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Localization of CD9 in pig oocytes and its effects on sperm–egg interaction

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Abstract

CD9 is a cell surface protein that participates in many cellular processes, such as cell adhesion. Fertilization involves sperm and oocyte interactions including sperm binding to oocytes and sperm–oocyte fusion. Thus CD9 may play an essential role during fertilization in mammals. The present study was conducted to examine whether CD9 is present in porcine gametes and whether it participates in the regulation of sperm–oocyte interactions. The presence of CD9 in ovarian tissues, oocytes and spermatozoa was examined by immunohistochemistry, immunofluorescence and immunoblotting. Sperm binding and penetration of oocytes treated with CD9 antibody were examined by *in vitro* fertilization. The results showed that CD9 was present on the plasma membrane of oocytes at different developmental stages. A 24 kDa protein was found in oocytes during *in vitro* maturation by immunoblotting and its quantity was significantly ($P < 0.001$) increased as oocytes underwent maturation and reached the highest level after the oocytes had been cultured for 44 h. No positive CD9 staining was found in the spermatozoa. Both sperm binding to ooplasma and sperm penetration into oocytes were significantly ($P < 0.01$) reduced in anti-CD9 antibody-treated oocytes (1.2 ± 0.2 per oocyte and 16.6% respectively) as compared with oocytes in the controls (2.5 ± 0.4 per oocyte and 70.3% respectively). These results indicated that CD9 is expressed in pig oocytes during early growth and meiotic maturation and that it participates in sperm–oocyte interactions during fertilization.

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Introduction

CD9 is a member of the tetraspanin family and is widely present on cell membranes in animals (Berditchevski 2001, Boucheix & Rubinstein 2001, Hemler 2001). Some tetraspanins participate in many physiological processes, such as cell adhesion, motility, proliferation and differentiation (Boucheix & Rubinstein 2001, Hemler 2001). Tetraspanins do not appear to function as receptors for extracellular ligands, but they do associate in the plane of the lipid bilayer with other membrane proteins, including other tetraspanins, integrins, immunoglobulin superfamily (IgSF) members, proteoglycans, complementary regulatory proteins, growth factor receptors and others (Hemler 1998, Berditchevski & Odintsova 1999, Woods & Couchman 2000). It has been found that CD9 also plays an important role in gamete membrane interactions. For example, oocytes from CD9 knockout mice were rarely fertilized (Kaji *et al.* 2000) although ovulation and maturation were normal. Further evidence has indicated that sperm were able to adhere to the plasma membrane of zona pellucida (ZP)-free oocytes from CD9 knockout mice; however, very few sperm could fuse with the oocyte membrane (Le Naour *et al.* 2000, Miyado *et al.* 2000). Reduced sperm binding was also found in ZP-free mouse oocytes treated with anti-CD9 monoclonal antibodies (Chen *et al.* 1999, Takhashi *et al.* 2001, Wong *et al.* 2001, Zhu & Evans 2002). Sperm–oocyte fusion was also decreased when the oocytes treated with anti-CD9 antibody were inseminated (Miller *et al.* 2000). These results indicate that CD9 participates in regulating fertilization in the mice. However, its role(s) in other mammals is still unknown. In the present study, experiments were conducted to examine: (1) whether CD9 is located in porcine ovarian tissues, oocytes and spermatozoa and (2) whether CD9 regulates sperm–oocyte interaction during fertilization in pigs.

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Materials and Methods

Media and chemicals

Chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless stated otherwise. The basic medium, designated TCM-199B (pH 7.4), used for the maturation of oocytes was tissue culture medium (TCM)-199 (with Earle's salts; Gibco; Grand Island, NY, USA) supplemented with 3.05 mmol D-glucose/l, 0.91 mmol sodium pyruvate/l, 75 μg potassium penicillin G/ml and 50 μg streptomycin sulfate/ml. This medium was essentially the same as that used by Wang *et al.* (1994, 1997*a*) except that calcium lactate was not included in the present study. Insemination medium was modified Brakett and Oliphant (mBO) medium which was exactly the same as that used in our previous report (Wang & Niwa 1997).

Immunohistochemical staining of CD9 on pig ovarian histological sections

Porcine ovaries from prepubertal gilts were cut into two to four pieces and then immersed in 10% formalin for 24 h at 4 °C. The tissues were washed in 0.01 M phosphate-buffered saline (PBS) for 3 days (replaced every 8 h), and then dehydrated and hyalinized in different concentrations of ethanol and dimethylbenzene. Finally, they were embedded in paraffin and sliced into 5–6 μm serial sections. The sections were placed on the slides, placed on copper shelves in an oven for 4 h at 50 °C and then deparaffinized in xylene and rehydrated in graded alcohol.

Immunohischemical staining was carried out according to the labeled avidin–biotin (LAB-SA) method with Histostain-SP kits (Beijing Zhongshan Biochemical Co., Beijing, China) and 3,3′-diaminobenzidine (DAB) as a peroxidase substrate. Sections were placed in 3.8% citrate acid solution and heated to 95 °C for 15 min to recover the antigens. After being cooled down to room temperature (RT), the sections were covered with blocking solution (5% of goat serum in 0.01 M PBS and incubated in a moist chamber for 15 min at 37 °C. After three washes in PBS, slides were treated with 3% H_2O_2 for 15 min to block the endogenous peroxidase reactivity. After another three washes in PBS, the slides were covered with the primary antibody of CD9 (mouse anti-human CD9 monoclonal antibody (mAb) from Monosan, Uden, The Netherlands) overnight at 4 °C, in a concentration of 1:50. After removing the superfluous primary antibody solution by spilling (with no washing), the second antibody conjugated with biotin (Beijing Zhongshan Biochemical Co.) was added for 15 min at 37 °C. After three washes in PBS, the slides were then covered with streptavidin–horseradish peroxidase for 15 min. After three washes, slides were stained by DAB containing 0.1% H_2O_2 for about 10 min. After being thoroughly washed with distilled water, the slides were dehydrated in graded alcohol baths, hyalinized in xylene, mounted and examined by phase contrast microscope at × 400. Positive staining of CD9 by DAB showed brown. To test the specificity of the immunohistochemical staining, control slides were also stained with 0.1 M PBS, instead of the primary antibody of CD9.

Oocyte maturation in vitro

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/w) NaCl solution containing 75 μg potassium penicillin G/ml and 50 μg streptomycin sulfate/ml at 30–35 °C. Cumulus–oocyte complexes (COCs) were aspirated from antral follicles of 3–5 mm in diameter with an 18 gauge needle fixed to a 10 ml disposable syringe. The COCs were washed three times with Hepes-buffered Tyrodes medium containing 0.1% (w/v) polyvinyl alcohol (PVA) (Sigma), and three times with maturation medium. Each set of 60 COCs was transferred into maturation medium into which 10 ng epidermal growth factor/ml, 10 IU human chorionic gonadotropin/ml and 10 IU pregnant mare serum gonadotropin/ml had been added. The medium had been previously covered with warm paraffin oil in a polystyrene culture dish 35 × 10 mm, Nunc; Roskilde, Denmark and equilibrated in an atmosphere of 5% CO_2 in air for at least 6 h. These COCs were cultured at 39 °C for 44 h under the same conditions. After culturing, oocytes were freed of cumulus cells in the maturation medium containing 0.1% (w/w) hyaluronidase obtained from bovine testis (Type I-S, H-3506; Sigma), and then washed three times before being used in the following experiments.

Immunostaining of CD9 in the oocytes

ZP in oocytes was removed by putting oocytes into M2 medium (pH 2.5; Sigma) for ~2 min. The ZP-free oocytes were washed three times in TCM-199B and then treated for 45 min in the same medium containing anti-CD9 mAb (1:40). After being washed three times in PBS–0.01% PVA, the oocytes were fixed with 4% paraformaldehyde in PBS–0.01% PVA (pH 7.4) for at least 15 min at RT. After another three washes, oocytes were stained with fluorescein isothiocyanate-conjugated goat anti-mouse antibody in a 100 μl drop (1:40) for 45 min. Stained oocytes were further washed three times in PBS–0.1% PVA, each for 5 min, before nuclear staining with 10 μg propidium iodide/ml in PBS for 2 min. Finally, the oocytes were mounted on slides with antifade solution and examined by a laser scanning confocal microscope.

Immunostaining of CD9 in sperm

Spermatozoa were obtained from three boars and were frozen and stored in liquid nitrogen according to the method reported previously (Wang *et al.* 1991). For the experiment, sperm pellets were thawed at 39 °C and washed three times in PBS–PVA solution. Two different immunostaining procedures were used to identify CD9 in

the sperm. The first was the same as that for oocyte immunostaining except that all procedures were conducted in a 0.5 ml Eppendoff tube and the washing was also in the Eppendoff tube by centrifuging at 1000 ***g*** for 3 min. In the second immunostaining procedure, spermatozoa were first fixed with 4% paraformaldehyde in PBS (pH 7.4) and permeabilized with 0.5% Triton X-100 for 5 min (RT) before primary CD9 antibody treatment. All other procedures were the same as the oocyte staining procedures.

Immunoblotting analysis of CD9 in oocytes during maturation

A total of 100 oocytes cultured for 0, 22 and 44 h was collected in sodium dodecyl sulfate (SDS) sample buffer and heated to 100 °C for 4.5 min. After being cooled on ice and centrifuged at 12 000 ***g*** for 5 min, samples were frozen at −80 °C until use. The total proteins were separated by SDS-PAGE with a 4% stacking gel and a 10% separating gel for 2.5 h at 120 V and then electrophoretically transferred onto nitrocellulose membrane for 2 h at 200 mA at 4 °C. After blocking for 1 h in TBST buffer (20 mmol Tris/l, 137 mmol NaCl/l, 0.1% Tween 20, pH, 7.4) containing 1% low-fat milk, the membrane was incubated overnight at 4 °C in TBST containing 1:2000 CD9 antibody. After three washes, each for 10 min in TBST, the membrane was incubated for 1 h at 37 °C with alkaline phosphatase-labeled rabbit anti-mouse IgG diluted 1:3000 in TBST. The membrane was washed three times in TBST and then processed using the NBT/BCIP detection system (Sigma). Specificity was confirmed by preincubating the antibodies with their blocking peptide before immunoblotting. Immunoblot density was determined by the system of Personal Densitometer SI and FragmeNT Analysis software produced by Molecular Dynamics Inc. (Sunnyvale, CA, USA).

In vitro *fertilization (IVF)*

ZP in oocytes were removed by putting oocytes into M2 medium (pH 2.5) for less than 2 min. Thereafter, oocytes were washed three times and each 40 oocytes treated or not treated with anti-CD9 antibody (1:40, 45 min) were transferred into a 50 μl droplet of mBO medium covered with paraffin oil. The dishes were kept in a CO_2 incubator until spermatozoa were added for insemination. For IVF, one 0.1 ml frozen semen pellet was thawed at 39 °C in Dulbecco's PBS (DPBS) containing 1 mg bovine serum albumin/ml (fraction V, A-8022; Sigma) and antibiotics. After washing three times, spermatozoa were resuspended with mBO medium containing 2 mmol caffeine/l to give a concentration of 1×10^6 cells/ml, and 50 μl of the sample was added to 50 μl of the fertilization drop containing the oocytes. The oocytes and sperm were co-cultured for 6 or 16 h at 39 °C in an atmosphere of 5% CO_2 in air until examination of sperm binding and fertilization.

Assessment of sperm–oocyte binding

At 6 h after insemination, oocytes were removed from the microdrops, and the loosely binding spermatozoa were removed completely by pipetting. After being washed three to four times in PBS–0.1% PVA, oocytes were stained with 10 μg *bis*-benzamide (Hoechst 33342; Sigma)/ml in PBS–0.1% PVA for 5 min, mounted on slides and then examined under a fluorescence microscope. The number of sperm bound to oocyte membrane was then counted.

Assessment of sperm penetration

Sperm penetration was assessed 16 h after insemination. Oocytes from each group were fixed in acetic acid:alcohol (1:3) for 48 h, stained with 1% (w/v) orcein for 5 min and examined for evidence of sperm penetration under a phase contrast microscope at a × 400 magnification.

Statistical analysis

All experiments were repeated four times except immunoblotting which was repeated only three times. All percentage data were subjected to arc sine transformation before statistical analysis. Data were analyzed by ANOVA.

Results

Localization of CD9 in pig ovarian tissues, oocytes and spermatozoa

As shown in Fig. 1, CD9 was present on the membrane of many kinds of ovarian cells by immunocytochemistry. The immunostaining was stronger on granulosa cell membrane than that on oocyte plasma membrane in preantral follicles (Fig. 1A), but the staining on oocyte plasma membrane was almost the same as that on granulosa cell membrane in the fully grown follicles (Fig. 1B). No staining was observed in the control section (Fig. 1C).

When the immature oocytes were isolated from antral follicles (3–5 mm in diameter) and cultured *in vitro* for various times, as shown in Fig. 1D–F, CD9 staining was observed on the membrane of oocytes at various stages including germinal vesicle (GV, 0 h), metaphase I (M-I, 22 h) and metaphase II (M-II, 44 h). The staining was stronger as the oocyte nuclear stage proceeded to M-II than at earlier stages. The staining was evenly distributed on the membrane of oocytes when oocytes were scanned on the surface (insert in Fig. 1F).

As shown in Fig. 1G and H, there was no immunostaining of CD9 on the membrane of the sperm when the sperm were stained by two different immunofluorescent procedures.

By immunoblotting, a 24 kDa protein was found in the oocytes at GV, M-I and M-II stages, and the density was increased significantly ($P < 0.001$) during oocyte

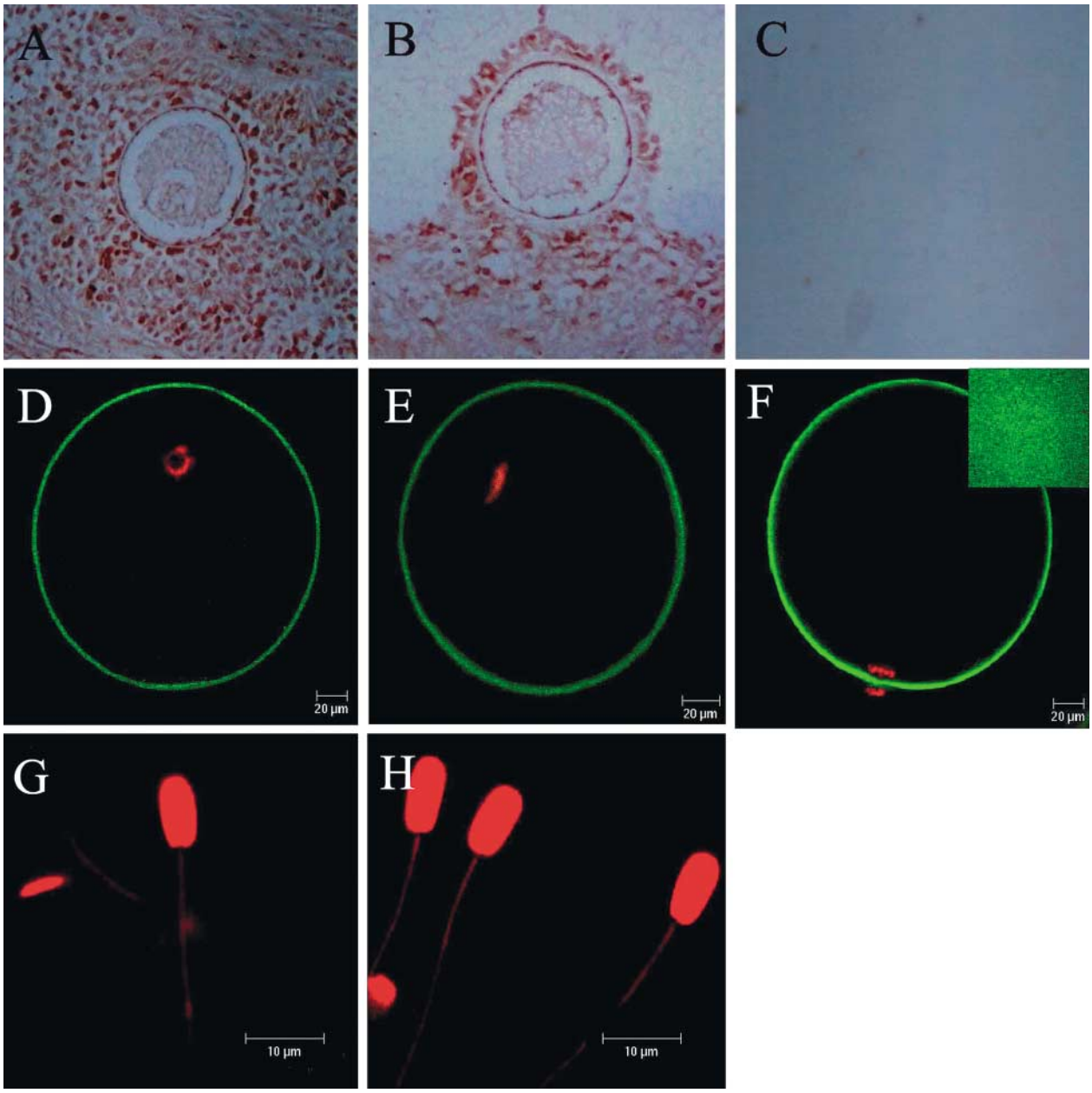

Figure 1 Localization of CD9 in pig ovarian tissues, oocytes during *in vitro* maturation and spermatozoa. Immunohistochemical staining of CD9 in (A) a preantral follicle, (B) a fully grown follicle and (C) a control section (×100). CD9 was distributed evenly on the membrane of oocytes at (D) GV, (E) M-I and (F) M-II stages. The insert in (F) indicates CD9 distribution on the oocyte surface, scanned by confocal microscopy. (G and H) Negative staining of CD9 was observed in boar spermatozoa by two different immunofluorescent staining procedures. The green images represent CD9 and the red images represent the nucleus from images D–H. This experiment was repeated four times.

maturation (Fig. 2) and it was ~2.5 times greater in the oocytes at the M-II stage than the oocytes at the GV stage. These results were consistent with those obtained by immunofluorescent staining.

Effect of anti-CD9 antibody on sperm–egg binding and sperm penetration

The rate of oocytes reaching the M-II stage after 44 h of *in vitro* maturation was 85.1 ± 2.3% ($n = 4$). When ZP-free oocytes were co-cultured for 45 min with anti-CD9 antibody and then co-cultured with frozen–thawed spermatozoa for 6 h, it was found that the number of spermatozoa bound to the oocytes was 1.2 ± 0.2 per oocyte, which was significantly ($P < 0.01$) fewer than that in the controls (2.5 ± 0.4 per oocyte). When the oocytes were co-cultured (for IVF) with spermatozoa for 16 h, as shown in Fig. 3, CD9 antibody treatment significantly ($P < 0.001$) reduced the rates of penetration (16.6 ± 3.3%) as compared with control oocytes (70.3% ± 6.2; $n = 4$).

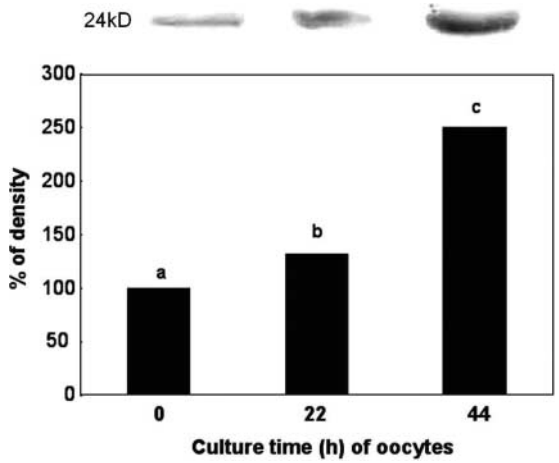

Figure 2 Quantification of CD9 in porcine oocytes during maturation. Immunoblots of CD9 in maturing oocytes at 0, 22, and 44 h of *in vitro* maturation. A 24 kDa protein was detected in the oocytes and its density was increased significantly during maturation. Different letters indicate statistically significant differences ($P < 0.001$). This experiment was repeated three times.

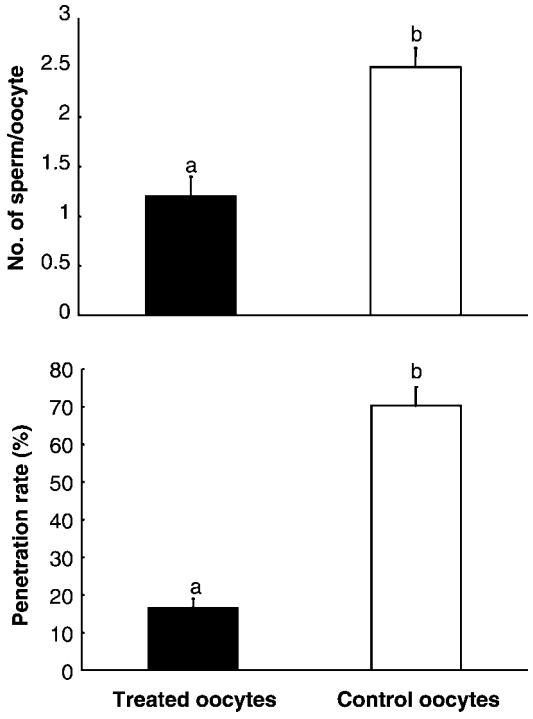

Figure 3 Effect of anti-CD9 mAb on sperm–egg binding at 6 h after insemination and sperm penetration in ZP-free oocytes. Different letters indicate statistically significant differences ($P < 0.001$). This experiment was repeated four times.

Discussion

As a member of the tetraspanin family, CD9 is extensively localized on the membrane of a variety of cells. CD9 is closely related to other tetraspanin proteins, integrins, IgSF members, glycoproteins, growth factor and other membrane proteins (Hemler, 1998, 2001, Berditchevski & Odintsova 1999, Woods & Couchman 2000, Boucheix & Rubinstein 2001). Some proteins in this network participate in many different cellular functions, such as adhesion, migration, differentiation, proliferation and signal transduction (Boucheix & Rubinstein 2001, Hemler 2001). In the present study, we found that CD9 was also located on the plasma membrane of porcine oocytes and other cells in preantral follicles and fully grown follicles. CD9 was significantly increased during the final oocyte maturation, indicating that it is associated with the competence of the oocyte to be fertilized. Our data indicated that the presence of CD9 is important for sperm to bind and fuse (penetration) with the oocytes, blocking CD9 by its antibody inhibits both sperm binding and penetration of oocytes. Our results also indicated that CD9 is not present on sperm membrane.

It has been found that there was a strong CD9 expression on the membrane of oocytes in developing follicles in the mouse and the strongest expression was on the membrane of oocytes in fully grown (developed) follicles (Chen *et al.* 1999). CD9 was also detected on some cells in the theca layer at the periphery of the immature (small) and mature (big) follicles, but not in surrounding ovarian tissue (Chen *et al.* 1999). Miller *et al.* (2000) reported that there was immunostaining of CD9 on both membrane of oocytes and membrane of cumulus cells in the mouse but there was no staining on ZP. Houle *et al.* (2002) also found that CD9 expression was in early but not late corpora lutea in the human ovary. In the present study, we found that CD9 was extensively expressed in porcine ovarian cells including oocytes, granulosa cells and theca cells. These results indicated that CD9 protein was already synthesized from early follicle development until oocyte maturation.

Most researchers have examined CD9 expression on the membrane of matured mouse oocytes (Chen *et al.* 1999, Le Naour *et al.* 2000, Miyado *et al.* 2000, Houle *et al.* 2002). Zhu *et al.* (2002) found that if CD9 mRNA was injected into CD9 knockout mouse oocytes CD9 could be expressed again on the egg membrane as revealed by immunofluorescent staining with anti-mouse CD9 mAb KMC8 or the anti-human CD9 mAb ALB6. Their results indicated that the localization of CD9 was not different from that in normal eggs: CD9 was present on the ooplasma where there were microvilli but was absent on the ooplasma over the metaphase plate. However, in the present study, we found that CD9 was distributed evenly on the membrane of the oocyte at M-II. There was no CD9-absent region. These differences in CD9 distribution between mouse and pig oocytes were the same as cortical granule (CG) distribution. There is a CG-free domain in mature mouse oocytes (Nicosia *et al.* 1977) but not in mature porcine oocytes (Wang *et al.* 1997*b*). It has been found that the adhesion, binding and fusion of the sperm with the egg only occur on the microvillus region not on the microvillus-free region (CG-free domain) in mouse oocytes (Ducibella 1991). However, it seems that boar

spermatozoa can bind oocytes at any area on the ooplasma. The localization of CD9 in accordance with the microvillus region in both the mouse and the pig provided further evidence that CD9 is involved in the process of fertilization.

Recently, it has been found that CD9 participates in sperm binding and sperm–egg fusion in the mouse (Kaji *et al.* 2000, Le Naour *et al.* 2000, Miyado *et al.* 2000). CD9 knockout female mice ovulate normally, and the ovulated oocytes mature to the M-II stage, but they are rarely fertilized (Kaji *et al.* 2000, Le Naour *et al.* 2000, Miyado *et al.* 2000). Further studies indicated that sperm were able to adhere to the plasma membrane of ZP-free oocytes from CD9 knockout mouse, but sperm could not fuse with the oocyte membrane (Miyado *et al.* 2000). These findings indicate that the CD9 on the membrane of oocytes has an important effect on fertilization. In the present study, we found that both sperm binding and sperm–oocyte fusion were significantly reduced in the ZP-free porcine oocytes when the CD9 was blocked by its antibody. These results are the same as those previously obtained in mice and they suggest that a similar mechanism may exist for CD9 to regulate fertilization in mammals. So far, however, evidence has only been obtained in mice (Le Naour *et al.* 2000, Miyado *et al.* 2000, Zhu *et al.* 2002, Zhu & Evans 2002) and pigs (present study); whether such a regulation by CD9 during fertilization is present in other mammals remains to be investigated.

The mechanisms by which CD9 participates in the sperm–oocyte interaction are not fully understood. Immunoprecipitation and other studies suggest that tetraspanins in the plasma membrane are associated with each other and with several other cell surface molecules, including a subunit of β1 integrins and IgSF members, to form a tetraspanin web (Nakamura *et al.* 1995, Berditchevski *et al.* 1996, Rubinstein *et al.* 1996, Maecker *et al.* 1997, Serru *et al.* 1999, Boucheix & Rubinstein 2001, Charrin *et al.* 2001, Stipp *et al.* 2001). They may organize specific cell-surface molecules to form functional macromolecular complexes on the surface of the cells that express the tetraspanin (Maeker *et al.* 1997, Boucheix & Rubinstein 2001). Zhu *et al.* (2002) found that CD9 acts by interaction with other proteins in the egg membrane. In addition, oocytes from CD9 knockout mice could be fertilized by intracytoplasmic sperm injection and these embryos developed to term (Miyado *et al.* 2000). These results suggested that CD9 might just function through extracellular loops not cytoplasmic elements. So Zhu *et al.* (2002) concluded that the residues S-F-Q in the CD9 large extracellular loop might be an active site that regulates the egg fusion machinery in mice (Zhu *et al.* 2002). Thus, the inhibition of fertilization by anti-CD9 mAb may be due to the blocking of sperm–egg adhesion and fusion during IVF of porcine oocytes.

It has been reported that another protein integrin (α6β1) may be the receptor of sperm on the mouse egg surface (Almeida *et al.* 1995). The binding of sperm to egg was achieved by the binding of integrin α6β1 with the disintegrin domain of fertilin β on the sperm surface in mice (Chen & Sampson 1999, Chen *et al.* 1999, Evans 2001). The receptors for sperm on oocytes in the pig are integrin subunits αv and β1 (Linfor & Berger 2000). Several anti-integrin antibodies could inhibit sperm–egg binding in mice, humans and pigs. For example, anti-β1 subunit antibody had a medium inhibitory effect on sperm–egg binding during fertilization and it could also inhibit the binding of recombinant fertilin β with mouse oocytes (Evans *et al.* 1997, Ji *et al.* 1998, Linfor & Berger 2000). However, more recent studies indicate that integrins αv, α3, α6, β1 and β3 on the mouse oocyte surface are not necessary proteins for sperm–egg fusion and fertilization (He *et al.* 2003). They might participate in sperm–egg adhesion, binding and fusion through forming complexes with CD9 or other tetraspanins (Gutierrez-Lopez *et al.* 2003).

In conclusion, our findings indicate that CD9 already exists on pig oocytes in preantral follicles and the oocytes continue to synthesize the CD9 until fully grown. CD9 synthesis was also observed during oocyte *in vitro* maturation and its quantity was significantly increased from the GV to the M-II stage. Fertilization can be blocked by anti-CD9 mAb. These results indicate that CD9 plays an important role in boar sperm–oocyte binding, fusion and fertilization.

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