RESEARCH

Possible involvement of annexin A6 in preferential sperm penetration in the germinal disk region

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Graphical abstract

Avian fertilization
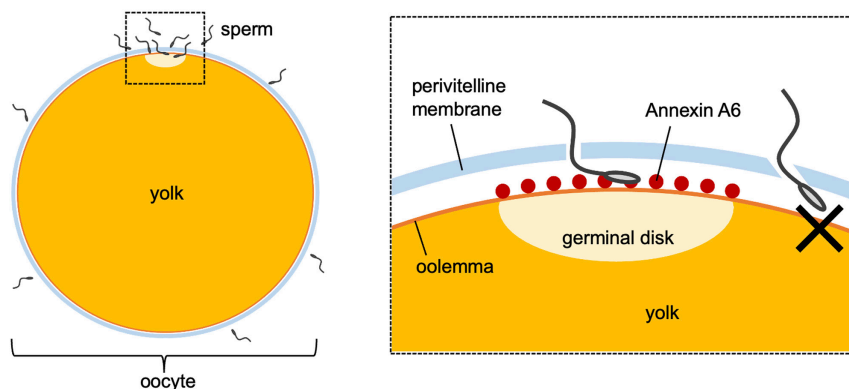
Abstract

During fertilization, avian sperm preferentially penetrate into the perivitelline membrane that covers the germinal disk region where the female nucleus is present. This phenomenon has been observed not only in domestic birds but also in wild birds; however, the mechanisms controlling sperm preference are still unclear. In this study, we investigated the possible involvement of annexin family protein in sperm–egg interaction in Japanese quail. Microscopic examination of fertilized eggs indicated that quail sperm penetration only occurred in the germinal disk region, and sperm localized outside the germinal disk were trapped in the perivitelline membrane. Western blot analysis and immunofluorescence microscopy revealed the presence of annexin A1 and A6 in the oocyte membrane, while annexin A6 localized in the perivitelline space of the germinal disk region. Further, our sperm binding assay using recombinant annexin A6 demonstrated that ejaculated sperm specifically bound to annexin A6 expressed in mammalian cell lines. These results suggest that annexin A6, which is expressed on the surface of oocytes, may function in sperm–egg interaction in the germinal disk region and that this binding may ensure sperm retention on the surface of the egg plasma membrane until fertilization takes place in Japanese quail.

Lay summary

In bird species, fertilization takes place immediately after ovulation of the egg. Sperm preferentially penetrate a specific area of the egg coating that covers the 'germinal disk region' – this area contains the cell that needs to be fertilized by a sperm. However, since the bird egg is extremely large in size and sperm must reach the 'germinal disk region' to achieve fertilization, it is unclear how this happens. Annexin proteins support fertilization in mammals, and we found that annexin A6 protein exhibits a unique localization in the germinal disk region in the eggs of Japanese quail. To test this interaction, we incubated quail sperm with cells that produced annexin A6 and found that ejaculated sperm bound to the cells. These results suggest that annexin A6 may have a role in the sperm–egg interaction in the germinal disk region in Japanese quail.

Introduction

Sperm–egg interaction is one of the crucial steps for fertilization in organisms, and various molecular events that are highly regulated occur to achieve proper fertilization. In comparison with those of mammalian species, avian oocytes are extremely large, and this leads to difficulty in direct observation of the sperm–egg interaction *in vitro*. In addition, no available method for *in vitro* insemination exists, and reverse genetic techniques such as gene knockout experiments are not easy to apply to birds.

Although knowledge regarding avian fertilization is limited, it exhibits several unique properties such as polyspermic fertilization (lack of a polyspermy block system) (Ichikawa *et al.* 2016), long-term oviductal sperm storage at normal body temperature (Sasanami *et al.* 2013, Matsuzaki & Sasanami 2017), as well as the fact that sperm do not need the capacitation process required for subsequent events such as the induction of an acrosome reaction (AR) (Howarth 1970). We have previously investigated these unique features of avian fertilization. For instance, in polyspermic fertilization, plural sperm need to penetrate the oocytes because sufficient sperm-borne egg-activating factors are required to resume meiosis of giant eggs. Also, we found three egg-activating factors (i.e. phospholipase C zeta, citrate synthase, and aconitase) that are essential for full-term egg activation in Japanese quail, in contrast to the fact that mammalian oocytes require only phospholipase C zeta (Mizushima *et al.* 2014). In addition, we found that oviductal sperm storage is indispensable for successful fertilization in Japanese quail. By storing the sperm in sperm storage tubules (SSTs), female birds are able to synchronize the timing of their own ovulation and sperm release from the SSTs by the stimulation of progesterone. It is considered that this mechanism facilitates the timely encounter of sperm and eggs at the site of fertilization, that is, the infundibulum part of the oviduct (Ito *et al.* 2011).

In birds, the oocyte, which encounters the spermatozoa in fertilization, is enclosed in a perivitelline membrane (PVM) constructed of several zona pellucida glycoproteins (ZP proteins: ZP1, ZP2, ZP3, ZP4, and ZPD) (Okumura 2017). Our previous study indicated that sperm binding to the PVM is, at least in part, mediated by the interaction of ZP1 and ZP3 with the sperm head during fertilization in Japanese quail (Sasanami & Mori 2005, Ichikawa *et al.* 2017). We also provided evidence that an *N*-linked oligosaccharide attached to ZP1 plays an important role in triggering the AR (Sasanami *et al.* 2007). However, no such molecule on the oocyte membrane, which is responsible for sperm–egg interaction, was reported in any birds.

As such, several important molecules and mechanisms that regulate avian fertilization have been discovered, but another important question remains unanswered. In chickens, Bramwell and Howarth reported that when sperm encountered oocytes, they preferentially penetrated the PVM that overlays the germinal disk region (Bramwell & Howarth 1992). This phenomenon was also reported in some species of wild birds (Birkhead *et al.* 1994). The mechanism of sperm preference is unknown; however, several hypotheses could be suggested: (1) the germinal disk may secret a sperm chemoattractant, and sperm sense such chemicals and then migrate toward the germinal disk; (2) the PVM overlaying on the geminal disk has a different property to that of the non-germinal disk region in terms of sperm-binding affinity or the capability of inducing AR; (3) the PVM surrounding the germinal disk may be sensitive to a sperm protease that is responsible for ZP protein degradation. To our knowledge, no investigation concerning the mechanism of preferential PVM

penetration has been done, and the specific mechanisms involved in this phenomenon have been a long-standing enigma.

In mammalian oviduct, it is known that the presence of sperm reservoir, is the specific structure that is responsible for sperm–oviductal binding (Suarez 2008). This structure is located in the utero-tubal junction of the oviduct and works as the temporal sperm retention site for timely sperm–egg encounter at the site of fertilization (Suarez 2008). Using bovine and porcine as the experimental animals, the series of elegant experiments by Suarez *et al.* demonstrated that annexin (ANX) protein family is the key constituent for this binding (Ignotz *et al.* 2007). Although the involvement of ANX protein family in the sperm–egg interaction is not known in many animals, our preliminary experiments indicated that several ANX proteins were expressed in the uterovaginal junction of the quail oviduct where sperm storage tubules, the structure for long-term sperm storage, exist (unpublished data). Since we had already produced antisera that are reactive to the ANX protein family, we decided to investigate the role of ANX proteins in sperm–oocyte interactions by examining whether they are actually present on the surface of oocyte.

In this study, we report that the ANX protein family exists on the surface of quail oocytes. Moreover, we also demonstrate the evidence that ANXA6 possesses sperm-binding capability when expressed in a mammalian cell line.

Materials and methods

Animals and tissue preparation

Male and female Japanese quail, *Coturnix japonica*, 8–20 weeks old (Quail Cosmos, Tahara, Japan), were maintained individually under a photoperiod of 14 h light: 10 h darkness (with the light on at 5:00 h) and were provided with water and a commercial diet (Motoki Corporation, Tokorozawa, Japan) *ad libitum*. The time of oviposition in each bird was recorded every day in order to estimate the time of ovulation (ovulation occurs approximately 30 min after oviposition) (Etches & Schoch 1984). We selected birds that regularly laid eggs. To collect the ovulated ova, females were decapitated at 2 h after oviposition, and the ovulated oocyte was obtained from the mid portion of the magnum. To isolate the preovulatory follicles, birds were decapitated at 8–10 h before the expected time of ovulation, and the largest follicle was excised. Fertilized eggs were obtained from the female birds, which were housed with male birds in the same cage (male:female = 1:3).

All experimental procedures for the care and use of animals were carried out in accordance with approved guidelines of the Animal Care Committees of Shizuoka University (approval number: 2018A-5).

Production of anti-ANXAs antiserum

A rabbit polyclonal anti-ANXAs antisera were used against bacterially expressed His-tagged ANXAs. Quail *ANXA1* cDNA was amplified by PCR (cycling conditions: 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min for 30 cycles) in order to introduce XhoI and XbaI sites on the N terminus and C terminus, respectively. The sense and antisense primers used were 5'-GGCTCGAGACTAAAAGAACAAATGCTCA-3' and 5'- GGTCTAGATAAAGCGACCAGGATGGTTT-3', respectively. ANXA2 was amplified by PCR (cycling conditions: 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min for 30 cycles) to introduce XhoI and SalI sites on N terminus and C terminus, respectively. The sense and antisense primers used were 5'-GGCTCGAGACAGTTAAGGCTTACTCAAA-3' and 5'-GGGTCGACTTCAGTCCTCTCCACCACAC-3', respectively. For the production of the ANXA5 expression construct, quail *ANXA5* cDNA was amplified by PCR (cycling conditions: 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min for 30 cycles) in order to introduce XhoI and SalI sites on the N terminus and C terminus, respectively. The sense and antisense primers used were 5'-GGCTCGAGATGGCGAAGTATACAAGAGG-3' and 5'-GGGTCGACTCTTTCTGAATCATCTGATA-3', respectively. For the ANXA6 construct, *ANXA6* cDNA was amplified by PCR (cycling conditions: 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min for 30 cycles) in order to introduce XhoI and SalI sites on the N terminus and C terminus, respectively. The sense and antisense primers used were 5'-GGCTCGAGCGGCTGATTGTAAGCTTGAT-3' and 5'-GGGTCGACTTGTAGAGCTTGTCAGCAAA-3', respectively. The PCR product containing ANXA1 was digested with XhoI and XbaI and ligated into a pCold TF DNA vector (Takara Bio) and treated with the same restriction enzymes. The PCR product of ANXA2, ANXA5, and ANXA6 was digested with XhoI and SalI and ligated into a pCold TF DNA vector treated with XhoI and SalI. The resulting construct was transformed into competent *Escherichia coli* strain *BL21* (Takara Bio), and an ampicillin-resistant clone was selected after the nucleotide sequence analysis was performed. All recombinant ANXAs were expressed in the presence of 1 mM isopropyl-β-thiogalactopyranoside at 15°C for 24 h, and the protein was purified from the cell lysate using nickel resin (Novagen, Sigma–Aldrich) according to the manufacturer's instructions.

The purity of the recombinant ANXAs was verified by SDS-PAGE followed by CBB staining.

A single female New Zealand White rabbit (SLC, Hamamatsu, Japan) was immunized with the recombinant ANXAs as described previously (Kuroki & Mori 1997). Briefly, the rabbit was injected subcutaneously at multiple sites along the back with a total of 1 mL of an emulsion made by mixing equal volumes of Freund's complete adjuvant (Sigma–Aldrich) and recombinant ANXAs (300 µg/mL). Booster injections with the same amount of antigen in Freund's incomplete adjuvant (Sigma–Aldrich) were made 6 and 8 weeks after the first immunization. Two weeks after the final injection, blood was collected, and serum aliquots were stored at 4°C.

Gel electrophoresis and Western blotting

The ovulated oocytes recovered from the magnum of the oviduct were placed in saline. The PVM was isolated by fine forceps and scissors, and adhering yolk was removed by a gentle water stream from a pasture pipette. The washed PVM was placed in PBS containing 0.1% TX-100 and extracted after 10 min of vigorous shaking. Debris was removed by centrifugation at 20,000 g for 10 min at 4°C. The supernatant of the sample was used as the oocyte membrane extract. The protein concentration of the sample was determined using a BCA Protein Assay kit (Pierce, ThermoFisher Scientific).

SDS-PAGE was carried out under reducing conditions as described previously (Laemmli 1970) using 12% (w/v) and 5% (w/v) polyacrylamide as the resolving and stacking gels, respectively. For Western blotting, proteins separated by SDS-PAGE were transferred to a PVDF membrane (Immobilon-P; Millipore, Merck) (Matsudaira 1987). The membrane was then incubated with blocking buffer containing 5% (w/v) skim milk in PBS supplemented with 0.1% (w/v) Tween 20 for 30 min. The membrane was reacted with anti-ANXA1, ANXA2, ANXA5, or ANXA6 antiserum (1:1000) or these antisera were preincubated with each antigen protein (1:1000) diluted with blocking buffer (Sasanami *et al.* 2002). A mouse anti-E cadherin antibody (BD Biosciences, NJ, USA) was also employed to detect the plasma membrane. The immune reactive signals were visualized using a chemiluminescent technique (Amersham™, GE Healthcare) using horseradish peroxidase-conjugated anti-rabbit IgG (Cappel™, MP Biomedicals, CA, USA) for ANXAs and horseradish peroxidase-conjugated anti-mouse IgG (Cappel™, MP Biomedicals) for E cadherin. Chemiluminescence was detected using an ImageQuant™ LAS 500 (GE Healthcare).

Immunohistochemistry

To detect ANXAs protein in the follicles, the largest follicles were fixed in Bouin's fixative and embedded in Paraplast (Oxford Labware, St. Louis, MO, USA). Sections of 4 µm thick were prepared and washed with xylene for deparaffinization. After washing with PBS, the sections were blocked with PBS containing 1% BSA and 10% normal goat serum for 1 h. Immunohistochemical techniques, which were the same as those described previously (Sasanami *et al.* 2002), used anti-ANXA1, ANXA2, ANXA5, or ANXA6 antiserum (1:100). To confirm the specificity of the antisera, each antiserum was preincubated with the respective antigen protein (1:100). After washing with PBS, specimens were incubated with Alexa546-conjugated goat anti-rabbit IgG (1:300) (Molecular probes, ThermoFisher Scientific). The specimens were stained with 1 µM Hoechst 33342 (FUJIFILM Wako Pure Chemical) and examined under a fluorescence microscope equipped with an interference-contrast apparatus and a 40× objective (BX 51, Olympus).

For detailed localization of ANXA6 protein in the follicles, the sections after blocking treatment were incubated with anti-ANXA6 antiserum (1:100) with rat anti-ZP1 antiserum (1:100) (Ohtsuki *et al.* 2004) or a mouse anti-E cadherin (1:100) antibody. After washing with PBS, specimens were incubated with Alexa546-conjugated goat anti-rabbit IgG (1:300) in the presence of FITC-conjugated goat anti-rat IgG (1:300) or Alexa488-conjugated goat anti-mouse IgG (1:300). Fluorescent microscopic observation was performed as described above.

Expression of ANXA6 in a mammalian cell line

The open-reading frame of quail ANXA6 was amplified by PCR (cycling conditions: 94°C for 30 s, 51.3°C for 30 s, and 72°C for 2 min, for 35 cycles) to introduce HindIII and XbaI sites upstream of the initiator methionine and downstream of the chain termination codons, respectively. The sense and antisense primers used were 5'-aaaaaagcttGC AGCTCTGGCACTTTGAGGA-3' and 5'-gctctagaTTAA TGTACCACATTTACC-3', respectively. The PCR product was digested with HindIII and XbaI and ligated into the mammalian expression plasmid vector pcDNA3.1(+) (Invitrogen, ThermoFisher Scientific) treated with the same restriction enzymes. The resulting quail ANXA6 expression construct was transformed into competent *E. coli*, strain *DH5α* (Takara Bio). The sequence of the constructs was verified with DNA sequence analysis.

Human embryonic kidney cells (HEK cells; generously provided by Dr Tetsuya Kohsaka, Department of Applied Life Sciences, Shizuoka University, Shizuoka, Japan) were cultured in D-MEM (Sigma–Aldrich) supplemented with 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2. Before transfection, cells were plated on a coverslip (22 mm × 22 mm) coated with poly-L-lysine, which was placed in a six-well culture plate (Falcon, Corning, NY, USA) and cultured for 48 h until cells had grown to approximately 75% confluency. DNA (2.5 μg) was introduced into the HEK cells using Lipofectamine Plus Reagent (Gibco, ThermoFisher Scientific) as suggested by the manufacturer, and the cells were cultured in fresh medium (3 mL/well) for an additional 48 h to express recombinant ANXA6. We used mammalian cell line because our previous study had demonstrated that mammalian cell lines are capable to produce functional protein of Japanese quail (Sasanami *et al.* 2003*a,b*).

Observation of sperm binding to ANXA6-expressing cells

After culture, the cells were washed twice with Hanks' balanced salts solution (HBSS, GE Healthcare) supplemented with 0.8 mmol/L of $MgSO_4$, 1.26 mmol/L of $CaCl_2$, and 4.2 mmol/L of $NaHCO_3$ (pH 7.4, 270–305 mOsm/kg). Semen was obtained from the male quails during mating with a teaser female prior to ejaculation according to the procedure by Kuroki and Mori (1997) and was suspended in 1 mL of HBSS. Sperm suspension was added to the HEK cell culture at a final concentration of 1×10^6 cells/mL. It was then incubated at 37°C in a humidified atmosphere with 5% CO_2 for 0, 10, or 30 min. After washing with PBS, sperm were stained with 1 μM Hoechst 33342, embedded in glycerol, and examined under a fluorescence microscope equipped with an interference-contrast apparatus with a 40× objective (BX 51, Olympus Optics). The number of sperm bound on the HEK cells was counted. At least five fields were randomly selected to enumerate the binding.

Statistical analysis

Data are expressed as means ± standard deviation and are statistically analyzed using the statistical package R version 3.4.2 (https://cran.r-project.org/bin/macosx/). Normality and homoscedasticity of the data were confirmed by the Shapiro–Wilk test and Bartlett tests, respectively. Means were compared using the Student's *t*-test or Tukey's test.

Differences were considered statistically significant when $P < 0.05$.

Results

Sperm penetration into the perivitelline membrane occurred in the germinal disk region

In the first experiment, we observed sperm head localization on a section of fertilized eggs recovered from the magnum part of the oviduct. As shown in Fig. 1, sperm were found inside the PVM in the germinal disk region (arrows in panel B), but no such sperm were observed in the non-germinal disk region (panel C). In the non-germinal disk region, sperm were found to be trapped on the PVM (arrow in panel C), although a hole due to hydrolyzation by sperm protease was evident. We counted the number of sperm that 'penetrated' or were 'trapped on the PVM' in this section in the fertilized eggs. As shown in Table 1, 34% of sperm penetrated the germinal disk region, and this percentage was significantly higher than that of the non-germinal disk region ($P < 0.001$). These results indicated that sperm were able to penetrate the PVM, which overlaid the germinal disk, but not the non-germinal disk region.

We next performed Western blot analysis on the lysates of a TX-100-soluble fraction of ovulated ova (Fig. 2) to confirm the presence of ANXAs proteins in this fraction where the plasma membrane of the oocyte is solubilized. As expected, E cadherin (plasma membrane marker protein) was detected, indicating that the lysates contained oocyte plasma membrane (panel A). We produced specific antisera reactive to quail ANXA1, ANXA2, ANXA5, or ANXA6 and detected the same lysates. As a result, the anti-ANXA1 antiserum strongly reacted with a 40 kDa band in the lysates under reducing conditions (panel B). Additionally, an immunoreactive 70 kDa band was also visible. When the blot was detected with anti-ANAX6 antiserum, a single 66 kDa band was visualized. Conversely, a faint band was detected when the blot was incubated with either anti-ANXA2 or ANXA5 antiserum. No such signals were observed when the blots were incubated with antisera preincubated with antigen proteins (panel B, lanes +). These results indicated that ANXAs proteins were dissolved in the TX-100-soluble fraction together with the plasma membrane.

Localization of ANXA6 at the germinal disk region

To investigate the localization of ANXAs proteins in the oocytes, we detected a section of preovulatory follicles
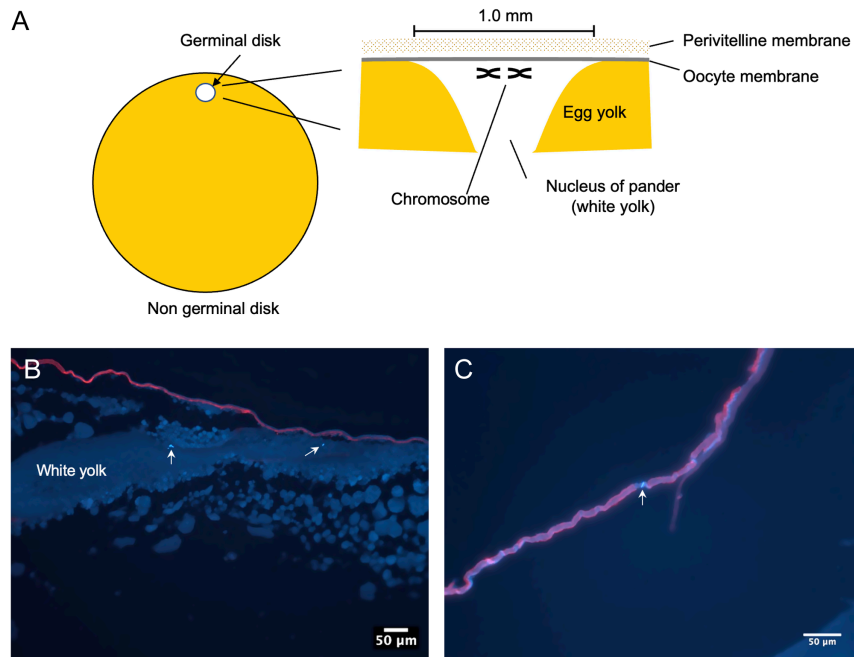
Figure 1 Sperm penetration into the perivitelline membrane in fertilized eggs. (A) The germinal disk of quail eggs. A schematic drawing of an egg and the cross-section of a germinal disk. The germinal disk of an oocyte is located at the region of animal pole, which is the small whitish area (white yolk) consisting of cytoplasm without egg yolk. The chromosomes are situated underneath the egg plasma membrane of the germinal disk. (B and C) Cross-section of the fertilized egg. The fertilized eggs were obtained from the magnum part of the oviduct after 2 h egg oviposition, and a paraffin section was stained with anti-ZP1 antiserum to show the PVM localization (red). Sperm nuclei were visualized with Hoechst 33342 (blue). Sperm nuclei were found inside the PVM of the germinal disk area (arrows in panel B), while no such sperm nuclei or sperm nuclei trapped in the PVM were observed in the non-germinal disk area (panel C). Autofluorescence of the white yolk was observed (panel B). Representative images are shown. Bar = 50μm.

with anti-ANXAs antisera (Fig. 3). Immunohistochemical analysis revealed that immunoreactive ANXA6 proteins were localized in the granulosa cells (GC) and just beneath the GC as narrow line (arrowheads in panel A) around the germinal disk; however, positive signals weakened and disappeared as the position shifted off from the center (double arrowheads in panel A). The same experiment was repeated on another sample to check the reliability of the results. The results showed that positive signals were detected in the germinal disk region (arrowheads in panel B) and suddenly faded away at the edge of the geminal disk (double arrowheads in panel B). When we observed a non-germinal disk region, no such signals were observed (panel C). As shown in panels D–F, anti-ANXA1, ANXA2, and ANXA5 failed to visualize such a unique staining pattern as observed by anti-ANXA6 antiserum. An immunoreaction was mainly observed in the round-shaped cells (arrows in panels D–F), which are presumably blood cells. Normal rabbit serum staining showed faint fluorescence (panel G).

We further pursued the detailed localization of ANXA6 in the follicle by means of double staining of the specimens with anti-ZP1 antiserum or an anti-E cadherin antibody, a major component of PVM and a plasma membrane marker protein, respectively. As shown in Fig. 4, a positive ANXA6 signal localized in both GC and just below the GC; however, the signal did not co-localize with anti-ZP1 (panels A–C), indicating that PVM did not contain ANXA6. In addition, immunoreactive E cadherin signals did not merge with those of ANXA6, and the ANXA6 signal was detected just
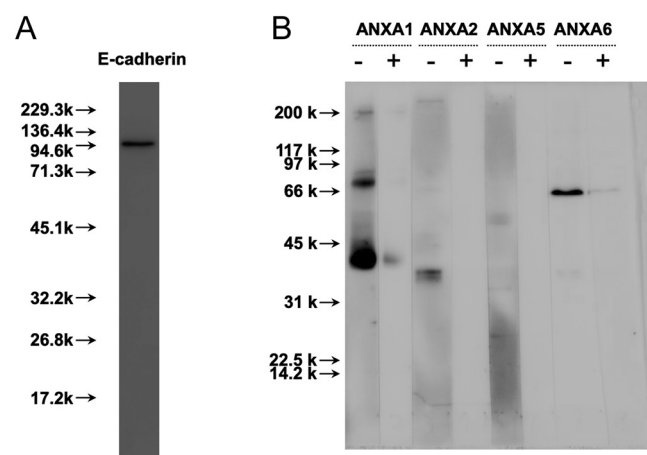
Table 1 Observation of spermatozoa in quail fertilized eggs.

Number of fertilized eggs	Area observed	Number of sperm		Penetration rate (%)
		Penetrated	Trapped	
5	Germinal disk	16	31	34**
5	Non germinal disk	0	131	0

**Significantly different from non-germinal disk (*P* < 0.01).

Figure 2 Western blot analysis of the oocyte membrane. A Triton-X100 solubilized oocyte membrane was separated by SDS-PAGE, transferred onto a PVDF membrane, and detected with (A) anti-E cadherin antiserum or (B) anti-ANXA1, ANXA2, ANXA5, or ANXA6 with (+) or without (−) respective antigen protein. Representative results of three experiments are shown.
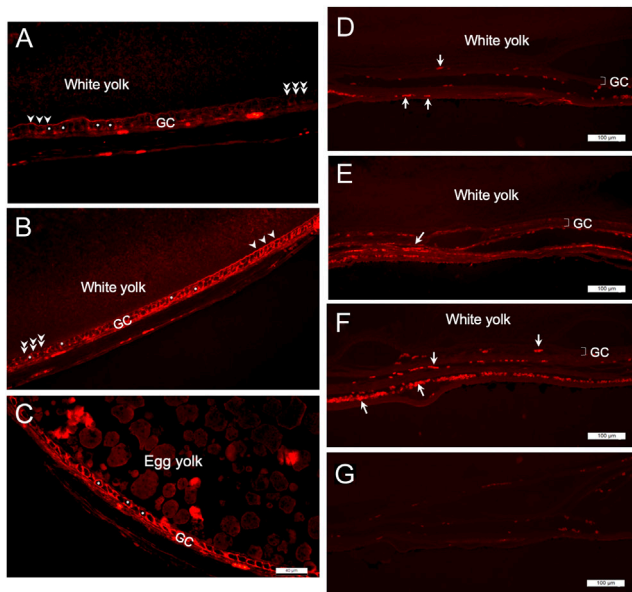
Figure 3 Immunohistochemical localization of ANXA proteins in preovulatory follicles. The largest follicles of laying birds were isolated and fixed overnight. The follicular walls of the germinal disk and non-germinal disk region were separately excised, prepared in paraffin sections, and detected using immunofluorescent microscopy. Sections of the germinal disk region (panels A and B) and non-germinal disk region (panel C) were detected with an anti-ANXA6 antibody. Sections of the germinal disk region were also detected with anti-ANXA1 (D), ANXA2 (E), ANXA5 (F), or normal rabbit serum (G). The immunoreactive signals to anti-ANXA6 were restricted to the germinal disk region (arrowhead in panels A and B), but the intensities of the signals faded away at the edge of the germinal disk (double arrowhead in panels A and B). No such staining was seen in the non-germinal disk region (panel C). Asterisks in panels A, B, and C indicate nucleus of GC. GC, granulosa cells. Arrows in panels D–F indicate round-shaped cells, presumably blood cells. Representative results of repeated experiments are shown. Scale Bars in panels A, B, C, D or E, F are 100 μm or 40 μm.

above the E cadherin signal (panels D–F). These results indicated that ANXA6 signals are located between PVM and the plasma membrane, in the perivitelline space.

Sperm binding to recombinant ANXA6 expressed in a mammalian cell line

To examine whether ANXA6 interacts with sperm directly, we expressed ANXA6 protein in HEK cells and incubated them with ejaculated sperm (Fig. 5). As shown in the figure, the number of sperm bound on the surface of ANXA6-expressed cells increased with time and became significantly higher than that of mock-transfected cells at 30 min of incubation (panels A–C). The addition of anti-ANXA6 antiserum inhibited the sperm binding, and the number of sperm bound on the surface of the cells decreased to the levels of mock-transfected cells (panel D). This neutralizing effect by anti-ANX6 antiserum indicates
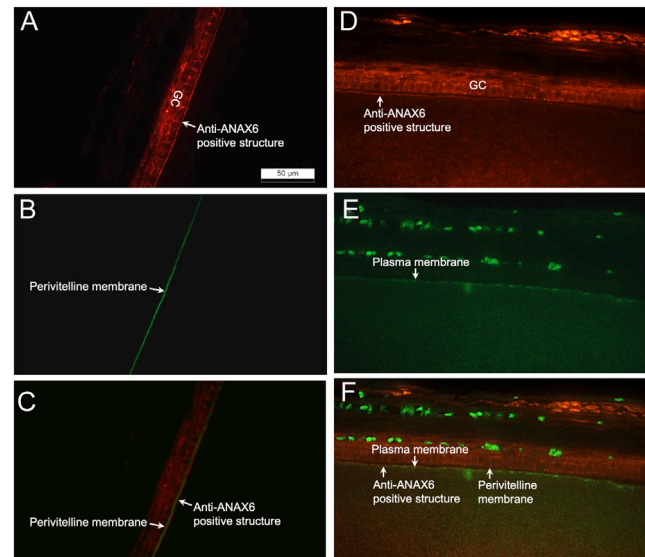

Figure 4 Detailed localization of ANXA6 in preovulatory follicles. (A, B, and C) Sections prepared from the largest preovulatory follicles were stained with anti-ANXA6 antiserum (A) or anti-ZP1 antiserum (B). A merged image is shown in panel C. (D, E, and F) The same specimens were stained with anti-ANXA6 antiserum (D) or anti-E cadherin (E). A merged image is shown in panel F. GC, granulosa cells. Representative results of three experiments are shown. Bar = 50 μm.

that sperm binding to ANXA6-expressed cells occurred via specific interaction between ANXA6 and sperm surface molecule. No such inhibitory effects were observed when anti-ANXA6 antiserum preincubated with antigen proteins was added (panel D).

Discussion

In this study, we found evidence that ANXA6 localized in quail oocytes specifically interacts with spermatozoa. To our knowledge, this is the first study to report the presence of sperm-binding protein on the surface of the egg plasma membrane in birds. The most interesting finding here is the localization pattern of ANXA6 on the oocytes; the plasma membrane that overlays the germinal disk was the main site of ANXA6 accumulation (Fig. 3). ANXAs are a family of Ca^{2+}-/lipid-binding proteins related to diverse events that occur outside cells, such as neutrophil extravasation and plasmin generation, by processes ranging from the control of membrane structures to membrane transport phenomena (Schloer *et al.* 2018). However, the classical signal sequence that could direct the protein to the cell surface is lacking (Rescher & Gerke 2004). Although the mechanism that regulates ANXA6 cell surface expression has not been elucidated, Deora *et al.* (2004) demonstrated that ANXA2
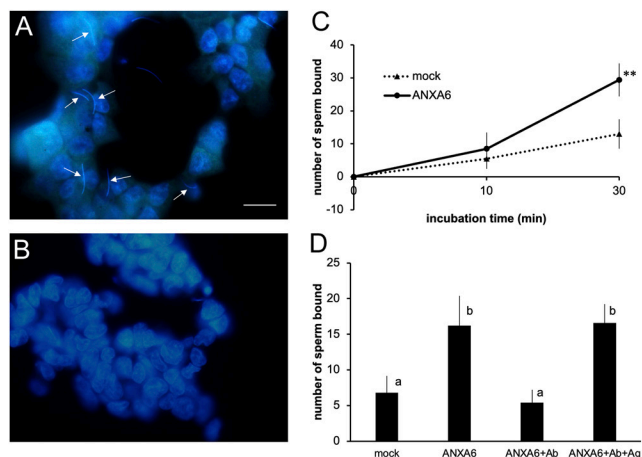
Figure 5 Specific binding of sperm on the surface of HEK cells expressed recombinant ANXA6. Cultured HEK cells were transfected with an ANXA6-expression construct (A) or vector alone (B) and incubated with ejaculated sperm. Arrows in panel A indicate bound sperm. Bar = 20 μm. (C) ANXA6-transfected and mock-transfected cells were incubated with sperm for 0, 10, or 30 min. After incubation, bound sperm were counted. **Significantly different from mock-transfected cells ($P < 0.01$). (D) ANXA6-transfected and mock-transfected cells were incubated with sperm for 30 min in the presence of an anti-ANXA6 antibody (ANXA6+Ab) or anti-ANXA6 antibody preabsorbed with antigen protein (ANXA6+Ab+Ag). Means ± standard deviation of three independent experiments. The means with different letters indicate significant difference ($P < 0.01$).

was translocated to the cell surface by phosphorylation of the protein (Deora *et al.* 2004). Also, at the time of the lactation onset, it is reported that ANXA6 was translocated from an intracellular to extracellular location in mouse mammary duct epithelial cells (Rocha *et al.* 1990). Because immunofluorescent microscopy indicated that ANXA6 was localized between the PVM and oocyte plasma membrane, ANXA6 is considered to have been transported outside the cells and accumulated in the perivitelline space. The origin of ANAX6 has not been identified. Although we did not examine the detailed structural observation, ANXA6 did not co-localize with E cadherin at light microscopical resolution and localized in perivitelline space. What is the nature of anti-ANXA6-positive structure? We think that ANXA6 may be associated with the oocyte plasma membrane, because this signal did not wash away from the PVM even after extensive washing. In addition, our Western blot analysis indicated that a TX-100 soluble fraction contained ANXA6 as well as E cadherin (Fig. 2A). These results suggested that ANXA6 may be tethered by an unknown molecule that localizes in the cell membrane and finally closely associated with the plasma membrane of the oocyte. Elucidation of the cell surface localization mechanism of ANXA6 is a target of future studies.

In our sperm-binding assay using ANXA6-expressed cells, we found that sperm-binding occurred, but the

sperm did not fuse with the plasma membrane of HEK cells (Fig. 5). Recent research in mice reported that JUNO, a glycosylphosphatidylinositol-anchored folate receptor family protein lacking the ability to carry folic acid, is the authentic fusogen that promotes the fusion of the plasma membrane between sperm and egg (Bianchi *et al.* 2014). To the best of our knowledge, there is no report describing a molecule involved in the sperm–egg fusion process in avian species (Nishio & Matsuda 2017). Although we do not know whether sperm–egg fusion actually occurs in avian fertilization, our results indicate that ANXA6 may function to retain sperm on the surface of the egg cell membrane prior to penetration.

In this study, we did not perform experiments to identify the binding partner of ANXA6 on the surface of spermatozoa. In bovines, binder of sperm 1 (BSP1), which was formerly referred to as PDC-109 (Ignotz *et al.* 2001), is synthesized in seminal vesicles and adsorbed on the sperm surface. Because BSP1 has been shown to facilitate sperm binding to the oviductal epithelium, and it interacts with Lewis[a], the key element of the sugar moiety of ANAXs, it is suggested that BSP1 is a candidate for the oviductal-binding protein on the sperm (Ignotz *et al.* 2007). It is not known if a component similar to BSP1 is present in quail sperm, but our previous preliminary experiments suggested that ANXAs are also present at the uterovaginal junction, where SSTs are located in laying quail (data not shown). The identification of sperm proteins responsible for ANXA6 binding remains a target for future studies.

As mentioned in the Introduction, sperm penetration sites in many species of birds are concentrated on the germinal disk (Bramwell & Howarth 1992, Birkhead *et al.* 1994). These findings suggested that sperm preferentially penetrate into the PVM on the germinal disk and it is believed that this phenomenon ensures sperm penetration into the germinal disk where the female nucleus is located. However, Yoshizaki *et al.* reported that it is not the case in quail fertilization because the holes produced by sperm are evenly localized on the egg surface, including non-germinal disk regions (Rabbani *et al.* 2006). Consistent with their observation, we also found that holes were ubiquitously localized on the PVM of fertilized eggs (data not shown). We have no idea if these discrepancies are due to species differences, but the mechanism of quail fertilization may be quite different from that of chicken. Actually, Nishio *et al.* demonstrated that ZP2 in chickens accumulates in the egg envelope of immature oocytes and remains in the germinal disk region of the mature egg (Nishio *et al.* 2014). These authors emphasized that this restricted localization of ZP2 on the germinal disk may be related to the sperm

perforation preference around the germinal disk region, although no relevant experiment was performed. In our previous study on Japanese quail, we reported that the ZP2 gene was expressed in immature follicles, and ZP2 protein was under the detection limit in the PVM of mature follicles in quail (Kinoshita *et al.* 2010). In this study, we found that ANXA6 accumulated exclusively in the germinal disk region, but it is unclear how this phenomenon enables the preferential entry of sperm into the germinal disk.

In conclusion, we showed that both sperm penetration sites and the localization of ANXA6 on the oocyte are limited to the germinal disk region of quail oocytes, although the relationship between the two was not elucidated. Because no sperm entry was observed in the non-germinal disk region, ANXA6 may play a role such as sperm retention on the surface of the egg cell membrane prior to penetration. Further studies are needed to clarify the molecular mechanism of sperm–egg interaction in avian species.

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