An integrase of endogenous retrovirus is involved in maternal mitochondrial DNA inheritance of the mouse

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Abstract

The mechanism of maternal mitochondrial DNA (mtDNA) inheritance in animals can be said to be the selective elimination of sperm mtDNA via the elimination factor of the egg and a sperm mitochondria-specific factor. In 2005, we clarified that t-tpis (Spag1 isoform 1) is a mitochondria-specific translocator and the sperm factor, and furthermore estimated that the elimination factor of the egg is the divalent cation-dependent endonuclease and s-tpis (Spag1 isoform 2 and isoform 3) the elimination system-specific chaperone [1]. This time, using a recombinant Spag1 isoform 1 protein, a pull-down assay of ovary cytosol was performed and the elimination factors searched for. Surprisingly an endogenous retroviral integrase fragment (Eri15) was identified using mass spectrometry of the electrophoresis band of the pull-down protein. Eri15 was detected as a complex of ~500 kDa with Spag1 isoform 2 or isoform 3 in native PAGE of the ovary cytosol. This strongly suggested that Eri15 is selectively transported into the sperm mitochondria matrix by Spag1 isoform 2 and 3 via Spag1 isoform 1 and that the sperm mtDNA is destroyed, thus causing the establishment of maternal mtDNA inheritance.

Maternal mitochondrial inheritance occurs in many eukaryotes [2]. With the exception of interspecific crosses, strict maternal inheritance is known to take place in mammals [3]. During fertilization, the entire spermatozoon, including the mitochondria of the midpiece, enters the egg [4, 5]. The sperm mitochondria are selectively destroyed, whereas those of the egg escape destruction [2]. Sutovsky et al. [6] reported that sperm mitochondria are ubiquitinated before fertilization in rhesus monkeys and cows. They assumed that an ubiquitin-proteasome system is the elimination factor in mammals, and speculated that ubiquitin directly recognizes prohibitin, which is an integral protein of the inner mitochondrial membrane.

Kaneda et al., [7] 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

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mtDNA. Maternal inheritance also occurs in chloroplast DNA. Nishimura et al. [8] separated the sexual chloroplasts from zygotes in *Chlamydomonas* immediately after mating. They detected gradually increasing Ca^{2+}-dependent nuclease activity during the disappearance of chloroplast DNA, but only in male derived chloroplasts. They therefore proposed that female chloroplasts are protected from this sequence-nonspecific endonuclease by an unknown mechanism.

We suggested that egg mtDNA is not protected from the elimination factor, but that the sperm mitochondria selectively take up the elimination factor, which would explain the question of selectivity. In 2005, we demonstrated that sperm associated antigen (Spag) 1 (t-tpis) to be the sperm mitochondria-specific translocator and that its antibody can block the elimination system of the mouse. Furthermore, as the elimination system could be blocked by processing fertilized eggs with EGTA as the metal chelator and aurintricarboxylic acd (ATA) as the nuclease inhibitor, it was estimated that one of the elimination factors of the egg is the divalent cation-dependent endonuclease. In addition, as the translocator of the mitochondrial outer membrane (Tom) 34 that has strong homology with

Spag1 was localized not only in the mitochondrial outer membrane but also the cytosol and also functioned as a chaperone, it was estimated that the Spag1 isoform (s-tpis) which developed in similar localizations in the ovary and which did not develop in spermatozoa would assure selectivity of the system as the elimination system-specific chaperone [1]. This time, using recombinant Spag1 protein, a pull-down assay of the ovary cytosol was performed and the elimination factors searched for. And with the expected results having occurred we report it here.

Materials and methods

Mouse strain, preparation of ovary and other organs and isolation of cytosol and mitochondria. The dissection of mature ovaries and other organs from C57BL/6J mice (SLC, Japan) killed through cervical dislocation and isolation of cytosol and mitochondria were performed as described previously [1]. Isolation of ovary cytosol was performed using a homogenization buffer without EDTA.

Protein expression and purification. Total RNA was extracted from

mature ovaries and testis using the acid guanidinium-phenol-chloroform method. After making cDNA with the random primer method, PCR was carried out, using a primer pair which was designed in accordance with the manufacturer's instructions for pENTR Directional TOPO Cloning Kits (Invitrogen) and KOD-plus-DNA polymerase (TOYOBO). Spag1-2; forward 5'-CACCATGATTCAGAAGAAGCTGATGG-3' and reverse 5'-TTAAAGCTCATACTGCCTTCTTAGGG-3', Spag1-1 N1; forward 5'-CACCATGACTGCTAAGGCTAAGG-3' and reverse 5'-TCAGGCCGCCTCATCCGAGCCACCATC-3', Spag1-1N2; forward 5'-CACCATGACTGCTAAGGCTAAGGAC-3' and reverse 5'-TCATTCCTTCTCAGTCAGACCTGCTGTTT-3, Eri15; forward 5'-CACCATGTCAGAGAGACCAG-3' and reverse 5'-CTAATCAATCCCCAATATCTTTGC-3'. A schematic illustration of isoforms and recombinant proteins is shown in Figure 1. After separating PCR products using 0.8 % GTG agarose gel the DNA was extracted with a High Pure PCR Product Purification Kit (Roche). After a TOPO cloning reaction of the purified PCR product into pENTR/SD/D-TOPO vector, the vector was transformed into One Shot TOP10 Chemically Competent *E.*

coli. Transformants were selected on LB plates containing 50 μ g / ml kanamycin and the plasmid DNA isolated using a QIAGEN Plasmid Mini Kit (QIAGEN) from a colony for which the correct insert had been confirmed with colony PCR. Sequencing was performed on the plasmid DNA with the dye deoxy terminator method using a 310 Genetic Analyser (Applied Biosystems) and the existence or non-existence of mutations confirmed. In accordance with the manufacturer's instructions for the *E. coli* Expression System with Gateway Technology (Invitrogen), a recombination of purified plasmid without mutation and pDEST 17 vector （6×His tag added to the N-terminal） through a LR recombination reaction was performed and then the vector transformed into competent Library Efficiency DH5 α cells. From the colony which was selected with LB plates containing 100 μ g / ml ampicillin, the correct inserted plasmid DNA was isolated and transformed into BL21-AI One Shot cells. The selected colony was incubated in LB medium at 37 ℃ until the OD600 reached 0.5～0.8, L-arabinose at a 0.2 % final concentration added, and after further incubation for 4～6 hours the *E. coli* collected.

5 μ l / mg of BugBuster protein extraction reagent (Novagen)

containing 0.5 mKU / ml lysozyme was added to the *E. coli* pellet and sonicated. Next the supernatant was transferred to a fresh tube after 20,800 ×g 10 min centrifugation. Though Spag1-1N1 and Spag1-1N2 formed an inclusion body and precipitated, this was solved by adding 8 M urea buffer (20 mM Na_2HPO_4, pH 7.8, 500 mM NaCl, 8M urea) to the pellet. The lysate, after electrophoresis in SDS-PAGE, was stained with Coomassie brilliant blue G250（BIO-RAD） and expression confirmed. In accordance with the manufacturer's instructions for a Ni-NTA Purification System (Invitrogen), the lysate and Ni-NTA resin were incubated overnight at 4 ℃. After washing the resin in wash buffer, the protein was eluted with imidasole elution buffer. The eluate was dialyzed against PBS containing 1 mM DTT, 1 mM PMSF with a Slide-A-Lyzer Dialysis Cassette (PIERCE) for buffer exchange and renaturation. It was then further dialyzed against store buffer (10 mM Tris-HCl, pH 8.5, 50 % glycerol, 0.01 % Triton X-100, 1 mM PMSF, 1 mM DTT) and stored at -20 ℃.

Pull-down assay and mass spectrometry. Ovary cytosol diluted with lysis buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM $MgCl_2$, 3mM $MnCl_2$) was incubated with His-tagged Spag1-1N1 protein combined

Ni-NTA resin overnight at 4 ℃. A control was made with two combinations of a resin which did not combine protein and ovary cytosol and a protein combined resin and lysis buffer. After incubation, the resin was washed twice in lysis buffer, four times in wash buffer A (50 mM Na2HPO4, pH 8.0, 500 mM NaCl, 20 mM imidazole, 0.5 % NP-40), four times in wash buffer B (50 mM Na2HPO4, pH 6.0, 500 mM NaCl, 20 mM imidazole, 0.5 % NP-40) and twice in wash buffer B without NP-40. 2× sample buffer (125 mM Tris-HCl, pH 6.8, 20 % glycerol, 4% SDS, 200 mM DTT and 0.005 % BPB) were added and heated for 5 min. at 95 ℃, and the eluate collected after centrifugation. After electrophoresis with 10~20 % gradient polyacrylamide gel (ATTO CORPORATION), it was stained with Coomassie brilliant blue G250（BIO-RAD）and several bands excised which were not in the control. In accordance with the manufacture's instructions for an ElutaTube Protein Extraction Kit (Fermentas), the excised gel and electrophoresis buffer (40 mM Tris base, 40 mM acetic acid, 1 mM EDTA) were placed in an ElutaTube and electrophoresis carried out. After transferring the eluate to a fresh ElutaTube and dialyzing against PBS, in accordance with the

manufacturer's instructions for a DNase Activity ELISA Kit (ORGENTEC GmbH, Germany), DNase activity was measured. After excising the band for which activity had been confirmed and in-gel digestion with trypsin, mass spectrometry was performed with a MALDI-TOF MS AXIMA-CFR （SHIMADZU BIOTECH, Japan） and the protein identified with a Mascot search.

Endonuclease activity assays of recombinant Eri15 (rEri15) protein. After isolated mitochondria from the ovary and testis was incubated with a protein lysis buffer (20 mM Tris-HCl, pH 7.5, 125 mM NaCl, 100 mM EDTA, 1 % SDS, 8M urea, 200μ g / ml proteinase K) overnight at room temperature and processed with the phenol-chloroform-isoamyl alcohol (PCI) method, isopropanol was used in precipitation. Furthermore, after incubating with 2 mg /ml DNase free RNaseA for 1 hour at 37 ℃ and precipitation again with the PCI method using isopropanol, it was then dissolved with TS buffer (10 mM Tris-HCl, pH 8.5, 150 mM NaCl). A final concentration of 50 mM Tris-HCl (pH 8.5), 30 μ M $MnCl_2$, 3 mM $MgCl_2$ was added to 200 ng mtDNA and 6.9μ g rEri15 and as a blocking test 300 μ M $ZnCl_2$ and 300μ M ATA were respectively added and incubated for 1

hour at 37 °C. After electrophoresis with 1.5 % agarose gel it was stained with ethidium bromide and observed with UV light.

In accordance with the manufacturer's instructions for the DNase Activity ELISA Kit (ORGENTEC GmbH, Germany), optimum pH and the requirements for divalent cation of Eri15 were checked. After putting 7.5 μ g rEri15 and reaction buffer for each pH condition and each divalent cation into microplates coated with a specific DNase substrate they were incubated for 1 hour at 37 °C . pH was set with the following solution of 50 mM final concentration ; MES-HCl (pH 5.5), bis-Tris-HCl (pH 6.5), Tris-HCl (pH 7.5, 8.5), Glycine-HCl (pH 9.5, 10.5). For the divalent cation, chloride salt was added of the following concentrations ; 30 μ M $MnCl_2$, 3 mM $MgCl_2$, 250 mM $MgCl_2$, 3 mM $CaCl_2$, 300 μ M $ZnCl_2$, 30 μ M $MnCl_2$+300 μ M $ZnCl_2$, 3 mM $MgCl_2$+300 μ M $ZnCl_2$. After washing in wash buffer, HRP-conjugate solution was added and incubated for 15 min. at room temp. After washing in wash buffer, TMB was added and incubated for 15 min. at room temp. , stop solution added and then the OD450 read.

Antibody generation. By immunizing a rabbit with recombinant Spag1-2 protein, recombinant Spag1-1N2 protein, and recombinant Eri15 protein

respectively, antiserum was obtained and refined with a peptide affinity column.

Localization of Eri15 protein. Western immunoblotting analysis by SDS-PAGE of various organs, pull-down eluates and native PAGE of mature ovary cytosol were performed as described previously [1]. Used respectively for the mature ovary and testis was cytosol protein, for other organs whole cells protein, and for first antibody anti-Eri15 polyclonal antibody, anti-Spag1-2 polyclonal antibody, anti-His-tag monoclonal antibody (Novagen), and anti-β-actin monoclonal antibody (SIGMA).

Immunohistochemical staining. After deparaffinizing and rehydrating 10 % phosphate buffered formalin (pH 7.4) fixed and paraffin-embedded sections of the mature ovary, for the antigen retrieval it was boiled in a pressure chamber containing 2 liters of 1mM EDTA (pH 8.0) solution for 2.5 min.. After quenching with 3 % H_2O_2 / methanol, blocking was carried out with 3 % BSA / PBS. The anti-Eri15 antibody, anti-Spag1-2 antibody as first antibody, and anti-Spag1-1N2 antibody as a negative control were respectively incubated overnight at 4°C. As a second antibody, HRP-conjugated anti-rabbit IgG antibody (Bio-Rad) was incubated for 45

min. at room temp. With DAB coloring it was then counterstained with hematoxylin.

Results and discussion

Pull-down assay of ovary cytosol by rSpag1-1 N1 protein combined resin

In the above treatise [1], it was considered that there were 2 protein sizes of 75 kDa and 64 kDa in Spag1 isoform (s-tpis) as splicing variants of Spag1-1, and 75 kDa protein was considered to be a post-translational modification of the 64 kDa protein. However, following that registration of *Spag1 isoform 3 (Spag1-3)* mRNA (UniProtKB accession no. **Q80ZX8-3**) occurred and it was thought that the 64 kDa protein was Spag1-2 and the 75 kDa protein Spag1-3, viewed from their molecular sizes (Fig. 1A). Using a pull-down assay of the ovary cytosol with rSpag1-1 N1 protein combined resin both Spag1-2 and Spag1-3 were pulled down (Fig. 2B).

No DNase activity was observed in the pull-down eluate with the control resin only, but DNase activity was observed in the pull-down

eluate with the rSpag1-1N1 protein combined resin（data not shown）. In the 113 kDa band extracts of several bands in the CBB stained SDS-PAGE gel (Fig. 2A) DNase activity was observed (data not shown）. Through mass spectrometry analysis of this band, rSpag1-1 N1 protein was detected as the major protein (57 % sequence coverage). As the only nuclease among the other minor proteins, Mus musculus similar to Retrovirus-related Pol polyprotein (Endonuclease) (GenBank accession no. **LOC641211**) was identified (34 % sequence coverage). This protein has an estimated molecular weight of 15 kDa and an integrase core domain sequence. Though generally a retroviral integrase is composed of three components of the zinc binding domain (HHCC motif), central catalytic core domain (DD35E motif) and DNA binding domain from N-terminal [9, 10, 11], the identified protein lacked a zinc binding domain and DNA binding domain. Furthermore, it lacked a C-terminal region which contains E out of DD35E as the core domain motif (Fig. 1B). From the fact that in the genomic sequence (GenBank accession no. **NW___ 001073458**) intron was not discovered, it was estimated that it derived from the retrovirus and was named <u>e</u>ndogenous <u>r</u>etrovirus <u>i</u>ntegrase <u>15</u>

kDa (Eri15).

Only Eri15 protein in the 60 kDa band (not detected in CBB stain) could be detected using a Western immunoblotting assay of the pull-down eluate by the anti-Eri15 antibody and anti-His-tag antibody, and Eri15 protein and His-tagged fusion protein in the 113 kDa band（Fig. 2B）. As the molecular weight of His-tagged Spag1-1 N1 protein is 53 kDa (Fig. 2A) and that of Eri15 protein of ovary cytosol is 60 kDa (Fig. 2B, Fig. 4A), it was thought that part of Eri15 formed a complex (53+60=113 kDa) with the His-tagged Spag1-1 N1 protein.

Characterization of rEri15 protein

Though integrase has exo- and endonuclease activity [9-18], in spite of the recombinant full-length Eri15 protein that developed in the *E. coli* lacking part of the integrase core domain and DNA binding domain, endonuclease activity was discovered which could digest mtDNA (Fig. 3A). The fact that it could digest not only testis mtDNA but also ovary mtDNA (Fig. 3A) leads to the conclusion that ovary mtDNA is not protected, at

least against Eri15.

Furthermore, activity was hampered by Zn^{2+} and ATA as the nuclease inhibitor (Fig. 3A, 3B). We reported that the elimination system could be blocked by processing fertilized eggs with Zn^{2+} or ATA [1], and the characteristics of the rEri15 protein coincides with this result. There is a report that although Zn^{2+} is needed to stabilize the folded state of the integrase and for enzymatic activity [12], the Mn^{2+}-dependent nicking activity of the avian sarcoma virus integrase catalytic domain is inhibited by Zn^{2+} [11]. The possibility does exist that Zn^{2+} blocking the elimination system of the egg could result from the fact that Eri15 was inhibited.

Many integrases have alkaline optimum pH [13, 14] and are Mn^{2+}, Mg^{2+}-dependent ($Mn^{2+} > Mg^{2+}$) [11, 15, 16]. rEri15 has an optimum pH of 8.5 and was Mn^{2+}, Mg^{2+} dependent ($Mn^{2+} > Mg^{2+}$) (Fig. 3B). The pH of the mitochondria matrix was 7.8～8.1 and has alkalinity comparable with the 7.4～7.5 of cytosol [19], and the Mn^{2+} content of mitochondria is rich and nearly double that of cytosol [20]. It can be said that Eri15 is adaptive to the internal environment of mitochondria.

Localization of Eri15 protein

Integrase normally forms a multimer and reveals activity in multimers [9, 10, 12, 17, 18]. Eri15 protein was not detected as a monomer of 15 kDa but was detected in the ovary and liver as a tetramer of 60 kDa and in testis as a trimer of 45 kDa (Fig. 4A). In the heart a 54kDa band was detected and in the cerebrum and cerebellum 2 bands of 54 kDa and 76 kDa. It is presumed that the 54 kDa band is perhaps a trimer of a monomer of 18 kDa which received a post-translational modification or an isoform of 18 kDa and that the 76 kDa band is a tetramer. In native PAGE of the ovary cytosol, Eri15 and Spag1-2 or Spag1-3 formed a complex of～500 kDa (Fig. 4B). The Spag1-1 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, and is involved in protein-protein interactions. The TPR domain mediates self-association and forms multimers of TPR protein [21, 22]. There is a possibility that the complex of Eri15 and Spag1-2 (or Spag1-3) is further forming a high-molecular weight complex via the TPR domain.

In immunohistochemical staining of the ovary, strong signals were found for Spag1-2 or Spag1-3 in the cytoplasm of stromal cells and eggs, and for Eri15 in the cytoplasm of stromal cells, eggs and cumulus cells (Fig. 4C). Integrase has a function that integrates viral DNA into host genomes and is localized in the nucleus [18]. Eri15 had many characteristics of integrase, but it was clearly not the nucleus and differed from integrase in that it was localized in the cytoplasm. Recently, an amazing report appeared that aminoterminally truncated isoform (UL12.5) of alkaline endonuclease (UL12) was involved in a recombination of the herpes simplex virus （though is a DNA virus） that eliminates the host mtDNA [23]. While the transport of UL12.5 to the mitochondria matrix is completely unclear it can be thought that the virus uses the mitochondria protein transport system of the host. UL12 is localized in the host nucleus, but UL12.5 gains mitochondria directivity by being truncated. The possibility exists that Eri15 could gain directivity to mitochondria by being truncated. It may be that the host started to use Eri15 for sperm mtDNA elimination, using a mechanism [23] whereby when a virus contaminates a host, the endonuclease of the virus destroys the mtDNA and shuts off the host

function.

Moriyama etc. [24] detected molecular weights of 13 kDa and 37 kDa, optimum pH 8.5, Mn^{2+}-dependent endonuclease activity forming a complex of 440-670 kDa that appears after mating by zymography via zygote extracts of true slime mold. They estimated that this endonuclease activity is a factor which is related to uniparental mtDNA inheritance in true slime mold as it is detected in zygote mitochondria with timing that coincides with that of selective digestion of mtDNA in the zygote. We consider the 37 kDa endonuclease to be the trimer of 13 kDa endonuclease. As they point out, given that the molecular size of many endonucleases is at least approximately 30 kDa and optimum pH is near physiological pH, the characteristics of this 13 kDa endonuclease are slightly peculiar. The above characteristics coincide with those of rEri15 to a large extent. But there is the difference that while rEri15 is Mg^{2+}-dependent next to Mn^{2+}, the 13 kDa endonuclease of Moriyama etc. is not Mg^{2+}-dependent. While activity in 2 mM MgCl2 for rEri15 could be confirmed, from the fact that in 250 mM performed by Moriyama etc. activity decreased (Fig. 3B), there is a possibility that the activity of 13 kDa endonuclease was decreased by too

high a MgCl2 concentration. From the fact that the characteristics of the elimination factor candidate endonuclease of true slime mold and mouse are very similar, there is a possibility that both are ortholog proteins and are strongly stored beyond species in the uniparental mtDNA inheritance system of eukaryotes.

Conclusion

From above, it turned out to be clear that Spag1-2 and Spag1-3 and Eri15 exist in egg cytoplasm, make complexes, and interact with the Spag1-1 specific region. It is considered that as Eri15 protein develops in testis cytoplasm too, what is assuring the selectivity to sperm mitochondria are Spag1-2 or Spag1-3, and that Eri15 is transported to the mitochondria matrix by the Spag1-1 on the sperm mitochondrial outer membrane, digests mtDNA, thus causing the establishment of maternal mtDNA inheritance.

8-10 % of mouse and human genomes are occupied by various sizes of the endogenous retrovirus from complete viruses up to truncated partial

sequences [25]. Though its expression has been confirmed in many species

and organs, there are few reports which show that expression protein is

directly involved in physiological events of the host [25, 26, 27]. We think

that Eri15 can add new knowledge concerning the use of the endogenous

retrovirus protein by the host. Also, mitochondria are thought to have

derived from eubacteria which lived symbiotically in archaeabacteria as

proeukaryotes [28]. It is very interesting that a retrovirus integrated to a

genome of eukaryotes is being used to eliminate the mitochondria when we

think of the role of symbiosis in the establishment and evolution of

eukaryotes.

Acknowledgments

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Figure 1. Schematic illustration of Spag1 isoforms, Eri15, and MLV integrase proteins. (A) Spag1-1 protein has three TPR domains: the 2 on the side of the C-terminal are common to three isoforms. rSpag1-2, recombinant His-tagged full-length Spag1-2; rSpag1-1N1, recombinant His-tagged N-terminal region not containing TPR domain 2, 3 but containing TPR domain 1; rSpag1-1N2, recombinant His-tagged N-terminal region not containing TPR domain at all specific to Spag1-1. (B) Comparison of murine leukemia virus (MLV) integrase and Eri15 protein; ▢ Region with homology of MLV integrase and Eri15; rEri15, recombinant His-tagged full-length Eri15.

Figure 2. Pull-down assay of ovary cytosol by His-tagged Spag1-1N1

protein combined Ni-NTA resin. (A) CBB stained SDS-PAGE gel of pull-down eluate. (-) resin, Resin which dose not combine protein; Arrow, Band not found in control; ※, Band which detected DNase activity and for which a mass spectrometry assay was made; M, protein molecular size marker. (B) Western immunoblotting assay of pull-down eluate by anti-Eri15 polyclonal antibody, anti-Spag1-2 polyclonal antibody and anti-His-tag monoclonal antibody.

Figure 3. Endonuclease activity of recombinant Eri15. (A) Digestion assay with substrate of mitochondrial DNA. ATA, aurintricarboxylic acid.

(B) DNase activity assay by ELISA. (-), Assay with buffer which does not contain divalent cation.

Figure 4. Localization of Eri15 and Spag1-2 (or Spag1-3) protein. (A) Western immunoblotting assay (SDS-PAGE) of various organs by anti-Eri15 antibody. Ovary and testis are cytosol proteins, other organs are whole cells protein. β-actin, Positive control by anti-β-actin monoclonal antibody. (B) Western immunoblotting assay (native PAGE) of ovary

cytosol by anti-Eri15 antibody and anti-Spag1-2 antibody. (C) Immunohistochemical staining of ovary by anti-Eri15 antibody and anti-Spag1-2 antibody. Spag1-1N2, negative control by anti-Spag1-1N2 antibody; Arrow, egg.

Fig. 1

Fig. 2

A

ovary mtDNA   testis mtDNA

Eri15   −   +   −   +   +   +

ZnCl2   ATA

B

Fig. 3

Fig. 4