学位論文

Studies on sperm capacitation of mouse regulated by seminal vesicle secretion proteins

（精嚢分泌タンパク質によるマウス精子受精能獲得の制御機構に関する研究）

平成 24 年 12 月　博士 (理学)　申請

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Studies on sperm capacitation of mouse regulated by seminal vesicle secretion proteins

Doctoral Thesis

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December, 2012

CONTENTS

ABBREVIATIONS

AR: acrosome reaction

B-Ch: BODIPY-cholesterol

BODIPY: boron dipyrromethene

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CBBR: Coomassie brilliant Blue R250

CCD: charge coupled device

CD: cyclodextrin

cDNA: complementary deoxyribonucleic acid

Ch: cholesterol

CTB: cholera toxin B subunit

DAPI: 4',6-diamidino-2-phenylindole

ΔM_{max}: maximum binding amounts

ELISA: enzyme-linked immunosorbent assay

ES cell: embryonic stem cell

FITC: fluorescein isothiocyanate

GM1: monosialotetrahexosylganglioside

Abbreviations

GST: glutathione S-transferase

HBS: hepes-buffered saline

HTF: human tubal fluid

IgG: immunoglobulin G

K_d: dissociation constant

MβCD: methyl-β-cyclodextrin

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PBS-T: phosphate-buffered saline with 0.1% (w/v) Tween-20

PFA: paraformaldehyde

p*I*: isoelectric point

PI: propidium iodide

QCM: quartz crystal microbalance

REST gene: rapidly evolving seminal vesicle-transcribed gene

RNA: ribonucleic acid

RT-PCR: reverse transcription-polymerase chain reaction

SD: standard deviation

SDS: sodium dodecyl sulfate

Abbreviations

SEMG: semenogelin

SVF: seminal vesicle fluid

SVS: seminal vesicle secretion

Trx: thioredoxin

UTJ: uterotubal junction

WT: wild type

ABSTRACT

Freshly ejaculated mammalian spermatozoa have no ability to fertilize an egg. They gain this ability after entering the female reproductive tract. This phenomenon is called as sperm capacitation. An efflux of sterols from the sperm plasma membrane and the accompanied reconstitution of membrane lipid rafts are essential for capacitation. On the other hand, capacitated spermatozoa reversibly lose their fertilizing ability when they are treated with seminal plasma. This phenomenon is called decapacitation. In mouse, the major components of seminal plasma are seminal vesicle secretions (SVSs), consisting of seven major proteins (SVS1−SVS7). Previous reports have shown that SVS2 acts as a decapacitation factor, inhibiting sterol efflux from uterine spermatozoa via binding to ganglioside GM1. Since GM1 localizes on membrane lipid rafts, SVS2 may affect the dynamics of lipid rafts, including the control of sterol dynamics. However, the precise regulatory mechanism of capacitation by SVS2 has not been elucidated.

In this thesis, I investigated the roles of SVS2, SVS3, and SVS4 in sperm capacitation and aimed to shed light on the regulatory mechanisms of capacitation by SVSs in general.

In Chapter I, I studied the inhibitory mechanism of capacitation by SVS2,

impeding the efflux of sterol from spermatozoa, retrieving cholesterol to spermatozoa in a cholesterol-dependent manner, and obstructing the reconstitution of lipid rafts accompanying capacitation. In the uterus, sterol levels of wild type spermatozoa were high, while sterol levels of *SVS2*$^{-/-}$ spermatozoa were decreased. These results indicate that SVS2 maintains the sterol level of spermatozoa to keep their incapacitated state *in vivo*.

In Chapter II, I studied the dynamics of SVS2 *in vivo* and the mechanism of *in vivo* capacitation. In mated female mice, SVS2 was found in the uterus and the uterotubal junction (UTJ), while no SVS2 was detected in the oviduct. Furthermore, GM1, which binds to SVS2, was detected along the uterine epithelium of estrus mice. These results indicate that SVS2 is excluded from the oviduct, where sperm capacitation occurs, and GM1 and SVS2 are released from spermatozoa at the UTJ. Since uterine fluid contains enough albumin to induce sperm capacitation, SVS2 actively inhibits capacitation of spermatozoa in the uterus.

In Chapter III, I studied the functions of SVSs other than SVS2. While SVS3 did not inhibit capacitation, it bound to SVS2 and facilitated the capacitation-inhibitory effect of SVS2. SVS4 had a capacitation-inhibiting effect, yet could not decapacitate spermatozoa, possibly owing to its lack of interaction with cholesterol. Therefore, these

SVSs can potentially control sperm capacitation.

This study revealed that *in vivo* capacitation of mouse spermatozoa is dynamically regulated by cooperative effects of SVS2, other SVSs, and the milieu in the female reproductive tract. Controlling the sterol level in spermatozoa is important for regulating sperm's fertilizing ability. Further studies on sterol level regulation by SVSs are expected to provide new insights into the mechanism of mammalian fertilization.

GENERAL INTRODUCTION

Effort to mimic *in vivo* fertilization in a test tube have been underway for a long time. The first observation of sperm penetrating an egg of an internal fertilizer was reported in the nematode *Ascaris* in 1851 (Nelson, 1851). In mammals, Schenk treated ovarian rabbit and guinea pig eggs with spermatozoa and observed their division (Schenk, 1878), before *in vitro* fertilization of many mammalian species, including humans, was described (Rock and Menkin, 1944). However, since *in vitro* fertilization required higher concentrations of spermatozoa than *in vivo* fertilization, events observed *in vitro* did not seem to reflect the *in vivo* situation. In addition, optimal conditions for mammalian fertilization were yet unknown.

In 1951, Austin and Chang reported that mammalian spermatozoa need to remain in the female reproductive tract for several hours before fertilization (Austin, 1951; Chang, 1951). This result indicated that spermatozoa must undergo some changes in the female reproductive tract to acquire fertilizing ability, a phenomenon called "capacitation" (Austin, 1952). While capacitation had been assumed to occur only under *in vivo* conditions, Yanagimachi and Chang showed that it could be induced *in vitro* (Yanagimachi and Chang, 1963). That study significantly advanced our understanding of fertilization mechanisms in mammals and technically improved *in vitro* fertilization.

Consequently, efficient *in vitro* fertilization methods were established for animals and humans (Edwards *et al*., 1970).

In addition, mammalian *in vivo* fertilization has been studied intensively, leading to the following scheme for its process (Fig. 1). Spermatozoa are ejaculated into the vagina (man, sheep, cow, and rabbit) or the uterus (horse, pig, dog, and rat), migrate to the oviduct, and are reserved at the oviductal isthmus until ovulation. Sperm capacitation seems to occur in the isthmic reservoir (Martinez, 2007). Capacitated spermatozoa undergo hyperactivation, gaining activated motility, accompanied by asymmetrical flagellar beats of large amplitude (Katz *et al.*, 1989), and are released from the reservoir (Demott and Suarez, 1992). Then the acrosome reaction is induced, changing the sperm plasma membrane in preparation for fusion with the egg. Finally spermatozoa penetrate the zona pellucida and fuse with the egg's plasma membrane.

During capacitation, sperm undergo various morphological and functional changes. The plasma membrane becomes hyperpolarized (Zeng *et al*., 1995), and the intracellular Ca^{2+} concentration increases (Baidi *et al.*, 1991; Florman *et al.*, 1998). Adenylyl cyclase and cAMP-dependent protein kinase A are activated (Visconti *et al*., 1995b), resulting in tyrosine phosphorylation of sperm proteins (Visconti *et al*., 1995a). Furthermore, properties of the sperm plasma membrane change drastically. The plasma

membrane of ejaculated spermatozoa is abundant in certain sterols, particularly cholesterol (Tesařík and Fléchon, 1986; Parks *et al*., 1987). Spermatozoa obtain sterols from principal cells during maturation in the epididymis (Keber *et al*., 2013). This keeps the fluidity and order of the membrane (Parks *et al*., 1987; Almeida *et al*., 1992), while, in contrast, sterols are decreased during capacitation (Davis, 1981), resulting in reconstitution and redistribution of the sperm plasma membrane (Shadan *et al*., 2004; Jones *et al*., 2010). Thus, sterol efflux is associated with the signal transduction pathway for sperm capacitation (Visconti *et al*., 1999a).

Sperm capacitation can be induced *in vitro* by a medium containing appropriate components (Byrd *et al*., 1979; Miyamoto and Chang, 1973) such as bovine serum albumin (BSA) (Brackett *et al*., 1972; Davis, 1976) and methyl-β-cyclodextrin (MβCD) (Choi and Toyoda, 1998). MβCD removes sterols from the plasma membrane (Choi and Toyoda, 1998), and BSA seems to act accordingly (Davis *et al*., 1979). Since sperm capacitation seems to occur in the oviductal fluid, where albumin concentrations are high (Miyamoto and Chang, 1973), albumin may act as an *in vivo* capacitation-inducing factor by liberating sterols from spermatozoa.

While sperm capacitation is essential for mammalian fertilization, the *in vitro* capacitated spermatozoa reversibly lose their fertilizing ability when treated with

seminal plasma (Chang, 1957). This phenomenon is called "decapacitation" and appears to be conserved among several species (Bedford and Chang, 1962). Certain molecules such as polysaccharide (Dukelow *et al.*, 1966), glycoprotein (Rayes *et al.*, 1975), and some membrane vesicle (Davis, 1974) are assumed to function as decapacitation factors. Considering the importance of sperm capacitation for accomplishing fertilization, the existence of decapacitation factors is a mystery. However, these factors may play an important role by inhibiting sperm capacitation.

The functions of spermatozoa are regulated by many secretions from male reproductive accessory glands; these secretions are accumulated in semen. In mice, the seminal vesicle is the predominant reproductive accessory gland, secreting seven major proteins called seminal vesicle secretions (SVSs) (Fawell *et al*., 1987). SVSs constitute a major component of seminal plasma and have many functions in copulation and fertilization; SVS1–SVS3 are cross-linked by transglutaminase and form the copulatory plug (Lin *et al*., 2002); SVS4 shows immunoregulatory activity (Romano-Carratelli *et al*., 1995); SVS5 and SVS6 may act as serine proteinase inhibitors (Clauss *et al*., 2005); and SVS7 enhances sperm motility (Luo *et al*., 2001).

SVS2 acts as a decapacitation factor (Kawano and Yoshida, 2007). While SVS2 is a major component of the copulatory plug, it partly enters the uterus, inhibits

sperm capacitation and also cancels acquired sperm fertility *in vitro* (decapacitation). In the uterus, SVS2 binds to spermatozoa via ganglioside GM1 on the sperm plasma membrane, whereas the spermatozoa entered the oviduct have neither SVS2 nor GM1 (Kawano *et al*., 2008). Recently, Kawano *et al*. generated *SVS2*$^{-/-}$ mice and reported that after mating, *SVS2*$^{-/-}$ spermatozoa were killed in the uterus and cannot enter the oviduct, resulting in male infertility (Kawano *et al*., 2014). Therefore, the *in vivo* suppression of capacitation by SVS2 seems to be essential for sperm viability in the uterus, but its regulatory mechanism is not clear. Moreover, it is not known whether other SVSs participate in the regulation of sperm capacitation.

In this study, I aimed to elucidate the roles of SVSs in the regulation of sperm capacitation. In Chapter I, I explicated the inhibitory mechanism of sperm capacitation by SVS2. In Chapter II, I analyzed the dynamics of SVS2 in the female reproductive tract and studied the *in vivo* capacitation mechanism. In Chapter III, I evaluated roles of SVS3 and SVS4 in capacitation. In terms of the participation of SVSs, I attempted to explain the "raison d'etre" of a decapacitation factor and the capacitation-regulatory mechanism *in vivo*.

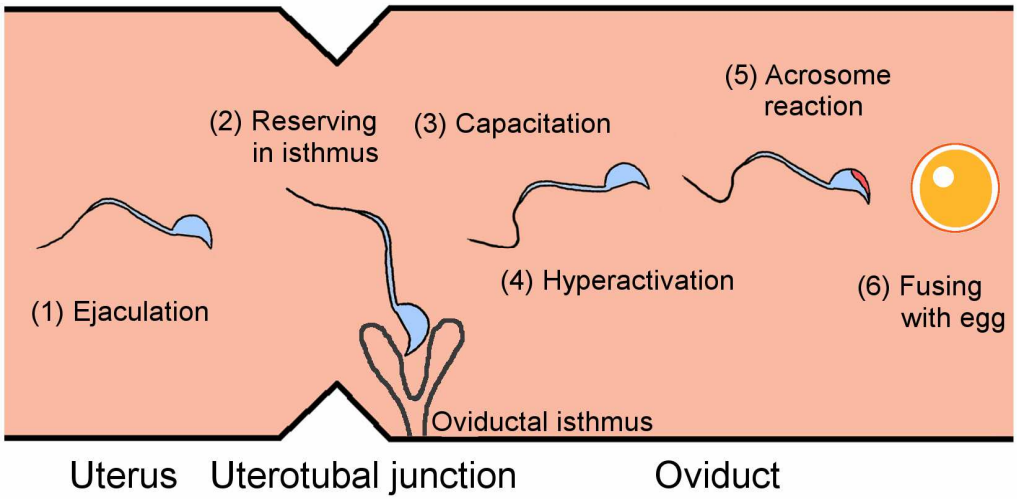

Figure 1. Schematic diagram depicting the journey of spermatozoa and fertilization in mammals.

(1) Spermatozoa are ejaculated into the vagina or the uterus. (2) Spermatozoa migrate to the oviduct and are reserved at the oviductal isthmus until ovulation. (3) Sperm capacitation occurs in the isthmic reservoir. (4) Capacitated spermatozoa undergo hyperactivation and are released from the reservoir. (5) Spermatozoa undergo acrosome reaction. (6) Spermatozoa penetrate the zona pellucida and fuse with the plasma membrane of the egg.

Chapter I

Regulatory mechanism of sperm capacitation by SVS2

Chapter I

ABSTRACT

The seminal vesicle secretion 2 (SVS2) functions as a decapacitation factor. SVS2 binds to ganglioside GM1 on the sperm plasma membrane and inhibits sperm capacitation. Furthermore, *SVS2*$^{-/-}$ male mice are infertile, whose spermatozoa are killed by some spermicidal activity in the uterus. Although its precise mechanism is still obscure, SVS2 seems to be involved in the dynamics of lipid rafts. Here, I examined the role of SVS2 on the regulatory mechanism of sperm capacitation, especially focused on the effect of SVS2 on sterol level in the sperm plasma membrane and capacitation-accompanied reconstitution of membrane lipid rafts. SVS2 prevented the sperm capacitation induced by methyl-β-cyclodextrin that liberates sterols from the sperm plasma membrane *in vitro*. SVS2 prevented cholesterol efflux from the sperm plasma membrane and incorporated liberated cholesterol in the sperm plasma membrane. SVS2 also inhibited capacitation-accompanied diffusion of GM1. *SVS2*$^{-/-}$ mice spermatozoa entered the uterine cavity of wild-type partners showed decreased sterol levels, while sterol level of wild type spermatozoa were maintained. These results suggest that SVS2 maintains sterol level of the sperm plasma membrane to keep their incapacitated state *in vivo*. SVS2 seems to protect sterols in the sperm plasma membrane from flowing out in the uterus.

Chapter I

INTRODUCTION

Mammalian spermatozoa need to experience a specific change called sperm capacitation in the female reproductive tract to acquire the fertilizing ability (Austin, 1951; Chang, 1951). One of important steps in capacitation is efflux of sterols from the sperm plasma membrane (Go and Wolf, 1985). Although main sterols in spermatozoa are cholesterol and desmosterol (Legault *et al*., 1979), cholesterol seems to be a main actor in sperm capacitation. Capacitation is associated with changes in the fluidity of the sperm plasma membrane accompanied by sterol efflux (Shadan *et al*., 2004). This yields changes in intracellular ion concentration, metabolism, tyrosine phosphorylation of sperm proteins, and sperm motility (Bailey, 2010; Visconti *et al*., 2011), which are all steps required to achieve sperm capacitation.

In general, the plasma membrane forms cholesterol- and sphingolipid-enriched microdomains known as lipid rafts, which functions in signaling, membrane trafficking, and polarization (Rajendran and Simons, 2005). Cholesterol is a major component of lipid rafts and plays significant roles in stability and function of the plasma membrane (Simons and Toomre, 2000; Fielding and Fielding, 2003). The lipid and sterol composition of the sperm plasma membrane changes drastically as a result of two main events. First, as spermatozoa undergo epididymal maturation, they obtain sterols from

their environment (Suzuki, 1988; Keber *et al.*, 2013). Second, in the female reproductive tract, sperm capacitation seems to be induced by a reduction of sterols in the plasma membrane (Davis, 1981). Cholesterol efflux from spermatozoa destabilizes lipid rafts (Shadan *et al.*, 2004), resulting in capacitation (Cross, 1998). This cholesterol efflux is associated with activation of the signal transduction pathway necessary for sperm capacitation (Visconti *et al.*, 1999a), and redistributes the molecules required for fertilization (Jones *et al.*, 2010).

Albumin (Brackett *et al.*, 1972; Davis, 1976) and methyl-β-cyclodextrin (MβCD; Choi and Toyoda, 1998) can liberate sterols from the sperm plasma membrane, which induces sperm capacitation *in vitro*. Although the importance of sterol efflux in capacitation has been recognized, the specific mechanism underlying *in vivo* capacitation, including the regulation of sterols in the sperm plasma membrane, is not well understood.

The seminal plasma protein, seminal vesicle secretion 2 (SVS2) is secreted from the seminal vesicle and is ejaculated with spermatozoa into the uterus. SVS2 functions as a decapacitation factor: it prevents spermatozoa from acquiring the fertilizing ability (Kawano and Yoshida, 2007). Recently it was shown that spermatozoa of *SVS2*$^{-/-}$ males are killed by some spermicidal activity in the uterus, resulting in

infertility (Kawano *et al*., 2014). SVS2 binds to the ganglioside GM1 in the sperm plasma membrane (Kawano *et al*., 2008) and was found attached to the surface of spermatozoa in the uterus (Kawano and Yoshida, 2007; Kawano *et al*., 2014). However, after entering the oviduct, spermatozoa are attached to neither SVS2 nor GM1 (Kawano and Yoshida, 2007; Kawano *et al*., 2008). Since GM1 is a marker of lipid rafts (Parton, 1994) and cholesterol efflux accompanied by sperm capacitation reconstitutes lipid rafts (Shadan *et al*., 2004; Jones *et al*., 2010), it is possible that SVS2 is involved in the dynamics of cholesterol and lipid rafts.

In this chapter, I tried to elucidate the role of SVS2 and sterols on sperm capacitation. I focused on the effect of SVS2 on the dynamics of lipid rafts and sterol levels in the sperm plasma membrane.

Chapter I

MATERIALS AND METHODS

Animals

All mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Mice were housed in cages under a 12 h: 12 h light-dark cycle. All animal experiments were carried out according to the University of Tokyo Guidelines on Animal Care.

Preparation of Recombinant SVS2 Protein

Because it was shown that the GST-tagged recombinant SVS2 protein has the same effect as the native SVS2 protein (Kawano and Yoshida, 2007), I used recombinant SVS2 protein. To increase the solubility of recombinant SVS2, I substituted the thioredoxin (Trx)-tag for a GST-tag. cDNA encoding SVS2 (NCBI accession No.NM_017390) was prepared by reverse transcription-polymerase chain reaction (RT-PCR) using RNA isolated from the mouse seminal vesicle (C57BL/6J). The cDNA was subcloned into the pET-32 vector (Takara Bio Inc.; Shiga, Japan). The Trx fusion protein was expressed in *Escherichia Coli* BL21-pLysS cells (Novagen; Madison, WI, USA) by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Recombinant SVS2 protein was recovered using Ni-NTA agarose (Qiagen;

Hilden, Germany) under denaturing conditions according to the manufacturer's instructions. After removing urea using a PD-10 column (GE Healthcare; Piscataway, NJ, USA), the protein was lyophilized and stored at −20°C until use. Immediately before the experiment, recombinant SVS2 was dissolved in human tubal fluid (HTF) medium without BSA (Nippon Medical & Chemical Instruments Co.; Osaka, Japan).

The purity of recombinant SVS2 protein was evaluated by western blotting with anti-SVS2 antibody produced by previous report (Kawano and Yoshida, 2007). Protein was separated by Laemmli SDS-PAGE using a 10% (w/v) polyacrylamide gel, and transferred onto a polyvinylidene fluoride membrane (Millipore; Billerica, MA, USA). The membrane was blocked with 5% (w/v) skim milk (Nacalai Tesque; Kyoto, Japan) in phosphate-buffered saline (PBS) with 0.1% (w/v) Tween-20 (Sigma-Aldrich; St. Louis, MO, USA) (PBS-T) for 30 min at room temperature. The membrane was treated with 20 μg/mL of anti-SVS2 antibody in PBS-T containing 5% (w/v) skim milk at 4°C for 12 h. After washing with PBS-T, the membrane was treated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Bethyl Laboratories; Montgomery, TX, USA) at a dilution of 1: 20,000 in PBS-T containing 5% (w/v) skim milk for 1 h at room temperature, and washed three times with PBS-T. Reacted proteins were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare). Proteins were

also visualized by staining with Coomassie Brilliant Blue R250.

Preparation of Epididymal Spermatozoa and Incubation for Capacitation

To evaluate the effect of SVS2 on sperm capacitation *in vitro*, spermatozoa were isolated from the cauda epididymis of sexually mature male CD-1 mice (9–15 weeks old), and were suspended in HTF medium at 37°C in a 5% CO_2 atmosphere.

I used methyl-β-cyclodextrin (MβCD; Sigma-Aldrich) and bovine serum albumin (BSA, A-4503; Sigma-Aldrich) to induce sperm capacitation. SVS2 was used at a concentration of 20 μM, which is similar to the physiological concentration in the uterus (Kawano and Yoshida, 2007). Schematic image of sperm incubating conditions was shown in Fig. 2. To wash the spermatozoa, HTF medium (37°C) was added to spermatozoa, which were gently suspended. After centrifugation at 600 × *g* for 3 min, the supernatant was removed. After repeating this procedure twice, the medium was replaced. Next, SVS2, BSA, MβCD, and cholesterol were added to the replaced medium. Cholesterol conditions were as follows: 50 mM cholesterol (Sigma-Aldrich) dissolved in 100% ethanol was added to obtain a final concentration of 500 μM.

Observation of Sterols by Filipin Staining

To visualize sterols, the filipin complex (Sigma-Aldrich) was used, which binds to sterols (Schroeder *et al*., 1971; Kessel *et al*., 1985). Spermatozoa were fixed with 0.004% (w/v) paraformaldehyde (PFA) for 10 min and washed twice with HEPES-buffered saline (HBS; 25 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 6 mM glucose, pH 7.1). Filipin solution (4% (w/v) in dimethyl sulfoxide) was added to the fixed spermatozoa at a final concentration of 0.2%, and the solution was incubated for 30 min. After washing with HBS, fluorescence was observed using a fluorescent microscope (Leica DMRBE; Solms, Germany) and images were acquired using a CCD camera (MicroPublisher 5.0; QImaging; BC, Canada). The fluorescence intensity of the sperm head was quantified using ImageJ software (NIH; Bethesda, MD, USA), and the normalized intensity of epididymal spermatozoa was set to 100. For each batch, the fluorescence intensities of 100 spermatozoa were quantified.

Visualization of Cholesterol Dynamics in the Sperm Plasma Membrane

To verify that SVS2 affects sterol dynamics, intercalation of fluorescent cholesterol into the sperm plasma membrane was studied. Spermatozoa were incubated in HTF medium containing 500 μM MβCD for 45 min. After washing as described

above, spermatozoa were incubated in replaced HTF medium containing 20 μM SVS2 and 500 μM BODIPY-cholesterol (TopFluor-Cholesterol; Avanti Polar Lipids; Alabaster, AL, USA) and incubated for 45 min. Spermatozoa were then stained with Hoechst 33342 (Sigma-Aldrich) at a final concentration of 5 μg/mL. After incubating for 5 min, fluorescence was visualized using a fluorescent microscope (Leica DMRBE; Solms, Germany) and images were acquired using a CCD camera (MicroPublisher 5.0; QImaging; BC, Canada).

Quantitation of Sperm Cholesterol

Lipid extraction was performed according to modified Bligh & Dyer as described by Jakop *et al*. (Jakop *et al*., 2009). Briefly, spermatozoa incubated in various conditions were centrifuged (1,000 × *g*, 5 min) after washed with HBS. 100 μL of sperm suspensions were mixed with 400 μL of chloroform/methanol (1: 2, v/v) and shaken for 30 min. The sperm were added with 130 μL chloroform and shaken for 0.5 min. 130 μL of 40 mM acetic acids was added and samples were shaken. After centrifugation (1,000 × *g*, 20 min, 4°C), samples were divided into the chloroform phase and the aqueous phase. The aqueous phase was treated with chloroform and acetic acids and centrifuged as described above. After repeating reextraction two times, whole chloroform phases

were combined and dried under reduced pressure.

Extracted lipids were suspended with 2-propanol containing 10% (v/v) Triton X-100. Enzymatic quantitation of cholesterol was carried out with Amplex red cholesterol assay kit (Molecular probes, Eugene, OR, USA). Fluorescence intensity was measured by a multilabel reader 2030 ARVO X4 (Perkin Elmer, Waltham, MA, USA).

Sperm Capacitation Assay

Sperm capacitation was evaluated based on acrosomal responsiveness to progesterone and ionomycin. Spermatozoa were treated with 100 μM progesterone (Wako; Kyoto, Japan) for 15 min at 37°C or with 15 μM ionomycin (Sigma-Aldrich) for 10 min at room temperature. Treated spermatozoa were spread onto a glass slide. Spermatozoa were fixed by 100% methanol for 10 min and stained with 100 μg/mL fluorescein isothiocyanate (FITC)-conjugated peanut lectin (Sigma-Aldrich) for 15 min at 37°C. Spermatozoa were washed with HBS, and FITC fluorescence was observed using a fluorescent microscope (Leica DMRBE; Solms, Germany) and images were acquired using a CCD camera (MicroPublisher 5.0; QImaging; Surrey, BC, Canada). Spermatozoa with no FITC fluorescence at the acrosome region were regarded as acrosome-reacted spermatozoa. Three fields were obtained for each batch, and 100

spermatozoa per field were evaluated.

Localization of GM1 in the Spermatozoa

The ganglioside GM1 (Galβ1 > 3GalNAcβ > 4(NeuAcα2>3)Galβ1 > 4Glcβ1 > 1'Cer) was visualized using FITC-conjugated cholera toxin B subunit (FITC-CTB) (Sigma-Aldrich) which is known to bind to GM1 specifically (van Heyningen, 1974). Spermatozoa were fixed with 0.004% (w/v) PFA for 10 min and washed three times with HBS. Fixed spermatozoa were added FITC-CTB at a final concentration of 0.5 mg/mL in HBS for 10 min. After washing with HBS, fluorescence was observed as described above. Localization of the GM1 signal was classified into four patterns based on previously described criteria (Selvaraj *et al*., 2006), and 100 signals were evaluated for each batch.

Evaluation of the Sterol Level of Spermatozoa *in vivo*

To evaluate the effect of SVS2 on the sperm sterol content in the female reproductive tract, I measured the sterol level of spermatozoa in the uterus and the oviduct. A 10–55-week-old male mouse (*SVS2*$^{+/+}$ or *SVS2*$^{-/-}$) was mated with a 9–12-week-old C57BL/6J female mouse. *SVS2*$^{-/-}$ mice were generated as described by

Kawano *et al.* (Kawano *et al*., 2014). Briefly, targeting vector designed to remove *SVS2* exons were electroporated into 129/Sv strain-derived ES cells. Recombinant ES lines were microinjected into C57BL/6J strain-derived blastocytes and the male chimeric mice were obtained. They were crossed with C57BL/6J female mice to produce heterozygote offspring. Then male and female heterozygotes were intercrossed and backcrossed to the C57BL/6N background. Females were sacrificed at 1.5 h post-coitus, and spermatozoa were obtained from the uterus and the oviduct by flushing with HBS. Obtained spermatozoa were fixed with 0.004% PFA for 10 min, stained with filipin, and the fluorescence intensity was quantified as described above.

Evaluation of Sperm Viability

To evaluate the viability of spermatozoa, propidium iodide (PI; Dojindo; Kumamoto, Japan) was added to sperm suspension with various treatments at a final concentration of 5 μg/mL, and incubated for 5 min at 37°C. After incubation, PI fluorescence was observed under a heating plate at 37°C using a fluorescence microscope (Leica DMRBE; Solms, Germany). Spermatozoa with no fluorescence were regarded as live. 100 spermatozoa were evaluated for each experiment.

Statistical Analysis

All experiments were repeated at least three times with different individuals. Data are expressed as mean ± SD. Statistical significance was calculated using the Student's *t*-test; $P < 0.01$ was considered statistically significant.

Chapter I

RESULTS

Sterol Level of *SVS2*$^{-/-}$ Mice Spermatozoa Deposited into the Female Reproductive Tract

At first, I visualized sterol level of mouse spermatozoa in the female reproductive tract with the sterol-staining dye filipin and confirmed whether sterol level of spermatozoa and fertilizing ability are correlated *in vivo*. Typical images of filipin staining were shown in Fig. 3. Intensity of filipin fluorescence of wild-type male spermatozoa entered the uterus after mating increased by 149 ± 40% compared to non-treated epididymal spermatozoa (n = 6) (Fig. 4). On the other hand, the fluorescence intensity of the spermatozoa in the oviduct was significantly reduced (35 ± 17% of that in the uterus) (n = 3, $P < 0.01$) (Fig. 4). This shows that sterol level of the mouse spermatozoa is really decreased when they enter the oviduct. On the other hand, the sperm sterol level in the uterus of females mated with *SVS2*$^{-/-}$ males was reduced to the level of wild spermatozoa in the oviduct (34 ± 7% of that of ejaculated wild-type spermatozoa in the uterus) (n = 8, $P < 0.01$) (Fig. 4). These results indicate that SVS2 preserves sterols on the sperm membrane in the female reproductive tract until entrance into the oviduct.

Effect of MβCD on Sperm Capacitation

MβCD is a potent liberator of sterols, and induces capacitation of mouse spermatozoa (Choi and Toyoda, 1998). I confirmed the effect of MβCD on sperm capacitation. Capacitation was evaluated by acrosomal responsiveness to progesterone and ionomycin (induced-AR rate) as described in Materials and Methods. Spermatozoa incubated with MβCD showed increased induced-AR rate and 500 μM MβCD induced the same level of the induced-AR rate as BSA (Fig. 5).

Effect of SVS2 on Sperm Capacitation Induced by MβCD

In order to examine the effect of SVS2 on sperm capacitation and sterol efflux, I examined the effect of SVS2 on the MβCD-treated spermatozoa. Expression and purification of recombinant SVS2 protein were checked with CBBR staining and western blotting with anti-SVS2 antibody (Fig. 6). Although recombinant SVS2 was expressed with thioredoxin (Trx)-tag, the tag itself has no effect on the induced-AR rate (Fig. 7).

The spermatozoa incubated with SVS2 and MβCD suppressed the induced-AR rate, which showed no significant differences from that of control spermatozoa (Fig. 8). The spermatozoa incubated with SVS2 prior to incubating with MβCD also suppressed

the induced-AR rate (Fig. 8). Furthermore, SVS2 cancelled sperm capacitation induced by MβCD: SVS2 showed decapacitation effect on MβCD-treated spermatozoa. When SVS2 was added to the MβCD-treated spermatozoa, which should be capacitated, the induced-AR rate decreased significantly (MβCD → SVS2 in Fig. 9). When SVS2 was added to BSA-treated spermatozoa, the induced-AR rate of spermatozoa did not show significant difference (Fig. 9). This may be because the incubation time of 45 min is not enough for capacitation induced by BSA.

Interestingly, the decapacitation effect of SVS2 was not observed when sperm incubation medium was washed out following MβCD-treatment. When MβCD-treated spermatozoa were washed with new HTF medium after MβCD-treatment, addition of SVS2 did not decrease the induced-AR rate (MβCD → wash → SVS2 in Fig. 10). However, the decapacitation effect of SVS2 on the washed MβCD-treated spermatozoa was rescued by addition of cholesterol (MβCD → wash → SVS2 + Ch in Fig. 10), though cholesterol itself and sterol liberators, MβCD and BSA had no effect on the washed MβCD-treated spermatozoa (Fig. 10). In addition, 1% ethanol, a vehicle of cholesterol, had no effect on sperm viability (Fig. 11) and capacitation (Fig. 12). These results indicate that the decapacitation effect of SVS2 requires cholesterol in the medium surrounding the spermatozoa.

Effect of SVS2 on Sterol Level in the Sperm Plasma Membrane

As describe above, sterol level on the *SVS2*$^{-/-}$ spermatozoa was significantly reduced in the uterus. Furthermore the decapacitation effect of SVS2 seems to closely relate to the existence of surrounding cholesterol. Thus, I examined the effect of SVS2 on sterol levels in the sperm plasma membrane *in vitro*.

Although recombinant SVS2 protein was expressed with Trx-tag, the tag itself has no effect on sterol level in the spermatozoa (Fig. 13). Fluorescence intensity of filipin in the sperm heads treated with 500 μM MβCD for 45 min decreased to approximately 55% of that of non-treated sperm heads (Fig. 14). On the other hand, SVS2 inhibited decrease in sterol content of the spermatozoa; the fluorescence intensity on sperm heads treated with SVS2 and MβCD showed no significant differences from that on non-treated sperm heads (Fig. 14). When spermatozoa were incubated with SVS2 prior to MβCD-treatment, the sterol level on the sperm head was maintained to show the same level as that of spermatozoa incubated HTF medium only (SVS2 → MβCD in Fig. 14). Furthermore, when SVS2 was added to the MβCD-treated spermatozoa, on which the membrane sterol level was low, sterol levels on the sperm heads recovered to that of incapacitated spermatozoa (MβCD → SVS2 in Fig. 14). These changes in sperm sterol levels were confirmed by enzymatic quantitating analysis.

Cholesterol content in the control spermatozoa was 142.3 ± 39.5 ng/10^6 sperm cells. MβCD-treatment decreased the sperm cholesterol by approximately 35%, whereas SVS2-treatment kept the sperm cholesterol to control level (MβCD + SVS2 and MβCD → SVS2 in Fig. 15). Thus, SVS2 appears to mediate sperm capacitation by controlling sterol levels in the plasma membrane.

When the MβCD-treated spermatozoa were washed with new HTF medium, a recovery effect of SVS2 on the sterol level in the spermatozoa was not observed by the filipin staining and the enzymatic quantitating assay (MβCD → wash → SVS2 in Fig. 14 and 15). However, addition of extrinsic cholesterol to the medium containing SVS2 rescued the sterol level in the sperm head (MβCD → wash → SVS2 + Ch in Fig. 14 and 15). Accordingly, SVS2 seems to prevent sterol efflux from the plasma membrane, thereby retrieving liberated sterols to the membrane and maintaining the spermatozoa in an incapacitated state.

Visualization of Cholesterol Dynamics in the Sperm Plasma Membrane

My results suggest that SVS2 does not only inhibit sterol efflux, but also incorporates external sterol into the sperm plasma membrane. To verify the effect of SVS2, I observed the dynamics of extrinsic BODIPY-cholesterol. When only

BODIPY-cholesterol was added to the washed MβCD-treated spermatozoa, minimal BODIPY fluorescence was observed (Fig. 16A). By contrast, when washed MβCD-treated spermatozoa were incubated with BODIPY-cholesterol and SVS2, BODIPY fluorescence was observed on many spermatozoa. BODIPY-cholesterol was incorporated into 69 ± 20% of spermatozoa (Fig. 16B). Furthermore, SVS2 slightly facilitated incorporation of BODIPY-cholesterol into the spermatozoa without MβCD-treatment (Fig. 16). Thus, SVS2 mediates incorporation of free cholesterol in the medium surrounding spermatozoa into the sperm plasma membrane.

Effect of SVS2 on Distribution of GM1 in Spermatozoa

Sterol efflux from spermatozoa changes fluidity of plasma membrane and reorganizes the lipid rafts to induce sperm capacitation (Shadan *et al*., 2004; Jones *et al*., 2010). Thus, I evaluated the effect of SVS2 on the distribution of ganglioside GM1, which is a well-known marker of lipid rafts. GM1 was visualized using FITC-conjugated cholera toxin B subunit (CTB), and the GM1 staining pattern was classified into four types based on criteria described by Selvaraj *et al*. (Selvaraj *et al*., 2006) (Fig. 17A). GM1 was primarily observed in the postacrosomal region, and occasionally on the entire sperm head (pattern PAPM or pattern APM/PAPM), when

spermatozoa were incubated in medium only; non-capacitated spermatozoa (Fig. 17B and Table 1). When spermatozoa were capacitated using MβCD, GM1 diffused through the entire head, appearing as pattern D (Fig. 17B and Table 1). When spermatozoa were co-incubated with SVS2 and MβCD, the pattern APM/PAPM was observed in a large proportion of spermatozoa and pattern D was seen in a low percentage of spermatozoa, which was similar to the patterns observed in non-capacitated spermatozoa (Fig. 17B and Table 1). In the spermatozoa pre-incubated with SVS2 prior to incubating with MβCD, distribution of GM1 was also similar to the non-capacitated spermatozoa (Fig. 17B and Table 1). Thus, SVS2 arrests the reconstitution of lipid rafts organization by preventing sterol efflux.

Furthermore, when the MβCD-treated spermatozoa were decapacitated using SVS2, GM1 was primarily distributed in the APM/PAPM pattern, similarly to the non-capacitated spermatozoa, although GM1 was dispersed over the entire sperm head (pattern D) before SVS2 addition (MβCD → SVS2 in Fig. 17B and Table 1). This suggests that SVS2 restores lipid rafts organization by retaining membrane sterols. On the other hand, wash of the MβCD-treated spermatozoa prevented the effects of SVS2 on the GM1 staining pattern; pattern D was observed in the highest percentage for the spermatozoa, which was similar to the capacitated spermatozoa (MβCD → wash →

SVS2 in Fig. 17B and Table 1). Addition of extrinsic cholesterol to the washed MβCD-treated spermatozoa rescued the effects of SVS2; the ratio of spermatozoa showing pattern APM/PAPM increased, while the ratio of spermatozoa showing pattern D decreased (MβCD → wash → SVS2 + Ch in Fig. 17B and Table 1). Cholesterol itself and cholesterol with MβCD showed no effect on GM1 patterns of the washed MβCD-treated spermatozoa. These results suggest that the effect of SVS2 on the reconstitution of lipid rafts requires free cholesterol in the medium surrounding spermatozoa. SVS2 appears to restrict sterol dynamics by altering sperm plasma membrane fluidity.

Chapter I

DISCUSSION

In this chapter, I demonstrated that SVS2 maintains sterols in the sperm plasma membrane to arrest sperm capacitation. Interestingly, SVS2 retrieved exogenous cholesterol into the spermatozoa and reset the distribution of lipid rafts, suggesting cholesterol is essential for decapacitation. These results are consistent with the results of previous studies demonstrating that cholesterol is required to prevent sperm capacitation (Davis, 1978; Zarintash and Cross, 1996).

The cholesterol concentration on the sperm plasma membrane increases during epididymal transit (Suzuki, 1988; Keber *et al*., 2013). Furthermore, seminal plasma contains a high concentration of cholesterol (Suarez, 2008), and spermatozoa appear to obtain cholesterol even after ejaculation. Thus, spermatozoa ejaculated into the uterus contain a high concentration of sterol and lose their sterol during capacitation. The cholesterol efflux triggers Ca^{2+} influx and acrosome exocytosis via GM1 dynamics (Cohen *et al*., 2014). Accordingly, SVS2 may function to maintain the sterol level of spermatozoa and suppress capacitation of spermatozoa in the uterus. The precise mechanism SVS2 maintains sterol in the sperm plasma membrane remains unknown. Seven consensus regions that recognize and interact with cholesterol are present in the SVS2 amino acid sequence (Fig. 18). Moreover, previous study showed that SVS2

directly interacts with GM1 (Kawano *et al*., 2008). Thus, SVS2 may bind both GM1 and cholesterol. GM1 may act as a membrane scaffold for SVS2, and SVS2 can inhibit sterol efflux and then retrieve liberated sterol into the sperm plasma membrane via binding to GM1.

It is interesting how sterols in the sperm plasma membrane are pulled out and how exogenous sterols are incorporated into the sperm plasma membrane. The mechanism which cyclodextrin (CD) removes cholesterol from the plasma membrane has been presumed by molecular dynamics simulation (López *et al*., 2011). CD has a rigid conical molecular structure and a cavity that can include various molecules. At first, four CD dimers bind to the membrane surface. Cholesterol is embedded in the cavity of CD and they are bound by hydrogen bond. The complex of CD and cholesterol desorbs from the membrane surface by a large free energy barrier. Finally, liberated cholesterol molecule is transferred to lipid vesicle or lipoprotein by a simple diffusion mechanism. Albumin is a potent candidate of the *in vivo* sterol liberator. Albumin has hydrophobic domains interacting with various hydrophobic ligands (Spector, 1986; He and Carter, 1992) and this structural feature also functions in liberating sterol from the plasma membrane.

Considering previous reports, SVS2 may regulate sterols in the sperm plasma

membrane as follows. Sterols in the sperm plasma membrane are removed by sterol liberators such as BSA and MβCD. After receiving sterols from sterol liberators, SVS2 binds to spermatozoa via GM1 and incorporates sterols into the sperm plasma membrane. Practically, since SVS2 appears to protect sperm sterols, high level of sterol is maintained in the sperm plasma membrane.

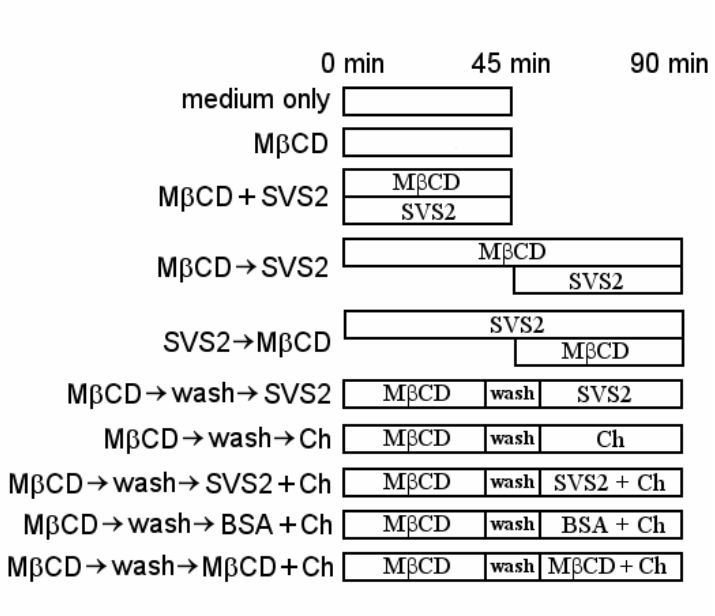

Figure 2. Schematic image of sperm incubating conditions.

MβCD: 0.5 mM, SVS2: 20 μM, Ch: 0.5 mM, BSA: 5 mg/mL, wash: spermatozoa are suspended with new medium and centrifuged for two times.

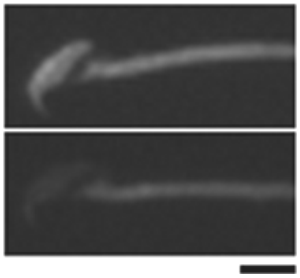

Figure 3. Spermatozoa stained with filipin.

Typical filipin staining of the non-treated spermatozoa with sterol intact (upper panel), and the capacitated spermatozoa after the efflux of sterol (lower panel) are shown. Scale bar: 5 μm.

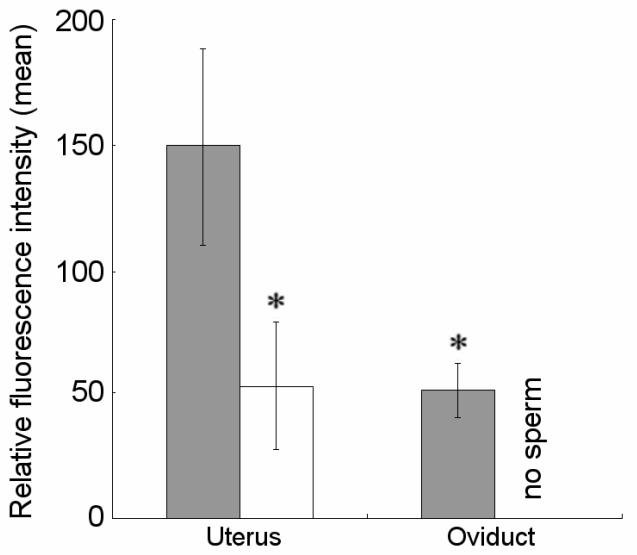

Figure 4. Sterol levels of *SVS2*$^{-/-}$ and wild type spermatozoa deposited into the female reproductive tract.

Sterol levels in the plasma membrane of spermatozoa of *SVS2*$^{-/-}$ (white bar) and wild type (WT) (gray bars) male mice collected from the uterus and the oviduct of wild-type female mice. The graph shows the relative filipin fluorescence intensity values compared to epididymal spermatozoa. Data are shown as mean ± SD. n = 8 (*SVS2*$^{-/-}$ spermatozoa in the uterus); n = 6 (WT spermatozoa in the uterus); and n = 3 (WT spermatozoa in the oviduct). No spermatozoa were found in the oviduct of the female mated with *SVS2*$^{-/-}$ male. The fluorescence intensity of the *SVS2*$^{-/-}$ spermatozoa showed a significant difference compared to that of the WT spermatozoa in the uterus (asterisks; $P < 0.01$, Student's *t*-test).

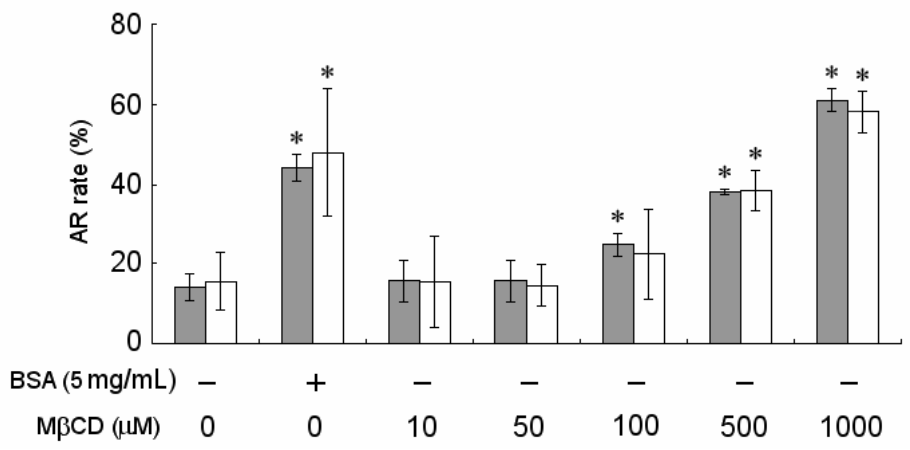

Figure 5. Effect of MβCD on sperm capacitation.

The rate of the acrosome reaction induced by progesterone (gray bars) or ionomycin (white bars) was evaluated. Data are shown as mean ± SD. Asterisks indicate significant differences compared with the spermatozoa incubated in HTF medium only ($P < 0.01$, Student's *t*-test) (n = 3).

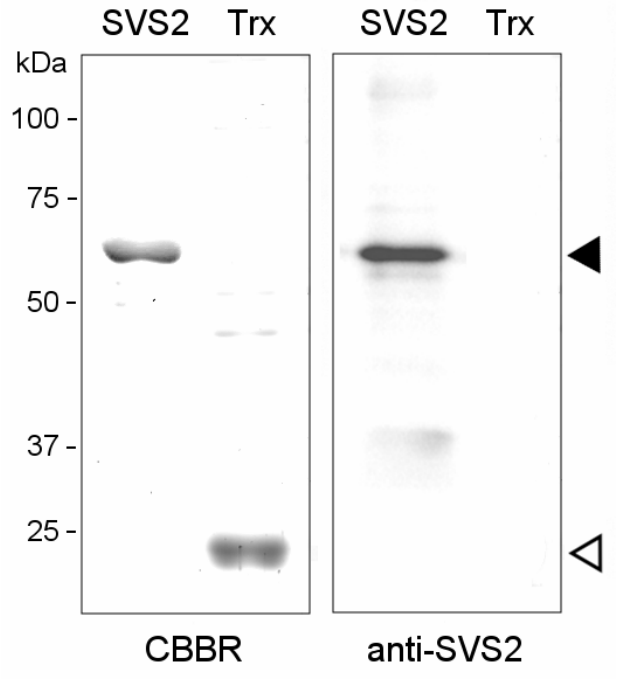

Figure 6. Western blotting of recombinant SVS2 protein.

Purified Trx-tagged SVS2 and Trx recombinant proteins were electrophoresed by SDS-PAGE and western blotting with anti-SVS2 antibody was performed. Proteins were also stained with CBBR, and the 60-kDa SVS2 (closed arrowhead) and the 20-kDa Trx (open arrowhead) were observed.

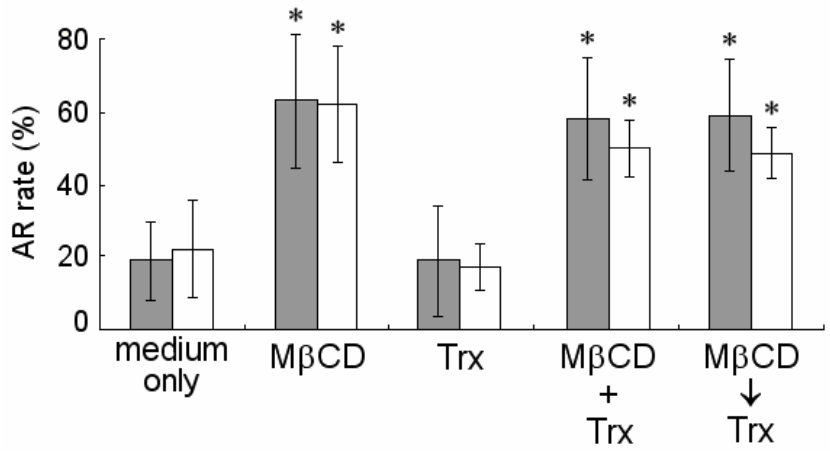

Figure 7. Effect of thioredoxin (Trx) on sperm capacitation induced by MβCD.

The rate of the acrosome reaction induced by progesterone (gray bars) or ionomycin (white bars) was evaluated. Conditions of sperm treatments are described in Figure 2. Data are shown as mean ± SD. Asterisks indicate significant differences compared with the spermatozoa incubated in HTF medium only ($P < 0.01$, Student's *t*-test) (n = 3).

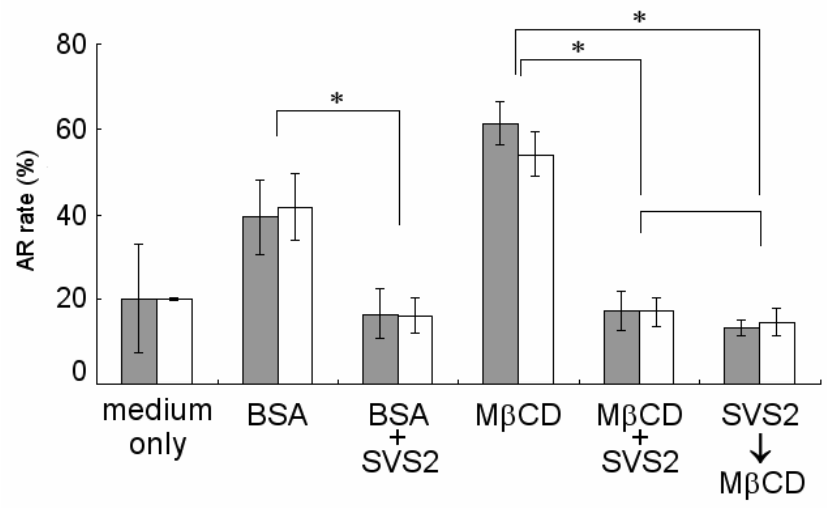

Figure 8. Effect of MβCD and SVS2 on sperm capacitation.

The rate of the acrosome reaction induced by progesterone (gray bars) or ionomycin (white bars) was evaluated. Conditions of sperm treatments are described in Figure 2. Data are shown as mean ± SD. Asterisks indicate significant differences between the two groups ($P < 0.01$, Student's *t*-test) (n = 3).

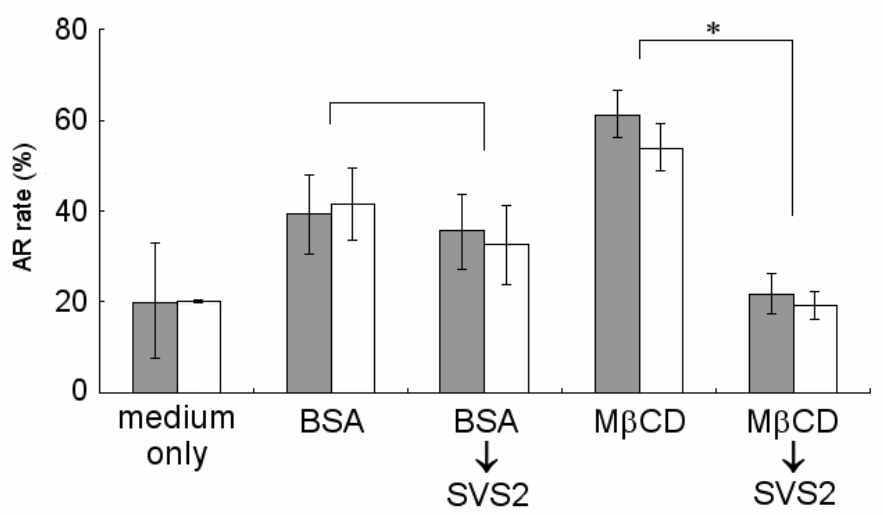

Figure 9. Effect of SVS2 on fertility induced by MβCD.

The rate of the acrosome reaction induced by progesterone (gray bars) or ionomycin (white bars) was evaluated. Conditions of sperm treatments are described in Figure 2. Data are shown as mean ± SD. Asterisks indicate significant differences between the two groups ($P < 0.01$, Student's *t*-test) (n = 3).

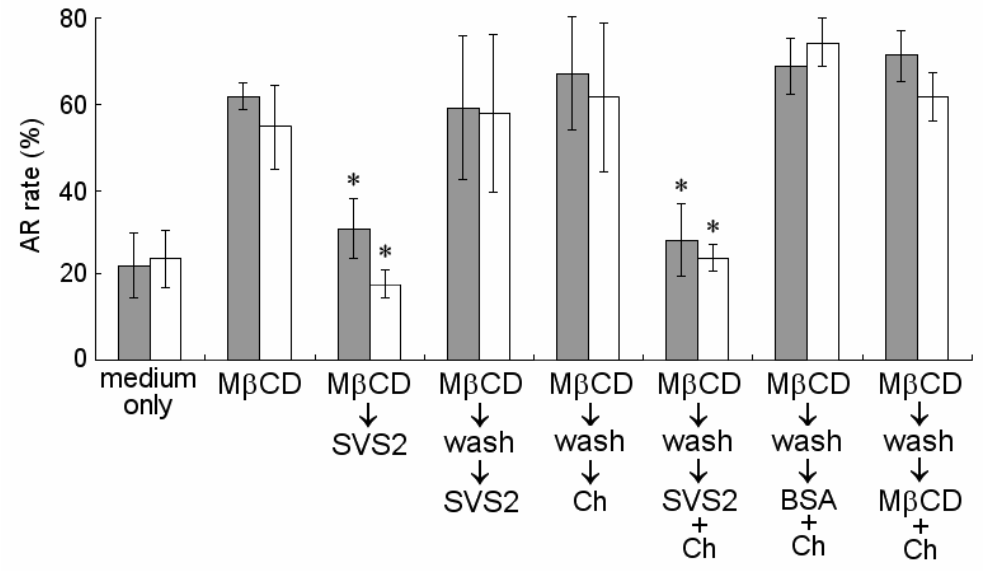

Figure 10. Influence of wash-treatment on the effect of SVS2.

The rate of the acrosome reaction induced by progesterone (gray bars) or ionomycin (white bars) was evaluated. Conditions of sperm treatments are described in Figure 2. Data are shown as mean ± SD. Asterisks indicate significant differences compared with the spermatozoa incubated with MβCD ($P < 0.01$, Student's *t*-test) (n = 3).

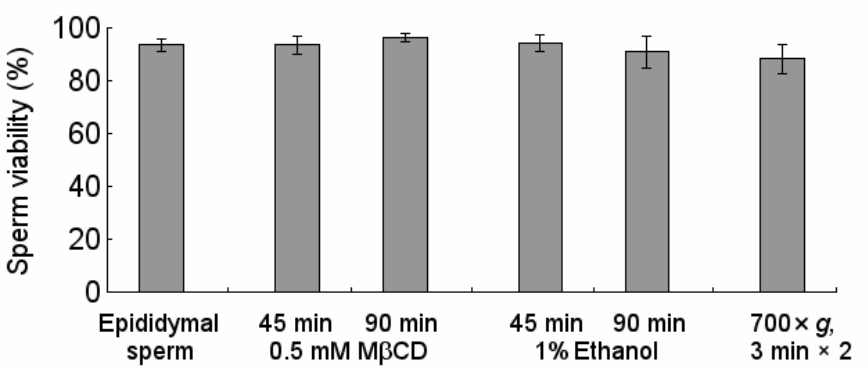

Figure 11. Evaluation of sperm viability.

Spermatozoa were stained with propidium iodide and spermatozoa with no fluorescence were regarded as live. Data are shown as mean ± SD (n = 3).

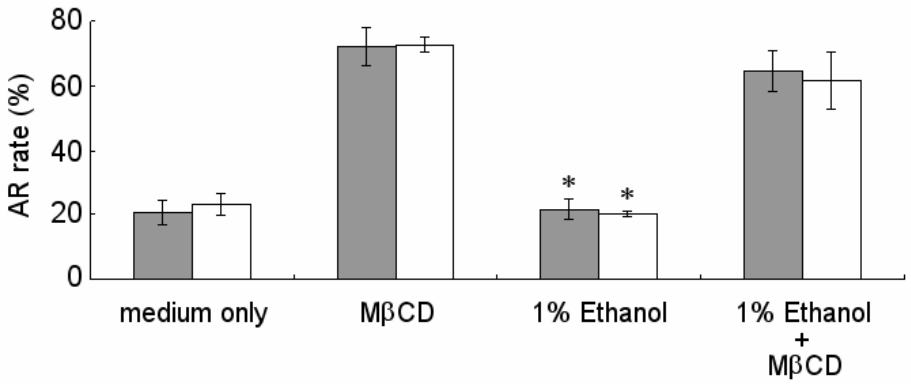

Figure 12. Effect of ethanol, vehicle of cholesterol, on sperm capacitation.

The rate of the acrosome reaction induced by progesterone (gray bars) or ionomycin (white bars) was evaluated. Data are shown as mean ± SD. Asterisks indicate significant differences compared with the spermatozoa incubated with MβCD ($P < 0.01$, Student's *t*-test) (n = 3).

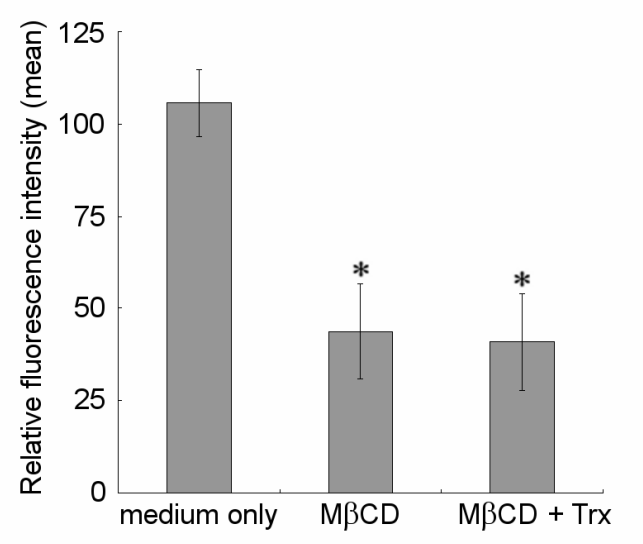

Figure 13. Effect of thioredoxin (Trx) on sterol levels in the sperm plasma membrane.

The sterol content of each sperm sample is shown as a relative value: the normalized sterol content of the spermatozoa treated with HTF medium only was set to 100. Data are shown as mean ± SD of the fluorescence intensity of spermatozoa for each treatment. Asterisks indicate significant differences compared with the spermatozoa incubated in HTF medium only ($P < 0.01$, Student's *t*-test) (n = 3).

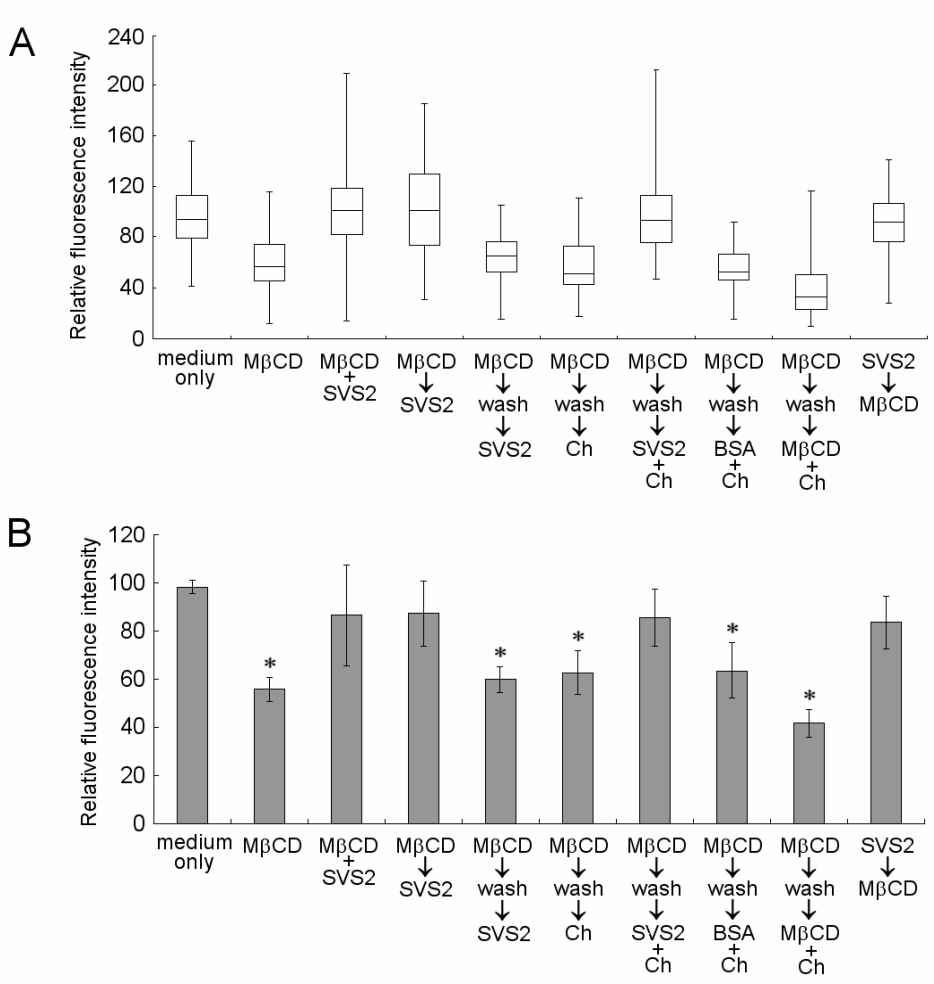

Figure 14. Effect of MβCD and SVS2 on sterol level of the sperm plasma membrane.

(A) A typical example of the distribution of fluorescence intensity of the spermatozoa from one batch. (B) Data are shown as mean ± SD of the fluorescence intensity of spermatozoa in each treatment. Conditions of sperm treatments are described in Figure 2. Asterisks indicate significant differences compared with the spermatozoa incubated in HTF medium only ($P < 0.01$, Student's *t*-test) (n = 3).

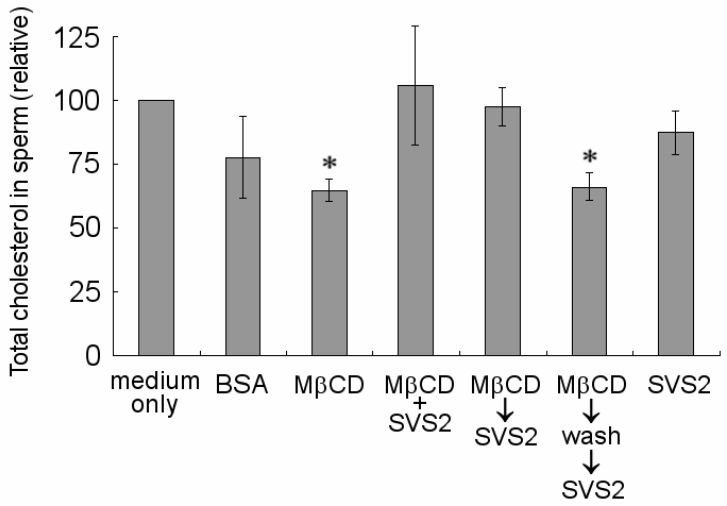

Figure 15. Enzymatic quantitation of cholesterol concentration in the sperm plasma membrane.

The cholesterol content of each sperm sample is shown as a relative value: the normalized cholesterol content of the spermatozoa treated with HTF medium only was set to 100. Absolute quantity of the cholesterol in the control spermatozoa was 142.3 ± 39.5 ng/10^6 sperm cells. Conditions of sperm treatments are described in Figure 2. Data are shown as mean ± SD, and asterisks indicate significant differences compared to spermatozoa incubated in HTF medium only (n = 4) ($P < 0.01$, Student's *t*-test).

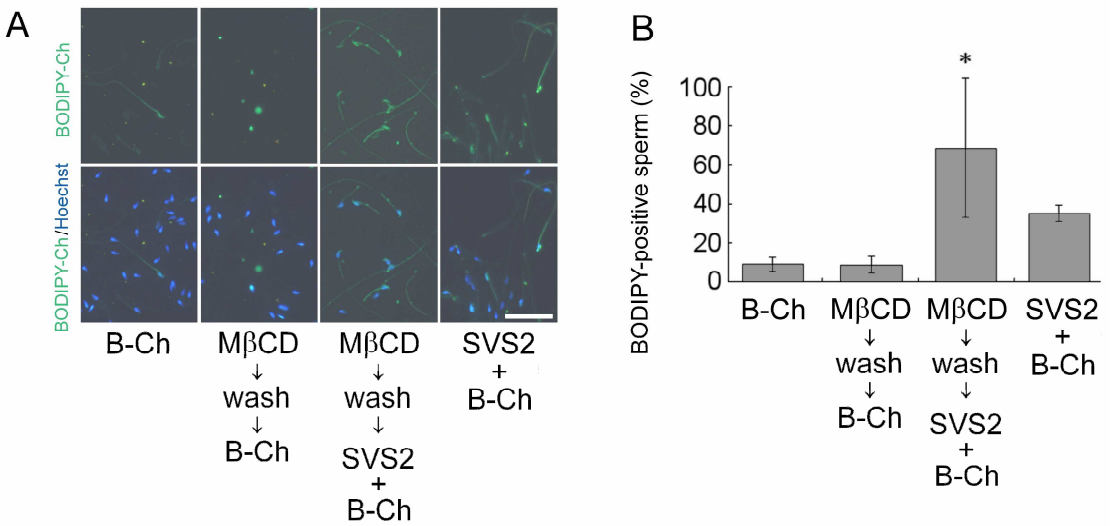

Figure 16. Visualization of cholesterol uptake into the sperm plasma membrane.

(A) Fluorescence images of spermatozoa with BODIPY-cholesterol (green). Sperm heads were visualized by staining with Hoechst 33342 (blue) in the merged images (lower panels). Scale bar: 50 μm. (B) Rate of BODIPY-cholesterol incorporation into spermatozoa. Data are shown as mean ± SD (n = 3). Conditions of sperm treatments are described in Figure 2. Asterisks indicate significant differences compared with the B-Ch groups ($P < 0.01$, Student's *t*-test).

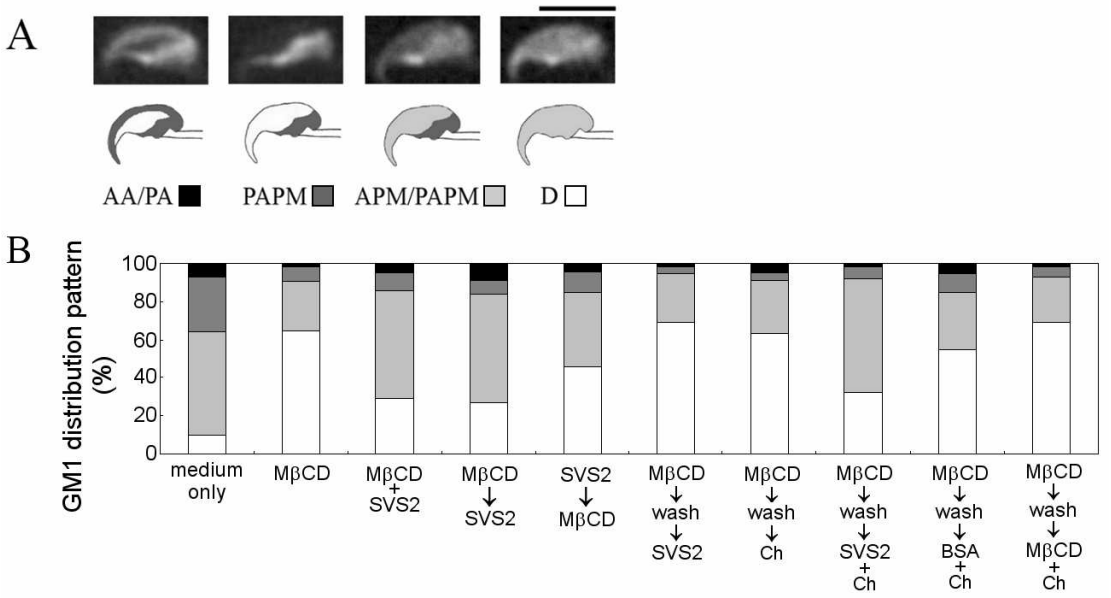

Figure 17. Effect of SVS2 on distribution of lipid rafts on the sperm head.

(A) Fluorescence images of GM1 signals observed in spermatozoa. Spermatozoa were fixed with PFA and GM1 was visualized using FITC-CTB. Pattern AA/PA: signal over anterior acrosome and postacrosome. Pattern PAPM: signal in postacrosome. Pattern APM/PAPM: weak signal in acrosome and remarkable signal in postacrosome. Pattern D: diffused signals through head. Scale bar: 5 μm. (B) Ratio of each GM1 localizing pattern in spermatozoa incubated under various conditions. Spermatozoa were obtained from one male mouse. Conditions of sperm treatments are described in Figure 2.

Table 1. Ratio of each GM1 pattern incubated under various conditions.

Conditions of sperm treatments are described in Figure 2. Data are shown as mean ± SD (n = 3).

	Percentage of staining pattern			
	AA/PA	APM	APM/PAPM	D
medium only	6.4 ± 1.2	62.4 ± 12.7	22.6 ± 7.2	8.6 ± 4.4
MβCD	4.2 ± 1.2	3.6 ± 2.1	26.0 ± 0.4	66.2 ± 1.2
MβCD+SVS2	7.2 ± 1.3	5.5 ± 2.8	55.5 ± 2.5	31.8 ± 5.3
MβCD→SVS2	8.4 ± 1.1	5.0 ± 2.5	56.4 ± 2.9	30.1 ± 6.4
SVS2→MβCD	5.3 ± 0.9	8.1 ± 2.2	50.1 ± 4.8	35.9 ± 2.2
MβCD→W→SVS2	5.4 ± 1.8	2.9 ± 0.7	30.0 ± 2.5	61.8 ± 4.2
MβCD→W→Ch	4.5 ± 0.5	4.0 ± 2.9	42.1 ± 22.2	49.5 ± 10.4
MβCD→W→SVS2+Ch	3.3 ± 1.0	7.5 ± 0.9	60.3 ± 2.4	28.9 ± 4.0
MβCD→W→BSA+Ch	4.8 ± 0.2	2.7 ± 1.1	31.5 ± 3.1	61.0 ± 3.3
MβCD→W→MβCD+Ch	2.4 ± 0.7	2.4 ± 1.4	24.1 ± 1.6	71.1 ± 1.1

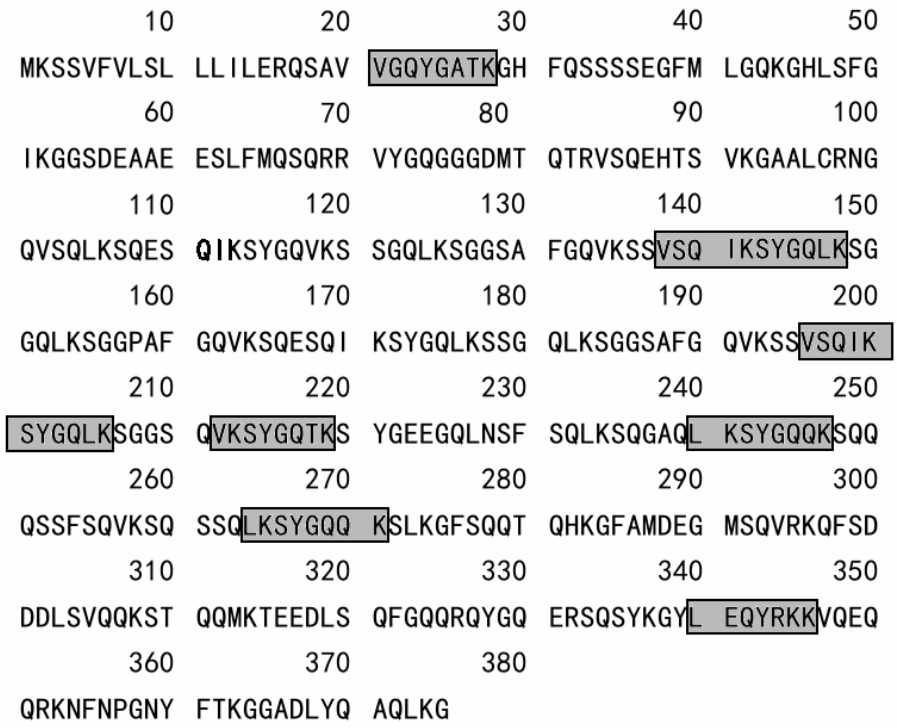

Figure 18. Amino acid sequence of mouse SVS2

The seven putative cholesterol recognition/interaction domains are shown as gray boxes.

Chapter II

Dynamics of SVS2 and sperm capacitation

in the female reproductive tract

ABSTRACT

Seminal vesicle secretion 2 (SVS2) suppresses sperm capacitation. In the uterus, SVS2 seems to inhibit sperm capacitation by interacting with ganglioside GM1 on the spermatozoa. Since sperm capacitation needs to be induced in the oviduct, there must be a mechanism for removing GM1 and/or SVS2 from the spermatozoa. Indeed, spermatozoa entering the oviduct have neither GM1 nor SVS2. However, the dynamics of SVS2 in the female reproductive tract is yet unknown. In Chapter II, I analyzed the distribution of SVS2 and GM1 in the female reproductive tract. Furthermore, I investigated the capacitation-inducing factor *in vivo*. In mated female mice, SVS2 was detected in the uterus and at the uterotubal junction, while it was not found in the oviduct. GM1, which is known to bind to SVS2, was detected along the uterine epithelium of the estrus stage. Although sperm capacitation may be induced by sterol liberators such as albumin in the oviduct, the uterine concentration of albumin was also sufficient for sperm capacitation throughout the estrous cycle. It seems plausible that while sperm capacitation can be induced in the entire female reproductive tract, SVS2 affects the uterine spermatozoa to keep in an incapacitated state. In summary, sperm capacitation might be regulated by SVS2-mediated inhibition and be induced by keeping SVS2 away from the spermatozoa.

Chapter II

INTRODUCTION

To acquire fertilizing ability, mammalian spermatozoa need to be capacitated in the female reproductive tract (Austin, 1951; Chang, 1951). Capacitated spermatozoa subsequently exhibit a change of flagellar motility, called hyperactivation, acrosome reaction, and penetration of the zona pellucida, before eventually fusing with the plasma membrane of the oocyte. Although sperm capacitation is indispensable for achieving mammalian fertilization, its regulatory mechanism is not clear.

Since albumin is required for inducing sperm capacitation *in vitro* (Brackett *et al*., 1972; Davis, 1976), it may also play an important role in capacitation *in vivo*. Albumin is present in the female reproductive tract (Miyamoto and Chang, 1973), and sperm capacitation is promoted by cholesterol transfer to albumin (Davis *et al*., 1979). On the other hand, there may be other factors concerned in sperm capacitation *in vivo*. Human follicular fluid contains high-density lipoprotein (Jaspard *et al*., 1996), while albumin mediates cholesterol transfer between cells and lipoproteins (Zhao and Marcel, 1996). Apolipoproteins A−I and J in the female reproductive tract have been reported as inducers of cholesterol efflux from the sperm plasma membrane (Thérien *et al*., 1997; Argraves and Morales, 2004). According to these previous studies, it seems likely that albumin, supported by other factors, induces sterol efflux from the sperm plasma

membrane, resulting in the induction of sperm capacitation. However, the detailed mechanism of *in vivo* capacitation, namely the regulation of sterol dynamics in the sperm plasma membrane, is unknown.

The seminal plasma protein seminal vesicle secretion 2 (SVS2) prevents sperm capacitation and acts as a decapacitation factor (Kawano and Yoshida, 2007). SVS2 is directly ejaculated into the uterus with spermatozoa; it binds to ganglioside GM1 in their postacrosomal region, yet spermatozoa entering the oviduct have neither GM1 nor SVS2 (Kawano *et al*., 2008). Since capacitated spermatozoa cannot enter the oviduct (Shalgi *et al*., 1992), sperm capacitation seems to occur there. In conclusion, the effect of SVS2 on spermatozoa via GM1 may regulate *in vivo* capacitation.

Sperm capacitation *in vivo* may occur via an interaction between spermatozoa and the milieu of the female reproductive tract. Mouse spermatozoa are deposited in the uterus and migrate to the uterotubal junction (UTJ). The UTJ functions as a barrier to sperm entrance into the oviduct and as a site of sperm selection (Suarez, 2008). Spermatozoa seem to be selected according to two features: sperm motility and membrane properties. In hamsters, artificially inseminated spermatozoa that are already capacitated cannot pass through the UTJ owing to their hyperactivated motility (Shalgi *et al*., 1992). In mice, the deficiency of certain proteins yields infertility caused by

incapability of the UTJ passage (Druart, 2012). Although the function of the UTJ is unknown, it appears to be important for fertilization via regulating sperm capacitation. Thus, GM1 and/or SVS2 might be released from spermatozoa around the UTJ, resulting in sperm capacitation. However, the dynamics of SVS2 in the female reproductive tract and interactions between SVS2, spermatozoa, and the genital epithelium have not been elucidated.

In this chapter, I tried to examine the dynamics of SVS2 in the female reproductive tract. Particularly, the interaction between SVS2 and the genital epithelium was studied. Furthermore, to substantiate that sperm capacitation can occur in the uterus, I detected the potent capacitation-inducing factor albumin in the uterine fluid.

Chapter II

MATERIALS AND METHODS

Immunohistochemistry of Female Reproductive Tracts

The anti-SVS2 polyclonal antibody was purified as described in Chapter I.

Nine–fifteen-week-old female mice (CD-1; Charles River Japan) were mated with 9–15-week-old male mice. At 1.5 h post-coitus, female reproductive tracts were obtained by dissection and fixed with 4% (w/v) PFA for 4–6 h. Samples were embedded in paraffin, and 10-μm-thick sections were cut according to standard protocols.

Immunohistochemistry was performed using the anti-SVS2 antibody (20 μg/mL), followed by an Alexa 488-conjugated secondary antibody (goat anti-rabbit IgG; 1:20,000; Life Technologies, Carlsbad, CA, USA) with 0.2 μg/mL 4',6-diamidino-2-phenylindole (DAPI) (Wako, Osaka, Japan). Anti-SVS2 antibody that was passed through a HiTrap NHS-activated HP column (GE Healthcare Japan, Tokyo, Japan) conjugated with recombinant SVS2 protein was used as the negative control. Stained sections were washed with PBS, analyzed using a fluorescent microscope (Leica DMRBE, Solms, Germany), and images were acquired using a CCD camera (MicroPublisher 5.0, QImaging, Surrey, BC, Canada). Exposure times were 5 s, 15 s, and 0.7s for anti-SVS2, control antibody, and DAPI staining, respectively.

Detection of GM1 in the Female Reproductive Tract

The estrous cycle stage of 9–15-week-old female mice was deduced by checking vaginal smears. Female reproductive tracts of each estrous cycle stage were obtained by dissection, fixed with 4% (w/v) PFA, embedded in paraffin, and 10-μm sections were cut as described above. Sections were stained with 0.1 mg/mL FITC-conjugated cholera toxin B subunit (FITC-CTB) in PBS for 12 h at 4°C. Stained sections were washed three times with PBS, visualized using a fluorescent microscope (Leica DMRBE) and images were acquired using a CCD camera (MicroPublisher 5.0, QImaging). Exposure time was 10 s for FITC-CTB staining.

Quantification of Albumin in Uterine Fluid

The uteri of each estrous cycle stage of 9–15-week-old female mice (deduced as described above) were eviscerated, and surface fluid and blood were thoroughly removed. The uterus was hung in a tube, and uterine fluid was obtained by centrifugation at 1,200 × *g* for 3 min. Albumin levels in uterine fluid were quantified using an ELISA kit (Shibayagi Co., Gunma, Japan).

Sperm Capacitation Assay

To evaluate the effect of SVS2 on sperm capacitation induced by high

concentrations of albumin, spermatozoa were isolated from the cauda epididymis of sexually mature 9–15-weeks-old male CD-1 mice, suspended in HTF medium, and incubated at 37°C in a 5% CO_2 atmosphere. Bovine serum albumin (BSA, A-4503; Sigma-Aldrich) was used to induce sperm capacitation. Recombinant SVS2 protein was prepared as described in Chapter I and used at a concentration of 20μM, which is similar to the physiological concentration in the uterus (Kawano and Yoshida, 2007). Sperm capacitation was evaluated based on acrosomal responsiveness to progesterone and ionomycin, as described in Chapter I.

Statistical Analysis

All experiments were repeated at least three times with different individuals. Data are expressed as mean ± SD. Statistical significance was calculated using the Student's *t*-test; $P < 0.01$ was considered statistically significant.

RESULTS

Distribution of SVS2 in the Female Reproductive Tract

To evaluate the role of SVS2 in *in vivo* sperm capacitation, the dynamics of SVS2 quantities in the female reproductive tract after copulation was examined. In the uterine tube, SVS2 was found on the sperm clot, which may have been derived from free SVS2 in semen (Fig. 19). Furthermore, SVS2 was found on the surface of the uterine wall and on the wall of the uterotubal junction (UTJ), a passage from the uterus to the oviduct (Fig. 19). However, little SVS2 was detected in the oviductal isthmus (Fig. 19). Although spermatozoa were seen in the isthmus, no SVS2 was detected on them.

Distribution of GM1 in the Uterus

Since SVS2 specifically binds to ganglioside GM1, the distribution of GM1 in the female reproductive tract was examined. Although the amount is different, GM1 localizes in many tissues (Iwamori and Nagai, 1981; Iwamori *et al*., 1984). While GM1 was localized all over the uterine tube, intense GM1 staining of the uterine epithelium was only detected at the estrus stage and not at the proestrus, metestrus, or diestrus stage (Fig. 20).

Albumin in the Uterus

Since serum albumin is a potent cholesterol acceptor *in vivo* (Davis, 1981), the quantity of albumin in the uterine fluid was examined. The detected range of 6–108 mg/mL throughout the estrous cycle (Table. 2) should be sufficient for inducing sperm capacitation.

Effect of SVS2 on Sperm Capacitation Induced by High Concentrations of Albumin

As described above, the quantity of albumin detected in the uterus exceeded its concentration in the *in vitro* capacitation medium (3–5 mg/mL; Davis, 1979) considerably. Therefore, I examined the ability of SVS2 to suppress sperm capacitation in the presence of such high concentrations of bovine serum albumin (BSA). First, I confirmed that 100 mg/mL BSA do not influence sperm viability, using propidium iodide as described in Chapter I (Fig. 21). Second, 100 mg/mL BSA induced the same level of capacitation as 5 mg/mL BSA (Fig. 22A). Third, the physiological concentration of SVS2 in the uterus of 20 μM (Kawano and Yoshida, 2007) inhibited sperm capacitation even in the presence of 100 mg/mL BSA (Fig. 22B). These results indicate that SVS2 prevents sperm capacitation in the uterus, and keeps sperm incapacitated until they reach the oviduct.

DISCUSSION

In this chapter, I showed that the distribution of SVS2 in the female reproductive tract is restricted to the uterus and the UTJ. It was also shown that GM1 is located along the uterine epithelium in estrus and seems to retain SVS2 on the uterine wall. While there is plenty of albumin in the uterine fluid for inducing sperm capacitation, SVS2 could suppress sperm capacitation *in vitro* even in the presence of high concentrations of albumin as detected in the uterine fluid. These results indicate that SVS2 inhibits sperm capacitation in the uterus, while the effect of SVS2 seems to be excluded from the oviduct.

Since spermatozoa recovered from the oviduct possess neither SVS2 nor its receptor GM1 (Kawano and Yoshida, 2007; Kawano *et al*., 2008), the final event of *in vivo* capacitation may be removal of GM1 from the sperm plasma membrane. Indeed, GM1 and SVS2 appear to be liberated from spermatozoa at the UTJ. While the mechanism of this release is not known, the UTJ seems to function as a checkpoint for spermatozoa. When the sperm surface protein ADAM3 was disrupted as in *Calmegin*$^{-/-}$, *Adam1a*$^{-/-}$, *Adam2*$^{-/-}$, and *Ace*$^{-/-}$ mice, spermatozoa could not migrate into the oviduct, resulting in infertility (Yamaguchi *et al*., 2009). As spermatozoa of *SVS2*$^{-/-}$ mice cannot enter the oviduct either (Kawano *et al*., 2014), these results indicate that the UTJ may

recognize physiological and molecular characteristics of spermatozoa and that only spermatozoa with specific protein(s) can interact with the epithelium and pass the UTJ.

In addition, the UTJ may be involved in the removal of molecules from spermatozoa. Spermadhesin AWN-1 localizes on spermatozoa and in seminal plasma and binds to the epithelium of the UTJ, while the oviductal fluid does not contain this protein (Calvete *et al*., 1997). This indicates that seminal plasma components are removed from spermatozoa in the UTJ. A previous study showed that GM1 binds to SVS2 via electrostatic interactions between the two molecules and that their affinity to each other changes depending on the pH (Kawano *et al*., 2008). As the pH of the oviductal fluid is higher than that of the uterine fluid in rhesus monkeys and cattle (Maas *et al*., 1977; Hugentobler *et al*., 2004), it seems possible that differences in environmental conditions such as pH can mediate the release of GM1 and/or SVS2 in the UTJ, ensuring that only naked spermatozoa pass through the UTJ and enter the oviduct.

Since high quantities of albumin were detected in the uterus (Table. 2), sterol liberators, which induce sperm capacitation, appear to be present throughout the entire female reproductive tract. Thus, in the female reproductive tract, spermatozoa may receive stimuli of opposite effects on sterols in their plasma membrane: an extracting

effect by liberators and a protecting effect by SVS2. In the uterus, SVS2 acts to inhibit sterol extraction and to intercalate sterols into the sperm plasma membrane, resulting in maintenance of sperm sterols. In the oviduct, the extracting effect surmounts the effect of SVS2, resulting in a decrease in sperm sterols. In other words, *in vivo* capacitation seems to be regulated not by a capacitation-inducing factor but by a decapacitation factor; SVS2 may be this factor regulating of sperm fertility *in vivo*.

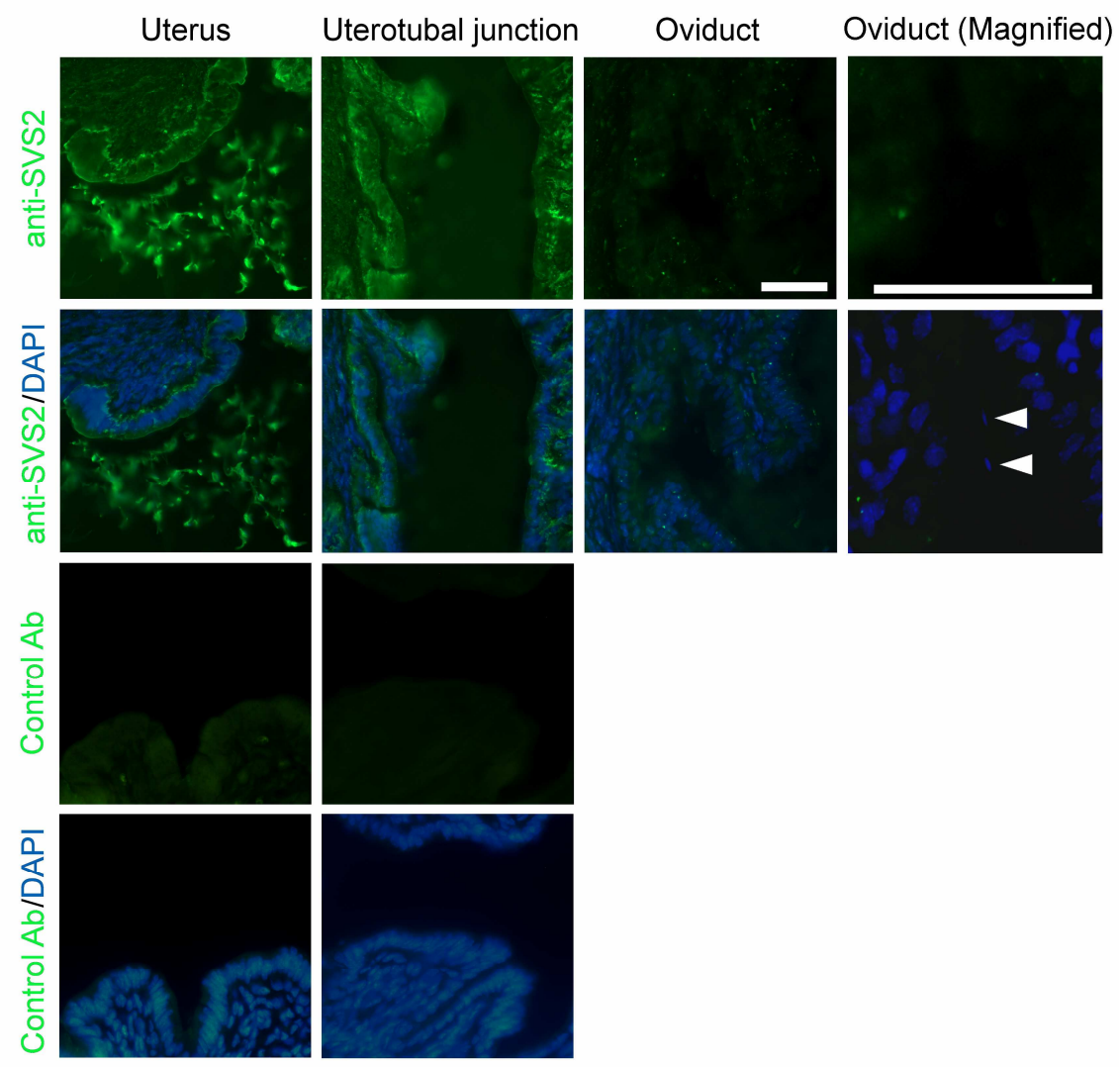

Figure 19. Immunolocalization of SVS2 in the female reproductive tract.

SVS2 in the female reproductive tract at 1.5 h post-coitus was stained with anti-SVS2 antibody (green). Images of nuclei stained with DAPI (blue) superimposed on the immunostained images are shown in the lower panel. SVS2 entered the uterus and the UTJ region, but a very low amount entered the oviduct. Spermatozoa in the oviductal isthmus (arrow heads) had no SVS2. No antibody staining was detected in the controls. Scale bar: 50 μm.

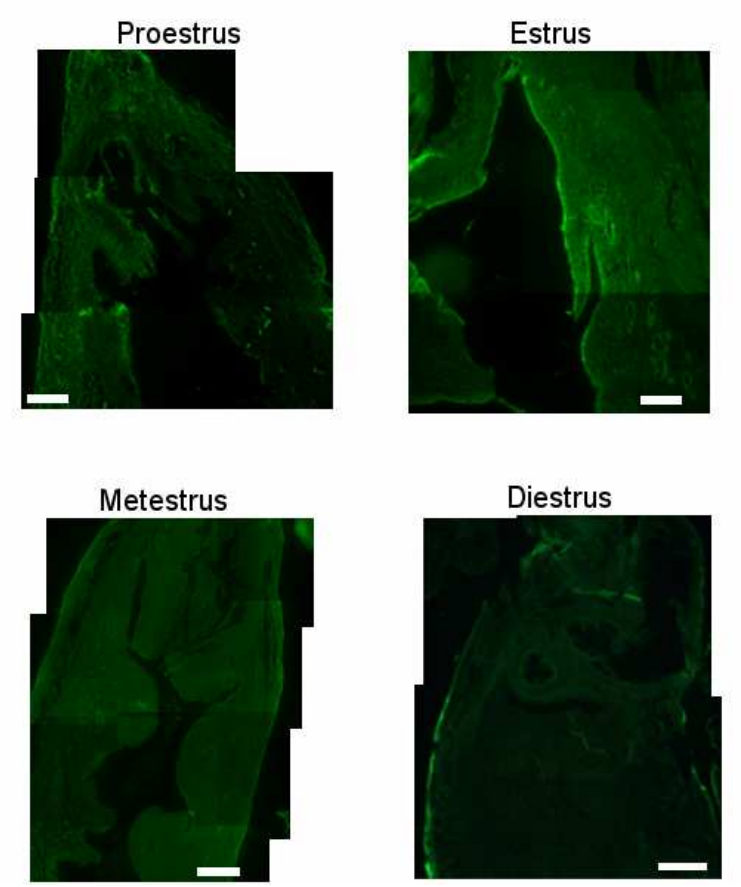

Figure 20. Detection of GM1 on the uterine epithelium.

Uteri of each estrous cycle stage were stained with FITC-CTB. Although GM1 localized all over the tissue in the uterine tube, intense GM1 staining of the uterine epithelium was only detected in estrus and not in proestrus, metestrus, or diestrus. Scale bar: 100 μm.

Table 2. Albumin in the uterine fluid.

Estrous cycle	Specimen #	Albumin in the uterine fluid (mg/mL)
Proestrus	1	11.8
	2	19.4
	3	107.7
Estrus	1	6.1
	2	19.4
	3	6.5
	4	57.8
Metestrus	1	9.3
	2	8.8
	3	49.6
Diestrus	1	14.7
	2	90.7
	3	56.0

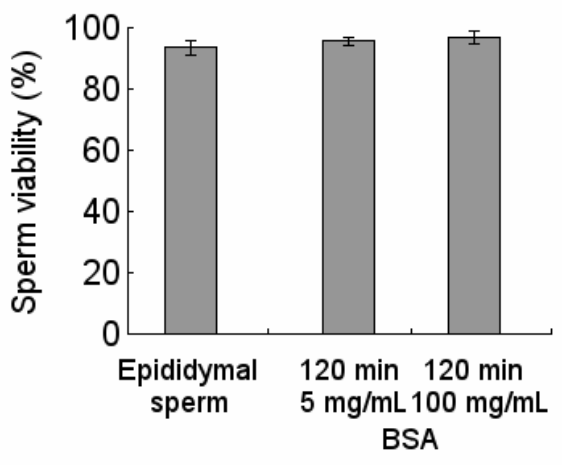

Figure 21. Evaluation of sperm viability.

Spermatozoa were stained with propidium iodide, and spermatozoa with no fluorescence were regarded as live. Data are shown as mean ± SD (n = 3).

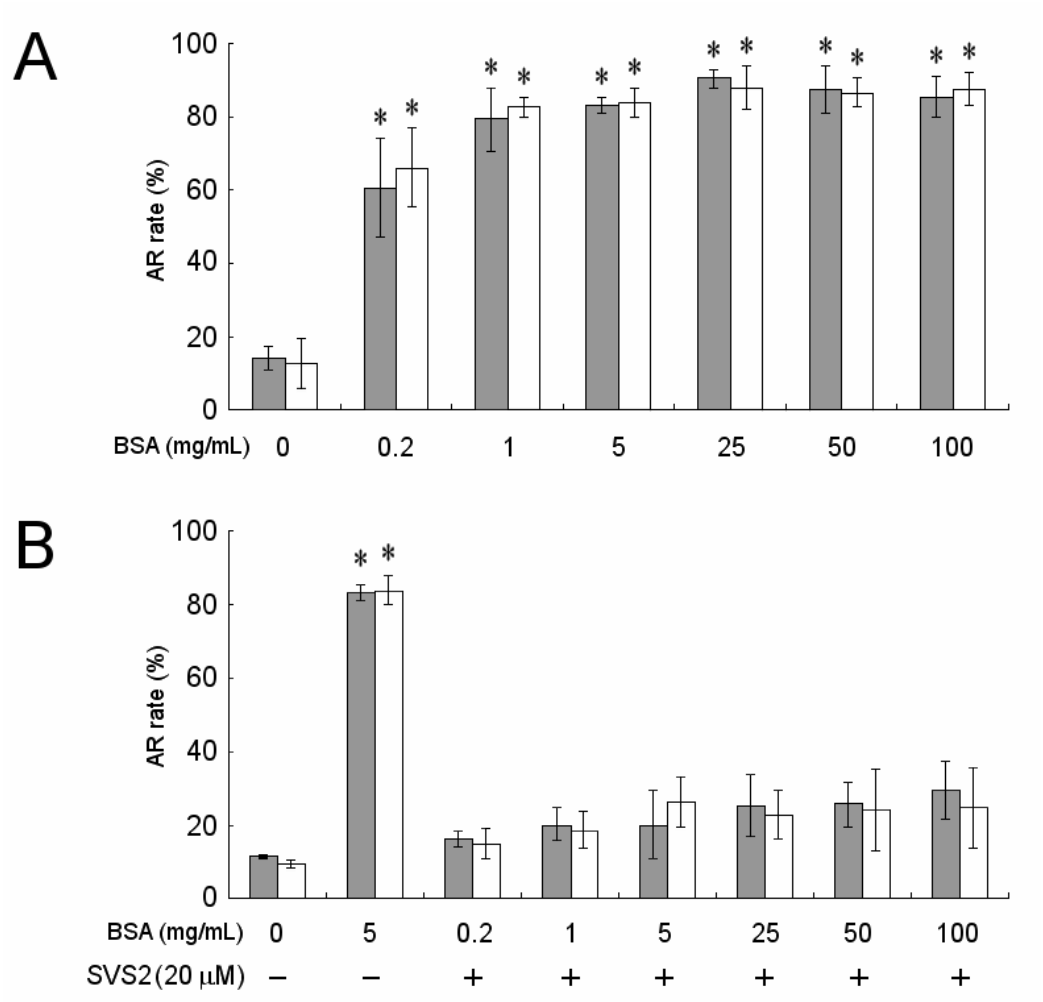

Figure 22. Effects of SVS2 on sperm capacitation induced by high concentrations of BSA.

Acrosome reaction (AR) rate induced by progesterone (gray bars) or ionomycin (white bars). (A) Sperm capacitation induced by various concentrations of bovine serum albumin (BSA). (B) Effects of SVS2 on capacitation induced by various concentrations of BSA. Data are shown as mean ± SD. Asterisks indicate significant differences compared with sperm incubated without BSA nor SVS2 ($P < 0.01$, Student's *t*-test) (n = 3).

Chapter III

Function and possible participation of SVS3 and SVS4 in sperm capacitation

ABSTRACT

Sperm capacitation in the female reproductive tract is essential for mammalian fertilization. Chapter I & II and our previous studies reported that seminal vesicle secretion 2 (SVS2) binds to ganglioside GM1 on the sperm plasma membrane to inhibit sterol efflux accompanying sperm capacitation. Furthermore, SVS2 has seven putative cholesterol-binding domains that may be important for preventing sperm fertility (decapacitation). While mouse *SVS2–SVS6* evolved by gene duplication and belong to same gene family, the effects of SVSs other than SVS2 on sperm capacitation have not been elucidated. In Chapter III, I examined the effects of SVS3 and SVS4, which are highly homologous to SVS2, on capacitation. SVS3 showed an affinity to spermatozoa and SVS2 and facilitated the effect of SVS2 on sperm capacitation. SVS4 bound to spermatozoa and inhibited capacitation. However, SVS3 and SVS4 could not decapacitate spermatozoa. Although SVS2 may interact with cholesterol, SVS3 and SVS4 seem to show weaker affinities to cholesterol than SVS2. Accordingly, SVS2 seems to be the main regulator of *in vivo* capacitation, while other SVSs can also be involved. The effect of SVSs on sperm capacitation may be determined by their ability to interact with spermatozoa and cholesterol.

Chapter III

INTRODUCTION

The function of mammalian spermatozoa is mediated by various male and female factors. The seminal vesicle, one of the male reproductive accessory glands, plays an important role in sperm motility and fertility. The removal of seminal vesicles from mice results in a decline of fecundability (Pang *et al.* 1979; Kawano *et al*, 2014). In human, the last portion of ejaculated semen is largely composed of secretions from the seminal vesicle (MacLeod and Hotchkiss, 1942). A semen-coagulating protein from the seminal vesicle (Lilja and Laurell, 1984) was identified as semenogelin (SEMG) (Lilja *et al*., 1989; Lilja and Lundwall, 1992). SEMG suppresses sperm motility and capacitation, making it a candidate for a decapacitation factor (de Lamirande *et al.*, 2001). It is a member of a gene family encoding semen-coagulating proteins, and genes of this family were found in various mammals such as mouse (Lundwall, 1996), rat (Harris *et al*., 1990), and pig (Iwamoto *et al.*, 1995).

In mouse, seminal vesicle secretions (SVSs) consist of seven major proteins (Fawell *et al*., 1987). *SVS2–SVS6* genes are located on chromosome 2 (Fig. 23) and belong to the REST (rapidly evolving seminal vesicle-transcribed) gene family (Lundwall and Lazeru, 1995; Lundwall, 1996), which are paralogous genes evolved by duplication from a proteinase gene ancestor (Clauss *et al.*, 2005).

SVSs evolve rapidly under selective pressure (Ramm *et al.*, 2009), indicating their adaptability to various reproductive strategies across species. Indeed, SVSs have many functions in copulation and fertilization. SVS1–SVS3 are cross-linked with each other by transglutaminase forming the copulatory plug (Lin *et al*., 2002). SVS1 is a putative copper amine oxidase (Lundwall *et al.*, 2003). SVS4 shows immunoregulatory activity (Romano-Carratelli *et al*., 1995). SVS5 and SVS6 may act as serine proteinase inhibitors (Clauss *et al*., 2005). SVS7 enhances sperm motility (Luo *et al*., 2001).

SVS2 is similar to SEMG (Kawano and Yoshida, 2007), showing 55% similarity in the 55 amino acids of the N-terminal region containing a signal peptide. Both SVS2 and SEMG are lysine-rich (SVS2, 10.4%; SEMG, 8.9–10.0%), highly basic proteins (p*I* values: SVS2, 10.68; SEMG, 9.45–9.68). In addition, they inhibit sperm capacitation, indicating their function as decapacitation factors. In conclusion, there may be an evolutionary conserved regulatory mechanism of capacitation by seminal vesicle proteins.

While SVSs are encoded by the same gene family, they have various functions. It is not known whether SVSs other than SVS2 acts as regulators of sperm capacitation. SVS3 and SVS2 compose the copulatory plug, and show high amino acid homology (31%) to each other. Therefore, the effects of SVS3 on sperm capacitation should be

examined. *SVS3* includes *SVS3a* and *SVS3b*, and the encoded amino acid similarity between them is 98%. However, since the lysine content of SVS3b (9.1%) is the same as that of SVS2 (9.1%) and *SVS2* localizes closer to *SVS3b* than to *SVS3a* on the chromosome, I studied the effects of SVS3b (hereafter designated as SVS3 only). In addition, SVS4 occupies a chromosomal location close to SVS2, and the homology between SVS4 and SVS2 is the next highest (21%) after SVS3. Accordingly, I also examined the effects of SVS4 on sperm capacitation.

In this chapter, I investigated inhibitory effects of SVS3 and SVS4 on capacitation and examined the affinity of SVS3 and SVS4 to spermatozoa. Finally, I discuss the possibility of these proteins' participation in *in vivo* capacitation.

Chapter III

MATERIALS AND METHODS

Preparation of Recombinant SVS Proteins

Recombinant SVS2 protein was produced as described in Chapter I. Preparation of cDNAs encoding SVS3b (NCBI accession No.NM_173377) and SVS4 (NCBI accession No.NM_009300) and expression of recombinant proteins were performed as described in Chapter I. Proteins were expressed with the thioredoxin (Trx)-tag, the Trx-tag itself has no effect on sperm capacitation (see Chapter I).

The purity of recombinant SVS3 and SVS4 proteins was checked by western blotting with anti-SVS3 and anti-SVS4 antibodies, respectively. Rabbit antiserum against SVS3 was produced by Operon Biotechnologies (Tokyo, Japan) by using peptide antigen (amino acids 75−88 of SVS3b). Anti-SVS3 polyclonal antibody was purified by affinity column chromatography (HiTrap HNS-activated HP; GE Healthcare) by using recombinant SVS3 protein as a ligand. Anti-SVS4 antibody was generously provided by Dr. N. Kawano. The specificity of anti-SVS3 and anti-SVS4 antibodies was evaluated by western blot analysis of seminal vesicle fluid (SVF). Electrophoresis of SVF, recombinant SVS3 and SVS4 by Laemmli SDS-PAGE using a 10% (SVS3) or 18% (SVF and SVS4) (w/v) polyacrylamide gel, and the following western blotting with anti-SVS3 antibody (2 μg/mL) or anti-SVS4 antibody (10 μg/mL)

were carried out as described in Chapter I.

Evaluating Concentrations of SVSs in Ejaculate

Fluid collected from seminal vesicles was dissolved in Laemmli sample buffer (Laemmli, 1970) and boiled for 5 min. Samples were electrophoresed by Laemmli SDS-PAGE by using a 15% (w/v) polyacrylamide gel, with bovine serum albumin (BSA) as the molecular weight standard. SVSs were densitometrically quantified by a CS analyzer (ATTO, Tokyo, Japan).

Detection of SVS3 and SVS4 Proteins in the Female Reproductive Tract

Female mice aged 9−15 weeks (CD-1; Charles River Japan) were mated with 9−15-week-old male mice. Females were sacrificed at 1.5 h post-coitus, and intratubular fluids were collected by flushing the following parts of the female reproductive tract with PBS: the vagina, the uterine region near the vagina, the uterine region near the oviduct, and the oviduct. Control samples were collected from the seminal vesicle, the copulatory plug and estrus-stage uterine fluid. The fluids were precipitated with acetone and dissolved in Laemmli sample buffer. Western blotting with anti-SVS3 or anti-SVS4 antibody was performed as described above.

Sperm Capacitation Assay

To evaluate the effects of SVSs on sperm capacitation *in vitro*, spermatozoa were isolated from the cauda epididymis of sexually mature male CD-1 mice (9–15 weeks old), suspended in HTF medium containing 5 mg/mL BSA (A-4503; Sigma-Aldrich), and incubated at 37°C in a 5% CO_2 atmosphere for 2 h. SVS protein(s) was added to the medium as indicated. Sperm capacitation was evaluated based on acrosomal responsiveness to progesterone and ionomycin as described in Chapter I.

Binding Assay of SVS3−GM1, SVS4−GM1, and SVS2−SVS3

Interaction between GM1 and SVS3/SVS4 was examined by a highly sensitive 30-MHz quartz crystal microbalance (QCM; NAPiCOS; Nihon Dempa Kogyo Co., Tokyo, Japan). One channel of a sensor chip was coated with 1,000 pmol SVS3 or SVS4 dissolved in PBS (pH 7.4). The second channel of the same sensor chip was coated with 1,000 pmol Trx dissolved in PBS as a reference. The sensor chip was washed three times with PBS, placed into the chamber, perfused with PBS until the frequency was stabilized, and blocked by perfusing twice with 1 mg/mL Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan) dissolved in PBS. Subsequently, the sensor chip was perfused with GM1 (2.5 nmol for SVS3; 5 nmol for SVS4) dissolved in

PBS, and the change of frequency was recorded. To evaluate the binding affinity of GM1 to SVS3/SVS4, the changes in the frequency of cumulative perfusion were analyzed. All experiments were carried out at 25°C with a flow rate of 50 μl/min.

Interactions between SVS2 and SVS3 were examined by QCM, as described above. One channel of a sensor chip was coated with 1,000 pmol SVS3 dissolved in PBS. The second channel on the same sensor chip was coated with 1,000 pmol Trx dissolved in PBS as a reference. After washing, perfusion, and blocking, the sensor chip was perfused with 100 pmol SVS2, and the change in frequency was recorded.

Affinities were evaluated by the Michaelis-Menten equation, and fitted curves were obtained by calculating the average of five experiments. Values of the dissociation constant (K_d) and maximum binding amounts (ΔM_{max}) were calculated with NAPiCOS Analysis software (Nihon Dempa Kogyo Co., Tokyo, Japan).

Statistical Analysis

All experiments were repeated at least three times with different individuals. Data are expressed as mean ± SD. Statistical significance was calculated using the Student's *t*-test; $P < 0.01$ was considered statistically significant.

Chapter III

RESULTS

Estimating Concentrations of SVSs in Ejaculate

To examine the functions of SVS3 and SVS4 in the female reproductive tract, their concentrations in the uterus were estimated. The uterine concentration of SVS2 was estimated to be 25 μM (Kawano and Yoshida, 2007). Providing that secretion volumes of SVSs are directly proportional to their abundance in the seminal vesicle, concentrations of SVS3 and SVS4 in ejaculate were estimated as 7.3 ± 0.5 μM and 30.0 ± 6.5 μM, respectively (n = 3).

Distribution of SVS3 and SVS4 in the Female Reproductive Tract after Copulation

After copulation, SVS2 enters the uterus, but is rarely detected in the oviduct (Chapter II). To examine whether SVS3 and SVS4 enter the female reproductive tract after copulation, I measured the quantities of SVS3 and SVS4 in the contents of each part of the female reproductive tract: the copulatory plug, the vagina, the uterine region near the vagina, the uterine region near the oviduct and the oviduct. Western blotting using anti-SVS3 antibody showed no signals in the entire female reproductive tract (Fig. 24), whereas the anti-SVS4 antibody showed a band of 13 kDa in the vagina and uterus, but not in the oviduct (Fig. 25).

Effects of SVS3 on Sperm Capacitation

To examine whether SVS3 can regulate sperm capacitation, the effects of SVS3 on the induced acrosome reaction (AR) rate were evaluated. Expression and purification of recombinant SVS3 protein were analyzed using CBBR staining and western blotting with anti-SVS3 antibody (Fig. 26A). As described in Chapter I, incubation of the spermatozoa with 5 mg/mL BSA for 2 h increased the induced-AR rate significantly (Fig. 27A). Spermatozoa incubated with BSA and 10−30 μM SVS3 showed no difference in the induced-AR rates from the spermatozoa treated with BSA alone (Fig. 27A). When spermatozoa were treated with SVS2 after having been capacitated by BSA, acrosomal responsiveness to progesterone or ionomycin was decreased (decapacitation effect). However, SVS3 did not show this decapacitation effect; if spermatozoa were incubated with SVS3 after BSA-treatment, the induced-AR rate did not decrease (Fig. 27B). These results indicate that SVS3 has neither capacitation-inhibiting nor decapacitation effect.

Subsequently, I examined whether SVS3 can influence the effect of SVS2 on sperm capacitation. When sperm capacitation was induced in medium containing 20 μM SVS2 and 20 μM SVS3, the reduction of the induced-AR rate was equal to spermatozoa incubated with 20 μM SVS2 alone (Fig. 28A). However, while 10 μM SVS2 showed

only a minor effect on the induced-AR rate (Fig. 28B), the rates of spermatozoa incubated with BSA, 10 μM SVS2, and 10−20 μM SVS3 decreased significantly (Fig. 28B). These results indicate that SVS3 enhances the inhibitory effect of SVS2 on sperm capacitation.

Effects of SVS4 on Sperm Capacitation

I examined whether SVS4 can regulate sperm capacitation. Expression and purification of recombinant SVS4 protein were analyzed using CBBR staining and western blotting with anti-SVS4 antibody (Fig. 26B). The induced-AR rate of spermatozoa incubated with BSA and 20−30 μM SVS4 decreased significantly compared to the spermatozoa incubated with BSA alone (Fig. 29A). However, capacitated sperm treated with SVS4 did not show a reduced induced-AR rate (Fig. 29B). These results indicate that SVS4 can inhibit sperm capacitation but does not have decapacitation effect.

Interactions of GM1 with SVS3 or SVS4

SVS2 binds to ganglioside GM1 on the sperm plasma membrane (K_d = 4.8 ± 0.5 μM; ΔM_{max} = 972.2 ± 35.1 ng; Kawano *et al*., 2008). To examine whether SVS3 and

SVS4 have binding affinity to spermatozoa via GM1, interactions between GM1 and SVS3/SVS4 were evaluated by the QCM assay, yielding the following results: K_d (GM1 to SVS3) = 94.1 ± 25.7 μM with ΔM_{max} = 808.3 ± 74.9 ng (Fig. 30), K_d (GM1 to SVS4) = 105.1 ± 23.2 μM with ΔM_{max} was 1599.7 ± 206.8 ng (Fig. 31). In contrast, the K_d value of GM1 to Trx was 285.8 ± 55.5 μM with ΔM_{max} was 527.3 ± 86.5 ng. Thus, some non-specific binding of GM1 to a negative control Trx was observed, but with much lower affinity than that observed to SVS3 and SVS4. These results indicate that GM1 has lower affinities to SVS3 or SVS4 than to SVS2.

Interactions between SVS2 and SVS3

Since SVS3 facilitated the inhibitory effect of SVS2 on sperm capacitation, SVS3 may directly interact with SVS2. Thus, the interactions between SVS2 and SVS3 were analyzed by the QCM assay, yielding a K_d value (SVS2 to SVS3) of 1.4 ± 0.2 μM with ΔM_{max} was 2395.9 ± 646.5 ng (Fig. 32), the K_d value of SVS2 to Trx was 9.9 ± 3.3 μM with ΔM_{max} was 536.4 ± 145.2 ng. As SVS3's affinity to SVS2 is higher than that to GM1, SVS3 seems to interact more tightly with SVS2 than with the sperm plasma membrane.

Chapter III

DISCUSSION

SVS2 interacts with GM1 on spermatozoa to inhibit sperm capacitation (Kawano and Yoshida, 2007; Kawano *et al*., 2008). In this chapter, I showed that SVS3 exhibits affinity to spermatozoa and SVS2 and facilitates the inhibitory effect of SVS2 on sperm capacitation *in vitro*. Intriguingly, SVS4 bound to spermatozoa and inhibited capacitation *in vitro*, while it could not decapacitate them. Accordingly, SVSs can regulate sperm capacitation in different manners.

Previous reports showed that SVS3 has a transglutaminase cross-linking site (Lin *et al*., 2002) and forms oligomers, including SVS1 and SVS2 (Wagner and Kistler, 1987). While SVS3 has some similarities with SVS2 (Table. 3), the effect of SVS3 on sperm capacitation was not known. In this study, I showed that SVS3 directly interacts with SVS2, apparently enhancing the inhibitory effect of SVS2 on sperm capacitation. SVS4 was shown to suppress immune responses to cellular antigens in rat (Romano-Carratelli *et al*., 1995). My study revealed that SVS4 has a certain inhibitory effect on sperm capacitation, indicating that SVS4 is partly involved in protecting spermatozoa from spontaneous capacitation in the uterus.

A decapacitation factor has two effects on sperm capacitation, namely, the inhibition of capacitation and the elimination of fertility (decapacitation). The results

indicate that these two inhibitory effects are caused by separate processes (Fig. 33). Capacitation is inhibited by binding of the factor to the spermatozoa to inhibit sterol efflux, decapacitation is attained by the factor interacting with both spermatozoa and sterols, resulting in intercalation of sterols into the sperm plasma membrane.

While SVS2 has seven cholesterol recognition/interaction domains (Epand, 2006; Scolari *et al*., 2010), SVS3 merely has two cholesterol-recognizing domains (Table. 4). This suggests that the affinity of SVS3 to cholesterol is lower than that of SVS2. As my results show that SVS3 interacts with SVS2, it seems plausible that SVS3 exhibits its effects not by interacting with sterols directly but by interacting with SVS2. SVS2 may inhibit sperm capacitation by binding to the sperm plasma membrane, reducing lipid rafts fluidity. This effect might be enhanced by SVS3, intensifying the interaction between SVS2 and spermatozoa.

SVS4 has no cholesterol recognition/interaction domain (Table. 4), suggesting that SVS4 cannot interact with sterols. Accordingly, SVS4 might not have decapacitated spermatozoa due to its incapability of retrieving sterols to the sperm plasma membrane. The concentration of SVS4 in seminal vesicle fluid is the same as that of SVS2, and SVS4 is detected in the uterus after copulation. These results indicate that SVS4 is a complementary factor to SVS2, which may participate in the regulation of *in vivo*

capacitation.

Since SVS3 and SVS4 had considerably lower affinity to GM1 than SVS2, it is possible that they have weak interactions with the sperm plasma membrane mediated by GM1. While SVS2 has a positive charge, which seems to be concerned in interacting with GM1, the isoelectric point of SVS4 is lower than that of SVS2. Thus, SVSs may be able to bind to molecules other than GM1 on the spermatozoa to regulate sperm capacitation. Comprehensive interaction between SVSs and the spermatozoa should be evaluated in the future.

The results of the present study suggest that sperm capacitation is inhibited not only by SVS2, but that other SVSs, at least SVS3 and SVS4 play a role in the process. Since *SVS2*$^{-/-}$ mice show male infertility (Kawano *et al.*, 2014), the main factor regulating *in vivo* capacitation may be SVS2, but it seems possible that other SVSs support this regulation. In human, semenogelin (SEMG) suppresses sperm motility and capacitation (de Lamirande *et al.*, 2001). Although mouse SVS2 shows considerable homology with SEMG, SVS2 does not inhibit sperm motility (data not shown). Interestingly, the chromosomal region occupied by *SEMG* in human is substituted for by *SVS2*−*SVS6* in mouse (Fig. 23; Clauss *et al.*, 2005), suggesting that SVSs substitute for the function of SEMG. Therefore, the roles of other SVSs in the regulation of sperm

function including capacitation should be elucidated.

While I showed roles of SVS3 and SVS4 on sperm capacitation in this study, the male reproductive tract and seminal plasma may contain other decapacitation factors. Although it is unknown whether all factors function *in vivo*, spermatozoa have to shut their effects down to acquire fertilizing ability eventually. I am particularly interested in the mechanism guaranteeing reliable capacitation, which will elucidate a mystery of mammalian fertilization and contribute to assisted reproductive technologies.

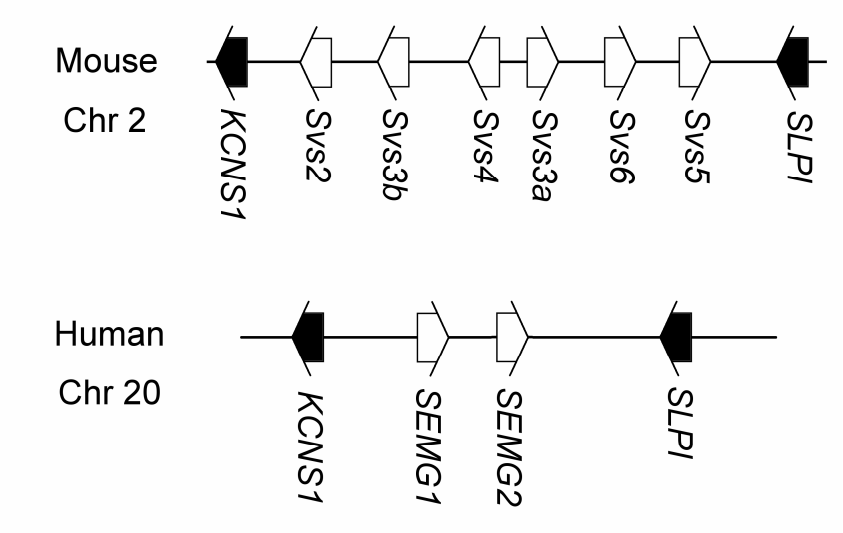

Figure 23. Schematic illustration of *SVS* loci in mouse and *SEMG* loci in human.

Arrows show approximate locations and transcriptional orientations of genes. *SVS2–SVS6* are located on chromosome 2, belonging to the same gene family. *SEMG* is located on chromosome 20, containing *SEMG1* and *SEMG2*. The figure was based on Clauss *et al*., 2005.

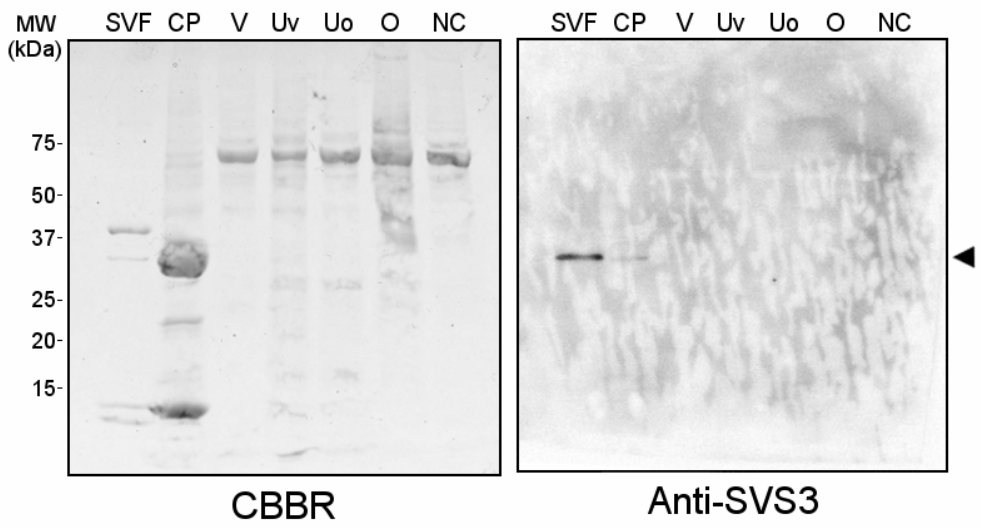

Figure 24. Detection of SVS3 in the female reproductive tract after copulation.

After copulation, intratubular fluids were collected from the vagina (V), the uterine region near the vagina (Uv), the uterine region near the oviduct (Uo), and the oviduct (O). Samples were also collected from the seminal vesicle (SVF), the copulatory plug (CP), and the uterine fluid of virgin females in estrus to be used as controls (NC). The distribution of SVS3 was analyzed by immunohistochemistry with anti-SVS3 antibody. SVS3 was detected in SVF and CP (arrow head), while no signals were obtained in the entire female reproductive tract.

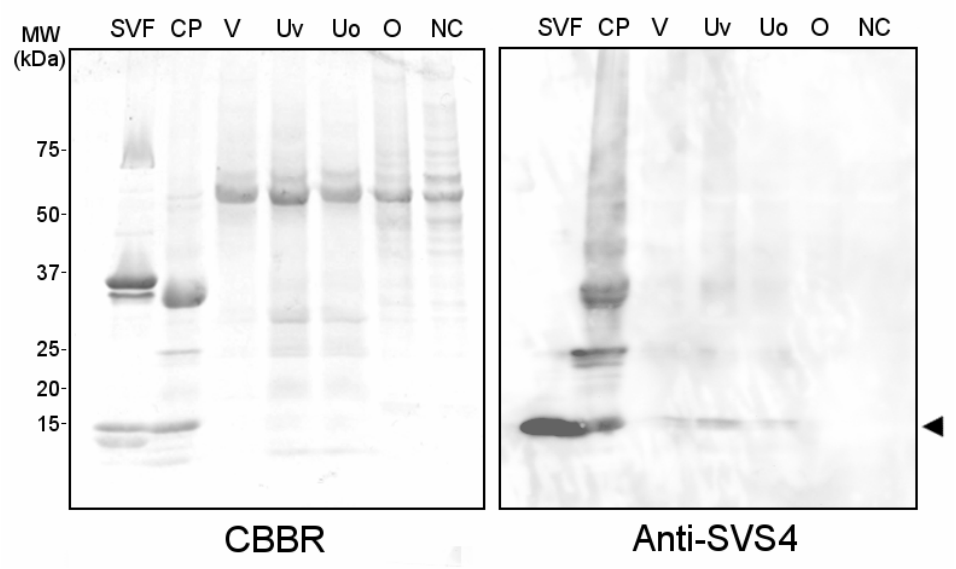

Figure 25. Detection of SVS4 in the female reproductive tract after copulation.

After copulation, intratubular fluids were collected from the vagina (V), the uterine region near the vagina (Uv), the uterine region near the oviduct (Uo), and the oviduct (O). Samples were also collected from the seminal vesicle (SVF), the copulatory plug (CP), and the uterine fluid of virgin females in estrus to be used controls (NC). The distribution of SVS4 was analyzed by immunohistochemistry with anti-SVS4 antibody. SVS4 was detected in SVF, CP, V, and the uterus (arrow head) but not in the oviduct.

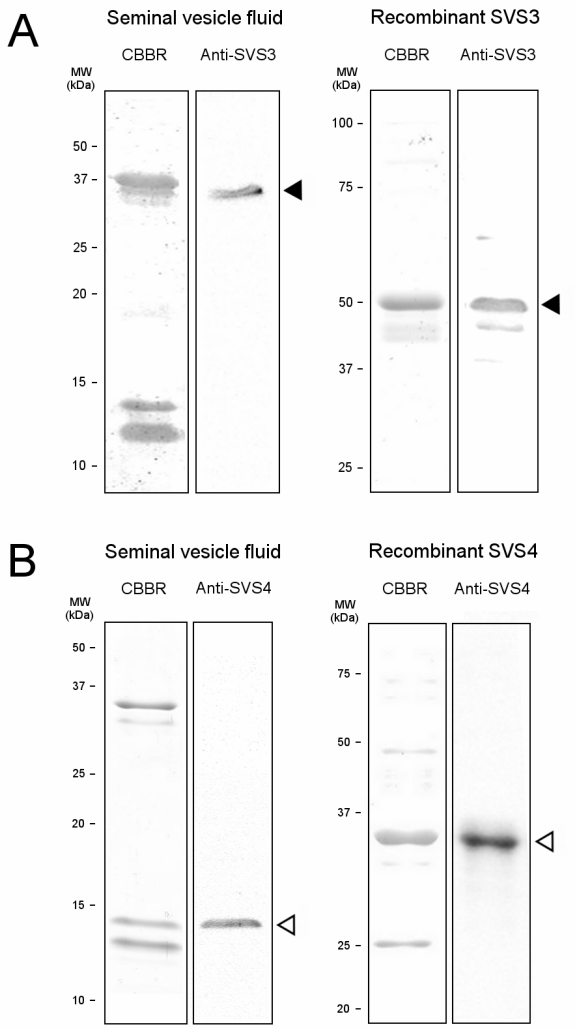

Figure 26. Western blotting of recombinant SVS3 and SVS4 proteins.

Seminal vesicle fluid (SVF), purified recombinant SVS3 (A), and SVS4 (B) were analyzed by SDS-PAGE and western blotting with anti-SVS3 (A) or anti-SVS4 (B) antibody. Proteins were also stained with CBBR. (A) SVS3 in SVF and recombinant SVS3 protein were detected at 35 and 50 kDa, respectively (closed arrowheads). (B) SVS4 in SVF and recombinant SVS4 protein were detected at 14 and 35 kDa, respectively (open arrowheads).

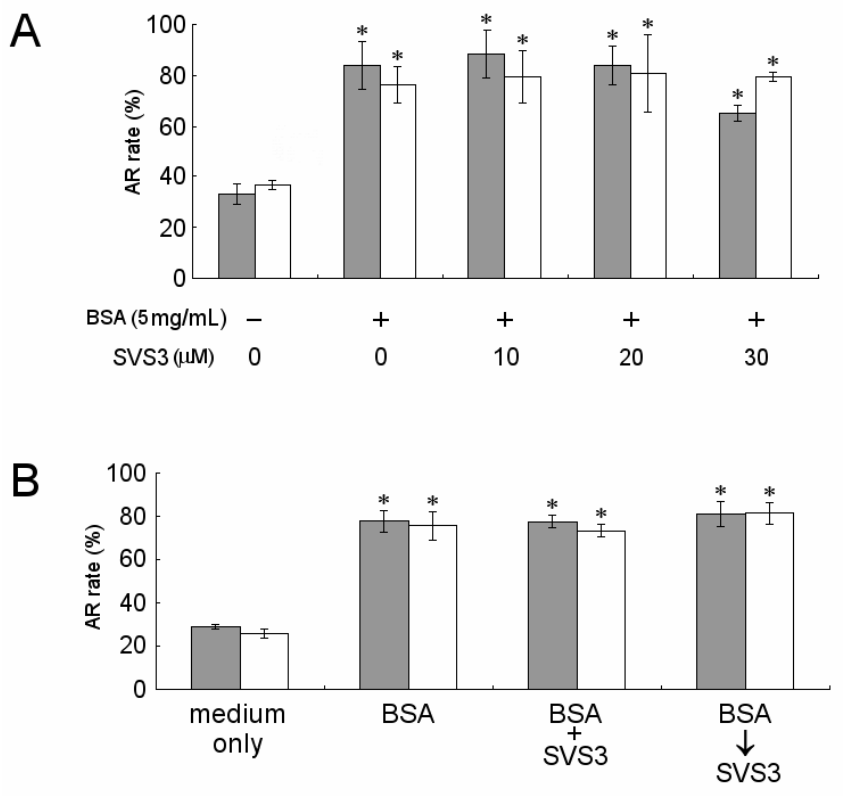

Figure 27. Effects of SVS3 on sperm capacitation induced by BSA.

Acrosome reaction (AR) rate induced by progesterone (gray bars) or ionomycin (white bars). (A) Sperm capacitation induced by BSA in medium containing 10−30 μM SVS3. (B) Effect of 20 μM SVS3 on capacitated spermatozoa. Data are shown as mean ± SD. Asterisks indicate significant differences compared with the spermatozoa incubated without BSA and SVS3 ($P < 0.01$, Student's *t*-test) (n = 3).

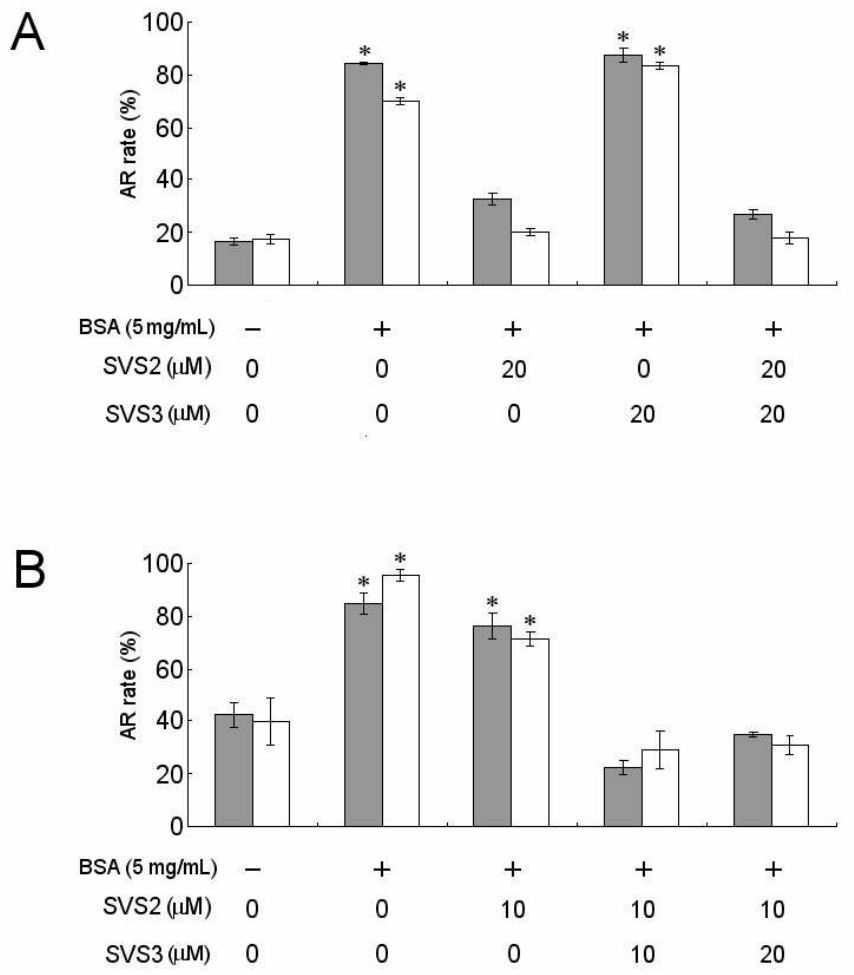

Figure 28. Cooperative effects of SVS2 and SVS3 on sperm capacitation.

Acrosome reaction (AR) rate induced by progesterone (gray bars) or ionomycin (white bars). (A) Sperm capacitation by BSA was induced in medium containing 20 μM SVS2 and 20 μM SVS3. (B) Sperm capacitation induced by BSA in medium containing 10 μM SVS2 and 10−20 μM SVS3. Data are shown as mean ± SD. Asterisks indicate significant differences compared with the spermatozoa incubated without BSA, SVS2, and SVS3 ($P < 0.01$, Student's *t*-test) (n = 3).

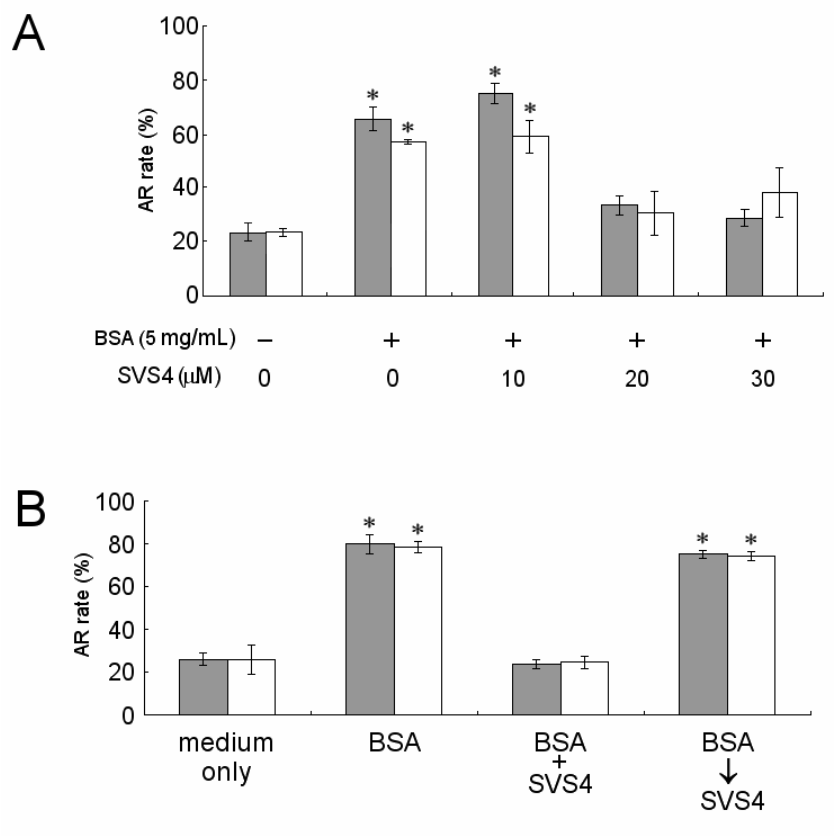

Figure 29. Effects of SVS4 on sperm capacitation induced by BSA.

Acrosome reaction (AR) rate induced by progesterone (gray bars) or ionomycin (white bars) was evaluated. (A) Sperm capacitation induced by BSA in medium containing 10−30 μM SVS4. (B) Effect of 20 μM SVS4 on capacitated spermatozoa. Data are shown as mean ± SD. Asterisks indicate significant differences compared with the sperm incubated without BSA and SVS4 ($P < 0.01$, Student's *t*-test) (n = 3).

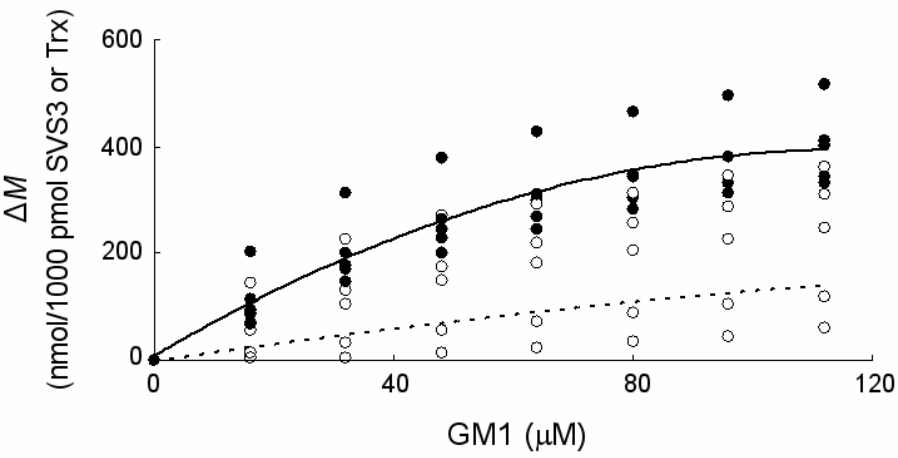

Figure 30. The interaction between GM1 and SVS3.

GM1 was continuously added to quartz-immobilized SVS3 (closed circles) or thioredoxin (Trx; open circles), and their interaction was evaluated by the Michaelis-Menten equation. Fitted curves (solid, SVS3; dashed, Trx) were obtained from five experiments. The affinity of GM1 to SVS3 is significantly different from that to Trx.

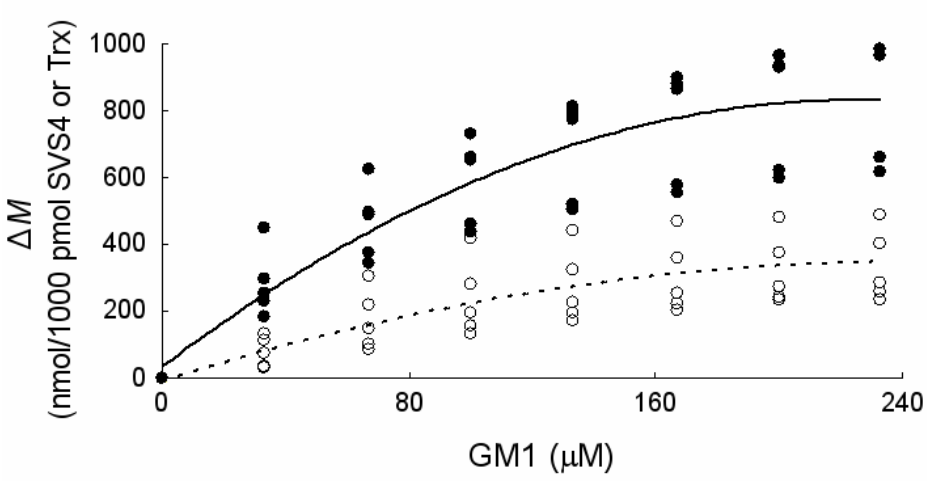

Figure 31. The interaction between GM1 and SVS4.

GM1 was continuously added to the quartz-immobilized SVS4 (closed circles) or thioredoxin (Trx; open circles), and their interaction was evaluated by the Michaelis-Menten equation. Fitted curves (solid, SVS4; dashed, Trx) were obtained from five experiments. The affinity of GM1 to SVS4 is significantly different from that to Trx.

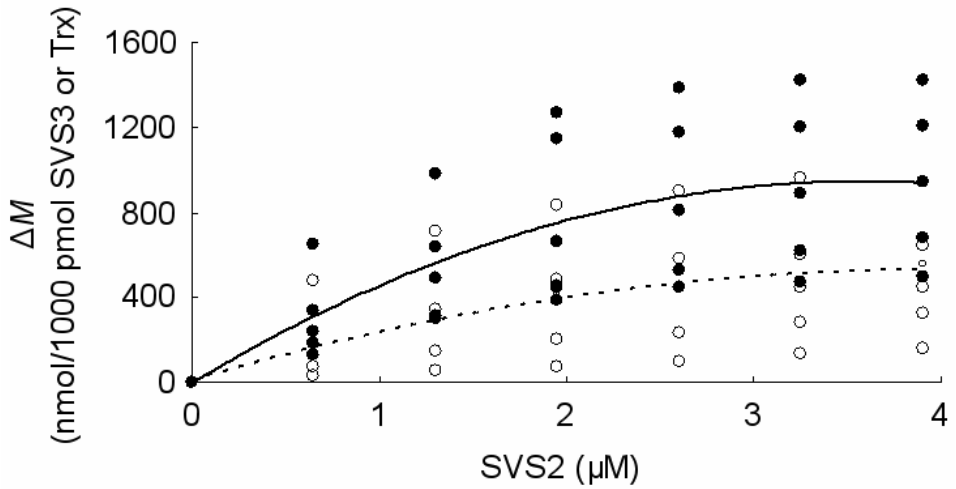

Figure 32. The interaction between SVS2 and SVS3.

SVS2 was continuously added to the quartz-immobilized SVS3 (closed circles) or thioredoxin (Trx; opened circles), and their interaction was evaluated by the Michaelis-Menten equation. Fitted curves (solid, SVS3; dashed, Trx) were obtained from five experiments. The affinity of SVS2 to SVS3 is significantly different from that to Trx.

Table 3. Comparison of SVSs

Protein	SVS1	SVS2	SVS3a	SVS3b
Chromosomal location	Chr6	Chr2	Chr2	Chr2
Amino acids (bps)	820	375	265	265
Molecular weight (kDa)	93.5	40.9	29.9	30.0
Lysine content	10.4%	9.1%	7.1%	9.1%
pI	8.98	9.89	9.39	9.37
Amino acids similarity against SVS2	11%	—	31%	31%
Forming copulatory plug	Yes	Yes	Yes	Yes
REST gene family	No	Yes	Yes	Yes
No. of cholesterol binding domain	7	7	1	2
Putative function	Cu amine oxidase	Decapacitation factor		

Protein	SVS4	SVS5	SVS6	SVS7
Chromosomal location	Chr2	Chr2	Chr2	Chr9
Amino acids (bps)	113	122	99	99
Molecular weight (kDa)	12.5	13.0	11.5	11.1
Lysine content	7.1%	5.7%	10.1%	10.1%
pI	8.03	9.52	5.54	8.93
Amino acids similarity against SVS2	21%	18%	16%	10%
Forming copulatory plug	No	No	No	No
REST gene family	Yes	Yes	Yes	No
No. of cholesterol binding domain	0	0	1	2
Putative function	Immune regulation	Serine proteinase inhibitors		Enhancing sperm motility

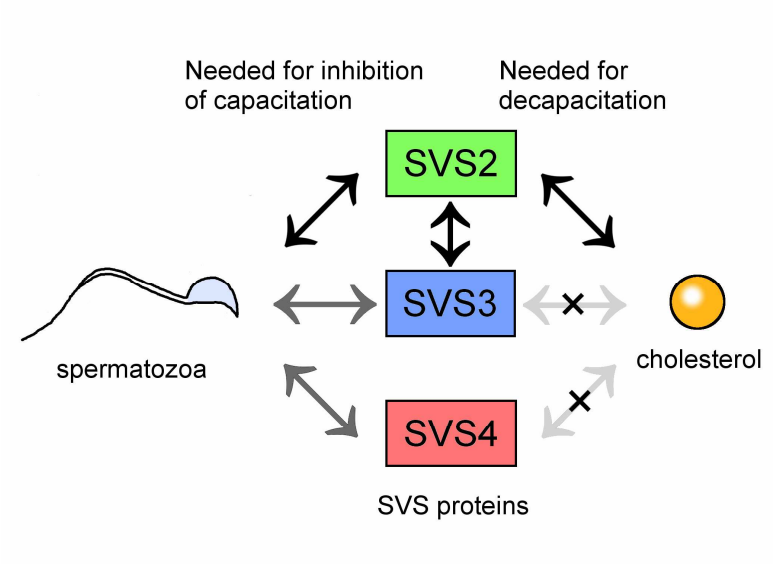

Figure 33. Interaction between spermatozoa, SVSs, and cholesterol in sperm capacitation.

SVSs show two-way interactions in sperm capacitation. The first way is an interaction with the sperm plasma membrane via GM1, which may be indispensable for the inhibition of sperm capacitation. The second way is an interaction with cholesterol via cholesterol-interacting domains, which may be required for decapacitation. SVS2 shows both interactions. SVS3 shows a stronger interaction with SVS2 than with spermatozoa and cholesterol, exerting a facilitating effect on SVS2. SVS4 does not interact with cholesterol but interacts with spermatozoa, providing an inhibition of capacitation but no decapacitation.

Table 4. Putative cholesterol recognition/interaction domains in SVSs.

Name	Position	Sequence
SVS1	124	V E Q P M Y M R
	250	V W Y N G K
	415	V P H Y R L K
	599	L L Y H S R
	655	L D K T Q Y S W E R
	725	V T K Y Q E S E R
	797	L Q P F D F Y N S F R
SVS2	30	V G Q Y G A T K
	138	V S Q I K S Y G Q L K
	196	V S Q I K S Y G Q L K
	212	V K S Y G Q T K
	240	L K S Y G Q Q K
	264	L K S Y G Q Q K
	340	L E Q Y R K
SVS3a	123	V K S Y A A Q L K
SVS3b	123	V K S Y A A Q J K
	156	L K S Y K A R
SVS4	—	
SVS5	—	
SVS6	49	V H E E V Y E E K
SVS7	59	V G T K H V Y S K
	79	L I Y I M C C E K

CONCLUSIONS and PERSPECTIVES

In the present thesis, I studied the role of SVSs proteins in sperm capacitation in mice. I have shown here that SVS2 maintains the sterol levels in the sperm plasma membrane to keep the spermatozoa in an incapacitated state. Since spermatozoa without SVS2 undergo ectopic sterol efflux *in vivo*, SVS2 must be a key factor that maintains sperm sterols and their fertilizing ability. I have also shown that SVS2 is prevented from entering the oviduct where sperm capacitation occurs. Thus, my study indicates that SVS2 is not only an inhibitor but also a regulator of sperm capacitation. Although SVS2 seems to be the main player in sperm capacitation, SVS3 and SVS4 appear to be also partly involved in the process.

Interestingly, removal of the seminal vesicles causes complete infertility, while *SVS2*$^{-/-}$ mice show a decrease in litter size (Kawano *et al*., 2014). Furthermore, spermatozoa treated with SVSs have thicker coatings than spermatozoa treated with SVS2, which may contribute to protection against uterus-derived cytotoxic factors (Kawano *et al*., 2014). Previous reports have shown that *SVS2−SVS6* are derived from one ancestor gene (Clauss *et al.*, 2005) and evolved rapidly to adapt to various reproductive strategies (Ramm *et al.*, 2009). Thus, while I showed roles of SVS3 and SVS4 on sperm capacitation in this study, SVSs other than SVS2 may have some roles

in sperm fertility and protection. Comprehensive functional analysis of SVSs will make us understand the significance of SVSs.

Several proteins suppressing sperm capacitation have been identified in the epididymis and the seminal vesicles (Yoshida *et al*., 2008). Some of those proteins also prevent cholesterol efflux from the spermatozoa. For example, glycodelin-S, a glycoprotein secreted from the seminal vesicles, inhibits the capacitation of the human spermatozoa by reducing the efflux of cholesterol and down-regulating the adenylyl cyclase/protein kinase A/tyrosine kinase signaling pathway (Chiu *et al*., 2005). Moreover, SERPINE2, a serine protease inhibitor, is expressed in the seminal vesicles (Vassalli *et al*., 1993) and it inhibits cholesterol efflux in the murine spermatozoa (Lu *et al*., 2011). Interestingly, *SERPINE2*$^{-/-}$ mice show loss of SVS2 and yield male infertility, suggesting that SVS2 is degraded by a protease (Murer *et al*., 2001). Since *SVS2*$^{-/-}$ mice showed the same phenotype as the *SERPINE2*$^{-/-}$ mice, SVS2 can be said to play an important role in sperm fertility. Although it is not known whether all potential decapacitation factors function *in vivo*, the induction of sperm capacitation may require the decontrol of multiple decapacitation factors. These multiple systems that guarantee reliable sperm capacitation are my areas of interest.

Since sperm capacitation is a complex phenomenon in which many signaling

pathways are intricately intertwined, the molecular mechanisms still remain elusive. My study showed that *in vivo* sperm capacitation is regulated by the dynamic changes of sterol levels in the sperm plasma membrane, which is mediated by SVS2 and capacitation-inducing factor(s). Hitherto, factors inducing sperm capacitation have been intensely investigated. However, considering that capacitation-inducing factor(s) seem to exist in the entire female reproductive tract (Chapter II), *in vivo* capacitation seems to be triggered not by the induction with inducing factor(s), but by suppressing the function of decapacitation factors. Therefore, SVS2 is the key factor regulating the sperm's fertilizing ability as it is able to control the inhibition and the induction of sperm capacitation *in vivo*.

Considering the current scientific knowledge, I propose the following model of *in vivo* sperm capacitation (Fig. 34): after mating, mouse spermatozoa that have high sterol levels enter the uterus. The ejaculated semen contains SVS2, and SVS2 attaches to the postacrosomal region of the spermatozoa via GM1 (Kawano and Yoshida, 2007; Kawano *et al*., 2008). While there are some capacitation-inducing factors such as albumin in the uterus, the sterol levels in the sperm plasma membrane are maintained by SVS2. When sterol is released from the sperm plasma membrane either spontaneously or through the function of capacitation-inducing factors, SVS2 retrieves free cholesterol,

resulting in maintaining spermatozoa in an incapacitated state. When spermatozoa enter the oviduct, both GM1 and SVS2 are detached from the spermatozoa, leading to the capacitation of oviductal spermatozoa by the capacitation-inducing factor. In this process, there are two indispensable steps for the accomplishment of murine fertilization: (1) the inhibition of capacitation in inappropriate sites, which is mediated by decapacitation factors, (2) the induction of capacitation by removing decapacitation factors and GM1, a possible wedge of lipid rafts. There must be mechanisms assuring the precise occurrence of both events and their elucidation will give us more insight into the mammalian fertilization process.

In the present thesis, I focused on sterol liberators such as albumin and methyl-β-cyclodextrin which seem to be needed for sperm capacitation. However, factors other than sterol liberators are also required for capacitation: for example, bicarbonate is essential for sperm capacitation (Boatman and Robins, 1991). Bicarbonate induces tyrosine phosphorylation of sperm proteins to trigger hyperactivation accompanied by sperm capacitation (Visconti *et al*., 1999b), and the oviductal fluid has a high concentration of bicarbonate (Okamura *et al*., 1985; Foley and Williams, 1991). Thus, the effect of bicarbonate on the regulation of sperm capacitation should be elucidated in the future.

Since I studied the function of secretory proteins from the seminal vesicles in mice, I am interested in whether these findings can be applied to other species. Some reports have shown a relationship between the seminal vesicles and fertility in humans. An insufficient volume of ejaculate, caused by an abnormality of the seminal vesicles, may be related to infertility (Lotti *et al*., 2012). While ejaculated spermatozoa of normal healthy men have little semenogelin (SEMG), ejaculated spermatozoa of asthenozoospermic patients have more SEMG, suggesting that it is related to infertility (Terai *et al*., 2010). Thus, the seminal vesicles in humans may have a similar relation to fertility as in mice. Interestingly, a number of carnivores such as cats and dogs have no seminal vesicles. In dogs, sperm capacitation is induced by glycosaminoglycans that exist in the uterine and oviductal fluid (Kawakami *et al*., 2000), suggesting that sperm capacitation in dogs may be regulated by factors other than SVSs proteins. Therefore, elaborate studies should be done to understand the regulatory mechanisms of capacitation in different species.

My study showed that proteins secreted from the seminal vesicles regulate sperm capacitation actively and coordinately, which can account for the significance of seminal vesicles and their secretions in mammals. I hope this study contributes to the understanding of the beginning of our life−mammalian fertilization.

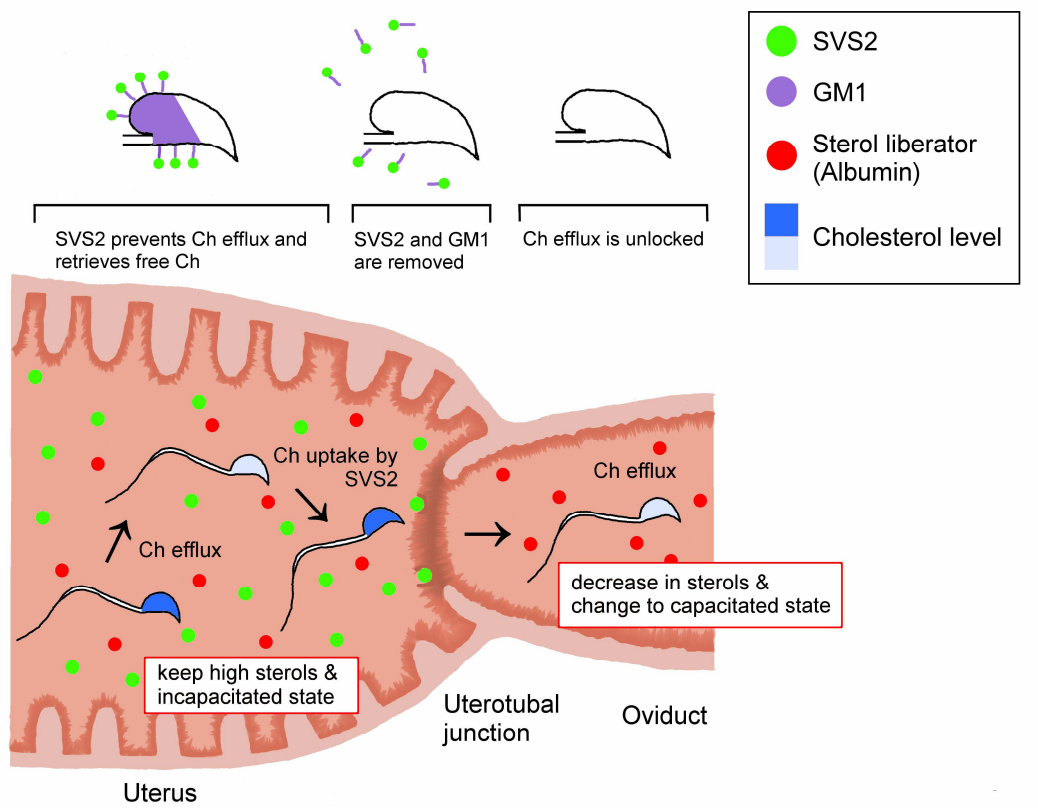

Figure 34. Working hypothesis for *in vivo* sperm capacitation regulated by SVS2.

In the uterus, SVS2 attaches to spermatozoa via GM1 and suppresses efflux of sterols such as cholesterol (Ch) to inhibit sperm capacitation. When sterols are occasionally released from spermatozoa, SVS2 retrieves them to the sperm plasma membrane. In the oviduct, SVS2 and GM1 are detached from spermatozoa. This induces sterol efflux, resulting in sperm capacitation.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Manabu Yoshida, Misaki Marine Biological Station, Graduate School of Science, the University of Tokyo for his dedicated direction and encouragement throughout the course of my study.

I would also like to express my cordial gratitude to Dr. Kaoru Yoshida, Biomedical Engineering Center, Toin University of Yokohama for her technical supports and helpful discussions.

I am thankful to Dr. Natsuko Kawano for laying the groundwork for my study and a lot of significant advice.

I also appreciate for Dr. Yoshitaka Oka, Department of Biological Sciences, Graduate School of Science, the University of Tokyo, Dr. Min Kyun Park, Department of Biological Sciences, Graduate School of Science, the University of Tokyo, Dr. Shoji Oda, Graduate School of Frontier Sciences, the University of Tokyo, and Dr. Kenji Miyado, National Center for Child Health and Development for their critical reading of my thesis and valuable discussion.

I thank students and staff of the Misaki Marine Biological Station, Graduate School of Science, the University of Tokyo for supporting harmonious research and giving a fulfilling life.

Acknowledgements

Finally, I am sincerely grateful to my parents, Takuji Araki and Yumiko Araki, for their generosity, encouragement, and support throughout the course of my study.

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