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Generation of transgenic zebrafish with liver-specific expression of *EGFP-Lc3*: A new *in vivo* model for investigation of liver autophagy

Jianzhou Cui [a,1], Tina Huey-Fen Sim [b,1], Zhiyuan Gong [b,*], Han-Ming Shen [a,c,*]

[a] Saw Swee Hock School of Public Health, National University of Singapore, Singapore
[b] Department of Biological Sciences, National University of Singapore, Singapore
[c] Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

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ABSTRACT

Transgenic expression of GFP-Lc3 is a useful tool for an *in vivo* model to monitor the formation of autophagosomes during the autophagy process. So far, two transgenic animals (mice and zebrafish) with expression of GFP-Lc3 have been reported. Liver is one of the most important organs for autophagy research. Here, we generated a transgenic zebrafish line with liver-specific *EGFP-Lc3* expression. By exposing transgenic larvae to the autophagy inducer, Torin1, we observed a substantial increase in the number of EGFP-Lc3 puncta in the liver as well as the increase of Lc3-II protein. Notably, addition of a chloroquine (CQ) led to further increase of EGFP-Lc3 puncta in liver cells due to the blockage of lysosomal function and degradation stage of autophagy. Thus, the newly established transgenic line will be a useful *in vivo* model to investigate liver autophagy, and, in particular, the involvement of autophagy in basic biology and diseases in the liver.

1. Introduction

Macroautophagy (referred as autophagy hereafter in this study) is an evolutionarily conserved process in which intracellular membrane structures sequester proteins and organelles for lysosomal degradation [1]. Autophagy is a dynamic process, consisting of several sequential stages (initiation, nucleation, elongation, and maturation) and controlled by distinct members of the autophagy-related (*Atg*) gene family and their respective upstream signaling pathways [2,3]. So far, more than 30 *Atg* genes have been identified in yeast, and many of them have homologues in higher vertebrates such as human, mouse, and fish [4].

The early steps of autophagosome formation require two conjugation systems for the covalent attachment: (1) ATG12 to the target protein ATG5; and (2) ATG8 (Lc3 in mammalian cells) to the lipid phosphotidylethanolamine (PE) [5]. Both ATG12 and ATG8 are activated by a common E1-like enzyme, ATG7, and transferred to two different E2-like enzymes, ATG10 and ATG3, respectively, before they are attached to ATG5 and PE. Hence, the ATG8/Lc3 system plays an essential role in the proper development of autophagic isolation membranes and the biochemical changes of ATG8/Lc3 (lipidation and membrane translocation) have been well established as an important autophagy marker [6].

The zebrafish has been used as a model organism for the study of vertebrate development due to its high fecundity, ease in husbandry and short generation times [7,8]. In recent years, the zebrafish has also gained popularity as a disease model such as cancer [9]. This is due to its close homologies to other vertebrate species including human and to the feasibility of high-throughput studies at a relative low cost. Now many experimental tools have been developed in the zebrafish, including forward genetic screens and reverse genetics, which allow zebrafish to be used to screen for genetic and chemical modifiers for diseases, in particular for cancers [10,11]. A wide spectrum of tumors can be induced in zebrafish, which are found to be similar to human tumors [12,13]. All the above advantages make zebrafish a favorable model organism for cancer research [10,14].

At present, several studies have been reported on autophagy in zebrafish [15–20]. He et al. constructed the first transgenic zebrafish line for autophagy study by using GFP-Lc3 and GFP-Gabarap which allowed the expression of GFP in the whole zebrafish [20]. The liver is a main target organ in autophagy study [21,22] and liver tumor is one of the main phenotypes in mice with deletion of *Atg* genes [23–25]. In order to investigate the relationship of autophagy and cancer (or other diseases) in zebrafish liver, we generated a transgenic line which specifically expressed *EGFP-Lc3*

* Corresponding authors. Address: Department of Biological Sciences, National University of Singapore, S2-Level 5, 14 Science Drive 4, Singapore 117543, Singapore (Z. Gong), Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, 16 Medical Drive, Singapore 117597, Singapore (H.M. Shen). Fax: +65 6779 1489.

E-mail addresses: dbsgzy@nus.edu.sg (Z. Gong), han-ming_shen@nuhs.edu.sg (H.-M. Shen).

[1] These authors contributed equally to this work.

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in the liver and the model was validated using chemical inducers and inhibitors of autophagy. Thus, this transgenic line will be a useful *in vivo* model to study the role of autophagy in liver physiology and pathology.

2. Materials and methods

2.1. Zebrafish and chemicals treatment

Zebrafish and embryos were maintained and staged as described [26]. The following treatment protocol has been modified from a published version [27]; all the chemicals dissolved in DMSO (Sigma, #D2650) were added into the egg water for the treatment in 24-well plates. The embryos were treated with Torin1 (Tor; Tocris Bioscience, 4247) (0.5 μM) and/or chloroquine (CQ; Sigma, #c6628) (50 μM) for 48 h respectively. Larvae treated with only DMSO were used as a negative control. All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of National University of Singapore.

2.2. Real-time PCR

The mRNA of zebrafish embryos at different stages (2 cells-24 hpf) and different adult tissues were extracted with RNeasy, and the RNA were reverse-transcribed to cDNA with Omiscript kit (Qiagen, # 205113) as per the manufacturer's protocols after DNase I (Invitrogen, # 18047-019) treatment. Real-time PCR was carried out according to the protocol found in the SSO Fast EvaGreen supermix kit (Bio-Rad). Sequences of the *lc3* primers are as follows: 5′-GACATTTGAGCAGCGGGTGGA-3′ and 5′-GGTTGGAGTTGAGTTGGAGGC-3′. The relative expression levels (RNA abundance) were estimated by calculation of the Ct value of *lc3* and *β-actin*. All data were expressed as the mean ± SD of the 3 replicates.

2.3. The pDs-fabp10-EGFP-Lc3 construction and generation of transgenic zebrafish

The pEGFP-Lc3 plasmid was a kind gift from Dr. Yoshimori [28]. The pAc and pMDS6 plasmids were kind gifts from Dr. Parinov [29]. Wild-type zebrafish embryos were used in establishing a stable transgenic line with liver-specific expression of EGFP-Lc3 fusion protein. The *EGFP-lc3* fragment was first sub-cloned into the plasmid containing the liver fatty acid binding protein *(Lfabp10)* promoter using Mlu I (NEB, # R0198L) and Nhe I (NEB, # R0131L). The resultant *fabp10-EGFP-Lc3* expression cassette was then sub-cloned into the pMDS6 plasmid using Mlu I (NEB, # R0198L) and Not I (NEB, # R0189L) such that the Ds elements are flanking the gene expression cassette.

To synthesize *p*Ac mRNA, the pAc plasmid was first linearised downstream of the polyA tail using BamH I (NEB, # R0136L). The resultant linearised plasmid was used in the generation of capped mRNA using the mMessage mMachine SP6 kit from Ambion (#AM1340). *pDS- fabp10-EGFP-Lc3* plasmid and *pAC* mRNA were mixed and co-injected into one-cell stage embryos. A volume of 1 containing 25 pg *pDS-fabp10-EGFP-Lc3* and 250 pg *pAC* mRNA were injected into each embryo at the one-cell stage.

Larvae with liver-specific EGFP-Lc3 expression were selected and bred to maturity and crossed with wild-type fish to screen for founders. To ascertain the germline transmission of the *fabp10*:*EGFP-Lc3* transgene, progeny were screened for *EGFP-Lc3* expression and their genomic DNA were extracted and subjected to PCR to amplify the *EGFP-Lc3* segment. PCR screening using genomic DNA of EGFP-Lc3 transgenic line had the following primer sequences: Forward: 5′-GTTCAAACAGCAGCAGGTCATTG-3′ and Reverse: 5′-GGTCTTCTCGGACGGTCTAGATCT-3′; *β*-actin primers were used as both loading and positive control and the primers had the following sequence: Forward: 5′-GAGAGAGGCTACAGCTTCAC-3′ and Reverse: 5′-ACTCCTGCTTGCTAATCCAC-3′. Founders with germline transmission of the transgene and liver-specific expression of EGFP-Lc3 were kept for further analysis.

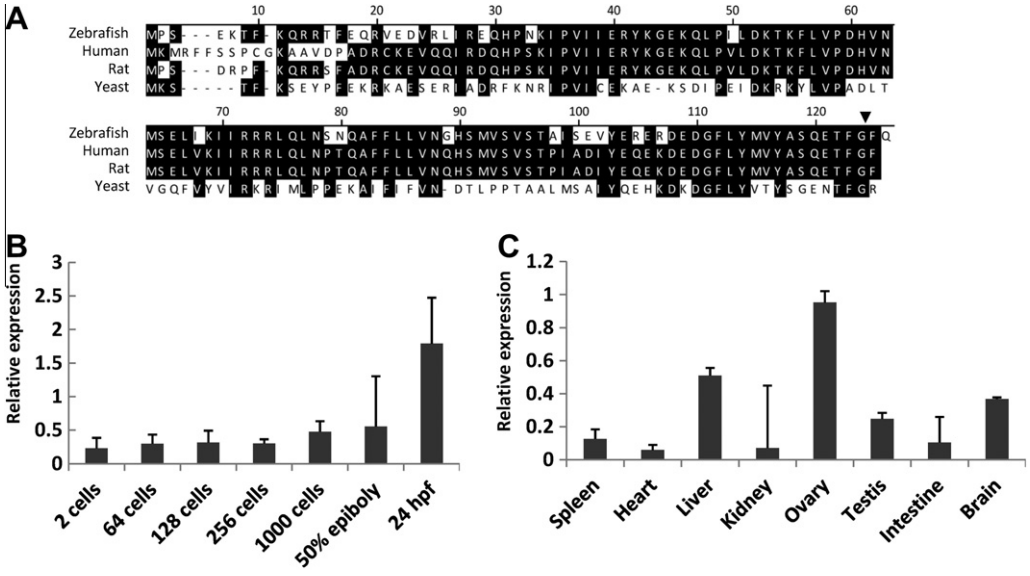

Fig. 1. Lc3 sequence alignment and expression of *lc3* mRNA in zebrafish. (A) Lc3 protein sequence alignment. Protein sequence of Lc3 from *D. rerio* (zebrafish), *H. sapiens* (human), *R. norvegicus* (rat) and *S. cerevisiae* (yeast) were aligned by using the ClustalW program. Identical amino acid residues are highlighted in black. An arrow head indicates the potential cleavage and lipidation site. (B) Quantity analysis of Zebrafish *lc3* mRNAs expression in early development. Real-time PCR was performed with RNA isolated from 2 cells to 24 hpf wild-type embryos. The observation suggesting that *lc3* mRNA was maternally deposited. hpf: hours post fertilization. (C) Quantity analysis of Zebrafish *lc3* mRNAs expression in adult tissues. The expression of zebrafish *lc3* in adult tissues measured by real-time PCR showed a ubiquities expression pattern in all 8 examined tissues with a relatively high level in the ovary and liver. The relative expression levels were investigated by calculation of the Ct value of *lc3* and *β-actin*. All data were expressed as the standard deviation (S.D.) of the 3 replicates.

2.4. Western blot analysis

For Western blot analysis, 40 μg proteins was fractionated on SDS–polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad) and blotted onto polyvinylidene difluoride membrane (Millipore). After blocking with 5% non-fat milk in TBST [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20], the membrane was probed with Lc3 (MBL, #PD014) antibody and signal detection was developed with enhanced chemiluminescence (Pierce, #1859024-025) using a Kodak Image Station (Kodak). Densitometric measurements of the bands in Western blot analysis were performed using digitalized scientific software program Kodak 1D 4.0 (Kodak).

2.5. Confocal microscopy

The embryos were treated with 0.003% 1-phenyl-2-thiourea (PTU; Sigma, #P7629) to reduce pigmentation at 12 hpf. After treatment, 4% Paraformaldehyde (PFU; Sigma, #158127) was added into each well to fix the EGFP signal before mounting. All samples were mounted in fluo-save prior to imaging. Images were taken using the Carl Zeiss LSM 510 Meta confocal system.

2.6. Statistical analysis

All data are presented as means ± standard deviations (S.D.). Comparisons of quantitative data were analyzed using Student's *t* -tests between two groups. Differences were considered significant if the *P* value was <.05.

3. Results

3.1. The expression pattern of zebrafish lc3

Sequence alignment indicated that the Atg8/Lc3 is highly conserved among different species, and zebrafish Lc3 showed high similarity with rat Lc3 (80.2%) and human (74.4%) (Fig. 1A). All the Lc3 proteins showed the same cleavage site at the C terminus (Fig. 1A). In addition, the phylogenetic analysis illustrated the common heritage of the Atg8/Lc3 in zebrafish and other species including human, rat and yeast. As expected, zebrafish Lc3 more closely related to mammalian Lc3 (data not show).

To understand the time when the *lc3* occurs in the zebrafish embryogenesis, we analyzed the temporal expression of *lc3* by real time PCR. As showed in Fig. 1B, *lc3* transcripts were constantly detected at a quite abundant level throughout the early development from 2 cell stage to 24 hpf suggesting that *lc3* mRNA was maternally deposited. Moreover, the expression of zebrafish *lc3* in adult tissues also showed a ubiquities expression pattern in all eight examined tissues with a relatively high level in the ovary and liver (Fig. 1C).

3.2. Basal autophagy levels in zebrafish embryonic development stages

To investigate whether the *lc3* transcripts expression is consistent with the protein level and if the conversion of Lc3 occurred during early development, we examined Lc3 with an anti-mammalian Lc3 antibody that cross-reacts with the zebrafish homolog. Lc3-I conversion to Lc3-II occurred from 2 dpf, as faint Lc3-II levels was detected from the immunoblot (Fig. 2A). This observation is consistent with the previous study which the conversion of Lc3-I to Lc3-II was evident at 48 hpf but not at 24 hpf [20]. Moreover, the level of Lc3-II increased from 2 dpf to 4 dpf, suggesting induction of autophagy during this developmental period. Having establishing that the zebrafish possesses Atg genes and expresses Atg proteins and that zebrafish Lc3 undergoes similar post-translational modification like its mammalian counterparts, we then assessed the functional conservation of the zebrafish autophagic machinery and its amenability to chemical manipulation. Larvae treated with an autophagy inducer, rapamycin, showed an increase of Lc3-II, indicating increased autophagic flux, while larvae treated with both rapamycin and chloroquine showed a significant increase of Lc3-II (Fig. 2B). Thus, the zebrafish autophagic machinery is conserved and is also amendable to chemical manipulation like mammalian cells.

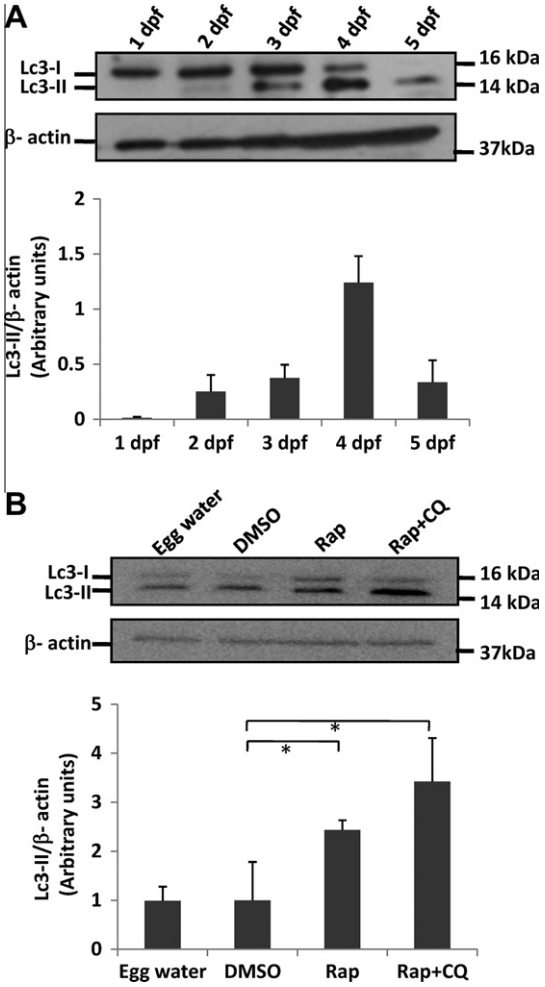

Fig. 2. Autophagy levels in early zebrafish development. (A) The basal autophagy level in early zebrafish development. Lc3-I converts to Lc3-II after 2 dpf. Protein extracts were prepared from different stages of wild-type larvae (1–5 dpf). The Lc3-II to ß-actin ratio was quantified using ImageJ software (http://rsbweb.nih.gov/ij/). Error bars represent the standard deviations of three independent experiments. dpf: days post fertilization. (B) Autophagic flux in zebrafish fry affected by chemical treatment. Three dpf larvae were treated with rapamycin and CQ showed an increase in Lc3-II levels. $^{*}p < 0.05$. β-Actin served as loading control. Rap: 1 μM Rapamycin; CQ: 50 μM chloroquine.

3.3. Generation of Tg(fabp10: EGFP-Lc3) transgenic zebrafish

To facilitate the investigation of autophagy in the liver, an EGFP-Lc3 fusion construct under the liver-specific zebrafish *fabp10* promoter was generated and injected into zebrafish embryos for establishing a stable transgenic line. Injected founders were crossed with wild type fish and their F1 offspring were screened for germline transmission by PCR, as shown in Fig. 3A. We screened 11 possible founders expressing *GFP-Lc3* after injection, and among

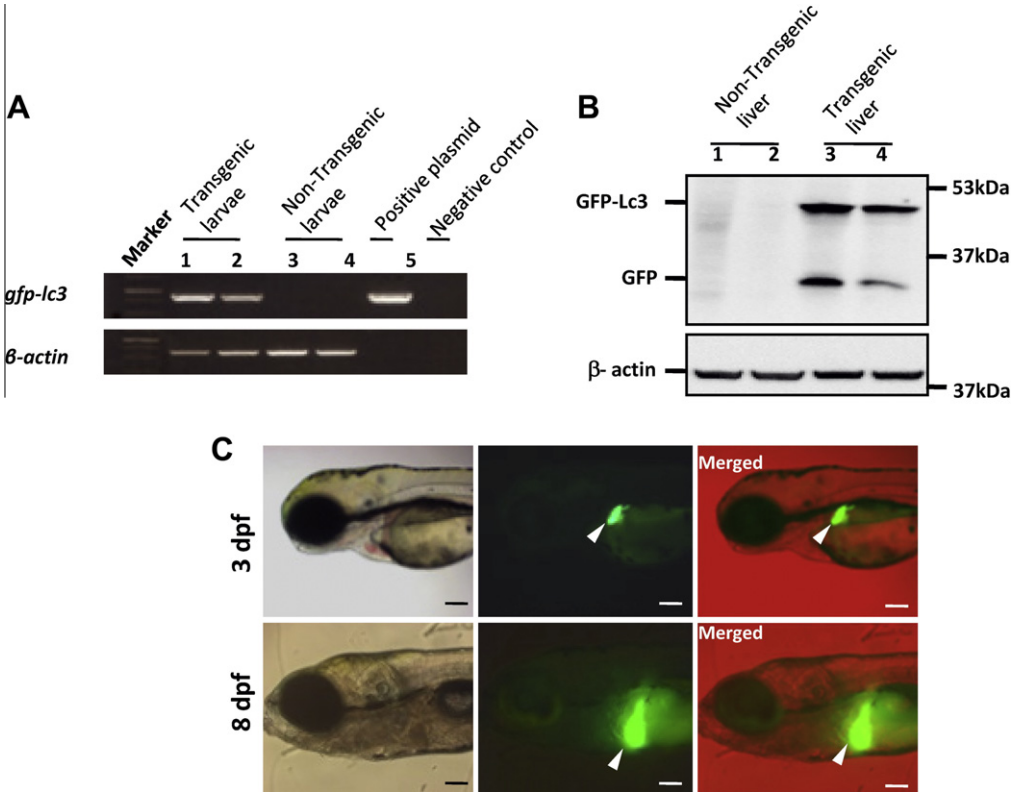

Fig. 3. Establishment and characterisation of Tg*(fabp10:EGFP-Lc3)* transgenic zebrafish. (A) PCR screening of F1 progeny for detection of the transgenic cassette *fabp10-EGFP-Lc3*. Genomic DNA were prepared from the transgenic larvae (line 1 and 2) and non-transgenic larvae (lines 3 and 4) for PCR analysis. (B) Detection of EGFP-Lc3 fusion protein from transgenic progeny. Protein extracts were prepared from the liver of wild type fish (lines 1 and 2) and transgenic fish (lines 3 and 4), and were subjected to Western blot analysis using anti-GFP antibody. (C) Liver-specific expression of EGFP from 3 dpf onwards in *Tg(fabp10: EGFP-Lc3)* transgenic zebrafish fry. The EGFP signal in the liver was observed under a fluorescence microscope and indicated with white arrows. Left panels: light field; middle panels: dark field pictures for observation of GFP fluorescence; right panels: merged pictures from the left and middle panels. Scale bar = 100 μm.

them, five produced progenies with *GFP-Lc3* expression in the liver were identified and showed germline transmission of the *fabp10:GFP-Lc3* gene. One founder was chosen for further characterization and chemical treatment experiments. From this founder, a specific band of expected size was amplified by PCR from genomic DNA derived from the transgenic F1 offspring but not from non-transgenic siblings (Fig. 3A). Further, the *EGFP-Lc3* transgene expression was confirmed by Western blot using anti-GFP antibody (Fig. 3B). Moreover, the *EGFP-Lc3* transgene expression in the liver was visualized from 3 dpf when the liver was well developed (Fig. 3C). All these observations supported the successful establishment of the transgenic zebrafish line, *Tg(fabp10: EGFP-Lc3)*, with liver-specific expression of EGFP-Lc3.

3.4. Autophagy in transgenic fish is up-regulated by Torin1 and suppressed by CQ

Since mTOR inhibitors are the most well-established autophagy inducers. In this study, to examine the potential role of the newly generated transgenic line in investigation of autophagy, we examined the autophagic flux affected by mTOR inhibitor Torin1 as well as a lysosomal inhibitor, CQ. The EGFP-Lc3 puncta (indicative of autophagosome formation) and the conversion of Lc3-I to Lc3-II were examined by confocal imaging and Western blot, respectively. While the control group with DMSO vehicle treatment did not show any obvious EGFP-Lc3 puncta (Fig. 4A), Torin1 treated liver cells showed a significant increase of EGFP-Lc3 puncta compared to the control group (Fig. 4B). Further, In order to confirm that the enhanced autophagy markers observed were indicative of increased autophagy flux, we also treated the transgenic larvae with a lysosomal inhibitor, CQ, to inhibit autophagy flux. As shown in Fig. 4C, there was an increase of EGFP-Lc3 puncta in liver cells with CQ treatment alone, due to the blockage of the basal level autophagy. By treatment with Torin1 and CQ together, there was a more significant increase in both the number and GFP intensity of Lc3 puncta than the groups treated with CQ and Torin1 alone (Fig. 4D). We quantified the number of GFP-Lc3-positive puncta in three independent fish and found that this number was slightly but significantly increased in the livers with chemical treatment (Fig. 4E). Consistently, treatment with Torin1 alone and/or combined with CQ resulted in a substantial increase in the Lc3-II to β-actin ratio (Fig. 4F). All these data clearly suggested that the changes of EGFP-Lc3 puncta reflect the autophagy level induced by TOR inhibition. Therefore, these data further suggested that *Tg(fabp10: EGFP-Lc3)* transgenic line could serve as a valuable tool to study autophagy in liver *in vivo*.

4. Discussion

The zebrafish has become a popular model system to study human diseases, especially cancer [30]. Moreover, many autophagy-related genes, including *ulk1/atg1*, *atg3* and *lc3/atg8* are present in the zebrafish genome, suggesting that the evolutionary conservation of *Atg* genes in autophagy regulatory pathway in zebrafish and thus the zebrafish may be useful as a suitable model system for studying the functional mechanism of autophagy [15]. Hence, for the first time, we generated a transgenic zebrafish line with liver specific expression of *EGFP-Lc3*. We used several approaches to characterize this *Tg(fabp10: EGFP-Lc3)* zebrafish transgenic line.

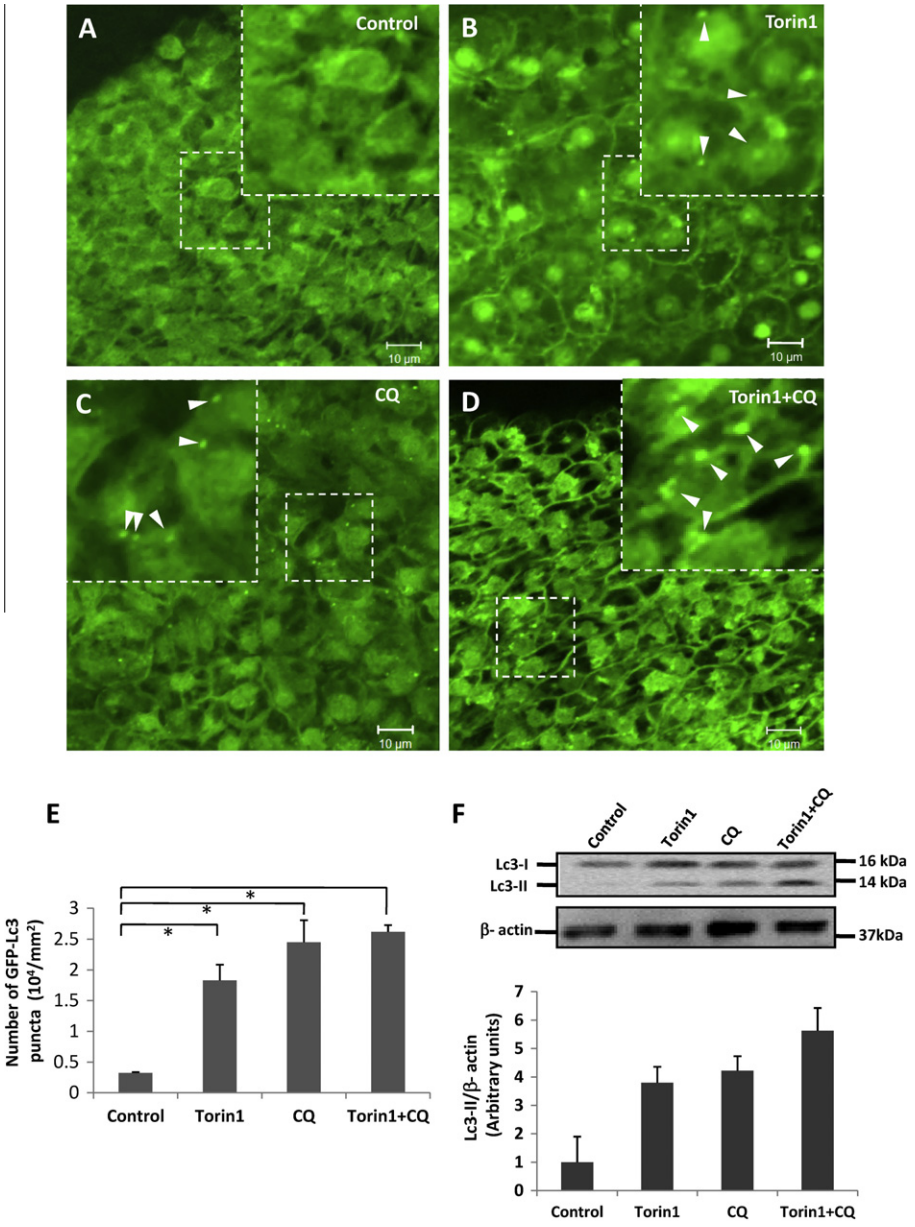

Fig. 4. Autophagy level in *Tg(fabp10: EGFP-Lc3)* transgenic zebrafish. EGFP-Lc3 larvae at 5 dpf were treated with autophagy inducer, Torin1 and lysosome inhibitor, CQ, as designated for 48 h. EGFP-Lc3 puncta in the liver were compared in the control group (A) and the groups treated with Torin1 (B), chloroquine (CQ) (C), and combination of Torin1 and CQ (D). (E) Quantitative analysis of the formation of GFP-Lc3 puncta in different treatment. The number of GFP-Lc3 puncta was counted and divided by the corresponding area. The *Y*-axis indicates the number of GFP-Lc3 puncta (10^4/mm^2). Each value represents the mean SD of at least three fish. $^*p < 0.05$ (F) Induction of Lc3 I to II conversion with control, Torin1 and CQ treatment in *Tg(fabp10: EGFP-Lc3)* larvae. Protein extracts were prepared from the different treatment groups were subjected to Western blot analysis using anti-Lc3 antibody. The Lc3-II to β-actin ratio was quantified using ImageJ software (http://rsbweb.nih.gov/ij/). The DMSO-treatment was taken as an internal control. Error bars represent the standard deviation (S.D.) of three independent experiments. The EGFP-Lc3 puncta was indicated by white arrow head.

Germ-line transmission of the transgene was determined by genomic PCR, and liver-specific EGFP expression was observed from 3dpf by fluorescence microscopy. In addition, EGFP-Lc3 protein was confirmed by Western blot analysis. Furthermore, with the autophagy inducer treatment, the important autophagic flux marker protein, Lc3, as well as the GFP-Lc3 puncta were significant increased in Torin1-treated group. Thus, the results of chemical treatment in this transgenic line indicated that the newly established transgenic zebrafish can be used as a good model system to study autophagy *in vivo*.

Several methods are now available for analyzing autophagy, and among them, use of marker proteins to monitor autophagosomes is widely used [6]. Detection changes of GFP-Lc3, a homolog of yeast Atg8, allows for the identification of autophagsomes and autophagy flux by fluorescence microscopy [28,31]. This method has been applied to whole animals by generating GFP-Lc3 transgenic animal models including mice and zebrafish [20,32]. Since the liver is one of the most critical organ in responsive to starvation and many other important autophagy-inducing factors [21,22], The current transgenic zebrafish line with specific expression of *EGFP-Lc3* in the liver should be a valuable tool in analyzing autophagy in an *in vivo* animal model. We believe that this model has several advantages. Firstly, using this transgenic line, we can examine the Lc3 puncta in the liver tissue in real time under a fluorescent microscope. Secondly, this transgenic line provides an *in vivo* model to study the role of autophagy in liver physiology and pathology.

Thirdly, using this transgenic line, we are able to create double-transgenic lines with liver specific expression of oncogenes to study liver cancer [33–35]. At last, this transgenic line would be an ideal organism for small molecule or drugs screens *in vivo* since drug administration can be used simply by aqueous exposure.

Recently, autophagy has been implicated in the etiology of a number of key diseases including cancer. Autophagy might play a dual role in cancer development: autophagy can exert important cancer suppressive functions according to many genetic evidences, while on the other hand, autophagy can be considered as a cancer-promoting mechanism by providing the survival edge for the cancer cells at the advanced stage [36]. However, most of the evidence supporting such a notion is from *in vitro* cell culture or cancer cells xenograft models, there is very limited information in animal models with primary tumor. To extend the usage of this transgenic line in cancer research, we have successfully crossed this EGFP-Lc3 transgenic line with another transgenic line with inducible liver-specific over-expression of X*mrk*, the homolog of mammalian epidermal growth factor receptor (EGFR) [33,37]. Our unpublished data also showed that autophagy plays an important role in the liver cancer development. Hence, we strongly believe that the availability of the *Tg(fabp10: EGFP-Lc3)* transgenic line will provide useful *in vivo* model systems to study the role of autophagy in cancer.

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