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Research Paper

LC3, an Autophagosome Marker, Can be Incorporated into Protein Aggregates Independent of Autophagy

Caution in the Interpretation of LC3 Localization

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KEY WORDS

autophagosome, LC3, aggregate, polyQ, ubiquitin, senescence

ABBREVIATIONS

LC3	microtuble-associated protein light chain 3
PE	phosphatidylethanolamine
GFP	green fluorescent protein
MEF	mouse embryonic fibroblast
polyQ	polyglutamine

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ABSTRACT

Autophagy is an intracellular bulk degradation system, through which a portion of the cytoplasm is delivered to lysosomes to be degraded. Microtuble-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, has been used as a specific marker to monitor autophagy. Upon induction of autophagy, LC3 is conjugated to phosphatidylethanolamine and targeted to autophagic membranes. Therefore, changes in LC3 localization have been used to measure autophagy. However, this method has some limitations. In this report, we show that LC3 protein tends to aggregate in an autophagy-independent manner when it is transiently overexpressed by transfection. In addition, LC3 is easily incorporated into intracellular protein aggregates, such as inclusion bodies induced by polyQ expression or formed in autophagy-deficient hepatocytes, neurons, or senescent fibroblasts. These findings demonstrate that punctate dots containing LC3 do not always represent autophagic structures. Therefore, LC3 localization should be carefully interpreted, particularly if LC3 is overexpressed by transient transfection or if aggregates are formed within cells.

INTRODUCTION

Autophagy is a degradation pathway that delivers cytoplasmic materials to lysosomes via double-membraned organelles termed autophagosomes that enclose a portion of the cytoplasm.[1] It has been determined that the primary role of autophagy in a variety of organisms is to adapt to nutrient starvation by producing amino acids.[2] The absence of autophagy genes results in rapid death in yeast[3] and *Dictyostelium discoideum* under starvation conditions,[4] early chlorosis in plants during starvation,[5-7] defective dauer development in *Caenorhabditis elegans*,[8] and early neonatal lethality in mice following interruption of the transplacental nutrient supply.[9,10] In addition to enhanced autophagy during starvation, basal autophagy is important for intracellular quality control in quiescent cells. Mice lacking autophagy genes exhibit intracellular accumulation of protein aggregates in neurons and hepatocytes.[10-12] Cells utilize the autophagy recycling system for both the constitutive removal of cytosolic proteins and organelles to maintain quality and nutrient supply under adverse conditions. Besides these fundamental roles, autophagy is thought to be involved in the degradation of intracellular bacteria, antigen presentation, tumor suppression, and cell survival and death.[2,13-16]

Recent progress in autophagy research has been led by the identification of a number of autophagy-related genes in the yeast, *Saccharomyces cerevisiae*.[3,17] The nomenclature of these genes has been recently unified, and they have been termed *ATG* genes. Analyses of the Atg proteins have identified two ubiquitination-like conjugation systems required for autophagosome formation.[18] One of these systems mediates the conjugation of Atg12 to Atg5[19] and the other mediates a covalent linkage between Atg8 and phosphatidylethanolamine (PE).[20]

Most of the Atg proteins, including the two conjugation systems, are highly conserved in mammals. Among these proteins, Atg5 and LC3 (a mammalian homolog of Atg8) have been analyzed in detail. An Atg12-Atg5 conjugate is present on the outer side of the isolation membrane and is required for elongation of the isolation membrane.[21] A PE-conjugated form of LC3 localizes on the isolation membrane and the autophagosome membrane.[22,23] The unconjugated (LC3-I) and conjugated forms (LC3-II) of LC3 can be easily separated by SDS-PAGE. Because the amount of LC3-II correlates with the number of autophagosomes, immunoblotting of endogenous LC3 can be used to measure autophagic activity.[22] In *Atg5*$^{-/-}$ embryonic stem cells, LC3-II is not detected.[21] Another

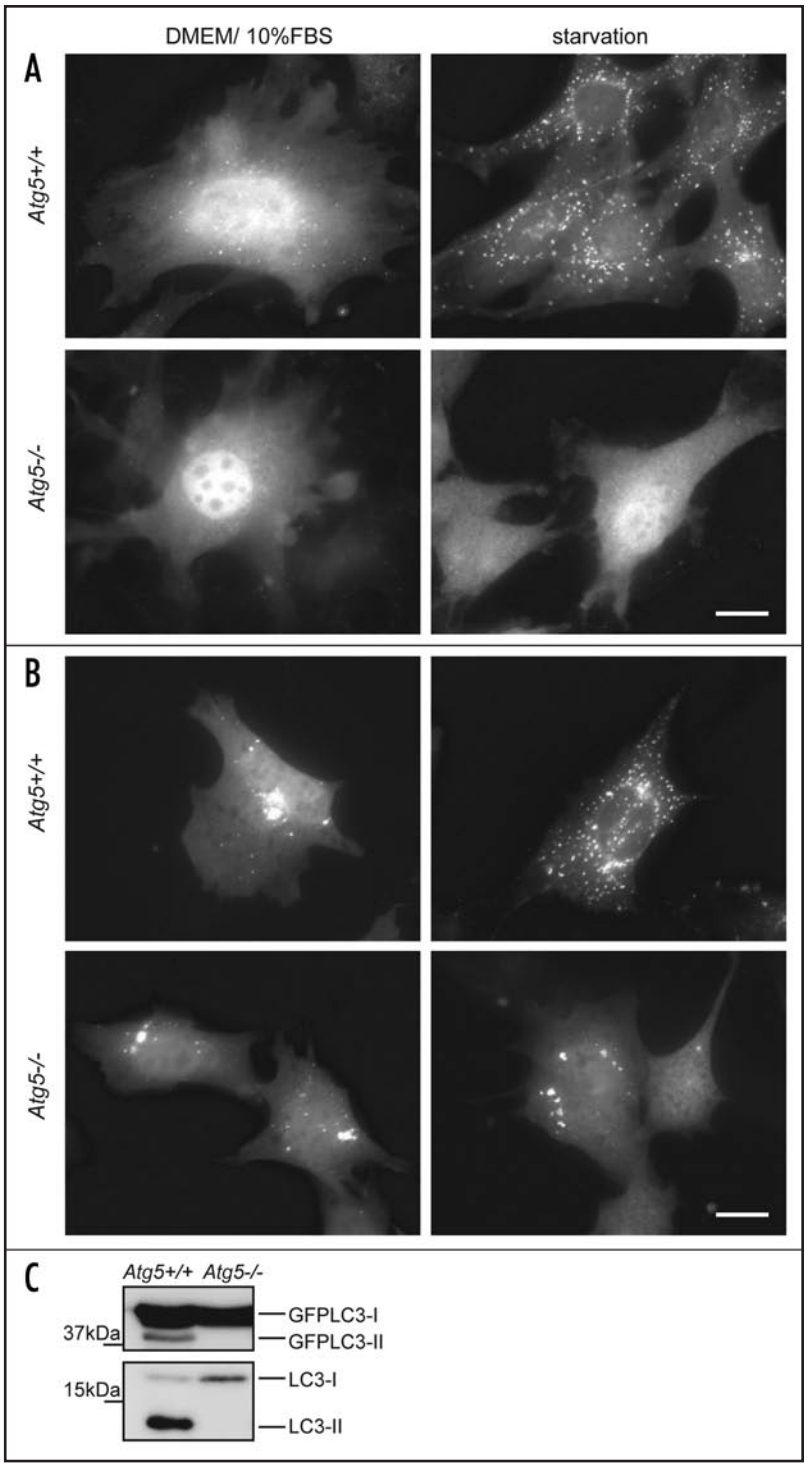

Figure 1. Localization changes of GFP-LC3 upon induction of autophagy in stable and transient transformants. (A and B) *Atg5*$^{+/+}$; GFP-LC3 and *Atg5*$^{-/-}$; GFP-LC3 MEFs (A) or *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs transiently expressing GFP-LC3 (B) were cultured in DMEM with 10% FBS or DMEM without amino acids and serum for 1.5 h. Cells were fixed with 3% PFA and analyzed by fluorescence microscopy. Scale bar 20 μm. (C) *Atg5*$^{+/+}$ and *Atg5*$^{-/-}$ MEFs transiently expressing GFP-LC3 were cultured in DMEM without amino acid and serum for 1.5 h, and then subjected to immunoblot analysis using anti-LC3 antibody. Positions of endogenous LC3 and GFP-LC3 are indicated.

widely-used method to measure autophagic activity is direct determination of LC3 localization, usually with green fluorescent protein (GFP)-LC3.[22,24] Autophagosomes are easily detected as fluorescent dots or sometimes ring-shaped structures. This method has been applied to animal studies by generating GFP-LC3 transgenic mice.[25]

Although the LC3 localization method has been successfully used in many studies, we have recently realized that there are some limitations of this assay. Under certain conditions, LC3 is easily incorporated into protein aggregates; this localization change is independent of autophagy. It can be difficult to distinguish these aggregates from true autophagosomes.

MATERIALS AND METHODS

Antibodies. Rabbit polyclonal HA antibody was purchased from BAbCO. Mouse monoclonal anti-ubiquitin antibody (1B3) was purchased from Medical & Biological Laboratory (MBL). AlexaFluor 660-conjugated goat anti-mouse IgG (H+L) antibody was purchased from Molecular Probes. The polyclonal anti-LC3 antibody (#1) was described previously.[26]

Mice. *Atg5*$^{+/-}$ and *Atg5*$^{-/-}$ mice on the C57BL/6 background have been described previously.[9] *Atg5*$^{+/-}$ mice were crossed with GFP-LC3 transgenic mice[25] to produce *Atg5*$^{-/-}$ mice expressing GFP-LC3 (*Atg5*$^{-/-}$; GFP-LC3). For caesarian delivery, pregnant mothers were injected on 17.5 dpc and 18.5 dpc with 2 mg progesterone (Luteum Injection, Teikoku Hormone Mfg. Co., Ltd., Tokyo) to delay birth; neonates were obtained at 19.5 dpc. All animal experiments were approved by the institutional committees of Tokyo Medical and Dental University and the Tokyo Metropolitan Institute of Medical Science.

Cell culture and transfection. Wild-type and *Atg5*$^{-/-}$ mouse embryonic fibroblasts (MEFs) transformed with the SV40 large T antigen were generated previously.[9] Wild-type and *Atg5*$^{-/-}$ MEFs expressing GFP-LC3 were generated from *Atg5*$^{+/+}$; GFP-LC3 or *Atg5*$^{-/-}$; GFP-LC3 transgenic mice. These cells were transformed with the SV40 large T antigen (kindly provided by Dr. S. Sugano). All cells were maintained in Dulbecco's modified Eagle's medium (SIGMA) containing 10% fetal bovine serum (FBS). For starvation, cells were washed with PBS and incubated in amino acid free DMEM (kindly provided by Ajinomoto Co.) without FBS. Cells were transfected with GFP-tagged LC3 expression vector (pCAG-GFP-LC3) having the CAG promoter (cytomegalovirus immediate-early enhancer and chicken β-actin promoter) as previously reported,[25] and polyQ expression vector (pCMXHA-Q22 or pCMXHA-Q79, kind gifts of Dr. Kakizuka)[27] using FuGene 6 reagent (Roche) or lipofectamine 2000 reagent (Invitrogen). For analysis of senescent cells, serial cultures were performed according to the 3T9 protocol.[28] Briefly, 9 x 10^5 cells were plated on a 6-cm dish. Every 3 days, cells were trypsinized, counted, and replated at the starting density.

Immunocytochemistry. Cells grown on cover slips were washed with PBS and fixed in 3% paraformaldehyde (PFA) in PBS for 10 min at room temperature. Fixed cells were permeabilized by 0.25% Triton X-100, blocked with 5% BSA in PBS and incubated with anti-HA antibody for 1 h. After washing, cells were incubated with AlexaFluor 660-conjugated secondary antibody for 30 min

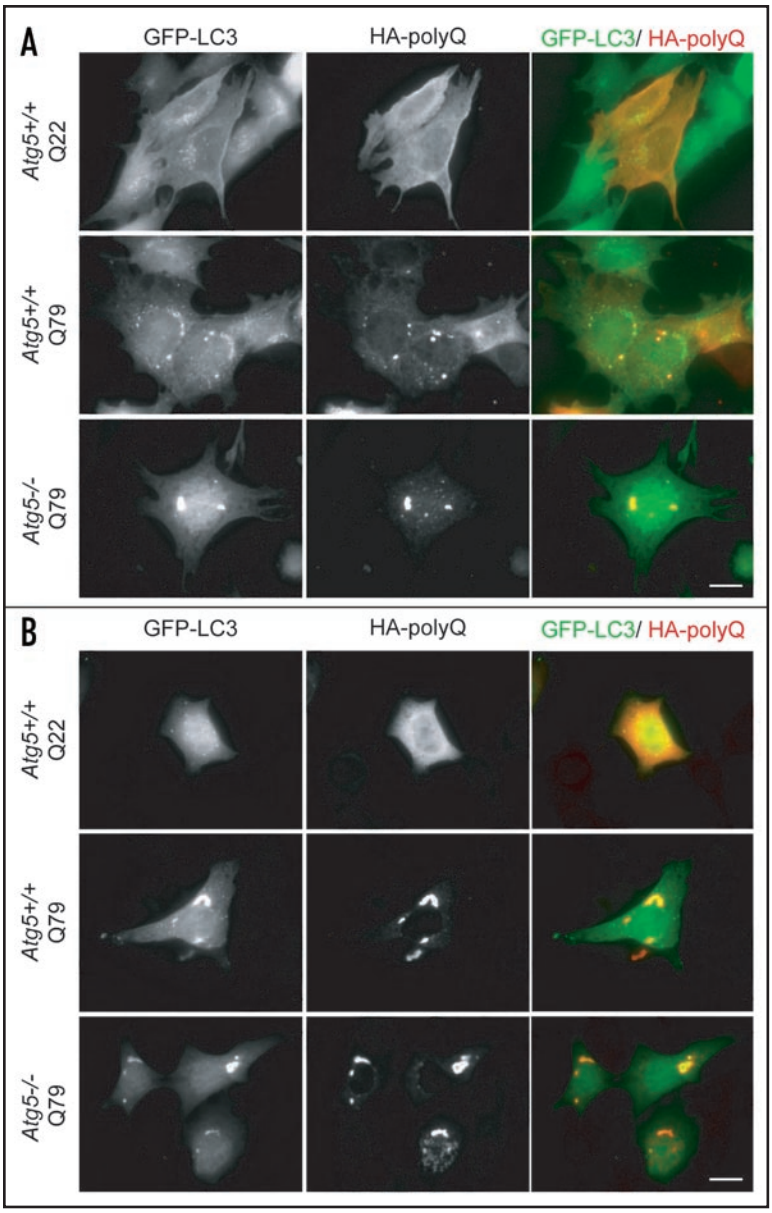

Figure 2. GFP-LC3 is incorporated into PolyQ protein aggregates. (A) Wild-type and *Atg5*$^{-/-}$ MEFs stably expressing GFP-LC3 were transiently transfected with HA-tagged normal polyQ repeat (pCMXHA-Q22) or expanded polyQ repeat (pCMXHA-Q79) expression plasmids. After 48 h, cells were fixed with 3% PFA and HA-tagged polyQ was stained with anti-HA polyclonal antibody and Cy5-conjugated secondary antibody. Scale bar 20 μm. (B) Wild-type and *Atg5*$^{-/-}$ MEFs were transiently cotransfected with GFP-LC3 and pCMXHA-Q22 or pCMXHA-Q79 and analyzed as in (A).

at room temperature and examined under a fluorescence microscope (Olympus IX81) equipped with a CCD camera (ORCA ER, Hamamatsu Photonics). To quantify the number of GFP-LC3 dots, the dot signals were extracted using the Top Hat program of Meta Morph Series Version 6 (Molecular Device). The number of the dots was counted at least for five different fields.

Immunohistochemical analysis. Mice were perfused transcardially with 4% PFA in PBS (pH 7.4). Tissues were postfixed overnight in the same fixative and embedded in either Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Ltd.) or paraffin. GFP-LC3 localization was analyzed as previously described.[25] For immunohistochemistry, tissue sections were subjected to antigen retrieval by the microwave method (in 0.01 M citrate buffer for 10 min). After blocking in BEAT Blocking solution (Zymed Laboratories Inc.), sections were incubated with primary antibody for 1 h, followed by a 30 min incubation with fluorescent-labeled secondary antibody.

RESULTS

Transient overexpression of GFP-LC3 causes aggregation. MEFs stably expressing GFP-LC3, a marker of autophagosomes, have been frequently used to monitor autophagy because they have relatively large and numerous autophagosomes and genetically-modified mutants are available.[24] When these cells are cultured in regular DMEM supplemented with 10% FBS, only a small number of GFP-LC3 dots were detected (26 ± 16 per cell) (Fig. 1A). The number of these GFP-LC3 dots increased to 152 ± 30 per cell after 1.5-h starvation for both amino acids and serum. In *Atg5*$^{-/-}$; GFP-LC3 MEFs, GFP-LC3 was diffusely distributed in the cytoplasm and no punctate dots were observed (Fig. 1A). These data confirm that GFP-LC3 is a specific marker for autophagosomes.

However, when we examined cells overexpressing GFP-LC3 by transient transfection, larger fluorescent dots were observed in some nonstarved wild-type MEFs (Fig. 1B). These dots were homogeneously fluorescent, and were not ring-shaped, suggesting that these dots are not likely to be autophagosomes. When GFP-LC3 was transiently expressed in *Atg5*$^{-/-}$ MEFs, larger florescent dots also appeared in these cells (Fig. 1B). As PE-conjugation of LC3 and LC3 targeting to autophagic membranes depends on Atg5, the large fluorescent dots do not represent true autophagic structures. Indeed the conversion of both endogenous LC3 and transfected GFP-LC3 was completely suppressed in *Atg5*$^{-/-}$ cells even in the presence of GFP-LC3-positive structures (Fig. 1C). These dots were not observed in stable transformants at moderate expression levels, suggesting that overexpression of GFP-LC3 may have caused the artificial aggregation of GFP-LC3 protein.

GFP-LC3 is incorporated into polyQ aggregates independent of autophagy. We next determined whether GFP-LC3 can be incorporated into protein aggregates composed of unrelated proteins. We utilized expanded polyglutamine (polyQ) to generate intracellular protein aggregates.[27] Typically, the presence of more than 40 repeats causes expanded polyQ proteins to form insoluble aggregates within cells. When expanded polyglutamine (Q79) was expressed in *Atg5*$^{+/+}$; GFP-LC3 MEFs, large GFP-LC3 dots appeared, most of which colocalized with the polyQ aggregates (Fig. 2A). One possible explanation is that autophagosomes were sequestering the polyQ aggregates. However, the colocalization of polyQ aggregates with GFP-LC3 was also observed in *Atg5*$^{-/-}$; GFP-LC3 MEFs (Fig. 2A). In contrast, if polyQ with fewer repeats (Q22) was expressed, polyQ aggregates were not formed and GFP-LC3 was mainly distributed diffusely in the cytosol with a few punctuate dots, which likely indicate genuine autophagosomes.

The polyQ and GFP-LC3 colocalization was more prominent if polyQ and GFP-LC3 were transiently transfected simultaneously into MEFs; most of the polyQ (Q79) aggregates were positive for GFP-LC3 in both *Atg5*$^{-/-}$ and *Atg5*$^{+/+}$ MEFs (Fig. 2B). These results suggest that GFP-LC3 is easily incorporated into protein aggregates in an autophagy independent manner.

Incorporation of GFP-LC3 and endogenous LC3 into aggregates in *Atg5*$^{-/-}$ mouse tissues. We previously reported that autophagy occurs at low levels during embryogenesis, and is upregulated in various tissues immediately after birth (Fig. 3A and E).[9] The small GFP-LC3 dots representing autophagosomes were not detected in most tissues in *Atg5*$^{-/-}$; GFP-LC3 neonatal mice (Fig. 3F). Larger, more intense fluorescent dots, however, appeared frequently in hepatocytes and subsets of neurons such as the dorsal root ganglion (DRG) neurons (Fig. 3B and D). These large dots appeared occasionally in cells of the anterior lobe of the pituitary gland (Fig. 3H). Such distribution was consistent with our previous report that ubiquitin-positive inclusion bodies accumulated in hepatocytes and a subset of neurons in *Atg5*$^{-/-}$ neonatal mice.[11] Similar GFP-LC3 dot structures were also observed in primary cultured hepatocytes from mice bearing a liver-specific knockout of *Atg7*.[10] It was observed that the LC3 conversion was suppressed in tissues of these mice.[9-12] We thus speculated that GFP-LC3 was incorporated into these ubiquitin-positive inclusion bodies. Indeed, clear colocalization of GFP-LC3 and ubiquitin was identified in *Atg5*$^{-/-}$ hepatocytes by indirect fluorescence microscopy using anti-ubiquitin antibody (Fig. 4A). Therefore, these large fluorescent spots represented inclusion bodies containing the unconjugated GFP-LC3 protein, not membranous structures.

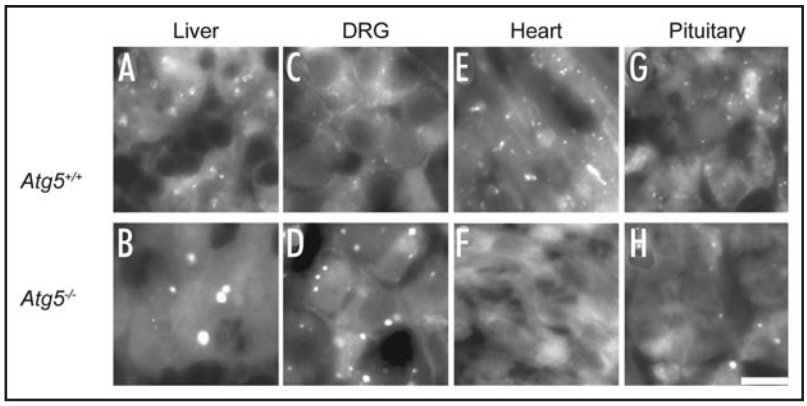

Figure 3. GFP-LC3 localization in tissues of wild-type and *Atg5*$^{-/-}$ neonatal mice. Various organs were isolated from 6-h *Atg5*$^{+/+}$; GFP-LC3 or *Atg5*$^{-/-}$; GFP-LC3 neonates. Tissues were immediately fixed, sectioned, and analyzed by fluorescence microscopy. (A and B) Liver, (C and D) dorsal root ganglia, (E and F) heart, (G and H) anterior lobe of the pituitary gland. Scale bar, 10 μm.

Overexpression of GFP-LC3 is well tolerated in wild-type mouse tissues. We did not observe large fluorescent dots representative of aggregates in *Atg5*$^{+/+}$; GFP-LC3 mice, even in tissues that highly express GFP-LC3, such as the brain, heart, liver, and pancreas.[25] However, in the absence of autophagy, the overexpression of GFP-LC3 might have facilitated the artificial aggregation of GFP-LC3 protein. We thus tested whether endogenous LC3 was also incorporated into those aggregates in *Atg5*$^{-/-}$ mice not expressing GFP-LC3. In DRG neurons of *Atg5*$^{-/-}$ neonates, endogenous LC3 protein was also detected in these aggregates (Fig. 4B), suggesting that LC3, rather than the GFP moiety, tends to be incorporated into protein aggregates regardless of its PE-conjugation status.

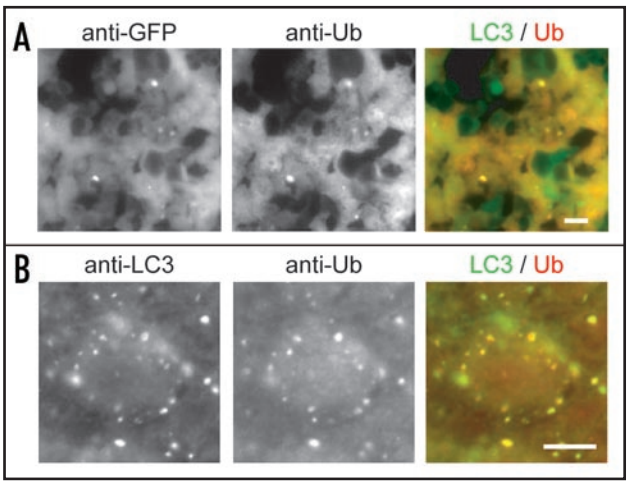

Figure 4. Inclusion bodies in *Atg5*$^{-/-}$ mouse tissues incorporate endogenous LC3. Neonatal livers from *Atg5*$^{-/-}$; GFP-LC3 (A) and dorsal root ganglia from *Atg5*$^{-/-}$ (without GFP-LC3 expression) mice (B) were examined by immunohistochemistry. OTC-embedded (A) and paraffin-embedded (B) sections were antigen-retrieved and stained with anti-GFP (A, left), anti-ubiquitin (the middle panel of A and B), or anti-LC3 antibodies (the left panel of B). Merged images are shown (the right panels of A and B). Scale bar, 10 μm.

GFP-LC3 aggregates in senescent *Atg5*$^{-/-}$ MEFs. Although a subset of *Atg5*$^{-/-}$ cells showed cytoplasmic protein aggregation in vivo, immortalized MEFs and ES cells do not exhibit aggregation, even when they are autophagy-deficient. This might be due to rapid dilution of abnormal proteins through cell division; misfolded proteins generally accumulate slowly over time, and continued cell division, especially in immortalized cells without replicative limitations, can keep the level of these proteins below a threshold that favors aggregation. To test whether aggregates can be formed in cultured cells, we analyzed primary cultured embryonic fibroblasts with limited replicative lifespan: most eukaryotic cells can divide only a finite number of times, after which they enter senescence. Primary MEFs were prepared from day 13.5 *Atg5*$^{-/-}$; GFP-LC3 embryos. Cells were passaged every third day and cultured according to the 3T9 protocol. At early passages, GFP-LC3 was present diffusely in the cytoplasm (data not shown). At the 10th passage, when cells had become senescent, large fluorescent GFP dots appeared in some *Atg5*$^{-/-}$ cells, but not in *Atg5*$^{+/+}$ cells (Fig. 5). These dots did not appear until the 8th passage. These results suggest that protein aggregates can be formed in cultured cells lacking *Atg5* if they are senescent, and GFP-LC3 can be incorporated into these aggregates independent of autophagy.

DISCUSSION

Detection of LC3-positive structures, either by immunostaining of endogenous LC3 or localization of transfected GFP-LC3, is the most commonly used method to detect autophagosomes by light microscopy. However, the results reported herein shed light on some limitations of this method. We have demonstrated that LC3-positive dots sometimes represent protein aggregation, particularly when GFP-LC3 is overexpressed. Thus, LC3 itself could be an aggregation-prone protein. However, cells derived from GFP-LC3 transgenic mice or cells stably-transfected with GFP-LC3 (data not shown) do not posses GFP-LC3 aggregates, suggesting that either overexpression by transient transfection may cause aggregation or long-term expression may be tolerated by cells. Based on these findings, we strongly recommend using only stable GFP-LC3 transformants.

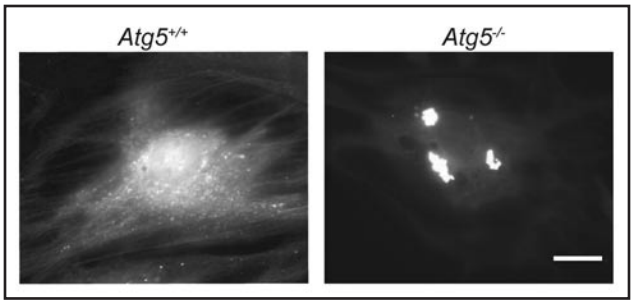

Figure 5. GFP-LC3 aggregates are generated in senescent *Atg5*$^{-/-}$ MEFs. Primary MEFs prepared from *Atg5*$^{+/+}$; GFP-LC3 or *Atg5*$^{-/-}$; GFP-LC3 transgenic mice were passaged by the 3T9 protocol. At 10 passages, cells were seeded in glass dishes and analyzed by fluorescence microscopy. Scale bar, 20 μm.

A second important case of LC3 aggregation is incorporation into inclusion bodies. We have demonstrated that two different types of inclusion bodies, one formed by polyQ expression and the other induced by autophagy-defective conditions, can recruit GFP-LC3 and even endogenous LC3. We have recently identified another example of LC3 incorporation into a type of aggregate called aggresome-like induced structures (ALIS), which could be autophagy-independent.[29] Therefore, this incorporation may be a general phenomenon. It is usually difficult to distinguish aggregate related GFP-LC3 dots from autophagosomes, even though the aggregated structures never exhibit the cup-shaped structure characteristic of authentic autophagosomes. This may represent a serious problem in experiments testing whether protein aggregates are sequestered by autophagosomes. Because the accumulation of misfolded proteins is considered to be the cause of cell degeneration in many neurodegenerative diseases, understanding the mechanisms by which cells eliminate these mutant or misfolded proteins is important. While human genetic studies have revealed a number of mutations in genes related to the ubiquitin-proteasome system, the involvement of autophagy in the pathogenesis of these diseases has been also suggested.[30-32] However, our data imply that it would be difficult to address this question using the LC3 marker. To demonstrate the direct sequestration of aggregates by autophagosomes, electron microscopic analysis will be required.

Our data suggest that LC3 itself could be an aggregation-prone protein, but another possible mechanism for LC3 dot formation is p62/sequestosome1-mediated incorporation into protein aggregates. Recently, it was demonstrated that p62/sequestosome 1, which is a common component of protein aggregates, can bind LC3.[33] LC3 is the only protein to be identified on the inner and outer membranes of autophagosomes. Therefore, the LC3-p62/sequestosome1 interaction may mediate selective recognition of protein aggregates by autophagosomes. Again, this hypothesis needs to be addressed by electron microscopy.

Our analyses have demonstrated that the requirement of autophagy for preventing intracellular protein aggregation differs among tissue and cell types (Fig. 3).[11] Although the reason is unclear at the moment, one possible explanation is that intracellular quality control is not very important in rapidly dividing cells, in which damaged materials can be quickly diluted. Yeast *atg* mutant cells[3] as well as autophagy-deficient mammalian cells, such as *Atg5*$^{-/-}$ embryonic stem cells[21] and immortalized embryonic fibroblasts,[9] do not exhibit significant abnormalities under normal conditions, despite the absence of low levels of constitutive autophagy. Basal autophagy may be more important in quiescent cells. This hypothesis is supported by our observations that senescent fibroblasts tend to form GFP-LC3 aggregates if autophagic activity is inhibited (Fig. 5). As autophagy is thought to exert an anti-aging function,[8,34,35] our data provide additional information for this hypothesis.

In summary, our results emphasize that, although LC3 is a good marker for autophagosomes, it can also indicate protein aggregates, particularly if overexpressed by transient transfection or if aggregates are formed within cells. Under these conditions, LC3 localization should be interpreted with caution and the occurrence of autophagy should be examined with additional methods such as immunoelectron microscopy against GFP-LC3 or endogenous LC3. In any cases, use of GFP-LC3 stable transformants is preferred.

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