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Targeting Autophagy in Cancer Therapy

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Targeting Autophagy in Cancer Therapy

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Preface

Over the past decade or so, an ever-increasing body of scientific evidence points to the functional role and unmistakable importance of autophagy in cancer. But can autophagy be successfully exploited as a target in effective cancer therapy? It is now widely believed that modulating the activity of autophagy through targeting regulatory components in the autophagy machinery may impact the development, progression, and therapeutic outcome of cancer. Therefore, autophagy has been considered a novel and promising target for drug discovery/development and therapeutic intervention for cancer; in fact, targeting of autophagy as a therapeutic strategy in cancer has already been explored in-depth and has shown great promise. The purpose of this volume is to provide the latest updates on the current status and a unique perspective on autophagy-based cancer therapy. This volume in the Springer series, *Current Cancer Research,* will cover a wide range of topics, including an overview of autophagy as a therapeutic target in cancer, autophagy modulators as cancer therapeutic agents, implications of micro RNA-regulated autophagy in cancer therapy, modulation of autophagy through targeting PI3 kinase in cancer therapy, targeting autophagy in cancer stem cells, and the roles of autophagy in cancer immunotherapy. In addition, this volume presents a chapter on the application of system biology and bioinformatics approaches to discovering cancer therapeutic targets in the autophagy regulatory network. This comprehensive volume is intended to be useful to a wide range of basic and clinical scientists, including cancer biologists, autophagy researchers, pharmacologists, and clinical oncologists who wish to delve more deeply into this exciting new research area.

Although there are already several excellent books that cover the biology and molecular biology of autophagy and their association with cancer development and progression, this is the first book devoted solely to dealing with targeting autophagy in cancer therapy. As the implications and importance of autophagy in cancer therapy have been increasingly appreciated, this timely and unique volume assembled by leading scientists in this field should prove its usefulness and value in understanding, exploring, developing, and promoting autophagy-based cancer therapy. This volume has the following distinguishing features: (1) it is the first book solely focusing on autophagy as a target in cancer therapy; (2) it is a comprehensive

discussion on the roles of autophagy in currently available cancer treatments; (3) it is a timely complement to the book (volume 8): *Autophagy and Cancer*, 2013, in this series. Finally, I want to sincerely thank all of the authors for their contribution. It is my earnest hope that this volume will serve as a catalyst for further exploration and investigation of autophagy-based cancer therapy.

Hershey, PA, USA Jin-Ming Yang

Contents

Chapter 1
Autophagy as a Therapeutic Target in Cancer

Jenny Mae Samson and Andrew Thorburn

Abstract Autophagy is the process by which cellular material is delivered to the lysosome for degradation and recycling. Macroautophagy involves delivery of macromolecules and organelles to double membrane vesicles called autophagosomes that fuse with lysosomes leading to degradation of the contents of the autophagosomes. Chaperone-mediated autophagy involves direct recognition of specific proteins by chaperone complexes that then directly deliver the protein target to the lysosome. Microautophagy involves direct lysosomal capture of cytoplasmic material. Of these three types, macroautophagy is by far the most studied and is known to have multiple roles in cancer development, progression and response to therapy. This has led to autophagy being widely viewed as a potential therapeutic target in cancer. Important questions that must be answered include: Which tumors should or should not be treated by direct autophagy inhibition? And, what is the best way to target autophagy for cancer therapy? In this overview we summarize the background and some current ideas about the answers to such questions.

Keywords Autophagy • Apoptosis • Cancer therapy • ATG7 • BRAF • KRAS

Autophagy is the process through which proteins, organelles, and other cellular contents are degraded in lysosomes. Macroautophagy involves the formation of double membrane vesicles called autophagosomes that engulf and sequester cellular material. The autophagosomes then fuse with lysosomes, generating autophagolysosomes, in which the lysosomal hydrolases degrade the delivered material into their macromolecular precursors for reuse. While the process of autophagy was first described in the early 1960s, it is only in the past 10–15 years that its role in cellular homeostasis (Kaur and Debnath 2015), as well as in many diseases (Kroemer 2015; Rubinsztein et al. 2012) has been recognized. Two other types of autophagy that do

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not involve autophagosomes have been characterized: chaperone-mediated autophagy and microautophagy. Chaperone-mediated autophagy (CMA) involves the direct recognition of proteins by heat shock protein hsc70 through an exposed amino acid (KFERQ) motif and subsequent delivery of the bound pair to the lysosome through the lysosomal protein LAMP2A (Arias and Cuervo 2011; Kaushik et al. 2011). Microautophagy is less well understood than either CMA or macroautophagy and may involve components of the autophagic machinery and endocytic pathways that allow direct engulfment of cytoplasmic material into the lysosome (Sahu et al. 2011). Most of the work related to autophagy in the context of cancer refers to macroautophagy, though recent work has demonstrated the importance of CMA in tumor growth and progression. Hereafter we use the term "autophagy" to mean macroautophagy.

As we will discuss, autophagy's involvement in cancer is confusing and oftentimes contradictory with both pro- and anti-tumor effects found in different contexts (Hippert et al. 2006; White 2012; Galluzzi et al. 2015) and during cancer therapy (Thorburn et al. 2014). In January 2016 a search of the ClinicalTrials.gov website with the search term "autophagy" returned 60 clinical studies across the world. The majority of these clinical studies deliberately attempt to inhibit autophagy during cancer therapy usually together other anti-cancer treatments. The first cancer clinical trials of autophagy inhibitors were reported in 2014 (Barnard et al. 2014; Rangwala et al. 2014a, b; Rosenfeld et al. 2014; Vogl et al. 2014; Wolpin et al. 2014). These attempts to target autophagy in cancer therapy contrasts with only a few examples where deliberate autophagy manipulation is being attempted to treat other diseases (Kroemer 2015). Thus, despite the fact that arguments can be made for and against inhibiting autophagy in cancer and for the utility of autophagy manipulation in infectious disease, neurodegenerative disease, metabolic disease and many others (Kroemer 2015), it is in cancer treatment where we are furthest along in trying to apply these ideas in a clinical setting. It is also important to note that many current anti-cancer treatments themselves induce autophagy (Shen et al. 2011; Levy and Thorburn 2011). Conversely, some microtubule-targeting drugs such as paclitaxel inhibit autophagy (Veldhoen et al. 2013). This means that we are routinely affecting autophagy in cancer patients through their course of treatment whether we intend to or not. In this chapter, we focus on the deliberate targeting of autophagy and provide an overview of arguments for and against the direct manipulation of autophagy in cancer therapy.

Autophagy is regulated by a large set of evolutionarily conserved genes called *ATG* genes (Mizushima et al. 2011). The ATG proteins represent a variety of types of molecules including lipid and protein kinases and protein conjugating enzymes and scaffolding proteins many of which may represent novel drug targets. Indeed selective inhibitors of a lipid kinase, VPS34, (Bago et al. 2014; Dowdle et al. 2014; Ronan et al. 2014) and the protein kinase ULK1 (Egan et al. 2015; Petherick et al. 2015) were recently shown to inhibit autophagy and to have anti-tumor effects. One important source of confusion in the literature comes from the fact that all known autophagy regulators (i.e. ATG proteins) have other cellular roles as well (Subramani and Malhotra 2013). For example, loss of ATG7 inhibits autophagy, but ATG7 also

regulates p53 via autophagy-independent mechanisms (Lee et al. 2012). So, if *Atg7* deletion in a mouse model of cancer alters tumor growth (Guo et al. 2013a; Karsli-Uzunbas et al. 2014; Strohecker et al. 2013; Xie et al. 2015; Rosenfeldt et al. 2013), is this due to autophagy being inhibited or could it be due to an effect on p53? Similar examples arise with other essential autophagy regulators—e.g. ATG12 regulates apoptosis (Radoshevich et al. 2010; Rubinstein et al. 2011), ATG5 controls MAP kinases (Martinez-Lopez et al. 2013) and mitotic catastrophe (Maskey et al. 2013), while BECN1 controls cytokinesis (Thoresen et al. 2010). These effects are all autophagy-independent and could also affect tumor cell growth/survival. Without a known molecule that only regulates autophagy without affecting other biological activities, current best practice for *in vitro* experiments is to target multiple autophagy regulators and ensure that they all have similar effects on the phenotype being studied before concluding that autophagy affects that phenotype (Thorburn 2008, 2011). Such experimental rigor is more difficult *in vivo* but is, if anything, even more important if we are to avoid misinterpretation of experimental results. For example, it was believed that autophagy is critical for tuberculosis infection based on studies where mice lacking ATG5 were very susceptible to infection. However, more extensive studies targeting multiple ATGs in mice demonstrated that this susceptibility is not due to ATG5's role in autophagy but rather a unique function that is not seen when other ATGs are targeted (Kimmey et al. 2015).

Autophagy is often described as a mostly a non-selective process whereby any cellular material in the vicinity of the forming autophagosomes can be sequestered and eventually degraded. This idea is mistaken and oversimplified, as there are several types of selective autophagy. In particular, there are specific autophagic mechanisms for the degradation of mitochondria (mitophagy), intracellular bacteria (xenophagy) (Randow and Youle 2014), the endoplasmic reticulum and contents of the nucleus (Mochida et al. 2015), lipid droplets (lipophagy) (Singh et al. 2009), and damaged lysosomes (Maejima et al. 2013). These specific forms of autophagy potentially have important effects on tumors; for example, defective mitophagy has been shown to promote breast cancer metastasis (Chourasia et al. 2015). Specific proteins are also targeted for autophagic degradation, such as under conditions of iron depletion, where specific targeting of ferritin to autophagosomes takes place to allow release of iron (Mancias et al. 2014). Even in conditions where one might think that non-selective autophagy would be favored, e.g. amino acid starvation where autophagic degradation of any proteins would, at least in principle, provide amino acids to the cell, autophagy is highly selective such that some proteins are degraded while others are protected (Mathew et al. 2014). Thus, although we currently have a poor understanding of how cells determine which autophagy cargos are degraded under different circumstances, it seems likely that autophagy is largely—if not entirely—selective. This specificity in cargo delivery to autophagosomes is critical in understanding the biological effects of autophagy. For instance, it can explain how autophagy can promote apoptosis for one apoptosis inducer but not another (Gump et al. 2014; Thorburn 2014). Although understanding selective autophagy may be vital to effectively target autophagy therapeutically, at present we have no way to selectively affect cargo-specific autophagy. All the current clinical

trials mentioned above use lysosome-targeted pharmacological agents to target autophagy, namely chloroquine (CQ) or its derivative hydroxychloroquine (HCQ), which both inhibit the lysosome. An important caveat to bear in mind is that CQ can chemosensitize to other anti-cancer drugs through autophagy-independent mechanisms as well as by inhibiting autophagy (Maycotte et al. 2012; Eng et al. 2016), adding another layer to the complexity underlying the debate.

Autophagy is induced by diverse stresses such as nutrient deprivation, hypoxia, metabolic stress and many others and in most cases the induction of autophagy serves to protect cells from the insult. Thus, if cells are starved of amino acids they rapidly induce autophagy and, if that autophagy induction is prevented using either pharmacological inhibitors or genetic interference of the ATG genes that regulate autophagy, many more cells die as a result of the amino acid starvation. Such experiments clearly show that autophagy is protective in this context. Moreover, because such effects are seen in response to a wide variety of pro-apoptotic stimuli, autophagy is widely thought to protect against apoptosis. This protective effect is generally the basis for the idea that autophagy inhibition will chemosensitize tumor cells to other drugs that underlies the numerous clinical trials mentioned above (Thorburn et al. 2014). Contrarily, early papers that considered autophagy's roles in the cancer chemotherapy response (e.g. to the anti-estrogen tamoxifen (Bursch et al. 1996), or in apoptosis-deficient cells treated with DNA damaging drugs (Shimizu et al. 2004)), often concluded that the induction of autophagy by the therapeutic agent caused tumor cell death. One of the first clear demonstrations that autophagy can protect against chemotherapy came from studies in a Myc-driven lymphoma model (Amaravadi et al. 2007). More recently, many studies with diverse anti-cancer drugs including DNA damaging agents and other traditional cytotoxics as well as newer "targeted" agents have tended to conclude that autophagy is primarily protective against cancer therapy (Thorburn et al. 2014). In fact, it is clear that both in response to physiological signals (e.g. during development) and exogenous pro-death stimuli, autophagy can both promote and inhibit cell death/apoptosis (Fitzwalter and Thorburn 2015).

As mentioned above, in the 60-odd ongoing clinical trials identified using the search term "autophagy," the majority are attempting to inhibit autophagy with HCQ. The basis for these studies is twofold. First, an idea that inhibition of autophagy will, by itself, inhibit tumor growth. Second the idea that autophagy inhibition will make another anti-cancer treatment more effective. Let's next consider the rationales for both ideas.

1.1 Inhibiting Autophagy on Its Own for Anti-cancer Treatment

Why think that autophagy inhibition could have an anti-tumor effect even in the absence of other treatments? This concept is based on a large body of data showing that direct interference with autophagy (e.g. by knocking down or knocking out *ATG* genes) can, by itself, inhibit tumor growth and/or promote tumor cell death

(Guo et al. 2013b). The first such demonstration from Jay Debnath's group showed that autophagy was important for transformation by KRAS (Lock et al. 2011) and many of the other studies identifying tumors that require autophagy have also tended to focus on tumors with RAS pathway mutations. In fact, a series of studies in genetically engineered mouse models from Eileen White and colleagues (e.g. Guo et al. 2011, 2013a; Karsli-Uzunbas et al. 2014; Strohecker et al. 2013; Xie et al. 2015), Alec Kimmelman (Yang et al. 2011, 2014), Kevin Ryan (Rosenfeldt et al. 2013), and Josef Penninger (Rao et al. 2014) all focused on tumors driven by mutant KRAS or BRAF and demonstrated anti-tumor effects upon genetic inhibition of autophagy by knock out of an essential ATG. Recent studies in flies also showed autophagy-dependence of RAS-driven tissue overgrowth, however, when tissue growth was driven by the Notch pathway, autophagy had the opposite effect (Pérez et al. 2015). This study is important because it establishes that autophagy's roles in controlling tissue growth can be different in different contexts. An important role for BRAF mutation was demonstrated in pediatric brain tumors where brain tumor cells with wild-type BRAF demonstrated no dependency on autophagy (Levy and Thorburn 2012) (i.e. autophagy inhibition had little effect on tumor cell growth) whereas similar tumor cells that harbored BRAF mutations displayed a high degree of autophagy-dependence such that genetic or pharmacological inhibition of autophagy was sufficient to kill them (Levy et al. 2014).

In some of the mouse studies, autophagy inhibition switched the tumor from an adenoma or adenocarcinoma to a less aggressive tumor type called an oncocytoma (Guo et al. 2013a; Strohecker et al. 2013). In humans, oncocytomas are known to display defects in autophagy (Joshi et al. 2015). The majority of the tumor studies listed above involved activation of an oncogene at the same time and in the same cells that autophagy was inhibited by tissue-specific knockout of an essential ATG; consequently in these cases tumor development and progression all took place without the ability of the tumor cells to perform canonical autophagy. This observation begs the question, what happens if a tumor is allowed to form first, then autophagy is inhibited? Such studies are important because they mimic what a therapeutic intervention might look like (if we had a perfectly effective inhibitor of autophagy that worked as well as knockout of an essential ATG). In one study (Karsli-Uzunbas et al. 2014) such an experiment was done. This work showed that although complete, inducible knockout of ATG7 in adult mice is eventually toxic (the mice die of infection or eventual neurodegeneration consistent with known functions of autophagy that protect organisms), when autophagy was inhibited in the whole animal, this blocked the growth and promoted regression, as well as switching to more benign oncocytomas of pre-existing KRAS mutant lung tumors. An important concern raised by this study is that because all the mice eventually died of neurodegeneration and others were more susceptible to bacterial infection, we must be cautious about autophagy inhibition as a therapeutic strategy. In humans we could presumably never achieve as efficient and irreversible an inhibition of autophagy as we get with the complete knockout of a gene in a mouse so such concerns may be alleviated given two points: first, with pharmacological autophagy inhibitors that would be used in people we could stop treatment to allow recovery from side effects, and second, we would be unlikely to have as complete inhibition of the process.

These studies have led to the suggestion that KRAS mutant or BRAF mutant tumors are the best candidates for autophagy inhibition therapy (Mancias and Kimmelman 2011; Thorburn and Morgan 2015). However some studies have shown that KRAS mutation does not always lead to tumor cells being more sensitive to autophagy inhibition. In an aforementioned mouse study described above, it was demonstrated that p53 status switched autophagy from being tumor promoting in KRAS-driven pancreas cancer to being tumor inhibiting. Therefore, when KRAS-driven pancreas tumors developed with germline loss of p53, autophagy inhibition caused increase growth of the tumors while the same genetic manipulations demonstrated an anti-tumor effect of autophagy inhibition in p53 wildtype mice (Rosenfeldt et al. 2013). It is important to note that germline loss of p53 is not the way that p53 is inactivated during human pancreas cancer development, and that another study where p53 loss occurred in a manner more analogous to what occurs during human pancreas cancer found that p53 status did not alter the beneficial effect of autophagy inhibition (Yang et al. 2014). The explanation for these differences is unknown but imply an important role for p53 function during the development of a tumor in determining whether autophagy promotes or inhibits tumor growth. Other evidence suggests that RAS mutation by itself does not predict whether a tumor cell will be inhibited or increased in its growth when autophagy is blocked. In genetically defined human tumor cells where normal cells are immortalized then transformed by sequential introduction of telomerase, inhibition of p53 and RB then transformed with oncogenic HRAS, some cells showed that transformation was associated with increased dependence on autophagy (i.e. autophagy inhibition reduced growth) whereas other cells transformed in exactly the same stepwise fashion showed increased growth when autophagy was inhibited (Morgan et al. 2014). More recent analysis of a large number of RAS-mutant cell lines also concluded that growth of RAS mutant cell lines was not necessarily inhibited when autophagy was blocked (Eng et al. 2016). A recent study of pancreas tumors demonstrated a critical role for autophagy that was linked not to RAS mutation per se (which nevertheless occurs in the vast majority of pancreas tumors), but instead to increased activity of transcription factors that drive autophagy and allow efficient tumor cell metabolism that is necessary for sustaining cancer growth (Perera et al. 2015).

Although many studies have focused on RAS pathway driven tumors, an anti-tumor effect of genetic inhibition of autophagy is also seen in murine tumors driven by different oncogenic drivers (Huo et al. 2013; Wei et al. 2011, 2014). This raises the question of whether some tumor cells are indeed highly dependent on autophagy but that this dependency can occur with or without RAS mutation. A study in breast cells (Maycotte et al. 2014) where over 100 different autophagy regulators were targeted with pooled shRNAs attempted to circumvent the problem noted above whereby non-autophagy functions of ATG genes confound conclusions of autophagy being important for a biological effect. This is important because all the studies described above where autophagy was targeted and shown to be critical for tumor growth came to this conclusion after inhibiting only one or two ATGs.

The Maycotte et.al. study (Maycotte et al. 2014) found that some breast cancer cell lines were highly dependent on autophagy for growth in the absence of added

stress such as amino acid starvation. These cells tended to lose shRNAs that target positive regulators of autophagy. In other words, when autophagy was inhibited the cells had a selective disadvantage for continued growth. Other breast cancer cell lines could be grown for weeks with no apparent selection against shRNAs that target autophagy suggesting that these cells don't care about autophagy unless they are stressed (e.g. by amino acid starvation). Importantly, only tumors grown from autophagy-dependent tumor cells displayed any inhibition of growth *in vivo* when autophagy was inhibited with CQ. These effects were associated with changes in STAT3 signaling such that in autophagy-dependent breast cancer cells STAT3 signaling and cell growth required autophagy, while in autophagy-independent breast cancer cells STAT3 activity was not controlled by autophagy. In a follow-up paper (Maycotte et al. 2015), it was shown that autophagy-dependent cells require autophagy to promote secretion of the cytokine IL6, which is critical for promoting tumor cell growth and cancer stem cell activity. In contrast, autophagy-independent cells demonstrated no decrease in IL6 secretion when autophagy was inhibited, instead secreting more IL6 when autophagy was inhibited. These effects were also associated with markedly different changes in gene expression patterns upon autophagy inhibition between autophagy-dependent and autophagy-independent tumor cells. Another study showed that autophagy-dependent secretion of IL6 and, most likely of other signaling molecules, is critical for breast cancer cell invasion and metastasis (Lock et al. 2014). Although we have a very poor understanding of the full nature of the differences between autophagy-dependent and autophagy-independent cancer cells, these experiments suggest that the central differences of behavior in response to targeting autophagy reveal themselves because autophagy controls completely different and sometimes opposing pathways in different cancer cells. These studies indicate that in some tumor cells (i.e. the ones that are highly dependent on autophagy) continued tumor growth, survival and perhaps invasion all depend on autophagy, making a strong argument for autophagy inhibition as a therapeutic approach in cancer. However, it is imperative to understand that this only occurs in *some* tumor cells. In others, not only might autophagy inhibition be ineffective, it may be counterproductive and actually increase tumor growth. It will be critical to dissect the biology that underlies these differences if we are to know which tumors to target and which not to target with autophagy inhibitors.

1.2 Inhibiting Autophagy to Make Other Treatments More Effective

The majority of the clinical trials where autophagy is deliberately targeted involve an autophagy inhibitor used in combination with another drug. A large amount of literature describes chemosensitization effects of autophagy inhibition (Levy and Thorburn 2011; Maycotte and Thorburn 2011; Thorburn et al. 2014; Rebecca and Amaravadi 2015). Some of these effects may be due to the other anti-cancer drug itself increasing autophagy. For example, mTOR inhibitors are potent inducers of

autophagy and it can be shown that co-ordinate inhibition of autophagy can sensitize to mTOR inhibitors (Xie et al. 2013). The interpretation of such studies is that the autophagy induced by the drug reduces its ability to kill the cancer cells, so that the addition of an autophagy inhibitor (such as CQ) blocks this protection thus sensitizing to the other drug. This finding has led to clinical studies of such combinations (Rangwala et al. 2014a). However, as with the findings of opposing effects when autophagy is targeted on its own in different contexts, recent work suggests that the even the same combination of drugs in autophagy-dependent and -independent tumors can show different effects. Thus, in the autophagy-dependent and autophagy-independent breast cell lines described in the previous section (Maycotte et al. 2014), the same drug combination (doxorubicin plus the autophagy inhibitor chloroquine) was only synergistic in autophagy-dependent breast cancer cells and was sometimes actually antagonistic in autophagy-independent breast cancer cells. Similar results were found in autophagy-dependent BRAF mutant versus autophagy-independent BRAF wild-type brain cancer cells (Levy et al. 2014). There are also cases where specific anti-cancer drugs have been reported to require autophagy in order to elicit their anti-tumor effect. Epidermal Growth Factor Receptor (EGFR) signaling was reported to inhibit autophagy by phosphorylating and disrupting the activity of the autophagy regulator Beclin 1 (BECN1) (Wei et al. 2013). Moreover, EGFR inhibitors, which are commonly used to treat EGFR mutant tumors, were found to restore this autophagy activity. The resultant anti-tumor effect was found to require autophagy restoration, implying that in this case, adding on an autophagy inhibitor would prevent the EGFR inhibitor from working. Such studies suggest that choosing the correct drug to combine with autophagy inhibitors will be important and, possibly even more critical, will be selecting such a combination for the appropriate tumor cells.

The aforementioned examples are attempting to increase the efficacy of a drug that has at least some activity. One of the major problems in cancer therapy comes when tumors acquire resistance to a drug, which may develop in myriad ways (Holohan et al. 2013), including the increased expression of drug efflux pumps and the reduced ability of the tumor cell to undergo apoptosis. For targeted therapies such as kinase inhibitors that block specific signaling pathways, resistance commonly arises due to activation of the same pathway downstream of the inhibited kinase or activation of a parallel signaling pathway. In some cases we are starting to obtain evidence that autophagy inhibition can be used as a way to circumvent such acquired resistance. The best examples to date come from studies in BRAF mutant tumors. It has been shown that autophagy inhibitors can synergize with BRAF inhibitors (Goodall et al. 2014). However, autophagy inhibitors may also be able to do more: they can also overcome resistance to the BRAF inhibitor. In BRAF mutant melanoma, the acquisition of resistance in the clinic to the RAF inhibitor vemurafenib was shown to correlate with higher numbers of autophagosomes, suggesting that increased autophagy occurs as the tumors evolve to become resistant against the BRAF inhibitor and undergo more endoplasmic reticulum stress (Ma et al. 2014). Moreover, *in vitro* experimental selection for vemurafenib resistance could be reversed in this situation via autophagy inhibition. We also have at least one case

where such an effect—adding an autophagy inhibitor reverses resistance to the BRAF inhibitor—may be true in a patient. In this case (Levy et al. 2014), a patient with a BRAF mutant brain tumor was treated for almost a year with vemurafenib but then had a recurrence indicating that her tumor had acquired resistance to the drug. The patient was then treated with a combination of vemurafenib and CQ, which caused tumor regression. Importantly, this particular patient was taken off the BRAF inhibitor for periods of time while continuing treatment with CQ and this caused the tumor to start growing again. Thus, in this patient, neither BRAF inhibitor alone nor the autophagy inhibitor alone was effective at inhibiting tumor growth and causing regression; only the combination works. These data are consistent with the idea that it is the reversal of resistance that is the key benefit of autophagy inhibition. This patient also demonstrates that autophagy inhibition therapy can be done for extensive periods of time (in this case more than 2 years as of the time of writing) without signs of toxicity due to the autophagy inhibitor. Therefore, the concerns raised with the mice where inducible knockout of the *Atg7* gene led to death caused by neurodegeneration within a few months (Karsli-Uzunbas et al. 2014) may be less significant in practice when we are incompletely inhibiting autophagy in the clinic.

1.3 Potential Reasons Not to Inhibit Autophagy in Cancer Therapy

The previous discussion argues that autophagy inhibition may be worthwhile as an anti-cancer therapy alone or together with other drugs but only in some already existing tumors. Other studies raise different issues that have been used to argue against autophagy inhibition therapy. One possible reason is that autophagy may serve to suppress the development of new cancers. The rationale for this argument rests on the observation that several autophagy genes function as tumor suppressors when they knocked out in mice. For instance, BECN1 homozygous deletion leads to early embryonic lethality but heterozygous deletion causes increased incidence of cancer (Qu et al. 2003; Yue et al. 2003), suggesting that BECN1 is a haploinsufficient tumor suppressor. In human tumors, this interpretation has been challenged and it has been suggested that the apparent loss of BECN1 in human cancers is primarily due to loss of an adjacent gene, BRCA1 (Laddha et al. 2014). However, other studies suggest that such a bystander effect is not in play and that BECN1 is functioning as a tumor suppressor in some human breast cancers (Tang et al. 2015). In mice, mosaic deletion of ATG5 or liver-specific deletion of ATG7 leads to the development of benign liver adenomas that do not progress to aggressive cancer (Takamura et al. 2011). Deletion of other autophagy regulators in mice can also cause spontaneous cancer development. Examples include BIF-1 (Takahashi et al. 2007), ATG4C (Marino et al. 2007) and UVRAG (Liang et al. 2006), although this last example could be due to autophagy-independent functions in maintaining chromosome stability (Zhao et al. 2012). Studies similar to these have led to the

suggestion that autophagy may suppress the development of cancer even when it can promote cancer progression. In this case, one might expect that general inhibition of autophagy would cause pre-neoplastic lesions to progress faster.

The above examples consider the effect of autophagy in cancer to be primarily an autonomous effect on the behavior of the tumor cell itself; that is, autophagy may promote or inhibit growth of the cancer cell, cause it to be more or less likely to die, or affect the cell's ability to migrate and invade other tissues. Autophagy manipulation in one cell may also alter the way that neighboring cells behave. This could have repercussions for cancer development, progression, and response to therapy. The best example here concerns how dying tumor cells do or do not affect and engage the immune system. It was demonstrated that chemotherapy-induced immunogenic cell death of cancer cells requires that autophagy be functional in the dying tumor cells (Michaud et al. 2011). This effect was necessary for effective treatment of the tumor in immune competent mice but not in immune deficient animals demonstrating that the difference was due to how the immune system recognized the dying cancer cells rather than an effect on the efficiency of tumor cell killing by the chemotherapeutic itself. A mechanism was traced to a requirement for autophagy in the release of ATP from the dying cells. In other circumstances autophagy may be important in controlling the release of other immune stimulators such as the Damage Associated Molecular Pattern (DAMP) molecule HMGB1 (Thorburn et al. 2009). Autophagy may also be important in tumor antigen presentation (Li et al. 2012). Together, these findings would tend to suggest that autophagy inhibition during cancer therapy should reduce immunogenic tumor cell killing, i.e. arguing against trying to target autophagy. However, even here the situation is complicated. It has been shown that autophagy inhibition with CQ significantly enhances T cell-mediated tumor killing after Interleukin 2 immunotherapy (Liang et al. 2012). Hypoxia-induced autophagy impairs natural killer (NK) cell-mediated killing of tumor cells and autophagy inhibition was shown to enhance tumor elimination by NK cells *in vivo* (Baginska et al. 2013). Thus, as with the other competing effects discussed above, the benefits and caveats of targeting autophagy are also manifested when it comes to immunogenic tumor cell killing. These studies further emphasize how crucial it will be to understand the full spectrum of effects—both good and bad—that occur when autophagy is targeted during cancer therapy.

1.4 Conclusions

What should be clear from the above discussion (which is by no means definitive, many other studies arguing both for and against autophagy as a therapeutic target in cancer could have been discussed) is that there is no straightforward conclusion as to how, or even whether, we should try to target autophagy as a therapeutic approach to cancer. Numerous important questions remain to be answered and there is evidence both for and against the idea of targeting autophagy that we need to make sense of. Moreover, it is unclear how we should go about targeting autophagy.

Current clinical approaches focus on targeting the lysosome with drugs like HCQ, and other, more potent lysosomal inhibitors are also being developed (Goodall et al. 2014; Mcafee et al. 2012). The ability of lysosome inhibitors to chemosensitize through autophagy-independent mechanisms may also be useful (Maycotte et al. 2012; Eng et al. 2016). Earlier steps in the autophagy pathway can also be therapeutically targeted (Bago et al. 2014; Dowdle et al. 2014; Ronan et al. 2014; Egan et al. 2015; Petherick et al. 2015). Will these be better than lysosome-targeted drugs for cancer therapy? Perhaps the most critical issue is to determine which tumors should be targeted and which should not. This is a pressing issue because accumulating evidence suggests that not only might targeting autophagy be ineffective in some tumors, in those tumors that are not highly dependent on autophagy, it may be counterproductive to do so. If we try to inhibit autophagy in the wrong tumor, this may not only fail to slow tumor growth, it might enhance growth. Targeting autophagy in the wrong tumor may not only fail to make another drug more efficacious, it might make that drug less effective. Added complexity comes when one considers how altering autophagy in cancer cells may affect how other cells (e.g. immune cells) recognize the tumor cells. It will require a much more sophisticated understanding of how these effects work and how their balance determines the final outcome if we are to effectively pursue autophagy as a therapeutic target in cancer.

Given this complexity, one might propose not even to try targeting autophagy in cancer therapy. However, this is not an option; not only do we have ongoing clinical trials whose interpretation will require that we better understand this process and what it means for cancer cell behavior, we already know that even if we wanted to avoid targeting autophagy we couldn't do so. Most of our current anti-cancer treatments themselves affect autophagy, so we are routinely affecting autophagy during cancer therapy whether we like it or not. The way forward is to understand how the various competing effects of autophagy on cancer treatment and cancer/tumor behavior occur. Fortunately, the field is now poised to do so.

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Chapter 2
Autophagy in Cancer Cells vs. Cancer Tissues: Two Different Stories

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Abstract Autophagy has been considered strongly associated with cancer development and possibly playing important roles in cancer progression. Here we present a computational study of transcriptomic data of cancer tissues, totaling 6317 tissue samples of 11 cancer types along with tissues of inflammatory diseases and cell line based experiments for comparative purposes. Our study clearly revealed that some widely held beliefs and speculations regarding autophagy in cancer may not be well founded, knowing that many of the previous observations were made on cancer cells cultured in man-made environments rather than actual cancer tissues. Our major findings include: (i) the widely used assumption that cancer tissue cells are nutrient depleted is not supported by our tissue-based gene-expression data analysis; (ii) the 11 cancer types studied fall into 2 distinct groups: those with low macro-autophagy (LM) activities and those with high lysosome (HL) activities but induced by

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micro-autophagy and chaperon-mediated autophagy; (ii) co-reduction in autophagy and apoptosis are widely observed in cancer tissues; (iii) down-regulated autophagy strongly correlates with up-regulated cell-cycle progression genes across all cancer types, with one possible functional link detected that repressed autophagosome formation may reduce the degradation of cellular organelles that are essential to cytokinesis, hence contributing to cell cycle progression; (iv) significant correlation is observed between autophagy and immune activities; (v) the down-regulated macroautophagy genes negatively correlate with the total mutation rates in cancer genomes in LM cancers; and (vi) conditional correlation analyses point to a very unexpected direction: cellular Fenton reactions may be the cause of the decreased macroautophagy and its co-expression with apoptosis, increased cell proliferation, genomic mutation rate and even possibly immune response. The information derived here may shed new light on elucidation of fundamental relationships between cancer and autophagy as well as on how to take advantage of the derived relationship for improved treatment of cancer.

2.1 Examining Autophagy in Cancer via Cancer Cell Line *Versus* Cancer Tissues

Autophagy is a cellular survival process under nutrient deprivation and metabolic stress. It degrades cellular proteins, other macromolecules, organelles and cytoplasm; and recycles the nutritious elements to support cell survival. Basal autophagy is a constitutive process that plays a homeostatic function, acting in parallel with the ubiquitin-directed proteasome degradation pathway to maintain the integrity of cellular proteins and organelles. In terms of its role in cancer, the current understanding is: autophagy has a role in supporting cancer cell survival under metabolic stress and in hypoxic regions (Degenhardt et al. 2006). Interestingly, a few essential autophagy genes are found to have high mutation rates across a few types of cancers. For instance, allelic loss of beclin1 gene (BECN1, also known as ATG6) is reported to be among commonly mutated genes in breast, ovarian and prostate cancers (Liang et al. 1999), suggesting that these cancers try to avoid autophagy.

A widely accepted model is that autophagy plays a major survival role throughout cancer initiation and early-stage development by helping cells overcome nutrient deprivation and metabolic stress (Mathew et al. 2007), which are believed to take place in cancer. Its role in more advanced cancers seems to be in stimulation of necrotic cell death, leading to persistent inflammation and repeated wound-healing, an environment that cancer development generally requires (Degenhardt et al. 2006). On the other hand, autophagy is believed to have an important role in maintaining genome integrity by limiting genome damages (Mathew et al. 2007), suggesting that cancers may tend to repress autophagy, knowing that mutations are essential to cancer cell survival. To sort out these complex and conflicting relationships between cancer and autophagy derived through cell line based studies as well as genome

analyses of cancer tissue cells, we need to take a more systematic approach to study a large number of actual cancer tissue samples (*versus* cancer cell lines).

It is noteworthy that there may be fundamental differences between cancer cell line biology and the actual cancer biology. For example, while it has been speculated that cancer cells tend to be low in ATP production based on cell line studies (Lim et al. 2011) (may not be accurate), tissue-based analyses suggest that cancer tissue cells may not be short of ATPs (Gottesman et al. 2002) and our recent study provides an explanation of why this is the case (Hui Yan et al. 2016), suggesting that nutrient deprivation might not necessarily be a valid assumption for autophagy study in cancer tissues.

Here, we present a computational analysis of transcriptomic and genomic data of cancer tissues in the TCGA database (Weinstein et al. 2013), covering 6317 samples of 11 cancer types, aiming to gain a coherent understanding about the relationship between autophagy and cancer. To put our study in a comparative setting, we have also included transcriptomic data of 14 types of inflammatory diseases with 8 being cancer prone and 6 being cancer independent along with control samples plus 12 cell-lined based datasets with experimentally induced autophagy along with controls, to gain a deeper understanding about the actual roles played by autophagy in disease tissues. The detailed information of the omic data used in our study is given in the Sect. 2.9.

The rest of the chapter is organized as follows. Section 2.2 characterizes autophagy in disease tissues and cell lines by comparing transcriptomic profiles of autophagy related genes in both systems. Section 2.3 assesses the level of nutrient deprivation and associated autophagy in cancer and inflammatory disease tissues. Section 2.4 analyzes the interplay between autophagy and apoptosis. Section 2.5 discusses two novel associations between autophagy and cell cycle progression as well as autophagy and immune system. Section 2.6 demonstrates the impact of mutations of autophagy related genes. Section 2.7 discusses about general biological processes that correlate with autophagy. Section 2.8 concludes our prediction of functional roles of autophagy in cancer and inflammatory disease tissues. Section 2.9 covers the methods and data used in this chapter.

2.2 Gene Expression of Autophagy in Disease Tissues

We have conducted differential gene-expression analyses over a total of 6317 disease *versus* control tissue samples of 11 cancer types, 8 cancer-prone inflammatory diseases and 6 cancer-independent inflammatory diseases plus 12 cell-based datasets collected under serum depletion or other metabolic stress conditions for induction of autophagy. An inflammatory disease is considered as *cancer-prone* or *cancer-independent* if the cancer occurrence rate is elevated with statistical significance or not in the disease sites based on published statistics. The details of these diseases and datasets are given in Sect. 2.9. A pathway is considered as *up-* or *down-regulated* if it is enriched with up- or down-regulated genes assessed using a hypergeometric test with p-value = 0.05 as the statistical significance cut-off.

Three types of autophagy have been defined, namely *macro-autophagy*, *micro-autophagy* and *chaperon-mediated autophagy*, which differ in both their induction and execution processes (Glick et al. 2010). To the best of our knowledge, more than 95 % published studies of autophagy in cancer are focused on macro-autophagy (Mizushima 2007).

Eleven autophagy-related pathways are considered in our pathway enrichment analyses, namely: (1) the core gene set of macro-autophagy induction, which consists of the essential genes involved in autophagy initiation, nucleation and expansion; (2) cargo recognition and selectivity genes, covering genes involved in selective autophagy with a recognition function; (3) cytoplasm-to-vacuole targeting pathway (CVT), which is similar to the bulk autophagy (Scott et al. 1996) except that it is activated constitutively under normal growth conditions; (4) autophagy-induction genes, consisting of early response genes that can lead to the induction of autophagy; (5) nucleation assembly genes for the formation of an autophagosome complex; (6) vesicle formation and autophagosome breakdown genes; (7) micro-autophagy invagination pathway (Li et al. 2012); (8) chaperone mediated autophagy genes; (9) lysosome genes; (10) lysosome degradation pathways; and (11) proteasome genes for protein degradation in an autophagy independent manner. The pathway enrichment results in all the aforementioned disease types are listed in Table 2.1.

Our finding is quite surprising as the analysis clearly shows that the expression patterns of the 11 autophagy pathways are substantially different, specifically the macro-autophagy pathways (i.e., the first 6 pathways above) in cancer cell lines *versus* cancer tissues. Specifically, the macro-autophagy and lysosome related pathways are consistently up-regulated in cell-line under nutrient deprivation conditions. However, the macro-autophagy pathways, especially the autophagosome formation genes, including MAP1LC3A, MAP1LC3B, MAP1LC3C, WIPI1, BNIP1, GABARAP, GABARAPL1, GABARAPL2, PARK2, SRPX, LRRK2, ULK2, FYCO1, and TP53INP2, are consistently down-regulated in 7 out of the 11 cancer types, namely THCA, BRCA, HNSC, BLCA, COAD, LUAD and LUSC; and the lysosome pathway is also down-regulated in COAD, LUAD and LUSC. The macro-autophagy recognition, induction and fusion pathways are up-regulated in KICH and STAD; and nucleation assembly pathways are up-regulated in KIRC. Interestingly, KICH, STAD, LICH and BRCA have their micro-autophagy related genes up-regulated; and STAD has its chaperone-mediated autophagy genes up-regulated while none of them are up-regulated in the aforementioned cell-line datasets. Cancer types with up-regulation of at least one autophagy type, namely KICH, STAD, KIRC, LICH and BRCA, all have up-regulated lysosome activity, hence providing a cross-validation between the two predictions and making the them more trustworthy. Interestingly, proteasome genes are generally more up-regulated in cancer types with down-regulated macro-autophagy, suggesting that they may serve similar purposes and hence are mutually exclusive between macro-autophagy and proteasome based protein degradation. Based on these observations, we classify the 11 cancer types into 2 groups: those with low macro-autophagy (LM): BRCA, HNSC, BLCA, COAD, LUAD and LUSC and those with high lysosome (HL): KICH, STAD, KIRC, LIHC and THCA. It is noteworthy that the ATG genes, key autophagy genes, are generally un-differentially expressed across all cancer types.

Table 2.1 Differential expressions of autophagy related pathways across different cancer cells, cancer tissues of different types, and disease tissues of different chronic inflammatory diseases, where cell line represent the overall statistics of the 12 cell-line data, CII and CPI are for cancer-independent and cancer-prone chronic inflammatory diseases, respectively

	Cell line	Cancer											Inflammatory diseases			
	Metabolic stress	KICH	STAD	KIRC	LIHC	THCA	BRCA	HNSC	BLCA	COAD	LUAD	LUSC	CII: PSO, AH, IBS	CII: AST,CS, NAS	CPI: COPD,HCV, CIR, IFP	CPI: UC,CD, BE,AD
Cargo recognition and selectivity	Up						Down			Down	Down	Down	Up			Down
Core	Up						Down	Down	Down	Down	Down	Down	Up			Down
CVT pathway	Up	Up	Up				Down	Down	Down	Down	Down	Down				Down
Induction and fusion	Up	Up	Up							Down			Up			Down
Nucleation assembly	Up			Up		Down	Down	Down	Down	Down	Down	Down	Up			Down
Autophagosomal structure	Up					Down	Down	Down	Down	Down	Down	Down				
Micro-autophagy invagination		Up	Up		Up		Up						Up			
Chaperone mediated autophagy				Up												
Lysosome	Up	Up	Up	Up	Up	Up	Up			Down	Down	Down	Up	Down	Up	Down
Lysosome degradation	Up	Up	Up	Up		Up				Down	Down	Down	Up	Down	Up	Down
Proteasome							Up	Up	Up	Up	Up	Up				

Detailed information of the data is given in Sect. 2.9

Down-regulated macro-autophagy and lysosome are also observed in four types of cancer-prone inflammatory diseases, namely UC, CD, BE and AD while up-regulated lysosome is observed in the other four types of cancer-prone inflammatory diseases: COPD, IFP, HCV and CIR. In comparison, up-regulated macro-autophagy and lysosome pathways are observed in three cancer-independent diseases: PSO, AH and IBS; and down-regulated lysosome is found in the other three cancer-independent diseases: AST, CS and NAS. These strongly suggest that there are no intrinsic relations between cancer and autophagy.

In the remaining portion of the chapter, we focus on elucidation of the possible reasons and functional roles of down-regulation of macro-autophagy and up-regulation of lysosome in LM and HL diseases, respectively.

2.3 Nutrient Deprivation Is Unlikely in Cancer Tissue

It has been repeatedly observed that macro-autophagy can be induced in cancer cells by nutrient deprivation (Mizushima et al. 2004). Hence it has been naturally assumed that cancer tissue cells are also nutrient depleted based on such cell-line studies coupled with observations that cancer tissue cells tend to have substantially increased uptake of glucose (Adekola et al. 2012). However no experimental studies have reliably established that cancer tissue cells are indeed nutrient depleted, to the best of our knowledge. Actually, recent metabolomic studies of cancer tissues suggest that the opposite may be true, i.e., cancer tissue cells are rich in nutrients and in ATPs (Coller 2014). Our recent research provides a possible explanation of where the plentiful ATPs may come from in cancer tissue cells (Hui Yan et al. 2016).

We have recently conducted a modeling analysis based on gene-expression data of 6600+ tissue samples of 14 types of cancer *versus* controls, aiming to assess if there may be Fenton reactions in mitochondria of cancer tissue cells, as strongly suggested by various hints (Hui Yan et al. 2016). Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$, a non-enzymatic reaction, can take place when the concentrations of Fe^{2+} and H_2O_2 are sufficiently high, which is generally true for chronic inflammatory sites (Winterbourn 1995). The products of the reaction are Fe^{3+}, OH^- and $\bullet OH$. When there are also plentiful reducing elements at or near the reaction sites such as Vitamin C, sulfur or NADH, Fe^{3+} can be reduced to Fe^{2+}, which will enable the reaction to continue. We have developed a computational method to demonstrate if a specific subcellular component may have elevated Fenton reactions or not (Hui Yan et al. 2016), with its basic idea summarized below.

We can rewrite (continuous) Fenton reactions as: $RA + H_2O_2 \rightarrow OH^- + \bullet OH + X$ with Fe^{2+} as the catalyst since Fe^{2+} is not consumed by the (continuous) reaction, with RA and X representing the reducing element and its oxidized form, respectively. We have identified marker genes for each of the five relevant quantities: [RA], [H_2O_2], [OH^-], [$\bullet OH$] and [X] in three cellular compartments: cytosol, mitochondria, and extracellular region, whose expression levels reflect these quantities. We have shown that in tissues without Fenton reactions, these five quantities (for each cellular compartment) are largely independent of each other; and in tissues

having Fenton reactions, they are strongly correlated with each other as measured via the Michaelis-Menton equation (Berg et al. 2002). Using this analysis tool, we have demonstrated that all cancer tissues we studied have Fenton reactions in the three cellular compartments with high statistical significance. Furthermore, we have demonstrated that the OH^- molecules continuously produced by the reaction in mitochondria will lead to reduced concentration of mitochondrial protons, hence leading to a proton gradient on the two sides of the inner membrane of mitochondria, as well as proton influx via the ATP synthase and ATP production just like in a respiration process. The difference is that there is no need for NADHs to push their electrons through the electron transport chain to produce a proton gradient. In sum, when mitochondrial Fenton reactions continue, they will produce ATPs just like in respiration but it does not consume NADHs instead it consumes some reducing elements such as Vitamin C or sulfur.

Using this modeling approach, we also discovered that LM cancers generally have higher levels of cytosolic and extracellular Fenton reactions in comparison with the HL cancers, which tend to have higher mitochondrial but less increased cytosolic Fenton reactions. Knowing that damaged mitochondria can be engulfed by autophagosome and then degraded by lysosome, we posit that up-regulated lysosome-degradation pathway may be involved in the removal of the oxidatively damaged mitochondrial components (Zhou et al. 2011). As a comparison, the down-regulated autophagosome-formation genes in the LM cancers (and inflammatory diseases) are mostly over-expressed in cell line data. All these revealed that the differentially expressed autophagy genes in both of the LM and HL cancers are highly different to metabolic stress induced autophagy in cell lines.

We have also examined in cancer tissues and cell line experiments the expression levels of nine sets of metabolic deprivation responsive genes, which are identified through experiments independent of the expression data used here, under the conditions of deprivation of methionine, leucine, glutamine, amino acids, glucose, and serum (see Sect. 2.9), where the level of differential expression for each gene set reflects the deprivation of a specific metabolite. On average, ~85% (p-value < 1e−30) of the relevant marker genes are differentially expressed in the cell lines treated with each such depletion while <15 % (p-value = 0.6) are differentially expressed in cancer tissues, suggesting a big difference in the level of metabolic deprivation between the cell line based experiments and cancer tissues.

All these observations strongly suggest that nutrient depletion-induced macroautophagy is highly unlikely in cancer tissues.

2.4 Autophagy and Apoptosis

Cross-talks between autophagy and apoptosis have long been known and extensively studied (Maiuri et al. 2007); and both are considered as having tumor suppression functions (Su et al. 2013). We present a computational analysis of expressions of genes involved in both autophagy and apoptosis to assess their

co-expression relations with other autophagy and apoptotic genes to elucidate possible relationships between the two processes in cancer.

Our analyses have detected that a number of core macro-autophagy regulatory genes are down-regulated in cancer tissues but up-regulated in cancer cell line data. Similar patterns of down-regulated core apoptotic regulatory genes such as BCL2L1, BAD, BAG1 and BCL2L11 are observed in tissues of most cancer types, but up-regulated in cancer cell lines. Our co-expression analyses observed positive co-expression among the down-regulated autophagy and apoptosis signaling genes and negative co-expression between the autophagy and proteasome genes in LM cancers. Interestingly, most of the autophagy co-expressed apoptosis genes are up-stream signaling genes, proteasome and ubiquitination genes. The extrinsic and intrinsic apoptotic pathways are largely independent to autophagy in cancer tissues.

We have also observed significant under-expression of several regulatory genes involved in both autophagy and apoptosis such as (1) BH3 binding genes (and complex) BECN1, BNIP3, UVRAG, VPS34, and BCL2L1; (2) DAPK genes: DAPK1, DAPK2 and DAPK3; and (3) ATG5, in both LM and HL cancers. Our co-expression analysis revealed that these genes in LM cancers are strongly co-expressed with a number of proteasome genes, whose up-regulation tends to be strongly associated with cytosolic Fenton reactions, hence supporting our hypothesis that Fenton reaction may be a common reason for the differentially expressed apoptosis regulation and down-regulated autophagy as detailed in Discussion.

2.5 Novel Biological Processes Related to Autophagy

Gene co-expression networks are constructed for each disease type under study, including both cancer and inflammatory diseases, to identify novel biological processes that may associate with the observed down-regulation of macro-autophagy in the LM diseases and up-regulation of lysosome in the HL diseases. We have previously developed a Mutual Rank (MR) based method to detect highly co-expressed gene clusters, also referred to as *co-expression modules* in a global gene co-expression network (Zhang et al. 2015) (see Sect. 2.9).

The method first applies a rank based statistic to detect the significant hub genes in a given co-expression network and then identifies the co-expression module surrounding each hub gene, where hub gene is intuitively defined as a gene with substantially more interaction partners than its neighboring genes in the given co-expression network. The method tends to identify strongly co-expressed gene modules, allowing a gene to be part of multiple modules, hence sensitive to identify novel biological processes correlated with specific targets, say autophagy in the current study. Here, we have identified numerous co-expression modules that are significantly enriched by autophagy genes. Functions of non-autophagy genes in each module are functionally analyzed to reveal novel biological processes strongly associated with autophagy in each disease class. In addition, we

Table 2.2 Information of the data analyzed in this chapter

Cancer type	Cancer label	#Tumor	#Control	Data source	Analyzed mutations
Bladder urothelial carcinoma	BLCA	408	19	TCGA	TP53, PIK3CA, ARID1A, MUC17, MUC16, TTN
Breast invasive carcinoma	BRCA	1095	113	TCGA	TP53, PIK3CA, PTEN, MUC17, MUC16, TTN, MUC4
Colon adenocarcinoma	COAD	285	41	TCGA	TP53, TTN
Head and neck squamous cell carcinoma	HNSC	520	44	TCGA	TP53, PIK3CA, NAV3, MUC17, MUC16, TTN, MUC4
Kidney chromophobe	KICH	66	25	TCGA	TP53
Kidney renal clear cell carcinoma	KIRC	533	72	TCGA	ARID1A, PTEN, NAV3, MUC17, MUC16, TTN
Liver hepatocellular carcinoma	LIHC	371	50	TCGA	TP53, MUC16, TTN
Lung adenocarcinoma	LUAD	515	59	TCGA	TP53, PIK3CA, NAV3, KRAS, MUC17, MUC16, TTN, MUC4
Lung squamous cell carcinoma	LUSC	501	51	TCGA	TP53, PIK3CA, NAV3, MUC17, MUC16, TTN, MUC4
Stomach adenocarcinoma	STAD	238	33	TCGA	TP53, PIK3CA, ARID1A, PTEN, NAV3, KRAS, MUC17, MUC16, TTN, MUC4
Thyroid carcinoma	THCA	505	59	TCGA	

Inflammatory disease types	Disease label	#Disease	#Control	Data source	Relevance to cancer
Crohn's disease	CD	37	12	GSE16879	Risking
Ulcerative colitis	UC	40	12	GSE16879	Risking
Liver cirrhosis	CIR	13	10	GSE6764	Risking
Barrett's esophagus	BE	20	19	GSE26886	Risking
HCV infection	HCV	16	2	GSE11190	Risking
Chronic obstructive pulmonary disease	COPD	35	63	GSE11784	Risking
Idiopathic pulmonary fibrosis	IFP	23	6	GSE21369	Risking
Atopic dermatitis	AD	13	8	GSE32924	Risking
Psoriasis	PSO	33	21	GSE14905	Independent
Asthmatics	AST	42	28	GSE4302	Independent
Alcohol hepatitis	AH	15	7	GSE28619	Independent
Non-alcoholic steatohepatitis	NAS	9	7	GSE63067	Independent

(continued)

Table 2.2 (continued)

Inflammatory disease types	Disease label	#Disease	#Control	Data source	Relevance to cancer
Irritable bowel syndrome	IBS	28	77	GSE36701	Independent
Cutaneous sarcoidosis	CS	15	5	GSE32887	Independent

Cell line experiments	Cell type	#Treated	#Control	Data source
Serum deprivation	T98G cell	3	3	GSE1692
Glucose deprivation	MCF7 cell	5	6	GSE19123
Starvation-induced autophagy	Lymphoblastoid cell line	3	3	GSE2435
Serum starvation	Lymphoblastoid cell line	6	6	GSE31040
Glucose deprivation	HCT116 cell	9	9	GSE38061
Tunicamycin treatment	PC3 cell	1	1	GSE38643
Induction of autophagy by atorvastatin	PC3 cell	2	2	GSE46376
GANT61 treatment	ES2 and H4	2	2	GSE54936
Glucose deprivation	A549 cell	2	2	GSE56843
4-hydroxytamoxifen treatment	IMR90 cell	6	6	GSE59522
Serum deprivation	LoVo cell	3	3	GSE70976
Serum deprivation	T cell	3	10	GSE7497

have also conducted a similar analysis but on co-expression modules enriched by lysosome genes. These two classes of modules are referred to as autophagy- and lysosome-centric modules in the following. Table 2.2 lists all such modules, along with their annotated functions for each disease type under study.

2.5.1 Autophagy and Cell Cycle Control

We noted that cell cycle genes, specifically the G2-M transition genes, enrich at least 30 % of the autophagy-centric co-expression modules in each LM cancer type while G1-S transition genes and other cell cycle genes enrich ~20 % of the lysosome-centric modules in HL cancers. Further analysis revealed that cell-cycle genes are negatively correlated with the down-regulated autophagy genes in LM cancers and positively correlated with the up-regulated lysosome genes in HL cancers. Figure 2.1 shows the co-expression networks among autophagy and cell cycle genes identified in each cancer type.

Macro-autophagy may suppress cell-cycle progression through blocking the transition from G2 to M (Kuo et al. 2011; Matsui et al. 2013). A recent study suggests that autophagy may also have important roles in suppression of cytokinesis (Kuo et al. 2011). Our analysis detected a consistent negative correlation between

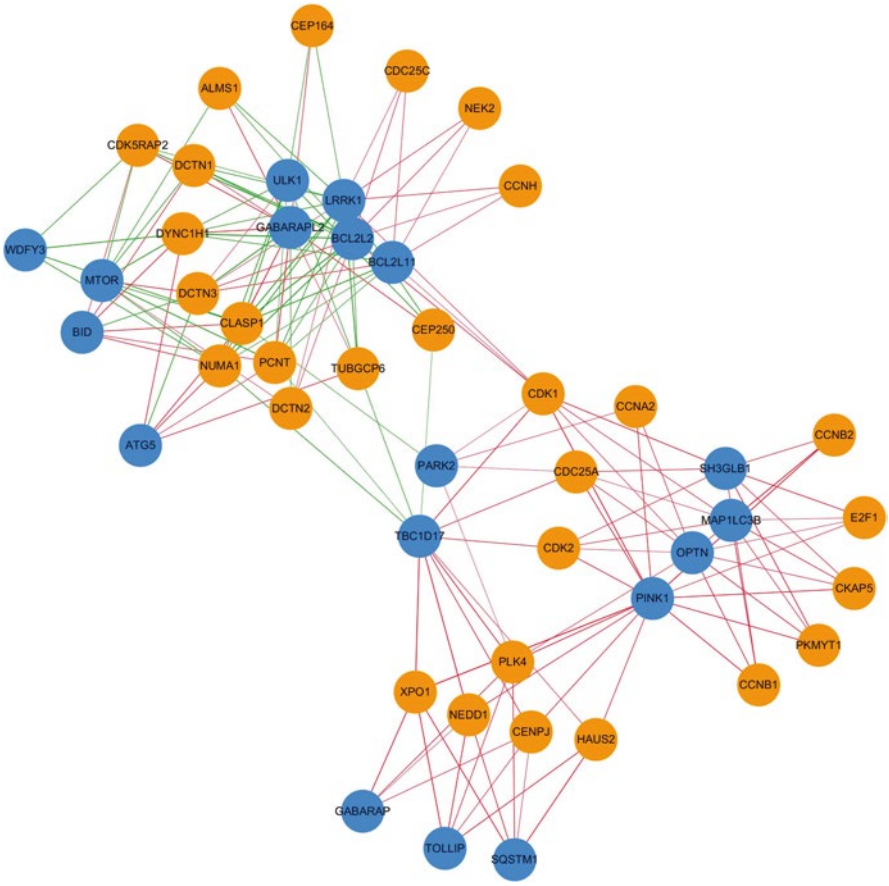

Fig. 2.1 Co-expression network among down-regulated autophagy genes and up-regulated cell cycle genes in COAD. The autophagy genes are represented by *blue* nodes and the cell cycle genes are in *orange*. The *red* and *green edges* represent negative and positive co-expression, respectively, and the width of the edge denotes the level of co-expression

the over-expressed cyclins CCNB1 and CCNB2, cyclin dependent kinases CDK1 and CDK2, a number of centrosomal protein genes, other G2-M transition genes *versus* all the under-expressed macro-autophagy genes: ATG5, ATG7, ATG10, ATG12, GABARAP, GABARAPL2, MAP1LC3B, and PARK2, all related to formation and maturation of autophagosome in the following LM cancers: COAD, LUAD and LUSC. With the knowledge that autophagic degradation may be involved in the cleaning of midbody derivatives after cytokinesis (Pohl and Jentsch 2009; Kuo et al. 2011), we speculate that suppression of the autophagosome formation may preserve the organelles that are necessary for cell proliferation in cancer tissues.

Further analysis revealed that most of the up-regulated G1-S transition genes in HL cancers are highly co-expressed with the over-expressed lysosome genes are proteasome genes. The cyclins, cyclin dependent kinases, DNA polymerases and other cell-cycle regulatory genes are largely independent of the lysosome genes. We speculate that the co-expression between lysosome and proteasome just reflect a normal relationship between the two protein degradation systems, which are both up-regulated in cancer probably due to the increased damage to proteins possibly by ROS (Waris and Ahsan 2006).

2.5.2 *Autophagy and Immune Response*

Our analysis has identified that the down-regulated genes involved in autophagosome formation and maturation, such as MAP1LC3C, SNCA, ATG7, ATG4C, ATG12, ATG5, OPTN, GABARAP, PARK2, and SH3GLB1 are significantly

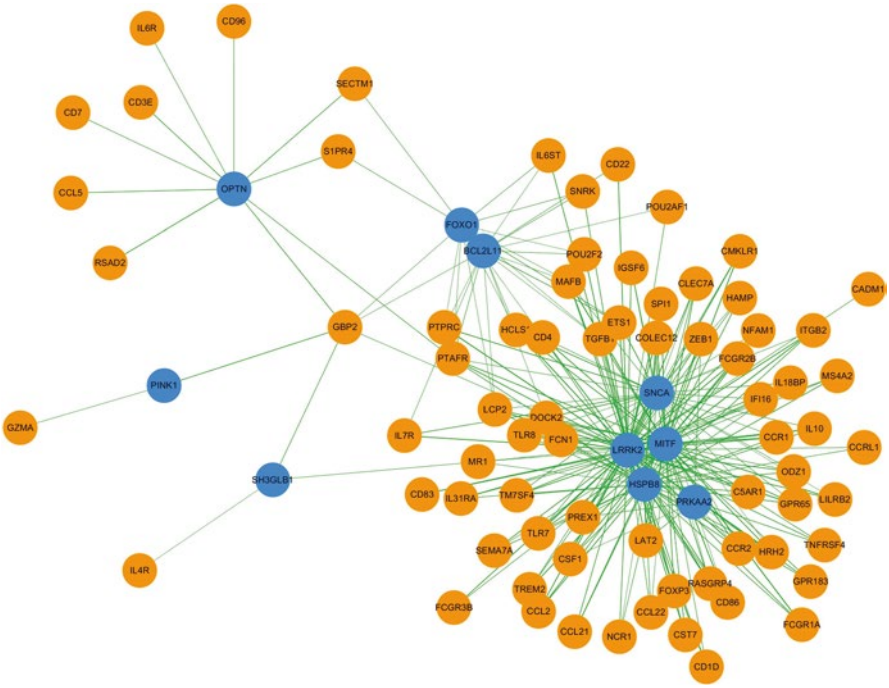

Fig. 2.2 Co-expression network among autophagy and immune response genes in COAD. The autophagy genes are represented by *blue* nodes and the immune response genes by *orange* nodes. The *green* edges represent positive co-expressions and the width of an edge denotes the level of co-expression

co-expressed with 81 under-expressed immune response genes in LM cancers. Similarly, significant co-expression is observed between the down-regulated autophagy genes and immune response genes in LM (and cancer prone) inflammatory diseases. In comparison, 57 up-regulated lysosome genes are strongly co-expressed with 178 up-regulated immune response genes in HL cancers. Interestingly, immune response genes that are co-expressed with autophagy genes have substantial overlap between LM and HL cancers but with opposite differential expression patterns, i.e., down vs. up-regulation. These immune response genes include CD markers, chemokine ligands, chemokine receptors, interleukins, interleukin receptors and other immune genes. The co-expression modules consisting of both autophagy and immune response genes are shown in Fig. 2.2 for selected diseases.

From Fig. 2.2, we can see that these co-expression modules contain a large number of genes related to multiple immune cell types such as CD4+ and CD8+ T cells, B cells, dendritic cells, natural killer cells and macrophages. This is consistent with the observation that the co-expression modules enriched with immune response and autophagy genes are also substantially enriched by lipid binding, lipid metabolism and glycosaminoglycan metabolic genes as it is known that increased lipid metabolism tends to trigger increased immune response (Fritsche 2006) and the same with increased synthesis of cell-surface glycan (Zhang 2006).

Previous studies have identified various invading microbes such as HBV and H. Pylori have developed ways to evade autophagy by suppressing autophagosome formation and fusion to lysosome (Tang et al. 2012), which is consistent with what we observed in the LM cancers and cancer-prone inflammation. The autophagy evasion mechanism has been observed in host cells causing a failed degradation of the infected cells hence less antigen presenting (Paludan et al. 2005). We speculate that normal autophagy in cancer tissue can degrade damaged macromolecules and organelles to promote immune response through induction of antigen presenting. Such a mechanism can be hindered in LM cancers by the suppressed autophagosome formation that is possibly caused by extracellular and cytosolic Fenton reaction, as discussed in Discussion.

2.6 Autophagy and Genomic Mutation

We have conducted a comparative analysis between the level of autophagy and genomic mutation rate using cancer genomic sequences and matching transcriptomic data in TCGA, to assess correlations between genomic mutations and the expression levels of autophagy genes.

We have computed correlation between the somatic mutation rate and gene expression level of autophagy-related genes for each cancer type to evaluate if there is any correlation between the level of differentiation of autophagy genes and the mutation rate at the whole genome level for each cancer type under

study. Interestingly, while correlations are detected, they are different for different cancer types. We noted: the expression levels of lysosome genes are generally positively correlated with the mutation rate in KIRC, KICH and STAD cancers while some sets of macro-autophagy genes are either positively or negatively correlated with the mutation rate in COAD, LUAD, BLCA, and BRCA with strong statistical significance. This correlation is insignificant in LICH, THCA, LUSC, and HNSC.

Genes involved in autophagosome formation such as ATG2B, TP53INP2, SQSTM1, FYCO1, GABARAPL1, GABARAPL2, GABARAPL3, MAP1LC3A, MAP1LC3B and MAP1LC3C are negatively correlated with the mutation rates. They are under-expressed in 8 out of the 11 cancer types, covering all LM cancers. We noted that a number of pro-autophagy and pro-apoptosis signaling genes such as ATG16L1, SKP2, BAX, BID, RPS6KB1, and PIK3R2 are positively correlated with the mutation rate; and they are up-regulated in eight cancer types. Seven autophagy core signaling genes: ATG3, ATG4A, ATG4C, ATG4D, ATG5, ATG7, and ATG12 are positively correlated with the mutation rate but they are not significantly differentially expressed. In addition, the proteasome genes are generally up-regulated and positively correlated with the mutation rate in LM cancers. One possible explanation is that increased proteasome activities tend to be associated with increased Fenton reactions, which can lead to increased mutations, as further discussed in Sect. 2.7.

We have also examined correlations between expressions of autophagy genes and non-synonymous mutation of six specific cancer gene mutations: TP53, KRAS, NAV3, PTEN, ARID1A, and PIK3CA and four frequently mutated genes namely MUC4, TTN, MUC16, and MUC17. TP53 mutation rate is highly positively correlated with the expression levels of a large number of autophagy signaling genes such as ATG genes and BCL-2 genes in BRCA and COAD. WIPI2, RB1CC1 and ATG9A, involved in regulation of the autophagosome formation, are down-regulated in BRCA, COAD and STAD tissues harboring PI3KCA mutations, which are known to affect autophagy responses through the PI3K pathway (Shanware et al. 2013). No significant association has been observed between the expressions of autophagy genes and the other examined gene mutations.

2.7 Discussion

The role of autophagy in cancer has long been debated as it is proposed to promote cancer cells' survival under certain stresses and also possibly to have tumor-suppression roles (Mathew et al. 2007). These studies have been predominantly conducted on cell lines with induced autophagy, hence naturally raising a question: are such observations applicable to cancer tissues, knowing

that the environments in cancer tissues could be substantially different from cell line studies?

We have recently conducted a comparative analysis of gene-expression of cancer tissues and cancer cell lines of the matching cancer types, namely BLCA, BRCA, COAD, KIRC, LIHC, LUAD, LUSC, PRAD, STAD and THCA, under multiple conditions to assess the expression patterns of 896 well-characterized biological pathways, covering 48.83 % (10,010/20,501) of human genes in the two systems (Wei Du and Xu 2016). The following interesting and informative observations are made: (1) 83.15 % of the pathways are found to have similar expression patterns under multiple conditions across different cell lines for the same cancer type; (2) 96.98 % of the pathways share similar expression patterns across different gene-expression datasets for the same cancer type; and (3) only 20.42 % of the pathways share similar expression patterns between cancer tissue samples and cell line datasets. This clearly raised a legitimate concern regarding the applicability of cell-line based observations, particularly about autophagy since our study has clearly shown that there are fundamental differences between autophagy in the two systems.

From the comparative analyses presented throughout this chapter, we noted that the following main differences between cancer tissues and cancer cell based studies:

- The 11 cancer types and 8 cancer prone inflammatory diseases studied here all have reduced or unchanged macro-autophagy activities compared to their basal level activities in the control samples, which is opposite to what has been observed in cancer cell lines under induced metabolic stresses;
- The 11 cancer types full into 2 classes, 1 with reduced macro-autophagy activities and the other with elevated lysosome activities associated with increased micro-autophagy or chaperon-mediated autophagy; reduced macro-autophagy is also observed in 4 cancer prone chronic inflammatory diseases;
- The differentially expressed autophagy genes are largely independent of the enzymes involved in energy metabolism across all the examined disease tissues. Furthermore, there does not seem to be any nutrient-deprivation induced macro-autophagy in cancer tissues;
- Co-expression between altered autophagy activity levels and each of the following: apoptosis, genomic mutation rate and to some level immune response seems to have a strong influence from Fenton reactions in cancer tissues, which cancer cells generally do not have.

Through co-expression analyses, we have identified numerous non-autophagy genes that are strongly co-expressed with various autophagy genes. Out of these genes, some could be the direct causes or results while others may co-occur with the altered autophagy activities, both as results of some common causes. We have conducted further computational analyses to assess if some of these co-expressed genes with autophagy genes may be the result of one specific common cause, Fenton reactions.

As discussed earlier, we consider Fenton reactions as one of the root causes of malignant transformation from normal cells to neoplastic cells. Specifically, we have demonstrated that all cancer tissues have Fenton reactions in cytosol, mitochondria and extracellular space (Hui Yan et al. 2016) and cancer-prone inflammatory tissue cells have the reaction in some but not all three subcellular compartments (Chi Zhang and Xu 2016). By using the Michaelis-Menton equation, we have developed a computational procedure to assess if a specific subcellular location may have Fenton reaction via estimating the four relevant quantities modeled by Michaelis-Mention equation (see below) using gene-expression data. Specifically, the following equation represents the model for estimating the quantity of OH produced by Fenton reaction in a specific subcellular location:

$$[\cdot OH] = \sum_{XH_2} \frac{K_{cat}^{RA}\left(\sum_i a_i X_i^{Fe} + a_0\right)}{\frac{K_1^{XH_2}}{1} + \frac{K_2^{RA}}{\sum_j b_j X_j^{ROS} + b_0} + \frac{K_3^{RA}}{\sum_k c_k X_k^{RA} + c_0} + \frac{K_4^{RA}}{\left(\sum_j b_j X_j^{ROS} + b_0\right)\left(\sum_k c_k X_k^{RA} + c_0\right)}} + \varepsilon,$$

where X_i^{Fe}, X_j^{ROS}, and X_k^{RA} denote the gene expression of marker genes to estimate the level of Fe^{2+}, H_2O_2 and RA by the following linear models:

$$\left[Fe^{2+}\right] = \sum_i a_i X_i^{Fe} + a_0 + \varepsilon_{Fe}$$

$$\left[H_2O_2\right] = \sum_j b_j X_j^{ROS} + b_0 + \varepsilon_{ROS}$$

$$[RA] = \sum_k c_k X_k^{RA} + c_0 + \varepsilon_{RA},$$

where a_i, b_i, and c_i are regression parameters and ε, ε_{Fe}, ε_{ROS}, *and* ε_{RA} are errors.

We have checked if the observed significant co-expressions between autophagy and other biological processes: cell cycle process, mutation rate, and immune response, are possibly causally linked with each other or are common results of Fenton reactions. Statistically, we have checked the co-expression level between A (autophagy) and B (one of the other biological processes), denoted as cor(A, B), and compared this with the same co-expression level but under condition of C (Fenton reaction), denoted as cor(A, B | C). The contribution of C to the co-expression between A and B is evaluated by the *Ratio of Significant Conditional Dependence* (RSCD) defined as the ratio of the number of significantly correlated gene pairs from A and B *versus* the number of significantly correlated gene pairs from A and B under condition of C (see

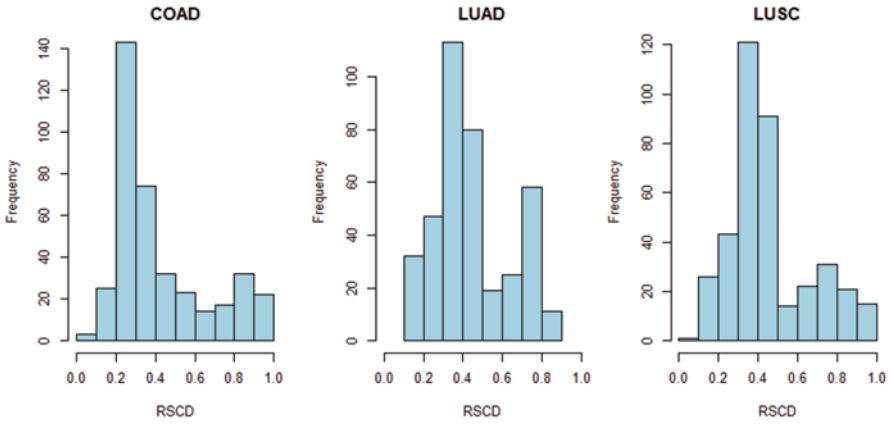

Fig. 2.3 Distribution of the RSCD (A,B|C) values with A = down-regulated autophagosome formation genes, B = the top 400 biological processes co-expressed with the autophagosome formation genes, and C = cytosolic Fenton reaction in three LM cancer types

Sect. 2.9). Smaller RSCD values indicate higher impact of C on the correlation between A and B.

We have computed the RSCD values for A = down-regulated autophagosome formation genes, B = the top 400 biological processes co-expressed with A, and C = cytosolic Fenton reaction in the LM cancer types. Histograms of the RSCD values for COAD, LUAD and LUSC are plotted in Fig. 2.3. From the figure, we can see that the RSCD values in the three cancer types are consistently distributed as bimodal distributions with one large peak for low RSCD values (<0.6) and a small peak for high RSCD (>0.6) values, strongly suggesting most of the biological processes co-expressed with the down-regulation of autophagosome formation genes are dependent on cytosolic Fenton reactions. Further analysis revealed that the biological processes with low RSCD values are quite consistent among the three cancer types, namely apoptosis, cell cycle, DNA binding, ion binding, mitochondria, nucleotide synthesis, Golgi apparatus, mRNA transcription, translation and organelles. It is noteworthy that the centrosome genes have the highest RSCD values among all the proliferation related pathways, substantiating our hypothesis that other than being the common results of cytosolic Fenton reactions, decreased autophagosome formation may directly influence cell cycle process. Considering the biological properties of A, B, and C, we speculate that the best explanation of the observations is that cytosolic Fenton reactions are a common reason for the down-regulated autophagosome formation and other co-expressed biological processes.

Interestingly, most of the immune related genes and pathways do not seem to have influence from cytosolic Fenton reactions as revealed by the above analysis (with high RSCD values). Hence we then conducted a similar analysis but

using extracellular Fenton reactions, and have the following results. The average RSCD value of the immune and inflammation related pathways conditional to cytosolic Fenton reaction is around 0.8 in the three cancer types while the average RSCD value of the pathways conditional to extracellular Fenton reaction is 0.5, suggesting a significant contribution of extracellular Fenton reaction on the correlation between immune response and autophagy but still directly interactions between them exist there. Possible explanations of the observation include (1) the macro-autophagy contribute to cytokine production as in infectious diseases, hence suppressed autophagosome cause less cytokine releasing and immune surveillance (Harris 2011); (2) the macro-autophagy is suppressed by signaling pathways of certain interleukins include IL-4, IL-10 and IL-13 as a result of deregulated immune response (Lapaquette et al. 2015); and (3) extracellular Fenton reaction is a common reason for the immune response and suppressed autophagosome in the LM cancers.

2.8 Conclusion

Our comparative analysis of transcriptomic data of cancer tissues *versus* cancer cell lines revealed that (1) cancer tissues generally do not have metabolic stress and its induced macro-autophagy; (2) while some cancer tissues have increased lysosome activity, it is largely induced micro-autophagy or chaperon-mediated autophagy for degradation of oxidatively damaged macromolecules and organelles due to Fenton reactions; and (3) various cellular processes are found to be co-expressed with autophagy genes; the majority of them may represent co-occurring events with autophagy as common results of Fenton reactions, rather than causal relations with autophagy. Overall, new information is revealed, which is clearly subject to further experimental validation and may possibly lead to improved understanding about the biology of autophagy in cancer tissues.

2.9 Material and Methods

2.9.1 Data Used

We have conducted a differential gene expression analysis measured using normalized fold change in 11 TCGA cancer types: namely bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), stomach adenocarcinoma (STAD), and thyroid carcinoma (THCA);

eight cancer-prone inflammatory diseases: cirrhosis (CIR), Barrett's esophagus (BE), ulcerative colitis (UC), Crohn's disease (CD), chronic HCV infection (HCV), idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), and atopic dermatitis (AD) that have significant increased risk for cancer development; and six cancer-independent inflammatory diseases namely alcohol hepatitis (AH), non-alcoholic steatohepatitis (NAS), cystitis (CS), asthmatics (AST), and psoriasis (PSO) whose occurrence does not increase the risk of cancer development as reported in literature; and 12 datasets of cell line-based gene-expression data collected under serum depletion or other metabolic stress conditions to induce autophagy.

RNA-seq and genomic data of these tissue and cell samples are collected from TCGA and GEO databases. Detailed information of the datasets are listed in Table 2.2. The RNA-seq data are normalized using the RSEM method while all the microarray data are measured by UA133 plus 2.0 array and normalized by the RMA method.

2.9.2 Gene Differential Expression and Pathway Enrichment Analysis

Differentially expressed genes in cancer and inflammatory disease are assessed by using the Mann-Whitney test with p-value adjusted by the FDR method and FDR = 0.05 is used as the significance cut-off. Average Fold Change (FC) is used on cell line gene expression under autophagy-inducing condition *versus* controls for determination of differentially expressed genes due to their limited sample size. We use log (FC) = 0.5 and –0.5 as the cut-off for over and under expression, respectively.

Eleven autophagy related pathways are manually generated, as detailed in the main text. 2775 pathways covering major biological processes including cell proliferation, apoptosis, and immune response among a few others are collected from the MsigDB database. In addition, nine sets of metabolic deprivation marker genes responsive to glucose, leucine, methionine, glutamate, other amino acids as well as serum deprivation are also retrieved from the MsigDB chemical perturbation responsive gene sets.

Pathway enrichment is assessed using a hypergeometric test and the p-values are adjusted by the FDR method with FDR = 0.05 as the significance cut-off.

2.9.3 Gene Co-expression Analysis

We have previously developed a Rank-based gene co-expression module extraction method (Zhang et al. 2015). The method first identifies hub genes in a given gene network and then expand the hubs to co-expression modules. The method is applied

to the cancer and inflammatory disease data to identify co-expression modules that are significantly enriched by autophagy genes. The co-expression modules enriched by down-regulated macro-autophagy genes in LM diseases and up-regulated lysosome genes in HL diseases are specifically analyzed to elucidate biological processes related to the differentially expressed autophagy genes.

2.9.4 Correlate Gene Expression Data to Genomic Mutations

We define the total mutation rate of each sample as the total number of non-synonyms point mutations. Pearson correlation coefficient between the expression level of each gene and the mutation rate for each disease type is calculated. Significance of the correlation is assessed by using the *t*-test. Six cancer genes and four frequently mutated genes are selected and analyzed. Association between each mutation and autophagy gene is tested by comparing the gene expression level in sample with the mutation *versus* the mutation free samples by Mann Whitney test. FDR = 0.05 is used as the significance cut-off.

2.9.5 Ratio of Significant Conditional Dependence

The Ratio of Significant Conditional Dependence (RSCD) is defined by the ratio of the number of significantly correlated gene pairs from sets A and B *versus* the number of significantly correlated gene pairs from A and B under condition C. The RSCD is defined by following:

$$\mathrm{RSCD}(\mathrm{A},\mathrm{B}\mid\mathrm{C}) = \frac{\#\left\{\mathrm{p.cor}\left(G_a, G_b\right)\alpha \mid G_a \in A, G_b \in B\right\}}{\#\left\{\mathrm{p.cor}\left(G_a, G_b \mid C\right) < \alpha \mid G_a \in A, G_b \in B\right\}},$$

where G_a and G_b are a pair of genes from pathway A and B, p.cor is the p-value of the co-expression and α is the significance level. We use Pearson correlation to evaluate the co-expression level between gene pairs. The Pearson correlation product-moment function is applied to access the p-value for each correlation (and conditional correlation) and $\alpha = 0.05$ is used as the statistical significance cut-off. There is a significant contribution by C to the correlation between A and B if the conditional correlation level Cor (A, B | C) is substantially lower than Cor (A, B), and there is no significant contribution by C to the correlation between A and B if Cor (A, B | C) is comparable with Cor (A, B). Hence smaller RSCD values imply higher impact of C on the correlation between A and B.

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Chapter 3
Small-Molecule Regulators of Autophagy as Potential Anti-cancer Therapy

Qing Li, Mi Zhou, and Renxiao Wang

Abstract Autophagy is an evolutionary conserved lysosomal pathway functioned in the turnover of cellular macromolecules and organelles. It is known that autophagy can have a cytoprotective effect in tumor cells under therapeutic treatment. Autophagy inhibitors thus may be used as auxiliary drugs to augment the anti-tumor activity of cancer therapies. On the other hand, autophagy is a cytotoxic event that can kill tumor cells. Autophagy inducers that increase the level of autophagy thus may be developed as a new class of anti-cancer therapy. This chapter will describe the known pathway of autophagy and its relationship to cancer. The focus of this chapter is to give a summary of the known small-molecule regulators of autophagy, including inhibitors and inducers, discovered as potential therapies for cancer treatment.

Keywords Autophagy • Cell death • Autophagy inhibitor • Autophagy inducer • Anti-cancer treatment

3.1 Introduction

Autophagy plays an essential role in normal physiology. Under normal conditions, autophagy occurs at basal levels to maintain cellular homeostasis by removing long-lived or misfolded proteins and clearing damaged or dysfunctional organelles. Under starved conditions, autophagy can be induced to digest dysfunctional proteins and organelles more rapidly, which will help cell to survive (Green and Levine 2014). It is also well known that autophagy may protect cell by overcoming adversities,

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such as starvation, chemotherapies or radiotherapies. Autophagy can also exhibit cytotoxicity in certain condition, e.g. when apoptosis is blocked (Gewirtz 2014).

In this chapter, we will briefly introduce the molecular mechanism of autophagy and its dual role in anti-cancer therapy development. We will also review the public reported small-molecule regulators of autophagy, including autophagy inhibitors and autophagy inducers, discovered as potential anti-cancer therapies.

3.2 The Process of Autophagy

The known pathway of autophagy includes induction, formation and elongation of isolation membrane, autophagosome completion, fusion of autophagosome and lysosome, and degradation in autolysosome (Fig. 3.1). Autophagy is regulated by a "pre-initiation" ULK complex, which includes ULK1, FIP200 and ATG13. The ULK complex then activates Class III PI3K complex, which requires the disruption of binding of anti-apoptotic Bcl-2 proteins to Beclin 1 and is also regulated by AMPK. The Class III PI3K complex generates Phosphatidylinositol 3-phosphate

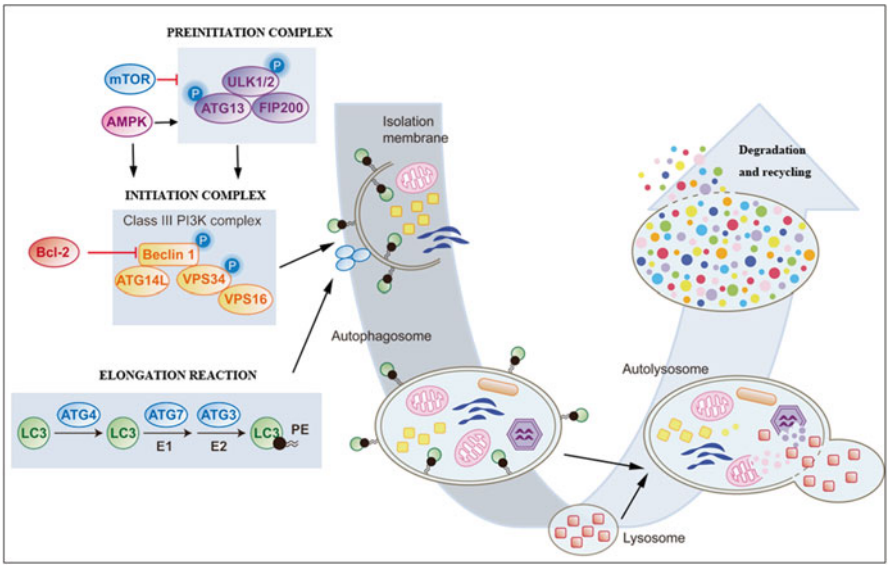


Fig. 3.1 The process of autophagy. The process of autophagy starts with the formation of an isolation membrane, which is regulated by the initiation Class III PI3K complex. The Class III PI3K complex is activated by the pre-initiation ULK complex which is negatively and positively regulated by upstream kinases mTOR and AMPK, respectively. The activated Class III PI3K complex generates PI3P at the site of nucleation of the isolation membrane. This event leads to the binding of proteins involved in the "elongation reaction" to the isolation membrane, resulting in formation of autophagosome. Then, autophagosome fuses with lysosome to form autolysosome, in which its contents undergo degradation and recycling

(PI3P) at the site of nucleation of the isolation membrane (also called the phagophore). The elongation reaction of the isolation membrane is a complicated process to form LC3-II. LC3-II generated by the ATG4-dependent proteolytic cleavage of LC3, and required of ATG7, ATG3, and the ATG12/ATG5/ATG16L complex, which is associated with the mature autophagosome. The autophagosome fuses with a lysosome to form an autolysosome, in which the surrounded contents are degraded and released into the cytoplasm for recycling (Marino et al. 2014).

In the context of nutrient starvation, AMPK is activated and/or mTORC1 is inhibited, which in turn activated the ULK complex to engage autophagy. During starvation-induced autophagy, AMPK is required to release negative regulators of the Beclin 1-VPS34 initiation complex, such as Bcl-2/Bcl-xL (Wirth et al. 2013).

3.3 Role of Autophagy in Cancer

Autophagy has been shown to act as a tumor suppressor, but its role in cancer treatment is still controversial (Maycotte and Thorburn 2011). Almost all of traditional anti-cancer therapies, such as anti-cancer drugs and ionizing radiation, affect autophagy. Most of anti-cancer drugs increase autophagy, which protects treated tumor cells to survive. However, it also has been reported that autophagy is a cell death mechanism when apoptosis is blocked, known as autophagic cell death (Maycotte and Thorburn 2011; Thorburn et al. 2014).

3.3.1 Tumor Promotion

In tumorigenesis, the rapid growth of tumor tissue puts cancer cells under harsh and continuous metabolic stress that results in nutrient deprivation, growth factor limitation, and hypoxia (Zhou and Wang 2013). Nutrient deprivation induces autophagy by mTORC1 inhibition and AMPK activation. Autophagy can also be induced by hypoxia, it has been found to localize to hypoxic tumor regions, supporting cell survival through elimination of autophagic substrate p62, damaged mitochondria and reactive oxygen species (ROS) (Maycotte and Thorburn 2011; Zhou and Wang 2013).

Autophagy may promote tumor cell metastasis by preventing anoikis. When cells are detached from the extracellular matrix (ECM), they may undergo anoikis. However, metastatic tumor cells may escape from anoikis and invade other organs (Frisch and Screaton 2001). Loss of clonogenic capacity is a foundational factor during tumorigenesis, autophagy can be induced to reduce clonogenic capacity after anoikis (Fung et al. 2008).

In tumor treatment, autophagy has been proposed as a protective mechanism to resist chemo- or radio-therapy and to help residual tumor cells to enter dormancy. It is well known that autophagy can function as a survival mechanism which is activated after cancer treatment. In certain instances, tumor can relapse and metas-

tasize after primary tumor treatment in many years later, suggesting residual tumor cells may remain in a dormant state. A recent study showed overexpression of tumor suppressor *aplasia Ras homolog member I* (*ARHI*) promotes the formation of dormant tumors, which was reduced by autophagy inhibitor CQ (Sosa et al. 2013).

3.3.2 Tumor Suppression

Autophagy occurs at basal levels during nutrient rich conditions. The basal autophagy has been shown to be a tumor suppressor mechanism. Cell-cycle check-points are inactivated in tumor cells, but autophagy limits the accumulation of DNA damage and suppresses the mutation rate. It confirms the role for autophagy in protecting the genome in a cellular spontaneous mechanism of tumor suppression (Mathew et al. 2007).

A direct link between autophagy and tumor suppression is the discovery that Beclin 1 could function as a tumor suppressor (Liang et al. 1999). The autophagy gene *Beclin 1* is mono-allelic deleted in 40–75 % of cases of human sporadic breast, ovarian, and prostate cancer. Disruption of *Beclin 1* increases the frequency of spontaneous malignancies and accelerates the development of *hepatitis B* virus-induced premalignant lesions in a targeted mutant mouse model (Qu et al. 2003). In addition, animals deficient in autophagy-related *Atg4C* show an increased susceptibility to develop fibrosarcomas induced by chemical carcinogens (Marino et al. 2007).

In apoptosis-deficient cancer cells, autophagy has been induced to maintain cell metabolism and viability during nutrient starvation and protect cells from necrosis. Finally, if the nutrient deprivation persists, continuous autophagy may lead to autophagic cell death, which is type II programmed cell death (PCD) (the others are type I PCD apoptosis and type III PCD necrosis) (Clarke 1990). Autophagic cell death can be suppressed by autophagy inhibitors (e.g., 3-methyladenine and wortmannin) or genetic knockout/knockdown of essential autophagy genes (Shimizu et al. 2014). A recent study indicated that JNK activation is crucial for the autophagic cell death of *Bax*/*Bak* double knockout cells (Shimizu et al. 2010).

3.4 Small-Molecule Regulators of Autophagy

A good number of small-molecule regulators of autophagy have been reported in literature. They have been used either as chemical tools in basic research on autophagy, or developed as drug candidates for cancer treatment (Zhou and Wang 2013; Baek et al. 2012; Fleming et al. 2011; Wu and Yan 2011; Levy and Thorburn 2011; Nagelkerke et al. 2015).

3.4.1 Autophagy Inhibitors

There is extensive and relatively definite evidence showed that the level of autophagy increased in tumor cells. Considering the tumor promotion mechanism of autophagy, many compounds have been developed to treat cancers based on their autophagy inhibition function (Table 3.1).

Multiple clinical trials are currently on-going at every phase by combining autophagy inhibitors with various conventional treatment methods in order to enhance the response to treatment (Gewirtz 2014; Kumar et al. 2015).

3.4.1.1 Class III PI3K Inhibitors

The Class III PI3K, Vps34, shows the positive relationship with autophagy and generates PI3P at the site of nucleation of the isolation membrane by forming a complex with Beclin 1 and other cofactors (Green and Levine 2014). A number of PI3K inhibitors have been developed as autophagy inhibitors, including wortmannin, LY294002, 3-methyladenine (3-MA), and SAR405.

Wortmannin, a steroid metabolite of the fungi *Penicillium funiculosum*, is a nonspecific covalent PI3K inhibitor (Powis et al. 1994). LY294002 is a morpholino derivative of quercetin (Vlahos et al. 1994). Wortmannin derivative PX-866 and LY294002 (Arg-Gly-Asp-Ser)-conjugated SF1126 were shown to be active against various cancer xenografts (Maira et al. 2009). Treatment with wortmannin or LY294002 resulted in a strong inhibition of proteolysis in amino acids-deprivation rat hepatocytes (Blommaart et al. 1997). 3-MA inhibited endogenous protein degradation by about 60 % at 5 mM, and suppressed the formation of autophagosomes (Seglen and Gordon 1982). These three PI3K inhibitors act on PI3K nonselectively, regarding as tools to study PI3K/mTOR pathway and autophagy.

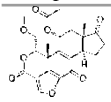
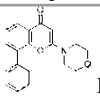
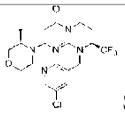
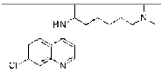
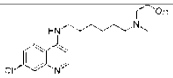
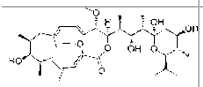
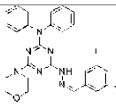
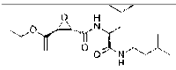
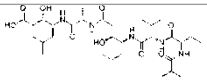
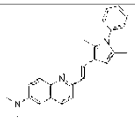
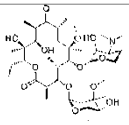
SAR405, a derivative of pyrimidinones, is a first reported selective inhibitor of class III PI3K Vps34. Inhibition of Vps34 by SAR405 affects late endosome-lysosome compartments and prevents autophagy, co-treatment with SAR405 and mTOR inhibitor everolimus results in synergistic anti-proliferative activity in renal tumor cell lines (Ronan et al. 2014).

3.4.1.2 Compounds Disrupting Lysosomal Homeostasis

Lysosome is a membrane-bound cell organelle found in most animal cells. It contains hydrolytic enzymes capable of breaking down virtually all kinds of biomolecules. In the late stage of autophagy, lysosomes fuse with autophagosomes to digest the contents of autophagosomes.

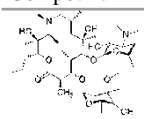
Chloroquine (CQ) and its derivative hydroxychloroquine (HCQ) are widely used as the first choice drug for malaria treatment. CQ seems to exert its effects through the weak-base feature by enriching in acidic lysosomes, and thereby destroyed lyso-

Table 3.1 Small-molecule inhibitors of autophagy

Compound	Mechanism and references	Compound	Mechanism and references
 Wortmannin	Pan-PI3K inhibitor (Powis et al. 1994; Maira et al. 2009; Blommaart et al. 1997)	 LY294002	Pan-PI3K inhibitor (Maira et al. 2009; Blommaart et al. 1997; Vlahos et al. 1994)
 3-methyladenine (3-MA)	Pan-PI3K inhibitor (Maira et al. 2009; Seglen and Gordon 1982)	 SAR405	Class III PI3K inhibitor (Ronan et al. 2014)
 Chloroquine (CQ)	Disrupts lysosomal homeostasis (Homewood et al. 1972; Fukuda et al. 2015; Balic et al. 2014; Kimura et al. 2013)	 Hydroxychloroquine (HCQ)	Disrupts lysosomal homeostasis (Homewood et al. 1972)
 Bafilomycin A1	ATPase inhibitor, disrupts lysosomal homeostasis (Harada et al. 1996; Mauvezin and Neufeld 2015)	 Vacuolin-1	Disrupts lysosomal homeostasis (Cerny et al. 2004; Lu et al. 2014)
 Matrine	Disrupts lysosomal homeostasis (Chen et al. 2006; Liu et al. 2010; Wang et al. 2013)	 Thymoquinone	Disrupts lysosomal homeostasis (Racoma et al. 2013)
 E-64d	Cathepsin inhibitor, disrupts lysosomal homeostasis (Tamai et al. 1986; Tanida et al. 2005)	 Pepstatin A	Cathepsin inhibitor, disrupts lysosomal homeostasis (Tanida et al. 2005; Umezawa et al. 1970)
 Lucanthone	Topoisomerase inhibitor, disrupts lysosomal homeostasis (Bases and Mendez 1997; Carew et al. 2011)	 Pyrvinium	Casein kinase activator, inhibits the transcription of autophagy genes (Thorne et al. 2010; Deng et al. 2013)
 NSC185058	ATG4B inhibitor, inhibits formation of LC3-II (Akin et al. 2014)	 Clarithromycin	Macrolide antibiotic (Nakamura et al. 2010)

(continued)

Table 3.1 (continued)

Compound	Mechanism and references	Compound	Mechanism and references
 Azithromycin	Macrolide antibiotic (Renna et al. 2011)		

somal function (Homewood et al. 1972). It shows the antitumor activity in many kind of tumor cells, such as endometrial cancer cells (Fukuda et al. 2015), pancreatic cancer stem cells (Balic et al. 2014). Inhibition of autophagy by CQ could sensitize cisplatin-tolerant cancer cells, as well as injure kidney cells in chemotherapy, leading to acute kidney injury (Kimura et al. 2013).

Bafilomycin A1 is an inhibitor of V-ATPase, which is necessary for acidification of the endocytic compartments (Harada et al. 1996). It can also disrupt autophagic flux by inhibiting calcium ATPase-dependent autophagosome-lysosome fusion (Mauvezin and Neufeld 2015).

Vacuolin-1 has been discovered in an image-based phenotypic screen for inhibitors of the secretory pathway, by blocking the Ca^{2+}-dependent exocytosis of lysosomes (Cerny et al. 2004). Treatment with vacuolin-1 alkalinized lysosomal pH and decreased lysosomal Ca^{2+} content in HeLa cells (Lu et al. 2014).

Matrine, derived from traditional Chinese medicine *Sophora flavescens*, has been reported to improve the immune function and life quality of cancer patients by combining standard therapies (Chen et al. 2006). It can also inhibit proliferation and induce apoptosis of pancreatic cancer cells (Liu et al. 2010). Recently matrine has been reported to block autophagic degradation by impairing the activities of lysosomal proteases, and elevating pH values in endosomes/lysosomes (Wang et al. 2013).

Thymoquinone, derived from *Nigella sativa* seed, was reported to inhibit proliferation in glioblastoma cells. It induced lysosomal membrane permeabilization, resulting in a leakage of cathepsin B into the cytosol, which mediates caspase-independent cell death (Racoma et al. 2013).

Cathepsins are proteases distributed in almost all mammalian cells, with functions in tumor progression (Nomura and Katunuma 2005). Most of the members of cathepsins become activated at the low pH level in lysosomes. Their activities are closely linked with the lysosomal function. Cathepsins inhibitors E64d (Tamai et al. 1986) and pepstatin (Umezawa et al. 1970) are frequently used in autophagy-related research as autophagy inhibitors (Tanida et al. 2005).

Lucanthone, an inhibitor of topoisomerase, has been used as an adjuvant in radiation therapy (Bases and Mendez 1997). It induces lysosomal membrane permeabilization to break lysosomal homeostasis, and possesses significantly more potent activity in breast cancer models compared with CQ (Carew et al. 2011).

3.4.1.3 Others Types of Autophagy Inhibitors

An FDA-approved antihelminthic drug pyrvinium shows wide-ranging anti-cancer activity during glucose starvation. It binds casein kinase 1 and increases the kinase activity of casein kinase 1α, which is a negative regulator of Wnt1 pathway (Thorne et al. 2010). Pyrvinium was reported to inhibit autophagy by suppressing the transcription of autophagy genes, such as *Beclin1* and *Vps34*. The inhibition of autophagy by pyrvinium increases the anti-cancer activity of 2-deoxy-D-glucose (Deng et al. 2013).

ATG4B is an essential cysteine proteinase to activate LC3 produce LC3-II. Knockdown of *Atg4b* in Osteosarcoma Saos-2 cells resulted in a failure of forming tumors in mouse models. The antagonist of ATG4B NSC185058 shows a negative impact on the development of Saos-2 osteosarcoma tumors *in vivo* (Akin et al. 2014).

Macrolide antibiotic clarithromycin and aithromycin were reported to block autophagy, whose mechanisms still remain unclear. Clarithromycin increased the anti-tumor activity of thalidomide against multiple myeloma cells (Nakamura et al. 2010). Azithromycin mediated autophagy increases the risk of infection with drug-resistant pathogens (Renna et al. 2011).

3.4.2 Autophagy Inducers

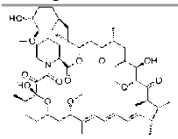
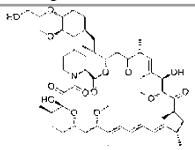
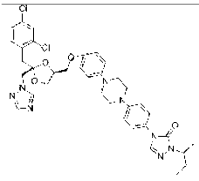
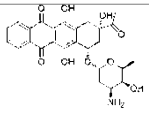
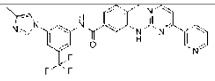
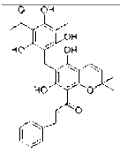
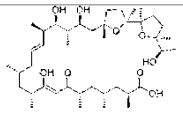
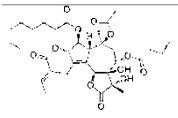
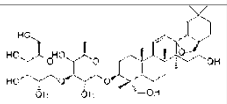
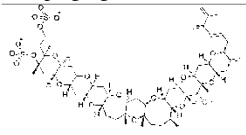
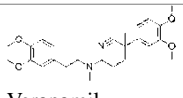
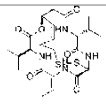
Based on the role of autophagy in tumor suppression, many compounds are used as anti-cancer reagents by inducing autophagy (Fulda and Kogel 2015) (Table 3.2). It should be noted that not all autophagy inducers may be used as anti-cancer reagents. It is because some of them induce protective autophagy, which leads to tumor resistance. These compounds are combined with autophagy inhibitors to treat cancer usually, so they are not discussed in this chapter.

3.4.2.1 mTOR Inhibitors

mTOR (mammalian Target Of Rapamycin) senses cellular nutrient and energy levels, and negatively regulates autophagy. mTOR forms two distinct signaling complexes, mTORC1 and mTORC2. The molecular mechanism of how mTORC2 is regulated by its upstream effectors is largely unknown. mTORC1 (hereafter mTOR) is a master regulator of cellular metabolism and autophagy, regulated by the growth factor/PI3K/AKT signaling pathway. It integrates nutrient and growth factors which signal to promote anabolic metabolism, such as protein synthesis and lipid synthesis, and to inhibit catabolic pathways, such as lysosomal biogenesis and autophagy (Laplante and Sabatini 2012).

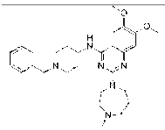
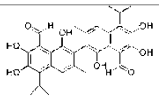
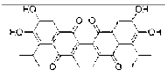
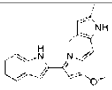
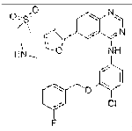
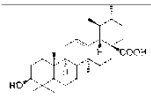
Inhibition of mTOR leads to activation of ULK1, which then phosphorylates other critical subunits of the ULK1 complex, ATG13 and FIP200 (Jung et al.

Table 3.2 small-molecule inducers of autophagy

Compound	Mechanism and references	Compound	Mechanism and References
 Rapamycin	mTOR inhibitor (Kim and Guan 2015; Nam et al. 2013)	 Everolimus	mTOR inhibitor (Kim and Guan 2015; Albert et al. 2006; Cao et al. 2006)
 Itraconazole	Antifungal drug, inhibits mTOR/AKT/PI3K signaling pathway (Liu et al. 2014)	 Idarubicin	Topoisomerase inhibitor, inhibits mTOR activity (Ristic et al. 2014; Plumbridge and Brown 1978)
 Metformin	AMPK activator (Takahashi et al. 2014)	 Nilotinib	Tyrosine kinase inhibitor, activates AMPK (Yu et al. 2013)
 Rottlerin	Protein kinase C inhibitor, activates AMPK (Kumar et al. 2014)	 Ionomycin	Disrupts calcium homeostasis (Hoyer-Hansen et al. 2007; Hoyer-Hansen and Jaattela 2007)
 Thapsigargin	Disrupts calcium homeostasis (Hoyer-Hansen et al. 2007; Hoyer-Hansen and Jaattela 2007)	 Saikosaponin-d	Disrupts calcium homeostasis (Wong et al. 2013)
 Yessotoxin	Disrupts calcium homeostasis (Rubiolo et al. 2014; Azad et al. 2008)	 Verapamil	Disrupts calcium homeostasis (Salabei et al. 2012)
 Valproic acid	HDACi, induces ROS-dependent autophagy (Shao et al. 2004; Fu et al. 2010)	 Vorinostat	HDACi, induces autophagic cell death (Shao et al. 2004; Zhang et al. 2005; Yamamoto et al. 2008; Wei et al. 2010)
 Sodium butyrate	HDACi, induces autophagic cell death (Shao et al. 2004; Hamer et al. 2008)	 FK228	HDACi, induces autophagic cell death (Watanabe et al. 2009)

(continued)

Table 3.2 (continued)

Compound	Mechanism and references	Compound	Mechanism and References
Arsenic trioxide (As_2O_3)	Induces autophagic cell death (Miller et al. 2002; Goussetis et al. 2010)	Sodium arsenite ($NaAsO_2$)	Induces ROS-dependent autophagic cell death (Miller et al. 2002; Zhu et al. 2014; You et al. 2015)
 BIX-01294	EHMT2 inhibitor, induces autophagic cell death (Kim et al. 2013a)	 Gossypol	Bcl-2 inhibitor, induces autophagy by releasing Beclin 1 (Shimizu et al. 2004; Voss et al. 2010)
 Apogossypolone	Bcl-2 inhibitor, induces autophagy by releasing Beclin 1 (Zhang et al. 2010; Arnold et al. 2008; He et al. 2014; Niu et al. 2014)	 Obatoclax	Bcl-2 inhibitor, induces autophagic cell death (Heidari et al. 2010; Bonapace et al. 2010)
 Lapatinib	Tyrosine kinase inhibitor, induces autophagic cell death (Chen et al. 2015; Chen et al. 2014)	 Gemcitabine	Induces autophagic cell death (Wang et al. 2014; Donadelli et al. 2011)
 Ursolic acid	Induces ATG-5-dependent autophagy (Leng et al. 2013)	Salinomycin	Cation ionophore, activates autophagic flux

2009). The growth factor/PI3K/AKT/mTOR pathway is the main pathway regulated by mTOR, its activation is associated with malignant transformation and apoptotic resistance, and hence represents a cell survival mechanism (Polivka and Janku 2014).

In earlier studies, mTOR inhibitors, such as rapamycin and everolimus, were reported to induce autophagy in various model systems. The induction of autophagy by mTOR inhibitors are more inclined to protect cancer cells survival, not death (Kim and Guan 2015). However, recent reports showed rapamycin-induced autophagy may sensitize cancer cells to radiotherapy. Everolimus inhibited radiation-induced AKT/mTOR signaling pathway and enhances the cytotoxic effects of radiation in breast cancer cell models (Albert et al. 2006). It increased the radio-sensitization of PTEN-null prostate cancer cells, and enhances radiation-induced mortality in apoptosis deficient cells, which means everolimus may induce autophagic cell death in certain condition (Cao et al. 2006). In addition, persistent activation of autophagy by mTOR inhibitor rapamycin leads radio-resistant cancer cells into senescence in head and neck cancer cells and a xenograft model (Nam et al. 2013).

Itraconazole, a traditional broad-spectrum antifungal drug, inhibited cell proliferation and induced autophagic progression in glioblastoma cells by repression of AKT/mTOR signaling pathway. Its anti-proliferative activity was inhibited by the blockage of autophagy, suggesting autophagy is responsible for itraconazole-induced inhibition of proliferation (Liu et al. 2014).

Idarubicin is a DNA-binding antileukemic drug (Plumbridge and Brown 1978), has been showed to induce apoptosis and cytotoxic autophagy through mTOR repression. Autophagy inhibitor wortmannin or CQ partially reduced the cytotoxicity of idarubicin in the acute lymphocytic leukemia REH cells (Ristic et al. 2014).

3.4.2.2 AMPK Activators

AMPK are known to induce autophagy. Under starvation condition, AMPK induction and/or mTOR inhibition will lead to autophagy by ULK1 phosphorylation. AMPK can also act directly on the Beclin 1/Vps34 complex (Kim et al. 2013b).

Metformin, a prescribed drug for type 2 diabetes, activated AMPK and reduced cell proliferation, leading to the induction of apoptosis and autophagy. Inhibition of autophagy by knockdown of *Beclin 1* or by 3-MA suppressed the anti-proliferative effects of metformin on endometrial cancer cells, indicating that the anti-proliferative effects of metformin are partially or completely dependent on autophagy (Takahashi et al. 2014).

Nilotinib, a tyrosine kinase inhibitor, significantly reduced cell viability in hepatocellular carcinoma cell lines through autophagy by AMPK activation instead of apoptosis. Knock-down of *Atg5* reduced the effect of nilotinib on autophagy and cell death significantly, co-treatment of nilotinib with a known AMPK activator metformin enhanced the effect of nilotinib on autophagy and cell death (Yu et al. 2013).

Rottlerin, a protein kinase C inhibitor, showed anti-cancer activity in prostate cancer. It induced early stage autophagy via AMPK activation and apoptosis via inhibiting PI3K/AKT/mTOR pathway in human prostate cancer stem cells. Co-treatment with autophagy inhibitors bafilomycin or 3-MA inhibited rottlerin-induced apoptosis. It illustrated that autophagy was required in rottlerin mediated prostate cancer treatment (Kumar et al. 2014).

3.4.2.3 Compounds Disrupting Calcium Homeostasis

Calcium (Ca^{2+}) is one of the most important cellular second messengers. The disorder of Ca^{2+} homeostasis can evoke different types of cell death in cancer cells. Autophagic cell death can be induced by vitamin D3, ATP, ionomycin and thapsigargin, which increased the cytosolic Ca^{2+} in MCF-7 breast cancer cells (Hoyer-Hansen et al. 2007). One of the best investigated mechanisms of calcium-mediated autophagy induction is mTOR-mediated endoplasmic reticulum (ER) stress and unfolded protein response (UPR) activation (Hoyer-Hansen and Jaattela 2007).

Saikosaponin-d is an inhibitor of Ca^{2+} ATPase which induces autophagy by activating the Ca^{2+}/calmodulin-dependent AMPK/mTOR signaling pathway, ER stress and UPR. It leaded to autophagic cell death especially in apoptosis-resistant MEFs cells, which either lack *Caspases 3*, *7* or *8* or have the *Bax/Bak* double knockout (Wong et al. 2013).

Yessotoxin has been shown to modulate Ca^{2+} gating resulted in increasing cytosolic calcium in human lymphocytes. In glioma cells, it induced autophagic cell death mediated by BNIP3 (Rubiolo et al. 2014). BNIP3 inhibits the mTOR pathway through RHEB, which has been shown to induce cell death by autophagy inhibitor 3-MA but not by caspase inhibitor z-VAD-fmk (Azad et al. 2008).

Verapamil is an L-type Ca^{2+} channel antagonist. Treatment with verapamil did not affect cell viability in vascular smooth muscle cells, but inhibited cell proliferation and induced morphological alterations, such as karyokinesis and accumulated perinuclear vacuoles due to enhanced mitochondrial damage and upregulated autophagy (Salabei et al. 2012).

3.4.2.4 Histone Deacetylase Inhibitors

Histone deacetylase (HDAC) is a class of enzymes that remove acetyl groups from an lysine on histone, allowing the histones to wrap the DNA more tightly, leading to chromatin remodeling and transcriptional suppression of key apoptosis and cell cycle regulatory genes (Jazirehi 2010).

It has been reported that HDAC inhibitors preferentially kill transformed cells or cancer cells. According to their chemical structures, HDAC inhibitors can be classified into several groups, including (i) short-chain fatty acids, such as sodium butyrate and valproic acid ; (ii) hydroxamic acids, such as vorinostat; and (iii) cyclic tetrapeptides, such as FK228 (Shao et al. 2004).

Valproic acid, a widely used anti-epilepsy drug, induces autophagy by ROS-dependent pathway in glioma cells. Combination with other autophagy inducers (such as rapamycin, LY294002) increased valproic acid-induced autophagic cell death (Fu et al. 2010). Sodium butyrate exerts potent effects on the inhibition of inflammation and carcinogenesis in colon tissue (Hamer et al. 2008). It induces mitochondria-mediated apoptosis and autophagic cell death in HeLa cells (Shao et al. 2004).

Vorinostat (also known as suberoylanilide Hydroxamic Acid) is approved by FDA to treat cutaneous T cell lymphoma (Zhang et al. 2005). It was reported to induce apoptosis and autophagic cell death in chondrosarcoma cell lines and HeLa cells (Shao et al. 2004; Yamamoto et al. 2008). Combination with vorinostat and BH3-mimetic GX15-070 has synergistic effects in acute myeloid leukemia (AML) cell lines and primary AML cells by activating both apoptosis and autophagy (Wei et al. 2010).

Another HDAC inhibitor FK228 can also induce autophagy. Disrupting autophagy with CQ enhanced FK228-induced cell death, which means FK228-induced autophagy is cytoprotective (Watanabe et al. 2009).

3.4.2.5 BH3 Mimetics

Anti-apoptotic Bcl-2 proteins show its anti-apoptotic activity by binding pro-apoptotic protein Bax/Bak, they inhibit autophagy by binding Beclin 1 through their BH3 domain. Consequently, BH3 mimetics are able to activate apoptosis and autophagy (Pattingre and Levine 2006). They have a role in control of autophagic cell death depends on the autophagy genes *Beclin 1* and *Atg5* (Shimizu et al. 2004).

Bcl-2 inhibitor gossypol was reported to induce autophagic cell death by releasing Beclin 1 in malignant glioma, it potentiated the anti-cancer activity of temozolomide, which was used as a first-line treatment of glioblastoma multiforme (Voss et al. 2010). Apogossypolone is a derivative of gossypol, exhibits a higher antitumor activity and lower toxicity than gossypol (Zhang et al. 2010; Arnold et al. 2008). It can also inhibit the binding of Bcl-2 to Beclin 1, it induces autophagy and radiosensitizing in nasopharyngeal carcinoma cells *in vitro* and *in vivo* (He et al. 2014). Moreover, it reduced Bcl-2 expression, and enhanced the expression of Bax and Beclin 1 in MCF-7 cells (Niu et al. 2014).

Obatoclax (also known as GX15-070) has been reported to overcome glucocorticoid resistance in acute lymphoblastic leukemia (ALL) by inducing apoptosis and autophagy, which can be inhibited by downregulation of ATG5 or Beclin 1 (Heidari et al. 2010). In childhood ALL cells, obatoclax plays a role in autophagy-dependent necroptosis, which is required for overcoming glucocorticoid resistance (Bonapace et al. 2010).

3.4.2.6 Other Types of Autophagy Inducers

Inorganic arsenic is a worldwide environmental pollutant. It is widely known as carcinogens that induce cancers in many human tissues (Miller et al. 2002). Arsenic trioxide (As_2O_3) has been used to treat acute promyelocytic leukemia, and also acts as a potent inducer of autophagy in leukemia cells. Treatment with autophagy inhibitors or knockdown of *Beclin 1* or *Atg7* resulted in the decreased inhibition of arsenic trioxide on leukemic cell lines and primary leukemic progenitors from AML patients (Goussetis et al. 2010). Another trivalent arsenicals sodium arsenite ($NaAsO_2$) has showed the positive relationship with the incidence of type 2 diabetes. This phenomenon may be attributed to the ability of sodium arsenite in inducing ROS-dependent autophagic cell death in pancreatic β-cells. Autophagy inhibitor 3-MA protected the cells against sodium arsenite cytotoxicity, and autophagy inducer rapamycin further decreased the cell viability of sodium arsenite-treated INS-1 rat insulinoma cells (Zhu et al. 2014). Sodium arsenite showed an anti-proliferative effect on DU145 prostate cancer cells in xenograft mice, it induced both apoptosis and autophagic cell death via ROS (You et al. 2015).

BIX-01294, a selective inhibitor of euchromatic histone-lysine N-methyltransferase 2 (EHMT2), induced autophagic cell death via EHMT2 dysfunction and intracellular ROS production, and increased autophagy-dependent and caspase-independent cell death in primary human breast and colon cancer cells (Kim et al. 2013a).

Lapatinib, a tyrosine kinase inhibitor, has been widely accepted in the treatment of breast cancer. In breast cancer cells, it induced apoptosis and protective autophagy related with lapatinib resistance (Chen et al. 2015). But in human hepatoma cells, researchers found that lapatinib induced autophagy, a higher percent of dead cells and a lower percent of hypodiploid cells, that suggesting non-apoptotic cell death but autophagic cell death in lapatinib-treated hepatoma cells (Chen et al. 2014).

Gemcitabine (GEM) is currently the first-line treatment for pancreatic cancer. GEM elevated autophagic progress by the MEK/ERK signaling pathway. The autophagic activity was reduced in GEM-resistant human pancreatic cell line KLM1-R compared to GEM-sensitive KLM1 cells, suggesting autophagy was required in GEM-mediated pancreatic cancer treatment (Wang et al. 2014). Combination with GEM and cannabinoid triggers autophagic cell death in pancreatic cancer cells through a ROS-mediated mechanism (Donadelli et al. 2011).

Ursolic acid (UA), a natural pentacyclic triterpenoid carboxylic acid, has been showed potent anti-cancer activity. UA was reported to promote cervical cell lines TC-1 cell death, not through apoptosis but ATG5-dependent autophagy. Treatment with autophagy inhibitor and *Atg5* knockdown increased the survival of TC-1 cells treated with UA (Leng et al. 2013).

Salinomycin, a cation ionophore, has been showed to reduce the viability of breast cancer stem-like/progenitor cells by inhibiting autophagy (Yue et al. 2013). However, it has been reported to induce autophagy in human non-small cell lung cancer cells (Li et al. 2013). In recent study by Li et al. (Jangamreddy et al. 2015), treatment with lower concentration of salinomycin activates autophagic flux in prostate cancer cells while murine embryonic fibroblasts (MEFs) show an inhibition of autophagic flux. But it inhibits autophagic flux in both cell types at a higher concentration, which means salinomycin seems like autophagy inducer instead of inhibitor.

3.5 Summary

Autophagy is a catabolic mechanism mediated by lysosomal degradation, which is required for cellular homeostasis. Disruption of autophagy is associated with various human diseases. Autophagy is regulated by a series of proteins, such as some kinases, some of which in turn may be used as the molecular targets for the development of autophagy inhibitors and inducers. The most notable characteristics of autophagy related to cancer is its dual role, i.e. it can act as a tumor suppressing mechanism as well as a tumor promoting mechanism. Using autophagy inhibitors as an adjuvant therapy in combination with cytotoxic anti-cancer drugs is a promising way to overcome resistance in cancer therapies. On the other hand, a number of compounds achieve their anti-cancer activities by inducing autophagic cell death, presenting another possible strategy for developing novel anti-cancer drugs. Considering the complex context of autophagy, it is important to specify the indication and potential side effects of each type of small-molecule autophagy regulator in anti-cancer drug discovery.

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Chapter 4
Regulation of Autophagy by microRNAs: Implications in Cancer Therapy

Hua Zhu and Jin-Ming Yang

Abstract As an emerging hallmark of cancer, aberrant energy metabolism has drawn increasing attention in both basic research and clinical study. Autophagy is one of the main mechanisms for cells to maintain metabolic homeostasis, and cancer cells often display altered autophagic activity. Thus, autophagy is now pursued as a target for anti-cancer therapies. The current approaches to modulating autophagy include manipulation of either expressions or functions of the proteins that are key components of autophagic pathways. As a main post-transcriptional regulatory factor, microRNAs play important roles in various physiological and pathophysiological processes including cancers. Since miR-30a was first reported to regulate autophagy through targeting 3′ untranslated region (3′ UTR) of *Beclin*-1, a key autophagy regulatory gene, numerous miRNAs involved in autophagy regulation have been reported. Here we overview the current knowledge regarding the roles of miRNAs in regulation of autophagy and their implication in cancer therapy.

Keywords Autophagy • microRNA • Post-transcriptional regulation • Cancer therapy • Cancer metabolism

4.1 Introduction

Autophagy is one of the key processes in maintaining cellular homeostasis. In response to various stresses such as metabolic and therapeutic stress, autophagy promotes the elimination of abnormal proteins or organelles and the refurbishment of energy for cell survival. Most of the time, there is a basal level of autophagy responsible for degradation of cytoplasmic aggregates. However, prolonged

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autophagy under stressful condition (e.g., nutrient deprivation) could result in cell death, i.e., cannibalistic cell death. Dysregulation of autophagy is implicated in many pathological conditions including neurodegenerative diseases and cancer. Therefore, understanding the molecular mechanism of autophagy regulation would be important for development of novel therapeutic strategies for those diseases. MicroRNA (miRNA) a group of non-coding RNAs able to target to 3′ untranslated region (3′ UTR) of mRNAs to suppress protein expression. Increasing evidences have demonstrated that miRNAs have important roles in a variety of physiological and pathophysiological processes. For instance, connections between miRNAs and autophagy have been revealed in many studies. miRNAs can manipulate autophagy by targeting the expression of key proteins involved in autophagy regulation. It has become clear that there is an abundance of miRNAs, which are either tumor suppressive or tumor promotive, are involved in autophagic control and impact cancer development and progression. In the chapter, we will discuss the role of miRNA in regulation of autophagy and the related therapies for cancer treatment.

4.2 Autophagy and Its Regulatory Proteins

4.2.1 Autophagy Is an Intrinsic Process to Maintain Cellular Homeostasis

The cycle of protein synthesis and degradation is essential in sustaining cellular homeostasis. Autophagy is maintained at a basal level under physiological conditions (Mizushima et al. 2008; Denton et al. 2009). Increases in autophagic activity often occur as a response to various stresses such as glucose deprivation and hypoxia (Kroemer et al. 2010), serving as a survival or protective mechanism. Prolonged autophagy, however, can eventually result in cell death (cannibalistic cell death) (Pan et al. 2013). Autophagy, a process of "eating of self" (Glick et al. 2010; Levine and Kroemer 2008, 2009), is a means of cell survival through eliminating protein aggregates and removing damaged cell organelles (Gottlieb et al. 2010). It was first clearly demonstrated in the rat livers exposed to the pancreatic hormone glucagon (Deter and De Duve 1967) that the mitochondria and other cytoplasmic material were subjected to lysosome-mediated degradation in double-membrane structures referred to as autophagosomes (Zhang and Baehrecke 2015; Glick et al. 2010). Various forms of autophagy have been identified, including macro- or micro-autophagy, and chaperone-mediated autophagy (Mizushima et al. 2008; Glick et al. 2010; Yang and Klionsky 2010). Macroautophagy is the most extensively defined type that is characterized by the formation of autophagosome. Autophagy prevents dysfunctional intracellular proteins from accumulating, subsequently recycling the proteins to produce source of energy. Therefore, autophagy is appreciated as an intrinsic pathway to promote cell survival under stress conditions. However, as mentioned above, prolonged autophagy can cause cell death. Indeed, autophagy was originally considered as a type of programmed cell death that plays an

important role in controlling the balance of organism health (Wang and Klionsky 2003; Zhang and Baehrecke 2015), in addition to other interactive types of cell death such as apoptosis and necrosis (Gordy and He 2012; Amaravadi et al. 2007; Schweichel and Merker 1973). Differently, autophagy is an intracellular procedure in which defective or excess cytoplasmic components are degraded (Wu et al. 2015).

Autophagy consists of five stages: induction, vesicle nucleation, elongation, retrieval, and fusion for cargo degradation (Sui et al. 2011; Jing et al. 2015). During autophagy, various organelles such as the endoplasmic reticulum, mitochondria, or plasma membrane are utilized to form autophagosomal vacuoles (Puri et al. 2013; Longatti et al. 2012; Hailey et al. 2010; Ravikumar et al. 2010; Maiuri et al. 2007; Levine and Klionsky 2004). The vacuoles fuse with the lysosomes to initiate protein degradation via lysosomal hydrolases (Gottlieb et al. 2010; Maiuri et al. 2007; Levine and Klionsky 2004). Microautophagy occurs when there is an uptake of whole organelles or cytoplasm directly at vacuole surface. These two processes are seemingly nonspecific for bulk degradation, although other more selective degradation pathways are seen in chaperone-mediated autophagy (Wang and Klionsky 2003).

4.2.2 Regulation of Autophagy by ATG

Genetic studies in yeast uncovered a set of autophagy-related, termed ATG genes, which are required for autophagosome formation (Feng et al. 2014; Nakatogawa et al. 2009). Further investigations revealed the preservation of the ATG genes across yeast and humans (Mizushima and Komatsu 2011). The formation of autophagosome involves ATG genes (such as ATG5, ATG7, and ATG10) and anti-apoptotic proteins (e.g., Bcl-2/Bcl-xL) (Mizushima et al. 2008). Other important regulators of autophagy include mammalian target of rapamycin (mTOR) and AMP-responsive protein kinase (AMPK) (Sui et al. 2015). Autophagy is closely associated with nutrition status of cells because it is inhibited by mTOR, a metabolic sensor, and activated by AMPK (Zhang and Baehrecke 2015; Gottlieb et al. 2010). mTOR senses amino acid levels and insulin signaling to inhibit autophagy through phosphorylating unc-51-like kinase ½ (ULK1/2) and ATG proteins (Klionsky 2007). On the other hand, AMPK can exert a negative effect on mTOR signaling through TSC1/2 phosphorylation in response to an increased AMP/ATP ratio or mitogen-activated protein kinases (MAPKs) (Sui et al. 2011; Li et al. 2013; Radogna et al. 2015; Russell et al. 2014). Nutrient deficiency inhibits mTOR and promotes the binding of dephosphorylated ATG13 to ATG1, triggering the creation of autophagosomal membranes (Jing et al. 2015).

Induction of autophagy is initiated by the activation of the ULK complex (ULK ½, ATG13, FIP200 and ATG101) (Jing et al. 2015). Subsequently, vesicle nucleation is induced by the formation of a complex of Beclin-1 and a myristylated kinase to activate Vps34 class III phosphatidylinositol 3-kinase (PI3K) (Jing et al. 2015). Elongation is mediated by the ubiquitin-like conjugation systems with a number of ATG proteins

(ATG12-ATG5-ATG16L and ATG8-phosphatidylethanolamine system), along with the retrieval and fusion process regulated by ATG2, ATG9, ATG18 and the UV irradiation resistance-associated tumor suppressor gene (UVRAG). This process is further facilitated by the microtubule-associated protein 1 light chain 3 (LC3), which couples with phosphatidylethanolamine (PE) to form a membrane-bound conjugation system (Su et al. 2015). Finally, the autophagosome fuses with lysosome to form autophagolysosome via ATG9 and vacuole membrane protein 1 (VMP1), where intracellular proteins are degraded by the lyososomal cathepsins (Wang and Klionsky 2003; Gottlieb et al. 2010; Yang and Klionsky 2010; Jing et al. 2015; Levine and Klionsky 2004; Ichimura et al. 2000; Mizushima et al. 1999; Kaminskyy and Zhivotovsky 2012). Recycled macromolecules and organelles are then turned into their original building blocks of amino acid and fatty acid (Mizushima and Komatsu 2011; Li and Vierstra 2012).

4.2.3 Other Autophagy-Regulatory Proteins

There are a variety of other proteins that participate in autophagy regulation (Gibbings et al. 2013). For instance, apoptosis repressor with caspase recruit domain (ARC) is an anti-autophagic protein (Bo et al. 2014). Immunity-related GTPase family M gene (IRGM) manipulates autophagy to alter the innate immune response (Singh et al. 2010). Histone acetyltransferases (HATs) and deacetylases (HDACs) are critical in protein acetylation to promote or suppress gene transcription (Jing et al. 2015; Sui et al. 2015). Selective autophagy can be facilitated by the SQSTM1/p62-like receptors (SLRs), which identify and target substrates for autophagosomes (Gibbings et al. 2013).

The Bcl-2 protein family is a group of key autophagy regulators. Bcl-2 can bind to Beclin-1 to inhibit autophagy (Jing et al. 2015). The interaction between Bcl-2 and Beclin-1 determines the coordination of autophagy and apoptosis (Kang et al. 2011). Beclin-1 is a 60 kDa-coiled protein, and its aberrant expression has been observed in certain diseases including cancer and neurodegenerative disease (Wang and Klionsky 2003). Beclin-1 is frequently monoallelically mutated in various cancers such as breast or ovarian cancers, with decreased expression of the protein in several types of tumors (Itakura et al. 2012; Yue et al. 2003). A study showed that Beclin-1 is required in the embryonic phase and for autophagy but not for apoptosis (Yue et al. 2003).

4.2.4 Implication of Autophagy in Human Diseases

Dysregulation of autophagy is related to pathogenesis of various human diseases (Klionsky 2007; Zhou et al. 2012; Lavandero et al. 2013) such as aging, cancer (Radogna et al. 2015), and neurodegenerative disease (Wu et al. 2015; Zhu et al.

2009). Beclin-1 has been reported as a candidate tumor suppressor (Wang and Klionsky 2003; Yue et al. 2003), and mutation or inactivation of one allele of Beclin-1 is frequently detected in many types of human cancers (Gottlieb et al. 2010). Induction of autophagy is believed to be beneficial in certain pathological conditions. However, whether autophagy is a gateway to or a protection from cell death remains to be fully elucidated. While some studies propose that hyperactivation of autophagy may segue into apoptosis or necrosis (Maiuri et al. 2007; Denton et al. 2015; Berry and Baehrecke 2008; Clarke and Puyal 2012), others suggest that autophagy is an adaptive response to counter cell death (Singh et al. 2010; Brest et al. 2011). It is likely that autophagy plays roles in both of cell death and survival, similar to inflammation that can act as an organism-protective immune reaction but when overactive, it can result in cannibalistic cell death (Mizushima 2007). Understanding the mechanisms of the crosstalk between autophagy and other cellular processes (e.g., apoptosis, necrosis) would be important for further dissecting the contribution of autophagy to pathogenesis of some human diseases.

4.3 Molecular Basis of miRNAs

4.3.1 MicroRNAs in Human Genome

Eukaryotic cells utilize protein regulatory mechanisms that have been relatively conserved through evolution (Zhao and Srivastava 2007). Researches in the past a few decades have progressed toward the direction of studying small non-coding RNAs in the regulatory of cellular activity. Four of these RNAs have been identified: microRNA (miRNA), short interfering RNAs (siRNA), repeat-associated small interfering RNAs (rasiRNAs) and piwi-interacting RNAs (piRNAs) (Aravin and Tuschl 2005; Kim 2006). Among these small non-coding RNAs, miRNAs are the most phylogenetically conserved in various physiological processes—one of the first miRNAs discovered, let-7, is conserved from worms to mammals (Pasquinelli et al. 2000; Reinhart et al. 2000). MicroRNAs are small RNAs of about 22 nucleotides in length that can regulate specific messenger RNAs (mRNAs) (Zhao and Srivastava 2007; Clark et al. 2015). There are more than 1000 miRNAs encoded in the human genome and are expressed in a tissue-specific manner (Zhao and Srivastava 2007). Each strand has a specific function in silencing post-transcriptional gene expression (Clark et al. 2015). They have been found to regulate approximately 50 % of all protein-coding genes and facilitate many cellular processes including apoptosis, differentiation, and cell proliferation (Zhai et al. 2013a; Krol et al. 2010; Huang et al. 2011a). Recent studies have implied a great potential of targeting miRNA as a novel therapeutic strategy for diseases such as cancer, neurodegenerative diseases, heart disease, and many others (Zhao and Srivastava 2007; Clark et al. 2015; Ding et al. 2015; Gupta et al. 2015).

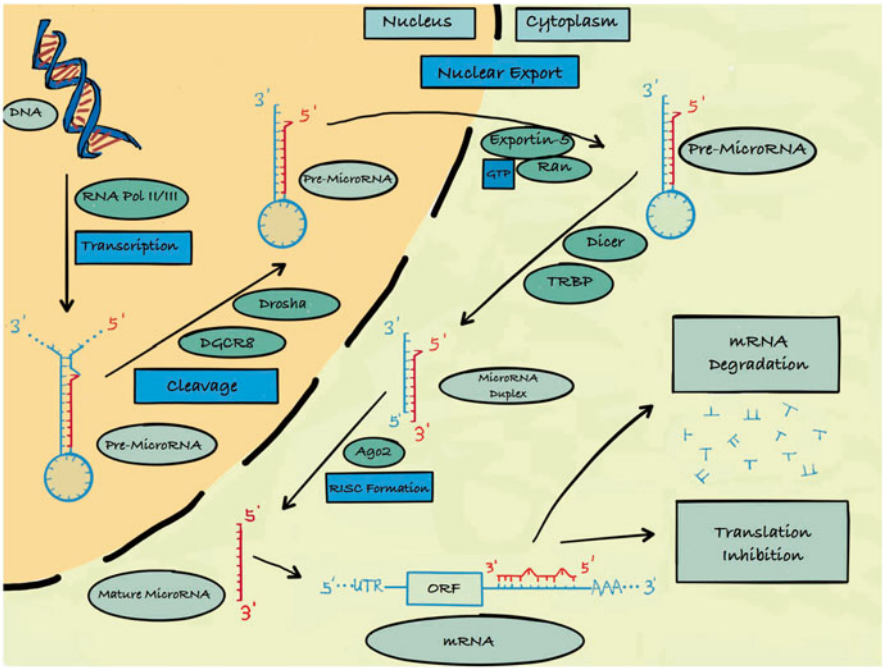

Fig. 4.1 Biogenesis of microRNAs and their intracellular functions

4.3.2 Biogenesis and Function of miRNAs

As shown in Fig. 4.1, majority of miRNA is transcribed by RNA polymerase II in the nucleus. Transcripts are then processed in the nucleus by a Class 2 ribonuclease III enzyme, Drosha, to become one or more precursor miRNAs (pre-miRNAs) with a hairpin structure of about 70 nucleotides (Lee et al. 2003). At the 3′ end, there is a 2-nucleotide overhang that is recognized by exportin 5 for export to the cytoplasm (Leung 2015). An enzyme of the RNAse III family, Dicer, processes the pre-miRNAs into an approximately 22-nucleotide duplex, and then incorporated into RISC (Gibbings et al. 2013; Zhao and Srivastava 2007; Leung 2015; Bernstein et al. 2001; Jing et al. 2005). EIF2C complex is required for protecting the miRNAs from degradation by cytoplasmic RNases (Gibbings et al. 2013). The duplex is then loaded by an Ago protein that retains a specific single-stranded mature miRNA (Leung 2015). When miRNA-mRNA complementarity is identified by the 'seed sequence', AGO binds the protein GW182 to activate RISC for translation suppression or deadenylation/degradation of mRNAs (Leung 2015).

The processing bodies (P-Bodies) located in the cytoplasm are rich in Ago proteins. Fluorescent microscopy observations indicate that P-Bodies are full of Ago and mRNA decay factors, insinuating that miRNAs and their complementary

mRNAs are processed in P-Bodies (Cougot et al. 2004; Liu et al. 2005; Sen and Blau 2005). However, the quantity of the splicer Ago2 in P-Bodies accounts for <1 % of cytoplasmic Ago2, indicating that mRNA decay occurs elsewhere in the cytoplasm as well (Leung 2015). Ago2 is phosphorylated at Ser-387 by mitogen-activated protein kinase (MAPK), which is essential for its localization to P-bodies to repress translation. Precise complementarity of miRNA-mRNA allows Ago2 protein to cleave the targeted mRNA (Leung 2015). During stress, some Ago proteins and polymerases can re-localize to the cytoplasmic structures known as stress granules (Leung et al. 2006, 2011). Because Ago proteins are crucial in orchestrating miRNA activities, post-translational modifications of these proteins (e.g., under hypoxia or immune stimulation) can largely alter miRNA actions (Leung 2015). Therefore, a better understanding of protein modifications in the RISC complex and their upstream signaling would help further elucidate the miRNA regulatory pathways.

4.3.3 Regulation of Messenger RNA by microRNAs

miRNAs are generated based on nucleolytic targets for distinct genes (Weil et al. 2015) through binding to 3′-untranslated region (3′ UTR) of matching mRNAs. The end caps of mRNA are essential for strand stability (Weil et al. 2015). The 5′ cap regulates nuclear export (Visa et al. 1996; Lewis and Izaurralde 1997), prevent degradation by exonucleases (Evdokimova et al. 2001; Gao et al. 2000), and promote translation (Sonenberg and Gingras 1998). In the 3′ end, structures including the poly(A) tail can increase or decrease the stability of mRNAs. It was believed that absence of poly(A) tail results in mRNA destabilization (Weil et al. 2015). 3′-untranslated region (3′ UTR) contains putative miRNA target sites for down-regulation of mRNAs (van Solinge et al. 2015). Of particular interest, the AU-rich elements (AREs) are observed in the 3′ UTR of many mRNAs with short half-lives, such as proto-oncogenes and cytokines (Jing et al. 2005; Weil et al. 2015). There are various proteins able to bind to ARE and responsible for the stability of mRNAs (Jing et al. 2005). It has been reported that miR-16 holds the complementary sequence (UAAAUAUU) to the ARE of 3′ UTR (Jing et al. 2005). Down-regulation or overexpression of miR-16 respectively increases or decreases mRNA stability, suggesting that mRNA degradation is dependent on ARE pairing with miRNA (Jing et al. 2005). Various ARE sequences are present in mRNAs, allowing for simultaneous miRNA manipulation (Jing et al. 2005).

Two modes of complementary mRNA silencing are mediated by miRNAs: by mRNA decay or by translational repression (Ameres and Zamore 2013). miRNAs form the RNA-induced silencing complex (RISC), a ribonucleoprotein complex (Leung 2015; Wilson and Doudna 2013) that includes Argonaute (Ago) proteins to facilitate gene-silencing pathways (Hock and Meister 2008). In mammals, Ago1 through Ago4 are all active in the miRNA pathway. When miRNAs are precisely complementary to the target mRNA through partial base pairing (usually through

the 'seed' sequence, nucleotides 2–7), the RISC cleaves the mRNA at the position facing nucleotides 10 and 11 of the small RNA (Leung 2015; Iwakawa and Tomari 2015; Lai 2002; Stark et al. 2003; Lewis et al. 2005). Current evidence suggests that each miRNA may affect specific targets independently or cooperatively (Zhao and Srivastava 2007). There are miRNA clusters that reside in corresponding introns of paralogous genes, and miRNAs of the same family can regulate sequential events (Zhao and Srivastava 2007).

However, the complementary base-pairing of miRNA-mRNA is not the only criterion for targeting mRNA for silencing (Zhao and Srivastava 2007). Additional effector proteins are recruited to induce other modes of mRNA silencing or degradation distinct from endonucleolytic cleavage (Zhao and Srivastava 2007; Iwakawa and Tomari 2015). miRNAs can also induce mRNA destabilization through the GW182 protein and Ago, which attracts deadenylases onto target mRNAs (Iwakawa and Tomari 2015). GW182 is known to play a role in both of mRNA decay and translational repression (Iwakawa and Tomari 2015). miRNAs can also promote the accessibility of mRNA poly(A) tail to deadenylases (Iyer et al. 2006). Decapping factors, the decapping complex (DCP2) and activators (DCP1, RCK/p54/DDX6), are then recruited and attached to the target mRNAs. Finally, RISC proceeds with the decapping and decay of target mRNAs, which is promoted by the binding of 4E-T to eIF4E (Artavanis-Tsakonas et al. 1999; Johnston et al. 2005; Johnston and Hobert 2003). Degradation occurs in the traditional 5′-to-3′ fashion in the decay pathway (Lai 2002; Du and Zamore 2005). It has been proposed that mRNA decay is the dominant mechanism of mRNA silencing over translational repression (Iwakawa and Tomari 2015). Further studies are warranted to determine the physiological importance of both of the miRNA functions in cells.

4.4 MicroRNA and Autophagy: Connections in Cancer Biology

4.4.1 miRNA in Cancers

Alternations in gene expression via DNA methylation, histone acetylation, and microRNAs have been implicated in carcinogenesis and progression (Sui et al. 2015). Involvement of miRNAs in tumor formation was first reported in 2002 (Dai and Tan 2015), and since then it has been widely investigated. Studies have shown opposing spectrums of miRNA functions: miRNAs can act either as tumor suppressors or as oncogenes. Targeting miRNA has been emerging as a novel therapeutic strategy in cancer treatment. Mechanisms of miRNA-based cancer therapeutics include: inducing apoptosis, suppressing tumor angiogenesis, and down-regulating adenosine triphosphate (ATP)-binding cassette (ABC) transporters (Dai and Tan 2015). Post-transcriptional modification of autophagy by miRNAs is a new area of investigation (Zhai et al. 2013a). The role of miRNA in regulating autophagy and its impact on anticancer treatment will be discussed in this chapter.

4.4.2 The Role of miRNAs in Various Steps of Autophagy

Dysregulation of programmed cell death is a hallmark of cancer development and progression (Dai and Tan 2015). The first connection between miRNA and autophagy was made by Zhu et al. who reported that miR-30a could negatively affect autophagic activity by regulating beclin-1 expression in cancer cells (Zhu et al. 2009). Further researches showed that various miRNAs could affect different stages of autophagy. For example, induction of autophagy via the ULK complex is affected by a variety of miRNAs including miR-20a, miR-101 and miR-106a/b, which can directly target ULK1/2 (Pan et al. 2013; Ciuffreda et al. 2010; Wu et al. 2012). Several binding sites for miR-885-3p were found in ATG13, ATG9A, and ATG2B (Huang et al. 2011b). Multiple components in the mTOR signaling including RHEB and RICTOR are modulated by miR-155 (Wang et al. 2013a). AMPK, which can inhibit mTOR, is targeted by miR-148b (Zhao et al. 2013). The Class III PI3K/Beclin-1 complex in vesicle nucleation is regulated by miR-30a, miR-519a, miR-216a, and miR-376b (Pan et al. 2013; Su et al. 2015; Menghini et al. 2014; Huang et al. 2012; Korkmaz et al. 2012; Mikhaylova et al. 2012). Furthermore, the expression of PI3K catalytic unit is thought to be silenced by miR-338-5p (PIK3C3 or Vps34) (Su et al. 2015). Phosphatase and tensin homolog (PTEN), a known inhibitor of PI3K, is a target for a number of miRNAs, including miR-21, miR-214, miR-216a, miR-217, miR-221, miR-222, miR-26a, and miR-18a (Su et al. 2015; Dai and Tan 2015). In the elongation step, UVRAG is modulated by miR-374a and miR-630 (Xu et al. 2013). A pathway of the ubiquitin-like conjugation system involving the Atg12 and Atg5 covalent conjugation that requires Atg7 and Atg10 is modulated by a number of miRNAs. MiR-375 and miR-17 target 3′ UTR of Atg7 gene to regulate its expression (Su et al. 2015; Comincini et al. 2013; Chang et al. 2012a). miR-204 can inhibit autophagy and suppress cancer progression via the LC3 conjugation system (Su et al. 2015). Other possible miRNAs involved in modulators of autophagy include miR-30a, miR-181a, miR-374a and miR-630 (Frankel and Lund 2012). Finally, the autophagosome fusion to lysosomes can be regulated by miR-34a and miR-130a (Christoffersen et al. 2010). MiR-21, miR-155, and miR-221/222 influence programmed cell death in similar types of cancer cells. Specifically, miR-21 can confer radio-sensitivity through inhibition of PI3K/AKT pathway (Chen et al. 2014) and regulate anti-apoptotic Bcl-2 in glioma cells (Liu et al. 2014). Furthermore, single miRNA may play a role in multiple pathways. An example of this case is miR-30a, which can target Beclin-1, ATG5, and p53 (Su et al. 2015). The regulation of autophagy by various microRNAs is summarized in Fig. 4.2.

4.4.3 Oncogenic miRNA (Oncomir)

Oncogenic miRNAs are often implicated in modulation of autophagic pathways. miR-30a can bind to 3′ UTR of Beclin-1 to suppress its expression and autophagy (Zhu et al. 2009; Chen et al. 2014). Downregulation of miR-30a and miR-19a-5p

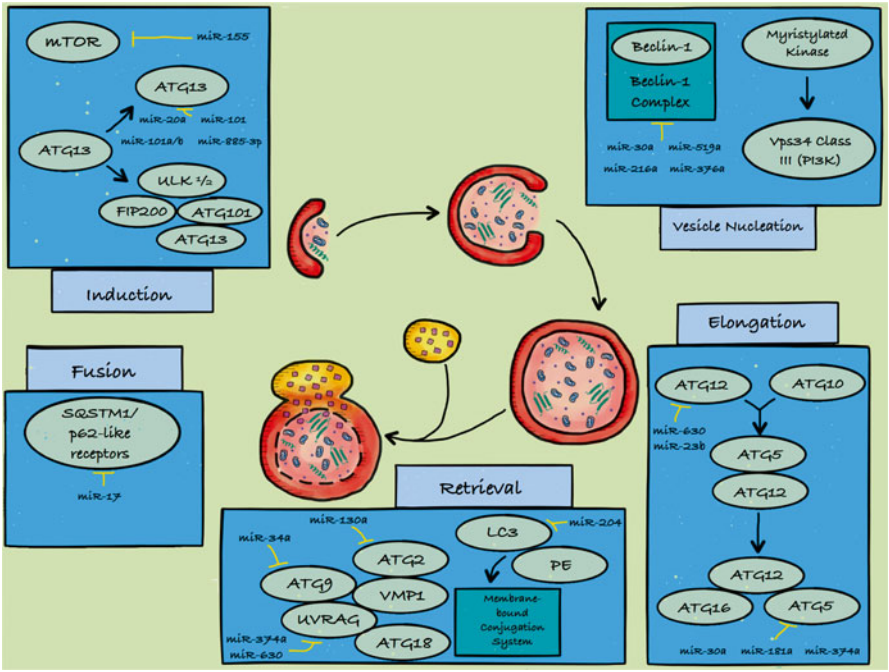

Fig. 4.2 Summary of identified microRNAs that target key proteins of autophagic pathway for regulation of autophagy

may decrease tumor cell chemosensitivity by activating autophagy and suppressing apoptosis (Xu et al. 2012; Zou et al. 2012). In nasopharyngeal cancer and cervical cancer cells, knockout of the endogenous miR-155 targeting mTOR signaling components inhibits hypoxia-induced autophagy (Wan et al. 2014). miR-31 and miR-34c respond to oxidative stress by activating cytoprotective autophagy, sustaining cancer progression and metastasis (Pavlides et al. 2010). A therapeutic strategy has been proposed using anti-miR-21 to augment autophagy and increase apoptosis in cancer treated with radiation (Liu et al. 2014).

4.4.4 Tumor Suppressive miRNA

Tumor suppressive miRNAs may have equally important roles in regulating autophagic pathways. When miR-23b is overexpressed in pancreatic cancer cells, its target ATG12 is found to be down-regulated, which leads to lower autophagic activity. This phenomenon has also been observed in bladder cancer, where overexpression of miR-23b correlates to a longer overall survival rate (Majid et al. 2013; Wang et al. 2013b). Tumor suppressor miR-101 can inhibit etoposide- or rapamycin-induced autophagy in MCF-7 breast cancer cells (Frankel et al. 2011), and its expression is down-regulated in various types of cancer, including breast, liver, and prostate cancer. In castration-resistant mesenchymal prostate cancer cells,

down-regulation of miR-205 led to increased cytotoxicity of cisplatin and interfered with the adaptive ability of cancer cells to cisplatin treatment (Pennati et al. 2014). Replacing miR-375 in liver cancers inhibited hypoxia-induced autophagy by targeting ATG7 and ATG4D (Chang et al. 2012b). Mimicking the tumor suppressive miRNAs or augmenting their effects in autophagy may have important implication in therapeutic intervention against cancer.

4.4.5 Regulation of miRNAs by Autophagy

While miRNAs can control autophagy, this important cellular process may also regulate miRNAs to maintain their homeostasis. Gibbings et al. first reported that the key components of miRNA biogenesis complexes, Dicer and Ago2, are selectively degraded by the NDP52-mediated autophagy (Gibbings et al. 2012). Cells with low autophagic activity exhibits increased Ago2 and Dicer, with less Ago-binding to miRNAs. As the inactive Dicer-Ago2 complexes can suppress the active complexes, autophagy is an important means in eliminating the inactive complexes to promote miRNA activity (Gibbings et al. 2013). Autophagy has also been shown to degrade specific miRNAs or the RISC components. Down-regulation of autophagic activity may be associated with up-regulation of oncogenic miRNA and carcinogenesis (Jing et al. 2015).

4.4.6 Role of Autophagy and miRNA in Cancer Therapy

During tumor development, autophagic activity may fluctuate in different stages (Guo et al. 2013; Jiang and Mizushima 2014; Rao et al. 2014). Autophagy may act as a tumor suppressor in the initial stages of tumoregenesis and therefore, but it is often down-regulated (Jing et al. 2015). Beclin-1, a key autophagy regulator, is considered as a tumor suppressor in breast cancer (Liang et al. 1999). However, many cancer cells exhibit increased autophagic activity in response to therapeutic or metabolic stress, implying a cyto-protective role of autophagy. It was observed in pancreatic cancer cells that inhibiting autophagy can elevate the amount of intracellular reactive oxygen species, which increase DNA damage (Yang et al. 2011). Interestingly, apoptosis is usually inhibited in cancer of all stages and as a result, tumor size is increased and drug resistance occurs. Using autophagy as an alternative method to facilitate cell death in non-apoptotic cancer cells is a new therapeutic strategy being explored (Li et al. 2013). Modulation of autophagy by miRNAs has been used to decrease drug resistance of cancer cells (Pan et al. 2013). miR-30a can inhibit Beclin-1-dependent autophagy and increase the sensitivity to imatinib in chronic myeloid leukemia (CML) cells (Yu et al. 2012a). miR-30a and a number of other miRNAs can sensitize tumor cells to cisplatin both *in vitro* and *in vivo* (Jing et al. 2015). Future research on miRNA-based therapeutic strategies should focus on tumor type, tumor environment, and disease context (Jing et al. 2015).

4.5 Targeting miRNA-Mediated Autophagy in Cancer Treatment

4.5.1 Current Status

Autophagy can act either as a tumor promoter or tumor suppressor, depending on context. Inhibiting the tumor suppressor role of autophagy may pave a way for growth of precancerous cells; on the other hand, malignant cells may require autophagy to survive under various stressful conditions (Li et al. 2013; Shintani and Klionsky 2004). To certain extent, the dual functions of autophagy in cancer may complicate the application of modulating autophagy in therapeutic intervention. Still, emerging evidence suggest that targeting miRNAs may hold promises as a novel strategy in cancer therapy (Table 4.1).

4.5.2 Breast Cancer

miR-30a inhibits autophagy by down-regulating Beclin-1 expression and suppressing tumor growth (Zhu et al. 2009). Similarly, miR-376b targets Beclin-1 and ATG4 to suppress starvation-induced autophagy (Zhai et al. 2013a; Korkmaz et al. 2012). In the HER2/neu+ MCF-7 breast cancer cells, autophagic cell death can be induced by miR-221/222, which inhibits $p27^{kip1}$ to regulate PI3K/Akt pathway (Miller et al. 2008). miR-148b can regulate the PI3K pathway that involves the catalytic subunit p110α, reducing breast cancer aggressiveness (Cimino et al. 2013). Also, in MCF-7 cells, miR-101 acts as a potent inhibitor of basal, etoposide-induced or rapamycin-induced autophagy (Frankel et al. 2011). Suppression of miR-21 in the HER2+ breast cancer cells induced PTEN expression and increased trastuzumab sensitivity (Braconi et al. 2010). In the Cav(−/−) breast tumor stromal cells, there was an upregulation of both miR-31 and miR-34 that is associated with autophagy induction that supports cancer cell survival (Zhai et al. 2013a). miR-200c-mediated inhibition of autophagy can enhance radio-sensitivity in breast cancer cells (Sun et al. 2015).

4.5.3 Prostate Cancer

Increased levels of miR-205 in castration-resistant mesenchymal prostate cancer cells can cause an impairment of autophagy and an increase of cisplatin toxicity (Pennati et al. 2014). In the prostate cancer tissues and serum from patients, miR-212 was found down-regulated. Further study demonstrated that miR-212 could suppress the starvation-induced autophagy by targeting sirtuin 1 (SIRT1) and induce cellular senescence and anti-angiogenic effect (Ramalinga et al. 2015).

Table 4.1 Roles of miRs in autophagy in cancer

Cancer types	miR(s)	Target of miR(s)	Effects	References
Breast cancer	miR-30a	Beclin-1	Slows cancer progression	Zhu et al. (2009)
	miR-376b	Beclin-1 and ATG4	Down-regulate starvation-induced autophagy	Zhai et al. (2013a), Korkmaz et al. 2012
	miR-221/222	$p27^{kip1}$	Induce autophagic cell death in HER2/neu+ MCF-7 breast cancer cells	Miller et al. (2008)
			Inhibit $p27^{kip1}$ to regulate PI3K/Akt down stream	
	miR-148b	PI3K pathway	Reducing breast cancer aggressiveness	Cimino et al. (2013)
	miR-101	MCF-7 cells	Potent inhibitor of basal, etoposide-induced and rapamycin-induced autophagy	Frankel et al. (2011)
	miR-21	HER2+ breast cancer cells	Suppression of miR-21 induce PTEN expression and increased trastuzumab sensitivity	Braconi et al. (2010)
	miR-31 and miR-34	Cav(−/−) breast tumor stromal cells	Promote autophagy	Zhai et al. (2013a)
	miR-200c	Breast cancer cells	Inhibition of autophagy and enhancement of radiosensitivity	Sun et al. (2015)
Prostate cancer	miR-205	Castration-resistant mesenchymal prostate cancer cells	Induce autophagy impairment that potentiated cisplatin toxicity	Pennati et al. (2014)
	miR-212	SIRT1	Suppress starvation-induced autophagy	Ramalinga et al. (2015)
Ovarian cancer	miR-214	PTEN	Positively regulate autophagy	Yang et al. (2008)
	miR-29b	Expression of high MAPK and ATG9a protein levels	Negatively regulate autophagy	Dai et al. (2014)

(continued)

Table 4.1 (continued)

Cancer types	miR(s)	Target of miR(s)	Effects	References
Lung cancer	miR-99a	mTOR	Suppress the tumorigenicity of cancer cells	Oneyama et al. (2011)
	miR-503	Non-small-cell lung cancer (NSCLC)	Suppress proliferation and metastasis	Yang et al. (2014)
	miR-193a-5p	Class I PI3K pathway	Inhibit metastasis	Yu et al. (2014)
	miR-17-5p	Beclin-1	Confer paclitaxel resistance	Chatterjee et al. (2014)
	miR-143	Non-small-cell lung cancer (NSCLC) and H1299 cells	Halt cell proliferation	Wei et al. (2015)
			Modulate autophagy	
	miR-7	Epidermal growth factor receptor (EGFR)	Induce autophagy	Tazawa et al. (2012)
Colorectal cancer	miR-30b	Human colorectal cancer cells	Regulate the PI3K pathway	Liao et al. (2014)
	miR-18a	HCT116 cells	Increase autophagy in response to radiation	Qased et al. (2013)
			Apoptosis of colon cancer cells	Seoudi et al. (2012), Fujiya et al. (2014)
		mTOR	Suppression	Qased et al. (2013)
	miR-22	Cancer cells	Enhance sensitivity to 5-fluorouracil by inhibiting autophagy and promoting apoptosis	Zhang et al. (2015a)
	miR-502	RAB1B	Hinder autophagy	Adlakha and Saini (2011), Zhai et al. (2013b)
	miR-204-5p	LC3BII and Bcl2	Suppress autophagy	Sumbul et al. (2014))
Renal cell carcinoma	miR-204	LC3	Inhibit autophagy and suppress renal clear cell carcinoma development	Su et al. (2015), Mikhaylova et al. (2012), Hall et al. (2014)

(continued)

Table 4.1 (continued)

Cancer types	miR(s)	Target of miR(s)	Effects	References
Hepatocellular carcinoma	miR-101	Hepatocellular carcinoma (HCC) and oncogene eZH2	Activate apoptosis, synergize with doxorubicin or fluorouracil	Yu et al. (2012a), Xu et al. (2014)
	miR-199a-3p	mTOR	Reduce cell invasion and sensitizes HCC to doxorubicin	Xu et al. (2012), Fornari et al. (2010)
	miR-26b	AMPK	Enhances chemosensitivity of the cancer cells	Zhao et al. (2014)
	miR-375	ATG7	Inhibit autophagy under hypoxic conditions	Chang et al. (2012a, b)
	miR-423-5p	Cells treated with sorafenib	Promote autophagy	Stiuso et al. (2015)
	miR-224	HCC	Induce autophagy	Lan et al. (2014a, b)
Pancreatic cancer	miR-23b / miR-630	Pancreatic cancer cells	Increase autophagic activity, promote ATG 12 expression and increase radioresistance	Wang et al. (2013b, c), Donadelli and Palmieri (2013)
	miR-155	Pancreatic cancer cells	Protect from programmed cell death	Shahbazi et al. (2013)
	miR-216a	Beclin-1	Increase the radiosensitivity of pancreatic cancer cells	Zhang et al. (2015b)
	miR-182	Pancreatic cancer cells	Suppression of Bcl2	Peng et al. (2013)
Glioma	miR-17	ATG7	Increase the sensitivity of cancer cells to chemotherapy and radiation	Comincini et al. (2013)
	miR-663	Cancer cells	Regulate the PI3K pathway	Shi et al. (2014)
	miR-21	Human glioma cells LN18 and LN428	Suppress radiosensitivity	Gwak et al. (2012)

4.5.4 Ovarian Cancer

Cisplatin resistance in ovarian cancer cells can be promoted by miR-214, which targets PTEN to positively regulate autophagy (Yang et al. 2008). The negative correlation of low miR-29b expression to high MAPK and ATG9a protein levels was associated with poor prognosis in patients with ovarian cancer (Dai et al. 2014).

4.5.5 Lung Cancer

Lung tumorigenesis can be suppressed by miR-99a that targets mTOR (Oneyama et al. 2011). The ectopic expression of miR-503 in non-small-cell lung cancer (NSCLC) leads to suppression of proliferation and metastasis of tumor cell (Yang et al. 2014). Metastasis can also be inhibited by miR-193a-5p-mediated inactivation of the class I PI3K pathway (Yu et al. 2014). Down-regulation of miR-17-5p can confer paclitaxel resistance through altering Beclin-1 expression (Chatterjee et al. 2014). Cell proliferation in NSCLC can be halted by miR-143, which modulates autophagy in tumor cells (Wei et al. 2015). Ectopic expression of miR-7 induces autophagy by limiting the expression of epidermal growth factor receptor (EGFR), and this also occurs in esophageal cancer (Tazawa et al. 2012).

4.5.6 Colorectal Cancer and Renal Cell Carcinoma

miR-30b directly regulates the PI3K pathway in human colorectal cancer cells, thereby modulating autophagy (Liao et al. 2014). Oncogenic miR-18a increases autophagy in HCT116 cells by interacting with the ataxia telangiectasia mutated (ATM) gene in response to radiation (Qased et al. 2013). However, prolonging this action can lead to apoptosis in colon cancer cells (Seoudi et al. 2012; Fujiya et al. 2014). miR-18a can also suppress mTOR to impact autophagy (Qased et al. 2013). miR-22 can enhance tumor cell sensitivity to 5-fluorouracil by inhibiting autophagy and promoting apoptosis (Zhang et al. 2015a). miR-502 can block autophagy by decreasing RAB1B, a GTPase (Adlakha and Saini 2011; Zhai et al. 2013b). Tumor suppressor miR-204-5p can suppress the activity of LC3B-II in autophagy (Sumbul et al. 2014). Via the LC3 conjugation system, miR-204 can inhibit autophagy and suppress the development of renal clear cell carcinoma (Su et al. 2015; Mikhaylova et al. 2012; Hall et al. 2014).

4.5.7 Hepatocellular Carcinoma

miR-101, which is considered as a tumor suppressor, can inhibit autophagy in hepatocellular carcinoma (HCC) cells and target the oncogene EZH2 to activate apoptosis, thereby effectively synergizing with doxorubicin or fluorouracil (Yu et al. 2012a; Xu et al. 2014). miR-199a-3p can regulate mTOR and autophagy to reduce cell invasion and sensitize HCC to doxorubicin (Xu et al. 2012; Fornari et al. 2010). miR-26b can modulate autophagy through directly affecting AMPK, thereby enhancing chemosensitivity of the cancer cells (Zhao et al. 2014). Down-regulation of miR-375 can inhibit autophagy through targeting ATG7 in tumor cells subjected to hypoxic (Chang et al. 2012a, b). On the other hand, miR-423-5p can promote

autophagy in cells treated with sorafenib (Stiuso et al. 2015). In a study by Lan et al., using amiodarone as an autophagy inducer, autophagy-mediated degradation of miR-224 suppressed HCC tumorigenesis (Lan et al. 2014a, b).

4.5.8 Pancreatic Cancer

Autophagic activity was found to be increased in pancreatic cancer cells when miR-23b or miR-630 expression was decreased, with an up-regulation of ATG12 expression and increased resistance to radiation therapy (Wang et al. 2013b, c; Donadelli and Palmieri 2013). Oncogenic miR-155 can protect pancreatic cancer cells from programmed cell death (both apoptosis and autophagy) by targeting p53 pathway (Shahbazi et al. 2013). miR-216a can inhibit the Beclin-1-mediated autophagy and increase the sensitivity of pancreatic cancer cells to radiation therapy (Zhang et al. 2015b). Up-regulation of miR-182 is correlative with the suppression of Bcl2 in pancreatic cancer cells (Peng et al. 2013).

4.5.9 Glioma

It has been reported that miR-17 has the ability to enhance the sensitivity of glioma cells to chemotherapy and radiotherapy through interfering with the E1-like enzyme, ATG7 (Comincini et al. 2013). miR-663 can directly regulate the PI3K-mediated autophagy signaling (Shi et al. 2014). Overexpression of oncogenic miR-21 can suppress radiosensitivity in human glioma cell lines LN18 and LN428 (Gwak et al. 2012).

4.5.10 Perspective

Although the roles of the miRNA-regulated autophagy in cancer development and progression remains to be further elucidated, a growing body of evidence indicate that targeting miRNAs to modulate autophagy may have important implication in cancer treatment. Many recent studies have demonstrated the potential of using miRNA mimics or anti-miRs as anticancer therapeutics. The miRNA-based therapy may complement conventional treatments by reinforce their efficacy. Increased cell death resulting from suppression of autophagy was observed in breast cancer cells treated with miR-101 (Frankel et al. 2011). Cisplatin treatment induces autophagy in various types of cancer, accompanied by down-regulations of several miRNAs (Claerhout et al. 2010; Harhaji-Trajkovic et al. 2009; Ren et al. 2010). Apoptosis in tumor cells induced by cisplatin can be enhanced by miR-30a through suppression of Beclin-1 (Zou et al. 2012). Additionally, miR-30a can enhance the therapeutic

efficacy in imatinib-resistant chronic myelogenous leukemia (Yu et al. 2012b), and is considered a putative biomarker in a variety of cancer. Contrastingly, there are known miRNAs that can increase drug resistance and radio-resistance in cancer cells, for instance, miR-221/222 in breast cancer cells (Miller et al. 2008) and miR-21 in glioblastoma cells (Maiuri et al. 2007).

A common barrier of miRNA-based therapy is the drug delivery problem. This includes inadequate cellular uptake, short half-life, and rapid renal clearance of miRNAs (Aliabadi et al. 2012). A number of strategies are being employed to overcome these issues. Chemical modification (Bader et al. 2011; Janssen et al. 2013), biodegradable nanocarriers (Chen et al. 2014; Aliabadi et al. 2012; Bouchie 2013; Daka and Peer 2012), and conjugations of miRNAs to conventional cytotoxins (Schroeder et al. 2012), are being developed at present. Future studies on autophagy-related miRNAs should take into account the following issues: (1) are the miRNAs a single tumor-specific or present in multiple tumors? (2) are they tumor suppressive or oncogenic? (3) are they promoting or suppressing autophagy, and in which specific step do they regulate autophagy? (4) what are the specific targets for these miRNAs? (5) how can we take advantage of the knowledge of miRNAs for treatment of different cancer?. The increasing understanding of the molecular mechanisms behind miRNA regulation of autophagy will help overcome various barriers in developing the miRNA-based cancer therapy against multi-drug resistance, radio-resistance, cancer metastasis, and other malignant phenotypes.

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Chapter 5
Targeting PI3-Kinases in Modulating Autophagy and Anti-cancer Therapy

Zhixun Dou and Wei-Xing Zong

Abstract Phosphoinositide 3-kinases (hereafter referred to as PI3-kinases) are lipid kinases that phosphorylate the 3′-hydroxyl group of inositol lipids. The generated phospholipids are critical signaling molecules that recruit proteins to specific intracellular membranes leading to localized activation of these proteins. PI3-kinases regulate many cellular activities, and are closely linked to human diseases, including cancer. One molecular event regulated by PI3-kinases is autophagy, an evolutionarily conserved membrane trafficking process that degrades and recycles cellular constituents to maintain cell and tissue homeostasis. Over the past two decades, our understanding of PI3-kinases has progressed from pan-PI3-kinase inhibitor studies to isoform-specific genetic knockout and systems biology interactome analyses. Our view of autophagy has emerged from unicellular yeast vesicle trafficking to mammalian physiology and human diseases. In this chapter we summarize the major discoveries on autophagy regulation by PI3-kinases and discuss the therapeutic potentials of targeting PI3-kinases in modulating autophagy and in cancer therapy.

Keywords Autophagy • PI3K • Cancer therapy • Chloroquine • p110α • p110β • Vps34

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5.1 Introduction to PI3-Kinases

In late 1980s, Lewis Cantley and coworkers discovered a new lipid kinase that phosphorylates the 3′-hydroxyl group of inositol lipids to produce a new lipid PtdIns(3)P (Whitman et al. 1988). The lipid showed slight different biochemical signatures from PtdIns(4)P and PtdIns(5)P, which were the known lipids at that time. The importance of PI3-kinase was not clear until the discovery that it associates with growth factor receptors, such as G protein-coupled receptor (GPCR), and that growth factor, including insulin, stimulation results in production of a novel lipid PtdIns(3,4,5)P_3 from PtdIns(4,5)P_2 (Auger et al. 1989; Ruderman et al. 1990; Traynor-Kaplan et al. 1988). In early 1990s, the first PI3-kinase, p110α, was cloned, and it was found to be in a complex with p85 (Hiles et al. 1992). Later years in the 1990s have witnessed an expansion of the PI3-kinase field, as more subunits and isoforms were cloned (Vanhaesebroeck et al. 2012). It has since become clear that PI3-kinases are central players of cellular metabolism, growth, proliferation, and survival. The research on PI3-kinases was largely facilitated by the discovery of wortmannin, a steroid metabolite of the fungus *Talaromyces wortmannii*, as a non-specific covalent inhibitor of all PI3-kinase isoforms (termed as pan-PI3-kinase inhibitor). In conjugation with other available molecular biology tools, the use of wortmannin has helped identify numerous cellular activities regulated by PI3-kinases, including autophagy (Engelman et al. 2006).

Based on substrate specificity and sequence homology, PI3-kinases are grouped into three classes: Class I, Class II, and Class III (Engelman et al. 2006) (Table 5.1). Class I PI3-kinases are among the first identified PI3-kinases, and are comprised of a 110 kDa catalytic subunit and a regulatory subunit. When this group of enzymes were first discovered, they showed in vitro activity to phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2. In vivo, activation of Class I PI3-kinases by receptor tyrosine kinases (RTKs) and GPCRs induces production of PtdIns(3,4,5)P_3, but not PtdIns(3)P or PtdIns(3,4)P_2 (Hawkins et al. 1992; Stephens et al. 1991). Hence, PtdIns(4,5)P_2 is more likely to be the preferred in vivo substrate of Class I

Table 5.1 Components and catalytic preference of three classes of PI3-kinases

Class	Catalytic subunit	Regulatory subunit	In vivo substrate	Catalytic product
IA	p110α	p85α p85β	PtdIns(4,5)P_2	PtdIns(3,4,5)P_3
	p110β	p50α p55α		
	p110δ	p55γ		
IB	p110γ	p101 p84/p87	PtdIns(4,5)P_2	PtdIns(3,4,5)P_3
II	PIK3C2α	None	PtdIns	PtdIns(3)P
	PIK3C2β			
	PIK3C2γ			
III	Vps34	Vps15	PtdIns	PtdIns(3)P

PI3-kinases. Class I PI3Ks are further divided into two classes, Class IA and Class IB. Class IA PI3Ks respond to both RTKs and GPCRs, and are composed of a 110 kDa catalytic subunit (p110α, β, δ) and an 85 kDa regulatory subunit (p85). In some cases, a p50 or p55 subunit is present as the regulatory subunit. Class IB PI3Ks respond only to GPCRs and consist of the p110γ catalytic subunit and the p101 or p84/p87 regulatory subunit. Among the p110 catalytic subunits, p110α and p110β isoforms are ubiquitously expressed, whereas the p110δ and p110γ expressions are restricted in hematopoietic cells (Engelman et al. 2006).

Class II PI3-kinases are comprised of three members, PIK3C2α, β, and γ. The preferred in vivo substrate is PtdIns. Class II PI3-kinases are monomeric and lack a regulatory subunit. PIK3C2α and PIK3C2β are ubiquitously expressed, whereas PIK3C2γ is highly expressed in the liver and prostate (Engelman et al. 2006).

Class III PI3K consists of only one member, Vps34. It was first identified in a yeast mutant that is defective in vacuole protein sorting (Herman and Emr 1990; Schu et al. 1993) and is the only PI3-kinase evolutionary conserved from yeast to mammals. Vps34 phosphorylates PtdIns into PtdIns(3)P. Vps34 associates with its regulatory subunit Vps15, and is ubiquitously expressed in mammalian tissues.

5.2 Class I PI3-Kinases as Essential Regulators of Growth, Proliferation, and Cancer

The functional significance of Class I PI3-kinases is suggested by the early evidence that they are associated with growth factor receptors, and thus may be involved in cell stimulation, growth, and metabolism. In support of this notion, inhibition of PI3-kinases by pharmacological inhibitors or genetic approaches leads to impaired cellular activities signaled through growth factor receptors (Auger et al. 1989; Cantley 2002; Ruderman et al. 1990). The identification of p85 regulatory subunits also helped the understanding of the regulation of PI3-kinase activity. p85 and p110α exist as a heterodimer, with a strong binding affinity. While p85 does not possess PI3-kinase activity, p110α was shown to be a bona-fide PI3-kinase. The p85 regulatory subunit is composed of an N-terminal SH3 domain, a BH domain, and the p110 binding domain flanked by two SH2 domains (Cantley 2002; Engelman et al. 2006). Since SH2 domains bind to phosphorylated-tyrosine peptides, this provides a clue to the recruitment of PI3-kinases to RTKs. The p110 catalytic subunit possesses an N-terminal p85 binding domain, a Ras binding domain, a C2 domain, a helical domain, and a C-terminal catalytic domain. The interaction of p110s with p85s stabilizes p110s, and inactivates their catalytic activity. In vivo, p110s are obligated heterodimers with p85s (Geering et al. 2007). Upon RTK activation, the SH2 domains of p85 associate with activated receptor and recruits p110s to the localized plasma membrane. The p110-p85 interaction with receptor relieves p110s from the inhibition posed by p85, which produce PtdIns(3,4,5)P_3 at the localized plasma membrane. The mechanisms of recruitment and activation of p110-p85 by GPCRs are distinct from those of RTKs, and are involved in direct GPCR-p110 interaction (Engelman et al. 2006).

The Class I PI3-kinases mediate growth factor signaling mainly through the localized production of PtdIns(3,4,5)P_3 at the plasma membrane. Several other PH domain-containing proteins regulated by PtdIns(3,4,5)P_3 have been identified, including Akt/PKB, PDK1, Rac, and SGK3 (Engelman et al. 2006). Akt, also known as PKB, was the first identified effector of PI3-kinase (Burgering and Coffer 1995; Franke et al. 1995; Stephens et al. 1998). Akt possesses a pleckstrin homology (PH) domain, which binds to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. The catalytic product of PI3-kinases recruits Akt to the plasma membrane, where it is phosphorylated by PDK1 at Thr308, a step required for its activation. Akt regulates several signaling pathways, including the activation of glucose transporters that mediates glucose uptake, FOXO family transcription factors that regulates cell cycle and metabolism, and tuberous sclerosis complex (TSC) that regulates activation of mammalian target of rapamycin (mTOR) (Cantley 2002; Engelman et al. 2006). mTOR complex activation leads to phosphorylation of S6 and 4-EBP which ultimately promote protein synthesis and cell growth (Laplante and Sabatini 2012).

Class I PI3-kinases and their signaling pathways are widely mutated in human cancer (Wong et al. 2010). Several pieces of evidence directly connect PI3-kinase pathways to cancer. First, oncogenic Ras can directly interact with and activate PI3-kinase via the Ras-binding domain on p110α. In vivo mouse models demonstrate that the interaction of Ras and p110α is required for tumorigenesis (Gupta et al. 2007). Second, the tumor suppressor PTEN is a phosphatase for PtdIns(3,4,5)P_3 that functions to antagonize PI3-kinase signalings (Maehama and Dixon 1998; Myers et al. 1998). Loss of PTEN was found in many cancers. Third, p110α activation mutations have been identified in a large number of cancer patients. The p110α hyper-activation mutations are the most frequent onco-protein activation event in all human cancers. They bypass the need for growth factor stimulation, resulting in uncontrolled activation of cell growth and proliferation (Engelman et al. 2006; Wong et al. 2010). Given the strong connection of Class I PI3-kinases and cancer, there is tremendous pharmaceutical interest in developing PI3-kinase inhibitors as targeted anti-cancer therapies. Several drugs are in clinical trials and have shown promising results in treating cancers bearing PI3-kinase mutations.

5.3 PI3-Kinases and Autophagy: Class I and Class III PI3-Kinases Differentially Regulate Autophagy

The connection between PI3-kinases and autophagy was first reported by Blommaart et al. in 1997 (Blommaart et al. 1997). Using rat hepatocytes as a model, the authors investigated autophagy triggered by amino acid starvation, in the presence of two structurally unrelated pan-PI3-kinase inhibitors, wortmaninn and LY294002. Both inhibitors strongly suppressed autophagy, at the autophagy membrane sequestration step, while did not affect lysosomal pH. In addition, the authors demonstrated that 3-methyladenine, a commonly used inhibitor of autophagy membrane sequestration, is an inhibitor of PI3-kinases (Blommaart et al. 1997). Petiot et al. made a similar

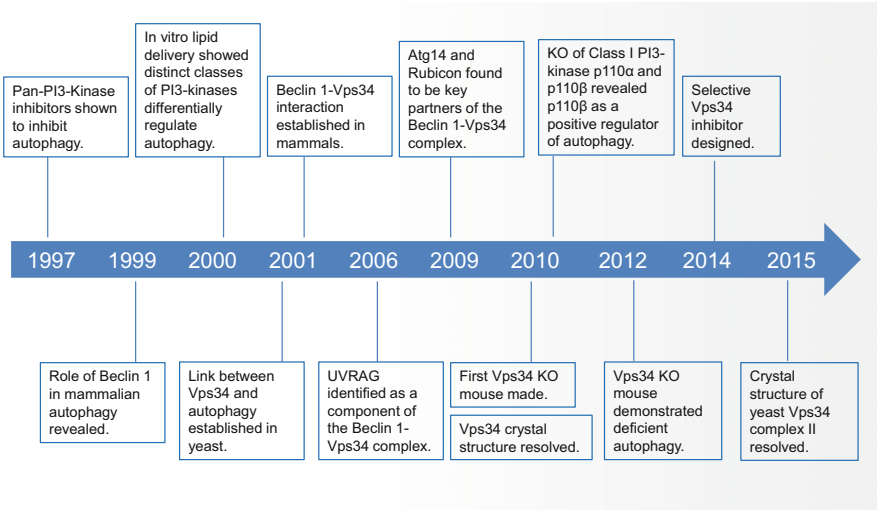

Fig. 5.1 Key events related to PI3-kinases in modulating autophagy are highlighted

observation that pan-PI3-kinase inhibitors and 3-methyladenine suppress autophagy (Petiot et al. 2000), and further evaluated the effects of distinct classes of PI3-kinases in autophagy regulation. Through direct intracellular delivery of phospho-lipids, the authors discovered that the product of Class I PI3-kinase, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, inhibited autophagy, whereas the product of Class III PI3-kinase, PtdIns(3)P, stimulated autophagy. PtdIns(4)P and PtdIns(4,5)P_2 had little or no effect on autophagy. In addition, 3-methyladenine was demonstrated to be an inhibitor of Class III PI3-kinase (Petiot et al. 2000). These early studies indicated that Class I PI3-kinases inhibit autophagy, whereas Class III PI3-kinase promotes autophagy (Fig. 5.1).

In 2001, the role of Class III PI3-kinase, Vps34, in autophagy was established in yeast (Kihara et al. 2001b). Vps34 was co-precipitated with Vps30/Atg6. Furthermore, Vps34 was shown to be in two distinct complexes: one consists of Vps34, Vps15, Vps30/Atg6, and Atg14, which regulates autophagy (termed as complex I), and the other consists of Vps34, Vps15, Vps30/Atg6, and Vps38, which regulates carboxypeptidase Y (CPY) sorting (termed as complex II). Genetic deletion of Vps34 or Vps15 impairs both autophagy and CPY sorting (Kihara et al. 2001b). This work provided the first genetic evidence that Vps34 is involved in autophagy (Fig. 5.1).

In 1999, Beclin 1, the first mammalian specific autophagy gene, was identified (Liang et al. 1999). Beclin 1 is the mammalian ortholog of yeast Vps30/Atg6. Its ectopic expression was able to rescue the autophagy defect in Vps30/Atg6 deficient yeast. Monoallelic deletion of Beclin 1 gene was found to be associated with human breast cancer, opening up a functional link between autophagy and cancer (Liang et al. 1999) (Fig. 5.1). Given the interaction of yeast Atg6 and Vps34, Tamotsu Yoshimori and coworkers first demonstrated a Beclin 1-Vps34 interaction in mammalian cells (Kihara et al. 2001a) (Fig. 5.1). The work also showed that while all

Beclin 1 associates with Vps34, only 50 % of Vps34 associates with Beclin 1 (Kihara et al. 2001a). This is consistent with the current understanding that Vps34 has functions other than autophagy.

Vps34 exists in a stable complex with Vps15. Vps15 itself does not possess PI3-kinase activity but is an essential regulatory subunit for Vps34. Approximately 50 % of the Vps34-Vps15 complex interacts with Beclin 1, which is likely to be the pool involved in autophagy (Kihara et al. 2001a). Like in yeast, the Beclin 1-Vps34-Vps15 core complex interacts with distinct partners which positively or negatively regulate autophagy.

The Atg14-containing pool of the Beclin 1-Vps34-Vps15 complex is termed complex I, which specifically regulates autophagy (Fig. 5.1). Atg14 stimulates Vps34 catalytic activity and is required for autophagosome formation (Matsunaga et al. 2009; Sun et al. 2008; Zhong et al. 2009). Atg14 is able to sense curved membrane (Fan et al. 2011), and localizes to the precursor membrane of autophagosome (Matsunaga et al. 2010), which directs autophagosome formation from ER and mitochondrial contact regions, termed as mitochondria-associated ER membrane (Hamasaki et al. 2013).

The UVRAG-containing Beclin 1-Vps34-Vps15 complex pool is termed complex II, which regulates autophagosome maturation (Fig. 5.1). UVRAG is the ortholog of yeast Vps38, which mediates CPY sorting. Mammalian UVRAG stimulates Vps34 catalytic activity and primarily functions at the endosome and lysosome steps, which promotes endosomal and lysosomal activities and hence autophagy flux (Liang et al. 2006, 2008). The crystal structure of Vps34 was resolved in 2010 (Miller et al. 2010) and that of the yeast Vps34 complex II in 2015 (Rostislavleva et al. 2015) (Fig. 5.1). Another interacting partner in the complex, Rubicon, is a negative regulator of autophagy (Fig. 5.1). Rubicon primarily localizes to the late endosome, and inhibits Vps34 kinase activity, thus suppressing autophagosome maturation (Matsunaga et al. 2009; Zhong et al. 2009).

In addition to the above major components, several other proteins have been described to interact with the Beclin 1-Vps34-Vps15 core complex, although at a weaker affinity or in a condition-dependent manner, such as Ambra1, Bif-1, NRBF2, and Bcl-2 (Funderburk et al. 2010).

5.4 PI3-Kinases and Autophagy: Views from Genetically Modified Mice

While early studies using PI3-kinase inhibitors paved the road for autophagy study, it should be noted that most of the inhibitors used are not specific for the various PI3-kinase isoforms. For example, wortmaninn and LY294002, the first two inhibitors shown to inhibit autophagy, are pan-PI3-kinase inhibitors. 3-methyladenine inhibits both Class I and Class III PI3-kinases (Wu et al. 2010). Several PI3-kinase knockout mouse lines have been generated to elucidate the physiological roles of

PI3-kinases, including the two ubiquitously expressed Class I isoforms p110α and p110β, and the Class III PI3-kinase Vps34 (Fig. 5.1). As whole body knockout of these isoforms are embryonic lethal, most of the information was obtained from tissue-specific knockout strategy.

The Class I PI3-kinase p110α and p110β subunits are downstream of growth factor receptors that stimulate cell growth and proliferation. This is mainly mediated through the Akt-mTOR pathway that activates nutrient uptake and macromolecule synthesis. Therefore the Class I PI3-kinases were expected to suppress autophagy. While this is indeed the case for p110α, genetic ablation of p110β impairs autophagy (Dou et al. 2010). Further mechanistic studies revealed that p110β does not act on the Akt-mTOR pathway to regulate autophagy. Rather, p110β stimulates Vps34 catalytic activity through a scaffolding mechanism that does not require the catalytic activity of p110β (Dou et al. 2010).

The kinase-independent function of p110β has been well noticed. While p110β knockout mice are embryonic lethal, mice bearing both alleles of kinase-dead mutants of p110β are viable (Ciraolo et al. 2008). Further, the kinase-dead p110β mutant is able to rescue some of the defects of p110β knockout cells with respect to cell growth, endocytosis, and mTOR activity (Jia et al. 2008). These observations support a scaffold function of p110β. p110β is known to be a Rab5 effector (Christoforidis et al. 1999). Through competition of Rab5 GAP that stimulates hydrolysis of GTP, p110β keeps Rab5 at its GTP-bound form, which is the active form (Dou et al. 2013). The GTP-bond active Rab5 interacts with the Beclin 1-Vps34 complex, and stimulates autophagy through activation of Vps34 (Ravikumar et al. 2008).

A direct evidence of Vps34 in autophagy in mammals was established by the conditional genetic ablation of Vps34, which showed deficient autophagy in mouse embryonic fibroblast, liver, and heart (Jaber et al. 2012) (Fig. 5.1). Consistent with the results in yeast, deletion of Vps34 leads to defects in both autophagy and endosomal trafficking. Electron microscopy imaging demonstrated that Vps34 knockout cells do not form double-membrane autophagosome, and show abnormal endosomes and multivesicular bodies. Functional analysis proved that Vps34 deletion led to impaired autophagy flux, both in vitro and in vivo. In liver, loss of Vps34 largely phenocopies Atg7 deficiency, including accumulation of lipid droplets, protein aggregations, loss of glycogen, and impaired starvation-induced autophagosome formation and autophagy flux (Jaber et al. 2012). Similarly, Vps34 was also deleted in immune cells, where deficient autophagy is observed in the absence of Vps34 (Willinger and Flavell 2012).

5.5 PI3-Kinases Integrate Nutrient and Growth Factor Signals to Modulate Autophagy

Autophagy as an evolutionarily conserved stress response and cell renewal process is tightly regulated at the molecular level. Both positive and negative signals balance the fine-tuning machinery of autophagy, at the steps of autophagosome initiation,

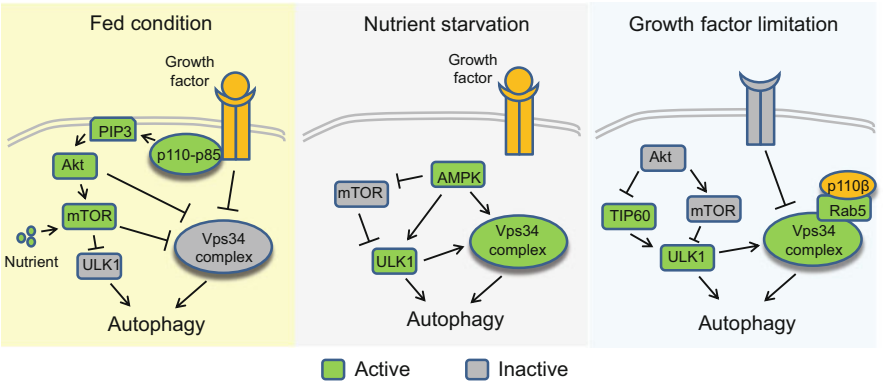

Fig. 5.2 Schematics of autophagy regulation by Class I and Class III PI3-kinases. In fed condition, Class I PI3-kinases are activated while Class III PI3-kinase is inactivated, resulting in suppressed autophagy. Upon nutrient or growth factor starvation, PI3-kinases are differentially regulated to promote autophagy

maturation, degradation and lysosome recycling. In this perspective, PI3-kinases are not only the key machinery of autophagy, but are also the critical signal transducers of environmental clues that instruct differential cellular responses.

Amino acids are potent activators of mTOR (Laplante and Sabatini 2012), which efficiently suppresses autophagy (Fig. 5.2). Amino acid deprivation inhibits mTOR and stimulates autophagy. mTOR regulates autophagy through multiple mechanisms (Fig. 5.2). It has been shown to directly phosphorylate and inhibit ULK1/Atg1 complex (Kim et al. 2011). mTOR can also directly phosphorylates Atg14 at five Ser/Thr sites, and inhibits the Atg14-containing Vps34 complex I. In support of mTOR-mediated inhibitory Atg14 phosphorylation, mutation of the phosphorylation sites on Atg14 results in an enhanced Atg14-pool of Vps34 kinase activity and autophagy even under fed condition (Fig. 5.2) (Yuan et al. 2013).

Glucose is another essential mediator of autophagy. Glucose scarcity results in reduced cellular ATP and increased ADP and AMP, which activates AMP-activated protein kinase (AMPK) (Mihaylova and Shaw 2011). AMPK directly phosphorylates ULK1/Atg1 and stimulates the kinase activity of the complex, thereby promoting autophagy (Kim et al. 2011; Mihaylova and Shaw 2011) (Fig. 5.2). In addition, AMPK directly phosphorylates multiple players of the Vps34 complex, including Vps34, Beclin 1, Atg14, and UVRAG (Kim et al. 2013) (Fig. 5.2). Upon glucose withdrawal, while total Vps34 kinase activity is reduced, Atg14- and UVRAG-associated Vps34 activity is induced. Beclin 1 phosphorylation by AMPK is necessary to promote the activity of Atg14-containing Vps34, which is required for glucose starvation-induced autophagy. Further, AMPK also phosphorylates Vps34 that does not participate in autophagy, leading to a reduction of Vps34 kinase activity of this pool (Kim et al. 2013). These observations support a central role of Vps34 in autophagy triggered by glucose starvation.

While autophagy in unicellular organisms is mainly regulated by nutrient availability, metazoan cells depend on growth factors to regulate nutrient uptake, growth, proliferation, and metabolism. An essential player transducing growth factor signals is PI3-kinase. Growth factors and their signalings are able to regulate autophagy through several mechanisms. One is through the activation of the Class I PI3-kinase-Akt-mTOR signaling pathway. Upon growth factor limitation, mTOR is suppressed, resulting in activation of autophagy. The other is through Akt-mediated phosphorylation of autophagy molecules (Fig. 5.2). Akt directly phosphorylates Beclin 1 to suppress autophagy (Wang et al. 2012). Mutation of residues on Beclin 1 phosphorylated by Akt induces autophagy (Wang et al. 2012). Akt can also signal through GSK3-TIP60 to regulate ULK1 acetylation (Lin et al. 2012). Growth factor limitation leads to decreased Akt activity and enhanced ULK1 acetylation and autophagy (Lin et al. 2012). It is interesting to note that there exist mechanisms in multi-cellular organisms to regulate autophagy in a manner that is more proximal to growth factor receptors (Fig. 5.2). Active EGFR has been shown to directly bind to Beclin 1 and lead to tyrosine phosphorylation of Beclin 1 at multiple residues, which inhibits Beclin 1 and Vps34 activity and autophagy (Wei et al. 2013). Another mechanism connecting growth factor receptors and the Vps34 complex is through the Class I PI3-kinase p110β subunit. In response to growth factor deprivation, p110β dissociates from the growth factor receptor complex, and translocates to the Rab5-positive subcellular compartments to stimulate Rab5-GTP and Vps34 activity, leading to enhanced autophagy (Dou et al. 2013). These mechanisms provide a more direct and rapid way in multi-cellular organisms to activate autophagy when a cell senses growth factor scarcity.

5.6 PI3-Kinase Inhibitors in Targeting Cancer and Autophagy

The Class I PI3-kinase-Akt-mTOR pathway is frequently mutated in human cancers (Engelman et al. 2006; Wong et al. 2010). It is also often interconnected with other oncogenic signaling events. Much effort has been put on developing pharmacological inhibitors for anti-cancer therapy. Several classes of agents targeting the Class I PI3-kinase-Akt-mTOR pathway have been approved by the US Food and Drug Administration (FDA) (for a complete database of clinical trials, visit ClinicalTrials.gov) or are in clinical trials. These include isoform specific PI3-kinase inhibitors, pan-Class I PI3-kinase inhibitors, mTOR inhibitors, Akt inhibitors, and inhibitors that target both Class I PI3-kinases and mTOR.

The design of isoform-specific PI3-kinase inhibitors is based on the discovery that many tumors harbor specific PI3-kinase mutations. The most common example is the prevalent p110α hotspot mutations, such as the H1047R, E542K and E545K mutations. Thus, inhibitors specifically blocking p110α catalytic activity should be effective against these types of tumors. In addition to p110α inhibitors, p110δ selective inhibitors have shown very promising outcome for the treatment of hematopoietic malignancies (Fruman and Rommel 2014).

Several potential caveats for isoform specific Class I PI3-kinase inhibitors are noted. One issue is the presence of feedback loops. The growth factor receptor-Class I PI3-kinase-Akt-mTOR signaling negatively regulate the growth factor receptor complex, leading to reduced growth factor receptor activity, to prevent over-activation. Inhibition of specific isoform of PI3-kinase, such as p110α, results in reduced Akt-mTOR signaling, thus relieves the inhibition of the growth factor receptor, resulting in signalings through other mechanisms, such as p110β and Ras pathways. To overcome this, pan-Class I PI3-kinase inhibitors that targets multiple isoforms were designed and are in clinical trials. Such inhibitors also show high efficacy against PTEN deficient cancers, which signal through both p110α and p110β isoforms. Inhibitors that simultaneously target PI3-kinases and mTOR are also in clinical trials. While these combinatorial approaches should be more effective than single selective inhibitors, potential side effects should be taken into consideration, because Class I PI3-kinases are critical for a variety of normal cell and tissue activities, including heart contractility and glucose metabolism (Fruman and Rommel 2014).

Autophagy is often induced as a stress response during oncogenesis and upon many therapeutic treatments to confer survival advantage cancer cells (Galluzzi et al. 2015). Inhibition of autophagy has been considered a reasonable way to treat cancer especially in combination with other therapeutics to overcome resistance (Amaravadi et al. 2011). A well-pursued autophagy inhibitory drug in cancer treatment is chloroquine, as well as its derivative hydroxy-chloroquine (HCQ). Chloroquine is a well-tolerated FDA-approved drug for treating malaria, rheumatoid arthritis, and lupus. Pre-clinical studies support the notion that combination therapy with HCQ is an efficient anti-cancer strategy. In recent years, clinical trials with HCQ are actively ongoing for treatment of several cancers, in combination with other therapies, including radiation, mTOR inhibitors, BRAF inhibitors, and others (Rangwala et al. 2014; Rosenfeld et al. 2014). Besides chloroquine and HCQ, other autophagy inhibitors are being pursued. Lys05, a chloroquine-based lysosomal inhibitor, is tenfold more potent than HCQ in inhibiting autophagy (McAfee et al. 2012). Unlike HCQ which does not show anti-tumor activity by itself, Lys05 has anti-tumor activity as a single agent (McAfee et al. 2012). Another set of drug candidates are inhibitors specific to Class III PI3-kinase (Vps34). Several new lines of Vps34 inhibitors have been synthesized, which show specific inhibitory effect on Vps34 but not other PI3-kinases. Such inhibitors potently inhibit autophagy and endosomal trafficking (Dowdle et al. 2014; Ronan et al. 2014). Cautions are noted for the potential side effects of Vps34 inhibitors. Deletion of Vps34 in mouse heart results in contractility dysfunction and heart failure, and ablation of Vps34 in liver results in fatty liver accompanied by metabolic dysfunctions (Jaber et al. 2012). Other inhibitors are also being pursued, including small molecules inhibiting ULK1 and Atg7. The safety and antitumor activities of these compounds awaits further evaluation. Nonetheless, as PI3-kinase signaling and autophagy are largely involved in human cancers and are heavily interconnected with each other, more effective and selective approaches to target these molecular events in combination with other emerging therapeutics will offer new opportunities to eradicate the most deadly malignant disease.

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Chapter 6
Adult and Cancer Stem Cells: Perspectives on Autophagic Fate Determinations and Molecular Intervention

Kevin G. Chen and Richard Calderone

Abstract Autophagy is a highly conserved mechanism for the maintenance of cellular homeostasis and functionality in pluripotent stem cells, adult stem cells, and normal somatic cells. Cytoprotective roles of autophagy are essential for eliminating damaged subcellular organelles like mitochondria and protein aggregates, thereby reducing reactive oxygen species (ROS) levels and promoting normal or cancer cell survival. We clarify multiple autophagic inducers, default pathway sensors, and regulators in various stages of stem cells. Of note, with autophagy deficiency, there are two major autophagy-associated outcomes, including pro-autophagic cell survival and death. Clearly, the fates of autophagic determination are tightly regulated by their microenvironments, cell types, and the interplay among multiple cell death machineries related to autophagy, apoptosis, and necrosis. Based on the above fundamental autophagic differences among various cell types, we propose a new concept, balanced autophagy, which sheds light on an equilibrium state between pro-autophagic cell survival and death. We further suggest new strategies targeting therapeutic-resistant cancer stem cells that emphasize the modulatory effects of pro-autophagic cell death in intractable cancer cells.

Keywords Autophagy • Mitophagy • Adult stem cells • Cancer stem cells • Mitochondria

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6.1 Introduction

Autophagy is how eukaryotic cells remove severely injured subcellular organelles, free-radical impaired proteins, aggregated macromolecules, and other cytotoxic substances (Kroemer et al. 2010; Rubinsztein et al. 2005, 2011; Ravikumar et al. 2010; Cheng et al. 2013). Autophagic processes are usually implemented in several sequential steps (i.e., induction, vesicle nucleation, and expansion), involving multiple protein complexes (Rubinsztein et al. 2011). Briefly, cytoplasmic substrates such as damaged organelles or protein aggregates are isolated in a double-membraned vesicle (known as an autophagosome), which are subsequently fused to lysosomes to form autolysosomes where autophagic cargoes are degraded. Moreover, the degraded small molecules or peptides can be recycled into the cytoplasm as new energy resources.

There are various autophagic mechanisms based on the types of cytoplasmic substrates (of autophagosomes), which include subcellular organelles (such as mitochondria, peroxisomes, and endoplasmic reticulum), various sizes of vesicles, and part of the cell nucleus (Kroemer et al. 2010). Ultrastructural analysis reveals that the diagnostic feature of autophagy is the formation of either autophagosomes or autophagolysosomes. Other associated changes include mitochondrial damage, dilation of the rough endoplasmic reticulum (ER) and Golgi complex, and cytoplasmic lipid droplets (Kroemer et al. 2010; Rubinsztein et al. 2005, 2011; Ravikumar et al. 2010). Specific autophagosomes containing melanosomes (termed melano-autophagosomes) were also reported in melanoma cells induced by cytotoxic drugs (Chen et al. 2009). Here, we focus on mitophagosome-related autophagy, a specific autophagy that involves damaged mitochondria digested by macroautophagy.

Mitochondria-based mitophagy is of particular interest. Because mitochondria are the critical sites for oxidative phosphorylation, a predominant resource of energy (ATP) production in eukaryotic cells. Under oxidative stress and pathological conditions, “bad” mitochondria discharge highly reactive oxygen species (ROS). Cytotoxic mitochondrial metabolites such as ROS are able to induce mitochondrial DNA mutations, mitochondrial dysfunction, cytoplasmic and nuclear damages, subsequently leading to mitochondria-related diseases. Mitochondrial damage also triggers programmed cell death or apoptosis. Current data support that mitophagy is an efficient way to halt many pathological-condition-driven processes and to increase the turnover of functional mitochondria. Moreover, mitophagy may be the most efficient type of autophagy against physiological or pathological aging in neurons, cardiomyocytes, and many types of mammalian cells including cancer cells (Cheng et al. 2013; Kubli and Gustafsson 2012; Zhang 2013; Ding and Yin 2012; Chen and Chan 2009).

Nonetheless, the fates of mitophagy in vivo are not well elucidated due to a lack of powerful live imaging assays in humans. A growing body of evidence suggests a pivotal role for mitophagy in the maintenance of the stemness of adult stem cells.

However. it is not well understood how mitophagy is regulated in cancer-initiating cells, which are also known as "cancer stem cells." The cancer stem cell hypothesis is an elusive but potentially important theory in cancer therapies (Clarke et al. 2006; Tan et al. 2006). It is important to verify how cancer stem cells evolve to utilize mitochondria to balance ATP production under various stress conditions. A deep understanding of these complicated natures of mitophagy would enable new strategies to combat aggressive cancer.

Here, we briefly review the evolution of diverse autophagic mechanisms in various organisms, dissect essential autophagic machineries with a focus on mitophagy, and evaluate the functionality of mitophagy based on its defense mechanisms. We further elucidate major autophagic inducers, energetic sensors, transducers, and molecular regulators of oxidative stress-based mitophagy, particularly in adult and cancer stem cells. With respect to cancer therapeutics based on targeting mitophagy, we concisely analyze current treatments. Finally, we propose new therapeutic concepts and strategies concerning how to treat intractable cancer *via* precision intervention of autophagy dynamics.

6.2 Evolutionarily Conserved Mitophagic Machineries

In yeasts, autophagy is an ancient method to obtain recycled energy-rich molecules during nutrient deprivation. Among many model organisms, stimulation of autophagy activity via caloric restriction, Sirtuin 1 activation, and pharmacological interventions (e.g. treatments with rapamycin, resveratrol, or spermidine) increases life span in these organisms (Rubinsztein et al. 2011). Genetically, short-lived mutants in *Saccharomyces cerevisiae* were associated with autophagy defects (Matecic et al. 2010). Upon dietary restriction, activation of the translational repressor 4E-BP prolongs life span by refining mitochondrial function, likely through inactivation of S6K and eIF4F in the cytoplasm in yeasts and *Drosophila* (Wang et al. 2008a; Zid et al. 2009). Transgenic expression of the autophagy-related gene *Atg8a* in the brain of *Drosophila* directly augmented autophagic activity in neurons, which decreased insoluble ubiquitinated proteins and increased cellular resistance to hydrogen peroxide (H_2O_2), thus increasing life span up to 56% in female *Drosophila* (Simonsen et al. 2008).

In mammalian cells, caloric restriction, decreased protein synthesis, reduced ROS, and augmented autophagic activity play an important role in prolonging life span. Genetic inhibition of autophagy-responsive genes diminishes autophagic potentials and induces degenerative changes in various tissues, thereby accelerating physiological and pathological aging (Ravikumar et al. 2010). In recent years, mutations of autophagy-related genes were found to underlie miscellaneous human diseases, including static encephalopathy of childhood with neural degeneration in adulthood (SENDA), Vici syndrome, hereditary spastic paraparesis, Parkinson's disease, lysosomal storage disorders, Crohn's disease, and different types of cancer

(Jiang and Mizushima 2014). Given that autophagy is associated with diverse human diseases, there is a great need to precisely define the functionality of autophagy, especially mitophagy, in mammalian cells.

6.3 Functions of Mitophagy

It is generally believed that autophagy, a critical regulator of mitochondrial homeostasis, confers cytoprotective effects in various tissues or organs by degrading detrimental cargoes, bacteria, and viruses (Kroemer et al. 2010; Jiang et al. 2010; Ravikumar et al. 2005; Zhang and Cuervo 2008). Damaged mitochondria with a missing membrane potential discharge ROS and noxious apoptotic intermediates, which are subjected to removal by mitophagy, thus maintaining physiologically relevant numbers of mitochondria. Exemplified in apoptotic T-cells, the maintenance of mitochondrial turnover is essential for promoting mature T-cell survival (Kovacs et al. 2012). The molecular basis of this mitophagy was often facilitated by serine/threonine protein kinases-interventional phosphorylation of mitochondria and subsequently by the E3 ubiquitin ligases-mediated ubiquitination of mitochondrial membrane proteins. For instance, in neurons, the bad mitochondria are usually tagged by the PTEN induced putative kinase 1 (PINK1), ubiquinated by the E3 ubiquitin ligase Parkin, and then degraded by (macro)autophagy. Thus, deletion of PINK1 results in deficiency of Complex I and II of mitochondria in the stratum of knockout mice (Gautier et al. 2008), suggesting a potential role of PINK1 in brain function as well as neurogenesis.

Genetic manipulation of autophagy-related (ATG or Atg) genes has been frequently used to identify potential mitophagy functions. Tissues with deletion of ATG genes regularly exhibit some aging-related phenotypes, including intracellular accumulation of inclusion bodies, lysosomes containing the aging lipofuscin pigment, and morphologically damaged mitochondria. For example, *Atg7*-knockout in mesoendodermal lineages (such as liver, skeletal muscle, and islet β cells), mitochondrial dysfunction was a common feature, which includes accumulation of deformed and swelling mitochondria (Ebato et al. 2008; Jung et al. 2008; Masiero et al. 2009; Komatsu et al. 2006, 2007; Wu et al. 2009). Other noticeable changes were reflected in subcellular organelles (e.g. accumulation of peroxisomes, ER distension, and vacuolar changes), mesodermally muscle-related changes (e.g. muscle atrophy and sarcopenia), and altered endocrine processes (e.g. degeneration of islets and impaired glucose tolerance). In addition, pronounced molecular signatures include ubiquitin-positive inclusions in hepatocytes, accrual of an autophagy receptor (p62/STQM or p62) concomitant with NRF2 activation, and an increase in ubiquitinated protein aggregates colocalized with p62 (Ebato et al. 2008; Jung et al. 2008; Masiero et al. 2009; Wu et al. 2009; Komatsu et al. 2010). These structural and biochemical changes that resulted from Atg gene deletion in mice are consistent with the autophagic phenotypes displayed from yeasts to human beings. Thus, mitophagy is a default program that may be accurately regulated by different inducers, energetic sensors, and signal transducers.

6.4 Mitophagy: Inducers and Energetic Sensors

Collectively, inducers, metabolic or energetic sensors, transducers, and regulators function cooperatively in multiple pathways. Nutrient starvation, caloric restriction, hypoxia, and oxidative stress are potent autophagy inducers. Specific energetic sensors (on membrane and in the nuclei) receive signals from both exogenous and endogenous inducers (e.g. starvation, low glucose, oxidative stress, and low ATP). These sensors are able to transactivate core transducers such as 5′ adenosine monophosphate-activated protein kinase (AMPK). Consecutively, signal transduction pathways activate regulatory networks, which intertwine transcriptional programs with proteomics to positively or negatively regulate the input signals (Fig. 6.1).

Nutrient Starvation and Caloric Restriction as Generic Inducers: Caloric restriction or reduced nutrient consumption has been proven to be an effective anti-aging intervention in many model organisms including primates (Colman et al. 2009). Caloric restriction induces autophagy through multiple pathways that include activation of AMPK, SIRT1 (an NAD^+-dependent deacetylase), PHA4, and the inhibition of the IGF1-mTOR pathway (Egan et al. 2011; Canto et al. 2010; Kenyon 2010; Hansen et al. 2008). These manifold pathway-driven anti-aging mechanisms are perhaps carried out in a spatial-temporal manner, which significantly increase the autophagic diversity and complexity. Interestingly, caloric restriction or starvation induces metabolic stress and activates autophagy in a cancer microenvironment, which is partially guided by the ROS-mediated AMPK activation (Li et al. 2013). These data provide a strong link between nutrient starvation and oxidative stress in autophagy.

Oxidative Stress Inducers: The tumor suppressor role of autophagy has been confirmed in genetically modified mouse models with targeted deletion of Beclin1 (Qu et al. 2003; White et al. 2010; Yue et al. 2003). The underlying mechanism includes autophagy that removes damaged mitochondria and reduces high levels of ROS, thus minimizing DNA damage, enhancing chromosomal stability, and mitigating protein quality control (Mathew et al. 2007, 2009). Interestingly, ROS (e.g. hydrogen peroxide) induces becline-1-independent autophagy, known as non-canonical autophagy. Moreover, hydrogen peroxide was also shown to direct oxidation of ATG4, a protease, upon starvation (Scherz-Shouval and Elazar 2011). Thus, autophagy inhibits oxidative stress through both canonical and non-canonical autophagy pathways, therefore offering efficient quality control of mitochondrial activity.

Mitochondrial fusion and asymmetric fission offer alternative quality control to remove damaged mitochondria (as indicated by low membrane potential, $\Delta\Psi_m$) and to enable the functional ones to enter the next fusion-fission cycle (Twig et al. 2008). Given that asymmetrical fission is compromised or discontinued in cells, it would certainly increase the accumulation of endogenous ROS rendering cells bioenergetic deficient (e.g. defective oxidative phosphorylation). However, it is not clear whether mitophagy deficiency-induced defective oxidative phosphorylation would encourage cancer cells to adapt to the lower energy-producing glycolysis. Nonetheless, the above mitophagy mechanisms might provide new insights into the Warburg effect, which describes how cancer cells utilize low-efficiency glycolysis as the predomi-

nant energy source, regardless of the availability of aerobic and anaerobic (or hypoxic) conditions (Vander Heiden et al. 2010; Gottlieb and Vousden 2010). Hypoxia, one of the cancer hallmarks, represents a blood supply deficiency related to oxygen deprivation in a tumor mass. Hypoxia has been an active area of cancer research because it confers cancer therapeutic resistance and thus is an adverse prognostic factor in cancer therapy (Shannon et al. 2003).

Hypoxia as a Mitophagic Inducer: Under hypoxic conditions, induction of autophagy enables cancer cells to survive in certain cellular stress conditions. In general, this process seems to be regulated by HIF1α (Thiery et al. 2009). Specifically, HIF1α induces autophagy-associated expression of BNIP3 and BNIP3L (Mazure and Pouyssegur 2009), which are responsible for stimulating mitophagy to cope with ROS. Under clinically relevant hypoxia, AMPK-independent autophagy is able to support human tumor cells resistant to radiotherapy (Chaachouay et al. 2015). Recently, HIF1α has also been shown to regulate the viability of prostate cancer stem cells *via* mTOR signaling (Marhold et al. 2015). Furthermore, an AMP/ATP-AMPK-TSC-mTOR pathway was implicated in the control of HIF1α-independent hypoxia-induced autophagy (Papandreou et al. 2008; Yu et al. 2011). These studies highlight the importance of intracellular energy sensors such as AMP and ATP in the regulation of autophagic response (Fig. 6.1).

AMP/ATP as intracellular Sensors: Energy utilization by cancer cells has been an enigma since the discovery of the Warburg effect. The role of ATP signaling in autophagy is not well understood. Remarkably, the energy levels in cells can be accurately measured by the AMP/ATP ratios. An increase in AMP/ATP ratios and nutrient depletion were found to induce AMPK activation, which subsequently activates autophagy via Unc-51-like autophagy activating kinase 1 (ULK1) and the inhibition of mTOR (Alers et al. 2012). Moreover, exhaustion of cellular ATP in

Fig. 6.1 (continued) LKB1 might function as a cellular context-dependent switch between a regulator of AMP/ATP ratios and a modulator of AMPK-dependent and AMPK-independent pathways (*g*). Under clinical relevant hypoxia, HIF1α stimulates the AMPK-independent autophagy through inhibition of mTOR, which renders adult and cancer stem cells resistant to the detrimental effects of DNA-damaging agents and ROS (*h*). HIF1α also induces autophagy-related BNIP3 and BNIP3L expression to enhance its protective effects (*c*). Finally, frequent oncogenic, genotoxic, and oxidative stress stabilize and activate p53, which might also stimulate or inhibit autophagy by activating or inhibiting AMPK respectively and by coordinately regulating downstream transcriptional programs (*i*, *c*). *Abbreviations*: *ACD* autophagy-induced cell death or autophagic cell death, *AMP* adenosine monophosphate, *AMPK* 5′ adenosine monophosphate-activated protein kinase, *ATG* autophagy-related genes, *ATP* adenosine triphosphate, *HIF1α* hypoxia-inducible factor 1-alpha, *LKB1* serine/Threonine Kinase 11 encoded by the *STK11* gene, *MAPK* mitogen-activated protein kinases, *mTOR* the mammalian target of rapamycin, *p53* tumor protein p53, *PM* plasma membrane, *ULK1* Unc-51 like autophagy activating kinase 1. *Pathway diagram descriptions*: "?" undefined signal transduction pathways, "↑" increased ratios; lines or curves with *arrowheads* indicating enhanced effects or activation, whereas lines or curves with *round ends* denoting decreased effects or inhibition; the thickness of the lines (or curves) corresponding to the magnitude of the effects (i.e., *thicker lines* indicate stronger effects); and the *dotted lines* (or curves) specifying reduced or weak contributions to downstream outcomes

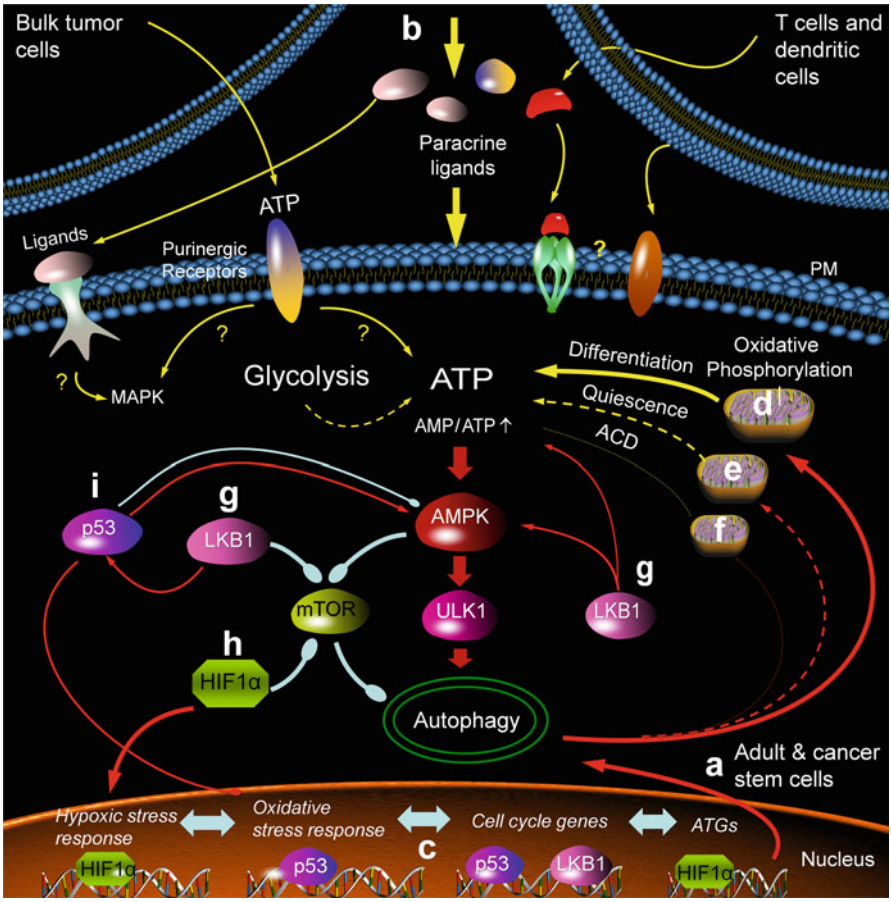

Fig. 6.1 An integrative model of energy states with autophagic fate outcomes in adult and cancer cells: cellular and molecular mechanisms by which adult and cancer stem cells sense both intracellular (*a*) and extracellular energetic changes (*b*) integrate mitochondrial autophagy pathways with core transcriptional programs (such as hypoxic, oxidative stress, and cell cycle control) (*c*). The proposed model emphasizes the prevalence of glycolysis in stem cells (including pluripotent stem cells, adult and cancer stem cells). Altered ATP energetic states ultimately alter proteomics and transcriptional networks to determine autophagic cell fates in different types of stem cells. These interconnections provide the possibility to precisely formulate autophagy-related cancer therapies using anti-autophagic cell survival or pro-autophagic cell death strategies. In this model, we designate that cellular lineage differentiation is an energy-consuming process that requires sufficient ATP production through the oxidative phosphorylation of mitochondria *via* the tricarboxylic acid (TCA). The reactive oxygen species (ROS) byproducts generated during this process might cooperatively stimulate lineage differentiation (*d*). ROS might also suppress cancer cell metastasis by inhibiting the epithelial-mesenchymal transition (EMT). Moreover, a low energy-consuming state is required for stem cell quiescence and self-renewal (*e*). Under the conditions of autophagy-induced cell death (ACD) conditions, mitophagic events produce extremely low or no ATP, thus incapable of maintaining the mitochondrial membrane potential (*f*). With respect to the regulation, diverse functionality of LKB1 might directly or indirectly control ATP levels, cell cycle checkpoints, AMPK, and mTOR, thus placing this factor as one of the central regulators. Intriguingly,

defective mitochondria was found in Lkb1-deficient bone marrow cells (Gurumurthy et al. 2010). Lkb1 is a new cell-cycle restrictive checkpoint that is independent of its regulation of AMPK and mTOR signaling. Further inactivation of Lkb1 led to rapid depletion of hematopoietic stem cells (Gurumurthy et al. 2010). These data validate AMP/ATP dynamics as major intracellular autophagic sensors. Moreover, AMP/ATP signals might control a tentative autophagic switch between an LKB1-ATP-AMPK-dependent and -independent pathways (Fig. 6.1).

Interestingly, the immune system exemplifies autophagy regulation of ATP. In activated $CD4^+$ T cells, suppressed autophagy led to reduced energy metabolism characterized by reduced ATP production, glycolysis, and fatty acid utilization (Hubbard et al. 2010). Autophagy appears to provide metabolite resources needed to produce ATP (Lum et al. 2005), which facilitates cell survival under sustained withdrawal of growth factors in bone marrow hematopoietic cells. Thus, autophagy might play an essential role in the maintenance of intracellular ATP levels during the course of apoptosis concomitant with caspase activation. A stable intracellular level of ATP may be indispensable for secretion of "find-me" signals (e.g. lysophosphatidylcholine) as well as "eat-me" signals (e.g. phosphatidylserine). In addition, cancer chemotherapy-induced autophagy also promotes ATP release from cancer cells (Fig. 6.1). This process can be blocked by the inhibition of autophagy. Does ATP modulate antitumor immune response in T-cells? Indeed, extracellular ATP released from tumor cells recruits dendritic cells and triggers a T-cell response to tumor cells (Michaud et al. 2011). On the other hand, ATP-mediated immunoresponse, together with T-cell mediated autophagy, may enhance cancer cell survival by evading conventional therapeutic regimens (Buchser et al. 2012).

6.5 Stem Cells and Mitophagy

Numerous types of stem cells exist in mammalian life, ranging from embryonic development to adulthood under both physiological and pathological conditions. Briefly, stem cells can be classified into two major groups (i.e., pluripotent and multipotent stem cells). Pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which can potentially produce any cell or tissue type in mammals (Chen et al. 2014a). With respect to multipotent stem cells, they share some basic features of pluripotent stem cells. However, multipotent adult stem cells only have limited diversification potential, usually differentiating to two or more cell types (Wagers and Weissman 2004).

Embryonic Stem Cells: In mammalian development, ESCs generate the embryo and ultimately the fetus. The inner cell mass (ICM), an ESC cluster inside the blastocyst from preimplantation-stage embryos, could be isolated and cultured in cell culture dishes *in vitro* (Evans and Kaufman 1981; Thomson et al. 1998; Chen et al. 2014b). These ESCs possess the capacities of self-renewal (i.e. replicating themselves) and differentiation to all cell types of the three germ layers (i.e. ectoderm, mesoderm, and endoderm). The ectoderm, the outermost germ layer of cells derived from the ICM, develops into the nervous system, sensory organs, and skin. The

endoderm generates respiratory and digestive organs (e.g. lung, liver, and pancreas), whereas the mesoderm gives rise to bone, muscle, connective tissue, kidney, and hematopoietic cells.

Induced Pluripotent Stem Cells: These iPSCs are derived by directly reprogramming somatic cells using multiple transcriptional factors (e.g. Oct4, Sox2, Klf4, and c-Myc). The introduction of four specific genes encoding the above transcription factors into somatic cells (e.g. fibroblasts) could convert these cells into a pluripotent state, similar to ESCs (Takahashi and Yamanaka 2006). Essentially, iPSCs can be propagated indefinitely *in vitro* and differentiated into all cell types of the three germ layers. Hence, iPSCs hold great promise in the fields of regenerative medicine, disease modeling, drug discovery, and cancer research (Robinton and Daley 2012).

Multipotent Adult Stem Cells: Multipotent adult stem cells, also known as somatic stem cells, are undifferentiated cells that are found within differentiated tissues or organs. Adult stem cells exist in the bone marrow, brain, liver, heart, and many other organs. The primary roles of adult stem cells in a living organism may be associated with cellular maintenance, tissue repair, and cell replacement. Interestingly, adult stem cells also possess the capacities of self-renewal and differentiation into some specialized cell types. The origins of adult stem cells are different in terms of their tissue or organ types. The adult brain contains stem cells that are able to differentiate into astrocytes, oligodendrocytes, and neurons. Some tissues or organs may have one or more types of adult stem cells. For example, the bone marrow comprises at least two stem cells niches that nurture hematopoietic stem cells (that form all types of blood cells in the body) and skeletal stem cells (that generate cartilage, bone, hematopoiesis-supportive stroma, and marrow adipocytes) (Morrison and Scadden 2014; Bianco and Robey 2015). Encouragingly, hematopoietic stem cells from the bone marrow have been used in allogenic bone marrow transplants for more than 40 years. It is conceivable that many other types of adult stem cells could be also useful for stem cell-based therapies in the future.

Nonetheless, adult stem cells only exist as a rare population of cells within a tissue or organ. Due to a lack of definitive surface markers, isolation of these stem cells represents a big challenge. Additionally, large-scale amplification of these adult stem cells may be also an obstacle that impedes future stem-cell based therapies. Moreover, there is little known about how adult stem cells are regulated both *in vivo* and *in vitro*. Some adult stem cells as well as cancer stem cells typically exist in a quiescent state with a longer lifespan in specified stem cell niches. Autophagy is thought to be critical for maintaining stem cell homeostasis in cells that have undertaken tissue regeneration and cellular reprogramming (Pan et al. 2013; Phadwal et al. 2013). The exact role of autophagy in the regulation of pluripotent, adult, and cancer stem cells remains to be determined.

The Role of Mitophagy in Pluripotent Stem Cells: Mitophagy has been implicated in facilitating reprogramming of somatic cells to iPSCs. Vazquez-Martin et al. reported that reprogramming of somatic cells (e.g. mouse fibroblasts) by the Yamanaka factors (i.e., Oct4, Klf4, and Sox2) to the pluripotent state was drastically reduced by 95 % by pharmacologically induced mitochondrial fusion using the mitochondrial

division inhibitor mdivi-1 (Vazquez-Martin et al. 2012). Mechanistically, mitochondrial division by mdivi-1 discriminatorily impedes the self-assembly of a dynamin family member (termed DRP1) of the large GTPases, thereby preventing the renewal of functional mitochondria via mitochondrial fission. Furthermore, Sox2-dependent temporal inhibition of mTOR activates autophagy, which is also a key step to initiating cellular reprogramming (Wang et al. 2013). Additionally, human iPSCs have been generated from patients carrying m.3243A>G, the most common mitochondrial DNA mutation found in many human diseases. The iPSC-derived neurons and various tissues showed specific complex I defects in the respiratory chain of mitochondria. Complex I was subjected to degrading via mitophagy as indicated by explicit expression of both PINK1 and Parkin in perinuclearly sequestered autophagosomes (Hamalainen et al. 2013). Taken together, the above studies provide new insights into the role of autophagy in the regulation of reprogramming efficiency. Clearly, mitochondrial fusion, a surveillance mechanism to ensure sufficient numbers of functional mitochondria and Complex I activity, is essential for mitochondrial homeostasis and normal cell differentiation.

The Role of Mitophagy in Adult Stem Cells: Adult stem cells or progenitors are found in the tissues derived from the three-lineage differentiation. The involvement of autophagy in the maintenance of self-renewal and differentiation of these stem cells might be essential for these cells to meet the minimal metabolic requirements (Ito and Suda 2014). With respect to adult stem cells derived from neuroectoderm, p53 negatively regulates the self-renewal and proliferation of adult neural stem cells (NSCs) through the cell cycle regulator p21 (CDKN1A) (Meletis et al. 2006). A recent report indicates that deletion of RB1-Inducible Coiled-Coil 1 (*RB1CC1*), a gene essential for autophagy induction in mammalian cells, leads to the loss of postnatal NSCs and impaired neuronal differentiation in the postnatal brain (Wang et al. 2008b). Interestingly, p53-dependent apoptosis and cell cycle arrest were associated with postnatal NSC death, but not with the impaired neuronal differentiation. Thus, down-regulation of an oxidative state by *N*-acetylcysteine was able to salvage the weakened neuronal differentiation (Wang et al. 2008b). Moreover, after insulin withdrawal, adult hippocampal NSC had a caspase-independent autophagic cell death related to the essential autophagy gene *Atg7* (Yu et al. 2008). Systemic Atg7 ablation in mice instigated neurodegeneration, increased susceptibility to infection, and reduced survival to 3 months (Karsli-Uzunbas et al. 2014).

Regarding adult stem cells from mesoendoderm, *ex vivo* cytokine withdrawal and *in vivo* caloric restriction induce a protective autophagy against metabolic stress, which is mediated by FOXO3A in mouse hematopoietic stem cells (Warr et al. 2013). Hematopoietic stem cell compartment sustains FOXO3A-driven gene expression profiles that poise hematopoietic stem cells for rapid induction of autophagy upon the emergence of an energy crisis. Endoderm-derived bipotent liver progenitors are believed to activate liver regeneration in the adult liver upon hepatectomy or massive liver damage. However, the mechanism that maintains the progenitors' stemness is not well studied. A recent report revealed that autophagy was also required for the maintenance of liver progenitor cells under both physiological

and pathological conditions (Cheng et al. 2015). Collectively, these data support the essential roles of autophagy in ensuring adult stem cell survival and death under specific conditions. These results also offer consistent examples that shed light on the molecular interplay among oxidative states, autophagy, autophagic cell death, and apoptosis.

The Role of Mitophagy in Cancer Stem Cells: There are likely two-compartment autophagy mechanisms: autophagy metabolism in cancer cells and in tumor microenvironments (including normal tissues and adult stem cells). In cancer cells, canonical autophagy is mediated by Beclin 1 and essential for the tumorigenicity of mammary cancer stem-cell like progenitors (Gong et al. 2013). Autophagy is essential for glucose homeostasis and the maintenance of lung tumors (Karsli-Uzunbas et al. 2014). Noticeably, autophagy is also able to suppress tumor progression by maintaining tumor cells in a quiescent state. Moreover, the tumor suppressor protein p53 induces autophagy under genomic stress conditions. The p53-dependent activation of autophagy is coordinately regulated by mTOR and AMPK (Feng et al. 2005, 2007; Crighton et al. 2006). Essentially, it is the nuclear p53 that transactivates autophagy-related genes (Tasdemir et al. 2008a). Under certain conditions, p53 is also a negative regulator of autophagy. Genetic or pharmacological inhibition of the tumor suppressor protein p53 activates autophagy (Fleming et al. 2011; Tasdemir et al. 2008b, c). Many autophagic inducers induce autophagy through the E3 ubiquitin ligase MDM2-mediated degradation of p53. Under nutrient depletion and hypoxic conditions, increased autophagy activity maintains higher ATP levels, thus enhancing the survival of p53-deficient cancer cells. However, cytoplasmic p53, instead of nuclear p53, was able to repress enhanced autophagy in p53 (−/−) cells (Tasdemir et al. 2008a). Thus, p53 plays a dual role in the regulation of autophagy, in which p53 protein modifications, intracellular localizations, and functional states are critical for this distinct regulation. Verification of the detailed cellular and molecular context related to p53 is an important step to understand the complexity of autophagy regulation in malignant tumors.

With regard to autophagy in tumor microenvironments, an increasing body of evidence indicates that there are complicated signaling interactions among normal tissues, adult stem cells, and cancer stem cells. The interactions between endocrine and paracrine signals are implicated in the regulation of pluripotent stem cell niches (Chen et al. 2014a). Presumably, parallel endocrine-paracrine signals would also regulate cancer stem cell survival and growth (Fig. 6.1). Another possible mechanism is that endocrine-paracrine interactive signals regulate normal adult stem cells, stromal cells, immunological response cells, and fibroblast-like cells at the periphery of cancer stem cell niches. It is conceivable that increased autophagy may interfere with the function of endocrine cells (such as insulin-producing β cells) and hormonal response of microenvironmental stem cell niches in a non-cell-autonomous way.

Nevertheless, caution must be taken when we study a rare population of multipotent and cancer stem-like cells with the capacity of self-renewal and tumor-initiating in vitro and in animal models. For example, breast cancer stem-like cells can be propagated as suspended colonies termed “mammospheres.” Enhanced autophagic expression of Beclin 1 was found in aldehyde dehydrogenase 1-positive ($ALDH1^+$)

cells within mammospheres (Gong et al. 2013). These results may reflect a preventive response of cancer stem cell-like cells under altered growth conditions in vitro, not necessarily the properties of mammary cancer stem cells. Cancer stem cell-like cells usually have different degrees of quiescence, sometime referred to as slow-cycling cells (Roesch et al. 2010). These cellular behaviors would make it difficult to distinguish them from normal adult stem cells or normal tissues. Furthermore, such cellular quiescence may also benefit cancer stem cell to survive in a low energy state, potentially facilitating the development of intractable cancer properties. Unraveling these autophagy-related behaviors is particularly important when we consider a strategy for treating cancer patients by targeting cancer stem cells-based autophagy.

6.6 Cancer Therapeutics Targeting Mitophagy

It is clear now that the inhibition of autophagy may lead to at least three different outcomes (i.e., cell differentiation or survival, cellular quiescence, and autophagic cell death) (Fig. 6.1d–f). Autophagy-mediated differentiation and cell survival render cancer cells resistant to oxidative stress, apoptosis, and necrosis. Therefore, inhibition of autophagy sensitizes cancer cells to DNA-damaging agents such as cisplatin and 5-fluorouracil in esophageal and colorectal cancer cells (Li et al. 2010; Liu et al. 2011). It has been noted that the cyclosporine A analogue SDZ PSC-833, a potent multidrug resistance (MDR) inhibitor, has a pro-autophagic cell death effect in pigmented melanoma cells (Chen et al. 2009). However, there is not a well-defined interphase between pro-survival and pro-death autophagy, which we designate here as "balanced autophagy." We reason that treating cancer patients by targeting different phases of autophagy may achieve correspondingly different outcomes.

Anti-autophagy Based Cancer Therapy: Inhibition of autophagy in cancer seems an emerging cancer therapy (Cheng et al. 2013). There is a broad spectrum of small molecules that belong to autophagy stage-specific inhibitors or modulators. For example, class III PI3K (Vps34) inhibitors (e.g. 3-methyladenine, wortmannin, and LY294002) interfere with early-stage autophagic recruitment to the membranes and with lysosomal structures. Chloroquine, hydroxychloroquine, and bafilomycin A1 belong to late-stage inhibitors of autophagy. Both chloroquine and hydroxychloroquine are lysosomotropic drugs that inhibit lysosomal acidification (Ruiz-Irastorza et al. 2010). Of note, bafilomycin A1, a specific inhibitor of vacuolar-H^+-ATPases, inhibits the acidification of endocytic structures (Shacka et al. 2006). Lysosomal inhibition of vacuolar-H^+-ATPase activity by proton pump inhibitors increases both extracellular and lysosomal organelle pH. As a result, these proton pump inhibitors significantly escalate cytoplasmic retention and nuclear import of some chemotherapeutic agents, thus vividly sensitizing solid tumor cells to the effects of cisplatin, 5-fluorouracil, and vinblastine (Luciani et al. 2004). Therefore, lysosomotropic drugs and proton pump inhibitors might share an antitumor mechanism through the inhibition of lysosomal acidification-based autophagy.

Microtubule-stabilizing (e.g. taxanes) and disrupting agents (e.g. colchicine and *Vinca* alkaloids) interfere with subcellular organelle transport along microtubules, thereby indirectly inhibiting the fusion between autophagosomes and lysosomes. We need to point out that the majority of these inhibitors including chloroquine and microtubule-stabilizing and -destabilizing agents are widely used anticancer drugs. These cytotoxic drugs frequently cause multidrug resistant phenotypes in cancer cells, which are mediated by a cluster of ATP-binding cassette (ABC) transporters, particularly, ABCB1, ABCG2, and ABCC1 (Chen and Sikic 2012; Gottesman et al. 2002). Nonetheless, some anti-malaria-based drugs such as chloroquine and quinacrine have been assessed in humans and showed greater cytotoxicity, which should be modified for future clinical trials, perhaps in combination with other pathway inhibitors to enhance autophagic effects. Finally, ionizing radiation preferentially induces expression of LC3, Atg5, and Atg12 in $CD133^+$ glioma initiating stem cells, but not in $CD133^-$ cells (Lomonaco et al. 2009), suggesting the utility of the induction of a specific anti-autophagy in eradicating cancer stem cells, particularly in those quiescent and slow-cycling stem cells.

Balanced Autophagy Initiates Homeostasis and Sustains Cell Dormancy: Balanced autophagy represents an equilibrium state, often with cell dormancy, between pro-autophagic cell survival and cell death. Fundamentally, cell dormancy with reversible cell cycle arrest or slow-cycling cells, low metabolic rates, reduced protein synthesis, and autophagic activation provide essential resources for cancer stem cell survival, repair, and self-renewal. As discussed in previous sections, a well-characterized signaling pathway of dormancy is the inhibition of the mTOR pathway and subsequently decreased biosynthesis. These properties are essential for cancer stem cells to develop therapeutic resistance (Fig. 6.1e). It is imperative to accurately define the cellular and molecular states of cancer, which enable us to determine potential therapeutic resistance, tolerance, and predictive response.

Pro-autophagic Cell Death Based Cancer Therapy: Teleologically, we may consider pro-autophagic cell death to enhance cancer therapies under certain circumstances (Fig. 6.1f). In pigment-producing melanoma cells, the use of the MDR inhibitor SDZ PSC-833 promotes melanoautophagic cell death (Chen et al. 2009). This study provides the rationale for the combined use of potent MDR inhibitors with other pro-autophagic death inducers to treat patients with drug-resistant cancer stem cells.

Managements of Oxidative Stress and Metastasis of Cancer: Metastatic cancer cells with upregulated autophagy exhibit an apoptosis-resistant phenotype (e.g. resistant to TRAIL-induced apoptosis) when compared to non-metastasizing cells (Glinsky and Glinsky 1996; Han et al. 2008). Administration of inhibitors of Beclin1 and ATG7 restore TRAIL-induced apoptotic cell death in cancer (Han et al. 2008; He et al. 2012). The influence of oxidative stress levels on therapeutic response in metastatic cancer cells is not well elucidated. A recent study found that metastatic melanoma cells had high levels of oxidative stress that is associated with low viability of this type of metastasizing cells. Therefore, antioxidant treatments of metastatic

melanoma in the mouse model promote melanoma cell survival (Piskounova et al. 2015). This study raises the possibility of using pro-oxidants to modulate oxidative stress in cancer, which might prevent cancer metastasis in future clinical trials.

6.7 Conclusions

The cytoprotective roles of autophagy may function as a double-edge sword, which promotes both normal and cancer cell survival. We show that autophagic inducers, sensors, transducers, and regulators function cooperatively in multiple default pathways. Noticeably, the inhibition of autophagy may cause three possible autophagic outcomes, including pro-autophagic cell survival, quiescence, and death. Moreover, the cellular determination of a given autophagic outcome is dependent on cellular context and complicated spatial-temporal relationships among autophagy, apoptosis, and necrosis. Importantly, these different outcomes provide a fundamental basis for clinical intervention of cancer and should be considered when designing a therapeutic regimen. Finally, we propose new concepts such as balanced autophagy related to the quiescent state of cancer stem cells and the rationale of pro-autophagic cell death, a previously unappreciated strategy, for future cancer therapy.Acknowledgments We would like to thank our colleague Professor R. Padmanabhan for discussion and Ms. Verma Walker, NIH Library Editing Service, for reviewing the manuscript.

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Chapter 7
Role of Autophagy in Tumor Progression and Regression

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Abstract Depending on tumor type, stage, and genetic context, autophagy can play an opposite role in cancer by promoting tumor progression or regression. It is now well established that autophagy limits tumor initiation, however, it promotes the progression of well-established tumors. In the context of tumor progression and immune response, experimental evidence indicate that autophagy plays a key role in maintaining survival of tumor cells under stress condition such as hypoxia. Indeed, by activating autophagy, tumor cells are able to escape immunosurveillance by activating several overlapping mechanisms in cancer cells. Such findings have inspired significant interest to develop autophagy inhibitor molecules as an entirely new approach to cancer treatment. While much remains to be learned mechanistically, it is now widely established that modulation of this process will be an attractive avenue for future anticancer therapeutic approaches. In this chapter, we will briefly describe the role of autophagy in tumor regression in the context of inflammation, necrosis, oxidative stress and genomic instability. We will also focus on recent reports highlighting the role of autophagy in the impairment of the anti-tumor immune response. In keeping with this, we believe that targeting autophagy may represent a conceptual realm for new anti-tumor strategies aiming to block immune escape.

Keywords Autophagy • Tumor immunity • Inflammation • Hypoxia • Tumor progression • Tumor regression • Tumor therapy

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7.1 Autophagy Regulation in Physiological and Pathological Conditions

Autophagy acts as a catabolic process crucial for cellular homeostasis and maintenance of cell integrity under stressful conditions (Mizushima 2007; Yang and Klionsky 2010). Autophagy is a degradation mechanism of cell components which allows the recycling of essential amino acids, nucleotides, and fatty acids necessary for energy and macromolecule biosynthesis (Corcelle et al. 2009; Glick et al. 2010). During cancer progression, autophagy can be induced by different stresses, particularly hypoxia, nutrient deprivation, or extracellular matrix detachment (Rosenfeldt and Ryan 2009; Yang and Klionsky 2009). The autophagic process is characterized by the formation of phagophore or isolation membrane mainly dependent on Beclin-1 (BECN1) complexes. Following this so-called nucleation stage, the phagophore is elongated by several Autophagy-related proteins (ATG) and the Microtubule-associated protein 1A/1B-light chain 3 (LC3)-I is lipidated into LC3-II. Maturation of the phagophore, through the action of LC3-II and BECN1 proteins, enables the sequestration of cell constituents into well-characterized vesicles named autophagosomes. Fusion of autophagosomes with lysosomes leads to the formation of autolysosomes and the degradation of their contents by lysosomal hydrolases (Kang et al. 2011).

Under physiological conditions, autophagy is constitutively executed at basal level in all cells to promote cell homeostasis. However, in tumor cells autophagy is activated in response to various cellular stresses and environmental factors including hypoxia (Mathew and White 2011). Therefore, the major consensus that emerge is that autophagy can act as tumor suppressor and tumor promoter. Such opposite role of autophagy in cancer seems to be related to the stage of the tumor. In fact, autophagy clearly suppresses the initiation and the development of tumors, however, it is considered as a key survival pathway in response to stress, and many established tumors require autophagy to survive.

7.2 Autophagy as a Tumor Regression Mechanism

The role of autophagy in tumor suppression relies on its effect on several oncogenic pathways such as the activation of the PI3K/Akt pathway *via* activating *PI3K* mutations, *AKT* amplifications, or *PTEN* loss of function. (Guertin and Sabatini 2007). Moreover, the amplification of the apoptosis inhibitor Bcl-2 has been reported in some circumstances to inhibit autophagy through its binding to beclin1 (Sinha and Levine 2008; Maiuri et al. 2007). The involvement of p53 in the regulation of autophagy seems to be complex. Indeed, the activation of p53 by nutrient deprivation or genotoxic stress leads to the activation of autophagy through the inhibition of mTOR or by the activation of DRAM (damage-regulated autophagy modulator) (Balaburski et al. 2010; Crighton et al. 2006; Feng et al. 2005). However, consistent with the role

of autophagy as tumor suppressor, the functional loss of p53 was expected to decrease autophagy or suppress basal autophagy. The later effect seems to depend on the cytoplasmic, not the nuclear, pool of p53 (Tasdemir et al. 2008).

In addition to the indirect evidence described above, several direct evidences support the tumor suppressing properties of autophagy. Indeed the autophagy execution protein Beclin1 is a haplo-insufficient tumor suppressor protein. Monoallelic deletion of *BECLIN1* are reported in sporadic human breast and ovarian carcinoma (Aita et al. 1999), and heterozygous deletion of *BECLIN1* predisposed mice to a variety of tumors including mammary neoplastic lesions, lung adenocarcinomas, hepatocellular carcinomas and B cell lymphomas (Qu et al. 2003). These results indicate that functional autophagy may be constraining tumor initiation (Liang et al. 1999). Similarly, homozygote deletion of *ATG5* was shown to predisposed mice specifically to liver tumors with high penetrance (Takamura et al. 2011). The tumor suppressive properties of autophagy have been extensively investigated. Below we will provide some mechanistic insights into the tumor-suppressive functions of autophagy.

7.2.1 *Autophagy Inhibition Regulates Tumor Necrosis and Inflammation*

It has been reported that autophagy can modulate the inflammatory microenvironment that play a major role in tumor development and considered as a common future of early cancer development. Thus, experimental evidence suggest that autophagy-deficient tumors displayed an increased level of necrosis and inflammation. The activation of autophagy in tumor cells inhibits necrotic cell death which subsequently stimulates a robust inflammatory response *in vivo* (Kono and Rock 2008). In addition, it has been proposed that the impairment of both apoptosis and autophagy promotes necrotic cell death, *in vitro* and *in vivo*, associated with an inflammatory response and an accelerated tumor growth (Degenhardt et al. 2006). These results highlight that autophagy regulates necrosis-induced cell death and inflammation. Furthermore, autophagy also prevents necroptosis which is a form of caspase-independent cell death mediated by cell death ligands (*i.e.* TNF-α and FasL) (Degterev and Yuan 2008; Shen and Codogno 2012). Indeed, autophagy is essential to overcome zVAD-induced necroptosis in L929 cells. Activation of PI3K-Akt-mTOR pathway, a well-known autophagy inhibitor pathway, can sensitize L929 cells to zVAD-induced necroptosis, while amino-acid and serum starvation protect these cells (Wu et al. 2009). Similarly, autophagy prevents poly-(ADP-ribose) polymerase (PARP)-mediated cell death. Such cytoprotective role of autophagy in PARP-mediated necrosis was illustrated by showing that DNA damages induced by doxorubicin in fibroblasts lead to PARP-1 activation and autophagy induction which protects cells against necrosis. Targeting autophagic genes ATG5 or BECLIN1, sensitizes cells to doxorubicin-induced necrotic cell death (Munoz-Gamez et al. 2009).

Autophagy is also a key process for the maintenance of intracellular ATP level required for the secretion of lysophosphatidylcholine (LPC). Secretion of LPC is associated with the acute phase of the inflammatory response and is involved in the development of chronic inflammation. It has been shown that autophagy-deficient cells fail to generate phosphatidylserine on the outer membrane surface—an important anti-inflammatory pro-apoptotic marker. Such observation could explain why defect in autophagy stimulates inflammatory response subsequently to insufficient clearance of dead cells (Pierdominici et al. 2012).

Following autophagy inhibition, the accumulation of the autophagy cargo protein p62/SQSTM1 activates the pro-inflammatory transcription factor NF-kB and the stress-responsive transcription factor NRF2, thus favoring inflammation and tissue injury (Levine et al. 2011). The transcription factors NF-kB family members regulate the expression of a broad range of genes involved in development, proliferation, and survival of tumor cells. The activation of these transcription factors leads to the regulation of inflammation and innate and adaptive immune responses (Smale 2011). As the activation of NF-kB is mediated by the IkB kinase (IKK) complexes, it has been reported that IKK complexes are targets for degradation by autophagy when the heat shock protein 90 (Hsp90) function is inhibited (Xu et al. 2011). Another mechanism of regulation of NF-kB by autophagy is mediated by the Kelch-like ECH-associated protein 1 (Keap1). Keap1 interacts with the kinase domain of IKKβ through its C-terminal domain. This domain is also required for the binding of Keap1 to the transcription factor NRF2, which controls the expression of some antioxidant genes. In response to tumor necrosis factor (TNF), Keap1 negatively regulates the activation of NF-kB through inhibition of the IKKβ phosphorylation and induction of IKKβ degradation by autophagy pathway (Fan et al. 2010). The E3 ubiquitin ligase Ro52 is another signaling molecule that targets IKKβ for degradation through the autophagy pathway. In response to distinct stimuli, specific interactions of Hsp90, Keap1 and Ro52 with IKKs regulate NF-kB activity through their ability to activate or repress the degradation of IKKs by autophagy (Trocoli and Djavaheri-Mergny 2011). It has been suggested that the crosstalk between NF-kB and autophagy regulates inflammasome activity leading to the modulation of the activation of caspase-1 and subsequently the secretion of potent pro-inflammatory cytokines (Strowig et al. 2012). Based on the studies discussed above, it appears that autophagy is an important modulator of cancer pathogenesis through its ability to regulate inflammation.

7.2.2 Autophagy Prevents Oxidative Stress and Genomic Instability

The role of autophagy in cancer suppression has been reported by several *in vivo* studies (White et al. 2010). Thus, Beclin1-defective mice showed an increased susceptibility to develop cancer (Qu et al. 2003; Yue et al. 2003). This could be related to the involvement of autophagy in the management of oxidative stress and in the

maintenance of the genomic integrity. In this regard, it has been described that autophagy can limit DNA damage, chromosomal instability and aneuploidy (Mathew et al. 2007). Several studies suggested that the ubiquitin- and LC3-binding protein p62 may play a determinant role (Komatsu et al. 2007; Mathew et al. 2009). Indeed, the inability of autophagy-deficient cells to degrade p62 lead to the aberrant accumulation of this protein, which is sufficient to promote tumorigenesis (Mathew et al. 2009). Thus, p62 activates the transcription factor NRF2 through the direct inhibition of Keap1 (Komatsu et al. 2010; Lau et al. 2010). However, the role of NRF2 in DNA damage promotion is not clearly understood so far. In addition, p62 may act as an important NF-kB modulator in tumorigenesis (Duran et al. 2008). This study highlights that the increase in DNA damage in autophagy-deficient cells is associated with high levels of damaged mitochondria and reactive oxygen species (ROS), accumulation of ER chaperones and protein disulfide isomerases. DNA alterations were suppressed by ROS scavengers, confirming the essential role of autophagy in oxidative stress management and, subsequently, in protein quality control (Mathew et al. 2009).

Excessive exposure to ROS alters the function of multiple cellular macromolecules by oxidation (*e.g.* nucleic acids, lipids, proteins). However, oxidative stress is closely linked to mitochondria dysfunction. Since autophagy is the only process allowing the mitochondrial turnover by a mechanism so-called mitophagy, preventing the accumulation of damaged mitochondria highly reduces the risk of oxidative stress. Moreover, mitochondria produce the bulk of ATP required for vital cellular functions (*e.g.* DNA replication, mitosis, transcription). In this regard, the ability of autophagy to control proteins/organelles quality and to maintain cellular energy homeostasis highlights its antitumorigenic activity (Jin 2006). Such a role has been demonstrated in autophagy-defective cells, where the presence of damaged proteins is crucial in DNA replication, mitosis or centrosome function. Moreover, autophagy defective cells displaying defect in mitochondrial clearance and subsequently an alteration in ATP production may also alter DNA replication or repair by affecting the arrest of the replication forks and the generation of breakage/fusion/bridge cycles responsible for gene amplification (Jin and White 2008). Finally, the implication of autophagy in the physiological protein turnover may also influence the occurrence of DNA damage. Indeed, cell cycle progression is driven by the periodic activity of proteins including Cyclin-dependent kinases (CDKs), Cyclins, CDKs inhibitors. Therefore, it stands to reason that a deregulation in the physiological protein turnover in autophagy-deficient cells may alter the correct sequence of the cell cycle progression (Jin and White 2008). Taken together, it has become clear that autophagy helps normal cells to overcome several types of stresses (*e.g.* metabolic, oncogenic), that directly limits their oncogenic transformation. In contrast, such management of cellular stresses is also observed in cancer cells, and leads in this case to cancer promotion (Rosenfeldt and Ryan 2011).

Senescence is an irreversible cell cycle arrest associated with an active metabolism, which can limit the proliferation of abnormal cells. In this context, autophagy is also able to mitigate the accumulation of genomic alteration by inducing the mitotic senescence transition. Young et al. reported an accumulation of autophagosomes in

Ras-induced IMR90 senescent fibroblasts, suggesting that autophagy is required for tumor senescence. In addition, targeting *ATG5/7* delayed the senescent phenotype, while induction of autophagy clearly enhanced the protein turnover that contributed to synthesis of pro-senescence cytokines (*e.g.* IL-6, IL-8) (Young et al. 2009). This study suggests that autophagy not only facilitates the entry into senescence but also reinforces the senescent phenotype of cells.

7.2.3 Autophagy Contributes to Tumor Cell Death

The role of autophagy in promoting tumor cell death has been proposed based on the observation that apoptosis occurs concomitantly with features of autophagy (Kroemer and Levine 2008) and that prolonged stress and progressive autophagy can lead to cell death (Mathew and White 2007). Together with apoptosis (type I cell death) and necrosis (type III cell death) (Schweichel and Merker 1973), autophagy was first described as type II cell death. The relevance of autophagic cell death in development has been established in lower eukaryotes and invertebrates such as *Dictyostelium discoideum* and *Drosophila melanogaster* (Denton et al. 2009; Kosta et al. 2004). Evidence has been reported that mammalian development does not require autophagy, as newborn mice lacking essential autophagy genes show any anatomical or histological defects and no impairment of the cell death (Mizushima et al. 2008). This evidence is supported by the fact that the depletion of autophagy genes in human or mice mammalian cells induces apoptosis rather than protects cell against death induced by different stresses (Boya et al. 2005; Gonzalez-Polo et al. 2005). The role of autophagy in cell death induction is not clear, and needs further investigation. However, the more convincing evidence highlighting the role of autophagy in cell death has been reported in mammal's neuronal cells. Indeed, following insulin starvation, hippocampal neural stem cells undergo autophagic cell death, while targeting autophagy by silencing *ATG7* blocks this process. It is worthy to note that autophagic cell death occurs only in cells with functional apoptosis and is caspase-independent (Yu et al. 2008). Currently, the majority of experimental evidence showing autophagic cell death in mammalian cells were mainly conducted *in vitro* and in cells defective in apoptosis machinery. It has been shown that DAPK (death associated protein kinase) plays an important role in the regulation of both autophagy and apoptosis. Indeed, DAPK induces autophagy by phosphorylation of Beclin1, and is associated with the induction of apoptosis. However this type of DAPK-dependent autophagic death is caspase dependent, and it remains to be elucidated whether DAPK-mediated cell death is a real autophagic cell death, or whether autophagy only assists in the apoptosis execution phase (Gozuacik et al. 2008). It has been proposed that cells rather die *with* autophagy, and not *by* autophagy as they showed that none of 1400 compounds, evaluated for their ability to induce autophagic puncta and increase autophagic flux, killed tumor cells through the induction of autophagy (Shen and Codogno 2012). Moreover a careful determination of the autophagic flux is needed to differentiate autophagic cell death from

other forms of non-apoptotic programmed-cell death, such as necroptosis. These examples illustrate that autophagy may be involved in lethal signaling although the role of autophagy itself in cell killing remains unclear. Thus, further studies are required in order to define the exact role of autophagic cell death mechanism.

7.3 Autophagy Modulates the Anti-tumor Immune Response

Recently, autophagy has emerged as a new critical mechanism activated in tumor cells in hypoxic microenvironment that mediates tumor resistance to innate and adaptive anti-tumor immune responses. Several reports demonstrate that autophagy activation not only enables tumor cells to survive stress conditions during cancer development but also provides them an intrinsic resistance mechanism to escape anti-tumor immune response.

7.3.1 Role of Autophagy in Tumor Cell Resistance to CTL-Mediated Killing

The first evidence for such a role of autophagy was provided by Noman et al. who demonstrated that hypoxic lung carcinoma cells can evade cytolytic T lymphocyte (CTL)-mediated lysis through autophagy induction (Noman et al. 2011, 2012). Indeed, inhibition of autophagy using small interfering RNA (siRNA) directed against ATG5 or BECN1 restored tumor cells sensibility to CTL-mediated lysis which correlated with a decrease in hypoxia-dependent induction of the phosphorylation of Signal Transducer and Activator of Transcription (STAT)-3. This result allowed the prediction that blocking autophagy would inhibit pSTAT3-dependent survival mechanism making tumor cells more susceptible to CTL attack under hypoxia. However, considering the degradation role of autophagy, it is difficult to perceive that autophagy is involved in the stabilization of pSTAT3 under hypoxia. Focusing on the crosstalk between the adaptor protein p62/SQSTM1, the ubiquitin-proteasome system (UPS) and autophagy, this study revealed that the induction of hypoxia inducible factor (HIF)-1α has two effects in tumor cells: (i) HIF-1α triggers the phosphorylation of Src which subsequently phosphorylates the tyrosine residue Y705 of STAT3 (ii) HIF-1α activates autophagy by a mechanism implicating the increased expression of BCL2/adenovirus E1B 19 kDa protein-interacting protein (BNIP)3/BNIP3L and the dissociation of the BECN1-BCL2 (B cell lymphoma 2) complex. Autophagy activation results in degradation of the p62 protein. Knowing that p62 is the receptor/adaptor protein responsible for targeting pSTAT3 to the UPS, the autophagy-dependent degradation of p62 leads to the accumulation of pSTAT3. When autophagy is inhibited in tumor cells, the degradation of p62 is blocked and therefore accumulates in tumor cells. This accumulation accelerates the UPS-dependent degradation of pSTAT3 (Noman et al. 2009) (Fig. 7.1a).

Epithelial to mesenchymal transition (EMT) is a trans-differentiation process necessary for the morphogenesis of tissue during embryonic development (Nieto 2013). While its role in cancer cell invasion, metastasis and drug resistance is well established, recent report described that autophagy can be activated in tumor cells undergoing EMT and that such EMT-induced autophagy represents another mechanism of cancer cell resistance to CTL-mediated lysis (Akalay et al. 2013a, b). In this study, the authors showed that the induction of EMT program by overexpression of SNAI1 in breast cancer cells coincides with a drastic change in cell morphology and the activation of autophagy flux most likely through the overexpression of BECN1 in mesenchymal cells. Although the exact molecular mechanism by which the EMT affects the expression of BECN1 remained to be addressed, several lines of evidence indicate that this may be related to SNAI1- or EMT-dependent repression of microRNA(s) involved in modulation of BECN1 expression (Siemens et al. 2011; Yu et al. 2012). This result extended the role of SNAI1 as a regulator of autophagy and paves the way to investigate the functional role of EMT-induced autophagy in tumor cells. In this context, results described in this study showed that targeting BECN1 in mesenchymal cells was sufficient to restore CTL-mediated tumor cell lysis, without affecting the mesenchymal morphology and the expression of EMT markers. This finding implies that autophagy is a downstream target of the EMT program in breast cancer cells. Overall, this study suggests that EMT-induced autophagy is a novel mechanism by which tumor cells regulate CTL reactivity and impede their cytotoxic activity, and further points to the complex relationship between the tumor and the immune system (Fig. 7.1b).

7.3.2 Role of Autophagy in Tumor Cell Resistance to NK-Mediated Killing

It is now well established that several resistance mechanisms are regulated in tumor cells to escape immune surveillance in hypoxic tumor microenvironment. Recent evidence described how tumor cells can escape natural killer (NK)-mediated immune surveillance by activating autophagy under hypoxia (Baginska et al. 2013; Viry et al. 2014). Indeed, NK cells recognize and kill their targets by several mechanisms including the release of cytotoxic granules containing perforin (PRF1) and serine protease granzyme B (GZMB). It has been recently proposed that PRF1 and GZMB enter target cells by endocytosis and traffic to large endosomes named "gigantosomes" (Thiery et al. 2010, 2011). Subsequently, PRF1 is involved in the formation of pores in the membrane of the "gigantosome", leading to the gradual release of GZMB and the initiation of apoptotic cell death. The formation of amphisomes following the fusion between autophagic vacuoles and early endosomes appears to be necessary in some cases for the generation of autolysosomes. In this report (Baginska et al. 2013), the authors described that the pro-apoptotic protein GZMB is selectively degraded upon autophagy activation in hypoxic cells, thereby blocking NK-mediated target cell apoptosis (Fig. 7.1c). In line with this, they showed that GZMB is detected in autophagosomes and provided evidence that

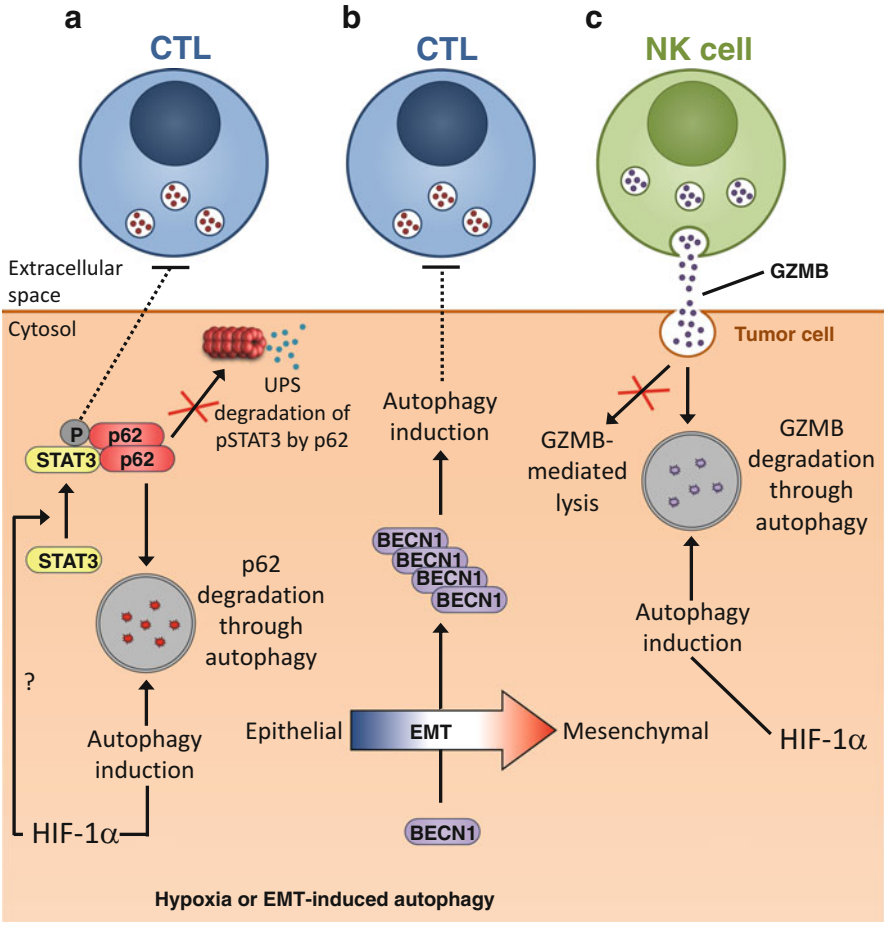

Fig. 7.1 Autophagy activation in tumor cell acts as an intrinsic resistance mechanism against anti-tumor immune response. The tumor microenvironment and/or EMT program activate autophagy in target cells. The induction of autophagy operates as a cell resistance mechanism leading to tumor escape from CTL- or NK-mediated lysis. (**a**) Hypoxic stress leads to the accumulation of HIF-1α. HIF-1α activates autophagy and simultaneously increases the phosphorylation level of STAT3 at the Tyr705 residue. As an autophagic substrate, p62/SQSTM1 is degraded in the autophagosomes following their fusion with lysosomes. As p62 is involved in targeting pSTAT3 to the UPS, its degradation leads to the accumulation of pSTAT3 in cells and such accumulation constitutes a cell survival mechanism. In autophagy-defective cells, p62 is no longer degraded, and its accumulation accelerates the UPS-dependent degradation of pSTAT3 and thereby restores CTL-mediated tumor cell lysis. (**b**) The acquisition of an EMT phenotype confers resistance to CTL-mediated lysis through autophagy induction. The increase in mesenchymal markers following the activation of EMT program leads to the up-regulation of BECN1 by a yet undefined mechanism. Such upregulation induces autophagy and impairs CTL-mediated tumor cell lysis. In mesenchymal cells, targeting BECN1 is sufficient to restore CTL-mediated lysis. (**c**) Following the recognition of their targets, NK cells secrete cytotoxic granules containing PRF1, GZMB, and other hydrolytic enzymes that enter target cells, traffic to enlarged endosomes, and initiate tumor cell death. Under hypoxia, excessive autophagy in target cells leads to the fusion of autophagosomes with vesicles containing GZMB leading to its specific degradation by autophagy, thereby inhibiting NK-mediated lysis. Targeting autophagy prevents the degradation of GZMB and thereby restores NK-mediated tumor cell killing

GZMB level is significantly decreased in hypoxic compared to normoxic target cells. Furthermore, targeting autophagy genetically or inhibiting lysosomal hydrolases by pharmacological approaches restored GZMB level which ultimately leads to the recovery of hypoxic cells lysis by NK cells in vitro and in vivo. Based on these results, the authors stated that tumor regression can be achieved by inhibiting autophagy in hypoxic cancer cells, thus enabling their NK-mediated lysis (Baginska et al. 2013; Viry et al. 2014).

Overall, studies described above underline the activation of autophagy as a key mechanism in tumor escape from immune cell attack within the tumor microenvironment. However, an important issue that arises from these studies is whether hypoxia is the only microenvironmental factor involved in the induction of autophagy in tumor cells. An interesting recent report provided strong evidence that lymphoid effectors not only provide lytic signals but also promote autophagy in the remaining target cells, a process called cell-mediated autophagy (C-MA) (Buchser et al. 2012). Thus, C-MA has been reported in different human epithelial tumors after interaction with immune cells at high ratio of effectors to targets. Importantly, it has been showed that C-MA not only acts as a mechanism of resistance to immune cell-mediated lysis but also limits the cytotoxic activity of stress factors such as γ-radiation (Buchser et al. 2012).

These studies highlight that the activation of autophagy plays a critical role in tumor cell escape from both adaptive and innate immunity. Therefore, targeting autophagy has been proposed to improve CTL- and NK-based immunotherapy in experimental mouse model (Baginska et al. 2013; Noman et al. 2011, 2012). Intense research efforts are currently focusing on the development of autophagy inhibitors that could improve tumor immunotherapy.

7.4 Targeting Autophagy in Cancer Therapy

Currently, there are several clinical trials registered in the National Cancer Institute (www.cancer.gov/clinicaltrials) exploring anti-autophagy strategies in a variety of human cancers. Most of these trials are ongoing, with minimal published results available, and nearly all use Hydroxychloroquine (HCQ) or Chloroquine (CQ). It is worthy to note that CQ or HCQ are lysosomotropic agents that act at the level of the lysosome by inhibiting acidification, thereby impairing autophagosome degradation. These clinical trials were initiated based on the fact that autophagy is induced as a survival mechanism in a variety of tumor cells and preclinical models by several types of chemotherapeutic agents. Because only a subpopulation of tumor cells undergo autophagy, it is unlikely that autophagy inhibitors are used in cancer therapy as single agent. Indeed, most of these clinical trials used HCQ in combination with other anti-cancer therapies. While these preclinical data are generally supportive of incorporating anti-autophagy therapies in cancer treatment trials, it has been observed, in some circumstances, that inhibition of autophagy decreases therapeutic efficacy. Understanding the circumstances in which autophagy inhibition impairs the therapeutic effect will be

of great importance. Importantly, while CQ and HCQ are effective inhibitors of autophagy in vitro, whether they will do so at doses used in current clinical trials is still uncertain. An important issue related to the use of these autophagy inhibitors concerns the micromolar concentration that is required to inhibit autophagy and show anti-tumor efficacy in preclinical models. While this is theoretically achievable at tolerated doses after prolonged dosing, it should be better optimized in clinic (Tett et al. 1993; Munster et al. 2002). Trials combining HCQ as neoadjuvant treatment will provide tumor tissues available for analysis both before and after HCQ treatment. However, the effectiveness of HCQ in the inhibition of autophagy still prove difficult, as HCQ is often combined with other therapies (chemotherapy and radiotherapy) that are also known to modulate autophagy. Alternative biomarkers to predict for autophagy activation as well as autophagy dependence are currently an area of intense investigation (Kimmelman 2011). A recently reported phase I trial of HCQ in combination with adjuvant temozolomide and radiation in patients with glioblastoma found that the maximum tolerated dose of HCQ was 600 mg per day, and this dose achieved concentrations of HCQ required for autophagy inhibition in preclinical studies. In this trial, investigators observed a dose-dependent inhibition of autophagy, as indicated by increases in autophagic vesicles (revealed by electron microscopy), and detected elevations in LC3-II in peripheral blood mononuclear cells. In addition, in a phase I trial of 2-deoxyglucose, an agent that blocks glucose metabolism, autophagy occurred in association with a reduction in p62/SQSTM1 in peripheral blood mononuclear cells (Stein et al. 2010). These data suggest the potential interest of such biomarkers in the evaluation of autophagy modulation during therapy and in the correlation with treatment outcome.

CQ inhibits the last step of autophagy at the level of the lysosome, thereby impacting lysosomal function. Therefore, its effects are not entirely specific to autophagy. Currently, there is a great deal of interest in developing new inhibitors of autophagy. In this regards, and given the complexity of the autophagic process, multiple proteins involved in this process could be good candidates for developing others autophagy inhibitors. It is likely that kinases would be prime candidates for inhibition such as Vps34, a class III PI3K, which has a critical early role in autophagosome development. This is particularly attractive, as there has been significant success in designing effective class I PI3K inhibitors (Wong et al. 2010). However, one potential issue which needs to be considered is that Vps34 has roles in other aspects of endosome trafficking, and this may lead to unwanted effects and toxicity (Backer 2008). The mammalian orthologs of yeast ATG1, ULK1/2, which acts downstream from AMPK and the TOR complex, have been recently shown as critical proteins for autophagy activation (Hara et al. 2008; Egan et al. 2011). Other potential targets for autophagy inhibitors would be LC3 proteases, such as ATG4b, which are necessary for LC3 processing. However, whichever approach is taken, the delicate balance between potency and toxicity must be determined to achieve a clinical success. While there are still uncertainties of how autophagy inhibition will fare as an anti-cancer therapy, the preclinical data generally support this approach. The current clinical trials will hopefully provide insight into whether this will be a viable therapeutic paradigm (Kimmelman 2011).

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