STAT3-Mediated Autophagy Dependence Identifies Subtypes of Breast Cancer Where Autophagy Inhibition Can Be Efficacious

Paola Maycotte1, Christy M. Gearheart2, Rebecca Barnard3, Suraj Aryal1, Jean M. Mulcahy Levy2, Susan P. Fosmire2, Ryan J. Hansen3, Michael J. Morgan1, Christopher C. Porter2, Daniel L. Gustafson3, and Andrew Thorburn1

Abstract

Autophagy is a protein and organelle degradation pathway that is involved in diverse diseases, including cancer. Recent evidence suggests that autophagy is a cell survival mechanism in tumor cells and that its inhibition, especially in combination with other therapy, could be beneficial but it remains unclear if all cancer cells behave the same way when autophagy is inhibited. We inhibited autophagy in a panel of breast cancer cell lines and found that some of them are dependent on autophagy for survival even in nutrient rich conditions without any additional stress, whereas others need autophagy only when stressed. Survival under unstressed conditions is due to cell type–specific autophagy regulation of STAT3 activity and this phenotype is enriched in triple-negative cell lines. This autophagy-dependency affects response to therapy because autophagy inhibition reduced tumor growth in vivo in autophagy-dependent but not in autophagy-independent breast tumors, whereas combination treatment with autophagy inhibitors and other agent was preferentially synergistic in autophagy-dependent cells. These results imply that autophagy-dependence represents a tumor cell–specific characteristic where autophagy inhibition will be more effective. Moreover, our results suggest that autophagy inhibition might be a potential therapeutic strategy for triple-negative breast cancers, which currently lack an effective targeted treatment.

Cancer Res; 74(9); 2579–90. © 2014 AACR.

Introduction

Autophagy is a long-lived protein and organelle degradation pathway in which cytoplasmic material is engulfed in a double membrane structure known as the autophagosome and later delivered to the lysosome for degradation. Macroautophagy (referred herein as autophagy) is thought to be the major type of autophagy (1). It has been extensively studied in recent years, increasing our understanding of the major constituents of the autophagic pathway, encoded by the ATG genes, and unraveling many of its roles in homeostasis and development. Most importantly, defects in the autophagic pathway have been involved in diverse diseases, including cancer (1–4).

Under normal conditions, basal autophagy has been proposed to function as a tumor suppressor mechanism by reducing oxidative stress, inflammation, and genome instability (2). However, autophagy has also been suggested to act as a survival mechanism in established tumors. It is well known that in cells under stress—including starvation, growth factor deprivation, hypoxia, radio- and chemotherapy—autophagy is upregulated to recycle cytoplasmic components and provide the cell with amino acids and ATP to support essential metabolic pathways and protein synthesis (2). This is critical in tumor cells, which are constantly exposed to both metabolic stress via hypoxia, inadequate glucose supply and increased energetic demands of rapidly proliferating cells as well as proteotoxic stress induced by high levels of genomic instability found in cancers. Although this autophagy requirement could be a generally important mechanism of survival in tumor cells, recent evidence suggests that certain tumors are "autophagy addicted." In this regard, RAS transformation is known to induce high levels of basal autophagy in cancer cell lines; autophagy is required for efficient RAS-induced tumorigenesis, and many, but not all, RAS-transformed cell lines are highly dependent on autophagy (5–7).

Despite implementation of prevention programs and novel therapeutic strategies, breast cancer remains the second leading cause of cancer-related death among women in the United States (8). One of the biggest recognized barriers to progress in prevention, diagnosis, and treatment is the clinical and genetic heterogeneity of the disease (9). In this regard, gene expression analyses have led to the definition of five molecular "intrinsic" subtypes of breast cancer (luminal A, luminal B, HER2-
enriched, basal-like, and claudin-low), which have differences in incidence, survival, and response to treatment (10). Basal-like and claudin-low tumors comprise the majority of triple-negative breast cancers (TNBC; ref. 10), a subgroup of tumors that do not express clinically significant levels of estrogen receptor, progesterone receptor, and HER2 and thus cannot be treated with endocrine or anti–HER2-targeted therapies. They include 10% to 24% of invasive breast cancers and have a worse prognosis when compared with other tumor subtypes. Importantly, although patients with TNBC do benefit from chemotherapy, there are no known targeted agents for this type of cancer and after therapy, they tend to relapse with distant metastases, resulting in a worse overall survival (11) and underscoring the need to develop better, less toxic treatment approaches.

Among many other distinctive characteristics, TNBC cells are known to have high levels of activation of the JAK–STAT pathway (12). STATs are transcription factors that are activated in the cytoplasm by tyrosine phosphorylation in response to cytokine receptor activation (IFN or interleukin6) and their associated Janus-activated kinase (JAK) or to growth factor receptor signaling (EGF and platelet-derived growth factor), either directly or through recruitment of associated proteins (13). Cytoplasmic kinases such as SRC and ABL1 can also phosphorylate and activate STATs (14). Phosphorylated STATs dimerize and translocate to the nucleus-activating transcription. Ligand-dependent activation of STATs is associated with cellular differentiation and growth regulation. Conversely, their constitutive activation is associated with tumorigenesis by inducing the transcription of genes that promote cell-cycle progression, prevention of apoptosis (BCL2L1, CCND1, and MYC), cancer inflammation, and inhibition of antitumor immunity (14–16).

We found that autophagy inhibition is much more important in some breast lines than others and that cancer cell lines with constitutively high levels of STAT3 activity were more sensitive to autophagy inhibition than those with low levels of basal STAT3 activity. The most autophagy-dependent lines require autophagy to survive even under conditions with no added metabolic stress and are enriched in the basal-like and claudin-low subtypes of breast cancer (10). These effects were due to autophagy-dependent STAT3 activation and were also associated with increased ability to synergize when autophagy was inhibited in combination with another anticancer drug. Our results suggest that autophagy inhibition might prove beneficial especially for "basal-like" and "claudin-low" breast cancers, which mostly constitute the TNBC subgroup and that currently lack effective treatment strategies.

Materials and Methods

Cell culture

All cell lines were acquired from the University of Colorado Tissue Culture Core in 2012 and were authenticated by allele testing (date of authentication is as follows: MCF10A, MCF7, T47D, HCC1937, MDA-MB-468, and MDA-MB-231: October 2011; MCF12A and BT549: May 2012). After acquisition, cells were grown, frozen, and each time they were thawed, they were not used for longer than 6 months. Cell culture media used are shown in Supplementary Materials and Methods. STAT3c plasmid and its empty vector (Addgene plasmids 24983 and 14883) were a gift from Linzhao Cheng (17) and David Baltimore (18), respectively. MDA-MB-231 and MDA-MB–468 cells were transduced with lentiviruses expressing these plasmids and sorted for a high green fluorescent population.

The autophagy short hairpin RNA library

We designed a lentiviral short hairpin RNA (shRNA) library with 629 shRNAs targeting autophagy proteins from the autophagic core machinery, proteins described in the autophagy interactome (19) and apoptosis-related proteins. A pool of shRNA-expressing plasmids from The RNAi Consortium (TRC) library (pLKO.1 vector) was prepared at the Functional Genomics Core of the University of Colorado Cancer Center (Boulder, CO). Of note, three to five shRNAs per gene target were used. Lentiviruses were generated according to protocols from TRC (http://www.broadinstitute.org/rna/trlc/lib) and cells were transduced at a multiplicity of infection (MOI) of 0.3 and selected with puromycin. Posttransduction samples were collected 18 hours after the addition of virus in quintuplicate. Another sample of transduced cells was replated with puromycin and allowed to grow for 15 days, after which the growth sample was collected in quintuplicate. Samples were barcoded as described previously (20) and analyzed in an Illumina Genome Analyzer IIX.

Functional genetic screening data were analyzed using our in-house bioinformatics pipeline (20–24), and as described in Supplementary Materials and Methods.

shRNA lentiviral transduction

Lentiviruses were prepared according to protocols published at TRC webpage (http://www.broadinstitute.org/rna/trlc/lib). Viruses (containing either nonsilencing, ATG5 TRCN0000151474, ATG7 TRCN0000007587, Becn1 TRCN000003549, STAT3 TRCN0000020840, or STAT3 TRCN0000020842 pLKO.1 vectors) were added to achieve an MOI of 5 to10. Cells were selected with puromycin according to a dose–response curve and with the concentrations shown on Supplementary Materials and Methods for 2 to 3 days, then tripinized and used for experiments.

Viability assays

For long-term clonogenic assays, cells were plated in 12-well plates and allowed to grow for 8 to 10 days. They were fixed and stained with crystal violet (Becton, Dickinson and Company). Stain was solubilized and absorbance was measured at 540 nm. For proliferation curves, cells were counted at 3, 6, 8, or 10 days after plating. Trypan blue–negative cells were considered viable.

For MTS assays, cells were plated in 96-well plates and allowed to grow for 2 to 6 days. Cells were treated with MTS reagent (Promega) according to the manufacturer’s instructions. For cell death assessment, media were collected and evaluated for lactate dehydrogenase (LDH) activity with the LDH Cytoscan Cytotoxicity Kit (G-Biosciences) according to the manufacturer’s instructions. For time-lapse movies, cells were plated at equal densities and imaged for 48 hours. For propidium iodide (PI) staining, cells transduced with the
different shRNAs were plated, allowed to grow for 48 hours, stained with PI, and imaged.

**Protein isolation and Western blots**

Of note, 2 to 3 days after plating, cells were lysed with RIPA buffer containing protease inhibitor (Roche) and Halt phosphatase inhibitor cocktail (Thermo Scientific). Protein was quantitated using Bradford reagent. Antibodies used are listed in Supplementary Materials and Methods. Western blot analysis figures show a portion of the membranes containing the corresponding bands cropped for clarity.

**Animal studies**

All animal studies were performed in accordance with the Colorado State University Animal Care and Use Committee. Female nude nu/nu mice were purchased from the National Cancer Institute (Frederick, MD) and challenged with $5 \times 10^6$ MDA-MB-231 cells. Female nude nu/nu mice were first ovariectomized and received a subcutaneous, 60 day release, 0.25-mg estradiol implant (Innovative Research). Mice were then challenged, 7 days later, with $5 \times 10^6$ MCF7 cells. Cells (100 μL) in 50% serum-free media and 50% Matrigel (BD Biosciences) were injected into the fourth mammary fat pad. Upon reaching a tumor volume of 100 mm$^3$, mice received either 60 mg/kg chloroquine diphosphate salt or 0.9% saline given by intraperitoneal injection, once daily for the duration of the study. The study was followed until tumors reached four times initial volume (TV$^4$; ref. 25).

**Statistical analysis**

Statistical differences were determined using a two-sample equal variance Student t test. Log-rank analysis for the Kaplan-Meier curves was done in Prism v5.0a. Three MCF7 xenografts did not quite reach four times initial volume (TV$^4$), so time to reach TV$^4$ was extrapolated using calculated tumor doubling time. The combination index (CI) was calculated using CompuSyn (ComboSyn, Inc.).

A more detailed description of the Materials and Methods section was included in Supplementary Materials and Methods.

**Results**

**The autophagy-focused shRNA lentiviral library**

We designed an autophagy-specific shRNA library, targeting the core components of the autophagic pathway (ATG genes) as well as some of their interacting proteins (19) and used this library to evaluate “autophagy dependence” in a panel of breast cancer cell lines. Because functions independent from autophagy have been described for ATG genes (26–28), we reasoned that if autophagy is important for tumor cell survival and proliferation to different extents in different cell lines, this would be manifested by differential selection for or against shRNAs that target the autophagy pathway during cell growth, leading to overall differences in the pattern of shRNA representation of the library in different breast cell populations, and not to changes in the shRNA of a particular ATG protein. On the other hand, if autophagy is not important for the growth or survival of cells, shRNA representation after growth selection should be similar to the starting population. Because the approach examines large numbers of shRNAs whose only similarity is their connection to autophagy, differences in the overall representation of the whole library should represent the importance of autophagy itself rather than any other function for individual autophagy regulators (Fig. 1A). We used a nontumorigenic cell line (MCF12A), two luminal (MCF7 and T47D), and two triple-negative cell lines, one basal (MDA-MB-468) and one claudin-low (MDA-MB-231), according to the classification by Prat and colleagues (10). One DNA sample was collected 18 hours after transduction (posttransduction) and another sample collected 15 days following cell growth in complete medium, both in quintuplicate. Samples were bar-coded and analyzed by next-generation sequencing as described previously (20). Hierarchical clustering of the shRNA readouts produced two main clusters (Fig. 1B). The first cluster contained all the posttransduction samples combined with the MCF7 and MCF12A growth samples, indicating a similar shRNA representation among these conditions. The second cluster included MDA-MB-231, T47D, and MDA-MB-468 growth samples, suggesting differences in shRNA representation between this group of samples and the other cluster, but a similar representation among them. Those shRNAs “lost” in the growth sample but present in the posttransduction represent proteins essential for growth, because their knockdown causes decreased proliferation or cell death and a lack of representation of the shRNA in the growth sample (as in Fig. 1A, panel 1). One condition cluster in the heatmap showed shRNAs that had less reads in growth samples of MDA-MB-231, T47D, and MDA-MB-468 cell lines when compared with the posttransduction cluster (Supplementary Fig. S1). This cluster included mostly shRNAs that target autophagy at the nucleation and expansion steps, indicating that MDA-MB-231, T47D, and MDA-MB-468 had a decreased proliferation or death induction when transduced with these shRNAs, suggesting these cells were sensitive to autophagy inhibition. On the other hand, MCF7 and MCF12A cell lines clustered with the posttransduction samples with no significant selection for or against the shRNAs during the 15-day growth period, indicating they proliferate normally despite having shRNAs that target the autophagic pathway. These results suggest that breast cancer cell lines behave differently when autophagy is modulated and that some breast cancer cell lines are dependent on autophagy for proliferation or survival, whereas others are much less dependent on it. A complete list of the shRNAs in the library and their representation per cell line is included in Supplementary Table S1.

**Breast cancer cell lines have a differential sensitivity to autophagy inhibition**

We validated our shRNA library results with individual shRNAs targeting the core autophagy proteins ATG5, ATG7, and BECN1 that were each independently validated as inhibiting autophagy. Figure 2A shows a panel of breast cell lines organized in increasing sensitivity to autophagy inhibition in complete medium when measured by a clonogenic survival assay. These cell lines were differentially affected in their...
growth/survival when autophagy was inhibited. MCF10A cells were the least affected by autophagy inhibition, whereas MDA-MB-468 were the most affected. To test how inhibition of autophagy was affecting cell number, we performed a growth curve (Fig. 2B). MCF10A cells were resistant to autophagy inhibition; in fact, they proliferated faster. Although there was a
decrease in cell number in MCF7, MCF12A, and T47D cells with autophagy inhibition, the fact that cell number continued to increase over time in the knockdown cells suggested that their proliferation rate was only decreased. The most affected cell lines were MDA-MB-231, HCC1937, BT549, and MDA-MB-468 (Fig. 2A and B), in which autophagy inhibition greatly decreased proliferation, or completely inhibited it. Importantly, the three shRNAs showed a similar response in each cell line. These results, together with the autophagy library screen, suggest that inhibition of the autophagic pathway and not an unspecific effect of the inhibition of a certain ATG gene differentially affects breast cancer cell proliferation or survival. Analysis of clonogenic experiments and growth curves from all the cell lines indicated that TNBC cell lines (basal and claudin-low) were the most sensitive to autophagy inhibition (Fig. 2A and B). Conversely, in nontumorigenic...
and luminal cells, proliferation was induced (MCF10A) or was decreased to a much lesser extent (MCF12A, MCF7, and T47D) than TNBC cell lines, suggesting a delay in cell proliferation rather than cell death. The shRNAs were effective at knocking down their target proteins in all cell lines, showing markedly decreased starvation-induced autophagy as measured by LC3-II accumulation when autophagic flux was blocked (Supplementary Fig. S2).

Decreased clonogenic growth could be caused by decreased proliferation or cell death. To test these possibilities, we chose a luminal line with only a modest effect of autophagy inhibition (MCF7) and two TNBC cell lines (MDA-MB-231 and MDA-MB-468) in which autophagy inhibition had a significant effect and imaged them by time-lapse microscopy after transduction with nonsilencing, ATG7 or BECN1 shRNAs (Fig. 3A and Supplementary Movies 1–6). The same number of cells were plated for all conditions and Fig. 3A shows the cells after 48 hours. ATG7 and BECN1 knockdown resulted in a decreased number of cells when compared with the nonsilencing controls in the three cell lines. MCF7 cells after ATG7 or BECN1 knockdown looked normal and only proliferated at a somewhat slower rate compared with the nonsilencing cells, resulting in smaller colonies. Conversely, MDA-MB-231 cells showed significantly decreased proliferation when compared with the nonsilencing controls and some cells died. The most affected cells were MDA-MB-468s, in which ATG7 and BECN1 knockdown cells did not proliferate and most of these cells died during the 48 hours period. In agreement with these observations, MCF7 cells did not show an increase in LDH release in a 6-day period when autophagy was inhibited (Fig. 3B). MDA-MB-231 cells showed increased LDH release after 6 days and MDA-MB-468 cells showed a maximum LDH release since day 3. All three cell lines had a similar knockdown when measured by Western blot analysis (Fig. 3C). Similar results were found in a PI staining experiment to measure cell death, in which the highest increase in PI staining was seen on the MDA-MB–468 cells in which autophagy had been inhibited (Supplementary Fig. S3).

Because autophagy inhibition did not induce cell death in MCF7 cells, we tested the functionality of the autophagic pathway in these cells by starving them with Earle’s Balanced Salt Solution, a standard treatment for autophagy induction. Pharmacologic inhibition of autophagy with chloroquine treatment (Fig. 3D) or inhibition of autophagy with ATG7 or BECN1 shRNAs (Supplementary Fig. S3B) greatly decreased cell viability in a clonogenic assay, suggesting that MCF7 cells need autophagy for survival but only under stressed conditions.

Chloroquine treatment had a similar effect as ATG7 or BECN1 knockdown when cells were grown in full medium (Fig. 4). A differential sensitivity to chloroquine was found among all the breast cancer cell lines, with the TNBC cells again being the most sensitive. An increase in LC3II was observed in all the cell lines with chloroquine treatment (Fig. 4). However, this increase in LC3II did not correlate with sensitivity to autophagy inhibition, suggesting that differences in basal autophagy among the cell lines are not the cause of autophagy dependence. Together, these data show that breast cancer cell lines differ greatly in their dependency on autophagy, with some lines barely affected when autophagy is inhibited, some showing only growth inhibition, and some dying. The most autophagy-dependent cells tended to be the triple negatives, which require autophagy for their survival even under conditions of no added stress.

**Autophagy regulates STAT3 phosphorylation and cell survival**

Autophagy has recently been linked to JAK/STAT pathway activation (29–31) and some breast cancer cell lines are known to have high levels of constitutively activated STAT3, particularly those belonging to the TNBC subgroup (12, 32).
Importantly, basal and claudin-low subtypes of breast cancer comprise most (70%–80%) of TNBC (10) and the basal and claudin-low cell lines used in this study are known to be triple negative (33). In agreement with previous reports (12), TNBC cell lines (MDA-MB-231, HCC1937, BT549, and MDA-MB-468) showed substantially higher levels of activated STAT3 by Tyr phosphorylation (Fig. 5A) than the luminal cell lines. These cells were determined to be STAT3 dependent, because genetic inhibition of STAT3 by gene knockdown showed a significant increase in LDH release as well as decreased MTS activity (Fig. 5B) and clonogenic growth (Fig. 5C) in both MDA-MB-231 and MDA-MB-468. Although MCF7 cells showed a decrease in MTS activity and clonogenic growth, STAT3 knockdown did not induce a substantial amount of LDH release, suggesting that proliferation was impaired but that MCF7 cells are not STAT3 dependent. Moreover, pharmacologic inhibition of STAT3 with Statistic, a STAT3 small-molecule inhibitor (34), killed breast cancer cell lines in a similar manner and with the same relative specificity to autophagy inhibition by chloroquine or ATG gene knockdown (Fig. 5D and Supplementary Fig. S4A).

Tyrosine-phosphorylated STAT3 could not be detected in MCF7 cells in basal conditions (Fig. 5A) or upon autophagy inhibition (Fig. 5E). On the other hand, ATG gene knockdown decreased tyrosine-phosphorylated STAT3 in both the MDA-MB-231 and MDA-MB-468 cells and led to a reduction in the levels of transcriptional targets of STAT3-like cyclin D and BCL2L1 (also known as BCL-XL; Fig. 5E). Similar results were observed in the other triple-negative cell lines (HCC1937 and BT549; Supplementary Fig. S4B). Pharmacologic inhibition of autophagy by chloroquine treatment (Fig. 5F) also decreased phosphorylation of STAT3 in MDA-MB-231 and MDA-MB-468 cell lines. Together, these data suggest that autophagy inhibition decreases STAT3 phosphorylation in TNBC cell lines and that this reduction in STAT3 activity induces cell death. To test this, we overexpressed a constitutively active form of STAT3 (STAT3C), which partially restored phospho-Tyr STAT3 levels (Fig. 6A) and decreased death induced by autophagy inhibition in both MDA-MB-231 and MDA-MB-468 cell lines (Fig. 6B).

Chloroquine increases the median survival of MDA-MB-231 mouse xenografts

To evaluate the effect of autophagy inhibition in vivo, we performed mouse xenograft studies with the MCF7 and MDA-MB-231 cell lines. Tumor growth was followed until tumors reached four times initial volume (TV/C4) as suggested by Teicher (25). Chloroquine treatment significantly delayed the median time to reach TV/C4 of mice injected with MDA-MB-231 cells and not in those having MCF7 xenografts (Fig. 7A). LC3 staining revealed more LC3-positive puncta in the chloroquine-treated tumors when compared with their respective vehicle-treated controls, indicating inhibition of autophagy in both MCF7 and MDA-MB-231 chloroquine-treated tumors. These results suggest that autophagy inhibition has differential effects on breast cancer tumors in vivo and that TNBC tumors similar to the MDA-MB-231 xenografts are more likely to benefit from autophagy inhibition even in the absence of treatment with other agents.

Autophagy inhibition with chloroquine synergizes with chemotherapy primarily in autophagy-dependent cells

Because MDA-MB-231 and MDA-MB-468 cells were the most affected by autophagy inhibition, we hypothesized that chloroquine treatment would synergize with chemotherapy in these cells. Figure 7B shows the CI for treatment with doxorubicin and chloroquine at different concentrations. MDA-MB-231 and MDA-MB-468 cells showed the highest synergy (log CI < 0), whereas the combined treatment in MCF7 cells was only weakly synergistic in most concentrations tested and even antagonistic in others (log CI > 0).
Discussion

Autophagy is frequently increased in tumors, particularly in areas of low oxygen and blood supply, where it serves as a survival mechanism that helps the cells survive periods of metabolic stress (33). Although autophagy could, in principle, serve as a general survival mechanism in all tumor cells, recent studies suggest that certain types of cancers are particularly sensitive to autophagy inhibition. In this regard, pancreatic and RAS transformed cancers seem to have high energetic requirements that are supported by autophagic mitigation of reactive oxygen species (6), and autophagic maintenance of mitochondrial (5) and glucose metabolism (7). However, even with RAS-driven cancers, the role of autophagy is context dependent because a recent study suggests that autophagy inhibition prevents Kras-driven tumor development when p53 is wild-type but promotes tumorigenesis and progression when p53 is deleted (36).

In this work, we studied autophagy dependence in a panel of breast cancer cell lines representing different intrinsic subtypes of the disease. Importantly, breast cancer has not previously been considered particularly addicted to autophagy. Only two previous studies have suggested that autophagy could be a therapeutic target in breast cancer: one using the MDA-MB-231 cell line, which is KRAS transformed (7), and another one using the MMTV (mouse mammary tumor virus)-PyMT mouse model of breast cancer, which
induces Ras, Src, and phosphoinositide 3-kinase activation (37). Importantly, Ras has not been found to be frequently activated in breast cancer (38).

Here, we found that different subtypes of breast cancer vary markedly in their dependence on autophagy, with TNBC cell lines displaying particular sensitivity to autophagy inhibition by ATG gene knockdown or chloroquine treatment when compared with luminal and nontransformed cell lines (Figs 1–3). These cells were dependent on autophagy for survival even in complete media, i.e., in the absence of stresses, which are known to stimulate autophagy to maintain cellular homeostasis. Conversely in autophagy-independent MCF7 cells autophagy was needed for survival upon starvation but not in unstressed conditions. Autophagy dependence was also independent of Ras pathway status because the only TNBC cell line that we used that is known to be Ras mutared is the MDA-MB-231 cell line (39). Instead, we found that STAT3 constitutive activity is regulated by autophagy only in some cells and that this is required and sufficient for autophagy dependence for survival (Figs 5 and 6). This differential requirement for autophagy has effects on tumor treatment because mice with chloroquine-treated MDA-MB-231 xenografts had a better event-free survival (when compared with their matched controls) than those with MCF7 tumors (Fig. 7A) and autophagy inhibition with chloroquine treatment synergized with chemotherapy in cell lines that were autophagy-dependent and not in the ones that were autophagy-independent (Fig. 7B).

The effect of autophagy inhibition during cancer treatment remains a controversial issue (1, 4, 40). Although numerous studies have shown a significant decrease in cell number with autophagy inhibition in combination with radio- or chemotherapy in breast cancer cell lines (41, 42) and in tumor xenografts (43–45); other studies show a limited or null effect (46). Although it makes sense that autophagy inhibition may be more beneficial when used with particular cancer therapies (e.g., those that themselves directly activate the autophagy pathway), our data suggest that in addition, only some cancer cells will really benefit from its suppression during treatment. Particularly, we propose that TNBCs with high constitutive levels of STAT3 phosphorylation will be autophagy dependent and will, therefore, respond the most to autophagy inhibition alone or in combination with chemotherapy. Therefore, synergy with chemotherapy and autophagy inhibition will work best in such tumor cells. The mechanism by which autophagy promotes STAT3 phosphorylation remains to be determined.

Recent studies have suggested a correlation between the autophagic marker LC3B and patient outcome in breast cancer, in which breast cancers with the highest LC3B expression were found to show increased markers for cell proliferation, aggressiveness, and had the worst outcome (47, 48). About breast cancer subtypes, Lazova and colleagues found that only luminal A tumors correlated between LC3B expression and poor outcome, whereas Chen and colleagues found this correlation only in TNBC. Although both studies suggest that increased LC3B total staining could be used as a marker of increased autophagy, this is not necessarily true because increased total LC3 or autophagosomal number could also reflect a block in autophagic flux (49). Our results suggest that regardless of the levels of autophagy in breast cancer cells, molecular markers like activated STAT3, could be used to determine which breast cancers would benefit the most from autophagy inhibition.

This work has potential therapeutic implications because TNBC has the worst prognosis among all breast cancers. There are currently no specific therapies for patients with TNBC and, although they can be treated with chemotherapy, they tend to relapse and metastasize early (11). Our results suggest that this subtype of the disease may be most responsive to autophagy inhibition and that screening for high levels of constitutive

---

**Figure 6.** Constitutively active STAT3 decreases cell death induced by autophagy inhibition. MDA-MB-231 and MDA-MB-468 cells were transduced with a constitutively active form of STAT3 (STAT3C). A, as shown by Western blot analysis, STAT3C increased p-Tyr STAT3 levels after autophagy inhibition with ATG7 (A7) or BECN1 (B1) shRNAs. B, cells were then evaluated for death with an LDH assay 3 days (MDA-MB-231) or 1 day (MDA-MB-468) after plating. Graphs, mean ± SE of three independent experiments performed in triplicate. *, different to its empty vector control, $P < 0.05$. 

A

<table>
<thead>
<tr>
<th>MDA-MB-231</th>
<th>Empty vector</th>
<th>STAT3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS A7 B1</td>
<td>NS A7 B1</td>
<td></td>
</tr>
<tr>
<td>95 kD</td>
<td>92 kD</td>
<td></td>
</tr>
<tr>
<td>72 kD</td>
<td>64 kD</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MDA-MB-468</th>
<th>Empty vector</th>
<th>STAT3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS A7 B1</td>
<td>NS A7 B1</td>
<td></td>
</tr>
<tr>
<td>95 kD</td>
<td>92 kD</td>
<td></td>
</tr>
<tr>
<td>72 kD</td>
<td>64 kD</td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>MDA-MB-231</th>
<th>Empty vector</th>
<th>STAT3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>ATG7</td>
<td></td>
</tr>
<tr>
<td>0–20 %</td>
<td>0–20 %</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MDA-MB-468</th>
<th>Empty vector</th>
<th>STAT3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>ATG7</td>
<td></td>
</tr>
<tr>
<td>0–20 %</td>
<td>0–20 %</td>
<td></td>
</tr>
</tbody>
</table>

---

www.aacjrournals.org Cancer Res; 74(9) May 1, 2014 2587

Published OnlineFirst March 3, 2014; DOI: 10.1158/0008-5472.CAN-13-3470

Downloaded from cancerres.aacrjournals.org on April 30, 2017. © 2014 American Association for Cancer Research.
STAT3 activation in tumors may serve as a selection strategy for treatment with autophagy inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: P. Maycotte, M.J. Morgan, D.L. Gustafson, A. Thorburn
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Maycotte, R. Barnard, S. Aryal, J.M. Mulcahy Levy, R.J. Hansen, D.L. Gustafson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Maycotte, C.M. Gearheart, R. Barnard, R.J. Hansen, C.C. Porter
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Aryal, S.P. Fosmire
Writing, review, and/or revision of the manuscript: P. Maycotte, C.M. Gearheart, R. Barnard, J.M. Mulcahy Levy, R.J. Hansen, M.J. Morgan, C.C. Porter
Study supervision: P. Maycotte, A. Thorburn

Acknowledgments

The authors are thankful for the contribution to this research made by the following: University of Colorado Cancer Center Research Cures: Protein Production/Mab/Tissue Culture, Flow Cytometry, Functional Genomics, and Research Histology Core (E. Erin Smith, April Otero, and Jenna Van Der Volgen).
Grant Support
This work was supported by NIH grant CA150925 and the following shared resources supported by P30 CA046934 Protein Production/Mab/Tissue Culture, Flow Cytometry, Functional Genomics, and Histology. This work was also supported (in part) by a research grant from the Cancer League of Colorado, Inc. (P. Maycotte); Elobe, Inc. St. Baldrick Foundation Scholar Award (C.M. Gearheart and J.M. Mukhaly Levy); and NIH/NICHD Child Health Research Career Development Award (K12 HD068572; J.M. Mukhaly Levy).

References


STAT3-Mediated Autophagy Dependence Identifies Subtypes of Breast Cancer Where Autophagy Inhibition Can Be Efficacious

Paola Maycotte, Christy M. Gearheart, Rebecca Barnard, et al.


**Updated version**
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-3470

**Supplementary Material**
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/03/04/0008-5472.CAN-13-3470.DC1

**Cited articles**
This article cites 46 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/9/2579.full.html#ref-list-1

**Citing articles**
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
/content/74/9/2579.full.html#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.