Real-time imaging of mitochondria in transgenic zebrafish expressing mitochondrially targeted GFP

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BioTechniques 45:331-334 (September 2008)
doi 10.2144/000112909

Mitochondria maintain a web-shaped network in cells through a balance between fusion and fission. Under certain physiological and pathological conditions, this balance is breached, and as a result, change in mitochondrial morphology ensues. Real-time monitoring of such change is of significant importance for studying mitochondrial physiology and pathology, such as apoptosis, aging, and neurodegeneration. Numerous studies have been conducted in animal cell culture systems concerning mitochondrial morphology change. However, very little is known to date about the real-time changes in mitochondrial morphology at the organism level due to difficulties in observation and administration of mitochondria-disrupting drugs. Here we report the generation of transgenic zebrafish (Danio rerio) expressing mitochondrially targeted green fluorescent protein (GFP). The transparency of transgenic zebrafish embryos make it possible to monitor mitochondrial morphology in real time and in vivo. Since zebrafish inhabit fresh water, incubating zebrafish in drug-dissolved water sufficed to administer drugs to the zebrafish. We observed real-time and in vivo fragmentation of mitochondria in the transgenic embryos upon incubation in water with the following apoptosis-inducing drugs: valinomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and staurosporine. Thus, the transgenic zebrafish we generated could provide a platform for research on apoptosis and mitochondrial physiology and a screen for apoptosis-modulating drugs. It could also facilitate study of the pathogenesis of apoptosis-related diseases.

Mitochondria are double membrane-bounded organelles that play an important role in energy production, apoptosis, and aging (1,2). In contrast to the classic textbook electron micrographs, which depict mitochondria as bean-shaped solitary organelles, mitochondria are interconnected and very dynamic organelles continuously undergoing fusion and fission, the balance between which is instrumental in maintaining a web-shaped mitochondrial network in cells (3,4). Apoptosis occurs during, among other things, embryonic development and disease, and tips the balance in favor of fission, turning reticular mitochondria into fragmented mitochondria (5,6).

During embryonic development, apoptosis refines embryo shape, thereby determining organ function. Thus, knowledge about when and where apoptosis occurs is key to understanding embryogenesis (7). This knowledge also helps to understand certain pathological conditions (8), an indicator of which is apoptosis.

In mammals, however, observation of mitochondrial morphology during embryonic development is not easy, in that development occurs in utero. Given the transparency and external development of zebrafish embryos, the zebrafish (Danio rerio) is an ideal organism to visualize mitochondria in real time and in vivo during development. In addition, apoptotic pathways and disease phenotypes are similar between mammals and zebrafish (9-11), implying that information gained from zebrafish mitochondrial research could translate into an understanding of mammalian mitochondrial physiology. Therefore, we set out to generate transgenic zebrafish expressing mitochondrially targeted green fluorescent protein (GFP).

To find a mitochondrial localization sequence (MLS) in the zebrafish, a tBlastn search was carried out with subunit VIII of human cytochrome c oxidase (COXVIII; GenBank accession no. CAG28615), a mitochondrial protein, against the zebrafish expressed sequence tags (EST) database. An EST clone (GenBank accession no. CO929675) was identified, and its nucleotides (35-127 bp) corresponding to the MLS of human COXVIII (12) were cloned between the 3′ end of an elongation factor-1α (EF-1α) promoter and the 5′ end of an enhanced GFP (EGFP) gene on a mini-Tol2 vector (13) (Figure 1A). The resulting plasmid was injected into one- to two-cell stage zebrafish embryos along with transposase messenger RNA (mRNA) as described previously (14). The injected embryos were raised to adulthood, and 10 founder fish were identified by outcross. Embryos from the founder fish appeared healthy (Figure 1B) and grew to adulthood without any significant morphological defects. To test if EGFP fused to the MLS of the zebrafish COXVIII (termed MLS-EGFP hereafter) resides in mitochondria, transgenic embryos expressing MLS-EGFP (termed MLS-EGFP embryos hereafter) were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), permeabilized in 0.3% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA), immunostained with anti-cytochrome c antibody (1:100 dilution; Invitrogen, Carlsbad, CA, USA), followed by anti-mouse Alexa Fluor 543 antibody (1:400; Invitrogen), and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were taken with a confocal laser-scanning microscope (Model no. LSM 510; Carl Zeiss, Thornwood, NY, USA) using a 100x/1.40 oil apochromat objective (Carl Zeiss). The excitation wavelengths were 365 nm for DAPI, 488 nm for EGFP, and 543 nm for Alexa Fluor 543. As expected, EGFP fluorescence co-localized with cytochrome c staining (Figure 1C). Of note, fragmented mitochondria were observed in some cells, which is not surprising, since approximately 6% of mammalian cells in culture were reported to harbor fragmented mitochondria even in the absence of apoptotic stimuli (15). In
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these cells, cytochrome c staining surrounded EGFP fluorescence (Figure 1D), consistent with mitochondrial matrix localization of a COXVIII MLS (12) and inner membrane localization of cytochrome c (16), thereby confirming mitochondrial localization of MLS-EGFP.

To determine whether MLS-EGFP zebrafish mitochondria change their morphology upon exposure to apoptosis-inducing reagents, MLS-EGFP embryos were treated with dimethyl sulfoxide (DMSO), which was used as a delivery vehicle for the following three chemicals: valinomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), or staurosporine (all from Sigma Aldrich). Subsequently, images were taken with a confocal laser-scanning microscope using a 63×1.20 water apochromat objective. The excitation wavelength was 488 nm for EGFP. Valinomycin, a potassium ionophore, induces rapid dissipation of mitochondrial membrane potential (ΔΨm), leading to apoptosis (17). Incubation of the transgenic embryos with 10 μM valinomycin for 2 h significantly fragmented mitochondria, as opposed to tubular mitochondria in DMSO-treated siblings (Figure 2). FCCP, which uncouples ATP synthesis and reduction of oxygen in oxidative phosphorylation, triggers abolition of ΔΨm, resulting in apoptosis (18). One-hour treatment of the transgenic embryos with 10 μM FCCP elicited mitochondrial fragmentation (Figure 2). Staurosporine, a potent inhibitor of protein kinase C, induces apoptosis (19). Upon a 6 h treatment with 3 μM staurosporine, fragmented mitochondria were noted in most of the cells in the transgenic embryos (Figure 2). To capture fragmentation of mitochondria in real time, the transgenic embryos were treated with either 10 μM DMSO or FCCP and then immediately imaged every 2 min for 1 h with a confocal laser-scanning microscope using a 63×1.20 water apochromat objective. The excitation wavelength was 488 nm for EGFP. Mitochondria in the FCCP-treated embryos started to fragment at 30 min, and almost all mitochondria fragmented at 1 h; however, mitochondria in the DMSO-treated embryos remained tubular throughout 1 h (see the Supplementary Movie S1 and S2 available online at www.BioTechniques.com).

At the time of each imaging described above, the number of heartbeats and velocity of blood circulation in drug-treated MLS-EGFP embryos were comparable to those in DMSO-treated embryos, demonstrating that it is less likely that the fragmented mitochondria observed were secondary to massive necrosis elicited by the administered drug. Taken together, these findings indicate that zebrafish mitochondria expressing MLS-EGFP change their morphology upon treatment with apoptosis-inducing reagents, as do mitochondria in cultured mammalian cells, and that morphological changes in zebrafish mitochondria can be imaged in real time and in vivo.

Here, we report transgenic zebrafish expressing EGFP in mitochondria. We show that apoptosis-inducing reagents fragment EGFP-expressing zebrafish mitochondria and that this fragmentation can be visualized in real time and in vivo by confocal microscopy. We believe MLS-EGFP zebrafish may have several implications for mitochondria-related research. First, the transparency and external development of zebrafish embryos allow for MLS-EGFP zebrafish to be used in imaging apoptosis in real time and in vivo, facilitating the study on the role for apoptosis in development. Second,
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the development of the embryos in water could accelerate a screen for drugs that induce or prevent apoptosis, as most drugs subject to testing can be directly added to water. Third, crossing MLS-EGFP zebrafish to disease model zebrafish and subsequent observation of mitochondria as the disease develops would be of help to explore in vivo how change in mitochondrial morphology, and thus apoptosis, affects the pathogenesis of the model disease.

ACKNOWLEDGMENTS

We thank Koichi Kawakami for the mini-Tol2 plasmid, Ajay Chitnis for support, Eun Young Choi, Hyunju Ro, Sang-Yeob Yeo, and Richard Youle for encouragement, Chongmin Wang for zebrafish maintenance, and Michael Frohman, Kyung Keun Kim, and Eunhee Kim for critical reading of the manuscript. This work was supported by the

Figure 2. Apoptosis-inducing reagents fragment in vivo zebrafish mitochondria expressing mitochondrially targeted enhanced green fluorescent protein (EGFP). Thirty-two-hour postfertilization (hpf) embryos expressing MLS-EGFP were incubated in egg water with dimethyl sulfoxide (DMSO) for 6 h, valinomycin for 2 h, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) for 1 h, or staurosporine for 6 h, then embedded in 0.8% low melting agarose, and examined by confocal laser-scanning microscopy. Images were taken from the 13th somite of each embryo indicated. Lateral views anterior to the left are shown. The boxed areas are magnified in the lower panels. Each image is representative of three independent experiments. Scale bar equals 20 μm. Note that the magnified image of a staurosporine-treated embryo was taken 10 min later than was the unmagnified one, which rendered the two images slightly different due to the dynamic nature of mitochondria. MLS-EGFP, EGFP fused to the mitochondrial localization sequence (MLS) of the zebrafish subunit VIII of human cytochrome c oxidase (COXVIII).
Intramural Research Program of the National Institute of Child Health and Human Development at the National Institutes of Health and the Korea Science & Engineering Foundation through the Medical Research Center for Gene Regulation (grant no. R13-2002-013-04001-0) at Chonnam National University. This paper is subject to the NIH Public Access Policy.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES


Received 6 March 2008; accepted 6 June 2008.

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