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Germ-line mitochondria exhibit suppressed respiratory activity to support their accurate transmission to the next generation

Naomi Kogo, Akira Tazaki [1], Yasuhiro Kashino, Keisuke Morichika [2], Hidefumi Orii, Makoto Mochii, Kenji Watanabe *

Graduate School of Life Science, University of Hyogo, 3-2-1 Koto, Kamigori, Akou-gun, Hyogo 678-1297, Japan

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ABSTRACT

Mitochondria are accurately transmitted to the next generation through a female germ cell in most animals. Mitochondria produce most ATP, accompanied by the generation of reactive oxygen species (ROS). A specialized mechanism should be necessary for inherited mitochondria to escape from impairments of mtDNA by ROS. Inherited mitochondria are named germ-line mitochondria, in contrast with somatic ones. We hypothesized that germ-line mitochondria are distinct from somatic ones. The protein profiles of germ-line and somatic mitochondria were compared, using oocytes at two different stages in *Xenopus laevis*. Some subunits of ATP synthase were at a low level in germ-line mitochondria, which was confirmed immunologically. Ultrastructural histochemistry using 3,3′-diaminobenzidine (DAB) showed that cytochrome *c* oxidase (COX) activity of germ-line mitochondria was also at a low level. Mitochondria in one oocyte were segregated into germ-line mitochondria and somatic mitochondria, during growth from stage I to VI oocytes. Respiratory activity represented by ATP synthase expression and COX activity was shown to be low during most of the long gametogenetic period. We propose that germ-line mitochondria that exhibit suppressed respiration alleviate production of ROS and enable transmission of accurate mtDNA from generation to generation.

Introduction

The mitochondrial genome in animal cells has been conserved throughout evolution for more than 800 million years; all mtDNAs in metazoic cells have a similar length, from 13 to 19 kbp, containing 37 genes (Saccone et al., 2002). Mitochondria are accurately transmitted to the next generation. However, mitochondria are organelles responsible for most ATP production. They are best known for housing the oxidative phosphorylation (OXPHOS) machinery. Mitochondria generate the majority of cellular ROS, which may impair mtDNA. In somatic cells, the mitochondrial genome is vulnerable to rapid accumulation of deleterious mutations during an individual animal's lifetime (Wallace et al., 1995).

Several investigators proposed a bottleneck hypothesis to explain the strict limitation of deleterious mutations in the mitochondrial genome through generations (Bergstrom and Pritchard, 1998; Hauswirth and Laipis, 1982). However, the mechanism remains contentious. It is thought that the bottleneck occurs during embryonic development, as a result of a marked reduction in germ-line mtDNA copy number (Cree et al., 2008). Otherwise, it occurs without a reduction in germ-line mtDNA content (Cao et al., 2007; Wai et al., 2008), and the mtDNA genetic bottleneck results from replication of a subpopulation of mtDNA(Wai et al., 2008). The selection of mitochondria may be necessary for the transmission of intact mitochondria to the next generation. We have hypothesized that inherited mitochondria (germ-line mitochondria) are distinct from those of non-inherited ones (somatic mitochondria).This is the first report to compare directly germ-line mitochondria with somatic ones.

Germ plasm is the cytoplasm found in germ-line cells of some animals, including *Xenopus laevis*. Germ plasm contains the molecules and organelles that probably function in the differentiation and maintenance of germ-line cells (Kloc et al., 2001). A remarkably large number of mitochondria are found in germ plasm. They are parceled out to a small number of cells together with other components during development and are finally transmitted to the next generation.

The process of oocyte growth in *Xenopus* is divided in 6 stages (Dumont, 1972). In stage I oocytes, germ plasm is called mitochondrial cloud or Balbiani body, which contains a large number of mitochondria (Heasman et al., 1984). The space between mitochondria is filled with mitochondrial cement, which is probably precursor of germinal granules. That is, most mitochondria in mitochondrial cloud are germ-line. In stage VI oocytes, small patches of germ plasm

* Corresponding author. Fax: +81 791 58 0187.
E-mail addresses: tazaki@mpi-cbg.de (A. Tazaki), keisuke_morichika@rikkyo.ac.jp (K. Morichika), kirowato@sci.u-hyogo.ac.jp (K. Watanabe).
[1] Present address: Center for Regenerative Therapies Dresden, University of Technology Dresden, Tatzberg 47/49, 01307 Dresden, Germany.
[2] Present address: Graduate School of Life Science, Faculty of Science, Rikkyo University, 3-34-1 Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan.

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with mitochondria are localized at the cortex of the vegetal hemisphere. In the animal hemisphere, there are a large number of mitochondria, which are somatic mitochondria distributed to somatic cells (Tourte et al., 1984). That is, most mitochondria in stage VI are somatic.

We compared the protein profiles of the mitochondrial cloud fraction from stage I oocytes and mitochondria fraction from stage VI oocytes. We found that ATP synthase was at a lower level in germ-line mitochondrial than somatic ones. In the most of oogenesis, the expression of ATP synthase in germ-line mitochondria was at a low level. COX as well as ATP synthase is one of major components of the respiratory chain. The COX activity was also suppressed in most of oogenetic period. It is important for animals to suppress respiratory activity of germ-line mitochondria during the long oogenetic period. We propose that suppressed respiration of germ-line mitochondria avoids producing ROS and enables the accurate transmission of mtDNA from generation to generation.

Materials and methods

Oocytes

Ovary was surgically obtained from a mature female, and stage VI oocytes were defolliculated by 0.2% collagenase (Wako) treatment (Cohen and Pante, 2005), with modifications. Stage I oocytes were isolated from froglets (body length 4–6 cm) as for stage VI oocytes.

Purification of stage VI oocyte mitochondria

We modified the protocol of (Bogenhagen et al., 2003; Wu and Dawid, 1972). All steps in the purification of mitochondria were conducted at 0–5 °C. The following buffers were used: TE (10 mM Tris–HCl, pH7.5, 1 mM EDTA); 0.25 M TES, 1 M TES, and 1.5 M TES were TE with 0.25 M, 1 M, and 1.5 M sucrose, respectively; and KMTD (0.125 M KCl, 7 mM $MgCl_2$, 30 mM Tris–HCl, pH 8.0, 0.03 mM dithiothreitol). Oocytes were homogenized in 0.25 M TES. The homogenate was filtered through cheesecloth to remove most of stage I–III oocytes. Mitochondria fraction was prepared by some rounds of centrifugation and resuspended in 1 ml of KMTD. The resuspended mitochondria were layered over two preformed sucrose step gradients containing 1.2 ml of 1 M TES over 1.2 ml of 1.5 M TES in Ultracentrifuge tubes. Gradients were spun at 92,600×*g* (BECKMAN TL-100 Ultracentrifuge, rotor TLA 100.3) for 30 min to sediment mitochondria to the 1 M/1.5 M TES interface. The mitochondrial layer was gently removed, leaving the 1.5 M TES layer behind. Mitochondria were diluted with 0.25 M TS (0.25 M TES without EDTA) buffer and repelleted and resuspended in 0.25 M TS.

Differential absorption spectrum (reduced - oxidized)

Differential absorption spectrum of air-oxidized and sodium dithionite (final concentration, 10 mM) reduced mitochondria of stage VI oocytes (25 mg/ml) was obtained using a homemade spectrophotometer, which was used for mammalian mitochondria (Takahashi et al., 2005).

Purification of mitochondrial cloud of stage I oocytes

We modified the protocol of Chan et al. (1999). 0.1 ml of stage I oocytes isolated by collagenase treatment was transferred to an 1.5 ml microtube and was homogenized on ice with plastic bar. The homogenate was diluted with 0.25 M TES up to 0.8 ml, and it was placed on top of centrifuge tube containing a step gradient of 1 ml of 1.5 M TES, 0.6 ml of 0.4 M TES, and 0.6 ml of 0.25 M TES. Centrifugation was carried out for 45 min at 110,000×*g*. After fractionation, a layer of grayish “fluffy” material obtained near the middle layer was collected as mitochondrial cloud fraction. The mitochondrial cloud fraction was diluted with 0.25 M TS and pelleted and resuspended in 0.25 M TS buffer.

Protein analysis

Protein separation was carried out by using a 18–24% gradient acrylamide gel containing 6 M urea (Kashino et al., 2001). Mitochondria of stage VI oocytes (20 μg of protein) or mitochondrial cloud of stage I oocytes (40 μg of protein) were mixed with an equal amount of denaturing solution and subjected to electrophoresis without further boiling (Kashino, 2003). For the N-terminal sequencing, proteins were blotted onto the PVDF membrane (Amersham Pharmacia Biotech) after electrophoresis. The proteins on the membrane were stained by 0.1% Amido Black-10B (Nacalai Tesque, Kyoto, Japan) in a solution containing 10% methanol and 2% acetic acid and were then treated overnight with 0.6 N HCl at room temperature for the deblockage of the formylated N-terminus (Ikeuchi et al., 1989). The amino acid sequences of proteins were determined by a polypeptide sequencer (Shimadzu PSQ-1 protein sequencer; Simazu, Kyoto, Japan). Determined amino acid sequences were identified by BLAST search (Altschul et al., 1997).

Production of antibodies

Overall procedures followed (Orii et al., 2002). F_0-b (xL058e21), F_1-β (xL043e02), GOT (xL026b18), and MDH (xL091c04) cDNAs were characterized using the NIBB/NIG/NBRP *X. laevis* EST database (XDB3; http://xenopus.nibb.ac.jp/). Almost full length of each protein was used as antigen. For F_0-b, the cDNA corresponding to the sequence 50–250 a.a. was amplified by polymerase chain reaction (PCR) using forward primer 5′-atagcatgcgtccgttttggtttgatccct-3′, containing a SphI site and reverse primer 5′-cgcgtcgacttaaactcttgcttgtgcagt-3′ containing a SalI site. The PCR product was cloned into the pQE30 vector (Qiagen) using these sites. The construct was introduced into *Escherichia coli* strain XL1Blue (Stratagene). This region of F_0-b was expressed as a fusion protein with dihydrofolate reductase and histidine tag, and purified using a Ni-NTA resin column according to the manufacturer's protocol (Qiagen). The protein was dialyzed against water, lyophilized, and dissolved in phosphate–puffered saline (PBS). An appropriate volume of this solution was emulsified with Freund's adjuvant complete or incomplete (Sigma) and injected into Balb/c mice or Japanese white rabbits at intervals of 1 month. For F_1-β, GOT, MDH, the cDNA corresponding to the sequence 9–525 a.a., 28–427 a.a., 1–338 a.a. was amplified by PCR using forward primer 5′-atagcatgctctgctggggctctgcgggct-3′, 5-atagagctctcctggtggtctcatgttgag-3′, 5′-ataggatccatgttctctcgcatcgccaga-3′, and reverse primer 5′-gcgaagcttatgagtgctcttctgcgagtt-3′, 5′-cgcaagctttcatttggtcacctggtgaat-3′, 5′-cgcaagcttcacttccggctcttgatgaat-3′, respectively. All other procedures for preparation of the antisera against these proteins were same as those for F_0-b.

Western blotting

Mitochondria of stage VI oocytes (1 μg of protein) or mitochondrial cloud of stage I oocytes (2 μg of protein) were mixed with an equal amount of denaturing solution. Proteins were separated by Laemmli's SDS-PAGE and blotted onto PVDF membrane after electrophoresis. The membrane was probed with primary antisera (anti F_0-b 1:1500, F_1-β 1:3000, GOT 1:6000, MDH 1:1000). After washing, peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) was used as secondary antibody, and detection was enhanced by super-signal CL-HRP substrate (PIERCE). For stripping primary and secondary antibodies from blots, the membrane was incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.7, 2% SDS, 0.1 M 2-mercaptoethanol)