ACADEMIC PRESS

Available online at www.sciencedirect.com
SCIENCE DIRECT®

DEVELOPMENTAL BIOLOGY

Developmental Biology 254 (2003) 226–237
www.elsevier.com/locate/ydbio

None of the integrins known to be present on the mouse egg or to be ADAM receptors are essential for sperm–egg binding and fusion

Zhi-Yong He,[a] Cord Brakebusch,[b] Reinhard Fässler,[c] Jordan A. Kreidberg,[d] Paul Primakoff,[e] and Diana G. Myles[a,*]

[a] *Section of Molecular and Cellular Biology, University of California Davis, Davis, CA 95616, USA*
[b] *Department of Experimental Pathology, Lund University, S-22185 Lund, Sweden*
[c] *Department of Molecular Medicine, Max Planck Institute for Biochemistry, 82152 Martinsried, Germany*
[d] *Department of Medicine, Children's Hospital, and Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA*
[e] *Department of Cell Biology and Human Anatomy, School of Medicine, University of California Davis, Davis, CA 95616, USA*

Received for publication 10 September 2002, revised 1 November 2002, accepted 6 November 2002

Abstract

Antibody inhibition and $\alpha6\beta1$ ligand binding experiments indicate that the egg integrin $\alpha6\beta1$ functions as a receptor for sperm during gamete fusion; yet, eggs null for the $\alpha6$ integrin exhibit normal fertilization. Alternative integrins may be involved in sperm–egg binding and fusion and could compensate for the absence of $\alpha6\beta1$. Various $\beta1$ integrins and αv integrins are present on mouse eggs. Some of these integrins are also reported to be receptors for ADAMs, which are expressed on sperm. Using $\alpha3$ integrin null eggs, we found that the $\alpha3\beta1$ integrin was not essential for sperm–egg binding and fusion. Oocyte-specific, $\beta1$ integrin conditional knockout mice allowed us to obtain mature eggs lacking all $\beta1$ integrins. We found that the $\beta1$ integrin null eggs were fully functional in fertilization both in vivo and in vitro. Furthermore, neither anti-mouse $\beta3$ integrin function-blocking monoclonal antibody (mAb) nor αv integrin function-blocking mAb inhibited sperm binding to or fusion with $\beta1$ integrin null eggs. Thus, function of $\beta3$ or αv integrins does not seem to be involved in compensating for the absence of $\beta1$ integrins. These results indicate that none of the integrins known to be present on mouse eggs or to be ADAM receptors are essential for sperm–egg binding/fusion, and thus, egg integrins may not play the role in gamete fusion previously attributed to them.

Keywords: Integrin; Sperm–egg binding; Membrane fusion; Null mutation; ADAMs

Introduction

It has become widely accepted that sperm binding to an egg integrin is a prerequisite adhesion step for sperm–egg membrane fusion in mammalian fertilization. More specifically, $\beta1$ integrins have been implicated as the egg integrins required for sperm–egg fusion. The concept that an egg integrin is required for membrane fusion originated with the identification of a sperm surface protein fertilin (originally termed PH-30) that was implicated in gamete fusion based on antibody inhibition studies (Primakoff et al., 1987). Fertilin is a heterodimer (α and β subunits), and both subunits belong to the ADAM (*A D*isintegrin *A*nd *M*etalloprotease) family of plasma membrane proteins (Blobel et al., 1992). Because it was known that the soluble disintegrin domains found in snake venom bind to the integrin αIIb$\beta3$ (Adler et al., 1991), it was proposed that disintegrin domains of sperm fertilin would bind to an egg integrin. Subsequently, it was shown that disintegrin domain peptides from fertilin β/ADAM2 and from another ADAM family protein, cyritestin/ADAM3, were able to bind to the egg plasma membrane and inhibit sperm–egg binding and fusion (Almeida et al., 1995; Bigler et al., 2000; Bronson et al., 1999; Evans et al., 1997, 1998; Gichuhi et al., 1997; Gupta et al., 2000; Linder and Heinlein, 1997; Mwethera et al., 1999; Myles et al., 1994; Takahashi et al.,

* Corresponding author. Fax: +1-530-752-7522.
E-mail address: dgmyles@ucdavis.edu (D.G. Myles).

0012-1606/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0012-1606(02)00043-X

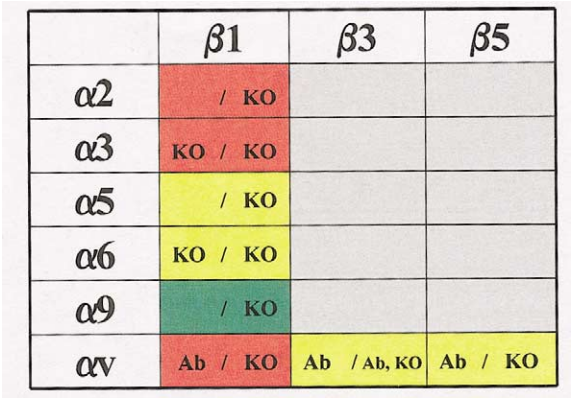

Fig. 1. Summary of integrins present on eggs or reported to be ADAM receptors. Each colored box is a specific α/β heterodimer. A red box represents an integrin reported to be expressed on mouse eggs; a green box represents an integrin reported to be an ADAM receptor, and a yellow box represents an integrin with both properties; gray boxes are irrelevant integrins. Ab represents no inhibition with function-blocking antibody, and KO represents no inhibition testing knockout eggs. If the word Ab or KO is on the left side of the box, it designates the α subunit. If the word Ab or KO is on the right side of the box, it designates the β subunit. [The data are from this study, except for the following published data: α6 KO (Miller et al., 2000); β3 KO (Hodivala-Dilke, et al., 1999); and β5 KO (Huang et al., 2000).]

2001; Waters and White, 1997; Yuan et al., 1997; Zhu et al., 2000).

Several different integrins have been reported on the egg (Almeida et al., 1995; Evans et al., 1995; Tarone et al., 1993) (Fig. 1). Based on the ability of a function-blocking monoclonal antibody (mAb), GoH3, against the α6 integrin subunit to inhibit sperm–egg binding and fusion, it was suggested that $\alpha6\beta1$ was the required integrin (Almeida et al., 1995). This evidence was strengthened by the finding that peptides with a sequence of part or all of the fertilin β disintegrin domain bound to the $\alpha6\beta1$ integrin (Almeida et al., 1995; Chen and Sampson, 1999; Chen et al., 1999; Takahashi et al., 2000). A case for a role for integrins was also made indirectly because integrins (especially α6 and α3) were found to be associated with CD9 in various cells (Nakamura et al., 1995; Rubinstein et al., 1997; Scherberich et al., 1998; Schmidt et al., 1996; Zhang et al., 2001), and eggs from CD9 null mice do not fuse with sperm (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000).

As additional research was reported, the role of the $\alpha6\beta1$ integrin in sperm–egg binding and fusion was called into question. Subsequent to the initial results (Almeida et al., 1995), new antibody inhibition studies using different conditions did not find inhibition of sperm–egg fusion by the GoH3 mAb (Evans et al., 1997; Evans, 1999; Miller et al., 2000). More decisively, α6 integrin KO eggs were fully functional in binding and fusion with sperm, demonstrating that the α6 integrin is not essential for gamete binding and fusion (Miller et al., 2000). Thus, it became clear that $\alpha6\beta1$ was either redundant with other egg integrins or had no role in binding and fusion. These studies led to the suggestion that other integrins, especially β1 integrins, including $\alpha4\beta1$ and $\alpha9\beta1$, may be required (Evans et al., 1997; Ji et al., 1998; Linfor and Berger, 2000; Zhu and Evans, 2002). Involvement of the $\alpha9\beta1$ integrin in sperm–egg membrane interaction was suggested (Eto et al., 2002; Zhu and Evans, 2002), although the α9 integrin has not yet clearly been shown to be present on eggs.

β1 integrins and αv integrins have been described as specifically binding to the disintegrin domain of ADAMs present on sperm (or other cells). Specifically, the β1 integrins include: $\alpha6\beta1$ as a receptor for fertilin β (ADAM2) and ADAM9 (Bigler et al., 2000; Chen and Sampson, 1999; Chen et al., 1999; Nath et al., 2000); $\alpha9\beta1$ as a receptor for ADAM12 and ADAM15 or all ADAMs containing the R(X6)DLPEF motif (Eto et al., 2000, 2002); and $\alpha5\beta1$ as a receptor for ADAM15 (Nath et al., 1999). αv integrins also apparently act as ADAM receptors including: $\alpha v\beta3$ as a receptor for ADAM15 and ADAM23 (Cal et al., 2000; Nath et al., 1999, 2000; Zhang et al., 1998), and $\alpha v\beta5$ as a receptor for ADAM9 (Zhou et al., 2001).

To test the hypothesis that alternative egg integrins are functioning in parallel with $\alpha6\beta1$ or in lieu of $\alpha6\beta1$ when it is deleted, we investigated whether other integrins are required for gamete fusion. We concentrated on integrins that are present on mouse eggs and/or have been demonstrated to be ADAM receptors (Fig. 1). First, we tested the ability of sperm to bind to and fuse with oocytes that were null for either the α3 integrin or all β1 integrins. We also used eggs null for the β1 integrins in combination with function-blocking antibodies for other integrins (αv and β3) to cover all previously unexplored integrins reported to be present on oocytes or known as ADAM receptors. Our data indicate that none of these integrins are essential for fertilization.

Materials and methods

Generation of mature α3 KO eggs

Because α3 integrin knockout pups die within a few hours of birth (Kreidberg et al., 1996), it is necessary to culture their ovaries to obtain mature oocytes. The ovaries were dissected from newborn pups and were cultured under the capsule of the adult female kidney as previously described (Miller et al., 2000). In brief, the ovaries of newborn knockout, heterozygous, or wild type (WT) pups were placed into minimum essential medium (MEM; GIBCO BRL) at 37°C. After most of the ovarian bursa was removed under the microscope, the ovaries were implanted under the kidney capsule of female C57/B16 mice. The ovaries were matured in the host mouse for 21 days. The recipient mice were injected with pregnant mares' serum gonadotropin (PMSG; Sigma), and 48 h later, the ovaries were removed and placed in medium M199 (GIBCO BRL) with 3.5 mM sodium pyruvate, 1000

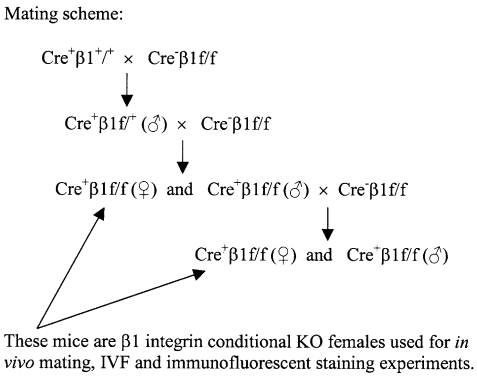

Fig. 2. Matings used to produce β1 integrin conditional knockout female mice. Cre^+ represents the mice with the ZP3-Cre transgene. Cre^- represents the mice without the ZP3-Cre transgene. β1f is the floxed β1 integrin allele, and $\beta1^+$ is the wild type β1 integrin allele.

IU penicillin–streptomycin (M199*), and 5% FBS (GIBCO BRL). Germinal vesicle-stage oocytes were teased from the ovaries. Granulosa cells were removed mechanically by pipetting. The oocytes were cultured in the M199* medium at 37°C and 5% CO_2 for 16–18 h. Mature metaphase II-arrested eggs were selected for in vitro fertilization (IVF) assays.

Generation of β1 integrin conditional knockout mice by the Cre/loxP recombinant system

Oocyte-specific β1 integrin conditional KO mice were generated by the *Cre/loxP* recombinant system. The floxed β1 integrin gene mice were previously described (Brakebusch et al., 2000; Potocnik et al., 2000). Transgenic mice expressing the Cre recombinase under the control of the ZP3 promoter (kind gift of Dr. Jamey Marth, UCSD) were mated with the floxed β1 integrin mice (Fig. 2). Offspring were genotyped by PCR to detect the ZP3-Cre transgene and the floxed β1 integrin gene. Female mice with both the ZP3-Cre transgene and homozygous floxed β1 integrin gene (Cre^+ β1f/f) were putative β1 integrin conditional knockout mice. These mice were used for in vivo mating, and eggs collected from these mice were used for immunofluorescent staining and IVF assays.

In vivo fertilization of the β1 integrin conditional KO mice

$Cre^+\beta$1f/f female mice and C57/B16 females (wild-type control) were mated with C57/B16 wild-type males to determine fertility. Mice were housed with one male and one female per cage. The females were checked for pregnancy at 14 days and for birth of pups from 20–30 days after mating. The litter size from each pair was counted, and the percentage of pregnancies and average litter size were calculated.

In vitro fertilization assay

Eggs, isolated from cultured ovaries, or normal superovulated eggs, after removal of cumulus cells (Yuan et al., 1997), were treated with 30 μg/ml chymotrypsin (Sigma) in M199* and 0.3% BSA (Sigma) for 3 min at 37°C and 5% CO_2. The treated zona pellucida was removed mechanically with a pipette, and the eggs were transferred through three wash drops of the same medium. The eggs were allowed to recover for 3 h 15 min by incubation in M199* at 37°C and 5% CO_2; 4′,5′-diamidino-2-phenylindole dihydrochloride (DAPI; Polysciences Inc.), at a final concentration of 10 μg/ml, was added for the final 15 min before insemination. The eggs were washed through three drops of M199* before insemination.

Sperm for use in the IVF assays were isolated from the cauda epididymis and the vas deferens of 10- to 12-week-old C57/B16 male mice. The dissected cauda and vas deferens were placed into M199* and 3% BSA for 15 min at 37°C in 5% CO_2 to release sperm, and the tissues were removed after 15 min of incubation. Sperm were capacitated for 3 h in the same medium. This procedure results in a population of 60-70% acrosome-reacted sperm (Moller et al., 1990).

Sperm and eggs were coincubated for 40 min at 37°C in 5% CO_2. The eggs were scored for the number of sperm bound and for fusion. Bound sperm were counted at the egg equator in a single plane of focus by using phase optics at a magnification of 20×. Fusion was scored by counting the fluorescently labeled sperm nuclei by DAPI transfer from inside the preloaded eggs after membrane fusion. The fertilization rate (FR, the percentage of eggs fused with at least one sperm) and the fertilization index (FI, the mean number of fused sperm per egg) were calculated.

The anti-β3 integrin mAb (2C9; Pharmingen) and anti-αv integrin mAb (H9.2; Pharmingen) were pretested and were found positive for immunofluorescent staining of mouse bone marrow cells (as described by Pharmingen). To test the effect of the mAb on the fertilization of the β1 knockout and wild type eggs, zona-free eggs were treated as described above and then preincubated for 30 min with the mAb in M199* with 0.3% BSA. Sperm were added to the drop containing eggs and antibodies (final sperm concentration 1-5 $\times$ 10^5/ml; final mAb concentration 200 μg/ml). The eggs and sperm were coincubated for 40 min. Binding and fusion were assessed as described above.

Indirect immunofluorescent staining of zona-free eggs

Zona-free eggs were prepared and allowed to recover as described above. After 3 h of recovery, both the β1 integrin knockout eggs and wild type eggs were incubated with 50 μg/ml of the rat anti-α6 integrin mAb GoH3 (isotype IgG2a; Chemicon) or 100 μg/ml rat anti-mouse β1 integrin mAb MB1.2 (isotype IgG2a; Chemicon) in PBS (pH 7.2) with 0.3% BSA at room temperature for 1 h. Nonspecific rat

IgG2a (100 μg/ml; Pharmingen) was used as the negative control. The eggs were washed through three drops of PBS. An Oregon green-conjugated goat anti-rat secondary antibody (Molecular Probes) was used to determine the localization of the primary antibody binding. Staining was visualized by using a laser scanning confocal microscope (LSM 410; Carl Zeiss).

β-galactosidase activity assay

A promoterless *lacZ* gene is present in the floxed β1 integrin gene (Brakebusch et al., 2000). The Cre-mediated deletion of a segment of the β1 integrin gene enabled the β1 integrin promoter to support transcription of the *lacZ* reporter gene. To determine the percentage of pups derived from eggs where the Cre recombinase had deleted the floxed β1 integrin gene, the pups from the β1 integrin conditional knockout females were assayed for lacZ activity with the β-galactosidase substrate X-gal. The enzyme assay was modified from Vernet et al. (1993). In brief, the E18-stage embryos were taken from pregnant conditional knockout mice, and 0.2 g of tissue excised from the head of each embryo was washed once in PBS and then homogenized in 1 ml PBS. The homogenate was centrifuged for 10 min at 5000 rpm, and the supernatant was collected. β-galactosidase activity was assayed by the addition of 10 μl 2 mg/ml X-gal (GIBCO BRL) to 500 μl of supernatant and incubated at 30°C for 3.5 h, and absorbance was measured at 590 nm on the Beckman DU 640 spectrophotometer.

Luminescence assay of β1 integrin mAb binding to WT and β1 integrin conditional KO eggs

The luminescence assay was carried out as previously described (Evans et al., 1997). Briefly, zona-free eggs were collected and allowed to recover for 3 h as described above. The eggs were fixed in freshly prepared 4% formaldehyde in PBS for 30 min. Fixed eggs were washed twice in PBS with 0.3% BSA and then blocked in alkaline phosphatase (AP)-blocking buffer [0.5% Casein (I-block; Tropix), 0.01% Tween 20, and 0.02% sodium azide in PBS] for 60 min at room temperature. The eggs were then incubated with rat anti-mouse β1 integrin mAb MB1.2 (Chemicon) in AP-blocking buffer at room temperature for 60 min and washed three times in 0.05% casein in PBS. A goat anti-rat IgG conjugated to AP (Chemicon) was applied to detect binding of the primary antibody. After incubation with the secondary antibody in 0.05% casein in PBS at room temperature for 30 min, the eggs were washed nine times in 0.05% casein in PBS and once in AP assay buffer (2 mM Tris–HCl, pH 9.5; 1 mM $MgCl_2$; Tropix). The egg-associated AP activity was then quantified in a luminometric assay with a substrate that, upon dephosphorylation, decomposes to an intermediate form with a concomitant emission of photons. The eggs were transferred to 25 μl AP assay buffer in separate wells of a Dynex Microlite 1^+ 96-well plate (Tropix) (1–10 eggs per well). Enhancer– substrate solution (100 μl; AP assay buffer containing 25 mM Sapphire II enhancer and 0.4 mM CSPD [disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate] substrate; Tropix) was added to each well. After incubation of the plate at room temperature for 20 min, photon emission was measured by an ML3000 microtiter plate luminometer (Dynatech Laboratories). We first calibrated the detection of AP activity associated with the goat anti-rat IgG secondary antibody alone. The luminescence increased linearly over the range of $1{:}10^8$ to $1{:}10^5$ dilution of the secondary antibody. Luminescence values as low as 0.0045 could be measured in this linear range.

Results

Eggs lacking the α3 integrin are fully functional in sperm–egg binding and fusion

We first asked whether the α3 integrin had a required role in sperm–egg binding or fusion. We considered that the α3 integrin might be a substitute for the α6 integrin for several reasons. First, the α3 integrin subunit has a closely related sequence to the α6 integrin subunit and, like α6, is a laminin receptor (Hemler, 1999). Second, also like α6β1, the α3β1 integrin is reported to associate with CD9 in somatic cells (Zhang et al., 2001) and CD9 is essential for gamete fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000).

Because mice lacking the α3 integrin die shortly after birth (Kreidberg et al., 1996), it was necessary to culture ovaries to obtain mature eggs. The ovaries of the newborn pups were removed and cultured under the kidney capsule of an adult female for 21 days. The immature α3 integrin knockout oocytes ($\alpha3^{-/-}$) and the control wild type ($\alpha3^{+/+}$) or heterozygous ($\alpha3^{+/-}$) oocytes were collected from the transplanted ovaries and matured in vitro. The mature $\alpha3^{-/-}$ eggs were normal in their morphology and produced first polar bodies. Zona-free eggs were tested for their ability to bind and fuse with sperm in an IVF assay. The following three parameters were measured in these assays: (1) the fertilization rate (FR), the percent of eggs fused with at least one sperm, (2) the fertilization index (FI), the total number of fused sperm/total number of eggs, and (3) the mean number of sperm bound to the egg. There was no significant difference in the number of sperm bound or fused with the $\alpha^{-/-}$ eggs compared with $\alpha3^+$ eggs (wild type and heterozygous eggs) (Fig. 3). These data indicate that the integrin α3β1 does not have a required role in sperm binding and fusion.

Eggs lacking β1 integrins are fertile in vivo

The finding that α3 KO eggs are fully functional, along with the previous α6 KO result, indicates that neither α3β1

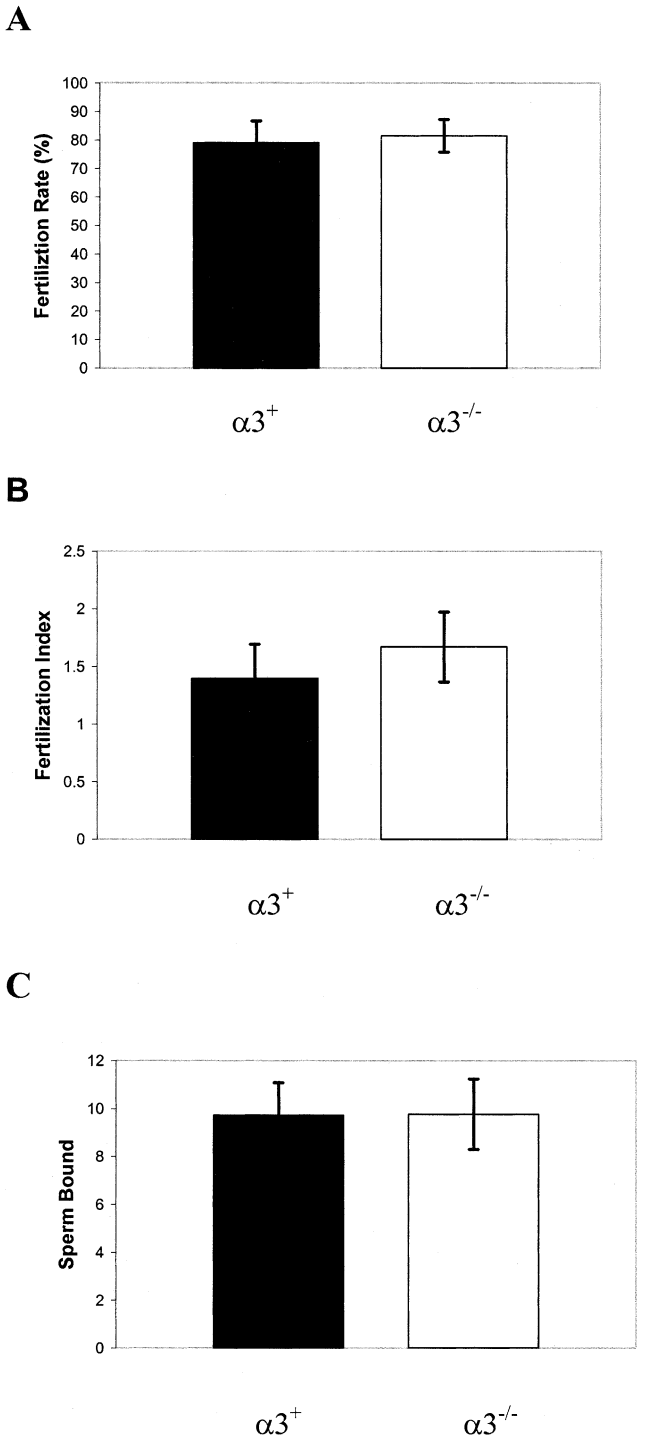

Fig. 3. A comparison of the function of wild type (WT) and the $\alpha 3$ integrin KO eggs in the in vitro fertilization assay. The dark bars show the results with the cultured WT eggs, and the white bars show the results with the cultured $\alpha 3$ integrin KO eggs. (A) Fertilization rate (FR, percentage of eggs fused with at least one sperm). The mean FR for $\alpha 3$ KO eggs was 81.5 ± 1.7% and 79.1 ± 7.7% for the $\alpha 3^+$ eggs. (B) Fertilization index (FI, the mean number of fused sperm per egg). The mean FI for $\alpha 3$ KO eggs is 1.7 ± 0.3, and 1.3 ± 0.3 for the $\alpha 3^+$ eggs. (C) Number of sperm bound per equator: 9.8 ± 1.5 and 9.7 ± 1.4 for $\alpha 3$ KO and $\alpha 3^+$, respectively. The data are the means and SEMs from 8 tests, using a total of 119 $\alpha 3$ KO eggs and 146 WT eggs obtained from littermates.

nor $\alpha 6\beta 1$ is essential for fertilization. Because these two integrins or other $\beta 1$ integrins could be substituting for one another, we next asked whether $\beta 1$ integrin null eggs could function in sperm–egg membrane binding and fusion. The $\beta 1$ integrin null mutation would simultaneously eliminate both $\alpha 3\beta 1$ and $\alpha 6\beta 1$ as well as the other $\beta 1$ integrins on mouse eggs (Fig. 1). Because $\beta 1$ integrin KO mice were periimplantation lethal (Fässler and Meyer, 1995; Stephens et al., 1995), it was necessary to make conditional KO mice that would express the $\beta 1$ integrin subunit normally in all tissues except the oocyte. To make conditional KO eggs, floxed $\beta 1$ integrin homozygous mice were mated with ZP3-Cre mice whose Cre-recombinase is expressed under the control of the ZP3 promoter. The ZP3 promoter is oocyte-specific and is activated at the beginning of oocyte development (1-day-old mice) (Philpott et al., 1987). $\beta 1$ integrin mRNA was not detected from 12-day oocytes by RT-PCR. Thus, the Cre-recombinase transgene controlled by the ZP3 promoter is activated earlier than the $\beta 1$ integrin promoter, allowing Cre-recombinase to delete the floxed $\beta 1$ integrin gene before it is expressed. Female mice containing the ZP3-Cre transgene (Cre^+) and homozygous floxed $\beta 1$ integrin alleles ($Cre^+\beta 1f/f$) are predicted to be conditional knockout mice with the floxed $\beta 1$ integrin gene being ablated in eggs. Indeed, we observed the absence of the $\beta 1$ subunit in eggs from females with this genotype (see below).

Fertility of the $\beta 1$ integrin conditional knockout females was tested in mating studies. Eleven $Cre^+\beta 1f/f$ females were mated with 11 wild type C57/B16 males (one-to-one mating). The $\beta 1$ integrin conditional knockout females were 100% fertile, and their mean litter size was the same as for wild type control females (Fig. 4).

Fully effective β1 integrin gene deletion in fertilized conditional KO oocytes

Because the $\beta 1$ integrin conditional KO mice showed full fertility, we considered that the Cre-recombinase might not always be effective and females were producing some eggs where the floxed $\beta 1$ integrin gene remained present, i.e., not deleted. In order to determine whether all the fertilized eggs from $Cre^+\beta 1f/f$ female mice were null for the $\beta 1$ integrin gene, we tested the genotype of the embryos resulting from the $Cre^+\beta 1f/f$ females. If the pups resulted from fertilization of an egg where the floxed $\beta 1$ integrin gene had been deleted, the embryo should contain a $\beta 1$ integrin KO allele from the egg and a wild-type allele from the sperm. To test the genotype of the embryos, we took advantage of the promoterless *lacZ* gene inserted in the floxed $\beta 1$ integrin gene. If Cre-mediated deletion of the $\beta 1$ gene is successful, the *lacZ* gene will be expressed under control of the $\beta 1$ integrin promoter (Potocnik et al., 2000; Brakebusch et al., 2000). The day 18 embryos of two $Cre^+\beta 1f/f$ females were therefore tested for β-galactosidase activ-

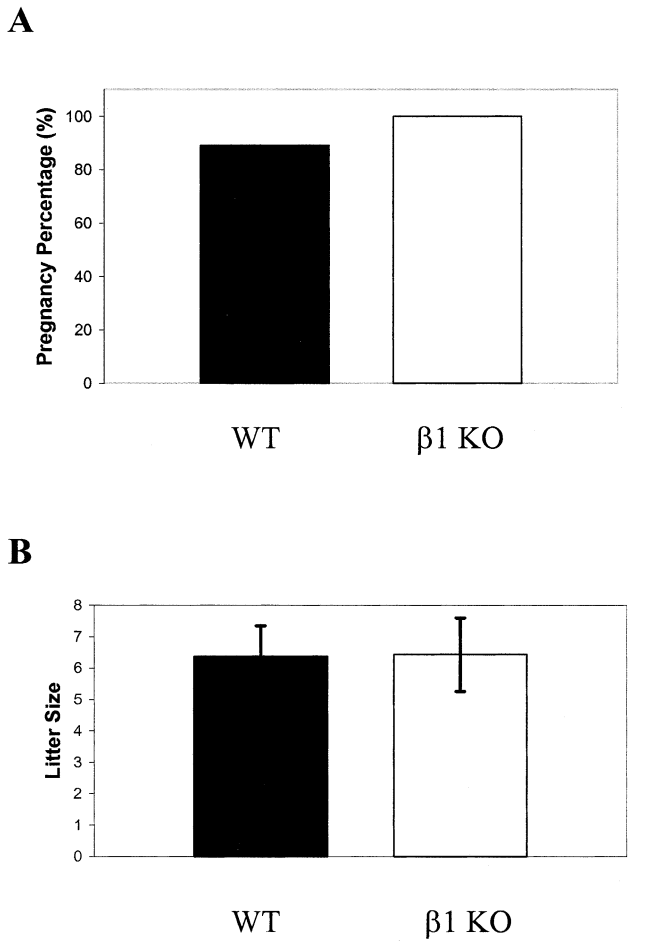

Fig. 4. In vivo fertility of β1 integrin conditional KO female mice. (A) The dark bar shows the pregnancy percentage (8/9 = 89%) of C57/B16 WT females, and the white bar represents the pregnancy percentage (11/11 = 100%) of β1 integrin conditional KO females. (B) The dark bar represents the mean litter size of 9 C57/B16 WT females, and the white bar shows the mean litter size of 11 β1 integrin conditional KO females.

ity. We found that all (16/16) embryos from KO mothers expressed elevated β-galactosidase, and on average, these embryos expressed about 7- to 9-fold more β-galactosidase activity than WT controls (Table 1). These data indicate that all the embryos in KO mothers developed from fertilized oocytes where the floxed β1 integrin gene had been ablated by the Cre-recombinase.

The β1 integrin subunit is not detected in the conditional KO eggs

We also tested the ovulated oocytes for the presence of the β1 integrin subunit protein, to check the possibility that β1 integrin was expressed prior to the Cre-mediated ablation and remained on the cell surface. First, we checked for β1 integrins by immunofluorescence using a rat anti-mouse β1 integrin monoclonal antibody (mAb), MB1.2. We used immunofluorescence because it allowed us to detect positive expression if only a minority of the

Table 1
β-galactosidase activity in embryos from mothers with β1 integrin conditional KO or wild type genotype

Genotype	β1 integrin conditional KO		WT
	Female 1	Female 2	
Embryo #	A_{650}	A_{650}	A_{650}
1	0.70	0.76	0.0881
2	0.73	1.58	0.1112
3	0.70	1.59	0.1265
4	0.82	0.81	0.1045
5	0.77	0.83	0.1502
6	0.90	0.83	0.1509
7	0.89		0.1078
8	0.85		0.0882
9	0.93		
10	0.82		
Mean ± SEM	0.81 ± 0.03*	1.07 ± 0.16*	0.12 ± 0.01

*, $P < 0.00005$ for difference between KO and WT.

egg population was expressing the protein. We did not detect the β1 integrin subunit on the eggs from the $Cre^{+}\beta$1f/f females with MB1.2 (Fig. 5C); the wild type control eggs showed the typical pattern of MB1.2 binding to the microvillar region (Fig. 5A). The α subunits, which are uniquely complexed with the β1 integrin subunit in eggs, were also expected to be absent on the β1 integrin conditional KO eggs. Therefore, we also looked for the presence of one α integrin subunit, α6 (Fig. 5D). [The α6 integrin subunit also associates with the β4 subunit, but the β4 subunit has not been found on mouse eggs

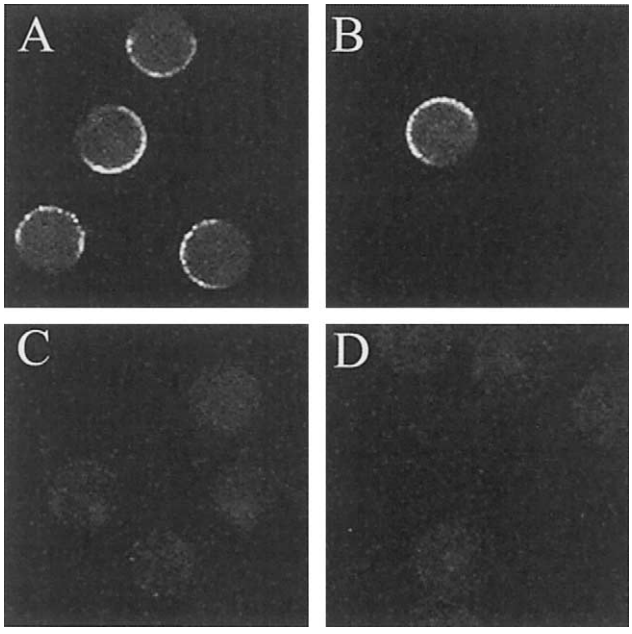

Fig. 5. Immunofluorescent staining of zona-free eggs using anti-β1 integrin mAb MB1.2 and the anti-α6 integrin mAb GoH3. (A) WT eggs stained with MB1.2; (B) WT eggs stained with GoH3; (C) β1 integrin KO eggs stained with MB1.2; and (D) β1 integrin KO eggs stained with GoH3.

(Almeida et al., 1995).] All of the β1 conditional KO eggs we observed lacked both the β1 integrin subunit (12 eggs) and the α6 integrin subunit (12 eggs).

To complement the data obtained from immunofluorescence microscopy, we used a highly sensitive luminescence assay (Evans et al., 1997) to quantify the amount of anti-β1 integrin antibody (MB1.2) bound to the eggs. Zona-free eggs were incubated with MB1.2 and then with alkaline phosphatase (AP)-conjugated goat anti-rat IgG secondary antibody. Nonspecific rat IgG_{2a} (the same isotype as MB1.2) was used as a negative control. We found that the β1 integrin subunit was not detected on the β1 KO eggs. The photon emission rate of the KO eggs was slightly lower than that of the negative control (not a significant difference) (Fig. 6). The photon emission rate of WT eggs was significantly higher than both the β1 integrin KO eggs and the negative control. The data indicated that specific MB1.2 binding to β1 integrin KO eggs was <0.5% of specific MB1.2 binding to WT eggs (Fig. 6). Thus, we conclude that the β1 integrin subunit was not expressed on the β1 integrin conditional KO eggs.

Eggs lacking β1 integrins function normally in binding and fusion with sperm in vitro

Eggs lacking β1 integrins were tested for sperm binding and fusion in an in vitro fertilization assay. The β1 integrin conditional KO mice (Cre^{+}β1f/f) developed normally, and their eggs lacking β1 integrins were normally superovulated. Control eggs were obtained by superovulation of WT littermates. Using IVF assays, we found that β1 KO eggs could not be distinguished from WT eggs in the ability to bind and fuse with sperm in vitro (Fig. 7).

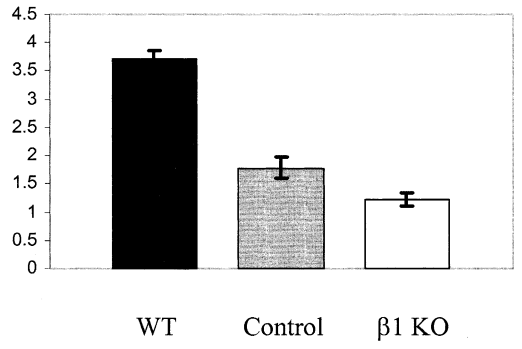

Fig. 6. Quantitative luminescence assay of MB1.2 (rat IgG_{2a}) binding to the β1 integrin conditional KO eggs compared with WT eggs. The rate of photon emission was measured for groups of 10 eggs (WT, $n = 6$ groups; Control, $n = 4$ groups; KO, $n = 5$ groups). The control is nonspecific rat IgG_{2a} binding to WT eggs. The dark bar shows the mean luminescence of MB1.2 binding to WT eggs; the gray bar shows luminescence of control rat IgG_{2a} binding to WT eggs; and the white bar shows luminescence of MB1.2 binding to β1 KO eggs. The WT relative specific luminescence (WT value with specific antibody minus WT value with control, nonspecific antibody) was 1.9281 units. The β1 KO relative specific luminescence (β1 KO value with specific antibody minus WT value with non-specific antibody) was 0, which is <0.0096 units, a measurable value in the linear range of luminescence (see Materials and methods), or <0.5% of wild type. **, $P < 0.005$ level of statistical difference comparing WT to control.

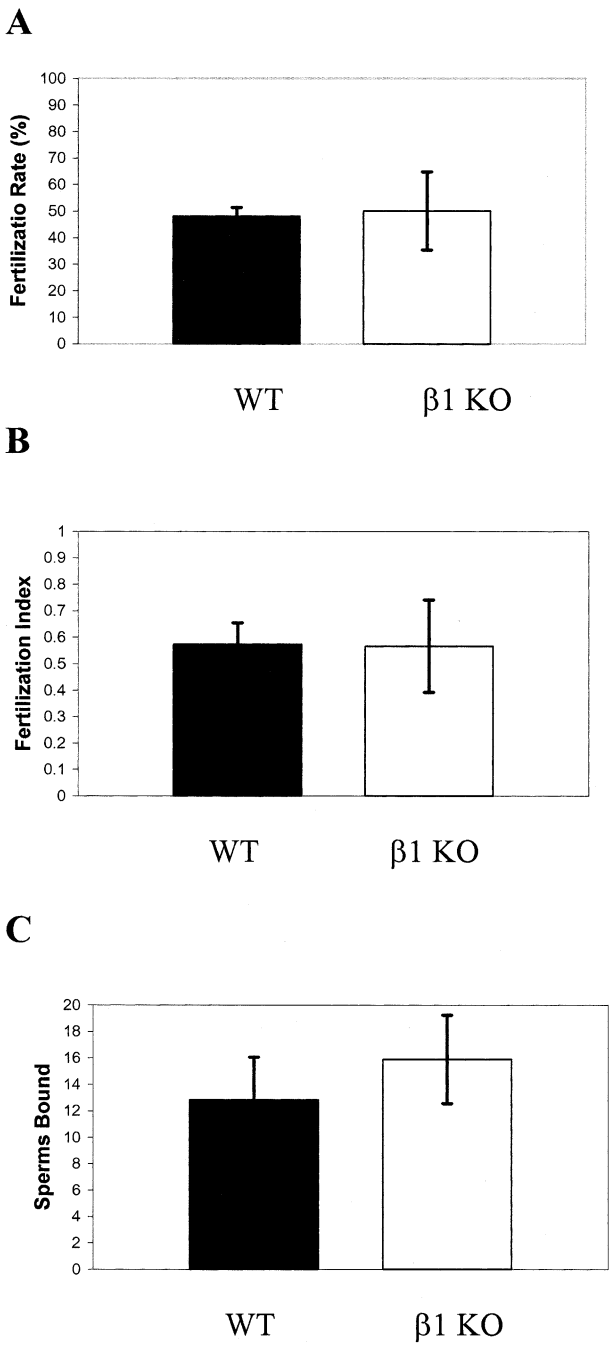

Fig. 7. A comparison between the function of β1 integrin KO and WT eggs in the in vitro fertilization assay. The dark bars show the results with the WT eggs, and the white bars show the results with β1 integrin KO eggs. (A) Fertilization rate (FR). The mean FR for β1 KO eggs was 50.1 ± 14.6% and 48.1 ± 3.4% for WT eggs. (B) Fertilization index (FI). FI for β1 KO eggs, 0.57 ± 0.17 and 0.57 ± 0.08 for WT eggs. (C) Number of sperm bound per equator: 15.9 ± 3.3 and 12.8 ± 3.2 for β1 KO and WT eggs, respectively. The difference observed for sperm bound is not significantly different between WT and KO; $P = 0.53$. The data are the means and SEM of 6 experiments from a total of 139 β1 integrin KO eggs and 138 WT eggs.

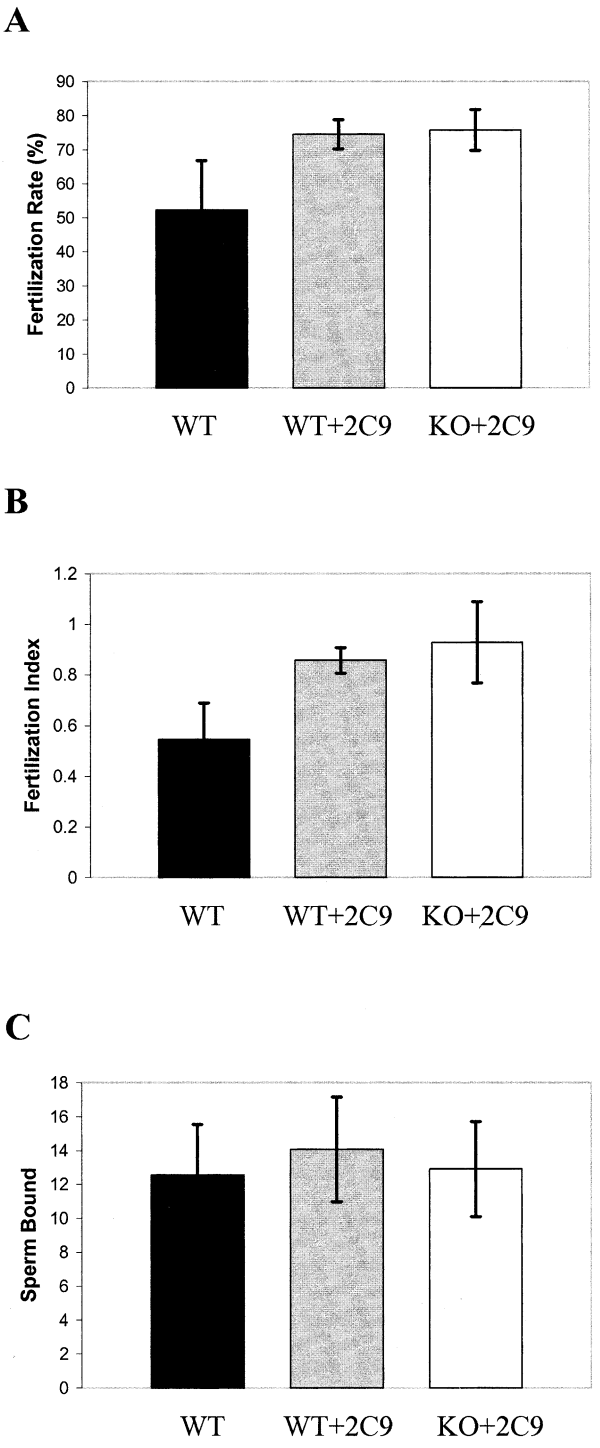

Fig. 8. Effect of the anti-β3 integrin mAb (2C9) on sperm binding to and fusion with the β1 integrin conditional KO eggs. The black bars show the results with the WT control. The gray bars show the results with WT eggs preincubated with mAb 2C9, and the white bars show the results with β1 integrin KO eggs preincubated with the 2C9. (A) Fertilization rate. In these assays, the mean FR of the β1 KO and WT eggs with 2C9 were 75.8 $\pm$ 6.0 and 74.5 $\pm$ 4.3, respectively, and the mean FR of the control eggs was 52.4 $\pm$ 14.5. (B) Fertilization index. The mean FI of the β1 KO eggs with 2C9 was 0.93 $\pm$ 0.16, and 0.86 $\pm$ 0.05 for the WT eggs with 2C9, and the mean FI for the control WT eggs was 0.55 $\pm$ 0.14. (C) Number of sperm bound. The mean number of sperm bound to the egg equator was almost same for the three groups: 12.9 $\pm$ 2.8, 14.1 $\pm$ 3.1, and 12.6 $\pm$ 3.0, respectively. The differences for FR and FI between the three groups are not significant; $P > 0.5$. The data are means and SEMs from 104 β1 integrin KO eggs incubated with 2C9, 130 WT eggs incubated with mAb 2C9, and 117 WT eggs.

Anti-mouse β3 integrin mAb does not inhibit binding and fusion of the β1 KO eggs with sperm

In addition to β1 integrin expression on mouse eggs, the β3 integrin has also been reported on eggs by both immunofluorescent staining and immunoprecipitation (Evans et al., 1995). β3 KO mice have normal fertility (Hodivala-Dilke et al., 1999), but a β3 integrin could potentially compensate for a β1 integrin (or vice versa) for sperm–egg binding and fusion. If this were the case, a function-blocking antibody against the β3 integrin subunit should be able to inhibit the ability of β1 integrin KO eggs to bind and fuse with sperm. We used the function-blocking anti-mouse β3 integrin mAb, 2C9, (Schultz and Armant, 1995; Yasuda et al., 1995) directly in IVF assays with the β1 integrin KO eggs as well as with wild type (WT) eggs. The final mAb concentration used (200 μg/ml) was 20 times higher than previously reported as function-blocking (Schultz and Armant, 1995). The mAb 2C9 did not inhibit the binding and fusion of the β1 integrin KO eggs or of the WT eggs (Fig. 8). These data indicate that the eggs can still bind and fuse with sperm even when neither β1 integrins nor β3 integrins can function on the egg membrane.

Anti-αv integrin function-blocking mAb does not inhibit sperm binding and fusion with the β1 integrin null eggs

According to published reports, the only non-β1 integrins reported to be expressed on the mouse egg are the αv integrins (αvβ3 and αvβ5) (Fig. 1). αv Integrins have been reported to be required for sperm–egg binding and fusion in pigs (Linfor and Berger, 2000) and as receptors for ADAMs in other cell types (Cal et al., 2000; Nath et al., 1999, 2000; Zhang et al., 1998; Zhou et al., 2001). Therefore, we tested the ability of β1 integrin conditional KO eggs to bind and fuse with sperm in the presence of a function-blocking mAb against the mouse αv integrin subunit (H9.2) (Maxfield et al., 1989; Moulder et al., 1991; Piali et al., 1995; Roberts et al., 1992; Schultz and Armant, 1995).

The antibody H9.2 did not inhibit sperm binding to or fusion with the β1 integrin conditional KO eggs (Fig. 9), even though we used a 20 times (200 μg/ml) higher concentration than reported to inhibit function on other cell types (Schultz and Armant, 1995). These data implied that αv integrins were unlikely candidates for redundancy with the β1 integrins and were not themselves required for sperm–egg binding or fusion in mouse.

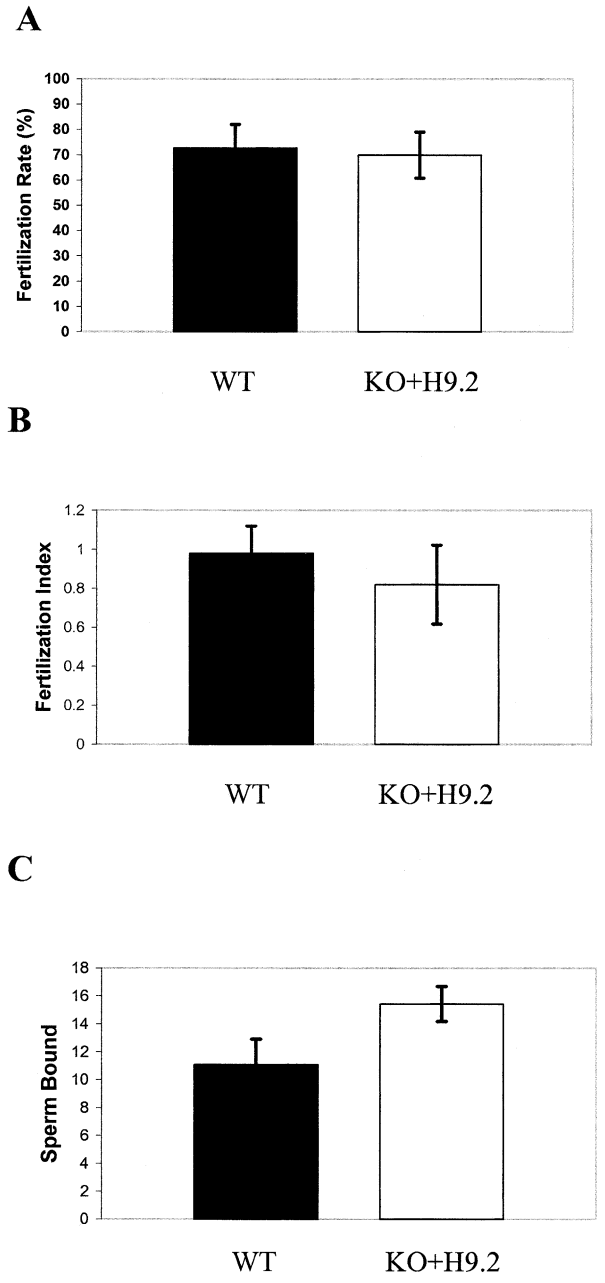

Fig. 9. Effect of anti-αv integrin subunit mAb (H9.2) on sperm binding to and fusion with the β1 integrin KO eggs. The black bars show results with WT eggs, and the white bars show the results with β1 integrin KO eggs incubated with the H9.2. (A) Fertilization rate. The mean FR for the β1 KO eggs incubated with H9.2 (200 μg/ml) was 70 $\pm$ 9.1%, and for the WT control without H9.2, 73 $\pm$ 9.4%. (B) Fertilization index. The mean FI of the KO + H9.2 was slightly lower than that of the WT: 0.82 $\pm$ 0.14 and 0.98 $\pm$ 0.2, respectively ($P > 0.5$). (C) Number of sperm bound per equator. The mean number of sperm bound of the KO + H9.2 was higher than that of WT, but this difference was not significant; $P = 0.14$. Data represent 3 separate experiments, 78 total eggs (β1 KO eggs incubated with H9.2), and 89 total eggs (WT control).

Discussion

The molecular mechanism of sperm–egg membrane binding and fusion is currently unknown. Four lines of evidence have been presented to support the idea that integrins act in this process. (1) Peptides or recombinant proteins, including part or all of the disintegrin domains of sperm ADAMs, are able to inhibit both sperm–egg membrane binding and fusion (Almeida et al., 1995; Bigler et al., 2000; Bronson et al., 1999; Evans et al., 1997, 1998; Gichuhi et al., 1997; Gupta et al., 2000; Linder and Heinlein, 1997; Mwethera et al., 1999; Myles et al., 1994; Takahashi et al., 2001; Waters and White, 1997; Yuan et al., 1997; Zhu et al., 2000). Because disintegrins are known integrin ligands, this implicated integrins on the egg surface as the receptor for sperm ADAMs (Bigler et al., 2000; Cal et al., 2000; Chen and Sampson, 1999; Chen et al., 1999; Eto et al., 2000; Nath et al., 1999, 2000; Zhang et al., 1998; Zhou et al., 2001). (2) A function-blocking antibody, GoH3, directed against the α6 integrin subunit, was observed to block sperm–egg binding and fusion (Almeida et al., 1995). Furthermore, sperm fertilin β disintegrin peptides specifically bind to the α6β1 integrin (Almeida et al., 1995; Chen and Sampson, 1999; Chen et al., 1999; Takahashi et al., 2000). (3) Antibodies against the β1 integrin subunit inhibit sperm–egg binding and fusion (Ji et al., 1998; Linfor and Berger, 2000) or inhibit recombinant fertilin β peptide binding to eggs (Evans et al., 1997). (4) CD9 null female mice are infertile and their eggs cannot fuse with sperm. Because there were reports that CD9 associates with integrins, it was suggested that the role of CD9 in sperm–egg fusion was to modify the function of an egg integrin (Miyado et al., 2000).

Several different integrins have been reported to be expressed on the egg surface (Almeida et al., 1995; Evans et al., 1995; Tarone et al., 1993). Integrins expressed on the murine egg surface can be divided into two groups: the β1 integrins (α2β1, α3β1, α5β1, α6β1, and possibly α9β1) and the αv integrins (αvβ1, αvβ3, αvβ5) (Fig. 1). The integrins on murine eggs were detected by immunoprecipitation, immunofluorescent staining, and RT-PCR.

To test, by genetic approaches, the expected role for integrins in sperm binding and fusion, we decided to investigate all integrins reported on the murine egg or suggested as ADAM receptors. In addition to α6 integrin KO mice (Miller et al., 2000), several lines of mice null for integrin subunits (α7, β3, and β5) have been reported to be normally fertile (Hodivala-Dilke et al., 1999; Huang et al., 2000; Mayer et al., 1997). In this study, integrin α3 null eggs were found to be fully functional in both sperm binding and fusion. We also generated oocyte-specific β1 integrin conditional KO mice to obtain β1 integrin null eggs. Our data indicated that sperm binding and fusion with the β1 null eggs was not impaired in vivo or in vitro. Thus, we concluded that no single integrin (α2β1, α3β1, α5β1, α6β1, α9β1, αvβ1, αvβ3, αvβ5) reported to be on the mouse egg surface or to be an ADAM receptor is essential for gamete binding and fusion (Fig. 1). Most of the other known integrin α and β subunits, α1, α4, α7, αM, β2, β4, β6, β7, have been reported to be absent from the mouse ovary (Burns et al., 2002).

Furthermore, we considered the question if integrins present on the mouse eggs could be redundant with each other. Clearly, the β1 integrins do not substitute for each other since gamete fusion is normal in the absence of all β1 integrins. We also tested the ability of β1 integrin KO eggs to bind and fuse with sperm in the presence of function-blocking monoclonal antibodies against the β3 integrin or αv integrin subunit. Neither antibody inhibited sperm binding and fusion with the β1 integrin null eggs. Thus, we conclude that the integrins on mouse eggs are unlikely to be redundant with each other in sperm–egg binding and fusion.

Sperm ADAMs (in particular fertilin β/ADAM2 and cyritestin/ADAM3) have been suggested to be the ligand(s) for an egg integrin during gamete fusion (Chen and Sampson, 1999; Takahashi et al., 2001). However, fertilin β KO sperm fuse with eggs at ~50% the WT rate, cyritestin KO sperm fuse at 100% the WT rate, and the double knockout sperm fuse at ~50% the WT rate (Cho et al., 1998; Nishimura et al., 2001). These results indicate that fertilin β and cyritestin individually or together are not essential for gamete fusion. The small (50%) reduction in fusion rates seen with fertilin β null sperm remains to be explained. It may be an indirect effect or represent a contribution of fertilin β interaction with a nonintegrin receptor.

The strongest evidence remaining that ADAMs are important in sperm–egg fusion comes from ADAM disintegrin domain peptide mimics or recombinant constructs that inhibit egg binding and fusion with sperm (Almeida et al., 1995; Bigler et al., 2000; Bronson et al., 1999; Gichuhi et al., 1997; Gupta et al., 2000; Linder and Heinlein, 1997; Mwethera et al., 1999; Myles et al., 1994; Takahashi et al., 2001; Waters and White, 1997; Yuan et al., 1997; Evans et al., 1997, 1998; Zhu et al., 2000). There are at least two possibilities to explain this discrepancy between studies using disintegrin peptides/constructs or knockout mice. First, the ADAM2/ADAM3 peptides may bind with low specificity to the eggs and saturate a nonintegrin receptor for a different ADAM, one that is actually required for binding and fusion. Since sperm bound and fused normally to the eggs with the integrins knocked out and/or blocked, there certainly could be other egg molecule(s) that function as sperm ADAM receptors. A second possibility is that the peptides/constructs bind to the eggs and change the egg membrane in a nonphysiological way so that it no longer can function normally in sperm binding or fusion. For example, disintegrin peptide/construct binding might lead to signaling that artifactually inhibits gamete fusion.

CD9 is essential for sperm–egg fusion (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000). One explanation of this result was that CD9 functions in sperm–egg fusion by modulating the function of an integrin, particularly $\alpha3\beta1$ or $\alpha6\beta1$. In fact, CD9 is not associated only with integrins, but forms even more stable complexes with other proteins, such as Ig superfamily members (Boucheix et al., 2001; Charrin et al., 2001; Stipp et al., 2001). An obvious possibility is that, on the egg plasma membrane, a CD9 partner(s) other than integrins could function as sperm receptor(s) to initiate the gamete fusion process.

In summary, we found that α3 null eggs and β1 integrin null eggs function normally in sperm–egg binding and fusion. Function blocking antibodies used in combination with β1 integrin null eggs indicated that neither β3 integrins nor αv integrins are likely to be substituting for the missing β1 integrins. Thus, our data, combined with previously reported KO data, show that none of the integrins reported on murine eggs or known as ADAM receptors are required for sperm–egg binding and fusion (Fig. 1). These findings should certainly encourage investigation of other possible players in the gamete fusion process.

Acknowledgments

We thank Dr. Brent J. Miller for help with the surgical techniques and Megan Choi for help with the genotyping. We also thank the members of the Myles– Primakoff lab for helpful discussions. This work was supported by a grant from the National Institutes of Health, HD 16580 (to D.G.M.).

References

Adler, M., Lazarus, R.A., Dennis, M.S., Wagner, G., 1991. Solution structure of kistrin, a potent platelet aggregation inhibitor and GP IIb-IIIa antagonist. Science 253, 445–448.

Almeida, E.A., Huovila, A.P., Sutherland, A.E., Stephens, L.E., Calarco, P.G., Shaw, L.M., Mercurio, A.M., Sonnenberg, A., Primakoff, P., Myles, D.G., White, J.M., 1995. Mouse egg integrin alpha 6 beta 1 functions as a sperm receptor. Cell 81, 1095–1104.

Bigler, D., Takahashi, Y., Chen, M.S., Almeida, E.A., Osbourne, L., White, J.M., 2000. Sequence-specific interaction between the disintegrin domain of mouse ADAM2 (fertilin beta) and murine eggs. Role of the alpha(6) integrin subunit. J. Biol. Chem. 275, 11576–11584.

Blobel, C.P., Wolfsberg, T.G., Turck, C.W., Myles, D.G., Primakoff, P., White, J.M., 1992. A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. Nature 356, 248–252.

Boucheix, C., Rubinstein, E., 2001. Tetraspanins. Cell Mol. Life Sci. 58, 1189–1205.

Brakebusch, C., Grose, R., Quondamatteo, F., Ramirez, A., Jorcano, J.L., Pirro, A., Svensson, M., Herken, R., Sasaki, T., Timpl, R., Werner, S., Fassler, R., 2000. Skin and hair follicle integrity is crucially dependent on beta 1 integrin expression on keratinocytes. EMBO J. 19, 3990–4003.

Bronson, R.A., Fusi, F.M., Calzi, F., Doldi, N., Ferrari, A., 1999. Evidence that a functional fertilin-like ADAM plays a role in human sperm–oolemmal interactions. Mol. Hum. Reprod. 5, 433–440.

Burns, K.H., Owens, G.E., Fernandez, J.M., Nilson, J.H., Matzuk, M.M., 2002. Characterization of integrin expression in the mouse ovary. Biol. Reprod. 67, 743–751.

Cal, S., Freije, J.M., Lopez, J.M., Takada, Y., Lopez-Otin, C., 2000. ADAM 23/MDC3, a human disintegrin that promotes cell adhesion via interaction with the alphavbeta3 integrin through an RGD-independent mechanism. Mol. Biol. Cell 11, 1457–1469.

Charrin, S., Le Naour, F., Oualid, M., Billard, M., Faure, G., Hanash, S.M., Boucheix, C., Rubinstein, E., 2001. The major CD9 and CD81 molecular partner. Identification and characterization of the complexes. J. Biol. Chem. 276, 14329–14337.

Chen, H., Sampson, N.S., 1999. Mediation of sperm–egg fusion: evidence that mouse egg alpha6beta1 integrin is the receptor for sperm fertilin-beta. Chem. Biol. 6, 1–10.

Chen, M.S., Almeida, E.A., Huovila, A.P., Takahashi, Y., Shaw, L.M., Mercurio, A.M., White, J.M., 1999. Evidence that distinct states of the integrin alpha6beta1 interact with laminin and an ADAM. J. Cell Biol. 144, 549–561.

Cho, C., Bunch, D.O., Faure, J.E., Goulding, E.H., Eddy, E.M., Primakoff, P., Myles, D.G., 1998. Fertilization defects in sperm from mice lacking fertilin beta. Science 281, 1857–1859.

Eto, K., Huet, C., Tarui, T., Kupriyanov, S., Liu, H.Z., Puzon-McLaughlin, W., Zhang, X.P., Sheppard, D., Engvall, E., Takada, Y., 2002. Functional classification of ADAMs based on a conserved motif for binding to integrin alpha 9beta 1: implications for sperm–egg binding and other cell interactions. J. Biol. Chem. 277, 17804–17810.

Eto, K., Puzon-McLaughlin, W., Sheppard, D., Sehara-Fujisawa, A., Zhang, X.P., Takada, Y., 2000. RGD-independent binding of integrin alpha9beta1 to the ADAM- 12 and -15 disintegrin domains mediates cell–cell interaction. J. Biol. Chem. 275, 34922–34930.

Evans, J.P., 1999. Sperm disintegrins, egg integrins, and other cell adhesion molecules of mammalian gamete plasma membrane interactions. Front. Biosci. 4, D114–D131.

Evans, J.P., Kopf, G.S., Schultz, R.M., 1997. Characterization of the binding of recombinant mouse sperm fertilin beta subunit to mouse eggs: evidence for adhesive activity via an egg beta1 integrin-mediated interaction. Dev. Biol. 187, 79–93.

Evans, J.P., Schultz, R.M., Kopf, G.S., 1995. Mouse sperm-egg plasma membrane interactions: analysis of roles of egg integrins and the mouse sperm homologue of PH-30 (fertilin) beta. J. Cell Sci. 108, 3267–3278.

Evans, J.P., Schultz, R.M., Kopf, G.S., 1998. Roles of the disintegrin domains of mouse fertilins alpha and beta in fertilization. Biol. Reprod. 59, 145–152.

Georges-Labouesse, E., Messaddeq, N., Yehia, G., Cadalbert, L., Dierich, A., Le Meur, M., 1996. Absence of integrin alpha 6 leads to epidermolysis bullosa and neonatal death in mice. Nat. Genet. 13, 370–373.

Gichuhi, P.M., Ford, W.C., Hall, L., 1997. Evidence that peptides derived from the disintegrin domain of primate fertilin and containing the ECD motif block the binding of human spermatozoa to the zona-free hamster oocyte. Int. J. Androl. 20, 165–170.

Gupta, S., Li, H., Sampson, N.S., 2000. Characterization of fertilin beta-disintegrin binding specificity in sperm–egg adhesion. Bioorg. Med. Chem. 8, 723–729.

Hemler, M.E., 1999. Integrins, in: Kreis, T., Vale, R. (Ed.), Guidebook to the Extracellular Matrix, Anchor and Adhesion Proteins, Oxford University Press, Oxford, pp. 196–212.

Hodivala-Dilke, K.M., McHugh, K.P., Tsakiris, D.A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F.P., Coller, B.S., Teitelbaum, S., Hynes, R.O., 1999. Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J. Clin. Invest. 103, 229–238.

Huang, X., Griffiths, M., Wu J., Farese Jr., R.V., Sheppard, D., 2000. Normal development, wound healing, and adenovirus susceptibility in beta5-deficient mice. Mol. Cell. Biol. 20, 755–759.

Hynes, R.O., 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69, 11–25.

Ji, Y.Z., Wolf, J.P., Jouannet, P., Bomsel, M., 1998. Human gamete fusion can bypass beta1 integrin requirement. Hum. Reprod. 13, 682–689.

Kaji, K., Oda, S., Shikano, T., Ohnuki, T., Uematsu, Y., Sakagami, J., Tada, N., Miyazaki, S., Kudo, A., 2000. The gamete fusion process is defective in eggs of Cd9-deficient mice. Nat. Genet. 24, 279–282.

Kelic, S., Levy, S., Suarez, C., Weinstein, D.E., 2001. CD81 regulates neuron-induced astrocyte cell-cycle exit. Mol. Cell. Neurosci. 17, 551–560.

Kreidberg, J.A., Donovan, M.J., Goldstein, S.L., Rennke, H., Shepherd, K., Jones, R.C., Jaenisch, R., 1996. Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. Development 122, 3537–3547.

Le Naour, F., Rubinstein, E., Jasmin, C., Prenant, M., Boucheix, C., 2000. Severely reduced female fertility in CD9-deficient mice. Science 287, 319–321.

Linder, B., Heinlein, U.A., 1997. Decreased in vitro fertilization efficiencies in the presence of specific cyritestin peptides. Dev. Growth Differ. 39, 243–247.

Linfor, J., Berger, T., 2000. Potential role of alphav and beta1 integrins as oocyte adhesion molecules during fertilization in pigs. J. Reprod. Fertil. 120, 65–72.

Maxfield, S.R., Moulder, K., Koning, F., Elbe, A., Stingl, G., Coligan, J.E., Shevach, E.M., Yokoyama, W.M., 1989. Murine T cells express a cell surface receptor for multiple extracellular matrix proteins. Identification and characterization with monoclonal antibodies. J. Exp. Med. 169, 2173–2190.

Mayer, U., Saher, G., Fassler, R., Bornemann, A., Echtermeyer, F., von der Mark, H., Miosge, N., Poschl, E., von der Mark, K., 1997. Absence of integrin alpha 7 causes a novel form of muscular dystrophy. Nat. Genet. 17, 318–323.

Miller, B.J.., Georges-Labouesse, E, Primakoff, P., Myles, D.G., 2000. Normal fertilization occurs with eggs lacking the integrin alpha6beta1 and is CD9-dependent. J. Cell Biol. 149, 1289–1296.

Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A., Okabe, M., Mekada, E., 2000. Requirement of CD9 on the egg plasma membrane for fertilization. Science 287, 321–324.

Moller, C.C., Bleil, J.D., Kinloch, R.A., Wassarman, P.M., 1990. Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. Dev. Biol. 137, 276–286.

Moulder, K., Roberts, K., Shevach, E.M., Coligan, J.E., 1991. The mouse vitronectin receptor is a T cell activation antigen. J. Exp. Med. 173, 343–347.

Mwethera, P.G., Makokha, A., Chai, D., 1999. Fertilin beta peptides inhibit sperm binding to zona-free eggs in a homologous baboon in vitro fertilization system. Contraception 59, 131–135.

Myles, D.G., Kimmel, L.H., Blobel, C.P., White, J.M., Primakoff, P., 1994. Identification of a binding site in the disintegrin domain of fertilin required for sperm–egg fusion. Proc. Natl. Acad. Sci. USA 91, 4195–4198.

Nakamura, K., Iwamoto, R., Mekada, E., 1995. Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin alpha 3 beta 1 at cell–cell contact sites. J. Cell Biol. 129, 1691–1705.

Nath, D., Slocombe, P.M., Stephens, P.E., Warn, A., Hutchinson, G.R., Yamada, K.M., Docherty, A.J., Murphy, G., 1999. Interaction of metargidin (ADAM-15) with alphavbeta3 and alpha5beta1 integrins on different haemopoietic cells. J. Cell Sci. 112, 579–587.

Nath, D., Slocombe, P.M., Webster, A., Stephens, P.E., Docherty, A.J., Murphy, G., 2000. Meltrin gamma(ADAM-9) mediates cellular adhesion through alpha(6)beta(1)integrin, leading to a marked induction of fibroblast cell motility. J. Cell Sci. 113, 2319–2328.

Nishimura, H., Cho, C., Branciforte, D.R., Myles, D.G., Primakoff, P., 2001. Analysis of loss of adhesive function in sperm lacking cyritestin or fertilin beta. Dev. Biol. 233, 204–213.

Park, K.R., Inoue, T., Ueda, M., Hirano, T., Higuchi, T., Maeda, M., Konishi, I., Fujiwara, H., Fujii, S., 2000. CD9 is expressed on human endometrial epithelial cells in association with integrins alpha(6), alpha(3) and beta(1). Mol. Hum. Reprod. 6, 252–257.

Philpott, C.C., Ringuette, M.J., Dean, J., 1987. Oocyte-specific expression and developmental regulation of ZP3, the sperm receptor of the mouse zona pellucida. Dev. Biol. 121, 568–575.

Piali, L., Hammel, P., Uherek, C., Bachmann, F., Gisler, R.H., Dunon, D., Imhof, B.A., 1995. CD31/PECAM-1 is a ligand for alpha v beta 3 integrin involved in adhesion of leukocytes to endothelium. J. Cell Biol. 130, 451–460.

Potocnik, A.J., Brakebusch, C., Fassler, R., 2000. Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. Immunity 12, 653–663.

Primakoff, P., Hyatt, H., Tredick-Kline, J., 1987. Identification and purification of a sperm surface protein with a potential role in sperm–egg membrane fusion. J. Cell Biol. 104, 141–149.

Roberts, K., Yokoyama, W.M., Kehn, P.J., Shevach, E.M., 1991. The vitronectin receptor serves as an accessory molecule for the activation of a subset of gamma/delta T cells. J. Exp. Med. 173, 231–240.

Rubinstein, E., Poindessous-Jazat, V., Le Naour, F., Billard, M., Boucheix, C., 1997. CD9, but not other tetraspans, associates with the beta1 integrin precursor. Eur. J. Immunol. 27, 1919–1927.

Scherberich, A., Moog, S., Haan-Archipoff, G., Azorsa, D.O., Lanza, F., Beretz, A., 1998. Tetraspanin CD9 is associated with very late-acting integrins in human vascular smooth muscle cells and modulates collagen matrix reorganization. Arterioscler. Thromb. Vasc. Biol. 18, 1691–1697.

Schmidt, C., Kunemund, V., Wintergerst, E.S., Schmitz, B., Schachner, M., 1996. CD9 of mouse brain is implicated in neurite outgrowth and cell migration in vitro and is associated with the alpha 6/beta 1 integrin and the neural adhesion molecule L1. J. Neurosci. Res. 43, 12–31.

Schultz, J.F., Armant, D.R., 1995. Beta 1- and beta 3-class integrins mediate fibronectin binding activity at the surface of developing mouse peri-implantation blastocysts. Regulation by ligand-induced mobilization of stored receptor. J. Biol. Chem. 270, 11522–11531.

Shamsadin, R., Adham, I.M., Nayernia, K., Heinlein, U.A., Oberwinkler, H., Engel, W., 1999. Male mice deficient for germ-cell cyritestin are infertile. Biol. Reprod. 61, 1445–1451.

Stephens, L.E., Sutherland, A.E., Klimanskaya, I.V., Andrieux, A., Meneses, J., Pedersen, R.A., Damsky, C.H., 1995. Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. Genes Dev. 9, 1883–1895.

Stipp, C.S., Orlicky, D., Hemler, M.E., 2001. FPRP, a major, highly stoichiometric, highly specific CD81- and CD9-associated protein. J. Biol. Chem. 276, 4853–4862.

Takahashi, Y., Bigler, D., Ito, Y., White, J.M., 2001. Sequence-specific interaction between the disintegrin domain of mouse ADAM 3 and murine eggs: role of beta1 integrin-associated proteins CD9, CD81, and CD98. Mol. Biol. Cell 12, 809–820.

Takahashi, Y., Yamakawa, N., Matsumoto, K., Toyoda, Y., Furukawa, K., Sato, E., 2000. Analysis of the role of egg integrins in sperm–egg binding and fusion. Mol. Reprod. Dev. 56, 412–423.

Tarone, G., Russo, M.A., Hirsch, E., Odorisio, T., Altruda, F., Silengo, L., Siracusa, G., 1993. Expression of beta 1 integrin complexes on the surface of unfertilized mouse oocyte. Development 117, 1369–1375.

Vernet, M., Cavard, C., Zider, A., Fergelot, P., Grimber, G., Briand, P., 1993. In vitro manipulation of early mouse embryos induces HIV1-LTRlacZ transgene expression. Development 119, 1293–1300.

Waters, S.L., White, J.M., 1997. Biochemical and molecular characterization of bovine fertilin alpha and beta (ADAM 1 and ADAM 2): a candidate sperm–egg binding/fusion complex. Biol. Reprod. 56, 1245–1254.

Wolfsberg, T.G., Bazan, J.F., Blobel, C.P., Myles, D.G., Primakoff, P., White, J.M., 1993. The precursor region of a protein active in sperm–egg fusion contains a metalloprotease and a disintegrin domain: structural, functional, and evolutionary implications. Proc. Natl. Acad. Sci. USA 90, 10783–10787.

Yasuda, M., Hasunuma, Y., Adachi, H., Sekine, C., Sakanishi, T., Hashimoto, H., Ra, C., Yagita, H., Okumura, K., 1995. Expression and function of fibronectin binding integrins on rat mast cells. Int. Immunol. 7, 251–258.

Yuan, R., Primakoff, P., Myles, D.G., 1997. A role for the disintegrin domain of cyritestin, a sperm surface protein belonging to the ADAM family, in mouse sperm–egg plasma membrane adhesion and fusion. J. Cell Biol. 137, 105–112.

Zhang, X.A., Bontrager, A.L., Hemler, M.E., 2001. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific beta(1) integrins. J. Biol. Chem. 276, 25005–25013.

Zhang, X.P., Kamata, T., Yokoyama, K., Puzon-McLaughlin, W., Takada, Y., 1998. Specific interaction of the recombinant disintegrin-like domain of MDC-15 (metargidin, ADAM-15) with integrin alphavbeta3. J. Biol. Chem. 273, 7345–7350.

Zhou, M., Graham, R., Russell, G., Croucher, P.I., 2001. MDC-9 (ADAM-9/Meltrin gamma) functions as an adhesion molecule by binding the alpha(v)beta(5) integrin. Biochem. Biophys. Res. Commun. 280, 574–580.

Zhu, X., Bansal, N.P., Evans, J.P., 2000. Identification of key functional aminoacids of the mouse fertilin beta (ADAM2) disintegrin loop for cell–cell adhesion during fertilization. J. Biol. Chem. 275, 7677–7683.

Zhu, X., Evans, J.P., 2002. Analysis of the roles of RGD-binding integrins, a4/a9 integrins, a6 integrins and CD9 in the interaction of the fertilin b (ADAM2) disintegrin domain with the mouse egg membrane. Biol. Reprod. 66, 1193–1202.