Non-invasive visualization of sperm mitochondria behavior in transgenic mice with introduced green fluorescent protein (GFP)

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Abstract Using high sensitive polymerase chain reaction (PCR), we previously demonstrated that selective elimination of sperm mitochondrial DNA occurred during early embryogenesis in mouse. To analyze the process morphologically in more detail, a non-invasive, real-time observation of sperm mitochondria was used. Transgenic mice that express green fluorescent protein (GFP) exclusively in mitochondria (mtGFP-tg mice) were generated. The fluorescence in mtGFP-tg mice was strong and stable enough to carry out repeated observations under confocal laser scanning microscopy. In these mtGFP-tg mice it was revealed that the sperm mitochondria were selectively eliminated from egg cytoplasm during the two-cell stage of early embryogenesis. Therefore, mtGFP-tg mice should contribute to studies on sequential or repeated analysis of mitochondria.

Key words: Mitochondrion; Mitochondrial DNA transmission; Green fluorescent protein; Transgenic mouse; Embryogenesis

1. Introduction

Mitochondrial DNA (mtDNA) shows strict maternal inheritance in mammals (for reviews, see [1,2]). In previous studies [3,4], using highly sensitive polymerase chain reaction (PCR) methods we showed that sperm mtDNA was eliminated from the cytoplasm of embryonic cells during the two-cell stage in early embryogenesis. This phenomenon could cause the maternal inheritance of mtDNA.

For direct, visual observation of the intracellular behavior of sperm mitochondria during early embryogenesis, the mitochondria were stained by Rhodamine 123 or MitoTracker [3,5–7]. Using these fluorescent dyes, the stained mitochondria were clearly and specifically stained in the middle piece of the sperm. However, there were several drawbacks that remained: (1) the dyes were easily bleached on irradiation with excitation light, (2) the procedures in the staining, such as centrifugation and resuspension, damaged the cells and mitochondria, and consequently the fertility of sperm was significantly reduced. Thus, these drawbacks make it difficult to do sequential or repeated observations.

Instead of vital staining dyes, green fluorescent protein (GFP) has been widely and successfully applied as a non-invasive chemiluminescent reporter molecule to visualize the localization and/or migration of proteins of interest to the intracellular compartmentation. GFP could also be used for real-time visualization of cellular organelles. Rizzuto et al. [8,9] reported that GFP was accumulated exclusively in the mitochondria of HeLa cells by transfection of a GFP cDNA clone tagged by the importing signal of the mitochondrial cytochrome *c* oxidase subunit VIII (mtGFP). The fluorescence was observed as a rod-like shape typical of mitochondria, evidencing the exclusive accumulation of GFP in the mitochondria. Moreover, little or no reduction of GFP fluorescence was observed on repeated irradiation, whereas the fluorescence of Rhodamine 123, a vital staining dye specific for mitochondria, was dramatically reduced under the same conditions. These results showed again that GFP tagged by an organelle-importing signal could have wide applications for real-time observation of the organelle.

We generated transgenic (Tg) mice, and visualized the mitochondria with GFP (mtGFP-tg mice). The fluorescence was ubiquitously and exclusively expressed in the mitochondria of the Tg mice. Moreover, the Tg mice enabled us to make non-invasive observation of the fate of sperm mitochondria during early embryogenesis.

2. Materials and methods

2.1. Construction of expression vector

The signal sequence of cytochrome *c* oxidase subunit VIII (COX8, GenBank U15541) was amplified by PCR with primers 5′-GCA GAA TTC TGC AGC GCC ACC ATG CCA AGG CTC CCC CC-3′ and 5′-GGC GGA TCC TAA GCT TGC ATA ATC AGG AAC ATC ATA ATG GGC TTT GGG AAC C-3′ using genomic DNA derived from C57BL/6J (B6) as a template. The coding region of EGFP was amplified by PCR with primers 5′-GAT GGA TCC ATC GCC ACC ATG GTG AGC AAG-3′ and 5′-CGG AAT TCT TAC TTG TAC AGC TCG TCC ATC CG-3′ using the recombinant plasmid pEGFP-N3 (Clontech, CA, USA) as a template. Both PCR products were digested with *Eco*RI and *Bam*HI, and ligase pCAGGS expression vector [10] digested with *Eco*RI.

2.2. Generation of transgenic mice

DNA fragments for transgenesis were removed by double digestion with *Sal*I and *Stu*I (Fig. 1a), separated by agarose gel electrophoresis from the cloning vector, and then purified by QIAEX II (Qiagen, CA, USA). The purified DNA fragments were injected into the pronuclei of the fertilized mouse (B6) oocytes according to the standard procedure [11]. To identify transgenic mice, genomic DNA was prepared from ear-punched pieces as described previously [4], and two sets of

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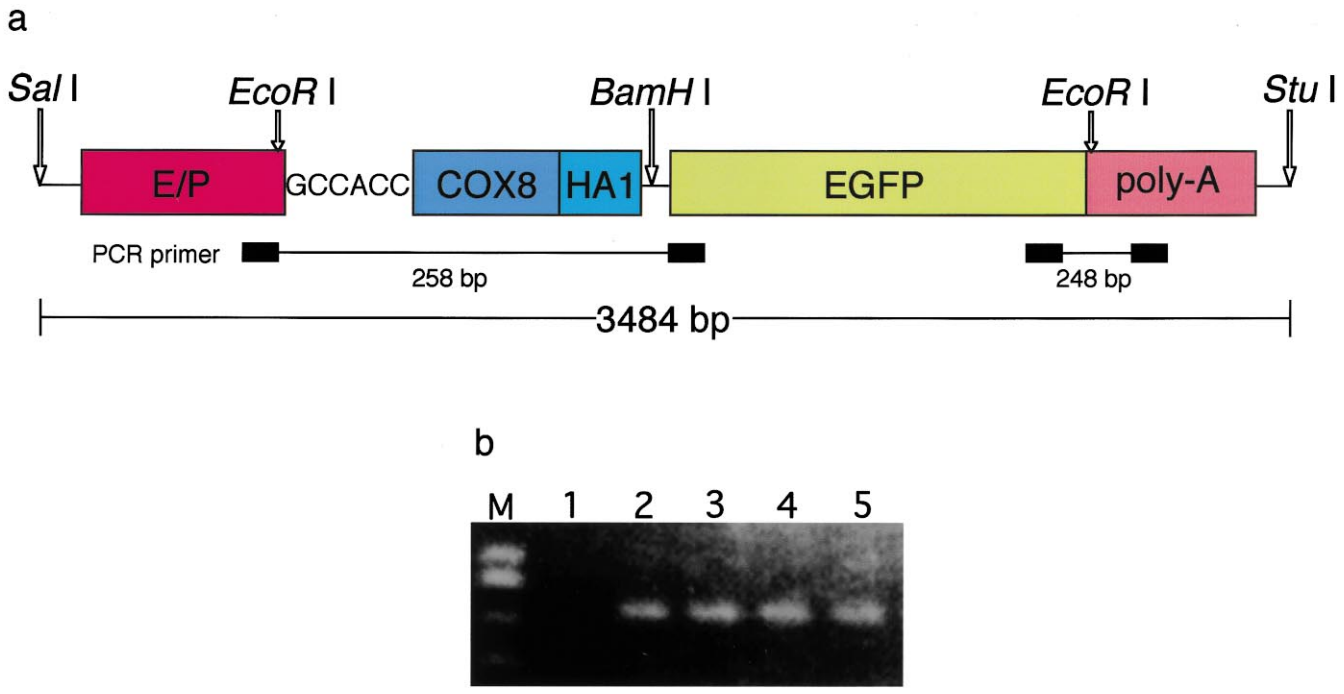
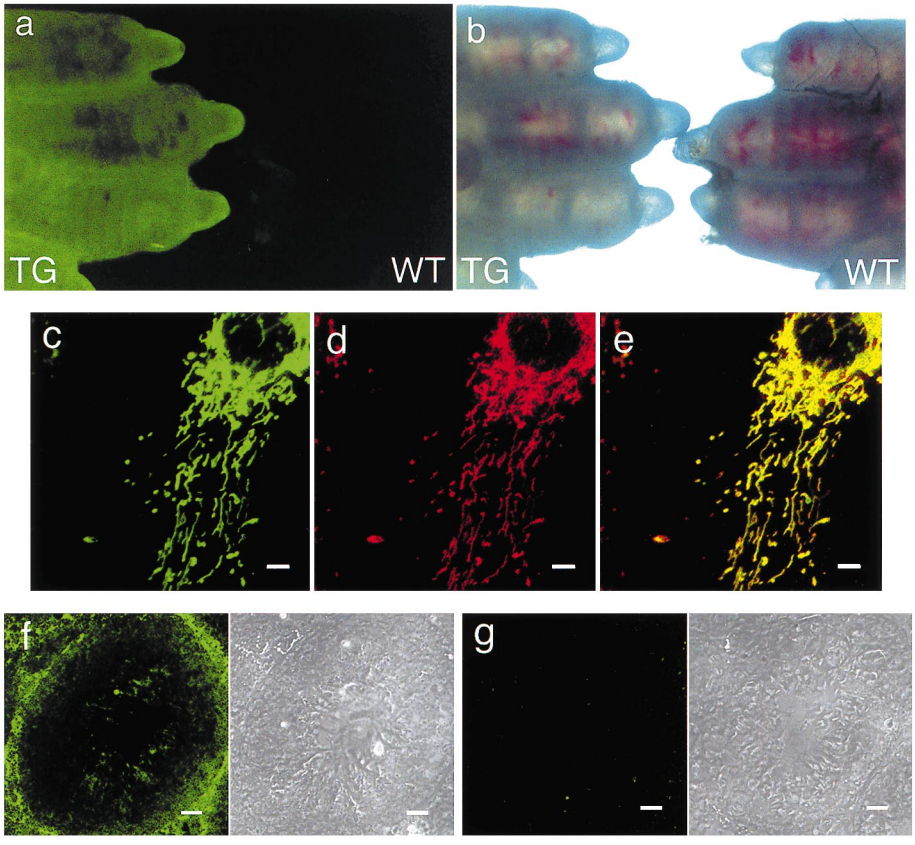


Fig. 1. Generation of transgenic mice. a: Schematic structure of the transgene (3484 bp). The Kozak sequence (GCCACC), transit peptide of cytochrome *c* oxidase subunit VIII (COX8) and HA1 epitope (HA1) nucleotide sequence were synthesized by PCR (see Section 2), and this fragment was ligated to the cDNA of EGFP. The synthesized fragment was introduced into a pCAGGS expression vector which possessed cytomegalovirus enhancer, chicken β-actin promoter (E/P) and rabbit β-globin poly-A (poly-A). For microinjection, the DNA fragment digested with *Sal*I and *Stu*I was isolated. b: Detection of transgene was done by PCR. The sequence specific for the transgene (258 bp) was amplified. Lane M, marker (fx174/*Hae*III digest); lane 1, B6 total DNA; lane 2, B6 total DNA and *Sal*I/*Stu*I fragment of transgene; lane 3, #20 total DNA; lane 4, #40 total DNA; lane 5, #49 total DNA.



primer pairs (A) 5′-GCT CTA GAG CCT CTG CTA ACC-3′, 5′-TGA ACA GCT CCT CGC CCT TGC TC-3′ and (B) 5′-TGA GCA AAG ACC CCA ACG AGA AGC-3′, 5′-TTA GCC AGA AGT CAG ATG CTC AAG-3′ were synthesized to amplify the PCR fragments (258 bp and 248 bp) specific for the transgene sequence (Fig. 1b).

2.3. Sample preparation

2.3.1. Fibroblast. A primary culture of fibroblasts was obtained according to the explant culture method [12]. The cultured fibroblasts were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 500 nM MitoTracker Red CMXRos (Molecular Probes, OR, USA; cat. M7512) for 15 min at 37°C. Then, the stained fibroblasts were rinsed twice with DMEM and used for observation by laser scanning confocal microscopy.

2.3.2. Sperm, eggs, embryos and testis. Sperm, eggs and embryos were collected as previously described [3]. Testis isolated from 12-week-old mtGFP-tg mice was frozen in liquid nitrogen and stored in a deep freezer (−80°C). 10 μm thick sections of frozen testes were sliced in a cryostat at −20°C and were directly mounted on slide glass.

2.4. Fluorescence imaging

Fluorescence of EGFP or of MitoTracker Red CMXRos was visualized under a Carl Zeiss laser scanning confocal microscope (LSM510). EGFP fluorescence was excited with a 488 nm argon-ion laser line and imaged through a 505–530 nm bandpass emission filter. MitoTracker Red CMXRos fluorescence was excited with a 543 nm HeNe laser and imaged through a 560 nm longpass emission filter.

3. Results

3.1. Generation of transgenic mice

To generate Tg mice expressing GFP exclusively in the mitochondria (mtGFP-tg mice), we applied the method of Rizzuto et al. [8,9]. The mtGFP recombinant DNA was introduced by microinjection of a DNA fragment double-digested with *Sal*I/*Stu*I (Fig. 1a) into pronucleus stage eggs derived from B6. After selection with two sets of PCR primers specific for the transgene sequences (see Section 2, Fig. 1b), we obtained three founder Tg mice (#20, #40, #49) and established the mtGFP-tg strains from the founders. We used the strain #49 for the following experiments, because its GFP expression was strong and stable throughout the generations of the Tg strain and the highest among the Tg strains. The selection of mtGFP-tg mice from their non-transgenic littermates also became much easier after the establishment of the #49 strain, because the GFP expression was clearly detected on the forelimb skin of the mtGFP-tg neonates under irradiation with excitation light (488 nm) (Fig. 2a).

3.2. GFP expression and labeling by mitochondria-specific dyes

We confirmed that the fluorescence was expressed exclusively in the mitochondria using cultured fibroblasts as mentioned above. Since the expression pattern of GFP fluorescence was typical for mitochondria, as previously reported [8,9,13] (Fig. 2c), the fibroblasts were stained with MitoTracker Red CMXRos, a vital staining dye specific for mitochondria (Fig. 2d), and then the image obtained by MitoTracker staining was merged with that of GFP in the same field. The two images completely overlapped (Fig. 2e), showing that GFP was expressed exclusively in the mitochondria.

Table 1
Detection of green fluorescence at embryo stages

Female strain	Positive/total no. of embryos	
	Pronucleus	Two-cell
C57BL/6J	29/29	4/29
JF1	25/25	15/25

JF1 inbred strain is derived from *Mus musculus molossinus*.

We also confirmed that the GFP expression was ubiquitous in tissues other than the skin, by observing GFP expressions in the testes (Fig. 2f), liver, thymus, muscle, heart and brain (data not shown). This is consistent with the report by Okabe et al., who have previously produced GFP-tg mice (green mice) [14,15]. The similar tissue distribution in both Tg mouse strains is reasonable, because they used the same expression vector and the only difference between their study and ours was the absence or presence of the mitochondria-importing signal in the DNA constructs. Therefore, we concluded that our mtGFP-tg mice are useful for observing the intracellular morphology and/or behavior of the mitochondria, because neither fixation nor staining processes are needed (Figs. 2 and 3).

3.3. Observation of sperm mitochondria in early embryos

We found that the testes of our mtGFP-tg mice showed linear fragment-like fluorescence in the zone where the late stage of spermatozoa is localized (center of the seminiferous tubule) (Fig. 2f). The shape was typical for sperm and much clearer in free sperm cells: the fluorescence of GFP was exclusively expressed in their middle piece, where the sperm mitochondria are located [3,6,7] (Fig. 3a). Then, we observed the fate of sperm mitochondria during early embryogenesis by *intra*specific cross. Female B6 mice after superovulation were mated to male mtGFP-tg mice. Under irradiation with excitation light, the middle piece of sperm from mtGFP-tg showed strong fluorescence, whereas unfertilized eggs of non-Tg (B6) mice did not (Fig. 3a). The fluorescence was detected from the step of sperm intrusion into the egg cytoplasm (Fig. 3b) to that of extrusion of the second polar body and the formation of the male and female pronuclei (Fig. 3c).

When *intra*specific crosses were generated, the GFP fluorescence could not be detected in most of the two-cell stage embryos (25/29). This clearly showed that selective elimination occurs at the two-cell stage (Fig. 3d, Table 1). However, in a few two-cell stage embryos (4/29) (Table 1), sperm mitochondria still remained in the cytoplasm, but their GFP intensities were much fainter than those observed in the pronucleus stage embryos (Fig. 3e, Table 1).

In contrast to *inter*subspecific crosses, when JF1 females, derived from Japanese fancy mice (*Mus musculus molossinus*), were mated to males of the transgenic mice [16,17], a high

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Fig. 2. GFP expression in the transgenic mouse. The foreleg of a transgenic mouse (TG, left) and a non-Tg littermate (WT, right) were observed by microscopy under irradiation with excitation light (a) and under a Nomarski differential interference system (b). Fibroblast fluorescent images of GFP (c), MitoTracker Red CMXRos (d), and overlay (e). Testis sections (seminiferous tubule) of the transgenic mouse (f) and wild-type (B6, g). The scale bars represent 5 μm in c, d and e and 20 μm in f and g.

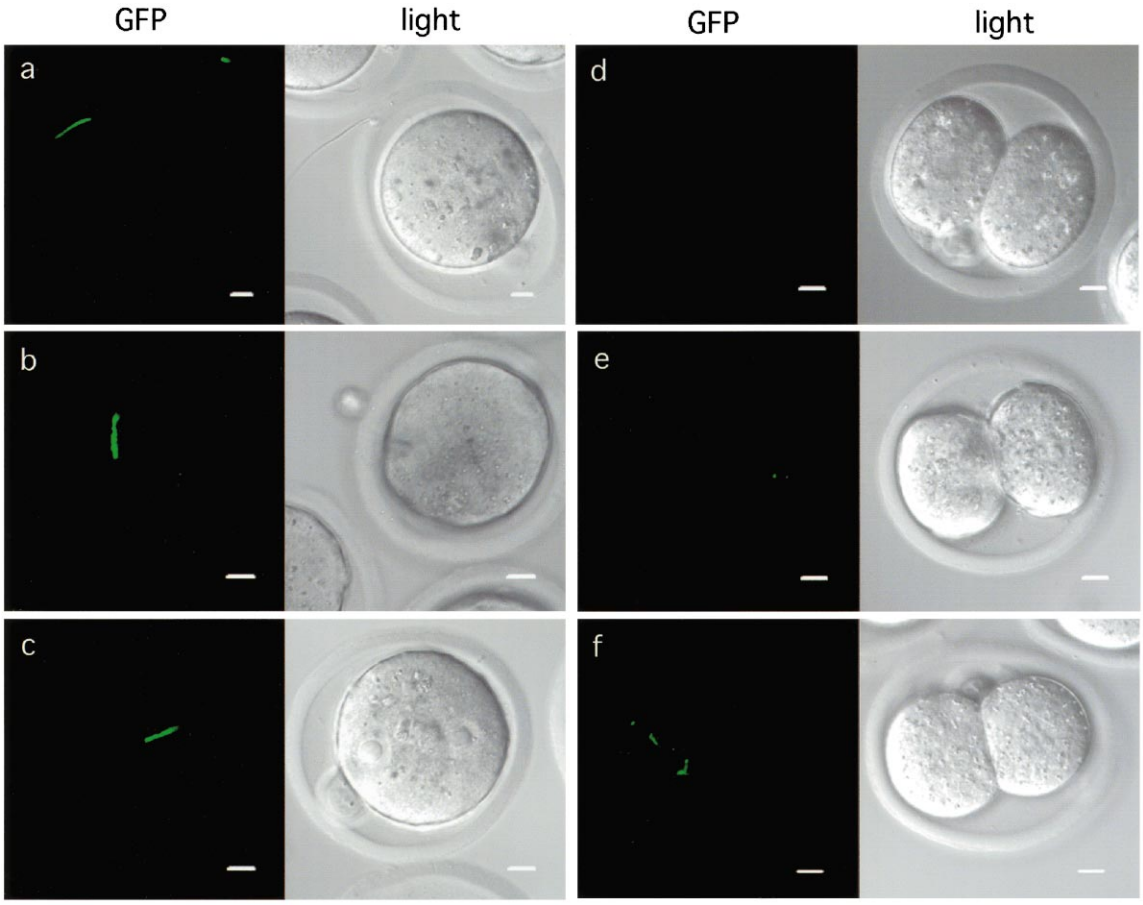

Fig. 3. Time course of early embryogenesis. ♀ B6×♂ #49 strain: a: Sperm bound on the zona pellucida of the unfertilized egg. b: Just after fertilization. c: Pronucleus stage. d: Two-cell stage; fluorescent negative. e: Two-cell stage; fluorescence could be observed. f: Two-cell stage. The scale bars represent 10 μm.

intensity of sperm mitochondria remained in half of the two-cell stage embryos (15/25) (Fig. 3, Table 1). These results are consistent with our previous results using the PCR methods [3,4].

4. Discussion

GFP has at least three advantages over vital staining dyes for observing the intracellular behavior of mitochondria: Firstly, staining by dyes is not needed, but the mitochondria can be directly observed immediately after tissue or cell preparation. This is beneficial for observing the fate or behavior of the sperm mitochondria after fertilization, because we only need to carry out in vitro fertilization (IVF) using sperm from the mtGFP-tg mice. We previously stained sperm mitochondria with fluorescent dyes to observe the fate of the sperm mitochondria during early embryogenesis [3]. However, the staining of mitochondria with dyes such as Rhodamine 123 or MitoTracker needs much more complex and skillful techniques as described previously [3]. These procedures may also damage the sperms and eggs, and consequently the number of gametes available for observation will be drastically decreased. Secondly, the intensity of GFP fluorescence shows little or no decrease on irradiation with excitation light, compared with those of the vital staining dyes [8]. Therefore, the sperm mitochondria could be used for real-time observation during early embryogenesis (Fig. 3). Thirdly, unlike vital staining dyes, fluorescence could not be dispersed from mitochondria by diffusion, since GFP has a much higher molecular weight than the dyes and its localization in the mitochondria was forced within import signal tag. This also elucidates the reason of why fluorescence-specific sperm could be detected in the presence of excess egg mitochondria.

GFP toxicity in cells [18] and mice [19] has been reported, but neither morphological abnormality nor delay in embryogenesis was observed in fertilized eggs or early stage embryos, showing that fertilization and successive processes occurred normally in the mtGFP-tg mice. This also suggested that GFP molecules in the mitochondria caused little or no delay in early embryogenesis.

We observed the elimination of sperm mitochondria in *intra*specific cross. In some cases, the delay of timing for the elimination of sperm mitochondria was inconsistent with our previous results by the PCR methods. Currently, we cannot determine whether these sperms were a result of a delayed elimination, because de novo expression of GFP began at the four-cell stage and all the mitochondria in their cytoplasm showed fluorescence (data not shown). The timing of paternally transmitted GFP gene expression was consistent with those green mice (GFP-tg mice) reported by Okabe and his colleagues [14]. In conclusion, because elimination of sperm mitochondria is finished by the two-cell stage, mtGFP-tg mice are useful for observing the fate of sperm mitochondria during early embryogenesis. However, further improvement of the

timing of the GFP transgene expression is needed to overcome the problem of how the expression of GFP transgene should be delayed.

Selective elimination of the sperm mitochondria causes maternal inheritance of mitochondrial DNA (mtDNA) in the mouse, which may be common in other animal species. Strictness of the inheritance depends upon that of the elimination; e.g. completeness and incompleteness were observed in *inter*specific and in *intra*specific hybrid mice, respectively. This elimination was controlled not by a cytoplasmic but nuclear encoded factor(s) [3,4,20]. Our remaining issues are (1) to know the exact time stage for the elimination of the sperm mitochondria in the egg cytoplasm, and (2) to clone the gene(s) responsible for the elimination to elucidate the mechanism of maternal inheritance. We expect that our mtGFP-tg mice will be useful to solve these issues, because the mtGFP-tg mice showed strong and stable fluorescent intensity sufficient to allow non-invasive observations on repeated irradiation with excitation light.

We also expect that the mtGFP-tg mice can be used to analyze mitochondria in several cells and tissues. For example, if we mate mtGFP-tg mice to the mitochondria deficient mice [21], we would be able to observe morphological changes in the mitochondria in the abnormal tissues of mitochondrial disease.

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