondialdehyde and 4-hydroxyalkenals, did not differ significantly between the genotypes (Supplemental Figure 8B).

Discussion

Epithelial cells of the epididymis provide the microenvironment necessary for structural remodeling of spermatozoa (1). Through regulated secretion and reabsorption, they control the pH, osmotic pressure, and composition of epididymal fluids and also the transfer of metabolites such as proteins, sugars, and lipids to and from the sperm surface. Interactions with the proximal epididymis are essential for the acquisition of fertilizing ability by sperm, and alteration of the genes encoding several proteins secreted from the epididymal epithelium often leads to male infertility as a result of defective sperm motility or morphology (10, 42, 56–59). We found that sPLA2-III is expressed in the proximal epididymal epithelium and secreted into the luminal fluid (Figures 1 and 2), that spermatozoa of Pla2g3–/– mice are numerically normal but functionally and structurally abnormal (Figures 3–5), and that epididymal sperm of Pla2g3+/+ mice have an altered phospholipid composition (Figure 6). Importantly, sperm malformation in Pla2g3–/– mice proceeds during epididymal transit from the caput to the cauda epididymis, at which time sperm membrane remodeling is severely impaired, suggesting that the reproductive defect of male Pla2g3–/– mice results primarily from altered lipid homeostasis in the epididymis. Our results shed some light on the reported changes in the proportion of DHA and DPA in epididymal maturing sperm in various species, including humans (18–23).

Male but not female Pla2g3–/– mice and to a lesser extent Pla2g3–/– mice had greatly reduced fertility (Table 1), accompanied by impaired sperm motility (Figure 3F and Supplemental Videos 1 and 2). In agreement, cauda spermatozoa from Pla2g3–/– mice had less ATP and lower capacitation-induced tyrosine phosphorylation levels than did those from Pla2g3+/+ mice (Figure 4, A–C). In addition, cauda spermatozoa from Pla2g3–/– mice had aberrant flagellar structures with unusual outer and inner dynein arms, a central pair of microtubules, and a fibrous sheath (Figure 5). In fact, spermatozoa from mice harboring abnormal flagella, due to defects in flagellar axoneme proteins such as Spag6, Spag16, Tekt2, Paerc, and Akap4, are commonly unable to swim and fail to reach the oviducts in females (49–53), events that are reminiscent of Pla2g3–/– mice. Interestingly, flagellar abnormality in Pla2g3–/– spermatozoa was less obvious in the testis and caput epididymis than in the cauda epididymis (Figure 5, E and F), suggesting that the observed structural changes in Pla2g3–/– spermatozoa mainly take place during epididymal transit from the caput to cauda epididymis. Furthermore, spermatozoa from Pla2g3–/– mice showed reduced ability to interact with oocytes (Figure 3, D and E), consistent with abnormal head morphology (globozoospermia) (Figure 4E and Figure 5C). Moreover, even if fertilization was successful, many of the eggs that had fused with...
In the present study, we found that transit of spermatozoa containing higher proportions of DHA have better fluidity of the sperm membrane (17), and as a matter of fact, midis, a process that is thought to contribute to increasing the fluidity of spermatozoa during their passage from the caput to the cauda of the epididymis (see text). Another sPLA₂ isoform, sPLA₂-X, is stored in acrosomes in spermatozoa and is released during capacitation and acrosome reaction to affect fertility (68). Thus, the 2 particular sPLA₂s (sPLA₂-III and -X), which are expressed in different locations within the male reproductive organs, exert nonredundant functions in 2 major steps of male fertility, one during epididymal sperm maturation and the other during capacitation. Dotted lines indicate sperm movement.

Figure 7
A proposed model for the sPLA₂ network in reproduction. During epididymal transit of maturing sperm cells, PC in the sperm membrane undergoes a dramatic remodeling of PUFAs, with more ω-3 and less ω-6/ω-9 PUFAs in mature spermatozoa. sPLA₂-III, which is secreted from epithelial cells into the luminal fluid, may regulate this membrane-remodeling process by facilitating deacylation of oleate (OA), linoleate (LA), and arachidonate (AA) from sperm membrane PC. Accordingly, Pla2g3−/− sperm contain less DHA/DPA than WT sperm, culminating in abnormal sperm motility and fertility. Both in the epididymis and testis (in which sPLA₂-III is located in supporting cells such as Sertoli cells and Leydig cells), significant portions of the LA and AA liberated by sPLA₂-III are metabolized to their oxygenated products by 12/15-lipoxygenase, whose biological significance in sperm maturation remains obscure. sPLA₂-III may also regulate lipid transport between spermatozoa and epithelial cells in the epididymis (see text). Another sPLA₂ isof orm, sPLA₂-X, is stored in acrosomes in spermatozoa and is released during capacitation and acrosome reaction to affect fertility (68). Thus, the 2 particular sPLA₂s (sPLA₂-III and -X), which are expressed in different locations within the male reproductive organs, exert nonredundant functions in 2 major steps of male fertility, one during epididymal sperm maturation and the other during capacitation. Dotted lines indicate sperm movement.

Pla2g3−/− sperm failed to develop properly into morulae (Figure 3, G and H). These results collectively suggest that spermatozoa from Pla2g3−/− mice have defects in multiple steps necessary for sperm fertility and even subsequent embryo development, most likely because of impaired sperm maturation.

Sperm cells leaving the testis must pass through the epididymal duct to acquire modifications necessary to reach, recognize, and fuse with the female gamete. Such modifications involve sperm lipid remodeling during interaction of spermatozoa with the epididymal luminal microenvironment, which is loaded with secretions from the epithelial cells (1). The relative abundance of DHA and DPA in mouse spermatozoa has been shown to increase during their passage from the caput to the cauda of the epididymis, a process that is thought to contribute to increasing the fluidity of the sperm membrane (17), and as a matter of fact, spermatozoa containing higher proportions of DHA have better fertilizing ability in various animal species, including humans (18–23). In the present study, we found that transit of spermatozoa from the caput to the cauda epididymis was accompanied by decreases in several PC species with oleic, linoleic, and arachidonic acids and by compensatory increases in those with DHA and DPA in the WT sperm membrane, whereas this membrane remodeling was markedly even if not entirely compromised in Pla2g3−/− mice (Figure 6, A–C). Accordingly, cauda epididymal spermatozoa in Pla2g3−/− mice had PC species containing more oleate and less DHA/DPA than did those in Pla2g3+/+ mice, a finding that appears to be consistent with previous reports indicating that sperm with higher proportions of DHA have better motility and fertility (17–23). On the basis of these observations, it is tempting to speculate that sPLA₂-III may participate in the hydrolysis of PC with oleic, linoleic, and arachidonic acids in the sperm membrane during epididymal transit and that this event may be followed by reacylation of lysophosphatidylcholine, a PLA₂ reaction product, with DHA and DPA, leading to an increase of PC with DPA/DHA in mature sperm. In the Pla2g3−/− epididymis, impairment of the deacylation step may eventually perturb the subsequent reconstitution of DPA/DHA in the sperm membrane, culminating in the asthenozoospermia phenotype. The alteration in sperm phospholipid composition in Pla2g3−/− mice
cells (e.g., testicular Sertoli and Leydig cells as well as epididymal epithelial cells) (62, 63) could be regarded as further evidence for the action of sPLA<sub>2</sub>-III on the sperm membrane, where the arachidonate/linoleate released by sPLA<sub>2</sub>-III would be more readily accessible to proximal 12/15-lipoxygenase than to distant cyclooxygenases. Although the role of 12/15-lipoxygenase in male fertility has not yet been fully established, expression of this enzyme in spermatogenic cells has led to the suggestion that it may participate in sperm maturation (62). In this context, the possibility that certain 12/15-lipoxygenase-derived lipid mediator(s) may be at least partly responsible for the regulation of sperm maturation by sPLA<sub>2</sub>-III should be taken into account.

Additionally, sPLA<sub>2</sub>-III may also affect lipid transport between spermatozoa and epididymal epithelial cells. Several lipoprotein components, such as apoB, apoE, apoA-I, and apoJ (clusterin), are secreted by epididymal epithelial cells (64) and associate with and dissociate from sperm membranes scheduled for endocytosis by epididymal principal cells (65). ApoE receptor-2 and the ABC lipid transporters are abundantly expressed on the epididymal epithelium and sperm membrane, respectively (10, 66). Male fertility can be impaired to various degrees by inactivation of these genes involved in lipoprotein metabolism (8–11). Moreover, membrane transport by epidydmosomes, a particular lipoprotein membrane particle emitted from caput epididymal principal cells into the lumen, is fundamental for the process of sperm cell maturation in the epididymis (17, 67). Our speculation that sPLA<sub>2</sub>-III may also affect this epididymal lipid transport is supported by the finding that the epididymal fluid from Pla2g3<sup>−/−</sup> mice contained more PC than that from Pla2g3<sup>+/+</sup> mice (suggesting impaired hydrolysis of lipoprotein-associated PC) (Figure 6F). Thus, although transgenenic overexpression of sPLA<sub>2</sub>-III in mice leads to systemic hydrolysis of PC in circulating lipoproteins (39), under physiological situations this reaction may operate within a local niche (i.e., the epididymal duct) in which endogenous sPLA<sub>2</sub>-III is intrinsically expressed.

Although sPLA<sub>2</sub>-III is expressed in the testis (Figure 1 and Supplemental Figure 1C), our data, including normal sperm counts, testosterone levels and testicular weight, unaltered testicular phospholipid profiles, and minimal alteration in testis histology in Pla2g3<sup>−/−</sup> mice, argue against the significant contribution of this enzyme to testicular spermatogenesis. The only notable alteration in the testis of Pla2g3<sup>−/−</sup> mice is the reduction of 12/15-lipoxygenase products, whose functions in the testis remain obscure in view of the current absence of a report describing the reproductive phenotype in Alox15<sup>−/−</sup> mice. However, our present results do not rule out the possibility that sPLA<sub>2</sub>-III might participate in certain steps of spermatogenesis or other events in the testis. Considering that several other sPLA<sub>2</sub>s, including sPLA<sub>2</sub>-Ic, -IId, -Ilf, -V, and -X, are also expressed in distinct cell populations in mouse testis (41), the absence of apparent phenotypes in the testis of Pla2g3<sup>−/−</sup> mice might be because of the redundant or compensatory actions of other sPLA<sub>2</sub>s in this tissue.

Targeted disruption of Pla2g6, which encodes iPLA<sub>2</sub>B, leads to male infertility because of impaired sperm motility (24), and ablation of Pla2g4a (cPLA<sub>2</sub>α), which plays a pivotal role in eicosanoid generation in various biological events, impairs female reproductive ability by preventing implantation and parturition (2, 3). However, despite the fact that various sPLA<sub>2</sub>s are expressed in male genital organs (41), little has been known about their roles in reproduction. The present work demonstrates for the first time to our knowledge that sPLA<sub>2</sub>-III, an atypical sPLA<sub>2</sub> that is supplied from gonadal microenvironments, controls sperm maturation. In addition, Escoffier et al., in collaboration with us, have very recently shown that sPLA<sub>2</sub>-X, a sperm acrosomal sPLA<sub>2</sub>, controls capacitation, acrosome reaction, and thereby fertility (68). Thus, various forms of intracellular and extracellular PLA<sub>2</sub> enzymes participate in the regulation of distinct steps of reproduction through distinct mechanisms. Of particular importance, the sequential control of epididymal sperm maturation and postepididymal sperm capacitation by sPLA<sub>2</sub>-III from supporting cells and sPLA<sub>2</sub>-X from sperm acrosomes, respectively, in the gonadal niche has opened new insights into the physiological role of this particular extracellular phospholipase family in the reproduction biology (Figure 7).

Finally, given the fact that sPLA<sub>2</sub>-III is expressed in human epididymal epithelium (Supplemental Figure 2), it is likely that the enzyme plays a similar role in humans. In this context, the question of whether sPLA<sub>2</sub>-III might also contribute to the regulation of epididymal sperm maturation in humans needs further elucidation. From a clinical standpoint, the reduced motility and fertilization competence of spermatozoa from male Pla2g3-null mice suggest that sPLA<sub>2</sub>-III is a potential target for the development of male contraceptive agents or a potential diagnosing marker for the male sterility. On the other hand, application of a particular agent that is capable of inhibiting sPLA<sub>2</sub>-III in patients with atherosclerosis or inflammation might necessitate caution, in light of the associated risk of inducing male infertility.

**Methods**

**Targeted disruption of the Pla2g3 gene.** The pla2g3<sup>−/−</sup>-targeting vector was constructed with a neo<sup>+</sup> gene driven by the polycloma enhancer/herpes simplex virus thymidine kinase (MC1) promoter. The MC1-neo<sup>+</sup> cassette was inserted into the exons encompassing the catalytic domain and introns of the Pla2g3 gene (Figure 1C). The 5.5-kb long arm and 1.7-kb short arm genomic fragments from the Pla2g3 gene were isolated from the 129SvEv genome using PCR. The diphtheria toxin A gene (rdtA, a synthetic construct modified diphtheria toxin fragment gene) was inserted at the 3′ end of the short arm of the targeting vector for negative selection. The targeting vector linearized by HindIII digestion was transfected into mouse ES cells of 129SvEv origin (Dainippon Sumitomo Pharma). Appropriately targeted ES cell clones were used to obtain chimeric mice that transmitted the disrupted locus through the germ line. The 10- to 15-week-old mice were backcrossed to the C57BL/6J background for 1 or 2 generations; WT littermates were used as controls. Genotyping was performed on genomic DNA isolated from tail biopsies by PCR using the following primers: 5′-TGAGTCAGCTCTCAAGGAAGA-CAAGTCC-3′ (WT forward), 5′-CCATCTGTACTGACTGAGATCCCAA-3′ (mutant reverse), and 5′-GTTGAGAGATGGTGAGAAAACGAAA-3′ (WT reverse). The primer set of WT forward and mutant reverse amplified a 627-bp fragment specific for the mutant allele, and the set of WT forward and WT reverse yielded a 375-bp fragment specific for the WT allele. The reaction was carried out using an ExTaq Kit (Takara Bio) with 35 cycles of amplification (95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 30 seconds). For Southern blot analysis, genomic DNA was digested with SphI and hybridized with a probe as indicated in Figure 1C.

Mice lacking sPLA<sub>2</sub>-X (Pla2g10<sup>−/−</sup>) are described elsewhere (68). All mice were housed in climate-controlled (21°C), specific pathogen–free facilities with a 12-hour-light/12-hour-dark cycle, with free access to standard laboratory food (Picolab mouse diet 20; Laboratory Diet) and water. All procedures involving animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of Showa University and of the Tokyo Metropolitan Institute of Medical Science, in accordance with the standards relating to the care and management of experimental animals in Japan.
Histological analyses. Immunohistochemistry of tissue sections was performed as described previously (38, 39). In brief, paraffin-embedded mouse testis and epididymis sections (4-μm thick) were incubated with Target Retrieval Solution (Dako Cytonation) and then with anti-sPLA2-III or control antibody at 1:100 dilution in 10 mM Tris-HCl (pH 7.4), containing 0.15 M NaCl overnight at 4°C. The sections were then treated with a CSA System Staining Kit (Dako Cytonation) with diaminobenzidine substract, followed by counterstaining with hematoxylin. Human epididymal sections (male, 16 years old) were obtained through surgery at Toho University School of Medicine, following approval by the ethical committee of the Faculty and informed consent from the patient.

For transmission EM, testis and epididymis samples were fixed with 2.5% glutaraldehyde, post-fixed with 2% OsO4, dehydrated through an ascending ethanol series, passed through propylene oxide, and then embedded in Queto812 resin (Nissin EM). Ultrathin sections (90-μm thick) on mesh grids were stained with uranyl acetate and lead and examined with an H-300 electron microscope (Hitachi). Real-time quantitative RT-PCR. First-strand cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR analysis was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), using Power SYBR Green PCR Master Mix (Applied Biosystems). For the detection of PLA2g10 mRNAs, TaqMan probes from Applied Biosystems were used: Mm00555594_m1 and Mm01191142_m1 (Pla2g3), Mm00448161_m1 (Pla2g5), Mm00449530_m1 (Pla2g10), Mm00447404_m1 (Pla2g4), Mm00479527_m1 (Pla2g6), and Mm00470656_m1 (Pla2g8). The Taqman probe X00686 (Rn18S; 18S ribosomal RNA) was used as a control. The thermal cycling conditions comprised an initial denaturation step at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds. Semiquantitative RT-PCR for mouse Pla2g3 mRNA was performed with a set of primers (5′-GGCTGGAGGCC-CACCTCTATACTCCTC-3′ and 5′-TCTTCTTGGCTCCAGCACCTAGA-3′), using EsTaq polymerase with 35 cycles of amplification (95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 30 seconds). The amplified product was electrophoresed on 1% (w/v) agarose gel, with ethidium bromide.

Measurement of PLA2 activity. PLA2 activity was assayed by measuring the amounts of radiolabeled linoleic acid released from the substrate 1-palmitoyl-2-[14C]linoleoyl-phosphatidylethanolamine (Perkin-Elmer), as described previously (37). Each reaction mixture consisted of appropriate amounts of the epididymal fluid, 100 mM Tris-HCl (pH 7.4), 4 mM CaCl2, and the substrate at 1 μM. After incubation for 30 minutes at 37°C, [14C]linoleic acid was extracted, and the radioactivity was quantified with a liquid scintillation counter.

Sperm motility. Male mice (8 weeks old) were sacrificed by cervical dislocation, and a tubule segment was dissected from the cauda epididymis. The luminal sperm were allowed to disperse in 200 μl of HTF medium (ARK Resource) for 10 minutes. After the tissue had been removed, the sperm were allowed to capacitate for 2 hours at 37°C in 5% CO2 in air. A 5-μl aliquot of the sperm suspension was then placed in a counting chamber (Standard Count; Laje) for assessment of motility by computer-assisted analysis, using a Sperm Motility Analysis System (KAGA Electronics). The parameters measured were the percentage of motile sperm cells, vigor of movement (curvilinear velocity), speed of forward progression (straight-line velocity), amplitude of lateral head displacement, beat cross-frequency, and linearity of swim path.

IVF. Female mice (10 weeks old) were injected intraperitoneally with 7.5 IU pregnant mare serum gonadotropin (Asuka Pharmacy), followed 48 hours later with 7.5 IU human chorionic gonadotropin (Asuka Pharmacy). Oviducts were collected 13 hours later, and the oocyte cumulus complexes were placed in 100 μl HTF medium in a 60-mm culture dish (Iwaki), and droplets were covered by embryo-tested mineral oil (Nakarai Tesque).

As required, oocytes were treated with 0.1% hyaluronidase (Sigma-Aldrich) for 30 minutes at 37°C to remove the cumulus mass, soaked in Tyrode’s buffer (Sigma-Aldrich) to remove the ZP, and resuspended in 100 μl HTF medium. Spermatozoa collected from the cauda epididymis were allowed to swim into 50 μl HTF medium, aspirated, incubated in 200 μl of HTF medium for 60 minutes at 37°C to permit capacitation, diluted, and added to oocyte droplets to achieve a concentration of 200 spermatozoa/μl. After the spermatozoa and oocytes had been coincubated for 6 hours at 37°C, the oocytes were washed and cultured for 24 hours before examination of oocyte fertilization, as demonstrated by the presence of a second polar body and 2 pronuclei, one near a sperm tail.

Sperm binding to the ZP. Cumulus-free oocytes were incubated with spermatozoa (200 cells/μl) in HTF drops for 3 hours at 37°C. After washing, nuclear DNA in the sperm-oocyte complexes was stained with 1 μg/ml Hoechst 33342 (Sigma-Aldrich), and the number of sperm bound to the oocytes was counted using a BX-9000 fluorescence microscope (Keyence).

Quantification of ATP content of spermatozoa. Cauda epididymal spermatozoa were incubated for 1 hour at 37°C under a 5% CO2 atmosphere in M16 medium (Sigma-Aldrich). Floating sperm cells (107 cells) were collected and resuspended in 50 μl M16 medium, to which 450 μM sample buffer (4 mM EDTA/0.1 M Tris-HCl, pH 7.8) was added, and boiled for 2 minutes. After centrifugation for 5 minutes at 20,000 g, ATP in the supernatants was quantified by an ATP Bioluminescence Assay Kit CLS II (Roche Applied Science) and Gene LIGHT S5 (Microtec Nichion).

Immunoblotting. Homogenates of testis and epididymis (20 μg protein equivalents) or cauda epididymal sperm (107 cell equivalents) were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). After blinding, the membranes were incubated with anti-sPLA2-III antibody (a gift from S. Hatakeyama, NOVARTIS Pharma, Tsukuba, Japan, which reacts specifically with sPLA2-III but not with other sPLA2s) (38, 39), anti-ADAM2 (fertilin β) antibody (clone 9D2.2) (Chemicon), anti-phospho-tyrosine antibody (PY20) (Santa Cruz Biotechnology Inc.), or anti-α-tubulin antibody (Santa Cruz Biotechnology Inc.) (1:5,000 dilution) and then with horseradish peroxidase-conjugated anti-lgg antibody (Zymed) (1:10,000 dilution) in Can Get Signal Solution (TOYOBO). After washing with TBS-Tween, the membranes were soaked using an ECL Western Blot Analysis System (Amersham) and examined with a Light-Capture Cooled CCD Camera System (ATTO).

Mass spectrometric analysis. Lipids were extracted from tissue or cell homogenates or fluids using the method of Bligh and Dyer (69). Before lipid extraction, PC with C28:0 (14:0–14:0) was added to each sample as an internal standard (2 nmol per epididymal [50 mg] or testicular [200 mg] tissue or whole sperm cells). The ESI-MS analysis was performed using a 4000Q-TRAP quadrupole-linear ion trap hybrid mass spectrometer (Applied Biosystems/MDS Sciex), with an Ultimate 3000 HPLC system (DIONEX) combined with an HTC PAL autosampler (CTC Analytics). The extracted lipids were subjected to ESI-MS analysis by flow injection (3 nmol phosphorus equivalent), with a mobile phase composition of acetonitrile/methanol/water (6:7:2, v/v), plus 0.1% ammonium formate, pH 6.8, at a flow rate of 10 μl/min. The scan range of the instrument was set at 200–1,000 m/z, at a scan speed of 1,000 Da/s in the positive ion mode. The trap fill time was set at 1 ms, the ion spray voltage was set at 5,500 V, and the declustering potential was set at 100 V. Nitrogen was used as the curtain gas (setting of 10 arbitrary units) and as the collision gas (set to “high”). The method used for detection of the proper precursors and neutral losses derived from phospholipids in ESI-MS/MS analysis has been described previously (70, 71).

For the assay of oxygenated lipids using LC-ESI-MS/MS analysis, tissues were soaked in 10 volumes of methanol and then homogenized with a Polytron homogenizer. After overnight incubation at –20°C, H2O
was added to the mixture for a final methanol concentration of 10%. 18: Labeled arachidonic acid (Cayman Chemicals) was added as an internal standard. The oxygenated lipids in the supernatant were extracted using Sep-Pak C18 cartridges (Waters). The LC-ESI-MS/MS analysis was performed using a 4000Q TRAP quadrupole-linear ion trap hybrid mass spectrometer (Applied Biosystems/MDS Sciex) with ACQUITY Ultra Performance LC (Waters). The sample was applied to the ACQUITY UPLC BEH C18 column (1 × 150 mm i.d., 0.17 µm particle) and then to ESI-MS/MS analysis. The samples injected by the autosampler (10 µl) were directly introduced and separated by a step gradient with mobile phase A (water containing 0.1% formic acid) and mobile phase B (acetonitrile/methanol, 4:1, v/v) at a flow rate of 50 µl/min and a column temperature of 30°C. The specific detection of individual lipids was performed by multiple reaction monitoring (54, 55).

Other procedures. Levels of serum testosterone and epididymal prostanoids were quantified using respective enzyme immunoassay kits (Cayman Chemicals). Lipid peroxidation was evaluated by quantifying malondialdehyde and 4-hydroxynonenals, using an LPO Assay Kit (BIOXYTECH LPO-586 (OXIS International)). Protein concentrations were determined using a BCA protein assay kit (Pierce). The immortalized mouse Sertoli cell line Sertoli B was provided by T. Hara (Tokyo Metropolitan Institute of Medical Science) (72). Data were statistically evaluated by unpaired Student’s t-test, and P values of less than 0.05 were considered significant.

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Address correspondence to: Makoto Murakami, Biomembrane Signaling Project, The Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan. Phone: 81.3.5316.3228; Fax: 81.3.5316.3125; E-mail: murakami-mk@igakukenu.or.jp.
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