Supporting Information

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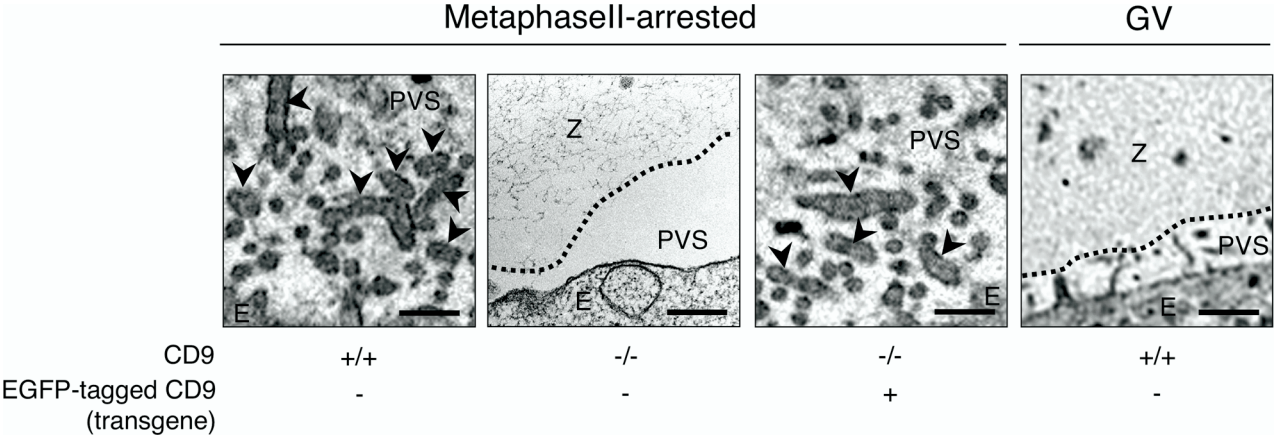

Fig. S1. Electron-microscopic analysis of vesicular structures inside the PVS in $CD9^{+/+}$, $CD9^{-/-}$, and $Tg^{+}CD9^{-/-}$ eggs, showing sectioned microvilli (arrowheads), zona pellucida (Z), the inner margin of zona pellucida (dotted line), egg (E), and perivitelline space (PVS). Metaphase II–arrested and germinal vesicle–stage eggs were collected from superovulated mice. Cumulus-free, zona-intact eggs were treated for electron-microscopic analysis. Scale bars: 350 nm.

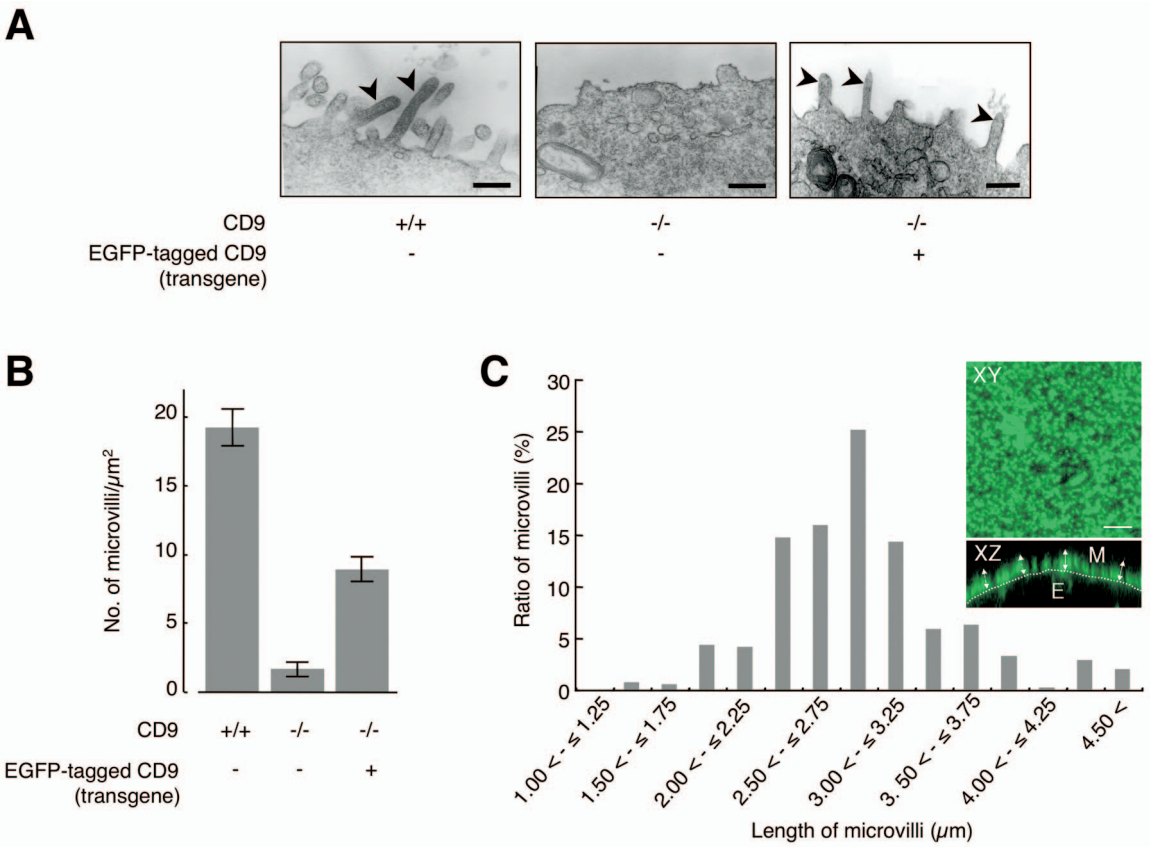

Fig. S2. Observation of microvilli impaired on CD9$^{-/-}$ egg plasma membrane and recovered by exogenous expression of CD9-EGFP in CD9$^{-/-}$ eggs. *A*, Ultrastructural comparison of the egg plasma membrane in CD9$^{+/+}$, CD9$^{-/-}$, and Tg^{+}CD9$^{-/-}$ eggs. Arrowheads indicate microvilli. Scale bar: 200 nm. *B*, Estimated number of microvilli in wild-type, CD9$^{-/-}$, and Tg^{+}CD9$^{-/-}$ eggs (mean ± standard error of the mean [SEM]). In each of 10 photographs, the microvilli were counted and the number of microvilli per μm^2 estimated. *C*, Distribution of microvillar length in zona-free Tg^{+}CD9$^{-/-}$ eggs without fixation (microvilli examined, n = 1,115; eggs examined, n = 20). The average length (2.93 ± 0.02 μm) was comparable to that of zona-free wild-type eggs treated with glutaraldehyde (2.82 ± 0.32 μm; microvilli examined, n = 222; eggs examined, n = 10). In the Tg^{+}CD9$^{-/-}$ eggs, 95% of the microvilli examined were within 4 μm in length. The inset shows representative microvilli observed from XY and XZ orientations (M, microvilli [double-headed arrows]; dotted line, membrane; E, egg cytoplasm). Scale bar: 5 μm.

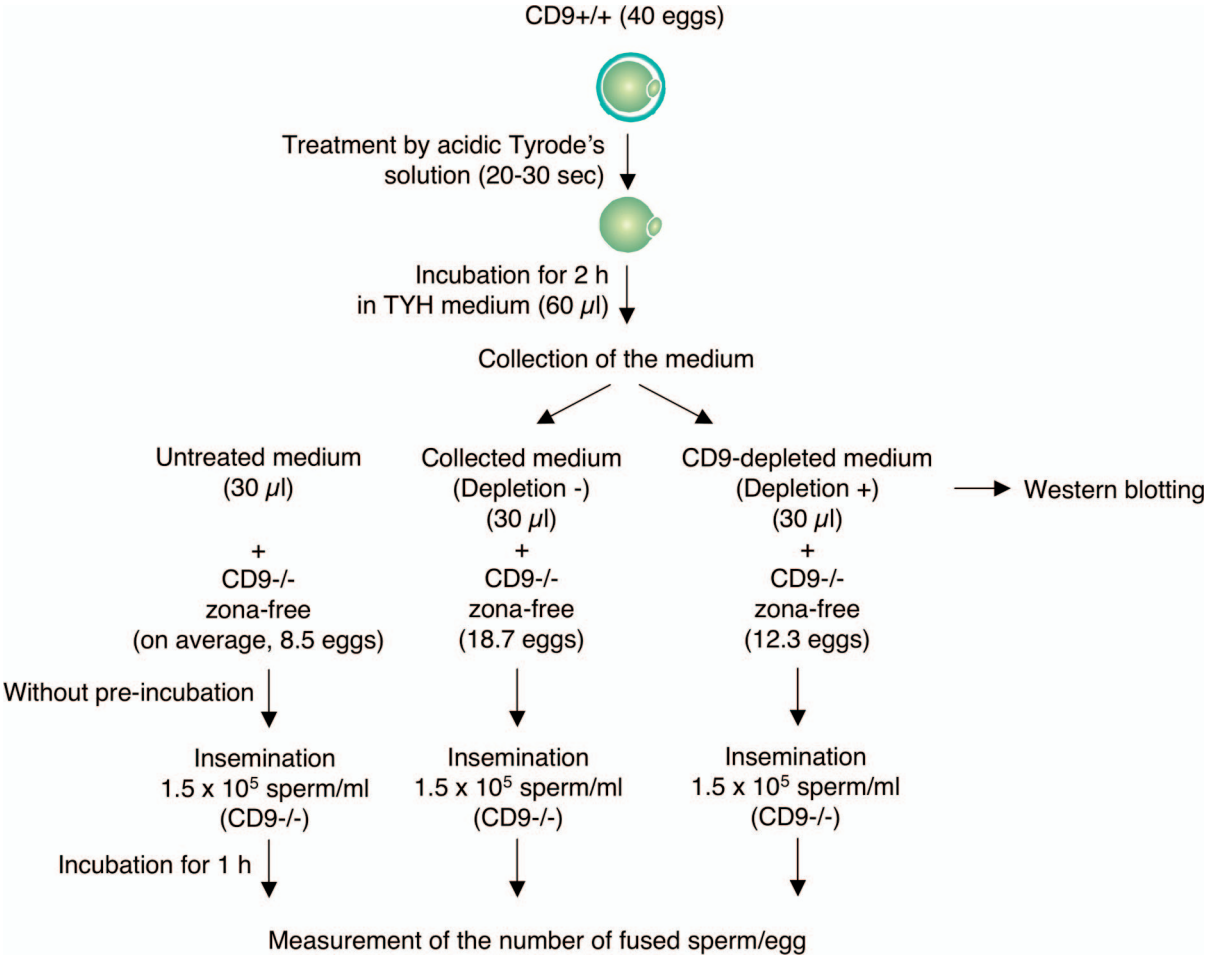

Fig. S3. Experimental procedure for analysis of egg-conditioned medium. To examine the involvement of CD9-incoporated vesicles in sperm–egg fusion, the egg-conditioned medium was collected from zona-free $CD9^{+/+}$ eggs. Soon after the zona pellucida was removed from the eggs using of acidic Tyrode's solution, 40 zona-free eggs were cultured in a 60-μl drop of medium for 2 h. The egg-conditioned medium was used for two subsequent experiments. In experiment 1, the sperm–egg fusion capability of egg conditioned–medium was examined, using $CD9^{-/-}$ eggs with the zona pellucida removed. To measure the number of sperm fused with $CD9^{-/-}$ eggs, the eggs were preloaded with DAPI for 20 min before zona pellucida removal. As the egg-conditioned medium was being collected, zona-free $CD9^{-/-}$ eggs were prepared and incubated in TYH medium. To remove the contamination of CD9-containing materials from males, we used the $CD9^{-/-}$ sperm. Before insemination, the sperm were incubated in TYH medium for 2 h. $CD9^{-/-}$ eggs were transferred into the egg-conditioned medium (30 μl) and, without no preincubation, incubated with $CD9^{-/-}$ sperm at a final concentration of 1.5×10^5 sperm/ml. At 1 h after incubation, the number of DAPI-incorporated sperm on the egg plasma membrane of zona-free $CD9^{-/-}$ eggs (the fused sperm) was measured. CD9-depleted medium was prepared to confirm the capability of the CD9-containing materials in sperm–egg fusion. Half of the collected medium was treated with beads (Protein Coupling Kit for COOH microparticles; Polysciences) conjugated with anti-CD9 mAb for 30 min at room temperature. After incubation in the medium, the beads were removed from the medium by centrifugation (3000 rpm at 20°C for 5 min). The CD9-depleted medium was used as a negative control for measuring the number of sperm fused with eggs. The numbers of eggs examined (average number/drop) were 8.5 $CD9^{-/-}$ eggs (as a negative control), 18.7 $CD9^{-/-}$ eggs (incubated in the egg-conditioned medium), and 12.3 $CD9^{-/-}$ eggs (incubated in the CD9-depleted medium). Alexa488 (green fluorescence)-conjugated anti-CD9 mAb was used to detect CD9 on the egg and sperm membranes. Experiment (2) involved analysis of the egg-conditioned medium by Western blot analysis. The egg-conditioned medium was collected as in experiment (1). The 60 μl of medium containing the remnant material from 40 eggs was precipitated with TCA and was loaded in each lane on the gel (medium volume containing the releasing vesicles from a single egg: 1.5 μl). The protein size and quantity were compared with those of 5 zona-intact eggs.

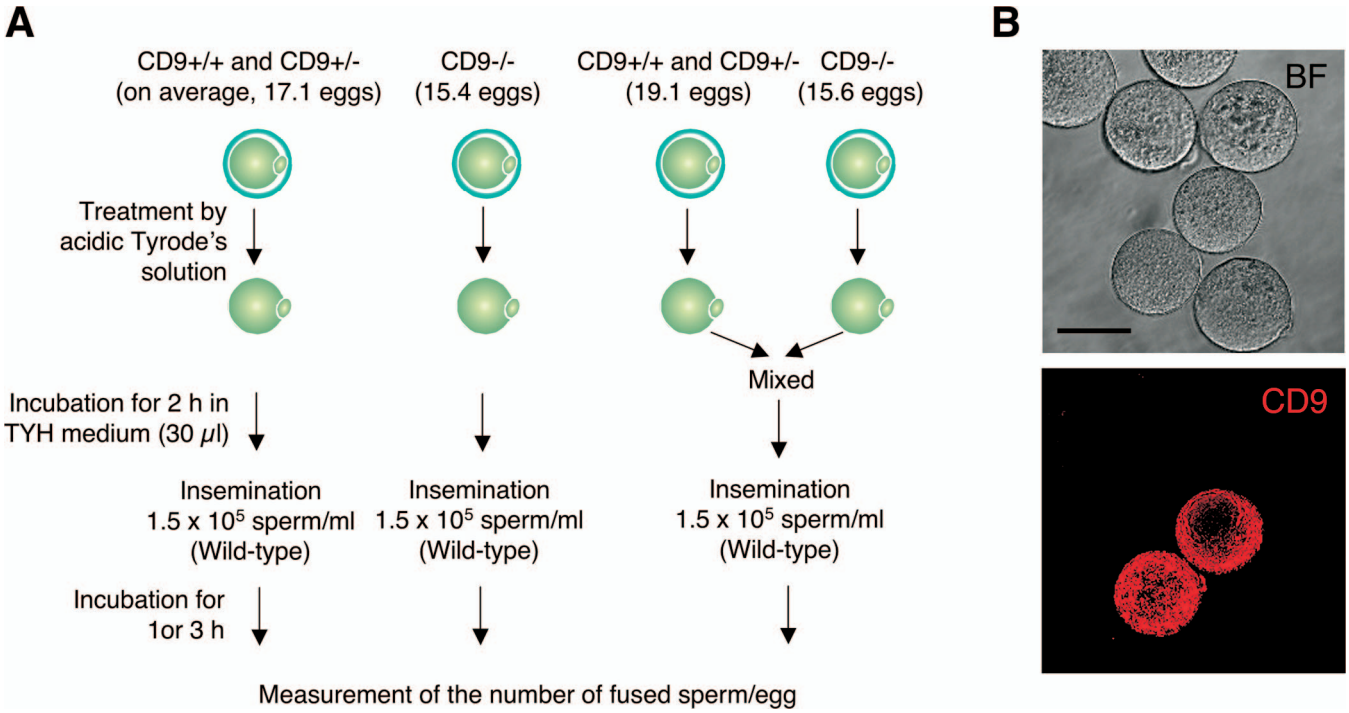

Fig. S4. Improvement of impaired fusion of $CD9^{-/-}$ eggs with sperm through incubation with CD9-expressing eggs. *A*, Experimental procedure. To measure the number of sperm fused with zona-free eggs, $CD9^{-/-}$ eggs were preloaded with DAPI for 20 min before the zona pellucida was removed form the eggs. To identify the egg genotype, $CD9^{-/-}$ eggs were prestained with FM4–64 for 20 min. The zona pellucida was removed from eggs by treatment with acidic Tyrode's solution. Before insemination, the mixed eggs were preincubated in the medium (30 μl) for 2 h. The numbers of eggs examined (average number/drop) were 17.1 $CD9^{+/+}$ and $CD9^{+/-}$ eggs (as a positive control), 15.4 $CD9^{-/-}$ eggs (as a negative control), 19.1 $CD9^{+/+}$ and $CD9^{+/-}$ eggs co-incubated with $CD9^{-/-}$ eggs, and 15.6 $CD9^{-/-}$ eggs co-incubated with $CD9^{+/+}$ and $CD9^{+/-}$ eggs. After preincubation, wild-type sperm were added into the medium (1.5×10^5 sperm/ml). Before insemination, the sperm were incubated in TYH medium for 2 h. After insemination, the mixed eggs were co-incubated in the medium (30 μl) for 1 or 3 h. After incubation, the numbers of DAPI-incorporated sperm on the egg plasma membranes were measured. Alexa488-conjugated anti-CD9 mAb was used for detection of CD9. *B*, Co-incubation of zona-free $CD9^{+/+}$ and $CD9^{-/-}$ eggs (10 h). Alexa546 (red fluorescence)-conjugated anti-CD9 mAb was used to detect CD9 on the egg membranes. Images were projected from *z*-series images. Scale bar: 50 μm.

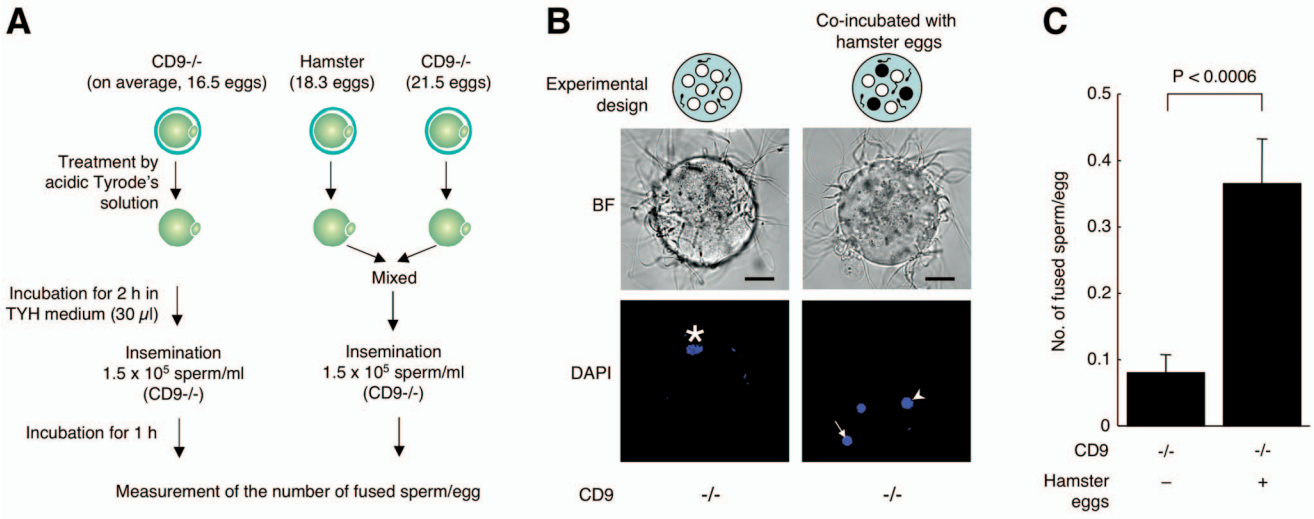

Fig. S5. Improvement of impaired fusion of CD9$^{-/-}$ eggs with sperm by incubation with hamster eggs. *A*, Experimental procedure. To measure the number of sperm fused with zona-free CD9$^{-/-}$ eggs, the eggs were preloaded with DAPI for 20 min before the zona pellucida was removed from the eggs. The zona pellucida was removed from the eggs by treatment with acidic Tyrode's solution. Before insemination, the mixed eggs were preincubated in the medium (30 μl) for 2 h. Mouse eggs are morphologically distinguishable from hamster eggs without staining with FM4–64. After preincubation, wild-type sperm (1.5×10^5 sperm/ml) were added into the medium. Before insemination, the sperm were incubated in TYH medium for 2 h. At 1 h after insemination, the numbers of DAPI-incorporated sperm on the egg plasma membranes were measured. The numbers of eggs examined (average number/drop) were16.5 CD9$^{-/-}$ eggs (as a negative control), 18.3 hamster eggs co-incubated with CD9$^{-/-}$ eggs, and 21.5 CD9$^{-/-}$ eggs co-incubated with hamster eggs. Alexa488 (green fluorescence)-conjugated anti-CD9 mAb was used to detect CD9 on the egg and the sperm membranes. *B*, Reversed fertility of CD9$^{-/-}$ eggs by co-incubation with hamster eggs. As shown in the experimental designs, CD9$^{-/-}$ eggs (white circles) were co-incubated with hamster eggs (black circles). The CD9$^{-/-}$ eggs were preloaded with DAPI to allow counting of the number of fused sperm (arrowhead). Sperm fused with the CD9$^{-/-}$ eggs were counted at 1 h after incubation. Metaphase II–arrested chromosomes are indicated by *; the extruded second polar body, by an arrow. Images were projected from z-series images. Scale bar: 20 μm. *C*, The sperm fused with the zona-free CD9$^{-/-}$ eggs were counted at 1 h after incubation (mean ± SEM) in sextuplicate experiments. The CD9$^{-/-}$ eggs were co-incubated with hamster eggs; as a negative control, CD9$^{-/-}$ eggs were incubated with sperm without the hamster eggs. The total numbers of eggs examined were 99 CD9$^{-/-}$ eggs (as a negative control) and 129 CD9$^{-/-}$ eggs co-incubated with hamster eggs ($n = 110$).

Table S1. Primer sets for genotyping and RT-PCR

Target	Primer name	Primer sequence
CD9 gene*	C1	5′-CCTCCCTCAGGAGTGTACATTC-3′
	C2	5′-GAGGAACCCGAAGAACTAGAAGAC-3′
	N1	5′-AATGGGCTGACCGC TTCCTCG-3′
CD9-EGFP transgene†	E1	5′-TGAACCGCATCGAGCTGAAGGG-3′
	E2	5′-GAATATCACCAAGAGGAACC-3′
GM3 synthase transcripts‡	SAT-1F	5′-AGGTACAGCATCAACTTGGAGCCT-3′
	SAT-1R	5′-TGGCGTAGTATTCAACGTCCGACA-3′
CD9 transcripts‡	CD9F	5′-GGCATTGCAGTGCTTGCTATTGGA-3′
	CD9R	5′-TCACCAAGAGGAACCCGAAGAACA-3′
GAPDH transcripts‡	GAPDHF	5′-TGTGATGGGTGTGAACCACGAGAA-3′
	GAPDHR	5′-TTGTCATTGAGAGCAATGCCAGCC-3′

*The three primers for genotyping were C1, C2, and N1; the C1C2 PCR product for the wild-type CD9 allele was 670 bp, and the C1N1 PCR product for the recombinant CD9 allele was 480 bp; 93°C for denaturation (30 s), 60°C for annealing (30 s), and 72°C for extension (30 s); 30 cycles.

†The two primers for the CD9-EGFP transgene were E1 and E2; the E1E2 PCR product was 580 bp; 93°C for denaturation (30 s), 60°C for annealing (30 s), and 72°C for extension (30 s); 30 cycles.

‡RT-PCR was performed to evaluate the expressions of GM3 synthase (ST3GalV/SAT-1), CD9, and GAPDH. Primers for mouse SAT-1 mRNA were SAT-1F and SAT-1R; the product of SAT-1F and SAT1R was 370 bp. Primers for mouse CD9 mRNA were CD9F and CD9R; the product of CD9F and CD9R was 220 bp. The set of primers for GAPDH was GAPDHF and GAPDHR; the product of GAPDHF and GAPDHR was 525 bp. 93°C for denaturation (30 s), 55°C for annealing (30 s), and 72°C for extension (30 s); 30 cycles.