Biochemical and Biophysical Research Communications 421 (2012) 768–772

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Autophagy in the intestinal epithelium is not involved in the pathogenesis of intestinal tumors

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ARTICLE INFO

Article history:
Received 12 April 2012
Available online 21 April 2012

Keywords:
Autophagy
Intestinal tumor
APC
Intestinal epithelium

ABSTRACT

Autophagy has been demonstrated to be associated with the pathogenesis of cancer, but no consensus has been reached about its precise role. Therefore, we investigated whether autophagy in the intestinal epithelium is involved in the pathogenesis of intestinal tumors. To evaluate the relationship between autophagy and intestinal tumors, GFP-LC3-APC$^{min/+}$ mice were generated by mating GFP-LC3 transgenic mice with APC$^{min/+}$ mice. Autophagy was weakly induced in the intestinal polyp regions of the mice in comparison to their non-polyp regions. Under starved conditions, autophagy was not induced in the polyp regions, whereas it was observed in the non-polyp regions. Then, to examine whether a lack of autophagy in the intestinal epithelium enhances the induction of intestinal tumor, Atg7flox/flox:vil-cre-APC$^{min/+}$ mice, in which Atg7 had been conditionally deleted in the intestinal epithelium, were generated by mating Atg7flox/flox:vil-cre mice with APC$^{min/+}$ mice. However, there was no significant difference in the number of intestinal polyps between the Atg7flox/flox:vil-cre-APC$^{min/+}$ and the corresponding control Atg7flox/flox-APC$^{min/+}$ mice. These results indicate that autophagy in the intestinal epithelium is not involved in the pathogenesis of intestinal tumors, and future research should focus on regulating autophagy as a form of cancer therapy.

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1. Introduction

Autophagy is the primary intracellular catabolic process responsible for the degradation and recycling of long-lived proteins and organelles, whereas the ubiquitin/proteosome system is the major cellular pathway responsible for the degradation of short-lived proteins [1]. The initial steps of autophagy are the formation and subsequent elongation of a structure called an isolation membrane, which envelops cytoplasmic constituents such as organelles. Subsequently, its edges fuse together resulting in the formation of a double membrane structure called an autophagosome. The autophagosome then fuses with a lysosome, and the enveloped cytoplasmic components are degraded [2]. Autophagy functions during the normal turnover of cellular components, particularly in response to starvation [3], and autophagy-defective yeast cells die quickly during starvation [4]. It was also reported that mice lacking the genes for Atg5 and Atg7, which are essential autophagic machinery components, died within 24 h of birth [5,6]. In addition, autophagy is known to be involved in many physiological processes including the regulation of cell growth, anti-aging effects, and innate immunity [1]. Autophagy is inhibited by the target of rapamycin (TOR), and reduced TOR-signaling led to lifespan extension in yeast, worms, and flies [7]. The TOR signaling pathway is a central regulator of protein homeostasis, and in drosophila, the inactivation of TOR signaling markedly inhibited cell growth [8]. It was also revealed that an interferon-γ (IFN-γ)-inducible GTPase promoted autophagy and that the autophagic machinery effectively eliminates mycobacteria in macrophages [9,10]. Thus, autophagy is of considerable significance in living organisms.

Autophagy has been reported to be associated with various pathological conditions [11]; however, studies of the relationship between cancer and autophagy have produced contradictory results. For example, the conditional deletion of Atg7 in the liver

Abbreviations: TOR, rapamycin; IFN-γ, interferon-γ; GFP, green fluorescence protein; LC3, microtubule-associated protein 1A/1B-light chain 3; H&E, hematoxylin and eosin; FAP, familial adenomatous polyposis; PI3K, phosphoinositide 3-kinase.

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http://dx.doi.org/10.1016/j.bbrc.2012.04.081

resulted in liver tumors [12], and the heterozygous disruption of beclin1 also promoted tumorigenesis in mice [13,14]. On the contrary, it was demonstrated that autophagy promoted tumor cell survival [15,16], and the administration of autophagy inhibitors in combination with chemotherapy suppressed tumor growth and triggered cell death [17,18]. Accordingly, the following points are generally agreed upon, although further research needs to be performed to confirm them: the suppressive actions of autophagy against tumors occur during tumor initiation, and autophagy acts as a survival strategy during tumor progression and metastasis.

In this study, we focused on the involvement of autophagy in intestinal tumors. First, green fluorescence protein (GFP)-microtubule-associated protein 1A/1B-light chain 3 (LC3)-APC$^{min/+}$ mice, which are an early stage colorectal cancer mouse model that overexpress GFP-LC3, were generated, and then the expression of autophagy in intestinal tumor tissue was evaluated by comparing the polyp and non-polyp regions of the mice. Second, Atg7flox/flox:vil-cre-APC$^{min/+}$ mice, in which Atg7 had been conditionally deleted in the intestinal epithelium, were prepared, and then it was investigated whether autophagy in the intestinal epithelium affects the pathogenesis of intestinal tumors.

2. Material and methods

2.1. Animal and experimental design

All treatments in this study were approved by the Institutional Animal Care and Use Committee and carried out in accordance with the Kobe University Animal Experimentation Regulations. All mice were housed and bred at the Animal Unit of the Kobe University School of Medicine in a specific-pathogen free facility under an approved experimental protocol. Female 6- to 8-week-old C57BL/6J mice were purchased from CLEA Japan (Shizuoka, Japan). APC$^{min/+}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). APC$^{min/+}$ mice with a C57BL/6J background were maintained by breeding male mice that were heterozygous for the Min allele with female wild-type C57BL/6J mice. GFP-LC3 transgenic mice (strain: GFP-LC3#53) were purchased from the Riken BioResource Center (Tsukuba, Japan) with the permission of Dr. N. Mizushima (Tokyo Medical and Dental University, Japan). B6.SJL-Tg(Vil-cre)997Gum/J (villin-cre) mice were obtained from The Jackson Laboratory. Atg7flox/flox mice were kindly provided by Dr. M. Komatsu and Dr. K. Tanaka (The Tokyo Metropolitan Institute Medical Science, Japan) [6]. Atg7flox/flox:vil-cre mice, in which Atg7 had been conditionally deleted in the intestinal epithelium, were generated as described in our previous report [19]. GFP-LC3-APC$^{min/+}$ mice were generated by mating GFP-LC3 transgenic mice with APC$^{min/+}$ mice, and Atg7flox/flox:vil-cre-APC$^{min/+}$ mice were produced by mating Atg7flox/flox:vil-cre mice with APC$^{min/+}$ mice. GFP-LC3-APC$^{min/+}$ mice and C57BL/6J mice were starved for 48 h, and then the mice were sacrificed. The small intestine was excised, the polyp and non-polyp regions were obtained under a microscope, and their protein fractions were subjected to Western Blotting analysis. The small intestines of the GFP-LC3-APC$^{min/+}$ mice were also subjected to histological examination or immunofluorescent staining. The 12-week-old Atg7flox/flox:vil-cre-APC$^{min/+}$ mice and the corresponding control APC$^{min/+}$ mice were also sacrificed, and then the number of polyps in their small intestines was counted. These experiments were carried out at least three times.

2.2. Histological examination

The intestinal tissues of the mice were dissected and fixed with 4% paraformaldehyde, and then the paraffin-embedded tissues were sliced at 4 μm and stained with hematoxylin and eosin (H&E) in a blinded manner. The sections were observed using a microscope (BX51; OLYMPUS, Tokyo, Japan).

2.3. Immunofluorescent staining

The intestinal tissues were frozen in O.C.T. compound (Sakura Finetek, Tokyo, Japan) and then sectioned into 10 μm-thick slices. The immunofluorescent staining (dilution ratio) was performed using Alexa Fluor 546-labeled phalloidin (1:400) (Invitrogen, Carlsbad, CA), Alexa Fluor 488-conjugated anti-GFP antibody (1:200) (Invitrogen), anti-β-catenin antibody (1:75) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-beclin1 antibody (1:100) (Sigma Aldrich, St. Louis MO), and anti-p62 antibody (1:500) (MBL, Nagoya, Japan), as described in our previous report [19].

2.4. Western Blotting

The extraction of proteins from the intestinal tissues and the subsequent Western Blotting analysis of LC3-I and LC3-II protein expression were performed as described in our previous report [19].

2.5. Statistical analysis

The results are expressed as the mean ± SE Statistical significance was analyzed using the Student's *t*-test, and a level of probability of 0.05 was used as the criterion for significance.

3. Results and discussion

3.1. Autophagy is readily induced in the non-polyp intestinal tissues of GFP-LC3-APC$^{min/+}$ mice compared with their polyp intestinal tissues

Previously, various research groups have examined the relationship between autophagy and cancer [20], but no consensus had been reached about its precise role. In genetic studies, it was demonstrated that autophagy suppresses tumorigenesis. For example, beclin1+/− mice developed spontaneous tumors, including lymphoma, hepatocellular carcinoma, lung adenocarcinoma, and mammary hyperplasia [13,14]. In addition, it was found that the tumor tissues of breast carcinoma patients displayed lower beclin1 expression than their normal tissues [21]. These results indicate that autophagy contributes to tumor suppression. On the contrary, it was revealed that autophagy is necessary for the survival of cancer cells. For example, the knockdown of essential autophagy genes in tumor cells led to the induction of cell death and the suppression of tumor cell growth [22,23]. Thus, it is unclear whether autophagy suppresses tumorigenesis or promotes tumor cell survival. Regarding colorectal cancer, the protein expression level of Atg5 in colorectal tumor tissues was lower than that in normal tissues, although there was a low incidence of Atg5 gene mutation [24]. Moreover, the decreased expression of Bif-1, an autophagy-related molecule, was detected in human colorectal adenocarcinoma [25]. In contrast, autophagosome formation was observed in the tumor tissues of colorectal cancer patients but not their non-tumor tissues [26]. However, the direct evaluation of autophagy in humans is technically difficult; therefore, we investigated whether autophagy is involved in the pathogenesis of intestinal tumors using a transgenic mouse model. In this study, GFP-LC3-APC$^{min/+}$ mice, which were generated by mating GFP-LC3 transgenic mice with APC$^{min/+}$ mice, were used. APC$^{min/+}$ mice develop numerous intestinal lesions that resemble human familial adenomatous polyposis (FAP) and are a useful model for investigating early stage colon tumorigenesis. The GFP-LC3-APC$^{min/+}$ mice were sacrificed under non-starved conditions. In a histopathological examination with

H&E staining (Fig. 1A, right), excessive adenomatous proliferation characterized by cell dysplasia was observed in the polyp regions of the GFP-LC3-APC$^{min/+}$ mice. A number of β-catenin-positive dots (red) were observed in the polyp epithelium (tumor) (Fig. 1A, right), whereas the non-polyp regions (normal) of the mice displayed an increased number of GFP-LC3-positive dots (green), which are an indicator of autophagosome formation, compared with the non-polyp regions (Fig. 1A, left). Beclin1, which is also known as Atg-6, is another autophagy-related gene. Beclin1 and its binding partner phosphoinositide 3-kinase (PI3K) are required for the formation of autophagosomes during autophagy. Many beclin1-positive dots (red) were also observed in the non-polyp regions (Fig. 1B, left). p62 is a selective substrate for autophagy, and p62-positive dots (red) were absent from the non-polyp regions (Fig. 1B, right). Using Western Blotting analysis, it was found that the conversion of LC3-I to LC3-II was induced in the non-polyp regions of GFP-LC3-APC$^{min/+}$ mice subjected to 48 h starvation (Fig. 2). In the non-starvation conditions, the ratio of LC3-II to LC-3-I was higher in the non-polyp regions than in the polyp regions (Fig. 2). These results strongly suggest that autophagy is more readily induced in non-polyp intestinal tissues than in polyp intestinal tissues.

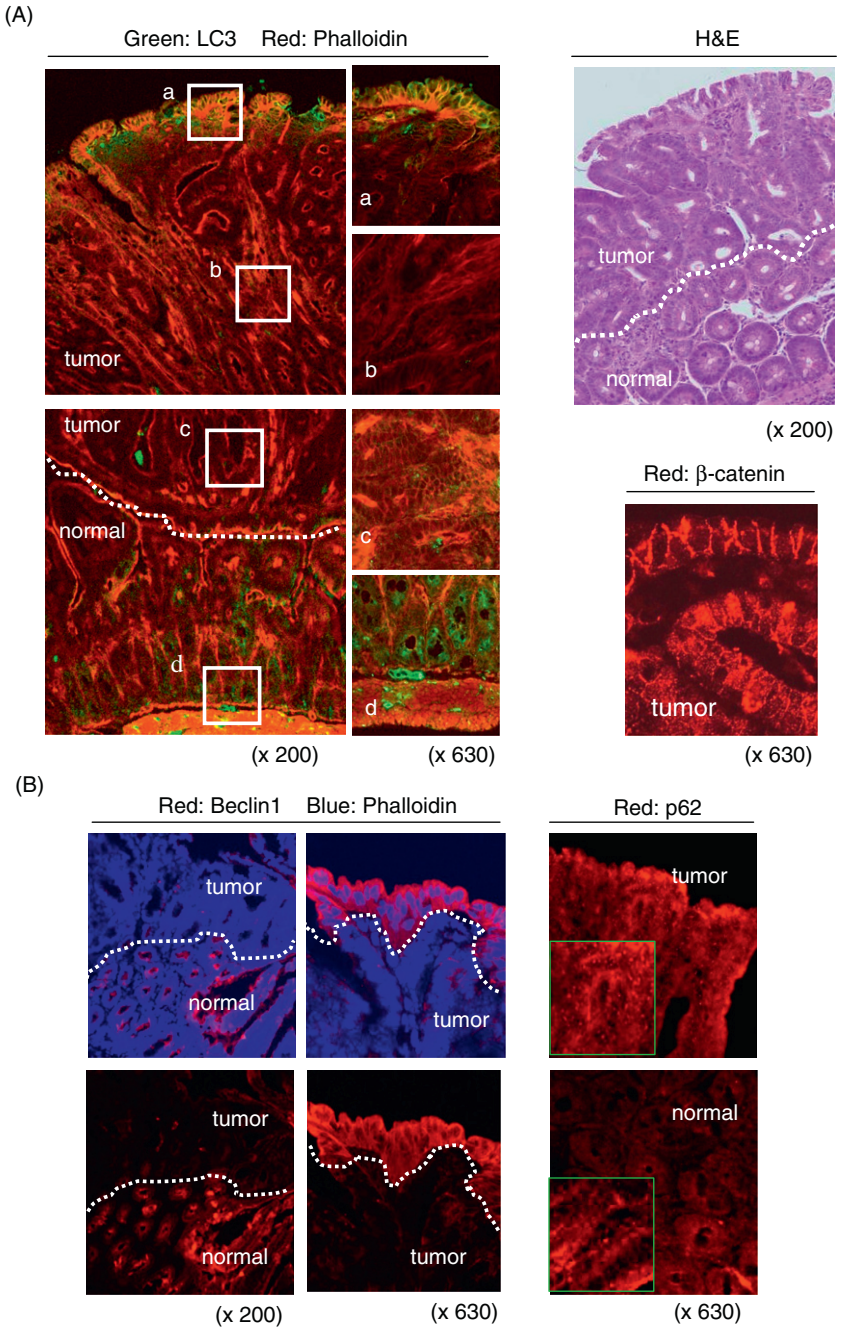

Fig. 1. Autophagy is readily induced in the non-polyp intestinal tissues of GFP-LC3-APC$^{min/+}$ mice compared with their polyp intestinal tissues. (A) An immunohistochemical examination with anti-GFP antibody and anti-β-catenin antibody and a histological examination using H&E staining were performed in the small intestines of GFP-LC3-APC$^{min/+}$ mice. Typical images are shown. Green: GFP-LC3; Red: phalloidin or β-catenin. Magnification: ×200 or ×630. (B) An immunohistochemical examination with anti-beclin1 antibody and anti-p62 antibody was carried out in the small intestines of GFP-LC3-APC$^{min/+}$ mice. Typical images are shown. Red: beclin1 or p62; Blue: phalloidin. Magnification: ×200 or ×630. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

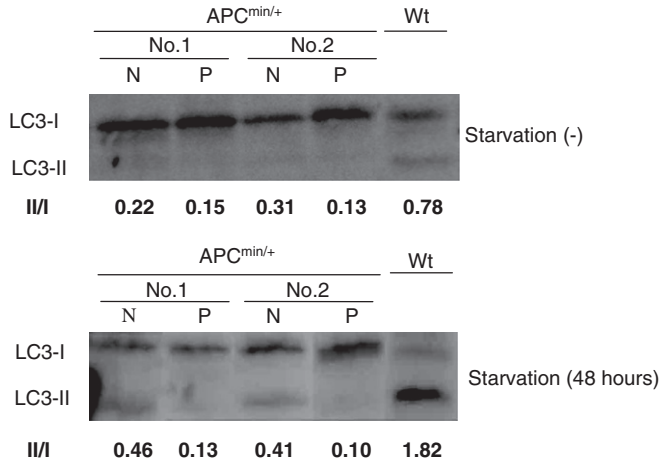

Fig. 2. Starvation-evoked autophagy is weakly induced in the polyp intestinal tissues of GFP-LC3-APC$^{min/+}$ mice. GFP-LC3-APC$^{min/+}$ mice and wild-type mice (Wt) were starved or not for 48 h, and then the proteins in their intestinal epithelial cells were subjected to Western Blotting analysis with anti-LC3 antibody. The relative band intensity of LC3-I compared with that of LC3-II was analyzed, and the values are shown below. N: non-polyp region; P: polyp region. Typical images from the results of two GFP-LC3-APC$^{min/+}$ mice and one wild-type mouse are shown.

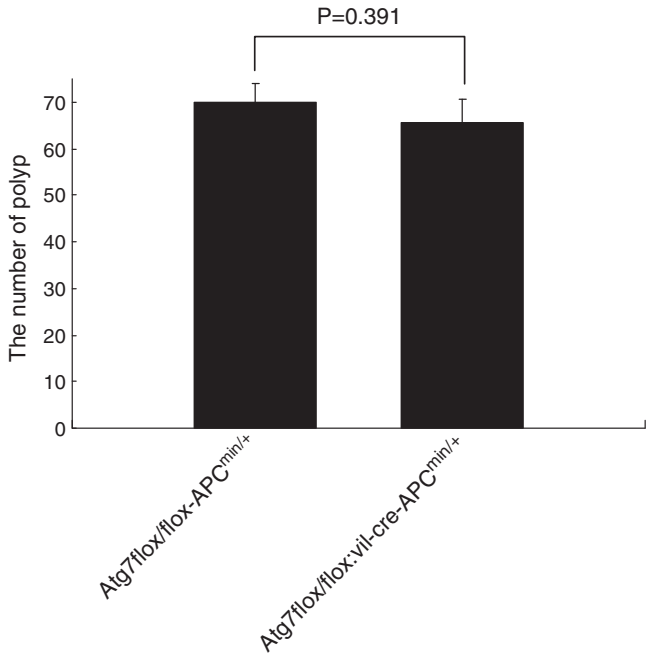

Fig. 3. Atg7flox/flox:vil-cre-APC$^{min/+}$ mice do not display a significantly different number of intestinal polyps. The numbers of polyps in the intestines of Atg7flox/flox:vil-cre-APC$^{min/+}$ mice and the corresponding control Atg7flox/flox-APC$^{min/+}$ mice were counted. Data are shown as the mean ± SE (n = 4). Statistical significance was analyzed using the Student's t-test, and a level of probability of 0.05 was used as the criterion of significance.

3.2. No significant changes were observed in the number of intestinal polyps in Atg7flox/flox:vil-cre-APC$^{min/+}$ mice

As shown in Figs. 1 and 2, the functional depression of autophagy was observed in the intestinal polyp regions of GFP-LC3-APC$^{min/+}$ mice, and decreased autophagy in the intestinal epithelium might lead to cancer initiation, promotion, and progression. Therefore, Atg7flox/flox:vil-cre-APC$^{min/+}$ mice were used to investigate the role of autophagy in the formation of intestinal polyps. Atg7flox/flox:vil-cre mice display a conditional Atg7 deficiency in the intestinal epithelium [19], and Atg7flox/flox:vil-cre-APC$^{min/+}$ mice were generated by mating Atg7flox/flox:vil-cre mice with APC$^{min/+}$ mice. The number of intestinal polyps in the mice was counted at 12 weeks after birth. As a result, it was found that there was no significant difference in the number of intestinal polyps between the Atg7flox/flox:vil-cre-APC$^{min/+}$ mice and the corresponding control Atg7flox/flox-APC$^{min/+}$ mice (Fig. 3), indicating that autophagy in the intestinal epithelium is not involved in cancer initiation, promotion, or progression.

3.3. Autophagy in the intestinal epithelium is not related to cancer initiation, promotion, or progression

In this study, it was demonstrated that autophagy in the intestinal epithelium is not involved in cancer initiation, promotion, or progression (Fig. 3), although autophagy was readily induced in the non-polyp regions of GFP-LC3-APC$^{min/+}$ mice compared with their polyp regions (Figs. 1 and 2). In our previous study, it was demonstrated that the levels of various amino acids were higher in the non-polyp regions of APC$^{min/+}$ mice than in their polyp regions [27]. Autophagy is a catabolic process involving the degradation of a cell's own components by lysosomal machinery, resulting in the production of peptides and amino acids by protein degradation. Therefore, the increased induction of autophagy in non-polyp regions (Figs. 1 and 2) would lead to higher levels of various amino acids in these regions [27]. The autophagic changes observed in this study were consistent with the amino acid alterations detected in our previous study [27]. In the normal state, decreases in the levels of amino acids lead to the induction of autophagy, but autophagy is weakly induced in intestinal polyp regions, resulting in decreased amino acid levels in these regions. The weak induction of autophagy in the polyp regions is also supported by the results obtained by subjecting GFP-LC3-APC$^{min/+}$ mice to starvation (Fig. 2). In a previous study, Hirayama et al. reported that the levels of various amino acids were increased in the tumor colon tissues of colorectal cancer patients compared with those in their normal colon tissues [28], and they proposed that autophagy might be involved in this phenomenon [28]. However, lower levels of autophagy-related molecules were detected in the tumor tissues of breast and colorectal cancer patients [21,24]. Generally, tumor tissues display angiogenesis, and this might greatly affect amino acid levels. The results obtained by Hirayama et al. were inconsistent with our results. The APC$^{min/+}$ mice used in this study are a model of early stage colorectal cancer, and β-catenin nuclear activation was observed in the polyp regions of the GFP-LC3-APC$^{min/+}$ mice (Fig. 1A). Therefore, our results might reflect biological alterations in intestinal tumor tissues because APC$^{min/+}$ mice have early stage colorectal cancer, and the influences described above are small in APC$^{min/+}$ mice.

In the experiments using Atg7flox/flox:vil-cre-APC$^{min/+}$ mice (Fig. 3), it was demonstrated that autophagy in the intestinal epithelium is not involved in the pathogenesis of intestinal tumors. On the contrary, autophagy was weakly induced in the polyp regions of the mice compared with their non-polyp regions (Figs. 1 and 2). These results raise the possibility that a functional decline of autophagy was induced as a result of the intestinal tissue damage caused by the tumor. In tumor tissues, a variety of genes undergo mutation, and such mutations can affect tumor promotion and progression. Mutations in the p53, K-ras, and APC genes are common in colorectal cancer. DNA methyltransferases are one of the factors responsible for these mutations [29], and it was revealed that the mRNA expression level of DNA methyltransferase 1 was significantly higher in colorectal tumor tissue than in the corresponding noncancerous mucosa tissue [30]. In addition, an increased level of DNA methyltransferase 1 was found to be significantly associated with the CpG island methylation phenotype in

colorectal cancer [30]. In a previous study, the administration of a DNA methyltransferase inhibitor to $APC^{min/+}$ mice led to a reduced number of polyps, suggesting that the hyperexpression of DNA methyltransferases is heavily involved in cancer initiation, promotion, and progression [31]. Taken together, the gene mutation of autophagy-related molecules by DNA methyltransferases and/or other factors and the subsequent decreases in their expression levels might lead to a functional decline of autophagy in polyp regions, but these decreases would not be associated with the pathogenesis of intestinal tumors because its decline is due to the intestinal tissue damage caused by the tumor.

In conclusion, our study found that autophagy is weakly induced in the polyp regions of intestinal tissues, but its poor induction is not related to cancer initiation, promotion, or progression. To the best of our knowledge, this is the first cancer study to use intestinal epithelium-specific autophagy-deficient mice, Atg7flox/flox:vil-cre-$APC^{min/+}$ mice. Many researchers have studied the suppression of cancer via autophagy regulation, but research into treatments for colorectal cancer, should pay particular attention to this concept.

Acknowledgments

This study was partly supported by grants for the Global COE Program, Global Center of Excellence for Education and Research on Signal Transduction Medicine in the Coming Generation from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan [T.A., and M.Y.]; a Grant-in-Aid for Scientific Research on Innovative Areas from the MEXT of Japan [Y.F., S.N., and T.A.]; and the Education Program for Specialized Clinicians in the Support Program for Improving Graduate School Education from the MEXT of Japan [Y.F.]. We also thank Dr. M. Komatsu and Dr. K. Tanaka (The Tokyo Metropolitan Institute Medical Science, Japan) for providing the Atg7flox/flox mice.

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