Normal Fertilization Occurs with Eggs Lacking the Integrin $\alpha_6\beta_1$ and Is CD9-dependent

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Abstract. Previous results, based on inhibition of fertilization by an anti-$\alpha_6$ integrin mAb (GoH3), suggest that the $\alpha_6\beta_1$ integrin on mouse eggs functions as the receptor for sperm (Almeida, E.A., A.P. Huovila, A.E. Sutherland, L.E. Stephens, P.G. Calarco, L.M. Shaw, A.M. Mencurcio, A. Sonnenberg, P. Primakoff, D.G. Myles, and J.M. White. 1995. Cell. 81:1095–1104). Because the egg surface tetraspanin CD9 is essential for gamete fusion (Kaji, K., S. Oda, T. Shikano, T. O huku, Y. U ematsu, J. Sakagami, N. Tada, S. M iyazaki, and A. Kudo. 2000. Nat. Genet. 24:279–282; Le Naour, F., E. R ubinstein, C. Jasmin, M. P renant, and C. Bouchex. 2000. Science. 287:319–321; Miyado, K., G. Y amada, S. Y amada, H. A suwa, Y. N akamura, F. R yu, K. S uzuki, K. K o sai, K. I noue, A. O gura, M. O kabe, and E. M ekada. 2000. Science. 287:321–324). Using eggs from cultured ovaries of mice lacking the $\alpha_6$ integrin subunit, we found that the fertilization rate, fertilization index, and sperm binding were not impaired compared with wild-type or heterozygous controls. Furthermore, a reexamination of antibody inhibition, using an assay that better simulates in vivo fertilization conditions, revealed no inhibition of fusion by the GoH3 mAb. We also found that an anti-CD9 mAb completely blocks sperm fusion with either wild-type eggs or eggs lacking $\alpha_6\beta_1$. Based on these results, we conclude that the $\alpha_6\beta_1$ integrin is not essential for sperm–egg fusion, and we suggest a new model in which CD9 acts by itself, or interacts with egg protein(s) other than $\alpha_6\beta_1$, to function in sperm–egg fusion.

Key words: sperm–egg fusion • tetraspanin • membrane adhesion • oocyte • ovarian culture

Introduction

Sperm–egg binding and fusion is critical to the initiation of development in many organisms, but identification of the molecules involved in the adhesion, fusion, and signaling is incomplete. In the present study, we examined the role of molecules on the egg surface that have been implicated in the process of gamete binding and fusion. On the surface of the mammalian egg, two proteins, the integrin $\alpha_6\beta_1$ and the tetraspanin family member CD9, have been reported to act in sperm–egg binding and fusion. Integrins are transmembrane $\alpha\beta$ heterodimers that play crucial roles in cell–cell adhesion, cell–extracellular matrix adhesion, and multiple signaling pathways (Yamada, 1997). The $\alpha_6\beta_1$ integrin was first proposed as the receptor for sperm on mouse eggs by Almeida et al. (1995). Previously, $\alpha_6\beta_1$ had been found to be a laminin receptor whose adhesion activity was blocked by the anti-$\alpha_6$ mAb GoH3 (Sonnenberg et al., 1988). Almeida et al. (1995) reported that GoH3 also inhibited sperm–egg binding and fusion. Subsequently, the role of the $\alpha_6\beta_1$ integrin in sperm–egg binding and fusion was called into question. All the binding and fusion assays of Almeida et al. (1995)
were done with eggs where the extracellular coat (the zona pellucida) was removed by protease digestion. Evidene suggests that the inhibitory effects of the GoH3 antibody on sperm–egg binding and fusion depends on zona removal techniques (E vans et al., 1997). In addition, GoH3 was found not to inhibit sperm–egg fusion in in vitro fertilization assays using zona intact eggs (E vans, 1999). The conflicting evicence from these different studies has left unclear whethe

Recently, another egg surface protein, CD 9, has been discovered to play an essential role in sperm–egg fusion (Chen et al., 1999; K aji et al., 2000; L e N aour et al., 2000; M iyado et al., 2000). CD 9 is a member of the transmembrane 4 superfamily, also called the tetraspanin family. Transmembrane 4 superfamilies contain four transmembrane domains, two extracellular loops, one cytoplasmic loop, and cytoplasmic amino and carboxyl termini. Chen et al. (1999) found that anti-CD 9 mAbs inhibited sperm–egg binding and fusion. The involvement of CD 9 in sperm–egg fusion has been confirmed using CD 9 knockout mice. The phenotype of the CD 9 mutant mice is restricted to infertility in females, despite the broad tissue distribution of CD 9. Eggs from the CD 9 knockout mice bind sperm normally, but are severely inhibited in their ability to fuse with sperm (K aji et al., 2000; L e N aour et al., 2000; M iyado et al., 2000). Moreover, the tetraspanins CD 9 and CD 81 appear to play a role in the fusion of myoblasts to form myotubes during muscle cell differentiation (Tachibana and H emler, 1999).

Tetraspanins are generally thought to work through various interactions with other cell-surface proteins such as CD 4, CD 8, CD 19, CD 21, major histocompatibility complexes I & II, integrins, and cytoplasmic signaling molecules (H orejsi and V icek, 1991; W right and T omlinson, 1994; C armo and W right, 1995; H emler et al., 1996; B erditchevski et al., 1997; M aeker et al., 1997; Y auch et al., 1998). Thius, in membrane fusion, tetraspanins may be best thought of as fusion facilitators rather than receptors, and may enlist/direct other cell-surface molecules to function in the fusion process. One reported exception to this concept is CD 81, a receptor for the hepatitis C virus (P ileri et al., 1998).

The physical association of tetraspanins and integrins has been established in various cell types (H emler, 1998; P orter and H ogg, 1999). In fertilization, it has been suggested that CD 9 may act through association with the α 6β 1 integrin in sperm–egg binding and fusion (Chen et al., 1999; K aji et al., 2000; L e N aour et al., 2000; M iyado et al., 2000). The data presented here show that gamete binding and fusion can proceed normally in the absence of the α 6 integrin, and are consistent with the conclusion that CD 9 is essential for sperm–egg fusion, but acts either alone or through interactions with proteins other than α 6β 1.

Materials and Methods

Genotyping of α 6 Knockout Mice

The generation of the total α 6 integrin knockout mice (with a deletion of both A and B forms) has been described previously (G eorges-L abouesse et al., 1996). The mice lacking the α 6 integrin subunit are born alive with severe skin blistering, reminiscent of epidermolysis bullosa (G eorges-L abouesse et al., 1996). This condition makes the identity of homozygous knockouts obvious. To confirm the genotypes of homozygotes and littermates, a PCR-based genotyping system was used. Two separate reactions were used to detect the mutant and wild-type alleles. The mutant allele was detected by using the forward primer S G-TG-ATACCAAATGTGGTCAAG-3′, which is located in the α 6 intron upstream from the neomycin cassette insert, and the reverse primer S G-TCAAGAGCCCATATGGC-3′, which is located in the neomycin cassette (G IBCO B RL). These two primers produce an 820-bp band in homozgyous and heterozygous mice. The wild-type allele was detected using the forward primer S G-TG-ATTAACTCCTGATGTCTACG-3′ and reverse primer S G-TCTG-CAGCGGGAGTGCTTC-3′ (G IBCO B RL). These primers are within the region deleted in the mutant allele and give a product of 500 bp in wild-type mice. The PCR parameters were as follows: 3-min denaturation at 94°C, 34 cycles of amplification at 94°C (for 1 min), 55°C (for 1 min), 72°C (for 1 min), followed by 72°C for (10 min) using a R obocycler (S trategene).

Egg Isolation

Because pups died shortly after birth, it was necessary to isolate ovaries within 1-2 h after birth. Ovaries were dissected from newborn wild-type, heterozygous, and homozygous C57/B16 pups and placed into minimum essential medium ( M EM; G IBCO B RL) at 37°C. They were prepared for grafting by removing the majority of the ovarian bursa. The ovaries were implanted under the kidney capsule of recipient females (C ox et al., 1996). In brief, adult female C57/B16 mice were anesthetized, their ovaries removed, and the newborn ovaries were placed under the kidney capsule. The ovaries were sutured and the mice revived. The grafted ovaries matured in the recipients for 21-30 d. 2 d before the isolation of the grafted ovaries, recipient mice were injected with 10 IU pregnant mares’ serum gonadotropin (Calbiochem or Sigma Chemical Co.) 48 h after the injection, the ovaries were surgically removed. The removed ovaries were placed into medium 199 ( M 199, G IBCO B RL), supplemented with 3.5 mM sodium pyruvate, 1.00 IU penicillin-streptomycin (G IBCO B RL), and 5% F BS (G IBCO B RL). M 199 containing sodium pyruvate and penicillin-streptomycin will be designated as M 199*. Germinal vesicle stage oocytes were teased from the ovaries using 26 gauge needles (B epton D ickson). Granulosa cells were removed from the oocytes by passing the oocytes through a thin bore pipette while transferring through two drops of clean medium. The oocytes were allowed to spontaneously mature for 16-18 h in M 199* + 5% F BS at 37°C in 5% CO 2 . M ature metaphase II eggs were selected for use in experiments.

In Vitro Sperm–Egg Binding and Fusion Assay with Wild-type and Mutant Oocytes Isolated from Ovaries

Eggs, isolated as described, were treated with 10 μg/ml chymotrypsin in M 199* + 0.3% B SA (S igna Chemical Co.) for 3 min to loosen the zona pellucidae. The zona pellucida was removed from the eggs because overnight culture can lead to modification (hardening) of the zona and, consequently, a reduction in fertilization rates. The treated eggs were transferred through three clean drops of medium using a narrow bore pipette to mechanically remove the loosened zonae. Zona-free eggs were pre-loaded with 4,6-diamidino-2-phenylindole dihydrochloride (D A PI) (P olysciences, I nc.) at 30 μg/ml in M 199* + 0.3% B SA for 15 min at 37°C, 5% CO 2. A fter D A PI treatment, the eggs were washed through three drops of clean M 199* + 0.3% B SA. The eggs were allowed to recover for 3 h before insemination.

Sperm for the in vitro adhesion and fusion assay were isolated from the cauda epididymis and the vas deferens of 10-12 wk-old male C57/B16 mice (H arrian Sprague D awley, I nc.). Dissected caudae and vas deferens were placed into M 199* + 3.0% B SA and were stripped of sperm. The sperm were allowed to dissociate for 15 min at 37°C in 5% CO 2 . Sperm were capacitated for 3 h in M 199* + 3.0% B SA at a 1:10 dilution of the initial sperm suspension. This procedure results in a population of 60-70% acrosome-reacted sperm (M oller et al., 1990).

Sperm and eggs were coincubated for 40 min at 37°C in 5% CO 2. The eggs were scored for binding and fusion. Binding was scored under the light microscope at a magnification of 20. Fusion was scored by the fluorescent labeling of sperm nuclei by D A PI transfer from inside the pre-loaded eggs. The fertilization index (F I, the number of fused sperm per egg), and the fertilization rate (F R, the percentage of eggs fused with at least one sperm) were calculated. To test the effect of the anti-CD 9

Abbreviations used in this paper: D A PI, 4,6-diamidino-2-phenylindole dihydrochloride; F I, fertilization index; F R, fertilization rate.
mAb KMC8.8 on the fertilization of mutant and wild-type eggs; zona-free eggs were preincubated for 30 min with 50 μg/ml KMC8.8 in M199 + 0.3% BSA. After the 30 min preincubation, sperm were added (final concentration 3 × 10^5 sperm/ml) to the drop containing the eggs and antibody. The gametes were co-incubated for 40 min. Binding and fusion were assessed as described above. The control antibodies used were of the same species (rat) and isotype (IgG2a,k) as KMC8.8.

**Cumulus-intact Egg Assay for In Vitro Fertilization**

To carry out in vitro fertilization assays with cumulus-intact eggs, 6-10-wk-old female ICR mice (Charles River) received pregnant mares’ serum gonadotropin and 48 h later human chorionic gonadotropin. 14 h after hCG injection, cumulus masses containing eggs were isolated from the ampulla. The cumulus masses were washed through two 500-μl drops of M199 + 0.3% BSA, and then placed into a 50-μl drop of the same medium. A nontoxic (500 μg/ml GoH3 or 50 μFg M199) were added to the drop with cumulus masses and preincubated for 45 min. A fter preincubation, sperm capacitated for 1.5 h at 37°C, 5% CO₂ in M199 + 3.0% BSA were added to the cumulus masses at a final concentration of 1-5 × 10⁸ sperm/ml. Gametes were allowed to coinubate overnight. Two cell embryos were counted to assess the FR. To determine if sperm penetrated the zona pellucida, the presence of sperm in the perivitelline space was scored using a Zeiss Axiophot microscope.

**Indirect Immunofluorescence with Zona-free Eggs**

Zona-free eggs were prepared and allowed to recover as described above. A fter the recovery period, eggs were incubated with either 50 μg/ml KMC8.8 (PharMingen) or 100 μg/ml GoH3 (PharMingen or Immunotech) for 45 min at 37°C, 5% CO₂. The medium used for the primary antibody incubation was M199 + 0.3% BSA. The eggs were transferred through two 100-μl wash drops containing PBS + 0.1% polyvinyl alcohol (PVA; Sigma Chemical Co.), fixed for 12 min using 4% paraformaldehyde in PBS + 0.1% PVA, and transferred through two wash drops containing M199 + 0.3% BSA. A n Oregon green™-conjugated goat anti-rat secondary antibody (Molecular Probes) was used to determine the localization of the primary antibody binding. Staining was visualized using a laser scanning confocal microscope (model LSM 410; Carl Zeiss). The control antibodies used were of the same species and isotype as the primary antibody being tested, unless otherwise noted.

**Indirect Immunofluorescence with Cumulus-intact Eggs**

Cumulus masses were collected as mentioned above except using M199 + 0.1% PVA. Cumulus masses were washed through three 500-μl drops of fresh medium. Primary antibody staining with either GoH3 (500 μg/ml), KMC8.8 (50 μg/ml), or irrelevant IgG was carried out in 50-μl drops of the same medium for 45 min, followed by washing the cumulus masses through three 500-μl drops of medium. Secondary antibody staining was done with an Oregon green™-conjugated goat anti-rat antibody in a 500-μl drop for 45 min, and cumulus masses were washed through three 500-μl drops. Cumulus-intact eggs were viewed using an LSM 410 confocal microscope.

**Calcium Imaging**

Zona-free eggs were mixed with 25 μg/ml Oregon green™-BA PTA A M (Molecular Probes) for 1 h at 37°C, 5% CO₂. Once loaded, the eggs were washed through three drops of M199 + 0.3% BSA. Eggs were inseminated on the microscope stage using an open perfusion microincubator (Medical Systems Corp.) to keep the fertilization drop at 37°C. Calcium oscillations were observed using an LSM 410 confocal microscope. Sperm-egg fusion was assessed by DAPI transfer. These techniques are described in detail in Faure et al. (1999).

**Results**

**Eggs from Mice Lacking the α6 Gene Are Morphologically Normal and Do Not Have the α6 Integrin Subunit on the Egg Surface**

Mice lacking the α6 gene die shortly after birth. Following previous work of Cox et al. (1996), we developed a method to remove ovaries from wild-type (α6 +/+) , heterozygous (α6 ++/−) , or homozygous (α6 −/−) females at birth, culture these ovaries for 3-4 wk, and recover fertilizable eggs (see Materials and Methods). E ggs from wild-type (α6 ++/+) or heterozygous (α6 ++/+ ) ovaries were equivalent to each other in fertilization assays, were used interchangeably, and are referred to as control eggs or α6 ++ eggs. E ggs from homozygous, mutant (α6 −/−) ovaries are termed α6 − eggs. Previous expression studies of the α6 integrin subunit in the developing embryo indicated a potential role in ovarian development (Fröjdman et al., 1995; Zuccotti et al., 1998). A fter maturation of eggs from α6 −/− females, we observed normal egg morphology. Cultured α6 ++ and α6 − eggs are morphologically similar (Fig. 1, A and B). The percentages of cultured control eggs or cultured α6 − eggs that produced first polar bodies after the overnight culture were 75 ± 5% and 76 ± 4%, respectively (mean ± SEM, n = 4). These data indicate that the integrin α6β1 does not have a required role in ovary development or oogenesis because apparently normal eggs, capable of fertilization (see below), can be isolated from α6 −/− pups via our culture method.

As expected, eggs isolated from α6 −/− ovaries do not bind the anti-α6 mAb GoH3 (Fig. 2 B). α6 − eggs, used as controls, showed the typical pattern of GoH3 binding to the zona pellucida (Fig. 2 A).

**Eggs Lacking the α6 Integrin Subunit Are Fully Functional in Sperm–Egg Binding and Fusion**

We directly tested the requirement for α6β1 in sperm–egg plasma membrane binding and fusion by testing the ability of mature eggs lacking the α6 subunit to bind to and fuse with sperm. Immature eggs were collected from transplanted ovaries of wild-type and α6 −/− mice, the granulosa cells were removed to induce oocyte maturation, and oocytes were cultured overnight to obtain metaphase II eggs for in vitro fertilization assays. A fter culturing, the zona pellucida was removed by chymotrypsin, and the eggs were used for in vitro fusion assays. The following three parameters in these assays were measured: (1) FR, the percent of eggs fused with at least one sperm; (2) FI, the total number of fused sperm/total number of eggs; and (3) the mean number of sperm bound to the equator of the egg. T here was no reduction in the number of sperm bound or fused with eggs lacking α6 compared with wild-type eggs (Fig. 3). In seven experiments, using a total of 284 eggs, the mean FR for the cultured control eggs was 56 ± 8% (FR ± SEM). T he fertilization rate for the α6 − eggs, in parallel experiments, was 59 ± 9%. T he FI of α6 − and α6 ++ eggs were equivalent (Fig. 3 B). In seven experiments, using a total of 230 eggs, the mean FI for both the cultured control eggs and the cultured α6 − eggs was 0.67 ± 0.09 and 0.70 ± 0.1 (FI ± SEM). T he mean number of sperm bound to the egg equator was 5.3 ± 2.8 sperm/egg with cultured control eggs and 7.8 ± 2.1 sperm/egg with α6 − eggs. T he increased level of sperm binding to α6 − eggs in this assay is not significantly different than control eggs.
In addition to the in vitro assays carried out using standard procedures, in some experiments we directly observed the interactions of sperm and egg binding and fusion using a microscope equipped with Hoffman modulation optics. The characteristics of sperm binding were similar in assays using α6-2 eggs and control eggs. Sperm were bound via both their tip and lateral head regions, presumably corresponding to the inner acrosomal and equatorial regions of the sperm membrane (data not shown).

Sperm Can Initiate Calcium Oscillations in Eggs Lacking the α6 Integrin Subunit

Even though α6β1 was not required for sperm–egg binding or fusion, we considered the possibility that α6β1 was a required component in sperm-egg signaling. A hallmark of early signaling in the egg is the series of transient \([\text{Ca}^{2+}]_{i}\) oscillations that occur directly after sperm binding/fusion. It has been suggested that integrins may participate in initiation and/or propagation of the calcium signal during egg activation (Fenichel and Durand-Clement, 1998). Therefore, we asked if sperm could initiate normal \([\text{Ca}^{2+}]_{i}\) oscillations in eggs lacking the α6 integrin subunit. α6-1 and α6-2 eggs were loaded with a calcium-sensitive dye, Oregon green™ BAPTA-AM, and fertilized under conditions where \([\text{Ca}^{2+}]_{i}\) oscillations could be observed. We observed no difference in the calcium oscillations of α6-2 eggs as compared with α6-1 eggs. Calcium oscillations in the α6-1 eggs showed typical time of onset, amplitude, and frequency (Fig. 4; Faure et al., 1999).

The Anti-α6 mAb GoH3 Has No Effect on Sperm Fusion with Cumulus-intact Eggs In Vitro

Our results with the α6-1 eggs led us to reexamine the interpretations of previous GoH3 inhibition studies (Almeida et al., 1995; Evans et al., 1997; Evans, 1999). In vivo, the egg is ovulated and fertilized while it is surrounded by its coat (the zona pellucida) and outside the zona, a shell of \(~3,000\) cumulus cells. The following three types of in vitro fertilization assays have been developed using: (1) zona-free eggs, obtained by removing the cumulus cells by treatment with crude preparations of hyaluronidase and removing the zona pellucida by brief acid treatment or protease digestion; (2) zona-intact eggs, obtained by removing only the cumulus cells by treatment with hyaluronidase; and (3) cumulus-intact eggs, which receive no

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**Figure 1.** Differential interference contrast micrographs of (A) cultured wild-type egg and (B) α6 knockout egg.

**Figure 2.** Indirect immunofluorescent staining of zona-free eggs using the anti-α6 integrin mAb GoH3. (A) Cultured α6-1 egg; arrows delineate the microvillus region from the area overlying the metaphase plate. (B) A cultured α6-2 egg. Eggs incubated with the secondary antibody alone showed no staining.
treatments. Use of zona-free eggs for sperm–egg fusion assays is common because it removes any possible influence of the zona pellucida and cumulus cells. However, the assay using cumulus-intact eggs most closely resembles in vivo fertilization. Because a question had been raised previously by Evans et al. (1997) about the in vitro assay using eggs, where the zona pellucida was removed with chymotrypsin (Almeida et al., 1995), we decided to test GoH3 with cumulus-intact eggs. Wild-type cumulus-intact eggs were preincubated with either GoH3 or control antibodies for 45 min and were inseminated with capacitated sperm. Sperm and eggs were allowed to coincubate overnight, and cultures were checked for two cell embryos 24 h later. The FR of the cumulus-intact eggs incubated with 500 μg/ml of GoH3 or of an irrelevant IgG were equivalent: 57 ± 14% and 58 ± 6%, respectively (FR ± SEM, n = 4, Fig. 5 A).

To determine if this assay could accurately measure an-
tibody inhibition, we used the anti-CD 9 antibody K M C 8 . 8 as a control. Using cumulus-intact wild-type eggs in the same assay, we measured a 95% inhibition of fertilization in the presence of 50 μg/ml K M C 8 . 8 antibody. The average fertilization rate for control eggs was 58% ± 12%, but for K M C 8 . 8 -treated eggs, the fertilization rate was reduced to 3% ± 1% (Fig. 5 B, n = 4). Neither the G O H 3 nor K M C 8 . 8 antibodies inhibited sperm passage through the cumulus or zona as sperm were observed in the perivitelline space of antibody-treated unfertilized eggs. Diffusion of G O H 3 or K M C 8 . 8 to the egg plasma membrane was apparently not impeded in cumulus-intact eggs as shown by indirect immunofluorescence staining of the egg plasma membrane by both antibodies (Fig. 5, C and D).

α6β1 Is Not Required for the Function of CD9 during Sperm–Egg Binding and Fusion

Previous studies have reported that CD 9 has a role in sperm–egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000) or binding and fusion (Chen et al., 2000). These same studies have suggested that CD 9 may function in cooperation with α6β1. To directly test the model that during sperm–egg binding and fusion CD 9 functions via associations with α6β1, we tested the effects of the anti-CD 9 antibody, K M C 8 . 8 , on cultured α6+ and α6− eggs. Sperm fusion with both α6+ and α6− eggs was completely inhibited in the presence of 50 μg/ml K M C 8 . 8 (Fig. 6). No apparent decrease in sperm binding was observed in the presence of either the control IgG or the K M C 8 . 8 antibodies (data not shown).

Discussion

Isolation of morphologically normal eggs from ovaries of α6 (−/−) knockout pups allowed us to measure if eggs lacking the α6β1 integrin bind and fuse with sperm. The fertilization rate and index both demonstrate that the α6β1 integrin is not essential for sperm–egg binding and fusion. Furthermore, the inhibition of sperm fusion with α6− eggs in the presence of an anti-CD 9 antibody shows that CD 9 acts alone or in association with proteins other than α6β1 to function in gamete binding and fusion.

Earlier evidence suggested the α6β1 integrin, on the egg surface, played a crucial role in sperm binding leading to fusion (Aimeida et al., 1995). The primary finding in support of this hypothesis was that in vitro fertilization as-

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Inhibition of fusion of both α6+ and α6− eggs by the anti-CD 9 mAb (K M C 8 ) at 50 μg/ml. Controls were carried out for both types of eggs with either no antibody or a rat control IgG (50 μg/ml), n = 3 for each condition, 181 eggs total.

In light of our current data, we asked why do the α6 knockout results not agree with previous findings implicating α6, specifically G O H 3 inhibition (Aimeida et al., 1995). Experiments that initially suggested a role for α6β1 during fertilization used a protease (chymotrypsin) treatment to prepare the zona-free eggs for the in vitro fertilization assays (Aimeida et al., 1995). The protease treatment may modify some egg plasma membrane proteins, resulting in a loss of function of some protein(s) and/or a modification of critical protein interactions. In either way, chymotrypsin could make the egg susceptible to inhibition by the G O H 3 antibody. E vans et al. (1997) have raised the question of whether the technique used to remove the zona may dictate G O H 3's effect on binding. The published data are as follows: (1) G O H 3 inhibits binding when the protease method is used to remove the zona (Aimeida et al., 1995; E vans et al., 1997); (2) G O H 3 does not inhibit binding when acid treatment is used to remove the zona (E vans et al., 1997); and (3) G O H 3 does not inhibit fertilization of zona-intact eggs (E vans, 1999).

Because fertilization could be inhibited in assays using chymotrypsin-treated eggs, but not acid-treated or zona-intact eggs, we chose to test the ability of the G O H 3 mAb GoH3 to inhibit fusion using cumulus-intact eggs. The assay with cumulus-intact eggs is the closest experimental system to in vivo fertilization as it avoids the use of both crude preparations of hyaluronidase to remove the cumulus cells and chymotrypsin or acid to remove the zona. Data from the assay with cumulus-intact eggs demonstrate that G O H 3 has no effect on fertilization under conditions where neither the cumulus cell layer nor the zona pellucida are disrupted. Experiments demonstrating the inhibitory effect of the anti-CD 9 antibody in the assay with cumulus-intact eggs show that the assay can detect antibody inhibition, and that the zona does not act as a barrier to antibody diffusion to the plasma membrane. Furthermore, immunofluorescent detection of G O H 3 and CD 9 on the plasma membrane of cumulus-intact eggs demonstrates that these antibodies can reach the plasma membrane.

Normal sperm fusion with eggs lacking the α6 integrin subunit could mean that another integrin or receptor can substitute for the α6β1 integrin in knockout eggs. However, our results with cumulus-intact eggs and previous results with zona-intact eggs (E vans, 1999) call into question the physiological significance of the inhibitory effects of G O H 3 on protease-treated, zona-free eggs (Aimeida et al., 1995). In light of the reassessment of inhibitory data with the G O H 3 antibody, and the finding that eggs lacking the α6 integrin subunit are not impaired in sperm–egg binding or fusion, we suggest that α6β1 is not required for sperm–egg binding and fusion.

What other egg surface protein might act as a receptor for sperm? One possibility is a different β1 integrin. A n anti-β1 integrin polyclonal antibody moderately inhibited sperm–egg binding (E vans et al., 1997) and other α integrin subunits, α2, α3, α5, and αV, known to pair with β1, have been reported to be present on the egg surface.
(Taroné et al., 1993; A Imeida et al., 1995; E vans et al., 1995). The possibility also exists that a novel integrin or receptor may be present on the surface of the egg and bind sperm.

A new and exciting development is the discovery of a role for the egg surface protein CD9. A nti-CD9 antibodies were reported to inhibit binding and fusion (Chen et al., 1999). With eggs from CD9−/− females, sperm fusion, but not sperm−egg binding, appeared to be the same at which the lack of CD9 blocked fertilization (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). Our anti-CD9 antibody data correlate well with the knockout data as we observed an inhibition of fusion, but no decrease in sperm binding, in the presence of the K M C8.8 antibody with either wild-type or α6 knockout eggs.

A Major Question to Be Answered Is How Does CD9 Function in the Fusion Process

In other systems, CD9 does not appear to function as a receptor itself, but has associations with several other cell-surface molecules (H orejši and V iclek, 1991; W right and T olminson, 1994; C armo and W right, 1995; H emler, et al., 1996; B erditchevski et al., 1997; M aeker et al., 1997; S chererich et al., 1998; Y auh et al., et al., 1998). Because CD9 associates with the α6β1 integrin in other systems (H emler, 1998) and in mouse eggs (M iyado et al., 2000), the interaction of CD9 and α6β1 has been suggested in models for sperm−egg binding leading to fusion (Chen et al., 1999; K aji et al., 2000; Le Naour et al., 2000) or sperm−egg fusion (M iyado et al., 2000). In the first model, direct or indirect interaction of CD9 with α6β1 would affect α6β1’s ability to bind its sperm surface ligand (Chen et al., 1999; K aji et al., 2000; Le Naour et al., 2000). In the second model, α6β1 transduces signals to CD9 to initiate/promote fusion (M iyado et al., 2000). Relevant to these hypotheses, our data suggest a different model in which CD9 acts by it-