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Cell-Based Assays for Evaluation of Autophagy in Cancers

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Abstract

Autophagy is a cellular mechanism that degrades damaged organelles and misfolded proteins to maintain cellular homeostasis. Autophagy in cancers is drawing increasing attentions due to its multifaceted roles in cancer development, progression, and treatment. There are several key autophagy effectors that are being extensively studied to understand the role of autophagy in cancer as well as their potential value as predictive and/or prognostic biomarkers and therapeutic target. These include ATG4A, ATG4B, Beclin-I, p62, LC3A, LC3B, LC3C, and LAMP. While having its own sophisticated pathway, autophagy has been reported to associate with multiple oncogenic pathways such as NF- κ B, mTOR, and PI3K signaling. This chapter aims to provide a detailed protocol for researchers to investigate the role of autophagy using in vitro cell line as model. Here, we demonstrate several techniques including Western blot (WB), immunofluorescence (IF), and small-interfering RNA (siRNA) knockdown using colorectal cancer cell lines as samples. This chapter provides information to researchers especially those in their early- and mid-career to plan and design their experiments to study the autophagy events in their area of interests.

Keywords: autophagy, cancer, Western blot, immunofluorescence, cell

1. Introduction

1.1. Brief history of autophagy

The term autophagy, or sometimes known as autophagocytosis, comes from the Greek language for “self-eating.” This term was coined by a Belgian biochemist, Christian de Duve in

1963. Prior to this term, however, autophagy was first observed, or at least hinted in as early as 1955 [1]. Kleinfeld and his colleagues found that the process involves three continuous stages of maturation and is seen as being used for the reutilization of cellular materials and organelle disposal. In Duve's definition, autophagy is a part of lysosomal function and glucagon being the main inducer of hepatic cell degradation. Together with his student, Russell Deter, they were the first to demonstrate that lysosomes play a central role for intracellular autophagy [2, 3]. Using Duve's work as reference, independent scientist groups discovered autophagy-related genes in yeast. In that period, Ohsumi and Michael Thumm studied on nonselective autophagy induced by starvation [4, 5]. At the same time, Klionsky discovered a form of selective autophagy called the cytoplasm-to-vacuole targeting (CVT) pathway [6, 7]. Not longer after, they discovered that their independent work actually revolves around the same pathway. Through collaborating, they published a paper titled "Cytoplasm-to-vacuole targeting and autophagy employ the same machinery to deliver proteins to the yeast vacuole" [8]. In 2003, a unified nomenclature was advocated by scientists in the field to use ATG for autophagy-related genes [9]. More than a decade later, in 2016, Ohsumi was finally awarded a Nobel Prize in Physiology or Medicine for his contribution toward the field of autophagy. While it is undeniable that Ohsumi deserves the Nobel Prize, some individuals have pointed out that the prize should have been more inclusive of other researchers who made Ohsumi's work possible [10]. In the second millennium, there was an accelerated growth of research in autophagy thanks to the work and contribution by these scientists on ATG genes. With the fundamentals on autophagy set strong, scientists began to study its association with human health and diseases. The first breakthrough discovery associating autophagy with cancer

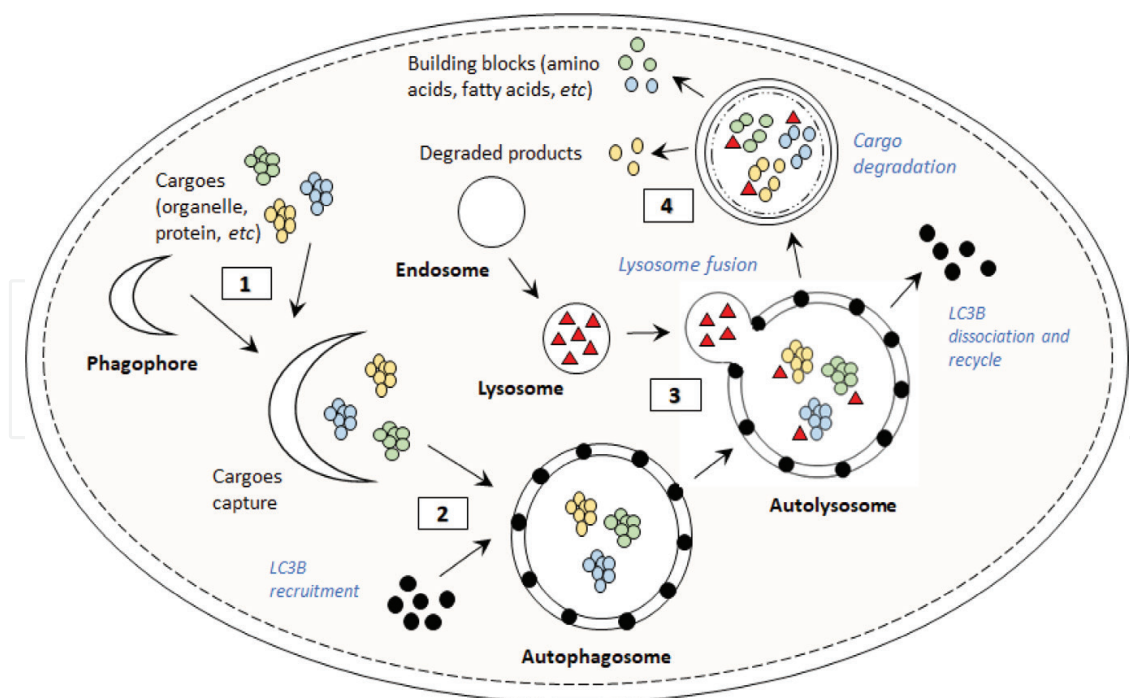


Figure 1. Cellular autophagy processes. (1) Phagophore forms and elongates to package the cargoes comprising damaged organelles and proteins. (2) LC3B proteins are then recruited and together with phagophore, it forms autophagosome. (3) LC3B proteins are then dissociated, and the cytoplasmic cargo is sequestered to fuse with endosome-derived lysosome to form autolysosome. (4) Hydrolytic enzymes then degrade the cargoes and release metabolic products and building blocks such as amino acids and fatty acids for nutrient recycling.

was landmarked by Beth Levine's group in 1999 [11]. To date, the link between cancer and autophagy remains to be the top focus of autophagy researchers.

1.2. Autophagy pathway

Autophagy is an evolutionary conserved mechanism involved in maintaining homeostasis and metabolism at the cellular level by degrading proteins with long half-life and clearing cytoplasmic organelles through lysosomes. Lipids, nucleotides, and glycogen are also subjected to lysosomal degradation via autophagy. Like other pathways involved in homeostasis, the cellular pathways are highly regulated. There are evidences that the recycling of proteins and other macromolecules may contribute to protective roles in normal development, senescence, cell death, and as a defense mechanism against intracellular infections. **Figure 1** shows the detailed processes involved in the autophagy pathway. The dysregulation of autophagy has been shown to cause cancer, inflammatory, metabolic, and neurodegenerative diseases [12, 13].

2. Role of autophagy in cancers

2.1. Autophagy as tumor suppressor

The suppression of autophagy has also been linked to an increase in oxidative stress, genome instability, and activation of the DNA damage response. The increase in oxidative stress leads to a cascade of reaction which may promote tumor growth [14]. A lack of autophagy in hepatocytes can also cause cell death and inflammation, which are known to progress to liver cancer [14]. Deficiency in autophagy has also been shown to promote the accumulation of p62, an autophagy substrate used as a reporter for autophagy activity [15, 16]. The aberrant accumulation of p62 is linked to increase toxicity and tumorigenesis [15]. In other conditions, the expression of p62 increased oxidative stress and tumor growth [17], while a suppression of p62 has been shown to suppress tumorigenesis in modified mouse with hereditary lung cancer [18]. Besides being associated to increased oxidative stress, p62 also acts as a signaling adaptor for the regulation of many oncogenic pathways, including NRF2, mTOR, and NF- κ B [19]. That being said, how the dysregulation of p62 contributes toward tumorigenesis is still not known.

Other than BECN1 and p62, other well-known oncogenes and tumor suppressor genes have also been reported to interrupt upon autophagic pathways, which include the PTEN/PI3K/Akt pathway, Ras, Myc, and DAPk. The autophagy-impinging activities from these genes may have been caused by malignant transformation [20]. PTEN is a tumor suppressor gene that inhibits the pro-proliferation PI3K/Akt pathway and has been shown to promote autophagy in HT-29 colon cancer cells [21]. Thus, the loss of PTEN or upregulation of PI3K can contribute to malignancy through the inhibition of both autophagy and apoptosis. Myc on the other hand has been shown to promote apoptosis, autophagic cell death, and even oncogenesis. In rat fibroblasts, the overexpression of Myc improved autophagic activities [22]. It is interesting to note that the autophagic-inducing domain of the Myc gene is different to that of apoptosis and oncogenesis. The oncogenic Ras protein, including KRAS and NRAS, has been implicated in the promotion of p53-independent non-apoptotic autophagic cell death, which cannot be blocked by Bcl-2 overexpression. This is exemplified in a study where non-apoptotic

neuroblastoma degeneration undergoing autophagic cell death showed high expressions of RAS [23]. Similarly, this phenomenon was also observed in neuroblastoma and HT-29 colon cancer cell lines in vitro [20]. DAPk was initially identified as a cell death-promoting protein before its role in mediating autophagy and tumor suppression was characterized [24]. Like other cases of tumor suppressors, DAPk expression was found to be reduced in several cancer cell lines including but not limited to bladder, breast, renal, lung, ovarian, cervix, colon, head and neck, prostate, and brain cancers. The reintroduction of DAPk into highly metastatic cell lines ameliorated metastasis and tumorigenesis and improved cell death. These data suggest that DAPk has antimetastasis and tumor suppressor properties [20].

2.2. Autophagy as tumor promoter

Contrary to what was discussed above, cancer cells also rely on autophagy for survival and in many cases can be more dependent than normal cells. This is possibly due to the heavy reliance of tumors on nutrient supplies for the maintenance of rapid cell proliferation. In tumors inflicted with hypoxia, autophagy is essential for tumor survival [25]. In RAS-transformed cancer cells, autophagy was shown to be upregulated, and this promotes their survival, growth, invasion, and metastasis. The upregulation of autophagy in RAS-driven cancers has also been reported to ameliorate mitochondrial metabolic defects and the resulting sensitivity to stress. The genetic mechanism on how autophagy dependency arises is still at its infant stage [15].

In a study specific on the role of autophagy in GEM models for RAS-driven non-small-cell lung cancer (NSCLC), the essential autophagy gene ATG7 was deleted together with mutant KRAS in tumor cells. They observed that the absence of ATG7 causes an aberrant accumulation of defective mitochondria, and this leads to the accelerated activation of p53, cell growth arrest, and cell death. ATG7 loss also results in reduced tumor burden by facilitating the conversion of adenomas and carcinomas to a rare form of benign neoplasms called oncocytomas, characterized by the accumulation of nonfunctional mitochondria [26]. While it may seem beneficial to reduce the expression of ATG7 in cancer cells, there is no extension to the life-span of the mice as they die of pneumonia, possibly triggered by inflammation caused by autophagy defects [15]. The deletion of an alternative autophagy gene ATG5 in the same setting also resulted in a similar reduction of tumorigenesis, suggesting that tumorigenesis is mediated by autophagy itself and not just by ATG7 alone. Similarly, the activation of autophagy in GEM models of RAS-driven pancreatic cancer also suppresses p53 activation [27]. In a separate study, it was found that the allelic loss of BECN1 promotes the activation of tumor suppressor p53, and this in turns reduced tumorigenesis [28]. However, large-scale genomic analysis to date has not been successful in identifying recurrent mutations in essential autophagy genes such as BECN1 [29].

2.3. Potential targets and biomarkers in cancers

Oftentimes, the pathways of important cellular processes such as apoptosis and inflammation that can be linked to diseases are studied extensively in hopes of searching for biomarkers or potential drug targets. Autophagy is no exception, as its role in cancer has been demonstrated in the previously mentioned studies; several groups are investigating their predictive and prognostic values. As of 2017, most of the characterized autophagy-related protein markers are prognostic in nature but are not approved for use in clinical settings. To date,

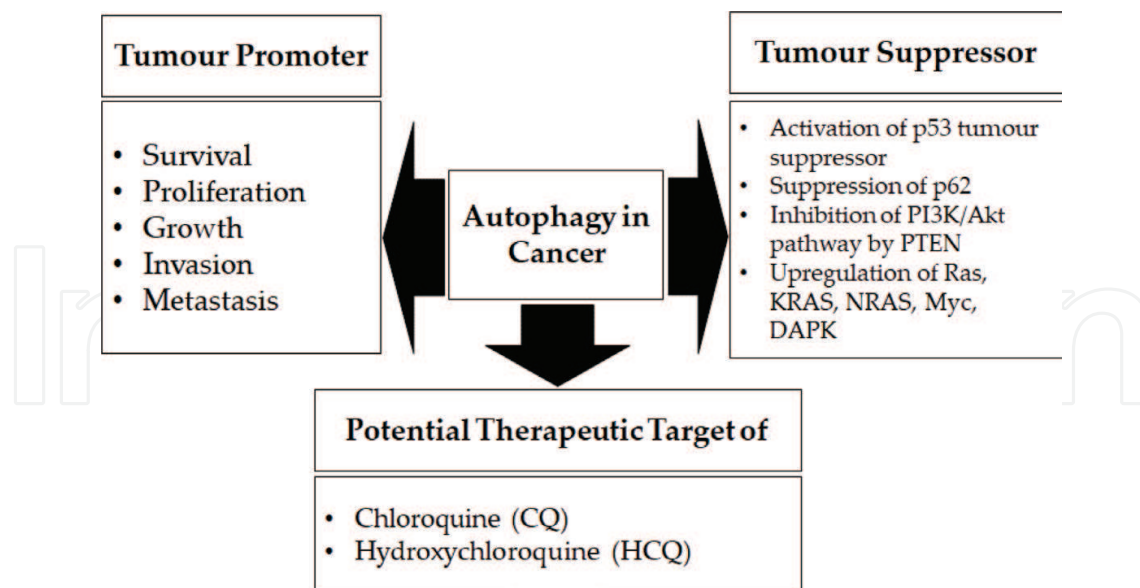


Figure 2. The role of autophagy in cancers and its therapeutic value.

drug development wecules targeting autophagy proteins. An example of lysosomal inhibitors will be the chloroquine (CQ) and hydroxychloroquine (HCQ), both of which are the main characters in clinical trials. The derivative of chloroquine Lys05 is currently going through optimization for clinical trials [30]. LC3B and p62 are common autophagy markers generally used in some assays to assess autophagy turnover, and these two can be useful to monitor the effectiveness of autophagy treatment. Potential targets for autophagy proteins to be discussed in the next section include LC3A, LC3B, p62, ULK, ATG4, ATG7, and Beclin-1. **Figure 2** summarizes the tumor promoting and suppressing roles of autophagy in cancers as well as their therapeutic potentials.

3. Autophagy markers

3.1. LC3B and LC3A

LC3B is an extensively studied autophagy-related protein and is the shortened name for microtubule-associated protein 1 light chain 3B. Together with p62, LC3B has been used as an autophagy marker in several laboratory assays to measure autophagy flux. While in general a high expression of LC3B in cancer patients represents a bad overall prognosis, there are exceptions. This is especially apparent in breast cancer, where the genetic disposition of the cancer subtype will determine the prognostic values of LC3B expression. In the context of colorectal cancer, poor prognosis with high LC3B expression was observed only in KRAS-mutated samples but showed no changes in KRAS-WT specimens [31]. Interestingly, a high expression of LC3B was found to be in favor of the patient's prognosis in NSCLC [31]. With these different findings, LC3B cannot be used as a general prognostic biomarker across all cancer types or even within cancer subgroups. It will be important to study and identify its prognostic value with attention to different cancer types and subtypes.

LC3A on the other hand is another autophagy marker that is being studied, albeit not as extensive as LC3B. IHC staining of LC3A can be defined into three distinct staining patterns: juxtanuclear staining, staining of “stone-like” structures (SLS), and diffuse cytoplasmic staining [32]. All three patterns translate to different prognostic values. For example, a high staining pattern of juxtanuclear LC3A in colorectal [33] and breast cancers [32] correlates with good prognosis; however, the opposite is true if it was SLS. Accumulation of SLS is also linked to poor prognosis in other cancer types including gastric [34], NSCLC [35], hepatocellular carcinoma [36], and clear cell ovarian carcinoma [30, 37].

3.2. p62/SQSTM1

p62/SQSTM1 was initially characterized as the mediator of NF κ B signaling and is now known as the signaling hub for a variety of cellular events including oxidative stress response and amino acid sensing [37]. As discussed previously, p62 is a signaling adaptor which links autophagic substrates to autophagic machineries. During autophagy, the degradation of p62 serves as a marker for functional autophagic activities. Thus, in a Western blot, the accumulation of p62 protein can be indicative of autophagy inhibition. While the principle of p62 assays seems straightforward, caution has to be taken during the interpretation by taking into account the regulation of p62 concentration at the transcriptional and post-translational stages. Taking autophagy out of the context, compilations of reports and studies demonstrate the link between high cytoplasmic but low nuclear expression levels with high tumor aggression and poor prognosis. The cancer types studied include endometrial cancer, oral squamous cell carcinoma, NSCLC, and epithelial ovarian cancer. Specifically, in breast cancer, high cytoplasmic expression levels of p62 have been shown to correlate with cancer grade, metastatic status, and reduction in five-year survival. Additionally, it was also significantly associated to high levels of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor-2/3/4 (HER2/3/4) expression. Poor prognosis due to high levels of cytoplasmic p62 expression also holds true in triple-negative breast cancer [38]. In general, the studies available to date indicate high cytoplasmic p62 expression as a marker for poor prognosis. More studies will have to be done on nuclear-localized p62 across cancer types to understand its prognostic values.

3.3. Beclin-1/VPS34

The Beclin-1/VPS34 complex is also one of the most popularly studied autophagy-related genes as it is the central coordinator of autophagy. Normally, Beclin-1 inhibits the anti-apoptotic Bcl-2 proteins; thus, the reduction of Beclin-1 expression can lead to enhanced anti-apoptotic pathways, consequently translating to poor prognosis. Overexpression on the other hand induces tumor hypoxia and acidity, which is likely to promote autophagy. Such events may increase aggressive tumor behavior [39]. Beclin-1 is a favorable prognostic marker in several cancers including lymphoma, lung, breast, and gastric cancer. In the context of colorectal cancer, the prognostic values can be split between poor and favorable. Poor prognosis in colorectal cancer patients was demonstrated in a study by Han et al. [40], and this finding was supported by additional studies by other researchers. The contradiction between colorectal cancer and other cancer types was addressed by Han et al. when he found that high expression level was associated to poor survival in patients undergoing chemotherapy, whereas patients without chemotherapy showed higher overall survival. Since most chemotherapeutic drugs actively induce

autophagy [41], it is possible that the increased autophagic rate caused by high expression levels promoted resistance to chemotherapy. As with p62, expression levels of Beclin-1 in different histologic and genetic subtypes of breast cancer must be examined. To date, Beclin-1 expression was shown to be significantly correlated to estrogen receptor (ER) negativity. Expression levels vary between subtypes, with maximum expression found in triple-negative breast cancer [42].

3.4. ULK-1/ULK-2

ULK-1 and ULK-2 are serine/threonine kinases that are the most upstream components of the autophagy pathway and as such became attractive drug targets. While ULK-1/ULK-2 small molecule inhibitors are being developed, the data on the prognostic value of ULK-1/ULK-2 in different cancers is contradictory and limited. In breast cancer [40] and gastric cancer [43], high expression of ULK-1 appears to associate to good patient prognosis. This contradicts the findings in colorectal cancer [44]. A separate study in colorectal cancer did not demonstrate any correlation to survivability, even after taking into account the status of KRAS. However, they found that high expression levels were linked to lymph node metastasis. High levels of ULK-1 expression were also translated to adverse prognosis in esophageal squamous cell carcinoma [45] and nasopharyngeal carcinoma [46]. As for ULK-2, high expression levels were reported in prostate cancer tissue compared to adjacent healthy prostate tissue, but no correlation to prognosis was possible from this study [47]. With these contradictions in mind, additional studies with bigger patient cohorts will be required to further understand the prognostic values of ULK-1 and ULK-2 in cancer.

3.5. ATG4B

ATG4B is a cysteine protease that plays a central role in autophagy. While it is one of the autophagy proteins that is being targeted as a potential therapeutic protein, not much is known about its prognostic value. Some studies showed elevated expression levels in chronic myeloid leukemia, colorectal, and lung cancer; however, there is no mention of its prognostic value in cancers, including members of the ATG4 family [30].

3.6. GABARAP

GABARAP is a part of the ATG8 protein homologs that consist of the subfamily LC3s and GABARAPs. While LC3 has been widely studied as the marker for autophagy, little is known about the GABARAP. GABARAP subfamily consists of GABARAP, GABARAPL1, and GABARAPL2 (GATE16) [48]. While LC3B is responsible in the elongation of autophagosome, Joachim et al. suggests that GABARAP lipidation onto the phagosome keeps the ULK-1 in an activated state until the dissociation of ULK-1 complex and the closing of the phagosome [49]. GABARAPL1 has been implicated in the formation of tumor in mice exposed to genotoxic DMBA [50]. Knockout GABARAPL1 mice showed a reduced expression of TGF- β 1, which acts as a suppressor of T helper 1 cells (T_H1). The reduced suppression on T_H1 results in increased expression of cytokines IL-2 and IFN- γ , which induces an immune response during the exposure of DMBA on the knockout mice. Salah et al. also suggest that the cytokines promote the expression of Xaf1, which inhibits Xiap, a negative regulator of apoptosis, thus promoting cell death and preventing formation of cancerous cells. On the other hand, Berthier et al. found that GABARAPL1 inhibits the growth of breast cancer cell line, MCF-7, and the expression of *GABARAPL1* gene is downregulated in breast cancer tissues [51].

4. In vitro studies of cellular autophagy

4.1. 2D and 3D establishment using CRC cell lines

CRC cell lines such as HT29, SW48, HCT116, and SW480 are adherent cell lines. By default, they will grow in normal tissue-cultured flat-bottom plates or flask in a monolayer, which is a 2D structure. However, monolayer morphology is not the natural appearance of all the established cell lines. Therefore, scientists have developed 3D models to bridge the difference between in vitro and in vivo experiments. For 3D model establishment, the cells were seeded into a round-bottom ultra-low-attachment 96-well plate and allowed to settle down and grow. The cells were observed every day until clear spheroids can be seen. It is important to capture images every day until clear spheroids can be seen. Some cell lines such as SW480 might not form clear rounded spheroids. Once the spheroids are formed, it is important to not disrupt spheroid formation when handling it in situations such as changing media. This is because the cells were seeded in ultra-low-attachment plate, which means they no longer attach to the bottom of the well. Careless handling of the spheroids might cause disruption to it or worse, the loss of spheroids. Although the process of 3D formation is straight forward, not all cell lines are able to form spheroids. Such example is the SW48, which when seeded into the well, remained in multiple clusters instead of forming spheroids as shown in **Figure 3**. Through the formation of 3D models, scientists can better study the gene expressions and cell behaviors [52], as well as carry out drug response experiments on a model that better mimics the natural

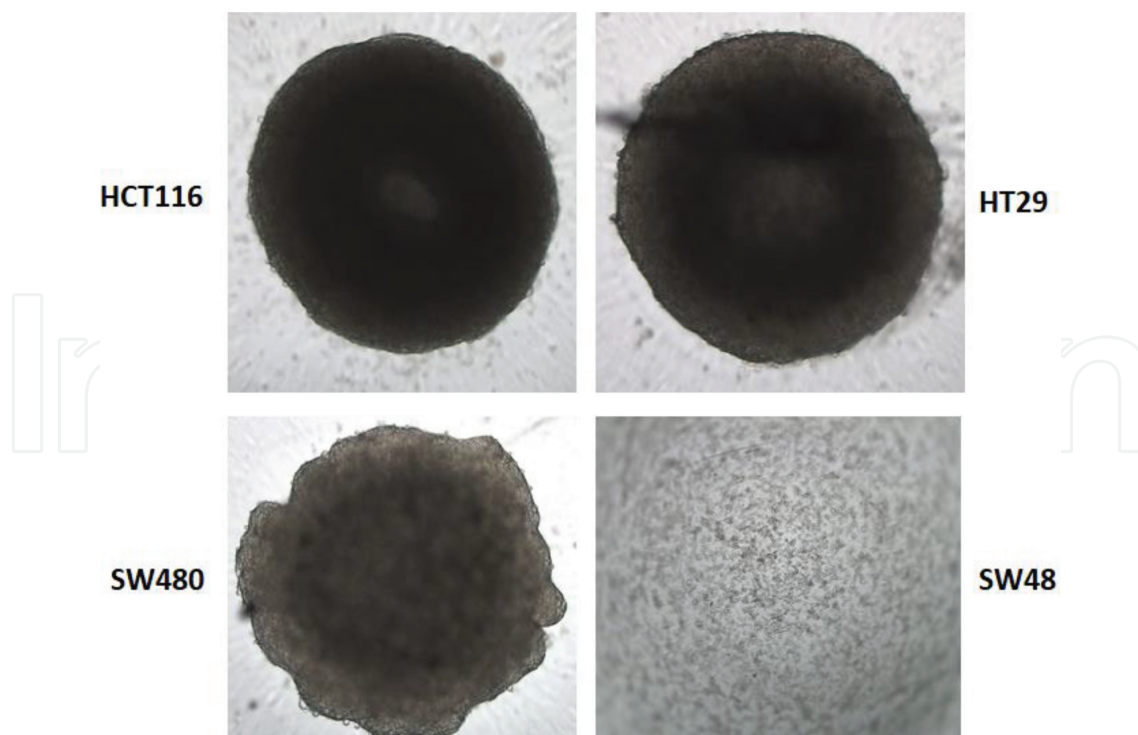


Figure 3. Spheroid formation of colorectal cancer (CRC) cell lines. 1×10^5 cells were seeded into round-bottom ultra-low-attachment 96-well plate and incubated at 37°C at 5% CO_2 with 95% humidity. The cells were observed every day until spheroids were formed. The images above were taken at 72 hours after seeding. HCT116 and HT29 both formed a round spheroid in the well, while SW480 formed an irregular spheroid. No spheroid was formed 72 hours after seeding.

tumor morphology in the human body [53]. 3D models can also be cocultured with other cells to emulate the tumor microenvironment and investigate cell-cell interactions.

4.2. Effect of starvation on autophagy

Starvation is one of the well-known methods that can positively induce autophagy for autophagy-related studies. Common methods of starvation include removal of amino acids as well as serum from the media. The effect of autophagy can be detected as early as 1 hour after removal of the amino acids from the media [54]. This effect can be studied using either Western blot or immunofluorescence (i.e. p62 accumulation).

4.3. Autophagy inhibitors and treatments

Autophagy-related activities can be inhibited by treating cell lines with inhibitors. In the market, there are multiple types of inhibitors that are manufactured to target specific pathways that are related to autophagy in cells. As shown in **Table 1** below, these inhibitors with known mechanisms can be used in vitro.

Cytotoxicity experiments should be carried out by researchers when using such inhibitors as they can reduce the viability of cells tested on. This can be carried out through serial-diluted inhibitors using MTT or any luminescence-based viability assays. The effect of inhibition can then be determined using the safe range of concentration obtained through the cytotoxicity assay followed by Western blot or immunofluorescence.

4.4. siRNA knockdown for autophagy

siRNAs have been developed by companies that specifically targets different genes that produce important proteins that play roles in the activation of autophagy. Some examples of currently available siRNAs are those that target Atg3, Atg5, Atg7, Atg10, Atg12, Atg13, Atg14, and Atg101. When researchers first receive the siRNA, the user should run an optimization experiment to determine a few characteristics of the siRNA. The toxicity of such siRNA should first be tested, and the IC₅₀ should be determined to better understand what concentration range might work the best and have the least impact on cell viability. Cytotoxicity assays such as

Name	Mechanism	References
LY294002	PI3K inhibitor	[55]
3-methyladenine	PI3K inhibitor	[56]
Wortmannin	PI3K inhibitor	[57]
SBI-0206965	ULK-1 inhibitor	[58]
Spautin-1	USP10 and USP13 inhibitors	[59]
SAR405	Vsp18 and Vsp34 inhibitor	[60]
NSC195058	ATG4 inhibitor	[61]

Table 1. Autophagy inhibitors that can be used in vitro.

MTT or any real-time luminescence-based assays can be carried out for this purpose. After that, the user should run the silencing experiments using serial-diluted siRNA followed by Western blot to determine what is the minimum effective siRNA concentration for the best gene-silencing results. These steps should be repeated for every cell line that is going to be tested using the siRNA because there will be some differences between cell lines.

4.5. Evaluation of autophagy by immunofluorescence

Immunofluorescence is a common method of immunostaining. This technique uses the specificity of antibodies to their antigen, a specific biomolecule target within or around a cell for the visualization of the distribution of the target molecules in the cells. Through this technique, researchers can visualize the location of the desired targets as well as qualitatively analyze protein concentration in the cells. The following protocols are carried out in tissue-culture flat-bottom 96-well plate.

4.5.1. Materials

1. LC3B (D11) XP® Rabbit mAb—Cell Signaling Technology, #3868
2. Anti-rabbit IgG (H + L), F(ab')₂ Fragment (Alexa Fluor® 488 conjugated)—Cell Signaling Technology, #4412
3. DAPI—Cell Signaling Technology, #4083
4. Phosphate buffered salts (PBS) tablets—Takara, #T900
5. Tissue culture-treated 96-well flat-bottom plate—TPP, #92096
6. Albumin, bovine serum, fraction V, low heavy metals—Merck, #12659-100GM
7. Methanol, methyl alcohol, Grade AR—Riendemann Chmidt, #M2097-1-2500
8. Triton X-100 (for molecular biology)—Sigma, #T8787

4.5.2. Methods

1. Cells were seeded into 96-well plates and grown to a confluency of ~50%.
2. Treatments such as starvation and autophagy inhibition were carried out in the well prior to fixation.
3. The spent media were removed and the cells were washed with 1X PBS once, before 200 uL ice-cold 100% methanol was added into each well, followed by incubation in -20°C for 15 minutes.
4. The methanol was removed from each well, and the wells were washed for three times with 200 uL 1X PBS incubated for 5 minutes between each wash on the bench.
5. 5% bovine serum albumin (BSA) dissolved in 1X PBS with 0.3% Triton X-100 was prepared, and 50 uL is added into each well for blocking. The cells were incubated for 1 hour at room temperature.
6. Antibody diluent buffer was prepared by dissolving 1% BSA in 1X PBS with 0.3% Triton X-100 while the cells are blocked.

7. Primary antibody was diluted in antibody diluent buffer at the recommended concentration by the manufacturer. LC3B was diluted 1:200 for this experiment.
8. After blocking, the blocking buffer was removed, and 50 uL of the diluted antibody was added into each well. The plate was incubated at 4°C overnight.
9. The primary antibody was removed the next day, and the wells were washed as mentioned in Step 4.
10. Fluorophore-conjugate secondary antibody targeting the primary antibody is diluted in antibody diluent buffer (Step 6) at the recommended concentration by the manufacturer. For this experiment, Alexa Flour 488-conjugated anti-Rabbit IgG antibody was diluted 1:1000. 50 uL of diluted secondary antibody was added into each well and incubated at room temperature for 2 hours in the dark.
11. The Secondary Antibody was removed and the wash step in Step 4 was repeated 5 times.
12. DAPI was diluted to a concentration of 0.5 ug/mL in 1X PBS, and 50 uL was added into each well in the dark. The wells were incubated for 5 minutes at RT in the dark.
13. The wells were washed 1 time according to Step 4 and viewed using a fluorescence microscope in the dark.

Cells were treated with LY294002, which is a cell-permeable inhibitor for phosphoinositide 3-kinase (PI3K) that acts on the enzyme ATP binding site (**Table 1**). As PI3K is strictly required for autophagy, PI3K inhibition sequentially leads to autophagy inhibition. As shown in **Figure 4**, both CCD112 and HT29 showed an increase in LC3B signal after starvation and LY294002 treatment. The signal is correlated to the increase in autophagosome formation after the treatment. However, such result was not obvious for HCT116, which has a high autophagosome formation in the untreated cell lines. This may be a result of continuous activation of autophagy pathway due to KRAS mutation.

4.6. Evaluation of autophagy by Western blot analysis

One of the most widely used method for the examination of autophagy activity is by elucidating the protein expression of the autophagy markers through immunoblotting. The fluctuation in the expression can help in showing the effect of different interventions such as gene silencing or inhibition on autophagy activity. The following protocol for determination of autophagy marker expressions is adapted from the general protocol for Western blotting (Bio-Rad).

4.6.1. Materials

1. Cell lines (ATCC):
 - a. HT-29 (ATCC HTB-38)
 - b. HCT 116 (ATCC CCL-247)
 - c. CCD-112CoN (ATCC CRL-1541)
2. RIPA lysis buffer, 10X—Cell Signaling Technology, #9806S

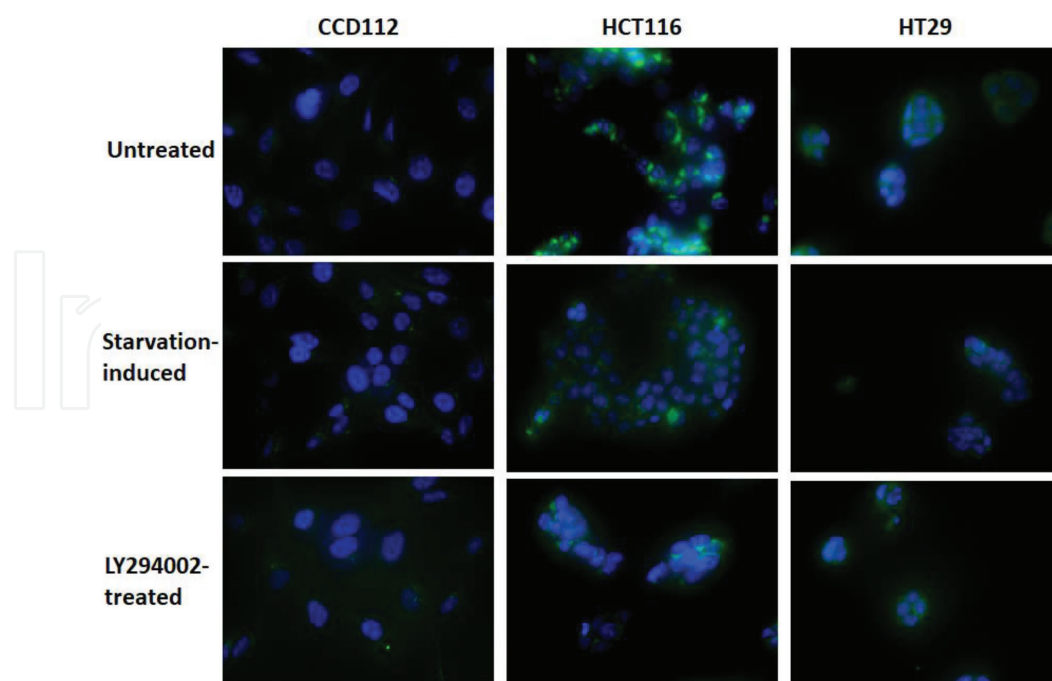


Figure 4. Immunofluorescence analysis of autophagy marker LC3B expression in colon cells after treatment. Immunofluorescence analysis of starvation-induced and PI3K-inhibited colorectal cancer cell lines: HT29, HCT116, and CCD112. Cells were seeded at 70,000 per well and incubated for 24 hours prior to treatment. Each cell line was subjected to two different treatments: starvation in serum-free media and inhibition of PI3K in media containing 50 μ M of LY294002. Cells were incubated for 24 hours prior to fixation and staining. The DAPI-stained nuclei is in blue, while the LC3B expression is in green (Alexa Fluor 488 conjugated antibody).

3. Protease inhibitor cocktail, 100X—Cell Signaling Technology, #5871
4. Pierce BCA protein assay kit—ThermoFisher Scientific, #23227
5. 2-mercaptoethanol—Merck, 60–24-2
6. Tris/Glycine/SDS running buffer, 10X—Bio-Rad, #1610772
7. 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well—Bio-Rad #4561096
8. FlashBlot transfer buffer—Advansta, 150,421–95
9. Immun-Blot PVDF membrane—Bio-Rad, #1620177
10. Blotting Grade Blocker—Bio-Rad, #170–6404
11. Primary antibody (Cell Signaling Technology)
 - a. β -Actin—#3700
 - b. LC3B—#4599
12. Secondary antibody (Cell Signaling Technology)
 - a. Anti-mouse IgG, HRP-linked antibody—#7076S
 - b. Anti-rabbit IgG, HRP-linked antibody—#7074S
13. WesternBright Sirius HRP substrate—Advansta, 170,501–39

4.6.2. Methods

1. In a 24-well plate, the cells were seeded at 50% confluency and left to incubate overnight.
2. The media were then changed accordingly, with normal media, serum-free media, and media with the inhibitor.
3. After 24-hour incubation, the cells were collected and rinsed with PBS.
4. The cells were then lysed with lysis cocktail consisting of 1X RIPA lysis buffer and 1X protease inhibitor cocktail.
5. The lysates were vortexed briefly every 5 minutes for 30 minutes and kept in ice in between mixing.
6. The lysates were then centrifuged at 16000×g for 15 minutes at 4°C, and the supernatants were kept.
7. The lysates were then quantified by using BCA protein assay and immediately diluted with deionized water to obtain 13 µL of 20 µg lysate and then added to 5 µL of 4X SDS loading buffer and 2 µL of 2-mercaptoethanol.
8. The samples were boiled at 90°C for 10 minutes, and 10 µL of the samples were loaded into the well of a gradient gel.
9. The lysates were separated by SDS electrophoresis by using Tris/Glycine/SDS running buffer at 150 V for approximately 45 minutes and transferred to a PVDF membrane by using FlashBlot transfer buffer at 55 V for 1 hour.
10. The membrane was then blocked by using 5% Blotting Grade Blocker in TBST for 1 hour to prevent non-specific binding of antibody.
11. The membrane was then incubated with primary antibody diluted with the Blocker in TBST at 1:2000 dilution.
12. The incubation was done overnight at 4°C or 1 hour at room temperature.
13. Any unbound antibodies were then removed by washing with TBST for 10 minutes once and 5 minutes for another 2 times.
14. Secondary antibodies conjugated with horseradish peroxidase (HRP) were then prepared by diluting the antibody in the Blocker in TBST at 1:3000 dilution.
15. The membrane was then incubated in the secondary antibody:
 - a. Anti-rabbit secondary antibody: LC3B
 - b. Anti-mouse secondary antibody: β-Actin
16. The membrane was incubated for 1 hour at room temperature, and unbound antibodies are washed away with TBST once for 10 minutes and for 5 minutes for another 4 times.
17. The WesternBright Sirius HRP substrate was then dropped on the membrane, and the proteins were viewed by using ImageQuant LAS 500.
18. The blot images were then analyzed using ImageJ software for densitometry analysis.

As shown in **Figure 5**, the level of the LC3B-II proteins is generally lower than LC3B-I in cells grown at normal condition. However, following the inhibition with LY294002, the conversion of the LC3B-I to LC3B-II increases dramatically especially for the cancer cell lines. This shows an increase in the formation of the autophagophore in response to the inhibition. This is similar to the findings of Luo et al. where the inactivation of the PI3K/Akt pathway results in an increase in expression of LC3B-II proteins [62]. Meanwhile, following starvation, the cell lines also showed a marked increase in conversion of LC3B-I to LC3B-II indicating increase in autophagy activity. The increase in formation of autophagosome in cells undergoing starvation and inhibition by LY294002 is due to the effect of the PI3K pathway on the mammalian target of rapamycin complex 1 (mTORC1). In nutrient-deprived cells, I κ B kinase (IKK) expression has been shown to be upregulated, while p85 regulatory subunit of PI3K has been shown to be a substrate of IKK. During starvation, the increase in IKK expression leads to the increase in phosphorylation of the p85 subunit of PI3K leading to inactivation of PI3K pathway [63]. The inactivation of the PI3K pathway inactivates mTORC1, which has an inhibitory effect on ULK1–Atg13–FIP200 complex, an autophagy initiation complex [64, 65]. So the inactivation of the PI3K pathway in both starvation and LY294002 treatment leads to the activation of autophagy by ULK-1 complex. However, the monitoring of only one protein marker is not enough to conclusively indicate the effect of the treatment.

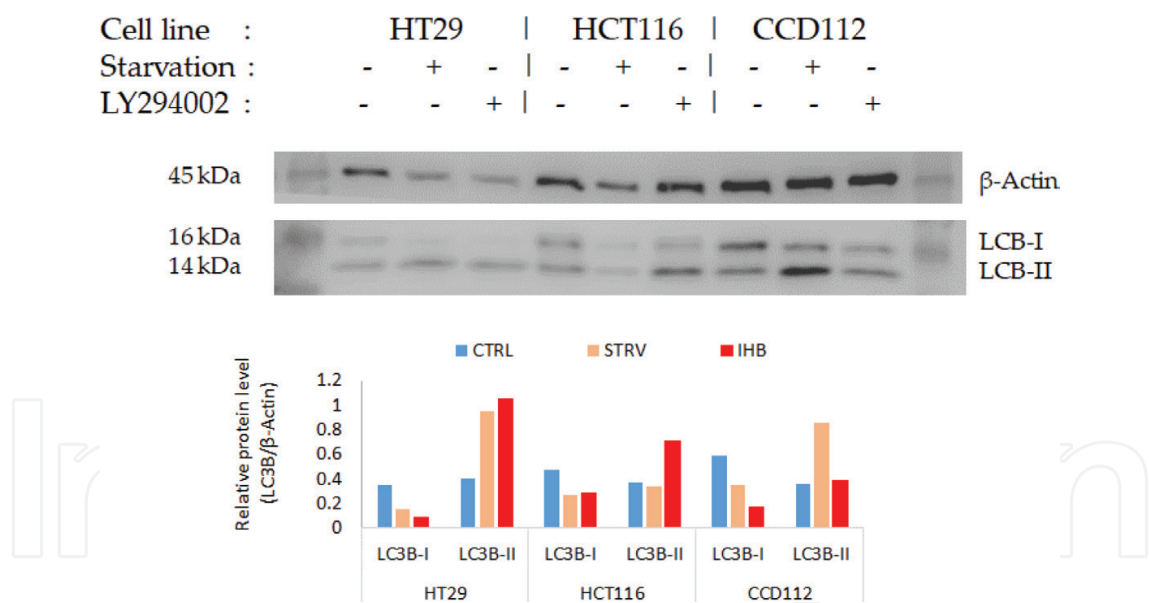


Figure 5. Western blot analysis of LCB-I and LCB-II. Top panel: Western blot analysis of starvation-induced and PI3K-inhibited colorectal cancer cell lines: HT29, HCT116, and CCD112. Cells were seeded at 70,000 per well and incubated for 24 hours prior to treatment. Each cell line was subjected to two different treatments: Starvation in serum-free media and inhibition of PI3K in media containing 50 μ M of LY294002. Cells were incubated for 24 hours prior to harvesting. Cell lysates were run on 4–20% gradient gels under reducing conditions, and proteins were immunodetected on a PVDF membrane with rabbit anti-LC3B Mab (#3868) and mouse anti- β -actin Mab (#3700) from cell signaling technology. Both antibodies were diluted to 1:2000 with 5% milk in TBST. The bands were subsequently visualized with HRP-labeled anti-rabbit IgG antibodies (#7074) for LC3B and anti-mouse IgG antibodies (#7076) for β -actin (cell signaling technology) diluted at 1:3000 with 5% milk in TBST. Bottom panel: Densitometry analysis of protein bands. The analysis was done by using image J, and the relative protein levels were calculated by dividing absolute protein level of LC3B with β -actin.

5. Notes and limitations

The enclosed protocols in this chapter focus on evaluating autophagy using in vitro cancer cell lines but not limited to them. It should be noted that this fundamental cellular mechanism can be detected and studied in other cell types such as leucocytes, fibroblasts, stem cells, and so on. Autophagy is also extensively studied in fixed and live tissues (which are not discussed here) with regard to cancers and other diseases. We have only included Western blot and immunofluorescence protocols because of their simplicity and cost-effectiveness. Due to availability, colorectal cancer cell lines were used as study models for autophagy in this chapter. It must be noted that the expression pattern of studied proteins may vary among cell lines and across different cell types. Hence, the enclosed data should only be used as a reference. Researchers are advised to perform their own optimization experiments and baseline studies based on the given protocols. There are numerous varying parameters that may contribute to varying outcomes including brand and manufacturer of reagents and consumables, ambient conditions, personnel, instrumentation, and so on. Here, we have only targeted one of the autophagy effectors, LC3B for demonstration. It should be noted that there is a list of autophagy-related proteins/mRNA/DNA (described in Section 3) that can be studied according to the researchers' target of interest with respect to the nature of the research project. In addition, a plethora of autophagy-associated inducer or inhibitor (described in Section 4) can be chosen to study a specific protein/mRNA or pathway in autophagy. Last but not least, to further understand how autophagy functions and its association with a disease or disorder, it is always more favorable to study two or more autophagy-related targets concurrently to maximize the gained output and cost-effectiveness. Our enhanced understanding on autophagy and the development of technology allowed the study of autophagy to be made easier through panel assays such as Autophagy Regulators Panel (Millipore), CYTO-ID Autophagy detection kit (Enzo Life Sciences), Autophagy Antibody Sampler Kit (Cell Signaling Technology), and Autophagy Detection Kit (Abcam). Newly engineered study models such as ATG, p62, and ULK-1 knockout cell lines and animals have also been generated for in-depth study of autophagy pathway. In addition, the advancement in bioinformatics also helps in data organization and analysis as well as deciphering the potential interaction of autophagy with other unexplored cellular pathways.

6. Conclusions

The phenomenon of autophagy has been hinted since decades ago and has been a hot area of research ever since the 1990s. Extensive studies have been done on the characterization, mechanism, function, and its association with a multitude of diseases including cancer. While a simple search of autophagy in Google may yield an insurmountable amount of information, its role, mechanism, and function in relation to cancer are still not fully understood and present multiple contradictions between and within different cancer types. The simplified literature review and the protocols enclosed in this book chapter will hopefully help researchers in further understanding the roles and mechanisms of autophagy in different cancer cell types,

identifying new therapeutic targets and predictive/prognostic biomarkers, and developing diagnostic assays and therapeutic drugs.

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Conflict of interest

The authors declare no conflict of interests for this article.

List of Abbreviations

ATG	autophagy-related
ATP	adenosine triphosphate
CQ	chloroquine
EGFR	epidermal growth factor
ER	estrogen receptor
HCQ	hydroxychloroquine
HER	human epidermal growth factor receptor
HRP	horseradish peroxidase
IF	immunofluorescence
IHC	immunohistochemistry
IKK	I κ B kinase
mTORC1	mammalian target of rapamycin complex 1
NF- κ B	nuclear factor kappa beta
NSCLC	non-small-cell lung cancer

PI3K	phosphoinositide 3-kinase
SLS	stone-like structure
WB	Western blot
WT	wild type

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