The sperm mitochondria-specific translocator has a key role in maternal mitochondrial inheritance

Kenji Hayashida*, Katsuhisa Omagari, Jun-ichi Masuda, Hiroaki Hazama, Yoshiko Kadokawa, Kazuo Ohba & Shigeru Kohno

Second Department of Internal Medicine, Nagasaki University School of Medicine,

Nagasaki 852-8501, Japan

*Corresponding author. Tel.: +81-95-849-7273. Fax: +81-95-849-7285,

E-mail address: **khayashida-ngs@umin.ac.jp** (K.Hayashida).

Abstrct

The mechanism of maternal mitochondrial inheritance in animals involves the selective elimination of sperm mitochondria by the elimination factor of the egg and the sperm mitochondria-specific factor. *In vitro* fertilization using sperm from congenic mice incorporating heterogeneous mitochondrial DNA (mtDNA) showed that the number of PCR positives of sperm mtDNA in two-cell embryos was significantly increased following sperm incubation with anti-*tpis*, anti-Tom22 and anti-Tom40 antibodies. The treatment of fertilized eggs with EGTA and endonuclease inhibitors increased the sperm mtDNA levels. We conclude that the elimination factor, which is probably an endonuclease, is selectively received by the *tpis* protein of the sperm mitochondrial outer membrane within the egg. It is then transported into the sperm mitochondria by Tom22 and Tom40, where it destroys the sperm mtDNA, establishing the maternal inheritance of mtDNA.

1. Introduction

Maternal mitochondrial inheritance occurs in many eukaryotes (Birky, 2001). With the exception of interspecific crosses, strict maternal inheritance is known to take place among mammals (Shitara et al., 1998). During fertilization, the entire spermatozoan, including the mitochondria of the midpiece, enters the egg (Szollosi, 1965; Yanagimachi and Noda, 1970). The sperm mitochondria are selectively destroyed, whereas those of the egg escape destruction (Birky, 2001). Kaneda et al., (1995) showed that sperm mitochondrial DNA (mtDNA) is lost by the late pronucleus stage immediately after incorporation, using intraspecific crosses of congenic mice that incorporated heterogeneous mtDNA (for detection by PCR). Observation of sperm mitochondria using rhodamine123-generated fluorescence, which is dependent upon the mitochondrial membrane action potential, showed the loss of fluorescence up to the late pronucleus stage (Kaneda et al., 1995). The microinjection of spermatid and liver cell mitochondria into eggs resulted in only the spermatid mitochondria being eliminated (Shitara et al., 2000). This suggests that a sperm mitochondria-specific substance might

be incorporated into the sperm mitochondria during spermatogenesis. Moreover, the mtDNA of sperm from mice with a heterogeneous mitochondrial and nuclear genome was not eliminated, whereas that from mice in which only the mitochondrial genome was heterogeneous was destroyed (Kaneda et al., 1995). This indicates that the sperm mitochondria-specific substance might be a protein encoded not by the mitochondrial genome but by the nuclear genome.

Based on the above information, we suggest that there are at least two factors involved in the selective elimination system: the elimination factor of the oocyte and the sperm factor. As this system does not operate in interspecific crosses, and the sperm mitochondria are not eliminated (Kaneda et al., 1995; Lee *et al*., 2002; Sutovsky et al. 2000), we believe that species specificity exists in this system. Hiraoka and Hirano (1988), using electron microscopy, showed that multivesicular bodies, which are a type of lysosome, gathered around the sperm mitochondria in the fertilized eggs of hamsters at the two-cell stage. Sutovsky et al. (1999, 2000) reported that sperm mitochondria were ubiquitinated before fertilization in rhesus monkeys and cows. They assumed that a ubiquitin-proteasome system was the elimination factor in mammals, and speculated

that ubiquitin directly recognizes prohibitin, which is an integral protein of the inner

mitochondrial membrane, in a species-specific manner. Moriyama and Kawano (2003)

detected the rapid selective digestion of mtDNA from one parent immediately after

nuclear fusion in the isogamous protist *Physarum polycephalum*, using vital

fluorescence staining and PCR analysis of mtDNA. They suggested that ubiquitylation

before gamete fusion was unlikely, as whether the strain became the donor or the

recipient of mtDNA was determined by the mating partner in accordance with the

sexual hierarchy of *Physarum*, which has many mating types. Vacant mitochondrial

sheaths were gradually eliminated after the fluorescence of mtDNA diminished;

therefore, the authors assumed that a ubiquitin-proteasome system might perform a role

in the destruction of the mitochondrial membrane after mtDNA digestion.

Maternal inheritance also occurs in chloroplasts. These organelles are considered to

have originated as photosynthetic bacteria co-existing with eukaryotes that were already

co-existing with mitochondria. It is therefore likely that a similar strategy exists in

chloroplasts and mitochondria in terms of the elimination system of mtDNA. In the

unicellular green algae *Chlamydomonas*, the chloroplast DNA (cpDNA) of one parent

disappears immediately after mating, before the fusion of the sexual pronucleus (Sager and Lane, 1972). Nishimura et al. (2002) separated the sexual chloroplasts from zygotes in *Chlamydomonas* immediately after mating. They detected gradually increasing Ca^{2+}-dependent nuclease activity during the disappearance of cpDNA, but only in male derived chloroplasts. They therefore proposed that female chloroplasts are protected from this sequence-nonspecific endonuclease by an unknown mechanism. As described above, the destruction of both organelles' DNA by elimination systems is commonly observed immediately after mating. However, there are no hypotheses at present to explain the common mechanism that might be involved in both systems.

Mitochondrial proteins are "selectively" transported into the mitochondrion via the translocator of mitochondrial outer membrane (Tom) complex (Hartl and Neupert, 1989). We suggested that egg mtDNA is not protected from the elimination factor, but that the sperm mitochondria selectively takes up the elimination factor, which would explain the question of selectivity. We therefore established a working hypothesis that included the following points: firstly, the elimination factor is a protein that directly or indirectly destroys mitochondrial protein or mtDNA within the mitochondria; and secondly, the

sperm factor is an elimination factor-specific receptor of mitochondria.

As the key to this system is the sperm factor that determines selectivity, we predicted the following characteristics of the protein and conducted a search within GenBank: first, that the protein's expression is specific to the testis and spermatozoa; second, that it is localized in the mitochondrial outer membrane; third, that it is a receptor or translocator protein; fourth, based on predictions two and three, that the similarity with the Tom complex might be high; fifth, based on prediction one, that it might be an antigen of an antisperm antibody in human females; sixth, that there is species specificity in binding with the elimination factor; and seventh, that the gene for the protein is encoded in the nuclear genome. As a result, the tetratricopeptide repeat(TPR)-containing protein involved in spermatogenesis (*tpis*) gene was found to meet predictions one, four and seven. According to a report by Takaishi and Huh (1999), the gene is expressed as both a skin type (s-*tpis*) (accession code AF181253) and a testis type (t-*tpis*) (accession code AF181252) of cDNA. s-*tpis* codes for 529 amino-acid residues, while t-*tpis* codes for 901 with 372 additional amino-acid residues at the 5′ end. The *tpis* gene is encoded by chromosome 15. The t-*tpis* protein showed 50%

identity with mouse Tom34. In Northern blotting analysis, s-*tpis* transcripts were detected in the cerebellum, tongue, esophagus and forestomach among various adult tissues as well as in the embryonic skin, while *t-tpis* transcripts were detected in the testis. *In situ* hybridization showed strong signals from *tpis* transcripts in testis spermatogenic cells. The function of *tpis* is unknown. The human homologue of t-*tpis* is assumed to be *HSD-3.8* (67% identity; accession code AF311312). The *HSD-3.8* protein meets predictions one, four, five and seven, and the gene lies on chromosome 22; it encodes 926 amino-acid residues that show testis-specific expression. *HSD-3.8* was identified using the serum of an infertile woman containing antisperm antibody (Lin et al., 2001; Zang et al., 1992).

This paper demonstrates that the *tpis* protein meets our predictions and appears to be the protein involved in the elimination system. We additionally discuss indications of a candidate elimination factor.

2. Materials and methods

2.1. Mouse strains

ICR mice were used for RT-PCR, protein extraction and immunofluorescence staining. Congenic strain C57BL/6J(B6)-mt^{spr} males (Shitara et al., 1998) provided by The Tokyo Metropolitan Institute of Medical Science and B6 females were used for the inhibition assay.

2.2. Detection of tpis mRNA (RT-PCR)

RNA was extracted from the testis, spermatozoa in the epididymis, and the cerebellum of a mature male ICR mouse using the acid guanidinium-phenol-chloroform method. A total of 50 ng of each was measured. Unfertilized eggs were collected by harvesting the mature ovaries of female ICR mice 48 hours after injecting them with pregnant-mare's serum gonadotropin (PMSG). One-cell and two-cell embryos were collected following the *in vitro* fertilization of spermatozoa and unfertilized eggs in the

oviduct, which were made to superovulate 24 hours after injecting human chorionic gonadotropin (hCG) 48 hours after the PMSG injection. Five of each type of egg were collected in the PCR tube. A few whole spermatozoa were sampled from the suspension. Based on the manufacturer's directions for the OneStep RT-PCR Kit (QIAGEN), RT-PCR was performed by treating the specimen, primer and dNTP at 70°C for 10 min, and then adding the enzyme mix followed by reverse transcription at 60°C for 60 min. The primer pair for the t-*tpis*-specific portion comprised the forward primer 5′-AAAACCATCCCGGAGTGGAA-3′ and the reverse primer 5′-CGGCCTGAGCTCGATTGTTA-3′. The primer pair for the s-*tpis* mRNA-specific portion comprised the forward primer 5′-CCGGTGCGCCCTTCTCTAC-3′ and the reverse primer 5′-CTCTCCCGCCATTTTGATCC-3′. A total of 10 μl of RT-PCR products was applied to NuSieve 3:1 agarose gel and electrophoresed in TBE buffer. Gels were stained with ethidium bromide.

2.3. *Preparation of anti-tpis, -Tom complex antibody*

Purified rabbit antiserum against *tpis* protein was kindly provided by Dr. M.

Takaishi of the Okayama University Graduate School of Medicine and Dentistry, Japan.

To prepare the antiserum, cDNAs encoding full-length s-*tpis* proteins were amplified by

RT-PCR from the skin of ICR mouse, subcloned into pGEX-6P-1 and transfected into

BL21 cells. Antiserum was affinity purified using a Hitrap NHS-activated column

(Amersham Pharmacia Biotec). Anti-Tom-complex antiserum was kindly provided by

Dr. M. Mori of the Department of Molecular Genetics, Kumamoto University School of

Medicine, Japan. Anti-human Tom20 (Yano et al., 2000), Tom22C (C-terminal region)

(Yano et al., 2000) and Tom34 (Chewawiwat et al., 1999) antiserum were prepared as

described. Antiserum against Tom70 was prepared using a histidine-tagged soluble

domain (region 60-608) of human Tom70, which was expressed in Escherichia coli and

purified using metal chelation chromatography. Antibodies to Tom40 were raised

against histidine-tagged human Tom40 proteins, which were purified using

metal-chelation chromatography under denaturing conditions.

2.4.Preparation of testis, spermatozoa, ovary and other organs proteins

The testis and cauda epididymis from a mature male ICR mouse, and the mature

ovary, cerebellum, liver, kidney and heart from a female ICR mouse, were dissected.

The testis and the spermatozoa obtained by dissecting the cauda epididymis were placed

in homogenization buffer A (0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5

mM benzamidine HCl, 2 mg/ml SBTI, 10 mM PMSF, 5 mg/ml leupeptin and 1 mg/ml

pepstatin A), and the other organs were placed in homogenization buffer B (0.25 M

sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 3 μg/ml

pepstatin A). They were homogenized and sampled as whole cells. Homogenates of the

testis, spermatozoa, ovary and cerebellum were centrifuged twice (600 × g for 15 min at

4°C). The supernatants were centrifuged twice more (7,000 × g for 15 min at 4°C) with

the resulting sediments considered as crude mitochondria. Crude mitochondria of the

testis were separated by sucrose density gradient centrifugation (82,000 × g for 200 min

at 4°C) and the mitochondria layer was centrifuged (7,000 × g for 15 min at 4°C). The

sediments obtained were considered to be purified mitochondria. The homogenates of

the cerebellum and ovary were centrifuged (140,000 × g for 60 min at 4°C). The

supernatants were sampled as cytosol. Each sample was dissolved in 2 × sample buffer

(125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 200 mM DTT and 0.005% BPB)

after the addition of 3% final concentration octyl β-D-glucoside. The sample derived from the testis and the spermatozoa was left overnight at 4°C, while the sample derived from the other organs was boiled at 100°C for 5 min.

2.5. Deglycosylation and dephosphorylation treatment of tpis protein

Based on the manufacturer's directions for the Enzymatic Protein Deglycosylation kit (SIGMA), deglycosylation treatment was performed by adding PNGase F, O-glycosidase and α-2(3,6,8,9) neuraminidase to the crude sperm mitochondria and mature ovary cytosol. For the dephosphorylation treatment, reaction buffer (0.1M Tris-HCl, pH 7.5 and 0.1 M $MgCl_2$) and calf-intestine alkaline phosphatase (250 mU/μg of protein) were added followed by 60 min incubation at 37°C, after which 2 × sample buffer was added.

2.6. Trypsination assay of mitochondria

A trypsination assay of testis mitochondria was carried out according to the method described by Werhahn et al. (2001). Crude mitochondria from testis separated by centrifugation were incubated at 20°C for 20 min with trypsin (0.25 mg/mg of mitochondrial protein). The reaction was stopped by adding trypsin inhibitor (10 mg/mg of trypsin). Homogenization buffer B was added, centrifuged at $7,000 \times g$ for 15 min and the sediments were removed. Centrifugation at $7,000 \times g$ for 15 min was performed again, followed by the addition of 3% octyl β-D-glucoside to the sediments, which were then dissolved in $1 \times$ sample buffer.

2.7. Western blotting analysis

Proteins were separated by SDS-PAGE using 5-20% gradient gel (ATTO CORPORATION). They were then transferred to a PVDF membrane. Following blocking in 3% skim milk with 0.1% Tween PBS for 90 min, the membranes were

incubated overnight at 4°C with each antiserum. They were incubated for 45 min at room temperature with a 1:5,000 dilution of HRP-conjugated anti-rabbit IgG antibody (BIO-RAD). The blot was then developed using the ECL plus system (Amersham Biosciences).

2.8.Native PAGE

Native PAGE was performed as described previously by Kunkele et al. (1998). Crude mitochondria were solubilized in solubilization buffer (0.5% digitonin, 50 mM K-acetate, 10 mM MOPS, pH 7.0, 20% glycerol and 1 mM PMSF). Insoluble material was removed by centrifugation for 30 minutes at $20,000 \times g$. The supernatant was electrophoresed through 2-15% polyacrylamide gradient gels (DAIICHI) prior to the immunoblotting analysis.

2.9.Immunofluorescence staining of spermatozoa

Spermatozoa of ICR mice were collected in 0.01 M PBS. For human spermatozoa, fresh ejaculates were collected in homogenization buffer A. The sperm suspension was placed on a slide by cytospin (SHANDON), fixed for 10 min with 100% cold acetone and incubated for 5 min with 0.1% TritonX-100 in PBS. After blocking for 20 min with 1% BSA, the slide was left overnight at 4°C to react with a 1:500 dilution of purified anti-*tpis* antiserum. It was allowed to react with a 1:80 dilution of FITC-conjugated anti-rabbit IgG for 2 hours, then observed using fluorescence microscopy.

Spermatozoa were incubated in human tubal fluid (HTF) medium (NK system) at 37°C in 5% CO_2 for 30 min for capacitation, after which purified anti-*tpis* antiserum and each anti-Tom-complex antiserum at 1:300 concentration were added, followed by 1-hour incubation. The spermatozoa were placed on a slide and the secondary antibodies were allowed to react without fixing and blocking.

2.10. In vitro fertilization and inhibition assay

Unfertilized eggs were harvested from the oviduct of superovulated female B6 mice and placed in HTF medium. The capacitated spermatozoa of B6-mt^{spr} were added to the eggs and insemination was allowed to occur. After 5-6 hours, the eggs were transferred to HTF medium containing 50 μM EDTA, and incubation was continued in the CO_2 incubator. Two-cell embryos were collected individually 24 hours later, placed in a PCR tube and frozen. This was the negative control with no inhibitors.

For the sperm-inhibition assay, 1:300 purified anti-*tpis* antiserum and each heat inactivated anti-Tom-complex antiserum were added to 300 μl HTF medium of capacitated spermatozoa, followed by 1-hour incubation. After washing/centrifuging with 7 ml HTF medium three times, the sediment was suspended with 100 μl HTF and incubated with the eggs. After 5-6 hours, the eggs were transferred to HTF medium containing 50 μM EDTA and incubation was continued in the CO_2 incubator. The two-cell embryos were collected after 24 hours.

For the inhibition assay of the fertilized eggs, the eggs were transferred to HTF

medium containing each inhibitor (500 μM EGTA, 100 μM ZnCl2 and 300 μM ATA) 1

hour after mixing the untreated spermatozoa and eggs. Eight hours later, they were

washed with HTF medium without inhibitor and EDTA, after which time incubation

was continued. The two-cell embryos were collected 24 hours later.

2.11. Detection of mtDNA (PCR)

Detection of the sperm mtDNA of B6-mt^{spr} was carried out according to the method

of Kaneda et al. (1995) using nested PCR. The frozen specimen was thawed, proteinase

K was added (final concentration: 50 μg/ml) and the final volume was made up to 20 μl

with distilled water. This was incubated at 37°C for 1 hour to digest the protein,

followed by incubation at 95°C for 10 min to deactivate the proteinase K. Mixtures for

PCR (AmpliTaq Gold) were added and the final volume was made up to 50 μl. For the

first round of PCR, 30 cycles were carried out at 94°C for 1 min for denaturation, 45°C

for 1 min for annealing and 72°C for 1 min for extension, with the outer

haplotype-specific primer 5′-AATTATATCAATGATCT-3′ and the common primer

5′-GGCCCGGAGCGAGAAGA-3′. The second round of PCR was carried out under

the same conditions as the first, with the inner haplotype-specific primer

5′-ATACTCAACATAATAT-3′ and the common primer

5′-TCACGGAGGATGGTAGA-3′, using 1 μl of the first-round PCR products as the

template. Single PCR was carried out under the same conditions using 1 μl of the

first-round PCR products as the template with the common forward primer

5′-AGCCCTAAGAAAACACA-3′ and the reverse primer

5′-TCTAAACACAGAGGTTTAA-3′ as a positive control. A total of 10 μl of the

second-round PCR products was applied to NuSieve 3:1 agarose gel and

electrophoresed in TBE buffer. The gels were stained with ethidium bromide to detect

the PCR products.

2.   Results

3.1. Cell specificity of tpis gene expression

To further investigate the sperm specificity of t-*tpis* gene expression in mouse

gametes, mRNA expression was studied using RT-PCR in the testis, spermatozoa, unfertilized eggs in the mature ovary, one-cell embryos, two-cell embryos and cerebellum of the mouse. A primer pair was designed with a forward primer in the s-*tpis* mRNA-specific 5′UTR, and another primer pair in the t-*tpis* mRNA-specific portion. Both types were detected in all cells examined (Fig. 1), and the sperm specificity of the t-*tpis* gene expression at the transcriptional level could not be determined. Contrary to Takaishi and Huh's findings (1999), s-*tpis* mRNA was detected in the testis and t-*tpis* mRNA was detected in the cerebellum. There was also expression of a small amount of mRNA that could not be detected by Northern blotting analysis, but was detected by PCR.

Western blotting analysis with anti-*tpis* antibody detected the following bands: 114 kDa in the testis, 166 kDa in the spermatozoa, 64 kDa and 75 kDa in the mature ovary, 64 kDa in the cerebellum, and 75 kDa in the liver, kidney and heart (Fig. 2A). The molecular weight predicted from the amino-acid sequence is 101 kDa for the t-*tpis* protein and 59 kDa for the s-*tpis* protein, suggesting that only the t-*tpis* protein is expressed in the testis and spermatozoa, and only the s-*tpis* protein in the ovary,

cerebellum, liver, kidney and heart.

3.2 Intracellular localization of the tpis protein

Crude mitochondria from the testis, spermatozoa, mature ovary and cerebellum, purified mitochondria from the crude testicular mitochondria, and the cytosol from the mature ovary and cerebellum were prepared by centrifugation. Western blotting analysis with anti-*tpis* antibody detected the following bands: 114 kDa in the purified mitochondria of the testis, 166 kDa in the mitochondria of the spermatozoa, 64 kDa and 75 kDa in the mitochondria and 75 kDa in the cytosol of the ovary, and 64 kDa only in the mitochondria of the cerebellum (Fig. 2A).

In immunofluorescence staining performed with anti-*tpis* antibody to mouse spermatozoa and human ejaculated spermatozoa, fluorescence was located in the midpiece in the area of the mitochondria (Fig. 3A and 3B). Based on the above findings, t-*tpis* protein is localized in the mitochondria of the testis and spermatozoa. According to Lin et al. (2001), the postacrosomal zone is stained when anti-*HSD-3.8* fragment

protein antibody is used for the immunoperoxidase staining of human spermatozoa. The

recognition sites of the two antibodies might be different because of differences in the

length of the antigen portion; alternatively, *HSD-3.8* might not be a human homologue

of *tpis*. However, whilst *HSD-3.8* protein was initially found to be 75 kDa using

Western blotting analysis (Zang et al., 1992), it was later reported to be 55 kDa by Lin et

al. (2001), which is far smaller than the predicted molecular weight of 104 kDa. This

indicates that there might be problems with the antibody being used.

Deglycosylation and dephosphorylation were performed in the spermatozoa

mitochondria protein and the ovary cytosol protein prior to Western blotting analysis.

Although a band shift was found following deglycosylation of the spermatozoa

mitochondria protein, it did not drop to 114 kDa and there was no phosphorylation (Fig.

2B). We assumed that the 166 kDa sperm protein is the t-*tpis* protein, and that it

undergoes post-translational modifications other than glycosylation or phosphorylation

during epididymal passage. A band shift to 64 kDa was found following deglycosylation

of the ovary cytosol protein (Fig. 2B), indicating that the 75-kDa protein might be the

glycosylated 64 kDa s-*tpis* protein. To examine whether the Tom complex also

undergoes post-translational modification in the spermatozoa, the testis and sperm crude mitochondria and the sperm mitochondria treated with deglycosylation and dephosphorylation were subjected to Western blotting analysis (Fig. 2C). The results indicated that while Tom40 was present as a 40-kDa protein in the testis, the sperm preparation displayed bands at 40 kDa and 87 kDa, indicating the presence of phosphorylated Tom40.

When crude mitochondrial fractions of the testis were treated with trypsin and analyzed using Western blotting analysis, the 114-kDa band disappeared and the degraded fragment could not be detected. Tom40, most of which is embedded within the mitochondrial outer membrane, did not disappear (Fig. 2D). We suggest that the t-*tpis* protein is localized in the mitochondrial outer membrane, with the majority of the protein facing the cytosolic side.

To demonstrate that the t-*tpis* protein is a member of the Tom complex, native PAGE was attempted using crude mitochondria. As the structures of mammalian spermatozoa are tightly packed due to disulfide bond cross-linking (Calvin and Bedford, 1971; Sutovsky et al., 1997), separation of the sperm mitochondrial proteins is difficult

under nonreducing conditions; therefore, testis mitochondria were used (Fig. 2E). The

results showed detection of bands for the tpis protein as well as Tom22 and Tom20 in

the ~500 kDa area (Fig. 2E). Therefore, it is possible to state that the t-*tpis* protein is a

constituent of the Tom complex.

3.3. Evaluation of the sperm factor

The spermatozoa of congenic strain C57BL/6J(B6)-mt^{spr}, incorporating the mtDNA

of *Mus spretus*, were incubated with anti-*tpis* and anti-Tom complex antibodies. The

unfertilized eggs extracted from a B6 female were inseminated *in vitro* with the

spermatozoa. The following day, two-cell embryos were sampled individually in PCR

tubes. We tried to determine whether the sperm mtDNA of B6-mt^{spr} in the two-cell

embryo could be detected by the method of Kaneda et al. (1995) using nested PCR, and

if the elimination system could be inhibited by the antibodies (Fig. 4). At this point,

there was a concern as to whether the antibody could bind to the mitochondria in vital

spermatozoa. Since the report by Alarcon-Segovia et al. (1978), there has been much

research regarding antibody penetration of the cell membrane of living cells and

translocation to subcellular compartments (Deng et al., 2000; Yanase et al., 1997). Most

of the information is on autoantibodies in autoimmune diseases, but one study reports

that antibodies can enter a variety of cell types from different animal species (Deng et

al., 2000). Although receptor-mediated endocytosis is assumed to be the mechanism of

translocation (Yanase et al., 1997), the details are unknown. When immunofluorescence

staining was performed on spermatozoa incubated in a medium containing each

antibody, fluorescence was always found in the midpiece (Fig. 3D-3I), confirming that

these antibodies bind to the mitochondria of the vital spermatozoa. Although

anti-Tom22C antibody recognizes the intermembrane-space end of Tom22, fluorescence

was found in the midpiece, similar to other antibodies. Although it was relatively weak,

the anti-Tom22C antibody could inhibit preprotein transport in the antibody-inhibition

assay using isolated mitochondria (Yano et al., 2000). As it also had a stronger affinity

than the anti-Tom22N antibody, which recognizes the cytosolic side of Tom22 in

Western blotting analysis, the anti-Tom22C antibody was selected. As a result, there was

no significant difference in anti-Tom20, -Tom34 and -Tom70 antibodies compared with

the 13.0% positive rate of the non-treated negative control; however, the rates were

significantly higher in anti-*tpis*, -Tom22C and -Tom40 antibodies with values of 77.8%,

41.2% and 46.2%, respectively (Table 1). No cross-reactions between anti-*tpis*,

-Tom22C and -Tom40 antibodies were found (Fig. 2F), and independence was

confirmed.

3.4. Evaluation of the elimination factor

B6 mouse eggs were inseminated with B6-mt^{spr} mouse spermatozoa. Immediately

after insemination the eggs were transferred to medium containing EGTA, which is a

metal chelating agent, and ZnCl2 and aurintricarboxylic acid (ATA), which are

inhibitors of endonuclease, at concentrations that did not influence embryogenesis. Nine

hours after insemination, which is equivalent to the start of the late pronucleus stage, the

egg was washed with medium containing no added reagents and incubation was

continued. The sperm mtDNA in two-cell embryos was detected by nested PCR. There

was no difference in the rate of two-cell formation between the eggs treated with each

reagent and the non-treated negative control (data not shown). The PCR

mtDNA-positive rate of the spermatozoa was significantly higher for all three compared

with the negative control (Table 1).

4. Discussion

In yeast, Tom40 functions as a channel protein while Tom20, 22, 37 and 70 function

as receptor proteins. In addition to its receptor function, Tom22 is thought to function as

a component of the channel that binds strongly to Tom40. Tom40 and Tom22 are

thought to be vital proteins (Mori and Terada, 1998). The Tom holocomplex, which

contains all the Tom-complex proteins, is detected as a ~500 kDa band by native PAGE

(Ahting et al., 1999; Kunkele et al., 1998). In mammals, the homologues of Tom20, 22,

40 and 70, as well as Tom34 (the mammal-specific receptor), have been identified

(Nuttall et al., 1997). The native PAGE of mouse testis mitochondria showed that the

t-*tpis* protein is a member of the Tom complex, and the trypsination assay demonstrated

its localization in the cytosolic side of the mitochondrial outer membrane. As a result, it

was assumed that the t-*tpis* protein is a receptor protein in the Tom complex. To satisfy

our hypothesis, which states that the sperm factor of sperm mitochondria selectively

incorporates the elimination factor in the fertilized egg, sperm mitochondria must retain

protein-translocation functions after fertilization. Based on the findings that sperm

mtDNA is not eliminated in interspecific crosses, is detected in the neonate (Kaneda et

al., 1995) and partially survives if sperm mitochondria are transplanted into

mtDNA-free Rho0 cells (Manfredi et al., 1997), we assumed that spermatozoa

mitochondria retain normal functions. Furthermore, our Western blotting assay showed

that Tom40 undergoes post-translational modification in the spermatozoa, suggesting

that the Tom complex is a necessary apparatus in sperm mitochondria; it would

therefore maintain its function as a protein translocator. The t-*tpis* protein is expressed

in spermatogenic cells, and also undergoes glycosylation prior to its function in the

spermatozoa. We were able to inhibit the elimination system by treating the

spermatozoa prior to fertilization with anti-*tpis*, -Tom40 and -Tom22C antibodies,

suggesting that the elimination factor is a protein that is transferred into the

mitochondria through the Tom complex of the sperm mitochondria. The receptor

proteins of Tom20, 70 and 34 were not involved in the elimination system. Coupled

with findings from localization studies of the t-*tpis* protein in mitochondria, this

indicates that the t-*tpis* protein functions as a receptor in the elimination-system pathway.

Western blotting analysis demonstrated that, at least in mouse gametes, the t-*tpis* protein

is expressed in sperm but not in oocyte. Therefore, it is the t-*tpis* protein that enables

selectivity in sperm mitochondria. This indicates that the t-*tpis* protein is the sperm

factor.

As the elimination system was inhibited by the treatment of fertilized eggs with

EGTA, $ZnCl_2$ and ATA, we suggest that divalent cation-dependent endonuclease is

involved in the elimination system. This is unlikely to be due to the effects of these

substances on embryo metabolism, as the rate of two-cell formation showed no

difference relative to that of the control. However, the possibility of small effects cannot

be ruled out. It is possible for EGTA to inhibit proteases of the ubiquitin-proteasome

system, but DNase is not involved in this system and there are no reports of $ZnCl_2$ and

ATA blocking it. Lysosomes contain DNase, but only the DNase II of divalent

cation-independent endonuclease has been verified (Arsenis et al., 1970). Therefore, it is

likely that the three substances block another factor that operates in the initial stage of

the elimination system. As the divalent cation-dependent endonuclease also exists in the

mitochondria (Cote et al., 1989), the elimination factor might be a protein that activates

the endonuclease.

In general, the presequence of precursor proteins is not enough for the orientation of

mitochondrial protein import and cytosolic factors; either heat-shock cognate 70 protein

(Hsc70) or mitochondrial import stimulation factor (MSF) is needed (Hachiya et al.,

1995; Murakami et al., 1988). In addition, it is thought that the precursor protein with

strong affinity to MSF is transported to Tom70-Tom37 and then sent to Tom40 via

Tom20-Tom22, while the precursor protein with strong affinity to Hsc70 is sent directly

to Tom40 from Tom20-Tom22 (Hachiya et al., 1995; Mihara and Omura 1996). The

cytosolic factor thus selects the receptor, indicating that the presence of a cytosolic

factor that specifically binds to t-*tpis* protein is needed in the elimination system as well.

It has been reported that the part of Tom34 that has a strong homology to t-*tpis* is found

in the mitochondrial outer membrane, with the majority in the cytosol. Tom34 functions

as a chaperone-like protein and not just as a receptor (Chewawiwat et al., 1999). The

glycosylated s-*tpis* protein was expressed in the cytosol of ovaries, but not in

spermatozoa (Fig. 2A). This suggests the possibility that the cytosol s-*tpis* protein is a

candidate specific chaperone or MSF in the elimination system. The expression of s-*tpis*

mRNA in two-cell embryos (Fig. 1), and s-*tpis* protein in some somatic cells (Fig. 2A),

does not conflict with the observations that the elimination system occurs throughout

embryogenesis (Shitara et al., 2000) and in somatic cells (Manfredi et al., 1997).

The t-*tpis* protein has eight tetratricopeptide repeat (TPR) motifs. The TPR motif is

a degenerate 34 amino-acid sequence, which can sometimes form domains made by

several TPR-motif tandem arrays (t-*tpis* protein has a total of three domains: one in the

t-*tpis*-specific region and two in the region shared with s-*tpis*). Each motif has diverged,

but eight amino-acid residues have been preserved at a relatively high frequency.

Although found in various proteins, among the Tom complexes, Tom20, 34 and 70 have

a TPR motif and form a TPR family. They are involved in protein-protein interactions

through TPR-TPR or TPR-non-TPR interactions. It is believed that each TPR motif

within a protein specializes in different protein-protein interactions (Lamb et al., 1995).

In terms of TPR-TPR interactions, there is a 'knob and hole' model involving the

hydrophobic surfaces formed by the eight consensus residues (Goebl and Yanagida,

1991; *Lamb et al.,* 1995). There is also a report that suggests that other conserved

residues are potentially important for these interactions (Blatch and Lassle 1999). This is thought to be a motif that can exercise sufficient diversity to maintain species specificity.

There have been reports of cDNA segments that are thought to be homologous with the *tpis* gene. It is believed that the *tpis* gene is widely preserved, as it is found in a range of species including plants; for example, *Xenopus laevis* (frog; accession code BG347694), *Danio rerio* (zebrafish; accession code BG727710) and *Glycine max* (soybean; accession code BE803270). Therefore, it is possible that an elimination system with the *tpis* protein as the key protein is common to the eukaryotes.

antiserum and Dr. Takeo Imanishi of Koebaru Central Hospital for support.

References

Ahting U, Thun C, Hegerl R, Typke D, Nargang FE, Neupert W et al. The TOM core complex: the general protein import pore of the outer membrane of mitochondria. J Cell Biol 1999;147:959-68.

Alarcon-Segovia D, Ruiz-Arguelles A, Fishbein E. Antibody to nuclear ribonucleoprotein penetrates live human mononuclear cells through Fc receptors. Nature 1978;271: 67-9.

Arsenis C, Gordon JS, Touster O. Degradation of nucleic acids by lysosomal extracts of rat liver and Ehrlich ascites tumor cells. J Biol Chem 1970;245:205-11.

Birky CWJr. The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. Annu Rev Genet 2001;35:125-48.

Blatch GL, Lassle M. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. BioEssays 1999;21: 932-9.

Calvin HI, Bedford JM. Formation of disulphide bonds in the nucleus and accessory

structures of mammalian spermatozoa during maturation in the epididymis. J Reprod Fertil Suppl 1971;13: 65-75.

Chewawiwat N, Yano M, Terada K, Hoogenraad NJ, Mori M. Characterization of the novel mitochondrial protein import component, Tom34, in mammalian cells. J. Biochem 1999;125:721-7.

Cote J, Renaud J, Ruiz-Carrillo A. Recognition of (dG)n•(dC)n sequences by endonuclease G. J Biol Chem 1989;264:3301-10.

Deng SX, Hanson E, Sanz I. In vitro cell penetration and intracellular transport of anti-Sm and anti-La autoantibodies. Int Immunol 2000;12:415-23.

Goebl M, Yanagida M. The TPR snap helix: a novel protein repeat motif from mitosis to transcription. Trends Biochem Sci 1991;16:173-7.

Hachiya N, Mihara K, Suda K, Horst M, Schatz G, Lithgow T. Reconstitution of the initial steps of mitochondrial protein import. Nature 1995;376:705-9.

Hartl FU, Neupert W. Import of proteins into the various submitochondrial compartments. J Cell Sci Suppl 1989;11:187-98.

Hiraoka J, Hirao Y. Fate of sperm tail components after incorporation into the hamster

egg. Gamete Res 1988;19:369-80.

Kaneda H, Hayashi JI, Takahama S, Taya C, Lindahl KF, Yonekawa H. Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. Proc Natl Acad Sci USA 1995;92:4542-6.

Kunkele KP, Heins S, Dembowski M, Nargang FE, Benz R, Thieffry M et al. The preprotein translocation channel of the outer membrane of mitochondria. Cell 1998;93:1009-19.

Lamb JR, Tugendreich S, Hieter P. Tetratrico peptide repeat interactions: to TPR or not to TPR? Trends Biochem Sci 1995;20:257-9.

Lee YJ, Kim BK, Cui XS, Kim NH. Male pronuclear formation and sperm mitochondria in porcine oocytes following intracytoplasmic injection of pig or mouse sperm. Zygote 2002;10:117-22.

Lin W, Zhou X, Zhang M, Li Y, Miao S, Wang L et al. Expression and function of the HSD-3.8 gene encoding a testis-specific protein. Mol Hum Reprod 2001;7:811-8.

Manfredi G, Thyagarajan D, Papadopoulou LC, Pallotti F, Schon EA. The fate of human sperm-derived mtDNA in somatic cells. Am J Hum Genet 1997;61:953-60

Mihara K, Omura T. Cytoplasmic chaperones in precursor targeting to mitochondria: the role of MSF and hsp70. Trends Cell Biol 1996;6:104-8.

Mori M, Terada K. Mitochondrial protein import in animals. Biochim Biophys Acta 1998;1403:12-27.

Moriyama Y, Kawano S. Rapid, selective digestion of mitochondrial DNA in accordance with the matA hierarchy of multiallelic mating types in the mitochondrial inheritance of Physarum polycephalum. Genetics 2003;164:963-75.

Murakami H, Pain D, Blobel G. 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J Cell Biol 1988;107:2051-7.

Nishimura Y, Misumi O, Kato K, Inada N, Higashiyama T, Momoyama Y et al. An mt^{+} gamete-specific nuclease that targets mt^{-} chloroplasts during sexual reproduction in C. reinhardtii. Genes Dev 2002;16:1116-28.

Nuttall SD, Hanson BJ, Mori M, Hoogenraad NJ. hTom34: a novel translocase for the import of proteins into human mitochondria. DNA Cell Biol 1997;16:1067-1074

Sager R, Lane D. Molecular basis of maternal inheritance. Proc Natl Acad Sci USA

1972;69:2410-3.

Shitara H, Hayashi JI, Takahama S, Kaneda H, Yonekawa H. Maternal inheritance of mouse mtDNA in interspecific hybrids: segregation of the leaked paternal mtDNA followed by the prevention of subsequent paternal leakage. Genetics 1998;148:851-7.

Shitara H, Kaneda H, Sato A, Inoue K, Ogura A, Yonekawa H et al. Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. Genetics 2000;156:1277-84.

Sutovsky P, Tengowski MW, Navara CS, Zoran SS, Schatten G. Mitochondrial sheath movement and detachment in mammalian, but not nonmammalian, sperm induced by disulfide bond reduction. Mol Reprod Develop 1997;47:79-86.

Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. Ubiquitin tag for sperm mitochondria. Nature 1999;402:371-2.

Sutovsky P, Moreno RD, Ramalho-Santos J, Simerly C, Schatten G. Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial

inheritance in mammalian embryos. Biol Reprod 2000;63:582-90.

Szollosi D. The fate of sperm middle-piece mitochondria in the rat egg. J Exp Zool 1965;159:367-77.

Takaishi M, Huh N. A tetratricopeptide repeat-containing protein gene, tpis, whose expression is induced with differentiation of spermatogenic cells. Biochem Biophys Res Commun 1999;264:81-5.

Werhahn W, Niemeyer A, Jansch L, Kruft V, Schmitz UK, Braun HP. Purification and characterization of the preprotein translocase of the outer mitochondrial membrane from Arabidopsis. Identification of multiple forms of Tom20. Plant Physiol 2001;125:943-54.

Yanagimachi R, Noda YD. Electron microscope studies of sperm incorporation into the golden hamster egg. Am J Anat 1970;128:429-62.

Yanase K, Smith RM, Puccetti A, Jarett L, Madaio MP. Receptor-mediated cellular entry of nuclear localizing anti-DNA antibodies via myosin 1. J Clin Invest 1997;100:25-31.

Yano M, Hoogenraad N, Terada K, Mori M. Identification and functional analysis of

human Tom22 for protein import into mitochondria. Mol Cell Biol 2000;20:7205-13.

Zang ML, Wang LF, Miao SY, Koide SS. Isolation and sequencing of the cDNA encoding the 75-kD human sperm protein related to infertility. Chinese Med J 1992;105:998-1003.
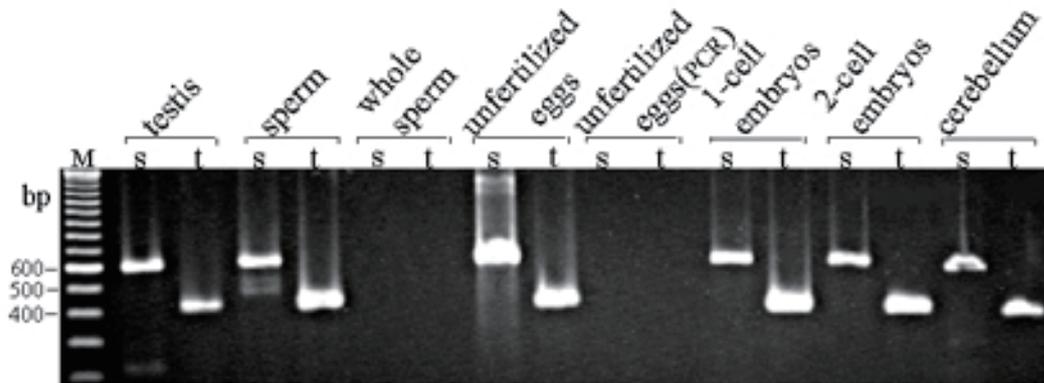
Figure Legends

Fig. 1. Detection of *tpis* mRNA (RT-PCR). Both types of *tpis* mRNA were detected in RNA extracted from the spermatozoa, but were not detected in a few whole spermatozoa. It is not likely that contamination by spermatozoa caused a false positive in the embryo. M, DNA size marker; s: s-*tpis*-specific RT-PCR products (601 bp); t, t-*tpis*-specific RT-PCR products (405 bp); testis, spermatozoa, cerebellum, RNA extracted from each organ was measured; whole spermatozoa, unfertilized eggs, one-cell embryos, two-cell embryos, whole cells were measured; unfertilized eggs (PCR), whole unfertilized eggs were measured from PCR step after pre-treating at 70°C for 10 min.
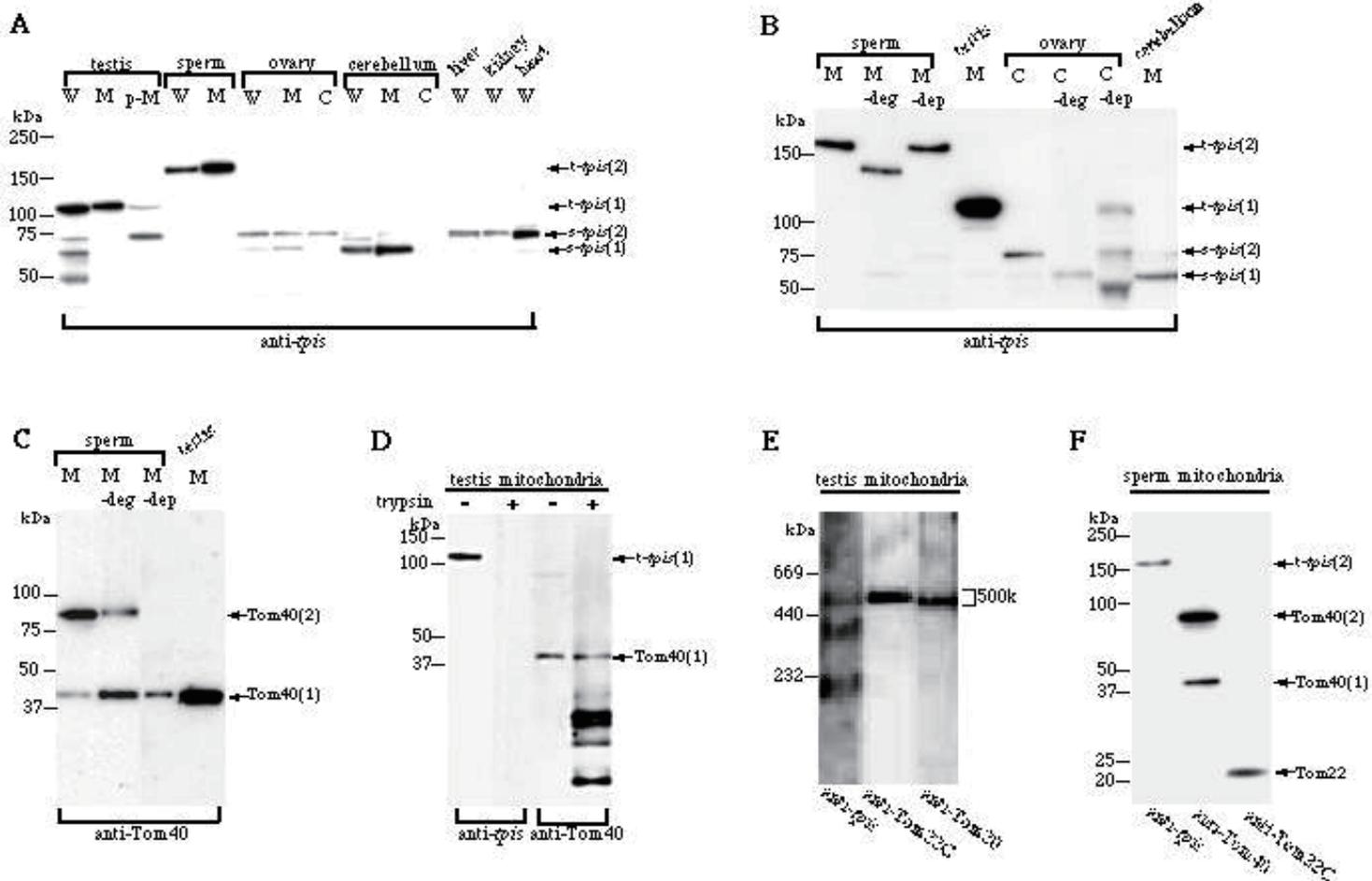
Fig. 2. Western Blotting Analysis. (A) Localization of the *tpis* protein in cells of the testis, spermatozoa, ovary, cerebellum, liver, kidney and heart. (B) Post-translational modification of the *tpis* protein. (C) Post-translational modification of sperm Tom40. (D) A trypsination assay of the *tpis* protein and Tom40. (E) Native PAGE of mitochondria from the testis. (F) Study of the cross-reaction of anti-*tpis*, -Tom22C and -Tom40 antibodies against sperm mitochondria. W, whole cells; M, crude mitochondria;

C, cytosol; p-M, purified mitochondria; -deg, deglycosylation treated; -dep, dephosphorylation treated; s-*tpis* (1), 64-kDa s-*tpis* protein; s-*tpis* (2), 75-kDa s-*tpis* protein ; t-*tpis* (1), t-*tpis* protein of the testis; t-*tpis* (2), t-*tpis* protein of the spermatozoa; trypsin-, trypsination untreated; trypsin+, trypsination treated.
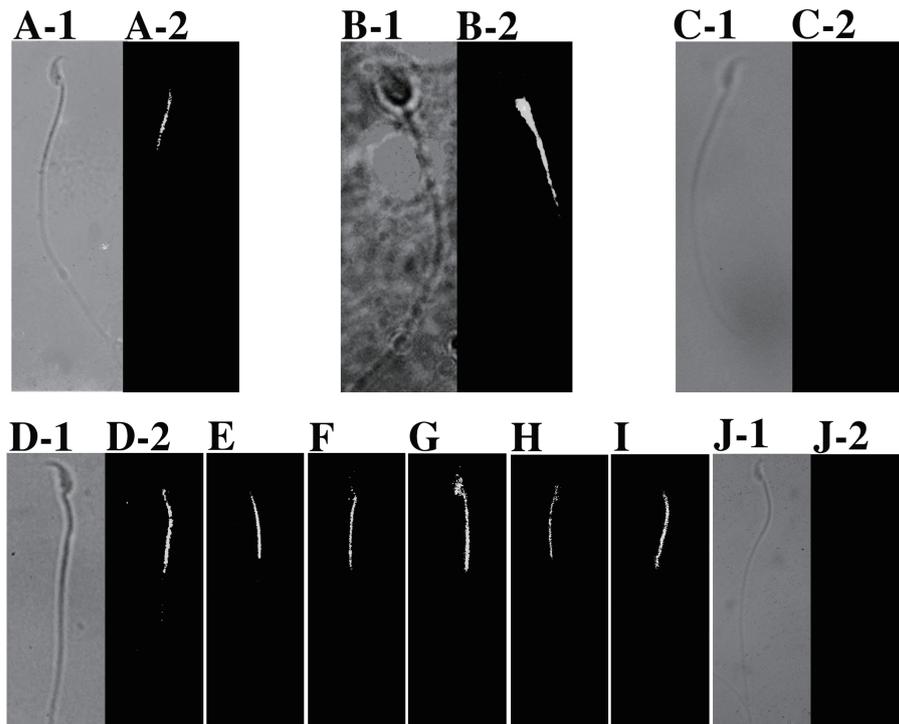
Fig. 3. Immunofluorescence Staining of Spermatozoa. (A-C) Staining of fixed spermatozoa. (A-1 and A-2) Light and fluorescent microscopic images, respectively, of mouse spermatozoa treated with anti-*tpis* antiserum. (B-1 and B-2) Light and fluorescent microscopic images, respectively, of human spermatozoa treated with anti-*tpis* antiserum. (C-1 and C-2) Light and fluorescent microscopic images, respectively, of mouse spermatozoa treated with normal rabbit IgG (negative control). (D-I) Staining of vital spermatozoa. (D-1 and D-2) Light and fluorescent microscopic images, respectively, of mouse vital spermatozoa treated with anti-*tpis* antiserum. (E) Mouse vital spermatozoa treated with anti-Tom40 antiserum. (F) Mouse vital

spermatozoa treated with anti-Tom22C antiserum. (G) Mouse vital spermatozoa treated with anti-Tom20 antiserum. (H) Mouse vital spermatozoa treated with anti-Tom34 antiserum. (I) Mouse vital spermatozoa treated with anti-Tom70 antiserum. (J-1 and J-2) Light and fluorescent microscopic images, respectively, of mouse vital spermatozoa treated with normal rabbit serum (negative control); no fluorescence was observed in the negative control, but fluorescence was present in the midpiece of spermatozoa treated with each of the antisera.
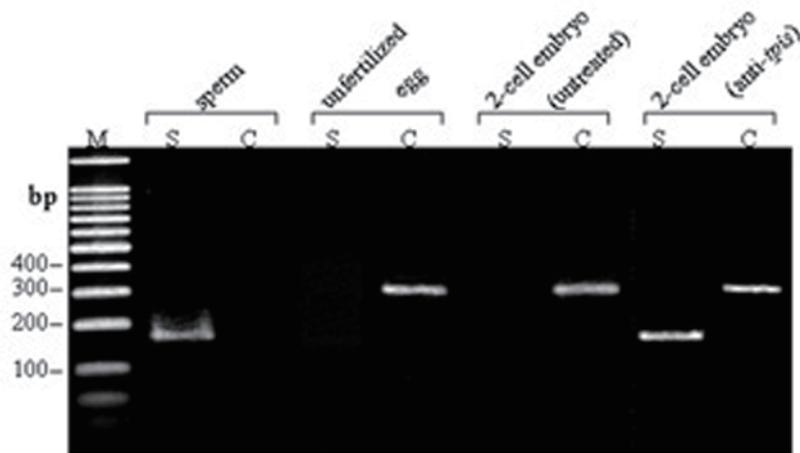
Fig. 4. Detection of Mitochondrial DNA. Spermatozoa, eggs and embryos were analyzed

by single-cell PCR. M, DNA size marker; S, nested PCR products (168 bp) from a

B6-mt^{spr}-mouse mtDNA-specific primer; C, single PCR products (325 bp) from the

primer for the commonly shared portion of the mtDNA of B6 and B6-mt^{spr} mice. The

mtDNA of a single spermatozoan was substantially reduced compared to that of a single

oocyte. Therefore, a single oocyte could be detected, but not a single spermatozoan,

using single PCR. This was used as a positive control of the two-cell embryos.

Table 1

Inhibition assay of spermatozoa and fertilized eggs

Treatment	Total number of two-cell embryos examined	Positive number of sperm mtDNA (%)
Negative control	23	3 (13.0)
Treatment of spermatozoa		
Anti-*tpis* antibody	18	14 (77.8)*
Anti-Tom20 antibody	9	1 (11.1)
Anti-Tom34 antibody	18	1 (5.6)
Anti-Tom70 antibody	16	1 (6.3)
Anti-Tom22C antibody	17	7 (41.2) ***
Anti-Tom40 antibody	13	6 (46.2) ***
Treatment of fertilized eggs		
EGTA (500 μM)	14	12 (85.7)*
ZnCl$_2$ (100 μM)	15	8 (53.3) ***
ATA (300 μM)	15	10 (66.7) **

χ^2 test :*P <0.0001; ** P <0.01; ***P <0.05.