utophagy is an intracellular bulk degradation process in which various cytoplasmic components, such as unfolded proteins and damaged organelles, are transported in double-membrane vesicles called autophagosomes through the cytoplasm to lysosomes. The autophagosome acquires hydrolytic enzymes by fusing with the lysosome to generate an autolysosome, whose contents are then degraded and recycled. Constitutive autophagy in the heart under baseline conditions is a homeostatic mechanism for maintaining cardiomyocyte size and global cardiac structure and function. Autophagy is induced by various stresses including starvation, ischemia/reperfusion, and pressure-overload. Autophagy is detected in cardiomyocytes from patients with aortic stenosis and dilated cardiomyopathy. It has been reported that upregulation of autophagy in pressure overload-induced heart failure is an adaptive response for protecting cells from hemodynamic stress. However, cardiac autophagy has been believed to be a maladaptive response that contributes to heart failure progression. Although the possible involvement of autophagy in heart disease has been documented, experimental results are controversial.

Background: The involvement of autophagy in heart disease has been reported. Transgenic mice expressing GFP-LC3 have been a useful tool in detecting autophagosomes systemically. It is difficult to differentiate increased formation of autophagosomes from decreased clearance of autophagosomes in the heart using GFP-LC3 mice.

Methods and Results: We generated transgenic mice expressing mCherry-LC3 under αMyHC promoter and crossed the mice with transgenic mice expressing GFP-LC3. The deference of resistance to acidic conditions between GFP and mCherry overcame the limitation.

Conclusions: This method is an innovative approach to examine the role of autophagy and to analyze autophagosome maturation in cardiomyocytes. (Circ J 2010; 74: 203–206)

Key Words: Autophagy; Cardiomyocytes; Transgenic mouse

Double Transgenic Mice Crossed GFP-LC3 Transgenic Mice With αMyHC-mCherry-LC3 Transgenic Mice Are a New and Useful Tool to Examine the Role of Autophagy in the Heart

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Autophagy is an intracellular bulk degradation process in which various cytoplasmic components, such as unfolded proteins and damaged organelles, are transported in double-membrane vesicles called autophagosomes through the cytoplasm to lysosomes. The autophagosome acquires hydrolytic enzymes by fusing with the lysosome to generate an autolysosome, whose contents are then degraded and recycled. Constitutive autophagy in the heart under baseline conditions is a homeostatic mechanism for maintaining cardiomyocyte size and global cardiac structure and function. Autophagy is induced by various stresses including starvation, ischemia/reperfusion, and pressure-overload. Autophagy is detected in cardiomyocytes from patients with aortic stenosis and dilated cardiomyopathy. It has been reported that upregulation of autophagy in pressure overload-induced heart failure is an adaptive response for protecting cells from hemodynamic stress. However, cardiac autophagy has been believed to be a maladaptive response that contributes to heart failure progression. Although the possible involvement of autophagy in heart disease has been documented, experimental results are controversial.

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All rights are reserved to the Japanese Circulation Society. For permissions, please e-mail: cj@j-circ.or.jp
The development of useful tools and methods to evaluate autophagy in the heart is needed.

Methods

mCherry-LC3 (a gift from R.A. Gottlieb and R.Y. Tsien) was subcloned into the αMyHC promoter expression vector (a gift from J. Robbins and M.D. Schneider). The 7.7 kb BamHI and BamHI fragment was isolated and microinjected into C57BL/6 fertilized eggs at Tsukuba University (S. Takahashi, F. Sugiyama). Genotyping of αMyHC-mCherry-LC3-mice was carried out by PCR using primers F (5’-ATGTTGAGCATGCGGCAGAAGATAAC-3’) and R (5’-CTTGTAC-AGCTCCTCATGCGCCGGA-3’). GFP-LC3#53 mouse strain (N. Mizushima, Tokyo Medical and Dental University) was provided by RIKEN BRC (RBRC00806). The protocols for animal experimentation described in this paper were approved by the Animal Research Committee, Akita University.

Results

To overcome this limitation, we have generated transgenic mice expressing mCherry fused to LC3 under the control of α myosin heavy chain promoter (αMyHC-mCherry-LC3-mice) (Figure 1A). Because mRFP does not lose fluorescence under acidic conditions, we used mCherry, improved-mRFP, to detect both autophagosomes and autolysosomes. We confirmed cardiac specific expression of the transgene by Western blot. Antibody against mCherry detected 2 bands and they are suspected to be an appropriate size for mCherry-LC3-I and mCherry-LC3-II.

Figure 1. Transgenic mice expressing mCherry fused to LC3 under the control of α myosin heavy chain promoter (α MyHC-mCherry-LC3-mice) to detect autophagy only in cardiomyocytes. (A) Schematic representation of the transgene containing α MyHC promoter, mCherry-LC3 cDNA, and human growth hormone polyadenylation signal. (B) Western blot analysis was performed using antibodies against mCherry (Clontech, Cat No. 632496), LC3 (MBL, code M115-3), and GAPDH (Santa Cruz, sc-25778). Samples were prepared from non-starved or 48 h-starved mice. Expression of mCherry-LC3 was confirmed in the heart of transgenic mice (Left panel). Expression level of mCherry-LC3 was compared to endogenous LC3 in αMyHC-mCherry-LC3-mice and GFP-LC3 in CAG-GFP-LC3-mice (Right panel). WT, wild type mice; #21, αMyHC-mCherry-LC3-mice line number 21; B, brain; H, heart; L, liver; K, kidney; Sp, spleen; Sk, skeletal muscle; T, testis. (C) Autophagy in response to starvation in the heart of αMyHC-mCherry-LC3-mice. Heart samples before and after starvation were fixed with 2% paraformaldehyde overnight and fixed with 15% sucrose in PBS for 4 h and then with 30% sucrose in PBS overnight. The samples were frozen and sectioned at 7μm thickness with cryostat. We stained cardiomyocytes with antibody against α-actinin (clone EA53; Sigma) and Zenon Alexa Fluor 647 Mouse IgG1 (Cat. No. Z-25008, Molecular Probes). Cryosections were analyzed by confocal laser scanning microscopy (LSM510 META, Zeiss). mCherry positive dots were detected only in cardiomyocytes (arrows). Scale bars: 10μm. (D) Heart samples were fixed with 3% glutaraldehyde. Double-membrane autophagic vacuoles were observed in the heart of wild type mice and αMyHC-mCherry-LC3-mice using electron microscopy after starvation. Arrows indicate autophagic vacuoles. N, nucleus; Mt, mitochondria. Scale bars: 500 nm.
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increased after starvation (Figure 1B). At the right panel, it is possible that mCherry-LC3-II and mCherry-LC3-I might not be separated in this gel, and both are contained in the band indicated as “mCherry-LC3”. The lower band A might be an unknown degradation product, which is also detected in CAG-GFP-LC3-mice. The expression level of mCherry-LC3 was higher than that of endogenous LC3, however, it was almost the same as that of GFP-LC3 in CAG-GFP-LC3-mice. It has been reported that GFP-LC3 expression level in CAG-GFP-LC3-mice is greater than endogenous LC3, however, it does not affect the endogenous autophagic process. These results suggest that the expression level of mCherry-LC3 would be appropriate. We confirmed induction of autophagy after starvation in cardiomyocytes of αMyHC-mCherry-LC3-mice. A few red mCherry-LC3 signals indicated autophagosomes or autolysosomes in the basal state. The number of red signals increased after starvation (Figure 1C). We obtained fourteen founder mice. Some of them occasionally developed large fluorescent aggregates that were morphologically inconsistent with autophagosomes. Thus, we chose line number 21 for the experiments in this paper. We have compared αMyHC-mCherry-LC3-mice to wild-type mice in order to rule out unexpected adverse effects of mCherry-LC3. Almost identical patterning of autophagy occurred after starvation in both αMyHC-mCherry-LC3-mice and wild-type mice using electron microscopy (Figure 1D). Cardiac function evaluated by echocardiography was not affected.

Taking advantage of the deference of resistance to acidic conditions between GFP-LC3 and mRFP-LC3, autophago-
some maturation process can be evaluated using the expression of both GFP-LC3 and mRFP-LC3.12 Thus, we have crossed αMyHC-mCherry-LC3-mice with CAG-GFP-LC3-mice (GFP/mCherry-LC3-mice) to detect activity of autophagy in the heart. Both GFP-LC3 and mCherry-LC3 can be detected in autophagosomes of cardiomyocytes. Only mCherry-LC3 can be detected in autolysosomes because mCherry does not lose fluorescence under acidic conditions. In other words, double positive signals indicate autophagosomes and only red signals indicate autolysosomes in cardiomyocytes. Only green signals indicate autophagosomes in non-cardiomyocytes. We confirmed induction of autophagy in response to starvation in the heart of GFP/mCherry-LC3-mice. Few autolysosomes (red signals) and autophagosomes (yellow, double positive signals) were detected before starvation, however, the number of red and yellow signals increased after starvation (Figure 2A). We can detect both autophagosomes and autolysosomes and distinguish between them simultaneously using GFP/mCherry-LC3-mice. We did not detect any green signals before and after starvation. We speculate that autophagic activity after starvation in non-cardiomyocytes of the heart is too low to be detected.

Some agents including bafilomycin A1 and chloroquine have been used to analyze autophagic activity.11 To validate the usefulness of our model in analyzing autophagosomal maturation, we used chloroquine treatment11 to accumulate autophagosomes after starvation using GFP/mCherry-LC3-mice and CAG-GFP-LC3-mice, and then quantified each puncta.10 Both the number of autophagosomes (yellow) in GFP/mCherry-LC3 and autophagosomes (green) in CAG-GFP-LC3 increased with chloroquine (Figure 2B).

**Discussion**

These results suggest that both GFP/mCherry-LC3-mice and CAG-GFP-LC3-mice with chloroquine treatment are useful in illustrating that starvation increases autophagic activity. However, there are some advantages in using GFP/mCherry-LC3-mice. Only autophagosomes can be detected in CAG-GFP-LC3-mice. Both autophagosomes and autolysosomes can be detected and distinguished simultaneously using GFP/mCherry-LC3-mice. Thus, we can analyze autophagosomal maturation and autophagic activity using GFP/mCherry-LC3-mice by measuring the number of autophagosomes and autolysosomes without chloroquine. Our method can rule out any possibility of adverse effects of chloroquine in the heart. By ignoring the chloroquine treatment in GFP/mCherry-LC3-mice we cut the number of mice by 50%, making our method more efficient.

Our new approach can overcome the limitation of monitoring autophagic activity in the heart. Because dysfunction during the fusing of autophagosomes with lysosomes might contribute to heart disease, we will evaluate the impaired function of fusion under various stresses such as ischemia/reperfusion and pressure-overload using this new method. In conclusion, this method we developed is an innovative approach in examining the role of autophagy in cardiomyocytes.

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**References**