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REVIEW

MicroRNA regulation of autophagy

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Macroautophagy (hereafter referred to as autophagy) is a tightly regulated intracellular catabolic pathway involving the lysosomal degradation of cytoplasmic organelles and proteins. Central to this process is the formation of the autophagosome, a double membrane–bound vesicle, which is responsible for the delivery of cytoplasmic cargo to the lysosomes. Autophagy levels are constantly changing, allowing adaptation to both immediate and long-term needs of the cell, underlining why tight control of this process is essential in order to prevent the development of pathological disorders. Substantial progress has recently contributed to our understanding of the molecular mechanisms of the autophagy machinery, yet several gaps remain in our knowledge of this process. The discovery of microRNAs (miRNAs) established a new paradigm of post-transcriptional gene regulation and during the past decade these small non-coding RNAs have been closely linked to virtually all known fundamental biological pathways. Deregulation of miRNAs can contribute to the development of human diseases, including cancer, where they can function as bona fide oncogenes or tumor suppressors. In this review, we highlight recent advances linking miRNAs to regulation of the autophagy pathway. This regulation occurs both through specific core pathway components as well as through less well-defined mechanisms. Although this field is still in its infancy, we are beginning to understand the potential implications of these initial findings, both from a pathological perspective, but also from a therapeutic view, where miRNAs can be harnessed experimentally to alter autophagy levels in human tumors, affecting parameters such as tumor survival and treatment sensitivity.

Introduction

Autophagy is an evolutionarily conserved cellular catabolic process in which proteins and organelles are eliminated through delivery to lysosomes (1). During autophagy, parts of the cytoplasm are sequestered into characteristic double-membrane vesicles, autophagosomes, which subsequently fuse with late endosomes or lysosomes, forming the autolysosome. Exposure of the inner compartment to lysosomal hydrolases causes degradation of the cytoplasmic cargo and the resulting degradation products are then released into the cytosol for recycling (1).

The recycling function of autophagy has varying impact on cellular physiology depending on the circumstances. Basal autophagy is essential for maintaining cellular homeostasis and quality control through the elimination of damaged/old organelles, proteins and protein aggregates (2). Autophagy is also acutely induced in response to cellular stresses including nutrient starvation, pathogen infection, hypoxia and anticancer drug treatment, where it provides a cytoprotective response resulting in cellular adaptation and survival. Autophagy, therefore, serves as a natural and essential defense mechanism against inflammatory, infectious, neurodegenerative and neoplastic disorders, and deregulation of this pathway has been implicated in the pathogenesis of numerous human diseases (2). This underlines why tight control of autophagy is essential and recent advances in this research field have begun to unveil the molecular mechanisms of autophagy regulation. Within the past decade, genetic screens in yeast have identified a large family of core autophagy regulators, the AuTophaGy (ATG)-related genes, many of which have known orthologs in mammalian cells, that serve to coordinately regulate the stepwise progression of this degradation pathway (3,4). In addition, a diverse and complex network of upstream signaling pathways contribute to autophagy regulation including the phosphatidylinositol 3 kinase (PI3K), RAS and AMP-activated protein kinase (AMPK) pathways, many of which converge at the mammalian target of rapamycin complex 1 (mTORC1), a key negative regulator of autophagy signaling (1,5).

miRNAs are a class of endogenously expressed, short non-coding RNAs, which post-transcriptionally regulate gene expression. They guide the binding of the RNA-induced silencing complex to regions of partial complementarity located mainly within 3′untranslated regions (UTRs) of target messenger RNA (mRNA) molecules, resulting in mRNA degradation and/or translational inhibition (6). Importantly, miRNAs play a critical role in a broad range of biological processes including proliferation, differentiation, apoptosis and stress response, linking them to numerous human diseases including cancer (7). There is ample evidence that miRNAs are differentially expressed in human cancers, where they can function as both tumor suppressors and oncogenes, and their regulation of tumorigenesis spans from initiation and progression to metastasis and treatment sensitivity. Importantly, single miRNAs can simultaneously regulate a multitude of targets and biological networks, providing a clear advantage from a clinical viewpoint, and extensive research is now focused on exploiting miRNA-based treatment strategies for cancer therapy (7,8).

In this review, we will focus on recent findings from the last 4 years, which have implicated novel roles for miRNAs in the regulation of the autophagy process. In particular, we will focus on miRNAs with direct implications in autophagy, either through known core components of the autophagy machinery or through less well-characterized mechanisms. Links between miRNAs and upstream autophagy signaling pathways, including Bcl-2, p53, AMPK and PI3K/PTEN signaling axes, which are complicated by their involvement in multiple other cellular functions, are not discussed here.

The physiological importance of the miRNA-autophagy interconnection is only beginning to be elucidated; however, considering the widespread importance of both miRNAs and autophagy in stress response, adaptation and in the development of human disease, it will be intriguing to further understand these interactions in the coming years. Importantly, many of these findings present promising possibilities for future treatment strategies.

miRNAs impacting the core autophagy pathway

The core pathway of mammalian autophagy (Figure 1) begins with the formation of the isolation membrane (also called the phagophore) and comprises at least five defined molecular steps including (i) autophagy induction, regulated by the ATG1/unc51-like kinase (ULK) complex; (ii) vesicle nucleation, mainly involving the Beclin-1/class III PI3K complex; (iii) vesicle elongation, coordinately controlled by two ubiquitin-like conjugation systems (ATG12 and ATG8/LC3); (iv) retrieval, a poorly defined event in mammalian cells in which the transmembrane ATG9 and associated proteins provide lipids and recruit other ATGs to the phagophore site; (v) fusion between

Abbreviations: AMPK, AMP-activated protein kinase; ATG, AuTophaGy; HCC, hepatocellular carcinoma; miRNAs, microRNAs; mRNA, messenger RNA; mTORC1, mammalian target of rapamycin complex 1; PAS, phagophore assembly site; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3 kinase; RCC, renal clear cell carcinoma; siRNA, small interfering RNA; ULK, unc51-like kinase; UTR, untranslated region; UVRAG, UV irradiation resistance-associated gene.

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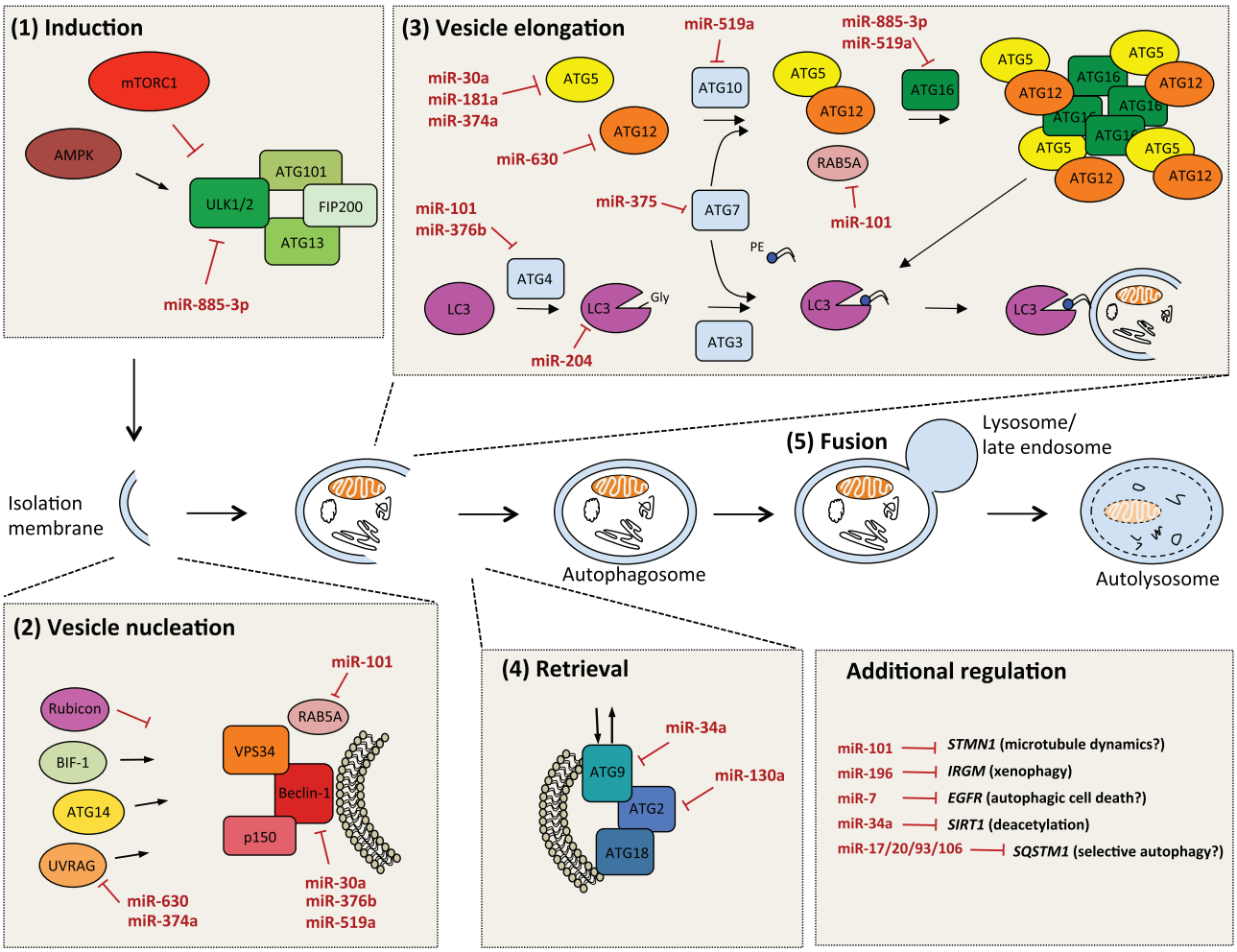

Fig. 1. Overview of the mammalian core autophagy pathway and its regulation by miRNAs. (1) Autophagy induction is regulated by the ULK complex, composed of ULK1/2, FIP200, ATG101 and ATG13; this complex is in turn regulated by upstream mTORC1 and AMPK signaling pathways. miR-885-3p directly regulates ULK2. (2) Vesicle nucleation mainly involves the Beclin-1 complex, which is positively or negatively regulated by a number of associated proteins. Beclin-1 itself is regulated by miR-30a, miR-376b and miR-519a. Additional regulators of this step are miR-101 (RAB5A), miR-630 and miR-374a (UVRAG). (3) Vesicle elongation is controlled by two ubiquitin-like conjugation systems. LC3 is cleaved by ATG4 to expose a C-terminal glycine allowing conjugation to PE by E1 and E2-like enzymes, ATG7 and ATG3, respectively. ATG5 is conjugated to ATG12 by E1-like ATG7 and E2-like ATG10. ATG5-ATG12 forms a large multimeric complex with ATG16, which functions as the E3 ligase for LC3. Several miRNA regulators of this step have been identified including miR-30a, miR-181a and miR-374a (ATG5), miR-630 (ATG12), miR-376b (ATG4), miR-204 (LC3), miR-375 (ATG7), miR-519a (ATG10 and ATG16), miR-885-3p (ATG16) and miR-101 (ATG4 and RAB5A). (4) Retrieval is a poorly defined event in mammalian cells in which the ATG9-ATG2-ATG18 complex participates and probably recruits lipids and other regulatory proteins to the growing phagophore. miRNAs identified to regulate this step include miR-34a (ATG9) and miR-130a (ATG2).(5) Fusion between autophagosomes and lysosomes results in vesicle breakdown and cargo degradation in autolysosomes by lysosomal hydrolases. To date, no miRNAs are experimentally confirmed to regulate at this step. Additional regulation of autophagy by miRNAs through less well-defined mechanisms include miR-101, miR-196, miR-7, miR-34a and the miR-17/20/93/106 family. See text for details.

autophagosomes and lysosomes, involving proteins such as LAMP2 and RAB7, resulting in vesicle breakdown and cargo degradation in autolysosomes by lysosomal hydrolases.

Autophagy induction

In mammalian cells, the induction of autophagy is initiated by the ULK complex, composed of the mammalian ATG1 homologs ULK1 or ULK2, ATG13, focal adhesion kinase family interacting protein of 200 kDa (FIP200) and ATG101 (9–11). The complex acts immediately downstream mTORC1, a key negative autophagy-regulating complex that, under nutrient-rich conditions, associates with and phosphorylates ULK1 (or ULK2) and ATG13, resulting in inhibited activity of the complex and a block in autophagy activation. Under conditions that induce autophagy, mTORC1 is dissociated from the complex causing dephosphorylation of specific residues on ULK1/2 and ATG13, catalytic activation of ULK1/2 and an ULK1/2-mediated increased phosphorylation of FIP200 and ATG13 (9,11). Recently, AMPK was shown to promote autophagy by direct phosphorylation of ULK1 during glucose starvation (12). It remains poorly understood how the ULK complex activates components of the downstream machinery.

A recent study identified *ULK2* as a direct target of miR-885-3p (13). Interestingly, this miRNA was significantly upregulated in a squamous cell carcinoma cell line following cisplatin exposure, and although ectopic expression of miR-885-3p reduced cellular survival, its inhibition could reverse a cisplatin-mediated reduction in viability. These effects were partly phenocopied by small interfering RNA (siRNA)-mediated ULK2 knockdown and overexpression, indicating the potential importance for autophagy regulation by miR-885-3p in controlling cellular viability (13). Interestingly, conserved, predicted binding sites for miR-885-3p exist in additional early autophagy-regulating genes, including the ULK-binding partner ATG13, as well as ATG9A and ATG2B, best known from yeast for their regulation of the retrieval step, where lipid and proteins are recruited to the phagophore assembly site (PAS) (see below (3,14)). Although this additional regulation remains

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to be confirmed, this would suggest a clear role for miR-885-3p in the regulation of early autophagy induction.

Vesicle nucleation

Vesicle nucleation is the initial step in which proteins and lipids are recruited for construction of the autophagosomal membrane. In mammalian cells, this process is initiated by the activation of the class III PI3K/Beclin-1 complex, including the core members hVPS34, Beclin-1 and p150 (15). Numerous additional binding partners of this complex function as either positive or negative regulators and include BAX-interacting factor-1, ATG14L, UV irradiation resistance-associated gene (UVRAG), activating molecule in Beclin-1-regulated autophagy protein 1 (Ambra1) and Rubicon (16).

The first published link between miRNAs and autophagy came from Zhu *et al.* (17) showing that *BECN1,* encoding Beclin-1, is a direct target for miR-30a. Overexpression of miR-30a could reduce rapamycin-induced autophagy, and endogenous miR-30a levels were affected by autophagy induction, indicating a possible physiological role for this miRNA in autophagy regulation. In support of these initial findings, miR-30a levels were found downregulated after cisplatin or taxol treatment in Hela cells, miR-30a overexpression could reduce cisplatin-induced autophagy and intravenous-injected miR-30a could effectively reduce tumor size and autophagy levels of liver-derived implanted tumors from mice treated with cisplatin (18). miR-30a also inhibited autophagic flux in human chronic myeloid leukemia cells where it regulated both Beclin-1 and ATG5 levels (19). Similar to its combined effect with cisplatin, miR-30a could in this setting increase sensitivity to imatinib-induced cytotoxicity, an effect that was phenocopied by short hairpin RNA shRNA-mediated *BECN1* or *ATG5* knockdown. Importantly, reduced sensitivity to imatinib treatment by a miR-30a inhibitor was restored by *BECN1* and *ATG5* knockdown (19). Together, these data indicate the importance of miR-30a-mediated autophagic regulation with regards to altering chemotherapeutic sensitivity in different human cancer types. The identification of *BECN1* as a direct target indicates that this regulation probably occurs at the level of vesicle nucleation.

A second miRNA regulating *BECN1* is miR-376b, identified in a screen based on miRNA overexpression in MCF-7 cells using GFP-LC3 as a readout during starvation conditions (20). Besides *BECN1*, *ATG4C* was also identified as a direct target (see below), and rescue experiments using Beclin-1 and ATG4C overexpression either alone or combined evidenced the functional importance of these targets (20). Finally, miR-519a, which similarly to miR-30a was downregulated by cisplatin treatment, was also found to regulate *BECN1* in a 3′UTR reporter–based assay and inhibition of this miRNA could mimic the effect of cisplatin treatment on the *BECN1* 3′UTR (21). This same study showed direct regulation of *UVRAG*, the Beclin-1 binding partner, by both miR-630 and miR-374a (21), suggesting regulatory effects for these miRNAs at the nucleation step, which remains to be further characterized.

In a functional screen for detection of miRNAs regulating autophagic flux, we identified miR-101 as a potent inhibitor of both basal, rapamycin- and etoposide-induced autophagy (22). One of the targets identified, RAB5A, is a small guanosine triphosphatase, which can induce autophagosome formation, probably in part via its direct interaction with hVPS34 (23) and Beclin-1 (24), implicating a function for miR-101 regulation at the step of vesicle nucleation. Interestingly, endogenous levels of both screen-identified early autophagic regulators, miR-376b and miR-101, were induced by various autophagic stimuli, supportive of their physiological relevance in autophagic regulation (20,22). Since both of these miRNAs inhibit early autophagic membrane formation, we speculate that induction of their endogenous levels may represent an important negative feedback mechanism to keep the level of autophagy initiation in check.

Vesicle elongation

Two unique ubiquitin-like conjugation systems are involved in vesicle expansion. One pathway involves the covalent conjugation of ATG12 to ATG5 in a reaction, which requires the E1- and E2-like enzymes, ATG7 and ATG10, respectively. The ATG12–ATG5 conjugate then forms a large multimeric complex with ATG16L (16). The second pathway involves the conjugation of LC3 to phosphatidylethanolamine (PE) and is initiated through the cleavage of LC3 by the ATG4 protease at its C terminus to generate cytosolic LC3-I in which a C-terminal glycine residue is exposed, allowing subsequent conjugation to PE. This reaction requires ATG7 and the E2-like enzyme ATG3, as well as the ATG12-ATG5-ATG16L complex, which functions as the E3 ligase for LC3 (16). The lipidated form of LC3 (LC3II) is anchored to both inner and outer faces of the phagophore membrane and is a commonly used experimental marker of autophagy (25).

A recently identified miRNA regulator of the vesicle elongation process is miR-204. Its role in autophagy regulation was initially recognized in cardiomyocytes (26) and was further confirmed in the context of renal clear cell carcinoma (RCC) via its direct regulation of the main LC3 homolog, LC3B (27). In the latter study, LC3B-dependent autophagy was shown to be necessary for RCC tumor growth and, importantly, miR-204 levels were significantly decreased in human renal carcinomas relative to matched normal kidney tissue, suggesting a tumor-suppressive function for this miRNA. miR-204 overexpression arrested subcutaneous tumor growth relative to a control miRNA with a mutated seed sequence and its effects on both autophagy and viability were rescued upon re-expression of LC3B lacking the 3′UTR. In addition, supporting the validity of these data, a negative correlation between LC3B and miR-204 was shown in RCC tumors (27). Interestingly, miR-204 regulation of autophagy and cytotoxity occurs only in the absence of the von Hippel-Lindau tumor-suppressor gene (VHL(27)). The loss of effect in VHL+ cells was attributed, at least in part, to VHL-dependent upregulation of the LC3 homolog LC3C, which may protect from the effect of LC3B loss. The mechanism for upregulation of LC3C is unknown but is probably transcriptional and dependent on HIFα levels (27).

Besides regulating LC3 levels themselves, microRNA regulation of the LC3 processors, the ATG4 proteases, was recently elucidated—namely the abovementioned miR-376b and miR-101, which target the *ATG4C* and *ATG4D* homologs, respectively (20,22). The ATG4 family of endopeptidases are crucial regulatory components not only of vesicle expansion (through cleavage of newly synthesized LC3) but also in facilitating autophagosome closure (28), autophagosome–lysosome fusion and LC3 recycling by delipidating PE from outer membrane-bound LC3 (29). Previous studies have suggested differences in activity and substrate specificity among these protease family members (30) and genetic deletion of *Atg4b*, but not *Atg4c*, resulted in prominent defects in autophagy, underlining phenotypic variations among these family members (31,32). Interestingly, although previous studies have suggested a main role for ATG4B in human cells, the recently elucidated miRNA links to ATG4C and ATG4D suggest a previously unacknowledged importance of these homologs (20,22). Indeed, we have observed that siRNA-mediated depletion of *ATG4D* in MCF-7 cells resulted in non-detectable levels of both LC3I and LC3II by western blotting (unpublished).

ATG7, the E1-like enzyme required for the initial step in both conjugation systems, was identified as a direct target of miR-375 in hepatocellular carcinoma (HCC) cells (33). Among a panel of miRNAs tested, miR-375 reduced LC3I to LC3II conversion and further quantification of autophagic flux, GFP-LC3 punctae and autophagosomes by transmission electron microscopy indicated that miR-375 effectively inhibited hypoxia-induced autophagy in HCC cells. In this setting, autophagy was activated as protective mechanism against hypoxic stress, and miR-375, which was significantly downregulated in human HCC relative to background livers, probably exerts tumor-suppressive activity via inhibition of autophagic vesicle elongation triggered by hypoxia in HCC.

The miR-101 target, *RAB5A*, has besides its association with the Beclin-1 complex, also been implicated in ATG5-ATG12 conjugation (24). RAB5A co-localizes with ATG5 and dominant negative inhibition of RAB5A led to the redistribution of unconjugated ATG12, similarly to treatment with the autophagic inhibitor 3-MA (24). Therefore, miR-101, via its target RAB5A, probably interferes both at the level of vesicle nucleation and elongation. Other potential regulators of ATG5-ATG12 conjugation include miR-30a, miR-181a,

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miR-374a and miR-630 (19,21). Although miR-30a overexpression and inhibition regulated ATG5 protein and mRNA levels, an *ATG5* short hairpin RNA shRNA restored sensitivity to imatinib-induced apoptosis after miR-30a inhibition (19). Although a direct regulation of this target was not shown, *ATG5* contains a conserved 8mer binding site for miR-30a in its 3′UTR. Moreover, miR-181a and miR-374a were shown to directly regulate *ATG5* through 3′UTR-based reporter assays and assessment of protein levels after miRNA overexpression and inhibition, whereas *ATG12* was regulated by miR-630 (21). Further analysis is required to address the status of ATG5-ATG12 conjugation in conditions where these miRNAs are altered.

Another likely regulator of vesicle elongation is miR-885-3p, shown to regulate *ATG16L2* (13). Thus, besides a role for this miRNA at autophagy induction via ULK2, miR-885-3p could have additional functions further downstream during vesicle elongation, via ATG16L2. However, a recent characterization of ATG16L2 indicated that this isoform, in contrast to ATG16L1, was not essential for mediating canonical autophagy, despite its ability to form a complex with ATG5 and ATG12 (34). Therefore, the importance of this interaction with regards to autophagy should be explored further.

An additional likely regulator of vesicle elongation is miR-519a, which besides regulating *BECN1*, was found to directly regulate *ATG16L1* and the E2-like enzyme *ATG10A* (21), although the significance of these interactions with respect to the autophagy phenotype was not investigated further.

Retrieval

The mechanism of retrieval, in which the ATG9 complex participates, involves the recruitment of lipids and proteins to the growing phagophore. However, the details with regards to mechanism and timing relative to other pathway steps are still unclear as this process is poorly studied and most information is limited to studies in yeast. The transmembrane protein, Atg9, moves bi-directionally between the PAS and so-called non-PAS structures, which in yeast consists of mitochondria (35), whereas in humans include late endosomes and the *trans*-Golgi network (36). The bi-directional movement is necessary for autophagosome formation and, potentially, this shuttling could contribute to the delivery of membrane to the PAS. Atg2 and Atg18 bind to Atg9 at the PAS and are required for proper Atg9 trafficking (14). Although ATG2 function in humans is not well understood, a recent characterization of mammalian ATG2A and ATG2B attributed a function for these proteins in autophagosomal closure (37). Interestingly, *ATG2B* was identified as a direct target of miR-130a by Kovaleva *et al*. (38) among a set of miRNAs found downregulated in human chronic lymphocytic leukemia and miR-130a effectively inhibited autophagic flux and induced cell death (38). miR-130a may therefore interfere with ATG9-ATG2-ATG18 complex formation and hereby retrieval of lipids and proteins to the growing phagophore, possibly also resulting in inefficient closure. However, the functional importance for *ATG2B* as a miR-130a target and the precise location of the resulting autophagy defect remains to be established.

Another potential regulator of the retrieval step is the tumor-suppressor miR-34a, which besides its well-established effects related to cell-cycle arrest and senescence (39–41) has recently been identified as an inhibitor of autophagic flux and a direct regulator of *ATG9A* in mammalian cells (42). Interestingly, this study reported that a miR-34 loss-of-function mutation in *Caenorabditis elegans* extended lifespan and increased resistance to oxidative stress and that RNAi against *Atg4, Bec-1* or *Atg9* reversed the lifespan-extending effect of the miR-34a mutant (42).

Fusion

The lysosomes are the final destination for autophagosomes, the outer membrane of which ultimately fuses with the lysosomal membrane forming the autolysosome where autophagic cargo is degraded. The fusion process has been closely studied in yeast, as autophagosome to vacuole fusion, and involves among others, the Rab-SNARE system and the small Rab guanosine triphosphatase, Ypt7 (43). The mammalian homolog of Ypt7, RAB7, is involved in autophagosome–lysosome fusion in mammals (44,45) and additionally, the lysosomal membrane proteins LAMP1 and LAMP2 are required for the fusion step (46,47).

To date, no miRNAs directly affecting the fusion process have been identified. However, a computational systems biology approach identified a set of miRNAs with potential functional involvement in the autophagy-lysosomal pathway including miR-130, 98, 124, 204 and 142 (48). Among the predicted targets for these miRNAs were LAMP1, LAMP2 and the v-SNARE protein, vesicle-associated membrane protein 7, also shown to be important for autophagosome–lysosome fusion(49), suggesting a possible role for these miRNAs at the fusion step. Supporting the validity of these computational predictions, two of these miRNAs, miR-204 and miR-130 (described above), were independently confirmed in other groups as autophagic regulators via their targets *LC3B* and *ATG2B*, respectively (27,38). Another predicted regulator of autophagy, miR-124, potentially regulating 52 target genes in the autophagy-lysosomal pathway, remains to be experimentally validated. However, several of these predicted targets have been previously validated in the literature (48). Thus, despite the limitations in using systems biology-based approaches, including the large number of potential false positives, it is becoming increasingly clear that such studies, in combination with candidate gene approaches, provide a powerful tool in revealing novel interactions, which would otherwise remain unidentified.

Interestingly, the Beclin-1 binding partner, UVRAG, was assigned a Beclin-1 independent role in autophagy regulation through its interaction with the class C Vps complex, a key component of the fusion machinery (50). This interaction stimulated RAB7 recruitment and activity and facilitated autophagosome fusion with late endosomes/lysosomes (50). Therefore, the *UVRAG*-regulating miRNAs, miR-630 and miR-374a (21), may have functional implications at the autophagosome–lysosome fusion step.

Additional miRNA regulators of autophagy

Although the miRNAs and targets described above have relatively well-defined locations of action within the autophagy signaling pathway, there are several examples of miRNAs where the exact location of function is more difficult to pinpoint.

Immunity-related GTPase family M protein IRGM, a human immunity–related guanosine triphosphatase, has been shown to be necessary for the execution of autophagy, particularly xenophagy, where it confers autophagic defense against intracellular mycobacteria in human cells (51). The mechanism for this remains unknown; however, it has recently been linked to the regulation of mitochondrial dynamics (52). miR-196 was found to be overexpressed in inflamed mucosa from patients with Crohn's disease and correlated with decreased IRGM expression levels (53). Interestingly, a risk-associated polymorphism of *IRGM* located in the predicted seed-binding region for miR-196, resulted in decreased binding efficiency of this miRNA to its target. Similarly to an siRNA against *IRGM*, miR-196 significantly reduced LC3I to LC3II conversion both in the presence and absence of lysosomal inhibitors, indicating an inhibition of autophagy at the level of its initiation. This decrease in autophagic flux was associated with decreased numbers of LC3II-associated bacteria, indicating that miR-196-mediated control of *IRGM* provides an important means of regulating autophagy-mediated intracellular pathogen clearance in human cells (53). The allele-specific regulation of *IRGM* by miR-196 provides an attractive explanation for how a genetic polymorphism can alter tissue-specific expression levels of IRGM and thereby influence predisposition to inflammatory bowel disease; however, further analysis is required to define the precise mechanism of IRGM-regulated autophagy.

Stathmin, encoded by *STMN1*, was identified as a novel regulator of autophagy and a direct target of the tumor-suppressive miR-101. Importantly, overexpression of a 3′UTR-less STMN1 partially rescued miR-101-mediated inhibition of autophagy, confirming the functional

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importance of this target (22). The mechanism for Stathmin-mediated autophagy regulation remains unknown. However, the fact that stathmin is a potent regulator of microtubule dynamics presents an interesting hypothesis, since a role for microtubules in autophagy regulation of mammalian cells is well documented (54–56). Efficient autophagosome formation requires an intact microtubular network (55) and autophagosomes are transported bi-directionally along microtubule tracks, allowing them to dock and fuse with late endosomes and lysosomes (54,55). Jahreiss *et al*. (54) 2008 showed by live cell time-lapse video microscopy that microtubule dissolution delays the arrival of autophagosomes in the proximity of late endosomes and lysosomes, preventing their efficient fusion with these organelles. Further studies are necessary to establish whether the role of miR-101 and stathmin in autophagy regulation is linked to microtubule function.

Sirtuins are nicotinamide adenine dinucleotide-dependent deacetylases and full induction of autophagy by starvation requires Sirt1(57). Accordingly, *Sirt1–/–* mice display a phenotype consistent with defective autophagy and similar to *Atg5–/–* mice, including increased p62, accumulation of damaged organelles and early perinatal lethality (57). Sirt1 can interact with and de-acetylate several components of the autophagic machinery, including Atg5, Atg7 and LC3, further supporting a direct role in autophagy regulation (57). The exact function for this de-acetylation remains unknown. miR-34a, which as described above has been linked to autophagy via *ATG9A*, is a direct regulator of *SIRT1* (58), adding an additional potential explanation for miR-34a-mediated regulation of autophagy.

The miR-17/20/93/106 family of miRNAs, sharing a common seed sequence, was identified as direct regulators of *SQSTM1*, encoding the poly-ubiquitin binding protein p62, which also binds to LC3 and acts as a selective autophagy receptor and molecular carrier of cargo to be degraded by autophagy (59,60). Ectopic expression of these miRNAs increased proliferation, colony outgrowth and replating capacity of myeloid progenitors (61). In this setting, p62 may regulate stability of human colony-stimulating factor 3 receptor, important for myeloid differentiation, by targeting it to endosomes/lysosomes for degradation (61). However, a potential direct role in autophagy regulation for these miRNAs was not addressed and remains to be established.

Much of the literature described so far assigns negative regulatory roles for miRNAs in autophagy via direct regulation of core autophagy machinery components (17,27,38). In contrast, miR-7 is an example of a miRNA, which may induce autophagy, concordant with a decrease in cellular viability in human non-small-cell lung cancer cells. Although the precise mechanism behind these observations remains unknown, the authors suggest that this could involve a miR-7-mediated reduction of epidermal growth factor receptor (62). Epidermal growth factor receptor is an established target of this miRNA (63) and has previously been implicated in the prevention of autophagic cell death (64). Interestingly, miR-7 has been characterized as a tumor-suppressive miRNA known to inhibit tumor growth and metastasis through its negative regulation of the PI3K/AKT-mTOR pathway in hepatocellular carcinoma (65), which may also account for the increased autophagic activity induced by this miRNA. The concept of miR-7-induced autophagic cell death is uncertain and should be further adressed, since it has become clear over recent years that the concept of 'autophagic cell death' in itself is a highly questionable process (66). Indeed, autophagy usually constitutes a futile attempt of dying cells to adapt to lethal stress rather than serving as a mechanism to execute cell death, and therefore, caution should be taken when interpreting data where cell death is accompanied by increased autophagy (66).

miRNAs—yet another layer of regulation

Although our molecular understanding of autophagy is far from complete, recent studies have elucidated important details concerning the autophagy core machinery and the means whereby it is regulated (12,67,68).

Much of the known regulation of autophagy occurs at the post-translational level, involving protein modifications such as phosphorylation, acetylation and ubiquitin-like conjugation. Key phosphorylations on members of the ULK complex are crucial, for instance during glucose starvation, where AMPK promotes autophagy by phosphorylating ULK1 on two specific residues, whereas during nutrient sufficiency, where mTORC1 prevents ULK1 activity by phosphorylating a third site (12). Phosphorylations can also cause steric hindrance of protein interactions, for example the Bcl-2-Beclin-1 interaction, which inhibits the pro-autophagic activity of Beclin-1 and is dissociated by phosphorylation on three Bcl-2 residues (69,70). Recently, acetylation of Atg3, the E2-like enzyme, was attributed an essential role in autophagy regulation in *Saccharomyces cerevisiae* by controlling the Atg3-Atg8 interaction and thereby the lipidation of Atg8 (71). The ubiquitin-like conjugation systems, resulting in ATG5 conjugation to ATG12 and LC3 conjugation to PE, are yet another example of post-translational modifications, which are essential for autophagy.

Transcriptional control of autophagy genes, however, is an area much less explored. The forkhead box transcription factor 3 was one of the first identified transcriptional regulators of autophagy genes (72,73) and a recent study identified the transcription factor EB as a crucial coordinator of autophagic/lysosomal gene expression (74).

Importantly, regulation of autophagy by non-coding RNAs, and in particular by miRNAs, represents a new post-transcriptional regulatory layer, the understanding of which is still only in its infancy. Since miRNA expression is altered during conditions of stress and disease, the complexity and dynamics of this regulation and its potential consequences for disease pathogenesis are widespread.

Many aspects of this regulation remain to be explored, for example variations in target 3′UTR length and altered target structure and accessibility are known to affect miRNA-mediated gene regulation (75,76). Interestingly, the miR-376b targets, *ATG4C* and *BECN1*, are more efficiently regulated during conditions of starvation- or rapamycin-induced autophagy, relative to their regulation during basal autophagy (20), indicating a potentially altered miRNA affinity or accessibility to its targets during autophagy. Indeed, conditions of stress have previously been shown to affect miRNA-target accessibility; the cationic amino acid transporter 1 mRNA is relieved from miR-122-mediated repression during starvation, a mechanism that is dependent on the HuR RNA-binding protein, which binds to the *cationic amino acid transporter 1* 3′UTR and causes relocation of the transcript (77). In addition, existence of alternative 3′UTR lengths, which can differ in various biological settings, can result in altered availability of miRNA binding sites (75). *ATG4C* has alternative annotated 3′UTR lengths, only the longer of which contain the miR-376b binding site (20). However, potential biological implications for this observation will require further investigation. Finally, disease-associated gene polymorphisms have been shown to alter miRNA binding sites (78,79) exemplified by miR-196 regulation of the protective *IRGM* variant (c.313C) but not the risk-associated allele (c.313T), causing lost control of IRGM expression and compromised regulation of intracellular *Escherichia coli* replication during Crohn's disease by autophagy (53).

Interconnecting miRNA and autophagy pathways

Although specific subsets of miRNAs have increased expression levels during tumorigenesis, cancer cells generally present a global downregulation of miRNAs. This has, in part, been attributed to reduced levels or loss of function of key miRNA biogenesis regulators, including the primary and mature miRNA processors, Drosha and Dicer, respectively (7). These and other biogenesis proteins can function as tumor suppressors, which when lost can cause repression of miRNA processing and acceleration of tumorigenesis *in vivo* (80,81). It has been shown that siRNA-mediated knockdown of Dicer reduced LC3I and LC3II levels in the presence and absence of the lysosomal inhibitor bafilomycinA, possibly implicating a role for Dicer in autophagy (38); however, this finding requires further substantiation. Interestingly, comparison of *in vivo* models for defective autophagy and defective miRNA processing reveals several similarities. Both Dicer and Beclin-1 are haplo-insufficient tumor

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suppressors, whereas full ablation of these genes leads to lethality early in embryonic development (82,83). In addition, murine systems of conditional Dicer deletion showed a requirement for Dicer in the proliferation and survival of various neuronal cell types (84,85) and the absence of functional Dicer in the adult forebrain was accompanied by neurodegeneration and inflammation (86). Indeed, targeted deletion of *Atg5* and *Atg7* in the mouse brain causes profound neurodegeneration (87,88) and defective autophagy is known to contribute to neurodegerative diseases such as Parkinson's, Alzheimer's and Huntington's diseases (89). It would therefore be highly interesting to investigate autophagy function during conditions of dysfunctional miRNA processing, for example upon Dicer deletion.

Many other questions remain unanswered. Are miRNA expression levels themselves altered during autophagy? Indeed, fluctuations in miRNA expression have been demonstrated during conditions of autophagy, triggered by for instance starvation, rapamycin or different chemotherapeutics (18–20,22); however, caution should be taken when interpreting these results, since these stimuli induce general stress effects and the resulting changes in miRNA expression may not be directly linked to autophagy. Nevertheless, the possibility for intricate feedback loops between autophagy and miRNA networks is intriguing and should be investigated further. Profiling miRNA expression levels in autophagy-deficient cells, that is genetically deleted for specific *ATGs*, could provide useful information to address this issue.

Selective autophagy, in which specific subgroups of cargo are selectively degraded, is a well-known but poorly understood phenomenon, and a potential role for miRNAs in orchestrating the selection of autophagic cargo is an appealing possibility. Interestingly, miR-375 seems to specifically inhibit hypoxia-induced mitophagy in HCC cells, as demonstrated by cytoplasmic accumulation of mitochondria by transmission electron microscopy and by decreased co-localization between LC3 and the mitochondrial translocase of outer membrane 20 kDa subunit (TOM20 (33)). Moreover, miR-196, via its regulation of *IRGM*, inhibits xenophagy (53). It was recently suggested that ubiquitin represents a selective degradation signal, which targets various cargo types, including ribosomes, mitochondria and intracellular bacteria to the autophagosomes, via the autophagy receptor p62, which simultaneously binds both ubiquitin and LC3 (90). Therefore, regulators of p62 (*SQSTM1*), such as the miR-17/20/93/106 family (61) or the related receptor, neighbor of BRCA1 gene 1 (*NBR1*), may have influential roles in selective autophagy, which should be investigated further.

The context-dependent role for autophagy in cancer—a role for miRNAs?

Autophagy has both tumor-suppressive and tumor-promoting functions, complicating the understanding of this process in cancer development. Nevertheless, we are now beginning to understand how such contradictory roles for autophagy in cancer can make sense (91).

In initial tumor development, autophagy plays an important role in protecting cells from the accumulation of damaged organelles and protein aggregates—in this way autophagy limits inflammation, genome instability and tissue damage that can otherwise promote cancer initiation (91). Therefore, autophagy can, in specific settings, be considered a tumor-suppressive mechanism in early cancer initiation. In line with this, human cancers frequently display inactivating mutations in pro-autophagy genes. On the contrary, autophagy is robustly activated in established tumors by damaging stimuli, growth factor deprivation, hypoxia and oncogene activation and thus many established cancers present high basal autophagy levels or so-called autophagy addiction in which autophagy is required for continued growth and survival (92–96).

Although examples of inverse correlations between autophagy-regulating miRNAs and their targets in human cancers are emerging, including miR-375 and *ATG7* in human HCC (33) and miR-204 and *LC3B* in RCC (27), little is known regarding the dynamics of the miRNA-autophagy interplay during tumor progression. Considering the vast implications for miRNAs in stress responses, including DNA damage and inflammation (97) and during tumorigenesis (7), it is tempting to speculate that miRNAs could contribute to autophagy dynamics during cancer progression. The autophagy-inhibitory miR-101 is progressively lost through the transition from clinically localized disease to metastatic prostate cancer, where one or both of its two genomic loci were lost in 37.5% of clinically localized prostate cancer cells and 66.7% were lost in cells from metastatic disease (98). Whether this progressive loss impacts on the autophagy status of early versus late disease is an appealing possibility that requires further investigation.

Most of the described miRNA inhibitors of autophagy (e.g. miR-101, miR-30a, miR-34a, miR-204, miR-375) are known tumor suppressors, which are downregulated or lost in cancer. The mechanisms for their tumor-suppressive activities are context dependent and mediated via regulation of several targets and pathways. miR-375, for instance, is involved in glucose metabolism (99), apoptosis (100) and autophagy regulation (33), all of which probably contribute to tumor suppression. So although autophagy regulation will probably not stand alone, it is tempting to speculate that a general miRNA 'safeguarding' of the autophagy system by tumor-suppressive miRNAs may serve to limit excessive autophagy activation, thereby providing a means to protect against oncogenesis.

miRNA-modulated autophagy for limiting therapeutic resistance

Since the realization that autophagy is primarily a survival mechanism in already established tumors, there has been a great focus on inhibiting autophagy for cancer therapy (101,102).

The ability of miRNAs to simultaneously target multiple genes and pathways provides a potential advantage from a treatment perspective, since it allows for a robust inhibition, which is less affected by single target mutations and cell-to-cell heterogeneity than conventional approaches. miRNA inhibition strategies, including the use of locked nucleic acids, have achieved good success *in vivo* in non-human primates (103,104) and although the use of miRNA mimics has yielded positive results in mouse models (105,106), delivery issues must be improved before this approach can be implemented for clinical use.

miR-101 and miR-376b are examples of miRNAs, which negatively regulate multiple steps in the autophagy pathway (20,22) potentially providing a therapeutic advantage. Indeed, miR-101 can effectively reduce tamoxifen-induced autophagy and enhance the sensitivity of breast cancer cells to tamoxifen treatment (22). Furthermore, overexpression of miR-101 suppressed tumor development in a xenograft mouse model (107), efficiently reduced tumor size and sensitized human lung carcinoma cells to radiation treatment (108). Further examples from cell culture and mouse studies indicate a clear potential for combining miRNA-based manipulation of autophagy with conventional chemotherapeutics, which becomes highly relevant with regards to battling treatment-resistance issues. miR-30a, via its *BECN1*-mediated inhibition of autophagy, can enhance sensitivity to imatinib-induced cytotoxicity in chronic myeloid leukemia CML cells (19) and can sensitize tumor cells to cisplatin *in vitro* and *in vivo* (18). Moreover, modulation of miR-885-3p levels altered cisplatin-mediated effects on cell viability (13). Importantly, miR-101, miR-30a and miR-885-3p inhibit the early pathway steps of induction, vesicle nucleation and elongation, possibly providing an attractive therapeutic advantage as opposed to targeting late lysosomal-related steps, which would instead lead to a more general degradation block and a massive autophagosome accumulation, which could cause deleterious stress-related effects.

Perspectives

As outlined in this review, important ties exist between miRNAs and the core autophagy machinery. However, although a number of studies have demonstrated miRNA-mediated regulation of proteins in the autophagy pathway, only few studies investigate the effect of the miRNA on autophagic flux or attempt to identify the physiological context in which the miRNA may be linked to autophagy. Hence, for a comprehensive understanding of the importance of miRNA regulation of autophagy, there is a substantial need for more thorough functional investigations.

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Despite the obvious differences between a core catabolic pathway and a genome-wide regulatory network, the autophagy and miRNA systems share interesting and important commonalities in facilitating cellular responses to stresses. Intriguingly, many of the miRNAs negatively regulating autophagy, such as miR-30a, miR-34a and miR-101, are also demonstrated to be tumor repressive suggesting a causal link between downregulation of these miRs and the requirement of certain tumor types to activate autophagy. Although still limited, there are indications that the miRNA regulatory network per se may be important for controlling autophagy as suggested by Dicer knockdown experiments (38). Also here, there is a need for detailed genetic studies involving analysis of autophagy function following tissue-specific ablation of core enzymes in the miRNA biogenesis pathway. In this context, it would be of particular interest to explore functional links between miRNA regulation and the involvement of autophagy in neuronal degeneration.

Interestingly, the miRNAs so far demonstrated to regulate proteins involved in autophagy predominantly target early stages of the pathway during vesicle nucleation and elongation. Whereas this could reflect an evolutionary adaptation to prevent accumulation of autophagosomes and a total block of lysosomal function, it certainly points to the need for studies directed at investigating miRNA-mediated regulation of autophagosome fusion and the function of the autolysosome.

Finally, several studies suggest that miRNAs may be exploited therapeutically to block autophagy in cancer and although several small chemical inhibitors of autophagy or lysosome function exist, miRNA mimetics or inhibitors may present an advantage in targeting the autophagy pathway at several different stages.

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