MIR376A Is a Regulator of Starvation-Induced Autophagy

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Abstract

Background: Autophagy is a vesicular trafficking process responsible for the degradation of long-lived, misfolded or abnormal proteins, as well as damaged or surplus organelles. Abnormalities of the autophagic activity may result in the accumulation of protein aggregates, organellar dysfunction, and autophagy disorders were associated with various diseases. Hence, mechanisms of autophagy regulation are under exploration.

Methods: Over-expression of hsa-miR-376a (shortly MIR376A) was performed to evaluate its effects on autophagy. Autophagy-related targets of the miRNA were predicted using Microcosm Targets and MiRanda bioinformatics tools and experimentally validated. Endogenous miRNA was blocked using antagonirs and the effects on target expression and autophagy were analyzed. Luciferase tests were performed to confirm that 3' UTR sequences in target genes were functional. Differential expression of MIR376A and the related MIR376B was compared using TaqMan quantitative PCR.

Results: Here, we demonstrated that, a microRNA (miRNA) from the DLK1/GTL2 gene cluster, MIR376A, played an important role in autophagy regulation. We showed that, amino acid and serum starvation-induced autophagy was blocked by MIR376A overexpression in MCF-7 and Huh7 cells. MIR376A shared the same seed sequence and had overlapping targets with MIR376B, and similarly blocked the expression of key autophagy proteins ATG4C and BECN1 (Beclin 1). Indeed, 3' UTR sequences in the mRNA of these autophagy proteins were responsive to MIR376A in luciferase assays. Antagomir tests showed that, endogenous MIR376A was participating to the control of ATG4C and BECN1 transcript and protein levels. Moreover, blockage of endogenous MIR376A accelerated starvation-induced autophagic activity. Interestingly, MIR376A and MIR376B levels were increased with different kinetics in response to starvation stress and tissue-specific level differences were also observed, pointing out to an overlapping but miRNA-specific biological role.

Conclusions: Our findings underline the importance of miRNAs encoded by the DLK1/GTL2 gene cluster in stress-response control mechanisms, and introduce MIR376A as a new regulator of autophagy.

Introduction

Two major degradation pathways, namely macroautophagy (autophagy herein) and the ubiquitin-proteasome system, are operational in the maintenance of cellular homeostasis. Functional at a basal level for long-lived protein degradation and organelle turnover under normal conditions, autophagy is rapidly upregulated in response to both extracellular (e.g. nutrient starvation, hypoxia) and intracellular (e.g. accumulation of unfolded proteins, damaged organelles, pathogens) stress factors.[1] Convergent action of several protein complexes formed by at least 32 different autophagy (ATG) proteins result in the formation of double- or multi-membrane vesicles called autophagic vesicles or autophagosomes.[2] These vesicles enwrap cargo molecules and carry them to lysosomes for degradation, resulting in the recycling of their constituents for reuse by the cell.[3,4]

Protein complexes playing a role in autophagosome formation are numerous. A key event is the accumulation of a modified lipid molecule, phosphoinositol 3-phosphate on the ER and mitochondrial membranes, marking the autophagic vesicle nucleation centers.[2] A phosphoinositol 3-kinase, VPS34, is responsible for the conversion of membrane associated inositol lipids into phosphoinositol 3-phosphate (PI3-P). BECN1 was discovered as a master regulator of the VPS34 activity and autophagosome formation.[2] Autophagic vesicle membrane elongation, growth and closure occur through the action of two ubiquitination-like protein conjugation systems.[5] The first system is rather regulatory, resulting in the covalent conjugation of a ubiquitin-like protein ATG12 to ATG5, and in the eventual formation of a
lager complex including the ATG16 protein. The ATG12-5-16 complex serves as a E3 ubiquitin ligase-like enzyme for the second reaction involving covalent attachment of a lipid, phosphatidylethanolamine (PE), to a carboxy-terminal (C-ter) glycine residue of the autophagy-related MAP1LC3 or simply LC3 protein. [6] To expose the key glycine residue for conjugation, prior C-ter cleavage of pro-LC3 by ATG4 proteins is required. Lipid conjugated LC3 is necessary for the elongation of autophagic membranes and completion of the vesicles. [7] Indeed, cells lacking one of the conjugation reaction components were shown to harbor autophagy defects. [8]

Recent studies introduced microRNAs (miRNAs) as novel regulators of autophagy. [9,10,11] miRNAs are small non-coding RNAs serving as negative regulators of gene expression. [12] By base pairing with sequences found mainly in the 3' untranslated region (3' UTR) of specific mRNAs, miRNAs lead to mRNA instability and/or translation inhibition resulting in a decrease in target gene expression. [13] A single miRNA may target tens to hundreds of mRNAs, hence may co-regulate and coordinate a number of cellular proteins and pathways at once. [14] So far, only a handful of miRNAs were shown to directly affect the autophagic activity. Among them, MIR376B was introduced as a new regulator of starvation and mTOR-inhibition-related autophagy. [10] MIR376B blocked autophagy by affecting the expression of two key autophagy proteins, namely ATG4C and BECN1. MIR376B belongs to a miRNA gene family encoded from a gene cluster region in the human chromosome 14q32, called the DKL1/GTL2 region. [15,16,17] Therefore, we wondered whether other miRNAs from the same region could play a role in autophagy regulation.

Here, we report that another miRNA from the DKL1/GTL2 region, namely hsa-miR-376a1 (hereafter MIR376A) containing a seed sequence similar to that of MIR376B, is a novel regulator of autophagy. Overexpression of MIR376A attenuated starvation-induced autophagic activity and did so by modulating cellular ATG4C and BECN1 mRNA and protein levels. We showed that miRNA response elements (MRE) in the 3'UTR region of these genes were direct targets of MIR376A. Importantly, antagonir-mediated suppression of endogenous MIR376A levels led to an increase in ATG4C and BECN1 expression and resulted in autophagy stimulation. Our findings underline the importance of miRNAs coded by the DKL1/GTL2 genomic region in physiological regulation and control of the autophagic activity.

Results

**MIR376A overexpression blocked autophagy**

In an unbiased screen, we discovered several miRNAs, including MIR376B and MIR181A as inhibitors of starvation-induced autophagy [10,11]. MIR376B is encoded from a gene region called, DKL1/GTL2 containing several miRNA genes (Figure 1A). Although the nucleotide sequences of at least seven miRNA genes found in this region differed considerably, the seed sequences (around 8 nucleotide long miRNA core sequences responsible for target mRNA recognition) of MIR376A1, MIR376A2 and MIRB2 were identical to that of the MIR376B (Figure 1B and C). Since in our screen, MIR376A1 was also a hit, we wondered whether this miRNA would regulate autophagy.

Firstly, we transiently overexpressed MIR376A1 (MIR376A herein) together with the autophagy marker GFP-LC3 in MCF-7 breast cancer cells. Detection of cytoplasmic puncta formation by otherwise soluble GFP-fused MAP1LC3 (shortly LC3) protein is a commonly used method to follow autophagy activation using microscopy. As shown in Figure 2A and B, overexpressed MIR376A could repress starvation-induced GFP-LC3 puncta formation. To confirm these results with a complementary technique, we analyzed the effect of MIR376A overexpression on the conversion of endogenous free LC3 protein (LC3-I) to its lipidated and autophagic vesicle-associated form (LC3-II). As seen in Figure 2C, LC3-I conversion to LC3-II was decreased in miRNA transfected MCF-7 cells compared to controls.

Autophagic cargo receptor protein p62/SQSTM1 is also carried by autophagosomes to lysosomes and degraded there during the process. [18] Indeed, starvation resulted in SQSTM1 degradation in cells transfected with the control miRNAs, and inhibition of the lysosomal enzymes blocked its degradation (Figure 2D). Yet, in starved cells overexpressing MIR376A, SQSTM1 was neither degraded following starvation, nor accumulated after lysosomal inhibition, confirming autophagy blocking activity of the miRNA.

We also performed tests in Huh7 hepatocellular carcinoma cells. Similar to the results obtained in MCF-7 cells, MIR376A overexpression could block starvation-induced GFP-LC3 dot formation (Figure 3A and B) and LC3 lipiddation (Figure 3C) in Huh7 cells as well.

All these results showed that, MIR376A, another gene encoded by the miRNA gene cluster in the DKL1/GTL2 genomic region, was a new miRNA regulator of starvation-activated autophagy.

**Autophagy-related targets of MIR376A**

We have previously described ATG4C and BECN1 as autophagy-related targets of MIR376B. Since MIR376A seed sequence was identical to that of MIR376B, it could potentially target the MRE sequences found in the 3' UTR regions of these autophagy genes (Figure 4A and B). Indeed, mRNA levels of both genes were decreased upon MIR376A overexpression (Figure 4C and D). Additionally, MIR376A overexpression led to a decrease in the levels of both ATG4C (Figure 4E) and BECN1 (Figure 4F) proteins. Therefore, similar to MIR376B, MIR376A could also regulate expression levels of two key proteins in the autophagy pathway.

**Effect of MIR376A antagonors on ATG4C and BECN1 levels**

To check whether blockage of the endogenous miRNA would affect ATG4C and BECN1 expression levels, we used antagonors (chemically engineered oligonucleotide anti-miRNAs) specifically neutralizing MIR376A. While control antagonors (CNT-Ant) showed no significant effect, introduction of MIR376A-specific antagonors (Ant-376A) into cells led to a significant increase in ATG4C mRNA (Figure 5A) and protein levels (Figure 5B). Similar antagonor-related changes were observed in BECN1 mRNA (Figure 5B) and protein (Figure 5D) levels. Therefore, endogenously expressed cellular MIR376A also played a role in the suppression of autophagy-related gene expression.

**MIR376A directly targeted ATG4C and BECN1 3' UTR sequences**

To prove that negative regulation of the autophagy proteins by MIR376A was a result of a direct effect of the miRNA, reporter luciferase vectors containing predicted 3’ UTR MRE sequences in ATG4C and BECN1 mRNAs were prepared. Mutant versions of these vectors were constructed as well (Figure 6A and B). 293T cells were transfected with these constructs together with MIR-CNT or MIR376A, and luciferase activities were measured. Overexpression of MIR376A but not MIR-CNT resulted in a decrease in luciferase expression from vectors containing wild-type MRE sequences (Figure 6C and D). Wild-type. Mutation of the
MREs in ATG4C or BECN1 sequences abolished the effect of the miRNA on luciferase expression (Figure 6C and D, Mutant). Therefore MIR376A controlled ATG4C and BECN1 levels by directly affecting specific MRE sequences in the 3' UTR regions of these autophagy genes.

Role of endogenous MIR376A in the control of starvation-induced autophagic activity

To further reveal the role of endogenous MIR376A in autophagy regulation, we transfected cells with control or MIR376A antagomirs, and analyzed conversion of LC3-I to LC3-II and SQSTM1 degradation as markers of the autophagic activity. Interestingly, we observed that blockage of endogenous MIR376A further increased LC3-II/I ratios (Figure 7A). Moreover, starvation-induced degradation of the autophagy receptor SQSTM1 was further increased in cells transfected with MIR376A antagomirs compared to controls (Figure 7B). These results underlined the importance of endogenous MIR376A activity in the control of the amplitude of starvation-induced autophagy responses of cells.

Response of endogenous MIR376A and MIR376B levels to starvation

We have previously reported that autophagy-related miRNAs accumulated following starvation or mTOR inhibition.[10,11] To check whether endogenous MIR376A and MIR376B levels were similarly increased during starvation stress, we performed a kinetic analysis using TaqMan qPCR. Although cellular levels of MIR376A and MIR376B were increased in cells exposed to starvation stress, kinetics of the upregulation were different between individual miRNAs. In starved cells, while endogenous MIR376A reached peak levels following a 6 hours lag period (Figure 8A), endogenous MIR376B levels rapidly increased after 1 hr of stress, but the response was transient (Figure 8B). Therefore, the two miRNAs responded to autophagy-inducing starvation signals with different kinetics.

Discussion

In this study, we demonstrated that another miRNA encoded by the DLK1/GTL2 region and belonging to the MIR376 family, namely MIR376A, was an autophagy regulatory miRNA. Inhibitory effect of MIR376A on autophagy was demonstrated using various autophagic tests including GFP-LC3 puncta analysis, LC3II/LC3I gel shift and p62/SQSTM1 degradation tests. The miRNA affected ATG4C and BECN1 protein levels as well as the mRNA levels. MIR376A directly and specifically affected the mRNAs of the autophagy genes, since MREs in the 3' UTR of both ATG4C and BECN1 were responsive to the inhibitory effects of the miRNA in luciferase tests, and mutations in the relevant regions abolished the suppressive effects. Contribution of the endogenous MIR376A to the control of basal and stress-activated autophagy was documented as well. Specific antagomirs against the miRNA led to a clear increase in both ATG4C and BECN1 mRNA and protein levels, and stimulated autophagic activity in cells.

Since ATG4C and BECN1 function at two different steps of autophagic vesicle formation, targeting of both of these key autophagy proteins at once by the MIR376 family of miRNAs allows a robust decrease in the autophagic activity. In fact, while BECN1 controls the production of PI3-P and regulates of
**Figure 2. Effect of MIR376A overexpression on autophagy.** (A) MCF-7 cells were co-transfected with either MIR-CNT (control plasmid) or MIR376A and GFP-LC3 plasmid, and GFP-LC3 dot formation was analyzed. White arrows indicate clusters of the GFP-LC3 dots in cells. (B) Quantification of the experiments in A. MIR376A overexpression, but not MIR-CNT expression, blocked starvation (STV)-induced autophagy (mean ± S.D. of independent experiments, n = 4, ***p < 0.01). NON STV, non-starved (C) Overexpression of MIR376A resulted in a decrease in the autophagic activity of MCF-7 cells. Starvation-induced conversion of LC3-I to LC3-II in MCF-7 cells was analyzed. Tests were performed in the presence or absence of E64d (10 μg/ml) and Pepstatin A (10 μg/ml) (E+P). LC3-II/LC3-I densitometric ratios were marked. ACTB was used as a loading control. (D) MIR376A blocked starvation induced SQSTM1 degradation in MCF-7 cells. ACTB was used as a loading control. SQSTM1/ACTIN densitometric ratios were marked.

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**Figure 3. MIR376A overexpression blocked autophagy in Huh-7 cells.** (A) MIR376A blocked GFP-LC3 dot formation under starvation condition. (B) Quantitative analysis of experiments in A (mean ± S.D. of independent experiments, n = 3, **p < 0.01). (C) Overexpression of MIR376A resulted in decreased autophagic flux in Huh-7 cells. Starvation-induced conversion of LC3-I to LC3-II was analyzed. Tests were performed in the presence or absence of E64d (10 μg/ml) and Pepstatin A (10 μg/ml) (E+P). LC3-II/LC3-I densitometric ratios were marked. ACTB was used as a loading control.

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Figure 4. ATG4C and BECN1 were targets of MIR376A. (A and B) MIR376A mature miRNA sequence and MIR376A binding target sequences in the 3' UTR of ATG4C (A) and BECN1 (B) mRNAs were depicted. MIR376A seed sequence was shown in bold letters and and complementary sequences were indicated. (C and D) qPCR analysis of ATG4C and BECN1 mRNA expression in control miRNA (MIR-CONT) or MIR376A overexpressing cells under non-starved (NON STV) or starved (STV) condition (mean ± S.D. of independent experiments, n = 3, *p<0.05). (E and F) Immunoblots showing ATG4C and BECN1 protein levels following control miRNA (MIR-CONT) or MIR376A overexpression. ACTB was used as a loading control. ATG4C/ACTB or BECN1/ACTB densitometric ratios were marked.

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Figure 5. Effect of antagomirs on MIR376A target expression. (A and B) qPCR analysis of ATG4C and BECN1 mRNA levels in MCF-7 cells transfected with control antagomirs (CNT-Ant) or antagomir-376a (Ant-376a) (mean ± S.D. of independent experiments, n = 3, **p<0.03 for ATG4C, and n = 3, **p<0.03 for BECN1). Results were expressed as fold changes against GAPDH mRNA levels. (C and D) ATG4C and BECN1 protein levels were increased following antagomir-376a (Ant-376a) transfection. ATG4C/ACTB or BECN1/ACTB densitometric ratios were marked.

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autophagic vesicle nucleation sites, ATG4 is important for LC3 lipidation and in autophagic vesicle membrane elongation. In line with the importance of these proteins in autophagy control, both proteins were reported to be regulated by diverse signals and pathways. In fact, BECN1 was subject to regulation by transcriptional upregulation, protein-protein interactions, phosphorylation and ubiquitination.[19,20,21] ATG4 was also reported to be regulated in various ways including transcriptional upregulation and reactive oxygen species [22] Moreover, miRNAs other than MIR376 family were shown to target BECN1 or ATG4 as well. For example, miR-30a targeted BECN1 3'9 UTR but at a different and unrelated MRE. Similarly, ATG4 was targeted by autophagy-related MIR101.[23]

Although MIR376A and MIR376B genes are separated from each other by only hundreds of bases and their products are not identical but closely-related (Figure 1B and 1C). Hence, their cellular functions could be overlapping. Indeed, we have previously shown that MIR376B was also blocking starvation-induced autophagy through its effects on ATG4C and BECN1 levels. Yet, we observed in this study that kinetics of miRNA responses during starvation stress were divergent. While endogenous levels of MIR376A increased after 6 hours of starvation, MIR376B levels increased following a short starvation period, but declined rapidly. The observed difference in the response of MIR376A and MIR376B might be relevant to miRNA function during stress. Results presented here provide evidence that, the physiological function of these miRNAs is to control and limit the amplitude of autophagic activity which is strongly stimulated by starvation stress. A sequential accumulation of individual miRNAs might allow a sustained and a two-step control of the autophagic activity depending on the duration of stress. Since uncontrolled autophagic activity could be detrimental for cells, and under certain conditions lead to cell death [24] restriction of aberrant activation of autophagy by miRNAs under stressful conditions might prevent cellular demise and give cells a chance to recover a transient stress.

Data presented here point out to the presence of distinct control mechanisms resulting in dissimilar MIR376A and MIR376B response kinetics. Transcriptional and/or post-transcriptional regulatory mechanisms might be in the origin of these observa-

Figure 6. MIR376A controlled miRNA-response elements (MREs) in ATG4C and BECN1 mRNA 3' UTRs. Schemes showing ATG4C (A) and (B) BECN1 MRE (top), or their mutated versions (bottom) placed in the 3' UTR of the luciferase gene in the pGL3 vector. Mutated residues were italicized. MIR376A seed target sequence was underlined. (C and D) Normalized luciferase activity measurements of lysates from 293T cells that were cotransfected with either MIR-CNT or MIR376A plasmids together with the wild-type or mutant luciferase constructs (mean ± S.D. of independent experiments, n = 3 for ATG4C tests, *p<0.05 and n = 5 for BECN1 tests, ***p<0.01).

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Figure 7. Endogenous MIR376A limits starvation-induced autophagy. (A) Blockage of endogenous MIR376A by Ant-376a, but not CNT-Ant further stimulated starvation (STV)-activated LC3-I to LC3-II conversion in MCF-7 cells. ACTB was used as a loading control. LC3-II/LC3-I densitometric ratios were marked. (B) Ant-376a, but not CNT-Ant resulted in further activation of SQSTM1 protein degradation following starvation in MCF-7 cells. SQSTM1/ACTB ratios were marked.

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![Figure 8.](image)

Figure 8. **MIR376A and MIR376B level changes in response to starvation stress.** (A and B) TaqMan quantitative PCR (qPCR) analysis of endogenous MIR376A (A) and MIR376B (B) levels under control (CNT, no starvation) or starvation (STV, 1 to 8 hours) conditions. TaqMan qPCR data was normalized using U6 small nuclear 1 (RNU6-1) (U6) mRNA levels (mean ± S.D. of independent experiments, n = 5 for both A and B, ***p<0.01). doi:10.1371/journal.pone.0082556.g008

Autophagy serves as a cellular defense mechanism against various pathogens including viruses.[44,45] Not surprisingly, as a countermeasure, viruses evolved ways of modulating autophagy during productive and latent stages of the viral infection.[46] miRNAs of both viral and cellular origins were shown to affect cellular responses to viruses such as apoptosis, therefore, it is possible that autophagy-regulating miRNAs play a role in the battle between viruses and the host. In line with a possible role of miR-376 family members was reported to be abundant in the brain and uterus (Fig. S1).[30] A number of studies in the literature, showed the importance of MIR376A during development and differentiation-related events including erythroid differentiation [31], keratinocyte differentiation [32] and skeletal muscle development.[33] MIR376A was also shown to be upregulated during chemical and replicative senescence in human fibroblasts.[34]

Additionally, changes in MIR376 levels were observed under pathological conditions. While MIR376A was downregulated in esophageal cancer [35] and melanomas [36] and upregulated in salivary gland adenomas,[37] and pancreatic carcinomas,[38,39] MIR376B was differentially expressed in uterine leiomyomas [40] and renal cell carcinomas.[41] Moreover, changes in MIR376A levels could be used as a marker to distinguish acute myeloid leukemia subtypes, but MIR376B could serve as a breast cancer subtype marker.[42] Additionally, MIR376A and MIR376B were subjected to variable levels of editing in glioblastomas.[43]

Differential editing of MIR376 family members was reported in a number of tissues.[17] For example, while +44 site in the seed sequence of MIR376A was not edited in the mouse cortex, in the same tissue MIR376B was highly edited (around 50–60%) at this position.[17] Since editing in the seed region was shown to change target specificity of miRNAs including MIR376A and MIR376B,[17,29] in addition to relative ratios of MIR376A and MIR376B, ratios between edited versus non-edited versions of MIR376 family members might be important factors leading to changes in autophagy levels in different tissues and cells. Additionally, MIR376A might be compensating autophagy-related functions of MIR376B in tissues where the latter was predominantly edited and, vice versa. Overall, editing frequencies of MIR376 family members and their relative ratios might be critical factors determining the kinetics and the amplitude of autophagic responses in individual tissue and cell types. Further studies are required to analyze and integrate these observations to cellular and organismal autophagy responses.
revealed differential expression of MIR376A and MIR376B in control versus HIV-1 positive peripheral blood mononuclear cells.[47] In another study, an interesting interplay between viral miRNAs and MIR376A was reported.[48] MREs for MIR376A in the 3′ UTR of the MICB mRNA (a stress-induced ligand necessary for host cell recognition and destruction by natural killer cells) overlapped with the MRE of Kaposi's sarcoma-associated herpesvirus (KSHV) miRNA, miR-K12-7. The MIR376A MRE was also in the vicinity of that of miR-UL112 of human cytomegalovirus (HCMV). Surprisingly, when MIR376A was used in combination with KSHV or HCMV miRNA, no antagonism but an increase in target MICB downregulation was observed. In the light of our data, here contribution of MIR376A to viral infection might not be limited to the attenuation of immune responses through MICB downregulation, but it might also involve blockade of antiviral autophagic degradation, autophagy-related antigen presentation on MHC molecules and perhaps autophagic cell death. Indeed, KSHV was previously shown to downregulate autophagy, apoptosis and cell death using its viral FLIP and BCL-2 proteins that target autophagy-related proteins ATG3 and BECN1, respectively. [49,50] MIR376A and possibly other autophagy-regulating miRNAs might be usurped by viruses to overcome the antiviral effects of autophagy.

Altogether, our results underline the importance of MIR376A and MIR376B, and miRNAs in general, in the control of autophagic responses of cells and tissues. miRNA-mediated regulation provides a flexible and dynamic mechanism for the regulation of autophagy under various stress conditions, and adds another layer of regulation for critical cell death and survival decisions in health and disease.

Materials and Methods

Plasmid constructs

The pMSCV-blast-miR plasmids, containing either hsa-miR-376a1 human miRNA or control miRNA (hTR-human telomerase RNA), were constructed as described previously.[51] For luciferase tests, miRNA response elements (MRE) located in the 3′ UTR of BECN1 or ATG4C genes (ATG4C Genbank accession number: AK027773, bases 2383-2403, and BECN1 Genbank accession number: NM_003766, bases 2030-2051), or their mutant versions were cloned into the 3′ UTR region of the luciferase gene in the pGL3 vector (Promega, E1741) as previously described.[10] ATG4C plasmid was purchased from Origene (SC126496). BECN1 cDNA ORF was cloned into the pcDNA3 mammalian expression plasmid using RT-PCR.

Cell culture and transfection

Dulbecco’s modified Eagle’s medium (DMEM; Sigma, D5671) supplemented with 10% (v/v) fetal bovine serum (FBS; Biochrom KG, S0115) and antibiotics (Penicillin/Streptomycin; Biological Industries, 03-031-1B) were used to culture MCF-7 human mammary carcinoma cells, Huh7 human hepatocellular carcinoma cells and 293T human embryonic kidney cells in a 5% CO2-humidified incubator at 37°C. Cells were cultured in Earl’s balanced salt solution (EBSS; Biological Industries, B102-010-1A) to activate starvation-induced autophagy. For autophagic flux analyses, cells were cultured in the absence or presence of lysosomal protease inhibitors E64d (10 μg/ml) (Santa Cruz, SC201280A) and pepstatin A (10 μg/ml) (Sigma, P5318).

Polyethyleneimine (PEI; Polysciences Inc., 29966) transfection method was used to transiently transfect MCF-7 and Huh7 cells.[52] For transfection of 293T cells, calcium phosphate co-precipitation method was used according to standard protocols.

Quantitative GFP-LC3 analyses

48h post-transfection, GFP-LC3 dot positivity was quantified following 2 hours (MCF-7 cells) or 4 hours (Huh7 cells) starvation in the EBSS medium. 10 or 15 GFP-LC3 dots per cell were considered as a threshold for the basal autophagic activity in MCF-7 and Huh7 cells, respectively. Minimum 150 GFP positive cells were counted under each condition, and percentage of GFP-LC3 positivity was expressed as a percentage of GFP-LC3 dot positive cells within the total transfected cell population.

miRNA target prediction

Bioinformatics tools, Microcosm Targets (http://www.ebi.ac.uk/entrez-srv/microcosm/cgi-bin/targets/v3/search.pl), and MiRanda (http://www.microrna.org/microrna/home.do) were utilized to determine miRNA potential target miRNAs.

Immunoblotting and antibodies

Protein extracts from cells were prepared and immunoblotted as previously described [53] using antibodies specific to BECN1 (Santa Cruz, sc-11427), LC3B (Novus, NB100-2331), ATG4C (Sigma-Aldrich, AB75056), SQSTM1 (Abnova, H0000878) and ACTB (Sigma-Aldrich, A5441). ImageJ software was used to quantitatively determine band intensities.[54]

RNA isolation, RT-PCR analysis and Real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Sigma-Aldrich, T9424) according to the manufacturer’s instructions. SYBR® Green Quantitative RT-PCR kit (Roche, 04-913-914-001) was utilized for single step qRT-PCR reactions. The 2-ΔΔCt method was applied for the quantification of miRNA changes, and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) miRNA was used as control. Primers used in the study were: BECN1 primers 5′-AGGTTGAGAAGCAGGACACA-3′; 5′-GCTTTGTGTCCTGCTTC-3′; ATG4C primers 5′-GCATAAAGGATTCCCC-TCTTGA-3′; 5′-GCTGGGATCCATTTTTCG-3′, and GAPDH primers 5′-AGCCCAATCGCTGAGCACAG-3′; 5′-GCCCACATAAGCAGATTTCC-3′. Reactions were performed in duplicates in independent experiment repeat numbers (n) were marked.

Endogenous miRNA quantification by TaqMan RT-qPCR

FastStart Universal Probe Master kit (ROCHE, 04919957001) and iCycler iQ thermal cycler (BioRad) was utilized for TaqMan qPCR reactions. Reactions were previously described.[11] Primers used in this study were: Stemloop primer for MIR376A: 5′-GTGCTGATATCCAGTGCAAGGTTGCTGAGTACTG-GATACGACAGGTGATTTTGCTGATCTATGAT-3′; Forward primer for MIR376A: 5′-ATTAATCATAGAGGAAATC-CAAG-3′; Reverse primer for MIR376A: 5′-GTGCGAGGTCGGAGGT-3′; Probe for MIR376A: 5′(6-FAM)-TGACGACTGAGA-TAAGAACCATTCA-GATATC-3′. Primers for U6 small nuclear 1, and MIR376B were previously described.[10]

Dual luciferase reporter assay

Firefly and renilla activities in cell extracts were measured using dual-luciferase reporter assay system (Promega, E1910) according to manufacturer’s instructions. Results were expressed as firefly luciferase activity normalized to renilla luciferase activity and analyzed as described previously.[10,11]

Antagomir tests

miRIDIAN® microRNA Hairpin Inhibitors (antagomirs) against hsa-miR-376a1 (IH-300683-03-0005) and a control antagomir (miRIDIAN microRNA Hairpin Inhibitor Negative
Control (IN-001005-01-05) were purchased from Dharmacon. Antagomir transfections (200 nM) were performed using the PEI transfection method.[52]

Statistical analyses
Statistical analyses were performed using Student’s two-tailed t-test. Data were represented as means of ± SD of n independent experiments. Values of p<0.05 were considered as significant.

Supporting Information
Figure S1 Data extracted and analyzed using miRNA body map online website (http://www.mirnabodymap.org/)

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Author Contributions
Conceived and designed the experiments: DG. Performed the experiments: GK KAT. Analyzed the data: GK KAT DGO OUS DG. Contributed reagents/materials/analysis tools: AK OUS. Wrote the paper: KAT DG.
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