Oncophagy: harnessing regulation of autophagy in cancer therapy

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Abstract

Autophagy is an increasingly well-characterised process of cell component auto-digestion and recycling thought necessary for cellular subsistence. As we gain a more thorough understanding of the mechanisms underlying autophagy, its relevance to human disease and therapeutic potential are being clarified. This review summarises the evidence implicating autophagy in the pathogenesis and potential treatment of malignant disease. In addition, we explore the molecular role of microRNAs as key regulators in what we propose should now become known as ‘oncophagy’.

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Introduction

Autophagy is a eukaryotic cell process of self-catabolic degradation originally described in the 1960’s (Deter & De Duve 1967). The term autophagy is derived from Greek terminology ‘auto’ (meaning self) and ‘phagy’ (eating) to describe the process of cell component auto-digestion and recycling required for cellular survival. Beyond such origins, dual utility has also been proposed whereby autophagy plays somewhat of a Janus-like role in cell fate (Liu *et al.* 2011) being as important to cell death as it is to survival. Now that we are gaining a more thorough understanding of the mechanisms underlying autophagy, its relevance to human disease and burgeoning therapeutic potential are being unravelled (Ding *et al.* 2008, Chen *et al.* 2009, Li *et al.* 2009, 2012, Chen & White 2011, Gundara *et al.* 2011). Here, we review the potential pathogenic, prognostic and therapeutic roles of autophagy within the context of human malignancy and with a special reference to microRNA-mediated regulation.

Autophagy: the mechanism

Cellular homeostasis is a complex process dependent on close regulation of synthesis and degradation of both structural and functional elements. The

ubiquitin–proteasome pathway is one such mechanism whereby cellular proteins can be broken down and disposed of or recycled. Alternatively, the autophagy–lysosome pathway maintains particular importance in management of longer lived or larger proteins, potentially including entire organelles such as mitochondria (Klionsky & Emr 2000).

Autophagy has been sub-classified into three discrete sub-types (macro-, micro- and chaperone-mediated autophagy), the central theme involving proteolysis of cytosol components at the lysosome (Glick *et al.* 2010). The majority of current scientific interest focuses on macroautophagy and its relation to cellular processes in health and disease (Glick *et al.* 2010). As such, the term autophagy is generally considered to be a reference to macroautophagy specifically (Klionsky *et al.* 2008).

Autophagy commences with the phagophore (an isolation membrane) that originates from the endoplasmic reticulum, golgi apparatus and endosomes. The phagophore consumes intra-cellular, cytoplasmic contents (damaged/dysfunctional DNA, cell organelles and proteins) and sequesters them within the double-membrane autophagosome. This then fuses with the lysosome and lysosomal acid proteases induce degradation of autophagosomal contents (thence known as

the autolysosome). The resulting component nucleotides, fatty acids and amino acids may then be recycled as cellular building blocks or removed as a waste product. Given such a mechanism, autophagy has been known as a ‘recycling factory’, enabling efficient energy production and removal of potentially toxic waste products of metabolic processes (Glick *et al.* 2010). Therein lies the importance of autophagy, which, when defective, can result in a multitude of human diseases (White *et al.* 2010).

Autophagy: regulation

Regulation of autophagy is complex. However, a basic understanding of the key regulatory steps is crucial to an understanding of potential autophagy-based biomarkers and therapies. In their review of autophagy regulation, Glick *et al.* (2010) describe five key points at which regulation is imposed:

1. phagophore formation,
2. Atg5–Atg12 conjugation, interaction with Atg16L and multimerisation at the phagophore,
3. LC3 processing and insertion into the extending phagophore membrane,
4. capture of random or selective targets for degradation,
5. fusion of the autophagosome with the lysosome, followed by proteolytic degradation by lysosomal proteases of engulfed molecules.

Numerous key regulators are involved in maintenance of the pathway from phagophore to autolysosome. Within this regulatory milieu reside *ATGs* or autophagy-related genes and their associated *Atgs* (autophagy gene products). Our understanding of autophagy regulation was relatively superficial until the 1990’s when *ATGs* were discovered and have formed the crux of our understanding of regulatory processes since (Li *et al.* 2012). Beclin-1 was the first *ATG* discovered to be of importance in mammalian autophagy (Liang *et al.* 1999) and there are now over 30 *ATGs* known to be involved. *ATGs* (and their protein products) have subsequently been the target of specific investigation in an attempt to further delineate, and employ, the molecular utility of autophagy (Li *et al.* 2012).

Embedded within the myriad of regulatory processes governing autophagy is mammalian target of rapamycin (mTOR), which itself is part of the class I phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/Akt) signalling pathway. mTOR acts to inhibit autophagy under growth-promoting conditions, and

repression of its activity has been found in states of nutrient deprivation and hypoxia, conditions also associated with autophagy (Yang & Klionsky 2010). More specifically, class I PI3K is activated by growth factor receptors, which then lead to downstream mTOR activation and ultimately autophagy inhibition (Yang & Klionsky 2010). Thus, it can be reasoned that in states of nutrient deprivation, a lack of growth factors will release mTOR inhibitory signalling and result in autophagy induction to recycle cellular constituents and maintain cell viability.

An understanding of the mechanistic pathways involved is of great interest as it opens opportunities for potential therapeutic intervention. The ability to manipulate these complex pathways is, however, dependent on an understanding of the clinical importance of autophagy in disease, and cancer in particular. Beyond a homeostatic role, autophagy has been shown to be of importance in normal development (Kuma *et al.* 2004), and from a clinicopathologic viewpoint, it has been associated with neurodegenerative (Yang & Mao 2010), metabolic (Jung & Lee 2010), infectious (Gutierrez *et al.* 2007), inflammatory (White *et al.* 2010) and malignant diseases (Chen *et al.* 2009, White *et al.* 2010, Li *et al.* 2012).

The harnessing of a self-catabolic process like autophagy against malignancy is a novel concept and is the one that is fast gaining broad scientific and clinical interest (White & DiPaola 2009, Ravikumar *et al.* 2010, White *et al.* 2010, Li *et al.* 2012). The remainder of this review focuses primarily on our current understanding of the role of autophagy in cancer biology and the steps that have already been taken to utilise this process as a potential therapeutic strategy in cancer treatment.

Autophagy: role in oncogenesis and cancer therapy

Currently, conflicting evidence regarding the role of autophagy in tumour biology has led to a likening of this process to a double-edged sword (White & DiPaola 2009). Autophagy has been shown to be both inhibitory and beneficial to the malignant process (Ravikumar *et al.* 2010). Some investigators have suggested that it may suppress early tumour development, serving as an anti-neoplastic, biological defence mechanism in the early stages of oncogenesis (Karantza-Wadsworth *et al.* 2007), while paradoxically supporting tumour cell survival later, once true malignancy has evolved (White *et al.* 2010, Yang & Kimmelman 2011). Enhanced sensitivity of cancer

cells to chemo- and radiotherapy has also been associated with enhanced autophagic flux (Mukubou *et al.* 2010), although discerning objectively what reflects an efficacious therapeutic effect, rather than evidence of collateral cellular stress is a contentious issue (Klionsky *et al.* 2008, Shen *et al.* 2012). Additionally, direct quantification of basal tumour tissue autophagic flux has also proven difficult and has led some investigators to suggest that direct evidence linking reduced autophagic flux to tumorigenesis is still lacking (Chen & Debnath 2010). These observations reinforce the notion that the role of autophagy in oncogenesis is not only highly context dependent (White *et al.* 2010), but it is also still poorly understood.

Autophagic cell death

The association between oncogenesis and autophagy is typified by the finding of several *ATGs* and associated proteins that have been touted as potential tumour suppressors. Beclin-1 (Qu *et al.* 2003), PTEN (Arico *et al.* 2001) and p53 (Feng *et al.* 2005) have been found to be significant in autophagy pathways, with Beclin-1 gene suppression, for example, being linked directly to tumorigenesis (Qu *et al.* 2003). More specifically, reduced Beclin-1 expression has been identified in sporadic human breast cancers (Liang *et al.* 1999), higher grade brain tumours (Miracco *et al.* 2007) and epithelial ovarian cancers (Shen *et al.* 2008). The Beclin-1 genomic locus (chromosome 17q21) has also been shown to be deleted in up to 50% of breast (Saito *et al.* 1993), 75% of ovarian (Russell *et al.* 1990, Saito *et al.* 1993) and 40% of prostate (Gao *et al.* 1995) cancers. Conversely, elevated levels of Beclin-1 expression correlate significantly with disease-free and overall survival in colon (Li *et al.* 2009), hepatocellular (Ding *et al.* 2008) and oesophageal (squamous cell) cancers (Chen *et al.* 2009), and functional experimentation demonstrates that Beclin-1 overexpression can reduce *in vitro* colon (Koneri *et al.* 2007) and cervical (Wang *et al.* 2007) cancer cell proliferation and *in vivo* xenograft tumour growth (Wang *et al.* 2007). This effect is reversed when Beclin-1 is suppressed using RNA interference methods (Wang *et al.* 2007).

It has also been suggested that the tumour suppressor role of PTEN is enacted indirectly through positive regulation of autophagy (Arico *et al.* 2001). This is thought to occur secondary to PTEN inhibition of the PI3K/Akt pathway, as originally demonstrated in a colon cancer cell line by Arico *et al.* (2001). Consistent with this mechanism, heterozygous loss of PTEN in mice has been shown to lead to breast and thyroid

tumours, among others (Kishimoto *et al.* 2003). Additionally, *PTEN* mutations have also been identified in a range of hereditary cancer syndromes (Pezzolesi *et al.* 2008) and aggressive sporadic malignancies, such as castration-resistant prostate cancer (Grasso *et al.* 2012). A similar positive regulatory role in autophagy has also been proven for the commonly implicated tumour suppressor, p53 (Feng *et al.* 2005).

These findings support the theory that ongoing and continual autophagic flux may be tumoricidal and necessary to avoid neoplastic transformation, during the earliest stages of cancer initiation (Shen *et al.* 2008, White *et al.* 2010). However, if autophagy fails to maintain this housekeeper function, neoplasia ensues. This effect has been demonstrated in breast cancer and defines the role of Beclin-1 as a haplo-insufficient tumour suppressor (Karantza-Wadsworth *et al.* 2007). Having demonstrated such an effect, Karantza-Wadsworth *et al.* (2007) concluded that defective autophagy leaves cells susceptible to metabolic stress and DNA damage that ultimately leads to genomic instability and cancer initiation. It may be that many tumour suppressors, not unlike Beclin-1, PTEN or p53, exert their ‘suppressor’ effect through such an autophagic avenue.

In keeping with this theoretical continuum, associations between therapeutic response to cytotoxic agents and elevations in markers of autophagic flux have led some investigators to claim that iatrogenic autophagy induction may be a valid therapeutic strategy, by taking advantage of what was previously labelled ‘autophagic cell death’ (Ullen *et al.* 2010, Abraham *et al.* 2011). Ullen *et al.* (2010), in examining the *in vitro* efficacy of the tyrosine kinase inhibitor, sorafenib on prostate cancer cells, identified a dose-dependent impact on cell viability in parallel with evidence of increased autophagic flux. This led them to suggest that autophagy manipulation (in combination with established therapies) may be a valid approach to treating cancer.

Numerous other malignancies have also been investigated regarding the potential therapeutic harnessing of autophagic cell death. Using a variety of agents, claims of autophagic cell death have been made in a vast range of malignancies including hepatocellular carcinoma (HCC; Wang *et al.* 2010), oestrogen receptor-negative breast cancer (Vanderlaag *et al.* 2010), non-small-cell lung cancer (Li *et al.* 2010b), ovarian cancer (Le *et al.* 2010), pancreatic cancer (Mujumdar *et al.* 2010), gastric cancer (Hashimoto *et al.* 2008), malignant glioma (Takeuchi *et al.* 2005) and papillary thyroid cancer (Lin *et al.* 2009). Many of these investigations demonstrate

an apoptosis-independent therapeutic *in vitro* effect on cellular proliferation in association with elevations in markers of autophagy. In reasoning such results, investigators have previously concluded that autophagic cell death is the mechanism by which therapeutic efficacy is being enacted.

The role of the tyrosine kinase receptor pathway and autophagy has also been highlighted more recently by Xu & Weihua (2011), who demonstrated that EGFR receptor knockdown leads to autophagy induction. However, and in direct contrast to Ullen *et al.*, they concluded that the beneficial effect on prostate cancer cells related to EGFR protein suppression rather than inhibition of tyrosine kinase activity.

Table 1 summarises a broad, representative selection of basic scientific and clinicopathologic literature examining the pathophysiological, prognostic and therapeutic themes of what can now be labelled 'oncophagy' (i.e. cancer-related autophagy).

The alternative hypothesis

One of the lasting problems in identification of what may be considered autophagic cell death is confirming that this is the definitive, lethal process cells are committing to, rather than being an observation of a collateral response to stress that accompanies cellular demise. This is a present-day challenge for autophagy investigators and is a concept that has recently been challenged strongly (Shen *et al.* 2012).

Shen *et al.* (2012) questioned whether or not autophagic cell death exists at all. Following exhaustive experimentation involving treatment of osteosarcoma cells with 1400 different agents, they were unable to identify definitive evidence of autophagic cell death (Shen *et al.* 2011). They concluded that markers of autophagic flux may well be present within the context of experimentation attempting to demonstrate a therapeutic effect, but this does not constitute evidence of cell death 'by' autophagy, but rather, death 'with' autophagy. This is an important point, and one that has led to abandonment of older terminology whereby autophagy was otherwise known as programmed cell death type II (Kroemer *et al.* 2009). Despite this, the possibility of autophagy still being intimately involved in 'lethal signalling' pathways towards cell death is likely, but the precise role it plays requires further investigation and definition.

Given that the case for induced autophagic cell death is becoming less compelling, an alternative hypothesis requires consideration. In keeping with the homeostatic role of autophagy in cellular health, there is

evolving evidence that this may also be a potent strategy employed by tumour cells to ensure survival, thus paving the way for therapeutic manipulation (White & DiPaola 2009). White & DiPaola articulate this concept well by suggesting that tumour cells, with their rapid proliferation and poor blood supply, are some of the most metabolically stressed. This is justified by data showing that tumour tissues generally possess an increased state of autophagic flux (Degenhardt *et al.* 2006), therefore supporting the theory of a heightened reliance on mechanisms such as autophagy for survival and ultimately enhanced levels of stress tolerance when compared to cells in health (White & DiPaola 2009). Suppressing or removing this tumour cell survival mechanism would thus seem an attractive therapeutic manoeuvre.

The at times conflicting, two faced nature of autophagy involvement in oncogenesis is exemplified by the tumour suppressor gene *ARHI* (DIRAS3) (Lu *et al.* 2008). In attempting to explore *ARHI* gene suppression in ovarian cancer, Lu *et al.* induced re-expression of *ARHI* in a cell culture model and demonstrated cell death in association with autophagy induction. When cells were xenotransplanted into a mouse model without *ARHI* induction, tumours predictably grew, and when *ARHI* was induced tumour growth was inhibited, as expected. When *ARHI* induction was withdrawn after 30 days, tumours began to grow promptly indicating that xenografted cells had remained dormant but still viable and capable of proliferation. Additional experimentation with autophagy blockade (using chloroquine) led to retarded tumour growth following *ARHI* induction withdrawal. Cells were again viable but apparently dormant. The authors suggest that this is indicative of the establishment of an adaptive dormancy state that is dependent on *ARHI*-mediated autophagy. Lu *et al.* concluded that autophagy, while maintaining an efficacious tumoricidal effect *in vitro*, seemingly appears to aid maintenance of tumour cell dormancy in the *in vivo* model, thus implicating it as a tumour cell survival strategy. This provided some of the first evidence of therapeutic benefit when employing autophagy blockade, rather than induction, in a model of established malignancy.

A role for autophagic tumour cell survival has also been investigated in HCC cell lines and xenografts (Ding *et al.* 2011). Ding *et al.* treated HCC cell lines with oxaliplatin, a known chemotherapeutic agent efficacious in HCC. Treated cells and mouse model xenografts demonstrated evidence of autophagy induction in association with cell death and tumour

regression. However, when treatment was supplemented with autophagy blockade (using pharmacological and small interfering RNA methods), oxaliplatin treatment was potentiated with an increase in cell death and tumour regression being observed. These results suggest that autophagy may well be an adaptive tumour cell survival mechanism rather than promoting cell death. Similar findings have been reported by Li *et al.* (2010a) when examining the increased effect of 5-FU on colon cancer cell lines and xenografts with autophagy blockade (employing 3-methyladenine and siRNA to Atg7). Interestingly, this was also observed in association with an increase in flux through the apoptotic pathway, implying a degree of crosstalk between these two pathways of cell fate, a link that has been suggested previously (Shen *et al.* 2011). In exploring the cross talk between apoptosis, autophagy and necrosis, Shen *et al.* (2012) also showed enhanced cellular toxicity during treatment with autophagy blockade (using siRNA to *ATG5* and *ATG7*) to further reinforce the theory of autophagy being a cytoprotective mechanism. The alternative hypothesis therefore follows: namely that if this cytoprotective mechanism is disabled, tumour cells are vulnerable to toxic insults and death.

This novel theory has been expanded on both in the laboratory and also clinically through the commencement of several clinical trials that are attempting to exploit pharmacologic autophagy blockade in combination with recognised chemotherapeutics for cancer. Adjuvant autophagy blockade has been shown to be therapeutic during *in vitro* studies investigating various treatments for myeloid leukaemia (Bellodi *et al.* 2009), colon cancer (Carew *et al.* 2010), glioma (Lomonaco *et al.* 2009) and breast cancer (Vazquez-Martin *et al.* 2009). In addition, and while it is beyond the scope of this review to go into detail, it is important to note that such discoveries are now being translated into several phase II trials that are underway and are employing chloroquine as the autophagy blocking agent of choice. Efficacy in a number of cancer types is being explored, ranging from haematological malignancies to solid adult tumours, prostate and lung cancer (White & DiPaola 2009). The specifics of these trials are summarised in White & DiPaola (2009).

Thus, it would appear that there are a number of important factors within the realm of experimental therapeutic oncophagy that investigators should be aware of. The cell, tissue or tumour type; stage of malignant transformation and nature of treatment employed are important contextual factors of significance. The White *et al.* group focus on these concepts

in their eloquent reviews of autophagy in inflammation and cancer (White & DiPaola 2009, White *et al.* 2010). They also promote the ‘double-edged’ nature of autophagy by suggesting that autophagy generally promotes cellular survival and an anti-neoplastic housekeeping function that, when defective, is potentially carcinogenic. More specifically, they cite the inability of an impaired autophagic process to clear damaged proteins and organelles, leaving a toxic stimulus within the cellular milieu that may induce genetic instability and ultimately malignant transformation. They go on to suggest that while autophagy blockade may be therapeutically useful in managing progression of malignant disease, it may also be logical and advantageous to induce autophagy as a protective strategy in the early stages of pre-malignant transformation. This could even be considered in a prophylactic context.

The supposed paradoxical nature of autophagy manipulation may therefore be a redundant concept, or rather, it should be recognised that autophagy is a dynamic biological process. Autophagy induction is prophylactic before malignancy evolves, and blockade is therapeutic for established disease, which may well be the mantra of oncophagy as we continue exploration in an attempt to harness this normal physiological process for management of a highly abnormal one.

Novel therapeutic approaches: the autophagamiR

While the genetic and molecular basis of autophagy is now coming under increasing scrutiny, there remains a dearth of knowledge regarding the influence of microRNA's (miRNAs). miRNAs are short (~22nt) non-coding RNAs that affect regulation at a post-transcriptional level through target mRNA suppression (Shenouda & Alahari 2009). This is achieved through recognition of the miRNA response element present in the 3' UTR domain of the target mRNA. Given the exponential increase in interest regarding miRNA profiles of malignant diseases (and associated mRNA targets), the question regarding miRNA regulation of autophagy within the cancer context requires clarification (Gundara *et al.* 2011). More specifically, the negative regulatory nature of miRNA function is especially attractive within the domain of oncophagy, given increasing interest in strategies employing blockade of cytoprotective, autophagic flux.

One of the earliest studies considering this issue demonstrated a role for miR-30a targeting of Beclin-1 and subsequently autophagic flux in breast, brain and

Table 1 Evidence implicating autophagy in the malignant pathogenesis and prognosis of various cancers

Cancer	Study focus	Methods	Conclusions	Reference
Pancreatic	Therapy	Pancreatic cancer cell lines treated with gemcitabine and ionising radiation; +/- autophagy blockade	Treatment associated with cell death and autophagy induction; effect abolished with autophagy blockade	Mukubou <i>et al.</i> (2010)
Pancreatic	Therapy	Pancreatic cancer cell lines treated with triptolide and downstream cell death assays performed +/- autophagy blockade (using siRNA to Atg5 + Beclin-1)	Treatment associated with cell death and autophagy induction in three metastatic cell lines; autophagy blockade → effect abolished, conclude that autophagy is an important mechanism of triptolide-induced cell death	Mujumdar <i>et al.</i> (2010)
Lung hepatocellular lymphoma	Oncogenesis	Monoclonal deletion of Beclin-1 in pre-malignant mouse model	Reduced autophagic flux and spontaneous <i>in vivo</i> tumour development following Beclin-1 deletion conclude that Beclin-1 is a key regulator of cell growth and tumour suppression	Qu <i>et al.</i> (2003)
Colon	Prognosis	Beclin-1 IHC on stage IIIB tumours (n=115)	Beclin-1 expression correlated positively with improved survival	Li <i>et al.</i> (2009)
Colon	Oncogenesis therapy	Quantified Beclin-1 expression in four different colon cancer cell lines, transfected low expressing cell line (HT29 cells) with Beclin-1 and observed cell proliferation and performed cell cycle analysis	Beclin-1 expression variable in cell lines; Beclin-1 transfection led to growth inhibition and cell cycle arrest (at G1 phase) in colon cancer cells	Koneri <i>et al.</i> (2007)
Colon	Oncogenesis therapy	Autophagy blockade using siRNA and 3-MA with 5-FU treatment of colon cancer cell lines + mouse xenografts	Autophagy blockade enhances therapeutic efficacy of <i>in vitro</i> + <i>in vivo</i> 5-FU with increased cell death and increased apoptosis concludes that autophagy is a tumour cell survival strategy	Li <i>et al.</i> (2010a,b)
Breast	Oncogenesis	Determination of Beclin-1 protein expression in sporadic human breast cancers; mouse model xenograft of Beclin-1 knockout cell line	Reduced Beclin-1 expression in human breast carcinomas and cell lines; enhanced cellular proliferation in Beclin-1 knockout xenograft tumours	Liang <i>et al.</i> (1999)
Breast	Oncogenesis	Beclin-1 knockout mouse mammary epithelial cells subjected to metabolic stress <i>in vitro</i> and <i>in vivo</i>	Allelic Beclin-1 loss leads to <i>in vivo</i> mammary cell tumorigenesis and drug resistance conclude that defective autophagy allows DNA damage, genomic instability and cancer cell progression	Karantza-Wadsworth <i>et al.</i> (2007)
Breast	Therapy	Treatment of invasive oestrogen receptor-negative breast cancer cell lines (MDA-MB-231 + MDA-MB-453) + mouse model cell line xenografts with a phytochemical derivative; effect on cell proliferation, apoptosis, necrosis and autophagy examined	Apoptosis-independent reductions in cellular proliferation; in association with elevated lysosomal activity, GFP-LC3 vacuole staining, LC3B II + Beclin-1 protein expression; increased Beclin-1 expression in treated mouse model xenografts; conclude that phytochemical treatment induces cell death partly through autophagy induction in oestrogen receptor-negative breast cancer cell lines	Vanderlaag <i>et al.</i> (2010)
Hepatocellular	Prognosis	Determination of Beclin-1 gene (n=44) and IHC expression (n=300) in human HCC tumours and HCC cell lines	Reduced Beclin-1 expression in tumours and cell lines; reduced autophagic flux in HCC cell line vs normal; reduced Beclin-1 expression correlated with recurrent disease and reduced survival	Ding <i>et al.</i> (2008)

Table 1 continued

Cancer	Study focus	Methods	Conclusions	Reference
Hepatocellular	Therapy	Treatment of HCC cell line (HepG2 and MHCC97-L) with berberine with and without autophagy blockade (using 3-MA or Atg5 siRNA); effect on cell viability, apoptosis + autophagic flux examined	Cell death with berberine treatment in association with increases in both apoptosis + autophagy; effect diminished with autophagy blockade → suggests a role for berberine autophagic cell death; potentially mediated through Beclin-1 activation +/or mTOR inhibition	Wang <i>et al.</i> (2010)
Hepatocellular	Therapy	Determination of cell/tumour death and autophagy induction following oxaliplatin treatment of HCC cell lines and mouse model xenografts; effect of autophagy blockade (pharmacological/siRNA) examined	Cell death and tumour regression in association with autophagy induction observed post oxaliplatin treatment; oxaliplatin efficacy enhanced with autophagy blockade suggesting that autophagy is a tumour survival strategy	Ding <i>et al.</i> (2011)
Oesophageal (SCC)	Prognosis	Beclin-1 IHC on human oesophageal (SCC) tumours ($n=54$)	Beclin-1 expression correlates positively with improved survival	Chen <i>et al.</i> (2009)
Brain	Therapy	Rapamycin treatment of malignant glioma cell lines (U87MG, T98G and U373MG); effect on cellular proliferation, autophagy, mTOR and PI3K/Akt pathways examined	Rapamycin induced cell death with no evidence of increased apoptosis or elevated autophagic flux through proven mTOR inhibition; effect enhanced through PI3K/Akt signalling pathway inhibitors; conclude that rapamycin induces autophagic cell death in gliomas, an effect that may be enhanced through additional PI3K/Akt inhibition	Takeuchi <i>et al.</i> (2005)
Brain	Oncogenesis	Determination of Beclin-1 protein ($n=212$) and mRNA expression ($n=94$) in human brain tumours	Reduced Beclin-1 protein expression in high-grade astrocytic, ependymal neoplasms and atypical meningiomas; mRNA was reduced in higher grade glioblastomas, astrocytomas, meningiomas and in all glial tumours vs meningiomas	Miracco <i>et al.</i> (2007)
Adrenal	Therapy	Determination of Beclin-1 protein expression post-rosiglitazone treatment of an ACC cell line	Reduced cell proliferation post-treatment with rosiglitazone in association with increased Beclin-1 expression	Cerquetti <i>et al.</i> (2011)
Lung	Therapy	Treatment of non-small-cell lung cancer cell line (A549) with tephrosin; effect on cell viability, apoptosis + autophagy examined	Dose-dependent reduction in cell viability and cell cycle (G2/M) arrest independent of markers of apoptosis; elevations in lysosomal activity and LC3B II expression; conclude that autophagic cell death is induced by tephrosin	Li <i>et al.</i> (2010a,b)
Ovarian	Therapy	Dasatinib treatment of ovarian Ca cell lines (HEY+SKOV3) and mouse model xenografts; effect on cellular proliferation + markers of autophagy examined	Dasatinib suppresses cellular proliferation with little evidence of increased apoptosis; elevations in lysosomal activity, GFP-LC3 vacuoles, LC3B II expression + reduced expression/activity of AKT, Bcl-2, mTOR; effect diminished with Beclin-1 and Atg12 knockdown; conclude that dasatinib induces autophagic cell death	Le <i>et al.</i> (2010)

continued

Table 1 continued

Cancer	Study focus	Methods	Conclusions	Reference
Ovarian	Oncogenesis therapy	Transient re-expression of <i>ARHI</i> in <i>vitro</i> (ovarian cancer cell line) and <i>in vivo</i> with cell line xenografts +/- autophagy blockade	Autophagic cell death post- <i>ARHI</i> re-expression <i>in vitro</i> ; induction of dormant tumour cell survival <i>in vivo</i> (?protective strategy)	Lu et al. (2008)
Ovarian	Oncogenesis prognosis	Determination of Beclin-1 and LC3 protein expression in malignant epithelial ovarian cancers vs benign tumours	Reduced Beclin-1 and LC3 expression in ovarian cancers; association between reduced expression and grade/stage of disease; authors propose that reduced autophagic flux may lead to oncogenesis	Shen et al. (2008)
Cervical	Oncogenesis	Beclin-1 silencing using siRNA <i>in vitro</i> (HeLa cervical cancer cells) and <i>in vivo</i> (nude mice); cell proliferation and cell cycle analysis performed	Beclin-1 silencing induced cellular proliferation and <i>in vivo</i> tumour growth of cervical cancer cell line; Beclin-1 over-expression inhibited cell proliferation and <i>in vivo</i> tumour growth; conclude that defective autophagy or apoptosis may be critical to tumorigenesis	Wang et al. (2007)
Prostate	Oncogenesis therapy	Knockdown of EGFR expression in two hormone-resistant prostate cancer cell lines (DU145 and PC3); effect on autophagy and cell response to adriamycin observed	EGFR knockdown induces autophagy <i>in vitro</i> ; loss of EGFR knockdown-related autophagy enhances the therapeutic response to adriamycin	Xu & Weihua (2011)
Prostate	Oncogenesis	Homozygous deletion of SIRT1 in mouse model of prostate cancer; effect on tumour growth and autophagy flux quantified	SIRT1 expression induces autophagy; homozygous deletion in a mouse model reduces autophagic flux in association with prostate intraepithelial neoplasia	Powell et al. (2011)
Prostate	Oncogenesis prognosis	Examined prostate cancers for ATG5 expression using IHC (<i>n</i> =107); correlated expression with clinical outcome	ATG5 IHC positivity found in 100% of prostate intraepithelial neoplasia and 90% of cancers; expression did not correlate with tumour size, Gleason grade or stage of disease	Kim et al. (2011)
Prostate	Therapy	Sorafenib treatment of prostate cancer cell lines (DU145 and PC3); effect on cell proliferation and autophagic flux examined	Sorafenib treatment reduced cellular proliferation in a dose-dependent fashion in association with an increase in markers of autophagy; authors conclude that autophagy induction may be a therapeutic strategy in cancer	Ullen et al. (2010)
Osteosarcoma	Therapy	Treatment of U2OS cells with 1400 different agents and autophagic flux examined (using GFP-LC3); ATG5+7 knockdown followed by treatment with 80 different autophagy 'inducers'	Evidence of true autophagic flux with 59 different agents but no evidence of 'autophagic cell death'; ATG5+7 knockdown followed by treatment with autophagy inducers reduced GFP-LC3 puncta formation in association with enhanced cell death → suggesting that autophagy inhibition accelerates cell death; conclude that 'autophagic cell death' does not exist and autophagy inhibition may be a therapeutic strategy	Shen et al. (2011)

continued

Table 1 continued

Cancer	Study focus	Methods	Conclusions	Reference
Gastric	Therapy	Rapamycin treatment of a gastric cancer cell line (NUGC4); effect on cellular proliferation + autophagy examined	Reduced cellular proliferation with rapamycin treatment in association with elevated markers of autophagy; effect mediated through mTOR inhibition; conclude that rapamycin → autophagic cell death	Hashimoto <i>et al.</i> (2008)
Thyroid (papillary)	Therapy	Papillary thyroid cancer cell lines (TPC1 + 8505C) treated with doxorubicin or radiation +/- autophagy blockade (using 3-MA)	Cell death with both treatments in association with elevated LC3B II expression; effect diminished with autophagy blockade; conclude that autophagy inhibition promotes treatment resistance; enhancing autophagy may be a valid therapeutic strategy	Lin <i>et al.</i> (2009)

ACC, adrenocortical cancer; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; LC3, microtubule-associated light chain; mTOR, mammalian target of rapamycin; MTC, medullary thyroid carcinoma; SCC, squamous cell carcinoma; 3-MA, 3 methyladenine.

lung cancer cell lines (Zhu *et al.* 2009). Functional experimentation showed that miR-30a negatively regulated Beclin-1 mRNA and protein expression and subsequently inhibited autophagic flux. This effect was maintained despite attempts to induce autophagy with rapamycin. Unfortunately, the downstream influence on cellular proliferation was not examined in this study, making it difficult to comment further regarding the theory of autophagy blockade as a therapeutic strategy. However, Zou *et al.* (2012) have recently confirmed this hypothesis by showing that miR-30a inhibition of Beclin-1 can enhance the efficacy of platinum-based chemotherapy *in vitro*, effectively sensitising cancer cells to therapy through active inhibition of autophagy.

Subsequent to the original studies of Zhu *et al.*, Frankel *et al.* (2011) have demonstrated that miR-101 negatively regulates autophagy in MCF-7, breast cancer cells. Overexpression of miR-101 was shown to inhibit autophagy, and when knocked down, autophagy induction was observed. Importantly, Frankel *et al.* were also able to identify several miR-101 targets. This was achieved through an mRNA array study, 48 h post-miR-101 transfection of MCF-7 cells. Following filtering, normalisation, statistical analyses and qPCR validation, 14 potential targets were identified. Those deemed to be of interest within the context of miRNA regulation of autophagy (*STMN1*, *RAB5A* and *ATG4D*) were then knocked down using siRNAs in an attempt to mimic the effect of miR-101 on MCF-7 cells. As hypothesised, knock-down of gene targets inhibited basal levels of autophagy and also blocked rapamycin-induced autophagy to a similar extent to that induced by miR-101 transfection. These studies reinforce the therapeutic benefit of miRNA-mediated inhibition of autophagy.

miR-375 has also been shown to regulate the response to hypoxic stress-induced autophagy (Chang *et al.* 2012). Using HCC cell lines and mouse model xenografts, Chang *et al.* demonstrated that hypoxia induced evidence of autophagic flux that was abolished with transfection of a miRNA shown to be down-regulated in HCC cell lines (i.e. miR-375). The ability of cancer cells to employ autophagy as a tumour cell survival strategy was similarly compromised following miR-375 transfection and numbers of viable cells were reduced accordingly. Additionally, a predicted miR-375 target, *ATG7*, was validated through luciferase reporter experimentation.

Similar findings that support the importance of what are now known as autophagamiRs (Gundara *et al.* 2011) have been reported more recently, further

Table 2 Evidence implicating miRNA regulation of autophagy in the malignant pathogenesis and prognosis of various cancers

Cancer	Study focus	Methods	Conclusions	Reference
Breast	Oncogenesis	miRNA array following autophagy induction (rapamycin + HBSS) in breast (MDA-MB-468), brain (T98G) and lung (H1299) cancer cell lines; validation of purported miRNA targets with qPCR + luciferase reporter assay	miR30a differential expression with autophagy induction; predicted Beclin-1 target validated with functional experiments; miR-30a negatively regulates Beclin-1 expression and autophagic flux	Zhu et al. (2009)
Brain Lung Lymphoma (B cell)	Oncogenesis therapy	RNA extracted from lymphoma cell lines (BCWM1, WMWSU, MEC1, RL) + microarray performed; transfection of under + overexpressed miRNAs then performed; effect on miR-target histone acetylation, cell proliferation + death mechanisms examined	Array identified significant under + overexpression of miR-9* + miR-206 respectively; pre-miR-9* cell line transfection → cell cycle arrest, reduced DNA synthesis, reduced proliferation, increased apoptosis and elevated LC3B protein expression; conclude that miR-9* transfection reduces cell proliferation possibly through autophagic cell death	Roccaro et al. (2010)
Thyroid (medullary)	Therapy	Determination of LC3B protein expression post-anti-miR-183 treatment of MTC cell line (TT cells)	Reduced cell proliferation post-treatment with anti-miR-183 in association with altered LC3B expression and no change in markers of apoptosis	Abraham et al. (2011)
Chronic lymphocytic leukaemia	Oncogenesis therapy	miR-130a transfection of CLL cell line	Autophagy inhibition + reduced cell survival with miR-130a transfection; effect mediated by validated miR-130a gene targets: <i>ATG2B</i> + <i>DICER1</i>	Kovaleva et al. (2012)
Chronic myeloid leukaemia	Therapy	Quantification of K562 cell line and primary CML cell miR-30a expression; functional experimentation using a variety of combinatory treatment with imatinib +/- miR-30a/anti-miR-30a and knockdown of Beclin-1 and Atg5	Imatinib therapy of CML cell lines induces autophagic flux + suppresses miR-30a expression; miR-30a transfection specifically reduces Beclin-1/Atg5 expression and inhibits autophagic flux; miR-30a mimic or Beclin-1/Atg5 knockdown → enhanced imatinib-mediated cytotoxicity and elevated apoptosis; anti-miR-30a → increased autophagic flux and reduces the effect of imatinib; conclude that miR-30a dysregulation (and effect on autophagic flux) may contribute to the efficacy of imatinib-mediated apoptosis	Yu et al. (2012)
Hepatocellular carcinoma	Oncogenesis	Functional miR-376b experimentation using MCF-7 + Huh-7 cells with autophagy induction (starvation + rapamycin)	miR-376b expression inhibits induced autophagy; evidence of effect mediation through validated targets: <i>ATG4C</i> + <i>BECN1</i>	Korkmaz et al. (2012)
Hepatocellular carcinoma	Oncogenesis therapy	Determination of cell/tumour death and autophagy induction following hypoxic treatment of HCC cell lines and mouse model xenografts; effect of miR-375 transfection on cell viability + autophagy examined	miR-375 is down-regulated in HCC tissue and cell lines; miR-375 transfection → negatively regulates Atg7, reduces autophagic flux and slows HCC cell line proliferation under hypoxic conditions	Chang et al. (2012)

Table 2 continued

Cancer	Study focus	Methods	Conclusions	Reference
Colon cancer	Oncogenesis therapy	Functional cell line + xenograft studies employing miR-502 transfection; a number of downstream assays performed	miR-502 underexpressed in tumour vs normal colon tissue; miR-502 transfection reduces <i>in vitro</i> cell/ <i>in vivo</i> cell/tumour growth, induces G1/2 cell cycle arrest and inhibits autophagy through targeting <i>Rab1B</i> ; <i>Rab1B</i> also found to be overexpressed in tumour vs normal colon	Zhai <i>et al.</i> (2012)
Head+neck (SCC)	Oncogenesis therapy	Treated an SCC cell line (JHU029) with cisplatin	Cisplatin exposure → p-ΔNp63α-dependent changes in Atg1/ULK1, Atg3, Atg4A, Atg5, Atg6/Becn1, Atg7, Atg9A, Atg10; p-ΔNp63α miRNA targets (miR-181a, miR-519a, miR-374a, miR-630) modulate autophagy (ATG5, ATG6/BECN1, ATG10, ATG12, ATG16L1, UVRAG) protein levels; reveals miR regulated autophagy related mechanisms of chemotherapy resistance	Huang <i>et al.</i> (2012)
Breast	Therapy	Cell line + xenograft treatment with platinum-based chemotherapy; effect on autophagy and miRNA expression examined; subsequent miR-30a transfection of a breast cancer cell line (MCF-7); effect on cell viability assessed	Chemotherapy treatment of cell lines → autophagic flux in association with reduced miR-30a expression; transfection of miR-30a → reduced autophagic flux + improved treatment response; concluded that miR-30a inhibition of autophagy can sensitise tumour cells to chemotherapy	Zou <i>et al.</i> (2012)

validating this concept. Roles for miR-130a and miR-143 have been demonstrated in chronic lymphocytic leukaemia, with autophagy inhibition shown to be affected through repression of validated miR-130a gene targets, *ATG2B* and *DICER1* (Kovaleva *et al.* 2012). miR-376b has also been shown to inhibit autophagy through *ATG4C* and *BECN1* repression in both breast cancer (MCF-7) and hepatoma (Huh-7) cell lines (Korkmaz *et al.* 2012). Comparable results have been reproduced by other groups using miR-181a, a result facilitated through *ATG5* inhibition of a cisplatin-resistant squamous cell carcinoma cell line (Huang *et al.* 2012). Furthermore, colon cancer miR-502 under-expression, when ‘replaced’ using both *in vitro* and *in vivo* models, has shown evidence of autophagy inhibition and cell death through *Rab1B* repression (shown to be overexpressed in human colon cancer tissue samples) (Zhai *et al.* 2012). Lastly, a role for miR-9* regulation of autophagy has also been reported as one mechanism for cell toxicity in a cell culture model of Waldenström macroglobulinaemia; a form of B-cell lymphoma (Roccaro *et al.* 2010).

Huang *et al.*, while demonstrating the link between miR-181a and *ATG5*, were also able to identify the role of autophagamiR in drug resistance. More specifically, they showed that exposure of a squamous cell carcinoma cells to cisplatin resulted in altered expression of a number of markers of autophagic flux, an effect mediated by p-ΔNp63α-dependent transcriptional regulation. Additionally, miR-181a was also shown to be a downstream p-ΔNp63α effector target. Taken together, these results have revealed some of the first evidence of autophagamiR-mediated drug resistance mechanisms, thus enabling further work geared towards manipulation of these evolving treatment resistance strategies.

Similarly, the studies of Zou *et al.* have also showed that platinum-based chemotherapy leads not only to evidence of autophagic flux but also to reduced miR-30a tumour expression. Forced expression of miR-30a in cell culture and xenograft-treated cells subsequently resulted in enhanced treatment response, in association with reduced autophagic flux (Zou *et al.* 2012). Similar themes have been explored by Yu *et al.* (2012) in chronic myeloid leukaemia, whereby a role for miR-30a-induced autophagic repression in enhancing the response to imatinib therapy (an autophagy inducing insult) has also been proven. These findings reaffirm suggestions of tumour cell drug resistance mechanisms being reliant on autophagy, which, when manipulated through an improved understanding of miRNA regulation, results in enhanced treatment sensitivity.

Clearly then, investigation of miRNAs that govern autophagy is well and truly in evolution and the present body of evidence is in favour of miRNA inhibition of autophagic flux as the mechanism of therapeutic benefit. Not only is autophagy an important process within tumour biology but miRNAs are now becoming important players in regulation of what may ultimately be shown to be a fundamental tumour cell survival strategy and one that may even be of importance in acquired drug resistance. The translational impact of these findings is now more obvious than ever and miRNAs may represent the key to clinical application of the principles governing oncophagy. A summary of studies focusing on investigation of the autophagamiR in cancer is detailed in Table 2.

Conclusion

Our philosophical interpretation of the aforementioned findings is of great significance. Focussing too heavily on the inner conflict regarding whether autophagy is pro- or anti-tumour is to lose sight of the ultimate objective, namely, harnessing therapeutic potential regardless of the specific mechanism or method employed. An alternative viewpoint would maintain that these results do not provide contradictory evidence, but rather unveil the theme of manipulating autophagy dynamics within context, meaning that the self-catabolic process should not necessarily be inextricably linked to cell death, but rather be seen as a process that is intrinsic to the cell's response to any foreign (or native) insult. Autophagy induction has been associated with therapeutic benefit and this may well prove to be an effective strategy, but we must also take advantage of a context in which autophagy is cytoprotective and employ blockade as an alternative treatment strategy. The negative regulatory nature of miRNAs makes them a logical and important candidate for blockade initiation. The over-riding challenge, however, lies in identifying which approach to employ, and when.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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