

Role of Autophagy as a Survival Mechanism for Hypoxic Cells in Tumors^{1,2}



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Abstract

Enhanced autophagy has been observed in hypoxic regions of solid tumors. Here we address the hypothesis that autophagy is required for survival of hypoxic cells. We evaluated sensitivity to hypoxia of three human tumor cell lines (MCF7, PC3, and LNCaP) and their autophagy-deficient variants with shRNA knockdown of the genes ATG7 and BECLIN1. Hypoxia-induced cell death was more rapid for autophagy-deficient cells and was increased in the presence of the proton pump inhibitor pantoprazole that inhibits autophagy. Autophagy-deficient cells had a lower rate of oxygen consumption than wild-type cells. In xenografts derived from the three cell lines, autophagy (as determined by increased LC3 and reduced p62/SQSTM1) colocalized with hypoxic regions (identified by EF5). Xenografts derived from autophagy-deficient cells grew more slowly than wild-type tumors. Both LC3 expression and hypoxia were decreased in xenografts generated from single-knockdown cells and absent in double-knockdown tumors. Our results are consistent with the hypothesis that autophagy facilitates the survival of hypoxic cells, although reduced oxygen consumption of autophagy-deficient cells may contribute to lack of hypoxia in tumors derived from them. Because hypoxia is associated with resistance to anticancer therapy, inhibition of autophagy has potential to enhance the effectiveness of cancer treatment.

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Introduction

Tumor hypoxia is associated with a decrease in the effectiveness of radiation therapy and chemotherapy, and with a poor prognosis [1]. Autophagy is a cellular mechanism used to digest old or damaged cellular constituents into component residues, which may be recycled to generate essential macromolecules. All cells undergo autophagy, but it is upregulated in stressed cells such as those with nutrient or growth factor depletion; such conditions are common in the microenvironment of solid tumors, and autophagy colocalizes with regions of hypoxia [2]. In cancer development, autophagy has been shown to have a dual role [3]: in some studies, autophagy promotes tumorigenesis, but in others, autophagy is a tumor-suppressive mechanism.

Autophagy involves the formation of autophagosomes, which have a double membrane enclosing cytoplasmic cellular components; this then fuses with a lysosome to produce a mature autolysosome in which cellular proteins are degraded by cathepsins [4]. A series of autophagy-related proteins (known as ATGs) is responsible for the

induction and regulation of autophagy [5], and some of them can be used as markers of autophagy that can be quantified in Western Blots or by immunohistochemistry (IHC) applied to tumor sections. *Beclin 1*, the mouse homolog of yeast *ATG6*, encodes a Bcl-2–interacting candidate tumor suppressor and antiviral protein [6]. Molecular alterations in Beclin 1 are common in human cancers, and *beclin 1*

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gene knockouts in mice cause a marked increase in epithelial and hematopoietic malignancies [7]. Beclin1 and its binding partner class III phosphoinositide 3-kinase are required for vesicle nucleation and the formation of autophagosomes in the early phase of autophagy [8]. During autophagosome maturation, microtubule-associated protein light-chain 3 (LC3-I) is cleaved and then conjugated with phosphatidylethanolamine into LC3-II, a process mediated by the proteins ATG7 and ATG3 that mediate later stages of autophagy [9]. Lipidated LC3-II is bound to the membrane of the autophagosome until fusion with the lysosome is complete. It is then broken down and recycled. Thus, LC3-II acts as a biochemical marker for induction of autophagy, although agents that inhibit later stages of autophagy, including the acidification of lysosomes or their fusion with autophagosomes, will prevent breakdown of LC3-II and lead to increased levels of this protein [10]. An additional autophagy marker, p62/SQSTM1 (p62), is recruited with LC3-II to autophagosomes and degraded within the mature autolysosome [11]. Thus, observation of increased p62 is indicative of a build-up of the protein due to inhibition of lysosomal fusion to the autophagosome, i.e., to inhibition of autophagy.

Hypoxia occurs in tissue when the oxygen demand exceeds oxygen supply. Tumor hypoxia often correlates with poor outcome, and although hypoxic cells in tumors eventually die, there is evidence that they are resistant to apoptosis [12]. The mechanisms of hypoxia-induced cell death remain unclear because apoptosis, necrosis, and autophagy have all been reported in response to hypoxia [13]. Hypoxia has been shown to induce autophagy in different cellular settings [14], and autophagy might act as a survival mechanism for hypoxic cells through recycling of cellular constituents. Pharmacological inhibition of autophagy has been shown to enhance apoptosis and cell death under hypoxic conditions [15]. Autophagy levels were found to correlate with the expression of hypoxia-inducible factor 1 α and have been associated with early invasion in colon cancer [16]. Recent reports also showed that hypoxia is able to upregulate autophagy, leading to cell survival and resistance to anticancer therapies [17].

Agents which inhibit endosomal acidification, including (hydroxy)chloroquine and proton pump inhibitors such as pantoprazole, lansoprazole, and omeprazole, can suppress the later stages of autophagy [18,19], thereby inhibiting breakdown of LC3-II. Some studies, including those from our laboratory, have shown that proton pump inhibitors can sensitize cancer cells and solid tumors to different chemotherapeutic agents [20,21]. We have also shown that treatment of cancer cells *in vitro* and of solid tumors in mice with a wide variety of anticancer agents induces autophagy, suggesting that it is a common survival mechanism for drug-treated cells and therefore an important cause of drug resistance. It is not known how hypoxia and autophagy interact to modulate cancer cell response to hypoxia-induced or chemotherapy drug-induced cell death. The aim of the present study was to understand the implications of hypoxia and autophagy in tumors and to address the hypothesis that autophagy is a survival mechanism for hypoxic cells.

Methods

Cell Lines

Human prostate cancer PC3 and LNCaP cells and human breast carcinoma MCF7 cells were purchased from the American Type Culture Collection (Manassas, VA) in 2011. PC3 and LNCaP cells

were grown in Ham's F-12K medium (Life Technologies Inc, Burlington, ON, Canada); MCF7 cells were grown in α -minimum essential medium supplemented with 10% FBS (Hyclone, Logan, UT). All cells were cultured in a humidified atmosphere of 95% air/5% CO₂ at 37°C, and experiments were performed on fourth and fifth passages generated from frozen stock. Routine tests to exclude mycoplasma in all cell lines were conducted at least once per year. Short tandem repeat analysis was conducted to ensure that cells (PC3, LNCaP, and MCF-7) were of human origin in May 2013.

Generation of Autophagy-Deficient Cells

Lentiviral shRNA (ATG7 and BECLIN1) constructs were purchased from Open Biosystems (RMM4534_019584 and RMM4534_028835) (Ottawa, ON, Canada). ATG7 and BECLIN1 shRNAs were transfected into PC3, LNCaP, or MCF7 cells either alone or together with packaging plasmids following the manufacturer's protocol (Invitrogen ViraPower Lentiviral Expression Systems kit, Carlsbad, CA). Cells were passaged and plated in a six-well plate and allowed to adhere for 24 hours before infection. The silencing efficacy of the various shRNAs was assessed by Western blot analysis of ATG7 and BECLIN1 proteins using polyclonal antibodies (Sigma-Aldrich, Oakville, ON, Canada).

Cell Survival under Hypoxic Conditions

PC3, LNCaP, and MCF7 cells (wild type and autophagy deficient) were plated at a density of 1×10^5 cells in 10-cm³ plates and cultured for 24 hours in RPMI or alpha-MEM supplemented with 10% FBS. Cells were then exposed to aerobic conditions (20% oxygen) or hypoxia (0.2% oxygen) for 0, 12, 24, 36, and 48 hours. For exposure to hypoxia or control (aerobic) conditions, cells were incubated in a humidified HypOxygen H35 workstation; the atmosphere in the chamber consisted of 5% H₂, 5% CO₂, the desired % O₂, and residual N₂. Samples were removed as a function of time, centrifuged, washed, and plated in serial dilution in plastic tissue culture dishes. Colonies generated 8 to 14 days later were stained with methylene blue and counted.

The short-term MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium CellTiter 96 Aqueous one Solution Cell proliferation Assay; Promega Corporation, Madison, WI) was used to evaluate the effects of pantoprazole on viability of PC3, LNCaP, and MCF7 cells exposed under aerobic or hypoxic (0.2% oxygen) conditions. Pantoprazole (PANTO IV) was purchased from Sanofi Inc. (Laval, Quebec, Canada) as a lyophilized powder and dissolved in 0.9% saline. Cells were seeded at 2000 to 4000 cells per well, depending on the optimal conditions for each cell line, into 96-well plates and incubated overnight. Cells were treated with or without pantoprazole (100 μ M) under aerobic conditions or 0.2% oxygen for 24 hours. After 24-hour treatment, medium was removed and the cells were washed twice with PBS. Cell viability was determined according to the manufacturer's protocol.

Evaluation of Autophagy by Western Blot Analysis

Wild-type and autophagy-deficient PC3, LNCaP, and MCF7 cells treated with or without pantoprazole under aerobic or hypoxic conditions for 24 hours were lysed in RIPA buffer and centrifuged at 13,000g at 4°C for 30 minutes to collect the supernatant. Protein concentration was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). A total of 20 μ g of protein

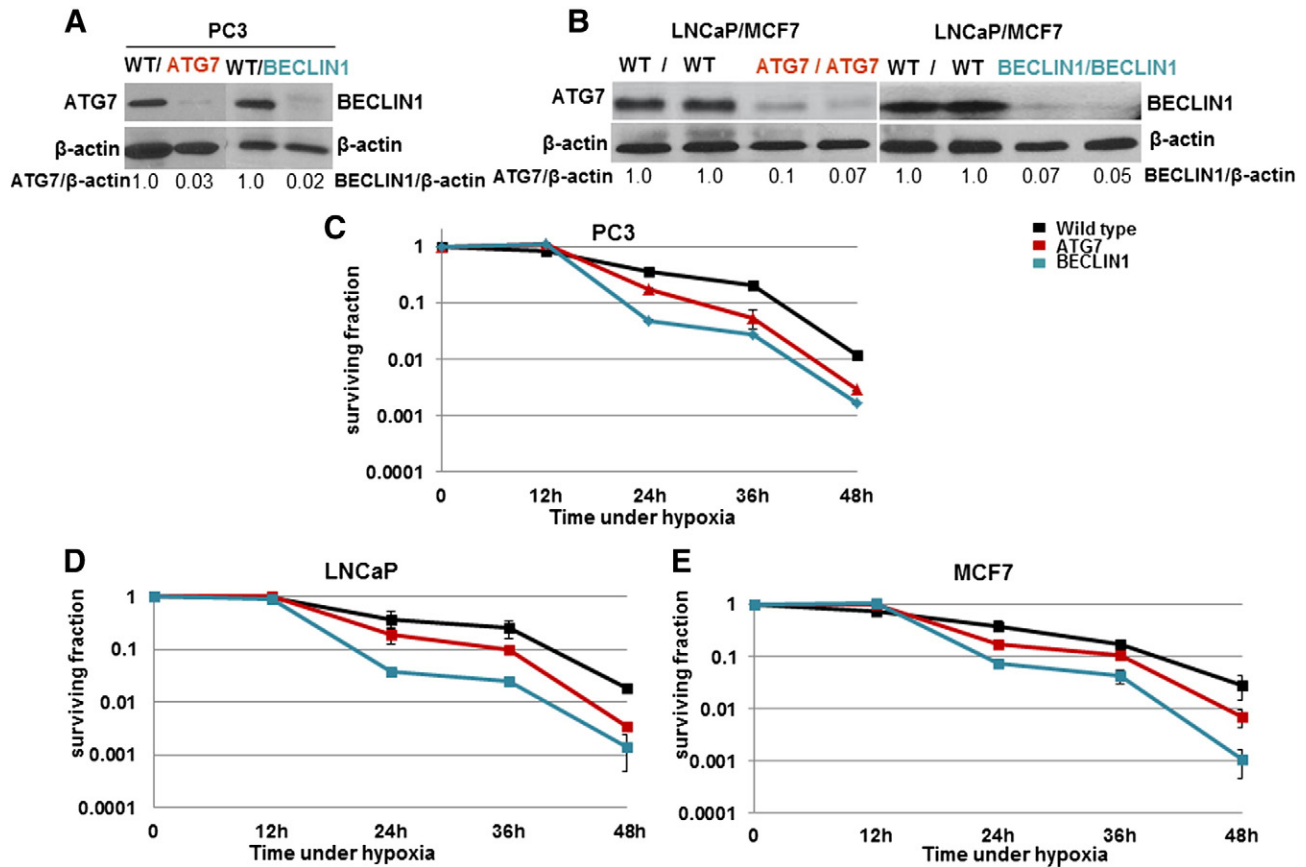


Figure 1. (A and B) Western blots to confirm knockdown of ATG7 and BECLIN1 in each cell line (numbers indicate relative ATG7 and BECLIN1 protein level compared with wild-type (WT) cell line). The surviving fraction of PC3 (C), LNCaP (D), and MCF7 (E) WT and autophagy-deficient cells as a function of time under hypoxic conditions as determined by a colony-forming assay (zero time indicates control aerobic condition). Data represent mean \pm SEM ($n = 3$). For each cell line, differences between curves for WT and each autophagy-deficient cell line are significant ($P < .001$).

were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Etobicoke, ON, Canada). The membrane was washed in Tris-buffered saline and Tween 20 and blocked with 5% skim-milk + 2% FBS in Tris-buffered saline and Tween 20. Membranes were incubated with primary antibodies overnight at 4°C followed by appropriate horseradish peroxidase-conjugated secondary antibodies. Protein concentration in the supernatant was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) to quantify LC3-II and p62. Protein levels were quantified using Image Pro software (Version premier 9).

Xenograft Experiments

Animal experiments were carried out using Animal Use Protocol (AUP1232.15, 09/05/14) approved by Princess Margaret Cancer Centre, University Health Network Animal Care Committee under the guidelines of the Canadian Council on Animal Care.

To generate xenografts, 4- to 6-week-old male athymic nude (Nu/Nu) mice (Jackson Laboratory, Bar Harbor, Maine) were injected subcutaneously in both flanks with 2×10^6 wild-type or autophagy-deficient PC3 or LNCaP cells. Four- to 6-week-old female athymic nude (Nu/Nu) mice (Harlan Sprague-Dawley, Madison, WI) with implanted 17 β -estradiol tablets (60-day release; Innovative Research of America, Sarasota, FL) were injected

subcutaneously with 5×10^6 wild-type or autophagy-deficient MCF7 cells per side.

Mice bearing xenografts were divided into groups of six mice to measure tumor growth rate. Two perpendicular diameters of tumors growing in the flanks of mice were measured with a caliper every 2 to 3 days. Measurements were taken until tumors reached a maximum diameter of 1.5 cm or began to ulcerate, when mice were killed humanely. Tumor volume was estimated using the formula $0.5(ab^2)$, where a is the longest diameter and b is the shortest diameter. To verify that xenografts generated from autophagy-deficient cells retained their phenotype, RNA was extracted from mature xenografts using TRI Reagent with DNase I digestion of contaminating DNA. cDNA was synthesized using a reverse transcriptase kit. Primer-probe sets for quantitative polymerase chain reaction (qPCR) and PCR master mix were supplied by Sigma (Oakville, ON, Canada). Samples were quantified against serial dilutions of standards of plasmids containing the target sequences to provide copy number.

At the end of the growth rate assay, tumors were excised for IHC to detect hypoxia and other biomarkers in sections of xenografts (mean cross-section area for wild-type tumors was 1.2-1.3 cm², ATG7 and BECLIN1 knockdown tumors were 0.5-0.6 cm², double-knockdown tumors were 0.2-0.3 cm²). Wild-type tumors might have more hypoxia because of their larger size. To avoid bias, we therefore also measured the hypoxic fraction in smaller wild-type tumors with mean

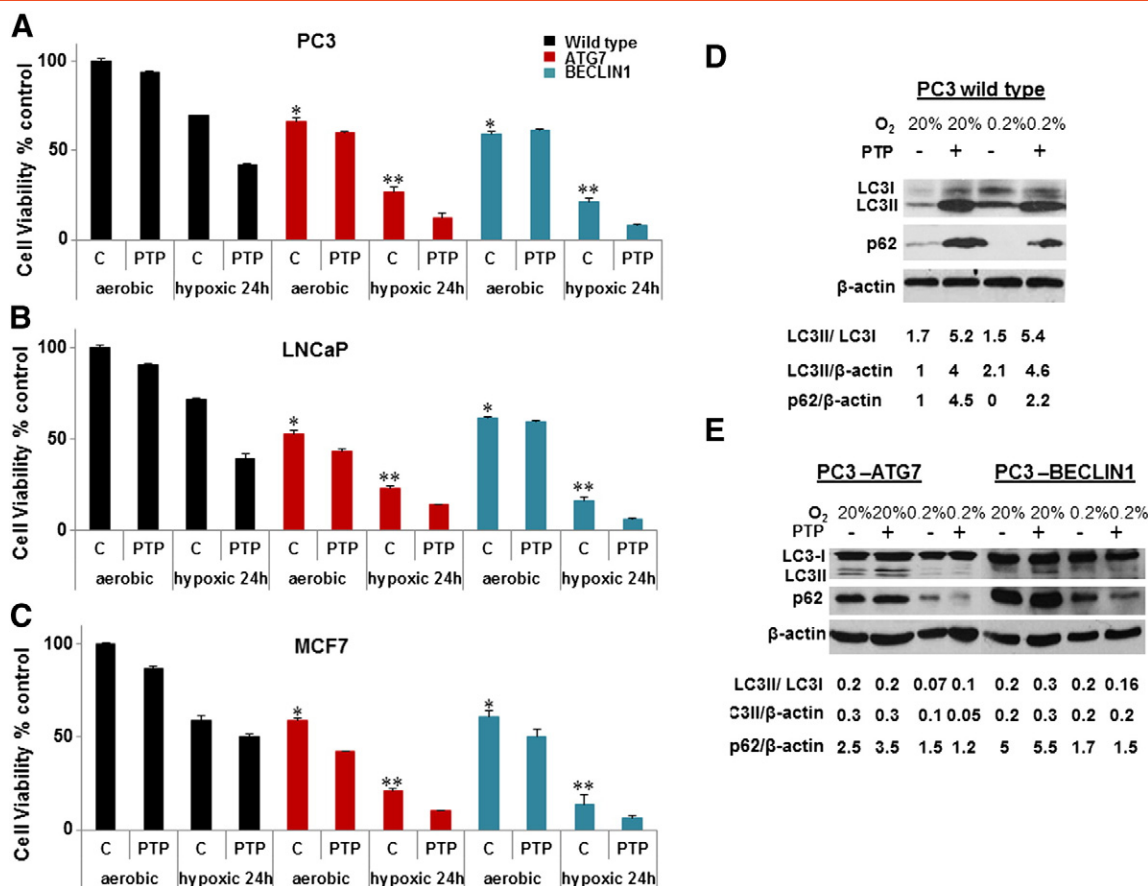


Figure 2. Effect of pantoprazole on viability of WT and autophagy-deficient PC3 (A), LNCaP (B), and MCF7 (C) cells following 24-hour exposure to aerobic or hypoxic conditions as evaluated by the MTS assay. Data represent mean \pm SEM ($n = 3$). *Significant difference ($P < .01$) between autophagy-deficient and WT cells under aerobic conditions. **Significant difference ($P < .005$) between autophagy-deficient and WT cells under hypoxic conditions. The difference in viability of hypoxic cells in the presence or absence of pantoprazole is significant ($P < .05$). (D and E) Effect of pantoprazole on expression of LC3-II and p62 by Western blot assay. (LC3-II, p62, and β -actin intensities were normalized to control.)

cross-sectional area of 0.4–0.6 cm², similar to that of tumors grown from the single-knockdown cells

The hypoxia marker EF5 was injected intraperitoneally approximately 2 hours before killing the mice (0.2 ml of a 10-mM stock per mouse). EF5 was provided by the National Cancer Institute as a powder and then dissolved in distilled water supplemented with 2.4% ethanol and 5% dextrose to make a 10-mM stock solution that was stored at room temperature. To detect functional blood vessels, the perfusion marker DiOC7 (1 mg/kg) was injected intravenously 1 minute before killing the mice. DiOC7 was purchased from AnaSpec (San Jose, CA), and a stock solution (2.5 mg/ml) was made by dissolving in dimethyl sulfoxide; this stock was diluted 1:10 in phosphate-buffered saline and 10% Solutol HS 15 (Sigma-Aldrich, Oakville, ON, Canada). Tumors were excised, embedded in OCT compound, frozen in liquid nitrogen, and stored at -70°C . Whole cryostat sections (10 μm thick) were analyzed, and artifacts and regions of necrosis were excluded. Tumor sections were first imaged for DiOC7 using a FITC filter set. Sections were then stained for hypoxic regions using a Cy5-conjugated mouse anti-EF5 antibody (1:50) purchased from Dr. Cameron Koch, University of Pennsylvania, Philadelphia, PA. Hypoxic regions were quantified by calculating the area of pixels with anti-EF5 fluorescence and dividing this region by the area of the entire tumor section (artifacts and regions of necrosis were omitted).

The same tumor sections were also stained with appropriate antibodies to the biomarkers LC3A and p62 (to quantify autophagy) and Ki-67 (to quantify cell proliferation). Ki-67 was identified with a primary rabbit anti-human Ki-67 antibody (Novus Biologicals, Oakville, ON, Canada). LC3A was recognized with a rabbit anti-human LC3A primary antibody, and p62 was recognized with a rabbit anti-human p62 primary antibody (ABGENT, San Diego, CA). Application of all primary antibodies was followed by Cy3-conjugated goat anti-rabbit IgG secondary antibody, and the sections were imaged with a Cy3 filter set (530–560 nm excitation/573–746 nm emission) using an Olympus fluorescent upright microscope. Image analysis and quantification of biomarker distribution in relation to blood vessels and regions of hypoxia were performed as described previously [22].

Measurement of Basal Oxygen Consumption Rate

Oxygen consumption rates were measured using Seahorse Biosciences extracellular flux analyzer (XFe 96, North Billerica, MA). Cells were seeded at 2.3×10^4 cells per well (0.32 cm²) in XFe 96 plates in 250 μl of Dulbecco's modified Eagle's medium (10% FBS, 1% Pen-Strep) and incubated for 24 hours at 37°C and 8.5% CO₂ before XF assay. Oxygen consumption was measured under basal conditions, in the presence of the mitochondrial inhibitor

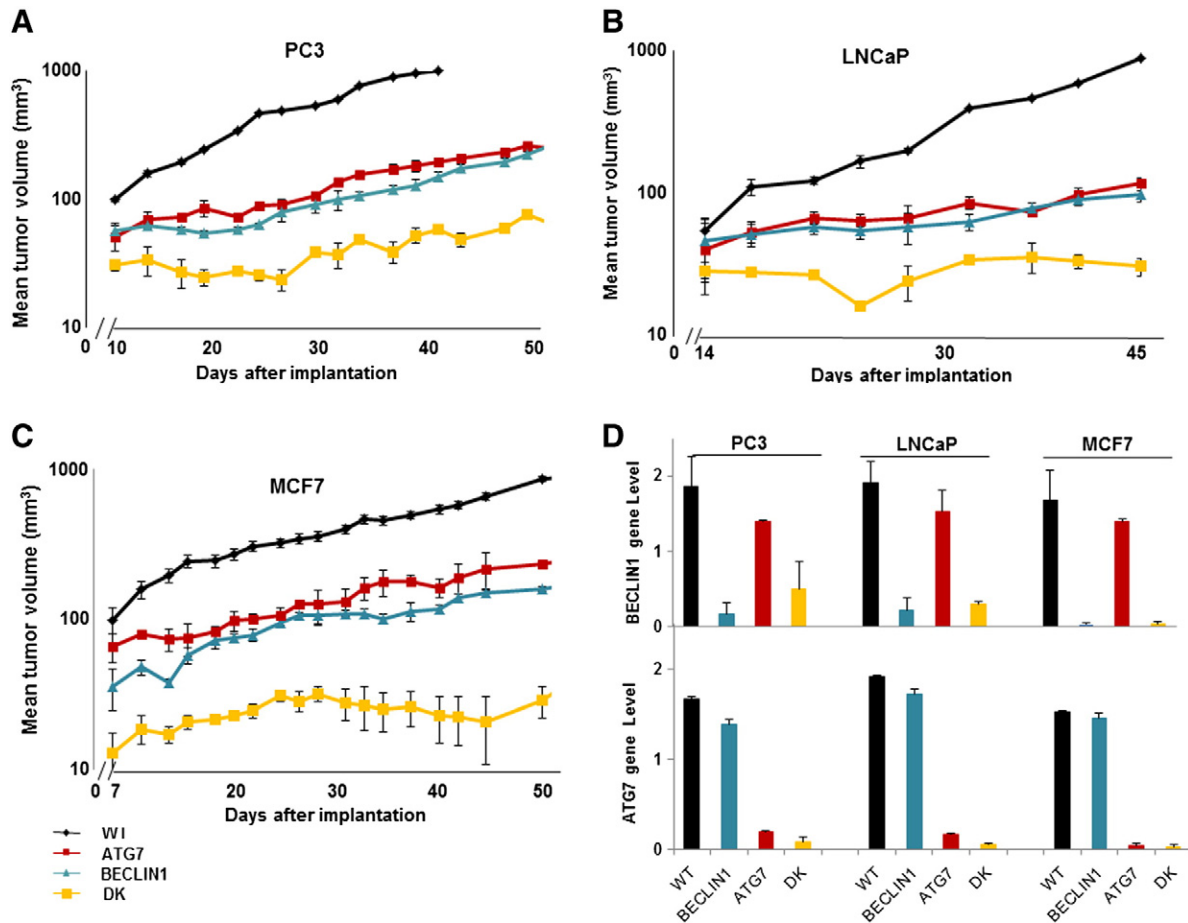


Figure 3. Growth curves for PC3 (A), LNCaP (B), and MCF7 (C) WT and autophagy-deficient xenografts (10-12 tumors in 6 mice per group, experiments were repeated twice). (D) qPCR was used to confirm knockdown status of tumors generated from autophagy-deficient cells of the indicated genotypes (WT represents wild type; DK represents double knockdown). Data represent mean \pm SEM, $n = 3$.

oligomycin (0.5 μ M), or in the presence of the mitochondrial uncoupler p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP, 1 μ M) to assess maximal oxidative capacity.

Statistical Analysis

One-way analysis of variance followed by Tukey's *post hoc* test determined statistical differences between treatment groups. For two-group comparisons, the Student's *t* test was used. $P < .05$ was used to indicate statistical significance. All experiments were repeated at least three times. Drug and biomarker distributions are represented as mean values \pm SEM.

Results

Autophagy-Deficient Cells More Sensitive to Hypoxia

The silencing efficacy of the various shRNA was assessed by western blot analysis of ATG7 and BECLIN1 proteins (Figure 1, A and B). Double-knockdown cells were also generated, but they did not grow in cell culture, although they did generate xenografts (see below). The survival of wild-type and autophagy-deficient PC-3, LNCaP, and MCF7 cells under hypoxic conditions (relative to aerobic conditions) is shown in Figure 1, C-E. All three cell lines had a decrease in cell survival under hypoxic conditions after 24 to 48 hours of exposure, and this hypoxia-induced cell death was more

rapid for autophagy-deficient cells, with the BECLIN1 knockdown cells being most sensitive ($P < .001$ for each comparison).

To obtain further evidence about the role of autophagy in hypoxic cells, we used the short-term MTS assay to evaluate viability of wild-type and autophagy-deficient cells exposed for 24 hours to aerobic or hypoxic conditions in the presence or absence of pantoprazole, an inhibitor of the late stages of autophagy. The knockdown cells had lower viability than the wild-type cells ($P < .01$ for ATG7 or BECLIN1 knockdown compared with wild type) under aerobic conditions but relatively greater loss of viability under hypoxic conditions (Figure 2, A and C). Pantoprazole (100 μ M) was found to reduce viability in the hypoxic cell population ($P < .05$) as well as under aerobic conditions ($P < .05$, Figure 2, A and C), suggesting that autophagy is a cytoprotective mechanism.

The influence of pantoprazole on expression of the autophagy markers LC3-II and p62 in PC3 wild-type cells under aerobic and hypoxic conditions is shown in Figure 2D. Hypoxia induced ~two-fold accumulation of LC3II and degradation of p62 consistent with induction of autophagy. Pantoprazole, which inhibits autophagy and leads to inhibition of breakdown of LC3-II and p62 degradation [23], increased LC3-II and p62 under both hypoxic and aerobic conditions. Figure 2E shows parallel results for PC3 knockdown cells deficient in ATG7 and BECLIN1. As expected, the Western blots indicate decreased levels of LC3-II and increased p62 under all

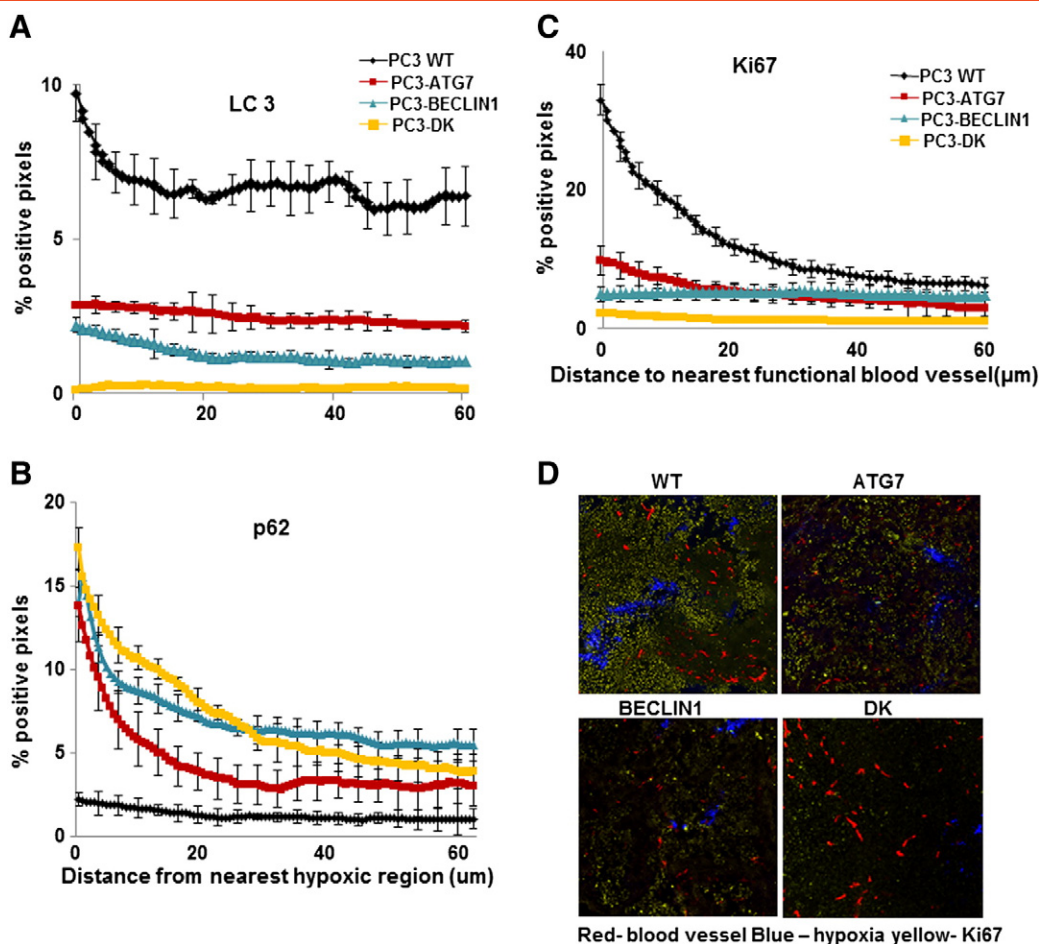


Figure 4. (A and B) LC3 and p62 in relation to hypoxic regions in WT, ATG7, BECLIN1 knockdown, and double-knockdown (DK) PC3 xenografts. (C) Ki67 in relation to functional blood vessels (indicated by DiOC7) in WT and knockdown PC3 xenografts. (D) Photomicrographs of Ki67 (yellow) in relation to functional blood vessels (recognized by DiOC7 red) and hypoxia (recognized by EF5 blue) in WT and knockdown xenografts. Means and SEs are for 10 tumors per group; bar, SE. Differences between WT and autophagy-deficient xenografts are significant ($P < .0001$).

conditions with no significant added effect of pantoprazole. Western blots to evaluate LC3-II in LNCaP and MCF7 cells showed similar results (data not shown).

Xenografts Generated from Wild-Type and Autophagy-Deficient Cells

Xenografts generated from the autophagy-deficient PC3, LNCaP, and MCF7 cells grew more slowly than those derived from wild-type cells (Figure 3, A and C). The double-knockdown xenografts had the slowest growth ($P < .005$ compared with wild type), whereas there was no significant difference between the growth curves for xenografts derived from ATG7 and BECLIN1 knockdown cells for any of the three human tumor xenografts. When animals were killed at the end of the growth experiment and their tumors were isolated and freshly prepared, qPCR was used to confirm their genotype (Figure 3D).

We used IHC to quantify the markers of autophagy LC3 and p62 in relation to the closest functional blood vessel (recognized by DiOC7, data not shown) and closest region of hypoxia (recognized by EF5) in PC3 wild-type and autophagy-deficient xenografts (Figure 4, A and B). LC3 expression was maximal in hypoxic and poorly nourished regions in wild-type tumors, markedly decreased in those generated from ATG7 and BECLIN1 knockdowns, and absent in

double-knockdown tumors. As expected, p62 showed least expression in wild-type xenografts and maximal expression in the double-knockdown tumors. We also used IHC to evaluate the spatial distribution of the proliferation marker Ki67. Cell proliferation decreased with increasing distance from functional blood vessels in wild-type tumors, but consistent with their slower growth rate, expression of Ki67 was reduced in xenografts generated from single- and double-knockdown PC3 cells (Figure 4, C and D).

The proportion of hypoxia was compared in sections of xenografts of the three cell lines excised at the end of the growth rate experiment where median volumes of the excised single- and double-knockdown xenografts were ~40% and ~15%, respectively, relative to that of the wild-type xenografts (Figure 5A). The proportion of hypoxia in tumors derived from knockdown cells with that in smaller wild-type tumors of similar volume is shown in Figure 5B. Hypoxic regions (recognized by EF5) were reduced in the autophagy-deficient xenografts, with minimal hypoxia in the double-knockdowns of all three cell lines.

Oxygen Consumption by Wild-Type and Autophagy-Deficient Cells

Measurements of oxygen consumption rates of wild-type and autophagy-deficient PC3, LNCaP, and MCF7 cells are shown in

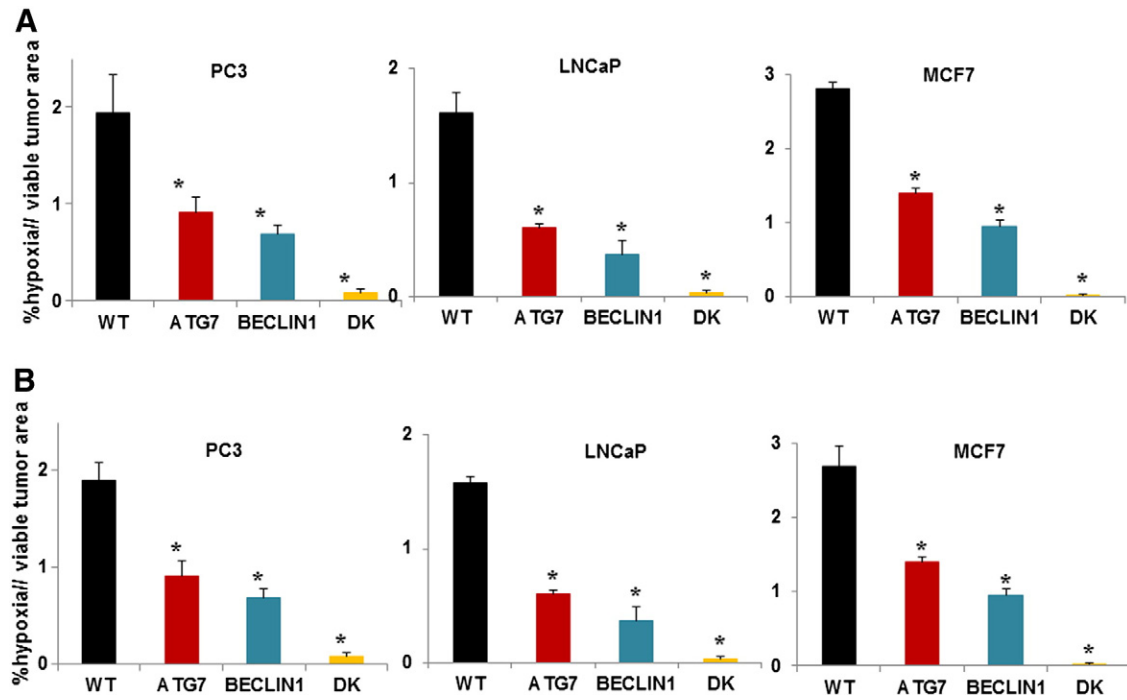


Figure 5. Proportion of hypoxia in WT and knockdown xenografts as a proportion of viable tissue in tumor sections. (A) Tumors were excised at the end of the growth rate experiment (mean cross-section areas: for WT tumors 1.2-1.3 cm², ATG7 and BECLIN1 knockdown tumors 0.5-0.6 cm², double-knockdown tumors 0.2-0.3 cm²). (B) WT xenografts were excised at smaller size (mean cross sectional: 0.4-0.6 cm²). Means and SEs are for 10 tumors per group; bar, SE. Differences between WT and autophagy-deficient xenografts are significant ($P < .001$).

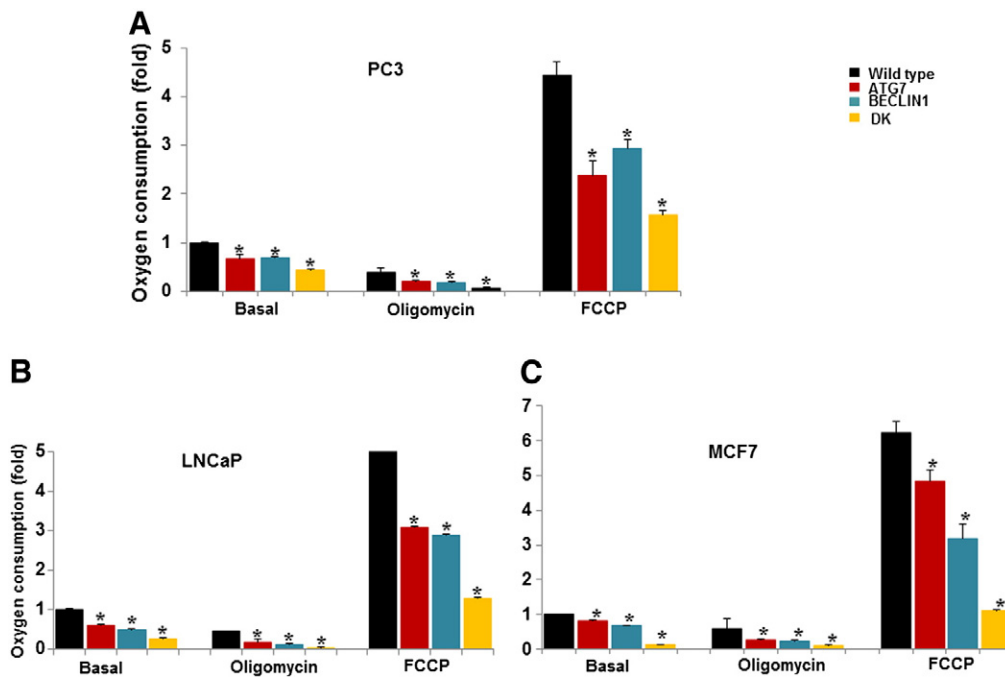


Figure 6. Oxygen consumption rates in WT and knockdown cells (A) PC3, (B) LNCaP, and (C) MCF7. Shown is the mean fold difference \pm SEM in oxygen consumption under basal conditions and following the addition of oligomycin (0.5 μ M) or the pharmacological uncoupler FCCP (1 μ M) (WT basal oxygen consumption rates = 1) obtained from experiments each performed in triplicate. Differences between WT and single-knockdown cells are significant ($P < .05$). Differences between WT and double-knockdown cells are significant ($P < .001$).

Figure 6. Respiration was measured under control conditions, in the presence of the ATPase inhibitor oligomycin to block respiration, and in the presence of the uncoupler FCCP to induce maximum respiration. Autophagy-deficient cells had significantly lower rates of oxygen consumption under both basal and modified conditions.

Discussion

Results in the present paper show that if autophagy is inhibited by RNA interference of the key regulators ATG7 or BECLIN1 or by treatment with pantoprazole, different types of human tumor cells die more quickly when exposed to hypoxic conditions in tissue culture (Figures 1 and 2). Autophagy-deficient cells also consumed less oxygen than wild-type cancer cells (Figure 6) consistent with previous reports that silencing of ATG7, ATG5, and BECLIN1 promotes the accumulation of dysfunctional mitochondria and low oxygen consumption in prostate, lung, and pancreatic cancers [24,25]. Xenografts derived from autophagy-deficient cells grew more slowly than those derived from wild-type cells and had lower rates of cell proliferation, consistent with their lower rate of oxidative metabolism (Figures 3 and 4C). The autophagy marker LC3 was increased (and p62 decreased) in hypoxic regions of wild-type PC3 tumors, expression of LC3 was markedly reduced (and p62 increased) in xenografts derived from single-knockdown cells, and LC3 was not detected (and p62 was maximal) in those derived from double-knockdown cells (Figure 4, A and B). Moreover, the proportion of hypoxia in tumor sections was reduced in xenografts derived from single-knockdown cells, and there was no detectable hypoxia in xenografts derived from double-knockdown cells (Figure 5). These data support the hypothesis that autophagy is a survival mechanism for hypoxic cells, although the reduced oxygen consumption of autophagy-deficient cells may contribute to reduced hypoxia in tumors generated from them.

The development of hypoxic regions within tumors promotes resistance against radiotherapy and chemotherapy and is associated with poor outcome of human tumors, even when treated by surgery. Likewise, autophagy, a process that allows recycling of constituent molecules in cells, has been associated with resistance to therapy and poor outcome. Although basal autophagy occurs in most cells, autophagy is activated by various stresses such as nutrient depletion (including hypoxia) or treatment with chemotherapy [26]. Markers of autophagy are increased in many types of cancer, and most evidence suggests that they function to promote survival of tumor cells. It has been shown that autophagy is induced in hypoxic regions of tumors where it promotes survival [13]. Further studies have confirmed the molecular connections between hypoxia and the induction of autophagy. Hypoxia-inducible factor 1 α is a positive regulator of autophagy; stimulates tumor metabolism, angiogenesis, and metastasis; and is associated with resistance to therapy in hypoxic tumors [27]. Induction of BNIP3 and BNIP3L in hypoxic cells disrupts the BECLIN1-BCL-2 complex, thereby releasing BECLIN1 to induce autophagy, and BNIP3-induced autophagy is an adaptive survival response during hypoxia [14].

Several cancer therapies induce autophagy, and the autophagic response to some treatments has been shown to be cytoprotective [28]. Our previous work and that of others indicate that autophagy is upregulated by treatment with a variety of anticancer drugs and is probably a general mechanism leading to drug resistance [29]. Inhibiting autophagy therefore has the potential to restore sensitivity of tumor cells, especially nutrient-deprived and hypoxic cells, to cancer treatment.

Strengths of our study are that it includes both *in vitro* and *in vivo* studies of three different human tumor cell lines that add to the current understanding of how autophagy contributes to the survival of hypoxic cells and thus to limitations of cancer treatment. It also uses both genetic and pharmacologic inhibition of autophagy to demonstrate increased sensitivity of cancer cells. Potential weaknesses of our study include failure to generate double-knockdown cells that can grow in tissue culture. Also, we assessed the efficacy of shRNA knockdown of autophagy by testing three commercially available shRNAs and picking the most deficient knockdown cell lines. We did not clone these knockdown cell lines, but we demonstrated that the phenotype remained stable following their growth as xenografts.

Conclusion

In summary, our data support the hypothesis that autophagy is a survival mechanism for hypoxic cells both in tissue culture and in experimental tumors. Our findings have substantial implications because both hypoxia and autophagy are associated with poor outcome for human cancers and are associated with resistance to treatment. Inhibiting autophagy in tumors might be exploited to increase the efficacy of anticancer treatment.

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