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Hybrid male sterility is caused by mitochondrial DNA deletion

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Abstract Although it is known that the hybrid male mouse is sterile just like any other animal's heterogametic sex, the reason why only the male germ cells are impaired has yet to be discovered. TdT-mediated dUTP nick end labeling assay using a confocal fluorescence microscope and DNA fragmentation assay of hybrid testis indicated destruction of the mitochondrial DNA (mtDNA) rather than the nuclear DNA. Previously we reported that maternal mtDNA inheritance is through selective sperm mtDNA elimination based on the sperm factor and two egg factors, and expression of these three factors was recognized in the hybrid testis. It was thereby assumed that mtDNA destruction caused by the expression of maternal mtDNA inheritance system in male germ cells is implicated in the hybrid male sterility of mice.

Keywords hybrid male sterility • mitochondrial DNA deletion • maternal mitochondrial DNA inheritance • Spag1 • Eri15 • speciation

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Introduction

Even though crossing between closely related species is possible, the gene flow will be blocked by the sterility of hybrids of heterogametic sex (male in the case of mammals) [1]. It can be said that postzygotic reproductive isolation by hybrid sterility is strict and basal. In other words, the initiative driver for speciation is hybrid sterility, and thus it can be said that hybrid sterility genes are speciation genes. In the case of mice, the female of an interspecific hybrid is fertile, while the male is sterile. Usually the hybrid male grows normally, except for having impaired spermatogenesis in its testis [2]. However, that mechanism is yet to be elucidated.

We previously reported that maternal mitochondrial DNA (mtDNA) inheritance of the mouse is through selective sperm mtDNA elimination based on the sperm-specific translocator of the mitochondrial outer membrane (Spag1-1), two egg factors of the system-specific endonuclease (Eri15) and the system-specific chaperone (Spag1-2 or 1-3) [3, 4]. In the male germ cells the expression of Spag1-2 (or 1-3) is possibly suppressed by epistatic gene(s). In the case of hybrid offspring of a female with a *Spag1-2 (or 1-3)* genetic mutation of the type that inhibits the suppression of the expression of Spag1-2 (or 1-3) and a wild type male, it is speculated that while there is no effect on the female germ cells the mtDNA of the male germ cells do get destroyed. Mitochondrial respiration defects through

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mutant mtDNA with a deletion result in spermatogenic meiotic arrest and cause male sterility [5]. In this study we discuss the maternal mtDNA inheritance system being involved in hybrid male sterility and report the below described “as-expected” result obtained.

Materials and Methods

Mouse strains and crosses

Mice of the inbred strain C57BL/6J (B6) (*Mus musculus domesticus*) were purchased from SLC (Japan). The wild-derived inbred strain PWK (*Mus musculus musculus*) and *Mus spretus* were provided by RIKEN BRC (Japan). We crossed female PWK with male B6 to make intersubspecific hybrids (PB-F1), female B6 with male *Mus spretus*, and female PWK with male *Mus spretus* to make interspecific hybrids (BS-F1, PS-F1 respectively).

Fertility was checked by backcrossing between the F1 male and the parent female, and the presence of spermatozoa in sections of F1 hybrid testis.

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Creation of cryptorchidism

To induce cryptorchidism mice were anesthetized with ether inhalation and the testes manipulated through the inguinal canal into the abdomen and sutured to the abdominal wall through the epididymal body with thread. The mice were killed through cervical dislocation and the testes sampled on 6 days after the operation.

Preparation of testis and ovary

The dissection of testis and mature ovary from mice killed through cervical dislocation and isolation of cytosol and mitochondria were performed as previously described [3].

Trypsination assay of mitochondria

Trypsination of isolated testis mitochondria was performed as previously described [3].

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TdT-mediated dUTP nick end labeling (TUNEL) assay

After deparaffinizing and rehydrating the fixed and paraffin-embedded sections of testis, the sections were treated with 20 μ g/ml proteinase K at room temperature for 15 minutes followed by labeling with fluorescein-dUTP and terminal deoxynucleotidyl transferase (TdT) 37 °C for 2 hours. Labeled sections were quenched with 2 % H₂O₂ / methanol on ice for 5 minutes prior to being incubated with HRP-anti-fluorescein antibody at 37 °C for 30 minutes and visualized using diaminobenzidine (DAB). The sections were counterstained with hematoxyline. With the negative control fluorescein-dUTP was omitted.

For confocal fluorescence microscopic image (Zeiss LSM 510) the sections were labeled with biotin-dUTP prior to incubate with avidin-Alexa 488 (invitrogen) without quenching. Nucleus and mitochondria is stained with TO-PRO3 (invitrogen) and MitoTracker Red CMXRos (invitrogen) respectively. With the negative control biotin-dUTP was omitted.

DNA fragmentation assay

Nucleus and mitochondria isolated by sucrose density gradient centrifugation were digested in TES buffer

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6 (10 mM Tris-HCl, pH7.5, 10 mM EDTA, 100 mM NaCl) containing 1 % SDS, 200 μ g / ml protenase
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9 K at 37 °C overnight and 200 μ g / ml RNase at 37 °C for 60 minutes. DNA was extracted using the
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12 phenol-chloroform-isoamyl alcohol method. The DNA was precipitated with ammonium acetate and
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15 ethanol and dissolved in TE buffer (10 mM Tris-Hcl, pH 7.5, 1 mM EDTA). An aliquot of DNA (2 μ g)
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18 from each sample was labeled at the 3' end with biotin-dUTP and TdT at 37 °C for 30 minutes. Labeled
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21 DNA was separated on 1.5 % agarose gel followed by blotting on a nylon membrane overnight with 10
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24 \times SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7.0). The DNA was fixed by being baked at 80 °C for
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27 60 minutes followed by UV cross-linking. The membrane was placed in blocking buffer (Roche)
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30 containing avidin-HRP at room temperature for 30 minutes prior to being washed with TBST buffer (10
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33 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 % Tween-20). The blot was developed using an ECL plus
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39 system (Amersham Biosciences) and exposed to X-ray film.
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45 Western immunoblotting analysis

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52 Western immunoblotting by SDS-PAGE of ovary and testis proteins with anti-Spag1-2 polyclonal
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55 antibody and anti-Eri15 polyclonal antibody was performed as previously described [3, 4]. Anti- β -actin
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59 monoclonal antibody (Sigma) and anti-VDAC1/porin polyclonal antibody (abcam) were used as the
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6 loading control of cytosol and mitochondria respectively.
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10 11 12 Immunohistochemical staining 13 14 15 16 17 18

19 After deparaffinizing and rehydrating the fixed and paraffin-embedded sections of BS-F1 testis,
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21 immunohistochemical staining with anti-Spag1-2 polyclonal antibody and anti-Eri15 polyclonal antibody
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23 was performed as previously described [4]. Normal rabbit serum was used as a negative control.
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32 **Results and discussion** 33 34 35 36 37 38

39 Mitochondrial DNA destruction in degenerated spermatocytes of hybrid male testis 40 41 42 43 44

45 All BS-F1, PB-F1, PS-F1 males were sterile. Although degenerated spermatocytes showing nuclear
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47 condensation and an eosinophilic cytoplasm through H.E staining (Fig. 1a) are stained in the TUNEL
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49 assay of all F1 hybrid testis (Fig. 1b), apoptotic bodies or nuclear fragmentations that are typical to
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52 apoptosis were not observed. As reported by T. Kaku *et al.* [6], BS-F1 testis are of the two types and
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56 include type I that suffer spermatogenic arrest before the pachytene stage in prophase and have missing
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6 spermatids (Fig. 1a), and type 2 that are disrupted from metaphase I to the maturing stage of spermatid
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9 and that have a small amount of spermatids of abnormal morphology (Fig. 3B(a)). Both PB-F1 and PS-F1
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12 testis indicated type 1 (Fig 1).
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16 The cytoplasm appears to be stained in a TUNEL assay using a light microscope. However, it is
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19 difficult to distinguish it from the nucleus (Fig. 1b). Hence type 1 hybrid testis, which would possibly
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22 have degeneration from the early stage of prophase in which the nuclear membrane is clear, were used to
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25 observe the TUNEL assay with a confocal fluorescence microscope. And as a result a stain corresponding
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28 to the cytoplasm was recognized not only in BS-F1, but also in PB-F1 and PS-F1 testis (Fig. 1).
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33 Furthermore, destruction of mtDNA was confirmed in the DNA fragmentation assay using BS-F1 testis
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36 DNA (Fig. 2).
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39 From the above it was suggested that mitochondrial DNA destruction occurs in degenerated
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42 spermatocytes of hybrid male testis. As mentioned above, mitochondrial respiration defects through
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45 mutant mtDNA with a deletion result in spermatogenic meiotic arrest and cause male sterility [5].
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48 Therefore hybrid male sterility can be considered to be a result of mtDNA deletion in the spermatocyte.
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56 Expression of maternal mtDNA inheritance system in hybrid male germ cells
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6 Normally, nuclear DNA (ncDNA) is destroyed in apoptosis while mtDNA is not [7, 8]. TUNEL assay of
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9 cryptorchid testis, which cause apoptosis [9], indicated a stain clearly corresponding to the nucleus (Fig.
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13 h,l,p). In addition it differed from necrosis in which both ncDNA and mtDNA are destroyed [8].
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16 Western immunoblotting assay of the BS-F1 ovary indicated expression of only Spag1-2, 1-3 and Eri15
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19 protein while Spag1-1 was not indicated, however, in the BS-F1 testis, Spag1-2 was also detected in
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22 addition to Spag1-1 and Eri15 protein (Fig. 3A). Eri15 has two monomers including 15 kDa, and its
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25 isoform or its post-translational modification of 18 kDa. Although a tetramer of 60 kDa and a trimer of 45
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28 kDa have been expressed respectively in ovary and testis [4], dimers of 30 (15×2) kDa and 36 (18×2)
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31 kDa were further detected common to both in this research. Among these, only 36 kDa Eri15 was
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35 detected in the BS-F1 testis mitochondria that were processed with trypsin while Eri15 was not detected
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38 in *Mus spretus* testis mitochondria (Fig. 3A), and thus Eri15 was thought to be transported into
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41 mitochondria in the hybrid testis.
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46 In order to study tissue localization type 2 hybrid testis was used as it displays recognizable stages of
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49 arrest cells. Part of metaphase spermatocytes was stained in the TUNEL assay (Fig. 3B(a)), and thus the
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52 metaphase was thought to be an initial stage of arrest in type 2 hybrid testis. In immunohistochemical
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55 staining too both Spag1 and Eri15 (both form a complex in the ovary cytosol [4]) were detected in the
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58 cytoplasm of some metaphase spermatocytes in a similar pattern (Fig. 3B(c)(d)).
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6 From the above it was suggested that maternal mtDNA inheritance system is expressed in hybrid male
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9 germ cells. Maternal mtDNA inheritance system is selective mtDNA elimination system [3, 4]. Therefore
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12 it was speculated that the expression of maternal mtDNA inheritance system in male germ cells is
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16 involved in mtDNA destruction in murine hybrid testis
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22 Conclusion

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29 From the above it was assumed that mtDNA destruction caused by the expression of maternal mtDNA
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32 inheritance system in male germ cells is implicated in the hybrid male sterility of mice.
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35 Sperm mtDNA elimination by the egg is a system with high species specificity [10]. It is considered
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38 that this system evolved in order to eliminate parasites imported along with the sperm [11] and sperm
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42 mtDNA damaged by reactive oxygen species (ROS) [12]. However, it would be more suitable for all
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45 purposes if the species specificity did not increase. Hence the system may have originally evolved in
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48 situations where species specific reactions such as speciation were necessary.
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52 Most mitochondrial genes get transferred to the nuclear genome except for the small portion of them
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55 that are necessary for the mitochondrial function [13]. However, the reason why all the genes do not get
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58 transferred is still unknown. The presence of a system of losing the mitochondrial function through
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6 mtDNA elimination to control the development of its cells may have been selective pressure to preserve
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9 the necessary genes for the mitochondrial function in mitochondria.
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Fig. 1 HE staining and TUNEL assay of testes. (a) HE staining of BS-F1 testes (type1). (b) Light microscopic image of TUNEL assay of BS-F1 testes (type1). (c-p) confocal fluorescence microscopic image. (c) negative control. TUNEL assay of C57BL/6J intact testes (merged image). (d) negative control. TUNEL assay without biotin-dUTP of BS-F1 testes (type1) (merged image). (e-h) TUNEL assay (green). (i-l) nucleus stained with TO-PRO3 (blue). (m-p) merged image. (insert in e-p) a higher magnification image of the boxed area. Red signals are mitochondria stained with MitoTracker Red CMXRos. (e, i, m) BS-F1 hybrids testes (type1). TUNEL positive fluorescence is observed at the cytoplasm of the proliferative leptotene spermatocytes. (f, j, n) PB-F1 testes (type1). (g, k, o) PS-F1 testes (type1). (h, l, p) C57BL/6J testes on 6 days after the cryptorchidism operation. TUNEL positive fluorescence is observed at the nucleus of the various stage of germ cells

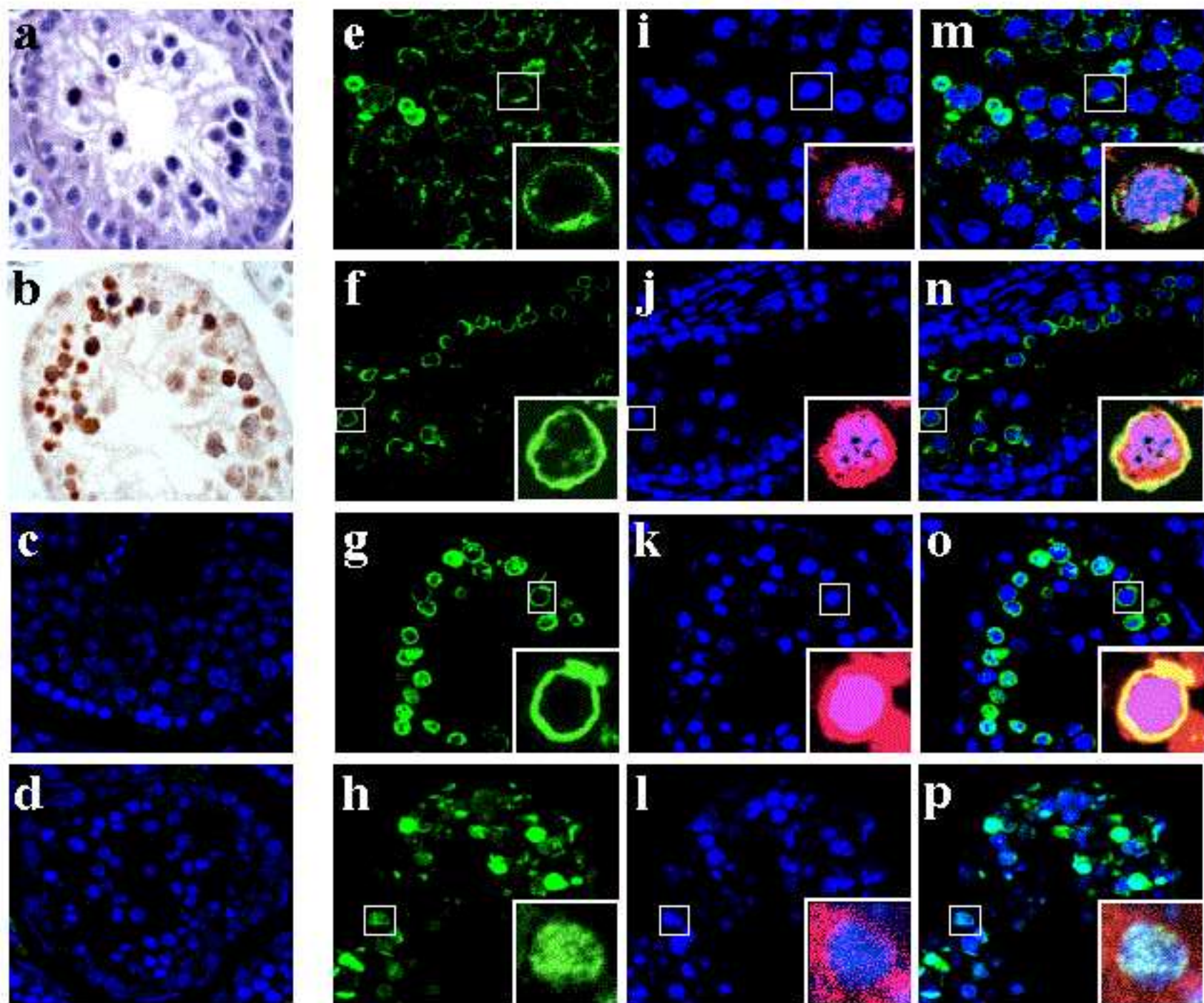
Fig. 2 DNA fragmentation assay. MM, positive control (blunt-ended DNA ladder marker); B6, C57BL/6J; BS, C57BL/6J ♀ × *Mus spretus* ♂ F1 hybrids; N, nuclear DNA; M, mitochondrial DNA

Fig. 3 (A) Western immunoblotting assay. B6, C57BL/6J; Ms, *Mus spretus*; BS, C57BL/6J ♀ × *Mus spretus* ♂ F1 hybrids; OM, ovary mitochondria; TM, testis mitochondria; OC, ovary cytosol; TC, testis

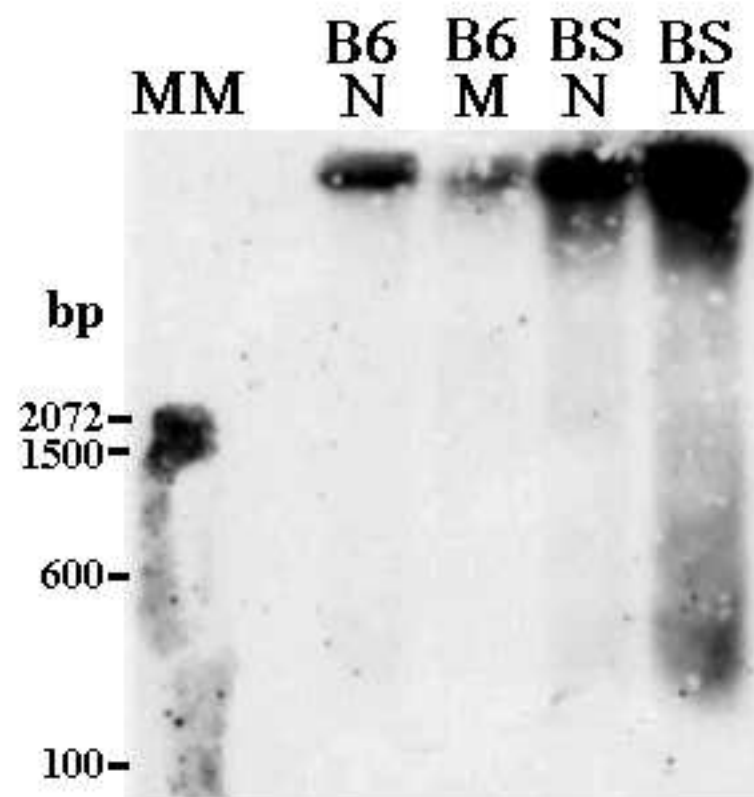
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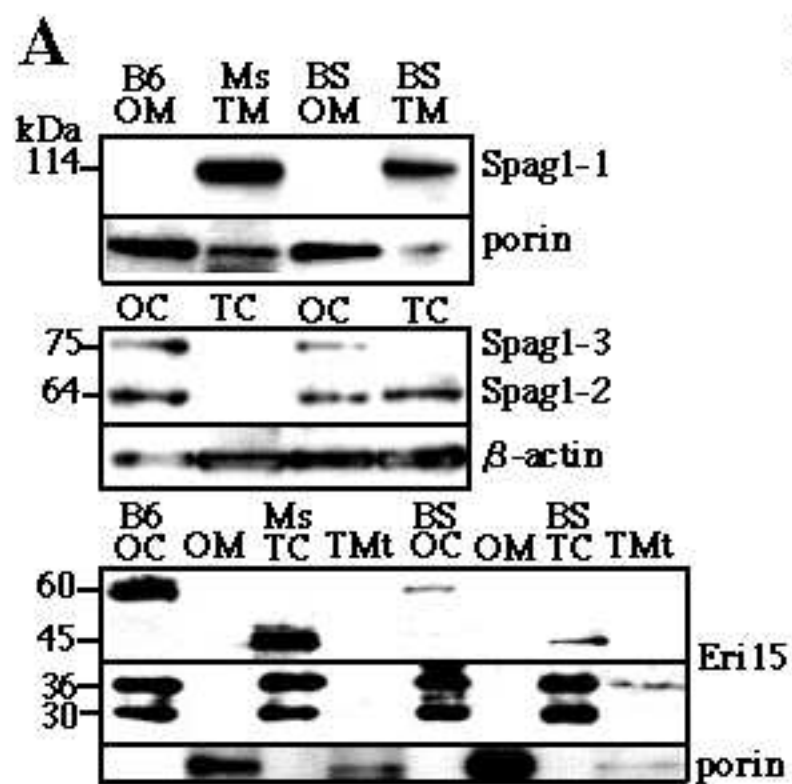
cytosol; TMT, testis mitochondria treated with trypsin. (B) Light microscopic image of TUNEL assay and immunohistochemical staining of BS-F1 testis (type 2). TUNEL assay (a). Part of metaphase spermatocytes was stained. immunohistochemical staining with normal rabbit serum (b). immunohistochemical staining with anti-Spag1-2 antibody (c). immunohistochemical staining with anti-Eri15 antibody (d). solid arrow, positive stained metaphase spermatocyte; open arrow, negative stained metaphase spermatocyte

Figure



Figure





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