

# CD9 protein appears on growing mouse oocytes at the time when they develop the ability to fuse with spermatozoa

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## Summary

CD9 is a member of the tetraspanin superfamily proteins and is the only protein on the mouse oocyte which is known to be indispensable in sperm–egg fusion. Here, using indirect immunofluorescence we show that CD9 appears on the oolemma during the early stages of the growth of the oocyte, when it measures 13–22  $\mu\text{m}$  in diameter. When the oocyte reaches a diameter of 17–22  $\mu\text{m}$ , the density of CD9 in its oolemma is similar to the density of this protein in the cell membrane of the fully grown secondary oocyte. The appearance of CD9 in growing oocytes correlates with the previously reported time of the acquisition of fusibility between the spermatozoon and the egg. Accordingly we propose that during oogenesis the development of the ability of the oolemma to fuse with sperm may be regulated by synthesis of CD9 by the oocyte.

Keywords: CD9, Fertilization, Oocyte cell membrane, Oogenesis, Tetraspanin

## Introduction

During fertilization the membrane of the spermatozoon fuses with the oolemma (the oocyte's plasma membrane). The problem of when the oolemma acquires the ability to fuse with spermatozoa has been studied in the hamster (Zuccotti *et al.*, 1991) and in the mouse (Zuccotti *et al.*, 1994). These studies demonstrated that in both species small primary oocytes were unable to fuse with spermatozoa. Hamster and mouse oocytes acquired the ability to fuse with spermatozoa during oogenesis, and first became fusion-competent when they reached about 20  $\mu\text{m}$  in diameter (Zuccotti *et al.*, 1991, 1994).

More recent findings have demonstrated that a crucial role in gamete fusion is played by the integral oolemma tetraspanin CD9 (Chen *et al.*, 1999; Miyado *et al.*, 2000). Female mice lacking CD9 are infertile due to the failure of sperm–egg fusion (Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miyado *et al.*, 2000). The aim of our study was to verify the hypothesis that the acquisition of fusibility with spermatozoa by growing oocytes

is correlated with the appearance of CD9 protein on their surfaces. To achieve this goal we used indirect immunofluorescence to detect the CD9 protein on oolemma of growing mouse oocytes.

## Materials and methods

### Reagents

All chemicals, unless otherwise stated, were obtained from Sigma-Aldrich (Poznan, Poland).

### Animals

F1 (C57Bl/10  $\times$  CBA/H and C57Bl/6  $\times$  CBA/H) female mice between 1 and 15 days after birth and adult F1 female mice (3–6 months old) were used for the experiments.

### Collection of growing oocytes

To induce superovulation adult females were injected with 10 IU of pregnant mare serum gonadotrophin (PMSG, Folligon, Intervet, The Netherlands) followed 48 h later by injection of 10 IU of human chorionic gonadotrophin (hCG, Chorulon, Intervet, The Netherlands). Ovaries were isolated into M2 (M-16

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medium buffered with HEPES; Fulton & Whittingham 1978). Oocytes were released from ovaries of females which were 1–3 days old by puncturing ovaries with a fine needle. Ovaries isolated from older mice (6–15 days old) were fragmented and exposed to mixture of enzymes (collagenase 0.5 mg/ml, hyaluronidase 0.5 mg/ml, lysozyme 0.5 mg/ml phosphate-buffered saline (PBS); Mangia & Epstein, 1975) to release oocytes from growing follicles. Subsequently follicular cells were removed from oocytes by pipetting with a fine capillary. The diameter of oocytes was measured before subsequent fixation and staining.

### Collection of ovulated oocytes

Ovulated secondary oocytes were collected from the oviducts 15–18 h after hCG injection, and were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (200 IU/mg) in PBS for a few minutes. The oocytes were subsequently rinsed thoroughly in M2.

### Removal of the zona pellucida

Growing oocytes obtained from females 6–15 days old and ovulated secondary oocytes were freed of zona pellucida by brief exposure to acidic Tyrode solution (pH 2.5, Nicolson *et al.*, 1975). Oocytes isolated from younger females (1–3 days old) did not have a visible zona pellucida and were not exposed to acidic Tyrode solution.

### Indirect immunofluorescence

Oocytes were fixed with 4% paraformaldehyde (20 min at room temperature), then washed twice in PBS and once in PBS with 10 mM glycine for 10 min. To block non-specific antibody binding sites, oocytes were incubated for 1 h in 2% bovine serum albumin (BSA) solution in PBS at room temperature. Oocytes were then incubated with monoclonal rat antibody raised against mouse CD9 (Clone KMC8, PharMingen, 50 µg/ml in 1% BSA solution in PBS) for 1 h at room temperature or overnight at 4 °C, and washed three times (15 min per wash) in PBS with 1% BSA. Then eggs were exposed to secondary antibody (fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat IgG, Clone MRK-1, PharMingen, 1.25 µg/ml in 1% BSA solution in PBS) during a 1 h incubation at room temperature. Oocytes were washed three times in PBS with 1% BSA (15 min per wash) and incubated for 20 min with propidium iodide (50 µg/ml in PBS) to stain chromatin. Then the oocytes were washed for 20 min in 1% BSA solution in PBS and mounted on glass slides in a drop of antifading mounting medium (Citifluor AF1, Citifluor Ltd). To check the specificity of staining, control oocytes were exposed to the secondary

antibody without previous treatment with the primary antibody. Oocytes were examined with confocal laser microscopy (LSM 510 Zeiss).

## Results

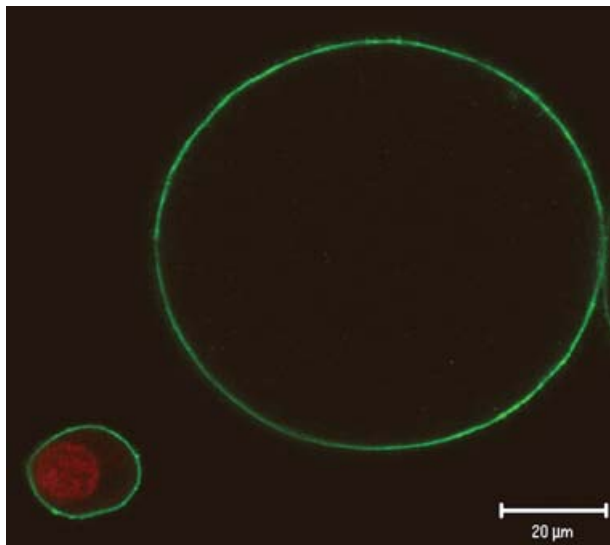
We isolated primary oocytes from female mice between 1 and 15 days of age to collect growing oocytes of different sizes. At birth the mouse ovary is populated with non-growing oocytes arrested in meiosis. It is known that during the first 2 weeks following birth more oocytes begin to grow (~5%) than at any other period in the lifetime of the mouse (Wassarman & Albertini, 1994). In our experiments we used growing oocytes which belonged to this first 'wave' of oocyte growth.

We found that in the ovaries of 1- to 3-day-old females only oocytes at the very early stages of growth are present (diameter between 10 and 24 µm). Larger oocytes (25–74 µm) appeared in the ovaries of females which were 6–9 days old, and the largest oocytes (75–80 µm) dominated among the growing oocytes isolated from ovaries of 15-day-old females. The relation between the age of females and the diameter of growing oocytes is shown in Table 1.

We next used immunocytochemistry to examine whether CD9 is present in growing oocytes. We observed expression of CD9 only in the oolemma and not in the cytoplasm of the eggs. Subsequently the amount of CD9 in the oolemma of growing oocytes, reflected by the intensity of immunostaining, was analysed. In each experiment ovulated eggs were collected and stained together with growing oocytes. The intensity of staining of oocytes isolated from the ovary was subjectively compared with the fluorescence of the antibody-stained, ovulated secondary oocytes (Fig. 1). We found that the intensity of staining increased during the earliest stages of the growth of oocytes, when they enlarged approximately from 10

**Table 1** The relationship between the age of newborn mice and the diameter of growing oocytes isolated from their ovaries

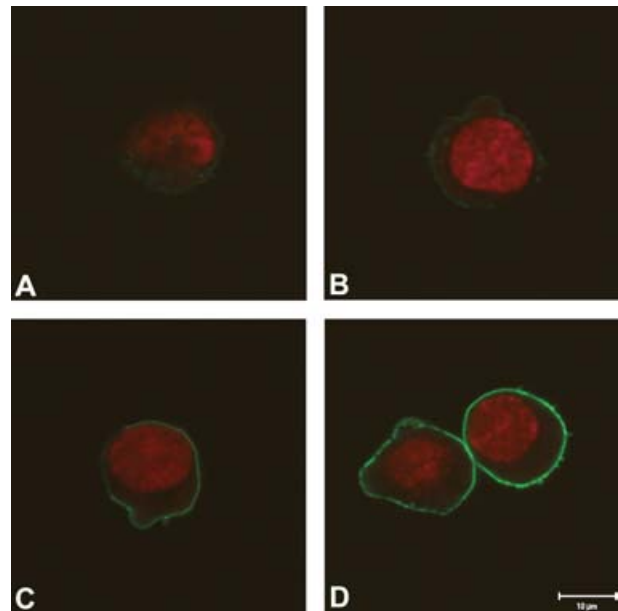
Age of mice (days)	No. of oocytes	Diameter of oocytes (µm)				
		No. (%) of oocytes of this size				
		10–24	25–39	40–54	55–74	75–80
1	62	62 (100)				
3	80	80 (100)				
6	124	93 (75)	4 (3)	10 (8)	17 (14)	
9	71			13 (9)	50 (70)	8 (11)
12	59				47 (80)	12 (20)
15	63				18 (29)	45 (71)



**Figure 1** Comparison of the intensity of staining of CD9 protein between a growing oocyte of diameter 17–22  $\mu\text{m}$  (left) and an ovulated oocyte (right). Both oocytes were stained during the same experiment. Pictures are single slices taken at the level of the centre of the oocytes. CD9 labelling is visualized in green, the nucleus of the growing oocyte is shown in red. The metaphase chromosomes of the ovulated oocyte are not visible because they were located at a different level. Scale bar represents 20  $\mu\text{m}$ .

to 20  $\mu\text{m}$  in diameter. All oocytes larger than 20–22  $\mu\text{m}$  were stained with anti-CD9 antibody with the same intensity as ovulated secondary oocytes (Fig. 1). Three classes of growing oocytes were distinguished according to the intensity of staining in relation to the diameter of the oocyte:

- 10–13  $\mu\text{m}$ :** The majority of oocytes of this size (90%, 28/31) were not stained for CD9, or stained very weakly (Fig. 2A). Only a few oocytes in this group (3/31) were stained to a similar degree as secondary oocytes.
- 13–17  $\mu\text{m}$ :** All oocytes of this size were stained for CD9 but in this group of oocytes the greatest variation of the intensity of staining was observed. Some oocytes (29.1%, 32/110) were stained for CD9 in their oolemma, but staining was much weaker than that observed for ovulated eggs, and CD9 was not uniformly distributed on the egg surface (Fig. 2B). However, 70.9% (78/110) of oocytes in this group were stained as intensively as the secondary oocytes (Fig. 2C).
- 17–22  $\mu\text{m}$ :** All oocytes of this group were stained for CD9 on the cell membrane. The majority of them (85%, 33/39) were stained with the same intensity as ovulated mature eggs (Fig. 2D).



**Figure 2** Detection of CD9 protein on the surface of growing oocytes. (A) An oocyte in the diameter range 10–13  $\mu\text{m}$ . (B), (C) Oocytes between 13 and 17  $\mu\text{m}$  in diameter. (D) Oocytes in the diameter range 17–22  $\mu\text{m}$ . Pictures are single slices taken at the level of the centre of the oocytes. CD9 labelling is visualized in green, the nuclei of growing oocytes are shown in red. Scale bar represents 10  $\mu\text{m}$ .

**Table 2** The relation between the diameter of growing oocytes and the presence of CD9 in the oolemma

Diameter of oocytes ( $\mu\text{m}$ )	No. of oocytes	No. of oocytes (%) containing CD9 in oolemma	No. of oocytes (%) stained for CD9 as intensively as the secondary oocytes
10–13	31	3 (10)	3 (10)
13–17	110	110 (100)	78 (71)
17–22	39	39 (100)	33 (85)

The relation between the diameter of growing oocytes and the presence of CD9 in the oolemma is shown in Table 2.

Control oocytes, for which the incubation with the primary anti-CD9 antibody was omitted from the staining procedure, did not stain with secondary antibody (not shown). These data show that CD9 appears on the oolemma during the early stages of growth of the oocyte.

## Discussion

In the present study we investigated the time during mouse oogenesis at which CD9 protein appears on the surface of the oocytes. We found that CD9 emerges

on the oolemma during the early stages of growth of the oocyte, when it measures 13–22  $\mu\text{m}$  in diameter. It was shown previously that at this period of growth of the mouse oocyte, the oolemma acquires the ability to fuse with spermatozoa (Zuccotti *et al.*, 1994). Thus we hypothesize that the development of the fusibility of the oocyte with spermatozoa may be related to the threshold amount of CD9 present in the oolemma. We observed that enrichment of the oolemma with CD9 is accomplished when the oocyte reaches a diameter of approximately 17–22  $\mu\text{m}$ . At this stage of growth the density of CD9 in the oolemma, evaluated by the examination of the intensity of staining in indirect immunofluorescence, is similar to the density of this protein in the cell membrane of the fully grown secondary oocyte. During subsequent growth of the oocyte CD9 has to be continuously synthesized and incorporated into the expanding oolemma.

The presence of CD9 in immature oocytes ranging from 30 to 100  $\mu\text{m}$  in diameter has been demonstrated previously by Chen *et al.* (1999), but these authors did not study the time during oogenesis at which CD9 begins to appear on the oolemma. In the present study we show that CD9 starts to accumulate in the oolemma soon after the beginning of growth of the oocyte, and soon reaches a density sufficient to enable the oocyte to fuse with spermatozoa (Zuccotti *et al.*, 1994). However, the presence of CD9 on the egg membrane apparently does not itself suffice to achieve its fusibility with sperm. It was demonstrated for the mouse (Zuccotti *et al.*, 1994), as well as for the hamster (Zuccotti *et al.*, 1991), that the fusibility of growing oocytes with spermatozoa increases with increasing oocyte diameter, even though, according to our study, the concentration of CD9 seems to be the same in all oocytes larger than approximately 20  $\mu\text{m}$ . Since fusibility is measured by the number of spermatozoa fusing with a single oocyte, it could be expected that fusibility increases as the oocyte grows since the surface area of the oocyte increases with growth. However, it cannot be excluded that synthesis and incorporation, or modification, of elements other than CD9 in the oolemma or cortex of the oocyte is required for the development of oolemma's ability to fuse with spermatozoa. For instance, integrins of the oolemma (Chen *et al.*, 1999) or cortical actin filaments (McAvey *et al.*, 2002) could also be involved in the fusibility of oolemma with spermatozoa.

The tetraspanin CD9 is the only protein of the mouse oolemma currently known to function in the fusion of gametes (Chen *et al.*, 1999; Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miyado *et al.*, 2000). It is known that tetraspanins mediate protein interactions in the cell membrane to create unique cell-surface domains (Hemler, 2003; Stipp *et al.*, 2003). In our previous paper (Komorowski *et al.*, 2003) we demonstrated that the

concentration of CD9 in the oolemma of mouse zygotes which cannot be penetrated by additional spermatozoa due to the oolemma block to polyspermy is similar to the concentration of CD9 in the ovulated oocytes which are able to fuse with spermatozoa. When CD9 is removed from the oocytes by proteolytic enzymes, they lose fusibility with sperm (Komorowski *et al.*, 2003). We observed that after 17 h of culture *in vitro* CD9 is restored in the oolemma, but this does not restore the ability of pronase-treated oocytes to fuse with sperm (Bialecka, Komorowski and Maleszewski, unpublished data). Thus the presence of CD9 in the oolemma is not sufficient to make the oocyte able to fuse with spermatozoa.

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