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絲胺酸蛋白酶抑制蛋白 E2 在精子獲能作用與卵子成熟過程中所扮演的角色

The roles of the SERPINE2 protein in sperm capacitation and oocyte maturation

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本論文係呂仲浩君（d95642001）在國立臺灣大學生物科技所所完成之博士學位論文，於民國 102 年 11 月 29 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

絲胺酸蛋白酶抑制蛋白 E2 (SERPINE2)為一種可以抑制絲胺酸蛋白酶活性的蛋白質，其藉由抑制絲胺酸蛋白酶活性來參與許多生理功能。目前已知 SERPINE2 可以調控 plasminogen activator 及 thrombin 的酵素活性。在第一部份的研究中，首先我們從小鼠儲精囊分泌液中純化出具有可以抑制 plasminogen activator 活性的 SERPINE2 蛋白質。接著發現 SERPINE2 會結合在射出的小鼠精子以及已經游入輸卵管的精子，但是無法在已發生獲能反應的精子身上發現 SERPINE2 蛋白質的存在。此外，在體外試驗中發現 SERPINE2 也會抑制牛血清白蛋白(BSA)所引起的精子獲能反應，使得精子無法與卵子結合進而影響與卵子的受精作用。精子身上的膽固醇移除(cholesterol efflux)為發生獲能反應的重要步驟，而 SERPINE2 的存在會去抑制精子身上膽固醇的移除，因此 SERPINE2 可能扮演著精子去獲能因子(decapacitation factor)的角色。

Plasminogen activator 已被證實存在於排卵過程中的濾泡壁上，但其在卵子成熟過程中的相關功能仍然未知。在第二部份的研究中我們證實了 PLAU (urokinase plasminogen activator)及其抑制者 SERPINE2 參與小鼠卵丘細胞擴張(cumulus expansion)及卵子成熟。當高量的 SERPINE2 結合上卵丘細胞之細胞間質(ECM)時會降低 PLAU 的活性，導致卵丘細胞無法擴張，進而影響卵子成熟。因此在病人進行人工生殖技術過程中，額外加入 PLAU 蛋白質於體外培養系統中，或許可以幫助不成熟卵子進行最後階段之熟成而提高受精率。

綜合這些實驗結果，我們認為 SERPINE2 蛋白質可以在精子到達輸卵管之前保護精子並避免其過早發生獲能反應。此外，我們推測部分不孕症病人之卵丘細胞無法擴張及卵子無法成熟可能與 SERPINE2 蛋白質不正常的高量表現有關。

關鍵字:

絲胺酸蛋白酶抑制蛋白 E2、絲胺酸蛋白酶、精子、獲能作用、受精作用、卵丘細胞擴張、卵子成熟。

Abstract

SERPINE2, one of the potent serine protease inhibitors that modulate the activity of plasminogen activator and thrombin, is implicated in many biological processes. In the first study, we purified SERPINE2 from mouse seminal vesicle secretion based on its potent inhibitory activity against the urokinase-type plasminogen activator. A prominent amount of SERPINE2 was detected on ejaculated and oviductal spermatozoa, predominantly on uncapacitated sperm, suggesting the need to remove SERPINE2 before initiation of the capacitation process. Moreover, SERPINE2 could inhibit in vitro bovine serum albumin-induced sperm capacitation and prevent sperm binding to the egg, thus blocking fertilization. It acts through preventing cholesterol efflux, one of the initiation events of capacitation, from the sperm. These findings suggest that SERPINE2 protein may play a role as a sperm decapacitation factor.

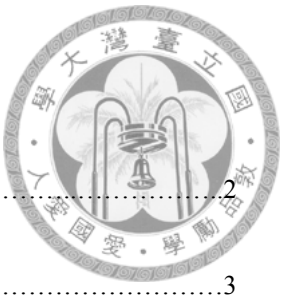
Plasminogen activators play a crucial role in follicle wall rupture during ovulation; however, their function in oocyte maturation during pre-ovulation remained unclear. Our second study provides the first evidence that PLAU (urokinase plasminogen activator) and its inhibitor SERPINE2 are involved in murine cumulus expansion and oocyte maturation. High SERPINE2 levels bound to the extracellular matrix of cumulus cells could reduce PLAU activity, and ultimately suppressing cumulus expansion and oocyte maturation. PLAU supplementation to culture medium

may assist the final maturation of the immature human oocytes collected during assisted reproductive technology procedures, thus providing a potential therapeutic strategy.

Based on these results, SERPINE2 protein possibly influencese on sperm to prevent precocious capacitation and the acrosome reaction before sperm reach the oviduct. In addition, we suppose that aberrantly high SERPINE2 protein levels in cumulus cells may be one of the etiologies for patients with defects in cumulus expansion and subsequent oocyte maturation.

Keywords:

SERPINE2, serine protease, sperm, capacitation, fertilization, plasminogen activator, cumulus expansion, oocyte maturation



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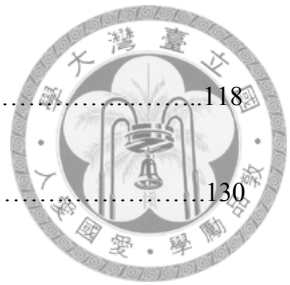

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Full name	Abbreviations
Protein kinase A	PKA
Seminal vesicle secretion	SVS
Urokinase-type plasminogen activator	PLAU
Prostasin	PRSS8
Tissue-type plasminogen activator	PLAT
Plasminogen activators	PAs
Cumulus-oocyte complexes	COCs
Extracellular matrix	ECM
Inter-alpha-trypsin inhibitor	ITIH
Pentraxin-3	PTX3
Tumor necrosis factor alpha-induced protein 6	TNFAIP6
Liquid chromatography/tandem mass spectrometry	LC-MS/MS
Bovine serum albumin	BSA
Dimethyl sulfoxide	DMSO
TRITC-conjugated peanut agglutinin lectin	PNA
Hyaluronan synthase 2	HAS2
Versican	Vcan
In vitro maturation	IVM
Germinal vesicle	GV
Metaphase I	MI
Metaphase II	MII
Quantitative real-time RT-PCR	qRT-PCR
Multiple cloning site	MCS

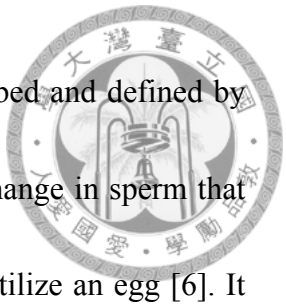
Chapter 1

Introduction

1.1 Prolegomenon

Germ cells are the vehicles for transmitting genetic materials to the next generation. Fully functional oocytes and sperms are therefore critical for reproduction. Infertility is a worldwide reproductive health problems, in which some infertility cases are caused by suboptimal quality of sperm and oocytes. Oocyte maturity and quality are key limiting factors in female fertility. Several studies indicated oocyte maturity and quality will affect subsequent embryo development or pregnancy [1-3]. On the other hand, ejaculated sperm are necessary to swim and reside in the female reproductive tract for a period of time to acquire the ability to fertilize the oocyte. Many events are involved in this process, such as capacitation and acrosome reaction. Only one capacitated sperm can penetrate the zona pellucida, and then fertilize the oocyte. In this study, we purified and characterized the SERPINE2 protein from seminal plasma where capacitation moderators reside. I will describe how the SERPINE2 protein affects sperm function and oocyte maturation.

1.2 Sperm Capacitation / Decapacitation and Fertility



Capacitation is a complex process first independently described and defined by Chang MC et al [4] and Austin CR et al [5]. It is a physiological change in sperm that occurs in the oviduct of some mammals to acquire the ability to fertilize an egg [6]. It can be mimicked *in vitro* in specifically defined medium [4,6]. Our current knowledge of capacitation largely originates from *in vitro* studies [7-11]. Capacitation is initiated by removal of cholesterol from the sperm plasma membrane [7,8,12-14]. Cholesterol efflux leads to changes in the membrane structure and fluidity, which then increases the permeability of sperm to calcium (Ca^{2+}) and bicarbonate (HCO^{3-}) ions, thus raising levels of sperm intracellular calcium ions ([Ca Ca^{2+}]$_i$) and the pH. Elevated levels of sperm intracellular Ca^{2+} and HCO^{3-} can activate adenyl cyclase and lead to the increases in intracellular levels of cAMP, activation of cAMP-dependent protein kinase (PKA), and finally induction of tyrosine phosphorylation of a subset of sperm proteins [9]. Sperm capacitation usually occurs in the mammalian oviduct at the right time, but the occasional premature capacitation that may affect sperm fertility. Therefore, decapacitation factor can modulate and protect fertilizing ability of sperm, which make sure sperm do the right thing in the right place at the right time.

The existence of decapacitation factors, which are removed from the sperm head surface during the capacitation process and are able to reverse sperm capacitation, in seminal plasma have been known for more than 50 years [15]. Since the discovery of

decapacitation factors existence, many proteins have been suggested to be decapacitation factors. The sperm's surface is immersed in a protein-rich solution of seminal plasma which is mixed with secretions from the accessory sexual glands. These seminal proteins interact with sperm and modulate changes in the sperm's physiology, and thus prevent them from premature capacitation [16]. The decapacitation factors, which cause capacitated sperm to lose the ability to fertilize, are present in seminal plasma [15]. They are removed from the surface of the sperm's head before or during the capacitation process. However, their identity and functions have not been fully characterized.

1.3 Identification and Characterization of Seminal Plasma Secretions Importance for Decapacitation Factor

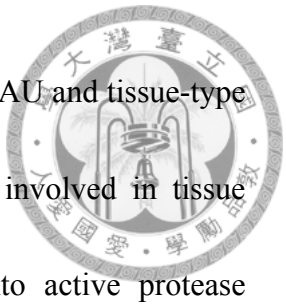
Most of the decapacitation factors identified so far are purified from seminal plasma secretion. The seminal vesicle is a male accessory sexual gland found in many mammalian species, which secretes a fluid called seminal vesicle secretion (SVS). SVS contributes a major portion to the liquid part of seminal plasma, which is a complex biological fluid formed from a mixture of secretions from various male reproductive tissues. Studies found that the removal of the seminal vesicle from mice and rats greatly reduced their fertility [17,18], thereby demonstrating the importance of SVS to male

fertility. Proteins from seminal plasma interact with sperm and modify the sperm's surface membrane, an essential process in maintaining sperm viability, thus modulating their functions [19]. In humans, several potential decapacitation factors that have been reported which include glycodelin-S [20], semenogelin I [21], a 130-kDa glycoprotein [22], and some mannosyl glycopeptides [23]. Several potential decapacitation factors from rodents were also identified, including a 40-kDa glycoprotein [24], phosphatidylethanolamine-binding protein 1 (PEBP1) [25]; three epididymal proteins, a cysteine-rich secretory protein 1 [26]; an acrosome-stabilizing factor [27]; and an epididymis-specific secretory protein, HongrES1 (symbol not official) [28,29]; and two secreted seminal vesicle proteins, SVA [30] and SVS2 [31,32]. Attempts have also been made to reveal sperm physiology modulating activities from other murine seminal vesicle secretory proteins. For example, a carcinoembryonic antigen-related cell adhesion molecule (CEACAM10) [33] and SVS7 [34] were found responsible for enhancing sperm motility. A kazal-type serine protease inhibitor (SPINK3), named P12, is able to suppress Ca2þ uptake by sperm [35]. Recently, the secreted LY6 protein (SSLP-1) was found to be expressed predominantly in SVS [36], although its function is unclear. In order to identify and characterize more factors important for decapacitation activities, we purified and characterized of the decapicitation factors in the mouse seminal plasma. In our previous study, we found a secreted serine protease inhibitor

Kazal-type-like (SPINKL) protein. The SPINKL protein was purified from mouse seminal vesicle secretions through a series of steps, including ion-exchange chromatography on a diethylaminoethyl-Sephacel column, gel filtration on a Sephadex G-75 column, and ion-exchange HPLC on a Q strong anion exchange column. The SPINKL protein is able to bind onto sperm and enhance sperm motility. Also, it was able to suppress BSA-stimulated sperm capacitation and block sperm-oocyte interactions *in vitro*, suggesting that SPINKL may be a decapacitation factor [37]. However, other important components of the SVS remain to be identified. Further investigation of the SVS proteome may enhance our understanding of normal and abnormal male reproductive physiology. We therefore performed another round of screening based on decapacitation activity in order to identify novel decapacitation factors. We purified SERPINE2 from mouse seminal vesicle secretions, based on its potential function in actively inhibiting capacitation process. I will describe our characterization of SERPINE2's involvement in sperm decapacitation in Chapter 2.

1.4 SERPINE2 Protein Characteristics and Functions

SERPINE2, also known as glia-derived nexin or protease nexin-1, belongs to the serine protease inhibitor superfamily. It has broad antiprotease activity specific to serine proteases, including trypsin, thrombin, plasmin, urokinase-type plasminogen activator



(PLAU) [38], and prostasin (PRSS8) [39]. SERPINE2 can inhibit PLAU and tissue-type plasminogen activator (PLAT). Plasminogen activators (PAs) are involved in tissue remodeling by converting abundant extracellular plasminogen into active protease plasmin, which degrades almost all matrix proteins [40]. The PA system is associated with many physiological processes, including ovulation, embryogenesis, and embryo implantation in female reproductive tissues [40,41], and pathological processes, such as neoplasia [40]. Two PA types, PLAT and PLAU, and four types of SERPINs, including SERPINA5, SERPINB2, SERPINE1, and SERPINE2, constitute the PA system [40]. Understanding how serpins modulate PLAT/PLAU proteolytic activities is considerably important in developing therapeutic strategies for PA-involved tissue remodeling.

1.5 The Potential Role of SERPINE2 in Reproduction

SERPINE2 protein is extensively expressed in reproductive tissues, e.g., the placenta [42,43], uterus [43,44] and ovary [45,46]. However, different species have different expression patterns of *Serpine2*/SERPINE2. Lin et al. reported that expression levels of SERPINE2 in the monkey endometrium and placenta during early pregnancy were weak or below the level of detection [47]. On the contrary, SERPINE2 was highly expressed in the human placenta throughout pregnancy [48]. In rats, *Serpine2* mRNA was exclusively detected in endometrial stromal cells of the uterus, in particular on day

6.5 postcoitally, suggesting that it may be involved in the implantation process [49]. Our previous study demonstrated that SERPINE2 was a major PAs inhibitor in the mouse placenta and uterus during the estrous cycle, pregnancy, and lactation. It may participate in the PA-modulated tissue remodeling process in the mouse placenta and uterus [44]. PAs are associated with many reproductive processes, e.g., ovulation [50,51], embryonic development [52], embryo implantation [53], and pathological processes [40]. The expression and activity of PLAT and PLAU were detected in female reproductive tissues, including the endometrium during cycling [54,55], implantation [53], and placentation [56-59]. Also, PLAU was found to be expressed during mouse placental development [59].

1.6 Cumulus Expansion and Oocyte Maturation

In most mammals, cumulus cells are specialized granulosa cells surrounding, touching the oocyte and nourishing the oocyte development. Cumulus cells surround the oocyte to form a cumulus-oocyte complex (COC) and that are required for the successful maturation of oocytes and fertilization. The absence of cumulus cells impairs embryo production. Denuded oocytes in culture cannot undergo normal fertilization and development. The structural integrity of the cumulus cell extracellular matrix (ECM) is essential for oocyte maturation. Several cumulus proteins linked to ECM hyaluronan,

e.g., heavy chain of inter-alpha-trypsin inhibitor (ITIH) [60], pentraxin-3 (PTX3) [61,62], and tumor necrosis factor alpha-induced protein 6 (TNFAIP6) [1], are required for maintaining cumulus integrity, thus ensuring cumulus expansion and oocyte maturation [2,61,63].

Oocyte maturation refers to the progression of the oocyte nucleus from the germinal vesicle to the metaphase II stage. Nuclear maturation involves GVBD, condensation of chromosomes, metaphase I spindle formation, separation of the homologous chromosomes with extrusion of the first polar body and arrest at metaphase II [64]. During oocyte maturation, cumulus cells change from a compact cell into a dispersed structure of cells for the synthesis of extracellular matrix, spaces between cumulus cells in cumulus–oocyte complexes become enlarged, and cells become embedded in a sticky, mucified matrix, This phenomenon is referred to as cumulus expansion. Cumulus expansion is thought to influence a variety of fundamental developmental changes during oocyte maturation. Expansion of the cumulus-oocyte complex correlates with the outcome of oocyte maturation, fertilization, and embryo development. Therefore, detailed functional studies of cumulus expansion seem to be required to elucidate the mechanism of oocyte maturation. Cumulus expansion involves hyaluronan accumulation in the intercellular spaces of cumulus cells, and its induction by gonadotropins is crucial for oocyte maturation [3]. Oocyte-secreted molecules, e.g.,

growth differentiation factor 9 and bone morphogenetic protein 15, also affect cumulus expansion [65,66].

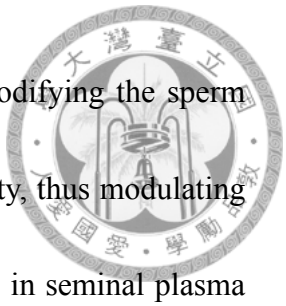
Thus, bidirectional intercellular communication between oocytes and their surrounding cumulus cells is important for the development of an egg that is competent to undergo fertilization and embryogenesis [3,67,68]. In chapter 3, I will describe our findings concerning the importance of cumulus cell expressing SERPINE2 in oocyte maturation.

Chapter 2

Study I: SERPINE2, a Serine Protease Inhibitor Extensively Expressed in Adult Male Mouse Reproductive Tissues, May Serve as a Murine Sperm Decapacitation Factor

2.1 Introduction

SERPINE2 protein is widely expressed in various tissues, whereas the highest level is found in the seminal vesicles [69]. The *Serpine2* gene knockout causes abnormal alterations in SVS components, which may result in an imbalance between PLAU, expressed in seminal vesicles [70], and SERPINE2. Thus, the vaginal plug becomes soft and shortened after mating and eventually impairs male fertility [71]. However, Pang et al. [17]found that removal of the coagulating glands, resulting in the absence of a vaginal plug, did not seem to explain the reduced fertility, which indicates that seminal vesicles may contribute certain factors to modulate fertility. Therefore, in addition to having a role in balancing the activity of proteases, SERPINE2 may also play a role in male reproduction. Although SERPINE2 was detected in portions of male reproductive tissues [45,69] the comprehensive expression patterns and functions of SERPINE2 in the male reproductive tract have not been characterized.



Proteins from the seminal plasma interact with sperm by modifying the sperm surface membrane, an essential process in maintaining sperm viability, thus modulating their functions [20]. Decapacitation factors are one of those proteins in seminal plasma important for preventing premature capacitation. The sperm capacitation and acrosome reaction are essential for sperm-egg fertilization but premature capacitation will shorten sperm's lifespan and potentially miss the fertilization opportunity. In the study I, we purified SERPINE2 protein from the mouse seminal vesicle to delineate its protein distribution in the male reproductive system and to study its effects on sperm function.

2.2 Materials and Methods

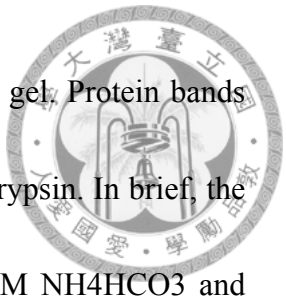
Animals

Specific pathogen-free outbred ICR mice were purchased from BioLASCO Taiwan (Taipei, Taiwan). The animals were bred based on the technology derived from Charles River Laboratories (Wilmington, MA) and were maintained in the Animal Center at the Department of Medical Research, Mackay Memorial Hospital, and were treated according to institutional guidelines for the care and use of experimental animals. They were housed under controlled lighting (12L:12D) at 21–22ºC and provided with water and NIH-31 laboratory chow ad libitum.

Protein Purification and Analysis

Adult male mice (10–12 weeks old) were killed by cervical dislocation. The SVS was successively collected, centrifuged, and fractionated by ion-exchange chromatography using a diethylaminoethyl Sephacel (GE Healthcare Life Sciences, Piscataway, NJ) column and gel filtration with a Sephadex G-75 (GE Healthcare Life Sciences) column as previously described [36]. The potential SERPINE2-containing peak II eluted from the Sephadex G-75 column was further subjected to a heparin Sepharose 6 Fast Flow (2.6- X 10-cm) column (GE Healthcare Life Sciences) preequilibrated with 0.1 M Tris-HCl, 0.01 M sodium citrate, and 0.225 M NaCl (pH 7.4). After the nonretarded fractions were washed out, the column was eluted with 0.5 M NaCl in 0.1 M Tris-HCl and 0.01 M sodium citrate at pH 7.4 at a flow rate of 0.5 ml/min; fractions (2 ml) were collected, and absorbance records are shown in Figure 1A. The protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The N-glycoconjugate was removed from a glycoprotein using a PNGase F kit (New England Biolabs, Beverly, MA) following the manufacturer's instructions.

Protein Identification by Mass Spectrometry



Purified protein was resolved by SDS-PAGE on a 10% slab gel. Protein bands on the SDS gel were excised and subjected to in-gel digestion with trypsin. In brief, the gel was washed in a solution of 50% (v/v) acetonitrile and 100 mM NH4HCO3 and digested by trypsin overnight at 37 ºC. The tryptic peptides were then extracted with a solution of 60% (v/v) acetonitrile and 1% (v/v) trifluoroacetic acid (TFA), lyophilized, resuspended in 0.1% (v/v) TFA, and analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) equipped with an 1100 series HPLC unit (Agilent Technologies, Palo Alto, CA) and an LTQ FT hybrid mass spectrometer (Thermo Electron, San Jose, CA). MS/MS data were used for protein identification, using MASCOT search engine software (http://www.matrixscience.com), based on the International Protein Index databases (http://www.ebi.ac.uk/IPI).

Activity Assay

The inhibitory activity of SERPINE2 toward PLAU (also named uPA) was assayed using a uPA colorimetric assay kit (Millipore, Billerica, MA) according to the manufacturer's protocol. In brief, 5 μg of purified SERPINE2 protein was incubated with 5 units of PLAU for 1 h at 37ºC. Subsequently, assay buffer and chromogenic substrate were added and incubated for 30 min at 37°C. The absorbance was read at 405 nm.

Antibody Production and Usage

Antisera against SERPINE2 were produced using New Zealand white rabbits. Purified SERPINE2 protein in normal saline (0.4 mg/ml) was emulsified with Freund's complete adjuvant (1:1, v/v). In total, 2 ml of the mixture was subcutaneously injected in multiple sites in individual rabbits. Two rabbits were boosted twice every 3 weeks with the mixture of the same amount of purified protein and Freund's incomplete adjuvant (1:1, v/v). Antisera were collected 10 days after the last injection. Purified SERPINE2 protein (200 μg) was conjugated to AminoLink beads (Pierce) according to the manufacturer's instructions. Antisera against SERPINE2 were adsorbed by the conjugated beads to remove the specific antibody against SERPINE2. The treated antiserum was used as the control antiserum. Antisera were also used to develop a custom-made sandwich-style ELISA kit by Taiwan Advanced Bio-Pharm (Taipei, Taiwan). The kit was used to estimate the SERPINE2 protein concentration in SVS, which was collected separately from 6 male mice at the age of 12 weeks.

Western Blotting

Proteins were resolved using SDS-PAGE on a 10% slab gel (8.2 × 7.3 × 0.075 cm) and stained with Coomassie Brilliant blue or transferred to a nitrocellulose

membrane for immunostaining. Membranes were blocked with 10% (w/v) skim milk in PBS (blocking solution) for 2 h and then incubated with anti-SERPINE2 antiserum or control antiserum (1:8000 dilution) in blocking solution for 1 h at room temperature. After gentle agitation over four changes of PBS for 10 min each, membranes were immunoreacted with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (GE Healthcare Life Sciences) diluted to 1:10 000 in blocking solution for 1 h. Immunoreactive bands were revealed using an enhanced chemiluminescence substrate according to the manufacturer's instructions (Pierce).

Immunohistochemical Staining of the Accessory Gland of Male Mice

Murine (~12 weeks old) reproductive tissues were collected, fixed in formalin, embedded in paraffin, and cut into 5-lm sections. After the slides were deparaffinized and hydrated, they were placed in a plastic slide holder filled with antigen retrieval AR-10 solution (BioGenex, San Ramon, CA), soaked in a 70°C water bath, rapidly boiled to 95°C, and maintained for 15 min. While cooling to room temperature for 30 min, the slides were treated with 3% (v/v) H2O2 in PBS for 15 min, blocked with 10% (v/v) normal goat serum in PBS (blocking solution) for 1 h at room temperature, and then incubated with anti-SERPINE2 antiserum or the control antiserum diluted 1:1000 in the blocking solution at 4 ºC for 16 h. After slides were washed, they were treated

with biotin-conjugated goat anti-rabbit IgG (~3 μg/ml) (Zymed Laboratories, South San Francisco, CA) in blocking solution for 1 h at room temperature. Slides were washed again and then incubated with HRP-conjugated streptavidin (~1 μg/ml) (Zymed Laboratories) in blocking solution for 40 min at room temperature. Protein signals were detected by 3-amino-9-ethylcarbazole staining (Zymed Laboratories). Slides were then counterstained with hematoxylin (Vector Laboratories, Burlingame, CA) and photographed using an Olympus BX 40 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP-70 digital camera.

Preparation of Spermatozoa

Epididymides and testes were immediately removed after male mice (;12 weeks old) were killed. Caudal epididymides were slit in prewarmed Biggers, Whitten, and Whittingham (BWW) medium and incubated at 37 ºC in 5% CO 2 for 15 min to allow motile sperm to swim upward. Motile sperm in the upper layer were collected. The caput and corpus regions of epididymides were treated in the same method as described above but were gently filtered through a 70 μm nylon cell strainer (BD Falcon, Franklin Lakes, NJ) to remove debris. Testes were decapsulated by cutting the tunica albuginea to expose seminiferous tubules. An 18-gauge needle was used to aspirate the seminiferous tubules and push them through. The dispersed seminiferous tubules were

cut into pieces and filtered through a 70-lm nylon cell strainer (BD Falcon) to collect the free seminiferous cells. To isolate ejaculated uterine and oviductal sperm, female mice (6 weeks old) were induced to superovulate by an intraperitoneal injection of 10 IU of equine chronic gonadotropin (China Chemical and Pharmaceutical, Hsinchu, Taiwan), followed by an intraperitoneal injection of 10 IU of human chorionic gonadotropin (China Chemical and Pharmaceutical) 48 h later, and were subsequently mated with male mice (~16 weeks old). Female mice with plugged vaginas were killed, and the ejaculated sperm in the uterine cavity were collected within 1 h. In brief, the semen filtered through a 70 μm nylon cell strainer (BD Falcon) was repeatedly agitated by pipetting with PBS. The sperm solution was washed by centrifuging it three times at 100 X g for 10 min. Sperm were then fixed using 4% (w/v) paraformaldehyde in an Eppendorf tube for 20 min at room temperature, transferred onto slides, and allowed to dry. Oviductal sperm were collected the next day after mating by flushing the oviduct with PBS. Sperm were transferred using a mouth pipette onto slides and fixed in 4% (w/v) paraformaldehyde for subsequent immunostaining analysis.

Immunolocalization of SERPINE2 on Spermatozoa

To determine whether SERPINE2 protein is originally a sperm-binding protein, freshly prepared epididymal and testicular spermatozoa were fixed using 4% (w/v)

paraformaldehyde and allowed to dry on a glass slide and washed twice with PBS. After slides were incubated in blocking solution of PBS containing 10% (v/v) normal goat serum for 1 h at room temperature, they were incubated with anti-SERPINE2 antiserum or control antiserum at a dilution of 1:100 in blocking solution for 1 h. The slides were washed three times with PBS to remove excess antibodies before they were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Vector Laboratories) diluted 1:500 in blocking solution for 40 min. All slides were then washed with PBS and counterstained with 5μg/ml Hoechst 33258 stain. After three brief rinses with PBS, the slides were mounted in 100μl of ProLong Gold antifade medium (Invitrogen Molecular Probes, Eugene, OR) and photographed using an epifluorescence microscope (Olympus BX 40) equipped with an Olympus DP-70 digital camera. To determine whether exogenous SERPINE2 protein can bind to epididymal sperm, 0.5 μM SERPINE2 was incubated with living sperm in Eppendorf tubes for 20 min at 37℃. Unbound SERPINE2 protein was washed away by centrifuging the sperm in the PBS solution at 100 x g for 5 min at room temperature. Sperm were then fixed using 4% (w/v) paraformaldehyde in an Eppendorf tube for 20 min at room temperature, transferred onto slides, and allowed to dry. Immunostaining was done as described above, except a dilution of 1:1000 was used for anti-SERPINE2 antiserum and control antiserum. The same dilution was also used to examine SERPINE2 on ejaculated and

oviductal sperm, without incubation with the exogenous SERPINE2 protein. To examine the correlation between SERPINE2-bound sperm and sperm capacitation, oviductal sperm were double fluorescence labeled by using indirect immunofluorescence and chlortetracycline (CTC) fluorescence staining, an empirical method used to morphologically assess sperm capacitation [72,73]. In brief, sperm slides were washed twice with PBS and incubated in blocking solution, as mentioned above, for 1 h at room temperature. Then the slides were incubated with anti-SERPINE2 antiserum or control antiserum at a dilution of 1:1000 in blocking solution for 1 h. After slides were washed three times with PBS, they were incubated with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in blocking solution for 40 min. All slides were then washed with PBS and counterstained with 5 μg/ml Hoechst 33258. After three brief rinses with PBS, the sperm on the slides were stained with a CTC solution prepared as previously described [72,73] by incubation at 4℃ overnight and photographed using an epifluorescence microscope (Olympus BX 40) equipped with an Olympus DP-70 digital camera.

Evaluation of Sperm Capacitation and the Acrosome Reaction

The molecular basis of sperm capacitation was examined by detecting any

capacitation-accompanied increase in protein tyrosine phosphorylation of a subset of proteins with molecular weights of 40,000–120,000, according to a previously described method [7]. In brief, about 5 x 10^6 spermatozoa/ml was incubated in modified Krebs-Ringer bicarbonate medium [74] with or without bovine serum albumin (BSA) (3 mg/ml), as the positive or negative control, respectively, or BSA replaced with SERPINE2 at 37℃ in an atmosphere of 5% (v/v) CO_2 in humidified air for 90 min. To assess the effect of SERPINE2 on BSA-induced capacitation, SERPINE2 was preincubated with sperm under the above-described conditions for 20 min, and BSA was added thereafter. Then, the soluble fraction of sperm protein extracts was subjected to SDS-PAGE on an 8% slab gel. Proteins on the gel were electrotransferred onto nitrocellulose paper. Western blot analyses were performed using an anti-phosphotyrosine antibody according to a method described previously [75]. To evaluate sperm capacitation by the CTC fluorescence-staining method, freshly prepared epididymal spermatozoa (10^6 cells/ml) were capacitated in 50 μl of BWW medium with or without BSA (3 mg/ml) as the positive or negative control, respectively, at 37℃ in an atmosphere of 5% (v/v) CO_2 in humidified air for 90 min. Medium was supplemented with SERPINE2 as described above, or the BSA was replaced with SERPINE2 to analyze the effects of SERPINE2 on sperm capacitation *in vitro*. CTC staining of sperm was carried out following the original method and examined using a

fluorescence microscope (BX 40 model; Olympus). To analyze the sperm acrosome reaction, the capacitated sperm, prepared as described above, were treated with 5 μM A23187 in dimethyl sulfoxide (DMSO) (0.2%) at 37℃ for 30 min. Sperm were smeared on the slide and fixed with methanol for 30 sec. The sperm acrosomal status was assessed by staining samples with 5 μg/ml TRITC-conjugated peanut agglutinin lectin (PNA; Sigma-Aldrich, St. Louis, MO) in the dark for 5 min and by counterstaining with 5 μg/ml Hoechst 33258. After three brief rinses in PBS, slides were mounted in 50 μl of ProLong Gold antifade medium (Invitrogen Molecular Probes) and immediately examined with a fluorescence microscope (BX 40; Olympus).

Sperm–Egg Binding and *In Vitro* Fertilization

Epididymal sperm (2 x 10^5 cells/ml) in 150μl of BWW medium under mineral oil with or without BSA and/or SERPINE2 were capacitated for 90 min at 37℃ in an atmosphere of 5% (v/v) C O 2 in humidified air. Oocyte-cumulus complexes collected by superovulation treatment, as described above, were added to the same medium. For the sperm–egg binding assay, treated sperm were inseminated with oocyte-cumulus complexes for 30 min and then gently transferred using a mouth pipette with a bore approximately 1.53 the diameter of the oocyte to 70μl of PBS under mineral oil. After allowing specimens to sit at room temperature for 5 min, the loosely bound sperm were

detached, and the tightly bound sperm on the oocyte were counted using a Zeiss Axiovert 100 microscope (Zeiss, Oberkochen, Germany). For *in vitro* fertilization, after 6 h of insemination, oocytes were washed with BWW medium, using a mouth pipette, fixed onto a slide with 4% (w/v) paraformaldehyde, and stained in 5μg/ml Hoechst 33258 for 3 min. Slides were observed with a fluorescence microscope (BX40; Olympus). Two pronuclei embryos were scored as fertilized.

Cholesterol Efflux Assay

Cholesterol content in the BWW medium with or without BSA (3 mg/ ml) and/or SERPINE2 (0.2 mg/ml) was assayed following the protocol described by Roberts et al. [26]. Freshly prepared epididymal spermatozoa (2 x 10^5 cells/ml) was capacitated in 150 μl of BWW medium as described above. After incubation, sperm were centrifuged at 10,000 x g to separately collect the supernatant and sperm pellets. Samples were mixed with chloroform and methanol in a chloroform-methanol-supernatant (or sperm) final ratio of 2:2:1.8. After vigorous vortexing, the mixture was centrifuged at 600 x g for 5 min, and the organic phase was transferred to a new Eppendorf tube and dried by speed vacuum. The cholesterol content was measured using an Amplex Red cholesterol assay kit (Invitrogen Molecular Probes) according the manufacturer's instructions. To calculate the cholesterol content

of the samples, a cholesterol standard curve was prepared using the cholesterol reference standard provided with the kit.

2.3 Results

Purification and Identification of SERPINE2 from Mouse SVS

To prepare SERPINE2 for functional analyses and antibody production, we prefractionated SVS by ion exchange and gel filtration chromatography. The possible SERPINE2-containing fraction, based on the molecular mass, was further purified to homogeneity using a heparin Sepharose column (Fig. 1A, peak II), as SERPINE2 is a heparin-binding protein [75]. The purity of the resulting protein was shown with SDS-PAGE (Fig. 1B). To identify this protein, the bands on the gel were excised and digested in-gel with trypsin, and the resulting tryptic peptides were subjected to LC-MS/MS analysis. Results showed that the purified protein had significant homology to SERPINE2, with the tryptic peptides matching 41%–43% of the protein sequences (Fig. 1C). The purified SERPINE2 protein showed potent inhibitory activity against PLAU (Fig. 1D), indicating that the purification procedures were not harmful to its protease-inhibitory activity. The SERPINE2 protein concentration in the SVS was estimated to be 0.6–0.8 mg/ml by the ELISA method.

Distribution of SERPINE2 Protein in Adult Male Mouse Reproductive Tissues

To study the tissue distribution of SERPINE2 protein in male reproductive tissues, we examined tissue homogenates, including the seminal vesicle, epididymis, testis, coagulating gland, vas deferens, and prostate, by Western blotting. The antibody against SERPINE2 recognized the purified 45-kDa band and at least three forms, 40-, 42-, and 45-kDa proteins, from thousands of protein components in the tissue extract Fig. 2). In addition, high-molecular-weight proteins were also detected in protein extract from the testes. These proteins may be aggregated forms as no signal was seen when the antiserum was removed from the blots and samples were reprobed with the antiserum that was pretreated with SERPINE2-conjugated beads (control antiserum), indicating the high specificity of the antibody. When protein database searching was conducted using basic Local Alignment Search Tool (BLAST) algorithms http://www.ncbi.nlm.nih.gov/BLAST) against a nonredundant database, using the SERPINE2 protein sequence (Swiss-Prot 07235) as the query, three isoforms were revealed, with accession numbers gb j EDL16269.1 j , gb j EDL16267.1 j , and gb j EDL16268.1 j. The theoretical molecular masses of the three isoforms were 30.812, 35.668, and 44.206 kDa, respectively. This was not processed by a signal peptidase. Thus, the mature protein would have the smaller molecular mass. However, the three

proteins recognized by the anti-SERPINE2 antiserum had greater molecular masses, indicating they might be the glycosylated forms. In fact, SERPINE2 expression was demonstrated as two forms of glycoproteins [76]. However, we treated the protein extract from seminal vesicles with -glycosidase F, only the 45-kDa protein was found to be deglycosylated. To reveal the cell types and subcellular compartments among male reproductive tissues that expressed the SERPINE2 protein, an immunolocalization study was conducted using the specific anti-SERPINE2 antiserum. The SERPINE2 protein was immunolocalized to epithelial cells of seminal vesicles, coagulating glands, vas deferens, and caput or caudal epididymides (Fig. 3, B–E and G). However, when slides were immunostained with control antiserum, no signal was detected (Fig. 3A). Signals on corpus epididymides and the rostate were relatively weaker (Fig. 3, F and H). The most prominent expression was found in the luminal fluid of seminal vesicles of adult mice. A signal on smooth muscle cells of seminal vesicles was also visible (Fig. 3B). Interestingly, ERPINE2 protein was identified on spermatogonia, spermatocytes, spermatids, Leydig cells, and spermatozoa (Fig. 3I), as revealed by the control slide treated with control antiserum (Fig. 3J).

Binding of the SERPINE2 Protein to Spermatozoa

A visible SERPINE2 protein signal was prominently present on sperm in the

lumen of the vas deferens and caudal epididymides (Fig. 3, D and G). To verify binding of the SERPINE2 protein onto sperm, sperm isolated from testes or epididymides were smeared on slides, as shown in the phasecontrast image for comparison (Fig. 4A, a). When slides were immunostained by control antiserum and an FITC-conjugated secondary antibody, no fluorescent signal was detected (Fig. 4A, b). In contrast, SERPINE2 was detected on the acrosomal caps of caput, corpus, and caudal epididymal sperm by using the anti-SERPINE2 antiserum (Fig. 4A, c–e). Likewise, apparent SERPINE2 protein signals were also visualized on the acrosomal cap of testicular sperm (Fig. 4A, f). These results suggested that the SERPINE2 protein is an intrinsic surface protein of sperm during spermiogenesis and sperm maturation. Exogenous SERPINE2 can apparently bind to caudal epididymal sperm, as demonstrated by incubation of the epididymal sperm with purified SERPINE2. The signal from this binding was so strong that it was prominently detected by a more-dilute anti-SERPINE2 antiserum (1:1000) (Fig. 4B, b). The binding was strong on the acrosomal cap and on the tails of living epididymal sperm. Under the same detection conditions, epididymal sperm showed only a very faint intrinsic SERPINE2 signal (Fig. 4B, a). Although faint, the intrinsic signal, as mentioned above, was detected using more highly concentrated antiserum (1:100) (Fig. 4A, e). The SERPINE2 protein derived from seminal plasma was also detected on ejaculated and oviductal sperm (Fig. 4C, b and d, respectively) as

demonstrated by control slides stained with control antiserum (Fig. 4C, a and c, respectively). The binding was strong on the acrosomal cap but weaker on the tail. These findings indicate that the exogenous SERPINE2 may be a sperm surface protein in vivo.

Removal of SERPINE2 from Capacitated Sperm in the Oviduct

To determine whether capacitated or uncapacitated sperm have a SERPINE2-binding zone, oviductal sperm were immunostained with anti-SERPINE2 antiserum, and then the same sperm were fluorescently stained with CTC. As shown in Figure 5, four staining types (Fig. 5, A–D) of sperm were found. Staining type A was defined as capacitated sperm without SERPINE2 on the acrosome; type B was uncapacitated sperm with SERPINE2 on the head; type C was capacitated sperm with less SERPINE2 on the head; and type D was uncapacitated sperm with no SERPINE2 on the sperm surface. About 40% of the SERPINE2-bound sperm were the uncapacitated B type, but only about 10% of the capacitated type C sperm were seen in the oviduct. In addition, about 50% of the sperm in the oviduct were capacitated and not bound by SERPINE2. Interestingly, SERPINE2 was prominently present on uncapacitated sperm. It seems that SERPINE2 was released from the acrosomal region when the sperm underwent capacitation.

Effects of SERPINE2 on Sperm Function *In Vitro*

To examine the effects of SERPINE2 on epididymal sperm capacitation, we assessed the protein tyrosine phosphorylation pattern of epididymal sperm after incubation with BSA and/or SERPINE2. As shown in Figure 6A, only a few sperm proteins were phosphorylated in control medium without supplementation with BSA or SERPINE2 (Fig. 6A, lane 1). However, BSA induced sperm capacitation accompanied by tyrosine phosphorylation of a group of proteins with a pattern similar to that found in previous studies (Fig. 6A, lane 2) [7]. The SERPINE2 protein prominently decreased the phosphorylation of the control medium (Fig. 6A, lane 3). In addition, the extent of BSA-induced protein tyrosine phosphorylation was successively suppressed by the increased concentration of SERPINE2 (Fig. 6A, lanes 4–7). Clearly, the characteristic capacitation specific protein tyrosine phosphorylation pattern induced by BSA was inhibited by SERPINE2. CTC fluorescence staining is often used to assess capacitation, as judged by the morphology of fluorescently stained sperm. In the control medium without BSA or SERPINE2, sperm showed a spontaneous capacitation rate of approximately 22%. The addition of SERPINE2 significantly decreased the spontaneous capacitation rate to 11%. The population of capacitated sperm remarkably increased (64%) after the control medium was supplemented with 3 mg/ml BSA. However,

SERPINE2 inhibited BSA-induced sperm capacitation significantly after 0.05, 0.1, or 0.2 mg/ml of SERPINE2 was added to BSA-containing medium (Fig. 6B). These observations are in accordance with the inhibition of BSA-induced tyrosine phosphorylation by SERPINE2 (Fig. 6A). Next, we examined the acrosome reaction induced by the calcium ionophore A23187. A spontaneous acrosome reaction was found (15%–26%) with *in vitro*-capacitated sperm in the incubation medium with or without BSA and/or SERPINE2 (Fig. 6C, white bars). The concentration of the vehicle used, 0.2% DMSO, did not increase the percentage of acrosome-reacted sperm. SERPINE2-treated sperm did not show an increased acrosome reaction compared to that of control medium. However, BSA-treated sperm showed remarkable enhancement of the acrosome reaction after A23187 induction. In contrast, the acrosome reaction was significantly inhibited when sperm were incubated with BSA and SERPINE2. An 85% reduction was observed after treatment with 0.2 mg/ml of SERPINE2 (Fig. 6C). Only capacitated sperm can be induced to undergo an acrosome reaction [16]. Thus, these results further indicated that SERPINE2 is able to inhibit sperm capacitation induced by BSA. Capacitated sperm can bind to the zona pellucida of oocytes and are induced to undergo an acrosome reaction. If SERPINE2 can inhibit sperm capacitation, this would affect sperm–egg binding and subsequent fertilization. As shown in Figure 7, epididymal sperm with no treatment had a low capacity to bind to oocytes, with a

fertilization rate of approximately 20% *in vitro*, which may have resulted from fertilization by sperm that reacted to acrosome spontaneously. In contrast, BSA-treated sperm showed strong binding to oocytes and had a higher fertilization rate (55%–66%). However, supplementation with 0.2 mg/ml SERPINE2 significantly reduced oocyte binding and the fertilization rates of BSA-capacitated epididymal spermatozoa by 82% and 64%, respectively. These findings further demonstrated that SERPINE2 can inhibit BSA-induced sperm capacitation and lead to failure of *in vitro* fertilization. BSA is used in most media for sperm capacitation. It is able to promote sperm membrane cholesterol efflux [12,77,78]. Cholesterol was released into the medium from BSA-treated sperm, while that release was significantly inhibited by SERPINE2 (Fig. 8A). SERPINE2-treated sperm retained an amount of cholesterol that was similar to that of untreated sperm (Fig. 8B).

2.4 Discussion

SERPINE2 antiserum, at a dilution of 1:50, had a maximal effect on epididymal sperm capacitation, with a small increase (14%–19%), compared to that in the control group without protein supplementation in the medium, as demonstrated by CTC fluorescence staining and *in vitro* fertilization, respectively. However, this slight

effect on sperm capacitation was reversed by 0.2 mg/ml SERPINE. A similar effect was found in a study by Ni et al. [28]. The antisera against HongrES1, a caudal epididymis-specific protein, displayed a more significant increase in guinea pig sperm capacitation, and HongrES1 significantly inhibited the effect [28]. In fact, our preliminary data also showed that BSA capacitation of sperm could be reversed by the addition of 0.2 mg/ml SERPINE2, further supporting the role of SERPINE2 as a decapacitation factor.

Cholesterol efflux is one of the initiation events of sperm capacitation. BSA is a cholesterol acceptor, which induces cholesterol efflux, leading to sperm capacitation [12,77,78]. In this study, we revealed that SERPINE2 inhibits BSA-induced cholesterol efflux; the precise mechanism that enables SERPINE2 to retain the cholesterol on the sperm warrants further investigation.

SERPINE2 seems to have protective effects toward sperm cultured *in vitro* by preventing spontaneous capacitation and the acrosome reaction of sperm. As our study demonstrates, the addition of SERPINE2 effectively lowered protein tyrosine phosphorylation levels (Fig. 6A, lanes 1 and 3), thus yielding a lower percentage of capacitated and acrosome-reacted sperm (Fig. 6, B and C). Additional evidence for this tendency is the fact that in the presence of SERPINE2, there is a reduced number of sperm that bind to an egg and a lower fertilization rate than that of the control medium

group (Fig. 7).

Our SERPINE2 antiserum detected two major and one minor SERPINE2 isoforms in the male reproductive tract. A BLASTP search revealed the existence of three SERPINE2 isoforms in the protein database. Many alternatively spliced SERPINE2 gene products can also be seen at the NCBI AceView website (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/). Previous reports have also supported the existence of multiple forms of SERPINE2 protein among mouse tissues [79].

The *Serpine2* mRNA and protein are predominantly expressed in seminal vesicles among mouse tissues [69]. The SERPINE2 protein was previously detected in the epithelium of bovine seminal vesicles and epididymides and in Leydig cells of the testes [45]. However, there is no comprehensive study that has specifically detected this protein in all male reproductive tissues. In this study, we found that if paraffin-embedded testicular sections were not treated with the antigen-retrieval solution, then SERPINE2 was immunostained only on Leydig cells. However, if the sections were retrieved to unmask the SERPINE2 antigen, staining was detected on spermatogonia, spermatocytes, spermatids, Leydig cells, and spermatozoa (Fig. 3). This demonstrates the existence of SERPINE2 protein and mRNA in germ cells of adult testes. The Serpine2 mRNA, in fact, exhibits a male-specific expression pattern in

developing gonads, suggesting its involvement in a testis-determining pathway [80].

Plasminogen activators and their cognate inhibitors, including SERPINE1, SERPINE2, SERPINB2, and SERPINA5, are all found in mouse testes. The level of Serpina5 mRNA is the highest among them, followed by, respectively, *Serpine2*, *Serpinb2*, and *Serpine1* mRNA levels [81]. However, murine testicular SERPINE2 seems to have more extensive localization than SERPINA5, as the latter is detected only in Leydig cells before birth and postnatally but is restricted to early spermatids within the acrosomal region in adult testes [82]. SERPINE2 and SERPINA5 are both members of the SERPIN family. Their gene expression levels are regulated by androgen [70,71], but they seem to work as counterparts in different species. A comprehensive proteomic study detected only the SERPINA5 protein but not the SERPINE2 protein in human seminal plasma [83]. However, only minor amounts of *Serpina5* mRNA were found in other accessory glands of mouse reproductive tissues, although it was prominently expressed in testes. SERPINE2 was expressed predominantly in the SVS (Fig. 3) and contributes to mouse seminal plasma. SERPINE2 would be the major plasminogen activator inhibitor in murine seminal plasma. Interestingly, they are all sperm surface-binding proteins. SERPINA5 binds to human sperm and may influence sperm–oocyte interactions [84], while SERPINE2 binds to mouse sperm and modulates sperm capacitation (Figs. 6–8). Interestingly, like SERPINE2, PDC-109 is also a

heparin-binding protein [85].

Some protease inhibitors are found on the sperm surface. SERPINA5 is present on the acrosomal surface of human sperm and has been suggested to prevent sperm from prematurely undergoing an acrosomal reaction [84]. SPINK3 was shown to exist on the acrosomal cap of murine sperm and is able to inhibit Ca^{2+} uptake by epididymal sperm [35]. A proteinase inhibitor of seminal vesicle origin was shown to block sperm–zona binding and the acrosome reaction [86]. In addition, SPINKL, found mainly on the midpiece, is able to inhibit sperm capacitation [37]. In this study, we showed that SERPINE2, another sperm acrosome-binding protein, also exhibits the ability to inhibit sperm capacitation *in vitro*. Interestingly, these proteins are all predominantly expressed in seminal vesicles.

SERPINE2 may be like PEBP1 in that it has the dual functions of serving as a decapacitation factor [25] and a serine protease inhibitor [87]. HongrES1, another SERPIN family protein, also displayed similar functions [28,29]. The inhibitory activity of a serine protease inhibitor and its role as a decapacitation factor might not necessarily be related. SPINKL inhibits sperm capacitation but does not seem to have inhibitory activities against serine proteases [37]. SPINK3 has no effects on sperm capacitation [88] while exhibiting trypsin inhibitory activity.

Glycosaminoglycans, including heparin, are moieties of the ECM. Sperm were

found to possess a surface sialic acid moiety [89], which is an anionic glycan residue like heparin. Thus, the binding of SERPINA5 and SERPINE2 to sperm may be like the case of protein binding to the ECM to protect sperm from protease attack in the testes and the epididymis during sperm maturation or in the female reproductive tract during transit toward fertilization. Whether they bind to sperm via sialic acid binding awaits further investigation.

2.5 Conclusion

We have demonstrated that SERPINE2 is expressed in nearly all of the male reproductive tissues examined, with the largest amount in seminal vesicles. SERPINE2 is intrinsically bound to the plasma membrane overlying the acrosome, while more SERPINE2 proteins derived from the seminal plasma were heavily bound to ejaculated and oviductal sperm in vivo. Nevertheless, SERPINE2 was predominantly detected on uncapacitated sperm, indicating that SERPINE2 is lost during the process of sperm capacitation. Furthermore, supplementation with purified SERPINE2 protein effectively suppressed BSA-induced sperm capacitation *in vitro* and blocked sperm–oocyte interactions, suggesting that the SERPINE2 protein may play a role as a sperm decapacitation factor. Our study has also demonstrated that SERPINE2 interferes with

the capacitation related signal transduction machinery by inhibiting cholesterol efflux of sperm. Further studies are warranted to elucidate its other possible mechanisms associated with sperm capacitation.

2.6 Figures

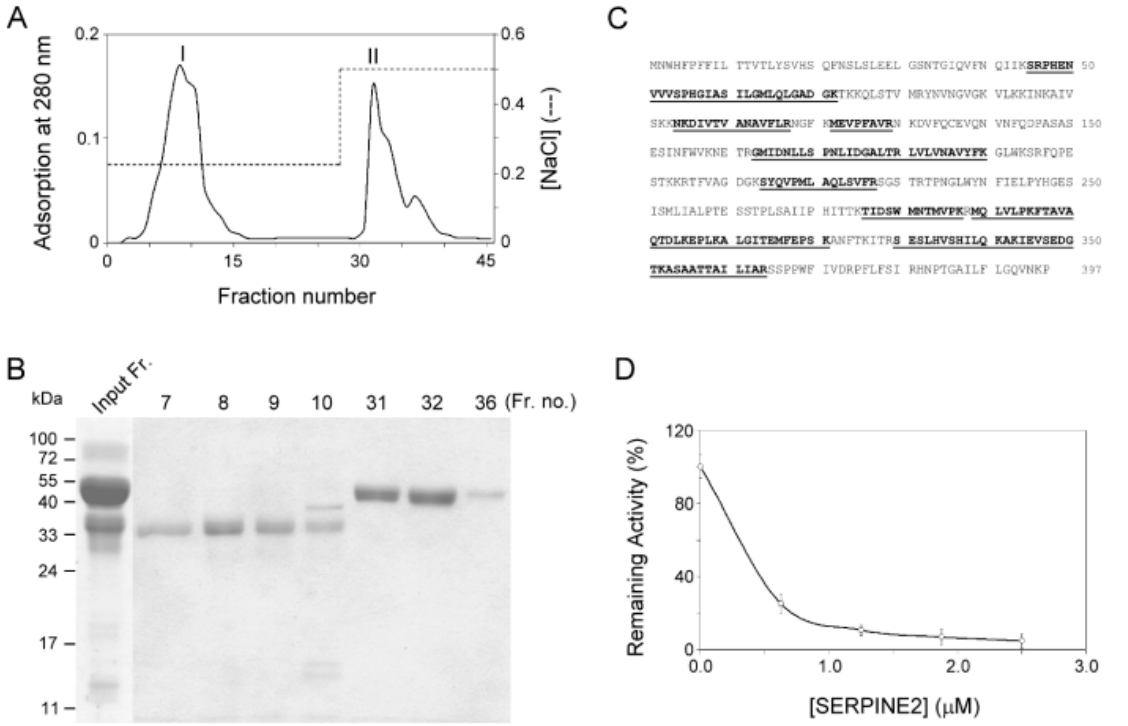


FIG. 1. Purification of SERPINE2 from mouse SVS. A) Resolution of peak II fraction from a Sephadex G-75 gel filtration column (see Materials and Methods) by affinity chromatography with a heparin-Sepharose CL 6B column is shown. B) Several fractions were resolved by 15% SDS-PAGE. Fraction numbers (Fr. no.) 30 to 35, purified SERPINE2, were collected and used as immunogen for antibody production. C) The cDNA-deduced amino acid sequences of mouse SERPINE2 are shown. The matched tryptic peptides found in the LC-MS/MS analyses are underlined and in boldface type. D) The inhibitory activity of SERPINE2 toward plasminogen activators.

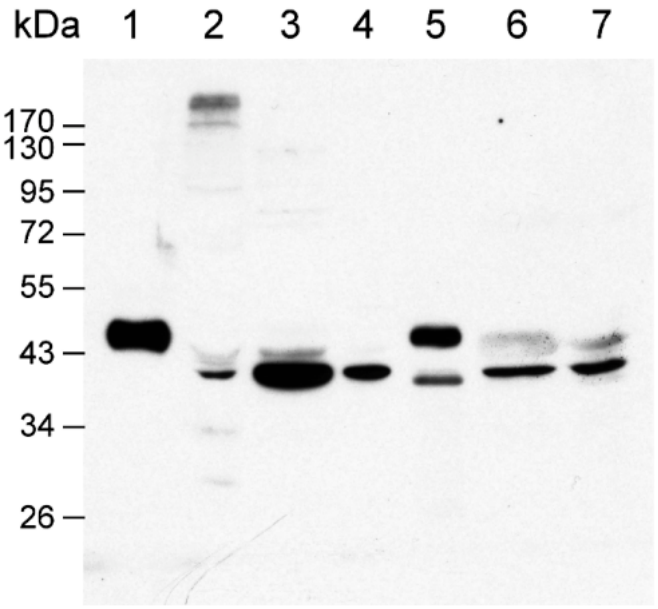


FIG. 2. Tissue distribution and antibody specificity. Total protein (100μg) prepared from the homogenates of each sexual tissue, except for 10μg from the seminal vesicle, was analyzed by Western blotting. Purified SERPINE2 (20 ng) was loaded as a positive control (lane 1). Lanes 2, testes; 3, epididymides; 4, vas deferens; 5, seminal vesicles; 6, prostate; 7, coagulating glands.

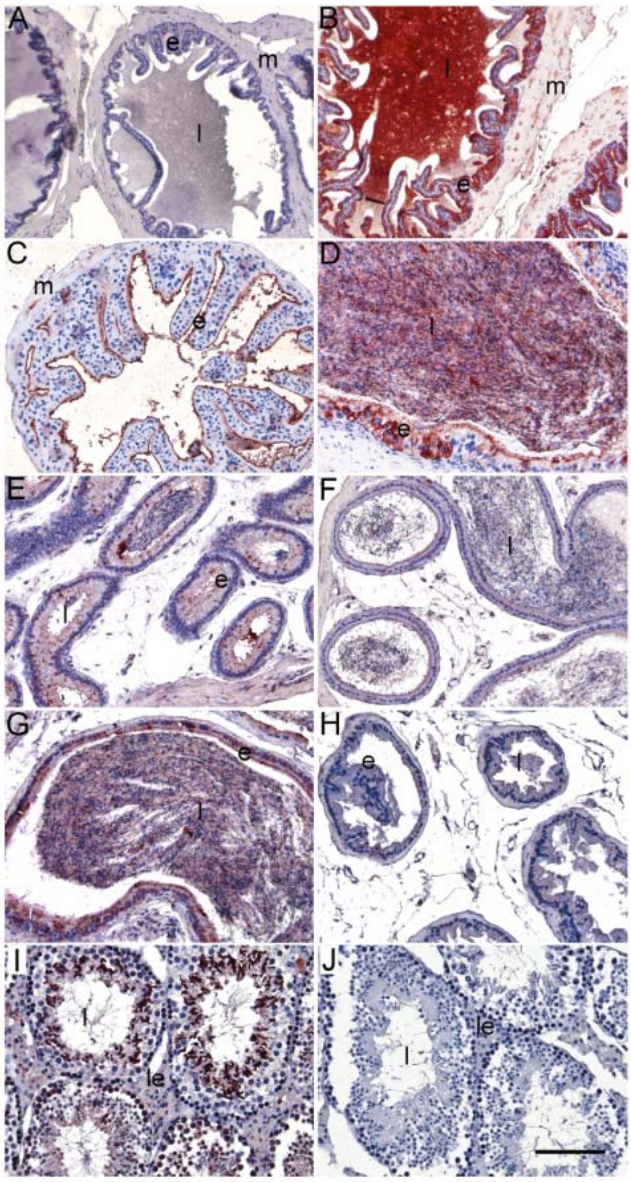


FIG. 3. Immunolocalization of SERPINE2 in male accessory reproductive tissues. Tissue slices from male accessory reproductive tissues, including the seminal vesicle (A and B), coagulating gland (C), vas deferens (D), caput (E), corpus (F), caudal (G) epididymis, prostate (H), and testes (I and J), were incubated with anti-SERPINE2 antiserum (B–I), or antiserum was pretreated with SERPINE2-conjugated beads for the control (A and J) and then treated with biotin-conjugated goat-anti-rabbit IgG and HRP-conjugated streptavidin (red). For contrast, specimens were further stained with hematoxylin (blue). Photomicrographs were taken under bright-field illumination. Bar = 200 μm. Tissue: e, epithelium; l, lumen; m, muscle; le, Leydig cell.

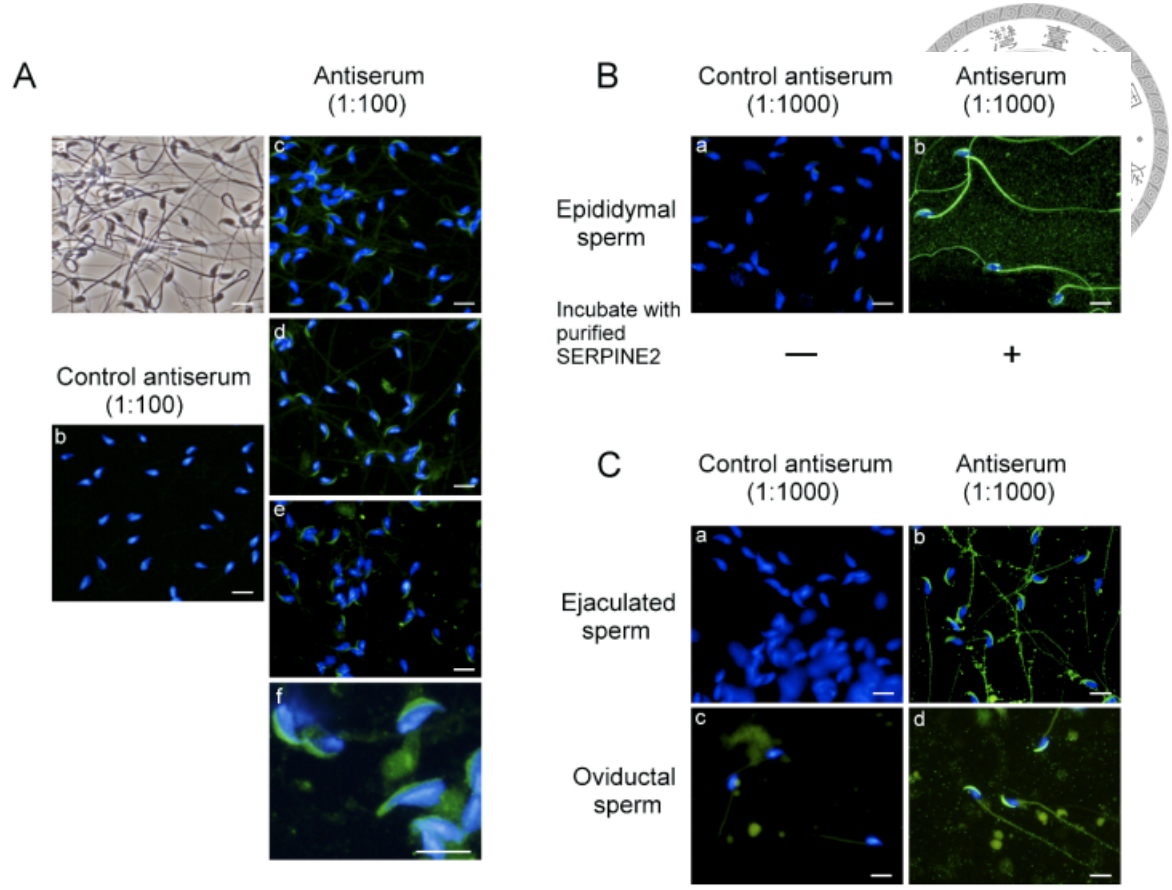


FIG. 4. Demonstration of the binding of SERPINE2 to sperm. A) Intrinsic SERPINE2 binding: sperm collected from the caput, corpus, and caudal epididymis, and testes were smeared on slides for immunolocalization of SERPINE2 on sperm. Slides were incubated with control antiserum (panel b) or anti-SERPINE2 antiserum (panels c–f) at a dilution of 1:100 and then treated with FITC-conjugated goat anti-rabbit IgG and counterstained with Hoechst dye to localize the nuclei for contrast. Phase-contrast photomicrography of sperm isolated from a caput epididymis reveals sperm morphology (panel a). SERPINE2 protein was detected on the acrosomal region of the caput epididymis sperm (panel c), corpus epididymis sperm (panel d), caudal epididymis sperm (panel e), and testicular sperm (panel f). Bar = 10μm. B) Exogenous SERPINE2 binding: living epididymal sperm incubated with (+ , panel b) or without (- , panel a) purified SERPINE2. After unbound SERPINE2 was washed away, epididymal sperm were fixed with 4% (w/v) paraformaldehyde in the tube and then transferred onto slides (panel b). C) Ejaculated sperm (panels a and b), with in vivo

binding of SERPINE2 derived from seminal plasma, were fixed with 4% (w/v) paraformaldehyde in the tube and then transferred onto slides (panel b). Oviductal sperm (panels c and d) flushed from the oviduct were directly fixed with 4% (w/v) paraformaldehyde on the slides. For immunolocalization of SERPINE2 on sperm, slides were incubated with anti-SERPINE2 antiserum (B, panel b, and C, panels b and d) or control antiserum (B, panel a, and C, panels a and c) at a dilution of 1:1000 and were immunostained as described above. Bar = 10 μm.

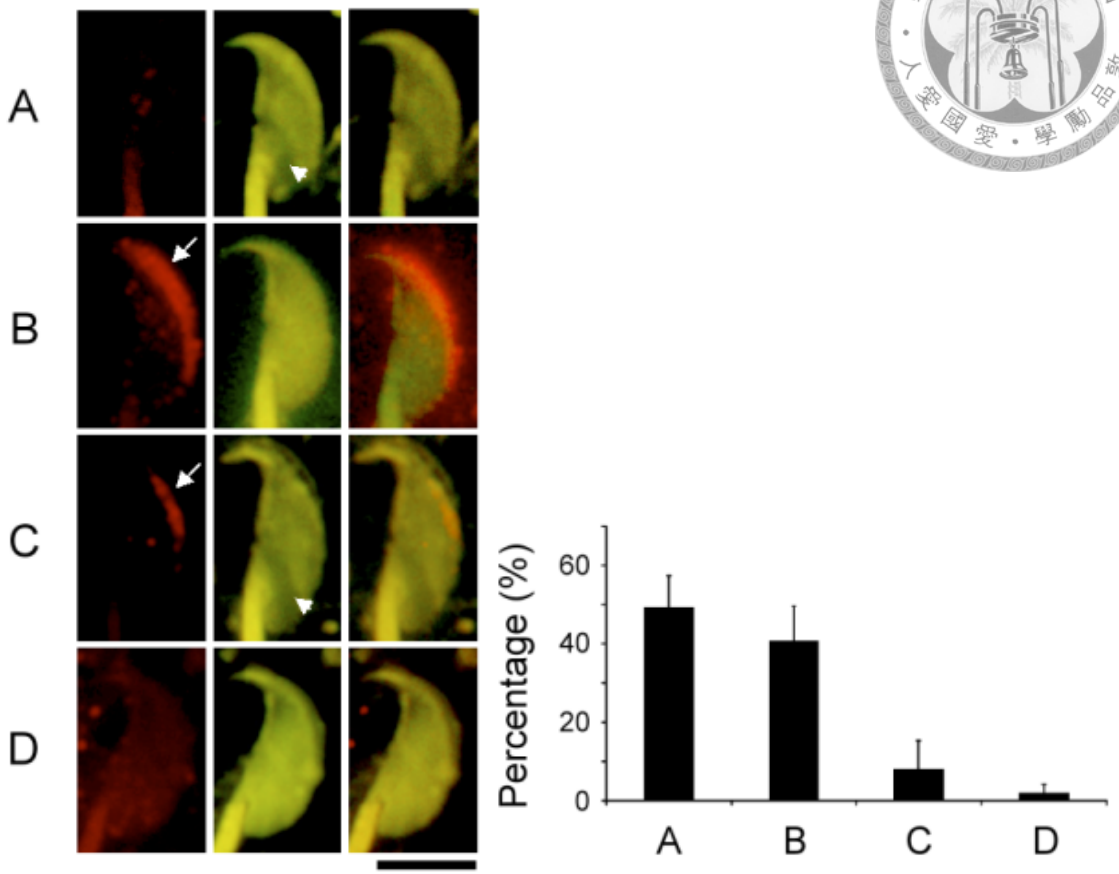


FIG. 5. Staining patterns of sperm in the oviduct. Sperm flushed from the oviduct were fixed in 4% (w/v) paraformaldehyde and immunostained with anti-SERPINE2 antiserum and TRITC-conjugated goat anti-rabbit IgG. Sperm were subsequently stained using a CTC fluorescence assay. Capacitated (A and C) and uncapacitated (B and D) sperm were observed under fluorescence microscopy. White arrowheads indicate the staining signal of capacitated sperm, while arrows show the SERPINE2 staining signal. Graph bars show percentages of sperm appearing under four different forms, type A to D. A random sample of 200 sperm per mouse was evaluated, and 9 mice were used in this experiment. Data are means ± SD of nine independent experiments. Bar = 5μm.

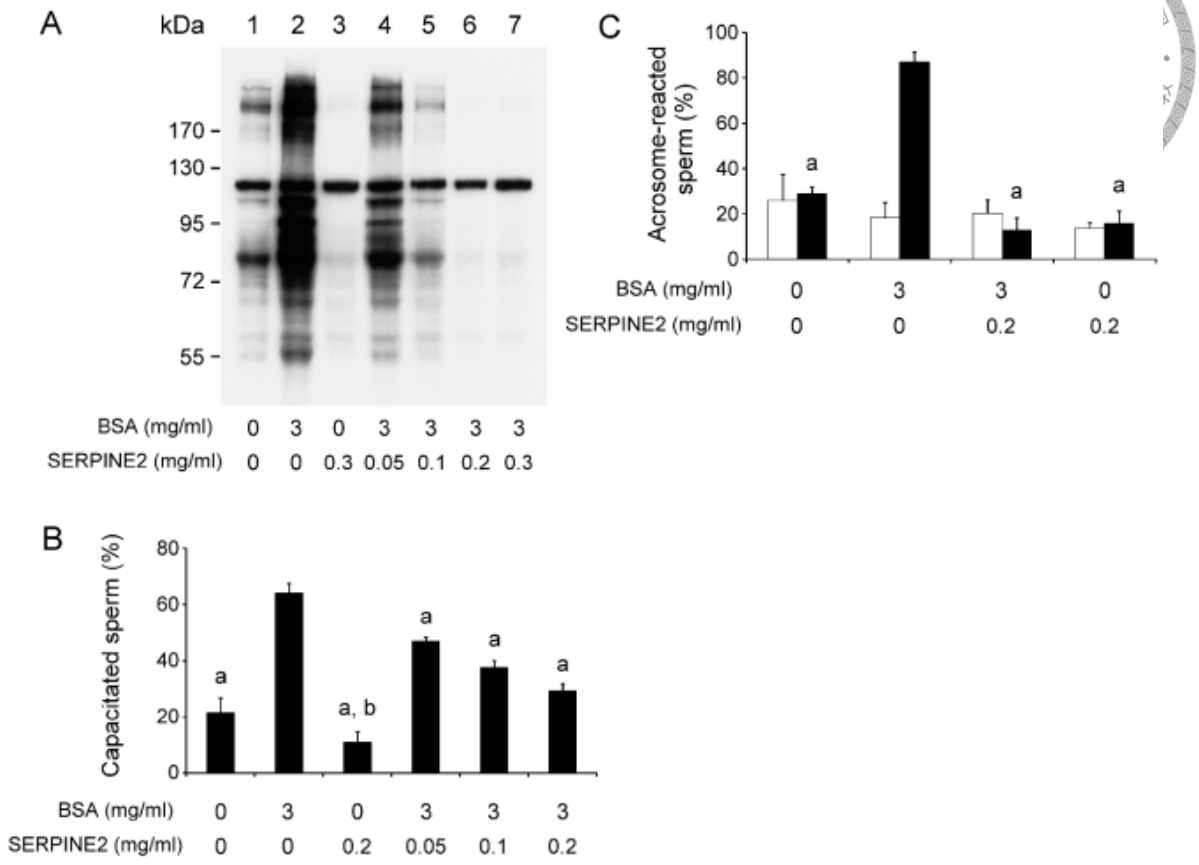


FIG. 6. Effects of SPERINE2 on murine sperm capacitation. Epididymal spermatozoa were incubated in the presence of 3 mg/ml BSA and/or different concentrations of SERPINE2 at 37℃ for 90 min. After treatment, the soluble fraction of the sperm lysate was resolved by SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and immunoblotted with anti-phosphotyrosine antibodies (A), or sperm smeared on slides were analyzed by CTC fluorescence staining to score the population of capacitated sperm (B); otherwise, the sperm acrosome reaction induced by the calcium ionophore A23187 (C, black bars) or uninduced (C, white bars) was evaluated by PNA staining (C). A minimum of 200 sperm per trial was evaluated. Data are means ± SD of three independent experiments. a, significant difference compared to the BSA-only group ($P < 0.001$); b, significant difference compared to the medium without protein supplementation ($P < 0.001$).

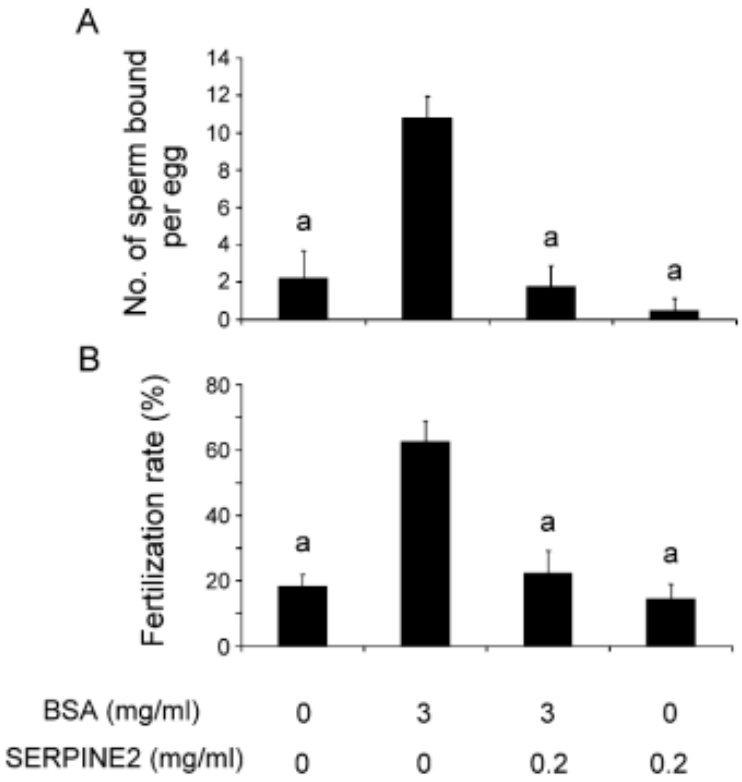


FIG. 7. Influence of SERPINE2 on sperm–egg interactions. Epididymal spermatozoa were capacitated in BWW medium with BSA and/or SERPINE2 for 90 min and subsequently inseminated with cumulus-intact oocytes for 30 min. Oocytes were gently transferred using a mouth pipette, and we waited 5 min to remove loosely attached sperm from the eggs. Numbers of tightly bound sperm per oocyte were counted (A). Otherwise, treated sperm were inseminated for 6 h to evaluate fertilization (B). Fertilized eggs that showed two pronuclei were identified by Hoechst 33258 staining. Data are means ± SD from four independent experiments. a, Significant difference compared relative to medium supplemented with BSA ($P < 0.001$).

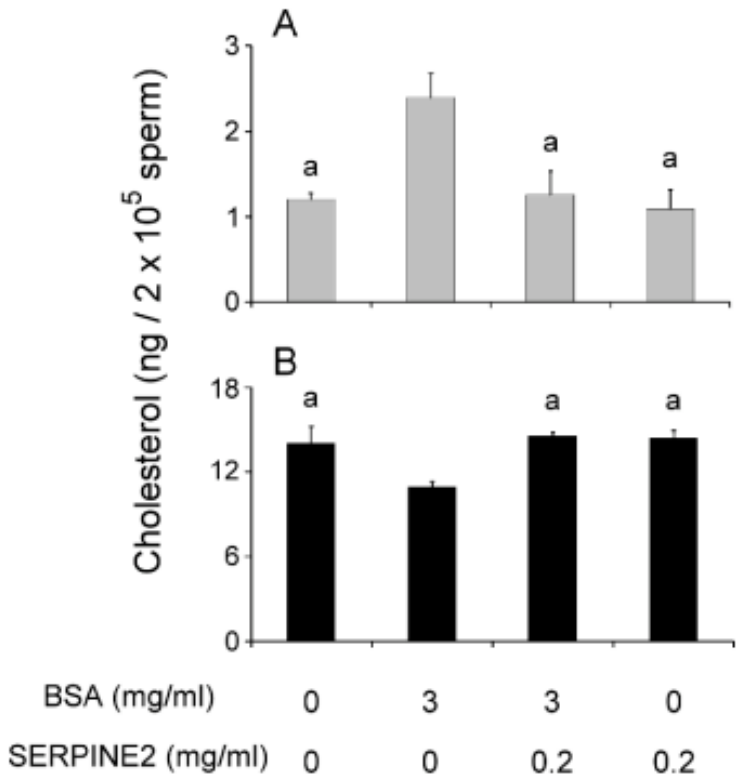


FIG. 8. Effect of SERPINE2 on BSA-induced cholesterol removal from capacitated sperm. Epididymal spermatozoa were capacitated in medium with BSA and/or SERPINE2 for 90 min. Cholesterol was extracted from the medium (A) and sperm (B). Data are means ± SD from four independent experiments. a, Significant difference compared to medium supplemented with BSA ($P < 0.01$).

Chapter 3

Study II: Involvement of the Serine Protease Inhibitor, SERPINE2, and the Urokinase Plasminogen Activator in Cumulus Expansion and Oocyte maturation

3.1 Introduction

During ovulation, SERPINE2 and PLAU expression is coordinated in mice [46], whereas SERPINE1 and PLAT expression is coordinated in monkeys and rats [90-92]. This indicates that the PA system has species-specific expression patterns in the ovary. PA expression levels are upregulated in cumulus cells just before ovulation [93] and are involved in follicle wall rupture during ovulation [38,47,48,69]. PAs and their cognate serpin inhibitors have been detected in cumulus cells [46]; however, their involvement in oocyte maturation during pre-ovulation needs clarification. Several studies have reported cumulus expansion is essential for oocyte maturation. Many cumulus proteins are required for regulating cumulus structure and cumulus expansion, such as *Hyaluronan synthase 2* (*Has2*), *PTX3*, *Versican* (*Vcan*) and *Tnfaip6*. In study II, higher SERPINE2 expression levels were detected in cumulus cells of human immature oocytes than in those of mature oocytes. Therefore, we here assumed that high

SERPINE2 levels were correlated with cumulus expansion and oocyte immaturity. To verify this, we used mouse cumulus–oocyte complexes (COCs) as a model for evaluating the association of SERPINE2 levels with cumulus expansion and subsequent oocyte maturation.

3.2 Materials and Methods

Ethics statement

This study was approved by the Mackay Memorial Hospital Institutional Review Board (reference number 09MMHIS024) with written consent for the use of human cumulus cells. Written consent for the use of cumulus cells was obtained from 20 patients undergoing intracytoplasmic sperm injection treatment. All animals contributed to this study were maintained in the Animal Center at the Department of Medical Research, Mackay Memorial Hospital. The animal use protocol has been reviewed and approved by the Mackay Memorial Hospital Institutional Animal Care and Use Committee with an approval number MMH-A-S-100-45. All efforts were made to minimize suffering.

Collection of human cumulus cells

Patients undergoing classical in vitro fertilization treatments at the Center of

Reproductive Medicine, Mackay Memorial Hospital, Taiwan received controlled ovarian hyperstimulation by application of the gonadotropin-releasing hormone antagonist protocol. COCs from follicles >14 mm were collected using transvaginal ultrasound and a 16-gauge needle and were exposed to 80 IU hyaluronidase in Quinn's Advantage Fertilization medium (Sage BioPharma, Bedminster, NJ) for 20 s at 37°C to dissolve hyaluronan. Of the 46 COCs, 26 and 20 had mature and immature oocytes, respectively. The cumulus cells were individually separated from the COCs under an Olympus SZX7 stereomicroscope (Tokyo, Japan). They were mixed with 20 μl of extraction buffer from the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, CA) for total RNA isolation and stored at −80°C until use. Cumulus cells individually collected from 10 other COCs were fixed on slides using 4% (v/v) paraformaldehyde for immunohistochemical staining.

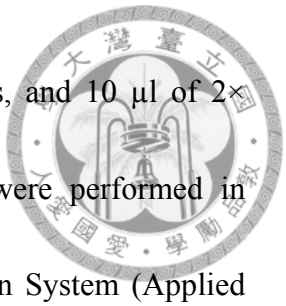
Collection of mouse cumulus cells

The mice (age, 21–24 days) were injected with 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich, St. Louis, MO) and sacrificed by cervical dislocation after 46 h. The ovaries were removed and briefly rinsed with PBS. COCs were isolated by puncturing antral follicles with a 30-gauge needle under an Olympus SZX7 stereomicroscope. To study the effect of luteinizing hormone on *Serpine2* and

Plau expression in cumulus cells during oocyte maturation, PMSG-primed mice were injected with 5 IU of human chorionic gonadotropin (hCG; Sigma-Aldrich). Ovaries were removed 3, 6, and 9 h after hCG treatment. COCs were isolated by puncturing antral follicles as described above. For post-ovulation COCs, the ovaries were removed 12 h after hCG injection, and the COCs were collected by flushing the oviducts with PBS. All COCs were treated with 150 IU hyaluronidase in PBS for 3 min at 37°C, the oocytes were removed, and cumulus cells were collected by centrifugation at 1000 ×*g* for 3 min at room temperature.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA of cumulus cells was extracted using the Arcturus PicoPure RNA Isolation Kit and directly reverse transcribed into a 50 µl first-strand cDNA pool using a High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. qRT-PCR was performed using primers (Table 1) [42]. The housekeeping genes, human ribosomal protein L19 and mouse hypoxanthine guanine phosphoribosyltransferase gene, were used as internal loading controls to normalize relative gene expression levels. PCR amplification efficiency for each tested gene was examined to ensure that it was equivalent to that of the housekeeping gene examined in a cDNA dilution series. PCR was performed in a total volume of 20 µl, containing 50 ng



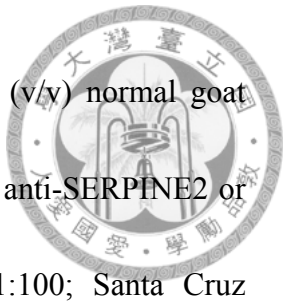
of tissue cDNA, 250 nM each of the forward and reverse primers, and 10 μl of 2× SYBR Green Master Mix (Applied Biosystems). All reactions were performed in triplicate and run on an ABI/PRISM 7500 Fast Sequence Detection System (Applied Biosystems) under the following conditions: 95°C for 20 s, and then 40 cycles at 95°C for 3 s and 60°C for 30 s. The threshold cycle (Ct) was defined as the fractional cycle number at which the reporter fluorescence, i.e., the number of amplified copies, reached a fixed threshold. Melting curve analysis was performed to verify that only a single product had formed in the reaction. The identity of the PCR products was confirmed by DNA sequencing. Relative quantification of mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method [90].

SERPINE2 proteins and anti-SERPINE2 antiserum

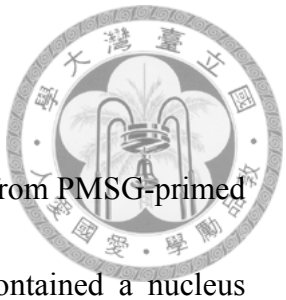
SERPINE2 proteins and anti-SERPINE2 antiserum were prepared (Chapter 1). To prepare control antiserum, anti-SERPINE2 antiserum was adsorbed onto SERPINE2-conjugated beads for removing the specific anti-SERPINE2 antibody (Chapter 1).

SERPINE2 and PLAU immunolocalization and hyaluronan status on COCs

COCs were transferred onto slides, air dried, and fixed in 4% paraformaldehyde



for 15 min. The slides were incubated in blocking solution [10% (v/v) normal goat serum in PBS for 1 h at room temperature and then incubated with anti-SERPINE2 or control antiserum (1:1000), with rabbit anti-PLAU antiserum (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), or with pre-immune rabbit serum (1:500; Jackson ImmunoResearch, West Grove, PA) in blocking solution at 4°C for 16 h. To assess the hyaluronan status in cumulus cells, slides were incubated with biotinylated hyaluronic acid binding protein (HABP; 1:200, Sigma-Aldrich, cat. no. H9910) in blocking solution at 4°C for 4 h. After washing three times in PBS with slight agitation for 5 min each, the slides were treated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch) or with tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch) in blocking solution for 1 h at room temperature or with streptavidin-conjugated Alexa Fluor 488 (1:1000; Jackson ImmunoResearch) in blocking solution for 2 h at room temperature. The slides were washed again and then counterstained with 5 μg/ml Hoechst 33258. After three brief rinses with PBS, the slides were mounted in 100 μl of ProLong Gold antifade medium (Invitrogen Molecular Probes, Eugene, OR) and photographed using an epifluorescence microscope (Olympus BX 40) equipped with an Olympus DP-70 digital camera.



***In vitro* maturation (IVM)**

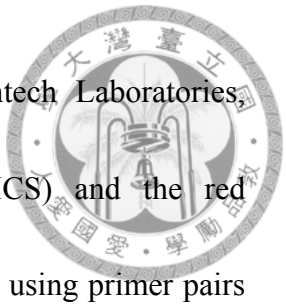
To assess the extent of cumulus cell expansion, COCs isolated from PMSG-primed ovaries that had even diameters of approximately 100 μm and contained a nucleus (germinal vesicle, GV) were cultured in IVM medium as described previously [94,95] with some modifications. The IVM medium consisted of MEMα medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 0.23 mM sodium pyruvate, 75 mU/ml follicle-stimulating hormone (FSH), 50 mg/l streptomycin, 60 mg/l penicillin, and 1 μg/l epidermal growth factor (EGF), pH 7.4. COCs were incubated in 150-μl microdrops of IVM medium supplemented with SERPINE2 (0.03, 0.06, or 0.12 mg/ml), anti-SERPINE2 antibody (1:1000), amiloride (300 μM), or PLAU (20 U; Millipore, Billerica, MA) and overlaid with mineral oil for approximately 16–20 h in a humidified 5% CO_2 atmosphere at 37°C. For control experiments, COCs were incubated in IVM medium without supplementation. After IVM, the diameters of expanded cumulus cells were scored. Next, the COCs were treated with 150 IU hyaluronidase in IVM medium for 3 min at 37°C, and cumulus cells were removed by repeated pipetting. The morphology of oocyte nuclei was observed, and the oocytes were classified as immature GV or metaphase I (MI) stage or mature (MII stage, with the extrusion of the first polar body). Oocyte maturation rate was determined after 16 h of culture by counting the number of

MII oocytes among the total oocytes used in an assay.

Treatment of COCs with small interfering RNA (siRNA)

siRNA against mouse *Serpine2* (catalog #20720-Serpine2; Dharmacon, Thermo Fisher Scientific, Lafayette, CO) and a non-targeting negative control siRNA (catalog #D-001206-05; Dharmacon, Thermo Fisher Scientific) dissolved in Accell siRNA delivery media were used according to the manufacturer's instructions. COCs isolated from PMSG-primed ovaries were incubated with 1, 2, or 3 μM siRNAs for 24 h in 150 μl MEMα medium supplemented with 10 μM milrinone (a phosphodiesterase inhibitor, Sigma-Aldrich, cat. no. M4659), 50 mg/l streptomycin, 60 mg/l penicillin, 0.23 mM pyruvate, and 3 mg/ml bovine serum albumin (Sigma-Aldrich). The optimal concentration for both siRNAs was 3 μM. After 24 h incubation, the COCs were transferred to IVM medium and cultured in a humidified 5% CO_2 atmosphere at 37°C for 16 h. Cumulus expansion and oocyte maturation were then assessed as described above. *Serpine2* mRNA levels in cumulus cells were examined by qRT-PCR. To analyze whether SERPINE2 protein was knocked down, COCs were transferred onto slides and examined by immunohistochemistry as described above.

Construction of the mouse *Serpine2* expression vector



The DNA fragment of the pIRES2-DsRed2 vector (Clontech Laboratories, Mountain View, CA) containing the multiple cloning site (MCS) and the red fluorescence protein coding region (DsRed2) was amplified by PCR using primer pairs bearing *EcoR*I sites (forward primer 5′-TTCGAATTCTGCAGTCGACGGTACC-3′, reverse primer 5′-TTTGAATTCATCTAGAGTCGCGGCCGC-3′; Fig 15a). Thirty-five PCR cycles were performed under the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR product was verified by agarose gel electrophoresis and DNA sequencing and ligated into an *EcoR*I-digested pCX-EGFP vector (Addgene, Cambridge, MA) to form the pCX-DsRed2 intermediate vector (Fig. 15b and c). Total RNA was extracted from mouse seminal vesicles using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA with a High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. The 1220-bp full-length mouse *Serpine2* cDNA (NCBI Reference Sequence NM_009255.4) was amplified by RT-PCR from the cDNA pool using a *Serpine2* primer pair (forward primer 5′-GAAGGAACCATGAATTGGC-3′, reverse primer 5′-TTCCTTTGTCTGTCCTTCAGG-3′). Thirty-five cycles of PCR were performed under the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR product was verified by agarose gel

electrophoresis and DNA sequencing and cloned into the pGEM-T Easy vector (Promega, Madison, WI) by TA cloning. The full-length *Serpine2* cDNA was excised with *Xma*I and cloned into MCS of the pCX-DsRed2 vector to create the *Serpine2* expression vector pCX-Serpine2-DsRed2 (Fig. 15d). The construct was sequenced to verify the sequence and orientation of the reading frame. This construct enabled the simultaneously translation of both SERPINE2 and DsRed2 for monitoring SERPINE2 protein expression by red fluorescence.

Serpine2 overexpression in COCs

COCs isolated from PMSG-primed ovaries were transfected with 500 ng of the *Serpine2* expression vector pCX-Serpine2-DsRed2 or the vehicle vector pCX-DsRed2 using PolyJet DNA *In Vitro* Transfection Reagent (SignaGen Laboratories, Gaithersburg, MD) in 150 μl of MEMα medium supplemented with 10 μM milrinone as mentioned above but without FSH and EGF, for 12 h. The COCs were washed three times using IVM medium, transferred to fresh medium, and cultured in a humidified 5% CO_2 atmosphere at 37°C for 16 h. Cumulus expansion and oocyte maturation were assessed as described above. *Serpine2* mRNA levels in cumulus cells were examined by qRT-PCR.

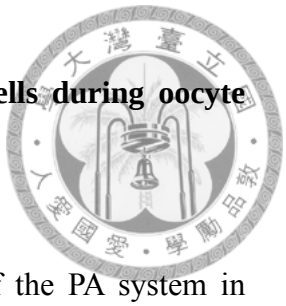
Statistical analysis

Data are presented as means ± SD. Differences were analyzed by one-way analysis of variance followed by the Bonferroni *post hoc* test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant.

3.3 Results

SERPINE2 was highly expressed in cumulus cells of immature human oocytes

We analyzed the expression levels of the four *SERPIN*s of the PA system in cumulus cells of the mature human oocyte by qRT-PCR and found that *SERPINE2* were the most highly expressed (Fig. 9A). Next, we compared *SERPINE2* mRNA expression levels in cumulus cells collected from mature and immature human oocytes. Cumulus cells from immature oocytes expressed significantly ($P < 0.0001$) higher *SERPINE2* mRNA levels than those from mature oocytes (Fig. 9B). Similarly, considerably more SERPINE2 protein was detected in cumulus cells from immature human oocytes at the GV and MI stages (Fig. 9C, a and b, respectively) than in those from mature MII oocytes or with the control staining of MII oocytes (Fig. 9C, c and d, respectively). Other similar cases are shown in Fig. 16.



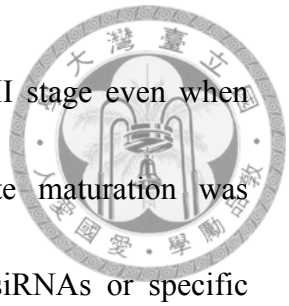
Serpine2 and _Plau_ were highly expressed in mouse cumulus cells during oocyte maturation

We analyzed the expression profiles of the four *Serpin*s of the PA system in cumulus cells surrounding mature mouse oocytes. Similar to the results with human cumulus cells, *Serpine2* mRNA was the most highly expressed in mouse cumulus cells (Fig. 10A). Next, we analyzed the gene expression patterns of SERPINE2-targeted serine proteases in the cumulus cells of mature mouse oocytes using qRT-PCR. *Plau* mRNA was the most highly expressed, followed by *Plat* and *Prss8* mRNAs. *F2* mRNA was almost undetectable (Fig. 10B). To examine the *in vivo* expression pattern of S*erpine2* and *Plau* mRNAs in mouse cumulus cells during oocyte maturation, the cumulus cells were collected at various intervals during gonadotropin-induced oocyte maturation. S*erpine2* mRNA was highly expressed 46 h after PMSG treatment and reached a maximum level 3 h after hCG administration, gradually decreasing to its lowest level 12 h after hCG administration (Fig. 10C). *Plau* mRNA was at a low level 46 h after PMSG treatment; however, it peaked 3 h and 6 h after hCG treatment and then gradually decreased to a low level after 12 h (Fig. 10D). The relative changes in *Plau* mRNA levels were much greater than the changes in *Serpine2* mRNA levels (Fig. 10C and 10D). Expression of SERPINE2 and PLAU proteins was consistent with their mRNA expression in cumulus cells. SERPINE2 was at a relatively high level following

PMSG administration and 3 h after hCG treatment (Fig. 10E, a and b). PLAU was at a relatively low level after PMSG treatment but peaked approximately 3–6 h after hCG treatment (Fig. 10F, a–c). From 6 h after hCG on, SERPINE2 protein levels were gradually decreased to a very lower level (Fig. 10E, c–f); on the contrary, PLAU protein levels were still at higher levels at 6 h after hCG (Fig. 10F, c) and then sharply decreased to a very low level thereafter (Fig. 10F, d–f).

Serpine2 silencing or SERPINE2 protein blockage had no effect on cumulus expansion and oocyte maturation _in vitro_

IVM is often used to culture compact immature oocytes collected from PMSG-primed ovaries (Fig. 11A, a) for developing MII mature oocytes with fully expanded cumulus cells (Fig. 11A, b). To examine the effect of *Serpine2* silencing on oocyte maturation in cumulus cells, siRNA was used to knockdown *Serpine2* mRNA expression during IVM. Cells were also treated with SERPINE2 antiserum to examine the effect of blocking SERPINE2 protein. No detrimental effects on COC structure or morphology (Fig. 11A, c–e) or on the extent of cumulus expansion (Fig. 11B) were observed, although *Serpine2* mRNA was significantly decreased ($P < 0.0001$) and SERPINE2 protein was knocked down by the introduction of *Serpine2* siRNA (Fig. 11C and Fig. 17, respectively). The treatments had no effect on oocyte maturation (Table 2).



As shown in Fig 11D, more than 70% of oocytes reached the MII stage even when S*erpine2* mRNA in cumulus cells was knocked down. Oocyte maturation was comparable in the media with and without control or *Serpine2* siRNAs or specific anti-SERPINE2 antiserum. Taken together, these findings indicated that eliminating SERPINE2 in cumulus cells had no apparent effect on cumulus expansion and oocyte maturation.

SERPINE2 overexpression in cumulus cells impaired cumulus expansion and oocyte maturation *in vitro*

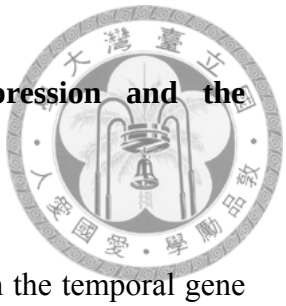
To test whether high SERPINE2 levels affected cumulus expansion and oocyte maturation, mouse COCs were transfected with a vector carrying *Serpine2*. The COC morphology was symmetrical with the outward expansion pattern of cumulus cells from the oocyte in both the untreated control and after transfection with control plasmid DNA (Fig. 12, a and b); however, the cumulus cell was compact or in an unexpanded state after transfection with the *Serpine2* plasmid (Fig. 12A, c). SERPINE2 protein was significantly overexpressed in cumulus cells after transfection and culturing for 16 h, although most of the protein expression was in the outer layer of cumulus cells (Fig. 12A, d). Similarly, exogenously added SERPINE2 resulted in compact, unexpanded cumulus cells that tightly encircled the oocyte (Fig. 12A, e–g). *Serpine2* mRNA was

significantly overexpressed in cumulus cells after transfection and culturing for 16 h (Fig. 12B). *Serpine2* overexpression significantly reduced the extent of cumulus expansion compared to transfection with the control plasmid ($P < 0.0001$; Fig. 12C, gray bars). Exogenous SERPINE2 also significantly inhibited cumulus cell expansion compared to the control (Fig. 12C, black bars). *Serpine2* overexpression in cumulus cells or exogenously added SERPINE2 significantly reduced oocyte maturation, with most oocytes halting at the MI stage (Table 3). Introduction of the *Serpine2* plasmid into cumulus cells significantly reduced oocyte maturation by approximately 45% compared with the control group (Fig. 12D, gray bars). SERPINE2 supplemented exogenously also significantly reduced oocyte maturation by approximately 26–42% (Fig. 12D, black bars).

PLAU protein was involved in cumulus expansion and oocyte maturation

Since PLAU was the most highly expressed serine protease in cumulus cells, we examined PLAU effects on cumulus expansion and oocyte maturation. COC expansion was visible at 6 h of culture (Fig. 13A, a) and had fully expanded cumuli with an average diameter of 236 μm after approximately 16–20 h of culture (Fig. 13A, b, and 13B, open bar). PLAU supplementation significantly expanded the COCs to an average diameter of 291 μm (Fig. 13A, d, and 13B, gray bar), and the expansion occurred earlier

at 6 h during IVM compared with that in the control group (Fig. 13A, a and c). Furthermore, the PLAU-supplemented cumulus cells degraded earlier (at 20 h) than the control cells (Fig. 13A, b and e), which generally degraded at 24 h. To determine whether the PLAU effect on oocyte maturation was specific, amiloride, a specific inhibitor of PLAU, was added to the IVM medium. As shown in Fig. 13A, f, and 5B (black bar), cumulus expansion was significantly diminished, and cumulus cells remained encircling the GV oocyte at 20 h, similar to the effects of SERPINE2 addition (Fig. 13A, g, and 4C, black bars). To further demonstrate that the inhibition of cumulus expansion was due to PLAU suppression, amiloride or SERPINE2 was coincubated with PLAU during IVM. Intriguingly, the COC morphology appeared normal (Fig. 13A, g and h), and the extent of cumulus cell expansion was comparable to that in the control group (Fig. 13B, hatched bars). PLAU significantly promoted oocyte maturation ($P < 0.05$), whereas amiloride significantly reduced oocyte maturation ($P < 0.0001$) (Fig. 13C and Table 4). Coincubation with PLAU and amiloride or SERPINE2 (Fig. 13C, hatched bars) reduced maturation to levels comparable with the control group (approximately 53% and 56%, respectively, vs. 67% for the control). Taken together, these data suggested that PLAU was involved in cumulus expansion and oocyte maturation and that its effects could be modulated by SERPINE2.



Excessive PLAU and SERPINE2 altered matrix gene expression and the hyaluronan status of cumulus cells during IVM

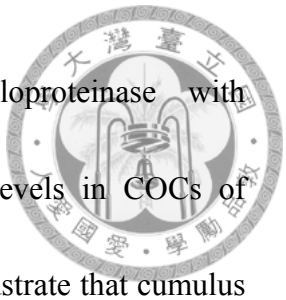
To examine the effect of excessive PLAU and SERPINE2 on the temporal gene expression pattern of the matrix genes in cumulus cells, cumulus cells at 3, 6, and 16 h, the critical time points, during IVM culture were collected from COCs and analyzed by qRT-PCR. PLAU significantly enhanced but SERPINE2 significantly down-regulated cumulus hyaluronan synthase 2 (*Has2*) mRNA levels at 3 and 6 h of IVM compared with that in the control IVM group (Fig. 14A, a). *Vcan* mRNA levels in cumulus cells were significantly diminished by exogenous SERPINE2 at 3 and 6 h and by PLAU at 6 h of IVM, but with a PLAU-induced surge at 16 h of IVM (Fig. 14A, b). Cumulus *Tnfaip6* mRNA levels were enhanced by exogenous PLAU after 3 h of IVM; however, this enhancement disappeared at 6 and 16 h of IVM culture. SERPINE2 showed no effect on *Tnfaip6* mRNA expression at all the time points (Fig. 14A, c). Furthermore, exogenous PLAU and SERPINE2 had no effect on *Ptx3* mRNA expression in cumulus cells (Fig. 14A, d). HAS2 is a critical enzyme required for matrix hyaluronan synthesis (63). Since *Has2* mRNA expression is the most affected, we analyzed the hyaluronan status of cumulus cells during IVM. After 6 h of culturing, mouse COCs showed moderate expansion with relatively high hyaluronan contents around the cumulus matrix (Fig. 14B, a and e), in contrast to the negative staining control (Fig. 14B, b and f).

Hyaluronan staining looked over the cell; however, the staining was clearly outside of the cumulus cell when ovarian tissue slides were stained. Thus, this staining pattern is probably caused by the steric stacking of cumulus cells (Fig. 18). Intriguingly, exogenous PLAU increased hyaluronan contents (Fig. 14B, c and g), while SERPINE2 supplementation decreased the contents on the cumulus matrix compared with that in the control group (Fig. 14B, d and h).

3.4 Discussion

PLAU and SERPINE2 were the most abundant PA and PA inhibitor, respectively, in murine cumulus cells, and their gene expression levels were coordinately regulated during gonadotropin-induced oocyte maturation (Fig. 10). SERPINE2 decreased rapidly 6 h after hCG administration, consistent with apparent cumulus expansion, whereas PLAU remained at a high level. Thus, the net proteolytic activity of PLAU may contribute to the initiation of cumulus expansion. This interplay appears to suggest that the net activity of PLAU may be crucial for cumulus expansion and subsequent oocyte maturation. Furthermore, we found that PLAU depletion via its specific inhibitor, amiloride, largely impaired these biological processes (Fig. 12). Hagglund *et al*. reported high *Serpine2* mRNA levels and low *Plau* mRNA levels in mouse cumulus

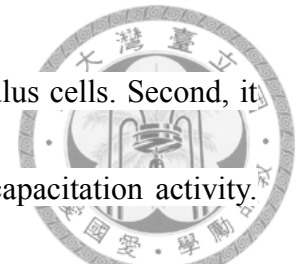
cells [46] and suggested that SERPINE2 may provide inhibitory activity for protecting the mucified COC matrix from proteolytic degradation. However, we found that cumulus cells expressed both *Plau* and *Serpine2* mRNA and protein during gonadotropin-induced oocyte maturation (Fig 10). Furthermore, *in vivo*, the granulosa cells, which are far more numerous, may also produce these proteins. Hence, we examined their relative expression in cumulus and granulosa cells after hCG 3, 6, and 9 h by immunohistochemistry. Granulosa cells expressed these proteins at levels similar to cumulus cells, especially at 3 and 6 h of hCG treatment (Fig. 19). PLAU and SERPINE2 detected in mural granulosa cells may also become associated with the COC matrix during cumulus expansion. We compared cumulus SERPINE2 and PLAU levels in COCs treated with hCG *in vivo* or cultured *in vitro*. Cumulus SERPINE2 levels had no significant difference at 3 h; however, they were significant higher at 6 and 9 h of culturing *in vitro* compared with hCG-treated *in vivo* (Fig. 20). This could be coordinated by FSH and EGF since it was found that FSH could enhance, while FSH coupled with EGF, 1 ng/ml in this study, could further enhance SERPINE2 expression in bovine granulosa cells cultured *in vitro*. Furthermore, cumulus PLAU levels were significant higher at 3 and 6 h of hCG-treated *in vivo* than culturing *in vitro* despite no changes found thereafter (Fig. 20). This was revealed that PLAU synthesis is repressed in FSH-stimulated COCs by an oocyte-soluble factor [50]. It is of interest that two



cumulus matrix genes, *Adamts1* (a disintegrin and metalloproteinase with thrombospondin-like motifs) and *Vcan*, have higher expression levels in COCs of hCG-treated *in vivo* than those culturing *in vitro* [96]. These data illustrate that cumulus matrix genes' expression is altered when COCs are cultured *in vitro*. Our results may suggest that SERPINE2 has only limited influence or that other inhibitors may be involved in the regulation of the activity of PLAU and other proteases. This is supported by our result that siRNA silencing of *Serpine2* in cumulus cells had no effect on cumulus expansion or oocyte maturation (Fig. 11). Since the siRNA efficiency is still not high enough, we cannot rule out the possibility that the remaining residual SERPINE2 is enough for maintaining normal function. Furthermore, Murer *et al*. reported that *Serpine2* knockout in mice did not result in female infertility [97]. These results appear to indicate that SERPINE2 can be depleted and compensated for by other factors, but that its overexpression hinders cumulus ECM remodeling by proteases and thus impairs cumulus expansion and subsequent oocyte maturation. Cumulus expansion includes limited ECM remodeling and requires sophisticated regulation of proteases and protease inhibitors. A convincingly characterized example is that ADAMTS1 cleavage of VCAM mediates essential remodeling of the COC matrix during ovulation [98]. PA expression in cumulus cells is species-specific. PLAT is the major PA expressed in bovine granulosa cells [99] and rat and human cumulus cells [90,91] (Fig. 21), whereas

PLAU is the major PA detected in murine granulosa and cumulus cells [46]. Ny *et al*. found that plasmin activity in the mouse ovary was increased 2–8 h after hCG treatment, and most of the activity was generated by PLAU [71]. D'Alessandris *et al*. found that mouse PLAT and PLAU activities dramatically increased between 16 h and 20 h of IVM and concluded that both PAs might function to destabilize the expanded COC matrix [50]. Several studies also demonstrated that PAs play a crucial role in follicle wall rupture during ovulation [50,51,90,100,101]; however, their function in oocyte maturation during pre-ovulation remained unclear. Our study provides the first evidence that PLAU and its inhibitor SERPINE2 are involved in murine cumulus expansion and oocyte maturation. Cumulus matrix genes, e.g., *Has2* and *Vcan*, are induced and these genes normally peak around 4–6 h after treatment with hCG *in vivo* or FSH/EGF *in vitro* [97]. The temporal cumulus matrix *Has2* and *Vcan* expression and hyaluronan status were altered by exogenous supplementation of SERPINE2 and PLAU during IVM (Fig. 14). *Has2* expression is correlated with hyaluronan synthesis, which is necessary for cumulus expansion [102]. SERPINE2 down-regulated *Has2* expression; thus, reducing the matrix hyaluronan contents. VCAM, an important cumulus matrix proteoglycan, is involved in cumulus expansion [98]. SERPINE2 also down-regulated *Vcan* expression at critical time points of cumulus expansion during IVM. These findings may explain why SERPINE2 resulted in compact, unexpanded cumulus cells

(Fig. 12A). On the other hand, PLAU up-regulated *Has2* expression and increased hyaluronan contents. These effects may cause earlier expansion and degradation than that seen in the control group (Fig. 13A). Many hyaluronan-binding proteins, e.g., ITIH [60], PTX3 [61,62], TNFAIP6 [1], and VCAN [98], as well as their interactions [2,61,63], have been shown to be crucial for cumulus structural integrity. We here demonstrate that two new components, SERPINE2 and PLAU, expressed in the cumulus ECM play roles in cumulus expansion and oocyte maturation. PLAU supplemented exogenously led to early cumulus expansion and matrix degradation and enhanced oocyte maturation (Fig. 13). This is probably through up-regulation of *Has2* expression and increased hyaluronan contents in the cumulus matrix (Fig 14). During fertilization, ejaculated sperm are necessary to penetrate the cumulus cells mass, and then bind to zona pellucida prior to fusion with the oocyte in the oviduct. Our first study demonstrated that SERPINE2 protein bound to sperm acrosome and inhibited sperm capacitation. Interestingly, we also found a small amount of SERPINE2 was still expressed in ECM of fully expanded cumulus cells in the oviduct after ovulation. Why doesn't the SERPINE2 of cumulus cells affect sperm capacitation when sperm penetration through cumulus mass in vivo? I propose the following explanation: First, Proteins often consist of multiple domains with different functions, so one protein may have multiple functions in different the cell and tissue. Base on our researches, we



suggest SERPINE2 may have different functions in sperm or cumulus cells. Second, it takes time and sufficient SERPINE2 to inhibit or reverse sperm capacitation activity. Sperm penetrate cumulus cell mass within 3-5 minutes, and the cumulus cells of mature oocytes express very little SERPINE2. These small amounts of SERPINE2 may not be enough to inhibit sperm capacitation within 5 minutes. Third, we observed that acrosome reaction has been triggered in most sperm penetrating cumulus cells, consistent with the findings demonstrated by Mayuko Jin et al in 2011 [64]. Therefore, SERPINE2 of cumulus cells may doesn't affect sperm capacitation.

3.5 Conclusion

The present results support the involvement of SERPINE2 and its cognate serine protease PLAU in cumulus expansion and subsequent oocyte maturation. Depletion or elimination of SERPINE2 expression has no effect on cumulus expansion and oocyte maturation; however, high SERPINE2 levels bound to the cumulus ECM could down-regulate *Has2* and *Vcan* expression and decrease matrix hyaluronan contents, leading to suppressed cumulus expansion and oocyte maturation. On the other hand, PLAU supplementation to IVM culture medium up-regulated *Has2* expression and increased matrix hyaluronan contents could be a potential therapeutic strategy for

rescuing immature human oocytes collected during ART procedures, although further study is required.

3.6 Figures

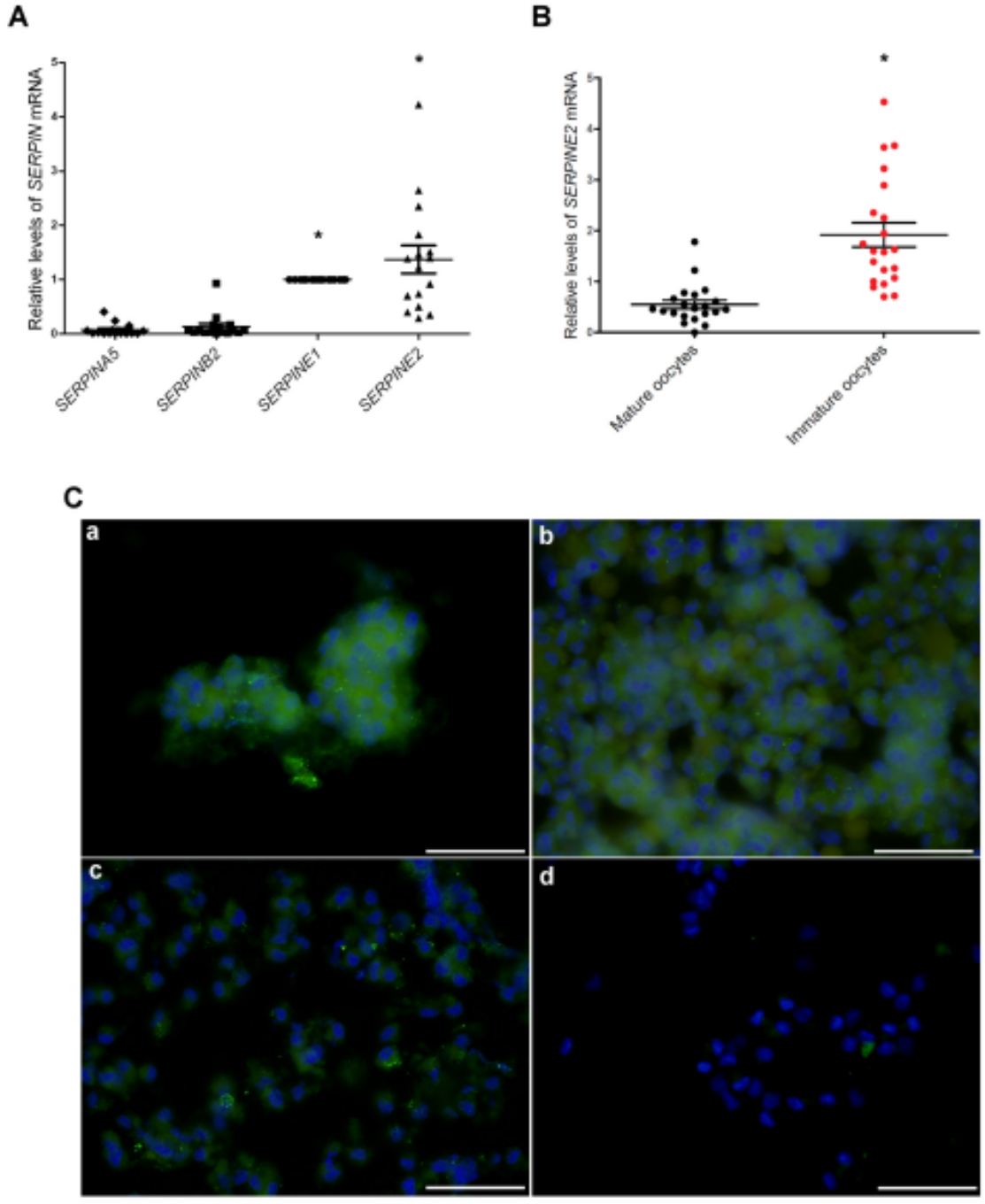


FIG. 9. SERPINE2 expression in cumulus cells of the human oocyte. A, qRT-PCR revealed the relative SERPIN mRNA levels in cumulus cells of mature (n = 16) human oocytes. Bars indicate means ± SD of sixteen independent experiments each. *P<0.0001

compared with SERPINA5 mRNA. B, qRT-PCR indicated SERPINE2 mRNA levels in cumulus cells of mature (n = 21) and immature (n = 21) human oocytes. Bars indicate means ± SD of twenty-one independent experiments each. *P<0.0001 compared with mature oocytes. C, Immunofluorescent staining showed SERPINE2 protein levels in cumulus cells. Cumulus cells collected from immature oocytes at the GV (a) or MI stages (b) and from mature oocytes at the MII stage (c) were immunostained with anti-SERPINE2 antibody, and cumulus cells of MII oocytes were immunostained with the control serum (d). Scale bars, 100 μm.

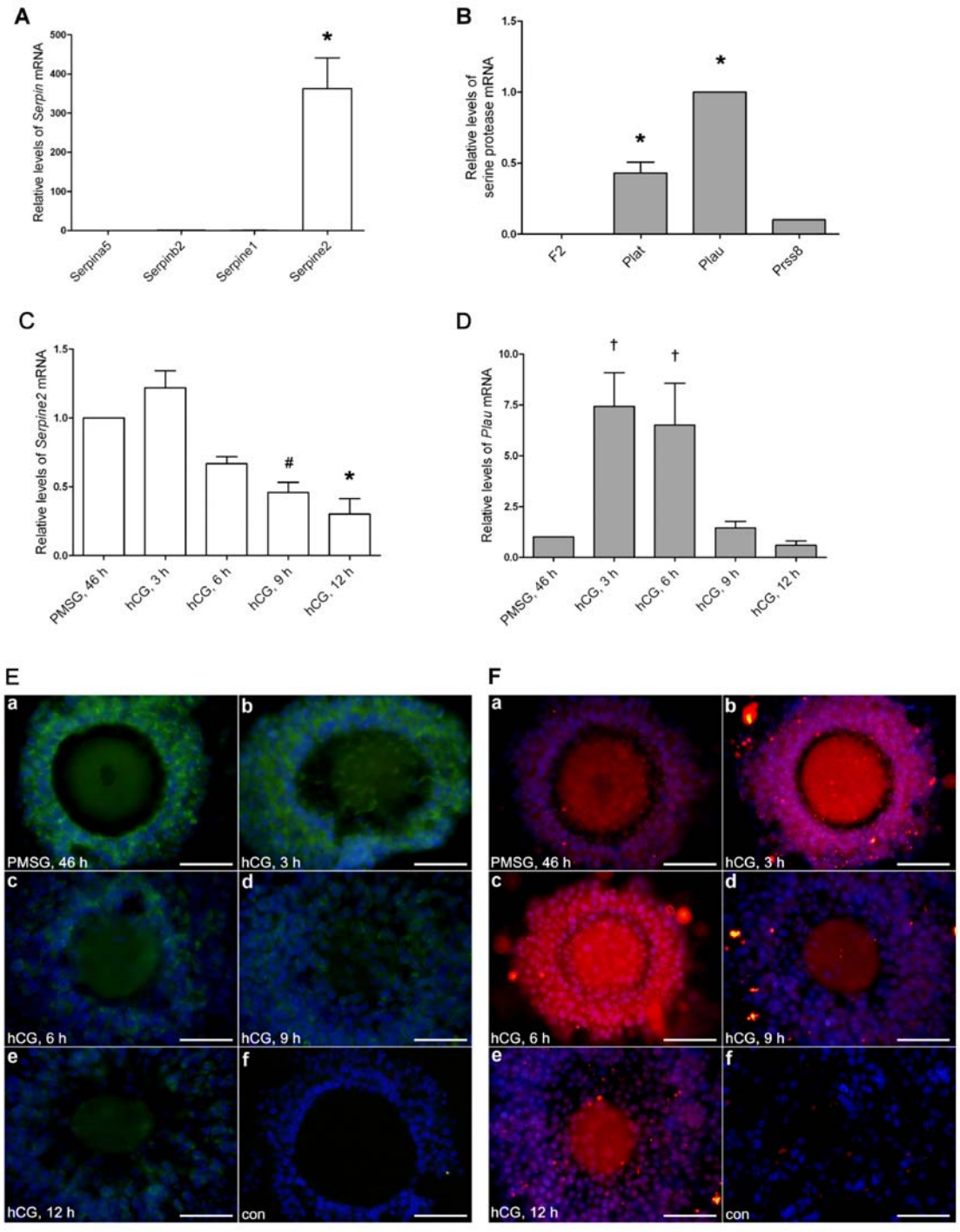


FIG. 10. *Serpine2* and *Plau* expression in mouse cumulus cells during oocyte maturation. qRT-PCR revealed the relative mRNA levels of *Serpin*s (A) and serine proteases (B) in cumulus cells surrounding mature oocytes (one mouse for one experiment). Bars indicate means ± SD of three independent experiments each. *$P<$

0.0001 compared with all *Serpin* mRNAs; **P*<0.0001 compared with *F2* mRNA. qRT-PCR revealed relative *Serpine2* (C) and *Plau* (D) mRNA levels in mouse cumulus cells surrounding developing oocytes (three mice for each group) during gonadotropin-induced oocyte maturation. Bars indicate means ± SD of three independent experiments each. $^{\dagger}P$<0.05, $^{\#}P$<0.001, **P*<0.0001 compared with the cumulus cells 46 h after PMSG injection alone (PMSG, 46 h). Immunofluorescent staining revealed SERPINE2 (E) and PLAU (F) protein levels in mouse COCs following gonadotropin treatments. COCs were fixed on slides and immunostained using anti-SERPINE2 antiserum and anti-PLAU antibody as described in the Materials and Methods: a, 46 h after PMSG; b, 3 h after hCG; c, 6 h after hCG; d, 9 h after hCG; e, 12 h after hCG administration; f, immunostaining with the control serum (con). Scale bars, 100 μm.

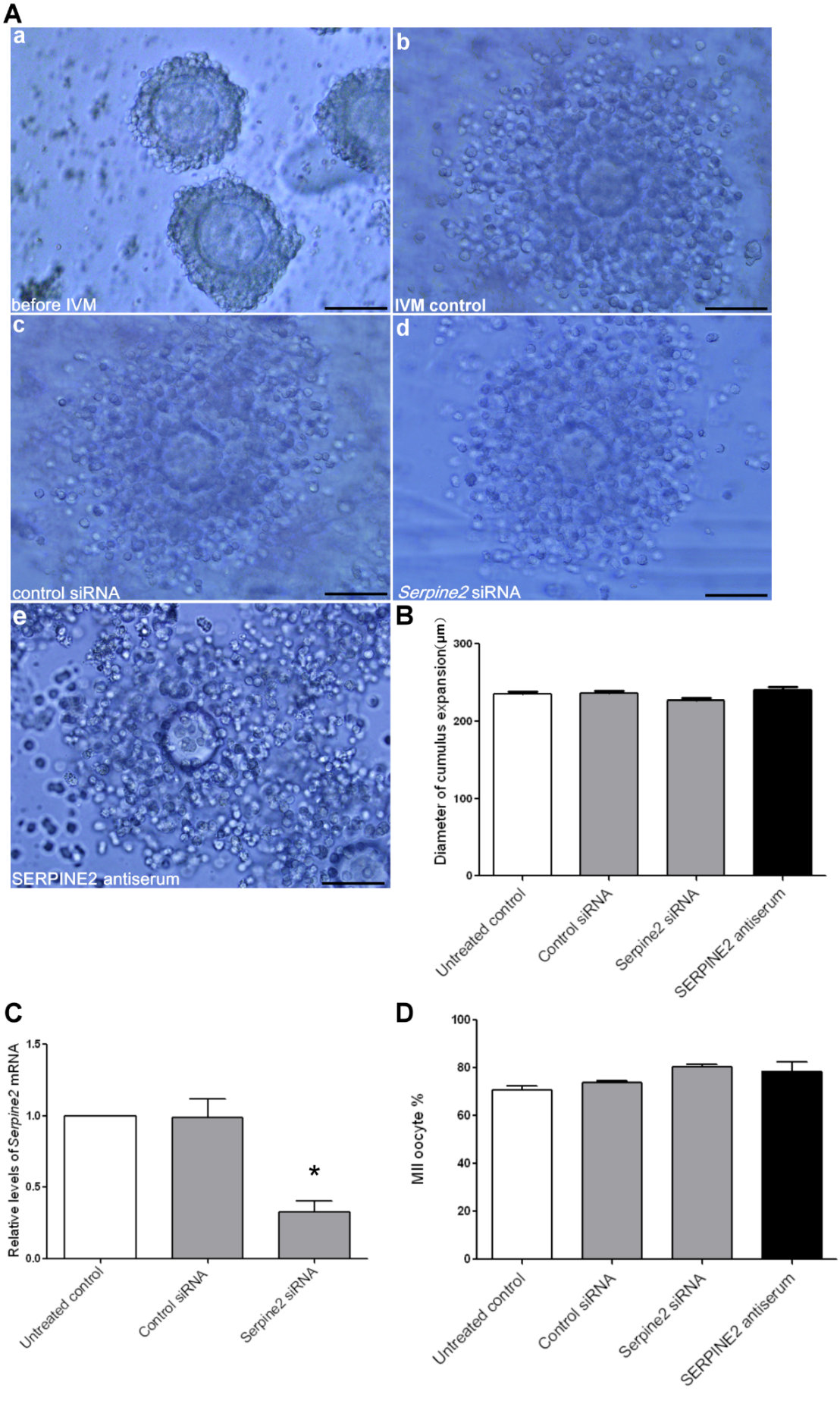

A
a
before IVM
b
IVM control
c
control siRNA
d
Serpine2 siRNA
e
SERPINE2 antiserum
B
Diameter of cumulus expansion(μm)
300
200
100
0
Untreated control
Control siRNA
Serpine2 siRNA
SERPINE2 antiserum
C
Relative levels of Serpine2 mRNA
1.5
1.0
0.5
0.0
*
Untreated control
Control siRNA
Serpine2 siRNA
D
MII oocyte %
100
80
60
40
20
0
Untreated control
Control siRNA
Serpine2 siRNA
SERPINE2 antiserum

FIG. 11. Silencing of *Serpine2* expression and antiserum blockage of SERPINE2 protein. A, The morphologies of COCs isolated from PMSG-primed ovaries (a), cultured in IVM medium for 16 h (b), and treated with control siRNA (c), *Serpine2* siRNA (d), or SERPINE2 antiserum (e) are shown. Scale bars, 100 μm. B, The extent of cumulus expansion in COCs treated as in A (b–e) are presented as the COC diameters after culturing (n = 100 for each). C, qRT-PCR revealed *Serpine2* mRNA levels in mouse cumulus cells with or without *Serpine2* siRNA. **P*<0.0001 vs. untreated control. D, MII oocyte maturation rate after 16 h of IVM culture are shown. B–D, Data represent means ± SD of four independent experiments each.

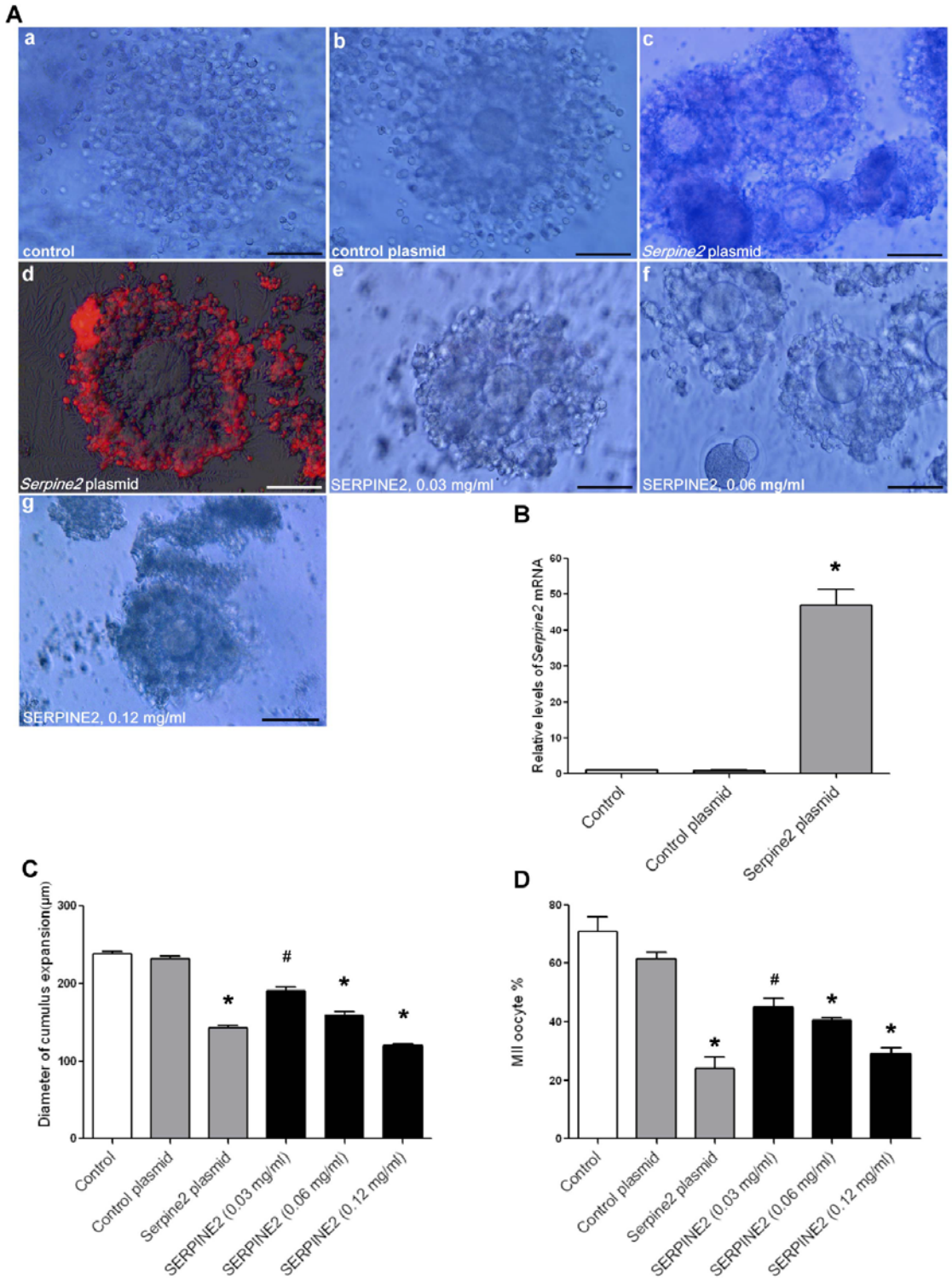


FIG. 12. Effects of *Serpine2* overexpression and addition of exogenous SERPINE2 on cumulus expansion. A, The morphologies of untreated and treated COCs after 16 h of IVM culture are shown: a, IVM control; b, transfected with the control plasmid; c,

transfected with the *Serpine2*-expressing plasmid. d, Cultured COCs transfected with the *Serpine2*-expressing plasmid were fixed on slides, and SERPINE2 protein expression was monitored by epifluorescence microscopy. The red fluorescence represents coexpression of SERPINE2 and the red fluorescent protein. e–g, COCs were incubated with 0.03, 0.06, and 0.12 mg/ml SERPINE2 protein, respectively. B, qRT-PCR revealed the relative *Serpine2* mRNA levels in cumulus cells transfected with *Serpine2*-expressing or vehicle plasmids. C, The extent of cumulus expansions are shown as COC diameters after 16 h of IVM culture (n = 100 for each group). D, MII oocyte maturation rate after 16 h of IVM culture. B–D, Data represent means ± SD for five independent experiments. $^{\dagger}P<0.05$, $^{\#}P<0.001$, $^{*}P<0.0001$ vs. untreated control or control plasmid.

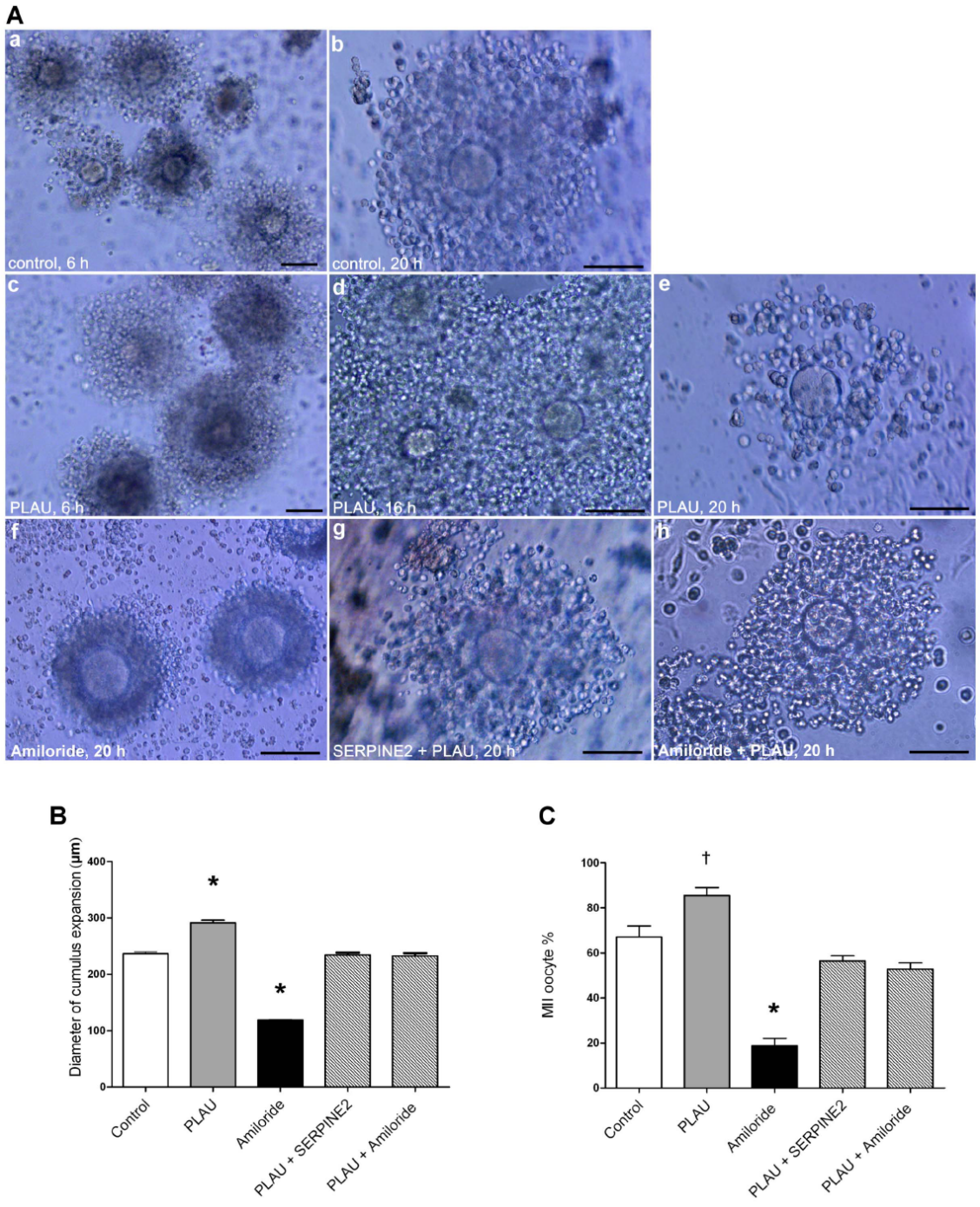


FIG. 13. Involvement of PLAU in cumulus expansion and oocyte maturation. A, COC morphologies after IVM culture under varying conditions are shown: a, control COCs after 20 h of culture; b, COCs incubated with 20 U of PLAU for 16 h; c, COCs incubated with 20 U of PLAU for 6 h; d, control COCs after 6 h of culture; e, COCs

incubated with 20 U of PLAU for 20 h; f, COCs incubated with 300 μM amiloride for 20 h; g, COCs coincubated with SERPINE2 (0.12 mg/ml) and PLAU (20 U) for 20 h; h, COCs coincubated with amiloride (300 μM) and PLAU (20 U) for 20 h. Scale bars, 100 μm. B, Cumulus expansion was measured as the COC diameters after 16 h of IVM (n = 100 each group). D, MII oocyte maturation rate after 16 h of IVM. C and D, Data represent means ± SD for six independent experiments; $^{\dagger}P<0.05$, $*P<0.0001$ vs. controls.

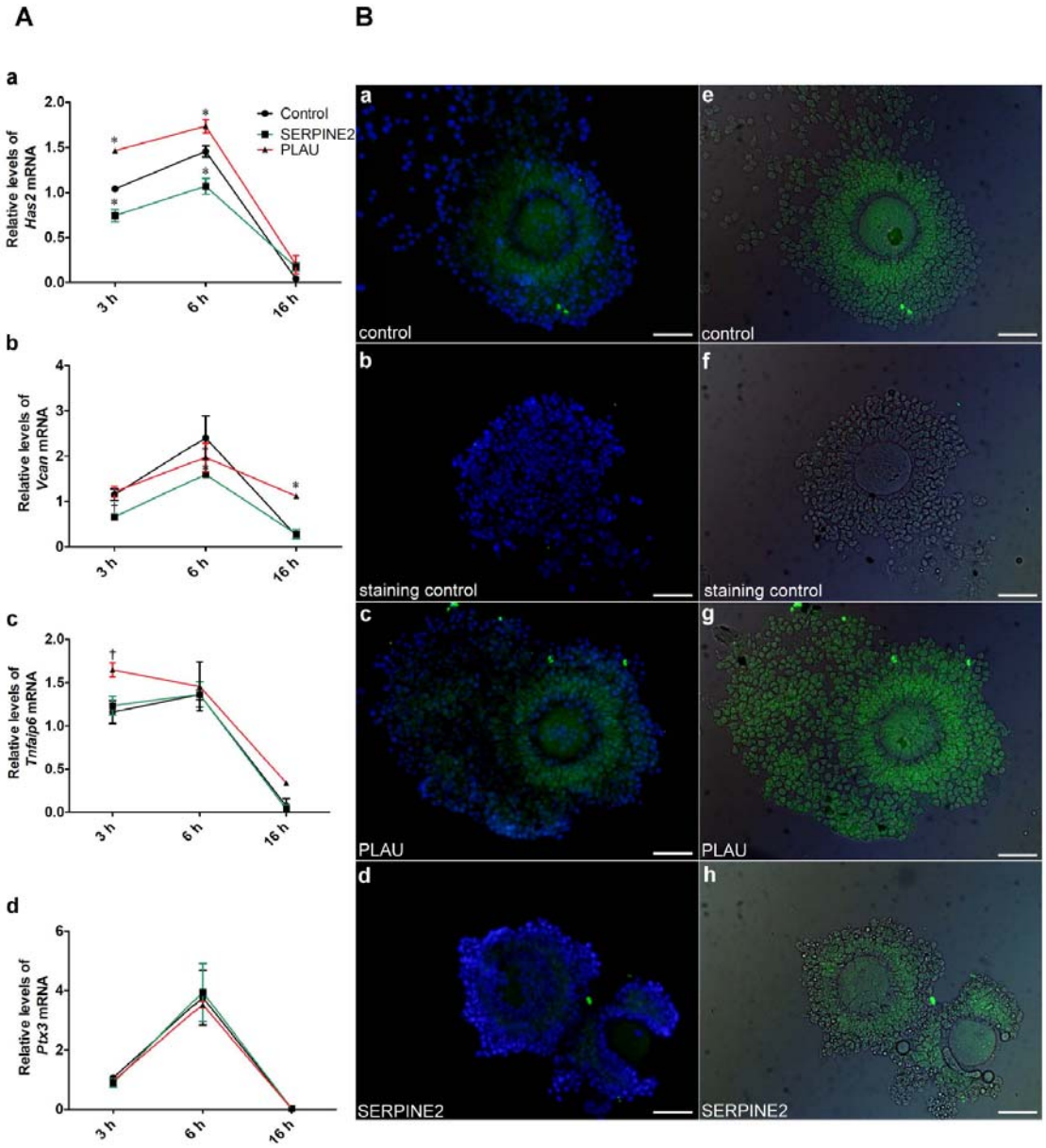


FIG. 14. Effect of exogenous PLAU and SERPINE2 on matrix gene expression and the hyaluronan status of cumulus cells during IVM. COCs were incubated with SERPINE2 (0.06 mg/ml) or PLAU (20 IU) in IVM medium for 3, 6, or 16 h. After culturing, cumulus cells were collected for qRT-PCR analyses or COCs were transferred onto slides and fixed for hyaluronan evaluation. A, qRT-PCR revealed *Has2* (a), *Vcan* (b), *Tnfaip6*, and *Ptx3* mRNA levels in cumulus cells. Bars indicate means 6 SD of three independent experiments each. $^{\dagger}P<0.05$, $*P<0.0001$ compared with the group that COCs cultured for 3 h in IVM medium. B, After culturing for 6 h, the hyaluronan

contents in untreated COCs (a and e) or COCs treated with PLAU (c and g) or SERPINE2 (d and h) were revealed by treatment with or without (staining control, b and f) HABP (green) as described in Materials and Methods. For contrast, the slides were counterstained with Hoechst 33258 (blue, a–d) or photographed under differential interference contrast microscopy (e–h). Scale bars, 100 μm.

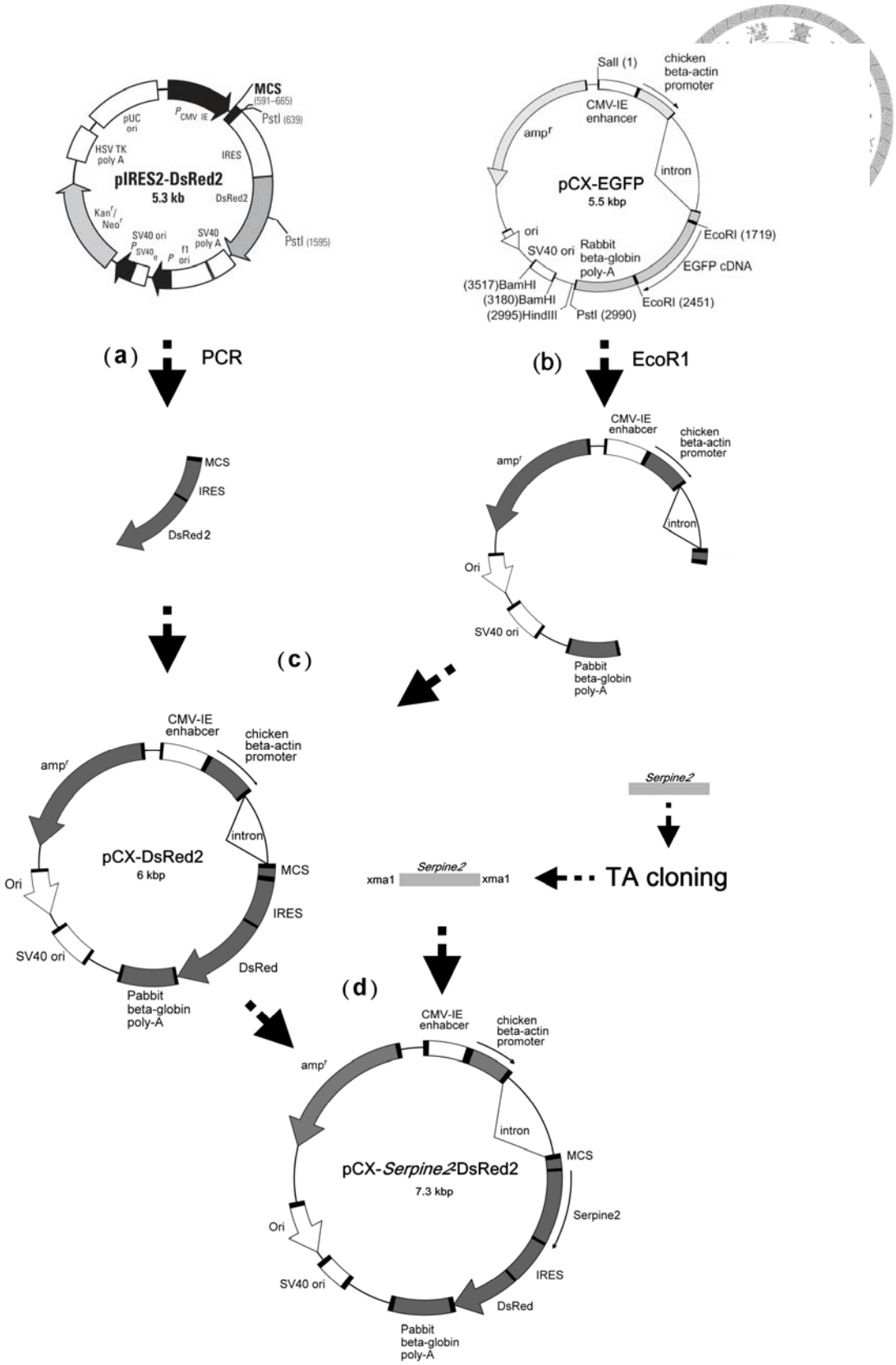
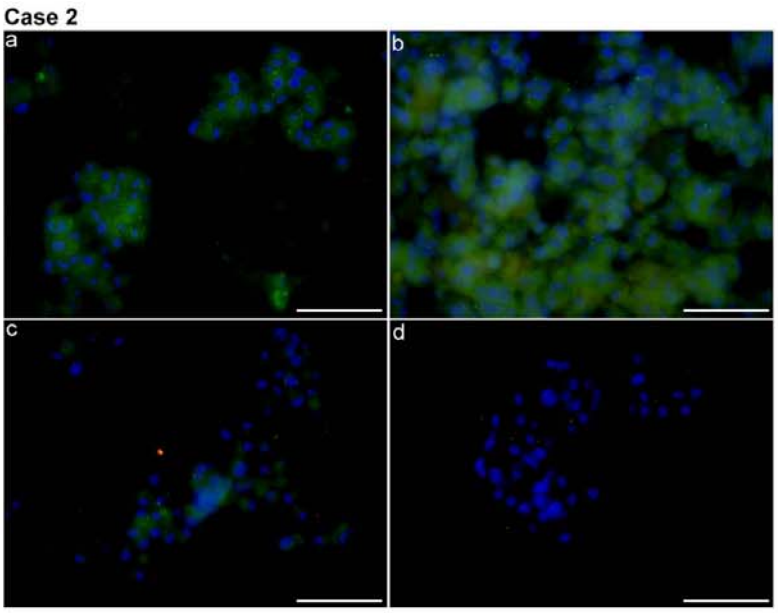
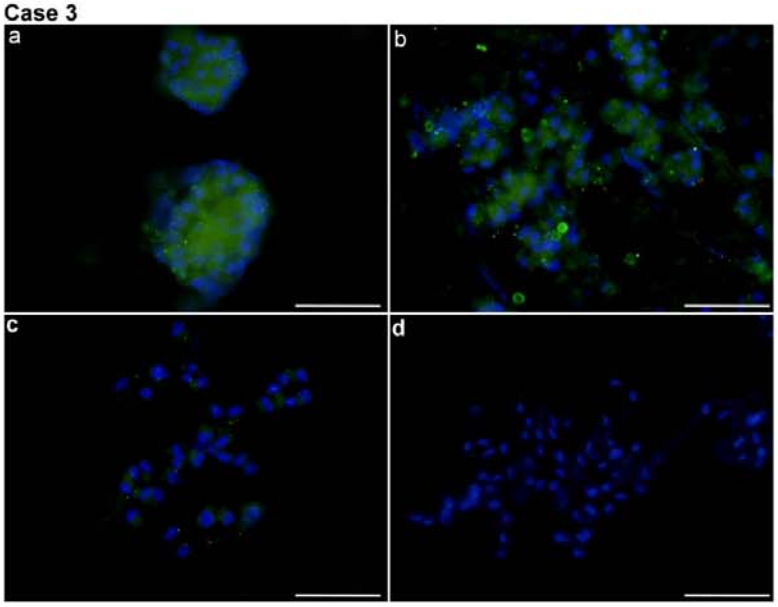


FIG. 15. Construction of the *Serpine2* expression vector. (a) A DNA fragment containing MCS and DsRed2 of the pIRES2-DsRed2 vector was amplified using primer pairs containing *Eco*RI sites. (b) The pCX-EGFP vector was digested with *Eco*RI. (c)

The DNA fragment from (a) was ligated into the *EcoR*I-digested pCX-EGFP vector to form an intermediate vector pCX-DsRed2. (d) A PCR-amplified full-length *Serpine2* cDNA propagated by TA cloning was digested with *Xma*I and cloned into the pCX-DsRed2 vector to create pCX-Serpine2-DsRed2.


Case 2
a
b
c
d


Case 3
a
b
c
d

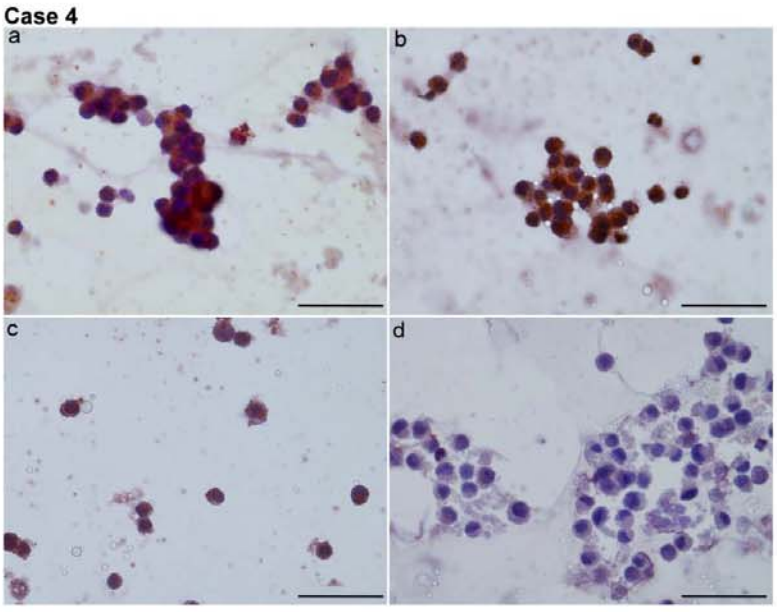

Case 4
a
b
c
d

FIG. 16. Immunofluorescence staining for SERPINE2 protein levels in human cumulus cells. COCs were collected from patients whose oocytes had all 3 nuclear stages (GV, MI, MII). Cumulus cells collected from immature oocytes at the GV (a) or MI stages (b) and from mature oocytes at the MII stages (c) were immunostained with anti-SERPINE2 antibody (green for case 2 and 3 or red for case 4), and cumulus cells of MII oocytes were immunostained with the control serum (d). For contrast, the slides were counterstained with Hoechst 33258 (blue, case 2 and 3) or hematoxylin (blue, case 4). Scale bars, 100 μm.

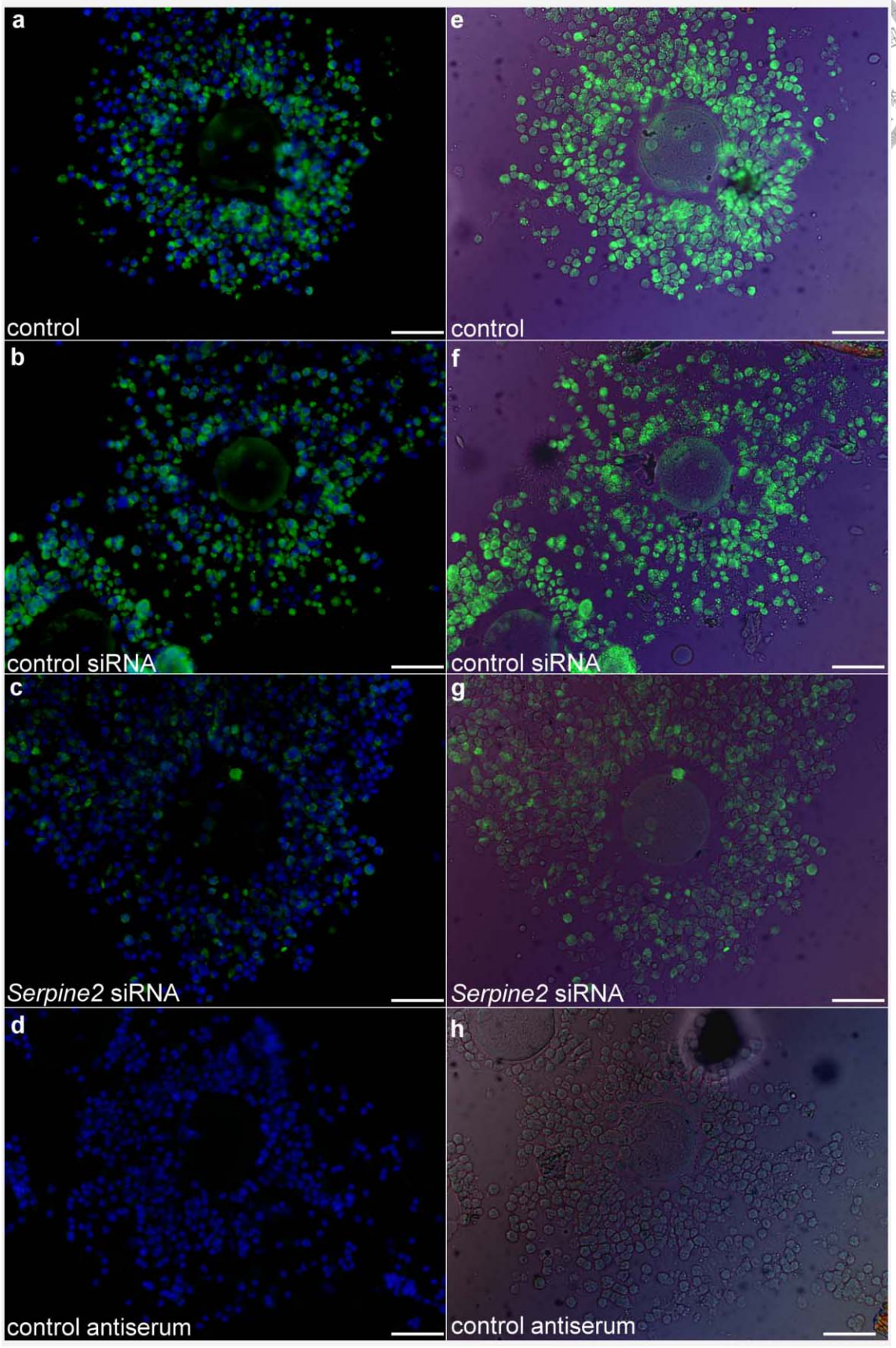


FIG. 17. Immunohistochemistry of SERPINE2 protein in cumulus cells treated with Serpine2 siRNA. COCs treated without (control, a and e) or with control siRNA (b and

f), or Serpine2 siRNA (c and g) were cultured in IVM medium for 16 h. After culturing, COCs were transferred and fixed onto slides and immunostained by anti-SERPINE2 (green) or control antiserum (d and h). For contrast, the slides were counterstained with Hoechst 33258 (blue, a–d) or photographed under differential interference contrast microscopy (e–h). Scale bars, 100 μm.

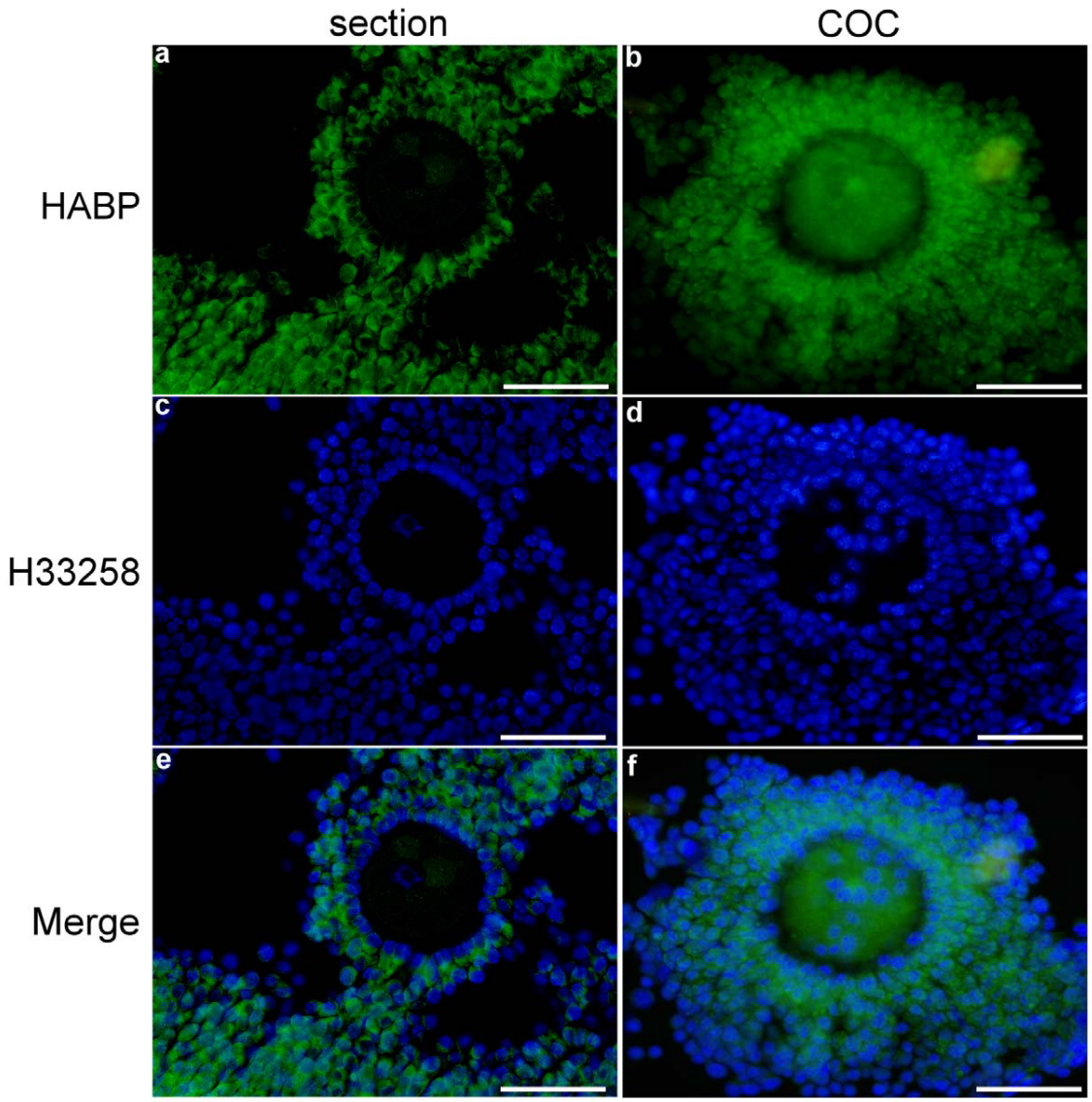


FIG. 18. Hyaluronan matrix staining of the tissue section and COC. The hyaluronan on ovarian sections that were PMSG-primed and treated with hCG for 3 h (a) or COCs that were cultured for 6 h *in vitro* and then transferred onto slides (b) were stained with HABP (green) as described in Materials and Methods. For contrast, the slides were counterstained with Hoechst 33258 (H33258, blue, c and d) and the merged images are also shown (e and f). Scale bars, 100 μm.

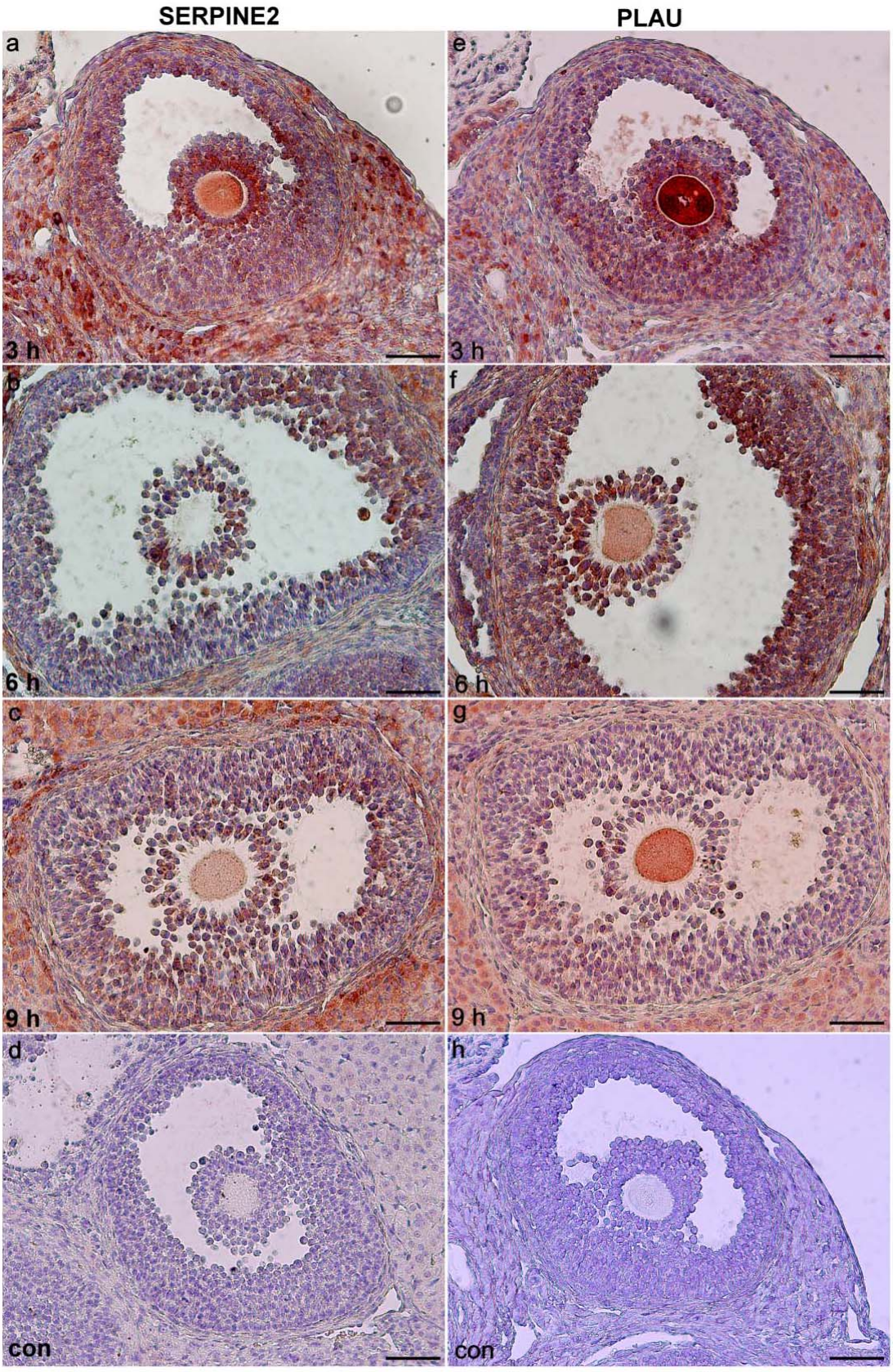


FIG. 19. Immunolocalization of SERPINE2 and PLAU in ovarian follicles during gonadotropin treatment. Ovarian sections from PMSG-primed and hCG administration

for 3, 6, and 9 h were immunostained using anti-SERPINE2 antiserum and anti-PLAU antibody as described in Materials and Methods: a and e, 3 h after hCG; b and f, 6 h after hCG; c and g, 9 h after hCG; d and h, immunostaining with the control serum (con). Scale bars, 100 μm.

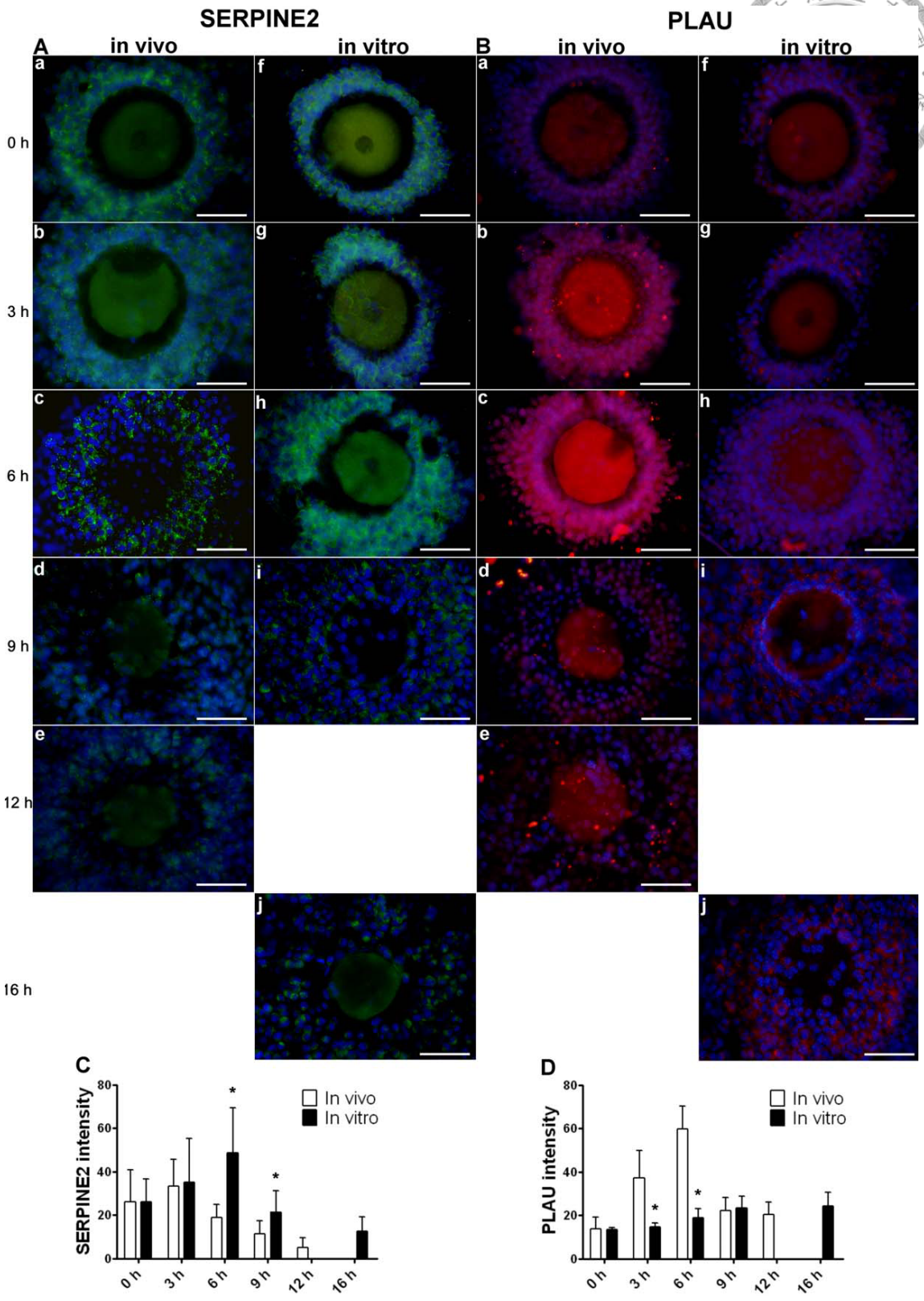


FIG. 20. Cumulus SERPINE2 and PLAU protein levels in COCs treated with hCG in vivo or cultured *in vitro*. COCs isolated from PMSG-primed ovaries (a and f), treated with hCG for 3, 6, 9, and 12 h (b–e, respectively) or IVM culture for 3, 6, 9, and 16 h

(g–j, respectively), were immunostained using anti-SERPINE2 antiserum and anti-PLAU antibody as described in Materials and Methods. The slides counterstained with Hoechst 33258 were photographed using a fluorescence microscope (Olympus BX 40) equipped with an Olympus DP-70 digital camera. The percentage of positively stained cells was determined using TissueQuest software (TissueGnostics, Vienna, Austria). A chi-square test was performed to independently compare the significance of difference in expression levels of SERPINE2 or PLAU in cumulus cells at different time points. *P<0.0001 compared with in vivo and *in vitro* samples at the same time point. Scale bars, 100 μm.

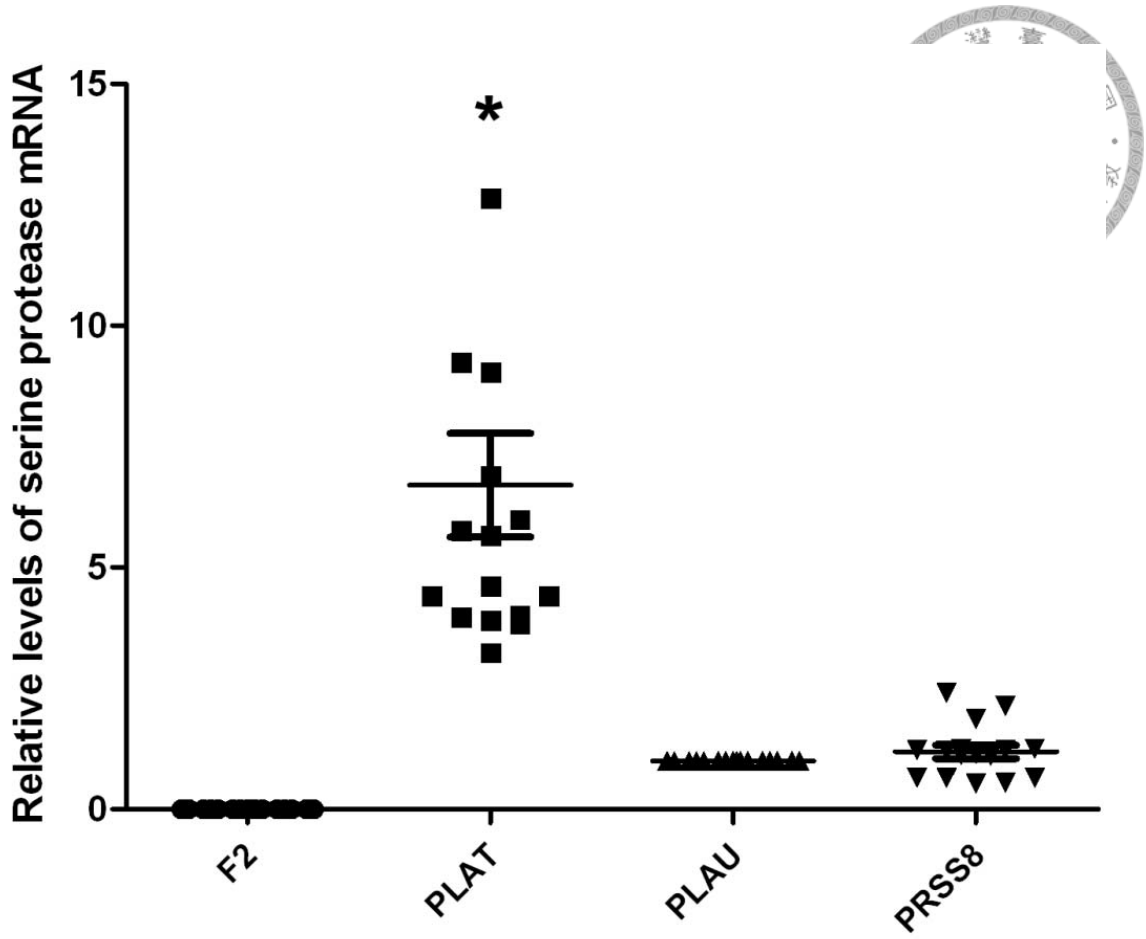


FIG. 21. PLAT expression in cumulus cells of human oocytes. qRT-PCR revealed the relative levels of serine protease mRNAs in cumulus cells of mature (n= 16) human oocytes. Bars indicate means 6 SD of sixteen independent experiments each. *P<0.0001 compared with F2 mRNA.

Table 1. Summary of real-time PCR primers.

Gene[a]	Primer	Sequence	Position	Product size (bp)
Human				
SERPINA5	F[b]	5'-CCCACACGACTGCAACATACAG-3'	1585-1606	118
	R[c]	5'-TGAGCAAGTCATCCAGCCTCTC-3'	1702-1681	
SERPINB2	F	5'-TTCTGAAGTGTTCCACCAAGCC-3'	1386-1407	100
	R	5'-TCCATGTCCAGTTCTCCCTGTC-3'	1485-1464	
SERPINE1	F	5'-TCTGTTCCAGTCACATTGCCAT-3'	1767-1788	115
	R	5'-TGCCACAGTGGACTCTGAGATG-3'	1881-1860	
SERPINE2	F	5'-TCTCATTGCAAGATCATCGCC-3'	1323-1343	97
	R	5'-CCCCATGAATAACACAGCACC-3'	1419-1399	
F2	F	5'-ATCCGCATCACTGACAACATG-3'	1667-1687	135
	R	5'-CCCATTTGATACCAGCGGTT-3'	1801-1782	
PLAT	F	5'-TCAGCTAAAGCCCAACCTCCT-3'	2055-2075	124
	R	5'-CTAATGCAATCCGTCTTTCCTG-3'	2178-2157	
PLAU	F	5'-TCACCACAACGACATTGCCTT-3'	959-979	134
	R	5'-TGATCTCACAGCTTGTGCCAAA-3'	1092-1071	
PRSS8	F	5'-TTCCCTGATGGCCTTTGGA-3'	1419-1437	120

	R	5'-CCCAAAAAGCACACCCAGAAG-3'	1538-1518	
RPL19	F	5'-TCAGCTTGTGGATGTGTTCCA-3'	406-426	93
	R	5'-TCGATCGCCACATGTATCACAG-3'	498-477	
Mouse				
Serpina5	F	5'-TCTCCATTGAGGCTACCTACAAACT-3'	1071-1095	131
	R	5'-GTGCACCATCTCAGACAACTTGA-3'	1201-1179	
Serpinb2	F	5'-TTCCGTGTGAACTCGCATGA-3'	664-683	143
	R	5'-GGAAGCAACAGGAGCATGCT-3'	806-787	
Serpine1	F	5'-CAGAGCAACAAGTTCAACTACACTGA-3'	810-835	106
	R	5'-CAGCGATGAACATGCTGAGG-3'	915-896	
Serpine2	F	5'-CAGATCATCAAGTCACGGCCT-3'	269-289	119
	R	5'-ACCGTGGAGAGCTGCTTCTTT-3'	387-367	
F2	F	5'-TTCTGTGCTGGCTTCAAGGTG-3'	1654-1674	115
	R	5'-ACCCATTTGATACCAGCGGTT-3'	1768-1748	
Plat	F	5'-AAGAGAGCAGCTCTGTTGGCAC-3'	1366-1387	112
	R	5'-AATGGAGACGATGCCTCATGC-3'	1477-1457	
Plau	F	5'-GAAGCGACCCTGGTGCTATG-3'	445-464	82
	R	5'-TTTGCTAAGAGAGCAGTCATGCA-3'	526-504	
Prss8	F	5'-AAGCTGTGACCATTCTGCTCCT-3'	117-138	63

	R	5'-CAGTCCCGTCAGCTCGGA-3'	179-162	
Has2	F	5'-AAGACCCTATGGTTGGAGGTG-3'	1245-1265	167
	R	5'-CATTCCCAGAGGACCGCTTAT-3'	1411-1391	
Vcan	F	5'-AACCAGGCGCTGATCCTTAAA-3'	10499-10519	129
	R	5'-CGGCAGTCCCATAATCCAAAC-3'	10627-10607	
Tnfaip6	F	5'-GATGGTCGTCCTCCTTTGCTT-3'	63-83	141
	R	5'-TATCTGCCAGCCCGAGCTT-3'	203-185	
Ptx3	F	5'-GGACAACGAAATAGACAATGGACTT-3'	265-289	109
	R	5'-CGAGTTCTCCAGCATGATGAAC-3'	373-352	
Hprt	F	5'-GAATCACGTTTGTGTCATTAGTGAAA-3'	752-777	62
	R	5'-TGCGCTCATCTTAGGCTTTGTA-3'	813-792	

[a]GenBank accession nos.: *SERPINA5*, NM_000624; *SERPINB2*, NM_001143818; *SERPINE1*, 3 NM_000602; *SERPINE2*, NM_001136528; *F2*, NM_000506; *PLAT*, NM_000930; *PLAU*, 4 NM_001145031; *PRSS8*, NM_002773; *RPL19*, NM_000981; *Serpina5*, NM_172953; *Serpinb2*, 5 NM_011111; *Serpine1*, NM_008871; *Serpine2*, NM_009255; *F2*, NM_010168; *Plat*, NM_008872; 6 *Plau*, NM_008873; *Prss35*, NM_178738; *Has2*, NM_008216.3; Vcan, NM_001081249.1; *Tnfaip6*, 7 NM_009398.2; Ptx3, NM_008987.3; *Hprt*, NM_013556. [b] F, forward primer. [c] R, reverse primer.

Table 2. Effects of Serpine2 siRNA and anti-SERPINE2 antiserum on oocyte maturation.

Oocyte stage	Control (%)	Control siRNA (%)	*Serpine2* siRNA (%)	SERPINE2 antiserum (%)
GV	8.49 ± 3.51	4.26 ± 1.74	4.64 ± 3.28	3.71 ± 1.58
MI	20.67 ± 2.33	22.04 ± 2.91	15.10 ± 3.26	19.96 ± 2.90
MII	70.84 ± 2.78	73.69 ± 2.51	78.42 ± 3.9	78.42 ± 1.97
Number of COCs	125	138	155	379

GV, germinal vesicle; MI, metaphase I; MII, metaphase II; COCs, cumulus–oocyte complexes. Data are means ± SD of three (groups of control, control siRNA, and Serpine2 siRNA) and four (the group of SERPINE2 antiserum) independent experiments. Percentages are based on the total number of oocytes examined.

Table 3. Effects of Serpine2 overexpression in cumulus cells and exogenously added SERPINE2 on oocyte maturation.

Oocyte stage	Control (%)	Control plasmid (%)	*Serpine2* plasmid (%)	SERPINE2[a] (%)	SERPINE2[b] (%)	SERPINE2[c] (%)
GV	7.14 ± 2.14	8.39 ± 0.65	9.52 ± 1.42	10.31 ± 2.28	7.35 ± 1.42	8.95 ± 2.34
MI	22.10 ± 1.50	17.35 ± 6.06	39.76 ± 8.59	44.67 ± 7.37	52.19 ± 3.22	62.03 ± 1.71
MII	70.76 ± 8.54	61.40 ± 5.40	26.07 ± 8.95*	45.02 ± 5.31#	40.46 ± 1.84*	29.01 ± 3.94*
Number of COCs	108	154	142	633	783	367

GV, germinal vesicle; MI, metaphase I; MII, metaphase II; COCs, cumulus–oocyte complexes. [a–c] SERPINE2 at 0.03, 0.06, and 0.12 mg/ml, respectively. Data are means ± SD of three (groups of control, control plasmid, and *Serpine2* plasmid) and five (groups of SERPINE2 in varying doses) independent experiments. Percentages are based on the total number of oocytes examined. Significant differences compared with the control: #$P<0.001$, *$P<0.0001$.

Table 4. Effects of PLAU protein on oocyte maturation.

Oocyte stage	Control (%)	PLAU, 20 U (%)	Amiloride 300 μM (%)	SERPINE2+PLAU (%)	Amiloride+PLAU (%)
GV	6.09 ± 2.43	3.53 ± 1.90	17.65 ± 4.72	4.55 ± 1.15	6.62 ± 3.13
MI	26.88 ± 4.52	10.96 ± 4.25	63.61 ± 10.24	38.97 ± 3.99	40.57 ± 4.76
MII	67.03 ± 6.48	85.51 ± 8.94†	18.73 ± 5.76*	56.48 ± 3.86	52.80 ± 4.87
Number of COCs	419	332	484	158	181

GV, germinal vesicle; MI, metaphase I; MII, metaphase II; COCs, cumulus–oocyte complexes. Data are means ± SD of six independent experiments. Percentages are based on the total number of oocytes examined. Significant differences compared with the control group: †$P<0.05$, *$P<0.0001$.

Chapter 4

Summary

To cut a long story short, we demonstrated that the SERPINE2 protein is extensively expressed in apical epithelial cells of male mouse reproductive tissues. It was present in germ cells and was found to be an intrinsic sperm surface protein. Also, more SERPINE2 proteins derived from the seminal plasma bound to ejaculated sperm, and the binding was still detected when sperm had reached the oviduct. Ejaculated sperm in the uterus seemed to be primarily uncapacitated. However, once sperm entered the oviduct, sperm capacitation occurred. Intriguingly, SERPINE2 was detected predominantly on uncapacitated sperm, indicating that SERPINE2 is lost when sperm capacitate. In addition, we demonstrated that exogenous SERPINE2 bound to epididymal sperm and inhibited BSA-induced sperm capacitation *in vitro*, thus blocking sperm–oocyte interactions. Thus, SERPINE2 possibly exerts its influence on sperm to prevent precocious capacitation and the acrosome reaction before sperm reach the oviduct where fertilization takes place. It is then removed as capacitation occurs. These findings suggest that SERPINE2 is possibly a decapacitation factor. In addition, SERPINE2 overexpression in mouse COCs or its exogenous addition to IVM medium remarkably impaired cumulus expansion, with the COCs exhibiting a compact morphology with a high proportion of GV and MI oocytes, leading to a significant

reduction in oocyte maturation. Coincubation of PLAU with amiloride or SERPINE2 neutralized the PLAU effects on cumulus expansion and subsequent oocyte maturation. Therefore, these results indicate that SERPINE2 can modulate PLAU activity. These findings from the mouse model may explain why cumulus cells surrounding immature human oocytes expressed more SERPINE2 than those surrounding mature oocytes and support our hypothesis that aberrantly high SERPINE2 levels correlate with oocyte immaturity.

Chapter 5

Future prospects

We have demonstrated that SERPINE2 possibly influences on sperm to prevent precocious capacitation and the acrosome reaction before sperm reach the oviduct. These findings suggest that the SERPINE2 protein may play a role as a sperm decapacitation factor. In addition, we also demonstrated high levels of SERPINE2 protein remarkably impaired cumulus expansion and oocyte maturation. Although our studies demonstrated the role of SERPINE2 in sperm capacitation and oocyte maturation, further mechanistic studies can provide novel opportunities in applying our findings in treating infertility couples. Several challenges and application will be achieve in the future. Future experiments can be planned as the following:

1. Capacitation is initiated by removal of cholesterol from the sperm plasma membrane. Our studies have demonstrated that SERPINE2 inhibited cholesterol efflux from the sperm plasma membrane, but more mechanisms are necessary to be investigated. Several molecular mechanisms involved in sperm capacitation such as (1) increased levels of sperm intracellular calcium ions (Ca^{2+}), (2) increased levels of sperm intracellular bicarbonate ions (HCO^{3-}), (3) increased levels of sperm intracellular cAMP, and (4) activation of cAMP-dependent protein kinase. Therefore, we will investigate the mechanisms underlying the suppressive effect of SERPINE2 on sperm

capacitation.

2. Several potential decapacitation factors of rodents have been identified. However, the decapacitation factors in the human seminal plasma remain to be investigated. We will focus on the decapacitation factors of human seminal plasma and propose three main approaches: (1) Purification and characterization of the decapacitation factors in the human seminal plasma. (2) Revealing the mechanisms of the decapacitation factors. (3) Assessment of the associations between the decapacitation factors and male infertility.

3. We demonstrated that PLAU and its inhibitor SERPINE2 are involved in murine cumulus expansion and oocyte maturation. Infertility patients undergoing superovulation treatments sometimes retrieve immature oocytes [103,104]. Our data indicated that PLAU significantly enhanced cumulus *Has2* mRNA levels and thus promote oocyte maturation. Thus, exogenous PLAU supplementation may be helpful for maturation of immature oocytes during IVM for clinical amplification.

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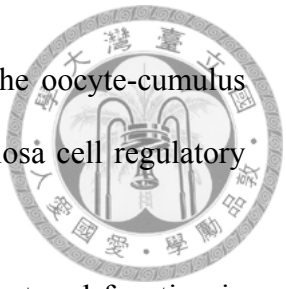

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Publication

1. **Lu CH**, Lee RK, Hwu YM, Lin MH, Yeh LY, Chen YJ, Lin SP, Li SH. Involvement of the Serine Protease Inhibitor, SERPINE2, and the Urokinase Plasminogen Activator in Cumulus Expansion and Oocyte Maturation. **PLoS One**. **2013,** 30;8(8):e74602.

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