Effect of a null mutation of the oviduct-specific glycoprotein gene on mouse fertilization

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The mammalian fertilization process takes place in a complex microenvironment within the female genital tract. A member of the chitinase protein family, oviduct-specific glycoprotein (OGP), has been identified in oviductal fluid from various mammalian species, including humans. Although OGP is widely believed to be involved in the process of mammalian fertilization, including spermatozoon function and gamete interactions, based on experimental results obtained *in vitro*, its physiological significance remains controversial. The present study established OGP gene-null ($ogp^{-/-}$) mice, and primarily characterized their reproductive properties to study the physiological function(s) of OGP. Results obtained from studies using an *in vivo* or *in vitro* system showed that the fertility of $ogp^{-/-}$ females was within normal limits. These results indicate that OGP is not essential for the process of *in vivo* fertilization, at least in mice.

Key words: fertilization, gene-knockout, glycoprotein, oviduct, oviduct-specific glycoprotein (OGP).

INTRODUCTION

During mammalian reproduction, the spermatozoon plasma membrane initially undergoes extensive biochemical changes as spermatozoa travel from the proximal to the distal region of the epididymis [1–4]. Besides molecular maturation in the male reproductive tract, mammalian spermatozoa must undergo functional changes between mating and fertilization. In fact, ejaculated spermatozoa cannot fertilize an oocyte immediately. During transit in the female genital tract, spermatozoa undergo a series of biochemical and functional modifications collectively referred to as capacitation [4]. Although the importance of capacitation has been known for several decades, the molecular mechanisms underlying these changes are not fully understood. It is generally considered that capacitation results from multiple molecular changes in spermatozoon plasma membrane proteins/glycoproteins and lipid components that probably modify ion channels on the spermatozoon plasma membrane [3]. These changes on the surface of spermatozoon plasma membrane allow the transmembrane flux of ions, which may be important in initiating events related to spermatozoon function, such as capacitation, hyperactivation and the acrosome reaction [3,4]. The first two events take place before the spermatozoon–zona pellucida (ZP) interaction, and the latter event is initiated after the spermatozoon–oocyte interaction, at least in the mouse [5,6]. These functional changes in spermatozoa and fertilization occur in the female reproductive tract. In addition to fertilization, early embryonic development also occurs in the proximal female reproductive tract, or oviduct [4]. It is therefore reasonable to assume that the oviductal microenvironment has biological function(s) that induce the gamete functions that are essential for fertilization *in vivo*.

In most mammals studied, spermatozoa become hyperactive in the isthmus of the oviduct, and the hyperactivated spermatozoa move to the ampulla, the site of fertilization *in vivo* [7,8]. One potential functional significance of hyperactivation could be that the hyperactivated beat pattern of a spermatozoon enhances its thrust at the site of its binding to the ZP [4]. This enhanced thrust, in part, is likely to be important in helping the spermatozoon to penetrate the ZP and fuse with the oocyte plasma membrane. Although normal fertilization and pre-implantation development are possible *in vitro* with recent progress in techniques in assisted reproduction, there is growing evidence that the oviduct is not just a passive conduit for gametes and embryonal transport, but that it provides a suitable microenvironment for the fertilization process [4,9,10].

Several published reports, including our previous studies, have shown that oviduct epithelial cells secrete specific glycoproteins into the lumen of the oviduct in various mammals (reviewed in [11–15]). These glycoproteins are expressed in a restricted manner within the oviduct during the post-ovulatory phase of the oestrous/menstrual cycle, and several investigators have indicated that ovarian steroids control the synthesis of oviduct-specific glycoprotein (OGP) [11,12,15,16]. OGP is associated with the surface of male/female gametes before fertilization, and the association is observed during early embryonic development [11–15]. Studies of cloned OGP cDNAs from various mammals [17–25] indicated that these glycoproteins are highly homologous, and belong to the chitinase protein family [26–30]; however, they do not hydrolyse the typical chitinase substrate [20,23]. The organization and structure of the genomic sequence of OGP have been reported for human [31], mouse [32] and hamster [33]. Although size variation in the mucin-type domain of OGP was

Abbreviations used: eCG, equine chorionic gonadotropin; ES cell, embryonic stem cell; GalTase, β-1,4-galactosyltransferase; hCG, human chorionic gonadotropin; HTF, human tubal fluid; i.p., intraperitoneal; IVF, *in vitro* fertilization; neo^r, neomycin-resistance gene; OGP, oviduct-specific glycoprotein; *tk*, herpes simplex thymidine kinase gene; WGA, wheat germ agglutinin; ZP, zona pellucida.

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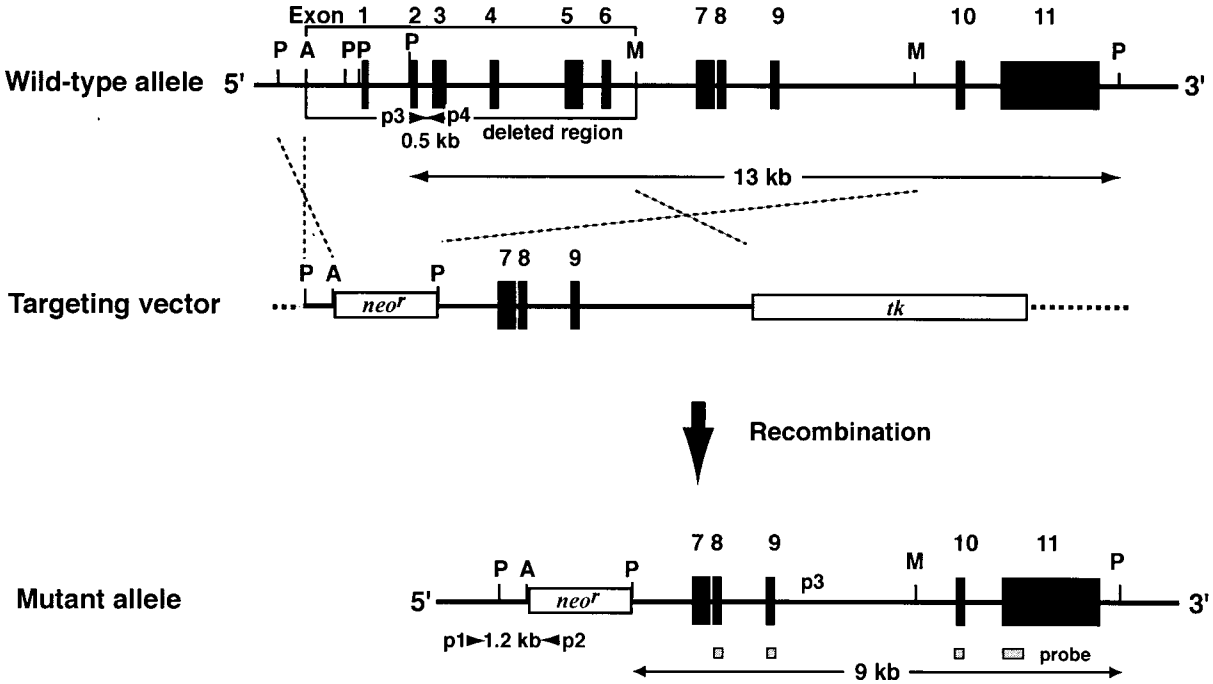

Figure 1 Targeting strategy of the mouse *ogp* gene

Shown are wild-type mouse *ogp* [32] locus (top), targeting vector (middle) and predicted mutant locus (bottom). The targeting vector was created by replacing exons 1–6 encoding the N-terminal portion of OGP with a phosphoglycerate kinase promoter (PGK)-*neo*^{r} cassette. The vector also includes a PGK-*tk* cassette at the 3′ end of the long homologous segment. Arrowheads represent the positions of mutant-allele-specific (p1–p2) and wild-type-allele-specific (p3–p4) PCR primers. p1, 5′-AGTGTCGGTCTTGGAGCTTCC-3′; p2, 5′-CATACACGGTGCCTGACTGCG-3′; p3, 5′-GTTCTTCTGATGAAACACAGTG-3′; p4, 5′-GCACACCAGTTAGTAGGCAG-3′. The probe position for Southern blot analysis is indicated by grey bars. P, *Pst*I; A, *Apa*I; M, *Mun*I digestive sites.

reported only in hamster [34], no evidence concerning subtype or more than one gene of OGP has been reported in other species. In mouse, OGP is encoded by a single gene located on R-positive F3 band of chromosome 3 ([32]; NCBI mouse genome database). Despite numerous advances in the molecular characterization of OGP, its physiological significance in the process of fertilization remains controversial. To date, several lines of evidence in various mammals, including humans, suggest that the association of OGP with the gamete may have a positive influence on fertilization and post-cleavage development to a blastocyst at least *in vitro* [35–42].

In the present study, we established OGP gene-null ($ogp^{-/-}$) mice to elucidate unequivocally the role of OGP in the process of fertilization *in vivo*, and primarily characterized their reproductive properties, especially the ability of fertilization.

EXPERIMENTAL

Chemicals

Restriction endonucleases, modifying enzymes, LA *Taq* DNA polymerase and proteinase K were purchased from either Takara Shuzo Co. (Otsu, Japan) or Roche Diagnostics Corp. (Indianapolis, IN, U.S.A.). SeaKem® GTG Agarose was from BioWhittaker Molecular Applications (Rockland, ME, U.S.A.). Human tubal fluid (HTF) medium [43] was obtained from Irvine Scientific (Santa Ana, CA, U.S.A.). BSA (fraction V) and equine chorionic gonadotropin (eCG) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and human chorionic gonadotropin (hCG) (Puberogen) was from Sankyo Chemical Co. (Tokyo, Japan). Silicone oil was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other chemicals were obtained from either Sigma or Wako Pure Chemical Industries (Osaka, Japan) and were of the highest purity available.

Vector construction for homologous recombination

Genomic clones of the mouse *ogp* gene were isolated from a genomic library constructed from the mouse embryonic stem (ES) cells (TT2) [44] in the phage vector λFIX II using a mouse OGP cDNA [21] probe as described previously [32,45]. A 13 kb genomic DNA fragment, λmg1 containing the *ogp* gene was subcloned into pZErO™-2.1 (Invitrogen, San Diego, CA, U.S.A.). A targeting vector was constructed using a 980 bp fragment of *ogp* containing upstream region of the exon 1 (short arm) and a 5.3 kb fragment containing partial intron F and downstream of the genomic DNA (long arm) (Figure 1). The 980 bp *Pst*I/*Apa*I fragment and the 5.3 kb *Mun*I fragment were subcloned into a pUK21 vector [46]. For short-arm construction, the pUK21-980 bp plasmid was digested with *Sal*I and *Xho*I, then introduced into the *Sal*I site of pLNTK [47] that contained the neomycin-resistance gene (*neo*^{r}) and the herpes simplex thymidine kinase gene (*tk*). To construct the targeting vector, the pUK-5.3 kb vector was cut with *Sal*I and *Xho*I, then the *Sal*I/*Xho*I fragment was subcloned into the *Xho*I site of the pLNTK-short arm. The plasmid DNA was purified using the Qiagen plasmid Midi kit (Qiagen, Hilden, Germany). The final construct (Figure 1) was linearized with *Sal*I and used for ES cell transfection.

Transfection and screening of *ogp* mutant ES cell clones

To generate heterozygous *ogp* mutant ES cells, 10–20 μg of linearized targeting vector was electroporated into $(1–2) \times 10^7$

TT2 ES cells at 300 V, 50 mF, according to a standard procedure as described previously [48]. Transfected ES cells were selected with G418 (200 μg/ml) and gancyclovir (1.0 μM) for 8 days on embryonic fibroblast feeder cells, then screened using PCR with a *ogp*-specific sense primer (p1) located 110 bases upstream of the targeting region and a *neo*^{r}-specific antisense primer (p2) (the positions of these primer sequences are shown in Figure 1) as follows: the template genomic DNA from ES cells was amplified with 25 μl of PCR mixture containing 2.0 mM $MgCl_2$, 200 μM dNTP, LA *Taq* DNA polymerase (125 units) and 1 μM of the primers. The sample was subjected to 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1 min with a programmable thermal controller (PTC-100, MJ Research, Waltham, MA, U.S.A.). Homologous recombination was confirmed by Southern hybridization as described below. ES cell clones containing one mutant *ogp* allele were injected into MCH/JCL strain mouse eight-cell-stage embryos and were transplanted into the uteri of pseudopregnant MCH/ICR strain foster mothers. Male chimaeras were mated with ICR females, and germline transmission of an ES cell gene background was judged by the eye colour of offspring. The chimaeric mice that showed germline transmission were subsequently mated with C57BL/6J females to produce heterozygous mutant mice. Offspring from breeding of the chimaeras were genotyped by PCR followed by Southern blot analysis as described below.

Generation of *ogp* knockout mice

Male and female heterozygous mutant ($ogp^{+/-}$) mice were mated, and their pups were analysed for their genotype by PCR using tale DNA. Briefly, 5 mM of tale biopsy samples were incubated for overnight at 55 °C in lysis buffer [50 mM Tris/HCl (pH 8.0), 0.1 M NaCl, 0.02 M EDTA, 1 % (w/v) SDS, 150 μg/ml proteinase K, 1 μg/ml pronase E]. DNA sample (40–80 ng), extracted with phenol/chloroform, was added to a 25 μl final volume of PCR buffer (2.5 μl of 10 × LA PCR buffer, 2.0 mM $MgCl_2$ and 0.2 mM dNTPs) containing 1 μM of the following primers: targeted allele, p1–p2, or wild allele, p3–p4 (Figure 1). After that, LA *Taq* DNA polymerase (125 units) was added. The PCR was cycled 35 times as described above, then the products were analysed by 1 % (w/v) agarose–Tris/acetate/EDTA gel electrophoresis to determine the genotype of the mice.

Southern and Northern blotting

Mouse genomic DNA (10 μg) was digested with *Pst*I, separated by 0.7 % (w/v) agarose–Tris/acetate/EDTA gel electrophoresis, transblotted on to a nylon membrane and probed with a ^{32}P-labelled mouse $OGP^{684-1329}$ cDNA [21] fragment. Total RNAs (8 μg) from sexually mature mice oviduct were separated by 1 % (w/v) agarose–formaldehyde gel electrophoresis, transblotted on to a nylon membrane and probed with ^{32}P-labelled full-length mouse OGP cDNA [32]. All the hybridized images were visualized with a BAS5000 Bio-Image Analyser (Fuji Film, Tokyo, Japan), as described previously [48].

Fertility of female *ogp* knockout mice *in vivo*

The experiments described in the present paper adhered to the Guide for the Care and Use of Laboratory Animals of the University of the Ryukyus and Yamagata University. Animals were maintained, bred, and given free access to food and water at the Animal Center in either the University of the Ryukyus or Yamagata University under a 12 h:12 h light/dark cycle. Female mice (7–8-week-old) were used for the experiments. Groups containing *ogp* mutant or control female mice were consecutively mated for 4 months with sexually mature male mice. Each pair was recorded for the total number of litters during the term and each litter size.

In vitro fertilization (IVF) for *ogp* knockout mice

HTF medium supplemented with 5 % (w/v) BSA was used for IVF. The cauda epididymidis was removed from 10–12-week-old male B6C3F1 mice and was placed into 500 μl of HTF medium. The distal epididymal tubules were punctured with a sterilized 23-gauge needle and cauda epididymal contents were extruded into the medium. The suspension was incubated at 37 °C in 5 % CO_2 in air for 10 min for spermatozoa dispersion. The spermatozoa suspension was transferred into 500 μl of fresh HTF medium to a final concentration of 10^4–10^6/ml, then the suspension was re-incubated for 2 h for spermatozoa capacitation. For superovulation and collection of oocytes, 7–9-week-old female mice were given an intraperitoneal (i.p.) injection of 5 units of eCG, followed by an i.p. injection of 5 units of hCG after a 48 h interval. At 14–16 h after the hCG injection, oocyte–cumulus complexes were removed from the oviducts and were then inseminated into 500 μl of pre-incubated spermatozoa suspensions. After 4 h of insemination, oocytes were mounted on slides, fixed with 2.5 % (w/v) glutaraldehyde for 20 min at room temperature (22–25 °C), and stained with 1 % (w/v) orcein, before examination under a phase-contrast microscope. Fertilization was assessed according to the formation of the female and male pronuclei, a fertilizing spermatozoon tail and a second polar body, as described previously [49,50].

Statistical analysis

The Mann–Whitney *U* test was used to examine the statistical evaluation of the results obtained from IVF studies. Differences at a probability of $P < 0.05$ were considered to be statistically significant. Data from IVF studies were analysed by χ^2 test and Fisher's exact test after applying the Bonferroni correction.

RESULTS

Targeted disruption of mouse *ogp* in ES cells and establishment of $ogp^{-/-}$ mice

To generate ES cells with one disrupted *ogp* allele, the *ogp*-targeting construct (Figure 1) was transfected into the (B6 × CBA) F1 ES cell line, TT2. A *neo*^{r} expression cassette replaced a fragment of *ogp*, which contains exons 1–6 encoding the N-terminal portion of mouse OGP. Out of roughly 400 ES clones screened, seven were selected by mutation-specific PCR analysis, and three independent clones (named 3-97, B-16 and B-133) were finally identified as being targeted by Southern blot analysis (Figures 2A and 2B). They were germline transmitted, and mice homozygous for the *ogp* deletion ($ogp^{-/-}$) were established by mating with $ogp^{+/-}$ mice. The genotype of the mice was confirmed by Southern blot analysis using tail DNA (Figure 3A). The $ogp^{-/-}$ mice were born in the expected Mendelian ratio and did not show any gross abnormalities (results not shown).

RNA analysis of the knockout mice

Previously, we demonstrated that mouse OGP mRNA was predominantly expressed within oviductal epithelial cells, but not in

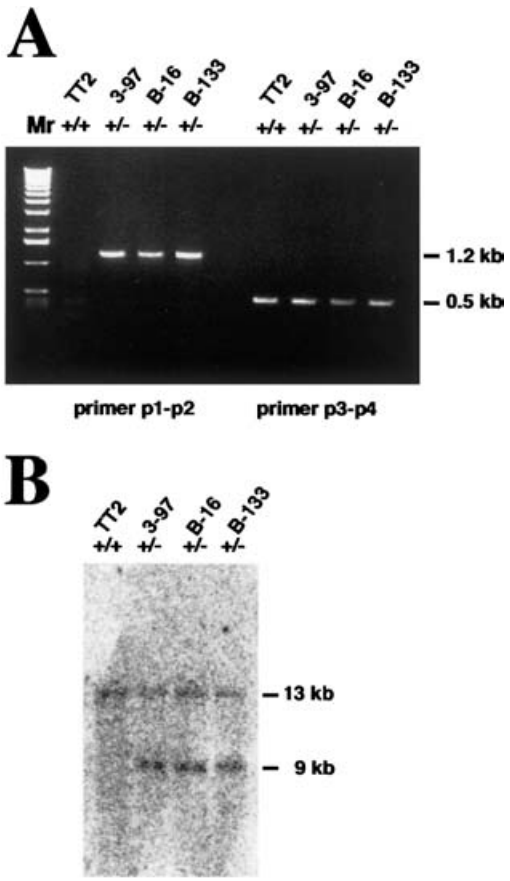

Figure 2 Genomic analysis of the targeted ES clones

(**A**) PCR. The PCR products are generated from the wild-type allele (0.5 kb) and the mutant allele (1.2 kb). Mr, size markers. (**B**) Southern blot. Genome DNA from wild-type ($^{+/+}$), or targeted ($^{+/-}$) ES cells (clones 3-97, B-16 and B-133) was digested with *Pst*I, and hybridized with the mouse OGP$^{684-1329}$ cDNA (including exons 8–10 and a part of exon 11) [21,32]. The positions of bands corresponding to the wild-type allele (13 kb) and the mutant allele (9 kb) are indicated.

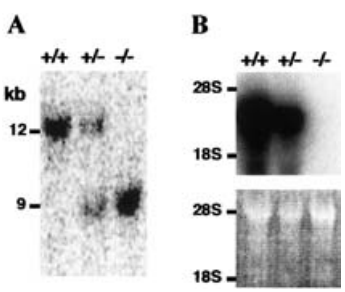

Figure 3 Genotyping and RNA analysis of *ogp*$^{-/-}$ mice

(**A**) Southern blot analysis. Genomic DNA was digested with *Pst*I and hybridized with the mouse OGP$^{684-1329}$ cDNA shown in Figure 1(A). The 12 kb *Pst*I fragment corresponding to the wild-type allele is decreased to 9 kb upon disruption of the *Pst*I digestive sites by integration of *neo*^{r}. (**B**) Northern blot of total RNA from mouse oviduct probed with the entire coding region of the mouse OGP cDNA [21]. The 28 S and 18 S rRNAs were stained with ethidium bromide on the same blot before probing as shown in the lower panel.

other tissues (uterus, ovary, liver, spleen, kidney, lung, brain or stomach), by either Northern blotting or *in situ* hybridization [21]. Since the modified *ogp* allele has a deletion of exons 1–6 of the gene, *ogp*$^{-/-}$ mice are expected to lack OGP mRNA. To confirm this, total RNA from the oviducts of wild-type (*ogp*$^{+/+}$), *ogp*$^{+/-}$ and *ogp*$^{-/-}$ mice was isolated and analysed. Northern blotting using ^{32}P-labelled full-length mouse OGP cDNA demonstrated that the *ogp* interruption abolished OGP mRNA in *ogp*$^{-/-}$ mice (Figure 3B). These results indicate that *ogp*$^{-/-}$ mice have an *ogp*-null-mutation phenotype, since no OGP mRNA including the truncated message was detected using full-length mouse OGP cDNA as a probe.

Table 1 Ability of *ogp*$^{-/-}$ female mice to be fertilized *in vivo*

ogp genotype	Number of animals	Number of litters	Number of fertile animals	Mean litter size (± S.E.M.)*	*P* value†
$^{-/-}$	9	34	9	7.38 ± 0.59	0.14
$^{+/-}$, $^{+/+}$	9	36	9	8.55 ± 0.49	

* Mean litter size from at least three, mostly four, deliveries per mouse during the mating period.

† Data were analysed on a litter basis, and differences between groups were examined statistically.

Effect of *ogp* inactivation on reproductive ability

Despite the lack of OGP mRNA expression as demonstrated above, *ogp*$^{-/-}$ mice developed normally. To access the possible physiological role of OGP in the process of fertilization, the fertility of *ogp*$^{-/-}$ mice was tested. *ogp*$^{-/-}$, *ogp*$^{+/-}$ or *ogp*$^{+/+}$ female mice were bred with sexually mature male mice. The results of these nine breeding experiments showed that *ogp*$^{-/-}$ females were fertile (Table 1). Although *ogp*$^{-/-}$ females had a slight decrease in litter size compared with control animals, no statistical difference was observed between the groups (Table 1).

Attempts were then made to examine the ability of oocytes from *ogp* mutant mice to be fertilized using an IVF system. Preliminary experiments using *ogp*$^{-/-}$ oocytes found no significant difference in the fertilization rate compared with that of oocytes from wild-type mice at normal spermatozoa concentrations (10^6/ml) for conventional IVF (results not shown). It is well-known that only a few spermatozoa reach the ampulla of the oviduct, the site of fertilization *in vivo* [4]. Since the spermatozoa concentration around oocytes is the most obvious difference between the *in vivo* and *in vitro* microenvironments at the time of fertilization, we performed IVF studies at various spermatozoa concentrations using *ogp*$^{-/-}$ oocytes. In the initial IVF studies at concentrations of 10^5–10^7 spermatozoa/ml, the fertilization rate of *ogp*$^{-/-}$ oocytes at a concentration of 10^5 spermatozoa/ml was diminished by approx. 30 % compared with that of control oocytes under the same conditions ($P = 0.14$; Table 1). However, the results of six independent replicates showed no significant difference in the fertilization rate among animal groups at any spermatozoa concentration tested (Table 2). These results indicated that the fertilizing ability of *ogp*$^{-/-}$ females is within normal limits.

DISCUSSION

In the last two decades, OGP has been identified as a protein that is secreted from non-ciliated secretary epithelial cells of the oviduct. This glycoprotein has been characterized as a specific molecule associated with the ZP and/or perivitelline space of oocytes as

Table 2 Fertilization rate of *ogp*$^{-/-}$ oocytes at various spermatozoa concentrations *in vitro*

Spermatozoa used for IVF were isolated from caudae epididymides of sexually mature male B6C3F1 mice.

Oocyte *ogp* genotype	Number of animals	Age (weeks)	Number of oocytes fertilized/number of oocytes examined (%) at spermatozoa concentration			
			10^4/ml	10^5/ml	10^6/ml	10^7/ml
Experiment 1						
−/−	3	8–9	N.T.*	6/17 (35.3)	12/17 (70.6)	14/17 (82.4)
+/+	5	7–9	N.T.	9/14 (64.3)	14/19 (73.7)	14/16 (87.5)
Experiment 2†						
−/−	12	7–9	9/48 (18.8)	56/89 (62.9)	42/58 (72.4)	N.T.
+/−	10	7–9	3/62 (4.8)	40/69 (58.0)	38/52 (73.1)	N.T.
+/+	8	7–9	8/38 (21.1)	32/59 (54.2)	24/35 (68.6)	N.T.

* N.T., not tested.
† Pooled results from six independent replicates.

well as the surface of spermatozoa in various mammals, including humans [11–15]. Since OGPs show high molecular homology across species and their secretion is regulated by sex hormones, this raises a question concerning the physiological significance of the molecule in the process of mammalian fertilization. From a clinical viewpoint, the oviduct is widely considered to be inessential for human reproduction, except as a conduit for gametes, because conventional IVF/embryo transfer programs do not require exposure to any factor(s) originating from the oviduct. To date, however, some experimental evidence suggests that an association of gametes with OGP has a positive role in fertilization. In the cow, for example, oocyte fertilization was enhanced in preparations enriched in OGP, presumably owing to direct effects on both the oocyte [41] and the spermatozoon [51]. Similarly, exposure to purified OGP before and during fertilization decreased the incidence of polyspermy in oocytes. This treatment also provided a decrease in the bound spermatozoa number and an increase in post-cleavage development to the blastocyst in a porcine system [42], although the positive influence of OGP on fertilization is thought to be a highly homogeneous system, i.e. gametes and oviductal glycoprotein from the same species are required [39,40].

The present study demonstrated that *ogp*$^{-/-}$ female mice were mostly fertile *in vivo* (Table 1). As expected, *ogp*$^{-/-}$ males were also fertile (results not shown). In addition, no statistically significant difference was noticed between the fertilization rates of oocytes from *ogp*$^{-/-}$ females and control animals, evaluated using a conventional IVF method (Table 2). These results strongly suggest that OGP is dispensable for the process of fertilization, at least in mice.

It should be noted, however, that information on mouse OGP is relatively limited compared with that for other mammals, especially at the protein level. Early studies by Kapur and Johnson [52] showed that a glycoprotein reactive with wheat germ agglutinin (WGA) in oviductal fluid originated from oviductal epithelial cells, associated with postovulatory mouse ova and early embryos [52]. The WGA-reactive glycoprotein, named GP215, was subsequently sequestered within the perivitelline space of oocytes and developing embryos [53]. Meanwhile, we cloned mouse OGP cDNA [21] as a homologue of bovine OGP [18] and the predicted mouse OGP contained a unique seven-residue, Ser/Thr-rich repeat sequence (21 repeats) at its C-terminal side [21]. In the case of other rodents, such as hamster, the computer-calculated molecular mass of the mature hamster OGP is 70890 Da. The large molecular-mass difference between the native form of the glycoprotein (> 200 kDa) [54,55] and the calculated value suggests that this molecule is a highly glycosylated glycoprotein. It is striking that the amino-acid sequence of hamster OGP contains eight potential N-glycosylation sites and numerous Ser/Thr residues, possible sites of O-glycosylation, and most of these potential glycosylation sites are located in the C-terminal region [22]. Similar to mouse OGP, this region of the hamster OGP contains a unique repeating structure, composed of 15 Ser/Thr-rich amino-acid repeats. These unique repeating structures in the C-terminal region are obviously found only in the mouse and hamster, suggesting that the tandem repeats may be characteristic of the rodent OGPs. Since no partial amino-acid sequence of mouse GP215 has yet been reported, the molecular identity between GP215 and mouse OGP is still unclear. Unlike other mammals, such as the hamster, cow, pig, baboon and human, the biological dynamics of the mouse OGP in the oviductal fluid, as well as its localization within the female reproductive tract, are still unknown, since no molecular probe for detecting mouse OGP is available. In addition, clarifying potential developmental alterations by OGP, including changes in cleavage rates and in the number of embryos reaching the blastocyst stage, is also important issue, so that further careful studies of mouse OGP, especially analyses at the protein level as well as the effect on early embryonic development, are needed, and care must be taken in designing and evaluating studies of OGP function(s) in other mammalian species, including cattle and humans.

Since fertilization in mammals requires the successful completion of many steps, involving many genes, inactivation of one gene may not have an obvious phenotypic effect. The ability to reproduce is essential to living organisms, so very high molecular redundancy is likely to be involved in fertilization. Considering spermatozoon–oocyte recognition, for example, spermatozoon β-1,4-galactosyltransferase (GalTase) I is expressed on the plasma membrane of mouse spermatozoa and acts as a receptor for the ZP glycoprotein, ZP3 [56–58]. GalTase I-null male mice are reported to be fertile [59]; however, careful consideration is needed to conclude whether the molecule has any physiological significance in the process of fertilization. Indeed, studies by Rodeheffer and Shur [60] suggested that GalTase I-null spermatozoa can bind ovulated oocytes via a ZP3-independent mechanism, i.e. mediated by a factor(s) added to the ZP on ovulation. This experimental evidence might reflect the complex molecular nature of the microenvironment at the time of fertilization, including the contribution of oviductal factor(s). Although simple disruption of a specific gene is still useful for studying the molecular mechanism underlying mammalian fertilization, in some cases, inactivating multiple genes (a combination of reproduction-specific genes, such as

ogp, and/or other ubiquitous genes) will provide more precise information on molecular regulation in mammalian fertilization.

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