Drugs of the Future 2011, 36(12): 919-925

CCC: 0377-8282/2011
DOI: 10.1358/dof.2011.36.12.1711892

THOMSON REUTERS

REVIEW ARTICLE

MODULATION OF AUTOPHAGY AND ITS POTENTIAL FOR CANCER THERAPY

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SUMMARY

Autophagy is a process whereby cellular contents are captured in specialized membrane-bound vesicles and delivered to lysosomes for final degradation. Most studies support an inherent connection between autophagy and survival, but increasing evidence also suggests an association between autophagy and cell death. The therapeutic potential of targeting the autophagy pathway in cancer appears clear, but specific strategies for achieving successful eradication of cancer cells are less obvious. Recent developments in the fields of autophagy and programmed cell death have nevertheless shed light on therapeutic strategies with significant potential. In this review, we provide an overview of the autophagy process, pathways that modulate autophagy and promising autophagy-based therapeutic strategies for cancer.

INTRODUCTION

The term autophagy was first used by Christian de Duve on the basis of electron microscopic observations (1). Autophagy, literally meaning “self-eating”, is an intracellular process in which a double membrane, or membrane cistern, isolates cellular constituents such as proteins and organelles, which are then degraded by lysosomes. Three reported subtypes of autophagy are macroautophagy, microautophagy (2) and chaperone-mediated autophagy (CMA) (3). Here, we focus specifically on macroautophagy, hereafter referred to as autophagy.

Under basal, nutrient-replete conditions, the main function of autophagy is to maintain cellular homeostasis by removing damaged proteins and organelles and by providing the cell with energy and new building blocks. During conditions of high metabolic demand or stress, including nutrient starvation, microenvironmental changes and DNA damage, the function of autophagy is ostensibly unchanged, but autophagic flux is dramatically increased in an attempt to maintain cell viability. Over 34 autophagy-related genes have been identified so far (4), and proteomic analysis recently uncovered a complex network of proteins that interact with and modulate autophagy (5).

AUTOPHAGY IS A MULTISTEP PROCESS

The autophagic process consists of four main steps: 1) *initiation*; 2) *nucleation* (mediated by phosphatidylinositol 3-kinase catalytic subunit type 3 [hVps34, *PIK3C3*] and beclin-1 [*BECN1*]); 3) *elongation and closure* (mediated by two ubiquitin-like systems); and 4) *fusion and degradation* (Fig. 1) (6).

Under basal, nutrient-replete conditions, mTOR complex 1 (TORC1) is active and negatively regulates *initiation* of autophagy, whereas cellular stresses such as hypoxia and nutrient starvation suppress mTOR function, thereby triggering autophagy initiation (7, 8). mTOR function can be decreased by three mechanisms: 1) tuberous sclerosis 2 (*TSC2*) phosphorylation by 5′-AMP-activated protein kinase (AMPK) deactivates GTP-binding protein Rheb and consequently inhibits TORC1 (9); 2) inhibition of regulatory-associated protein of mTOR (raptor, *RPTOR*), a component of TORC1, results in TORC1 recruitment to 14-3-3 proteins (10); and 3) amino acid starvation can decrease mTOR activity through Ras-related GTP-binding protein (Rag) GTPases (11). Although TORC1 is a key component of autophagy induction, there are also a number of TORC1-independent mechanisms that can induce autophagy (12, 13).

The *nucleation* step of autophagy serves to prevent unbalanced activation of autophagy via two major pathways downstream of mTOR –the serine/threonine-protein kinase ULK1/2 complex and the hVps34 “core complex” (Fig. 1). The mammalian ULK1/2 kinase complex consists of ULK1 or ULK2, autophagy related 13 homolog (*ATG13*), autophagy-related protein 101 (*ATG101/C12orf44*) and 200 kDa FAK family kinase-interacting protein FIP200 (*RB1CC1*) (6). Under nutrient replete conditions, TORC1 negatively regulates ULK1 and autophagy-related protein 13 by phosphorylation, which prevents ULK1 interaction with AMPK (14, 15); hence, the nutrients gen-

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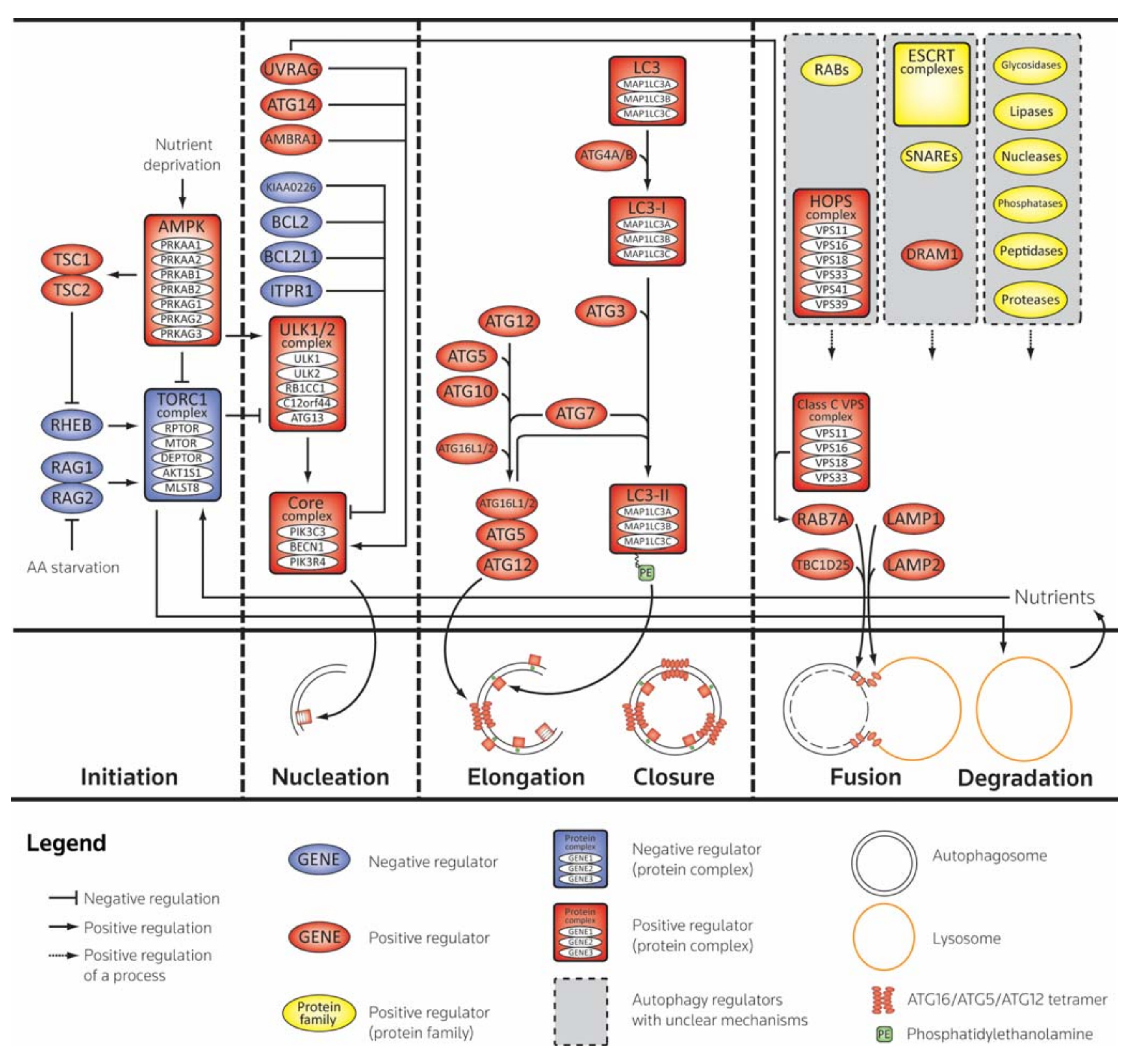

Figure 1. Molecular features of the autophagy process. The top panel shows specific entities (genes, proteins, complexes, and small molecules) associated with that process and, to the extent possible, their specific roles. The middle panel shows a schematic timeline of the macroautophagy process (four steps separated by vertical dashed lines). The bottom panel defines entities that appear in the top two panels.

erated by autophagy turn the pathway off through negative feedback. A series of recent publications propose an additional mechanism for ULK1 regulation in which AMPK activates autophagy by directly phosphorylating ULK1 (16). The other major regulator of *nucleation* is the core complex composed of hVps34, phosphoinositide 3-kinase regulatory subunit 4 (PI3-kinase p150 subunit, *PIK3R4*) and beclin-1. That complex is required for generation of phosphoinositide signals on autophagosomal source membranes (endoplasmic reticulum [ER], Golgi, plasma membrane, mitochondria or de novo) (17) and for recruitment of other autophagy-related proteins, for which beclin-1 functions as a scaffold. Binding of beclin-1 to autophagy/beclin-1 regulator 1 (*AMBRA1*), autophagy related 14 homolog (*ATG14*), UV radiation resistance-associated gene protein (*UVRAG*) and a number of additional proteins (18) activates autophagosome *nucleation*, whereas binding to Rubicon (*KIAA0226*), B-cell CLL/lymphoma 2 (*BCL2*), Bcl-2-like 1 (Bcl-X, *BCL2L1*) and inositol 1,4,5-trisphosphate receptor type 1 (IP3R1, *ITPR1*) inhibits autophagosome *nucleation* (19).

Autophagosome *elongation* is mediated by the autophagy protein 5 (APG5-like; *ATG5*)/ubiquitin-like protein ATG12 (*ATG12*) and microtubule-associated protein 1 light chain 3 (LC3, *MAP1LC3*) conjugation systems (20, 21). During the initial phase, ATG12 covalently binds APG5-like, which in turn promotes autophagosome *elongation* via recruitment of LC3 (Fig. 1). LC3 is cleaved by cysteine protease ATG4 and conjugated to phosphatidylethanolamine (PE) by an ubiquitin-like-conjugating enzyme ATG3/ubiquitin-like modifier-activating enzyme ATG7-dependent activation and transfer system. LC3 is then conjugated to the membrane and plays an important role in cargo recruitment and in the determination of autophagosome size. Cargo selection depends on cargo adaptor proteins, such as neighbor of BRCA1 gene 1 (*NBR1*), sequestosome-1 (p62, *SQSTM1*) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like (*BNIP3L*/*NIX*). Neighbor of BRCA1 gene 1 and sequestosome-1 contain ubiquitin binding domains and therefore can specifically recruit ubiquitinated cargo (22). Sequestosome-1 is degraded during the process of autophagy and accumulates when autophagy is impaired. BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like the other cargo adaptor protein, recruits mitochondria to the autophagosome (23). Autophagosome biogenesis ends with the *closure* of autophagosomal membranes, resulting in double membrane vesicles containing selected intracellular cargoes destined for degradation.

Autophagosomes then mature and *fuse* with endosome/lysosomes to form autolysosomes (Fig. 1). TBC1 domain family member 25 (*TBC1D25*/*OATL1*) regulates autophagosomal maturation through direct interaction with LC3 and is also involved in *fusion* of the outer membrane of autophagosomes with lysosomes (24). *Fusion* is additionally regulated by Ras-related protein Rab-7a (*RAB7*) (25) and lysosome-associated membrane glycoprotein 1 (LAMP, *LAMP1*) and 2 (LAMP, *LAMP2*) (26). Ultimately, lysosomal hydrolases degrade the luminal and inner membrane constituents, including most autophagy proteins, and the resulting products are then released into the cytosol by lysosomal efflux permeases (e.g., spinster homolog 1 [*SPNS1*] [27]) for reuse as building blocks or for the generation of energy. Finally, LC3 lipidation is reversed by ATG4, and lysosome homeostasis is restored by autophagic lysosome reformation (ALR) through activation of mTOR (28). Hence, the nutrients generated by autophagy turn the pathway off through negative feedback.

SURVIVAL VERSUS CELL DEATH BY AUTOPHAGY

It is well accepted that autophagy can promote cell survival. By providing energy and building blocks and serving as a disposal system, autophagy helps cells to cope with a wide variety of intracellular and extracellular stresses. However, a growing number of studies suggest that autophagy can also turn into a cell death mechanism. As suggested by others (29), however, caution must be exercised when using the term ''autophagic cell death'', since to date, death by autophagy has not been unequivocally demonstrated. As the pro-survival role of autophagy has been widely accepted, we will briefly discuss the contentious topic of autophagic cell death.

Autophagy associated with cell death

Autophagic cell death is considered to be one of three major cellular mechanisms of programmed cell death, along with apoptosis and necroptosis. Apoptosis is the best characterized, with a variety of hallmarks that have been clearly defined (30). Necroptosis –programmed necrosis– was identified in the last decade (31) and is becoming more clearly understood (32). In contrast, autophagic cell death is not clearly defined. In fact, its existence is questionable and highly dependent on exclusion of other cell death mechanisms (33-35). This has proven difficult due to the high degree of interconnection between programmed cell death mechanisms. For example, ATG3, ATG5, ATG12, Bcl-2 family members, beclin-1, caspase-3 (CASP-3, *CASP3*), caspase-8 (CASP-8, *CASP8*), death-associated protein kinase 1 (*DAPK1*), E2F transcription factor 1 (*E2F1*), CASP8 and FADD-like apoptosis regulator (*CFLAR*), mitogen-activated protein kinase 8 (JNK1, *MAPK8*), nuclear factor NF-kappa-B (*NFKB*), cyclin-dependent kinase inhibitor 1B (p27, *CDKN1B*), cellular tumor antigen p53 (*TP53*), sequestosome-1, retinoblastoma 1 (*RB1*), receptor-interacting serine/threonine-protein kinase 1 (*RIPK1*) and hVps34 have been found to play roles in both autophagy and apoptosis (36-40). In consideration of the overlap between autophagy and apoptosis, autophagic cell death was recently defined as cell death accompanied by: 1) absence of apoptosis markers; 2) increased autophagic flux; and 3) increased viability when autophagy is genetically or pharmacologically inhibited (41). As that definition lacks distinction from necroptosis, ''absence of necroptosis markers'' should be added to the list. To provide evidence that autophagic cell death is distinct from other modes of cell death, it will be necessary to investigate the dependence of the various modes of cell death on several factors: 1) level of autophagy induction; 2) duration of autophagy induction; 3) nature of stimulus; 4) genetic background; and 5) microenvironmental influences. Such investigations may facilitate the identification of biomarkers that clearly distinguish activation of each pathway, thereby enabling further refinement of the definition of autophagic cell death.

As there is still much to learn about the relationships between the various programmed cell death mechanisms, we conclude that it is not yet clear whether autophagic cell death exists, and hence, whether stimulating autophagy would have therapeutic utility. It should be clarified that many experiments have been misinterpreted; the use of autophagy inhibitors (either pharmacological or genetic) to demonstrate that autophagy is necessary to achieve cell death does not equate with demonstration of autophagic cell death. Under such conditions, the mode of cell death may technically be necroptosis. Developing a therapeutic strategy based on stimulation of autophagy will therefore require knowledge of the genetic context in which pharmacological stimulators of autophagy activate programmed cell death and which mode of cell death is being activated. Future studies will also need to address whether multiple cell death mechanisms are concurrently activated within a single cell and/or whether the pathways are mutually exclusive, either temporally or spatially.

AUTOPHAGY AS A TUMOR SUPPRESSOR AND TUMOR SUPPORTER

Autophagy has been proposed to mediate both tumor suppression and tumorigenesis, another seemingly contradictory pair of functions (34, 42). We refer the reader to a recent review by Debnath (43) for an extensive description of the multiple functions of autophagy during tumor initiation and progression. With regard to tumor suppression, studies investigating *ATG* genes and autophagy-associated signaling pathways have identified *ATG4C*, *ATG5*, *ATG7* (44), *BECN1* and the *BECN1* interactors *UVRAG* (45) and *SH3GLB1* as tumor suppressors (46-48). In addition, *PTEN*, *DRAM*, *DAPK*, *TSC1*, *TSC2* and *STK11* (*LKB1*), which are tumor suppressors that positively regulate autophagy, have been found to be lost or inactivated in many cancers (49). Further support for the role of autophagy as a tumor suppressor has been provided by the observation that defective autophagy is associated with genomic damage, which can promote tumor formation (44, 50). Finally, oncogenes such as *AKT*, *BCL2* and *PIK3CA*, which negatively regulate autophagy, are frequently upregulated and mutated in cancer (49). Possible mechanisms of tumor suppression by autophagy include suppression of inflammation (51, 52), mitigation of metabolic stress and genomic damage (53, 54), degradation of sequestosome-1 (55), facilitating oncogene-induced senescence (56), inhibition of necrosis (51), and autophagic cell death, although the latter is controversial, as previously stated. Altogether, these reports suggest that autophagy prevents tumor initiation and is a key feature in the homeostasis of normal tissue.

A role for autophagy in tumorigenesis, on the other hand, can be explained by the emerging concept of ‘‘autophagy addiction’’. Specifically, autophagy may serve as a tumor suppressor in normal cells, but once a tumor is formed, autophagy may be required for progression to an aggressive tumor, as suggested by the observation that aggressive tumors (e.g., those exhibiting oncogenic *RAS* activation) exhibit a high degree of autophagy activation (57, 58). Since cancer cells are exposed to increased cellular and metabolic stress, such as hypoxia (51) and anoikis (59), and since autophagy can prevent cell death by mitigating the effects of such stresses, it seems reasonable to hypothesize that an elevated autophagy rate could be selected during tumorigenesis in order to meet increasing metabolic demand and to tolerate the stress associated with that demand. Hence, the seemingly contradictory functions of autophagy as a tumor suppressor and tumor supporter may not be contradictory after all. Figure 2 illustrates those disparate functions.

AUTOPHAGY MODULATION AS A STRATEGY FOR ANTICANCER THERAPY

Modulation of autophagy in cancer therapy has come sharply into focus over the past couple of years. Armed with our current knowledge of autophagy-regulating molecules, the critical question is, ‘‘Should the anticancer strategy be based on stimulation or inhibition of autophagy?’’ Unfortunately, due to the molecular complexities involved, there is no one-size-fits-all answer; it will likely depend on context. However, the molecular determinants of ‘‘context’’ are still being defined; although autophagy has been a subject of investigation since the mid-1960s, the complex networks that regulate the process have only begun to be elucidated. A recent report described the combined use of protein expression, immunoprecipitation and mass spectrometry to identify an ‘‘autophagy interaction network’’ composed of 409 proteins and 751 interactions (5). However, for the purposes of identifying candidate drug targets, for which it is desirable to have a measure of a gene’s contribution to the autophagy process, siRNA screening represents a powerful alternative strategy. In this regard, only three siRNA screens that employed autophagy-specific endpoints in human cell lines have been reported to date (12, 60, 61), and only one of these was a genome-wide screen (12). Further limiting the utility of the existing data, LC3-II expression has been the only endpoint used; downstream markers of autophagic flux (e.g., sequestosome-1 turnover) have not been employed in siRNA screens. As LC3-II is formed early during autophagy, LC3-based autophagy screens may be limited to elucidation of early steps in the process and incomplete/abortive autophagy, which results in autophagosome accumulation. Hence, the existing siRNA screen data provide a starting point for identifying already-characterized autophagy drug targets, but they are probably quite limited in scope and coverage of the phenomenon.

It is not yet clear whether stimulating autophagy will be a useful anticancer strategy. Such a strategy will first depend on the existence of an autophagy/cell death threshold. Furthermore, successful eradication of cancer cells by stimulation of autophagy will require that the autophagy/cell death threshold be surmountable. For aggressive tumors that are addicted to autophagy and have adapted to survive with high autophagy rates, the autophagy/cell death threshold is likely to have a high set point. A chemotherapy strategy for stimulating autophagy might therefore be better suited for tumors with a low autophagy/cell death threshold. However, the molecular determinants of the autophagy/cell death set point are not yet clear and are likely to involve a variety of factors. For example, the death-inducing signaling complex (DISC), which mediates necroptosis, must be intact for cell death to occur by necroptosis. That complex includes tumor necrosis factor receptor type 1-associated DEATH domain protein (*TRADD*), Fas (TNFRSF6)-associated via death domain (*FADD*), CASP8 and FADD-like apoptosis regulator (*CFLAR*), caspase-8 (*CASP8*), receptor-interacting serine/threonine-protein kinase 1 (*RIPK1*) and receptor-interacting serine/threonine-protein kinase 3 (*RIPK3*) (32, 62).

Stimulation of autophagy has clearer potential in the context of cancer prevention, for which the efficacy of such a strategy would not depend on the existence of an autophagy/cell death threshold. Stimulation of autophagy in normal cells would be expected to repress the initiation of cancer through elimination of damaged proteins and abnormal mitochondria and by preventing accumulation of DNA damage. Despite the seemingly noncontroversial potential of such a strategy, more work will be needed to assess that potential, especially since it is conceivable that stimulation of autophagy may be found to support tumorigenesis by enabling premalignant cells to acquire a pro-survival mechanism and to become addicted to autophagy (63). Rapamycin, a well-known autophagy stimulator (and mTOR inhibitor), would be a model compound to aid in such studies.

Compared with stimulating autophagy, there is much better understanding of the contexts in which inhibiting autophagy holds promise for eradication of cancer cells. Increased levels of autophagy are almost always observed following anticancer treatment and are

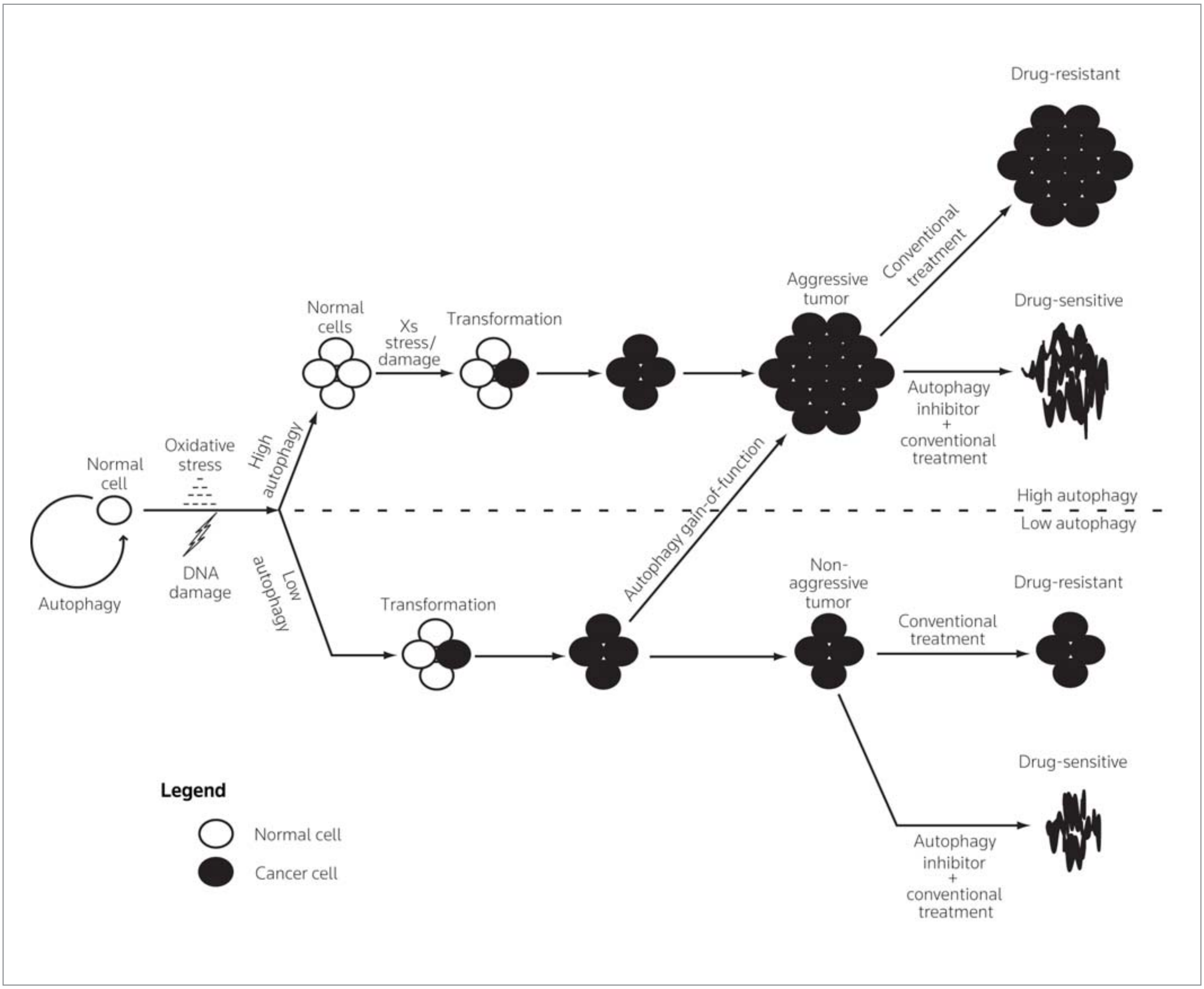

Figure 2. Conceptual schematic of autophagy as a tumor suppressor, tumor supporter and mediator of chemotherapy resistance. In the absence of stress signals, normal cells require autophagy to maintain homeostasis. Stress signals such as DNA damage or reactive oxygen species induce autophagy, which recycles damaged proteins and organelles and prevents transformation. Autophagy therefore serves as a tumor suppressor in normal cells. If the accumulation of damage exceeds autophagic capacity (flux), transformation to a cancer cell can occur. Thereafter, autophagy supports tumorigenesis by supplying recycled building blocks and energy to tumor cells while also clearing damage. Cells that sustain high autophagy or acquire a gain-of-function mutation are likely to progress to an aggressive tumor. A majority of conventional anticancer treatments stimulate autophagy, which imparts drug resistance. Therefore, combination therapies that include an inhibitor are expected to enhance anticancer efficacy.

thought to be associated with drug resistance. Not surprisingly, inhibition of autophagy has been found to augment drug efficacy (64). Toward the goal of achieving clinical efficacy, numerous trials are under way using chloroquine (CQ) or hydrochloroquine (HCQ) as an autophagy inhibitor (65) in combination with a variety of therapeutic agents. Of those trials, the one testing HCQ in combination with the rapamycin analogue temsirolimus is worth particular attention. As single agents, TORC1 inhibitors like temsirolimus have shown limited activity in clinical trials (66), presumably due to induction of autophagy and other pro-survival pathways. Therefore, the combination of a TORC1 inhibitor (e.g., temsirolimus) with an autophagy inhibitor like HCQ might be expected to achieve greater cancer cell death (see Fig. 2). Such a strategy may enable rapamycin (67), its analogues and other autophagy-inducing anticancer agents to reach their full therapeutic potential. It should be acknowledged, however, that chronic suppression of autophagy may stimulate tumorigenesis due, for example, to a buildup of reactive oxygen species-induced damage and genomic instability (68). It will therefore be necessary to optimize the time frame during which autophagy inhibitors are used clinically to minimize potential long-term side effects.

CONCLUSION

Autophagy appears to function as both a pro-survival mechanism and a cell death mechanism. Whether stimulating or inhibiting autophagy will improve patient outcomes will depend on context. The balance between the two mechanisms is a delicate one. Significant challenges remain in elucidating the complicated relationships between autophagy and programmed cell death, but current understanding suggests that inhibition of autophagy may have significant potential as an anticancer strategy, perhaps in combination with inhibition of mTOR.

ACKNOWLEDGMENTS

This work was supported in part by Komen Foundation grants KG 081694 and FAS0703849, Cancer Prevention and Research Institute of Texas, and the Ovarian Cancer Research Fund (GBM); the H.A. and Mary K. Chapman Foundation, The Michael & Susan Dell Foundation (honoring Lorraine Dell), the Ovarian Cancer Research Fund grant PPD/MDACC/01.08 03 (JNW); and U.S. National Cancer Institute (NCI) grant numbers CA143883 and CA083639 (JNW and PLL). Funding to SC as an Odyssey Fellow was supported by the Odyssey Program and the Theodore N. Law Endowment for Scientific Achievement at the University of Texas MD Anderson Cancer Center and NCI Breast SPORE Career Developmental Project award CA116199.

DISCLOSURES

The authors state no conflicts of interest.

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