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.

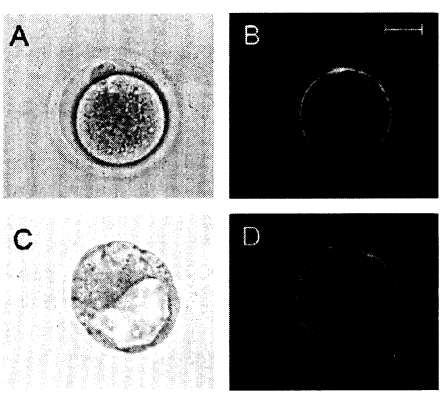


Fig. 3. CD9 expression on oocytes and embryos from wild-type mice. Staining was performed with a rat mAb to mouse CD9 4.1F12 (*15*) followed by FITC-coupled goat $F(ab')_2$ antibody to rat, and was then examined under a fluorescence microscope. $CD9^{+/+}$ oocytes denuded of cumulus cells (**A** and **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.

References and Notes

1. C. Boucheix *et al.*, *J. Biol. Chem.* **266**, 117 (1991); F. Lanza *et al.*, *J. Biol. Chem.* **266**, 10638 (1991).
2. H. T. Maecker, S. C. Todd, S. Levy, *FASEB J.* **11**, 428 (1997).
3. P. M. Sincock, G. Mayrhofer, L. K. Ashman, *J. Histochem. Cytochem.* **45**, 515 (1997).
4. S. Ikeyama, M. Koyama, M. Yamaoko, R. Sasada, M. Miyake, *J. Exp. Med.* **177**, 1231 (1993); J.-T. Dong *et al.*, *Science* **268**, 884 (1995); K. J. Radford, J. Mallesch, P. Hersey, *Int. J. Cancer* **62**, 631 (1995).
5. M. E. Hemler, B. A. Mannion, F. Berditchevski, *Biochim. Biophys. Acta* **1287**, 67 (1996).
6. C. Lagaudrière-Gesbert *et al.*, *Cell. Immunol.* **182**, 105 (1997).
7. E. Rubinstein, F. Le Naour, M. Billard, C. Boucheix, *Eur. J. Immunol.* **24**, 3005 (1994); F. Berditchevski, M. M. Zutter, M. E. Hemler, *Mol. Biol. Cell* **7**, 193 (1996). V. Serru *et al.*, *Biochem. J.* **340**, 103 (1999).
8. S. L. Mansour, K. R. Thomas, M. R. Capecchi, *Nature* **336**, 348 (1988).
9. M. R. Capecchi, *Science* **244**, 1288 (1989).
10. F. Le Naour, data not shown.
11. We have observed that in the human gene, a region comprising 200 base pairs 5′ of the translation initiation site had a strong promoter activity and was necessary for expression. The mouse gene has 60% identity with the human gene in this region [F. Le Naour *et al.*, *Oncogene* **13**, 481 (1996); F. Le Naour *et al.*, *Leukemia* **11**, 1290 (1997)].
12. Web fig. 1 and Web table 1 are available at *Science* Online at www.sciencemag.org/feature/data/1046233.
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–/–).
14. B. Hogan, R. Beddington, F. Costantini, E. Lacy, *Manipulating the Mouse Embryo, a Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1994).
15. The mAb to mouse CD9 was produced in collaboration with F. Lanza.
16. J. C. Conover and R. B. L. Gwatkin, *J. Reprod. Fertil.* **82**, 681 (1988).
17. P. Monget, S. Hembert, N. Binart, A. Gougeon, J. J. Panthier, *Med. Sci.* **15**, 141 (1999).
18. C. Liu *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5431 (1996); T. Rankin *et al.*, *Development* **122**, 2903 (1996).
19. K. Nakamura, R. Iwamoto, E. Mekada, *J. Cell Biol.* **129**, 1691 (1995).
20. G. Tarone *et al.*, *Development* **117**, 1369 (1993); J. P. Evans, R. M. Schultz, G. S. Kopf, *Mol. Reprod. Dev.* **40**, 211 (1995); G. Capmany, M. Mart, J. Santalo, V. N. Bolton, *Mol. Hum. Reprod.* **10**, 951 (1998); M. Zuccotti *et al.*, *Dev. Biol.* **200**, 27 (1998).
21. E. A. C. Almeida *et al.*, *Cell* **81**, 1095 (1995); P. M. Wassarman, *Cell* **96**, 175 (1999).
22. We checked that the tissue distribution of CD9 antigen in the mouse is similar to that in humans. In particular, it is strongly expressed on megakaryocytes and platelets, B lymphoid cells, epithelial cells of distal kidney tubules, epidermis, and smooth muscle cells.
23. J. Z. Kubiak, M. Veber, G. Géraud, B. Maro, *J. Cell. Sci.* **102**, 457 (1992).
24. J. V. Kilmartin, B. Wright, C. Milstein, *J. Cell. Biol.* **93**, 576 (1982).

25. We thank A. Tarakhovski, J. Di Santo, J. Chaker, M. Kress, D. Nogueira, J. Cosy, F. Lanza, D. Monniaux, I. Casal, and I. Cerruti for their help in setting up the experimental procedures and R. Kuhn for the gift of vector PTV-0. Supported by grants from Associations Recherche sur le Cancer, Nouvelle Recherche Biomédicale, and Institut de Cancérologie et Immunogénétique.

13 October 1999; accepted 29 November 1999

Requirement of CD9 on the Egg Plasma Membrane for Fertilization

Kenji Miyado,[1]* Gen Yamada,[2,3]* Shuichi Yamada,[4]* Hidetoshi Hasuwa,[1] Yasuhiro Nakamura,[5] Fuminori Ryu,[1] Kentaro Suzuki,[2] Kenichiro Kosai,[2] Kimiko Inoue,[6] Atsuo Ogura,[6] Masaru Okabe,[7] Eisuke Mekada[1,2]†

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,*

6). CD9 also associates with the membrane-anchored form of heparin-binding EGF-like growth factor (proHB-EGF) and up-regulates its biological activities (7).

To study the physiological role of CD9 in vivo, we produced CD9$^{-/-}$ mice by targeted disruption of the CD9 gene using embryonic stem (ES) cell technology (8). A part of the third exon and all of the fourth exon, which encode second to third membrane-spanning domains, were deleted in the targeting vector (Fig. 1A). Chimeric males were bred with C57BL/6J females to produce homozygous mice for the deletion. Homozygous mutant mice were identified by Southern blot analyses of genomic DNA (Fig. 1B). Two independent ES cell lines carrying a mutated allele generated chimeric mice transmitting the mutated alleles to the progeny. Both lines of mice had essentially identical results. Breeding yielded the predicted number of null mice at a mendelian frequency. Flow cytometrical analyses of bone marrow cells proved an absence of CD9 expression in the mice carrying the deletion (Fig. 1C). Both male and female homozygous mutant mice were born and grew normally, demonstrating that there were no detrimental effects in cell adhesion or cell growth in CD9$^{-/-}$ mice.

However, CD9$^{-/-}$ females were infertile, whereas CD9$^{-/-}$ males showed normal fertility when mated with wild-type mice. Mating experiments (9) showed that there was no difference in the frequency of copulation plugs between CD9$^{-/-}$ and CD9$^{+/-}$ females. However, the number of pups born from CD9$^{-/-}$ females was <2% of the rates of wild-type and heterozygote mice (Fig. 1D). Histochemical analysis of the ovaries of CD9$^{-/-}$ females showed no morphological abnormalities, and the follicles of all developmental stages were present, including corpora lutea indicative of past ovulation (10). The females responded to hormone treatment, and they ovulated as many eggs as wild-type females (average of 19 ± 1.9 in CD9$^{-/-}$ and 22 ± 3.0 in CD9$^{+/+}$ mice). First polar bodies were observed in 97% (37 of 38) of oocytes collected from CD9$^{-/-}$ females 12.5 to 13 hours after human chorionic gonadotropin (hCG) injection (Fig. 1E), and the oocyte chromosomes were confirmed to be in metaphase II by the observation of whole-mount specimens (11). Therefore, lack of ovulation or oocyte maturation did not cause the infertility.

We then conducted in vitro fertilization (IVF) experiments to analyze the infertility of CD9$^{-/-}$ female mice (12). Oocytes collected from CD9$^{-/-}$ and CD9$^{+/+}$ females were inseminated with wild-type sperm. When examined 6 hours after insemination, sperm penetrated the zona pellucida of eggs from both lines. However, almost all of the inseminated

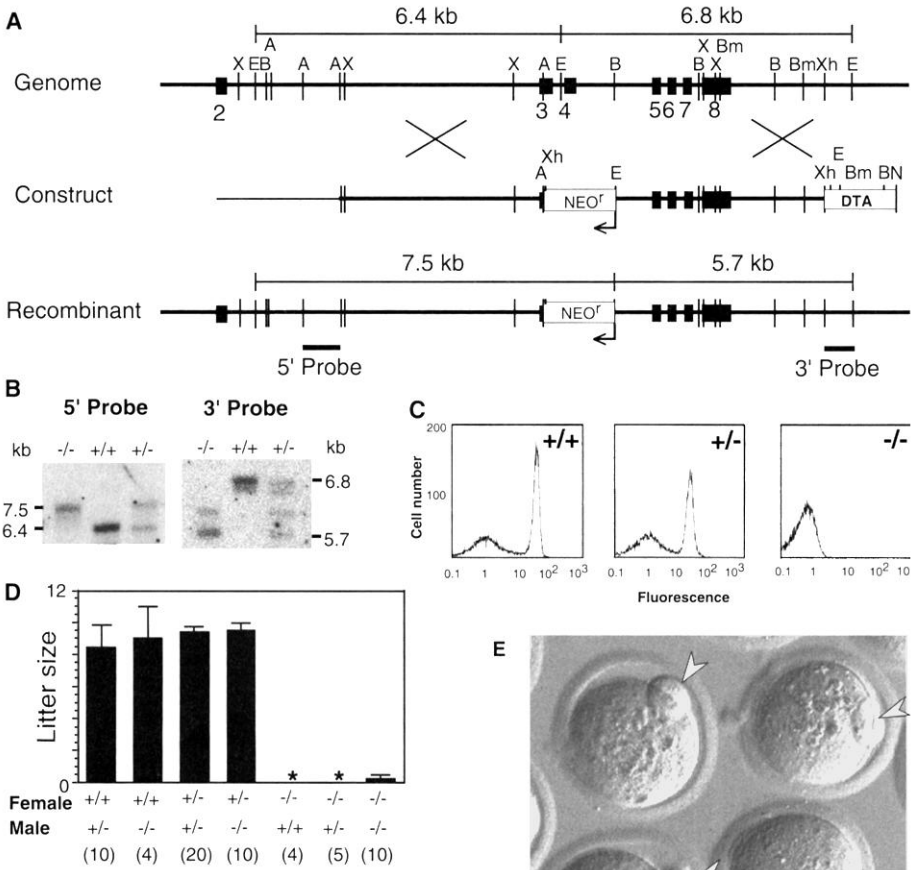


Fig. 1. Generation of CD9-deficient mice. (**A**) Gene-targeting strategy. Exons and introns are represented by vertical bars and horizontal lines, respectively. A neomycin-resistance gene driven by a PGK promoter (NEO^r) and a diphtheria toxin A fragment driven by a MC1 promoter (DTA) are indicated as white boxes. Restriction sites: A, Apa I; B, Bgl II; Bm, Bam HI; E, Eco RI; N, Not I; X, Xba I; Xh, Xho I. (**B**) Hybridization of Eco RI–digested genomic DNA from wild-type (+/+), heterozygous CD9 (+/–), or homozygous CD9 (–/–) mice with the 5′- and 3′-external probes. (**C**) Flow cytometry of bone marrow cells in CD9$^{+/+}$, CD9$^{+/-}$, and CD9$^{-/-}$ littermates stained with fluorescein isothiocyanate–conjugated anti-mouse CD9 antibody (KMC8.8). Data represents monocyte-rich fractions gated by forward scatter and side scatter. (**D**) Fecundity of CD9$^{+/+}$, CD9$^{+/-}$, and CD9$^{-/-}$ females and males was examined by caging one male to one or two females with various genetic backgrounds (9). The average litter size ± SEM is shown. The numbers in parentheses indicate the numbers of mating pairs. Asterisk indicates no litters. (**E**) Micrograph of oocytes collected from CD9$^{-/-}$ females. First polar bodies (arrowheads) are seen in all of the oocytes.

Table 1. Development of CD9$^{-/-}$ oocytes after direct injection of sperm into egg cytoplasm (ICSI) and embryo transfer. Results are from two replicate experiments.

Egg donor	No. of ICSI	No. of oocytes surviving after Injection	No. of oocytes developing to two-cell stage†	No. of embryos implanted‡	No. of embryos developing to term‡
CD9$^{+/+}$	55	35	25 (71%)§	13 (52%)	6 (24%)
CD9$^{-/-}$	50	37	33 (89%)	20 (61%)	14 (42%)

†All two-cell embryos were transferred into pseudopregnant females. ‡Among two-cell embryos transferred. §Percentages are from number of oocytes surviving injection.

[1]Institute of Life Science, Kurume University, Kurume, Fukuoka 839-0861, Japan. [2]Research Center for Innovative Cancer Therapy, Kurume University, Fukuoka 830-0011, Japan. [3]Center of Animal Resources and Development, Kumamoto University, Kumamoto 860-0811, Japan. [4]Program for Promotion of Basic Research Activities for Innovative Biosciences, Tokyo 105-0001, Japan. [5]St. Mary's Hospital, Kurume, Fukuoka 830-0047, Japan. [6]National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640, Japan. [7]Genome Information Research Center, Osaka University, Suita, Osaka 565-0861, Japan.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: emekada@lsi.kurume-u.ac.jp

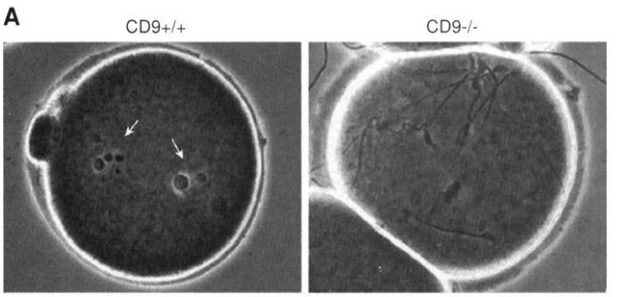
$CD9^{-/-}$ eggs (153 of 154) failed to fuse with sperm and to form pronuclei, whereas 57% of the $CD9^{+/+}$ eggs fused with sperm and reached the pronuclear stage (Fig. 2, A and B). In normal conditions, zona-penetrated sperm fuse with the egg plasma membrane and initiate egg activation that includes the release of cortical granule contents leading to modification of the zona pellucida to prevent subsequent sperm penetration (*13*). However, 65% of the $CD9^{-/-}$ eggs accumulated more than five sperm in their perivitelline space (Fig. 2A).

To define whether $CD9^{-/-}$ eggs have the ability to bind sperm at the plasma membrane, IVF was carried out using eggs that were freed from the zonae pellucidae (*12*). In this condition, many sperm can directly interact with the egg surface. No difference was seen in the number of sperm that bound to the surface of zona-free $CD9^{-/-}$ and $CD9^{+/+}$ eggs (Fig. 2C). In contrast, sperm-egg fusion (*14*) was greatly impaired in the $CD9^{-/-}$ eggs: the fusion rate dropped from 98 to 4% and from 100 to 21%, when judged 1 and 6 hours after insemination, respectively (Fig. 2D). Thus, CD9 functions in sperm-egg fusion (*15*).

The rare success of fertilization in vitro (Fig. 2) and the appearance of healthy offspring in vivo (Fig. 1D) suggest that the deficiency of CD9 affects only sperm-egg interaction but not the developmental processes that follow. To test this, sperm were injected directly into the cytoplasm of $CD9^{-/-}$ oocytes [intracytoplasmic sperm injection (ICSI)] (*16*). $CD9^{-/-}$ oocytes injected with wild-type sperm showed normal implantation efficiency in the uteri of pseudopregnant females, and the resulting embryos developed to term with rates similar to those of wild-type mice (Table 1).

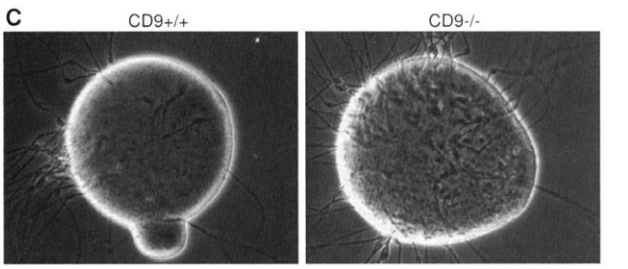
Protein immunoblot analysis with monoclonal antibody (mAb) to the CD9 antigen indicated that CD9 is expressed in the oocyte membrane, and the CD9 has a molecular size similar to that of CD9 in F9 mouse embryonal carcinoma cells (Fig. 3A). Immunofluorescent staining showed the localization of CD9 on the oocyte plasma membrane (Fig. 3B). When anti-CD9 mAb was added to the in vitro fertilization system, the fertilization of wild-type eggs was inhibited (Fig. 3C). Unlike antibodies to integrin α6 subunit (*17*), antibodies to CD9 did not block sperm binding to the egg plasma membrane. Although the inhibition of sperm-egg fusion was not as complete as the results seen in $CD9^{-/-}$ eggs, the antibodies to CD9 showed the inhibition of sperm-egg fusion, and as a result, an accumulation of many sperm inside the perivitelline space was observed. These results reinforce the involvement of CD9 in sperm-egg fusion.

It was postulated that the sperm surface protein fertilin, a member of the ADAM family, functions in sperm-egg fusion (*13, 18*). However, fertilin-β knockout mice showed that fertilin functions in sperm-egg binding (*19*). Integrin α6β1 has been noted as a sperm receptor on egg plasma membrane (*17, 20*) and binds to fertilin through fertilin's distintegrin domain. We examined whether CD9 forms a complex with integrin α6β1 on egg



B

Egg donor	No. of eggs examined (%)	Unfertilized (%)	Fertilized: Zona penetrated (%)	Fertilized: Pronuclear formation (%)	Degenerated (%)
CD9+/+	179 (100)	69 (39)	0 (0)	102 (57)	8 (4)
CD9 -/-	154 (100)	40 (26)	100 (65)	1 (0.6)	13 (8)



D

Egg donor	Time after insemination (hr)	No. of eggs examined	No. of sperm fused with oocyte (%): 0	1	2	3	4	Average no. of sperm / egg
CD9+/+	1	53	2	38	51	9	0	1.68
CD9 -/-	1	56	96	4	0	0	0	0.04
CD9+/+	6	26	0	11	62	23	4	2.19
CD9 -/-	6	19	79	21	0	0	0	0.21

Fig. 2. Phenotype of CD9-deficient eggs in in vitro fertilization experiments. [(A) and (B)] Zona-intact eggs were used. Oocytes, collected from $CD9^{-/-}$ and $CD9^{+/+}$ females 13 to 15 hours after the injection of hCG, were inseminated with wild-type sperm. (**A**) Representative micrographs and (**B**) a summary of the rates of fertilization of $CD9^{+/+}$ and $CD9^{-/-}$ eggs were obtained 6 hours after insemination. The number of sperm that penetrated the zona pellucida of $CD9^{+/+}$ or $CD9^{-/-}$ eggs was determined by staining with lacmoid (*24*). $CD9^{-/-}$ eggs failed to fuse with sperm and remained in metaphase II, whereas $CD9^{+/+}$ eggs fused with sperm, and pronuclei (arrows) were observed. [(C) and (D)] IVF was performed using zona-free eggs. (**C**) Similar numbers of sperm bound to the surface of $CD9^{+/+}$ and $CD9^{-/-}$ eggs following washing 1 hour after insemination. In (A) and (C), magnification (×400) shows live eggs under phase contrast view. (**D**) Fusing ability of $CD9^{+/+}$ and $CD9^{-/-}$ eggs with wild-type sperm. The number of sperm fused with $CD9^{+/+}$ or $CD9^{-/-}$ eggs was determined 1 and 6 hours after insemination, respectively.

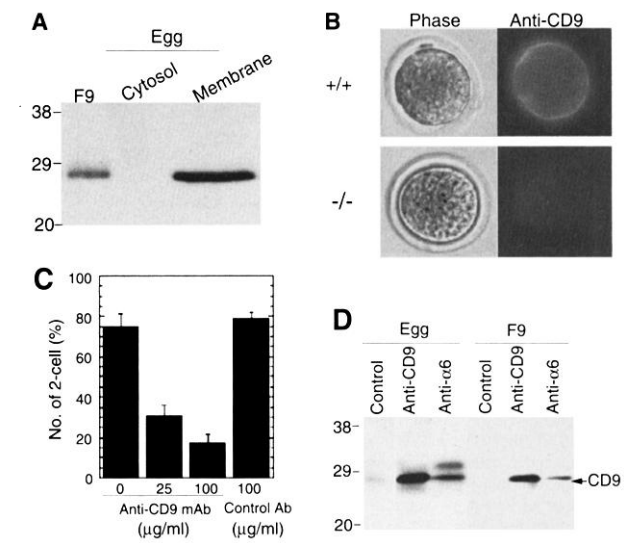


Fig. 3. Immunological studies of egg CD9 and the role of CD9 in fertilization. (**A**) Protein immunoblotting with an anti-CD9 mAb (KMC8.8). We lysed 37 eggs and 2×10^3 embryonic carcinoma F9 cells with 10 mM CHAPS solution (*5*) and loaded them on an SDS gel. (**B**) Immunofluorescence micrographs of CD9 on the egg plasma membranes. Zona-intact mouse eggs collected from $CD9^{+/+}$ and $CD9^{-/-}$ females were stained with anti-mouse CD9 mAb and the fluoresceinated second antibody. Original magnification, ×200. (**C**) Effect of anti-CD9 antibody on IVF of zona-intact eggs. Anti-CD9 mAb (KMC8.8) or control mAb (anti–Thy-1; PharMingen) was added at the concentrations indicated 30 min before insemination. The number of eggs at the two-cell stage were counted for assessment of fertilization 24 hours after insemination. (**D**) Co-immunoprecipitation of CD9 with integrin α6β1 from eggs and F9 cell lysates. We lysed 106 eggs or 3×10^5 F9 cells with 10 mM CHAPS solution, and then immunoprecipitated the lysates with anti-integrin α6β1 mAb (GoH3), anti-CD9 mAb, or control mAb (*25*). Immunoprecipitated materials were electrophoresed in SDS-gels under nonreducing conditions and immunoblotted by anti-CD9 antibodies.

plasma membrane by co-immunoprecipitation assay. CD9 co-precipitated with anti-integrin α6 antibody in lysates from both mouse eggs and F9 cells (Fig. 3D), indicating that CD9 physically associates with integrin α6β1 on egg plasma membrane, as shown in other cell lines (*7*).

The integrin family provides a physical link between the extracellular matrix and the cell cytoskeleton and transduces signals, eliciting changes in the intracellular pH, cytoplasmic calcium level, phospholipid metabolism, protein tyrosine and serine/threonine phosphorylation, and expression of certain genes (*21*). Recent studies suggest that integrin-associated transmembrane proteins, including CD9 and TM4, may also participate in integrin-mediated signaling (*22*). We have shown here that CD9 associates with integrin α6β1 in eggs. Therefore, integrin α6β1 may transduce signals to CD9 and initiate, or otherwise promote, fusion. However, CD9 may directly function in membrane fusion. In support of this possibility, it should be noted that some anti-CD9 or anti-TM4 antibodies block virus-mediated syncytium formations where the involvement of integrin is not clear (*23*).

Our results show that CD9 is a crucial factor for mouse oocytes in fertilization. $CD9^{-/-}$ mice may serve to elucidate the precise mechanism of sperm-egg fusion and the role of CD9-integrin complex.

References and Notes

1. R. Iwamoto, H. Senoh, Y. Okada, T. Uchida, E. Mekada, *J. Biol. Chem.* **266**, 20463 (1991).
2. J. H. Kersey *et al.*, *J. Exp. Med.* **153**, 726 (1981); R. A. Newman, D. R. Sutherland, T. W. Lebien, J. H. Kersey, M. F. Greaves, *Biochim. Biophys. Acta* **701**, 318 (1982); Y. Nakamura, R. Iwamoto, E. Mekada, *Am. J. Pathol.* **149**, 575 (1996); M. Higashiyama *et al.*, *Cancer Res.* **55**, 6040 (1995).
3. H. T. Maecker, S. C. Todd, S. Levy, *FASEB J.* **11**, 428 (1997).
4. A. R. Shaw *et al.*, *J. Biol. Chem.* **270**, 24092 (1995); M. Hadjiargyrou and P. H. Patterson, *J. Neurosci.* **15**, 574 (1995); E. S. Anton, M. Hadjiargyrou, P. H. Patterson, W. D. Matthew, *J. Neurosci.* **15**, 584 (1995); S. Ikeyama, M. Koyama, M. Yamaoko, R. Sasada, M. Miyake, *J. Exp. Med.* **177**, 1231 (1993).
5. K. Nakamura, R. Iwamoto, E. Mekada, *J. Cell Biol.* **129**, 1691 (1995).
6. F. Berditchevski, M. M. Zutter, M. E. Hemler, *Mol. Biol. Cell* **7**, 193 (1996).
7. R. Iwamoto *et al.*, *EMBO J.* **13**, 2322 (1994); S. Higashiyama *et al.*, *J. Cell Biol.* **128**, 929 (1995).
8. A mouse genomic DNA clone containing exons 2 through 8 of the CD9 gene was isolated by screening a genomic DNA library derived from the 129/Sv mouse strain. The targeting vector was prepared using a 10-kb Apa I–Xho I fragment containing exons 3 to 8 of the CD9 gene, PGK-neo-polyA cassette and pBluescript plasmid. This construct was designed to delete a ApaI-BglII fragment from the CD9 gene, encompassing a part of exon 3 to all of exon 4 as schematized in Fig. 1A. The Not I–linearized targeting vector was transfected into the ES line. Five of 300 G418-resistant clones underwent the desired homologous recombination. Two positive clones were injected into blastocysts, and chimeric offspring were mated to C57BL/6J females. Mice carrying the mutation in the heterozygous state ($CD9^{+/-}$) were intercrossed to produce homozygous mutants ($CD9^{-/-}$), and 4- to 8-week-old mice were used for analysis. To determine the genotype of the CD9 locus, DNA samples were extracted from adult tails after digestion with proteinase K and were analyzed by Southern blotting using a labeled probe (Fig. 1B).
9. $CD9^{+/+}$, $CD9^{+/-}$, and $CD9^{-/-}$ females (2 to 3 months old) were caged with one male for 10 days to 2 months, and the number of pups born was counted. Breeding capacities of each genetic background were determined using 4 to 20 pairs of mice of all the genetic combinations.
10. Ovaries dissected from mice were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.
11. K. Miyado, K. Inoue, A. Ogura, E. Mekada, data not shown.
12. Mature female mice were superovulated by injecting 5 IU of pregnant mare's serum and 5 IU of hCG at 48-hour intervals. Eggs were collected 13 to 15 hours after the hCG injection from the oviductal ampulla region under paraffin and were placed in a 400 μl drop of TYH medium (*24*) equilibrated with 5% CO_2 in air at 37°C. Zona-free eggs were prepared by acidic tyrode according to the method described [B. Hogan, F. Costantini, E. Lacy, *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986)]. In some of the experiments, eggs were incubated with antibodies for 30 min before sperm insemination. Antibodies were present throughout sperm-egg incubations. Sperm collected from the cauda epididymidis of mature male mice were suspended in 400 μl of TYH medium and preincubated for 90 min before adding to eggs. The final concentrations of sperm added to the eggs were 1×10^5 to 1.5×10^5 sperm/ml.
13. R. Yanagimachi, in *The Physiology of Reproduction*, E. Knobil and J. D. Neil, Eds. (Raven Press, New York, 1994), pp. 189–317; P. M. Wassarman, *Science* **235**, 553 (1987).
14. In order to observe the fusion, the eggs were fixed with 0.05% glutalaldehyde and 10% formaldehyde followed by staining with 0.5% lacmoid (*24*).
15. Fusing ability of $CD9^{-/-}$ and $CD9^{+/+}$ eggs with wild-type sperm was also determined by dye transfer assay (*17*). By this assay, fusion with sperm was not observed in $CD9^{-/-}$ eggs ($n = 9$), whereas 100% of $CD9^{+/+}$ eggs ($n = 10$) fused with sperm (average of 1.6 sperm per egg).
16. Mature eggs collected from $CD9^{+/+}$ or $CD9^{-/-}$ females, as described above (*12*), were freed from cumulus cells by treatment with bovine testicular hyaluronidase in CZB medium [C. L. Chatot *et al.*, *J. Reprod. Fertil.* **86**, 679 (1989)]. The eggs were each injected with the head of epididymal spermatozoa from mature C57BL/6J males by using Piezo micromanipulator as reported [T. Wakayama *et al.*, *Nature Biotechnol.* **16**, 639 (1998)]. After 24 hours of culture in CZB medium under 5% CO_2 in air at 37°C, eggs that developed to the two-cell stage were transferred into the oviducts of day-1 pseudopregnant ICR females. The recipient females were allowed to deliver young.
17. E. A. Almeida *et al.*, *Cell* **81**, 1095 (1995).
18. W. J. Snell and J. M. White, *Cell* **85**, 629 (1996); D. Bigler, M. Chen, S. Waters, J. M. White, *Trends. Cell Biol.* **7**, 220 (1997); J. P. Evans *et al.*, *Biol. Reprod.* **59**, 145 (1998); D. G. Myles and P. Primakoff, *Biol. Reprod.* **56**, 320 (1997).
19. C. Cho *et al.*, *Science* **140**, 1857 (1998).
20. H. Chen and N. S. Sampson, *Chem. Biol.* **6**, 1 (1998).
21. A. Howe, A. E. Aplin, S. K. Alahari, R. L. Juliano, *Curr. Opin. Cell Biol.* **10**, 220 (1998).
22. M. E. Hemler, *Curr. Opin. Cell Biol.* **10**, 578 (1998).
23. K. Fukudome *et al.*, *J. Virol.* **66**, 1394 (1992); S. Loffler *et al.*, *J. Virol.* **71**, 42 (1997).
24. Y. Toyoda, M. Yokoyama, T. Hoshi, *Jpn. J. Anim. Reprod.* **16**, 147 (1971).
25. Anti-diphtheria toxin antibody no. 2 [S. Hayakawa, T. Uchida, E. Mekada, M. R. Moynihan, Y. Okada, *J. Biol. Chem.* **258**, 4311 (1983)].

26. We thank S. Kaur and members of Chrysalis DNX Transgenic Science Inc. for producing knockout mice, K. Miyake for providing anti-CD9 antibody KMC8.8, M. Kimoto for suggestions, and the members of the department of Immunology, Saga Medical School, for suggestions and analysis of bone marrow cells. Supported in part by a grant from The Research for the Future Program, the Japan Society for the Promotion of Science (JSPS) (project 97L00303), and a Grant-in Aid for Scientific Research, The Ministry of Education, Science, Sports and Culture (grant 09480198) for E.M. All animal care and experiments were in accordance with Kurume University and Osaka University Animal Care and Use Committee guidelines.

6 October 1999; accepted 29 November 1999

Regulation of JNK by Src During *Drosophila* Development

Minoru Tateno, Yasuyoshi Nishida, Takashi Adachi-Yamada*

In *Drosophila*, the Jun amino-terminal kinase (JNK) homolog Basket (Bsk) is required for epidermal closure. Mutants for *Src42A*, a *Drosophila* c-*src* protooncogene homolog, are described. Src42A functions in epidermal closure during both embryogenesis and metamorphosis. The severity of the epidermal closure defect in the *Src42A* mutant depended on the amount of Bsk activity, and the amount of Bsk activity depended on the amount of Src42A. Thus, activation of the Bsk pathway is required downstream of Src42A in epidermal closure. This work confirms mammalian studies that demonstrated a physiological link between Src and JNK.

Genes that regulate cell shape changes in *Drosophila* are required for dorsal closure of the embryonic epidermis and thorax closure of the pupal epidermis (*1*). Mutations in genes such as *hemipterous* (*hep*) and *basket* (*bsk*, also known as *DJNK*) result in abnormal embryos with a dorsal hole or abnormal adults with a dorsal midline cleft (*1*, *2*). Hep and Bsk are homologous to the mammalian MKK7 (MAPK kinase 7) and JNK, and they are components of a MAPK (mitogen-activated protein kinase) cascade (*3*). Although the role of the Hep-Bsk cascade during dorsal closure has been extensively studied, the up-

Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan.

*To whom correspondence should be addressed. E-mail: adachi@bio.nagoya-u.ac.jp