Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy

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The mouse zona pellucida is composed of three glycoproteins (ZP1, ZP2, and ZP3), of which ZP2 is proteolytically cleaved after gamete fusion to prevent polyspermy. This cleavage is associated with exocytosis of cortical granules that are peripherally located subcellular organelles unique to ovulated eggs. Based on the cleavage site of ZP2, ovastacin was selected as a candidate protease. Encoded by the single-copy *Astl* gene, ovastacin is an oocyte-specific member of the astacin family of metalloendoproteases. Using specific antiserum, ovastacin was detected in cortical granules before, but not after, fertilization. Recombinant ovastacin cleaved ZP2 in native zonae pellucidae, documenting that ZP2 was a direct substrate of this metalloendoprotease. Female mice lacking ovastacin did not cleave ZP2 after fertilization, and mouse sperm bound as well to *Astl*-null two-cell embryos as they did to normal eggs. Ovastacin is a pioneer component of mouse cortical granules and plays a definitive role in the postfertilization block to sperm binding that ensures monospermic fertilization and successful development.

Introduction

Because polyspermy is an embryonic lethal, at least three postfertilization blocks to gamete interactions have evolved in mice. The first two occur rapidly after fertilization and prevent additional sperm from fusing with the egg's plasma membrane or penetrating the extracellular zona pellucida surrounding eggs and preimplantation embryos (Sato, 1979; Stewart-Savage and Bavister, 1988). The third and definitive block occurs over several hours and ensures that sperm do not bind to the surface of the zona pellucida (Inoue and Wolf, 1975; Baibakov et al., 2007). The molecular basis of the first two blocks remains largely unknown, and the third correlates with egg cortical granule exocytosis (Barros and Yanagimachi, 1971).

Cortical granules are Golgi apparatus–derived, membrane-bound vesicles (0.2–0.6 µm) that accumulate during oogenesis and form a uniform layer in the cortex of fully grown mouse eggs. The observed 15-fold increase in cortical granules during oocyte growth reflects both an increase in granule density and in the cortical area as oocytes increase in diameter from 40 to >80 µm (Zamboni, 1970; Nicosia et al., 1977; Ducibella et al., 1994). During meiotic maturation and germinal vesicle breakdown, cortical granules redistribute and are excluded from the region of the metaphase I spindle (Ducibella et al., 1988a; Deng et al., 2003). Cortical granules become competent to undergo exocytosis just before ovulation, and the ∼8,000 cortical granules observed in fully grown oocytes decline to ∼4,800 in ovulated eggs (Ducibella et al., 1994). Fertilization triggers cortical granule migration to the plasma membrane, where they fuse and exocytose their contents (Wessel et al., 2001; Ducibella et al., 2002).

Little is known about the contents of mouse cortical granules (Liu, 2011), and the only documented biological function is the postfertilization cleavage of ZP2 (Bleil et al., 1981), which, along with ZP1 and ZP3, forms a structured extracellular glycomatrix that surrounds mouse eggs (Bleil and Wassarman, 1980). Cleavage of ZP2 is N terminal of a diacidic residue (Gahlay et al., 2010), a known cleavage site for the astacin family of metalloendoproteases. Ovastacin (*Astl*, the official gene name) is expressed in growing mouse oocytes and has a signal peptide to direct it into a secretory pathway but has no known function (Quesada et al., 2004). We now localize ovastacin as a pioneer component of mouse egg cortical granules and document its ability to modify the zona pellucida to prevent postfertilization sperm binding and provide a definitive block to polyspermy.

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Abbreviations used in this paper: ES, embryonic stem; PVP, polyvinylpyrrolidone.

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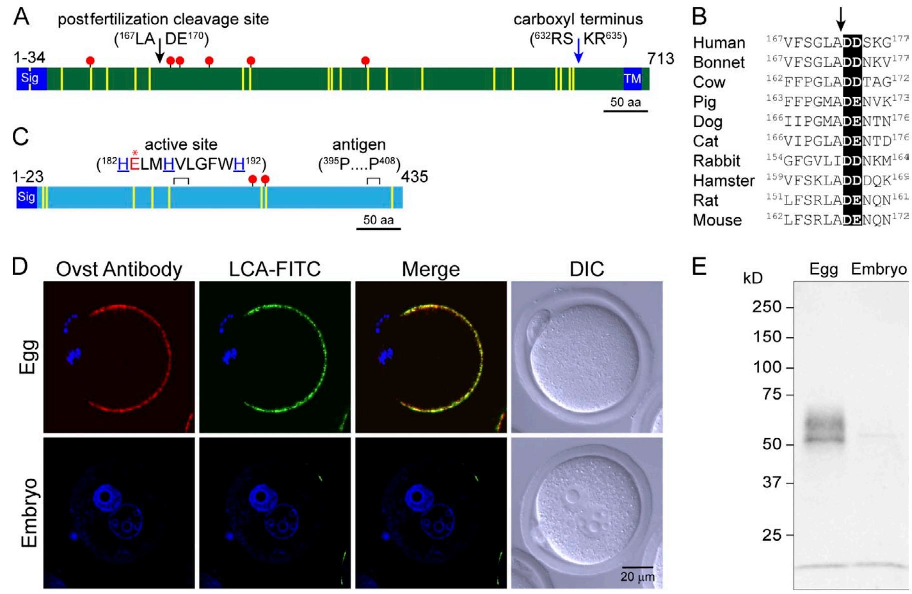


Figure 1. **Localization of ovastacin.** (A) Mouse ZP2 (713 aa) is proteolytically processed by cleavage of a signal peptide (Sig) and at a bibasic motif (blue arrow) upstream of a transmembrane (TM) domain to release the ectodomain (ZP2$^{35-633}$) that participates in the extracellular zona pellucida. After fertilization, ZP2 is cleaved upstream of a diacidic motif (^{169}DE^{170}). Conserved cysteine residues (yellow vertical lines) and potential *N*-linked glycosylation sites (red circles) are indicated. (B) The diacidic (shaded) proteolytic cleavage site (arrow) in the N-terminal region of mouse ZP2 is conserved in human, bonnet monkey, cow, pig, dog, cat, rabbit, hamster, and rat. (C) A schematic of mouse ovastacin including a signal peptide, a Zn^{+2}-binding (blue histidine residues) enzyme-active site (red glutamic acid with an asterisk), and the antigen (^{395}PLALFPEARDKPAP^{408}) used to generate a rabbit pAb. Cysteine residues and potential glycosylation sites are indicated as in A. (D) Unfertilized eggs and one-cell embryos from normal mice were imaged by confocal microscopy and differential interference contrast (DIC) after staining with rabbit anti-ovastacin (Ovst) antibody, LCA-FITC (a marker of cortical granules), and the nuclear stain Hoechst 33342. (E) An immunoblot of lysates from unfertilized eggs (150) and two-cell embryos (150) was probed with antibody to ovastacin.

Results and discussion

Ovastacin is present in mouse egg cortical granules

ZP1, ZP2, and ZP3 form the extracellular zona pellucida that surrounds mouse eggs and early embryos (Bleil and Wassarman, 1980). Sperm bind to eggs but not two-cell embryos, and the only documented biochemical change in the zona matrix is cleavage of ZP2 (Fig. 1 A; Bleil et al., 1981). This cleavage is associated with cortical granule exocytosis and is N terminal of a diacidic motif, ^{168}DE^{169} (Gahlay et al., 2010). The site is well conserved among mammals (Hasegawa et al., 1994; Tian et al., 1999; Lindsay and Hedrick, 2004), but the identity of the presumptive cortical granule protease has as of yet remained unknown (Fig. 1 B). Ovastacin (Fig. 1 C) is a member of the large astacin family of metalloendoproteases (Dumermuth et al., 1991; Bond and Beynon, 1995), which cleave upstream of diacidic residues. Because of its restricted expression in mouse oocytes (Quesada et al., 2004), ovastacin was selected as a candidate protease for the postfertilization cleavage of ZP2.

Taking advantage of the unique C-terminal extension of ovastacin, a peptide-specific (^{395}PLALFPEARDKPAP^{408}) rabbit antibody was used to image ovulated eggs by confocal microscopy. LCA-FITC (*Lens culinaris agglutinin* conjugated to FITC) is a marker of cortical granules (Ducibella et al., 1988b), and its colocalization in the periphery of ovulated eggs indicates the presence of ovastacin within these granules (Fig. 1 D). Disappearance of ovastacin after fertilization and cortical granule exocytosis was observed by confocal microscopy (Fig. 1 D) and confirmed by an immunoblot that detected the two known isoforms of the enzyme (Quesada et al., 2004) in eggs but not two-cell embryos (Fig. 1 E). From these observations, we conclude that ovastacin is expressed in eggs, where it localizes to peripheral cortical granules and is discharged during postfertilization cortical granule exocytosis.

In the absence of ovastacin, cortical granules persist in ovulated eggs

To determine its function, the single-copy *Astl* gene was successfully targeted for ablation in mouse embryonic stem (ES) cells using a neomycin cassette flanked 5′ and 3′ by 5.3 and 1.5 kbp of homology, respectively (Fig. 2 A). Colonies were initially screened by PCR, and 14 positive clones were confirmed by Southern blot analysis using 5′ and 3′ probes outside the regions of homology (Fig. 2 B). After blastocyst injection, two coat-color chimeric male mice were identified, and germline transmission of the null allele was confirmed by the genotype of tail DNA (Fig. 2 C). Mice, bred to homozygosity for the mutant *Astl* allele, were fertile. Although there was a modest decrease in fecundity, there was considerable overlap in the size of liters (Fig. 2 D), which may reflect effects of mixed genetic backgrounds.

To confirm the absence of ovastacin protein, eggs and two-cell embryos were stained with LCA-FITC or ovastacin antibodies and imaged by confocal microscopy. Colocalization in the periphery of eggs, but not two-cell embryos, was observed in normal mice (Fig. 3 A). Similar results were obtained for eggs and embryos from heterozygous null females, although the intensity of the signals was diminished. As anticipated, ovastacin was not detected in the homozygous null eggs, but, unexpectedly, LCA-FITC reactivity was lost as well (Fig. 3 A). To determine whether this reflected an absence of cortical granules, *Astl*^{Null} eggs were stained with WGA. Because WGA strongly reacts with the zona pellucida, it is difficult to detect peripheral

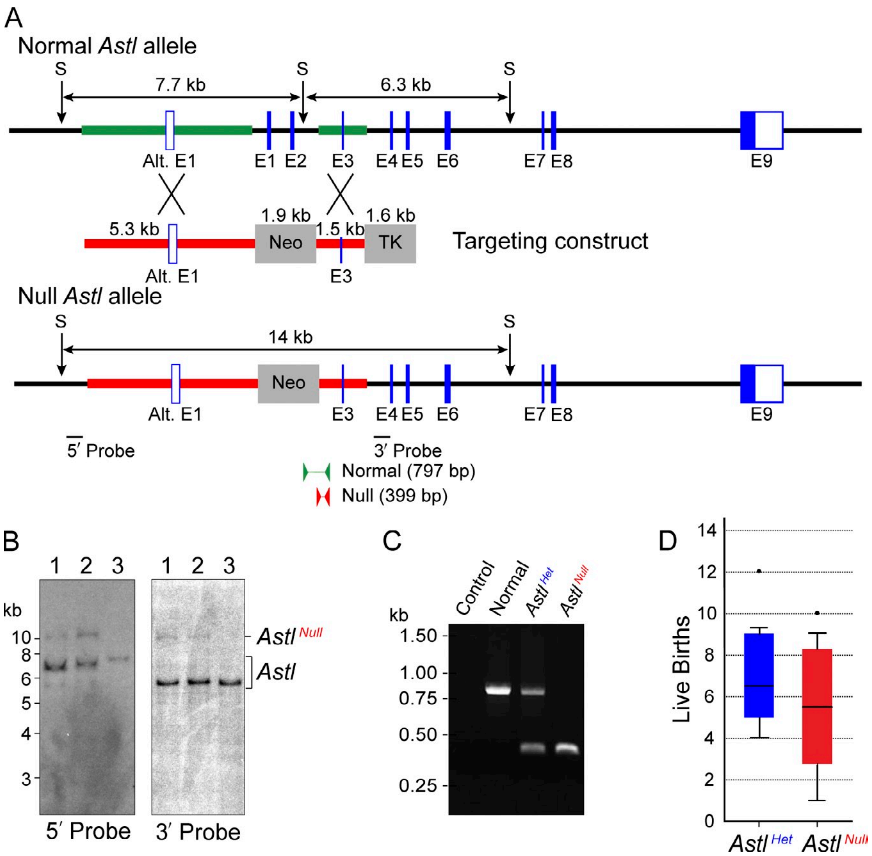


Figure 2. **Establishment of *Astl*^{Null} mice.** (A) A schematic of the normal and *Astl*^{Null} alleles after targeting with a construct containing positive (phosphoglucokinase [PGK]-Neo) and negative (MC1-TK) selectable markers. The thicker lines represent the 5.3- and 1.5-kbp homologous arms that are 5′ and 3′, respectively, to the Neo cassette. 5′ (407 bp; 5 bp outside of the targeting construct) and 3′ (368 bp; 97 bp outside of the targeting construct) probes were used for Southern blot hybridization. Arrows indicate SspI (S) restriction endonuclease sites. PCR genotyping was performed using primers for the normal allele (797 bp) and null allele (399 bp). Alt., alternative. (B) Southern blot hybridization of ES cell DNA from two successfully targeted clones (1 and 2) and one normal control (3) detected the normal allele as 7.7- and 6.3-kbp fragments with the 5′ and 3′ ^{32}P-labeled probes, respectively. The *Astl*^{Null} allele was detected as a 14-kbp fragment with either the 5′ or the 3′ probe. (C) PCR genotyping of mouse tail DNA detected the normal (797 bp) and null (399 bp) *Astl* alleles. (D) The size of litters from *Astl*^{Het} and *Astl*^{Null} female mice (five per group) mated with normal males over a period of 8–10 mo. Box plots reflect the median (line) and data points within the 10th and 90th percentiles (error bars). Boxes include the middle two quartiles, and outliers are indicated by dots.

cortical granules with great confidence. However, using EM, cortical granules were readily detected in *Astl*^{Het} and *Astl*^{Null} but not in two-cell embryos. Native ovastacin immunoprecipitated from ovaries or recombinant ovastacin expressed in insect cells did not react with LCA lectin (Fig. 3 C) despite LCA binding to a higher–molecular mass protein (not depicted) that validates the assay (Liu et al., 2003a). Thus, the molecular identity of the LCA-positive molecule in cortical granules remains to be determined. From these observations, we conclude that cortical granules remain present in the periphery of *Astl*^{Null} eggs (Fig. 3 B).

After fertilization, ZP2 remains uncleaved in the absence of ovastacin

Preimplantation development is a period of particular vulnerability for mammalian embryos. Biochemical or genetic removal of the protective zona pellucida causes resorption into the oviductal epithelium (Bronson and McLaren, 1970; Modliński, 1970; Rankin et al., 2001), and maternal effect genes that arrest or delay cleavage-stage development result in embryonic lethality (Li et al., 2010). Polyspermic aneuploidy is also a significant threat to embryonic survival, and mice have developed strategies to ensure monospermic fertilization. Prevention of sperm binding to the zona pellucida provides the ultimate postfertilization block to polyspermy. If sperm do not bind, they cannot penetrate the zona matrix, and they cannot fuse with the egg plasma membrane.

The postfertilization cleavage of ZP2 (120 kD) was initially reported to result in two fragments (30 and 90 kD) that remain disulfide bonded (Bleil et al., 1981). Using an mAb (M2c.2) that recognizes the 90-kD C-terminal fragment, this cleavage was detected by immunoblotting run under reducing conditions (Rankin et al., 2003) and occurs over 6–8 h after fertilization (Baibakov et al., 2007). ZP2 in *Astl*^{Null} eggs had a normal mass of 120 kD but was not cleaved in embryos isolated from *Astl*^{Null} females; ZP2, in two-cell embryos from normal and *Astl*^{Het} females, was cleaved and served as positive controls (Fig. 4 A). Using a 30-min de novo binding assay, capacitated mouse sperm bound to *Astl*^{Null} eggs (56.6 ± 2.8 SEM sperm/egg) but not normal two-cell embryos (3.8 ± 0.7 SEM sperm/embryo), which served as positive and negative controls, respectively. In sharp contrast, mouse sperm bound to two-egg embryos from *Astl*^{Null} mice (63.5 ± 3.0 SEM sperm/embryo) in a manner that was indistinguishable from the positive control (Fig. 4 B). Thus, the cleavage status of ZP2, independent of fertilization and cortical granule exocytosis, is the major determinant of sperm binding to the surface of the zona pellucida.

In reexamining the primary structure of mouse ZP2, two additional diacidic motifs were identified in the 30-kD N-terminal fragment (^{54}DE^{55} and ^{127}DD^{128}), and five were present in the 90-kD C-terminal fragment (Fig. 5 A). The detection of an intact 90-kD fragment on immunoblots of normal two-cell embryos probed with mAb M2c.2 (Fig. 4 A) suggests that additional C-terminal sites are not cleaved by ovastacin. To determine the cleavage status of the additional diacidic motifs in the 30-kD N terminus, immunoblots of eggs and two-cell embryos from normal and homozygous null *Astl* females were run under reducing conditions and probed with an mAb, IE-3, specific to ZP2$^{103-134}$ (East and Dean, 1984; Sun et al., 1999). As anticipated, ZP2 in

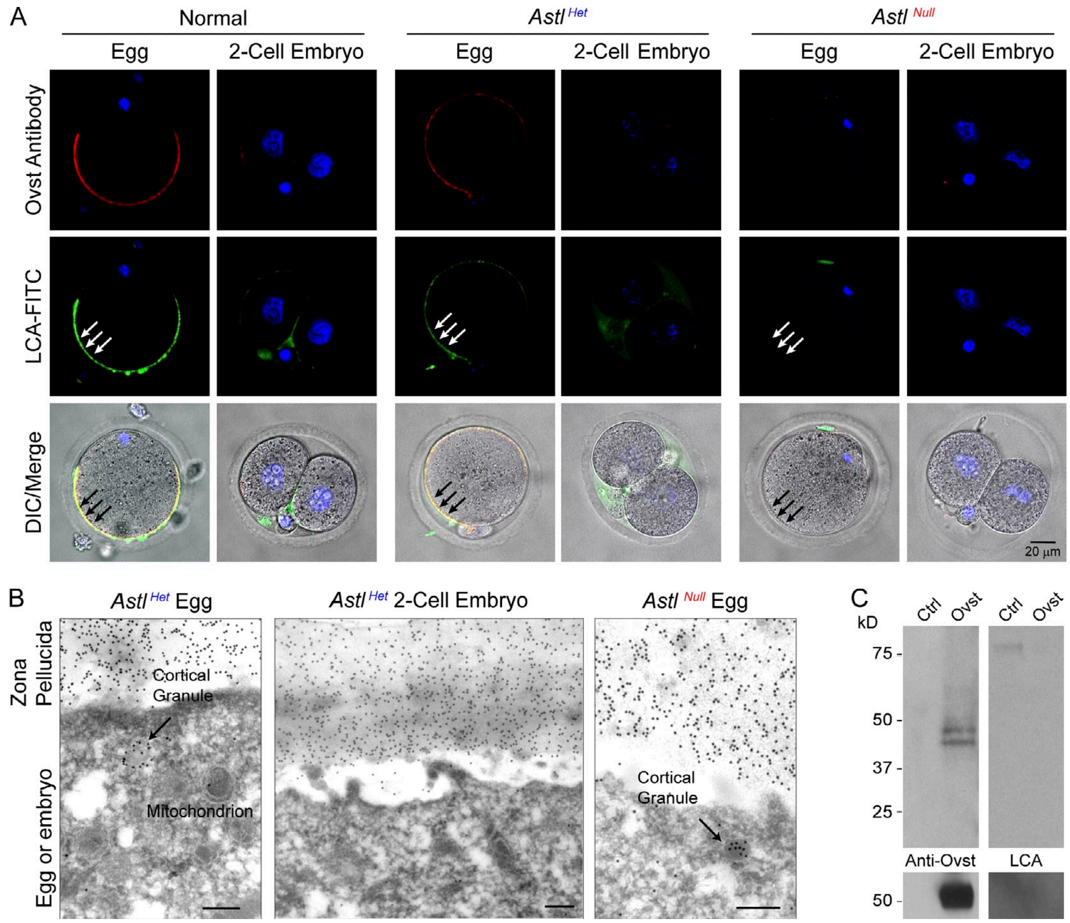
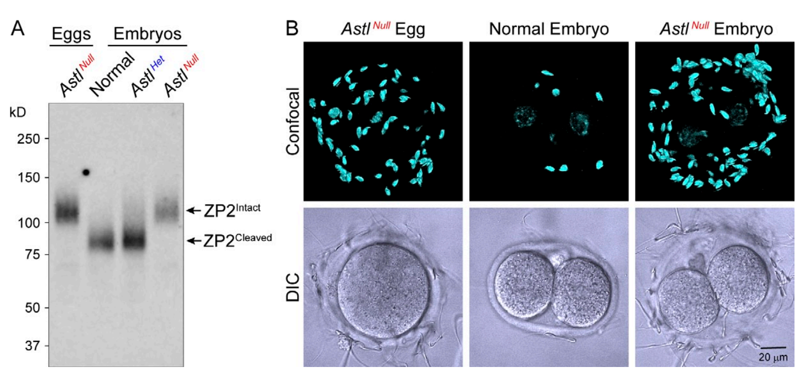


Figure 3. **Localization of ovastacin in normal and $Astl^{Null}$ mice.** (A) Unfertilized eggs and two-cell embryos from normal, $Astl^{Het}$, and $Astl^{Null}$ mice were collected and viewed by confocal and differential interference contrast (DIC) microscopy. Eggs and embryos were stained with rabbit anti-ovastacin (Ovst) antibody, LCA-FITC, and Hoechst 33342. Peripherally located cortical granules stained with LCA-FITC (white arrows) were observed in normal and $Astl^{Het}$ but not $Astl^{Null}$ eggs. The presence or absence of ovastacin correlated with the detection of LCA-FITC (black arrows in differential interference contrast/merge). (B) $Astl^{Het}$ and $Astl^{Null}$ eggs were stained with WGA lectin and imaged by EM. Cortical granules beneath the ooplasm (arrows) and the extracellular zona pellucida stained with WGA are shown. Mitochondria do not stain and serve as a negative control. After fertilization, cortical granules were absent from $Astl^{Het}$ two-cell embryos, although WGA continued to stain the zona pellucida. Bars, 0.5 µm. (C, top) Immunoblots of insect cell supernatant lacking (control [Ctrl]) or containing (Ovst) recombinant ovastacin probed with antibody to ovastacin (left) or with LCA lectin (right). (bottom) Immunoblots are the same after immunoprecipitation of normal ovarian lysates with antibody to ovastacin.

embryos from $Astl^{Null}$ females was not cleaved (Fig. 5 B). However, rather than a single 30-kD ZP2 fragment in embryos from normal females, four peptides were detected on the immunoblot probed with IE-3 (Fig. 5 B, arrows). One *N*-glycan (Arg^{83}), but no *O*-glycans, is present in the N terminus of ZP2, as determined by mass spectrometry (Boja et al., 2003). The largest glycopeptide observed on the immunoblot could represent the entire 30-kD N-terminal fragment, with the smaller peptides reflecting heterogeneity of cleavage at the three sites (^{54}DE^{55}, ^{127}DD^{128}, and ^{168}DE^{169}), given the binding site of the mAb (Fig. 5 A). Of note, all of the N-terminal ZP2 peptides remain disulfide bonded to the larger C-terminal fragment (Greenhouse et al., 1999). Thus, rather than a single cut, it now appears that the 30-kD fragment is further degraded through proteolysis.

Figure 4. **ZP2 cleavage and sperm binding to $Astl^{Null}$ two-cell embryos.** (A) Immunoblot of lysates from eggs (20) and two-cell embryos (20) from normal, $Astl^{Het}$, and $Astl^{Null}$ mice probed with an mAb specific for the C-terminal region of mouse ZP2 (M2c.2). Intact ZP2 is 120 kD, and the cleaved C-terminal fragment of ZP2 is 90 kD. (B) Eggs and two-cell embryos from $Astl^{Null}$ and two-cell embryos from normal mice were incubated (1 h) with capacitated sperm. After washing with a wide-bore pipette to remove all but two to six sperm on normal two-cell embryos (negative control), eggs and embryos were stained with Hoechst 33342, and bound sperm were presented as z projections of 5-µm confocal optical sections and differential interference contrast (DIC) images.



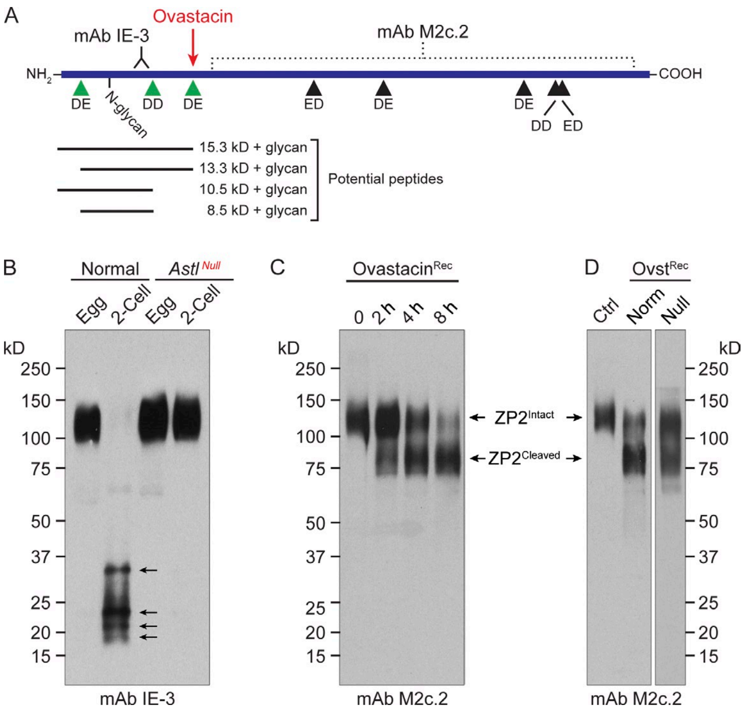


Figure 5. **Cleavage of ZP2 by ovastacin.** (A) A schematic of mouse ZP2 with eight diacidic motifs (triangles), the initial cleavage site of ovastacin, the binding sites of mAbs IE-3 and M2c.2 N terminal and C terminal to the initial cleavage site, and the localization of the single *N*-glycan in the N terminus. The potential size of peptides resulting from cleavage of the N-terminal fragment with ovastacin detected by IE-3 is shown on the bottom. (B) Immunoblot of eggs (15) or two-cell embryos (15) from normal and $Astl^{Null}$ females stained with IE-3 mAb that detects N-terminal fragments of ZP2. The top bands (120 kD) are intact ZP2, and bottom bands are a heterogeneous mixture of cleaved N-terminal fragments. Arrows indicate the four peptides detected on the immunoblot probed with IE-3. (C) Isolated zonae pellucidae (15–20) were incubated with partially purified recombinant ovastacin (Ovastacin^{Rec}) at 37°C for 0, 2, 4, and 8 h. (D) Same as in C but with insect cell supernatant lacking (control [Ctrl]) or containing (Ovst^{Rec}) recombinant ovastacin after 4 h of incubation with zonae pellucidae from normal (Norm) or $Astl^{Null}$ eggs. (C and D) Cleavage was detected by immunoblotting with M2c.2 to detect the ZP2 C-terminal fragment.

The $Astl^{Null}$ phenotype recapitulates mutation of the ZP2 cleavage site (^{166}LA↓DE^{169} → ^{166}LG↓AA^{169}; Gahlay et al., 2010), in which ZP2 remains intact in the zona pellucida surrounding two-cell embryos. Mutation of ^{168}DE^{169} prevents any cleavage of ZP2 (Gahlay et al., 2010), which indicates that it must be cut first before the ^{54}DE^{55} and ^{127}DD^{128} sites. In both $Zp2^{Mut}$ and $Astl^{Null}$ mouse lines, capacitated sperm bind to the zona pellucida surrounding two-cell embryos from homozygous mutant females despite fertilization and cortical granule exocytosis. However, the significant decrease in fecundity observed in $Zp2^{Mut}$ females was not present in $Astl^{Null}$ mice. A notable difference between the two mutant lines is a thinner zona pellucida (4 µm vs. 7 µm in normal mice) present in the $Zp2^{Mut}$ mice, which could result in precocious hatching from the protective zona matrix and embryonic lethality (Bronson and McLaren, 1970; Modliński, 1970; Rankin et al., 2001).

ZP2 is a direct target of ovastacin

These results indicate that ovastacin is exocytosed from cortical granules after fertilization and that the subsequent cleavage of ZP2 prevents sperm binding to the zona pellucida surrounding two-cell embryos. However, it was not clear whether ZP2 was the direct substrate for ovastacin or whether ovastacin acted indirectly through a cascade of proteolytic activators. Therefore, recombinant mouse ovastacin was expressed in insect cells and partially purified by column chromatography. The addition of the enzyme to zonae pellucidae isolated from normal eggs resulted in progressive cleavage of ZP2 over 8 h (Fig. 5 C), consistent with kinetics of cleavage observed in vivo (Baibakov et al., 2007). Similar cleavage of ZP2 was observed in the zona pellucida surrounding $Astl^{Null}$ as with normal eggs, and no cleavage was observed using cell supernatant from the same expression system not secreting ovastacin (Fig. 5 D). Thus, we conclude that ZP2 is the direct substrate for mouse ovastacin. Collectively, a simple explanation of these observations is that sperm bind to the N-terminal domain of ZP2 at the surface of the zona pellucida before penetration and fusion with the egg plasma membrane. After fertilization and cortical granule exocytosis, ovastacin diffuses through the zona matrix and fragments the N-terminal domain of ZP2. This proteolysis takes several hours and renders the zona pellucida unable to support sperm binding.

Although destruction of the sperm docking domain is definitive in preventing polyspermy, mice have additional strategies to ensure monospermic fertilization. Particularly striking is the ability to restrict the number of sperm that encounter ovulated eggs in the oviduct. Although millions of sperm are deposited in the female reproductive tract at coitus, only thousands traverse the utero–tubal junction, where they are initially confined to the oviductal isthmus by adherence to the epithelial lining (Suarez and Pacey, 2006). After capacitation and hyperactivation, individual sperm free themselves and ascend to fertilize ovulated eggs present in the oviductal ampulla. Gamete recognition occurs at low ratios in vivo, and the number of sperm does not exceed the number of eggs until 50% have been fertilized (Cummins and Yanagimachi, 1982). Nevertheless, the imperative of monospermy invokes additional defenses to polyspermy. The most immediate block prevents fusion of additional sperm in the perivitelline space with the egg plasma membrane. This block is independent of cortical granule exocytosis but requires fusion with sperm, as it can be bypassed by intracellular sperm injection (Horvath et al., 1993; Maleszewski et al., 1996) and does not depend on membrane depolarization (Jaffe et al., 1983). Within minutes of fertilization, a second block that is dependent on cortical granule exocytosis prevents additional sperm from penetrating through the zona pellucida. The rapidity of this block suggests diffusion of a small molecule or prompt propagation of a structural modification of the zona matrix. However, cleavage of ZP2 by ovastacin provides the most definitive block to polyspermy by destroying the sperm docking domain on the zona pellucida to ensure monospermic fertilization and successful development.

The *Astl* gene is well conserved in humans (Quesada et al., 2004) and presumably plays a similar role in the postfertilization cleavage of ZP2 (Bauskin et al., 1999). The modest decrease in fecundity observed with $Astl^{Null}$ mice lacking ovastacin could have a greater impact in humans with their preponderance of single births. If this leads to recurrent pregnancy loss, testing for mutations in the human *ASTL* gene could become relevant in the clinic. In this context, it will be important to determine whether the absence of ovastacin affects the incidence of polyspermy (rarely reported in humans) or the structural integrity of the zona pellucida that protects the embryo as it passes down the oviduct (Bronson and McLaren, 1970; Modliński, 1970; Rankin et al., 2001). It is also noteworthy that precocious release of ovastacin from cortical granules could prematurely cleave ZP2 to prevent sperm binding and fertilization. Thus, the mouse may provide a model in which to begin to test such contraceptive strategies for possible future use in human biology.

Materials and methods

Antibodies

A rabbit pAb was generated against peptide ^{395}PLALFPEARDKPAP^{408} of mouse ovastacin attached N terminal to a cysteine residue and conjugated to keyhole limpet hemocyanin (Sigma-Aldrich). mAbs IE-3 and M2c.2 that bind to the N- and C-terminal regions of ZP2, respectively, were previously described (East and Dean, 1984; Rankin et al., 2003), and the following antibodies and lectins were obtained commercially: LCA-FITC (Sigma-Aldrich), donkey anti–rabbit conjugated with Alexa Fluor 555 (Invitrogen), goat anti–rabbit DyLight 649 (Thermo Fisher Scientific), donkey anti–rabbit conjugated with HRP (Jackson ImmunoResearch Laboratories, Inc.), and goat anti–rat conjugated with HRP (Jackson ImmunoResearch Laboratories, Inc.).

Egg and embryo collection and culture

4–5-wk-old female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin followed by 5 IU of human chorionic gonadotropin 48 h later. Ovulated eggs and embryos were collected before and after mating, respectively, in M2 medium (Millipore) containing protease inhibitors (Roche). Embryos were subsequently cultured in potassium simplex optimized medium (Millipore) at 37°C in 5% CO_2. All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health under the Division of Intramural Research, National Institute of Diabetes and Digestive and Kidney Diseases approved animal study protocols.

Immunofluorescence and confocal microscopy

Oocytes and embryos were fixed in 2% PFA for 30 min at 37°C, washed in PBS containing 0.3% polyvinylpyrrolidone (PVP), and then blocked in 0.3% BSA/0.1 M glycine (three times for 10 min) followed by permeabilization in 0.2% Triton X-100 for 15 min (Baibakov et al., 2007). Oocytes and embryos were then incubated (1 h) with primary antibody (1:50), washed with 0.3% PVP/0.1% Tween (three times for 10 min), and incubated (45 min) with secondary antibody (1:100) followed by staining with Hoechst 33342 (Invitrogen) before imaging. Alternatively, eggs and embryos were stained with LCA-FITC (1:100). Samples were mounted in PBS, and confocal laser-scanning images were obtained on a confocal microscope (LSM 510; Carl Zeiss) with a 63× 1/2 W objective using the manufacture's software. LSM images were exported as full-resolution TIF files and processed in Photoshop (Adobe) to adjust brightness and contrast.

Immunoblot analysis

Eggs and two-cell embryos were lysed in 2 or 4× Tris-glycine SDS loading buffer with DTT, separated on 4–20% Tris-glycine gels by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Invitrogen), blocked in 3 or 5% nonfat milk in PBS, and probed with primary antibodies followed by secondary antibodies conjugated to HRP (Gahlay et al., 2010). Chemiluminescence was performed with ECL Plus (GE Healthcare), and signals were acquired by the Luminescent Image Analyzer LAS-3000 (Fujifilm) or with BioMax XAR film (Kodak).

For detection of ovastacin, blots were incubated with a 1:1,000 dilution of peptide-purified, rabbit anti–mouse ovastacin antibody (1.7 mg/ml) in 5% nonfat milk in TBS with 0.1% Tween 20 (TBST) at 4°C overnight. On the following day, blots were incubated with a 1:10,000 dilution of goat anti–rabbit HRP in TBST for 1 h at room temperature. For staining with LCA, blots were incubated with 10 µg/ml of the biotinylated LCA (US Biological) in 5% nonfat milk in TBST at 4°C overnight. Blots were then incubated with a 1:10,000 dilution of HRP-streptavidin (Thermo Fisher Scientific) in TBST and incubated for 1 h at room temperature.

Establishment of the *Astl*-null mouse line

Astl is a single-copy gene that encodes ovastacin. Mouse lines lacking ovastacin protein were established using DNA recombineering (Liu et al., 2003b) and targeted ablation in ES cells (Zheng and Dean, 2009). The targeting construct contained positive (neomycin resistance) and negative (herpes simplex virus thymidine kinase) selectable markers and replaced exons 2 and 3 of *Astl* and deleted the transcriptional and translational start sites. Correctly targeted ES cells were identified by Southern hybridization of SspI-digested genomic DNA using ^{32}P-labeled probes 5′ (−6,766 to −6,234 bp of the transcriptional start site) and 3′ (2,688–3,056 bp) to the targeting vector. Heterozygous null ES cells were injected into mouse blastocysts to establish chimeric founder lines. Germline transmission of the null allele and subsequent genotyping were determined by allele-specific PCR products of tail DNA. Primers P1 (5′-AGGCCTTGTCACCAGGTATG-3′) and P2 (5′-CCAGAGAATGAAGGGAGCAG-3′) were used to detect the normal allele (797 bp), and primers P2 and P3 (forward 5′-GGGAGGATT-GGGAAGACAAT-3′) were used to detect the null allele (399 bp) in PCR genotyping of tail DNA. The PCR condition consisted of one cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min and 30 s; and a full extension cycle at 72°C for 10 min.

Fertility

$Astl^{Het}$ and $Astl^{Null}$ females were cocaged with FVB males of proven fertility to determine the number and size of litters for a period of 8–10 mo.

EM

Oocytes and embryos from $Astl^{Het}$ and $Astl^{Null}$ females were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and incubated at 4°C for 2 h. After extensive washing in the cacodylate buffer, the oocytes and embryos were embedded in 2% agarose. The samples were then dehydrated through a graded series of ethanol and processed for embedding in London Resin white. Ultrathin sections were obtained with an ultramicrotome (MICROM International GmbH) and mounted on Formvar-coated nickel grids. For lectin cytochemistry (Jiménez-Movilla et al., 2004), grids were preincubated (for 10 min at room temperature) in PBS (1% BSA) and transferred to a drop of WGA-HRP lectin (Sigma-Aldrich) in PBS for 1 h. After rinsing in PBS, grids were floated on a drop of rabbit anti-HRP pAb (Sigma-Aldrich) diluted 1:500 in PBS for 1 h. Grids were then washed in PBS and floated on a drop of Protein A gold (15 nm)–conjugated antibody (1:60; Utrecht University) for 1 h. After washing in twice-distilled water, ultrathin sections were counterstained with uranyl acetate followed by lead citrate and imaged in a transmission electron microscope (Philips Tecnai 12; FEI). WGA-positive cortical granules were observed in five nonserial ultrathin sections (total; mean ± SEM/section) from three $Astl^{Null}$ oocytes (180; 12.0 ± 0.7). $Astl^{Het}$ (337; 22.5 ± 0.7) oocytes and two-cell embryos (no cortical granules) served as positive and negative controls, respectively.

Immunoprecipitation

Ovaries from 8-wk-old FVB mice were homogenized in cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol) with one tablet of protease inhibitor cocktail (Roche) added to every 10 ml of buffer. The sample was centrifuged (13,200 rpm at 4°C for 20 min), and supernatants were collected. 10 µg rabbit anti–mouse ovastacin antibody was added to the supernatant in a final volume of 800 µl and rotated overnight at 4°C. 30 µl protein G Sepharose beads (GE Healthcare) was equilibrated with lysis buffer and added to the protein–antibody mixture. This was rotated for an additional 2 h at 4°C. After a brief centrifugation, the supernatant was removed, and the beads were washed three times with 50 mM Tris, pH 8.0, 150 mM NaCl, and 1% NP-40 buffer. Two 30-µl aliquots of elution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and 1% SDS) were added to the beads for 10 min at 30°C. Eluates were separated on 12% SDS-PAGE gels before immunoblotting.

Sperm binding assay

Caudal epididymal sperm were isolated from wild-type FVB mice and placed under oil (Irvine Scientific) in human tubal fluid medium (Millipore) previously equilibrated with 90% N_2, 5% O_2, and 5% CO_2 and capacitated by an additional 30–60 min of incubation at 37°C (Baibakov et al., 2007). Sperm binding to 10–20 ovulated eggs and two-cell embryos isolated from normal and *Astl*^{Null} mice was quantified using capacitated sperm and normal two-cell embryos as wash controls. Samples were fixed in 2% PFA for 30 min, stained with Hoechst 33342, and imaged by confocal microscopy.

Recombinant ovastacin

pDonr253 is a Gateway Donor vector modified from pDonr201 (Life Technologies). pDonr253 replaces the kanamycin resistance gene with a gene encoding spectinomycin resistance and contains several sequencing primer sites to aid in sequence verification of Entry clones. The following oligonucleotides (Eurofins MWG Operon) were used: 4771, 5′-GGGGACAACTTTGTACAAGAAAGTTGATTAGCCTTTTTCGAATTGCGGATGGCTCC-3′; 6088, 5′-GGGGACAACTTTGTACAAAAAAGTTGGCACCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTTATGGTC-3′; 11154, 5′-CGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCGGCCGGAGCGCCCCTGGCCTCCAGCTGCG-3′; 11155, 5′-CGTTGCCCTTGTTTTTATGGTCGTATACATTTCTTACATCTATGCGGCCGGAGCACCCTCAGCATCCAGATGTTC-3′; 11156, 5′-GCCTTTTTCGAATTGCGGATGGCTCCAGGATCCATCTTCGGACATCCCCTTGAAATGATT-3′; and 11157, 5′-GCCTTTTTCGAATTGCGGATGGCTCCAGGATCCGTCTCTGGGCACCTCTCTAATGTGAC-3′.

Mouse ovastacin was cloned using PCR from cDNA templates for baculovirus expression. A honeybee melittin signal peptide leader sequence was added to the 5′ end of each construct to enhance secretion of the proteins in insect cells, and a C-terminal noncleavable Strep2 tag (GSWSHPQFEKG) was added for purification purposes. Initial PCR was performed using Phusion DNA polymerase (New England Biolabs, Inc.) under standard conditions using a 40-s extension time and 200 nM of flanking primers. After five cycles of amplification, 200 nM of each adapter primer was added, and amplification was continued for 20 additional cycles. The final PCR products are flanked by Gateway recombination signal sequences attB1 at the 5′ end and attB2 at the 3′ end. The PCR products were cleaned using the QIAquick PCR purification kit (QIAGEN) and recombined into pDonr253 using the Gateway BP recombination reaction (Life Technologies) and the manufacturer's protocols. BP reactions were transformed into *Escherichia coli* DH10B cells, and colonies were isolated on lysogeny broth plates containing 50 µg/ml spectinomycin. Plasmid DNA was prepared and sequenced using a variety of internal and external sequencing primers to verify the sequence.

The sequence-verified Entry clones were subcloned by Gateway LR recombination (Life Technologies) into pDest-8 for insect cell expression. Final expression clones were verified by size and restriction digest pattern. The expression clones were then transformed into *E. coli* DH10Bac (Life Technologies) and plated on selective media containing gentamycin, kanamycin, tetracycline, IPTG, and X-gal as per the manufacturer's protocols. White colonies were selected from these plates, and bacmid DNA was generated by alkaline lysis plasmid preparation and verified by PCR amplification across the bacmid junctions.

The bacmid DNAs were complexed with XpressNOW transfection reagent (Lonza) and transfected into 100 ml of Sf-9 insect cells at 1.5×10^6 ml^{-1} in SFX-Insect medium (HyClone; Thermo Fisher Scientific). At 5 d after transfection, the cultures were centrifuged at 1,100 *g*, and the virus-containing supernatant was collected. For expression, one liter of High Five cells was set in SFX medium in a three-liter Erlenmeyer (Corning) at a cell concentration of $\sim 1.5 \times 10^6$ ml^{-1} and infected (multiplicity of infection of three) with 40 ml of the recombinant baculovirus. The culture was grown in a shaker incubator at 21°C for 3 d, and the supernatant was collected after centrifugation at 1,100 *g*.

The conductivity of the supernatant was adjusted to 12.61 mS/cm with 20 mM Hepes, pH 7.3. The supernatant (41 ml) was applied to a 5-mL Q Sepharose column (GE Healthcare) equilibrated with 20 mM Hepes, pH 7.3, and 75 mM NaCl. A flow-through sample was collected, and then a 5-ml S Sepharose column (previously equilibrated in 20 mM Hepes, pH 7.3, and 75 mM NaCl) was added for subsequent purification on both columns. After completion of the load, the columns were washed to baseline in 20 mM Hepes, pH 7.3, and 75 mM NaCl. A 10-column volume elution from 75 mM to 1 M NaCl was completed by collecting 2.5-ml fractions across the gradient. Analysis was performed via SDS-PAGE/Coomassie staining and immunoblotting. The rabbit anti-ovastacin antibody was incubated overnight at 4°C, washed at room temperature with 1× TBS with Tween 20, incubated 1 h in 1:5,000 anti–rabbit secondary antibody, and washed five times in 1× TBS with Tween 20. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) was used to develop the signal. The fractions with the highest concentration of ovastacin were used in subsequent assays.

In vitro cleavage assay

Zonae pellucidae were isolated from 150 oocytes by freeze thawing four times in 100 µl PBS, pH 7.4, 0.1% IGEPAL CA-630 (Sigma-Aldrich), and 0.5 M NaCl. Isolated zonae were solubilized in 30 µl PBS, 0.4% PVP, and 0.1% SDS by heating at 60°C for 30 min. Solubilized zona samples were incubated with recombinant ovastacin at 37°C over time (0–8 h), and cleavage was analyzed by immunoblotting with mAb M2c.2.

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