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Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis

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At fertilization, spermatozoa bind to the zona pellucida (ZP1, ZP2, ZP3) surrounding ovulated mouse eggs, undergo acrosome exocytosis and penetrate the zona matrix before gamete fusion. Following fertilization, ZP2 is proteolytically cleaved and sperm no longer bind to embryos. We assessed *Acr3*-EGFP sperm binding to wild-type and hu*ZP2* rescue eggs in which human ZP2 replaces mouse ZP2 but remains uncleaved after fertilization. The observed de novo binding of *Acr3*-EGFP sperm to embryos derived from hu*ZP2* rescue mice supports a 'zona scaffold' model of sperm-egg recognition in which intact ZP2 dictates a three-dimensional structure supportive of sperm binding, independent of fertilization and cortical granule exocytosis. Surprisingly, the acrosomes of the bound sperm remain intact for at least 24 hours in the presence of uncleaved human ZP2 regardless of whether sperm are added before or after fertilization. The persistence of intact acrosomes indicates that sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. A filter penetration assay suggests an alternative mechanism in which penetration into the zona matrix initiates a mechanosensory signal transduction necessary to trigger the acrosome reaction.

KEY WORDS: Molecular mechanisms of mouse fertilization, Sperm acrosome exocytosis, Mechanosensory signal transduction, Zona pellucida matrix, Sperm-egg recognition, Postfertilization block to polyspermy

INTRODUCTION

Mouse spermatozoa navigate both the male and female reproductive tracts before encountering eggs in the upper oviduct. During this travel, spermatozoa must avoid inadvertent fusion with somatic cells while eventually fusing with the plasma membrane of the egg. These two competing demands are mediated in part by the integrity of the acrosome, a Golgi-derived exocytotic vesicle overlying the condensed sperm nucleus that releases its partially characterized contents during fertilization (Abou-Haila and Tulsiani, 2000). It has been reported that only acrosome-intact mouse sperm penetrate the cumulus oophorus (a hyaluronic acid matrix with interspersed somatic cells) surrounding ovulated eggs and bind to the zona pellucida, an extracellular matrix that encircles the egg (Storey et al., 1984; Cherr et al., 1986). However, only acrosome-reacted sperm are present in the perivitelline space (between the plasma membrane of the egg and the inner aspect of the zona pellucida), and only acrosome-reacted sperm fuse with the plasma membrane of the egg to effect fertilization (Austin, 1975; Saling et al., 1979). The presence of residual acrosomal shrouds on the zona pellucida (Yanagimachi and Phillips, 1984; Cherr et al., 1986; VandeVoort et al., 1997) suggests that induction of acrosome exocytosis (acrosome reaction) occurs on the surface of the zona matrix.

The induction of sperm acrosome exocytosis in mouse fertilization has long engaged investigative interest, but remains incompletely understood. Unlike recyclable exocytosis in somatic cells, the acrosome reaction occurs once and is biologically relevant only if it takes place in conjunction with sperm penetration of the zona pellucida. Exocytosis results from fusion between the outer acrosomal membrane of the sperm and the overlying plasma membrane, causing multiple membrane fenestrations and release of acrosomal contents (Barros et al., 1967). Although initially considered a binary event (acrosome-intact or acrosome-reacted), more recent data suggest that the acrosome reaction is a continuum, beginning with capacitation of sperm, and is dramatically accelerated by interactions with the zona pellucida (Lee and Storey, 1985; Kim and Gerton, 2003). The acrosome possesses a calcium store that is sufficient to trigger exocytosis (Herrick et al., 2005). Whether physiologic mobilization is initiated by G_i-protein mediated ligand-receptor interactions (Ward et al., 1994), extracellular Ca^{2+} influxes (Jungnickel et al., 2001), activation of internal inositol 1,4,5-triphosphate receptors (Herrick et al., 2005) or other mechanisms remains unclear. Although multiple agonists induce the acrosome reaction two- to threefold over background in vitro (Florman and Storey, 1982; Bleil and Wassarman, 1983; Meizel, 1985; Roldan et al., 1994; Shi and Roldan, 1995; Murase and Roldan, 1995; Sato et al., 2000; Son and Meizel, 2003), only binding or penetration of the zona pellucida during fertilization is 100% effective in triggering acrosome exocytosis (Austin, 1975; Saling et al., 1979; Yanagimachi, 1994).

The mouse zona pellucida is composed of three glycoproteins, ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980b; Boja et al., 2003). Following fertilization, egg cortical granules exocytose their contents, which modify the zona matrix so that additional sperm do not bind or penetrate (Austin and Braden, 1956; Ducibella, 1996). Although other biochemical changes have been inferred, only the proteolytic cleavage of ZP2 has been experimentally observed (Moller and Wassarman, 1989). The human homologs to the three mouse zona proteins are 62-71% identical (Hoodbhoy et al., 2005), and genetically altered mice in which human ZP2 replaces endogenous mouse ZP2 (hu*ZP2* rescue mice) form a zona pellucida and are fertile. Unexpectedly, sperm continue to bind to two-cell embryos after in vitro fertilization of eggs derived from hu*ZP2* rescue mice and this observation correlates with intact huZP2, which is not cleaved in the chimeric mouse-human zona pellucida. Nevertheless, the normal postfertilization block to zona penetration is observed and there is no increased incidence of polyspermy (Rankin et al., 2003). These observations form the basis of the 'zona

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scaffold' model of sperm binding, in which the cleavage status of ZP2 regulates the three-dimensional structure of the zona matrix, rendering it permissive (ZP2 intact) or non-permissive (ZP2 cleaved) for sperm binding, independent of fertilization and cortical granule exocytosis (Dean, 2004).

Using *Acr3*-EGFP sperm that accumulate soluble enhanced green fluorescent protein within the acrosome, we examined the acrosome status and binding of sperm to eggs and embryos derived from wild-type, hu*ZP2* transgenic and hu*ZP2* rescue mice. We observed persistent sperm binding to hu*ZP2* transgenic and hu*ZP2* rescue eggs and two-cell embryos as predicted by the 'zona scaffold' model. Unexpectedly, sperm acrosomes remained intact when bound to wild-type and hu*ZP2* rescue eggs or embryos for greater than 2 and 24 hours, respectively. These data have implications for the mechanisms by which fertilizing sperm bind, acrosome react and penetrate the zona pellucida.

MATERIALS AND METHODS

Establishment of transgenic mouse lines

Using a transgene supplied by Dr Masaru Okabe, Osaka University (Nakanishi et al., 1999), two transgenic mouse lines, TgN(*Acr3*-EGFP)1NIH and TgN(*Acr3*EGFP)2NIH, were established. Each accumulated EGFP in their acrosomes (see Fig. S1A in the supplementary material) and were fertile in vivo. Sperm were isolated from the caudal epididymis and incubated under capacitating conditions (see below). After treatment with the calcium ionophore A23187 (3 μmol/l), individual sperm underwent the acrosome reaction exposing the inner acrosomal membrane to which Alexa 568-conjugated soybean trypsin inhibitor (SBTI) bound (see Fig. S1B in the supplementary material). Motile *Acr3*-EGFP sperm bound to ovulated eggs as effectively as wild-type sperm (see Fig. S1C in the supplementary material) and eggs fertilized in vitro underwent the cortical granule exocytosis (see Fig. S1D in the supplementary material) (Rankin et al., 2003). TgN(*Acr3*-EGFP)1NIH was used in these studies.

Zp3-EGFP transgenic mice were established using *Bgl*II-*Ace*I and *Ace*I-*Hin*dIII fragments of the mouse *Zp3* promoter (1.5 kbp) isolated from pXPZP3WT (Millar et al., 1991) and cloned into the *Bgl*II-*Hin*dIII sites of pEGFP1 (Clontech, Palo Alto, CA). A purified *Bgl*II-*Afl*II DNA fragment was injected into the male pronucleus of fertilized FVB/N eggs (Rankin et al., 2003) to establish two transgenic lines that accumulate EGFP in the cytoplasm of growing oocytes and preimplantation embryos (to be fully described elsewhere). TgN(*Zp3*-EGFP)1NIH was used in these studies. All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the National Institutes of Health under a Division of Intramural Research, NIDDK approved animal study protocol.

Sperm capacitation

Unless noted otherwise, caudal epididymal sperm isolated from wild-type and *Acr3*-EGFP mice were placed under oil (Irvine Scientific, Santa Ana, CA) in M16 media (Specialty Media, Chemicon International, Phillipsburg, NJ) and incubated under capacitating conditions (1 hour, 37°C, 5% CO_2) before use in sperm binding and in vitro fertilization assays.

Confocal microscopy

Confocal laser scanning images were obtained on a Zeiss LSM 510 confocal microscope (Rankin et al., 2003). To detect acrosome-reacted sperm, samples were incubated for 15 minutes before fixation with soybean trypsin inhibitor (SBTI) (Worthington, Lakewood, NJ) conjugated with Alexa 568 (Invitrogen Molecular Probes, Carlsbad, CA) according to the manufacturer's protocol. Samples were fixed in 2% paraformaldehyde and stained with Hoechst 33342 before imaging.

In vitro fertilization

Wild-type or *Acr3*-EGFP sperm were incubated (1, 2, 4 or 24 hours) with ovulated eggs obtained from gonadotropin-stimulated mice (Rankin et al., 1998). Embryos were then isolated, fixed, stained with Hoechst 33342 and imaged by confocal microscopy. Fertilization was confirmed by the presence of Hoechst-positive sperm nuclei within the egg cytoplasm. To count sperm bound to the zona pellucida, consecutive 6 μm optical sections were collected through individual embryos and projected onto a single-plane image. Acrosome-intact (EGFP-positive), acrosome-reacted (Alexa 568-SBTI-positive) and the total number (Hoechst positive nuclei) of sperm were determined.

Immunoblot analyses

Eggs or two-cell embryos were isolated from 4-week-old wild-type, hu*ZP2* transgenic and hu*ZP2* rescue mice after stimulation with gonadotropins with or without in vivo mating (Rankin et al., 1998) and were prepared for immunoblot (Rankin et al., 2003). Chemiluminescence signals were acquired by Luminescent Image Analyzer LAS-3000 (Fuji Film Medical Systems, Stamford, CT). To present mouse and human ZP2 results on the same blot, digital images were displayed in green or red channels, respectively, using Adobe Photoshop (Adobe Systems, San Jose, CA) RGB color mode.

Electron microscopy

After washing three times with a wide bore pipette, embryos from in vitro fertilization with wild-type, hu*ZP2* transgenic and hu*ZP2* rescue eggs were embedded in Spurr's plastic (SPI-Chem Low Viscosity 'Spurr' Kit, SPI Supplies, West Chester, PA) and thin sections were cut using a diamond knife on a RMC MTX ultramicrotome (Boeckler Instruments, Tucson, AZ). Thin sections were stained with uranium and lead salts and examined on a Tecnai 12 transmission electron microscope (FEI, Hillsborrow, OR) (Rankin et al., 1999).

Sperm binding assay

Ovulated eggs and two-cell embryos were isolated from wild-type (FVB strain), hu*ZP2* rescue and hu*ZP2* transgenic mice for sperm binding assays (Rankin et al., 1998). As a wash control in these assays, two-cell embryos were isolated from TgN(*Zp3*-EGFP)1NIH mice. Wild-type and/or *Acr3*-EGFP sperm binding to the zona pellucida was assessed after 1 or 24 hours by confocal microscopy (Rankin et al., 1999).

Filter penetration assay

To enrich for acrosome-intact wild-type and *Acr3*-EGFP sperm (Bangham and Hancock, 1955), 450 μl epididymal sperm was applied to a column (55×8 mm) packed with the 250-320 μm glass beads and equilibrated with M16 media (1 hour, 37°C, 5% CO_2). After washing the column with M16 media (2 ml), sperm were collected in a second fraction (4 ml) and counted on a cellometer (Nexcelom Bioscience, Lawrence, MA).

The sperm penetration assay chamber was a 25 mm Swinnex Filter (Millipore, Bedford, MA) holder from which the syringe connector tip was removed to establish a 10 mm opening into the upper chamber. In the center of the filter support, a 10 mm opening was drilled and the holder outlet was blocked with a piece of plastic. Epididymal or glass-bead-washed sperm (2.1×10^4 sperm, 850 μl) were placed in the lower chamber through the opening in the filter support. Polycarbonate filters (1.2, 3, 5 μm pores, Millipore, Bedford, MA; 12 μm pores, Whatman, Florham Park, NJ) were degassed in PBS under vacuum and placed, glossy side up, over the opening in the filter support, ensuring the exclusion of trapped air. After assembly of the filter holder, 500 μl preincubated (1 hour, 37°C, 5% CO_2) M16 media was added on top of the filter in the upper chamber. Following a 30 minute incubation (37°C, 5% CO_2), 400 μl was removed sequentially from the upper and lower chambers, fixed with an equal amount of 4% paraformaldehyde in PBS, and stained with propidium iodide to visualize nuclei.

Aliquots (400 μl) of sperm from the upper and lower chambers were analyzed by fluorescence activated cell sorting (FACS) (Calibur, Becton Dickinson BD, San Jose, CA). Using forward or side scatter density data, a discrete population of cells was selected that excluded clumps as well as subcellular debris; events below cellular auto fluorescence on EGFP or forward scatter plots were not included. Regions used to determine acrosome-intact or -reacted sperm on PI/EGFP dot plot were selected from analysis of glass-bead washed sperm and confirmed morphologically by confocal microscopy.

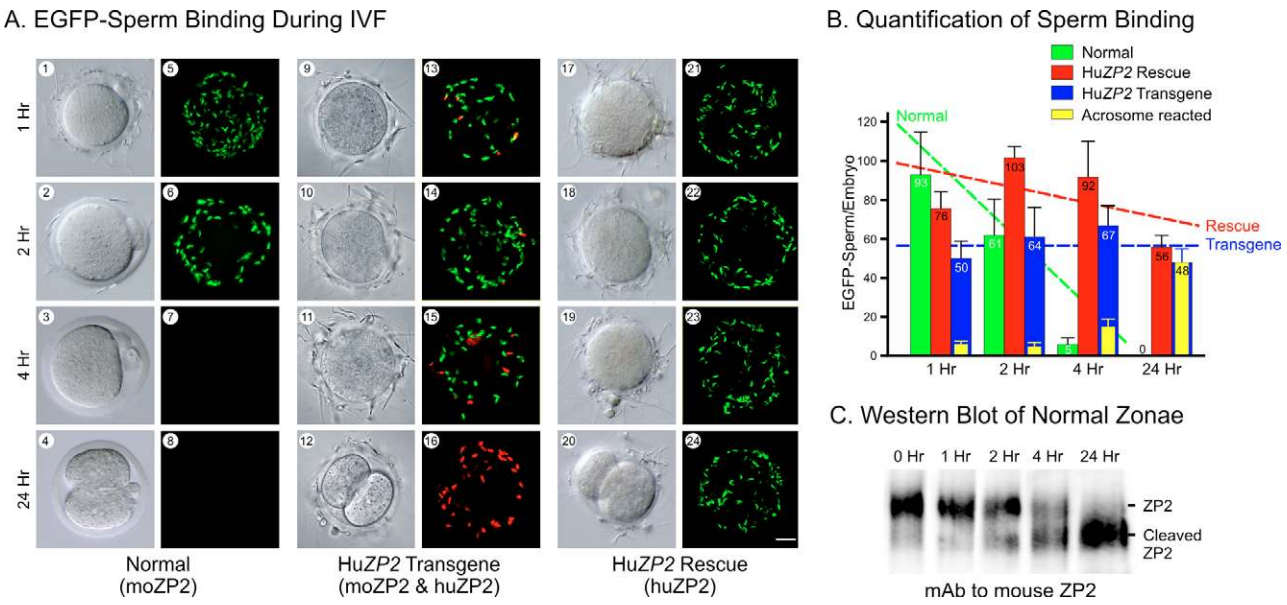

Fig. 1. Persistent sperm binding to hu*ZP2* rescue mouse embryos after fertilization. (**A**) Embryos were collected 1, 2, 4 and 24 hours after insemination and stained with Hoechst 33342 and Alexa 568-conjugated-soybean trypsin inhibitor (SBTI) before viewing by differential interference contrast (DIC) and confocal microscopy: (1-8) images of in vitro fertilization with *Acr3*-EGFP sperm (5×10^5) and wild-type (referred to as 'normal') eggs; (9-16) same as 1-8, but after in vitro fertilization of hu*ZP2* transgenic eggs; (17-24) same as 1-8, but after in vitro fertilization of hu*ZP2* rescue eggs. Only the green and red channels of each image are displayed. Intact acrosomes are indicated by green (EGFP) on the anterior surface of sperm heads; reacted acrosomes are indicated by red because of Alexa 568-SBTI binding to the inner acrosomal membrane. Fertilization rates (>85%) were comparable among genotypes as judged by decondensing Hoechst-positive sperm nuclei in the egg cytoplasm (data not shown). (**B**) Bar graph (mean and s.e.m.) of sperm binding to normal (A, 1-8) hu*ZP2* transgenic (A, 9-16) and hu*ZP2* rescue (A, 17-24) embryos. Dotted lines represent linear regression of averages of number of sperm binding to normal (green), hu*ZP2* transgenic (blue) or hu*ZP2* rescue (red) embryos. (**C**) Postfertilization cleavage of ZP2 detected by immunoblot using monoclonal antibody to mouse ZP2, and normal eggs or embryos isolated 0, 1, 2, 4 and 24 hours after in vitro fertilization with normal sperm. Scale bar: 20 μm.

RESULTS

Persistent sperm binding to hu*ZP2* rescue eggs

The zona pellucida surrounding wild-type (hereafter referred to as 'normal') mouse eggs contains three glycoproteins (moZP1, moZP2, moZP3), one of which (moZP2) is proteolytically cleaved (~120 kDa into 30 kDa and 90 kDa) following fertilization. The human *ZP2* transgenic zona contains the same three mouse proteins, plus human ZP2 (huZP2), and huZP2 entirely replaces endogenous moZP2 in human ZP2 rescue zonae (Rankin et al., 2003). After treatment with gonadotropins to stimulate ovulation, eggs were collected in cumulus from each of three genotypes (three independently obtained biological samples, 8-18 eggs each) and inseminated with 5×10^5 epididymal *Acr3*-EGFP sperm that had been incubated under capacitating conditions. During in vitro fertilization, a cohort of sperm approach the egg, and acrosome exocytosis of the 'fertilizing' sperm occurs shortly after sperm bind to the zona pellucida of normal, hu*ZP2* transgenic, and huZP2 rescue mice.

Acrosome-intact sperm (EGFP-positive) continued to bind to normal zonae pellucidae for several hours, but a progressive decrease in the number of sperm associated with fertilized eggs was observed over time and few bound to the embryo after 4 hours (Fig. 1A,B). The observed decrease in sperm binding correlated with initial cleavage of mouse ZP2, as detected by immunoblots using a monoclonal antibody specific to the larger (90 kDa), carboxyl fragment of ZP2 (Fig. 1C). Unexpectedly, *Acr3*-EGFP sperm remained acrosome-intact (EGFP-positive) for 2-3 hours, and few or no acrosome-reacted (SBTI-positive) sperm were observed (Fig. 1A, 5-6).

More strikingly, sperm bound for ⩾24 hours to fertilized eggs and early embryos derived from hu*ZP2* transgenic and hu*ZP2* rescue females (Fig. 1A) and by regression analysis, changes in sperm numbers were modest or non-existent (Fig. 1B). Sperm associated with hu*ZP2* rescue-derived embryos (Fig. 1A, 17-24) maintained an intact acrosome (EGFP-positive) for up to 24 hours after in vitro fertilization. Thus, the presence of intact human ZP2 provides a zona structure to which sperm will bind ⩾24 hours despite fertilization, and sperm binding to normal and hu*ZP2* rescue eggs or embryos was not sufficient to induce acrosome exocytosis. Sperm bound to hu*ZP2* transgenic eggs or embryos, however, underwent acrosome exocytosis beginning 4 hours after insemination, with all becoming EGFP-negative and SBTI-positive by 24 hours (Fig. 1A, 9-16 and Fig. 1B). The late acrosome reaction of these 'non-fertilizing' sperm may provide mechanistic insights, but is distinct from acrosome exocytosis induced by the initial interactions of 'fertilizing' sperm with ZP2-intact zonae pellucidae.

Electron microscopy of sperm bound to the zona pellucida

To better define the acrosome status of EGFP- and SBTI-positive sperm, embryos (3-5) derived from in vitro fertilization of normal (moZP1, moZP2, moZP3), hu*ZP2* transgenic (moZP1, moZP2, huZP2, moZP3) and hu*ZP2* rescue eggs (moZP1, huZP2, moZP3) were imaged by transmission electron microscopy (Fig. 2). Sperm bound to normal fertilized eggs (2 hours post-insemination), adhering to the zona pellucida with intact acrosomes contained within the inner and outer acrosomal membranes immediately apposed to the plasma

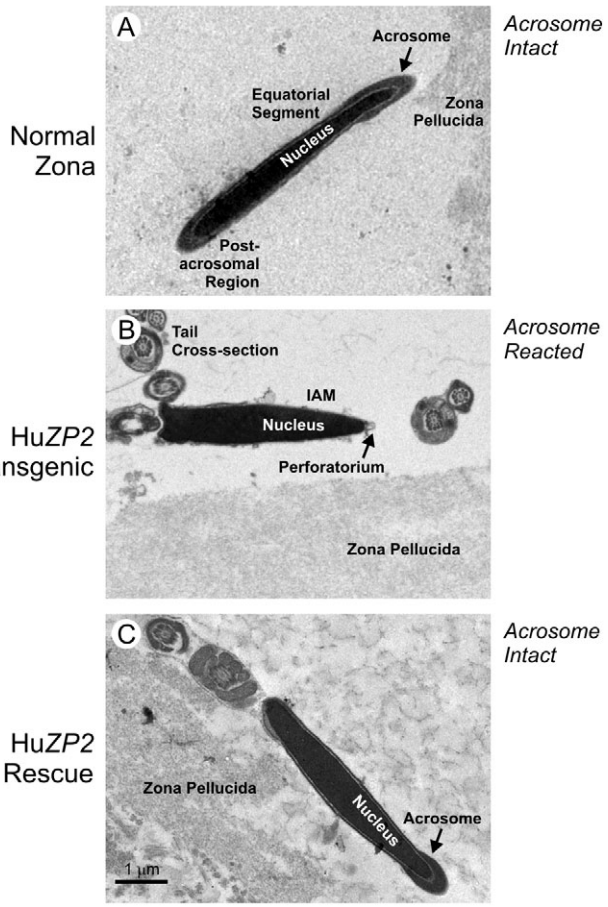

Fig. 2. Transmission electron photomicrographs of *Acr3*-EGFP sperm binding to mouse embryos. After in vitro fertilization, *Acr3*-EGFP sperm bound to: (**A**) the zona pellucida surrounding wild-type (referred to as 'normal') mouse embryos (2 hours post-insemination) with an intact acrosome; (**B**) hu*ZP2* transgenic embryos (12 hours post-insemination) having undergone acrosomal exocytosis; and (**C**) hu*ZP2* rescue embryos (12 hours post-insemination) with an intact acrosome. IAM, inner acrosomal membrane. Scale bar: 1 μm.

membrane overlying the sperm head (15 of 15 sperm, Fig. 2A). Sperm also bound to hu*ZP2* transgenic embryos (12 hours post-insemination), but for the most part (31 of 34 sperm, 91%) their plasma membrane and outer acrosomal membranes had fused (Fig. 2B), presumably leading to loss of EGFP through the resulting fenestrations and consistent with the similarly bound SBTI-positive sperm observed by confocal microscopy (Fig. 1A, 16). By contrast, sperm that bound to hu*ZP2* rescue embryos (12 hours post-insemination) were mostly acrosome-intact (42 of 51 sperm, 82%), and bound by intact plasma membrane (Fig. 2C), much like normal controls. Thus, confocal images of EGFP- and SBTI-positive sperm provide realistic proxies of the ultrastructure of the mouse acrosome, and both acrosome-intact and acrosome-reacted sperm can bind to early embryos containing human ZP2 in the zona pellucida.

Quantification of de novo sperm binding to two-cell embryos

Normally, sperm do not bind to two-cell embryos, and the persistence of sperm on the surface of hu*ZP2* rescue embryos has been ascribed to the ability of uncleaved human ZP2 to preserve a three-dimensional matrix architecture that is permissive for sperm binding (Rankin et al., 2003). To further examine this model, two-cell embryos derived from normal, hu*ZP2* transgenic and hu*ZP2* rescue mice were flushed from oviducts after in vivo mating. Additional *Acr3*-EGFP sperm (5×10^5) were added to these in vitro fertilized two-cell embryos (three independently obtained biological samples, ten embryos each) in a sperm binding assay using *Zp3*-EGFP two-cell embryos as wash controls.

After washing with a wide-bore pipette to remove loosely adherent sperm (Fig. 3A, 1,4,7), no sperm bound to normal two-cell embryos and comparable numbers bound to embryos derived from hu*ZP2* transgenic and hu*ZP2* rescue eggs (49±8 and 73±15, respectively). Virtually all of the sperm associated with embryos derived from both the hu*ZP2* transgenic and hu*ZP2* rescue eggs remained acrosome-intact (EGFP-positive), with few showing evidence of having undergone the acrosome reaction (SBTI-positive) (Fig. 3A, 4-9). If the embryos were incubated for an additional 24 hours, almost all of the sperm bound to hu*ZP2* rescue zonae remained acrosome-intact (Fig. 3B, 4-6), whereas all of the sperm bound to the hu*ZP2* transgenic zonae pellucidae underwent the acrosome reaction (Fig. 3B, 1-3). Thus, persistent sperm binding (up to 24 hours) to the zona pellucida is not sufficient to induce the acrosome reaction when human ZP2 replaces endogenous mouse ZP2, but does occur, albeit delayed, if both mouse and human ZP2 are present in the zona matrix.

Cleavage status of mouse and human ZP2 proteins

To investigate the mechanism of this dichotomy, the cleavage status of mouse and human ZP2 of eggs and two-cell embryos derived from normal, hu*ZP2* transgenic and hu*ZP2* rescue female mice was examined (Fig. 4). The primary structures of secreted mouse ZP2 (599 amino acids) and human ZP2 (602 amino acids) are 62% identical, but their molecular masses differ (120-140 kDa and 90-110 kDa, respectively) due to differences in post-translational modifications. Each ZP2 protein is normally cleaved following fertilization resulting in two fragments (~30 kDa, 90 kDa for mouse; ~35 kDa, 65 kDa for human) that remain linked by disulfide bands (Moller and Wassarman, 1989; Bauskin et al., 1999).

Eggs and two-cell embryos (10-15) were isolated from normal, hu*ZP2* transgenic and hu*ZP2* rescue mice. As determined by immunoblots (Fig. 4) using monoclonal antibodies specific to the carboxyl terminus of mouse (green) or human ZP2 (red), mouse ZP2 was intact in normal and hu*ZP2* transgenic eggs and cleaved in normal and hu*ZP2* transgenic two-cell embryos (Fig. 4, lanes 1,2,5,6). By contrast, human ZP2 remained uncleaved in both eggs and two-cell embryos derived from hu*ZP2* transgenic and hu*ZP2* rescue female mice (Fig. 4, lanes 3-6). Neither the mouse- nor the human-specific antibodies crossreacted with ZP2 from the other species (Fig. 4, lanes 1-4). Normally, ZP2 is cleaved by a cortical granule protease released by the egg following fertilization. The ability of the protease to cleave mouse, but not human, ZP2 in hu*ZP2* transgenic mice indicates access within the 'humanized' zona architecture and suggests that intrinsic differences in the two homologs prevent cleavage of human ZP2 (Rankin et al., 2003). Thus, uncleaved human ZP2 in the absence of mouse ZP2 is sufficient to support mouse sperm binding for ⩾24 hours without inducing the acrosome reaction.

Reversibility of sperm binding to ovulated eggs and two-cell embryos

The number of sperm binding to normal, hu*ZP2* transgenic and hu*ZP2* rescue eggs (three independently obtained biological samples, ten eggs each) was similar (100±10, 112±8, 103±5,

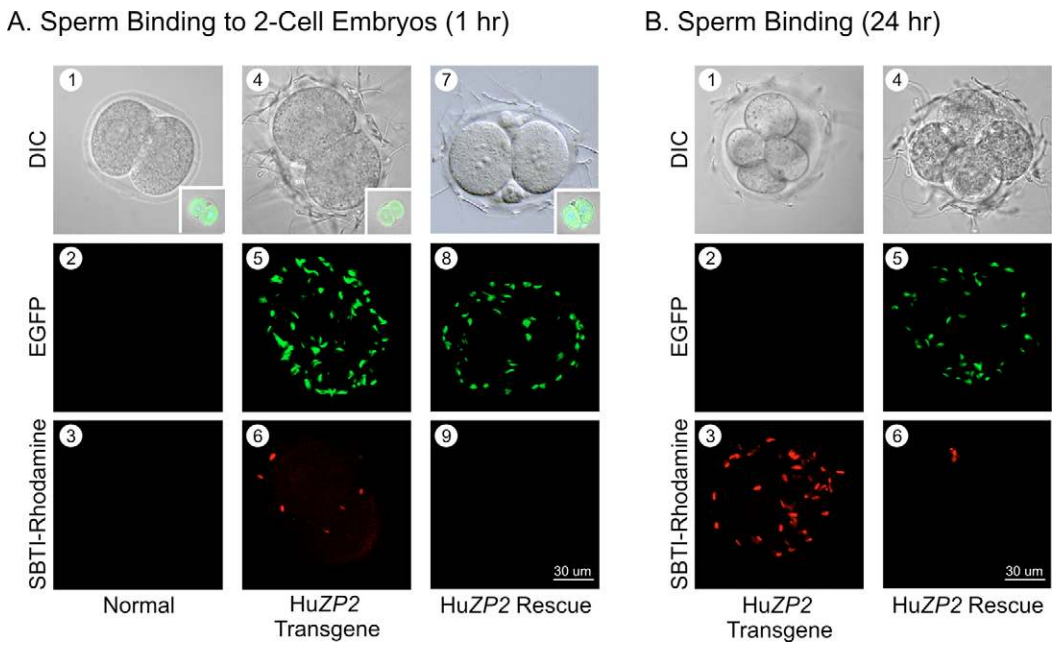

Fig. 3. De novo *Acr3*-EGFP sperm binding to two-cell embryos. (**A**) Two-cell embryos derived from wild-type (referred to as 'normal') (1-3), hu*ZP2* transgenic (4-6) and hu*ZP2* rescue (7-9) mice were incubated with 5×10^5 motile, capacitated *Acr3*-EGFP sperm for 1 hour and washed; *Zp3*-EGFP two-cell mouse embryos were used as controls (insets 1,4,7). Embryos were stained with Alexa 568-SBTI and fixed before imaging by DIC (1,4,7) and confocal microscopy to observe EGFP (2,5,8) or SBTI binding (3,6,9), reflecting acrosome-intact and acrosome-reacted sperm, respectively. (**B**) Aliquots of embryos from A were incubated for an additional 23 hours, and similar numbers of sperm bound to hu*ZP2* transgenic (1-3, 49±8 s.e.m. per embryo) and hu*ZP2* rescue (4-6, 73±15 s.e.m. per embryo) embryos. However, sperm binding to hu*ZP2*-transgenic-derived embryos were acrosome-reacted (97%), and those binding to hu*ZP2*-rescue-derived embryos remained acrosome-intact (94%). Scale bars: 30 μm.

respectively) in a 1 hour binding assay in which sperm were labeled with Hoechst dye (Fig. 5, 1,2,5,6,9,10). The fertilized eggs were rinsed by serial passage through three drops of media (30 μl) and challenged with *Acr3*-EGFP sperm (1 hour incubation) followed by washing with 0.2 mm pipettes using *Zp3*-EGFP two-cell embryos as controls (Fig. 5, 3,4,7,8,11,12). Although comparable numbers of total sperm bound normal, hu*ZP2* transgenic and hu*ZP2* rescue eggs (92±12, 115±17, 104±22, respectively), 84-88% of the initially bound sperm had been replaced with *Acr3*-EGFP sperm (Fig. 5, 4,8,12). Thus, under these assay conditions, a fairly constant number of sperm bound to the zona pellucida of normal, hu*ZP2* transgenic and hu*ZP2* rescue eggs, and the binding was almost completely reversible 1 hour after insemination.

A similar assay was performed with two-cell embryos (three independently obtained biological samples, ten embryos each) derived by in vitro fertilization with normal sperm and eggs isolated from each genotype. As expected from previous results, no sperm bound to normal embryos (data not shown) and comparable numbers of sperm initially bound to hu*ZP2* transgenic and hu*ZP2* rescue embryos (48±9, 56±7, respectively) (Fig. 5, 13,14,17,18). As previously observed (Fig. 1A), the sperm bound to hu*ZP2* transgenic zonae pellucidae were acrosome-reacted, and those bound to hu*ZP2* rescue zonae pellucidae were acrosome-intact. *Acr3*-EGFP sperm were added and embryos were washed (using *Zp3*-EGFP two-cell controls) after an additional hour of incubation. Comparable numbers of sperm remained bound to hu*ZP2* transgenic and hu*ZP2* rescue embryos (59±10, 68±16, respectively), and in each case most (>90%) were EGFP-positive (Fig. 5, 15,16,19,20). Thus, binding to the zona pellucida is reversible both for acrosome-intact (normal egg, hu*ZP2* transgenic egg, hu*ZP2* rescue egg, hu*ZP2* rescue embryos) and acrosome-reacted (hu*ZP2* transgenic embryos) sperm.

Mechanical induction of the acrosome reaction

The ability of *Acr3*-EGFP sperm to bind and remain acrosome-intact for 2-3 hours with normal or ⩾24 hours with huZP2 rescue eggs or embryos appears inconsistent with the induction of acrosomal exocytosis by a signal transduction pathway predicated on a zona

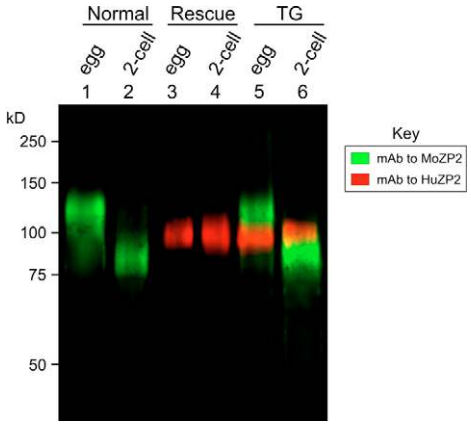

Fig. 4. Mouse, but not human, ZP2 is cleaved following fertilization. Immunoblot of eggs (lanes 1,3,5) and two-cell embryos (lanes 2,4,6) from wild-type (referred to as 'normal') (lanes 1 and 2), hu*ZP2* rescue (lanes 3 and 4) and hu*ZP2* transgenic (lanes 5-6) female mice. After SDS-PAGE run under reducing conditions (5% β-mercaptoethanol), immunoblots were incubated with monoclonal antibodies specific to the carboxyl fragment of mouse (faux colored green) or human (faux colored red) ZP2 and visualized with a secondary antibody and chemiluminescence. Molecular masses (kDa) are indicated on the left.

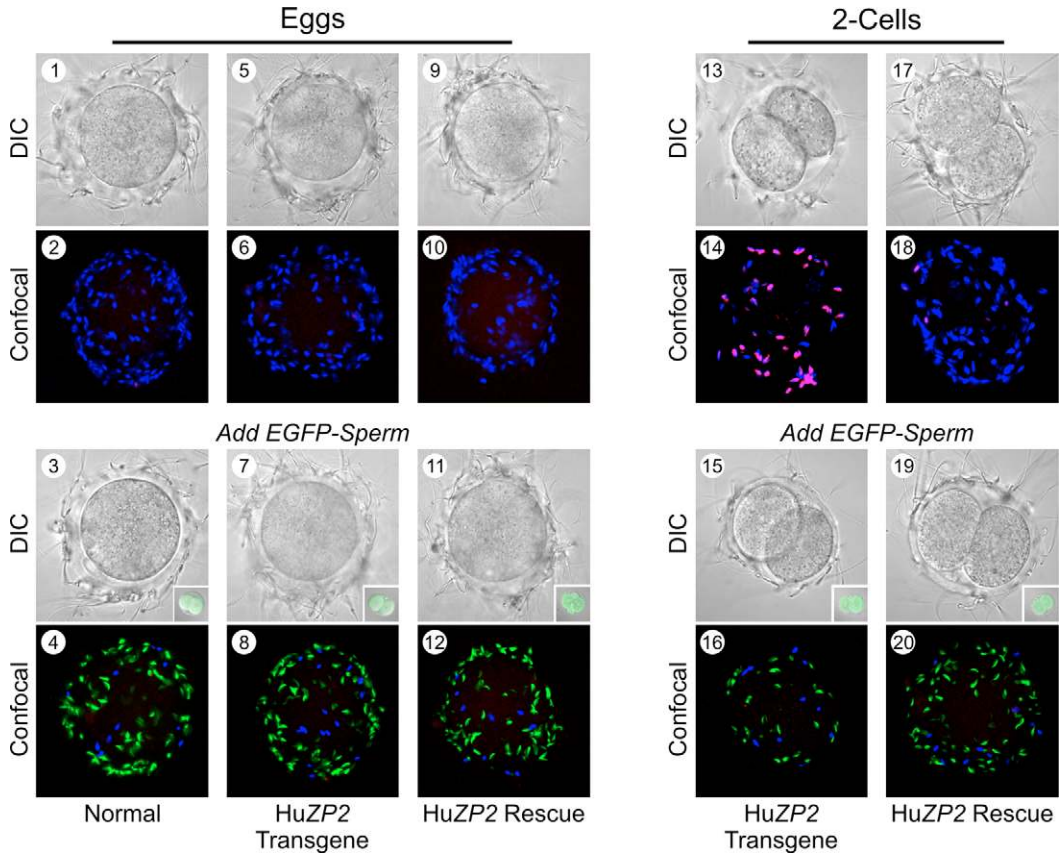

Fig. 5. Reversible sperm binding to wild-type, hu*ZP2* transgenic and hu*ZP2* rescue eggs and embryos. Wild-type (referred to as 'normal') (1,2), hu*ZP2* transgenic (5,6) and hu*ZP2* rescue (9,10) eggs or hu*ZP2* transgenic (13,14) and hu*ZP2* rescue (17,18) two-cell embryos were incubated with normal sperm for 1 hour (1,5,9) or 24 hours (13,17) and stained with Alexa 568-SBTI and Hoechst before imaging by DIC (1,5,9,13,17) and confocal microscopy (2,6,10,14,18). After a brief rinse, 5×10^5 motile, capacitated *Acr3*-EGFP sperm were added and incubated for an additional 1 hour. After washing to remove non-adherent sperm, eggs and embryos were stained with Alexa 568-SBTI before imaging by DIC (3,7,11,15,19) and confocal microscopy to observe SBTI and EGFP (4,8,12,16,20), reflecting acrosome-reacted and acrosome-intact sperm, respectively. Images were modified in Adobe Photoshop to remove nuclear staining from EGFP-positive sperm; thus, Hoechst-positive, EGFP-negative sperm (4,8,12,16,20) reflect those that were not displaced by EGFP-sperm. Insets (3,7,11,15,19), *Zp3*EGFP control two-cell mouse embryos from each incubation after washing.

ligand binding to a sperm surface receptor. However, initial penetration into the zona matrix might trigger a mechanosensory signal transduction, leading to exocytosis, and account for the presence of only acrosome-reacted sperm in the perivitelline space. This possibility has been investigated using a filter penetration assay with inert polycarbonate filters unadorned with zona pellucida ligands (Fig. 6). Normally, after 1 hour of incubation in capacitating conditions, ~70% of epididymal *Acr3*-EGFP sperm were acrosome-intact. However, passage through a column of glass beads, to which acrosome-reacted, non-viable sperm preferentially bind (Bangham and Hancock, 1955), resulted in a population that was 90-95% acrosome-intact. These glass-bead-treated sperm (three independently obtained biological samples) were used in a filter-penetration assay in which the progress of sperm was impeded by inert filters with pore sizes ranging from 1.2 to 12 μm (Fig. 6A). After incubation (30 minutes), aliquots of sperm from each side of the filters were obtained, fixed, treated with propidium iodide (nuclear stain) and analyzed by FACS. Two populations of sperm were observed according to the presence of EGFP and DNA or DNA alone and confirmed morphologically as acrosome-intact and acrosome-reacted, respectively (Fig. 6B). As the filter pore size decreased, the number of sperm in the lower chamber gradually increased from 1.5 to 1.9×10^4 (Fig. 7A), but spontaneous acrosome exocytosis remained comparable, ranging between 13-15% (Fig. 6C, Fig. 7B). As expected, the number of sperm recovered in the upper chamber after passage through filters decreased with smaller pore size (Fig. 6C, Fig. 7A), but the proportion of acrosome-reacted sperm increased dramatically to 85±5.3% in filters with 3 μm and 93±5.8% with 1.2 μm pores (Fig. 6C, Fig. 7B).

However, 'preferential passage' of spontaneously acrosome-reacted sperm through the polycarbonate filters might affect interpretation of these experimental results, even though acrosome exocytosis does little to decrease the maximal width of the mouse sperm head (Fig. 2). If the presence of acrosome-reacted sperm in the upper chamber reflected 'preferential passage', there should be a decrease in the percent of acrosome-reacted sperm in the lower chambers of the smaller, compared with the larger, pore-size filters (Fig. 7B). However, none was observed and the percent of acrosome-reacted sperm in the lower chambers remained constant (13-15%) as pore sizes decreased from 12 μm to 3 μm (Fig. 7B).

In addition, two capacitated *Acr3*-EGFP sperm aliquots from the same epididymal sperm sample were compared. One was pretreated with glass beads, the other was not, and each was observed after 30 minutes in the filter penetration assay. In three independently

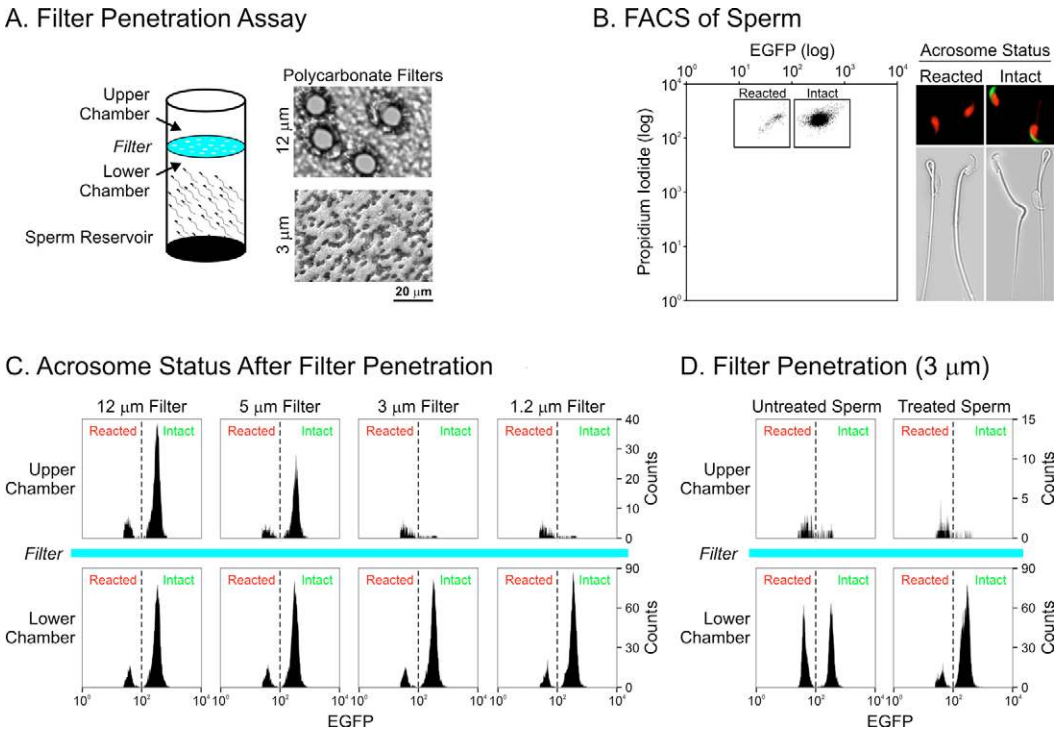

Fig. 6. Passage through inert polycarbonate filters induces the sperm acrosome reaction. (**A**) Capacitated *Acr3*-EGFP sperm (2.1×10^4) were placed in the lower chamber and incubated for 30 minutes. The sperm acrosome status was assayed before (lower chamber) and after (upper chamber) penetration of individual polycarbonate filters (pore size,1.2-12 μm); two example filters are imaged on the right. (**B**) Fixed sperm were stained with propidium iodide and divided into acrosome-intact and -reacted populations by FACS (left). The acrosome status of each population was confirmed by confocal microscopy (right). (**C**) *Acr3*-EGFP sperm, isolated before (lower chamber) and after (upper chamber) passage through individual filters (pore size, 1.2-12 μm), were fixed, stained with propidium iodide and scored by FACS for acrosome status. (**D**) Epididymal sperm were isolated and capacitated for 1 hour and an aliquot was passed over a glass bead column to remove acrosome-reacted sperm. The acrosome status of epididymal (left) and glass-bead-treated (right) sperm was assayed by FACS before (lower chamber) and after (upper chamber) passage through a 3 μm pore-size polycarbonate filter.

obtained biological samples, 21-31% of the untreated and 5-7% of the glass-bead-treated sperm in the lower chamber were acrosome-reacted, whereas more than 90% of sperm from each sample were acrosome-reacted in the upper chamber (Fig. 6D). If the presence of acrosome-reacted sperm in the upper chamber merely reflected 'preferential passage' of spontaneously acrosome-reacted sperm, an increased number of sperm would be anticipated in the untreated, compared with the glass-bead-treated, *Acr3*-EGFP sperm samples. However, no such increase was observed, and the number of acrosome-reacted sperm was similar (318.3±202.6, 141.7±81.0, respectively) between the glass-bead-treated and untreated samples, despite fourfold more untreated than treated sperm in the starting samples. Taken together, these observations suggest that the passage of motile sperm through the inert matrix is sufficient to induce the acrosome reaction and raise the possibility that similar mechanical forces may have physiological import for induction of the acrosome reaction during normal fertilization (Fig. 8).

DISCUSSION

Mouse fertilization requires a zona pellucida that is initially permissive for sperm binding to ovulated eggs and subsequent induction of the acrosome reaction. During in vitro fertilization, a cohort of capacitated sperm binds to the zona matrix via the plasma membrane overlying their anterior head. Current data suggest that the single 'fertilizing' sperm rapidly undergoes acrosomal exocytosis and the resultant acrosomal shroud stabilizes the sperm-zona interaction. The forward motility of the sperm then translates into scything movements of the dense head that facilitates complete penetration of the zona matrix. Once in the perivitelline space, the 'fertilizing' sperm fuses to the egg via the membrane of the posterior equatorial region. The rest of the cohort ('non-fertilizing' sperm) continues to associate with the surface of the zona pellucida with their acrosomes intact. However, shortly following fertilization the zona pellucida is altered in a yet-to-be-determined manner, and these bound sperm are unable to penetrate the matrix. Within hours, the more gradual enzymatic cleavage of ZP2 renders the zona matrix non-permissive for sperm binding. Major unresolved issues include: the molecular basis of sperm binding to the zona pellucida; the mechanism by which the sperm acrosome reaction is induced; and the process by which the zona matrix becomes impenetrable by sperm following fertilization.

Sperm-egg recognition

The zona pellucida surrounding ovulated eggs, but not two-cell embryos, is permissive for sperm binding. To account for this dichotomy, two models are considered. The 'glycan release' model postulates that sperm bind to a carbohydrate ligand, which is removed by a glycosidase released postfertilization by egg cortical granules, to account for the absence of sperm binding to the early embryo (Wassarman et al., 2005; Shur et al., 2006). The 'zona

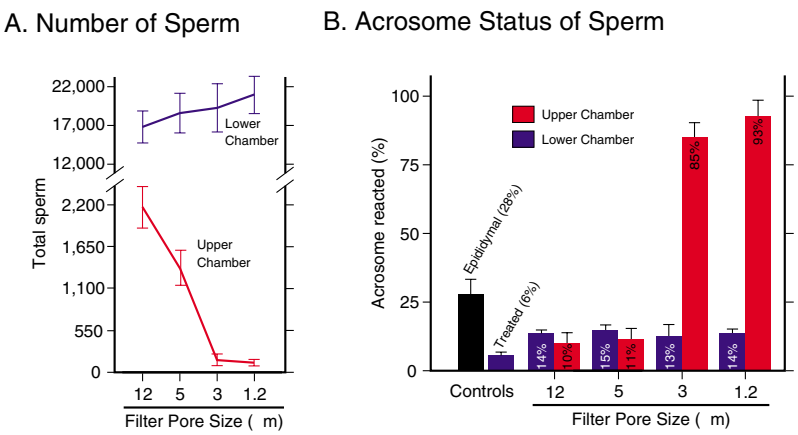

Fig. 7. Quantification of polycarbonate filter penetration assay. (**A**) Approximately 2.1×10^4 *Acr3*-EGFP sperm were assayed for penetration through polycarbonate filters. The average number of sperm (± s.e.m.) in the lower and upper chambers was determined by FACS and hand counting. The number of sperm recovered from the upper chamber was 123.3±38.1, 141.7±46.8, 1356.7±165.9, and 2165.0±188.0, after passage through filters with pore sizes 1.2, 3, 5 and 12 μm, respectively. (**B**) The acrosome status of *Acr3*-EGFP sperm was determined before (epididymal) and after (treated) passage through the glass bead column (controls) by FACS. Following the 30 minutes filter penetration assay, the percent of acrosome-reacted sperm in the lower (blue) and upper (red) chambers was determined by FACS and confirmed morphologically.

scaffold' model hypothesizes a three-dimensional zona matrix that is initially permissive for sperm binding and is rendered non-permissive by the postfertilization cleavage of ZP2 (Hoodbhoy and Dean, 2004). These two models make disparate predictions concerning de novo sperm binding to two-cell embryos derived from hu*ZP2* rescue mice in which ZP2 remains intact despite cortical granule exocytosis (Jungnickel et al., 2003). The first predicts no sperm binding because of postfertilization release of the glycan ligand, whereas the second predicts sperm binding to a zona matrix because of intact ZP2. The experimental observation of de novo *Acr3*-EGFP sperm binding to embryos derived from hu*ZP2* transgenic and hu*ZP2* rescue mice (Fig. 3) is not consistent with the 'glycan release' model and supports the 'zona scaffold' model by confirming earlier observations of persistent, postfertilization sperm binding to hu*ZP2* rescue eggs (Rankin et al., 2003).

Of note, the 'zona scaffold' model does not distinguish whether contacts between sperm and egg are based on protein, carbohydrate or both. However, biochemical and genetic results have placed increasingly severe limits on the range of possibilities for a single carbohydrate ligand (Nagdas et al., 1994; Liu et al., 1995; Thall et al., 1995; Lu and Shur, 1997; Ellies et al., 1998; Easton et al., 2000; Boja et al., 2003; Lowe and Marth, 2003; Shi et al., 2004), and genetic studies in which individual zona proteins are absent or replaced with human homologs do not support a single zona protein as a sperm adhesion molecule (Rankin et al., 1998; Rankin et al., 1999; Rankin et al., 2003). Thus, the three-dimensional structure of the zona pellucida probably plays a crucial role in forming the cognate binding site, and further high resolution structural definition of individual zona pellucida proteins and their relationship within the supramolecular architecture of the zona matrix should provide insights into the molecular basis of sperm-egg recognition.

Acrosome reaction

Acr3-EGFP sperm bind reversibly to normal eggs and embryos after in vitro fertilization, but binding is absent after 4 hours and correlates with partial proteolytic cleavage of moZP2. Those sperm initially bound to the surface of the zona pellucida remain acrosome-intact for several hours (Fig. 1A, 1-8). In eggs and embryos derived from hu*ZP2* rescue mice in which huZP2 is not cleaved, reversible *Acr3*-EGFP sperm binding persists for at least 24 hours after insemination, and again, sperm remain acrosome-intact (Fig. 1A, 17-24). The long dwell time of acrosome-intact sperm on the surface on the zona pellucida of normal (2-3 hours) and huZP2 rescue (≥24 hours) mice is seemingly inconsistent with a zona ligand interacting with a sperm receptor to induce acrosomal exocytosis via a classic ligand-receptor signal transduction pathway.

O-glycan ligands in the zona pellucida have been implicated as inducers of the acrosome reaction (Bleil and Wassarman, 1983; Leyton and Saling, 1989). However, ZP1 is not required for fertility (Rankin et al., 1999), and O-linked glycosylation of ZP2 and ZP3 is surprisingly sparse (Nagdas et al., 1994; Boja et al., 2003). Confidence in the role of O-glycans in acrosome exocytosis has been eroded by the inability to define definitively either the zona ligand or the sperm surface receptor as well as the observation that genetic ablation of leading candidates does not prevent in vivo fertility (Thall et al., 1995; Lu and Shur, 1997; Asano et al., 1997; Liu et al., 1997). Moreover, occupancy of putative attachments sites by O-glycans implicated as zona ligands (Chen et al., 1998) is not detected by microscale mass spectrometry of native ZP3 (Boja et al., 2003) and mutation of the sites to preclude glycosylation does not affect in vivo fertility in transgenic mice (Liu et al., 1995). The observed heterogeneity of O-glycan side chains (1-7 monosaccharide residues) in the zona pellucida (Easton et al., 2000; Chalabi et al., 2006) further complicates these models by suggesting that either not all glycan ligands at a particular attachment site are utilized or multiple sperm surface receptors are required to accommodate differing glycan isoforms.

Because sperm binding to normal or hu*ZP2* rescue zonae pellucidae is seemingly not sufficient to induce acrosome exocytosis, initial penetration into the zona pellucida matrix was explored as a possible alternative. In the filter penetration assay, *Acr3*-EGFP sperm were induced to undergo the acrosome reaction by passage through inert polycarbonate filters. In the absence of any zona ligand, 93±5.8% of the sperm underwent acrosome exocytosis if pore sizes were 1.2 μm, whereas only 10-11% were acrosome-reacted if the pore sizes were ≥5 μm. A simple explanation of these data is that passage through filter pores comparable in size to the zona pellucida matrix mechanically 'induces' acrosome exocytosis in virtually all sperm. This 'induction' model predicts that there would be little or no physical constraint until the pore size (3 μm) approaches the size of the acrosome-intact sperm (~3.5×8 μm) (Wyrobek et al., 1976; Cummins and Woodall, 1985). At that point, contact between motile sperm and filter would generate sufficient shear force to elicit a mechanosensory signal and acrosome exocytosis. The dramatic threshold increase in acrosome-reacted sperm in the upper chamber (Fig. 7B) as the pore size decreased (2

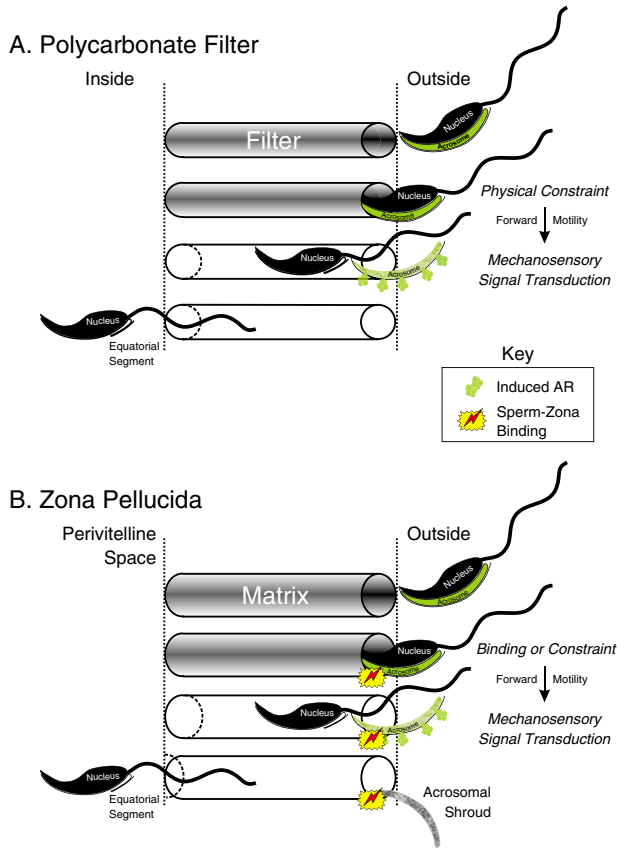

Fig. 8. Model of mechanosensory induction of sperm acrosome reaction. (**A**) Capacitated, motile and acrosome-intact sperm approach the inert polycarbonate filter lacking zona ligands. Depending on the size of the pore, the sperm is slowed or stopped as it seeks to penetrate the filter. The continued forward motility of the sperm transduces a mechanosensory signal that leads to increased intracellular Ca^{2+} and induction of the acrosome reaction. After passage through 3 μm or smaller pores, mostly acrosome-reacted sperm are observed on the far side of the filter. (**B**) Capacitated, motile and acrosome-intact sperm approach and bind to the zona pellucida. This binding (or the limiting size of the matrix interstices) immobilizes the sperm plasma membrane, inhibiting further progression of the sperm. However, the continued forward motility of the sperm transduces a mechanosensory signal that leads to increased intracellular Ca^{2+} and induction of the acrosome reaction. The residual acrosomal shroud is left behind bound to the surface of the zona pellucida matrix, and only acrosome-reacted sperm enter into the perivitelline space.

μm) from 5 μm (11% acrosome-reacted) to 3 μm (85% acrosome-reacted) is consistent with the 'induction' model of acrosome exocytosis.

If physiologically relevant, these results suggest that binding to the zona pellucida (or the limiting size of the matrix interstices) slows or stops the forward progression of motile sperm and the vigorous thrusting of the tail transduces a mechanosensory signal leading to mobilization of acrosomal Ca^{2+} stores (Herrick et al., 2005) and induction of the acrosome reaction (Fig. 8). Mechanosensory signaling is widespread among plants and animals, although molecular details have been more extensively described in bacteria (Kung, 2005). Unlike the lock and key binding of ligand to receptor transducing a signal, mechanically gated channels can respond to forces imposed on the lipid bilayer to regulate ion fluxes. This effect can be direct, as in stretch-activated channels, or indirect, whereby cytoskeletal or extracellular matrices augment minor perturbations in force. Multiple mechanotransducers have been described in animals (Orr et al., 2006), including polycystins (Delmas, 2004) and members of the transient receptor potential (TRP) family (Barritt and Rychkov, 2005). Sea urchin polycystin-2 has been implicated in the induction of the acrosome reaction of *Strongylocentrotus purpuratus* sperm (Neill et al., 2004) and TRPCl, 2, 3 and 5 are present in mouse sperm (Jungnickel et al., 2001; Trevino et al., 2001). Although TRPC2 has been incorporated into a ligand-receptor model of acrosome exocytosis (Arnoult et al., 1999; Jungnickel et al., 2001), mice lacking TRPC2 remain fertile (Stowers et al., 2002; Leypold et al., 2002) and the human homolog is a pseudo gene (Wes et al., 1995). Whether the other TRPC mechanosensory signal transducers present in mouse sperm play a role in the acrosome reaction remains to be determined.

Block to zona penetration

The 'fertilizing' sperm succeeds by binding to the zona pellucida, penetrating the matrix and fusing with the plasma membrane of the egg. The interaction of the 'fertilizing' sperm with the zona pellucida is rapid, and the binding to the zona matrix, induction of the sperm acrosome reaction, zona penetration and fusion with the plasma membrane of the egg occurs within minutes (Tollner et al., 2003; Bedford, 2004). After gamete fusion, there is a prompt, effective block to polyspermy at the plasma membrane of the egg (Wolf, 1978) and the observation of few, if any, supernumerary sperm in the perivitelline space (Sato, 1979) indicates that the postfertilization block to zona penetration is rapid as well. The observed accumulation of supernumerary sperm within the perivitelline space of *Cd9*-null eggs, deficient in sperm-egg fusion (Le Naour et al., 2000; Miyado et al., 2000), indicates that the block to penetration occurs after gamete fusion. Although this block has been ascribed to cleavage of ZP2 into two fragments that remain disulfide-bonded (Bleil et al., 1981), complete cleavage takes >4 hours (Fig. 1C) and hu*ZP2* rescue mice in which ZP2 is not cleaved have an effective postfertilization block to zona penetration (Rankin et al., 2003). Thus, the block to zona penetration appears to be independent of the relatively late-occurring cleavage of ZP2, and it seems likely that postfertilization release of stored materials, presumably small and highly diffusible, from the egg rapidly modify the zona pellucida to prevent sperm penetration.

The rapid block to zona penetration may account for the accumulation of reversibly adherent, 'non-fertilizing' *Acr3*-EGFP sperm on the zona pellucida (Fig. 5). Initial sperm-egg recognition has been described as 'loose' and then 'tight' (or 'primary' and 'secondary') sperm binding to the surface of the zona matrix (Hartmann et al., 1972; Hartmann and Hutchison, 1974; Bleil and Wassarman, 1980a; Bleil and Wassarman, 1986). However, without invoking repeated cycles of attachment and release, binding to zona glycoprotein(s) seemingly would impede the forward progression of the 'fertilizing' sperm through the zona pellucida. Alternatively, if sperm are attracted to the egg by chemoattractant guidance systems (Cohen-Dayag et al., 1995; Spehr et al., 2003; Eisenbach and Giojalas, 2006), then the initial penetration of the zona matrix by the 'fertilizing' sperm could induce acrosome exocytosis by mechanosensory signal transduction without requiring that sperm bind to the zona pellucida. In this formulation, the 'non-fertilizing' sperm would remain under the influence of the egg's chemoattractant, but be unable to progress through the zona matrix because of the rapid, effective postfertilization block to penetration. Thus, the perceived, reversible binding of 'non-fertilizing' sperm on the surface of the zona pellucida may reflect a conflict between chemoattraction or chemotaxis and the inability to penetrate the matrix.

Conclusions

These data support a 'zona scaffold' model of sperm-egg recognition in which the cleavage status of ZP2 determines whether the three-dimensional zona matrix will be permissive (ZP2 intact) or non-permissive (ZP2 cleaved) for sperm adherence, independent of fertilization and cortical granule exocytosis. The observed ability of sperm to remain acrosome-intact despite hours-long adherence to normal and hu*ZP2* rescue zona matrices is seemingly inconsistent with widely embraced involvement of ligand-receptor signal transduction in acrosome exocytosis. Thus, the 'zona scaffold' model has been extended to implicate initial penetration into the zona matrix as eliciting 'mechanosensory' signals sufficient for induction of acrosome exocytosis. This model differs from 'glycan release' models, in which ZP3 glycans act as ligands for sperm binding as well as the induction of acrosome exocytosis, with the postfertilization loss of the glycan accounting for the inability of sperm to adhere to embryos. The validity of these disparate models will require further investigations.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/5/933/DC1

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