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Organization of Membrane Rafts in Chicken Sperm

Atsushi Asano[1], Hitomi Kanbe[2], Ai Ushiyama[2] and Atsushi Tajima[1]

[1] Faculty of Life and Environmental Sciences, University of Tsukuba, Japan
[2] Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan

Being transcriptionally and translationally inactive, sperm must utilize preassembled pathways into specific compartments in which they function to fertilize ovum. Membrane rafts are specific membrane regions enriched in sterols and glycosphingolipids such as ganglioside G_{M1} (G_{M1}) and play an important role in a variety of cellular functions. Recent findings have demonstrated that membrane rafts are present in mammalian sperm and are involved in regulating the induction of acrosome exocytosis. However, no information is available on whether avian sperm possess membrane rafts. Thus, we investigated the organization of membrane rafts in chicken sperm. Our localization experiments for G_{M1} and sterols showed that the plasma membrane overlaying the sperm head possesses specific membrane domains enriched in both aforementioned lipids. Caveolin-1, which localizes into membrane rafts in other systems, was localized only to the sperm tail. Based on the biochemical definition that membrane rafts are insoluble membranes when subjected to a Triton X-100 treatment, we isolated detergent-insoluble membranes from chicken sperm and quantified the G_{M1} content, which showed an enrichment of G_{M1} in the membrane fraction relative to the detergent-soluble fraction. Together with the results of localization and biochemical experiments, we demonstrate for the first time that membrane rafts exist in chicken sperm. Thus, our results provide a foundation for investigating a novel cellular pathway inherent in avian sperm membranes that might be involved in functions necessary to achieve fertilization.

Key words: chicken, ganglioside G_{M1}, lipids, membrane rafts, sperm

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Introduction

Membrane rafts are specific regions of membranes enriched in sterols and glycosphingolipids such as ganglioside G_{M1} (G_{M1}), as opposed to phospholipids, and they act as foci for a wide variety of cellular functions (Simons and Toomre, 2000; Simons and Gerl, 2010). Because they are small and highly dynamic assemblies, demonstrating the existence of membrane rafts based only on the localization of raft-associated lipids is problematic. Therefore, many studies on the organization and function of membrane rafts have utilized another characteristic, i.e., their resistance to solubilization when incubated at low temperatures with detergents such as Triton X-100 (TX-100) to isolate them biochemically (Brown and Rose, 1992; Schroeder *et al*., 1994). Using these compositional and biochemical characteristics, the presence and function of membrane rafts have been investigated in diverse cell types (Rajendran and Simons, 2005; Simons and Gerl, 2010), including male germ cells (Bou Khalil *et al*., 2006; Selvaraj *et al*., 2006).

Cellular membranes represent an extensive diversity of lipids and proteins, and sperm are no exception. Terminally differentiated, sperm are highly polarized, with functions restricted to specific regions. They consist of a distinct tail and head; the latter contains one intracellular vesicle, i.e., the acrosome. In mammalian sperm, the plasma membrane overlaying the acrosome (APM) is unusual in composition. For example, multiple freeze–fracture studies using filipin to complex with membrane sterols in murine sperm have demonstrated that this membrane region is highly enriched in sterols (Lin and Kan, 1996; Visconti *et al*., 1999). We recently found that, in live murine sperm, the APM is composed of multiple membrane domains with focal enrichments of sterols (Selvaraj *et al*., 2009). Furthermore, using cholera toxin subunit B (CTB), which specifically binds to G_{M1}, we found that the APM is highly enriched in G_{M1} (Selvaraj *et al*., 2006), as seen with caveolin-1 (Travis *et al*., 2001). This characteristic of G_{M1} segregation into the APM is conserved across species, at least in mammals (Buttke *et al*., 2006). These localization studies, and the great functionality of the APM domain where acrosome exocytosis occurs in response to sterol efflux from the plasma membrane, suggest that there are membrane rafts within the APM domain that

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Correspondence: A. Asano, PhD, Faculty of Life and Environmental Sciences, University of Tsukuba, Tennoudai 1–1–1, Tsukuba, Ibaraki 305–8572, Japan. (E-mail: asano.atsushi.ft@u.tsukuba.ac.jp)

stimulate functions when needed. Several studies have attempted to determine the possible roles of membrane rafts in mammalian sperm and have demonstrated potential involvement in capacitation (Thaler *et al*., 2006), binding to the zona pellucida (Bou Khalil *et al*., 2006), and acrosomal exocytosis (Asano *et al*., 2013). Considering that sperm are transcriptionally and translationally inactive, this strategy using membrane rafts could be required for sperm to activate preassembled cellular pathways into a specific region that later functions to fertilize an ovum.

However, unlike mammalian sperm, avian sperm do not require a capacitation process to acquire the competency to fertilize; however, they share a need to undergo acrosomal exocytosis prior to penetration into the ovum by interacting with the inner perivitelline layer, which is considered to be analogous to the mammalian zona pellucida (Howarth, 1990). This similarity and distinction in functional changes in sperm between groups, and the functional requirement for the membrane to change to achieve fertilization, motivated us to investigate the nature of the cellular membrane in chicken sperm. Our findings demonstrate for the first time that membrane rafts exist in chicken sperm and will become a basis for investigations of novel cellular pathways pre-assembled in membranes where functions are excited.

Materials and Methods

Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Cholera toxin subunit B conjugated with Alexa Fluor 488 and horseradish peroxidase (CTB-488 and CTB-HRP, respectively) were obtained from Life Technologies (Carlsbad, CA, USA). Filipin III and antiserum against caveolin-1 were acquired from Cayman Chemical (Ann Arbor, MI, USA) and BD Transduction Laboratories (Lexington, KY, USA), respectively.

Sperm Collection

Ejaculated sperm were collected from sexually mature male Road Island Reds at the Agricultural Forestry Center, University of Tsukuba, using an abdominal massage method (Burrows, 1937) and then centrifuged at 1,000×*g* in phosphate-buffered saline (PBS) to remove seminal plasma. The testes were collected for preparing frozen sections. All animal work was performed with approval from the Institutional Animal Use Committees of the University of Tsukuba (Approval no. 15-018).

Localization of Lipids in Sperm and Testis

The localizations of G_{M1} and sterols in sperm and frozen testis sections were visualized using CTB-488 and filipin III, respectively, as previously described (Selvaraj *et al*., 2006). In brief, sperm were fixed with 4% paraformaldehyde and then washed with PBS. The sperm were incubated with 5 μg/mL CTB-488 for 15 min and then washed with PBS again. The samples were incubated with 10 μg/mL filipin for 15 min and mounted on a glass slide. Localizations of G_{M1} and sterols were viewed with a Leica DMI4000B microscope equipped with an AF6000 imaging system (Leica Microsystems, Wetzlar, Germany).

Frozen testis sections, which were previously sliced at 4 μm thickness, were incubated with PBS for 1 h to remove the OCT compound and fixed as described above. The tissue sections were incubated with 10 μg/mL CTB-488 for 1 h and then washed with PBS. Coverslips were mounted on glass slides using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA).

Isolation of Membrane Rafts

For the fractionation of membrane rafts, all steps were performed on ice or at 4℃ in the presence of protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). Cell membranes of sperm (2.5×10^8 cells) were first isolated by sonication in PBS and centrifuged for 10 min at 10,000×*g* to separate sperm debris. Membrane rafts are biochemically defined as being insoluble membranes when subjected to a TX-100 treatment at low temperatures (Brown and Rose, 1992). Utilizing this definition, the supernatant containing sperm membranes was mixed with 0.5% TX-100 and incubated for 15 min. The sample was centrifuged for 2 h at 20,000×*g* to fractionate insoluble and soluble membranes (representing membrane rafts and non-rafts, respectively).

G_{M1} Quantification

To determine the G_{M1} content in insoluble and soluble membrane fractions, both were subjected to slot blotting as described previously (Asano *et al*., 2009). In brief, samples were blotted using a Slot Blot Manifold (Hoefer, San Francisco, CA, USA) onto a PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA), and the membrane was blocked with 5% bovine serum albumin and incubated with 0.5 μg/mL CTB-HRP for 1 h at room temperature. The G_{M1} expression was detected by chemiluminescence using ChemiDoc XRS + (Bio-Rad, Hercules, CA, USA), and the resulting bands were subjected to densitometry using ImageJ 1.47v software downloaded from the NIH website (http://imagej.nih.gov/ij/).

Immunoblotting

Proteins were extracted from 1×10^7 sperm by boiling in sample buffer (Laemmli, 1970) and separated by SDS-PAGE. Transfer, blocking, and immunodetection of caveolin-1 were performed largely as previously described (Asano *et al*., 2009), except for the use of 5% fish gelatin as a blocking agent. Antiserum to caveolin-1 was utilized at a 1:500 dilution as a primary antibody. Anti-mouse IgG conjugated with HRP (GE Healthcare Life Sciences, Pittsburg, PA, USA) was utilized at a 1:2,500 dilution. Chemiluminescence was used to detect immunoreactivity.

Immunocytochemistry

Sperm were allowed to settle on MAS-coated glass slides (Matsunami Glass, Osaka, Japan) for 15 min and fixed. Caveolin-1 is a membrane-spanning hairpin-shaped protein which has both amino and carboxyl termini directed towards cytoplasm (Williams and Lisanti, 2004). Therefore, artificial permeabilization of membranes has been often given prior to immunostaining. In this study, sperm were permeabilized in 1% TX-100 for 1 min and blocked with 10% goat

serum for 1 h. Then, they were incubated with caveolin-1 antibody (1:100 dilution) overnight at 4℃ and incubated with anti-mouse IgG conjugated with Texas red (1:150 dilution) and 40 μg/mL FITC-PNA for 1 h. Coverslips were mounted as described above.

Statistical Analysis

Data for G_{M1} quantification were collected from six replicates. Statistical analyses for pairwise comparisons were carried out with a paired *t*-test when the *F*-test and Kolmogorov–Smirnov/Lilliefors test confirmed both equal variance and normality assumptions. Data are expressed as means±SEM.

Results

Localization of G_{M1} and Sterols in Chicken Germ Cells

In chicken testis, G_{M1} localization was observed in the sperm head region where nuclei were present (Fig. 1A, a-d). However, it was not present in the acrosomal membranes of condensed spermatids (Fig. 1A, a–d). Sterols were abundant in cytoplasm of elongating spermatids or more mature sperm relative to spermatocytes or spermatids of other stages, and the plasma membranes of sperm head appeared not to be enriched in sterols (Fig. 1B, a–e).

In ejaculated sperm, G_{M1} was localized to the sperm head region except for the apical ridge and was not observed in sperm tail (Fig. 2A and B), while sterols were highly abundant in the entire sperm head region (Fig. 2C and D). These results demonstrated that there are specific membrane domains enriched in both G_{M1} and sterols in the plasma membrane overlaying the sperm head region.

Expression and Localization of Caveolin-1 in Chicken Sperm

Western blotting showed caveolin-1 immunoreactivity at a predicted molecular weight (approx. 20 kDa; Fig. 3A). Unexpectedly, however, it was localized to the tail only and was not observed in any of the sperm head regions (Fig. 3B). These results demonstrated that caveolin-1 was only localized to sperm tail.

Biochemical Characterization of Membrane Rafts in Chicken Sperm

G_{M1} content in the TX-100-insoluble membrane fraction (108.9±15.0) was significantly greater than that of the TX-100-soluble membrane fraction (8.7±1.8, $P<0.05$; Fig. 4). Considering with the basic notion that membrane rafts are enriched with G_{M1}, this result demonstrated that there are membrane rafts enriched in G_{M1} in chicken sperm.

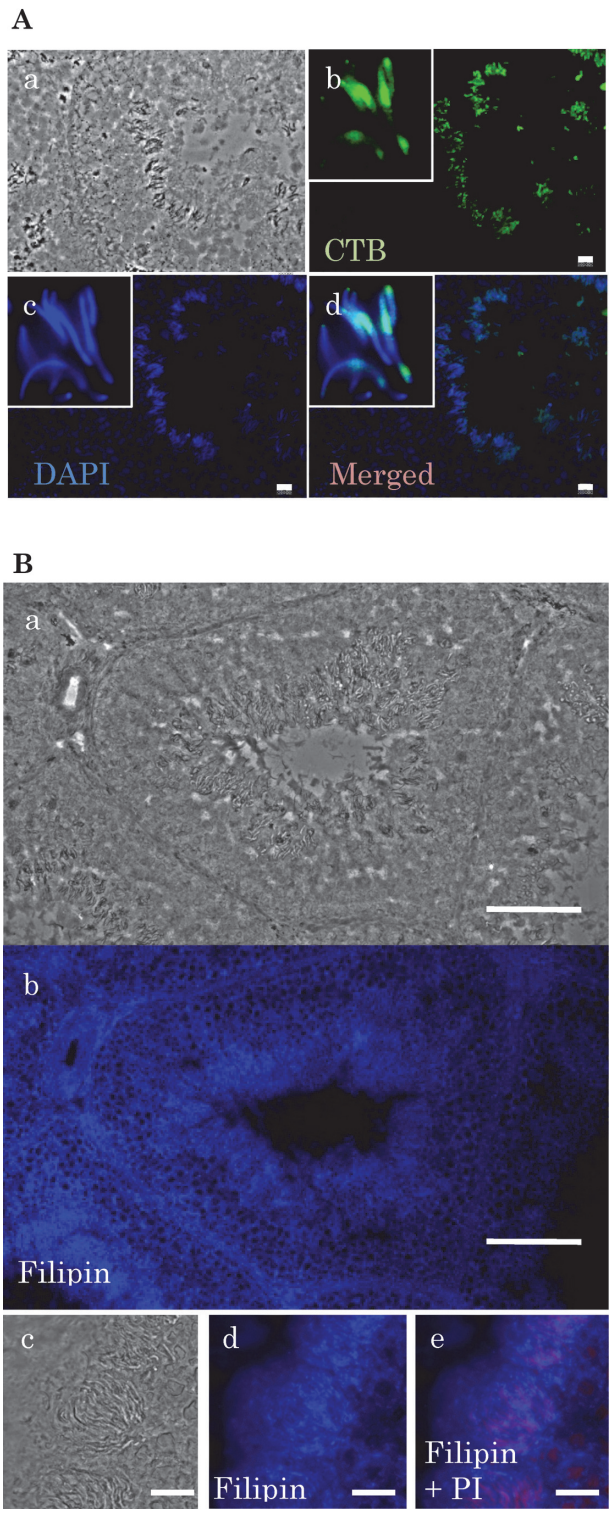

Fig. 1. **Localization of G_{M1} and sterols in chicken testis.** Frozen testicular sections were fixed with 4% paraformaldehyde in PBS and then labeled with CTB-Alexa 488 for the localization of G_{M1} (A) or filipin for the localization of sterols (B). The samples were stained with DAPI or propidium iodide (PI) for nuclear staining. Brightfield image showing representative seminiferous tubules (A, a). G_{M1}-enriched membranes (A, b) were localized to the area where the nuclei (A, c) of morphologically matured sperm were present. A merged image is shown in (A, d). Bars=10 μm (A, a–d). Bright field image showing representive seminiferous tubules (B, a and c). Sterols were abundant in elongating spermatids or more mature sperm (B, b, d and e). Bars=100 μm (B, a and b) or 20 μm (B, c–e).

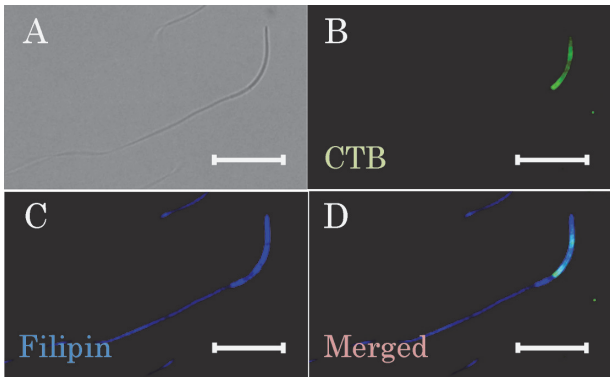

Fig. 2. **Localization of G_{M1} and sterols in chicken sperm.** Ejaculated sperm were fixed with 4% paraformaldehyde in PBS and then labeled with CTB-Alexa 488 and filipin for the localization of G_{M1} and sterols, respectively. Bright-field image showing representative chicken sperm (A). G_{M1} was localized to the plasma membrane overlaying the sperm head except for the apical ridge and posterior region (B). A sterol-enriched membrane was localized to the entire sperm head regions (C). A merged image is shown (D). Bars＝10 μm.

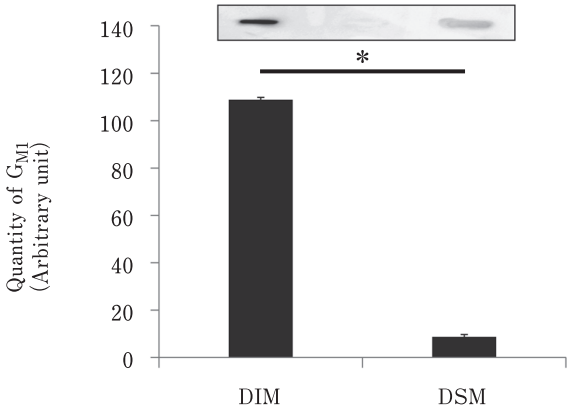

Fig. 4. **G_{M1} content in detergent-insoluble (DIM) and detergent-soluble (DSM) membrane fractions.** Sperm membranes were isolated by sonication, and centrifugally partitioned into the DIM and DSM following by 0.5% Triton X-100 treatment. Both fractions were subjected to slot blotting to quantify the G_{M1} content. G_{M1} content was significantly greater in the DIM than in the DSM. $*P<0.05$.

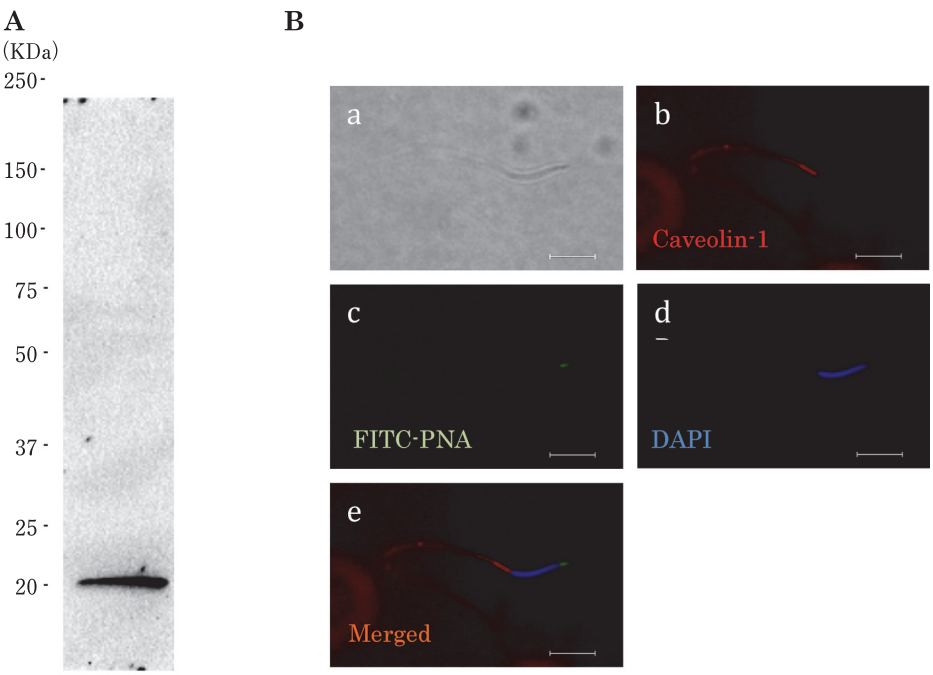

Fig. 3. **Expression and localization of caveolin-1 in chicken sperm.** Sperm (1×10^7 cells) were processed for SDS-PAGE and subjected to immunoblotting for the presence of caveolin-1. Caveolin-1 immunoreactivity was detected at a predicted molecular weight (A). Ejaculated sperm were fixed with 4% paraformaldehyde and subjected to immunostaining. Brightfield image showing representative chicken sperm (B−a). Caveolin-1 was exclusively localized to the Tail (B−b and B−e) and was not detected in the sperm head regions (B−c, d, and e). Bars＝10 μm.

Discussion

We demonstrated that the plasma membrane overlaying the sperm head of chicken sperm possesses a specific membrane domain that is enriched in G_{M1} and sterols. Previously, G_{M1} segregation into the APM domain was reported in murine, bull, and human sperm (Buttke *et al*., 2006; Selvaraj *et al*., 2006). The same membrane domain is enriched in sterols (Friend, 1982; Selvaraj *et al*., 2006). Together with these findings, our results suggest that the distribution of membrane rafts in the plasma membrane is largely conserved across animal groups. We previously showed in murine germ cells that G_{M1} is also abundant in acrosomal membranes (Asano *et al*., 2009; Selvaraj *et al*., 2009). However, our histochemical experiment using a chicken testicular section showed that the acrosomal membranes of developing spermatids do not show an enrichment of G_{M1}. This disparity could result from the difference in the acrosome exocytosis process between mammals and birds. In murine sperm, membrane rafts play important roles in acrosome exocytosis by stimulating membrane fusion between the APM and outer acrosomal membrane (Asano *et al*., 2013). However, acrosome exocytosis in chicken sperm appears to be induced without membrane fusion via the shedding of the acrosome cap in its intact form (Ahammad *et al*., 2013). Considering that membrane rafts regulate membrane fusion in diverse cell types (Salaun, *et al*., 2004), this difference in the machinery of acrosome exocytosis between mammalian and avian sperm might be attributable to distinctions in the functional roles of membrane rafts in sperm.

In this study, we found that sterols are abundant in cytoplasm of elongating spermatids or more mature sperm. This is consistent with the previous report that several proteins involved in cholesterol synthesis begin to be expressed in cytoplasm/residual bodies of post-meiotic spermatid or elongating sperm (Majdic *et al*., 2000). However, sterols was not detected in the membranes overlaying sperm head of testicular sperm although ejaculated sperm possessed sterol-enriched domain in sperm head region. Considering that sperm are transcriptionally and translationally inactive, this result suggests that chicken sperm might exogenously acquire sterols after leaving testis. In line with this, previous study in goat demonstrated that sterol content in sperm membrane dramatically increased during epididymal maturation (Rana *et al*., 1991). Although it is not known whether avian sperm undergo epididymal maturation or not, recent studies in chicken demonstrated that similar functional maturation of sperm occurs during their passage of male genital tract (Ahammad *et al*., 2011a; 2011b). Further experiments are required to determine whether chicken sperm acquire sterols when undergoing the functional maturation.

Membrane rafts have heterogeneous lipid and protein compositions (Pike, 2006). Membrane rafts in murine sperm have a subtype that is enriched in both sterols and G_{M1} and another subtype that is enriched in sterols only (Asano *et al*., 2009). Consistent with this finding, our localization experiments in this study showed that membranes of the apical ridge and the posterior region of the sperm head are rich in sterols but largely poor in G_{M1}, although the membrane of the central region of the sperm head is enriched in both sterols and G_{M1}, which suggests the presence of at least two distinct raft subtypes with different lipid compositions in chicken sperm. To determine whether sterol-enriched membranes of the apical ridge and those of the posterior region represent the APM and plasma membrane overlaying middle piece, respectively, further localization experiments using specific markers of subcellular organelles are required.

Caveolin-1, a sterol-binding protein enriched in membrane rafts, is believed to serve as a scaffold for a variety of signaling and metabolic molecules (Anderson, 1998). Because of these features, caveolin-1 is often utilized as a membrane raft marker. Therefore, we explored its localization in chicken sperm and found that it is localized to the tail. Previous studies on murine, stallion, and boar sperm have consistently demonstrated that caveolin-1 is localized to both the APM and the tail (Travis *et al*., 2001; Gamboa and Ramalho-Santos, 2005; van Gestel *et al*., 2005), suggesting differences among groups. Because caveolin-1 knockout mice are fertile(Razani *et al*., 2001), its functional role in sperm still remains unclear. Recent study using mutant mice demonstrated that caveolin-1 has a spatial interaction with calcium signaling complex tethered to sperm tail by CatSper1 which plays an important role in induction of hyperactivated motility (Chung *et al*., 2014). However, the genes of CatSper family are evolutionally lost in birds (Cai and Clapham, 2008), and sperm hyperactivation does not occur in bird sperm. Together with these, it is likely that the roles of caveolin-1 in sperm differ between mammals and birds. Although caveolin-1 is a sterol-binding protein (Murata *et al*., 1995), the finding that subcellular localization is different from that of sterols in chicken sperm is intriguing. This suggests that the localization of sterols does not require a binding interaction with caveolin-1 to be segregated into the plasma membrane overlaying the sperm head, which is consistent with the previous finding that murine sperm deficient in caveolin-1 showed no difference in sterol localization compared to wild-type sperm (Selvaraj *et al*., 2006).

Membrane rafts comprise a membrane region that is tightly packed with sterols, G_{M1}, and glycerophospholipids with saturated fatty acids compared to other membrane regions, resulting in resistance to TX-100-based solubilization (London and Brown, 2000). Despite a recent concern that detergent-insoluble membranes do not fully represent preexisting membrane rafts in the cellular membrane (Brown, 2006), the extraction of membranes using TX-100 has been a powerful method for characterizing the organization and compositional nature of membrane rafts (Kusumi and Suzuki, 2005; Lingwood and Simons, 2007). Therefore, we employed this method to biochemically demonstrate the organization of membrane rafts. Our results demonstrated that detergent-insoluble membranes of chicken sperm are highly abundant in G_{M1} relative to solubilized membranes. It should be noted that our methodology isolating detergent-insoluble membranes was designed to obtain membrane rafts

from all sub-cellular compartments of sperm. Therefore, it is not surprising that the membrane rafts isolated from chicken sperm contained membranes of intracellular organelles. In fact, there are several reports suggesting that mitochondria of murine sperm might possess membrane rafts (Nixon *et al*., 2009; Asano *et al*., 2010). Furthermore, we previously found in murine sperm that acrosome possess membrane rafts (Asano *et al*., 2009). Taking together with these, it is conceivable that membrane rafts are present in multiple intracellular organelles in sperm. Even under this context, our findings of lipid localization and biochemical analysis based on TX-100 insolubility consistently demonstrate that membrane rafts are present in the plasma membrane overlaying the sperm head. Thus, our results provide a foundation for investigating novel cellular pathways that might play an important role in avian sperm to achieve fertilization.

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