THE ROLE OF CYTOPROTECTIVE AND NON-PROTECTIVE AUTOPHAGY IN RADIATION SENSITIVITY IN BREAST TUMOR CELLS

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THE ROLE OF CYTOPROTECTIVE AND NON-PROTECTIVE AUTOPHAGY IN RADIATION SENSITIVITY IN BREAST TUMOR CELLS

A thesis in partial fulfillment of the requirements for the degree of

Master of Science in Pharmacology and Toxicology at

Virginia Commonwealth University, 2014

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May 2014
Acknowledgements

Foremost, I would like to express my sincere gratitude to those who have been supporting me during my M.S. study and research. I would like to thank my advisor, Dr. David Gewirtz for his patience, understanding, and continuous supports for me in all the time. I would like to thank him for accepting me into his lab, giving me chances to learn science, and more importantly, always having faith in me.

I would like to thank Dr. Hisashi Harada for his kindness, generosity, and tremendous encouragement. He has always treated me as one of his students, guided me to the best direction possible, and provided me with any means I needed to support my study.

I would like to thank my committee member, Dr. Joseph Ritter for his time, support, and insightful questions and comments.

I would like to thank all my lab members: Sean Emery, Moureq Alotaibi, Duaa Bakhshwin, Swheta Chakradeo, Aisha Alhaddad, and especially Khushboo Sharma for all the good and bad things they shared with me within the last two years. It was my great pleasure to know them and spend the time working with them.

My great appreciation goes out to Dr. Wataru Nakajima, a sincere friend of mine who has always been there for me, teaching me, supporting me, and
encouraging me. I would not have been coming to this stage without the tremendous help I got from him.

And as always, my love and deep appreciation goes to my beloved ones.
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LIST OF ABBREVIATIONS

HR Hormone receptor
ER Estrogen receptor
PR Progesterone receptor
HER2 Human epidermal growth factor receptor 2
TN Triple negative
NT No Treatment
IR Ionizing radiation
LC3 Light Chain 3
PI3K Phosphoinositide 3 kinase
PCD Programmed Cell Death
ABSTRACT

THE ROLE OF CYTOPROTECTIVE AND NON-PROTECTIVE AUTOPHAGY IN RADIATION SENSITIVITY IN BREAST TUMOR CELLS

Le, J; Nakajima, W; Harada, H; and Gewirtz, D.

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In general, ionizing radiation promotes cytoprotective autophagy in a majority of breast tumor cells. Previous studies from our laboratory indicated that radiation (5x2
Gy) induces cytoprotective autophagy in MCF-7 cells. In the current work, inhibition of autophagy by silencing of Beclin-1 in MCF-7 cells resulted in an increase in sensitivity to radiation based both on cell number and clonogenic survival; however, there was no increase in apoptosis and the basis for this sensitization is currently under investigation. Unexpectedly, enhancement of autophagy by silencing of Bcl-2 also led to an increase in sensitivity to radiation, possibly through the conversion of cytoprotective to cytostatic autophagy. In contrast to the MCF-7 cells, radiation (5x2 Gy) induces non-protective autophagy in Hs578t cells. Interference with autophagy through silencing of Beclin-1 or induction of Bcl-2 did not alter radiation sensitivity in the Hs578t cells. Since the induction of cytoprotective autophagy can represent an impediment to radiation therapy, it is important to understand the types of autophagy that occur in response to radiation in specific cellular settings and whether interference with autophagy can increase sensitivity to different forms of cancer treatment.
CHAPTER 1: INTRODUCTION

1.1 Cancer

Cancer is a major public health problem in the United States and other countries around the world. According to Siegel et al. (Siegel et al., 2013), one in four deaths in the United States are due to cancer. Cancer can be understood as a disease of malfunctioning cells that keep growing and accumulating. It is the general name for a group of more than 100 diseases, and can be grouped into five categories:

Carcinoma: cancer that arises from epithelial tissues that line the walls of cavities or in the case of skin covering the outside of the body and is responsible for more than 80% of the cancer-related death in the Western world. There are two main types of carcinoma that include squamous cell carcinomas—tumors that arise from epithelial cells that line and protect the underlying cells, and adenocarcinomas—tumors that arise from specialized epithelial cells that secrete substances into the ducts or cavities that they line (Weinberg, R., 2007).

Sarcoma: cancer that begins in bone, cartilage, fat, muscles, blood vessels, or other connective or supportive tissues (www.cancer.gov). The sarcomas constitute about 1% of the tumors encountered in the oncology clinic (Weinberg, R., 2007).

Leukemia: cancer arises from cell lineages that constitute the blood-forming tissues and move freely through the circulation.

Lymphomas: cancer begins in the cells of the immune system that aggregate to form solid tumors.
Central nervous system cancer: cancers arise from cells that form the central and peripheral nervous system.

1.2 Breast Cancer

Breast cancer is the second leading cause of cancer death in women in the United States (Jemal et al., 2011). According to the U.S. Breast Cancer Statistics 2013, about one in eight women in the U.S. will develop invasive breast cancer over the course of her life (www.breastcancer.org). Breast cancer can be divided into 2 large groups based on specific molecular markers including hormone-receptor [HR; estrogen receptor (ER) and progesterone receptor (PR)]-positive and HR-negative negative breast cancers. The HR-positive breast cancer can be sub-divided into two groups defined as luminal A and B tumors depending on the expression level of human epidermal growth factor receptor 2 (HER2). The HR-negative breast cancer can be sub-divided into two groups determined by HER2 expression including HER2-overexpressing tumors and HER2-negative tumors (Rakha et al., 2008).

About 65-70% of invasive breast cancers are HR-positive tumors, called luminal tumors, whose cells express high level of estrogen-hormone receptors and/or progesterone-hormone receptors and associated genes (Schnitt, 2010). The luminal type of breast cancer tends to exhibit a good prognosis since they are estrogen receptor-positive (ER+) and/or progesterone receptor-positive (PR+). The luminal A subtype is characterized by lacking the human epidermal growth factor receptor 2 (HER2-) which distinguishes it from the luminal B subtype. The first line clinical treatment of luminal type of breast cancer is endocrine therapy,
through targeting of hormone functions (tamoxifen to inhibit ER function) or blocking their synthesis (aromatase inhibitor to block estrogen synthesis). Usually, luminal A subtype exhibits better clinical outcomes in response to endocrine therapy than luminal B subtypes. Since 30% of ER+ breast cancers were reported resistant to hormonal therapy, additional therapeutic strategies are required for optimal outcomes (Zhang et al., 2014).

About 15% of invasive breast cancers are characterized by HER2 overexpression. HER2 overexpression in breast tumors is often associated with low expression of ER and associated genes (Schnitt, 2010). HER2- human epidermal growth factor 2- belongs to a family of receptors including EGFR/HER1, HER2, HER3, and HER4 and functions by stimulating the signaling pathway of growth factors (Gajria and Chandarlapaty, 2011). Overexpression of HER2 leads to the continued activation of growth signaling pathway and contributes to the growth of breast cancer cells. HER2-positive breast cancer tends to be aggressive, fast growing, and less responsive to hormonal therapy. However, treatments for HER2-positive breast cancer have been shown to be very effective when pharmacologically targeting at HER2. The most common pharmacologic treatment used is trastuzumab (Herceptin), a monoclonal antibody that binds to the extracellular domain of HER2 receptors, blocking cancer cells but not normal cells from receiving growth signals. The use of trastuzumab in combination with chemotherapy shows effectiveness in the case of advanced-stage breast cancer and reduces the risk of disease recurrence (Gajria and Chandarlapaty, 2011).
Another group belonging to the HR-negative subtype is triple negative (TN) breast cancer, which constitutes about 12-24% of breast cancer (Sorlie et al., 2001; Bertucci et al., 2000; Bertucci et al., 2004). TN breast cancer is defined as HR negative/PR negative/HER2 negative, and does not benefit from some of the most effective therapies against breast cancer such as hormonal therapy or HER2 targeting therapy (Rakha et al., 2008). TN breast cancer is associated with a worse prognosis, has a high rate of recurrence, and causes majority of death within the first 3 to 5 years after initial treatment (Ovcaricek et al., 2011). Chemotherapy is usually the only therapeutic option to treat TN breast cancer because of their lack of hormonal receptors and HER2. Anthracycline and anthracycline/taxane treatment usually results in good initial effects in treating of TN breast tumors, but they also yield a rapid relapse rate. Currently, there are several emerging agents under clinical trials for treatment of TN breast cancer such as platinum agent, poly (ADP-ribose) polymerase (PARP) inhibitors which interfere with DNA repair and are hoped to improve prognosis for patients with TN breast cancer (Ovcaricek et al., 2011).

1.3. Autophagy

Autophagy is a self-eating process by which cytoplasmic materials are delivered to and degraded in the lysosome (Mizushima and Komatsu, 2011). There are three major types of autophagy depending on the route by which the cytoplasmic materials delivered to the lysosome: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (Klionsky, 2005). In CMA, unfolded, soluble proteins are directly translocated across the lysosomal membrane
without involvement of membrane formation. In microautophagy, cytoplasmic materials are directly engulfed into the lysosome by inward invagination of the lysosomal membrane (Mizushima and Komatsu, 2011). Macroautophagy (hereafter referred to as autophagy), which is thought to be the major type of autophagy, involves the intermediate formation of autophagosomes, which sequester cytoplasmic materials, and finally fuse with the lysosome to degrade the components.

Autophagy is a dynamic and multi-step process. The Atg1/ULK1 complex is an essential initiator of autophagy, which is normally remained inactivated by binding with mammalian Target of rapamycin (mTORC1), a master regulator of nutrient signaling. Under stressful conditions such as nutrition starvation, mTORC1 dissociates from the Atg1/ULK1 complex, and allows the complex to phosphorylate downstream Atg proteins for autophagosome formation (Mizushima, 2010). The autophagosome is a double-membrane organelle that is derived from endoplasmic reticulum, Golgi apparatus, and/or mitochondria organelles. The autophagosome formation process consists of four major steps including isolation membrane formation, nucleation, elongation, and completion steps. Initially, the class III phosphoinositide 3-kinase (PI3K or Vps34) is associated with Beclin-1 (Atg6) and p150 to form the class III PI3K core complex. The complex triggers vesicle nucleation, which sequesters the cytoplasmic component into an isolation membrane called the phagophore. Next, complete sequestration and phagophore elongation are supported by two ubiquitin-like protein conjugation systems Atg12-Atg5 complex and LC3 (Atg8). The Atg12-
Atg5 complex consists of Atg12, Atg5, and Atg16L and plays role in recruiting additional membrane to complete the autophagosomes membrane formation. Then, lipidated LC3-II (a PE conjugated form of LC3 after the protein is cleaved by Atg4) associates with the newly formed autophagosomes to become the inner composition of autophagosomal membrane. Next, autophagosomes fuses with lysosomes, forming the autophagolysosome, to complete the autophagy pathway. This degradative process of autophagy is selective and mediated through p62/sequestosome 1 (SQSTM1), a mammalian protein that binds to LC3 (Bjorkoy et al., 2005; He and Klionsky, 2009). Finally, the inner membrane of the autophagosomes and the ubiquinated cargo are degraded by lysosomal hydrolase to complete the autophagic degradation (Pyo et al., 2012).

Autophagy is an important catabolic mechanism that occurs in cancer (Ouyang et al., 2012). The functions of autophagy in tumor cells are controversial and remained to be elucidated. In general, autophagy is well known for its cytoprotective function to maintain the cell homeostasis during period of starvation or stresses (He and Klionsky, 2009; Ouyang et al., 2012). It is also well established that autophagy can induce cell death independently of other types of programmed cell deaths such as apoptosis when coping with excessive stress (Bialik et al., 2010; Eisenberg-Lerner et al., 2009; Ouyang et al., 2012). Recently, it has been proposed that at least two more functions of autophagy may occur in response to stresses, including non-protective and cytostatic functions (Gewirtz, 2014). Thus, it is challenging but critical to analyze which
function autophagy plays in cancer cells in response to stressful conditions such as radiation and chemotherapy treatments.

![Diagram of autophagy](image)

**Figure 1.1.** Various steps involved in autophagy (Denton et al., 2012).

1.4 Apoptosis

Apoptosis, or type I programmed cell death (PCD), is a major cell death modality to control cell proliferation or in response to DNA damage (Ghobrial et al., 2005). Apoptosis is characterized by nuclear condensation and fragmentation, cell shrinkage, dynamic membrane blebbing, and loss of adhesion to the surroundings (Nishida et al., 2008; Ouyang et al., 2012) Apoptosis can occur through two main pathways: the extrinsic or cytoplasmic pathway, and the intrinsic or mitochondrial pathway. The two pathways will finally converge to the activation of a cascade of caspase proteases for execution of the apoptosis process (Ghobrial et al., 2005).
The extrinsic apoptotic pathway is triggered when extracellular ligand Fas-L binds to Fas plasma membrane death receptor (or other similar receptors). The Fas complex then recruits Fas-associated death domain protein (FADD) and pro-caspase-8 to activate caspase-8, which in turn proceeds to activate the rest of the downstream caspases (Wajant, 2002).

The intrinsic apoptotic pathway is involved in the activation and translocation of proapoptotic proteins to the mitochondria. The BCL-2 (B cell lymphoma gene-2) family proteins are key regulators of the intrinsic apoptotic pathway and can be classified into pro-apoptotic and anti-apoptotic functions (Weinberg, 2007). The pro-apoptotic proteins are divided into two groups: the BH-3 only proteins (Bad, Bid, Bim, Bmf, Bik, Hrk, Noxa, and Puma) and the multidomain proteins (Bax, Bak, and Bok). The anti-apoptotic proteins include Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 which antagonize Bax/Bak under normal conditions. In response to intrinsic apoptotic stimuli, the BH-3 only proteins are activated and subsequently block the Bcl-2 proteins anti-apoptotic function. Thus, Bax/Bak are activated and mediated the release cytochrome c from the mitochondria into the cytosol. Cytochrome c then recruits Apaf-1 and pro-caspase-9, leading to the activation of caspase-9, which in turn activates the rest of the downstream signaling cascade and leads to apoptosis (Ghobrial et al., 2005).
1.5 Bcl-2:Beclin-1 interaction regulates autophagy

Beclin-1 is a key regulator of autophagy in mammalian cells (Liang et al., 1999; Cao and Klionsky, 2007). Beclin-1 recruits key autophagic proteins to promote formation of a complex of Beclin 1, Vps34, and Vps15 which is important to initiate the autophagosome formation and induce autophagy (He and Levine,
Recently, Beclin-1 was classified as a BH-3 only protein, which binds to the anti-apoptotic Bcl-2 proteins (Oberstein et al., 2007). The Beclin-1/Bcl-2 interaction functions as a key regulatory mechanism in Beclin-1-dependent autophagy (Decuypere et al., 2012). In normal conditions, Bcl-2 binds to Beclin-1 and inhibits its autophagy function. When the cells are under stressful conditions, Beclin-1 dissociates from Bcl-2 and associates with Vps34, allowing subsequent activation of autophagy (Decuypere et al., 2012). It is also important to mention that not mitochondrial but only ER-localized Bcl-2 is able to regulate Beclin-1-dependent autophagy (Pattingre et al., 2005).

Figure 1.3. Bcl-2 as an anti-apoptosis and anti-autophagy protein (Marquez and Xu, 2012).
1.6 Previous studies

Previously our lab has demonstrated that MCF-7 breast cancer cells when irradiated with fractionated doses of 5x2 Gy induced cytoprotective autophagy which prevented radiation-induced cell death. However, when radiation was used in combination with EB1089, an analog of Vitamin D, cytoprotective autophagy was converted into cytotoxic form, which was associated with a reduction in the number of viable cells (Wilson et al., 2011; Bristol et al., 2012). Thereby, cytotoxic and cytoprotective functions of autophagy can occur in the same experimental system, which can be exploited to enhance the effects of cancer therapies. In addition, data from our lab (Chakradeo et al., manuscript in preparation) have demonstrated that fractionated doses of 5x2 Gy did not induce cytoprotective autophagy in Hs578t breast cancer cells. Instead, the autophagy induced in Hs578t when being blocked with autophagy inhibitors 3-methyladenine (3-MA) and Chloroquine (CQ) did not sensitize the cells to radiation treatment. Thereby, our lab has termed it non-protective autophagy. Furthermore, recent studies from our lab also demonstrated that another function of autophagy, termed cytostatic autophagy, could be induced and resulted in cellular growth arrest. Thus, we suggested that multiple functions of autophagy can occur between different cells lines and/or conversion can happen within one cellular system. Since autophagy can be a mode of cellular resistance to treatment, interference of autophagy, therefore, can increase sensitivity to treatment and can become therapeutic benefits to cancer therapy.

1.7 Hypothesis
Numerous studies have suggested that autophagy can function as either cytoprotective (pro-survival) or cytotoxic (pro-death) functions (Levine and Yuan, 2005; Eskelinen, 2005; Zong and Thompson, 2006; Nelson and White, 2004). In addition, studies from our lab have indicated that using the same radiation treatment condition (5 x 2Gy) resulted in the induction of cytoprotective autophagy in MCF-7 cells (Wilson et al., 2011; Bristol et al., 2012) and non-protective autophagy in Hs578t (manuscript in preparation). Here, I hypothesize that enhancement of radiation-induced autophagy in MCF-7 cells leads to a decrease in sensitivity to radiation. In contrast, interference in radiation-induced autophagy in Hs578t does not alter sensitivity to radiation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines and cell culture.

MCF-7 and Hs578t mammalian breast tumor cell lines were purchased from the American Type Culture Collection (ATCC). 293T cells were kindly provided by Dr. Hisashi Harada (Virginia Commonwealth University, VA, USA). MCF-7/shBcl-2, MCF-7/shBeclin-1, Hs578t/Bcl-2, and Hs578t/shBeclin-1 cells were maintained using (1ug/ml) puromycin (Sigma p8833) for selection. All the cells were cultured in DMEM supplemented with 10% heat-activated fetal bovine serum (FBS) and 5% of 100ug/ml penicillin G/streptomycin and maintained at 37°C in a humidified, 5% CO₂ incubator.

2.2 Plasmid construction and virus infection.
The lentiviral shRNA constructs to target Bcl-2 and Beclin-1 were kindly provided by Dr. Hisashi Harada (Virginia Commonwealth University, VA, USA). To generate recombinant lentivirus, the 293T cells (1 x 10^6 cells total) were plated on a 10mm cell culture dish. Transfection steps were carried out 2 days later as the 293T became approximately 70% confluence. First, 5μg of the shRNA constructs (including 2.5μg of interested shRNA and 2.5μg of lentiviral packaging plamids) were mixed into 200μL of RPMI medium (Gibco). In a separate tube, 15μL of Lipofectamine 2000 reagent (Invitrogen) was diluted into another 200μL of RPMI medium. Next, the diluted Lipofectamine reagent was added drop wise into the DNA solution while gently vortexing the DNA-containing tube. The mixture was incubated for 15-20 minutes at room temperature. Finally, the DNA-Lipofectamine mixture was added gently into the 293T cells. The medium was replaced after 14-18 hours overnight. After two days of incubation, the lentivirus shed into the medium was collected and used to infect the breast tumor cells of interest.

The retrovirus pBabe-puro Bcl-2 expressing vector was kindly provided by Dr. Wataru Nakajima (Nippon Medical School, Kawasaki, Japan). The vector was transfected into 293T cells along with the retrovirus packaging plasmids. The retrovirus shed into the medium was collected and used to infect Hs578t cells.

2.3 Cell viability and clonogenic survival.

Cell viability was determined by Trypan blue exclusion assay at different time points after treatment. The cells were seeded in triplicate in twelve well tissue
culture dishes with a concentration of $0.5 \times 10^5$ cells per well in 1ml medium. The cells were trypsinized and collected together with the supernatant, centrifuged at 4000 x g for 5 minutes, and washed twice with PBS. The viability was determined using 0.4% Trypan Blue dye (Sigma T8154), and counted using phase contrast microscopy. For clonogenic survival studies, treated cells were plated in six well tissue culture dishes with a concentration of $1 \times 10^4$ cells per well in 2ml medium. After 12 days, the cells were fixed with 100% methanol, air-dried, and stained with 0.1% crystal violet (Sigma C3886). Number of counted colonies was normalized relative to untreated controls, which were taken as 100% survival.

2.4 Detection and quantification of autophagic cells.

Acridine orange allows the visualization of an autophagy marker, which is the cellular acidic compartment. Cells were plated in triplicate in six well tissue culture dishes and treated as described above for the cell viability study. After treatment for appropriate times, the cells were incubated with medium containing 1µg/ml acridine orange (Invitrogen A3568) for 10 minutes. The medium containing acridine orange was removed, and the cells were washed once with PBS. Fresh PBS was added, and images were taken using an Olympus inverted fluorescence microscope. All images were taken under 20x magnifications. Flow cytometry was used to quantify for the number of cells with acidic vesicular organelles (AVO). The cells were treated in the same manner and analyzed by BD FACSCanto II using FCS Express 4 Flow Research Edition software. A minimum of 10000 cells within the gated region was analyzed.
2.5 Immunoprecipitation and Western blot analyses.

For immunoprecipitation, cells were lysed in CHAPS lysis buffer (20mM Tris (pH 7.4), 137 mM NaCl, 1 mM dithiothreitol (DTT), and 1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) containing 1:50 protease inhibitor and 1:100 phosphatase inhibitors in M-PER mammalian protein extraction reagent (Thermo Scientific). 500 µg of protein were captured with protein G Sepharose beads (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) and incubated with Beclin-1 antibody (Cell Signaling) at 4°C for 2 hours. After 3 times washing with the same CHAPS lysis buffer, the beads were re-suspended in sample buffer and separated by SDS-PAGE. For Western blotting analyses, cells were lysed with lysis buffer containing 1:50 protease inhibitor and 1:100 phosphatase inhibitor in M-PER mammalian protein extraction reagent (Thermo Scientific). Equal amounts of proteins were loaded on a SDS acrylamide gel, transferred onto a PVDF membrane using transfer buffer (700ml DI water, 200ml methanol, and 100ml 10X Tris/Glycine). The membrane was blocked for 30 minutes using blotto mixture (5% skim milk in 0.1% Tween-20 of 1 x PBS). After blocking, the membrane was incubated with respective antibodies overnight. After three times washing with TPBS (0.1% Tween-20 of 1 x PBS), the membrane was incubated with appropriate secondary antibodies followed by three times washing with TPBS. The membrane was developed using Pierce ECL Plus Western Blotting Substrate (Thermo Scientific #32132). Protein concentrations were determined using Bradford method. Primary antibodies used were cleaved PARP (Cell Signaling), p62 (BD Transduction), Beclin-1(Cell
Signaling), Mcl-1 (Enzo), Bcl-xL (Cell Signaling), Bcl-2 (Sigma), LC3 (Cell Signaling). All primary antibodies presented were used at a 1:1000 dilution.

2.6 Statistical analysis.

Values represent the means +/- SE for three separate experiments. The significance of differences between the experimental variables was analyzed using a one-way ANOVA followed by Bonferroni post-hoc test. Values with P<0.05 were considered as statistically different.

CHAPTER 3: RESULTS

Section 1: Radiation induced cytoprotective autophagy in MCF-7 cell.

3.1.1 Silencing of Beclin-1 inhibited autophagy and increased sensitivity in MCF-7 cell in response to radiation.

In MCF-7, ionizing radiation at 5x2 Gy promoted cytoprotective autophagy. Pharmacological inhibition of autophagy using 3-methyladenine (10mM) and chloroquine (25µM) resulted in growth inhibition or cell death (Wilson et al., 2011; Bristol et al., 2012). To further confirm previous results, I generated a stable MCF-7 cell line that expressed Beclin-1 short-hairpin RNA (shRNA). Figure 3.1.1a shows that Beclin-1 expression was successfully suppressed in MCF-7 cell. Acridine orange staining was utilized to monitor the induction of autophagy (which stains the late state of autophagosomes after fusion with lysosomes, indicated as acidic vesicular organelles). Figure 3.1.1b indicates that radiation alone induced autophagic vesicle formation in MCF-7/control cell. Silencing of
Beclin-1 reduced the increased level of autophagic vesicles caused by radiation. In Figure 3.1.1c, quantification of acidic vesicular organelles indicates that radiation-induced autophagy was statistically significant within 0 hour post radiation. Silencing of Beclin-1 could suppress or at least did not promote the formation of acidic vesicular organelles. To further assess whether silencing of Beclin-1 could inhibit the completion of autophagy, or autophagic flux, I performed Western blot analysis to examine the degradation of p62 protein. The protein p62, also mentioned as sequestosome 1 (SQSTM1), is an LC3-II binding protein whose degradation is indicative of autophagic flux. In Figure 3.1.1d, down regulation of p62 was observed at 24 hours post radiation in MCF-7. On the other hand, p62 level remained unchanged at 0 hour and 24 hours post radiation in MCF-7/shBeclin-1 as compared to the NT (none treatment) cells. Thus silencing of Beclin-1 could inhibit autophagy induced by radiation in MCF-7 cells.

Next, Trypan blue exclusion assay was performed to determine the number of viable cells after Beclin-1 silencing. In Figure 3.1.1e, radiation alone could induce about 50% of growth inhibition at 0 hour post radiation and 60% at 24 hours in MCF-7/control cell. When radiation was performed in combination with Beclin-1 silencing, the growth inhibition effects were increased to 80% at 0 hour and 70% at 24 hours post radiation. Percentage of cell viability was determined utilizing the same Trypan blue exclusion assay, where the number of viable cells was normalized to the total number of viable and dead cells. In Figure 3.1.1f, the percentage of viable cells were almost 97%, indicating minimum cell death occurred within 24 hours post treatment.
To further confirm the increase in growth inhibition after Beclin-1 silencing, I performed clonogenic survival assay to measure the ability of colony formation in MCF-7 after Beclin-1 silencing. A clonogenic survival assay is often used to measure the efficacy of a treatment by observing the ability of a single cell to form a colony. In Figure 3.1.1g, radiation alone could inhibit about 30% the ability of colony formation. However, when radiation was combined with Beclin-1 silencing, the inhibition effect was increased to about 50%. Thus, silencing of Beclin-1 could inhibit autophagy and significantly suppress the colony formation in the radiated MCF-7 cells. The data suggested that radiation-induced autophagy in MCF-7 performed cytoprotective function.
a. Western blot analyses of endogenous Beclin-1 in MCF-7 cells infected with control or Beclin-1 shRNA. β-actin was used as a loading control.

b. Acridine orange images were taken at 24 hours post radiation using an inverted fluorescent microscope. NT: no treatment. IR: radiation.

**Figure 3.1.1** Silencing of Beclin-1 inhibited radiation-induced autophagy in MCF-7 cell. (a) Western blot analyses of endogenous Beclin-1 in MCF-7 cells infected with control or Beclin-1 shRNA. β-actin was used as a loading control. (b) Acridine orange images were taken at 24 hours post radiation using an inverted fluorescent microscope. NT: no treatment. IR: radiation.
c. Figure 3.1.1 Silencing of Beclin-1 inhibited radiation-induced autophagy in MCF-7 cell. (c) Acridine orange staining of acidic vesicles quantified by flow cytometry. Measurement of autophagy induction based on % acidic vesicle formation was monitored by acridine orange staining and quantified by flow cytometry within 0 hour and 24 hours post radiation. *, p < 0.05; **, p < 0.01; ***, p < 0.001. One-way ANOVA was followed by Bonferroni post-hoc test. Results were taken from three independent experiments. (d) Cell lysates were collected at NT, 0 hour, and 24 hours post radiation and subjected to Western blot analyses with the indicated antibodies. β-actin was used as a loading control. NT: no treatment. IR: radiation.
Figure 3.1.1 Silencing of Beclin-1 inhibited radiation-induced autophagy in MCF-7 cell. (e) MCF-7 cells were plated with a concentration of $5 \times 10^4$ cells per well (six well tissue culture dish). The cells were exposed to radiation (5x2 Gy) over a period of 3 days. Number of viable cells was determined by Trypan Blue exclusion assays at NT, 0 hour and 24 hours post radiation. (f) Percentage of cell viability was determined by Trypan blue exclusion assay. The viable cell number was normalized to the total number of viable cells and dead cells. (g) After exposure to radiation, the cells were immediately plated with a concentration of $1 \times 10^4$ cells per well (six well tissue culture dish). Number of colonies was determined by clonogenic survival assays over a period of 12 days. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. One-way ANOVA was followed by Bonferroni post-hoc test. Results were taken from three independent experiments.
3.1.2 The interaction between Beclin-1 and the anti-apoptotic Bcl-2 proteins in MCF-7 cells

In order to enhance the level of radiation-induced autophagy in MCF-7 cell, I generated a MCF-7/shBcl-2 cell and examine the interaction between Bcl-2 family proteins and Beclin-1. In Figure 3.1.2a, silencing of Bcl-2 in MCF-7 was demonstrated by Western blot analyses. The levels of Bcl-xL, Mcl-1 and Beclin-1 remained unchanged after Bcl-2 was silenced as compared to the control cells. Next, co-immunoprecipitation assay was performed to determine the ability of Bcl-2 family proteins to interact with Beclin-1. In Figure 3.1.2b, the interaction of Beclin-1 to Bcl-xL or Mcl-1 was very weak or undetectable, respectively. However, endogenous Bcl-2 in MCF-7 clearly bound to Beclin-1, indicating that interfering with Bcl-2/Beclin-1 interaction could alter the level of radiation-induced autophagy in MCF-7 cell.
Figure 3.1.2 Endogenous Bcl-2 interacts with Beclin-1 in MCF-7 cells. (a) Western blots analyses for the silencing of Bcl-2. MCF-7 cells were infected with lentivirus-encoding shRNA for non-targeting control or Bcl-2. GAPDH was used as a loading control (b) Immunoprecipitation (IP) with Beclin-1. Total cell lysates were subjected to IP with anti-Beclin-1. Western blot analyses were carried out on the precipitated samples with the indicated antibodies.
3.1.3 Silencing of Bcl-2 enhanced the level of radiation-induced autophagy in MCF-7 cell.

To examine whether silencing of Bcl-2 could interfere with the level of autophagy induced by radiation in MCF-7 cell, I assessed the formation of acidic vesicular organelles by acridine orange and quantified by flow cytometry. Figure 3.1.3a demonstrates the Bcl-2 level after the introduction of Bcl-2 shRNA into MCF-7 cell. In Figure 3.2.3b, acridine orange images indicate that radiation alone induced autophagic vesicle formation in MCF-7 cell. Silencing of Bcl-2 further increased the formation of the autophagic vesicles caused by radiation. In Figure 3.1.3c, quantification of acidic vesicular organelles indicate that in MCF-7 cell the percentage of acidic vesicles came from 3.7% in no treatment condition to 10.5% at 0 hour and 18.8% at 24 hours post radiation. On the other hand, in MCF-7/shBcl-2 the percentage of acidic vesicles from 6.6% in no treatment condition was brought up to 21.6% at 0 hour and to 26.2% at 24 hours by radiation.

In addition to the acridine orange results indicating the enhancement of autophagy by Bcl-2 silencing, Western blot analyses in Figure 3.1.3d show that LC3-II accumulation was much higher in MCF-7/shBcl-2 cell as compared to MCF-7 cell, indicating higher level of autophagic vesicle formation. Furthermore, p62 degradation was observed earlier in MCF-7/shBcl-2 cell (started at 6 hour post radiation) as compared to MCF-7 cell (started at 24 hour post radiation).

In short, the data in Figure 3.1.3 suggest that silencing of Bcl-2 could enhance the level of radiation-induced autophagy in MCF-7 cell.
Figure 3.1.3 Silencing of Bcl-2 enhanced the level of radiation-induced autophagy in MCF-7 cell. (a) Bcl-2 level was analyzed using Western blotting in the control and Bcl-2 shRNAs transfected vectors. (b) Acridine orange images were taken 24 hours post radiation using an inverted fluorescent microscope.
Figure 3.1.3 Silencing of Bcl-2 enhanced the level of radiation-induced autophagy in MCF-7 cell. (c) Measurement of autophagy induction based on % acidic vesicle formation was monitored by acridine orange staining and quantified by flow cytometry within 0 hour and 24 hours post radiation. *, p < 0.05; **, p < 0.01; ***, p < 0.001. One-way ANOVA was followed by Bonferroni post-hoc test. Results were taken from three independent experiments. (d) MCF-7 cells were
exposed to radiation (5x2 Gy) over a period of 3 days. Cell lysates were collected at 0, 6, 12, and 24 hours post radiation and subjected to Western blot analyses with the indicated antibodies. β-actin was used as a loading control.
3.1.4 Enhancement of autophagy by Bcl-2 silencing increased growth inhibition effects produced by radiation in MCF-7 cell.

Trypan blue exclusion assays were performed to determine the number of viable cells after exposure to radiation. In Figure 3.1.4a, radiation alone could significantly inhibit cell growth in MCF-7/control cell at 0 hour and 24 hours post radiation. The growth inhibition effects of radiation were further increased in MCF-7/shBcl-2, in contrast with the hypothesis that enhancement of autophagy would further protect the cells from radiation. In Figure 3.1.4b, percentage cell viability was determined by normalizing the number of viable cells to the total number of viable cells and dead cells after 0 hour and 24 hours post radiation, indicating that minimum cell death was observed. Furthermore, Western blot analysis suggests that no cleaved-PARP expression was observed in either MCF-7/control or MCF-7/shBcl-2 cells, indicating minimum apoptosis happened within 24 hours post radiation (Figure 3.1.4c). In addition, data in Figure 3.1.4d taken from clonogenic survival assay indicates that radiation in combination with Bcl-2 silencing could significantly increase the inhibition effects on colony formation in MCF-7 cells as compared to radiation alone. In short, data in Figure 3.1.4 suggests that enhancement of autophagy through Bcl-2 silencing increased the growth inhibition effects produced by radiation.
Figure 3.1.4 Enhancement of autophagy through Bcl-2 silencing increased the growth inhibition effects produced by radiation. MCF-7 cells were exposed to radiation (5x2 Gy) over a period of 3 days. (a) Number of viable cells was determined by Trypan blue exclusion assays in NT cells, and at 0 hour and 24 hours post radiation. (b) Number of viable cells was normalized to the total number of viable and dead cells to calculate for the percentage of viable cells.
c. 

Figure 3.1.4 Enhancement of autophagy through Bcl-2 silencing increased the growth inhibition effects produced by radiation. (c) Cell lysates were collected and Western blot analyses were performed with the indicated antibodies. (d) Number of colonies was determined by clonogenic survival assays over a period of 12 days. *, p < 0.05; **, p < 0.01; ***, p < 0.001. One-way ANOVA was followed by Bonferroni post-hoc test. Results were taken from three independent experiments.
Section 2: Radiation induced non-protective autophagy in Hs578t cell.

3.2.1 Silencing of Beclin-1 inhibited radiation-induced autophagy and minimum changes in radiation sensitivity were observed.

Recent studies from our lab have proposed that 5x2 Gy radiation could induce non-protective autophagy in Hs578t cells. Pharmacological interference of autophagy using 3-methyladenine (10 mM) and chloroquine (25 uM) did not result in growth inhibition or cell death. To confirm these previous data, Hs578t/shBeclin-1 cells were generated. Figure 3.2.1a shows that Beclin-1 expression was suppressed in Hs578t. Cells were stained with acridine orange and subsequent quantification of acidic vesicular organelles was followed. In Figure 3.2.1b acridine orange images indicate that radiation alone strongly induced autophagic vesicle formation in Hs578t cell. The level of acridine orange staining was significantly attenuated after Beclin-1 was silenced. Figure 3.2.1c indicates that radiation alone could bring the autophagic vesicle level from 8.1% in NT condition to 40.4% at 0 hour post radiation. Silencing of Beclin-1 expression could suppress about 25% of the autophagic vesicle level, indicating that autophagy induction was successfully inhibited. To examine whether Beclin-1 silencing could inhibit autophagic flux, I performed Western blot to analyze p62 degradation. In Figure 3.2.1d, degradation of p62 started as early as 0 hour post radiation and continued to be clearly degraded at 24 hours post radiation in Hs578t cells. On the other hand, p62 level remained unchanged in Hs578t/shBeclin-1 cells. Thus, silencing of Beclin-1 could inhibit autophagy induction and completion in Hs578t cells.
Next, in order to determine the growth ability of the cells after interference with autophagy through Beclin-1 silencing, I performed Trypan blue exclusion assay, counting for the number of viable cells. Figure 3.2.1e indicates that no significant differences in growth inhibition effects between radiation alone and radiation in combination with Beclin-1 silencing were detected. Percentage of cell viability calculated in Figure 3.2.1f suggests that minimum cell death happened within 24 hours post radiation. Furthermore, clonogenic survival assay indicates that radiation alone could suppress about 20% of colony formation in Hs578t. Moreover, inhibition of autophagy by silencing of Beclin-1 did not alter the colony formation in the presence of radiation (Figure 3.2.1g), in agreement with our previous data (manuscript in preparation). In other words, inhibition of autophagy through Beclin-1 silencing did not change the sensitivity of Hs578t cells to radiation.
Figure 3.2.1 Silencing of Beclin-1 inhibited radiation-induced autophagy in Hs578t cell. (a) Western blot analyses of endogenous Beclin-1 in Hs578t cells transfected with control or Beclin-1 shRNAs lentivirus constructs. α–tubulin was used as a loading control. (b) Acridine orange images were taken at 24 hours post radiation using an inverted fluorescent microscope.
Figure 3.2.1 Silencing of Beclin-1 inhibited radiation-induced autophagy in Hs578t cell. (c) Acridine orange staining of acidic vesicles quantified by flow cytometry. Measurement of autophagy induction based on % acidic vesicle formation was monitored by acridine orange staining and quantified by flow cytometry within 0 hour and 24 hours post radiation. *, p < 0.05; **, p < 0.01; ***, p < 0.001. One-way ANOVA was followed by Bonferroni post-hoc test. Results were taken from three independent experiments. (d) Cell lysates were collected in NT cells, and 0 hour and 24 hours post radiation. The lysates were subjected to Western blot analyses with the indicated antibodies. β-actin was used as a loading control.
Figure 3.2.1 Silencing of Beclin-1 inhibited radiation-induced autophagy in Hs578t cell. (e) Hs578t cells were plated with a concentration of $5 \times 10^4$ cells per well (six well tissue culture dish). The cells were exposed to radiation (5x2 Gy) over a period of 3 days. Number of viable cells was determined by Trypan Blue exclusion assays in NT cells, and 0 hour and 24 hours post radiation. (f) Percentage of cell viability was determined by Trypan blue exclusion assay. The viable cell number was normalized to the total number of viable cells and dead cells. (g) After exposure to radiation, the cells were immediately plated with a concentration of $1 \times 10^4$ cells per well (six well tissue culture dish). Number of colonies was determined by clonogenic survival assays over a period of 12 days. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. One-way ANOVA was followed by Bonferroni post-hoc test. Results were taken from three independent experiments.
3.2.2 The expression levels of autophagy-related proteins in MCF-7 and Hs578t cells

To identify factors that could be responsible for the induction of different types of autophagy, I determined the expression levels of autophagy related proteins in MCF-7 and Hs578t cells. Western blot analyses in Figure 3.2.2 indicates that Atg-5, a critical regulator of autophagy, was similarly expressed in the two cell lines. On the other hand, the expression of Beclin-1, an essential initiator of autophagy, was much higher in Hs578t as compared to MCF-7 cells. Since Beclin-1 has been shown to interact with the anti-apoptotic Bcl-2 proteins, and the interaction inhibits Beclin-1 autophagic function (Liang et al., 1998), it is important to determine the expression levels of the anti-apoptotic Bcl-2 proteins. The Western blot indicates that Bcl-xL and Mcl-1 were similarly expressed in MCF-7 and Hs578t cells. Surprisingly, while MCF-7 expressed high level of Bcl-2, Bcl-2 expression was undetectable in Hs578t.
Figure 3.2.2 Western blot analyses demonstrated the expression levels of autophagy-related proteins in MCF-7 and Hs578t cells. Equal amounts of protein lysates were subjected to Western blot analyses using the indicated antibodies.
3.2.3 The interaction between Beclin-1 and the exogenous Bcl-2 in Hs578t cell.

In Figure 3.2.3a, Bcl-2 retrovirus construct was introduced into Hs5787t cells and the expression was demonstrated by Western blot analyses. The levels of Bcl-xL, Mcl-1 and Beclin-1 remained unchanged after Bcl-2 introduction as compared to the control cells. To examine the interaction between Beclin-1 and Bcl-2 proteins in Hs578t cells, I performed co-immunoprecipitation as demonstrated in Figure 3.2.3b. The interaction of Beclin-1 to Bcl-xL or Mcl-1 was very weak or non-detectable, respectively. However, the interaction between Beclin-1 and exogenous Bcl-2 was clearly observed.
Figure 3.2.3 The interaction between Beclin-1 and the exogenous Bcl-2 in Hs578t cell. (a) Western blot analyses for the introduction of Bcl-2. Hs578t cells were infected with retrovirus-encoding RNA constructs for non-targeting control or Bcl-2 expressing vector. (b) Co-immunoprecipitation (IP) with Beclin-1. Total cell lysates were subjected to IP with anti-Beclin-1. Western blot analyses were carried out on the precipitated samples with the indicated antibodies.
3.2.4 Introduction of Bcl-2 inhibited radiation-induced autophagy in Hs578t cell.

To examine whether the introduction of Bcl-2 contributed to the induction of autophagy in Hs578t cells, I determined the differences in autophagy level in Hs578t/control and Hs578t/Bcl-2 using acridine orange staining.

Figure 3.2.3a demonstrates the level of Bcl-2 in Hs578t/control and Hs578t/Bcl-2 cells. In Figure 3.2.3b acridine images indicate that radiation induced significant level of autophagic vesicles in Hs578t/control and lower level of the vesicles was detected in Hs578t/Bcl-2. Quantification of the acidic vesicles shown in Figure 3.2.3c indicates that the percentage of acidic vesicles in Hs578t/control increased from 8.1% in NT condition to 40.4% at 0 hour post radiation. At 24 hour, the amount of acidic vesicular organelles went down to 11.1%, suggesting that the vesicles were degraded and autophagy went to completion. In Hs578t/Bcl-2 cells, the percentage of acidic vesicular organelles started at 12.5% in NT conditions. The amount went up to 32.7% within 0 hour post radiation. However, at 24 hours post radiation, the amount of acidic vesicular organelles was 22.5%, indicating that degradation of acidic vesicular organelles in Hs578t/Bcl-2 was attenuated as compared to that in Hs578t/control cell. In addition, Western blot analyses in Figure 3.2.3d indicate that in Hs578t/control cell radiation could induce LC3-II accumulation as early as 0 hour post radiation. The level of LC3-II accumulation gradually went down together with the down-regulation of p62 protein, suggesting that autophagy was induced and went to completion within 24 hours post radiation. On the other hand, in Hs578t/Bcl-2 cell
radiation could promote the accumulation of LC3-II. However, down-regulation of LC3-II and p62 were not observed within 24 hours post radiation. The data in Figure 3.2.3 suggest that introduction of Bcl-2 into Hs578t cell could attenuate the formation of autophagic vesicles and inhibited the degradation step of autophagy induced by radiation.
Introduction of Bcl-2 inhibited radiation-induced autophagy in Hs578t cell. Hs578t cells were exposed to radiation (5x2 Gy) over a period of 3 days. (a) Bcl-2 level was analyzed by Western blot analyses comparing the control with Bcl-2 transfected vectors. (b) Acridine orange images were taken at 24 hours post radiation using an inverted fluorescent microscope.
Introduction of Bcl-2 inhibited radiation-induced autophagy in Hs578t cell. Hs578t cells were exposed to radiation (5x2 Gy) over a period of 3 days. (c) Acridine orange staining of acidic vesicles quantified by flow cytometry. Measurement of autophagy induction based on % acidic vesicular organelles was monitored by acridine orange staining and quantified by flow cytometry in NT cells and at 0 hour and 24 hours post radiation. *, p < 0.05; **, p < 0.01; ***, p < 0.001. One-way ANOVA was followed by Bonferroni post-hoc test. Results were taken from three independent experiments. (d) Cell lysates were collected in NT cells, and at 0,6,12 and 24 hours post radiation. The lysates were subjected to Western blot analyses with the indicated antibodies.
3.2.5 Inhibition of autophagy by exogenous expression of Bcl-2 produced minimum changes in growth inhibition effects produced by radiation in Hs578t cell.

Trypan blue exclusion assays were performed to determine the number of viable cells after exposure to radiation. In Figure 3.2.4a, radiation alone could mildly inhibit cell growth in Hs578t/control cell at 0 hour and 24 hours post radiation. Furthermore, stable expression of Bcl-2 in Hs578t did not alter the sensitivity of the cells to radiation. In Figure 3.2.4b, percentage cell viability was determined by normalizing the number of viable cells to the total number of viable cells and dead cells after 0 hour and 24 hours post radiation, indicating that minimum cell death was observed. Furthermore, Western blot analyses suggest that no cleaved-PARP expression was observed in either Hs578t/control or Hs578t/Bcl-2 cells, indicating minimum apoptosis happened within 24 hours post radiation (Figure 3.2.4c). In addition, data in Figure 3.2.4d taken from clonogenic survival assay indicates that radiation in combination with Bcl-2 stable expression did not change the sensitivity of Hs578t to the growth inhibition effects on colony formation after exposure to radiation. In short, data in Figure 3.2.4 suggests that inhibition of autophagy through Bcl-2 stable expression did not alter the sensitivity of Hs578t to radiation.
Inhibition of autophagy by exogenous expression of Bcl-2 produced minimum changes in growth inhibition effects produced by radiation in Hs578t cell. Hs578t cells were plated with a concentration of $5 \times 10^4$ cells per well (six well tissue culture dish). The cells were exposed to radiation (5x2 Gy) over a period of 3 days. (a) Number of viable cells was determined by Trypan Blue exclusion assays in NT cells, and 0 hour and 24 hours post radiation. (b) Percentage of cell viability was determined by Trypan blue exclusion assay. The viable cell number was normalized to the total number of viable cells and dead cells.

**Figure 3.2.5**
Inhibition of autophagy by exogenous expression of Bcl-2 produced minimum changes in growth inhibition effects produced by radiation in Hs578t cell. (c) Cell lysates were collected and Western blot analyses were performed with the indicated antibodies. (d) After exposure to radiation, the cells were immediately plated with a concentration of $1 \times 10^4$ cells per well (six well tissue culture dish). Number of colonies was determined by clonogenic survival assays over a period of 12 days. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. One-way
ANOVA was followed by Bonferroni post-hoc test. Results were taken from three independent experiments.
Section 4: Discussion

Most breast tumor cells undergo autophagy after exposure to radiation (Rodemann et al., 2011). Autophagy is an important catabolic process which removes damaged proteins and organelles to maintain cellular homeostasis (Mizushima and Komatsu, 2011). Even though pro-survival autophagy is the main function of autophagy in response to stresses (He and Klionsky, 2009; Ouyyyang et al., 2012), it has been suggested that autophagy can perform different functions in tumor cells.

Data from this study and previous studies suggested that radiation at 5x2 Gy induced cytoprotective autophagy in MCF-7 cells. Inhibition of autophagy by silencing of Beclin-1 led to an increase in growth inhibition produced by radiation in MCF-7 cells (Figure 3.1.1). Interestingly, enhancement of autophagy by silencing of Bcl-2 did not protect the cells from radiation growth inhibition effects. On the other hand, enhancement of autophagy by silencing of Bcl-2 sensitized MCF-7 cells to radiation (Figure 3.1.4). It was shown that cell death was not the mode of sensitization to radiation either in Beclin-1 silencing or Bcl-2 silencing MCF-7 cells. Thus further experiments were needed to determine the mode of sensitization to radiation growth inhibition effects.

Data from this work and previous works from the lab suggested that radiation-induced autophagy in Hs578t played non-protective function. Inhibition of autophagy by silencing of Beclin-1 could not sensitize the cells to radiation (Figure 3.2.1). Furthermore, Hs578t is a Bcl-2 non-expressing cell line (Figure 3.2.2). Stable expression of Bcl-2 resulted in Beclin-1/Bcl-2 interaction, which led
to inhibition of autophagy induced by radiation. Similarly, inhibition of autophagy by Bcl-2 stable expression did not alter the sensitivity of Hs578t cells to radiation (Figure 3.2.4).

In conclusion, data from current study suggested that interference with cytoprotective autophagy (in MCF-7 cell) could result in an increase in sensitivity to treatment (radiation). However, alterations in non-protective autophagy (in Hs578t cell) did not result in either increased or decreased radiation sensitivity.
Section 5: Future Studies

In MCF-7 cells, further studies are needed to determine modes of increased radiation sensitivity after Beclin-1 and Bcl-2 silencing. Since it was shown that cell death was not the mode of sensitization, it could be senescence growth arrest playing the role in the increase of growth inhibition after exposure to radiation. On the other hand, recent studies from our lab demonstrated that cytoprotective autophagy could be converted into cytostatic autophagy in the same cellular system. Cytostatic autophagy leads to cellular growth arrest.

Therefore, it is possible that silencing of Bcl-2 in MCF-7 cell led to a conversion from cytoprotective into cytostatic autophagy. Therefore, future studies could be involved in manipulating the level of autophagy induced in Bcl-2 silencing cells. Also, it is important to assess whether enhancement of autophagy by different approach such as through rapamycin treatment could lead to similar sensitivity as observed in Bcl-2 silencing cells.

In Hs578t, it looks like manipulating non-protective autophagy does not result in any change in radiation sensitivity. However, it is interesting that exogenous Bcl-2 did not inhibit autophagy at the autophagic vesicle formation level. It is possible that Bcl-2 regulates other autophagy-related proteins downstream of Beclin-1. Future studies could be done to understand whether Bcl-2 is interacting or regulating autophagy-related proteins other than Beclin-1.

It is also important to assess the sensitivity to treatment in other cell lines that induce cytoprotective autophagy and non-protective autophagy.
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VITA

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