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Plasminogen Improves Mouse IVF by Interactions with Inner Acrosomal Membrane-Bound MMP2 and SAMP14¹

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ABSTRACT

Spermatozoa must penetrate the outer investments of the oocyte, the cumulus oophorus and the zona pellucida (ZP), in order for fertilization to occur. This may require exposure of enzymes on the sperm's inner acrosomal membrane (IAM), one of which is matrix metalloproteinase (MMP) 2, to factors in oviductal fluid. Plasminogen is present in oviductal fluid and activates MMP2 in somatic tissues. The objectives of this study were: 1) to examine possible interactions between plasminogen and IAM-bound plasminogen activator receptor (SAMP14) and -MMP2, 2) to demonstrate plasminogen's presence in the extracellular environment at the site of fertilization, and 3) to provide evidence that plasminogen plays a role in fertilization. Zymographs of sonicated bull and rat sperm extracts incubated with plasmin and/or plasminogen (plasmin/ogen) showed acceleration of initiation of MMP2 activity in concentrations as low as 1 µg/ml. Immunohistochemical and immunofluorescence analysis of plasmin/ogen revealed its presence in the cytoplasm of mouse ovarian and oviductal oocytes, oviductal epithelium, around the ZP, and amongst the cumulus cells. We modified the standard *in vitro* fertilization (IVF) approach to more closely mimic natural fertilization by reducing sperm concentration during insemination by ~100× and also comparing cumulus-intact and denuded oocytes. In mice, addition of plasminogen in IVF medium significantly improved fertilization, while MMP2 antibody significantly inhibited sperm penetration in these conditions. IVF improvement by plasminogen was blocked by SAMP14 antibody. Furthermore, MMP2 antibody inhibition was coincident with a failure by spermatozoa to disperse the cumulus oophorus. We provide evidence that plasminogen on its own and through an MMP2-related mechanism improves the ability of oocytes to be fertilized, and demonstrate its effect in sperm penetration of oocyte investments.

acrosome, cumulus oophorus, fertilization, inner acrosomal membrane, IVF, matrix metalloproteinase 2, oocyte penetration, plasminogen, SAMP14, spermatozoa, zona pellucida

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INTRODUCTION

Mammalian fertilization requires a series of sequential and necessary steps to occur, one of which is the penetration of the outer investments of the oocyte, the cumulus oophorus and the zona pellucida (ZP), by spermatozoa. In order for penetration to occur, sperm must be induced to undergo the acrosome reaction. The acrosome is a cap-like vesicle covering the anterior of the sperm head, overlying the subacrosomal layer of the perinuclear theca and underlying the plasmalemma. In the acrosome reaction, membrane fusion between the outer acrosomal membrane and the overlying plasmalemma allows for the release of acrosomal contents and the exposure of the inner acrosomal membrane (IAM) to the extracellular environment of the female reproductive tract [1]. The prevailing model for penetration of the oocyte investments describes enzymatic digestion of the oocyte investments by enzymes exposed on the IAM after the acrosome reaction [2–5]. We have previously identified and characterized a ZP2 protein receptor, termed IAM38/ZP binding protein-1, which supports this model [6, 7]. Furthermore, we have identified and characterized two proteinases on the IAM, acrosin and matrix metalloproteinase (MMP) 2 [8].

Of acrosin and MMP2, acrosin is better characterized. Acrosin is the major serine protease found in the acrosome, and was formerly thought to be the effector of ZP penetration. However, acrosin knockout mice remain fertile [9, 10]. Although spermatozoa from acrosin knockout mice require more time to fertilize the oocyte *in vitro*, it appears that acrosin is not essential for ZP penetration. MMP2, also known as gelatinase A, is an MMP widely found in somatic tissues, and plays a role in digestion of extracellular matrix (ECM) [11]. Although secreted into the ECM as a zymogen (pro-MMP2) in somatic cells, its action is targeted to cell membranes [12]. Its activation requires binding of its C-terminal end to tissue inhibitor of MMPs (TIMP) 2, after which the TIMP2-pro-MMP2 complex binds via TIMP2 to cell membrane-bound membrane type 1-MMP (MT1-MMP, also MMP14) [13, 14]. MT1-MMP then cleaves the prosegment to activate MMP2 [13]. In excess, TIMP2 inhibits pro-MMP2 activation by binding free pro-MMP2 and MT1-MMP, preventing TIMP2-pro-MMP2-MT1-MMP complex formation. The membrane-specific activity of MMP2 allows it to play a role in cell migration by digesting ECM, and its activity is upregulated in many types of cancer. Its function in digesting ECM in a membrane-specific manner in somatic cells and its localization to the IAM of spermatozoa make it an excellent candidate for enzymatic digestion of the oocyte investments during sperm penetration.

We have previously demonstrated a reduced ability of spermatozoa to fertilize cumulus-free oocytes in the presence

of MMP2 inhibitors and anti-MMP2 antibody *in vitro* [8]. However, no reports of *in vivo* fertility deficiencies have been made in MMP2 knockout mice, which may be explained by the presence of other acrosomal proteases. A similar phenomenon is seen when examining other acrosomal proteases; for example, although sperm from acrosin knockout mice are fertile *in vitro*, acrosin/PRSS21 (also known as TESP5) double-knockout mice are infertile *in vitro* (with fertility rescued by the addition of oviductal fluid during insemination) and subfertile *in vivo* [9]. Thus, it appears that the female reproductive tract appears able to compensate for deficiencies in some acrosomal enzymes.

This idea led us to try to simulate the *in vivo* extracellular environment of the female reproductive tract during *in vitro* fertilization (IVF) and determine the subsequent effect on fertilization. We decreased the sperm concentration during insemination *in vitro* to recreate sperm scarcity in the fertilizing environment *in vivo*. We also experimented by adding plasminogen, normally present in oviductal fluid, and blocking its receptors on spermatozoa. We reveal that the activation of MMP2 enzymatic activity is accelerated in the presence of plasmin and/or plasminogen (plasmin/ogen), and possibly through this mechanism, plasmin/ogen enhances the ability of spermatozoa to fertilize oocytes. In addition, we provide evidence that plasminogen has other unresolved stimulatory effects on fertilization.

MATERIALS AND METHODS

Animals and Ethics

The use of animals for this study was conducted along guidelines approved by the Queen's University Animal Care Committee (Kingston, ON, Canada).

Sperm and Sample Collection and Treatment

Bull epididymides were obtained from the abattoir in Joyceville (ON, Canada) immediately after bull slaughter. Rat and bull cauda epididymides were submerged in 25 mM Tris-buffered saline (TBS; pH 7.5), containing PMSF. Epididymides were cut and squeezed gently to allow spermatozoa to diffuse into suspension. The suspension was filtered through 120- μ m nylon mesh netting to separate spermatozoa from any large pieces of tissue in suspension, and centrifuged at 1000 \times g. The resulting pellet was washed by resuspension in TBS and recentrifugation at 1000 \times g three times.

Spermatozoa suspended in TBS were sonicated on ice for three 15-sec bursts, with 1-min intervals on ice between bursts, using a small probe Vibra-Cell sonicator (Sonics and Materials, Danbury, CT). Sonicated spermatozoa were centrifuged at 4°C for 10 min at 14 000 \times g, and the supernatant was collected. Recombinant bovine plasmin or plasminogen (catalog ID BPLM and BPLG, respectively; Molecular Innovations Inc., Novi, MI) was added to the supernatant of sonicated sperm, which was then placed in a 37°C water bath for 40 min unless otherwise noted, and mixed with a nonreducing sample buffer (200 mM Tris [pH 6.8], 4% SDS, 0.1% bromophenol blue, 40% glycerol, 5% β -mercaptoethanol).

First-trimester human trophoblast HTR8/SV neo cell line conditioned media (donated by Dr. Charles Graham, Queen's University), known to contain MMP2 and stimulated with tumor necrosis factor to induce MMP9 expression, served as a positive control.

Zymography

Samples were loaded onto 10% SDS-polyacrylamide gels (2.3 ml ddH₂O, 1.25 ml 40% acrylamide, 1.25 ml 1.5 M Tris [pH 8.8], 50 μ l 10% SDS, 50 μ l 10% ammonium persulfate, 3 μ l N,N,N',N'-tetramethylethylenediamine) containing 5.24 mg gelatin per gel. Proteins in the samples were renatured after electrophoresis by washing the gel twice for 30 min and once for 1 h in 2.5% Triton X-100, 5 mM CaCl₂, and 50 mM Tris (pH 7.5) in ddH₂O at room temperature to remove SDS. Overnight incubation at 37°C followed, in the same solution, but without Triton. MMP gelatinase activity was blocked by including ethylenediaminetetra-acetic acid (EDTA)

in the rinse and incubation buffers, which chelates zinc ions required for MMP activity. The gel was subsequently stained with Coomassie blue and destained in 30% methanol, 10% glacial acetic acid, and 60% ddH₂O until clear bands were visible. Enzymatic digestion of gelatin is indicated by these clear bands. Results shown are typical of at least three different experiments.

Immunohistochemistry

Female CD-1 mice (8–10 wk old; Charles River, St. Constant, PQ, Canada) were superovulated by intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (catalog no. G4877; Sigma, St. Louis, MO) and a subsequent intraperitoneal injection of 10 IU human chorionic gonadotropin (Sigma catalog number CG10) 48 h later. Mice were killed 20 h after hCG injection. Ovaries, oviducts, and proximal uteri were harvested, fixed in Bouin fixative, and embedded in paraffin. Tissues were serially sectioned until cumulus-oocyte complexes were visible. Sections were deparaffinized in toluene and hydrated through serial dilutions of ethanol, during which sections were treated to abolish endogenous peroxidase activity, to neutralize residual picric acid and to block free aldehyde groups. Antigen retrieval occurred by microwaving sections in 0.1 M sodium citrate (pH 6). Immunolabeling was performed with an avidin-biotin complex kit from Vector Laboratories (Burlington, ON, Canada). Sections were treated with avidin blocking serum and biotin blocking serum, followed by 10% normal goat serum (NGS) to block nonspecific immunogenic sites. Primary antibody incubation followed at 4°C overnight with anti-plasminogen antibody (4 μ g/ml; catalog no. sc-25546; Santa Cruz Biotechnology, Santa Cruz, CA) or unrelated rabbit IgG (4 μ g/ml). After four 5-min washes in TBS containing 0.1% Tween 20 (TBST), sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody for 30 min at room temperature, followed by avidin-biotin complex for another 30 min.

In Vitro Fertilization

To obtain cumulus-free oocytes from superovulated mice, cumulus-oocyte complexes were collected by piercing oviducts in Embryomax M-2 medium (catalog no. MR-015-D; Millipore, Billerica, MA) 20 h after hCG injection and treating these isolated complexes briefly with 0.1% hyaluronidase in M-2 to disperse the cumulus. Denuded oocytes were then pooled, washed in M-2 and human tubal fluid (HTF; Millipore catalog no. MR-070-D), and allowed to recover in HTF at 37°C in 5% CO₂. To obtain cumulus-intact oocytes, oviducts were pierced in HTF and cumulus-oocyte complexes were immediately inseminated after collection.

Spermatozoa from young adult male CD-1 males were squeezed out of freshly isolated mouse cauda epididymides in HTF using sterile forceps and allowed to 'swim out' for 10 min at 37°C. Sperm were counted using a hemocytometer and incubated with any relevant enzymes or antibodies that would be present in the fertilization droplet for 30 min before insemination.

Insemination and subsequent wash and rest steps occurred in 50- μ l HTF drops overlaid by sterile mineral oil in a culture dish. Cumulus-oocyte complexes and denuded oocytes were inseminated in 10⁶ or 10⁴ sperm/ml in HTF, with or without anti-MMP2 antibody (0.04 μ g/ μ l raised in-house [8]), anti-plasminogen activator receptor (SAMP14) antibody [15], and plasmin/plasminogen (100 μ g/ml). After 8-h incubation (37°C, 5% CO₂), denuded oocytes and cumulus-oocyte complexes were washed once, photographed, and cultured overnight in HTF (37°C, 5% CO₂). Successful fertilization was assessed 24 h after insemination, indicated by the presence of a second polar body or cleavage.

Trials for each group were repeated at least three times. A chi-square test of homogeneity with the Bonferroni correction was performed to assess significant difference between treatment groups.

Immunofluorescence

Cumulus-oocyte complexes were fixed in 2% formaldehyde and washed with PBS-0.1% Triton X-100. Antigen retrieval was done by adding boiling 0.01 M sodium citrate (pH 6) in a six-well glass dish, with new boiling buffer added every minute. The cumulus-oocyte complexes were blocked with 5% NGS and incubated with or without 4 μ g/ml anti-plasminogen antibody in 1% NGS or unrelated rabbit IgG, in PBS-0.1% Triton X-100. The cells were then washed three times in PBS-0.1% Triton X-100, covered with goat anti-rabbit secondary antibody conjugated to Alexa Fluor 555 and 4',6-diamidino-2-phenylindole in PBS-0.1% Triton X-100, washed again three times, mounted onto slides, and visualized. Cumulus-free oocytes were treated similarly, but were attached to poly-L lysine coverslips after fixation.

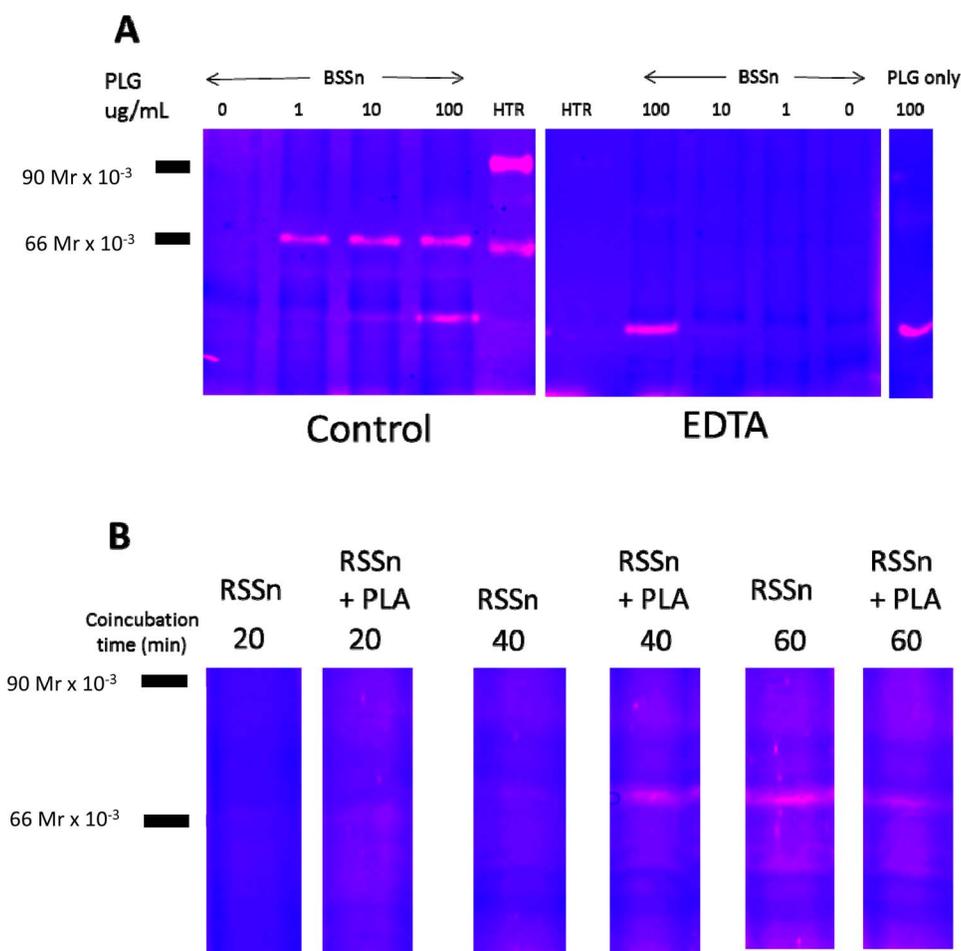


FIG. 1. Zymographs of sperm extracts and plasmin/ogen. Zymographs demonstrate that plasmin (PLA) and plasminogen (PLG) enhance MMP2 activity in sperm extracts. **A**) After coincubation for 40 min, MMP2 activity in sonicated bull sperm extract (BSSn) is induced by PLG concentrations as low as 1 µg/ml. EDTA, which binds zinc ions required for MMP2 activity, is used as a control. HTR8/SV neo conditioned media (HTR), known to contain MMP2 and MMP9, was used as a control. **B**) MMP2 activity in sonicated rat sperm extract (RSSn) appears after 60 min of incubation at 37°C, but is already visible after 40 min of coincubation with PLA.

RESULTS

Plasmin/ogen Reveals MMP2 Activity in Sperm Extracts

Since plasminogen is present in the extracellular environment during fertilization and can activate MMP2 in somatic tissues, we performed zymography on sperm extracts coincubated with or without plasmin/ogen to determine the effect of plasmin on MMP2 activation in sperm. Bull spermatozoa were sonicated without or with various dilutions of plasminogen and incubated for 40 min at 37°C and examined by zymography. No bands of enzymatic activity were seen in sonicated bull sperm extracts without plasminogen (Fig. 1). Concentrations of plasminogen as low as 1 µg/ml revealed bands of MMP2 enzymatic activity in the sperm extracts (Fig. 1A). When EDTA was added to the rinse and incubation steps of zymography, a negative control for MMP2 and MMP9 activity, no bands of clearance at 66 kDa were seen in any sample (Fig. 1A). HTR8/SV neo conditioned medium served as a positive control for MMP2 activity.

To find how effective plasmin/ogen is in shortening the incubation time required to reveal MMP2 enzymatic activity, rat sperm were sonicated with or without plasmin (100 µg/ml), incubated at 37°C, fractions collected every 20 min for 60 min,

and samples analyzed by zymography. Bands of digestion occurred within 40 min in the presence of plasmin and 60 min without plasmin (Fig. 1B).

Plasminogen Localization in the Female Reproductive Tract

We performed immunohistochemistry using anti-plasmin/ogen antibody on mouse oviducts and ovaries to examine plasmin expression in the female reproductive tract. By serial sectioning of oviducts until the superovulated oocytes were visible, we were also able to examine oocytes not only during their development, but also in situ during oviductal transit in the environment of fertilization.

Immunostaining was first observed in oocytes within primary follicles (Fig. 2A). Oocytes were also immunoreactive throughout their development in secondary follicles (Fig. 2B). Sections exposed to unrelated rabbit IgG served as controls (Fig. 2C). No staining was seen in oocytes within primordial follicles (data not shown). Antiplasminogen immunostaining continued to be present in the cytoplasm of superovulated oocytes, with less intense staining visible in the cumulus (Fig. 2D). When performing immunohistochemistry without counterstaining with methylene blue, immunostaining became more clearly visible on the surface of the ZP,

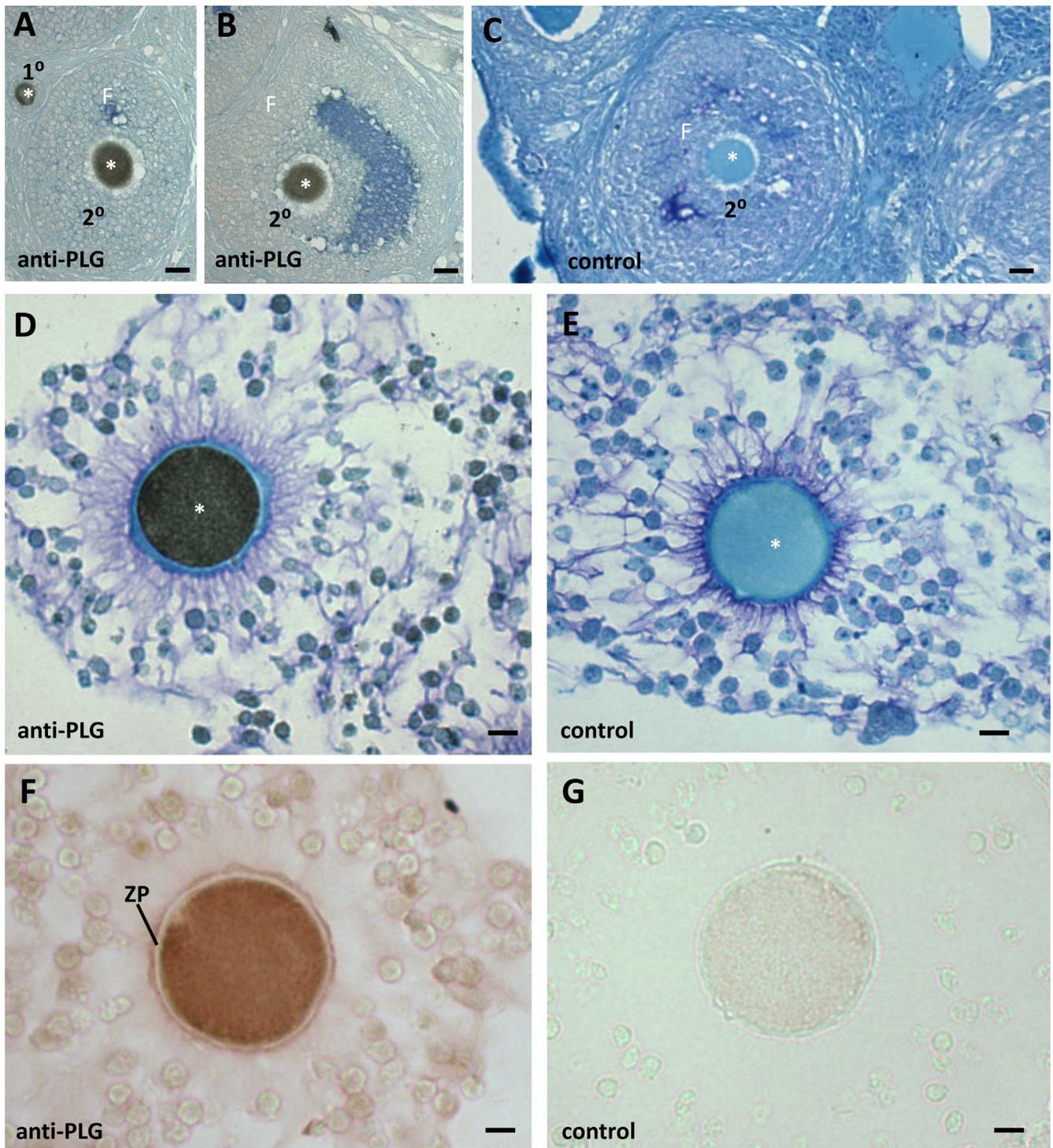


FIG. 2. Immunohistochemistry of ovarian and oviductal oocytes showing plasmin/ogen expression. Sections of mouse ovaries immunostained with anti-plasminogen antibody (A, B, D, and F) or unrelated rabbit IgG (C, E, and G) show that oocytes express plasmin/ogen throughout their development. Plasmin/ogen is present in oocytes within primary (A) and secondary (A and B) follicles. Ovulated oocytes and cumulus cells were also immunoreactive to anti-plasminogen antibody (D), with intense immunostaining apparent around some, but not all, cumulus cells. No immunoreactivity was seen when using secondary antibody only (data not shown) or unrelated rabbit IgG in ovarian (C) or oviductal (E) oocytes. When counterstain was avoided (F and G), immunostaining between cumulus cells and on the surface of the ZP became clearly visible when using anti-plasminogen (F) antibody, but not unrelated rabbit IgG (G). Asterisk, oocyte; F, follicular cells. Bar = 20 μ m.

in the ECM of the cumulus, and associated with the follicular cells (Fig. 2F). Control sections, serially cut and exposed to unrelated rabbit IgG, revealed no background or nonspecific staining (Fig. 2, E and G), and the lack of counterstain

necessitated using high-contrast settings of a phase-contrast microscope to visualize the cells (Fig. 2G).

Oviductal epithelial cells were immunoreactive against anti-plasminogen antibody (Fig. 3A), with secretory cells especially

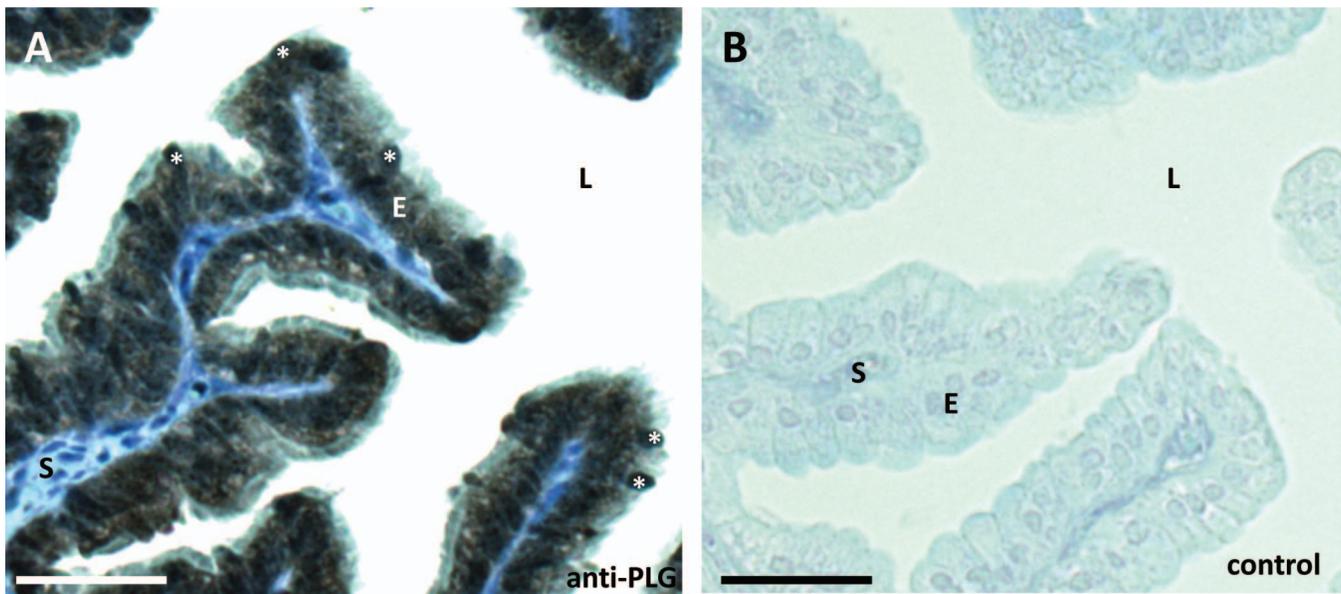


FIG. 3. Immunohistochemistry of oviductal epithelium showing plasmin/ogen expression. Sections of superovulated mouse oviducts immunostained with anti-plasminogen antibody show that oviductal epithelium is positively stained (A), while sections are unreactive when immunostained with unrelated rabbit IgG (B). Asterisk, secretory cell; E, epithelium; L, lumen; S, stroma. Bar = 50 μ m.

stained. Similar sections exposed to unrelated rabbit IgG were unreactive (Fig. 3B).

Plasmin/ogen Present in In Vitro Sperm-Penetration Environment of Cumulus-Intact, but Not Cumulus-Free, Oocyte

To inform us about the availability of plasminogen during IVF and examine changes to cells after their removal from the oviduct, whole cumulus-oocyte complexes and cumulus-free oocytes were processed for immunofluorescence. As expected, the cumulus and oocyte were found to be immunoreactive to anti-plasminogen antibody (Fig. 4), similar to in situ cumulus-oocyte complexes examined by immunohistochemistry in Figure 2. Unexpectedly, when the cumulus was removed with 0.1% hyaluronidase, no immunostaining was seen in the oocyte, indicating that plasminogen was no longer present (Fig. 4). Cumulus-oocyte complexes exposed to unrelated rabbit IgG or secondary antibody only were unlabeled (Fig. 4 and Supplemental Fig. S1; available online at www.biolreprod.org).

Plasminogen Rescues IVF Deficiency Due to Low Sperm Concentration and MMP2 Inhibition

IVF is typically performed with insemination of cumulus-free oocytes occurring in medium containing 5×10^5 to 1×10^6 sperm/ml. Since these sperm concentrations are higher than what would be seen in natural fertilization, we reduced the sperm concentration during insemination until we saw a significant deficiency in the IVF success rate, which we achieved at 10^4 sperm/ml ($\sim 1\%$ of typical IVF conditions; Fig. 5A).

Since we showed that plasminogen enhances or accelerates MMP2 gelatinase activity, and that it appears to be present in the penetration environment in vivo, we performed IVF with and without plasmin/ogen, to see if it would rescue the fertilization deficiency in low-sperm conditions typically seen in vivo. Although cumulus-free oocytes were significantly less

likely to be fertilized in vitro using 10^4 sperm/ml as compared to 10^6 sperm/ml (Fig. 5A), there was no significant difference between inseminating with 10^4 sperm/ml in the presence of plasminogen or plasmin than with 10^6 sperm/ml (Fig. 5A).

To test the possibility that plasminogen is activated via SAMP14, which resides on the IAM, anti-SAMP14 antibody was added to the IVF medium. Cumulus-free oocytes were significantly less likely to be fertilized when anti-SAMP14 antibody and plasminogen were present in the fertilization medium than when only plasminogen was present (Fig. 5B). The fertilization deficiency was not apparent when preimmune serum was used instead of anti-SAMP14 antibody (Fig. 5B). Note that the presence of both anti-SAMP14 antibody and plasminogen did not significantly affect the ability of oocytes to become fertilized by 10^4 sperm/ml (Fig. 5B).

Because immunohistochemical localization indicated that plasminogen resides in the ECM of the cumulus, we tested if cumulus-intact oocytes maintained a normal IVF rate under reduced sperm conditions. As shown, decreasing sperm concentrations used during IVF of cumulus-intact oocytes did not affect fertilization rates significantly from the standard 10^6 sperm/ml concentration, even when 10^4 sperm/ml was used (Fig. 5C). However, the addition of anti-MMP2 antibody to the IVF medium was able to reduce the fertilization rate of cumulus-intact oocytes (Fig. 5C). Note that there was no significant difference between the ability of cumulus-free oocytes to become fertilized by 10^4 sperm/ml and the ability of cumulus-intact oocytes to become fertilized by 10^6 sperm/ml in the presence of anti-MMP2 antibody (Fig. 5C).

We also performed IVF to determine the effect of inhibiting MMP2 in the presence of exogenous plasminogen in cumulus-intact oocytes, with the expectation that excess plasminogen would fail to compensate for the inhibition of MMP2 activity. Inhibition of MMP2 activity by anti-MMP2 antibody significantly inhibited the ability of cumulus-intact oocytes to become fertilized by both 10^4 sperm/ml and 10^6 sperm/ml, although the degree of inhibition was greater if using the lower sperm concentration (Fig. 5D). However, contrary to our expectations, the presence of exogenous plasminogen was able

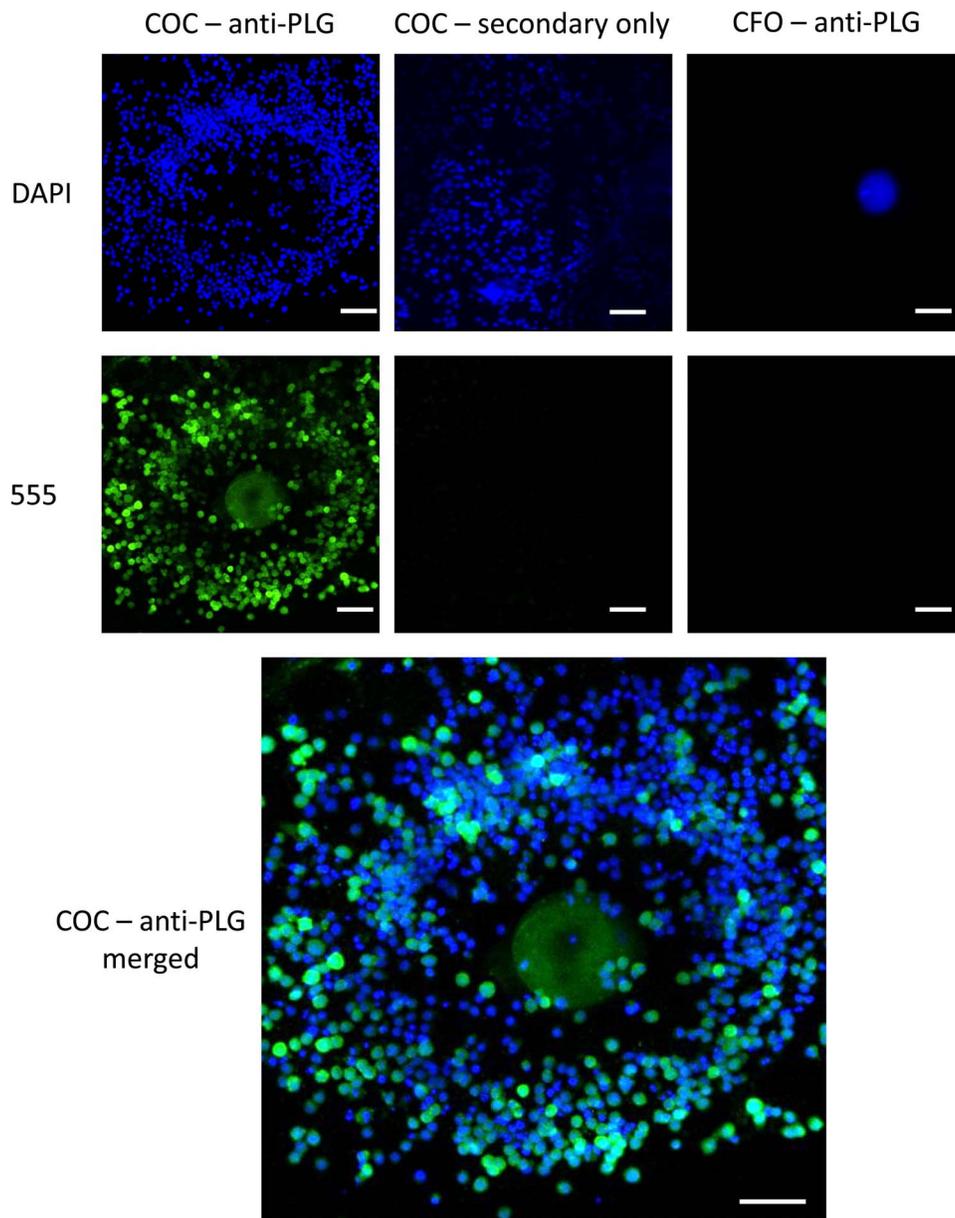


FIG. 4. Fluorescent micrograph of cumulus-free (CFO) and cumulus-intact (COC) oocytes showing plasmin/ogen expression. Fluorescent micrographs of cumulus-intact and cumulus-free mouse oocytes exposed to anti-plasminogen antibody (555) and 4',6-diamidino-2-phenylindole (DAPI) nuclear stain. Immunolabeling is visible in the cumulus-intact oocytes and in some, but not all, of the cumulus cells. No immunolabeling appears to be present in cumulus-free oocytes. Bar = 50 μ m.

to rescue the deficiency caused by anti-MMP2 inhibition (Fig. 5D).

MMP2 Inhibition Inhibits Cumulus Dispersal and Fertilization of Cumulus-Intact Oocytes

The macroscopic structure of the cumulus was found to be affected by MMP2 and plasminogen. Insemination with 10^6 sperm/ml effectively led to a full clearance of the matrix and cumulus cells around the oocyte after 8 h (Fig. 6A). Addition of anti-MMP2 antibody to the IVF medium with 10^6 sperm/ml also led to cumulus dispersal within this time frame (Fig. 6B). In contrast, in conditions more closely resembling physiological fertilization, insemination in vitro with 10^4 sperm/ml led only to partial or incomplete clearance of the cumulus (Fig. 6C). Furthermore, if anti-MMP2 antibody was added to the

IVF medium with 10^4 sperm/ml, dispersal of the cumulus matrix appeared completely inhibited (Fig. 6D), which is coincidental with a significant decrease in fertilization success rate compared to IVF without anti-MMP2 antibody (Fig. 5C). The addition of exogenous plasminogen when inseminating with 10^4 sperm/ml appeared to improve clearance of the cumulus around the oocyte (Fig. 6E). Plasminogen was also able to rescue the dispersal and clearance of the cumulus, even in the presence of anti-MMP2 antibody (Fig. 6F).

DISCUSSION

IVF laboratory methods utilizing cumulus-free oocytes and a very high sperm concentration have been optimized to maximize embryo yield, rather than to mimic physiologic conditions. The experiments we performed in this study were formulated to analyze conditions more closely resembling

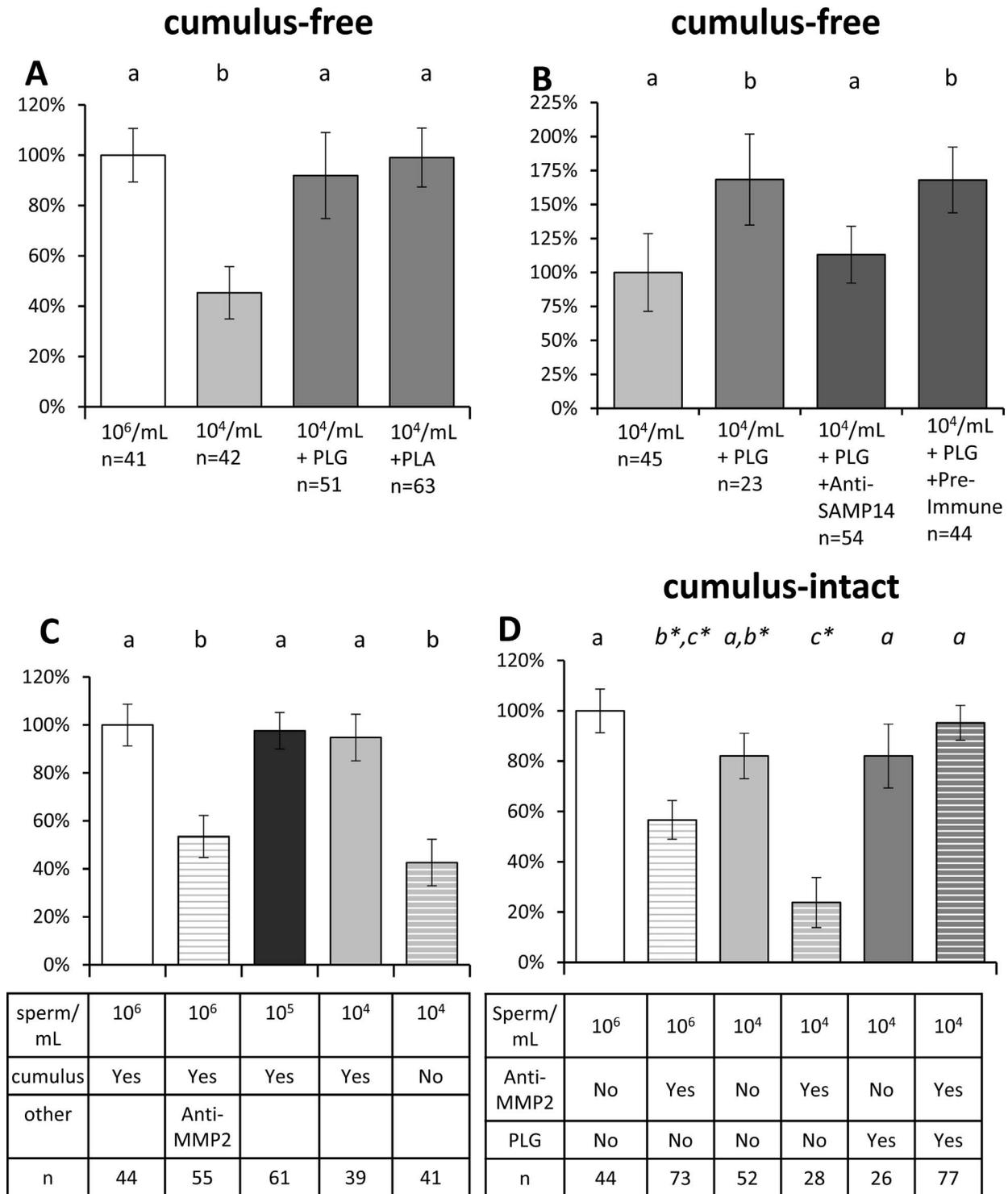


FIG. 5. Effect of sperm concentration, plasminogen, anti-SAMP14, and anti-MMP2 antibodies on mouse IVF. Block graphs depict the percentage of oocytes successfully fertilized over all trials compared to a control value adjusted to 100% for comparative purposes. Using the chi-square test, superscript letters indicate significant difference at $P < 0.02$ after the Bonferroni correction, and asterisks indicate a smaller degree of difference ($P < 0.05$) between groups with the same superscript letters. Each trial was repeated at least three times, and n indicates number of total oocytes. Error bars = SE. **A)** 10^4 mouse sperm/ml are significantly less able to fertilize cumulus-free oocytes than 10^6 sperm/ml, but the fertilization deficiency is rescued by the addition of PLA or PLG. **B)** The addition of anti-SAMP14 antibody to fertilization medium containing PLG nullifies the rescue of fertilization by PLG when using 10^4 mouse sperm/ml to fertilize cumulus-free oocytes. Note that the fertilization rate is not significantly different from using 10^4 mouse sperm/ml alone. PLG is able to rescue the fertilization deficiency when preimmune serum is used in lieu of anti-SAMP14 antibody. **C)** Although 10^4 sperm/ml is significantly less able to fertilize cumulus-free oocytes than cumulus-intact ones, there is no significant difference in the ability of 10^6 , 10^5 , or 10^4 sperm/ml to fertilize cumulus-intact oocytes. **D)** In the presence of anti-MMP2 antibody, both 10^6 and 10^4 sperm/ml are significantly less able to fertilize cumulus-intact oocytes, and the effect is more pronounced when using 10^4 sperm/ml than 10^6 sperm/ml. The inhibition of MMP2 is overcome or compensated for by the addition of PLG.

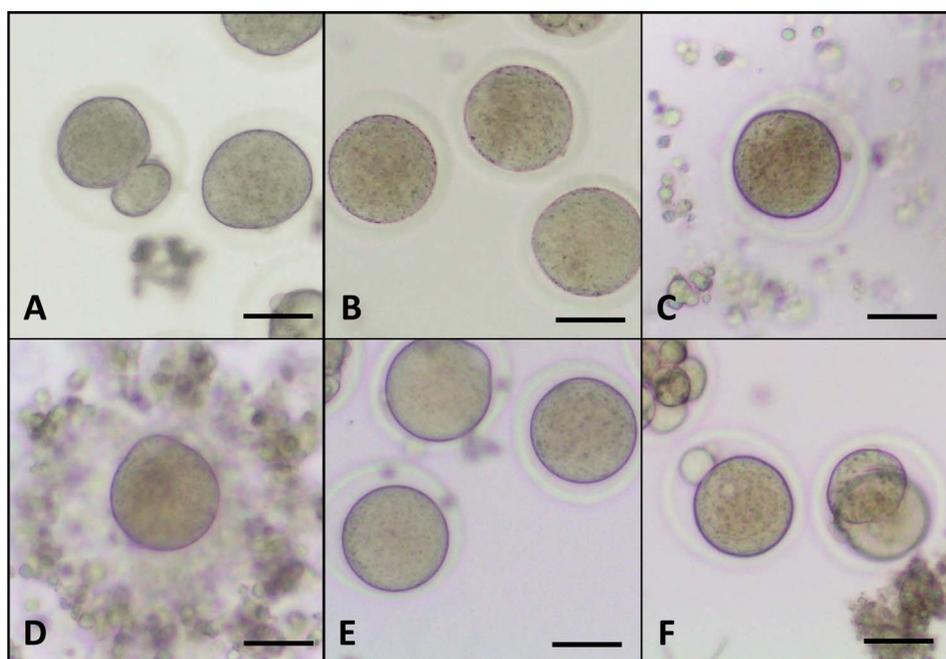


FIG. 6. Light micrographs of initially cumulus-intact oocytes 8 h after insemination. Mouse oocytes were coincubated with 10^6 sperm/ml (A), 10^6 sperm/ml and anti-MMP2 antibody (B), 10^4 sperm/ml (C), 10^4 sperm/ml and anti-MMP2 antibody (D), 10^4 sperm/ml and plasminogen (E), and 10^4 sperm/ml, anti-MMP2 antibody, and plasminogen (F). Plasminogen enables sperm clearance of the cumulus cells at low sperm concentrations, while anti-MMP2 antibody inhibits it. The addition of plasminogen overcomes the inhibition. Bar = 50 μ m.

physiological conditions than those seen in IVF. Our results confirm that plasmin/ogen enhances mammalian sperm MMP2 activity, and, possibly through this mechanism, also enhances mouse sperm fertilizing ability. Most IVF procedures involve insemination in conditions with an excess of sperm in order to maximize embryo production. A reduction in sperm concentration during insemination reduces the fertilization rate in vitro, but is closer to conditions expected in vivo. We demonstrate that the ability of low concentrations of spermatozoa to fertilize cumulus-free oocytes in vitro improves when exogenous plasmin/ogen is added, which is noteworthy, since plasmin/ogen has been reported to be a major component of oviductal fluid [16–18]. These parameters more closely represent in vivo conditions, and may explain previously published studies showing that IVF was rescued by the addition of oviductal fluid when inseminating cumulus-free oocytes with sperm from *Acr*^{-/-}*Prss21*^{-/-} mice [9]. Rescue of IVF by oviductal fluid indicates that a compensatory mechanism may be activated by factors present in the oviductal fluid, and our results strongly suggest that oviductal plasmin/ogen, acting through sperm-borne MMP2 and/or on its own, may be that mechanism. Our localization data suggest that the major source of plasminogen is likely secreted from the oviductal epithelium. Since plasminogen is found extracellularly in the cumulus, it would be interesting to test whether *Acr*^{-/-}*Prss21*^{-/-} sperm would be capable of fertilizing cumulus-intact oocytes in vitro without adding oviductal fluid. Thus, we propose a mechanism by which sperm-borne plasminogen activator released during the acrosome reaction [19, 20] binds to SAMP14 plasminogen activator receptor on the IAM [15], directing localized plasmin activity at the IAM and, at the same time, activating pro-MMP2 (Fig. 7A).

We previously identified the presence of MMP2 on the IAM of multiple species [8]. The present study builds on those comparative findings by demonstrating that the interactions observed are likely a conserved property within a diverse array

of at least eutherian species. We demonstrate that, although spermatozoa from bull and rat are able to initiate sperm-borne MMP2 activity on their own, addition of exogenous plasmin/ogen reduces the time required for this to occur. The ability of spermatozoa to initiate MMP2 activity without exogenous factors was expected, since neither plasmin/ogen nor oviductal fluid is added in most successful IVF procedures. Pancreatic trypsin is capable of activating MMP2 [21], so trypsin-like serine proteases, found in abundance in the acrosome, may also be the cause of endogenous MMP2 activation in spermatozoa. However, during natural fertilization where, at best, only a few spermatozoa are involved in penetrating the cumulus, there may not be sufficient amounts of endogenous serine proteases in the vicinity of the IAM to activate MMP2 after acrosomal exocytosis and diffusion of its contents.

Although our fertilization studies were restricted to the mouse, we observed that the plasmin-MMP2 accelerant effect is present in bull and rat sperm. MMP2 activation depends only on exposure of the catalytic site, and not necessarily on cleavage of the prosegment; the denaturing and refolding that occurs during zymography is possibly how uncleaved pro-MMP2 is enzymatically active in zymographs. Our findings indicated that, although MMP2 is present, little to no pro-MMP2/MMP2 gelatinase activity is detected in sperm samples that are freshly obtained and processed; activity gradually increases with time after sperm collection and incubation. Thus, the enhancement of MMP2 activity discovered by this study appears, at least in part, to be a novel description of the regulation of MMP2 activity by plasmin/ogen that is, at least partly, unrelated to prosegment cleavage.

Interestingly, our results indicate that spermatozoa are able to disperse the cumulus from the oocyte when used at typical IVF concentrations (10^6 sperm/ml), but are less able to do so at lower concentrations (10^4 sperm/ml). When anti-MMP2 antibody is added during insemination, spermatozoa appear even less able to disperse the cumulus, which is coincident with

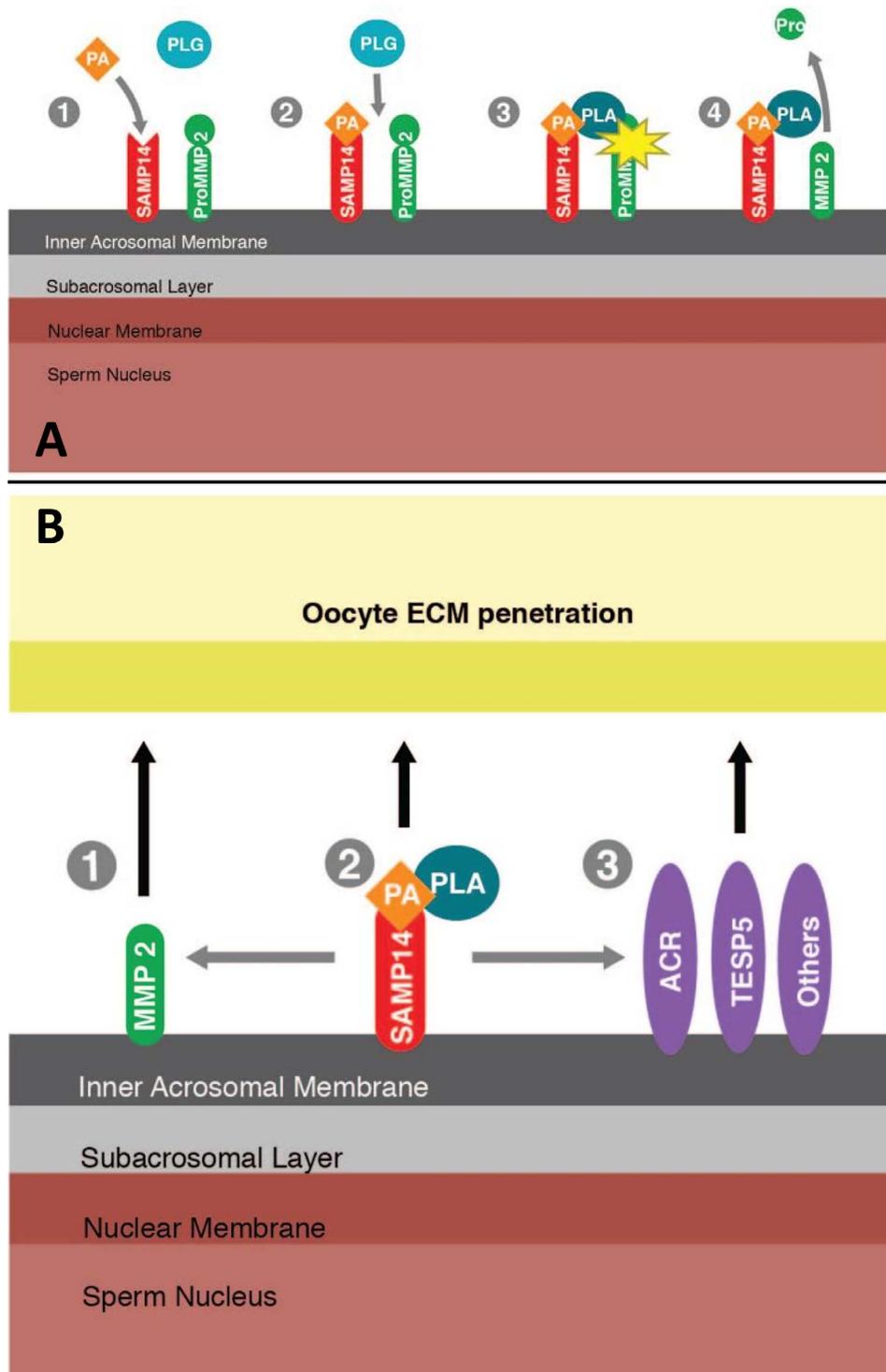


FIG. 7. Diagrams of proposed interactions on the IAM. **A)** Plasminogen activator binds to the SAMP14 plasminogen activator receptor on the IAM of acrosome-reacted spermatozoa. Plasminogen is then activated to plasmin. Plasmin then activates pro-MMP2 to MMP2 by cleavage of its pro segment. **B)** Our study answers the question of interactions between plasmin and MMP2 in oocyte ECM penetration (1), while raising the nonexclusive possibility that plasmin effects ECM penetration alone (2) and/or by acting on other sperm-borne IAM enzymes (3).

a decrease in the fertilization rate compared to insemination without anti-MMP2 antibody. It appears that, when MMP2 activity is blocked, the fertilization-enhancing effect of the cumulus is curtailed. Thus, our results suggest that at least part of the fertilization deficiency may be due to a failure of spermatozoa to penetrate the cumulus, suggesting that MMP2 may play a role not only in ZP penetration, but also in cumulus penetration. Also noteworthy was that the fertilization rate was significantly lowered in the presence of anti-MMP2 antibody as compared to in its absence, even though the cumulus appeared to be dispersed by 10^6 sperm/ml in both cases. This suggests that some of the fertilization deficiency occurs at a later stage of fertilization, possibly during zona penetration.

No fertilization deficiency has yet to be reported in MMP2 knockout mice, yet, in a previous study, we have reported that the fertilization rate of cumulus-free oocytes is decreased when MMP2 activity is inhibited during fertilization *in vitro* [8]. Thus, as our experimental conditions approached *in vivo* conditions, it became apparent that alternative pathways to MMP2-directed penetration were operating during fertilization. This is reflected by our findings that, in low-sperm conditions, addition of plasminogen in medium, but not the plasminogen reservoir within an intact cumulus, was able to overcome the inhibitory effect of anti-MMP2 antibody on fertilization rates *in vitro*. Our results suggest that plasminogen reservoirs in the medium and the intact cumulus are *in vivo* mechanisms, which can compensate for the lack of MMP2 activity in spermatozoa during fertilization.

The inability of spermatozoa to fertilize oocytes *in vitro* when various enzymes (Acr, PRSS21, MMP2) are not present or inhibited, together with the ability of those knockout mice to reproduce *in vivo*, suggests that the female reproductive tract may be a trigger for multiple and redundant pathways responsible for sperm penetration of the oocyte investments (Fig. 7B). Our results suggest that plasminogen is a factor present in the female reproductive tract that may be able to rescue the fertilizing deficiency of *Acr*^{-/-}*PRSS21*^{-/-} mouse sperm. The addition of plasminogen to IVF medium when inseminating with *Acr*^{-/-}*PRSS21*^{-/-} mouse sperm would yield an answer to this question. Our study also raises the possibility that plasmin may effect cumulus and/or zona penetration on its own (Fig. 7B). Assessing the fertility of sperm devoid of or with inhibited Acr/PRSS21/MMP2 *in vitro* and *in vivo* would reveal whether or not more pathways might exist. For example, another possible effector of penetration is the proteasome, which has previously been localized to the IAM [22]. ZP protein ubiquitination has been identified, making ZP proteins possible targets for proteasome processing, and various inhibitors of components of the proteasome as well as anti-proteasome antibody have also inhibited fertilization [23].

Up to 15% of couples are infertile, and an understanding of natural fertilization is key to identification of causes of infertility as well as in devising better treatments [24]. Assisted reproduction treatment (ART) in humans typically involves attempting IVF prior to performing intracytoplasmic sperm injection (ICSI) on unfertilized oocytes before *in vitro* embryo maturation and transfer into the birth mother. Barriers that stand in the way of IVF include low sperm concentration in samples collected from donors and failure of sperm to penetrate the ZP. In cases where these are the suspected cause of infertility, future research might examine modulating the insemination conditions by addition of plasmin/ogen to improve the success rate of IVF and reduce the time and cost of ART by reducing the need for ICSI after IVF.

Our results are the first to show an acceleration of the initiation of sperm-borne MMP2 activity by exogenous

plasmin/ogen, and suggest that the improved fertilization rate *in vitro* seen when plasmin is present during insemination may be due to this mechanism. The IVF experiments performed in this study differed from typical IVF conditions to emphasize better representation of *in vivo* conditions, rather than maximization of embryo production. Investigators studying fertilization should conduct IVF experiments in similar conditions to create a more accurate description of fertilization *in vivo*. Thus, we find that: 1) plasmin/ogen accelerates activation of MMP2 activity in spermatozoa, 2) plasmin/ogen is present in the cumulus and surface of the zona, the immediate environment of spermatozoa during penetration, 3) plasminogen or the intact cumulus are required for IVF in sperm-scarce conditions, 4) inhibiting MMP2 inhibits cumulus penetration and dispersal by spermatozoa, and 5) IAM-directed plasmin activity may, on its own, assist sperm penetration of the cumulus and/or zona.

REFERENCES

- Gerton GL. Function of the acrosome. In: Hardy EM (ed.), Fertilization. New York: Academic Press; 2002:265–302.
- Wassarman PM. Zona pellucida glycoproteins. *Annu Rev Biochem* 1988; 57:415–442.
- Huang TT Jr, Yanagimachi R. Inner acrosomal membrane of mammalian spermatozoa: its properties and possible functions in fertilization. *Am J Anat* 1985; 174:249–268.
- Yaganimachi R. Mammalian Fertilization. In: Knobil E, and Neil JD (eds.), *Physiology of Reproduction*. New York: Raven Press; 1994:189–317.
- Ferrer M, Xu W, Oko R. The composition, protein genesis and significance of the eutherian sperm's inner acrosomal membrane. *Cell Tissue Res* 2012; 349(3):733–748.
- Yu Y, Xu W, Yi YJ, Sutovsky P, Oko R. The extracellular protein coat of the inner acrosomal membrane is involved in zona pellucida binding and penetration during fertilization: characterization of its most prominent polypeptide (IAM38). *Dev Biol* 2006; 290:32–43.
- Yu Y, Vanhorne J, Oko R. The origin and assembly of a zona pellucida binding protein, IAM38, during spermiogenesis. *Microsc Res Tech* 2009; 72:558–565.
- Ferrer M, Rodriguez H, Zara L, Yu Y, Xu W, Oko R. MMP2 and acrosin are major proteinases associated with the inner acrosomal membrane and may cooperate in sperm penetration of the zona pellucida during fertilization. *Cell Tissue Res* 2012; 349(3):881–895.
- Kawano N, Kang W, Yamashita M, Koga Y, Yamazaki T, Hata T, Miyado K, Baba T. Mice lacking two sperm serine proteases, ACR and PRSS21, are subfertile, but the mutant sperm are infertile *in vitro*. *Biol Reprod* 2010; 83:359–369.
- Baba T, Azuma S, Kashiwabara S, Toyoda Y. Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *J Biol Chem* 1994; 269:31845–31849.
- Werb Z. ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 1997; 91:439–442.
- Sato H, Takino T. Coordinate action of membrane-type matrix metalloproteinase-1 (MT1-MMP) and MMP-2 enhances pericellular proteolysis and invasion. *Cancer Sci* 2010; 101:843–847.
- Cao J, Rehemtulla A, Bahou W, Zucker S. Membrane type matrix metalloproteinase 1 activates pro-gelatinase A without furin cleavage of the N-terminal domain. *J Biol Chem* 1996; 271:30174–30180.
- Strongin AY, Collier I, Bannikov G, Marmor BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase: isolation of the activated form of the membrane metalloprotease. *J Biol Chem* 1995; 270:5331–5338.
- Shetty J, Wolkowicz MJ, Digilio LC, Klotz KL, Jayes FL, Diekmann AB, Westbrook VA, Farris EM, Hao Z, Coonrod SA, Flickinger CJ, Herr JC. SAMP14, a novel, acrosomal membrane-associated, glycosylphosphatidylinositol-anchored member of the Ly-6/urokinase-type plasminogen activator receptor superfamily with a role in sperm-egg interaction. *J Biol Chem* 2003; 278:30506–30515.
- Tsantariotou MP, Zervos IA, Vatzias G, Billinis C, Taitzoglou IA, Kokolis NA. Plasminogen activator activity in the porcine oviduct during the oestrous cycle. *Theriogenology* 2005; 64:1007–1015.
- Roldán-Olarte M, Jiménez-Díaz M, Miceli DC. Plasminogen detection in

- oocytes and plasminogen activator activities in the porcine oviduct during the estrous cycle. *Zygote* 2005; 13:115–123.
18. Huarte J, Vassalli J, Belin D, Sakkas D. Involvement of the plasminogen activator/plasmin proteolytic cascade in fertilization. *Dev Biol* 1993; 157: 539–546.
 19. Taitzoglou I, Kokolis N, Smokovitis A. Release of plasminogen activator and plasminogen activator inhibitor from spermatozoa of man, bull, ram and boar during acrosome reaction. *Mol Androl* 1996; 8:187–197.
 20. Smokovitis A, Kokolis N, Taitzoglou I, Rekkas C. Plasminogen activator: the identification of an additional proteinase at the outer acrosomal membrane of human and boar spermatozoa. *Int J Fertil* 1992; 37:308–314.
 21. Lindstad RI, Sylte I, Mikalsen SO, Seglen PO, Berg E, Winberg JO. Pancreatic trypsin activates human promatrix metalloproteinase-2. *J Mol Biol* 2005; 350:682–698.
 22. Sutovsky P. Sperm proteasome and fertilization. *Reproduction* 2011; 142: 1–14.
 23. Yi Y, Manandhar G, Sutovsky M, Zimmerman S, Jonáková V, van Leeuwen F, Oko R, Park C, Sutovsky P. Interference with the 19S proteasomal regulatory complex subunit PSMD4 on the sperm surface inhibits sperm-zona pellucida penetration during porcine fertilization. *Cell Tissue Res* 2010; 341:325–340.
 24. Bolvin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod* 2007; 22(6):1506–1512.