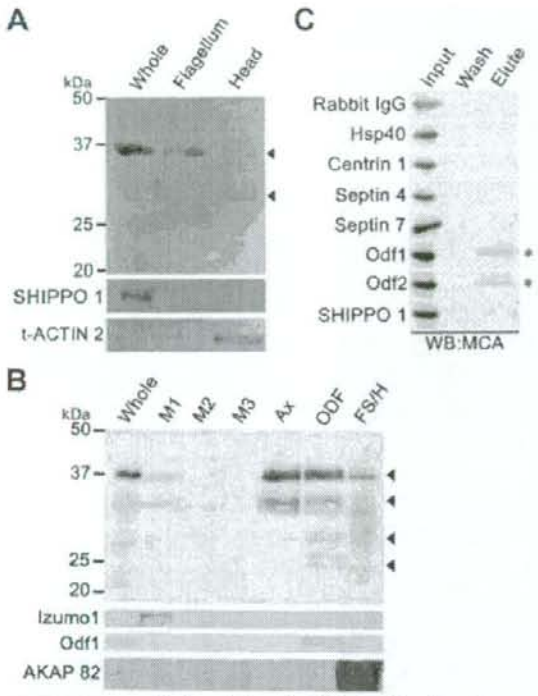

FIGURE 5. **Western blotting of the subcellular fractionation of sperm.** *A*, sperm was sonicated in PBS and fractionated by centrifugation. The *numbers* in the *left margin* indicate the molecular weights of the marker proteins. Anti-SHIPPO 1 or t-ACTIN 2 antibodies were used as controls in the flagellum or sperm head fractions (24, 37). *Arrowheads* indicate the signal of MCA. *B*, whole sperm fractions extracted with SEB. Approximately 20 μg of solubilized protein in each of the fractions obtained by sequential extraction with 1% Triton X-100 (*M1*, *M2*, and *M3*), potassium thiocyanate (*Ax*), and urea (*ODF*), and the remaining pellet (*FS/H*) extracted with SEB were tested for the presence of a sperm membrane protein (*Izumo1*), an FS component (*AKAP 82*), and an ODF protein (*Odf1*). Molecular weight marker bands are shown at the *left*. *Arrowheads* indicate the signal of MCA. *C*, lysate of testicular germ cells was immunoprecipitated with antibodies against flagellar proteins. Each antibody is indicated in the *left margin*. The Dynabeads-protein G treated with each antibody was reacted with the lysate (*Input*). After washing (*Wash*), the protein complexes were eluted from the Dynabeads using elution buffer (*Elute*). Each fraction was subjected to SDS-PAGE and Western blotting (*WB*) with anti-MCA antibodies. The *asterisk* indicates significant signals.

MCA was not detected in immunocomplexes with Hsp40 flagellum protein (6) and testis-specific centriole protein (*Centrin 1*, Fig. 5*C*) (23).

To examine the subcellular localization of MCA in detail, its presence in sperm treated with 0.05% CTAB and in samples from fractionation experiments following 1% Triton X-100 and 4 M urea treatment was determined using immunocytochemistry. Nontreated sperm displayed basic staining patterns for MCA (Fig. 6, *A–C* and see supplemental Fig. S5); the entire length of the sperm flagellum was stained homogeneously, and the sperm heads were stained weakly. In sperm treated with 1% Triton X-100, the membranes, organelles (including the mitochondrial sheath in the midsection), and cytosolic proteins are solubilized (24). These changes cause the sperm heads to bend because of the weakened support of the neck. In addition, the ODFs are sometimes observed to protrude from the weakened annulus region (10). In Triton X-100-treated sperm, the MCA signal was observed in the fibers along the rest of the flagellum and disappeared from the heads (Fig. 6, *D–H*, and see supplemental Fig. S5 in the supplemental materials). Therefore, MCA is associated with the ODF. As determined by fractionated Western blotting, the treatment of sperm with 4 M urea efficiently extracted most of the ODF protein together with MCA from the sperm flagellum. After only 15 min of exposure to urea, the MCA signal became scattered, and the fiber-like view of MCA in liberated ODF was lost (Fig. 6, *I–K*). Following 30 min of CTAB treatment, sperm released individual ODFs that were freed from the central core of the tail and dispersed distally to the connecting piece. Most sperm heads were separated from the tail, and resistant ODFs were frayed completely and joined together only at the connecting piece (14). MCA and Odf2 signals were clearly observed in individual fibers and the connecting piece (Fig. 6, *L–S*). Therefore, MCA is associated with ODFs and the connecting piece in a nonionic detergent-resistant form (Triton X-100). The MCA signal also appeared in the fibrous proteins that constitute the ODF, following exposure to an ionic detergent (CTAB).

Abnormal Sperm Might Be Phagocytosed by Sertoli Cells—The mass of *MCA* homozygous mutant testis was normal compared with that of wild-type mice (Table 2). However, there was no sperm in the epididymis of mutants. We also examined apoptotic cells in the testis. Apoptosis signals did not increase in germ cells, as determined by the detection of caspase-3 antibody (Fig. 7*A*). There were 0.33 ± 0.19 (average ± S.E.) and 0.20 ± 0.11 positive cells/tubule detected in the testes of homozygous and heterozygous mice, respectively. All cells in the centers of seminiferous tubules and Leydig cells were stained uniformly in testis sections of heterozygous and homozygous mutants. Therefore, these signals resulted from nonspecific staining. Using the TUNEL method, in the testes of heterozygous and homozygous mice, 0.15 ± 0.08 and 0.70 ± 0.19 TUNEL-positive cells/tubule, respectively, were detected (Fig. 7*B*). TUNEL-positive cells increased slightly in the germ cells of the homozygous mutant. There were 5.00 ± 0.75 strong signals with the shape of sperm nuclei per seminiferous tubule detected near the tubule walls, but not in the center, in the homozygous mutant mice only (Fig. 7*B*). The signal visualized using the TUNEL method was located near the nuclei of sperm in Sertoli cells (see supplemental Fig. S6). This signal may indicate the degraded products of sperm nuclei that were phagocytosed by Sertoli cells. *MCA* mutant sperm in the testis did not undergo apoptosis, but might be instead phagocytosed by Sertoli cells.

Sperm of MCA Mutant Mice Can Produce Viable Embryos—MCA was expressed from the spermatocyte through to sperm stages and was localized in meiotic metaphase chromosomes during meiosis (2). We examined whether the nuclei of *MCA* mutant sperm maintained the ability to produce viable embryos using testicular sperm extraction and intracytoplasmic sperm injection (TESE-ICSI). Sperm derived from heterozygous and homozygous *MCA* mutant mice was injected into eggs. Two-cell-stage embryos produced from heterozygous (n = 36) or

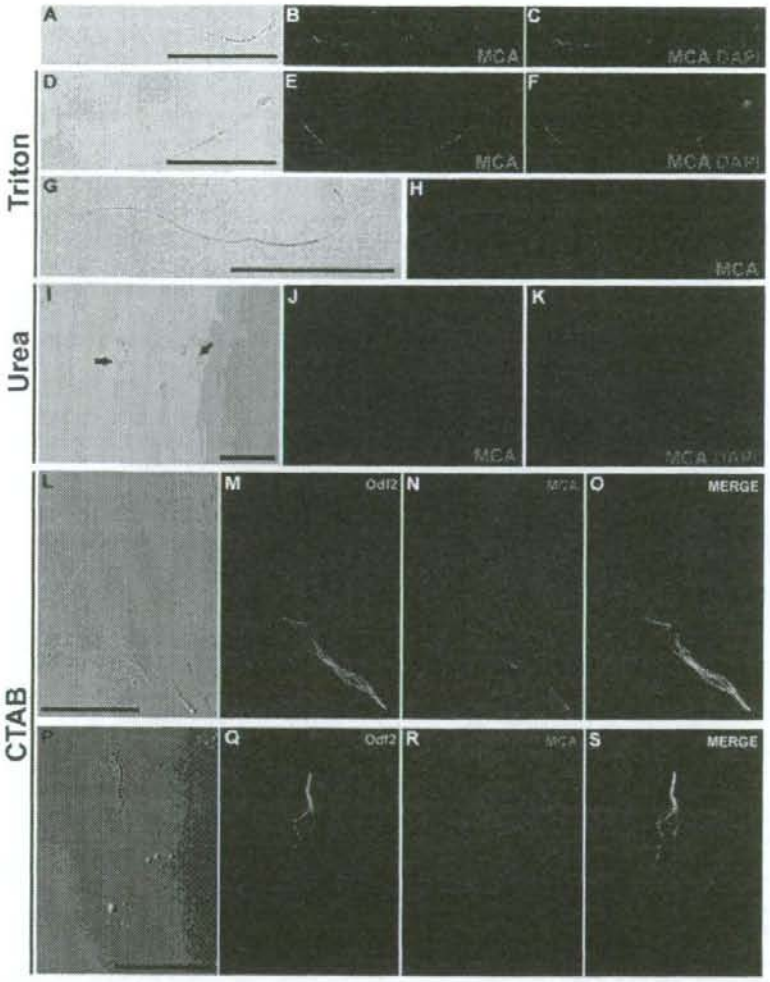

FIGURE 6. **Immunohistochemical examination of MCA in sperm.** Sperm and flagella from the cauda epididymis were stained with anti-MCA antibody (*A–C*). Following treatment with Triton X-100, the MCA signal disappeared from the sperm head but was maintained in the insoluble fractions of the flagellum (*D–H*). The signal disappeared from flagella subjected to urea treatment (*I–K*). Sperm treated with 0.05% CTAB to release the outer dense fibers (*L–S*) were stained with rhodamine-conjugated anti-MCA antibody (*N* and *R*). Samples were also reacted with anti-Odf2 antiserum and stained with FITC-conjugated secondary antibodies (*M* and *Q*). MCA signal was co-localized with Odf2 (*O* and *S*). *Bars* = 50 μm (*A–K*) or 25 μm (*L–S*).

homozygous ($n = 31$) *MCA* mutant sperm were transferred to the oviducts of pseudopregnant females. Nineteen (52%) and 10 (33%) pups were born from heterozygous and homozygous *MCA* mutant sperm, respectively, indicating that the nuclei of *MCA* mutant sperm were as capable of producing viable embryos as those of heterozygous sperm from fertile males.

SNPs in Human MCA—The analysis of human *MCA* sequences in more than 200 infertile male patients and in 172 proven-fertile male volunteers revealed two amino acid substitution-causing SNPs in the open reading frame of *MCA*. These SNPs were found in exon 2 in two of the azoospermia-infertile and in one of the oligospermia-infertile patients (Table 3 and see supplemental Fig. S2). The *a92c* SNP at amino acid 31, located within the first MORN motif in the N-terminal region, was found in three cases of heterozygosity and caused a shift from histidine to proline. The *g121a* SNP at amino acid 41, located between the first and second MORN motifs, was found in one case of heterozygosity and caused a shift from glycine to arginine. The *g393a* SNP in exon 5 at alanine 131 did not induce an amino acid substitution as seen by Rs2839536 in the dbSNP data base of the National Center for Biotechnology Information (NCBI). The prevalence of the *g393a* SNP was similar in proven fertile and infertile patients (Table 3).

DISCUSSION

In this study, we showed that the germ cell-specific *MCA* gene is essential for spermiogenesis and that two different SNPs in the human *MCA* gene (*h-MCA*) are associated with male infertility. A hydropathy plot (25) of the deduced MCA sequence revealed that MCA contains strongly hydrophilic regions throughout its total length (2). MCA is expressed predominantly in the cytoplasm of cells through a variety of stages, ranging from pachytene spermatocytes to sperm. MCA in sperm is localized throughout the cytoplasm and is specifically concentrated in the fibrous sheath (FS) and ODF of the flagellum. Apart from the axoneme and its associated proteins, the sperm flagellum consists of two exclusive cytoskeletal components as follows: the FS in the principal piece and the ODFs in the middle and principal pieces. Nine ODFs are anchored proximally at the connecting piece and run parallel to the tubulin doublets of the axoneme toward the distal end of the flagellum. Longitudinal columns and transversal ribs of the FS surround the ODF in the principal piece (26). The majority of proteins in the ODF and FS are hydrophobic and resist solubilization by ionic detergents (*e.g.* SDS) (10, 27, 28). Many proteins that were localized in this area have been identified previously (29). Of these proteins, glyceraldehyde-3-phosphate dehydrogenase-S was predominantly localized in the FS; however, it may be active only in the hydrophilic state

(30). There is little information available on how the hydrophilic FS and ODF are assembled in developing spermatids and how proteins in the FS and ODF maintain their functions. Even the hydrophilic protein MCA can occur in the FS and ODF, although it may be modified. Interactions between MCA and other proteins may support flagellum function and development.

MCA contains a set of seven MORN motifs. JPs, which are components of the junctional complexes that are expressed abundantly in the heart and brain, contain a conserved MORN motif (3, 4). The MORN motifs of JPs have tandem repeats of eight MORN motifs at the N-terminal region and a hydrophobic domain in the C-terminal region, which may function in anchoring the protein to the cellular membrane (see supplemental Fig. S7) (3). Other proteins containing a few MORN motifs have also been reported (see supplemental Fig. S7). MORN motifs may play an important role in protein complexes; however, the exact function of the MORN motif is not clear. The localization of the MORN motif in MCA was not significantly different from that in JPs. The exact function of the MORN motif may be more noticeable in MCA that consists of the MORN motif only.

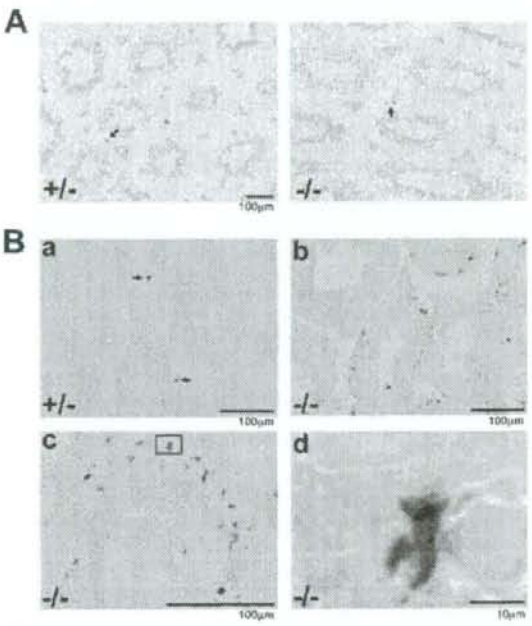

FIGURE 7. **Occurrence of apoptosis in the testis.** *A*, expression of active caspase 3 was observed in heterozygous (+/−) and homozygous (−/−) mutant mouse testis. *Arrows* indicate germ cells expressing active caspase 3. Cells in the centers of the tubules and Leydig cells were stained nonspecifically. *B*, TUNEL staining was performed on sections of testis. Apoptotic signals were observed in heterozygous mutant testis (*arrows*) (*panel a*). Signals indicated that phagocytosed cells were observed in the tubule walls of homozygous mutants (*panels b–d*). *Panels c* and *d*, high magnification of the signals found in mutant testis. *Panel d*, high magnification of the *box* in *panel c*.

Sperm in the testis of mutant mice appeared normal under light microscopy; however, detailed analysis using electron microscopy revealed some abnormalities. In other gene mutational mice that display abnormal sperm formation, sperm is sent to the epididymis even if it is abnormal (31). In addition, the abnormal sperm is observed in the vaginal plugs. However, the *MCA* mutant mice were azoospermic, and most defective sperm might be phagocytosed by the Sertoli cells. The absence of sperm in the epididimydis can be at least partly explained by Sertoli cell phagocytosis of defective sperms.

In the rat, epididymal ligation causes extensive degeneration of the seminiferous epithelium with loss of virtually the entire germ cell population and a significant decline in both testicular and epididymal weight (32), although ligation of the initial segment of the caput epididymis results in temporary testicular changes followed by evidence of recovery in the mouse (33). These testicular alterations differed from that of the *MCA* mutant testes. Furthermore, a few abnormally shaped sperm heads were occasionally observed in the cauda epididymis using light microscopy (data not shown). *MCA* mutant mice were nonobstructive azoospermic, although the relative size of the testis was normal. SNPs in the MORN motif of MCA only occurred in infertile men. These SNPs were heterozygotes of the major and minor SNP. *DMC1* is a *RecA* homolog that is specifically expressed during meiosis and is thought to play an important role. When one allele is not expressed in the DMC1 protein, the dysfunction does not appear (34). However, abnormal meiosis occurs when the mutant DMC protein is expressed from another allele (35). The male infertility-specific SNPs that we found may cause male infertility through their effects on mutant MCA protein expression. The *a92c* SNP may cause male infertility more readily than does the wild-type MCA and

TABLE 3
Prevalence of *MCA* SNPs in infertile and proven-fertile human populations

Exon	Position[a]			Genotype	No. of SNP				Reference (NCBI dbSNP)
	Nucleotide	Amino acid			Infertile		Proven fertile		
						%		%	
Exon 2	92	31	(H)	A/A	242	(99)	172	(100)	
			(H/P)	A/C	3	(1)	0	(0)	
			(P)	C/C	0	(0)	0	(0)	
	121	41	(G)	G/G	244	(99.5)	172	(100)	
			(G/R)	G/A	1	(0.5)	0	(0)	
			(R/R)	A/A	0	(0)	0	(0)	
Exon 5	393	131	(A)	G/G	141	(62)	82	(62)	
			(A)	G/A	79	(35)	41	(31)	Rs2839536
			(A)	A/A	7	(3)	9	(7)	
				ND[b]	20		40		

[a] Translation start site was +1.
[b] G393A haplotype in exon 5 was not decided exactly in 20 and 40 persons of infertile or proven fertile group, respectively.

may be transferred to the next generation via the female, as indicated by the results of the *MCA* mutant mouse study. Approximately 15% of couples that attempt to conceive over a 2-year period are unable to become pregnant (36). Recent technological developments in *in vitro* fertilization have ensured that even when sperm activity is low, pregnancy and birth are possible. The molecular mechanisms behind infertility remain uncertain. It is possible that SNPs in MCA are related to human infertility.

Collectively, our results demonstrate that MCA proteins containing the MORN motif play an important role in the construction of hydrophobic protein complexes in the sperm flagellum. These findings are valuable not only for understanding the molecular mechanisms of spermiogenesis but also for determining the function of proteins encoded by the MORN motif.

Acknowledgments—We thank Mayumi Ikenishi, Lina Fujita, and Mitsuko Yokota for technical assistance.

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Int. J. Dev. Biol. 52: 657-664 (2008)
doi: 10.1387/ijdb.072529mi

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Mechanisms of sperm-egg interactions emerging from gene-manipulated animals

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ABSTRACT Sperm-egg interactions have been studied for many years using biochemical approaches such as the employment of antibodies and ligands that interact with sperm or with eggs and their vestments. As a result, various factors that participate in fertilization have emerged. However, when animals were genetically manipulated to examine the roles of those factors, most of them were found, to our surprise, to be "not essential". Of course, all biological systems contain redundancies and compensatory mechanisms, but at least some factors were found to be "essential" after gene disruption. As a whole, the explanations of sperm-egg interactions require significant modification from the gene manipulation point of view. In this review, information about sperm-egg interactions obtained from genetically manipulated animals is mainly revisited in order to propose a new vision.

KEY WORDS: *fertilization, knockout mouse, sperm-zona interaction, sperm-egg fusion*

Introduction

About a billion years ago, living organisms devised sex (Margulis and Sagan, 1986) to facilitate a genetic shuffling for better survival and quick evolution. Since then enormous numbers of recognition and fusion of males and females have been successfully accomplished and the process has evolved immensely. In mammals, males produce innumerable tiny sperm while females produce much larger eggs with a thin glycoprotein layer: the zona pellucida. How do sperm and egg recognize each other, make contact, and achieve fusion? Various factors have been reported with convincing evidence. However, the recently-introduced, homologous gene disruption technique revealed many of the genes were dispensable because the animals devoid of those genes showed substantial fertilizing ability. One can argue that the disappearance of a factor could be compensated by adjusting the amount of equally functioning factors in gene disruption experiments. However, sperm are like "rockets" with limited resources available once launched. Sperm DNA are tightly packed with protamine and the transcriptions are shut down. Even if the sperm can carry out some *de novo* protein synthesis during capacitation using stored mRNA and mitochondrial ribosomes (Gur and Breitbart, 2006), it is difficult to imagine that the sperm rearrange their function by replacing one factor with others. One can also argue that, based on the importance of fertilization phenomena, various steps are made with backup systems. However, recent gene disruption experiments indicated that there are "essential" factors in fertilization, because in various cases, the lack of a factor leads to the complete loss of fertilizing ability of gametes. Thus the quest for more "essential" factors and analysis of the relationship among those factors represent the most promising path to elucidate the mechanism of fertilization in molecular bases. In the present review, we introduce essential factors in fertilization and clarify their relationships.

Eggs

Eggs are released into the peritoneal or bursal cavity and then are picked up by the cilia of the infundibulum. Mammalian eggs are almost invariably covered by numerous cumulus cells and an extensive extracellular matrix, which is recognized by specific receptors on the tips of the cilia. Pickup of this complex by the oviduct has been recorded in beautiful video pictures available online by Talbot *et al.* using hamsters (Talbot *et al.*, 1999) (Fig. 1).

After being picked up from the infundibulum, the eggs move to the ampulla of the oviduct and wait to be fertilized by sperm. It is not clear why or how they stay in this area, but they do, enveloped in the cumulus mass until fertilization occurs. Eggs have fertilizing ability depending on the nature of cumulus cells and zona pellucida (Yanagimachi, 1994), but surprisingly, fus-

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Published online: 4 July 2008

0214-6282/2008/$35.00

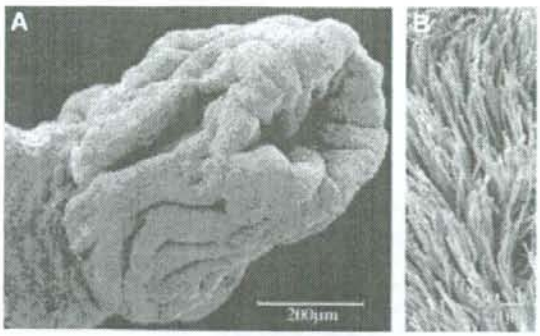

Fig. 1. Hamster infundibulum and magnified view of cilia on its surface *(Talbot et al., 1999). An online video showing the movement of ovulated eggs into the oviduct is available at: http://www.molbiolcell.org/content/vol10/issue1/images/video/mk0190776002b.mov*

ing ability with sperm is formed even as - the immature eggs - develop without cumulus cells inside mouse testis (Isotani *et al.*, 2005).

Sperm

Sperm and eggs move in different directions. Sperm need to ascend the female reproductive tract to find eggs residing in the oviduct, while the eggs descend the oviduct and enter the uterus after fertilization. Usually one egg in humans and about ten in mice await sperm in the oviduct. Compared to the numbers of eggs, an overwhelming number of sperm are ejaculated into the female reproductive tract. However, only a small number of sperm reach the fertilization site. The uterus and oviduct are connected at the uterotubal junction (UTJ). The outer portion of the oviduct hangs into the uterus and forms a colliculus in mice, pigs and cows. The tract is very narrow at the UTJ and sperm are not able to migrate freely into the oviduct through the UTJ, decreasing the number of sperm participating in fertilization. It is not known if only selected sperm can penetrate the UTJ, but various factors are known to be essential for sperm to pass through the UTJ (Cho *et al.*, 1998, Hagaman *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004). When we made chimeric mice that ejaculated a mixture of wild-type sperm and motile but not fertile sperm from calmegin-disrupted mice, only the former sperm in the same ejaculates could migrate to the oviduct. This result indicates a possibility of selection at the UTJ (Nakanishi *et al.*, 2004).

Sperm chemotaxis toward eggs before fertilization has been demonstrated clearly in ascidians (Yoshida *et al.*, 2002). In humans, olfactory receptors (ORs) on sperm might function in finding eggs. The hOR17-4 was demonstrated to function in human sperm chemotaxis (Spehr *et al.*, 2003). Mouse sperm might also locate eggs by sensing a chemoattractant (Fukuda *et al.*, 2004). The disruption of an ion channel expressed in VNO neurons are reported to cause females to behave like males (Kimchi *et al.*, 2007). The contribution of ORs in fertilization must be proven by gene disruption experiments.

Sperm are produced in the testis, transferred into the epididymis and remain stored in the cauda epididymis. Once ejaculated, they must be activated by stimuli from the female environment and start to swim vigorously. Only capacitated and acrosome-reacted sperm are competent to fertilize eggs, like matches being struck and activated before acquiring capability to cause fire (Fig. 2)

The nature of the capacitation process is not well understood, but there are many papers indicating the importance of protein phosphorylation and calcium ion influx upon release of "decapacitation factor(s)" from sperm (De Jonge, 2005). It should be noted that the acrosome reaction is a change that happens only in capacitated spermatozoa and gradually increases to 30–40% during 1–2h of incubation *in vitro* in mouse sperm population. This means sperm are not homogeneous but are individually different from each other. However, most reports treat sperm as a mixed mass to evaluate sperm status because there is not a convenient method to separate sperm at different stages of capacitation and/or acrosome reaction. We must be aware that this homogeneity problem exists in the experiments which analyze sperm as a combined mass.

Various methods are proposed to observe acrosome reaction (Cross and Meizel, 1989, Larson and Miller, 1999, Saling and Storey, 1979). Our strategy to observe the acrosomal status under microscope is to use transgenic mouse lines with green fluorescent protein (GFP) in their acrosome. We produced transgenic mice whose sperm have GFP in their acrosome; the green fluorescence is clearly seen with no previous treatment of sperm. After the acrosome reaction, GFP disappears within three seconds (Nakanishi *et al.*, 1999). Sperm from these mice could easily be analyzed using a flow cytometer, and real-time analysis of acrosome reaction is possible (Nakanishi *et al.*, 1999). Although the GFP disperses from acrosome rapidly, other acrosomal components such as MN7 antigen and MC41 antigen remain on sperm for at least 15 min. Thus, it was shown that the acrosome reaction is not a simple all-or-none phenomenon, but one with intermediate stages. Sperm–egg interactions must be investigated taking into account factors such as an intermediate stage of acrosome-reacted sperm (Kim and Gerton, 2003).

The acrosin-GFP mice are available to the public through RIKEN BRC or CARD, Kumamoto University under the registered name, B6;C3 Tg(acro3-EGFP)01Osb or C57BL/6-Tg(CAG/Acr-EGFP)C3-N01-FJ002Osb (http://www.brc.riken.jp/lab/animal/en).

Sperm-egg interactions: an original view

PH-20 has been indicated to have a role in sperm binding with the zona pellucida, based on the finding that two out of the three monoclonal antibodies raised against PH-20 inhibited sperm–zona binding (Primakoff *et al.*, 1988). In 1993, a group studying snake venom found a significant homology of hyaluronidase to PH-20 (Gmachl and Kreil, 1993). These structural data seemed to support the long-held view that hyaluronidase plays a role in fertilization. In fact, in macaque monkeys, zona penetration was completely blocked by anti-PH-20 IgG when present during sperm–oocyte interaction (Yudin *et al.*, 1999). However, when PH-20 gene-disrupted mice were produced and examined, the mice showed a reduced ability to disperse cumulus cells but were found to be fertile (Baba *et al.*, 2002). Baba's group found a remaining hyaluronidase activity in PH-20 disrupted mouse sperm, and they discovered an additional sperm specific hyaluronidase

gene Hyal5 very close to PH-20 in the genome (Kim *et al.*, 2005). The role of hyaluronidase in sperm-egg interaction will be concluded when we see the binding ability impaired sperm from Hyal5 knockout or PH-20 and Hyal5 double knockout mice.

There are more factors suggested to be involved in sperm–zona interactions. Beta 1,4-galactosyltransferase (GalTase) is reported to function not as an enzyme, but as a sperm-egg-binding factor. Various reports exist supporting this notion. However, when a GalTase-disrupted mouse line was produced by Shur's group, unexpectedly it was found that sperm lacking GalTase could fertilize eggs and the males were not sterile although there were some minor defects in sperm-egg interactions (Asano *et al.*, 1997, Lu and Shur, 1997). This could be interpreted as suggesting that the role of GalTase was compensated by other factors and another candidate SED1 was reported as a second zona-binding factor (Ensslin and Shur, 2003). When SED1 gene was disrupted, it was found that the mice were not sterile. Such an unexpected outcome by disrupting genes for sperm-egg interaction dates back to 1994. The first gene disruption aimed at studying sperm-egg interaction was the gene for acrosin. Despite hundreds of papers supporting the importance of sperm acrosin in fertilization, acrosin-null sperm can still fertilize eggs, albeit with a slight delay compared with wild type (Adham *et al.*, 1997, Baba *et al.*, 1994). Baba's group found that protease activity persists in the sperm of acrosin gene-disrupted mouse. Altogether they found five more testis-specific proteases, from TESP1 to TESP5 (Honda *et al.*, 2002), but the roles of these enzymes in fertilization await further investigation.

There are further examples that the disruption of "important" factors results in an unexpectedly mild phenotype or shows an unpredicted phenotype in fertilization. Fertilin was originally clarified as an antigen recognized by the anti-guinea pig sperm monoclonal antibody PH-30. As the PH-30 antibody inhibited sperm from fusing with eggs, the PH-30 gene was cloned and analyzed. The antigen was found to be a heterodimer and one of the monomer genes contains a fusogenic domain similar to viral fusogen. Moreover, the other half of the heterodimer was found to contain a disintegrin domain, which binds to integrin. Thus the antigen that reacts to PH-30 was speculated to be a fusion protein and named "fertilin," which consists of fertilin alpha and beta (ADAM1/2) heterodimer(Blobel *et al.*, 1992). After this report, various types of circumstantial evidence were reported to support the role of fertilin. However, when Adam2, which forms a fertilin heterodimer, disrupted mice were produced, they found that Adam2-null sperm was shown to have fusing ability with eggs but unexpectedly, the sperm failed to bind to zona pellucida (Cho *et al.*, 1998). Later, it was found that Adam1 consisted of two independent genes: Adam1a and Adam1b (Nishimura *et al.*, 2002). Both of the ADAM1 family proteins could make a heterodimer with ADAM2, but "fertilin" on sperm surface was found to be exclusively an ADAM1b/ADAM2 type. Baba's group disrupted the ADAM1b gene but the mice were fertile without fertilin on sperm (Kim *et al.*, 2006).(This will be discussed below.) Similar stories continue in disruptions of CD46 (Inoue *et al.*, 2003), ZPBP1 (Lin *et al.*, 2007) and PKD-REJ (our unpublished data) and in some more factors (private communications).

If the fertilization mechanism consists of–redundantly prepared factors, it is not possible to analyze the role of factors with a single gene disruption experiment. If this was the case, the above mentioned factors may represent the redundant factors. If there is a factor which is not redundant and not able to be compensated, the disruption of a concerned factor must produce sterile mice. Such cases are described below.

Sperm-egg interactions: an emerging view

The first case of normally swimming sperm with normal shape and numbers failing to fertilize eggs was our report on the calmegin gene-disrupted mouse (Ikawa *et al.*, 1997). Calmegin$^{-/-}$ males are almost sterile with a lack of sperm zona-binding ability. When sperm from such males were added to cumulus-free eggs and observed under the microscope, we noted that the sperm had lost their zona-binding ability and were bouncing off the zona pellucida. However the calmegin is not directly involved

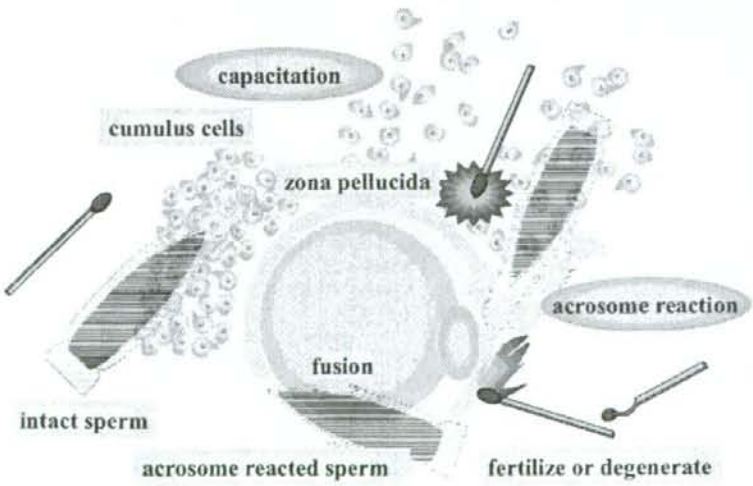

Fig. 2. Mechanism of sperm–egg interaction. *Sperm stored in the epididymis are kept metabolically inert to facilitate prolonged storage (left). Each sperm has a membranous sac over the nucleus called the acrosome which is filled with many kinds of hydrolytic enzymes. After sperm are exposed to the female reproductive environment, they become metabolically active. They undergo capacitation, which permits the acrosome reaction, and start to swim extremely vigorously (hyperactivation). Near the eggs, probably stimulated by the cumulus cells and zona pellucida, they undergo the acrosome reaction to release the acrosomal contents by exocytosis. Only acrosome-reacted sperm are known to fuse with eggs but their competency for fusion does not last long. The integrity of the acrosome in the mouse sperm can be monitored easily by using transgenic mice in which the GFP protein is targeted to the acrosomal contents (Nakanishi* et al., *1999).*

in sperm-egg interactions. Calmegin is a testis-specific homologue of the ubiquitously-expressed endoplasmic (ER) molecular chaperone calnexin. During spermatogenesis, sperm shed machineries for protein synthesis, including the ER. Thus, even in wild-type mice, there is no calmegin left on the sperm. Therefore, it is easily speculated that calmegin is acting to fold molecule(s) that are destined to function in sperm-egg binding. After we published the calmegin disruption, two other gene disruptions, Adam2 and angiotensin converting enzyme (ACE), were found to result in male sterility. Peculiarly, these knockout mouse lines and calmegin knockout mice share the phenotype of impaired zona-binding ability and, at the same time, an impaired UTJ penetrating ability. We thought the interaction of calmegin with ADAM2 was conceivable, so we immunoprecipitated calmegin from testicular lysate and examined the interaction of calmegin with ADAM2. Immunoprecipitation followed by western blot analysis revealed that ADAM1a, ADAM1b and ADAM2 formed complexes specifically with calmegin in the ER. The disruption of calmegin was shown to cause impaired heterodimerization of ADAM1a/2 and ADAM1b/2 leading to the complete loss of ADAM2 from mature sperm. Thus the reason why the calmegin and Adam2-disrupted mice share the same phenotypes became clear (Ikawa *et al.*, 2001).

To our surprise, the Adam3-disrupted male mice were also sterile with impaired zona-binding ability (Shamsadin *et al.*, 1999). Why did so many gene disruptions result in a single phenotype? This was explained by a simple reason. The amount of an ADAM family protein in sperm is easily affected by the disappearance of other ADAM family members. Baba's group found the testis specific ADAM1a was essential in fertilization, but they also found that when ADAM1a was eliminated, ADAM3 also disappeared from sperm, (Nishimura *et al.*, 2004). The synchronized disappearance of ADAM family proteins are reported by many researchers (Cho *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004, Stein *et al.*, 2005). A stunning result was reported by Baba's group. When they produced Adam1b (fertilin beta) disrupted mice, they found Adam2 also disappeared from sperm, but the sperm were fertile (Kim *et al.*, 2006). This result clearly indicates that the intensively studied protein "fertilin" is dispensable in fertilization. Combining these data together, ADAM3 emerged to be a putative key molecule in fertilization. The mechanism of infertility caused by ACE disruption remained unclear for many years. However, the relationship between ACE and ADAM3 was demonstrated. When we analyzed the ADAM3 in Ace-disrupted mice, we suggested the membrane micro-domain specific disappearance of ADAM3. We found a significant decrease of ADAM3 on Ace-/- sperm in the Triton X-114 detergent-enriched phase after phase separation, while ADAM2 remain unchanged on sperm (Yamaguchi *et al.*, 2006) (Fig. 3).

Combining these facts together, the most important factor that may participate in sperm-zona binding is tentatively ADAM3 (Yamaguchi *et al.*, 2006). However, multiple isoforms of Adam3 transcripts observed in the human were non-functional owing to the presence of deletions and in-frame termination codons (Frayne *et al.*, 1999). Therefore, ADAM3 cannot be an ultimate universal zona-binding factor among mammals.

In terms of other candidate factors for zona binding, sp56 was identified as having the characteristics expected of the sperm protein responsible for recognition of egg zona pellucida. The complementary DNA encoding sp56 was isolated and its primary sequence indicates that sp56 is a member of a superfamily of protein receptors (Bookbinder *et al.*, 1995). Zonadhesin is a multiple-domain transmembrane protein believed to function as a sperm–zona pellucida binding protein(Hardy and Garbers, 1995, Jansen *et al.*, 2001, Lea *et al.*, 2001, Wassarman, 1992). We must examine the fertilizing ability of sperm that lack these factors to reach a definite conclusion.

Moreover, it should be noted that all of the calmegin, ADAM1a-, ADAM2- and ACE-disrupted mouse sperm share the phenotype of inability, not only to bind to zona, but also to migrate into the oviduct. (Cho *et al.*, 1998, Hagaman *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004) The puzzle is why the two different inabilities of sperm–zona binding and oviduct migration run in parallel in these gene disruption experiments. We believe this phenomenon could be a good clue in helping to understand the molecular mechanisms of fertilization.

Membrane fusion

The lipid membrane has fluidity both horizontally and transversely and here are various mechanisms to maintain the lipid constitutions of both the outer and inner sides of the membrane. When the cell needs to divide, the membrane must be separated into two sections. Topologically, to achieve this, there must be a membrane break in the lipid bilayer at some point of the cytokinesis. If the egg membrane is broken artificially, as when we make a hole in the egg membrane to do intracytoplasmic sperm injection (ICSI), the opening normally seals back immediately. However, the ability for restoration is not consistent. It differs depending on the stage of the eggs. It also differs depending on the species. For example, mouse eggs are much more fragile than human eggs. We are not aware of the causes, but the adjustment and formation of a characteristic nature of the membrane must occur through a combination of various mechanisms such as the membrane undercoat and the constitution of lipids to form membrane. Sperm membrane is also known to have a complicated structure that contains cholesterol-dense "rafts" which are modified extensively during sperm capacitation (De Jonge, 2005), and evenly observed membrane by electron microscope is actually divided in some areas when examined by anti-sperm antibody.

Various important membrane fusion events exist in several tissues. For example, skeletal muscle cells are formed after myoblast cell fusion. Trophoblast cells fuse with each other to form syncytiotrophoblasts, which are necessary for implantation. Osteoclasts resorb the bone at multiple sites, occasionally containing more than 100 nuclei, and are formed by cell fusion(Yagi *et al.*, 2005). Mitochondrial membranes are known to fuse with neighboring mitochondrial membranes using mitofusin1 and mitofusion2 (Chen and Chan, 2005). One of the most precisely investigated fusion processes is exocytosis. SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors) and some kinds of viruses appear to mimic the fusion mechanism using SNAREs for invasion. (Chan *et al.*, 1997, Lu *et al.*, 1995, Weissenhorn *et al.*, 1997). The structures of viral fusion proteins suggest that the fusion machineries employ a fundamentally similar mechanism to coalesce lipid bilayers. Fertilization is the phenomenon that involves membrane fusion between the plasma membrane of an unfertilized egg and the freshly-rear-

ranged sperm membrane soon after the acrosome reaction. Both gametes have to be conditioned properly to accomplish membrane fusion, but the factors involved in fusion were not known for many years.

Sperm–egg fusion in gene disrupted mice

Gene disruption experiments not only destroy the predicted importance of various factors, but also introduce new factors in the mechanism of fertilization. Because all who produce gene knockout mouse lines need to breed them to keep the strain alive, if there is any defect in the fertilization process, it will inevitably draw the attention of researchers in any field. For example, CD9 was disrupted aiming to determine the effects on immune functions. However, the CD9-deficient mice looked healthy and lived normally, but surprisingly, the females lacking CD9 were sterile, while the male mice remain normal (Kaji *et al.*, 2000, Le Naour *et al.*, 2000, Miyado *et al.*, 2000). We analyzed the cause of the sterility using *in vitro* fertilization (IVF) and found that the eggs had no fusing ability with sperm (Miyado *et al.*, 2000). Since fusion did not take place, the cortical granules were not released to block the polyspermy (Barros and Yanagimachi, 1971, Yanagimachi, 1994) which allowed multiple sperm to penetrate into the perivitelline space as shown in Fig. 4A. Thus, the very first discovery of an essential factor in sperm–egg fusion was found serendipitously.

CD9 is a member of the "tetraspanin" family of proteins having four transmembrane domains and ability to bind with integrins. Since integrins α6 and β1 were found on the egg membrane, the effect of the addition of synthetic integrin peptides in the IVF system was examined and the inhibition of sperm-egg fusion was reported (Chen *et al.*, 1999). However, again the gene disruption experiments in integrin α6 and β1 revealed that both integrins are not essential for eggs to fuse with sperm (Miller *et al.*, 2000).

Sperm must have completed the acrosome reaction prior to fertilization. This may imply that fusogenic factors are latent in fresh sperm and exposed only after acrosome reaction. We raised monoclonal antibodies against human sperm and screened one which did not react to fresh sperm, but to acrosome- reacted sperm. If the antibody could inhibit sperm–egg fusion, the corresponding antigen could be a good candidate as a fusion factor. Based on this hypothesis, we raised the anti-human sperm monoclonal antibody MH61 to meet

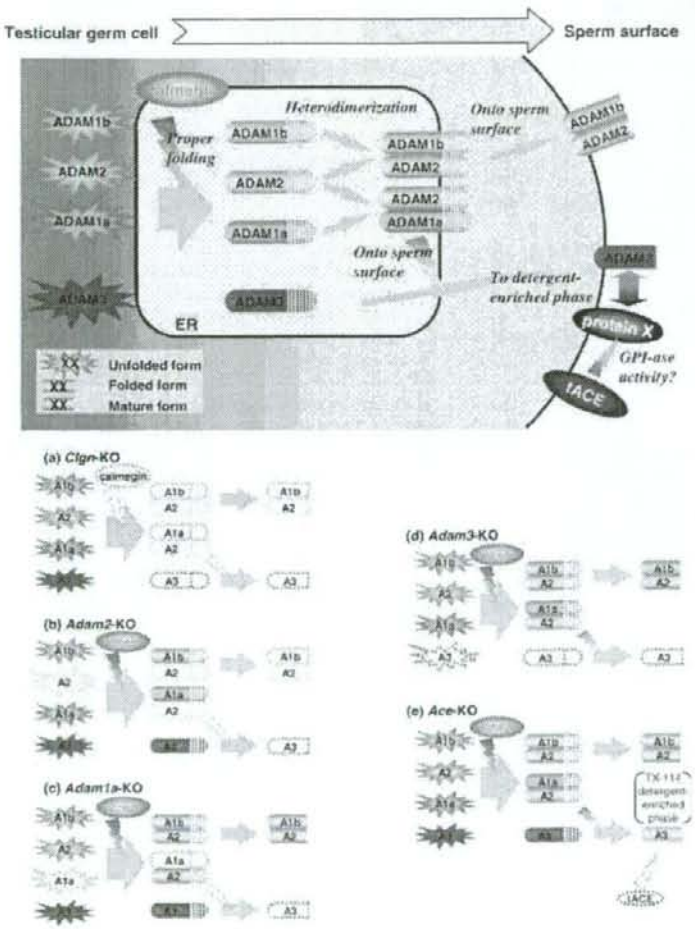

Fig. 3. Schematic model for ADAMs and their roles in sperm function. *The disruption of the genes that encode Adam1a, Adam2, and Adam3 results in impaired sperm-ZP binding. CLGN is required for the folding of ADAM1a, ADAM1b, and ADAM2 and the subsequent dimerization of these proteins. In Clgn-/-* **(a)** *and Adam2-/-* **(b)** *sperm, the disappearance of the ADAM1a/ADAM2 and ADAM1b/ADAM2 heterodimers results in the loss of ADAM1b, ADAM2, and ADAM3 from the sperm. ADAM1a is a testis-specific protein that is not found in sperm (Kim* et al., *2003). When ADAM1a is eliminated* **(c)**, *the ADAM1a/ADAM2 heterodimer disappears from the testis, whereas the expression of ADAM1b/ADAM2 is not affected. However, these sperm lack ADAM3 (Nishimura* et al., *2004). The disruption of ADAM3* **(d)** *is reported to have no significant effect on ADAM1a, ADAM1b or ADAM2 (Nishimura* et al., *2001). These findings suggest that ADAM3 is located downstream of these other ADAM proteins. The disruption of tACE leads to the aberrant localization of ADAM3* **(e)**, *most likely due to a different pathway from the one hypothesized for CLGN/ADAMs. These results indicate the importance of ADAM3 in sperm-ZP interaction and explain why disruption of the individual Ace, Clgn, Adam1a, Adam2, and Adam3 genes produces similar phenotypes.*

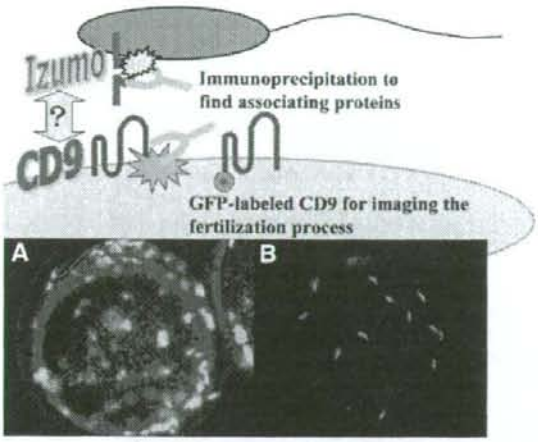

Fig. 4. Accumulation of sperm in the perivitelline space caused by failure of sperm–egg fusion. *Only one factor each on sperm and eggs is available at the moment. As in the case of live imaging acrosome reaction (ftp://ftp.gen-info.osaka-u.ac.jp/ARmovie/), the imaging of the fusion factors at the time of fertilization may be possible by producing fluorescent chimeric proteins. Immunoprecipitation will also clarify the new factors involved in the fusion event.* **(A)** *Sperm accumulated in the perivitelline space of CD9–/– mouse eggs. The sperm could penetrate the zona pellucida but failed to fuse with the egg surface. Many sperm were able to enter because of the lack of egg activation, which normally leads to cortical granule release and the zona block to polyspermy. Sperm nuclei were stained with Hoechst 33342(Miyado* et al., *2000).* **(B)** *Similarly, when eggs were inseminated with Izumo–/– sperm, the sperm could penetrate the zona pellucida but failed to fuse with the eggs. This also resulted in the accumulation of many sperm inside the perivitelline space (Inoue* et al., *2005). These penetrated sperm had clearly undergone the acrosome reaction, as they were all exposing the acrosome reacted sperm-specific antigen MN9 (Toshimori* et al., *1998).*

this criterion (Okabe *et al.*, 1990). The reacting antigen was identified as CD46, whose function is reported to be a complement receptor in human. However, when we cloned the CD46 gene in mouse, CD46 was found to be present only on sperm (Tsujimura *et al.*, 1998). We thought that this suggested the original role of CD46 was to function in sperm–egg interaction. Therefore, we produced CD46 disrupted mice with a strong expectation of causing sterility, but CD46 disrupted mice showed no visible damage to fertilizing ability in males or females (Inoue *et al.*, 2003).

We then analyzed OBF13 monoclonal antibody that was raised against mouse sperm, binds only to acrosome-reacted sperm, and inhibits sperm-egg fusion in the mouse (Okabe *et al.*, 1987). We recently identified the antigen by separation of crude extracts from mouse sperm by two-dimensional gel electrophoresis and subsequent immunoblotting with the monoclonal antibody. The identified spot was analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS). The antigen gene encodes an immunoglobulin superfamily (IgSF), type I membrane protein with an extracellular immunoglobulin domain that contains one putative glycosylation site. We termed the antigen "Izumo" after a Japanese Shinto shrine dedicated to marriage. We then produced an Izumo-disrupted mouse line. The Izumo-null males showed complete sterility despite normal mating behavior with normal vaginal plug formation. No offspring were fathered by these mice. When the sperm fertilizing ability was examined by *in vitro* fertilization system, many sperm were observed inside the perivitelline space indicating the fertilization was hampered at the sperm-egg fusion stage (Fig. 4B). However, when we performed intracytoplasmic sperm injection (ICSI) using Izumo $^{-/-}$sperm, they could activate the eggs, and the eggs were implanted normally and resulted in normal embryos. Therefore the deficiency of Izumo protein affects only to the sperm-egg fusion stage (Inoue *et al.*, 2005).

Relationship between the gene disruptions and their apparent phenotype

We need to be careful about the "off target" effects of gene disruption. A targeted disruption of the myogenic basic-helix-loop-helix gene Mrf4 is a good example. The phenotypes of three different Mrf4-disrupted mouse lines from three different laboratories with slightly different targeting vectors were very different, ranging from those showing complete viability of homozygotes to those displaying complete lethality. These three similar, but slightly different, targeting vectors had very different effects on expression of the adjacent Myf5 gene, which accounts for much of the phenotypic variation (Olson *et al.*, 1996). Another good example of the potential pitfalls of gene disruption is the case of the disruption of the Prion gene (PrP). Five independent PrP knockout mouse lines have been reported with no phenotype (Bueler *et al.*, 1992, Manson *et al.*, 1994) and with cerebellar symptoms (Moore *et al.*, 1999, Sakaguchi *et al.*, 1996, Silverman *et al.*, 2000). The discrepancy of the observation was associated with inter-gene splicing with neighboring Doppel gene in some of the targeting vectors (Flechsig *et al.*, 2003, Rossi *et al.*, 2001). To examine that the absence of Izumo directly caused the failure to fuse, we made a transgenic Izumo line driven by testis-specific calmegin promoter. The sterile phenotype was rescued with the transgenically-expressed Izumo on mouse sperm. Thus, we are certain that Izumo is the sperm factor shown to be essential for sperm-egg fusion.

Generality of sperm-egg fusion factors

In the sperm-zona binding event, ADAM3 holds the central position in the mouse, but the equivalent gene in human seems to be not producing functional protein (Frayne *et al.*, 1999). We were curious to see if Izumo is species-specific. Therefore, Izumo $^{-/-}$ sperm were mixed with hamster eggs which are able to fuse with sperm from different species. As a result, it was shown that Izumo is essential for mouse sperm to fuse with hamster eggs. Likewise, fusion of human sperm to hamster eggs was inhibited by the addition of anti-human Izumo antibody. This may suggest that Izumo is involved in sperm–egg fusion in humans as well. However, as indicated throughout this review, the addition of antibody in the IVF condition often provides us with different views obtained from various gene-manipulated animals. Therefore, it is too early to conclude that Izumo is functional in humans. It will become clear if men with mutations in their Izumo gene are found to be infertile with a symptom of fusion disability. In any case, the first

unambiguous fusion-related factors on sperm (Izumo) and on eggs (CD9) have been clarified. Again, the mice are available to the public through the Japanese animal distribution systems (indicated earlier). It is now a historic point in time, as we all are standing at the starting line of the elucidation of sperm-egg fusion.

Conclusions

Experiments using gene-manipulated animals are very powerful tools for judging the essentiality of concerned factors in fertilization. Of course, if a certain factor is judged as "not essential," it does not necessarily mean the factor is not functioning *in vivo*. However, the number of genes that are indispensable in fertilization is accumulating and their relationships with fertilization biology are emerging in the field of sperm-zona interaction. Gene disruption experiments are pursued in many research fields and the number of genes disrupted is increasing day by day. Thus, any genes that might affect reproduction will continue to be found even by researchers in different field. The day that we can draw a clear image of the sperm-egg fusion mechanism in molecular biology is definitely nearing.

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DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes

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Silencing of transposable elements occurs during fetal gametogenesis in males via de novo DNA methylation of their regulatory regions. The loss of MILI (miwi-like) and MIWI2 (mouse piwi 2), two mouse homologs of *Drosophila* Piwi, activates retrotransposon gene expression by impairing DNA methylation in the regulatory regions of the retrotransposons. However, as it is unclear whether the defective DNA methylation in the mutants is due to the impairment of de novo DNA methylation, we analyze DNA methylation and Piwi-interacting small RNA (piRNA) expression in wild-type, MILI-null, and MIWI2-null male fetal germ cells. We reveal that defective DNA methylation of the regulatory regions of the Line-1 (long interspersed nuclear elements) and IAP (intracisternal A particle) retrotransposons in the MILI-null and MIWI2-null male germ cells takes place at the level of de novo methylation. Comprehensive analysis shows that the piRNAs of fetal germ cells are distinct from those previously identified in neonatal and adult germ cells. The expression of piRNAs is reduced under MILI- and MIWI2-null conditions in fetal germ cells, although the extent of the reduction differs significantly between the two mutants. Our data strongly suggest that MILI and MIWI2 play essential roles in establishing de novo DNA methylation of retrotransposons in fetal male germ cells.

[*Keywords*: Piwi; piRNA; retrotransposon; DNA methylation; spermatogenesis]

Supplemental material is available at http://www.genesdev.org.

Received December 6, 2007; revised version accepted February 5, 2008.

Argonaute proteins, also known as PAZ Piwi domain (PPD) proteins, are members of a well-conserved family that is expressed in a variety of organisms, from fission yeasts to humans. The family can be divided into two subfamilies, Piwi and Ago, based on the primary sequence homology and expression pattern of each member. Piwi subfamily members are expressed only in germ lineage cells, whereas members of the Ago subfamily are expressed ubiquitously. The PPD proteins were initially characterized as essential molecules for stem cell self-renewal and maintenance in *Drosophila*, *Caenorhabditis elegans*, and certain plant species (Cox et al. 1998; Moussian et al. 1998), and a member of the family is essential for stem cell function during regeneration in planaria (Reddien et al. 2005). There are three Piwi subfamily genes in the mouse genome: *Miwi* (*mouse piwi*), *Miwi2*, and *Mili* (*miwi-like*), which are termed *Piwil1* (piwi-like homolog 1), *Piwil4*, and *Piwil2*, respectively, in the official nomenclature. Although they are ex-

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Article is online at http://www.genesdev.org/cgi/doi/10.1101/gad.1640708.

pressed only in germ lineage cells, the expression patterns of the three genes are different during germ cell differentiation. However, it is noteworthy that both *Mili*- and *Miwi2*-targeted mice are sterile because of impaired spermatogenesis at the pachytene stage (Kuramochi-Miyagawa et al. 2004; Carmell et al. 2007).

Spermatogenesis is one of the most dramatic biological processes, as it involves cellular proliferation, differentiation, and morphogenesis. Primordial germ cells (PGCs), which emerge at embryonic day 7.5 (E7.5), reach the genital ridges up to E11.5. Male germ cells enter mitotic arrest around E13.5 and restart cell proliferation on postnatal day 2 (Sakai et al. 2004). De novo DNA methylation occurs during the quiescent phase in the fetal testes. After birth, the first wave of spermatogenesis proceeds in a synchronous manner. Following mitotic division, the first meiotic division commences on day 10. Several stages of meiosis are distinguished on the basis of differences in the constitution and relationships of the pairs of homologous chromosomes and their degrees of condensation. During differentiation, male germ cells enter the preleptotene/leptotene, zygotene, pachytene, and diplotene stages on days 10, 12, 14, and 17, respectively (Bellve et al. 1977). Thereafter, the second meiotic division takes place, with the round spermatids first appearing around postnatal day 20, and spermatogenesis continues in the adult testis. Therefore, as shown in Figure 1A, the germ cells in male gonads are different in the fetal, neonatal, and adult stages.

Recently, it has been reported that PPD proteins play key roles in many gene-silencing phenomena involving small RNAs, including microRNA (miRNA)-directed or siRNA-directed target RNA cleavage, translational repression, and chromatin silencing. Post-transcriptional gene silencing by AGO subfamily-associated miRNAs or siRNAs has been reported (Peters and Meister 2007). MILI and MIWI were recently reported to bind 26-nucleoties (nt) to 31-nt Piwi-interacting small RNAs (piRNAs), respectively (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Watanabe et al. 2006) in the adult testis. Recently, MILI-associated piRNAs in the neonatal stage have been described as "prepachytene piRNAs" from day 10 testes (Aravin et al. 2007). However, the mechanisms of biogenesis and the biological functions of piRNAs are poorly understood compared with those of miRNAs and siRNAs.

Nearly half of the mammalian genome is composed of repeated sequences (Lander et al. 2001). Accumulating evidence suggests that PIWI-related mechanisms are involved in repressing the expression of retrotransposons, which are representative of the repeated sequences. Mutations in Piwi family genes have been shown to increase retrotransposon transcription in *Drosophila* (Sarot et al. 2004; Kalmykova et al. 2005), *Trypanosoma* (Shi et al.

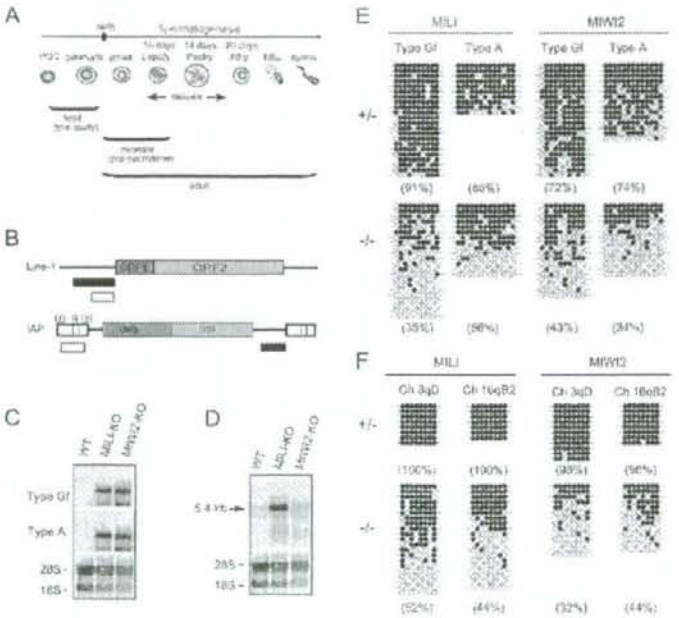

Figure 1. Expression of the IAP and Line-1 retrotransposons and methylation of their regulatory regions in neonatal prepachytene testes. (*A*) Scheme for the development of mouse male germ cells. (Gonia) Spermatogonia; (Lep) leptotene; (Zy) zygotene; (Pachy) pachytene; (RSp) round spermatid; (Esp) elongated spermatid. (*B*) Schematic diagram of the Line-1 and IAP genes. The locations of the probes used for Northern blotting and bisulfite sequencing are indicated by filled and open boxes, respectively. The sequences of the 5′-noncoding regions of the Line-1 genes are different for type Gf and type A. The probe for the 3′-noncoding region of IAP recognizes the full-length and all deletion derivatives of IAP. (*C*,*D*) Northern blotting showing transcription of the Line-1 (*C*) and IAP (*D*) retrotransposon genes in testes from 2-wk-old MILI- and MIWI2-deficient mice. The 5′-noncoding regions of type Gf and type A Line-1 and the 3′-noncoding region of IAP were used as probes. (*E*,*F*) Bisulfite sequencing of Line-1 (*E*) and IAP (*F*) in MILI- and MIWI2-deficient germ cells. Spermatogonial cells from 6- to 12-d-old mice were purified from Oct4-EGFP transgenic mouse (Yoshimizu et al. 1999) testes. (*E*) The 5′-noncoding regions of type Gf and type A Line-1 (nucleotides 874–1156; GenBank accession no. D84391) and nucleotides 1251–1542 of M13002 were analyzed. (*F*) Two LTR regions from the 5.4-kb IAI-type IAP in chromosomes 3qD and 16qB2 were arbitrarily chosen for analysis. Filled and open circles represent methylated and unmethylated CpGs, respectively. The percentage of methylated CpGs is shown in parentheses.

2004), *Neurospora* (Nolan et al. 2005), and mice (Aravin et al. 2007; Carmell et al. 2007). Small RNAs against transposon sequences have been cloned in germlines, and the loss of Piwi family gene expression causes down-regulation of these small RNAs in *Drosophila*, zebrafish, and mice (Saito et al. 2006; Aravin et al. 2007; Brennecke et al. 2007; Gunawardane et al. 2007; Houwing et al. 2007). Based on these results, it has been suggested that the murine Piwi family genes are involved in retrotransposon gene silencing via small RNAs, probably repeat-associated piRNAs.

Retrotransposons are thought to be maintained in a transcriptionally silent state by DNA methylation (Walsh et al. 1998). During spermatogenesis, the DNA methylation status of the regulatory regions in retrotransposons, such as IAP (intracisternal A particle) and Line-1 (long interspersed nuclear elements), which belong to the long terminal repeat (LTR) and non-LTR retrotransposon families, respectively, changes dynamically (Tanaka et al. 2000; Lane et al. 2003). These regions are demethylated in PGCs around E12.5–E13.5, and the reacquisition of DNA methylation (i.e., de novo methylation) takes place in nondividing prospermatogonia (i.e., gonocytes) in the fetal testis around E16.5–E18.5. This de novo methylation involves either of the two de novo DNA methyltransferases, Dnmt3a and Dnmt3b, as well as their activating factor Dnmt3L, and analyses of Dnmt3L-deficient mice clearly show that this molecule is essential for the process (Bourc'his and Bestor 2004; Hata et al. 2006; Kato et al. 2007). Recently, it was reported that *Mili*- and *Miwi2*-targeted mice exhibited enhanced IAP and Line-1 expression, and methylation of the 5′-untranslated region (UTR) region of Line-1 was shown to be reduced in these mice at the neonatal stage (Aravin et al. 2007; Carmell et al. 2007). However, the DNA methylation status and piRNA expression of retrotransposons during de novo DNA methylation have not been examined. Thus, in the present study, we analyzed the piRNA expression and DNA methylation profiles of the IAP and Line-1 retrotransposons in fetal MILI-null and MIWI2-null testes.

Results

Comparison of Line-1 and IAP gene expression in MILI$^{-/-}$ and MIWI2$^{-/-}$ testes

To understand the roles of the mouse PIWI family proteins, we generated and analyzed MILI- (Aravin et al. 2007; Carmell et al. 2007) and MIWI2-deficient mice (Supplemental Fig. S1). In MILI- and MIWI2-null testes, spermatogenesis was arrested at the early prophase of meiosis I (Supplemental Fig. S1E). Affymetrix GeneChip microarray hybridization showed that five genes were up-regulated more than threefold in the *MILI*$^{-/-}$ testes, and all of these genes belonged to the IAP retrotransposon (Supplemental Fig. S2). The Line-1 and IAP gene expression levels were subsequently examined in the testes of 2-wk-old mice—i.e., testes that contained both premeiotic and meiotic germ cells—by Northern blotting (Fig. 1C,D). The transcripts of two representative Line-1 species, type Gf and type A, which have similar and unique sequences in their coding and 5′-noncoding regions, respectively, were similarly accumulated in both the MILI- and MIWI2-deficient testes. Meanwhile, expression of IAP was strongly and only slightly enhanced in the MILI- and MIWI2-null testes, respectively. Although the mouse genome contains full-length (7.2-kb) IAP elements and variants with deletions of various sizes in the *gag-pol* area (Ishihara et al. 2004), only the 5.4-kb IΔ1-type IAP transcript was enhanced in both mutants.

Reduced CpG methylation of Line-1 and IΔ1-type IAP in MILI$^{-/-}$ and MIWI2$^{-/-}$ germ cells

We examined the methylation status of the regulatory regions of Line-1 and IAP in MILI- and MIWI2-deficient male germ cells sorted 8–12 d after birth; namely, premeiotic germ cells. The promoter regions of type Gf and type A Line-1 in the sorted male germ cells were examined by bisulfite sequencing. As shown in Figure 1E, a significant reduction in CpG methylation was observed in the MILI- and MIWI2-deficient male germ cells at this stage. The reduction in methylation of type Gf Line-1 was confirmed by methylation-sensitive Southern blotting (Supplemental Fig. S3). Next, we analyzed the methylation pattern of CpG in two arbitrarily chosen LTR regions of the 5.4-kb IΔ1-type IAP on chromosomes 3 and 16. These regions were almost completely methylated in the control germ cells, whereas the levels of methylation were much lower in the MILI- and MIWI2-deficient germ cells (Fig. 1F).

Dnmt3L is essential for the de novo methylation of retrotransposons in fetal testes, and the testicular phenotype of Dnmt3L-null mice is essentially the same as those of MILI- and MIWI2-null mice (Bourc'his and Bestor 2004; Webster et al. 2005; Hata et al. 2006). Thus, we examined the DNA methylation status of the Line-1 regulatory regions in MILI- and MIWI2-deficient neonatal testes, which contain premeiotic germ cells. Methylation-sensitive Southern blot analysis revealed that hypomethylation was already present on day 2 after birth (Fig. 2A), as was seen in Dnmt3L-mutant mice. Bisulfite sequencing confirmed the impaired CpG methylation status of the Line-1 and IΔ1-type IAP regulatory regions in MILI- and MIWI2-deficient germ cells on day 0–1 after birth (Fig. 2B). It seems unlikely that the defective methylation in the MILI-deficient mice was due to decreased expression of Dnmt3L and/or its presumptive cooperating DNA methyltransferase, Dnmt3a2, since the expression of these methylases was unaffected by the mutation in *MILI* (Supplemental Fig. S4).

It is possible that MILI and MIWI2, as well as Dnmt3L, have roles in de novo methylation during male germ cell differentiation. Therefore, we carried out bisulfite sequencing of the regulatory regions of the Line-1 and IAP retrotransposons in MILI-deficient fetal germ cells around de novo methylation. In control germ cells, almost all of the CpGs in the LTRs of IAP were methylated soon after birth (Figs. 1F, 2B), whereas only incomplete

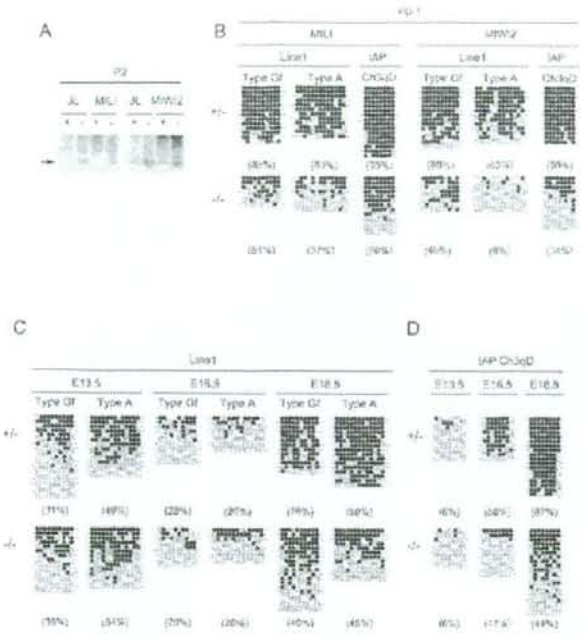

Figure 2. Methylation of the IAP and Line-1 regulatory regions in fetal testes. (*A*) Methylation-sensitive Southern blot analysis of the Line-1 promoter region. Whole-testis DNA was extracted from 2-d-old heterozygous (+) and homozygous (−) mice, followed by digestion with KpnI and the methylation-sensitive restriction enzyme HpaII. The probe for the type Gf Line-1 5′-noncoding region is the same as that in Figure 1C. (*B*) Bisulfite analysis of the Line-1 regulatory region in day 0–1 germ cells from MILI- and MIWI2-deficient and control testes. The germ cells were collected as described in Materials and Methods. (*C*,*D*) Bisulfite sequencing of the Line-1 (*C*) and IAP (*D*) regulatory regions in germ cells from MILI-deficient and control testes between E13.5 and E18.5. The germ cells were collected as described in Materials and Methods.

methylation was observed in E13.5, E16.5, and E18.5 germ cells (Fig. 2D). De novo methylation of the Line-1 and IAP regulatory regions of control mice occurred after E16.5 and E13.5, respectively (Fig. 2C,D). De novo methylation of Line-1 genes was slightly delayed compared with that of the IAP genes. In the MILI-deficient germ cells, hypomethylation of Line1 and IAP was observed at E18.5 and after E16.5, respectively. Differences in the methylation status of Line-1 in the MILI-deficient mice became apparent somewhat later than that of IAP, which seemingly reflects the delay of de novo methylation in the control germ cells. Our observations indicate that MILI plays an essential role in the timing of de novo methylation of the Line-1 and IAP regulatory regions.

Characterization of small RNAs in fetal testes

As discussed above, MILI and MIWI2 presumably function in gene silencing through DNA methylation in fetal testes. Based on the role of small RNAs from other organisms in gene silencing (Zaratiegui et al. 2007), it is possible that DNA methylation is mediated by small RNAs. Therefore, we analyzed many small RNA sequences (127,997 clones), 17–40 nt in length, from E12.5–E19.5 fetal male germ cells, to obtain a comprehensive picture of the piRNAs present at this stage, and compared our findings with the results of previous studies on piRNAs in neonatal (prepachytene) testes (Aravin et al. 2007) and adult testes (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Watanabe et al. 2006). The lengths of the small RNAs from the germ cells showed a bimodal pattern (Fig. 3A). One peak was observed at ~21 nt, which corresponds to the lengths of miRNAs, and a second was observed at 25–27 nt, which is consistent with the lengths of piRNAs.

Annotation of the small RNAs revealed that the library was enriched with retrotransposon sequences, with the exception of the breakdown products of rRNAs and other noncoding RNAs (tRNA/snRNA/snoRNA/scRNA/srpRNA) (Fig. 3B; Supplemental Table S1). The repeat-associated small RNAs (rasiRNAs) in the library, which reportedly bind MILI, showed a single peak at 25–27 nt (Fig. 3A). The relative abundance of repeat-associated RNAs (23%) was similar to that of the MILI-associated prepachytene piRNAs identified in neonatal testis (26%) (Fig. 3B; Supplemental Table S1; Aravin et al. 2007). However, detailed characterization of the repeat-derived small RNAs revealed that there were some differences between the fetal germ cell piRNAs and the reported prepachytene piRNAs (Aravin et al. 2007). As shown in Supplemental Table S2 and Figure 3C, the majority of the rasiRNAs in the E16.5 male germ cells were LTR retrotransposons (55%); namely, ERVK (37%), ERV1 (10%), MaLR (6%), and ERVL (2%). The others were LINEs (30%) and SINEs (11%). In contrast, among the prepachytene piRNAs, the SINE frequency (49%) was higher than the LTR (33.8%) or LINE (15.8%) (Aravin et al. 2007) frequency. Unique pachytene piRNAs were scarcely detected in the fetal male germ cells (0.1%)

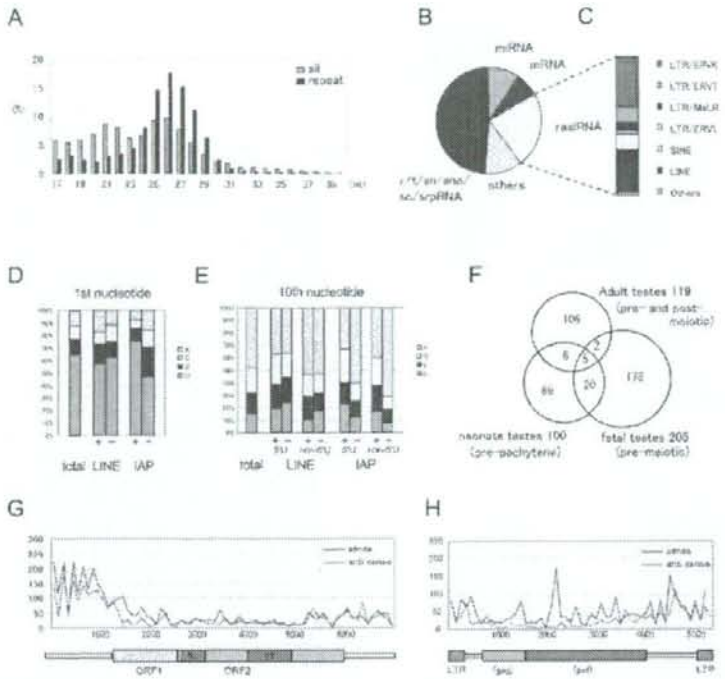

Figure 3. piRNAs in fetal premeiotic germ cells. (*A*) In total, 127,997 small RNAs were sequenced from E12.5–E19.5 fetal germ cells. The size distributions (in nucleotides) of the total small RNAs and rasiRNAs are shown by blue and purple bars, respectively. (*B*,*C*) Genomic annotation of the small RNAs (*B*) and the ratio of piRNA sequences of fetal premeiotic germ cells (*C*). Detailed results are listed in Supplemental Table S2. (*D*,*E*) Comparison of the first (*D*) and tenth (*E*) nucleotides of the total, sense (+), and antisense (−) piRNAs. Nucleotide biases were calculated for the Gf type Line-1 and IAP piRNAs analyzed in Supplemental Table S2. (*F*) The piRNA classes that contain or lack a 5′-end U are shown separately. (*F*) Venn diagram of the piRNAs in adult pre- and post-meiotic (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Watanabe et al. 2006), neonatal prepachytene (Aravin et al. 2007), and fetal (this study) testes. (*G*,*H*) Distribution of piRNAs corresponding to type Gf Line-1 (*G*) and IAPIΔ1 (*H*).

(Supplemental Table S1). Next, as the small RNAs that correspond to Line-1 and IAP were abundant, we examined the nucleotide composition of the piRNAs corresponding to type Gf Line-1 and IAP retrotransposons. The first and tenth nucleotides of the piRNAs are shown in Figure 3, D and E. Most of the piRNAs started with uridine and had an adenine at the tenth position, which is similar to the characteristics of prepachytene piRNAs (Aravin et al. 2007).

Adult and neonatal piRNAs are clustered within the genome (Aravin et al. 2006, 2007; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Watanabe et al. 2006). Therefore, we performed a cluster analysis of the male fetal gonadal small RNAs and detected 205 clusters (Supplemental Table S3), only seven and 25 of which were identified in adult and prepachytene testes, respectively (Fig. 3F). About 75% of the clustered small RNAs were 24–28 nt in length, and the percentage of small RNAs with U as the first nucleotide was high (71.5%). Therefore, we conclude that most of the clusters are piRNA clusters, and that the set of piRNAs expressed at the stage of de novo methylation is quite different from the piRNA sets expressed in the neonate and adult.

The distribution and frequency plots of the piRNAs that correspond to the type Gf Line-1 and IAPIΔ1 genes are shown in Figure 3, G and H, respectively. For Line-1, the number of piRNAs that corresponded to the regulatory region was higher than that corresponding to the coding region, and the distributions of sense and antisense piRNAs were indistinguishable. Meanwhile, for the IAPIΔ1 genes, the number of sense piRNAs was slightly higher than the number of antisense piRNAs, and there were many piRNAs that corresponded to the coding region as well as to the regulatory region.

Reduced levels of piRNAs in MILI- and MIWI2-deficient fetal testes

Although both *Mili* and *Miwi2* were expressed in fetal testes, the expression period of *Miwi2* was more re-

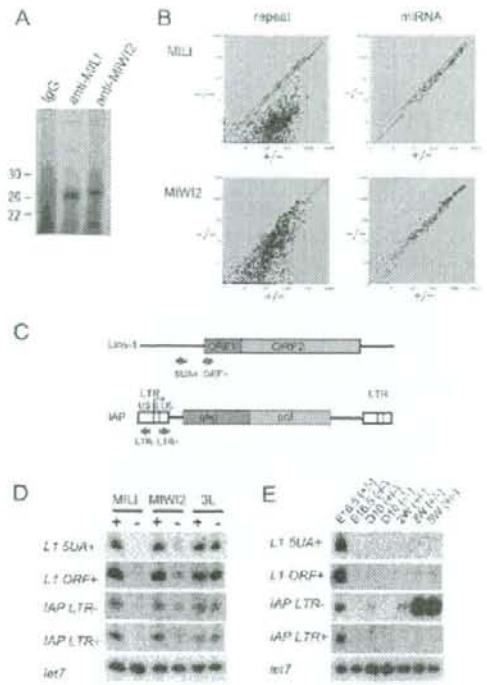

Figure 4. piRNA expression in MILI- and MIWI2-deficient fetal testes. (*A*) MILI- and MIWI2-bound piRNAs. The immunoprecipitated RNAs from E16.5 testicular lysates were ^{32}P-end-labeled and separated in a 15% denaturing urea–polyacrylamide gel. (*B*) Microarray analysis of the repeat-associated piRNAs and miRNAs. A microarray that contained 672 types of repeat-associated piRNAs and 150 types of miRNAs, both of which are expressed in fetal testes, was produced. RNA samples isolated from E16.5 control, MILI-deficient, and MIWI2-deficient mice were used. The expression of each small RNA was examined three times, and the mean values are plotted to compare the control and MILI- or MIWI2-deficient samples. Diagonal lines indicate a 1.5-fold difference in expression. (*C–E*) Expression of four arbitrarily selected piRNAs. piRNAs corresponding to the type A Line-1 promoter sense strand (5UA+), Line-1 ORF sense strand (ORF+), IAP LTR sense strand (LTR+), and IAP LTR antisense strand (LTR–) were arbitrarily selected. (*C*) The site and orientation of each piRNA is indicated by red arrows. (*D*) Northern blot analysis of the piRNAs in E16.5 control, MILI-deficient, MIWI2-deficient, and Dnmt3L-deficient testis. (*E*) Time-course analysis of the expression of the four piRNAs in control and MILI-null testes.

stricted (Supplemental Fig. S5) than that of *Mili* (Kuramochi-Miyagawa et al. 2001). *Miwi2* expression was reduced after birth, whereas *Mili* expression continued at essentially the same level throughout life. To examine the functions of MILI and MIWI2 in fetal testes, we determined whether the proteins bound to small RNAs. Ribonucleoprotein complexes from E16.5 testicular lysates were immunoprecipitated, the associated RNAs were isolated, and ^{32}P-labeling was carried out. As shown in Figure 4A, several RNAs of length 26–27 nt were coimmunoprecipitated with MILI and MIWI2.

Next, we used microarray analysis to test the roles of MILI and MIWI2 in the production of small RNAs in fetal testes. Using this method, we examined the expression patterns of 670 types of repeat-associated piRNAs and 150 types of miRNAs. As shown in Figure 4B, the vast majority of the piRNAs were significantly downregulated in MILI-null fetal testes. Although this downregulation was also observed in MIWI2-null testes, the mean fold reduction was smaller by one order of magnitude. In MILI-null fetal germ cells, ~88% of the piRNAs were present at less than one-eighth of the level in the control cells. In contrast, only 29% of the piRNAs in the MIWI2-null fetal germ cells were reduced to this level.

The relative levels of expression of the piRNAs that correspond to type Gf Line-1 and IAP are summarized in Table 1. In MILI-null testes, the expression levels of all of the type Gf Line-1 sense piRNAs and the vast majority of the type Gf Line-1 antisense piRNAs were less than one-quarter of the control. In contrast, the level of reduction was significantly lower in MIWI2-null testes. About 25% (27 of 101) and 14% (11 of 78) of the sense and antisense piRNAs, respectively, did not show significantly reduced expression in MIWI2-null testes. Notably, all of the piRNAs whose expression was unaffected by the MIWI2-null mutation were located in ORF1 and the endonuclease region of ORF2, whereas

Table 1. *Relative expression of the piRNAs for Line-1 Gf and IAP*

	Sense		Antisense	
Relative expression	MILI$^{-/-}$	MIWI2$^{-/-}$	MILI$^{-/-}$	MIWI2$^{-/-}$
(A) Line-1 Gf				
<1/64	63	0	44	3
1/64 ≦ <1/16	33	2	22	7
1/16 ≦ <1/4	5	23	10	26
1/4 ≦ <1/1.5	0	49	2	31
1/1.5 ≦ <1.5	0	22	0	10
1.5 ≦ <4	0	5	0	1
Total	101	101	78	78
(B) IAP				
<1/64	58	8	41	3
1/64 ≦ <1/16	42	7	10	3
1/16 ≦ <1/4	22	54	3	18
1/4 ≦ <1/1.5	1	43	0	22
1/1.5 ≦ <1.5	0	11	0	8
1.5 ≦ <4	0	0	0	0
Total	123	123	54	54

The relative expression levels of piRNAs that correspond to the type Gf Line-1 (A) and IAP (B) are shown. Relative expression is calculated from the microarray data and is shown as the ratio of the expression level of an individual piRNA in the MILI- and MIWI2-null mice to that in control mice. The numbers of piRNAs included in the relative expression range are described in the table.

none of these piRNAs were located in the reverse transcriptase region of ORF2. As with the type Gf Line-1 piRNAs, the expression levels of almost all of the piRNAs for IAP in the MILI-null testes were less than one-quarter of the control (Table 1), and the reduction was significantly less pronounced in the MIWI2-null testes. Unlike the case of Line-1, these piRNAs were not located in any special region.

We examined in greater detail the expression levels of four arbitrarily chosen piRNAs by Northern blot analysis using antisense oligonucleotides against small RNAs of 25–28 nt in length: the sense strand 5′-UTRs of type A (5UA+) and ORF (ORF+) for Line-1, and the sense and antisense strands of LTR (LTR+ and LTR−, respectively) for IAP (Fig. 3C). The sequences of LTR+ and LTR− were identical to those of the U3 region and R plus U5 region of LTR, respectively, and the expression levels of the four small RNAs were found to be quite similar in the E16.5 testes. As shown in Figure 4D, the four small RNAs were not detected in MILI-null testes, although they were weakly detected in MIWI2-null testes and expressed normally in Dnmt3L-null testes. The expression of the piRNAs was further examined in control and MILI-null testes during development, and 5UA+, ORF+, and LTR+ showed similar expression patterns (Fig. 4E). All three were expressed in fetal germ cells but were almost undetectable on day 10 and 2 wk after birth. Weak expression was detected 3 and 5 wk after birth. In contrast, the expression level of LTR− was higher 3 wk after birth than at E16.5.

Discussion

In the present study, we analyzed the mechanism of impaired retrotransposon silencing in MILI- and MIWI2-null male germ cells. DNA methylation of the regulatory regions of two retrotransposon species, Line-1 and IAP, was impaired from the stage of de novo methylation. Given that piRNAs are involved in gene silencing, we performed large-scale sequencing of the small RNAs in fetal male germ cells. Our data clearly indicate that the small RNA composition of fetal male germ cells is quite different from those of adult and neonatal prepachytene male germ cells. Finally, we compared the effects of MILI- and MIWI2-null mutations on the expression of repeat-associated piRNAs and found that these two mouse PIWI family proteins play similar but distinct roles in piRNA expression.

De novo DNA methylation and the murine PIWI proteins MILI and MIWI2

RT–PCR analysis showed that *Miwi2* expression commenced at E15.5 in male gonadal tissue (Supplemental Fig. S5). Meanwhile, *Mili* RNA was detected in male gonadal tissues beginning at E12.5. Therefore, both MILI and MIWI2 are expressed during de novo DNA methylation. Male germ cells from both MILI- and MIWI2-null mutant mice exhibited impaired DNA methylation in the regulatory regions of Line-1 and IAP soon after birth (Fig. 2A,B), which suggests that these two proteins play crucial roles in DNA methylation at an early stage of germ cell development. In addition, impaired de novo methylation was observed in MILI-deficient fetal germ cells. The significant reduction in piRNAs against the repeat-associated piRNAs in MILI- and MIWI2-deficient fetal testes implies that piRNAs are active in de novo DNA methylation.

Changes in piRNA content during male germ cell development

The compositions of the piRNAs and piRNA clusters were quite different during the process of male germ cell development, i.e., at the embryonic, neonatal prepachytene (Aravin et al. 2007), and adult stages (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Watanabe et al. 2006). Although the fetal and neonatal piRNAs were similar in that each included a significant number of repeat-associated RNAs, they shared only 10% of the species in their piRNA clusters. Recently, it has been proposed that piRNA production involves a ping-pong amplification cycle for retrotransposon-related piRNAs (Brennecke et al. 2007; Gunawardane et al. 2007). The high proportion of piRNAs with U at the first nucleotide position and A at the tenth nucleotide position in our analysis of fetal repeats corresponding to piRNAs fits well with this hypothesis. However, the different compositions of the embryonic, neonatal, and adult piRNAs suggest that the cycle does not continue throughout male germ cell development, since the piRNAs in fetal germ cells are not maintained in adult germ cells (Fig. 4E). Therefore, some other mechanism(s) of piRNA biogenesis must operate during the pachytene phase to establish piRNA expression in adult germ cells.

We arbitrarily chose four fetal piRNAs and examined their expression in detail (Fig. 4E). All four piRNAs were either undetectable or weakly expressed in neonatal germ cells, although expression was somewhat increased after a couple of weeks. This also suggests that the molecular mechanism of piRNA production changes during development. At the same time, it implies that the function of piRNAs in embryos is different from that in neonates and adults.

MILI and MIWI2 have different functions

Impaired de novo methylation was detected in both MILI- and MIWI2-null male fetal germ cells. Although a general reduction in piRNA expression was observed in the mutants, the extent of the reduction was significantly different. As shown in Figure 4B, the reduction in numbers of piRNAs against repetitive sequences was much greater in the fetal germ cells of MILI-null testis than in those of MIWI2-null testis. This may reflect a difference in the molecular mechanisms of piRNA biogenesis between MILI and MIWI2.

The expression of several piRNAs was not reduced at all in MIWI2-null germ cells. In fact, 27 of 101 sense and 11 of 78 antisense Line-1 piRNAs were found to belong to this group (i.e., relative expression $\geq 1/1.5$ in Table 1A). In contrast, the expression of these same piRNAs

was significantly reduced (i.e., relative expression <1/1.5 in Table 1A) in MILI-null germ cells. It is noteworthy that the vast majority of the piRNAs were located in ORF1 and the endonuclease region of ORF2 (Fig. 3G, ORF1 and Nu, respectively), although the significance of this result remains unclear. These data suggest that MILI and MIWI2 play different roles in the production, stabilization, and/or amplification cycle of piRNA.

Line-1 expression was similarly enhanced in MILI- and MIWI2-null testes (Fig. 1B), and bisulfite sequencing revealed that a significant reduction in DNA methylation occurred in both MILI- and MIWI2-null male germ cells (Fig. 2C). Considering that DNA methylation greatly influences retrotransposon transcription, the enhanced expression of Line-1 was correlated with DNA hypomethylation of the mutant male germ cells. The situation was a little different for IAPIΔ1 (Fig. 1B,D). Although defective DNA methylation was detected in both MILI- and MIWI2-null germ cells, IAPIΔ1expression was strongly increased in MILI-null testis, while it was either not increased or only slightly increased in MIWI2-null testis. These results imply different functions for MILI and MIWI2. Although it is difficult to explain the discrepancy, an as-yet-unknown post-transcriptional silencing mechanism may reduce IAP expression in MIWI2-null germ cells.

Molecular mechanisms of piRNA regulation

As described above, several aspects of piRNA regulation change significantly during development. For example, the piRNA composition differed among fetal, neonatal, and adult male germ cells. In addition, the first and tenth nucleotides and the distributions of piRNAs against the Line-1 or IAP sequence were different. Furthermore, although both MILI-null and MIWI2-null mice exhibited sterility owing to arrested spermatogenesis, the reductions in the percentages of piRNAs in their germ cells were significantly different. In addition, the regulatory mechanisms controlling IAPIΔ1 and Line-1 expression were different. It remains to be determined how the expression of IAPIΔ1, but not that of full-length IAP, is influenced by a null mutation of MILI or MIWI2. Finally, the mechanism of de novo methylation, presumably through piRNAs, remains an open question. Currently, it is difficult to propose a unified molecular mechanism to explain our results. Additional analyses will clarify the mechanisms underlying piRNA production and gene silencing.

Materials and methods

RNA extraction, RT–PCR, and Northern blot analysis

Total RNA samples were prepared from testes using Sepasol-RNA I Super (Nacalai Tesque), treated with DNase I, and subjected to RT–PCR with the ThermoScript RT–PCR System (Invitrogen) and random hexamers. Each reaction was performed using HotMaster Taq DNA polymerase and specific primers (Supplemental Table S6).

Northern blot analysis was performed at 65°C in 0.2 M $NaHPO_4$ (pH 7.2), 1 mM EDTA, 1% BSA, and 7% SDS. The membranes were washed with a 0.2× SSC/0.1% SDS solution at 65°C. The subcloned PCR products were labeled with [α-^{32}P]-dCTP and used as probes. The sequences used for PCR primers were as follows: the 3′-noncoding region of IAP (GenBank accession no. X04120), nucleotides 4489–4793; and the 5′-noncoding region of type Gf (D84391), nucleotides 874–1156, and A Line-1 (M13002), nucleotides 531–1642.

Methylation-sensitive Southern blot analysis

Whole-testis DNA was extracted from 2-d-old mice, and 3 µg of DNA were digested with KpnI and the methylation-sensitive restriction enzyme HpaII or the methylation-insensitive enzyme MspI. The type Gf Line-1 5′-noncoding region shown in Figure 1A was used as the probe.

Germ cell isolation

MILI mutant mice (Kuramochi-Miyagawa et al. 2004) and MIWI2 mutant mice were crossed with Oct-4/GFP transgenic mice (Yoshimizu et al. 1999) to obtain GFP-positive spermatogonia. Testis cells from embryos or pups were collected by two-step enzymatic digestion (Meistrich 1993), and the GFP-positive cells were sorted by FACS Aria (Becton Dickinson). Genomic DNA was extracted from the sorted germ cells.

Bisulfite methylation analysis

Bisulfite treatment of the genomic DNA isolated from fetal germ cells was performed using the EpiTect bisulfite kit (Qiagen). Two LTR regions of IAP (5.4-kb, IAI-type) on chromosomes 3qD and 16qB2 were arbitrarily selected for analysis by nested PCR, and the products were sequenced. The first and second rounds of PCR were carried out using the primers F1 and R1, and F2 and R2, respectively. PCR amplification of the 5′-region of Line-1 (types Gf and A) was carried out using specific primers. The sequence of each primer is listed in Supplemental Table S6.

Anti-MILI and anti-MIWI2 antibodies

Affinity-purified anti-MILI-N2 (anti-mouse PIWIL2 [MILI], PM044; MBL Co., Ltd.) and anti-MIWI2-N1 polyclonal antibodies against MILI and MIWI2 were generated by immunization with peptides derived from MILI (amino acids 107–122: VRKDREEPRSSLPDPS) and MIWI2 (amino acids 31–45: TSASPGDSEAGGGTSC), respectively (MBL).

Small RNA cloning and sequencing

To isolate small RNAs, 20 µg of total RNA from E12.5–E19.5 male germ cells were gel-fractionated, and species 17–40 nt in length were gel-purified. The small RNAs were sequentially ligated to 3′- and 5′-adapters and then amplified by RT–PCR using a small RNA cloning kit (RR065; Takara Bio). Sequencing of the small RNA library was achieved using the 454 Life Science sequencer.

Annotation of the small RNAs

To identify the small RNAs that corresponded to various repeats (e.g., rRNA, tRNA, retrotransposon, and DNA transposon), the genomic positions of the repeats were retrieved from the University of California at Santa Cruz (UCSC) Web site (http://hgdownload.cse.ucsc.edu/downloads.html) and compared with the genomic positions of the small RNAs. When the genomic position of a particular small RNA overlapped with that of any repeat by up to 15 nt, the small RNA was considered to be repeat-derived. Repeat names were retrieved from all the