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Research Paper

High-throughput functional screening for autophagy-related genes and identification of *TM9SF1* as an autophagosome-inducing gene

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Abbreviations: TMEM166, transmembrane protein 166; TMEM74, transmembrane protein 74; TM9SF1, transmembrane 9 superfamily member 1; TM9SF3, transmembrane 9 superfamily member 3; GFP, green fluorescent protein; MDC, monodansylcadaverine; LC3, microtubule-associated protein 1 light chain 3; FCS, fetal calf serum; TEM, transmission electron microscopy; PCR, polymerase chain reaction; LTR, lysotracker red; MTR, mitotracker red

Key words: high-throughput screening, autophagy, LC3, TM9SF1, automated fluorescence microscopy system

Autophagy, a tightly regulated process responsible for the bulk degradation of most long-lived proteins and some organelles, is associated with several forms of human diseases including cancer, neurodegenerative disease and cardiomyopathies. However, the molecular machinery involved in autophagy in mammalian cells remains poorly understood. Here, we describe a high-throughput, cell-based functional screening platform, based on an automated fluorescence microscopy system, which enables acquiring and quantitatively analyzing images of GFP-LC3 dots in cotransfected cells. From a library of 1,050 human cDNA clones, we identified three genes (*TM9SF1*, *TMEM166* and *TMEM74*) whose overexpression induced high levels of autophagosome formation. In particular, overexpression of *TM9SF1*, which colocalized with LC3 according to the confocal assay, led to a significant increase in the number of GFP-LC3 dots. The results of transmission electron microscopy and immunoblotting to examine LC3-II levels further confirmed the ability of *TM9SF1* to induce autophagy. Furthermore, knockdown of *TM9SF1* expression by RNA interference could hamper starvation-induced autophagy. The functional screening platform therefore can be applied to high-throughput genomic screening candidate autophagy-related genes, which would provide new insights into underlying molecular mechanisms that may regulate autophagy in mammalian cells.

Introduction

Autophagy, a highly regulated process, is responsible for the bulk degradation of most long-lived proteins and some organelles and is evolutionarily conserved among eukaryotes.[1-3] Autophagy is associated with neurodegenerative disease, cardiomyopathies, cancer, programmed cell death, and bacterial and viral infections.[4] There are three primary forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy.[5] Macroautophagy is considered the most prevalent form of autophagy. During macroautophagy (referred to as 'autophagy' hereafter), a cup-shaped structure called the preautophagosome engulfs cytosolic components, including organelles, and closes to form an autophagosome (also known as an 'autophagic vesicle containing undegraded cytoplasm') that subsequently fuses with a lysosome to form an autolysosome, leading to the proteolytic degradation of the internal components of the autolysosome by lysosomal enzymes.[5]

Autophagy has been investigated extensively in yeast, and because of the generation of autophagy-specific mutants in a variety of yeast cell lines, a number of yeast genes involved in autophagy (called 'ATG' genes) have been identified through genetic screens.[6] In mammalian, although some homologues of yeast ATG genes have been identified and remarkable progress have been made in understanding the molecular mechanisms involved in mammalian autophagy, there are still many questions remained to be answered.[5,7]

Microtubule-associated protein 1 light chain 3 (LC3), a homologue of yeast Atg8 (Aut7/Apg8), is considered a highly specific marker of autophagosomes.[8,9] Expression of a green fluorescent protein (GFP) fusion with LC3 (GFP-LC3) and endogenous LC3-II/LC3-I has been used extensively as a specific and sensitive autophagosomal marker used to monitor autophagic activity.[7] Until now, the GFP-LC3 autophagic marker has never been used in high-throughput assays to identify genes that regulate autophagy due to technical issues associated with the quantification of GFP-LC3 structures in large numbers of samples. Here we demonstrate that the GFP-LC3 structures can be identified and quantified using an automated approach for microscopic data acquisition and analysis using a high-throughput approach.

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In an effort to identify human genes involved in the induction of autophagy, we constructed a high-throughput, cell-based functional screening platform based on an automated fluorescence microscopy system, that enables acquiring and quantitatively analyzing images of GFP-LC3 dots in cotransfected cells. From a library of 1,050 human cDNA clones, we identified three genes (*TM9SF1*, *TMEM166* and *TMEM74*) whose overexpression led to increased numbers of GFP-LC3 dots in the initial screen, which were validated by transmission electron microscopy (TEM) analysis of autophagosome formation and by immunoblot analysis of LC3-II upregulation. Collectively, our data suggest that the genes are likely to play a significant role in autophagy induction. The screening platform we developed for the identification of autophagy-related genes based on automated fluorescence imaging can be applied readily for high-throughput genomic screening using a 384-well plate format or a cellular array, which would facilitate the identification of novel mammalian autophagy-associated genes and the elucidation of underlying molecular mechanisms involved in autophagy induction.

Results

Identification of three candidate genes that induce increased numbers of GFP-LC3 dots. To identify human genes involved in autophagy, we screened 1,050 genes of unknown function from our cDNA library using GFP-LC3 as a marker for autophagosomes (Fig. 1A, Suppl. Table 1). We evaluated the ability of candidate genes to induce autophagy according to the number of GFP-LC3 dots per cell. Cells cotransfected with the empty library vector and the GFP-LC3 vector with and without starvation induction served as positive and negative controls, respectively. The number of GFP-LC3 dots per cell was 0.53 ± 0.14 in negative control (unstarved) cells. For most cDNAs screened, the average number of GFP-LC3 dots per cell was very low (≤1.0 for 87.0% of the cDNAs screened). However, the average number of GFP-LC3 dots per cell increased to 2.65 ± 0.43 after starvation induction. Overexpression of three genes (*TM9SF1*, *TMEM166* and *TMEM74*) led to an increased number of GFP-LC3 dots (up to 2.0 per cell; Fig. 1C and D). The function of the three identified genes (*TM9SF1*, *TMEM166* and *TMEM74*) was further confirmed by carrying out three independent experiments (data not shown).

***TM9SF1*-overexpressed HeLa cells contain higher numbers of autophagic and acidic vesicles.** Transmission electron microscopic analysis, which is the primary and universally accepted method for the detection of autophagy,[7] showed that *TM9SF1*-overexpressed and starving HeLa cells contained extensive numbers of typical autophagic vesicles relative to control cells (Fig. 2A). Therefore, the results of our TEM studies provided additional evidence confirming the ability of *TM9SF1* to induce autophagosome production.

Furthermore, *TM9SF1* overexpression led to increased levels of MDC staining, which is a marker of acid vesicles, in HeLa cells (Fig. 2B). MDC was previously considered a marker for autophagic vacuoles, since it was shown to accumulate in acidic compartments enriched in lipids.[15] However, MDC is no longer considered a specific marker for autophagic vacuoles, because MDC stains acidic cell compartments.[16,17] The increased levels of MDC staining indicate that *TM9SF1* overexpression induces the formation of acidic vesicles in HeLa cells, which may correspond to acidic autophagic vacuoles or lysosomes.

***TM9SF1* overexpression leads to increased LC3-II levels in HeLa cells.** We found that *TM9SF1*-transfected HeLa cells showed both an increase in the amount of LC3-II and an increase in the LC3-II/LC3-I ratio as determined by immunoblot analysis (Fig. 2C). Furthermore, *TM9SF1* overexpression also led to both an increase in the amount of GFP-LC3-II and an increase in the GFP-LC3-II/GFP-LC3-I ratio (Fig. 2C); this was consistent with our previous results demonstrating that *TM9SF1* overexpression led to an increased number of GFP-LC3 dots and confirmed that *TM9SF1* overexpression induces autophagosome formation.

Previous studies showed that mutation of glycine at position 120 prevents the C-terminal cleavage and the conjugation of LC3 to the autophagosomal membranes.[9] We found that *TM9SF1* overexpression has no direct effect on aggregate of GFP-LC3(G120A), and GFP-LC3(G120A) was evenly distributed throughout the cell (Fig. 3A). This identified that *TM9SF1* overexpression induces conversion of GFP-LC3-I into GFP-LC3-II but not the aggregation of GFP-LC3 and the puncta are autophagosome-dependent. We also found that lysosomal inhibitor Bafilomycin A1 could significantly increase the dots of GFP-LC3 in *TM9SF1* overexpressed HeLa cells compared with vector overexpressed HeLa cells treated with Bafilomycin A1 (Fig. 3B). The progression of the autophagy is sensitive to the PI3K inhibitors such as wortmannin. We further found that wortmannin could partially inhibit the increment of GFP-LC3 dots caused by *TM9SF1* (Fig. 3C). These results collectively show that *TM9SF1* expression triggers autophagy in HeLa cells.

TM9SF1 localizes to autophagic vacuoles and lysosome in HeLa cells. The subcellular localization of proteins is critical to their biological function. Therefore, we determined the subcellular localization of TM9SF1 using confocal microscopy (Fig. 4). We found that TM9SF1-GFP colocalized completely with a DsRed fusion of LC3 (DsRed-LC3), which is associated specifically with autophagic vacuoles. With the treatment of Bafilomycin A1, an inhibitor of the vacuolar proton ATPase, TM9SF1 still partially colocalized with DsRed-LC3, indicating that TM9SF1 partially localized to the autophagosome. Furthermore, TM9SF1-GFP also colocalized extensively with LTR, which is a sensitive lysosomal/autolysosomal marker. In addition, TM9SF1 also can colocalize with lysosomal marker LAMP-1. TM9SF1-GFP did not colocalize with the mitochondrial marker MTR. In a proteomic analysis of lysosomal integral membrane proteins by Bagshaw et al.[18] TM9SF1 was found to localize in lysosome, which was consistent with our results. Taken together with the results of our earlier studies, the localization of TM9SF1 to autophagic vacuoles and lysosome supports a role for *TM9SF1* in autophagy induction.

Knockdown of *TM9SF1* endogenous expression reduces levels of starvation-induced autophagy. To further determine the role of *TM9SF1* in autophagy under physiological conditions, siRNA was designed to knockdown the expression of *TM9SF1* in HeLa cells. Nonsilencing siRNA or siRNA against *TM9SF1* (si-TM9SF1) was transfected into HeLa cells alone or combined with the TM9SF1-GFP vector. At 48 h after transfection, TM9SF1 mRNA and protein levels were significantly decreased in cells transfected with si-TM9SF1, as assessed by flow cytometry (Fig. 5A), RT-PCR and western blotting (Fig. 5B). To investigate whether si-TM9SF1 transfected HeLa cells have impact on general lysosomal function,