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Original Article

Localization of CD9 on sheep oocytes and early embryos

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Abstract: Gamete cells passing through the genital tracts of both male and female lose some specific structural component to acquire a new one and finish fertilization successfully. It has been identified that some molecules localized either on gamete cells or in the genital tract tissues involved in particular events of fertilization. Tetraspanin CD9 is considered to be a serious candidate molecule participating in these events. The aim of this study was to investigate-whether the molecule CD9 is expressed on sheep oocytes during their oogenesis and maturation as well as on the early embryos and in sheep reproductive organs. The expression of sheep CD9 was examined by immunofluorescence and immunoblotting. Sperm binding and penetration of oocytes treated with CD9 antibody were examined by fertilization in vitro. The immunofluorescence studies using an anti-CD9 monoclonal antibody showed strong staining on the plasma membrane of oocytes at different developmental stages and early embryonic blastomeres. Intensive tissue staining was observed in primary follicles and mature follicles. The Western blot analysis showed the 24 kDa molecule exists in immature and mature oocytes and early embryos protein obtained from 2-cells, 4-cells, and 8-cells of embryos. Both sperm binding to ooplasma and sperm penetration into oocytes were significantly reduced in anti-CD9 antibody-treated oocytes (4.4 ± 0.1/oocytes and 41.5% respectively) as compared with oocytes in the controls (6.0 ± 0.3/oocytes and 81.4% respectively) ($P < 0.01$). The obtained data could be considered in the interpretation of the role of CD9 in oogenesis , early embryonic growth and that it participates in sperm-oocyte interactions during fertilization.

Keywords: Tetraspanin CD9, oocyte, embryos, immunofluorescence, tissue

Introduction

Infertility is an emotionally devastating problem for married women at reproductive-age worldwide [1]. In vitro fertilization (IVF) is the best chance for reproductive success. Mammalian fertilization is a very complex process, depending on many events, including interaction between gametes mediated by multiple proteins on their surface. Successful binding of spermatozoa to zona pellucida followed by fusion is an essential step to zygote formation. Although fertilization process has been described in general, the molecules and their exact roles are not fully illustrated. Therefore, every molecule found in genital system or on the gamete surface is considered to be a potential candidate molecule involved in this process, and significant effort has been devoted to characterize and study it closer. The list of molecules known to be present on gametes is very broad and is still enlarging. Molecules known to be involved in mammalian sperm-egg fusion or a better interpretation of fertilization process complexity are also reviewed [2, 3]. At present, there are no morphological or physiological features of oocytes that can predict whether conventional fertilization will be successful. CD9 is a tetraspanin family membrane protein widely expressed on animal cell membranes [4-6] and implicated in many cellular functions such as adhesion, migration, co-stimulation, signal transduction, and sperm egg fusion [7, 8]. The presence of CD9 on the oocyte membrane was

found to be essential for sperm-egg fusion [9-11]. This function can be restored if CD9 deficient oocytes are injected with CD9 mRNA [12, 13]. Because CD9 is needed for gamete fusion, measuring the expression of CD9 in women could provide a useful marker for predicting conventional IVF fertilization in couples with normal sperm parameters. Potentially, high expression of CD9 could indicate a high probability of oocyte fertilization using conventional IVF whereas low expression might justify a need of ICSI instead, although such relationship has never been measured. CD9 is also expressed on blastocysts in mice and endometrium epithelial cells in human and cattle [10, 14, 15]. Given the striking similarities between embryogenesis and the biology of cancer cells, especially in the process of the invasion, CD9 might be involved in embryo-invasive behaviors. Embryo implantation is an important step in the establishment of pregnancy. Successful invasion or migration into the extracellular matrix environment is a fundamental property of embryo implantation. CD9 expression on granulose cells and embryos has been linked with female reproductive function [16-18], however, no study has tested whether CD9 expression on granulosa cells and embryos correlates with CD9 expression on sheep oocytes. The specific goal of this study, therefore, was to determine whether CD9 is expressed on sheep oocytes, early embryos and granulosa cells and whether CD9 is involved in sperm-oocyte interaction during fertilization and embryo implantation. It will be used to predict the fertilization success during a conventional IVF cycle and establishment of pregnancy.

Materials and methods

Reagents

Primary antibody anti-CD9 antibody and secondary antibody donkey anti-rabbit IgG H&L were purchased from Abcam Shanghai. Chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless stated otherwise. The basic medium, designated TCM-199 (pH 7.4), used for the maturation of oocytes, was tissue culture medium TCM-199 (Gibco; Grand Island, NY, USA).

Oocyte pick-up solution: TCM199 + 25 mmol/l HEPES + 2.2 mg/ml $NaHCO_3$ + 2% FCS + 100 IU/ml streptomycin + 100 μg/ml penicillin.

Maturation culture medium: TCM199 + 10 mmol/l HEPES + 2.2 mg/ml $NaHCO_3$ + 8 mg/ml BSA + 0.25 mmol/l sodium pyruvate + 2.75 mmol/l lactate + 100 IU/ml penicillin + 100 μg/ml-streptomycin + 50 ng/ml EGF + 1 μg/ml E2 + 10 μg/ml LH + 10 μg/ml FSH.

SOF working solution: SOF stock solution + 1 mmol/l glutamine + 0.3 mmol/l sodium pyruvate.

Oocyte washing solution: SOF working solution + 10 mmol/l HEPES + 5 mmol/l $NaHCO_3$ + 0.3% BSA + 100 IU/ml penicillin + 100 μg/ml streptomycin.

Sperm-washing solution: The same ingredients as oocyte washing solution, with a double amount of double antibody.

Capacitation solution: SOF working solution + 20% estrous sheep serum + 10 mmol/l enicillamine + 10 mmol/l hypotaurine + 10 μg/ml heparin + 0.5 mol/l calcium lactate + 100 IU/ml penicillin + 100 μg/ml streptomycin.

Embryo culture medium: SOF working solution + 10% FCS + 2% essential amino acids (BME-EAA) + 1% nonessential amino acids (MEM-NEAA) + 100 IU/ml penicillin + 100 μg/ml streptomycin.

Oocytes collection and in vitro culture

Sheep ovaries were collected from a local slaughterhouse and were immediately placed in 25-28°C normal saline containing penicillin and streptomycin, then transported to the laboratory within 2-4 h. The ovaries were washed three times in normal saline and trimmed to remove fat and the corpus luteum. Then, ovaries were placed in a petri dish containing oocyte pick-up solution to remove ovarian follicle. According to the test needs, cumulus-oocyte complexes (COCs) were sorted out under a stereomicroscope (Olympus SZ40, Tokyo, Japan). The selection criteria were as follows: complete morphology, dense cytoplasm, uniform color, at least three layers of granular cells, and dense encapsulation. Then the oocytes were respectively rinsed three times using maturation culture medium and transferred into 50 μl of pre-equilibrated maturation medium droplets (10 oocytes each), overlaid with paraffin oil. The incubation was performed

at 38.5°C in an atmosphere of 5% CO_2 under saturated humidity.

Removal of the zona pellucid

The cumulus cells were removed from COCs by vibrating in 0.1% hyaluronidase for 10 minutes and the mature oocytes with a first polar body were collected. Then the zona pellucid (ZP) was removed by dipping in 0.5 mg/ml pronase solution for about 1-2 min at 38°C. These ZP-free oocytes were washed three times in maturation medium and cultured in an incubator for 10 min and then selected for Immunofluorescence labeling of CD9.

Immunofluorescence staining of CD9 in oocytes

Different experimental groups of sheep oocytes were collected at varying maturity periods. The oocytes were digested in 0.1% hyaluronidase to completely remove granulosa cells, and the zona pellucida was removed with phosphate-buffered saline (PBS) (pH 2.5). The digested oocytes were fixed in 4% paraformaldehyde at room temperature for 20 min and then placed in 0.2% Triton-X100 (Sigma-Aldrich, MO, USA) for 30 min to osmosis. Thereafter, oocytes were incubated in a blocking agent (PBS + 2% BSA + 10% goat serum + 2% skim milk powder + 0.15 mol/l glycine) at 37°C for 1 h, followed by the addition of FITC-conjugated Goat Anti-Rabbit IgG (ab150077) with a final concentration of 1 μg/ml and incubation at 37°C for another 45 min. At the end of incubation, oocytes were thoroughly washed in 0.2% Triton-X100, 5 μg/ml propidium iodide (Sigma-Aldrich, MO, USA) was added, and then they were placed in a cassette for 10 min of nuclide labeling. Finally, oocytes were examined under a confocal microscope (C1/TE2000-U; Nikon, Tokyo, Japan). In this process, some of ZP-free oocytes without anti-sheep CD9 mAb treatment were used as a control.

In vitro fertilization of oocytes and embryo culture

Sperm capacitation: Fresh semen was washed twice with sperm-washing solution and centrifuged at 1500 rpm/min for 5 min. The supernatant was decanted and sperm at the bottom of the centrifuge tube was added to the pre-equilibrated capacitation solution for 30 min at 38.5°C in an atmosphere of 5% CO_2 under saturated humidity.

In vitro fertilization: Mature oocytes were digested with 0.1% hyaluronidase to partially remove granulosa cells. The oocytes were then washed thrice with the capacitation solution and transferred into fertilization droplets. Sperm was also added into the fertilization droplet. Each drop contained 10 μl of sperm with a density of 5×10^6 sperm/ml. The oocyte-sperm complex was incubated for 18 h at 38.5°C in a 5% CO_2 atmosphere under saturated humidity.

Embryo culture: After 18 h of oocyte-sperm co-incubation, zygotes were washed thrice with oocyte washing solution to remove granulosa cells and sperm. After twice washed with embryo culture medium, zygotes were transferred into droplet containing monolayer granulose cell and incubated at 38.5°C in a 5% CO_2 atmosphere under saturated humidity. Half the medium was exchanged with new medium every other day. The early embryos were collected at varying maturity periods.

Immunofluorescence staining of CD9 in embryos

The early embryos were obtained by IVF and rinsed three times in Ham's F-12 medium, then transferred into droplets of preheated Ham's F-12 medium supplemented with 0.5% BSA. For the CD9 block assay, the embryos were incubated with anti-CD9 mAb at concentrations of 10-1000 ng/ml. In control cultures,embryos were incubated with a purified isotype rat IgG at the same concentration as the treated blastocysts. To determine embryo attachment, the plate was shaken for 20 s with one rotation/s. If the blastocyst was found to stay at the same place, this blastocyst was designated as attachment; if not, it was designated as non-attachment. Each experiment was repeated three times.

Preparation of cryostat sections

Ovaries were taken from a local abattoir as described above. Then the tissues were fixed in 4% paraformaldehyde overnight (12-24 h) at 4°C until sinking into the bottom of the tube, and followed by dehydration with 30% sucrose at 4°C for 24-72 hours. The tissues were embedded in OTC and cut into 10 μm cryostat

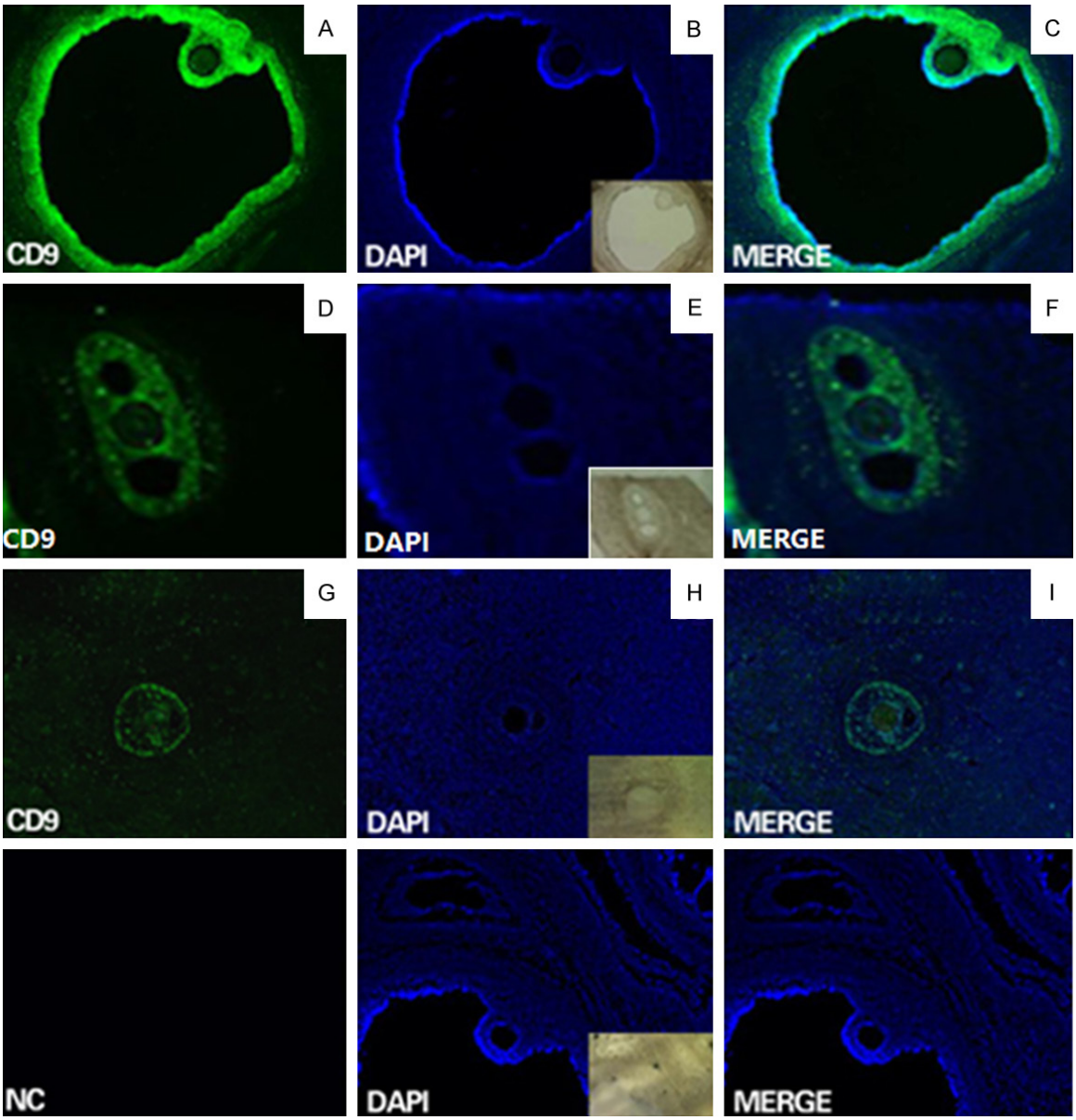

Figure 1. Localization of CD9 in sheep ovarian tissue. A. mature follicle (green); D. Tertiary follicle (green); G. Primary follicle (green); B, E, H. Nuclei of different stage follicle cells were counterstained with DAPI; C, F, I. Merged images; (NC) negative control; Magnification 40 ×.

sections. All sections were kept in -80°C for further analysis.

Protein localization by immunofluorescence

The prepared slices were washed with 0.01 M KPBS (pH 7.4) three times, 10 minutes at a time on shaker, then fixed with 0.01 M KPBS (+ 1% TritonX-100) for 2 hours at 4°C. After washed another three times, the unspecific staining is blocked by 5% BSA (bovine serum albumin) in 0.01 M KPBS (pH 7.4) for 30 min at room temperature. The sections were incubated overnight at 4°C with the primary antibody-Anti-CD9 antibody [EPR2949] diluted at 1:100 in KPBS (+ 0.1% Triton-100) followed by three times washing with KPBS (15 min/time). Then, the sections were incubated 1 h at room temperature with the Goat Anti-Rabbit IgG HandL-Alexa Flour 488 diluted at 1:200 followed by three times washing with KPBS (15 min/time). Finally, the samples were counterstained with DAPI and covered with antifade solution (DABCO). The staining was observed by confo-

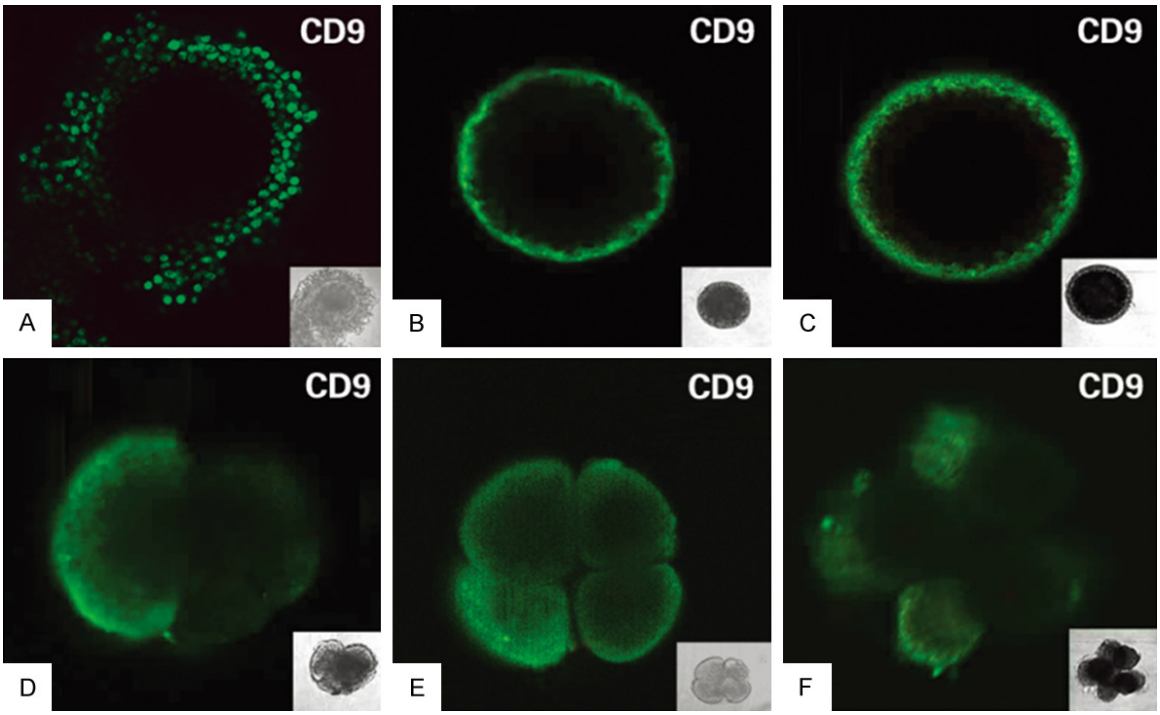

Figure 2. Localization of CD9 protein on cumulus cells, oocytes and early embryos. A. cumulus cells (green); B. ZP-free Immature oocyte (green); C. ZP-free Mature oocyte (green); D. 2-cells embryo (green); E. 4-cells embryo (green); F. 8-cells embryo (green).

cal laser scanning microscope (C1/TE2000-U; Nikon, Tokyo, Japan). To check the specificity of staining, control samples were exposed to the secondary antibody without previous treatment with the primary antibody.

Immunoblotting analysis of CD9 in oocytes and embryos

Total protein was isolated using Tri-Reagent. Proteins were separated in 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto a nitrocellulose membrane (Amersham Biosciences). The membranes were further kept in blocking buffer (Beyotime) for 1 h at room temperature, before incubation with an Anti-CD9 antibody [EPR2949] diluted at 1:1000 in primary antibody diluent (Beyotime) overnight at 4°C. Then incubate membrane in horseradish peroxidase conjugated Donkey Anti-Rabbit IgG H&L as secondary antibody (diluted at 1:2000) for 1 h at room temperature. The membrane was again cleaned three times with washing buffer (Beotime) before chemiluminesce assay by using the ECL western blotting detection system. The membrane was cut into two pieces; the one with ß-actin was used as internal control and for normalization.

In vitro fertilization (IVF)

Before in vitro fertilization,the ZP-free oocytes were treated for 45 min in an incubator filled with culture medium containing an anti-sheep CD9 mAb. They were then fertilized in vitro according to our previous description. In this process, the ZP-free oocytes without anti-sheep CD9 mAb treatment were used as a control.

Assessment of sperm-oocyte binding

At 8 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 DAPI in PBS-0.1% PVA for 5 min followed by washing 3 times for 10 min in PBS-0.1% PVA. Finally, the oocytes were mounted on slides and the number of sperm bound to oocyte membrane was counted under a fluorescence microscope.

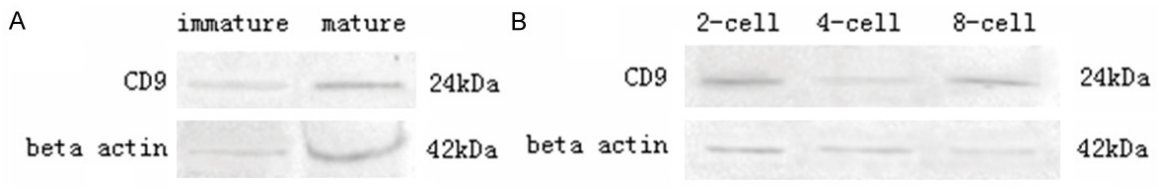

Figure 3. The CD9 in the different stages of oocytes and embryos by western blotting. A. Immature and mature oocytes; B. 2, 4, 8- cells early embryos.

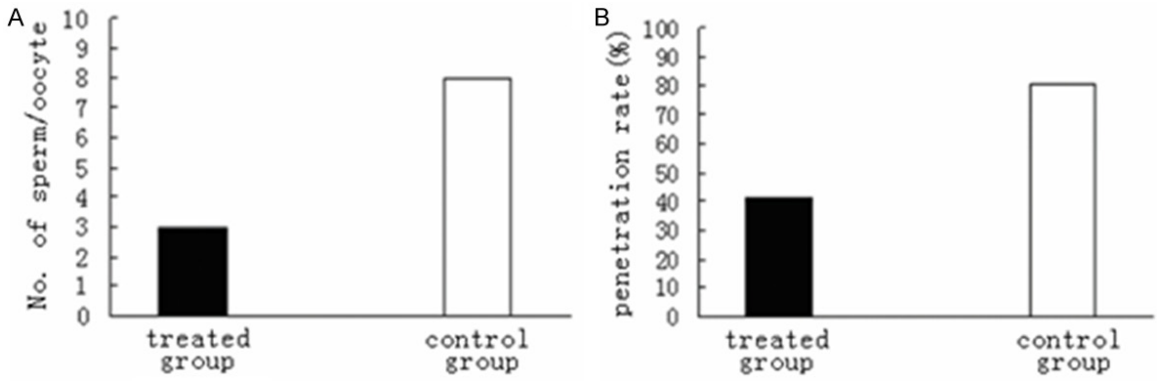

Figure 4. Effects of Anti-CD9 monoclonal antibody on sperm oocyte binding and sperm penetration. A. The number of spermatozoa binding to the oocytes; B. Sperm penetration rate.

Assessment of sperm penetration

Sperm penetration was assessed 18 h after insemination. Oocytes from each group were fixed in solution (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.

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

Distribution of CD9 in sheep ovarian tissue, oocytes and embryos

As shown in **Figure 1**, CD9 was present on the membrane at various stages of follicle cells by immunofluorescent staining. The immunostaining was stronger on granulosa cell membrane than that on oocyte plasma membrane in preantral follicles (**Figure 1G**), but the staining on oocyte plasma membrane was almost the same as that on granulosa cell membrane in the fully grown follicles (**Figure 1A** and **2A**). No staining was observed in the control section (**Figure 1B**, **1E**, **1H**).

When the immature oocytes were isolated from antral follicles (3-5 mm in diameter) and cultured in vitro for mature, as shown in **Figure 2B**, **2C**, CD9 staining was observed on the membrane of oocytes at germinal vesicle(immature) and metaphase II (mature). The staining was stronger as the oocyte nuclear stage proceeded to MII than at earlier stages.

By using immunofluorescence staining, CD9 antigen was detected to express strongly on the surface of blastomeres of two-, four-, and eight-cell embryos (**Figure 2D-F**). This expression pattern suggested that CD9 might play a role in sheep embryo development and embryo implantation.

By immunoblotting, a 24 kDa protein was found in the oocytes at GV and MII stages (**Figure 3A**), and also found in the embryos (**Figure 3B**). These results were consistent with those obtained by immunofluorescent staining.

Effects of anti-CD9 antibody on sperm-egg binding and sperm penetration

The rate of oocytes reaching the MII stage after 24 h of in vitro maturation was 87.3 ± 1.5%. When ZP-free oocytes were co-cultured for 45 min with anti-CD9 antibody and then co-cultured with frozen-thawed spermatozoa for 6-8 h, it was found that the number of spermatozoa bound to the oocytes was 4.4 ± 0.1 per oocyte, which was significantly ($P < 0.01$) fewer than that in the controls (9.0 ± 1.0 per oocyte). When the oocytes were co-cultured (for IVF) with spermatozoa for 20 h, as shown in **Figure 4**, sperm penetration rate CD9 antibody treatment group (41.5%) was significantly lower that of control group (81.4%) ($P < 0.01$).

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 [5, 6, 10, 20]. Some proteins in this network participate in many different cellular functions, such as adhesion, migration, differentiation, proliferation and signal transduction [5, 6]. In the present study, we found that CD9 also located on the plasma membrane of ovine 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 sperm from both binding with and penetrating of oocytes. It has been found that there was a strong CD9 expression on the membrane of oocytes in developing follicles in mouse and the strongest expression was on the membrane of oocytes in fully grown (developed) follicles [21-23]. 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 [21-24]. Miller et al. [24] reported that there was immunostaining of CD9 on both membrane of oocytes and membrane of cumulus cells but not on ZP in mouse. Houle et al. [25] 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 sheep 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 [10, 11, 13, 21, 25]. Zhu et al. [13] found that if CD9 mRNA was injected into CD9 knock out 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. It has been found that CD9 participates in sperm binding and sperm-egg fusion in mouse, pig and cattle [9-11, 23]. CD9 knockout female mice ovulate normally, and the ovulated oocytes mature to the MII stage, but they are rarely fertilized [9-11]. 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 [11]. These findings indicate that 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 sheep oocytes when the CD9 was blocked by its antibody. These results are the same as those previously obtained in mice,pig and cattle and they suggest that a similar mechanism may exist for CD9 regulating fertilization in mammals. So far, however, evidence has only been obtained in mice [10, 11, 13] and pigs [22], cattle [23] and sheep (present study); whether such a regulation by CD9 during fertilization is present in other mammals remains to be investigated. The mechanisms of 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. In addition, oocytes from CD9 knockout mice could be fertilized by intracytoplasmic sperm injection and these embryos developed to term [11]. These results suggested that CD9 might adjust function through extracellular loops instead of cytoplasmic elements. Thus, the inhibition of fertilization by anti-CD9 mAb

may be due to the blocking of sperm-egg adhesion and fusion during IVF of sheep oocytes. It has been reported that another protein integrin (α6β1) may be the receptor of sperm on the mouse egg surface [26]. 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 [21, 27]. Several anti-integrin antibodies could inhibit sperm-egg binding in mice, humans and pigs. For example, anti-b1 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 [28]. They might participate in sperm-egg adhesion, binding and fusion through forming complexes with CD9 or other tetraspanins [29].

Blastocyst adhesion and invasion into uterine endometrium are two important steps of successful embryo implantation. To gain an insight into the cellular and molecular mechanisms that control this process, we have demonstrated the expression profile of CD9 in pre-implantation embryos and the functional role that CD9 plays in embryo implantation in sheep. Results showed that CD9 was expressed on blastomeres of two-, four-, and eight-cell and morula embryos as well as trophoblast cells of blastocysts, and CD9 is involved in the regulation of embryo invasion.

Measurement of CD9 expression on sheep granulosa cells and platelets may not be a useful indicator for predicting the success of conventional fertilization in couples undergoing IVF. A weak negative relationship between surface density of CD9 on granulosa cells and fertilization rate of mature oocytes may reflect a downregulation of CD9 that accompanies follicle maturation at ovulation. More studies are necessary to determine the role of CD9 on granulosa cells during follicle maturity, and to assess if CD9 expression on human granulosa cells could be used as a factor to predict the success of conventional fertilization during IVF. Fertilization can be blocked by anti-CD9 mAb. These results indicate that CD9 plays an important role in sheep sperm-oocyte binding, fusion and fertilization.

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Disclosure of conflict of interest

None.

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