Human ZP4 is not sufficient for taxon-specific sperm recognition of the zona pellucida in transgenic mice

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Abstract

The molecular basis of human fertilization remains enigmatic. Mouse models are often used to study sperm–egg recognition, but the mouse zona pellucida surrounding ovulated eggs contains three proteins (ZP1, ZP2, and ZP3) whereas the human zona contains four (ZP1, ZP2, ZP3, and ZP4). Human sperm are fastidious and recognize human but not mouse eggs. Transgenic mouse lines were established to ascertain whether human ZP4 is the sole determinant of human sperm binding. Human ZP4 expressed in transgenic mice had a molecular mass similar to the range of native protein isoforms and was incorporated into the extracellular zona matrix. Transgenic females were fertile with normal litter sizes. Mouse sperm readily recognized transgenic ovulated eggs, but human sperm did not. We conclude that human ZP4 is not sufficient to support human sperm binding to the zona pellucida in transgenic mice and that other zona proteins may be needed for human gamete recognition.

Introduction

The zona pellucida, an acellular glycoprotein matrix surrounding mammalian eggs and early embryos, mediates sperm–egg interaction, provides a post-fertilization block to polyspermy, and protects the embryo prior to implantation (Yanagimachi 1994). The mouse zona contains three glycoproteins (ZP1, ZP2, and ZP3) and the human zona contains four (ZP1, ZP2, ZP3, and ZP4). Homologous genes encoding the four proteins are present on syntenic chromosomes in each taxon (Hoodbhoy *et al*. 2005), but mouse *Zp4* contains multiple stop codons and does not express the cognate protein (Lefièvre *et al*. 2004). Because of multiple pre-(habitat, physiognomy, and mating behavior) and post-zygotic (aneuploidy) barriers, the evolutionary basis of taxon-specific sperm–egg recognition among internally fertilizing mammals is not immediately obvious (O'Rand 1988). Nevertheless, human sperm exhibit stringent self-recognition and do not bind to mouse eggs. In contrast, mouse sperm recognize eggs from a taxonomically diverse group of mammals, including humans (Bedford 1977). Taking advantage of this dichotomy, it is possible to explore the molecular basis of sperm–egg interactions with 'loss-of-function' mouse lines deficient in single zona proteins and 'gain-of-function' transgenic mice expressing human zona proteins.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Using gene-targeting and embryonic stem-cell technologies, mice lacking individual zona proteins have been established. *Zp1* null female mice form a zona pellucida that is thinner than normal, but sperm bind and fertilize eggs *in vitro*. Thus, ZP1 is not essential for sperm–egg recognition in mice (Rankin *et al*. 1999). However, *Zp1* null females have decreased fecundity because pre-implantation embryos cannot survive precocious escape from the zona matrix during passage through the oviduct. *Zp2* and *Zp3* null mouse lines have also been established, but in the absence of either protein, no zona matrix is present surrounding ovulated eggs and the zona-free eggs are quickly absorbed to the epithelial lining of the oviduct. Therefore, the role of either ZP2 or ZP3 in sperm–egg recognition was indeterminate in these studies (Rankin *et al*. 1998, 2001). Transgenic mouse lines expressing either human *ZP2* or *ZP3* incorporate the human protein into the zona pellucida but, under the reported experimental conditions, the presence of either human protein was not sufficient to support human sperm binding even when crossed into the corresponding *Zp2* or *Zp3* null background (Rankin *et al*. 1998, 2003).

Although not detected in mice (Boja *et al*. 2003), ZP4 protein is present in multiple different species including humans, chimpanzees, cows, pigs, dogs, cats, and rats and has been reported to play a role in sperm–egg recognition in some (Prasad *et al*. 1996, Topper *et al*. 1997, Govind *et al*. 2000). The specificity of human sperm binding could reflect a taxon-independent need for four zona proteins to form a zona structure distinct from that formed with three zona proteins or a taxon-specific need for human ZP4 protein. The first hypothesis was tested with rats that have a zona pellucida composed of four zona proteins that are 62–70% identical to the human homologues. Although mouse and rat sperm bound to rat eggs, human sperm did not. Thus, a heterologous zona pellucida composed of four zona proteins is not sufficient for human sperm attachment under the experimentally described conditions (Hoodbhoy *et al*. 2005). We now test the second hypothesis by establishing a 'gain-of-function' assay in which human *ZP4* is expressed in transgenic mice to investigate the molecular basis of human and mouse gamete recognition.

Results

Establishment of human ZP4 transgenic mouse lines

Human *ZP4* (11.6 kb, including 2.4 kb of promoter) was isolated from a BAC and subcloned to provide a DNA fragment (Fig. 1A), which was injected into the pronucleus of one-cell embryos to establish transgenic mouse lines. The transgene was detected by PCR and Southern blot (data not shown) in 5 out of 25 pups (20%) and 3 (2 females and 1 male) were used to establish stable transgenic lines. Two founder lines were tested for taxon specificity of sperm binding and one (human *ZP4*Tg) was utilized in this study.

Tissue-specific expression of the transgene was assayed by RT-PCR of total RNA isolated from mouse brain, muscle, heart, lung, kidney, liver, uterus, spleen, testes, and ovary. Using primers specific for *ZP4* (Fig. 1A), the expression was detected only in the ovary of transgenic mice (Fig. 1B). Within the ovary, the expression was localized to growing oocytes by *in situ* hybridization of ovarian sections from 15-day-old transgenic females using human *ZP4*-specific ^{35}S-labeled anti-sense cRNA probes. The signal observed in normal oocytes (data not shown) or using control sense probes was no greater than background (Fig. 1C).

Ovarian histology, including the appearance of the zona pellucida, in transgenic animals was indistinguishable from normal controls (Fig. 1D). Eggs from normal and *ZP4* transgenic mice were analyzed on immunoblots probed with a MAB to human ZP4 (Fig. 1E). Although the band corresponding to ZP4 in the human sample was diffuse, the smaller isoforms appeared to co-migrate with the ZP4 expressed in transgenic mouse eggs. To determine

whether the lower size range of ZP4 in the human sample overlapped with the size of ZP4 expressed in the mouse, the two samples were mixed together and a single band was observed (Fig. 1E). Thus, native and recombinant ZP4 have an overlapping molecular mass 65–70 kDa. Using confocal microscopy and MABs to image ovulated eggs, human ZP4 co-localized with mouse ZP1, ZP2, and ZP3 in the extracellular zona pellucida matrix (Fig. 2). The MAB to human ZP4 antibody did not cross-react with native mouse zona proteins.

Fertility and taxon-specific recognition of human ZP4 transgenic eggs

Five human *ZP4* transgenic females and five normal controls were primed with pregnant mare serum gonadotropin and stimulated to ovulate eggs with human chorionic gonadotropin. Comparable numbers of eggs (average ± S.E.M.) were recovered from the oviduct of normal (23.5 ± 2.7) and human *ZP4* transgenic mice (22.8 ± 2.5). Fertility was determined by pairing five human *ZP4* transgenic with five normal FVB females of the same age and mating each pair with a normal male. Monitoring through several litters demonstrated that human *ZP4* transgenic females had litters (9.2 ± 0.84 pups) with a size comparable to normal females (7.1 ± 0.8 pups). The transgene was successfully transmitted to the next generation in 41% of the pups in eight litters, which is close to the expected 50% Mendelian ratio (Table 1).

Ovulated eggs and two-cell embryos (wash controls) were collected from human *ZP4* transgenic females after gonadotropin stimulation with or without mating, as appropriate. Eggs in cumulus were isolated from the oviduct and treated with hyaluronidase to remove somatic cells. Fertilized eggs were cultured overnight to obtain two-cell embryos. Groups of eggs and embryos were separated into two sets, which were incubated with either normal, capacitated epididymal mouse sperm, or capacitated human sperm from fertile male donors. After ~30 min, eggs were washed with a wide-bore pipette to remove loosely adherent sperm using two-cell embryos, to which sperm do not bind above background levels, as negative controls (Fig. 3A); uninseminated, immature human oocytes served as positive controls (Fig. 3B). Mouse sperm readily attached to transgenic eggs (37.4 ± 8.6/egg) and to normal (FVB) ovulated eggs (59.5 ± 1.4/egg) (Fig. 3). Only the occasional human sperm bound to human *ZP4* transgenic eggs (1.4 ± 0.28/egg), a number comparable to that observed binding to normal eggs (0.67 ± 0.22/egg). Both human and mouse sperm attached to uninseminated, immature human oocytes (Fig. 3B). Similar results were observed even after 22 h of incubation (data not shown). Human sperm did not penetrate the 'humanized' zona pellucida and were not observed in the perivitelline space of transgenic eggs.

Discussion

Human fertility remains a global issue in public health, both for those who wish to facilitate procreation through reproductive technologies and those who seek to limit population growth by contraception. A major step in fertilization is sperm–egg recognition that occurs between a motile, capacitated sperm and the surface of the zona pellucida surrounding recently ovulated eggs. It is an ideal point to affect human fertilization, but little is known about the molecular basis of this seminal event. In part because of ethical considerations and the practical challenges posed by the paucity of biological materials, it has been difficult to directly investigate fertilization in humans. Thus, investigators have sought to exploit other experimental systems to gain insights into the molecular basis of initial gamete interactions. The mouse model is particularly well established (Ikawa *et al*. 2010), but the recent observation that human zonae pellucidae contain an additional zona protein raises questions as to the adequacy of mice as a proxy for human fertility.

The human egg is larger than that of the mouse (120 vs 80 μm) and its zona pellucida is considerably thicker (12 vs 8 μm). Although the relative abundance of zona transcripts in the

mouse oocyte has been established (Epifano *et al.* 1995), determination of the stoichiometry of proteins within the zona matrix varies with the method of interrogation (Bleil & Wassarman 1980, Shimizu *et al.* 1983, Moos *et al.* 1995, Bauskin *et al.* 1999). At least three of the human zona proteins (ZP2, ZP3, and ZP4) are incorporated into the extracellular zona pellucida in transgenic mice and at least two (ZP2 and ZP3) can replace endogenous mouse proteins to support mouse fertilization. However, there are differences in how the human proteins participate in human–mouse chimeric zonae pellucidae. Normally, both mouse and human ZP2 are cleaved following fertilization (Moller & Wassarman 1989, Bauskin *et al.* 1999) and the cleavage status of mouse ZP2 determines sperm–egg recognition independent of fertilization and cortical granule exocytosis (Gahlay *et al.* 2010). The cleavage site in mice is upstream of a di-acidic motif (LA↓DEN) and is conserved in humans (LA↓DDS). However, human ZP2 present in the human–mouse chimeric zona pellucida is not cleaved following fertilization, which preserves a zona structure to which mouse sperm bind (Rankin *et al.* 2003, Baibakov *et al.* 2007). Either the mouse protease has taxon specificity or the structure of the chimeric zona renders the site inaccessible to the cleaving enzyme. In a similar vein, mice lacking ZP1 still form a zona pellucida composed of mouse ZP2 and ZP3, which mouse sperm recognize and fertilize. However, the expression of the homologous human proteins in transgenic mice lacking endogenous mouse zona proteins is not sufficient to form a corresponding zona pellucida matrix composed of just human ZP2 and ZP3 (Galina Baibakov, unpublished results).

Human ZP2 and ZP3 are 60–71% identical to their murine homologues and, when expressed in transgenic mice, they are post-translationally modified and secreted prior to incorporation into the zona pellucida. Although no direct comparison of *O*- or *N*-glycans in native and recombinant human zona proteins expressed in transgenic mice has been reported, the potential role of taxon-specific glycosylation in dictating the specificity of sperm–egg recognition has been an area of investigative interest (Dell *et al.* 2003, Chalabi *et al.* 2006). However, based on mobility on SDS-PAGE, human proteins expressed in transgenic mice are post-translationally modified to a similar extent to native human zona proteins (Rankin *et al.* 1998, 2003). In addition, there is accumulating evidence, based on mutation of putative attachment sites (Liu *et al.* 1995, Gahlay *et al.* 2010) and genetic disruptions of glycosyltransferases necessary to form candidate sugars (Ellies *et al.* 1998, Easton *et al.* 2000, Lowe & Marth 2003, Shi *et al.* 2004, Williams *et al.* 2007), that zona glycan ligands may not be essential for mouse sperm–egg recognition.

Since the discovery of the fourth human zona protein (Lefièvre *et al.* 2004), a similar complement of proteins has been described in other mammals (Conner *et al.* 2005). *ZP1* and *ZP4* appear to have arisen as a result of gene duplication early in the vertebrate lineage after its divergence from fish, ~650 million years ago. The two genes can be considered paralogues, which usually evolve to different functions. The persistence of both implies that each has its own specialized and important function for that particular taxon. Despite variations in nomenclature for zona proteins within the literature, it appears that ZP4 has been implicated in mediating sperm binding in several different species. For example, recombinant rabbit ZP4 binds to the acrosome of rabbit sperm and antibodies, but not Fab fragments, inhibiting sperm binding (Prasad *et al.* 1996). Likewise, sperm attachment to the zona matrix has been attributed to ZP4 in humans (Chiu *et al.* 2008) and heterocomplexes of ZP4 with ZP3 in pigs (Yurewicz *et al.* 1998, Kanai *et al.* 2007).

These observations suggested possible involvement of ZP4 in other species, but the current data indicate that recombinant human ZP4 is not sufficient to support recognition of transgenic mouse eggs by human sperm under conditions in which human sperm attach to human oocytes. Recombinant human ZP4 is secreted and incorporated into the extracellular zona pellucida that appears normal in ovarian sections and ovulated eggs of transgenic mice.

Based on their molecular mass, recombinant and native human ZP4 have overlapping isoforms, which may reflect subtle differences in glycosylation, but whether such differences affect structure or function remains to be determined. It will be important to complement these negative data (absence of human sperm binding) with positive results to validate mouse transgenesis as a physiological proxy for human gamete recognition. Thus, it will be of particular interest to determine whether improved assay conditions will support human sperm recognition of transgenic mouse eggs containing additional human zona proteins. Establishment of an experimental system in transgenic mice that supports human sperm recognition would not only provide insight into the molecular basis of gamete recognition, but would also establish a valuable diagnostic tool for the clinical evaluation of male infertility.

Materials and Methods

Establishment of transgenic mouse lines

Using DNA recombineering (recombination-mediated genetic engineering), human *ZP4* (chromosome 1:236,111,332–236,122,955) was subcloned from BAC RP11-484B19 (CHORI, Oakland, CA, USA) into a bacterial plasmid (Liu *et al*. 2003). Oligonucleotide primers were designed to PCR amplify left (forward 5′-GCGGCCGCAACTCCACCCATTG-3′; reverse 5′-AAGCTTATGTGGCTTCCCTGACAC-3′) and right (forward 5′-AAGCTTGCTGCTCAGCCATACTCT-3′; and reverse 5′-GTCGACCCTTCTCCCATTTACCC-3′) arms bounded by NotI and SalI and separated by HindIII restriction enzyme sites. The left and right arms were subcloned to establish the retrieving vector, the integrity of which was confirmed by DNA sequence. The BAC was electroporated into SW102 cells growing at 32 °C and its presence was confirmed by PCR of human *ZP4* exons. The isolated retrieving vector, linearized by digestion with HindIII, was then electroporated into SW102 cells containing BAC DNA and recombination was induced (15 min, 42 °C). Colonies were selected by antibiotic resistance and the retrieval of human *ZP4* was confirmed by DNA sequence.

After digestion with NotI and SalI, the human *ZP4* genomic DNA fragment was gel purified and injected into the pronucleus of one-cell embryos to establish transgenic mouse lines (Rankin *et al*. 1998). The transgene was detected by PCR of tail DNA using exon-specific oligonucleotides (forward primer 5′-GGTTTTCACTCTCCCACCAC-3′; reverse primer 5′-CAGGACAGGTCACCACACAG-3′). The presence of the transgene in 5 of 25 pups (20%) was confirmed by Southern hybridization using full-length human *ZP4* cDNA (Chakravarty *et al*. 2005) labeled with [^{32}P]dCTP (Rankin *et al*. 1998). For histological analysis, ovaries were collected from 3- to 4-week-old normal and transgenic females, fixed in 3% glutaraldehyde, embedded in plastic, and stained with periodic-acid Schiff's reagent (Rankin *et al*. 2001).

PCR

Total RNA was isolated from 4- to 8-week-old mouse tissue with RNeasy mini kit (Qiagen), treated with DnaseI, and used as a template for ssDNA synthesis with SuperScript First-Strand Synthesis (Invitrogen). Human *ZP4* (above exon primers) and *GAPDH* (forward primer 5′-ACTCCACTCACGGCAAATTC-3′; reverse primer 5′-CACATTGGGGGGTAGGAACAC-3′) were used to produce PCR products that were separated by gel electrophoresis and stained with ethidium bromide (Ohsugi *et al*. 2008).

In situ hybridization

Ovaries (15 days old) were isolated from normal and transgenic mice and fixed in OCT (Sakura-Finetek, Torrance, CA, USA). Sections (4 μm) were mounted on glass slides, fixed in 4% formaldehyde (Sigma–Aldrich) in PBS, blocked with triethanolamine/acetic anhydride, washed, and dehydrated through an ethanol series. Sense and anti-sense cRNA (1–778 nt, beginning at the AUG) derived from human ZP4 cDNA was labeled *in vitro* with ^{35}S-UTP according to the manufacturer's instructions (Ambion, Austin, TX, USA). Sections were hybridized overnight (55 °C, 50% formamide, 0.3 M NaCl, 20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 10 mM NaH_2PO_4, 10% dextran sulfate $1\times$ Denhardt's, 50 μg/ml total yeast RNA, and 5–8×10^7 c.p.m./ml ^{35}S-labeled probe (Phylogeny, Columbus, OH, USA)). After stringent washes, digestion with Rnase, and emulsion autoradiography, using Kodak D-19 as a developer (Kodak), slides were lightly counterstained with cresyl violet and imaged using bright- and darkfield microscopy (Rankin *et al*. 2003).

Immunoblots

Uninseminated, immature, nonviable human oocytes, obtained from Shady Grove Fertility Center, Rockville, MD, USA, were washed three times in PBS/protease inhibitors/0.3% polyvinyl-pyrrolidone prior to a quick freeze and storage in the vapor phase of liquid nitrogen. The human oocytes as well as ovulated eggs from normal and transgenic mice were used for immunoblots (Rankin *et al*. 2003) that were probed with a MAB specific to human ZP4 (1:500, overnight, 4 °C) (Bukovsky *et al*. 2008).

Confocal microscopy

Transgenic mice were stimulated with gonadotropins to recover ovulated eggs (three independently obtained samples of 6–8 eggs per mouse line), which were fixed in 2% paraformaldehyde and stained with MABs to mouse ZP1, ZP2, ZP3 (Rankin *et al*. 1998), and human ZP4 (Bukovsky *et al*. 2008). Images were obtained by confocal microscopy (Rankin *et al*. 2003).

Fertility and sperm binding to ovulated eggs

Fertility was assayed by mating transgenic and FVB females with FVB males (2:1). Taxon-specific sperm binding was initially determined in an assay in which mouse eggs and two-cell embryos were used as negative controls for human and mouse sperm binding respectively. Mouse eggs served as a positive control for mouse sperm binding, but these assays lacked a positive control for human sperm binding (Rankin *et al*. 2003, Hoodbhoy *et al*. 2005). Therefore, a modified assay was developed in which uninseminated, immature human oocytes served as a positive binding control for human sperm.

Mouse oocytes/embryos were collected as described earlier (Hoodbhoy *et al*. 2005) and uninseminated, immature human oocytes were obtained from Shady Grove Fertility Clinic, Rockville, MD, USA. Samples (1 ml) of liquefied human semen (Genetics and IVF Institute Cryobanks, Fairfax, VA, USA) were added to a column containing 500 μl of a mixture (80:20) of PureSperm silica suspension (Nidacon, Molndal, Sweden) and human tubal fluid (HTF, Specialty Media/Millipore, Billerica, MA, USA) supplemented with 5% BSA. After centrifugation (20 min×500 ***g***), sperm pellets were resuspended in 100–200 μl of the HTF/BSA. Two to four resuspended sperm samples from different donors were mixed and 300–400 μl were placed in a center-well organ culture dish (BD Falcon, 353037, Franklin Lakes, NJ, USA) surrounded by a ring of water. Mouse eggs/embryos and uninseminated, immature human oocytes were then added and co-incubated (90% N_2, 5% O_2, 5% CO_2, 37 °C) for 4 h to ensure capacitation of human sperm. Eggs/embryos/oocytes were washed by transfer pipette (0.35 mm tip) into a single fresh drop (20 μl) of HTF/BSA. Human oocytes and

mouse eggs served as positive and negative wash controls respectively. All experiments were conducted in triplicate. Even without the single wash, human sperm did not bind to mouse eggs.

Mouse sperm were collected from the cauda epididymis by macerating the tissue in a dish of HTF and capacitated (90% N_2, 5% O_2, 5% CO_2, 37 °C) for 40 min (Rankin *et al*. 2003). Aliquots (~50 μl) of capacitated sperm were added to center-well organ culture dishes already containing uninseminated, immature human oocytes, mouse eggs, and two-cell embryos in 350 μl of HTF. After incubation (30 min) with mouse sperm, mouse eggs/ embryos and human oocytes were washed sequentially by transfer pipette into fresh drops of HTF (20 μl) until only two to five sperm bound to two-cell mouse embryos, which served as negative controls. Mouse and human samples were fixed by adding 20 μl of 4% paraformaldehyde and stained with Hoechst to identify nuclei. Binding was assayed by collapsing the Z-stack of 5 μm optical sections to capture all bound sperm and results reflect the average ± S.E.M. of the mean from three independently obtained samples each containing 6–12 mouse eggs. These 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. The Office of Human Subject Research determined that federal regulations did not apply to the anonymous human sperm and egg samples.

Acknowledgments

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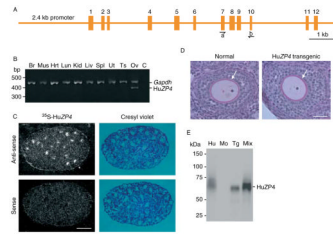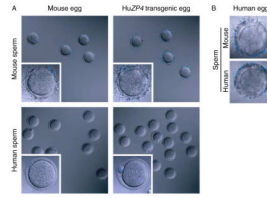
Figure 1.

Transgenic mice expressing human *ZP4*. (A) Schematic of 11.6 kb human *ZP4* gene locus composed of a 2.4 kb promoter, 8.2 kb coding region, and 1.0 kb 3′ of the last exon. Exons are indicated by Arabic numbers and PCR primers by arrowheads. (B) Tissue-specific expression of human *ZP4* was determined by RT-PCR. Total RNA was isolated from the brain (Br), muscle (Mus), heart (Hrt), lung (Lun), kidney (Kid), liver (Liv), spleen (Spl), uterus (Ut), testes (Ts), and ovary (Ov) of transgenic mice. Ovarian RNA without reverse transcriptase was a negative control (C). After RT to obtain ssDNA, PCR amplification was performed with exon-specific primers (A) to distinguish ssDNA (485 bp) from genomic DNA (1052 bp). *Gapdh* expression (572 bp) was used as an internal control for RNA integrity and quantity. Molecular mass markers are indicated at the left. (C) *In situ* hybridization of normal and human *ZP4* transgenic ovarian sections. Specific ^{35}S-labeled sense and anti-sense human *ZP4* cRNA probes were hybridized to OCT-embedded ovarian sections from 15-day-old transgenic females. Sections were viewed with bright- and darkfield microscopy. (D) Plastic-embedded ovarian sections from 3- to 4-week-old human *ZP4* transgenic and normal female mice. Sections were stained with periodic-acid Schiff's reagent to highlight the zona pellucida (arrow), surrounding developing oocytes and counterstained with hematoxylin. (E) Immunoblot of human and mouse eggs. Lane 1 (Hu), uninseminated, immature human oocyte (2); lane 2 (Mo), normal mouse eggs (30); lane 3 (Tg), human *ZP4* transgenic eggs (30); and lane 4 (Mix), mixture of lanes 1 (Hu) and 3 (Tg). Blot was probed with a MAB specific to human ZP4 and detected with HRP-conjugated secondary antibodies and chemiluminescence. Molecular weights are indicated to the left.
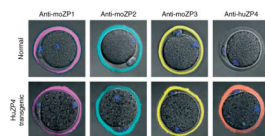
Figure 2.
Confocal microscopy of ovulated eggs. Ovulated eggs from normal and human *ZP4* transgenic females were stained with MABs specific to mouse ZP1, mouse ZP2, mouse ZP3, or human ZP4 and imaged by confocal microscopy. The zona pellucida surrounding human *ZP4* transgenic eggs contained ZP4, which was absent in normal eggs.

Figure 3.
Taxon-specific sperm binding to ovulated eggs. (A) Ovulated eggs and two-cell embryos from normal and human *ZP4* transgenic females were incubated with capacitated epididymal mouse sperm or capacitated human sperm from fertile donors. Washes to remove nonadherent sperm were performed until three or fewer sperm were bound to control two-cell embryos. Insets are higher magnification of individual eggs from lower magnification field. Eggs, embryos, and sperm were stained with DAPI to detect DNA. (B) Same as (A), but with uninseminated, immature human oocytes.

Table 1

Fertility of human *ZP4* transgenic mice.

	Ovulated eggs (average ± S.E.M.)	Litter size (average ± S.E.M.)	Transgenic pups (average ± S.E.M.)
Human *ZP4* (5)	22.8 ± 2.5[*]	8.6 ± 0.7[†]	41.1 ± 10.4 (%)
FVB (5)	23.5 ± 2.7[*]	7.1 ± 0.8[†]	ND

[*],[†]Student's *t*-test indicates no statistical difference; *P*<0.05.