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Autophagosomes in GFP-LC3 Transgenic Mice

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Summary

Recent studies of the molecular mechanism of autophagy have made available several marker proteins for autophagosomes. These marker proteins allow us to identify autophagic structures easily and accurately by fluorescent microscopy. The most widely used marker for autophagosome is LC3, a mammalian homolog of Atg8. To analyze autophagy in whole animals, we generated GFP-LC3 transgenic mice and describe here how we determine the occurrence of autophagy in vivo using this mouse model.

Key Words: Autophagosome; GFP; green fluorescent protein; LC3; Atg8.

1. Introduction

Although the autophagic vacuole was identified almost 50 years ago, specific markers for autophagosomes were not discovered until recently. In the 1990s, the autophagic pathway was dissected at the molecular level in the yeast *Saccharomyces cerevisiae*. To date, at least 16 genes have been found to be required for autophagosome formation. The nomenclature of these genes was unified under the term *ATG* genes, which also includes other autophagy-related genes ***(1)***. Seven of these *ATG* gene products function in two ubiquitination-like conjugation systems ***(2)***: one system mediating the conjugation of Atg12 to Atg5 ***(3)*** and the other mediating covalent linkage between Atg8 and phosphatidylethanolamine (PE) ***(4)***. These two systems are conserved in mammals. While the Atg12–Atg5 conjugate is present only on the autophagic isolation membrane or phagophore before complete enclosing, LC3 (a mammalian homolog of Atg8)–PE conjugate is present on both the phagophore and autophagosomes (but less on autolysosomes) ***(5–7)***. The localization of LC3 is usually determined with green fluorescent protein

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(GFP)-conjugated LC3 (GFP-LC3) ***(5,8)***. Autophagosomes are easily detected as fluorescent dots or sometimes ring-shaped structures if they are larger than 1 μm. In *Atg5*$^{-/-}$ cells, punctuate LC3 structures are not detected ***(6,9)***. Examination of GFP-LC3 localization is a very simple and highly specific method, requiring only a high-resolution fluorescence microscope. In addition, real-time observation in living cells is feasible. Thus, this method has been applied to animal studies by generating GFP-LC3 transgenic mice in which GFP-LC3 is overexpressed in almost all tissues under the control of the constitutive CAG promoter ***(10)***. Use of this transgenic mouse model enables the occurrence of autophagy in mouse tissues to be directly monitored by fluorescence microscopic analysis of cryosections.

2. Materials

2.1. Mice

1. GFP-LC3#53 mice: This mouse line is now distributed through the RIKEN Bio-Resource Center in Japan (http://www.brc.riken.jp/lab/animal/en/dist.shtml).
2. Wild-type C57BL/6 mice for colony maintenance.

2.2. Genotyping

1. Tail digestion solution: 10 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.45% NP-40, 0.45% Tween-20. Autoclave and store at room temperature. Add 1/100 vol of 20 mg/mL Proteinase K (final 0.2 mg/mL) prior to use.
2. PCR primers. Primer 1: 5'-ATAACTTGCTGGCCTTTCCACT-3';
   Primer 2: 5'-CGGGCCATTTACCGTAAGTTAT-3';
   Primer 3: 5'-GCAGCTC ATTGCTGTTCCTCAA -3'.
   Primer 1 and Primer 2 for amplification of the GFP-LC3 transgenic allele (about 250 bp).
   Primer 1 and Primer 3 for amplification of the wild-type allele (about 350 bp) ***(11)*** **(Fig. 1)**.
3. (optional) GFP macroscopy: Model GFsP-5 from Biological Laboratory Equipment, Maintenance and Service Ltd (http://www.bls-ltd.com/).

2.3. Sample Preparation

1. Perista pump (ATTO AC-2110).
2. Tissue-tek: OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan).
3. Cryostat: LEICA CM3050S.
4. SlowFade Light Antifade Kit (Molecular Probes).
5. Microscope: Fluorescence microscope (Olympus IX81, Tokyo, Japan) equipped with a 60X oil-immersion objective lens (Plan Apo, 1.40 NA) and a cooled CCD camera (Hamamatsu Photonics, ORCA-ER (1360x1024)) (*see* **Note 4**).
6. Software: MetaMorph Series Version 6 (Molecular Device, Sunnyvale, CA).

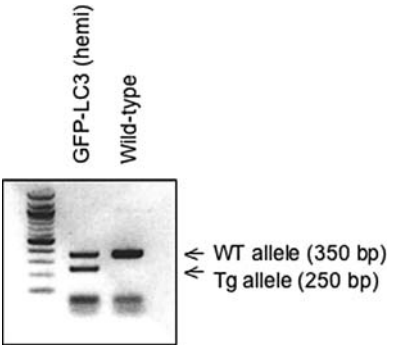

Fig. 1. PCR-based genotyping of GFP-LC3 mice. Genomic DNA was extracted from wild-type and hemizygous GFP-LC3 transgenic mouse tails and analyzed by PCR. Bands representing wild-type and GFP-LC3 allele are indicated.

3. Methods

3.1. Mouse Maintenance

1. It is recommended that GFP-LC3 mice be maintained as heterozygous, because it is very important to compare the transgenic mice with wild-type siblings to distinguish the GFP-LC3 signal from background autofluorescent signals.
2. If littermate control is not necessary, GFP-LC3 mice can be maintained as homozygous. No apparent abnormal phenotype has been observed in the homozygous mice.

3.2. Genotyping by Polymerase Chain Reaction (PCR)

1. Cut 0.2–0.5 cm of tail and place in a 1.5-mL tube.
2. Add 100–400 μL of tail digestion solution (with Proteinase K).
3. Incubate at 55°C for 8 h (or overnight). Mix occasionally.
4. Centrifuge briefly, then boil for 5 min.
6. Centrifuge at 13,000 g for 10 min.
7. Take 1 μL supernatant for PCR reaction. Mix 1 μL tail sample, 0.2 μL primer 1, 0.2 μL primer 2, 0.2 μL primer 3, 2 μL PCR buffer, 1.6 μL dNTP mix (2.5 m*M* each), 0.2 μL rTaq (Takara), 14.6 μL DW.
8. PCR reaction: Step 1: 94°C 4 min; step 2: 94°C 0.5 min; step 3: 60°C 0.5 min; step 4: 72°C 1 min; 30 cycles to step 2; step 5: 72°C 7 min; step 6: 4°C.

3.3. Genotyping by GFP Macroscopy (Optional)

1. GFP-LC3 transgenic neonates can be easily distinguished from wild-type neonates even in mouse cages using GFP macroscopy.
2. Adult mice can be genotyped by checking the GFP fluorescent signal of mouse palms.
3. A portable UV illuminator does not work for our GFP-LC3 mice.

3.4. Sample Preparation

1. Perfuse mice transcardially with about three times the volume (body weight) of 4% paraformaldehyde dissolved in 0.1 *M* Na-phosphate buffer (pH 7.4). Dipping tissues in PFA may be sufficient but quick fixation is important to avoid artificial induction of autophagy during sample preparation.
2. After perfusion, remove tissues and further fix them in the same fixative for an additional 4 h or overnight (depending on antibodies for double staining).
3. Immerse the fixed tissues in 15% sucrose/PBS for at least 4 h, then in 30% sucrose/PBS for at least an additional 4 h (or overnight).
4. Embed the tissue samples using OCT compound (Tissue-Tek) and store at –70°C.
5. Section the tissues at 5–7 μm thickness with a cryostat. Air-dry the sections at room temperature for 30 min. The sections can be stored at –70°C (or –20°C) until use.
6. Wash the well-dried cryosections in PBS and mount on glass slides using SlowFade Light Antifade Kit.

3.5. Fluorescence Microscopy

1. Select a 60X oil-immersion objective lens and put a small drop of immersion oil on the objective lens.
2. Place a glass slide and focus on cells by transmitted light imaging (usually differential interference contrast (DIC)).
3. Select an appropriate dichroic filter set (FITC or GFP).
4. Observe the sample by eye. This step should be as short as possible if the fluorescent signal is weak.
5. Capture images **(Fig. 2)**. It is recommended also to take images using unrelated filter sets such as RFP or Cy5 as controls (*see* **Note 2**).

3.6. Quantitative Analysis of GFP-LC3 Dots

The number or total area of GFP-LC3 structures can be quantified using software. However, uneven cytosolic background signals make conventional thresholding difficult or weak dot signals nonextractable. To better extract the dot signals, the "Top Hat" algorithm of the the MetaMorph Series Version 6 (Molecular Device) is useful. Small dot peaks can be extracted from the surrounding relatively lower background signals irrespective of absolute signal intensity (*see* **Notes 1** and **3**).

4. Notes

1. Although this transgenic mouse model is very useful for in vivo studies, there are a few possible limitations. First, GFP-LC3 localization represents only autophagosome formation. Since autolysosomes have less membrane-bound LC3

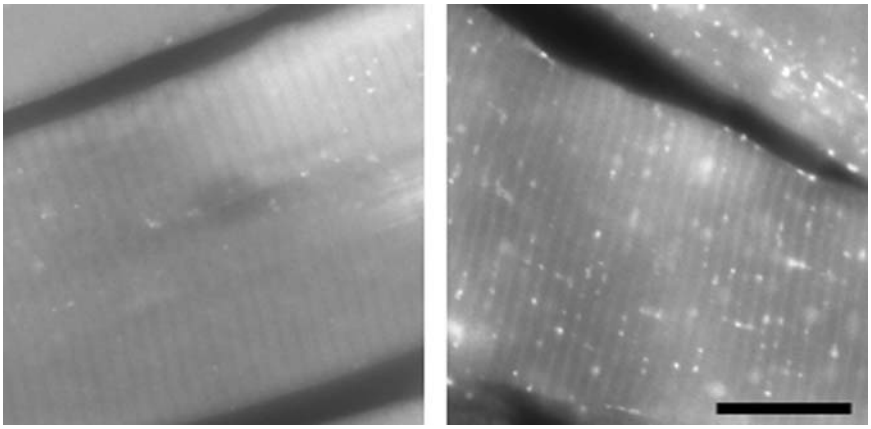

Fig. 2. Example of GFP-LC3 transgenic mouse analysis. Gastrocnemius muscle samples were prepared from GFP-LC3 transgenic mice before **(left)** or after 24-h starvation **(right)** and fixed with 4% paraformaldehyde. Cryosections were analyzed by fluorescence microscopy. GFP-LC3 dots represent autophagosomes. (Bar 10 μm.)

than autophagosomes, the appearance of GFP-LC3 dots does not definitely constitute autophagic "degradation." Too fast fusion of autophagosomes with lysosomes may result in a lower number of GFP-LC3 dots, which would underestimate autophagic activity.

2. It is also very important to distinguish true GFP-LC3 dot signals from autofluorescent signals. Certain cells such as neurons show autofluorescent dot structures like lipofuscin. Such artifacts may be avoided by the following two methods. First, it is particularly important to compare samples expressing GFP-LC3 with nontransgenic control samples. Second, specific GFP-LC3 signals should not be detected using other fluorescence filter sets such as rhodamine, Cy5, or UV. True GFP-LC3 signals should be detected specifically by the GFP or FITC filter set. We usually use the U-MGFPHQ unit for GFP observation and the U-MWIG2 unit to check autofluorescence.
3. GFP-LC3 can be incorporated into protein aggregates independent of autophagy. This phenomenon is particularly apparent in hepatocytes and neurons of autophagy-deficient mice such as *Atg5*$^{-/-}$ ***(12)*** and *Atg7*$^{-/-}$ mice ***(13,14)***, or cells having unrelated inclusion bodies such as those induced by polyglutamine expression ***(15)***. Additionally, transient transfection of GFP-LC3 often causes aggregation. In these conditions, GFP-LC3 dots should be carefully interpreted.
4. We usually use wide field microscopy rather than confocal laser scanning microscopy. Since the number of autophagosomes is not so large, the highest Z-axial resolution is generally not required to eliminate "out-of-focus" fluorescence.

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