[L. Hewitson *et al.*, *Hum. Reprod.* **13**, 2786 (1998)], zonae removed with pronase, and the zona-free embryos recovered individually for 20 min before splitting. After transferring into calcium- and magnesium-free TALP-Hepes medium, blastomeres were dissociated by aspiration through a 30-μm micropipet. Blastomeres were transferred into empty zonae produced by mechanical removal of oocyte cytoplasm after zona scoring. Each multiple was placed in its own zona to ensure blastomere aggregation. Consequently, zonae were limiting, and additional zonae recovered from bovine oocytes were used successfully. Surrogate females were selected on serum estradiol and progesterone.

11. Parentage assignments were performed by DNA typing for 13 microsatellite loci amplified by PCR with heterologous human primers for loci D3S1768, D6S276, D6S291, D6S1691, D7S513, D7S794, D8S1106, D10S1412, D11S925, D13S765, D16S403, D17S804, and D18S72 (Veterinary Genetics Laboratory, University of California, Davis).
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14. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL; Boehringer Mannheim) was used to assay apoptosis. Blastocysts were fixed (2% formaldehyde; pH 7.4; 30 min), rinsed, and permeabilized with 0.1% Triton X-100–0.1% Na citrate (4°C; 2 min). Hoechst 33258 (Sigma) was used to visualize total DNA. To retain their three-dimensionality, coverglass spacers (170 μm; >130 to 150) were placed alongside the blastocysts. Serial confocal imaging (3 μm) used Leica confocal TCS SP with ultraviolet argon and 488-argon lasers. These generated 3D reconstructions analyzed with Photoshop (Adobe Systems, Mountain View, CA). Reconstructions permitted total cell number by counting nuclei serially. TE cells were distinguished at the periphery, whereas ICM were interior.
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Severely Reduced Female Fertility in CD9-Deficient Mice

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CD9 is a widely expressed cell surface molecule that belongs to the tetraspanin superfamily of proteins. The tetraspanins CD9, KAI-1/CD82, and CD63 are involved in metastasis suppression, an effect that may be related to their association with β1 integrins. Knockout mice lacking CD9 were created to evaluate the physiological importance of CD9. $CD9^{-/-}$ females displayed a severe reduction of fertility. Oocytes were ovulated but were not successfully fertilized because sperm did not fuse with the oocytes from $CD9^{-/-}$ females. Thus, CD9 appears to be essential for sperm-egg fusion, a process involving the CD9-associated integrin α6β1.

Sequence analysis of CD9 predicts a structure with four transmembrane domains, two extracellular loops, and short intracytoplasmic amino and carboxyl tails (*1*). This structure is shared by all members of the tetraspanin superfamily of proteins (*2*). CD9 is expressed in multiple tissues but is not ubiquitous (*3*). The tetraspanins CD9, KAI-1/CD82, and CD63 act as metastasis suppressor molecules (*4*). Low expression of these molecules is correlated with an increased invasive and metastatic potential (*2*, *5*). In vitro studies show that tetraspanins function in cell adhesion, motility, proliferation, differentiation, and signal transduction (*2*, *6*). The tetraspanins are physically associated with each other and with several cell surface molecules, including a subset of β1 integrins, which are receptors for extracellular matrix proteins (*7*). Thus, tetraspanins may function as surface organizers or facilitators that group and interconnect specific cell surface proteins in macromolecular complexes and thus increase the formation or the stability of functional signaling complexes, or both (*2*, *7*).

To investigate the role of CD9 in vivo, we generated mice in which the CD9 gene was disrupted by gene targeting in embryonic stem (ES) cells (*8*, *9*). The murine CD9 gene consists of eight exons spanning more than 20 kb (*10*, *11*). The promoter and exon 1 were replaced by the neomycin resistance gene (Web fig. 1) (*12*). The recombination was verified by Southern (DNA) blot (*13*). Splenocytes from homozygous mice displayed no detectable CD9 surface expression as determined by flow cytometry analysis or by immunohistochemistry of kidney sections (*10*).

Heterozygous $CD9^{+/-}$ mice appeared normal and, when interbred, yielded litters of normal size with a Mendelian genetic distribution 25:50:25(%) and with an equal distribution between males and females. Homozygous adult $CD9^{-/-}$ mice showed no obvious abnormalities and appeared healthy. However, when the $CD9^{-/-}$ mice were intercrossed, fertility was severely reduced. Normal fertility (93 to 100% of pregnancies) was observed when $CD9^{-/-}$ males were mated with wild-type or heterozygous females. In contrast, only 50 to 60% of $CD9^{-/-}$ females produced litters after being maintained in the presence of fertile wild-type, heterozygous, or $CD9^{-/-}$ males for up to 2 months. The delay before the beginning of a successful pregnancy was 19 to 30 days on average for $CD9^{-/-}$ females, as compared with 4.5 days for wild-type animals. In addition, the litter size was reduced (2 ± 0.6 pups in $CD9^{-/-}$ females versus 8 ± 2.3 pups in $CD9^{+/+}$ females) and the initial mortality rate was increased to 32 to 55% as compared with less than 2% for wild-type females (Web table 1) (*12*).

No difference in the frequency of vaginal plugs was observed between wild-type and CD9-deficient mice, indicating normal mating behavior (16.9%; $n = 46$ for $CD9^{-/-}$ versus 17.4%; $n = 42$ in $CD9^{+/+}$). 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. Histological examination of ovaries of 6-week-old $CD9^{-/-}$ mice showed that there was no difference when compared to wild-type females (*10*) and that the number of ovulated oocytes was normal (Table 1). However, the naturally ovulated oocytes from $CD9^{-/-}$ mated females collected at day 0.5 did not divide and became fragmented if maintained in cell culture (Fig. 1A), whereas oocytes from $CD9^{+/+}$ mated females divided and 2 days later reached stage 4 (Fig. 1B). Similar fragmented oocytes (Fig. 1C), instead of blastocysts (Fig. 1D), were present in the uterus of $CD9^{-/-}$ females 3.5 days after mating.

The oocytes recovered from nonmated superovulated $CD9^{-/-}$ mice (Fig. 2A) or $CD9^{+/+}$ mice (Fig. 2C) 12 hours after human chorionic gonadotropin (post-hCG) injection had a similar response: 70 and 74%, respectively, showed a polar body (Web table 1)

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(*12*), which indicates completion of meiosis I. Labeling of these oocytes with a monoclonal antibody (mAb) to tubulin YL1/2 and propidium iodide showed that they were blocked in metaphase of the second meiotic division (Fig. 2, B and D). The first polar body is unstable because less than 25% of the oocytes collected at 17 hours post-hCG had a polar body (*10*). The oocytes recovered from mated $CD9^{-/-}$ females at day 0.5 frequently had several sperm (up to 10) in the perivitelline space (Fig. 2E) (Web table 1) (*12*), suggesting that they were not competent for fertilization. Few of these oocytes (17%) had a residual first polar body, but DNA-tubulin labeling showed that $CD9^{-/-}$ oocytes [12 out of 13 (12/13)] remained blocked in metaphase of the second meiotic division (Fig. 2F). One or two polar bodies were observed in 94% of oocytes from $CD9^{+/+}$ mated females (Table 1) (Fig. 2G) in which DNA-tubulin labeling showed that most oocytes (16/19) were fertilized and had exited the second meiotic metaphase. This was confirmed by the metaphase plate disappearance and by the extrusion of the second polar body (Fig. 2H).

CD9 was strongly expressed on ovulated oocytes (Fig. 3, A and B) and blastocysts (Fig. 3, C and D) from $CD9^{+/+}$ but not on oocytes from $CD9^{-/-}$ females. The expression of CD9 was also observed on follicular oocytes in the ovary, whereas no expression was found on follicular cells (*10*). Next, in vitro fertilization experiments (*14*) were performed with the oocytes of five superovulated C57BL/6 females. For each female, oocytes collected from the two oviducts were kept separate and were fertilized in the presence of either the mAb to mouse CD9 4.1F12 (*15*) or a control rat mAb. The fertilization rates, determined by the number of stage 2 to 4 embryos at 24 to 48 hours after fertilization, were 4% (n = 42) and 49% (n = 43), respectively, showing an inhibition of fertilization by the CD9 mAb.

To distinguish between a defect in fusion or in subsequent events, we performed a transfer test with the dye Hoechst 33342 (*16*). In 35 zona pellucida (ZP)–free oocytes of four superovulated $CD9^{-/-}$ females, we observed no sperm-egg fusion, whereas in five superovulated $CD9^{+/+}$ females (68 oocytes),

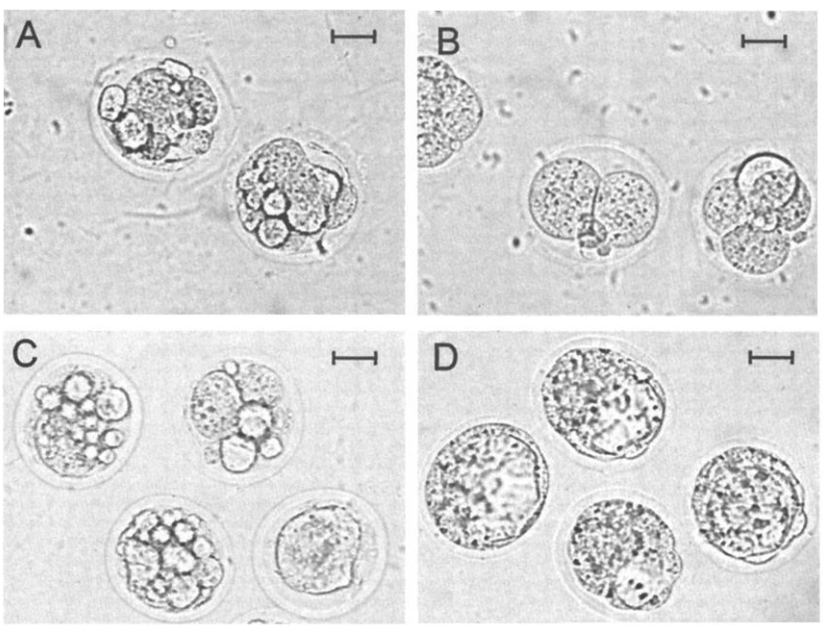

Fig. 1. Morphology of oocytes or embryos recovered after natural ovulation and mating of either $CD9^{+/+}$ or $CD9^{-/-}$ knockout females. Oocytes from $CD9^{-/-}$ (**A**) and $CD9^{+/+}$ (stage 2 or 4) (**B**) females were kept in culture for 2.5 days. Embryos or oocytes were recovered from the uterine horns 3.5 days after mating. (**C**) Fragmented oocytes obtained from $CD9^{-/-}$ females. (**D**) Embryos at blastocyst stage obtained from wild-type females. Scale bar, 30 μm.

Table 1. Effect of CD9 deficiency on ovulation rate and oocyte fertilization in vivo. Ovulated oocytes were recovered from the oviduct ampulla and examined by phase contrast microscopy. Granulosa cells were removed with type III hyaluronidase (0.1 mg/ml; Sigma). Oocytes from naturally ovulated $CD9^{+/+}$ females had one or two polar bodies: a residual first polar body in some and a second polar body in all cases. Values are the mean ± SEM. NA, not applicable.

	No. of females with an ampulla	No. of ovulated oocytes per mouse	Total no. of oocytes	Oocytes with a polar body (%)	No. of females with polyspermia	Oocytes with polyspermia (%)
		Superovulation, no mating, 12 hours post-hCG				
$CD9^{+/+}$	3	29.6 ± 15.3	89	74 ± 23	NA	NA
$CD9^{-/-}$	4	26.2 ± 11.7	105	70 ± 18.9	NA	NA
		Natural ovulation, mating with a fertile male				
$CD9^{+/+}$	7	8.7 ± 1.6	61	91 ± 10	1	3
$CD9^{-/-}$	13	8.8 ± 2.2	115	17 ± 18	7	23

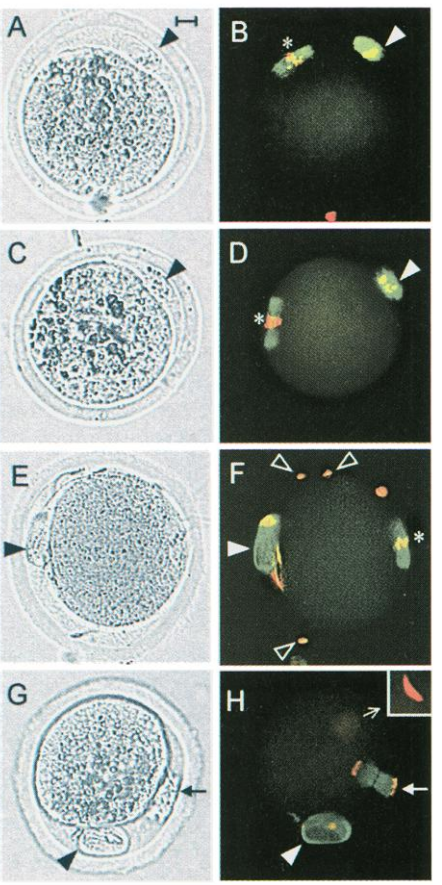

Fig. 2. Oocyte DNA-tubulin labeling. Oocytes were fixed (*23*) and incubated with mAb YL1/2 (*24*), followed by fluorescein isothiocyanate (FITC)–labeled goat $F(ab')_2$ antibody to mouse. Oocytes from nonmated superovulated $CD9^{-/-}$ (**A** and **B**) or $CD9^{+/+}$ (**C** and **D**) females were identically blocked in metaphase of meiosis II as confirmed by the presence of a metaphase spindle (*) and a first polar body containing residual DNA-tubulin material (solid arrowheads). (**E** and **F**) An oocyte from a $CD9^{-/-}$ naturally ovulated mated female blocked in metaphase of meiosis II with sperm in the perivitelline space (open arrowheads). (**G** and **H**) An oocyte from a $CD9^{+/+}$ naturally ovulated mated female that has progressed to telophase and has extruded the second polar body (arrow), whereas the first one remains visible. Inset shows partially decondensed sperm DNA in a different focal plane. Scale bar, 10 μm.

there was a mean sperm-egg fusion of 75 ± 19% (*10*). No difference in sperm-egg binding was observed. This shows that CD9 is involved in sperm-egg fusion.

Female infertility has previously been shown to be linked to oocyte growth and maturation or ovulation (*17*). A defect of fertilization was also observed in females lacking the ZP glycoprotein ZP3 (*18*). Here we have shown that in the absence of the tetraspanin CD9, the ovaries and ovulation rate are normal. However, most of the ovulated oocytes are not competent for fusion with sperm. As in other cells, CD9 may participate in oocytes in multimolecular complexes and may even play a role in the formation of these complexes. It should be noted that the integrins α3β1, α5β1, and α6β1, which are associated with CD9 (*7, 19*), are expressed on oocytes (*20*). Because the integrin α6β1 has been shown to bind to sperm fertilin and could function as a sperm receptor (*21*), it is possible that CD9 plays a role in sperm-egg fusion by modulating α6β1 receptor function.

The effect of the CD9 knockout on fertility contrasts with the lack of apparent phenotype in other tissues expressing high levels of this molecule (*22*). In tissues outside of the ovary, CD9 may be functionally replaced by one or several tetraspanins. Our data show the involvement of CD9 in oocyte fertilization and reveal a biological function of a tetraspanin. It now must be questioned whether certain human infertilities are caused by a defect of this molecule.

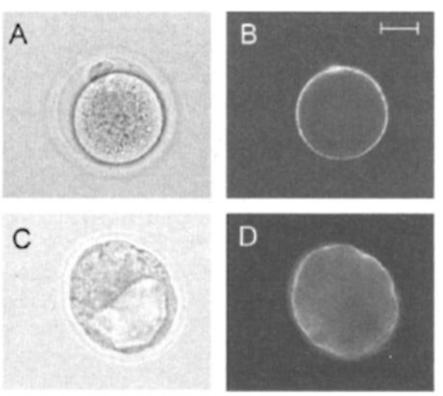

Fig. 3. CD9 expression on oocytes and embryos from wild-type mice. Staining was performed with a rat mAb to mouse CD9 4.1F12 (*15*) followed by FITC-coupled goat $F(ab')_2$ antibody to rat, and was then examined under a fluorescence microscope. $CD9^{+/+}$ oocytes denuded of cumulus cells (**A** and **B**) and $CD9^{+/+}$ blastocysts (3.5-day-old embryos) (**C** and **D**) were labeled and washed in microdrops of phosphate-buffered saline without fixation. Strong labeling of the cell surface was observed. The labeling of $CD9^{-/-}$ oocytes was identical to background, which was negligible and is not shown here. Scale bar, 30 μm.

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13. The construct was made linear by digestion at the unique Cla I site in the PTV-0 vector and was electroporated into E14 ES cells with 240 V and 500 μF. The neomycin- and gancyclovir-resistant cells were isolated by positive or negative selection (*8*) in medium containing G418 (300 μg/ml) and 2 μM gancyclovir. These cells were then screened by polymerase chain reaction (PCR) with primers that were complementary to sequences in the neomycin-resistant (3′ primer) gene and in the genomic region 5′ to the short arm (5′ primer). Two of 400 analyzed ES cell clones had undergone the desired homologous recombination. These clones were verified by Southern blotting with the indicated probe. The two clones were injected into 3.5-day-old C57BL/6 blastocysts, which were transferred into foster mothers. Chimeric males were crossed with C57BL/6 females and DNA samples from the tails of agouti offspring were analyzed by Southern blot. Mice carrying the mutation in the heterozygous state (CD9+/–) were intercrossed to produce homozygous mutants (CD9–/–).
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Requirement of CD9 on the Egg Plasma Membrane for Fertilization

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CD9 is an integral membrane protein associated with integrins and other membrane proteins. Mice lacking CD9 were produced by homologous recombination. Both male and female $CD9^{-/-}$ mice were born healthy and grew normally. However, the litter size from $CD9^{-/-}$ females was less than 2% of that of the wild type. In vitro fertilization experiments indicated that the cause of this infertility was due to the failure of sperm-egg fusion. When sperm were injected into oocytes with assisted microfertilization techniques, however, the fertilized eggs developed to term. These results indicate that CD9 has a crucial role in sperm-egg fusion.

CD9, expressed in a wide variety of cells (*1, 2*), is an integral membrane protein belonging to a family of tetraspan-membrane proteins (TM4) (*3*) and is reported to play a role in cell adhesion, cell motility, and tumor cell metastasis (*4*). CD9, which can associate with integrins such as α3β1 and α6β1, is suggested to be a possible co-factor of the integrins (*5,*