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Reduced fertility of female mice lacking CD81

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Abstract

In somatic cells, the tetraspanins CD81 and CD9 associate with each other, with additional tetraspanins and with non-tetraspanin molecules to form proteolipidic complexes. Here we show that CD81 is expressed on the surface of oocytes where it associates with tetraspanin-enriched membrane structures. A major CD9 and CD81 partner, CD9P-1, is also expressed by oocytes. Deletion of CD81 gene in mice results in a 40% reduction of female fertility. In vitro insemination indicated that this infertility is due to a deficiency of oocytes to fuse with sperm. While the fertility of CD9$^{-/-}$ mice is severely but not completely impaired, double knock-out CD9$^{-/-}$ CD81$^{-/-}$ mice were completely infertile indicating that CD9 and CD81 play complementary roles in sperm–egg fusion. Finally, a fraction of CD9 was transferred from CD81$^{-/-}$ oocytes to sperm present in the perivitelline space indicating that the defect of fusion of CD81$^{-/-}$ oocytes does not result from an impaired initial gamete interaction.
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Introduction

The molecular basis of mammalian sperm–egg fusion is still poorly understood. Several egg surface proteins have been implicated in this process but few have been confirmed by gene-targeting experiments (Evans, 2002; Stein et al., 2004). One group of egg surface proteins implicated is the GPI-anchored proteins. Indeed, treatment of mouse eggs with PI-PLC caused a strong reduction of sperm–egg fusion (Coonrod et al., 1999). Moreover, mice with oocyte-specific KO of GPI-anchored proteins were recently demonstrated to be infertile, and oocytes collected in these mice were severely deficient in their ability to fuse with sperm in vitro (Alfieri et al., 2003).

The only surface egg protein that was shown to be essential for fusion by gene KO is CD9. Indeed, CD9$^{-/-}$ females have a severe reduction of fertility, and CD9$^{-/-}$ oocytes failed to fuse in vitro (Le Naour et al., 2000; Miyado et al., 2000; Kaji et al., 2000). Additionally, several CD9 mAbs were demonstrated to inhibit the fusion process in vitro (Chen et al., 1999; Le Naour et al., 2000). CD9 is a member of the tetraspanin superfamily that comprises 32 members in human. Tetraspanins have 2 short N and C intracellular terminal tails and 4 transmembrane domains separating 2 extracellular domains of unequal size (Boucheix and Rubinstein, 2001; Hemler, 2003; Levy and Shoham, 2005). An isolated form of the largest of these extracellular domains was able to partially inhibit fusion when preincubated with eggs, not with sperm, indicating that somehow CD9 may function in cis on the oocyte surface, possibly by interacting with another surface protein (Zhu et al., 2002).

Tetraspanins have been implicated in various biological processes such as cell adhesion, migration, co-stimulation, signal transduction, and differentiation (reviewed in Boucheix and Rubinstein, 2001; Hemler, 2003; Levy and Shoham, 2005). A major characteristic of tetraspanins is their ability to interact with each other and with a large number of other surface proteins, thus building an extensive network of molecular interactions, the tetraspanin web. In a current model, each tetraspanin interacts directly with a limited

Abbreviations: ZP, zona pellucida; LED, large extracellular domain; KO, knock-out.

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number of molecular partners (a tetraspanin's molecular partner is a protein that interact with this tetraspanin under conditions in which no tetraspanin to tetraspanin interaction is observed), that they connect to other tetraspanins through tetraspanin/tetraspanin interactions. This second level of interaction is thought to occur in discrete microdomains different from the GPI-anchored proteins-enriched microdomains or rafts (Boucheix and Rubinstein, 2001; Hemler, 2003; Levy and Shoham, 2005). Two major molecular partners have been identified for CD9, CD9P-1 and EWI-2 (Charrin et al., 2001, 2003; Stipp et al., 2001a,b; Clark et al., 2001). They belong to a sub-family of proteins with Ig domains, and it is not known whether they are expressed by the oocyte. Among the molecules associated with tetraspanins, a subset of integrins are molecular partner of the tetraspanin CD151 (Yauch et al., 1998; Serru et al., 1999; Fitter et al., 1999). Integrins that are expressed on the oocyte surface include α6β1, which was shown to associate with CD9 in the egg (Miyado et al., 2000). The integrin α6β1 has long been considered to play a major role in sperm egg fusion by its ability to interact with proteins of the ADAM family expressed on the sperm, but this model was called into question after it was demonstrated that oocytes lacking either this integrin or possibly all β1 integrins could fuse with sperm (Miller et al., 2000; He et al., 2003).

CD9 is not the only tetraspanin expressed by oocytes. Human oocytes additionally express CD81 and CD151 (Ziyyat et al., in press), while the expression of CD81 on mouse eggs has been demonstrated (Takahashi et al., 2001). mAb to CD81 were shown to partially inhibit binding and fusion of sperm to the egg (Takahashi et al., 2001). Moreover, overexpression of CD81, following injection of mRNA in the eggs, could restore partially the ability of $CD9^{-/-}$ eggs to fuse with sperm (Kaji et al., 2002). It is however not known whether endogenous CD81 plays a role in sperm–egg fusion.

The aim of this work was to evaluate the extent of fertility impairment linked to the absence of CD81 and its relation with CD9 infertility. For that purpose, we compared $CD81^{-/-}$ mice with $CD9^{-/-}$ mice and double knockout mice.

Materials and methods

Animals

C57BL/6 $CD81^{-/-}$ (Maecker and Levy, 1997) and $CD9^{-/-}$ (Le Naour et al., 2000) mice were bred at the animal facility Lavoisier of Institute André Lwoff (Villejuif, France). They were bred as heterozygotes, giving rise to knockout and WT littermates. The animals were genotyped as previously described (Maecker and Levy, 1997; Le Naour et al., 2000). Double knockout $CD9^{-/-}$ $CD81^{-/-}$ mice were obtained by successive crossings of the 2 strains. Six- to 20-week-old mice (age and sex matched) were used in all experiments. All animal protocols were approved by the local Committee on Animal Welfare.

Preparation of oocytes

For natural ovulation studies, mice were mated at 8 p.m. and examined the next morning for the presence of a plug. Females with plugs were sacrificed by cervical dislocation. For in vitro fertilization experiments, they were superovulated by intraperitoneal injections of 5 IU PMSG (Folligon, Intervet, France) and 5 IU hCG (Chorulon, Intervet, France) 48 h apart. Fifteen to 16 h after hCG injection, the animals were sacrificed. Cumulus–oocyte complexes were collected by tearing the oviductal ampulla. Cumulus oophorus cells were removed by hyaluronidase (type IVs, 300 μg/ml, Sigma, St. Louis, MO) treatment and oocytes were examined by phase contrast microscopy. For in vitro fusion experiments with ZP-free oocytes, oocytes were transferred to a HEPES-buffered culture CZB medium (Chatot et al., 1989). The zona was removed by exposing the embryos to acid Tyrode's solution (pH 2.1–2.5) for a few seconds followed by immediate repeated rinsing in CZB medium.

Sperm preparation

Spermatozoa were collected after squeezing two caudae epididymis in wells with 500 μl Whittingham's medium supplemented with 30 mg/ml BSA under mineral oil (Whittingham, 1971). Spermatozoa were incubated at 37°C in 5% CO_2 for 20 min. The sperm at the periphery of the wells were carefully transferred to another well with the same medium. The plate was placed again in the CO_2 incubator and the sperm are counted in a Malassez count slide.

Oocyte DNA-tubulin labeling for meiosis stage assessment

Oocytes were fixed (Kubiak et al., 1992) and incubated with the anti-tubulin mAb YL1/2 (Serotec, Oxford, UK), followed by fluorescein isothiocyanate FITC-labeled goat F(ab)2 antibody to mouse and 1 μg/ml propidium iodide. The oocytes were then observed under a fluorescence microscope. Oocytes blocked in metaphase of meiosis II were recognized by the presence of a metaphase spindle and a first polar body containing residual DNA-tubulin material. Progression to telophase and extrusion of a second polar body could be observed following resumption of meiosis (Le Naour et al., 2000).

Sperm–egg fusion assay and in vitro fertilization

ZP free mature eggs were incubated for 10 min with 1 μg/ml Hoechst 33342 in Whittingham medium supplemented with 30 mg/ml BSA (Sigma, St. Louis, MO) followed by 5 washings of 5 min. in M16 medium. Oocytes were then added to wells containing 10^5 sperm in 500 μl Whittingham-BSA medium and incubated at 37°C 5% CO_2 for 90 min. We considered as fused the spermatozoa with Hoechst-stained nuclei (Conover and Gwatkin, 1988). Usually, the sperm DNA was already decondensed and oocytes had resumed meiosis. Alternatively inseminated ZP free eggs were kept at 37°C and 5% CO2 for 24 h and the number of two-cell embryos were determined.

mAbs and fluorescence staining of eggs

Oocytes were fixed by transfer into a drop of paraformaldehyde at 4% in PBS–BSA 1% for 30 min at room temperature. After washing, they were stained with 20 μg/ml rat primary mAb for 1 h and with Alexa 488-labeled goat anti-rat IgG (10 μg/ml) antibody for 1 h. The eggs were examined with a Leica DMR fluorescence microscope equipped with a camera. For confocal microscopy, the eggs were stained with an Alexa 488-coupled CD9 mAb. The eggs were deposited in a small drop of medium in a labteck coverslip and covered under mineral oil. Confocal analysis of CD9 expression was performed with a TCS SP2 confocal microscope (Leica, Wetzlar, Germany), using a 63× objective. About 100 sections were collected and superimposed using the maximum projection function of the LCS software (Leica). The mAbs used in this study were 4.1F12 (CD9, (Le Naour et al., 2000)), MT81 and MT81w (CD81, submitted for publication), EAT-1 (CD81, (Maecker et al., 2000)) and 8G1 (CD9P-1, submitted for publication).

Results

Reduced fertility of $CD81^{-/-}$ females

The fertility of $CD81^{-/-}$ mice was reduced. Reduced fertility was not apparent in these mice when originally derived

(Maecker and Levy, 1997), however, this impairment was noticed after the 4–5th backcrossing both onto the C57BL/6 and the BALB/c backgrounds (Deng and Levy, unpublished observations). This deficiency was not due to the male as normal fertility was observed when CD81$^{-/-}$ males were mated with wild-type or heterozygous females. We have previously shown that CD9$^{-/-}$ male had a normal fertility. CD9$^{-/-}$ CD81$^{-/-}$ males were also normally fertile (data not shown).

Only ~60% CD81$^{-/-}$ females produced litters after being maintained in the presence of fertile wild-type males for up to 2 months. This rate was similar to that of CD9$^{-/-}$ animals. However, the litter size was normal for CD81$^{-/-}$ mice, while it was reduced to 1.5 ± 0.8 for CD9$^{-/-}$ females. In addition, the delay between mating and the beginning of a successful pregnancy was also normal for CD81$^{-/-}$ mice while it was increased from 5.9 ± 3.4 days to 19 ± 17 days for CD9$^{-/-}$ mice. The important role of CD81 in female fertility was especially apparent using CD9$^{-/-}$ CD81$^{-/-}$ females for which no visible pregnancy or birth was observed (Table 1). In contrast, the fertility of CD9$^{+/-}$ or CD81$^{+/-}$ females and doubly heterozygous CD9$^{+/-}$ CD81$^{+/-}$ females was normal.

The initial postnatal mortality rate was high for pups produced by CD81$^{-/-}$ females (31%), as compared to less than 2% for wild type females. A decreased viability of CD81$^{-/-}$ animals beyond the first hours of life has been reported previously (Kelic et al., 2001).

Normal mating and ovulation but defective in vivo fertilization of CD81$^{-/-}$, CD9$^{-/-}$ and double KO CD9$^{-/-}$ CD81$^{-/-}$ females

No difference in the frequency of vaginal plugs was observed between the different groups of animals indicating that CD81$^{-/-}$ and double KO females had a normal mating behavior (data not shown), similar to what was described for CD9$^{-/-}$ females (Le Naour et al., 2000). The infertility was also not due to the absence of sperm at the site of fertilization, as shown by the presence of numerous sperm in the oviduct. Also, there was no abnormality of the ovaries as determined by histological examination of 6-week-old animals (data not shown). Natural ovulation of CD81$^{-/-}$ and double KO CD81$^{-/-}$ CD9$^{-/-}$ females was normal as the number of oocytes ovulated by each group of animals was similar (Table 2) and all were apparently normal. However precise assessment of the meiotic stage by tubulin/propidium iodide labeling of oocytes collected at day 0.5 after mating showed resumption of meiosis in 78% of oocytes collected in wild type females whereas it was reduced to 10% in CD81$^{-/-}$ females and null in CD9$^{-/-}$ or double knockout mice. We never observed a sperm nucleus in the cytoplasm of CD81$^{-/-}$ (or CD9$^{-/-}$) eggs that had not resumed meiosis, strongly suggesting that fusion had not occurred. Like the oocytes recovered from mated CD9$^{-/-}$ females at day 0.5, those recovered from CD81$^{-/-}$ and double KO mice had frequently several highly mobile sperm in the perivitelline space suggesting that there was no preclusion of multiple sperm from traversing the zona and entering the perivitelline space. This block occurs if a sperm fuses with the egg (Table 2).

Table 1
Effect of CD9 and CD81 deficiency on female fertility

Females	Number of matings	Females having offspring (%)	Delay before pregnancy (days)[a]	Mean litter size[a]	Dead offspring (% at 48 h)
WT	10	100	5.9 ± 3.4	7 ± 2.6	0
CD9$^{-/-}$	10	60	19 ± 17	1.5 ± 0.8	55
CD81$^{-/-}$	16	62	4 ± 6.5	6 ± 1.4	31
CD9$^{-/-}$ CD81$^{-/-}$	6	0	n.a.	n.a.	n.a.
CD9$^{+/-}$ CD81$^{+/-}$	11	100	3.9 ± 4.8	8.7 ± 2.6	0

Mice were continuously mated until pregnancy or for a period of at least 60 days.
[a] Values are the mean ± SEM; n.a. not applicable.

Table 2
Effect of CD9 and CD81 deficiency on oocyte fertilization in vivo

Female genotype	No. of females with an ampulla	No. of ovulated oocytes per mouse[a]	Meiotic stage	
			% oocytes with meisosis II resumption[b]	% oocytes blocked at metaphase II
CD9$^{+/+}$ CD81$^{+/+}$	5	10.2 ± 1.3	78 (51)	22
CD9$^{+/+}$ CD81$^{-/-}$	7	8.4 ± 1.8	10 (40)	90
CD9$^{-/-}$ CD81$^{+/+}$	4	8 ± 1.4	0 (32)	100
CD9$^{-/-}$ CD81$^{-/-}$	4	7.8 ± 2.2	0 (23)	100

Eggs were collected the day after mating and stained with propidium iodide and a mAb to tubulin to determine the meiotic stage.
[a] Values are the mean ± SEM.
[b] The number in parenthesis indicates the number of oocytes examined.

Altered fusion of CD81$^{-/-}$ eggs with WT sperm

The presence of several sperm in the perivitelline space and the lack of meiosis completion suggested that CD81$^{-/-}$ and double KO eggs were defective in their ability to fuse with sperm. To check this hypothesis, and eliminate a defect in events subsequent to fusion, in vitro fertilization experiments using the Hoechst dye transfer test were performed (Conover and Gwatkin, 1988). In these experiments, ZP-free oocytes are incubated with Hoechst 33342 before washing. The dye is retained in the cytoplasm, and labels the sperm DNA only after fusion. This test also allows the observation of sperm DNA decondensation and resumption of meiosis. Fusion was observed in more than 90% of wild type oocytes after 90 min insemination. In contrast, no fusion was observed in CD9$^{-/-}$ oocytes, and only 3% of CD81$^{-/-}$ oocytes fused with a capacitated sperm. This shows that CD81, like CD9, is involved in sperm–egg fusion. To examine whether fusion could occur with a delay, we counted the number of two-cell embryos 24 h after fertilization. While 85% of WT eggs were

fertilized using this criterion, only one out of 89 inseminated $CD9^{-/-}$ eggs produced a two-cell embryo. Five out of 44 $CD81^{-/-}$ eggs tested (11%, 4 females) produced two-cell embryos (Fig. 1B).

CD81 mAb do not prevent sperm–egg fusion

To our knowledge, all CD9 mAb tested so far strongly inhibit fusion (KMC8, JF9, 4.1F12) (Le Naour et al., 2000; Chen et al., 1999; Miller et al., 2000). In a previous report, the CD81 mAb 2F7 was found to block fusion by only 17%, while the CD81 mAb Eat-1 blocked fusion and sperm–egg binding by 40% (Takahashi et al., 2001). We tested whether other CD81 mAb could block the fusion process. In one set of experiments, the effect of a hamster mAb Eat-1 and of a CD9 mAb 4.1F12 were tested on the fusion of ZP-free eggs with sperm. As shown in Fig. 2, Eat-1 mAb (100 μg/ml) did not have any effect on fusion, while the CD9 mAb 4.1F12 inhibited fusion by 80%. In another set of experiments, a newly generated CD81 mAb, MT81 (200 μg/ml), also failed to inhibit the fusion of zona–intact oocytes with sperm (data not shown). The two CD81 mAb used in this study are functional since they inhibit the invasion of hepatocytes by the rodent malaria parasite *Plasmodium yoelii* (Silvie et al., 2003) (data not shown). Thus, in contrast to CD9, most CD81 mAb have a poor or no effect on sperm–egg fusion. Because CD9 and CD81 are closely related, and CD9 and CD81 mAb often produce similar effects (Boucheix and Rubinstein, 2001), this may reveal differences in the mechanism by which CD9 and CD81 participate in sperm–egg fusion.

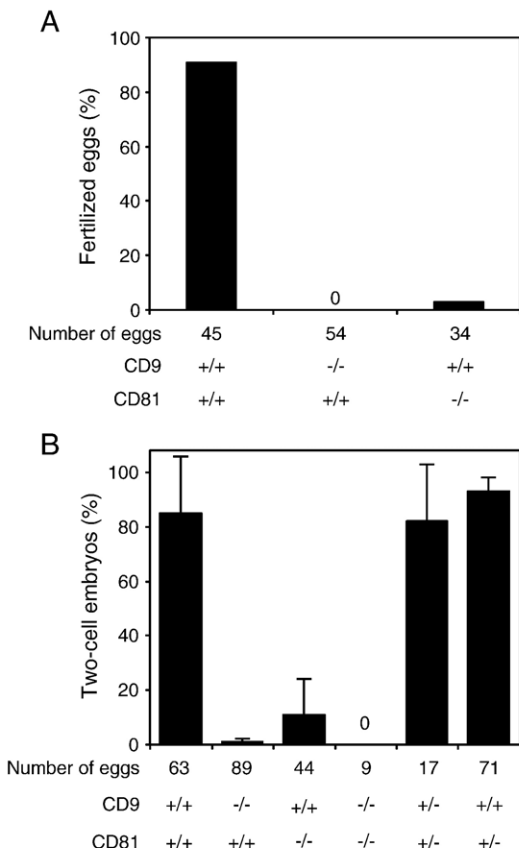

Fig. 1. In vitro fertilization assay. (A) After ZP removal, oocytes collected in mice lacking CD9 or CD81 were loaded with Hoechst 33342 and incubated with capacitated sperm for 90 min. Fertilized eggs were recognized by the presence of Hoechst-stained sperm nuclei. (B) The oocytes with the indicated genotype were incubated with sperm with 24 h, and the number of two-cell embryos was determined.

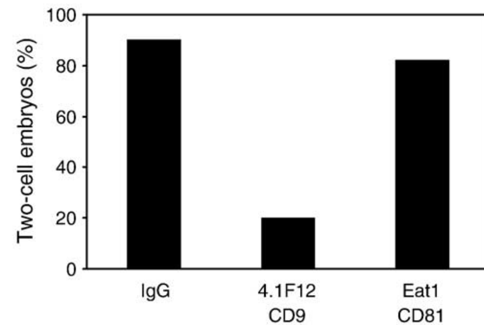

Fig. 2. Effect of CD9 and CD81 mAbs on sperm–egg fusion. After ZP removal, WT oocytes were incubated with capacitated sperm for 24 h, in the presence of CD9, CD81 or control mAb. The number of two-cell embryos was determined.

Expression of CD9, CD81 and CD9P-1 on mouse oocytes

To our knowledge, all anti mouse CD81 mAbs have been raised in hamster, while all anti-mouse CD9 mAb have been raised in rat. We have generated 2 rat anti-mouse CD81 mAb (submitted for publication). MT81 is directed against the bulk of CD81 molecules, and stains certain cell lines with the same intensity as the CD9 mAb, in contrast to previously described anti-CD81 hamster mAb such as Eat-1 and Eat-2 (data not shown). It is therefore a better tool to compare the expression levels of CD9 and CD81 by indirect immunofluorescence. The staining by the anti-CD9 mAb on the egg surface was much brighter than the staining by the anti-CD81 mAb, subsequently, detection of CD81 required fourfold exposure time by comparison to CD9 (Fig. 3). It became recently evident that certain anti-tetraspanin mAb were directed against sub-populations of the target tetraspanin (Serru et al., 1999; Gutierrez-Lopez et al., 2003). The other CD81 mAb, MT81w, recognizes CD81 only in association with other tetraspanins (submitted for publication) and therefore detection by this mAb indicates the localization of CD81 in tetraspanin-enriched microdomains. As shown in Fig. 3, the staining of eggs by the 2 CD81 mAb were very similar indicating that in the egg, the bulk of CD81 is in tetraspanin-enriched microdomains.

Two major molecular partners of CD9 and CD81 have recently been identified, CD9P-1 and EWI-2. As seen in Fig. 3, the anti-CD9P-1 mAb 8G1 stained the oocyte, indicating expression of CD9P-1. CD9P-1 expression was also observed on oocytes in the ovary (data not shown).

Sperm failing to fuse with zona-intact $CD81^{-/-}$ eggs acquire CD9

We have recently demonstrated that CD9 was subjected to a major redistribution in the course of fertilization (Ziyyat et al.,

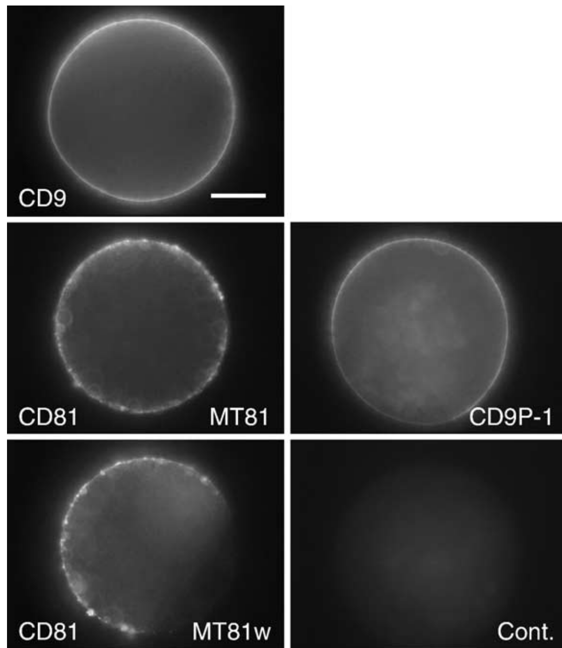

Fig. 3. Expression of CD9, CD81 and CD9P-1 on oocytes. After ZP removal, WT oocytes were stained with mAbs to CD9 (4.1F12), CD81 (MT81 and MT81w) and CD9P-1 (8G1). They were examined by fluorescence microscopy and images were taken using a high-resolution camera. The exposition times for CD9, CD81 and CD9P-1 labeling were 1/4 s, 1 s and 2 s, respectively. The control is shown after a 2-s exposure. Scale bar, 20 μm.

in press). We thus examined the localization of CD9 in non-inseminated and inseminated CD81$^{-/-}$ zona-intact oocytes. After 3 h, the eggs were fixed, labelled with the CD9 mAb, and analyzed by confocal microscopy. As seen in Fig. 4A, the distribution of CD9 in CD81$^{-/-}$ oocytes was finely punctuate in the microvillar region, probably reflecting a concentration on microvilli (Kaji et al., 2000). This is very similar to the distribution of CD9 in WT oocytes (Ziyyat et al., in press; Kaji et al., 2000). The distribution of CD9 in inseminated CD81$^{-/-}$ oocytes was still finely punctate but more heterogeneous (Fig. 4C). It is different from the distribution observed in inseminated WT eggs where CD9 formed large patches upon fertilization (Ziyyat et al., in press). As stated earlier, most CD81$^{-/-}$ were not fertilized and several sperm were frequently observed in the perivitelline space. Surprisingly, the sperm heads were strongly labeled by the CD9 mAb (Fig. 4C). The intensity of staining exceeds that of the egg membrane. An analysis of sperm in more equatorial sections (Figs. 4E–G), indicated that the labeling was indeed on the sperm head. No significant labeling could be observed on WT sperm present in the perivitelline space of CD9$^{-/-}$ eggs (Fig. 4H), indicating that 1, the labeling is specific and 2, this CD9 fraction present on sperm was transferred from the egg.

Discussion

CD9 is the only oocyte surface molecule whose role in sperm–egg fusion is clearly established. In this study we investigated the role of another tetraspanin present on eggs, CD81. We have demonstrated that the lack of CD81 produces a defect in fertility of the mouse female. This defect is actually linked to the absence of CD81 as transgenic expression of human CD81 in CD81$^{-/-}$ mice (Masciopinto et al., 2002) restored the fertility (S. Levy, unpublished data). Like CD9, CD81 plays a role in sperm–egg fusion as demonstrated by in vitro insemination. The defect of CD81$^{-/-}$ mice only became evident when mice were backcrossed onto homogenous background. Moreover, it was milder than that of CD9$^{-/-}$ mice as the overall reduction in fertility of CD81$^{-/-}$ mice was 40%. In CD9$^{-/-}$ mice, considering that a mean of 5 ovulatory cycles was necessary to produce 1.5 pups in 60% of the animals, the reduction of fertility is above 95%. In addition, a residual fertilization could be observed in vitro using oocytes from CD81$^{-/-}$ females whereas no fusion was observed with oocytes from CD9$^{-/-}$ females. Moreover, mice lacking both tetraspanins were completely infertile indicating that both tetraspanins act in concert for an effective fertilization.

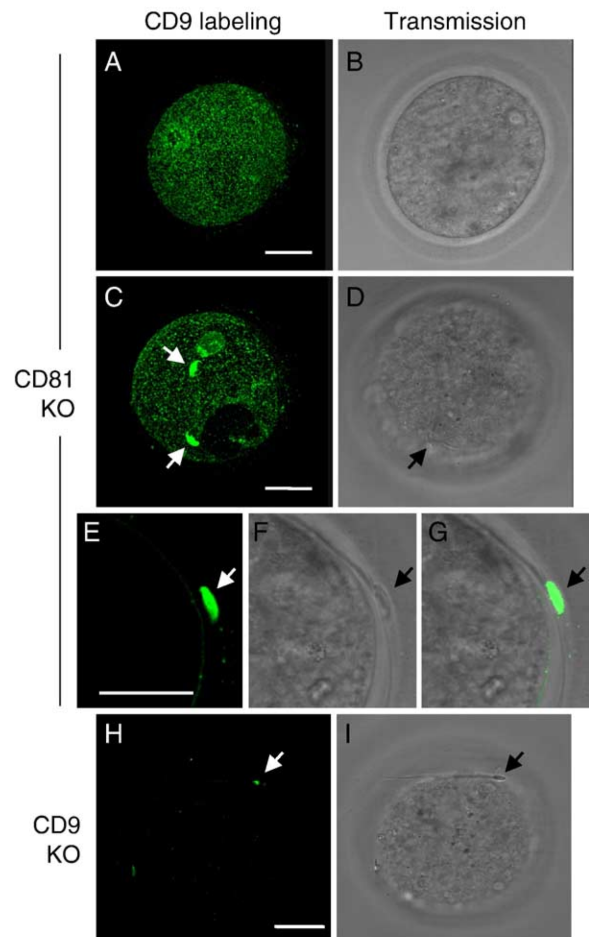

Fig. 4. Transfer of CD9 from CD81$^{-/-}$ oocytes to sperm head. CD81$^{-/-}$ or CD9$^{-/-}$ mouse intact oocytes were inseminated with wild-type sperms. After 3-h incubation, the eggs were fixed, labeled with the CD9 mAb 4.1F12 and analyzed by confocal microscopy as described in Materials and methods. Transmission images are also shown. (A, B): a non-inseminated CD81$^{-/-}$ egg; (C, D): an inseminated CD81$^{-/-}$ egg; (E–G): a single section showing that a sperm in the perivitelline space is labeled with the CD9 mAb. G is a combination of panels E and F. (H, I): an inseminated CD9$^{-/-}$ egg. Arrows indicate sperm heads. They are not all visible on the transmission images. Scale bar, 40 μm.

Among tetraspanins, CD9 and CD81 are closely related, having 45% identity to each other. The level of identity in the tetraspanin superfamily ranges from 7 to 57% identity (Boucheix and Rubinstein, 2001). Second, their association in tetraspanin-enriched microdomains is more stable than the association with any other tetraspanin studied (Charrin et al., 2002). Third, they share the ability to interact directly ("primary interactions") with at least two tetraspanin partners, CD9P-1 and EWI-2. These data raise the hypothesis that the roles of CD9 and CD81 might be completely redundant for certain functions. If this hypothesis was true for sperm–egg fusion, the ability of oocytes to fuse would be directly linked to the total level of expression of CD9 and CD81. Because oocytes express less CD81 than CD9, as determined by indirect immunofluorescence, the deletion of one allele of CD9 has a greater impact on the total level of CD9 plus CD81 than the deletion of one CD81 allele, and thus should have a greater impact on fertility. The data presented here show that this is not the case. Indeed, $CD9^{+/-}$ $CD81^{+/-}$ mice, that have a normal fertility (and oocytes collected in these mice have a normal ability to fuse in vitro), express a smaller quantity of CD9 plus CD81 than $CD9^{+/+}$, $CD81^{-/-}$ mice, that have a reduced fertility. A previous study has also shown that, following microinjection of mRNA, CD81 was less efficient in restoring the fusion ability of $CD9^{-/-}$ eggs (Kaji et al., 2002). This result indicates that CD81 cannot completely substitute for CD9, even, after overexpression. Altogether, these data suggest that CD9 and CD81 play complementary roles in sperm–egg fusion and cannot substitute for each other in this process.

The moderate effect of CD81 deletion on overall fertility contrasts with the strongly reduced number of fertilized eggs collected at day 0.5. In many of the eggs collected from mated $CD81^{-/-}$ mice, several sperm were present in the perivitelline space, but only 10% of eggs had resumed meiosis II. The simplest explanation for this discrepancy is that the delay necessary for fusion after ZP crossing by sperm is increased in $CD81^{-/-}$ oocytes. This may explain why the defect of $CD81^{-/-}$ oocytes is amplified in vitro, with only 3% of the eggs being fertilized after 90 min. Only a moderate increase of fertilization rate that reached 10% was observed when eggs were examined after 24 h but it is well known that the gametes loose their ability to fuse over time and this may be amplified in vitro. The average delay between ZP crossing and fusion in $CD81^{-/-}$ eggs may be prolonged beyond the period in which gametes remain competent for fusion.

At least 2 interpretations of the role of CD81 in gamete fusion may be proposed. First, it may contribute to the formation of a tetraspanin web on oocytes. In this regard, staining of oocytes with mAb MT81w which recognizes CD81 only in the context of tetraspanin-enriched microdomains (submitted for publication) indicated that the bulk of CD81 molecules were localized in these domains. If this hypothesis was true, altered expression of other tetraspanins expressed by oocytes should produce similar defects in fusion. PCR-SAGE analysis and immunostaining have demonstrated that CD151 is expressed by human oocytes (Neilson et al., 2000) (and data not shown). However, CD151 KO females have no fertility defect (Wright et al., 2004). Also, as stated earlier, the fact that $CD9^{+/-}$ $CD81^{+/-}$ mice have a normal fertility does not support the hypothesis that CD81 deficiency causes a global disorganization of the tetraspanin web. Alternatively, CD81 may associate with a partner molecule that plays an important role in the fusion process, and functionally regulate this partner, possibly in the context of the tetraspanin web. A similar hypothesis has been raised for CD9 (Le Naour et al., 2000; Zhu et al., 2002). Direct interactions of tetraspanins with partner molecules have so far been shown to involve the LED of tetraspanins (Stipp et al., 2003; Charrin et al., 2003). Consistent with a role of CD9 LED, a CD9 mutant with 3 adjacent mutations in the most variable region of the LED was not competent for fusion, and isolated CD9 LED could inhibit fusion when preincubated with oocytes, suggesting a role in cis for CD9 (Zhu et al., 2002). Recently, it was demonstrated that isolated CD81 LED could also inhibit the fusion of gametes, indicating a role for CD81 LED and possibly CD81-associated molecules (Higginbottom et al., 2003). CD9 and CD81 have two common partners, CD9P-1 and EWI-2, which belong to a new subfamily of proteins with Ig domains (Charrin et al., 2001, 2003; Stipp et al., 2001a,b; Clark et al., 2001). We have demonstrated for the first time that CD9P-1 is expressed both on freshly ovulated oocytes, and in the ovary. These data contrast with a previous study that failed to demonstrate the expression of CD9P-1/FPRP on the oocytes of rat ovaries (Orlicky et al., 1992). This discrepancy may be due to the use of a polyclonal antibody in this other study. It will be of special interest to determine whether CD9P-1 plays a role in sperm–egg fusion. In preliminary experiments, the mAb 8G1 directed against this molecule failed to inhibit fusion.

How CD9 and CD81 participate to sperm–egg fusion is unknown. The membrane fusion process can be divided into 2 successive events. The first step consists in an attachment of the two membranes and is achieved through protein–protein interaction. This step is followed by membrane apposition and then lipid mixing (Jahn et al., 2003; Chen and Olson, 2005; Stein et al., 2004). Sperm cells bind equally well to WT and $CD9^{-/-}$ oocytes after ZP removal (Le Naour et al., 2000; Miyado et al., 2000; Kaji et al., 2000). However, the binding of sperm to ZP-free oocytes may not be physiological for at least two reasons. First, most sperm bound to ZP-free oocytes cannot progress to fusion (Talbot et al., 2003). Second, the sperm accumulating into the perivitelline space of $CD9^{-/-}$ or $CD81^{-/-}$ oocytes do not stably bind to the oocyte plasma membrane. Instead, they move actively and attach transiently to the oocyte surface, sometimes triggering a rolling of the oocyte on itself (data not shown). Thus these data did not discriminate between a role of CD9 or CD81 in attachment or in fusion. In this study we have shown that a fraction of CD9 is transferred from $CD81^{-/-}$ oocyte to the sperm heads. Two hypotheses can be raised to explain this transfer. First, fusion between egg and sperm membranes may have been initiated but aborted before the nucleus was released into the egg cytoplasm, yielding some membrane exchange. Alternatively, the sperm may interact firmly with the egg in a way that does not lead to fusion. As stated in the results section,

the sperm is highly motile in the perivitelline space of CD81$^{-/-}$ (and CD9$^{-/-}$) eggs, making frequent contacts with the oocyte and sometimes triggering a rolling of the oocyte on itself. The sperm movements may therefore be so strong that the sperm detach from the egg pulling out plasma membrane fragments. In this hypothesis, the concentration of CD9 on sperm would increase with the number of contacts made, which is consistent with the sperm head labeling being much higher than the egg surface labeling. This hypothesis is also consistent with the altered CD9 labeling of inseminated CD81$^{-/-}$ eggs. Transfer of membrane fragments between two cells has already been described especially between antigen-presenting cells and lymphoid T cells (Hudrisier and Bongrand, 2002). In any case, our data strongly suggest that sperm binds to CD81$^{-/-}$ oocytes, in a way that does not lead to effective fusion.

Cell–cell fusion occurs in a variety of physiological and pathological situations, and CD9 and CD81 may play a general role in this phenomenon. This role was initially put in evidence by the demonstration that CD81 and CD82 mAb reduced the formation of syncytium by the retrovirus HTLV-1 (Imai et al., 1992). Then, CD9 mAb were demonstrated to inhibit the syncytium formation by canine distemper virus, a virus related to measles virus (Schmid et al., 2000). Both CD9 and CD81 mAb were shown to inhibit the fusion of myoblasts, and overexpression of CD9 in myoblast-derived sarcoma cells increases syncytium formation (Tachibana and Hemler, 1999). It was also recently reported that CD9 and CD81 could function as inhibitors of the fusion of mononuclear phagocytes in several infectious pathologies (Takeda et al., 2003). Interestingly, while enhanced cell fusion was observed after in vitro or vivo stimulation in the absence of CD9 or CD81, spontaneous multinucleated giant cell formation was detected in the lung only when these two tetraspanins were lacking. The mechanism by which tetraspanins interfere with fusion events is still poorly understood. The recent identification of Izumo, an Ig-domain molecule present on sperm that is essential for sperm egg fusion (Inoue et al., 2005) will help to better characterize this process. It will be of special interest to determine whether this molecule can interact with tetraspanins or tetraspanin-associated molecules.

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