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**Irradiation of Hs578t breast tumor cells induces non-cytoprotective
autophagy**

A thesis submitted in fulfillment of the requirements for the degree of Master of
Science in
Pharmacology and Toxicology at Virginia Commonwealth University

by

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May, 2014

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List of abbreviations

TNBC: Triple negative breast Cancer

HER-2: Human Epithelial Growth Factor Receptor\

AO: acridine orange

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

IR :Irradiation

Gy: Gray

NH₄CL: Ammonium Chloride

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Abstract

Cancer is the second most common cause of death in the US. The most frequently observed cancer type in women is breast cancer. A special type of breast cancer is triple negative (TNBC) cancer that is characterized by lacking three receptors: estrogen, progesterone and human epithelial growth factor (HER 2). The HS578t breast cell line is a model of TNBC that also has a mutation of the p53 protein.

Ionizing radiation is used widely in the clinic to debulk tumors before surgery as well as post-surgery to eliminate residual tumor cells outside the surgical field. Previous studies from our laboratory showed that inhibition of autophagy does sensitize p53 wild type MCF-7 and ZR-75 breast tumor cells to radiation. However, this is not necessarily the response in all breast cell lines. The Hs578t cells did not appear to be sensitized to radiation after inhibition of autophagy using chloroquine as a pharmacological inhibitor.

The present study was designed to build upon these previous findings and further confirm that the Hs578t breast cell line could not be sensitized to radiation through autophagy inhibition. Time course studies showed a reduction of viable cell number upon irradiation of Hs578t breast tumor cells and that both autophagy and senescence were induced. Acridine orange staining was used to examine the acidic vacuole formation while β -galactosidase staining indicated the promotion of senescence. Flow cytometry was used to quantify both autophagy and senescence. Inhibition of autophagy using pharmacological inhibitors such as ammonium chloride, or genetic silencing of autophagy by beclin1, which is a protein initiator of autophagy, did not sensitize Hs578t breast tumor cells to irradiation. It shows from these studies that autophagy is not necessarily cytoprotective in all breast cancer cell lines, which should be considered in current clinical trials designed to sensitize tumor cells to chemotherapy and radiation through inhibition of autophagy.

Section 1

Introduction

1.1 Cancer:

The body is comprised of billions of living cells that have the capability to grow, multiply and die in an organized manner. ^[1] Cancer is defined as cells growing abnormally and out of control. Cancer can be benign when it only spreads locally and does not invade other tissue, or it can be malignant if it metastasizes. Because there are many different types of cancer, treatments strategies will also differ. In general, cancer should be treated immediately upon diagnosis as otherwise it will lead to death.

In 2013, about 1,660,290 new cases of cancer were expected to be diagnosed and about 580,350 Americans were expected to die of cancer. ^[1] This leads to cancer being the second most common cause of death in the US.

There is no single cause of cancer; there are many genetic and environmental factors that might interact to cause cancer. Some types of cancer have been associated with environmental factors such as smoking, exposure to ultraviolet radiation and high-fat food. ^[3] Also viruses such as HPV (Human Papilloma Virus) have been associated with cervical and head and neck cancer. ^[4] Mutations in oncogenes, tumor suppressor genes and mis-match repair are thought to play central roles in the development of cancer. ^[5]

Telomerase, the specific DNA polymerase that inserts telomere repeat segments to the terminations of telomeric DNA, is lacking in normal cells but highly expressed in human cancer cells. [5] The expression of telomerase activity is associated with the immortalization of cancer cells and resistance to the development of senescence.

1.2 Breast Cancer

There are a number of different forms of breast cancer. When cancer originates in the ducts, it is known as ductal carcinoma and if it originates in lobules it is called lobular carcinoma. Inflammatory Breast Cancer is another type of the breast cancer that affects primarily the skin of breast. [2] In general breast cancer accounts for 22.9% of all cancers that affect women and it is more than 100 times more common in women than in men. Survival rates for breast cancer differ greatly based on the cancer type, stage, treatment, and health status of the patient.

The first symptom of breast cancer is typically the feeling of a lump that differs from the rest of the breast tissue. Earliest cancers can be detected by a mammogram. [2] Breast cancer can be a hereditary disease since in the United States, 10 to 20 percent of patients with breast cancer have a first- or second-degree relative with this disease. [6]

Breast cancers can be classified by several categories. Each has its influence on the prognosis and can affect treatment response. [7] Breast cancer can be classified according to histopathology in which most cancers are derived from epithelial cells. Alternatively, it can be classified according to the stage, where stage 1 represents cancer that is confined to the breast and stage 4 representing breast cancer that has metastasized to the lymph nodes and distant organs. Another classification of breast cancer is according to the receptor status, as it may or may not have three important receptors: estrogen receptor (ER), progesterone receptor (PR), and HER2. The presence of the estrogen receptor allows cancer cells to be treated with hormonal therapies such as Tamoxifen and to have a better prognosis. [2] Another type of breast tumor, one that does not express estrogen receptor (ER), progesterone receptor (PR), or HER2, is termed called Triple Negative Breast Cancer. [8]

1.3 Triple Negative Breast Cancer (TNBC)

As indicated above, TNBC is a type of breast cancer that does not express the estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor-2 Her2/neu. [9] Triple negative breast cancers are considered to be high aggressive with a poor prognosis. It accounts for approximately 15%-25% of all breast cancer cases and tends to affect younger women, African American and Hispanic women.

Great efforts have been devoted to identify novel therapeutic targets for TNBC; however, the heterogeneity and complexity of the biology of TNBC pose significant challenges to development of targeted agents. Agents that have been examined in clinical trials include the monoclonal antibody that targets the EGFR such as cetuximab, multityrosine kinase inhibitors and poly (ADP-ribose) polymerase (PARP) inhibitors. ^[10] Also anti-angiogenic therapies such as bevacizumab are used as adjuvant therapy for women with triple-negative breast cancer. ^[11]

1.4 Current Medical Treatment.

Treatment of breast cancer can include surgical intervention, radiation, and chemotherapy. ^[2] Treatment can be alone or in combination and will depend on many factors such as the type and stage of the cancer, whether the cancer is sensitive to certain hormones, and whether the cancer overexpresses some of the receptors such as HER2/neu. Amplification of HER2 plays a critical role in the development and progression of certain aggressive types of breast cancer. ^[12]

Surgery: Surgery is used to remove the primary tumor, whether or not it has metastasized. It is also used to test for cancer spread by checking lymph nodes under the arm for disease infiltration. Mastectomy removes the entire affected breast while lumpectomy removes only the breast lump and a surrounding margin of normal tissue. ^[2]

Chemotherapy: Chemotherapy refers to the use of medication to destroy or slow the growth of rapidly multiplying cancerous cells. [2] It is considered a systemic form of breast cancer treatment because drugs are given intravenously or orally and enter the bloodstream to travel to all areas of the body's in order to reach cancer cells. There are three major types of chemotherapy: neo-adjuvant which is used before surgery to shrink the tumor size, adjuvant which is given after surgery to reduce risk of recurrence, and palliative which is given when the cancer spreads beyond the breast and localized lymph nodes and is used to control but not to cure the cancer. Chemotherapy is given in cycles of treatment, followed by a recovery period. The total chemotherapy treatment usually takes several months to one year, depending on the type of drugs given. [2]

According to the breast cancer types and the patient condition, physicians usually try to personalize the breast cancer treatments. Drugs that are frequently used for adjuvant chemotherapy in breast cancer are cyclophosphamide, 5-fluorouracil, and taxol. [7]

Despite the improvement in the diagnostic tests that are used in early detection and understanding of basic molecular biology of breast cancer, around 30% of all patients with early-stage breast cancer have recurrent disease. Reduction in the effectiveness of chemotherapy is often due to development of resistance. [7] There are several mechanisms of drug resistance in breast cancer. For instance, resistance to Tamoxifen, which is an estrogen antagonist that binds to the estrogen receptor (ER) and inactivates it, has been developed by cancer cells by reducing estrogen receptor expression. [46]

Hormonal Therapy: Hormonal therapy is another form of systemic therapy that is used as adjuvant therapy to reduce the risk of cancer recurrence. [2] Estrogen stimulates the growth of cancers that are positive for the hormone receptor. Generally about two-thirds of breast tumors are hormone receptor-positive and express the receptors for the hormones estrogen and/or progesterone. The mechanism of action of hormonal therapy involves interfering with estrogen acting on breast cancer cells or lowering estrogen levels. Hormonal therapy is not effective for patients whose tumors are hormone receptor negative such as triple negative tumors. [2]

There are many drugs that are used as hormonal therapies. Selective estrogen receptor modulator or SERMs have estrogen-like agonist properties in some tissues but work as anti-estrogens in other tissues. [7] Tamoxifen is an estrogen antagonist that is an example of a SERM that can be given after surgery for 5 years to reduce the chances of recurrence of the cancer by about half, thereby significantly prolonging patient survival. Tamoxifen also reduces the risk of a new breast cancer in the other breast. [13]

Aromatase Inhibitors (AIs) represent another form of hormonal therapy that interferes with estrogen production in the breast tissue of post-menopausal women and which are used to treat both early and advanced breast cancer. [14] Because of their effectiveness, AIs are the most clinically used antihormonal treatment for breast cancer in postmenopausal women. However, AIs are ineffective in pre-menopausal patients where high levels of estrogens are being produced in the ovaries. [14]

Targeted therapy for breast cancer

Changes in the activity of specific genes have been associated with breast cancer development, and researchers have been able to discover newer drugs that specifically target these changes. The mechanisms of action of these targeted medications differ from that of standard chemotherapy drugs, and they often have less severe side effects. ^[15] About 1 in 5 patients with breast cancer has high levels of a growth-promoting protein known as HER2/neu on their surface. These breast cancers have a greater ability to grow and spread more aggressively and a number of drugs have been developed that target this protein. ^[2]

Trastuzumab is a monoclonal antibody that binds to HER2 and can help slow the growth of cancer cells that overexpress HER2. It may also stimulate the immune system to more effectively attack the cancer. ^[16] Trastuzumab is often used as adjuvant therapy for HER2-positive cancers with chemotherapy to reduce the risk of cancer recurrence. In the later course of treatment, trastuzumab can be given alone, usually for a total of a year of treatment. ^[2]

Bevacizumab (also known as Avastin) is another monoclonal antibody that can be used in patients with metastatic breast cancer. This antibody antagonizes the vascular endothelial growth factor, which is a protein that helps tumors to form new blood vessels. Bevacizumab is given by intravenous (IV) infusion. It is commonly used in combination with chemotherapy. ^[17]

Bevacizumab was first accepted by the US Food and Drug Administration (FDA) as part of the treatment for metastatic breast cancer in 2008, but is no longer approved for this purpose.^[18]

Another example of targeted therapy is Everolimus, which blocks mTOR (the mammalian target of Rapamycin) which is a protein in cells that normally promotes growth and division.^[15] By blocking mTOR, Everolimus can prevent cancer cells from growing. Everolimus may also prevent tumors from forming new blood vessels. It is approved to treat advanced tumor cells and earlier stage breast cancer, with other hormone therapy drugs, and in combination with other treatments. Bevacizumab is not effective in patients with triple-negative breast cancer (TNBC) because of the lack of targeted therapy for these patients.^[15]

Radiation therapy:

Radiation therapy involves the medical use of ionizing radiation to control or kill cells in cancer treatment.^[20] If the cancer is localized in one part of the body, then radiation therapy may be curative, or as adjuvant therapy to prevent tumor recurrence after surgery or with chemotherapy. The treatment goal of radiation therapy depends on the tumor type, location, and stage, as well as the general health of the patient. Ionizing radiation produces its effect by damaging the DNA of exposed tissue leading to cellular death. In photon therapy, the radiation produces double strand breaks which is more effective in promoting cancer death than single strand breaks.^[20]

In the Law of Bergonie and Trebondeau, radiation is more damaging for rapidly dividing cells such as epithelial cells of the gastrointestinal tract than non-dividing cells and neurons. [21] During IR exposure, both single-stranded DNA breaks (SSB) and double-stranded DNA breaks (DSB) occur. DSB have more lethal effects than SSB.[21]

The measure of radiation is in terms of Rads or Grays (Gy) where 100 rads is equivalent to 1 Gy. The radiation dose that will be administered depends on many factors including the stage and size of the tumor, as well as the patient's health.

[20]

In the clinic, the total doses for radiation of tumors range from 40 to 80 Gray, and usually these doses are given in the form of fractionated doses of 1.8 – 2 Gy over a period of time. [20] The rationale for using fractionated doses is to allow the healthy cells to recover since tumor cells are less effective in repairing damage to their DNA. [21] Also fractionated radiation will allow the specific cycle resistant tumor to enter to another cycle where they might be more radio-sensitive. With fractionated doses of radiation, hypoxic and more radio-resistant cells might re-oxygenate between fractions.

Radiation therapy is generally not of itself painful to the patient. However, the severity and longevity of side effects will depend on the organs involved in receiving the radiation, the co-administration of chemotherapy, and the patient's health.[20] The most common side effects of radiation are nausea, vomiting, gastrointestinal ulcers, edema, abdominal discomfort, and infertility in rare cases.

1.5 Cellular response in Cancer:

Apoptosis, autophagy, necrosis, and senescence are cellular responses to external stresses such as radiation and chemotherapy.^[22] Cellular responses may equilibrate cell death with survival of cells. This balance plays a critical role in the ultimate decision of cancer cell fate.

Apoptosis

Apoptosis, or type I programmed cell death, was first described by Kerr et al in 1972. Apoptosis is characterized by specific morphological and metabolic changes of dying cells, which includes membrane shrinkage, nuclear fragmentation, and loss of adhesion to extracellular matrix.^[23] Chromosomal DNA cleavage and externalization of phosphatidylserine, Annexin I and calreticulin are among specific biochemical abnormalities of apoptosis. In response to stress, cells are producing intracellular responses that initiate apoptosis and lead to cell death.

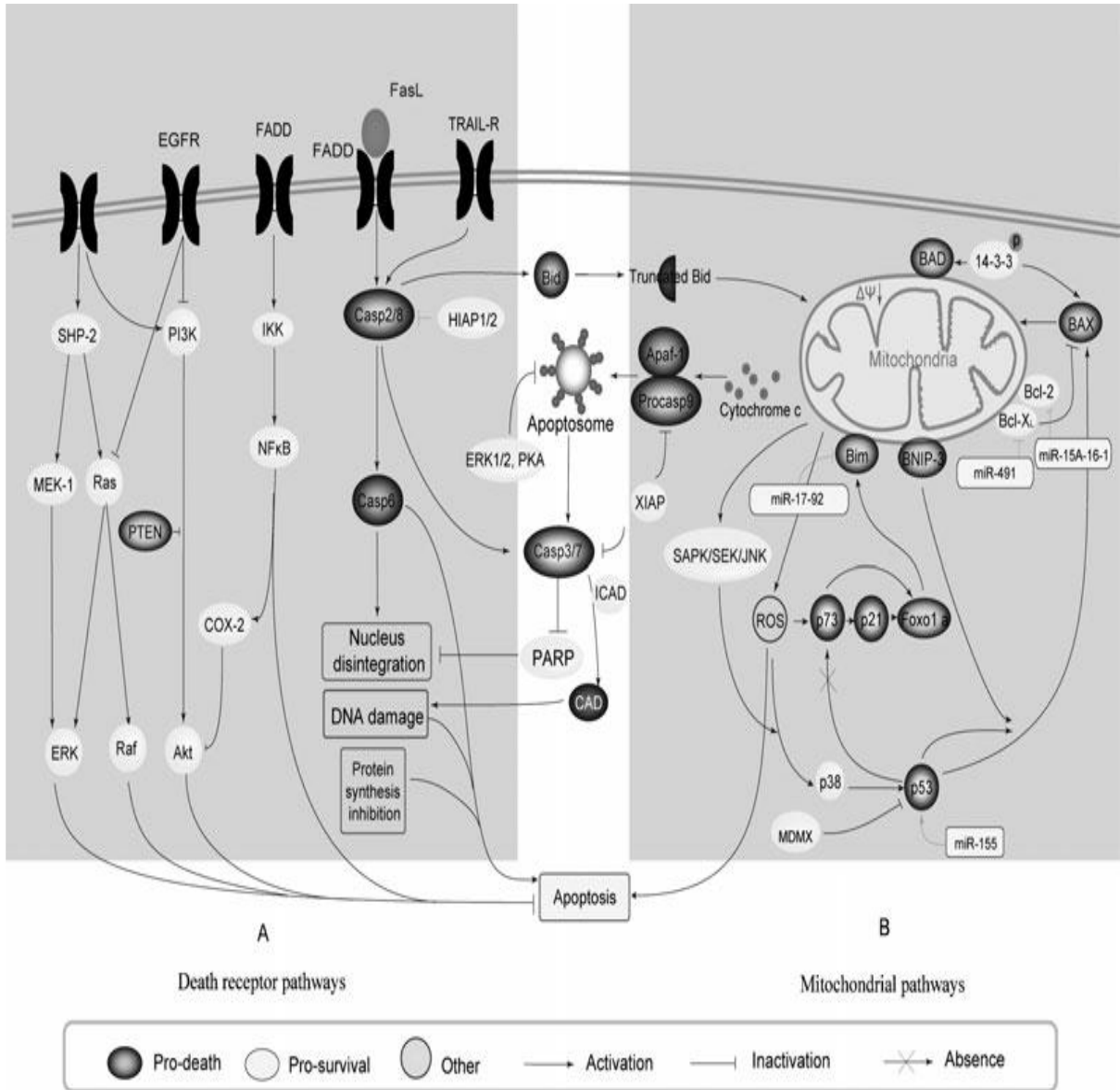
One of the most important regulators of apoptosis is the caspase family, which are endoproteases that hydrolyze peptide bonds and lead to activation of signaling pathways and ultimately apoptosis.^[23] Caspases that are involved in apoptosis have been subdivided according to their mechanism of action to either initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7).

There are two basic pathways that regulate apoptosis, the intrinsic or mitochondrial pathway and the extrinsic or receptor-mediated pathway. [22] In the extrinsic pathway, Fas, which is plasma membrane death receptor, binds with its extracellular ligand Fas-L. Formation of Fas/Fas-L complex initiates cell death and recruits death domain-containing protein (FADD) and caspase 8 to form the death-inducing signaling complex (DISC). DISC activates pro-caspase-8 and pro-caspase-3.

In the intrinsic pathway, activation of apoptosis is regulated by mitochondrial pro-enzymes. [22] When the cell is stimulated by external or internal stimuli, outer mitochondrial membranes become permeable and cytochrome c is then released into the cytosol. The release of cytochrome leads to recruitment of pro-caspase-9 and apaf-1 to form the apoptosome which downstream activates the caspase 9/3 signaling cascade of apoptosis.

In many types of cancer cells, the Bcl 2 family proteins are overexpressed. Bcl2 is one of the major regulators of apoptosis.[22] Reduction of Bcl-2 expression is permissive for apoptotic responses to anticancer drugs, while overexpression of Bcl-2 leads to resistance to chemotherapeutic drugs and radiation therapy. The Bcl-2 family consists of of pro-apoptotic members such as Bax, Bak, Bad, Bcl-XS, Bid, Bik, Bim and Hrk, plus further anti-apoptotic members such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1.

Another important proapoptotic factor and tumor inhibitor is p53, which acts as a tumor suppressor preventing cancer. Since p53 activates apoptotic cell death by promoting a number of positive regulators of apoptosis, the antitumor effects of many chemotherapeutic agents may be mediated by targeting p53-related signaling pathways. [24]



Ouyang L et al (2012)

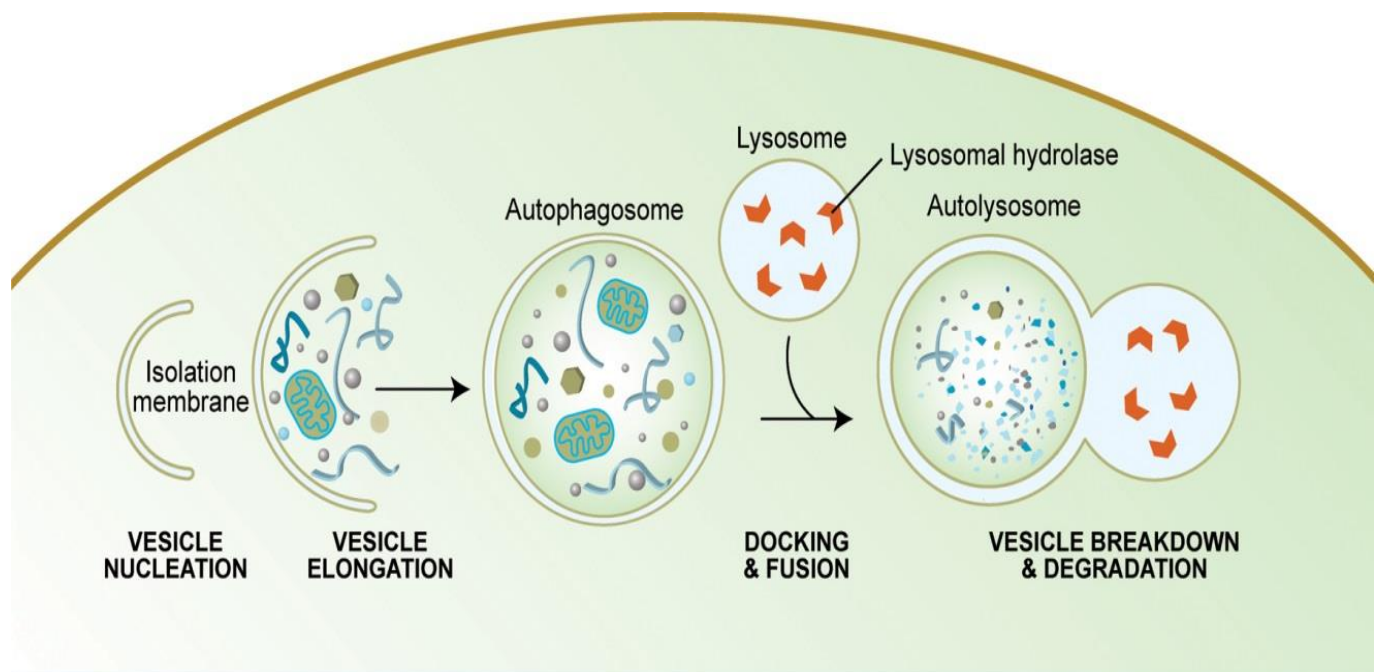
Autophagy

The process of autophagy was first revealed by Keith R. Porter and his postdoctoral student Thomas Ashford. ^[28] It involves the catabolism of unnecessary or dysfunctional cellular components, and is initiated by the formation of autophagosomes, double membrane-bound structures surrounding cytoplasmic macromolecules and organelles, designated for recycling. ^[26] Autophagy can promote cellular survival during starvation by maintaining cellular energy levels. ^[28] It plays a crucial role in a homeostasis by controlling physiological processes including cell differentiation, cell survival and death. ^[22]

The autophagy process consists of different steps, including induction step, cargo recognition and selection, vesicle creation, autophagosome-vacuole union, and analysis of the cargo followed by release of the degradation yields back into the cytosol. ^[22] Different ATG (Autophagy related genes) are essential in these steps. In the induction step, starvation or rapamycin treatment leads to activation of the Unc-51-like kinase 1 (ULK1) and -2 (ULK2), also FIP200 (the focal adhesion kinase family-interacting protein of 200 kD). FIP200 forms a complex with ULKs and mammalian Atg13 and identifies to the phagophore.

In the cargo recognition step, p62/sequestosome 1 (SQSTM1) binds both poly- or mono-ubiquitin through its ubiquitin-associated (UBA) domain and the mammalian Atg8 homolog, LC3 (microtubule-associated protein 1 light chain 3).^[27] In the formation of autophagosome step, multiple Atg proteins are recruited to the phagophore to take part in autophagosome formation. After completion of autophagosome formation, Atg8 attached to the outer membrane is cleaved from PE (phosphatidylethanolamine) by Atg4 leading to autophagosome-vacuole fusion.

The complete process of autophagy is called Autophagic flux. To confirm the autophagic flux, p62 may be used as a marker.^[27] p62 accumulates when autophagy is inhibited, and degraded levels of p62 can be observed when autophagy is induced



One of the crucial regulators of autophagy is p53, which is the tumor suppressor gene most commonly mutated in human cancers. p53 has a dual effect on autophagy.^[25] One way for p53 to induce autophagy is through activation of AMPK kinase, which leads to activation of TSC1 and TSC2 kinases, and to the acute inhibition of mTOR kinase (mammalian target of rapamycin). Kroemer et al reported that cytoplasmic but not nuclear p53 has the autophagy-inhibiting function.^[47]

Autophagy has many different roles in cancer.^[29] In the literature, it has been shown that chemotherapeutic drugs and radiation can lead to a cytoprotective form of autophagy in tumor cells, in which the sensitivity of tumor to radiation or chemotherapy is increased upon inhibition of autophagy. This increased in sensitivity after blockage of autophagy occurs by promoting apoptosis. Cytoprotective autophagy has its clinical implication, in that theoretically a patient can take an autophagy inhibitor such as chloroquine that leads to sensitization of tumor cells to radiation or chemotherapy.

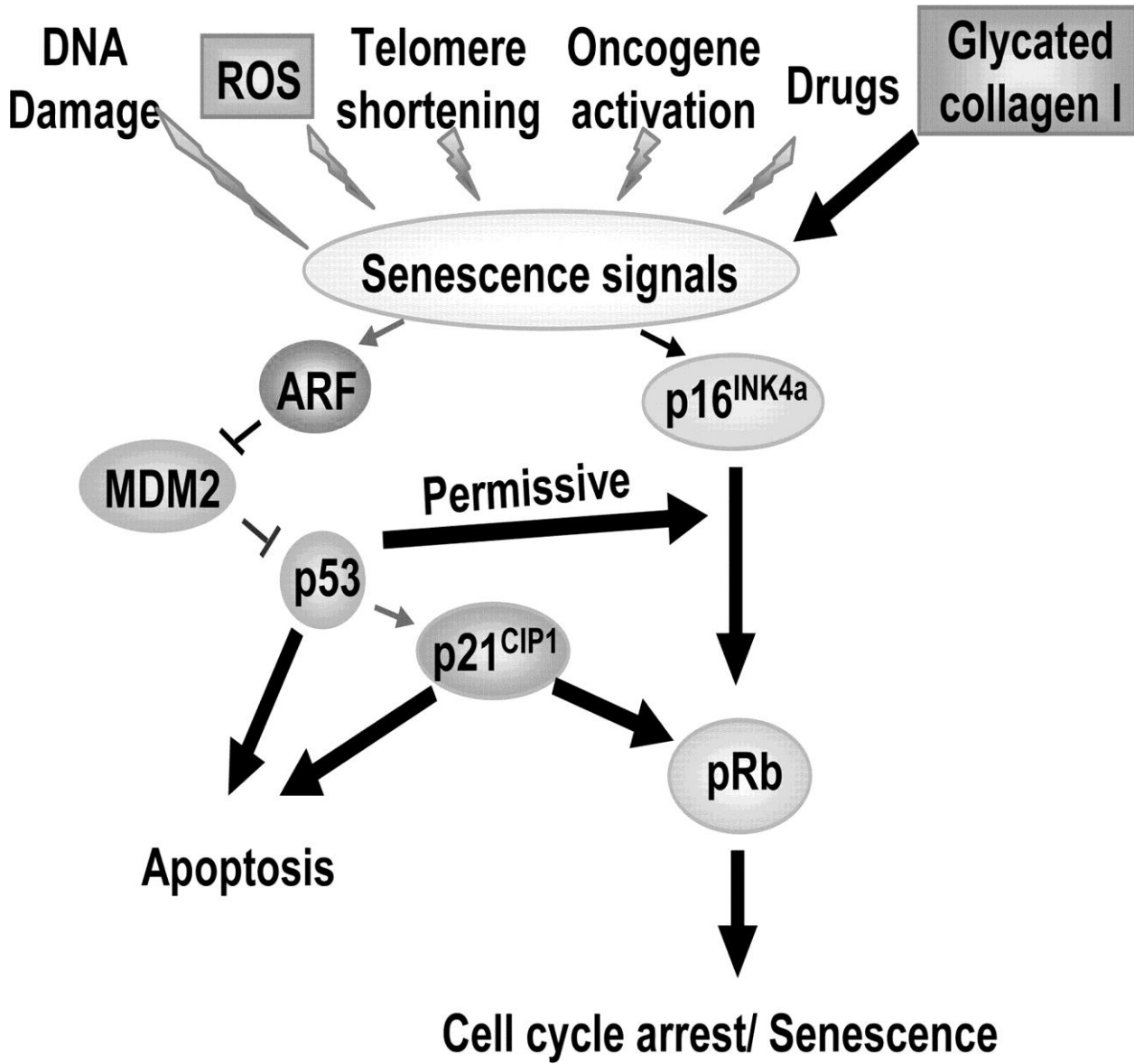
Another (lack of) function of autophagy is to be non-cytoprotective, in which inhibition of autophagy does not sensitize tumor cells to radiation or chemotherapy. ^[29] Cytotoxic effect is the third role of autophagy in cancer that leads to either killing of cells of itself or acts as a precursor to apoptosis. The main basic difference between cytotoxic and cytoprotective autophagy is that upon inhibition of autophagy in cytoprotective autophagy, the cells are sensitized to the treatment, while when cytotoxic autophagy is inhibited, the cells become less sensitive to the treatment.

The fourth function of autophagy in cancer is Cytostatic Autophagy. ^[29] In our laboratory, it has been shown that the combination therapy of vitamin D and radiation leads to a more pronounced growth inhibition of non-small cell lung cancer cells than for radiation alone. Also pharmacologic inhibition of autophagy protects the cells from the sensitization to radiation by vitamin D. The relationship of cytostatic autophagy with senescence is currently under investigation in our laboratory.

1.5.3 Senescence

Cellular senescence refers to the irreversible arrest of cell proliferation that occurs when cells experience oncogenic-induced stress or other forms of stress such as radiation and chemotherapy.^[30] Since there is no known physiological inducer that has the ability to stimulate senescent cells to reenter the cell cycle, the senescence is considered irreversible.^[32] One of the reasons why normal cells do not proliferate indefinitely is dysfunction of telomeres. Telomeres cap the ends of linear chromosomes and protect them from degradation or fusion by DNA-repair processes. In some cases, telomeres become short and dysfunctional which trigger a classical DNA damage response (DDR). DNA damage and dysfunctional telomeres induce p16, which acts as a tumor suppressor that is implicated in the prevention of cancers and offers a second obstacle to prevent the growth of cells.

Maintenance of the senescence growth arrested state is controlled mainly by either or both of the p53/p21 and p16INK4a/pRB tumor suppressive pathways.^[31] Both pathways have multiple upstream and downstream regulators. p53 and pRB are transcriptional regulators. p21 is a downstream effector of p53, while p16INK4a is a positive upstream regulator of pRB effectors. p21 and pRB are cyclin-dependent kinase inhibitors and strong negative regulators of cell cycle progression.

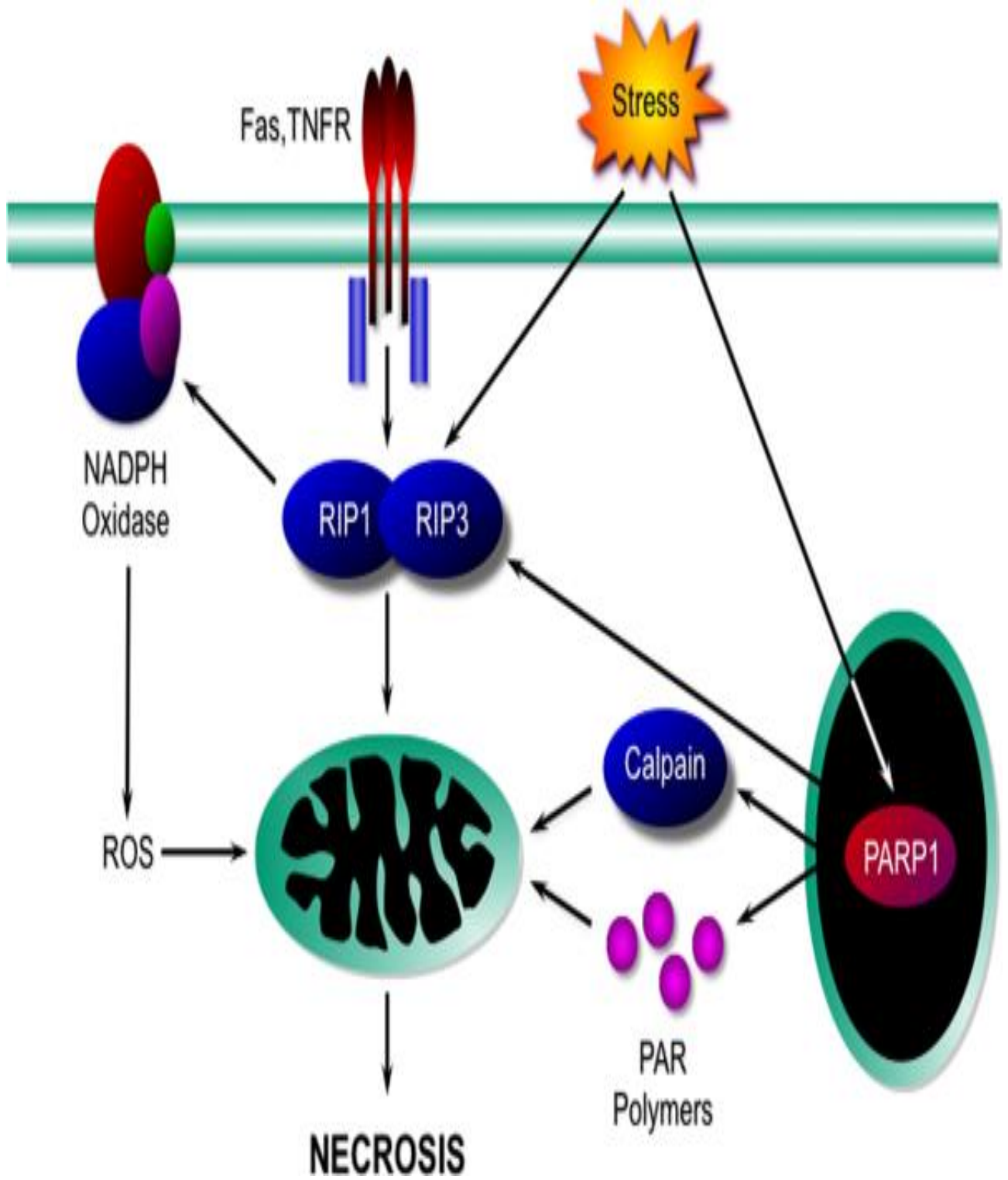


Campisi J et al (2007).

1.5.4 Necrosis

Necrosis is a result of cell injury that leads to the premature death of cells by autolysis. Infection, toxins, or trauma are external factors that lead to necrosis. [33] During necrosis, cells initially swell then the plasma membrane breaks down, and cells are rapidly lysed. Necrosis can be tested in vitro by measuring permeability of the cell plasma membrane to vital dyes like trypan blue, and efflux of cytosolic enzymes such as lactate dehydrogenase, or creatine kinase.

Binding of death ligands such as TNF α , TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) to their receptors leads to assembly of caspase-8 and receptor-interacting serine threonine kinase RIP1 and RIP3. The RIP family plays a critical role in necrosis and leads to stimulation of metabolic enzymes like glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1). [34] Enhancement of metabolic enzymes is accompanied by production of reactive oxygen species (ROS) which is dependent on the NADPH oxidase to form complexes with the adaptor molecules such as Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD).



Ouyang L et al (2012)

1.5.5 Mitotic catastrophe

The term mitotic catastrophe is defined as incorrect entrance of cells into mitosis which can be caused by chemical or physical stresses. ^[35] Agents that affect the stability of microtubules, anti-cancer agents, and ionizing radiation are considered to trigger mitotic catastrophe. Cells that undergo mitotic catastrophe are characterised by abnormalities of chromosome segregation.

Mitotic catastrophe is characterized by elevation of cyclin B1 which is a regulatory protein involved in mitosis, activation of p53 which is a tumor suppressor protein, persistent activation of SAC (Spindle- assembly checkpoint) which inhibits anaphase causing chromosome missegregation, and caspase-2 activation. ^[35]

1.6 Previous studies

In our laboratory, it has been shown that irradiation of p53 wild type, and estrogen receptor-positive MCF-7 and ZR-75-1 breast tumor cells induce autophagy. The autophagy produced by radiation alone was found to be cytoprotective in that both pharmacological and genetic inhibition of autophagy increased sensitivity to radiation. The combination therapies of vitamin D3 and radiation promote cytotoxic autophagy that upon inhibition the radio-sensitization by vitamin D is markedly attenuated. ^[36] ^[38]

It has been shown in much of the current literature that the cellular response to radiation in many cell lines is that of autophagy. ^[37] It has been shown in our laboratory that the inhibition of autophagy does sensitize MCF-7 and ZR-75 breast tumor cells to radiation. ^[36] However, this is not necessarily the response in all breast cell lines. In the 4T1 syngeneic murine breast tumor cells could not be sensitized to radiation in vitro by using pharmacological inhibitor of autophagy with chloroquine treatment, by silencing of the autophagy- related gene Atg12, or by chloroquine in vivo. ^[49] Also in Hs578t cells, which are a mutant p53 triple negative cell line, pharmacological autophagy inhibition did not increase radiation sensitivity.

Upon irradiation, Hs578t cells undergo autophagy; however, the combination of radiation with the autophagy inhibitor, chloroquine, does not sensitize this cell lines to radiation. Also, Hs578t cells show minimal apoptosis upon radiation alone and furthermore apoptosis does not increase with autophagy inhibition.

(Chakradeo S under revision)

1.7 Hypothesis

Since previous data using pharmacological inhibition of autophagy shows that the Hs578t cell line does not become sensitized to radiation, the effect of autophagy inhibition on radiation sensitivity using another pharmacological inhibitor and genetic approaches were recommended to be performed. (Chakradeo S under revision). In addition, to interfere with autophagy, beclin 1 was silenced using siRNA techniques.

Based on previous results in Hs578t, the hypothesis for this project was that Autophagy is a general response of breast cancer cells to radiation, but that inhibition of autophagy may not sensitize breast tumor cells to radiation (non-protective autophagy).

Our aims were:

AIM 1: Evaluation of the response of Hs578t to radiation

A- Determination of the effect of radiation on autophagy

B-Determination of the effect of radiation on apoptosis

C-Determination of the effect of radiation on senescence

AIM 2: Inhibition of autophagy genetically and pharmacologically

A-Assessment of the effect of autophagy inhibition on radiation sensitivity

B- Assessment of the effect of autophagy inhibition on senescence

C- Assessment of the effect of autophagy inhibition on apoptosis

Section 2: Materials and Methods

2.1 Material:

MEM Alpha media- L glutamate was purchased from Gibco. Trypsin-EDTA (0.25% trypsin, 0.53 mmol EDTA-4Na) was obtained from HyColne. PBS (pH=7) was obtaining from Gibco. X-gal (5-bromo chloro-3) was purchased from Fermentas Life Sciences. Fetal Bovine Serum was obtained from Serum source international. Acridine Orange was purchased from Invitrogen.

2.2 Cell lines:

Breast tumor cells Hs578t were obtained from American Type Culture collection (ATCC). Hs578t cells were developed to silence the expression of Beclin1. The lentiviral shRNA constructs to target Beclin-1 were kindly provided by Dr. Hisashi Harada (Virginia Commonwealth University, VA, USA). The shRNA constructs were transfected into 293T cells along with lentiviral packaging plasmids. The lentivirus shed into the medium was then used to infect the breast tumor cells. The retrovirus pBp-RNA expressing constructs were kindly provided by Dr. Wataru Nakajima (Nippon Medical School, Kawasaki, Japan). The constructs were transfected into 293T cells along with the packaging plasmids. The retrovirus shed into the medium was collected and used to infect Hs578t cells.

2.3 Cell Culture and Treatment:

Hs578t cells were grown from frozen stocks in MEM-alpha supplemented with 20% FBS, and penicillin/ streptomycin (0.5ml) in 100 ml medium. All cells were maintained at 37°C under a humidified 5% CO₂ atmosphere. Cells were kept in T75 flasks (Cellstar), sub-cultured and trypsinized with (0.25% trypsin, 0.03% EDTA) for 4- 6 minutes upon reaching confluence and then deactivated with complete MEM alpha media. Prior to any experiments, cells were examined by microscope for any fungal and bacterial infections.

2.4 Cell Viability:

Trypan blue exclusion dye was used to identify viable cells. Cells were plated in triplicate at a density of 6,000 to 10,000 cells per well and allow to adhere overnight. The following day, cells were treated with ammonium chloride, CQ, or 3-MA for 3 hours and then followed by a radiation dose 5x2 Gy. After radiation, media was changed and then cells were counted at various time points. Trypsin (0.25% Trypsin-EDTA) was added to cells and was incubated for 5-7 min at 36 C⁰. Then cells were stained with Trypan Blue (0.4% Trypan Blue). Haemocytometer with phase contrast microscopy was used to count cells on Days 1, 3, 5, and 7 post-radiation.

2.5 MTT Assay To determine the effective dose of Ammonium Chloride

The MTT assay was used to determine the effective dose of ammonium chloride for further experiments. Cells were plated in 96 well plates at a density of 5,000 cells/well in 200µL of MEM alpha Medium. Cells were allowed to adhere to the plates overnight and then treated with different doses of ammonium chloride. The doses of ammonium Chloride ranged from 1mM-10mM. Cells were incubated with drug for 72 hours. Then media was aspirated and cells were incubated for 3 hours with the MTT solution (2mg/ml PBS) at 37 C⁰. After removal of the MTT solution, autoclaved DMSO was added and plates were shaken for 10 min.

In MTT assay, MTT dye ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) becomes reduced to an insoluble formazan inside living cells. DMSO was used as solubilizing agent to dissolve insoluble purple formazan product into a colored solution. Then Absorbance was read at 490 nm using (KC Junior software, EL800 Universal Microplate Reader).

2.6 Clonogenic Survival Assay:

To evaluate the ability of a single cell to form a colony, clonogenic assay was used. In triplicate plates, cells were plated at a density of 150 cells per well. After 14 days, cells were fixed with 100% methanol, air-dried and stained with 0.1% v/v crystal violet (Sigma 3886)

2.7 Evaluation of Autophagy by Acridine Orange Staining

10,000 cells were plated in 6-wells plates and allowed to adhere for 24 hours prior to being treated and irradiated. At various time points at day 1, 3, 5, and 7 post-radiation, media was removed and cells were washed twice with PBS. Acridine orange dye was prepared in the dark at a dilution of 1 to 10,000 of media and incubated with cells for 15 minutes. Cells then were washed with PBS. Fluorescent micrograph images were taken using an inverted fluorescence microscope (Olympus). Increased numbers of acidic vesicular organelles were used as an indication of autophagy.

2.8 Evaluation of Senescence by Beta Galactosidase Staining Assay

The state in which cells stop growing but stay metabolically active is called senescence or biological aging. -Galactosidase staining is used to detect the senescence after exposure of cells to radiation. In senescence B-galactosidase enzyme is overexpressed and cleaves the substrate X-Gal. Cells were plated and allowed to adhere overnight then were treated and radiated with dose 5x2 Gy. At different time points on Day 1, 3, 5, and 7 post radiation, cells were washed twice with 1XPBS and fixed with fixing buffer that consists of 0.2 glutaraldehyde/2% formaldehyde for 10 minutes followed by aspiration of the fixing solution. Cells were washed twice by 1XPBS followed by adding staining buffer which consists of 1 mg/mL 5-bromo-4-chloro-3-inolyl- β -galactosidase in dimethylformamide (20 mg/mL stock), 5 mM potassium ferricyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, 2 mM MgCl₂. Staining buffer was incubated with cells overnight in CO₂

incubator to maintain pH of solution at 6. On the next day, cells were washed with 1XPBS and images were taken using light microscope.

2.9 Western Immunoblotting

A- Protein Collection

Western immunoblotting is used to indicate the expressions of different proteins. To isolate proteins, cells were plated at cell density of 70,000-100,000 cells per 10mm cell culture dish. Then cells were treated with the appropriate treatment and washed with 1XPBS at assigned time points. After washing with PBS, 100 to 500 μ l of lysis buffer (1:100 phosphatase inhibitors and 1:50 protease inhibitors suspended in M-PER mammalian protein extraction reagent (Thermo scientific #78501) was added for 5 minutes. The lysate was then centrifuged for 5 minutes at 10,000 rpm.

B-Determination of Protein Concentration by using Bradford method

Bradford reagent (Bio-Rad 500-0205) is used in the Bradford method to determine the concentration of proteins. 20 μ g of equal aliquots of total cell lysate was loaded onto a polyacrylamide gel (10-12% based on the molecular weight of proteins that being analyzed). By using SDS-PAGE running buffer and 170 milliamps constant current, proteins were separated for 1-2 hours. Then proteins were transferred onto a PVDF membrane for 1.5 hours and washed 4 times with PBS containing 0.01% Tween for 15 minutes in each time.

C-Immunoblotting

The membrane was blocked with TBS-Tween buffer containing 5% dry milk for 0.5 hour. Then primary antibody was added on the membrane and left overnight in 4 C⁰. The following day, the primary antibody was washed out with TBS-Tween buffer for three times each for 15 minutes. Then secondary antibody was added and incubated with membrane for one hour at room temperature and then washed with TBS-Tween buffer. West Femto Maximum Sensitivity substrate (Thermo Scientific # 34095) and Premium quality X-Ray films (PHENIX) were used to develop the membrane. The dilution of primary antibody was 1:1000 in blotto and the dilution of secondary antibody and beta- actin antibody were 1:5000 in blotto.

2.10 Flow cytometer Analysis (FACS)

2.10.1 Quantification of acridine orange staining by flow cytometry for autophagy

FACS analysis was used to quantify the amount of autophagic vacuoles which are positively stained with acridine orange staining. After radiation of the cells for three days, cells were collected and suspended with acridine orange staining solution of 1 in 10,000 dilution in PBS for 10 minutes. In FACS analysis, for each gated region at least 10,000 cells should be collected.

2.10.2 Quantification of β -galactosidase staining by flow cytometry for Senescence

Confluent plates with hs578t cells line were treated with radiation for three days and on the day of staining cells were incubated with 100 nM of bafilomycin A1 in fresh medium to induce lysosomal alkalization and left incubated at 37C⁰ and 5% CO₂ for 1 hour. After incubation, C12FDG working solution was added to each well in amount to make the final concentration 33 μ M and was continued incubation for another 1 hour. Media was then aspirated and cells were washed twice with PBS. Cells were harvested and collected for centrifugation at speed 1500 rpm and resuspended in PBS. C12FDG is a substance that is hydrolyzed by upregulated β -galactosidase enzyme and becomes fluorescent at wavelength of 500–510 nM.

Section 3: Results

3.1 Influence of Radiation on the growth of Hs578t breast tumor cells

As described in the Introduction, radiation is used as adjuvant therapy in breast cancer to eradicate any remaining cancer cells post-surgery. Fractionated radiation doses are used in the clinic, where patients receive multiple small doses of 2 Gy. Previous studies from our laboratory have shown that radiation promotes protective autophagy in MCF-7 and ZR-75 breast tumor cells and that pharmacological or genetic inhibition of the autophagy induced by radiation leads to growth suppression and cell death, primarily through apoptosis. ^[36] ^[38] In contrast, the 4T1 syngeneic murine breast tumor cells could not be sensitized to radiation in vitro by using pharmacological inhibitor of autophagy with chloroquine treatment, by silencing of the autophagy- related gene Atg12, or by chloroquine in vivo. ^[49]

In this study, cells were plated, irradiated over period of three days, and then counted on Days 1, 3, 5, and 7-post radiation. In Figure 3.1, irradiated cells show initial cell death with slight recovery from days 3 to day 5 but primarily growth arrest. The results represent the average of three experiments with standard errors.

Cells Viability Assay

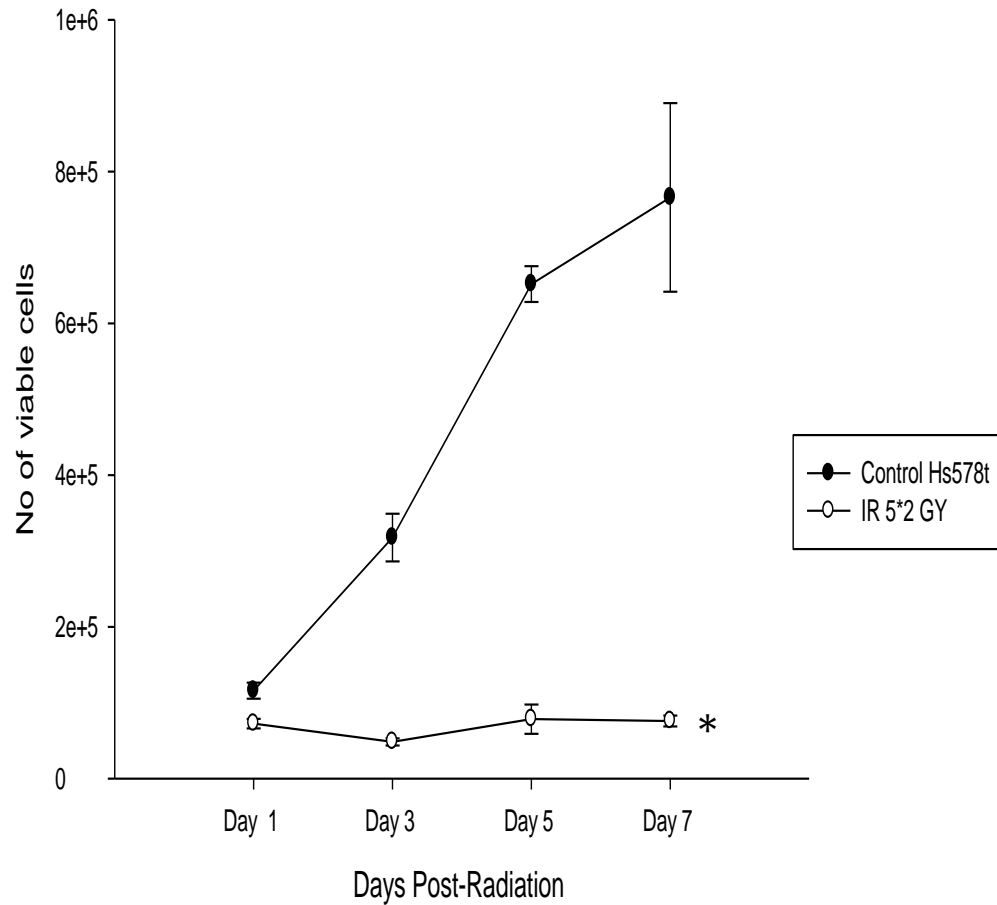


Figure 3.1 Effect of radiation on viable cell numbers. Upon radiation with 5 X 2 Gy, Hs578t cells showed growth arrest post day 5 of radiation. In each experiment, triplicates were used. Three different experiments were done to confirm the results. (* p value < 0.05 compared IR to control using ANOVA followed by tukey)

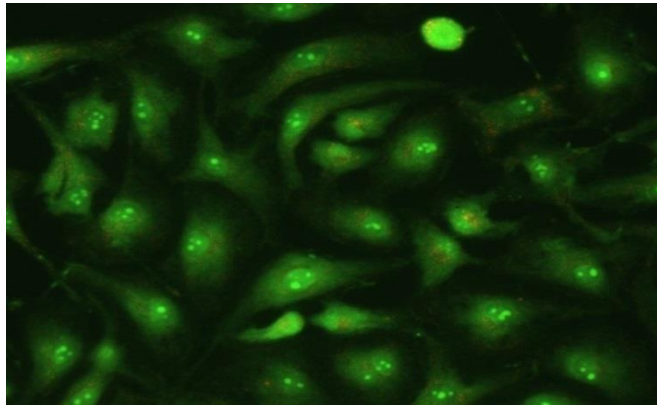
3.2 Effect of radiation on Hs578t breast tumor cells.

Autophagy is a homeostatic catabolic process that degrades impaired proteins and organelles and recycles them to sustain metabolic activity. Autophagy has a different effects in cancer, one role as tumor suppressor by preventing the accumulation of mutant proteins and organelles, and the other role (Cytoprotective) helping the existing tumor cells to survive by provide them with nutrients and metabolic precursors.^[40] Previous studies from our lab have shown that in MCF-7 breast tumor cells radiation induces a cytoprotective autophagy because interference with that autophagy leads to growth suppression and cell death.^[38]

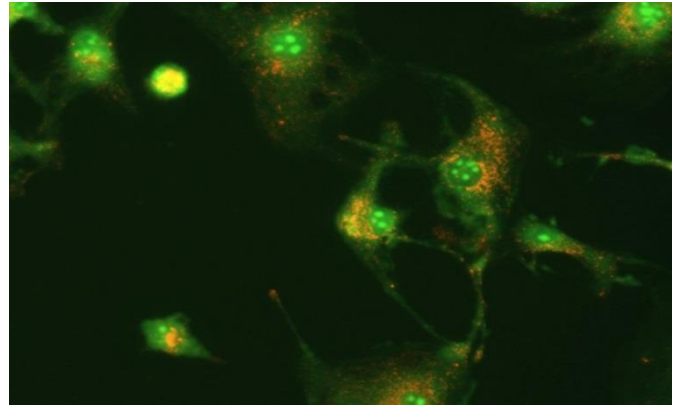
Acridine orange staining was used to evaluate the effect of radiation on autophagy in Hs578t breast tumor cells as it is widely used to detect the presence of autophagic vacuoles. Acridine orange is a lysomotropic dye that becomes trapped upon entering acidic compartments. Under low pH conditions, Acridine orange emits orange light when excited by blue light.

In Figure 3.2, it is clear that the Hs578t breast cancer cells have minimum basal autophagy while irradiated cells showed an increased acridine orange staining and development of the orange colored puncta, which is indicative of the induction of autophagy. Serum starved cells were used as positive control.

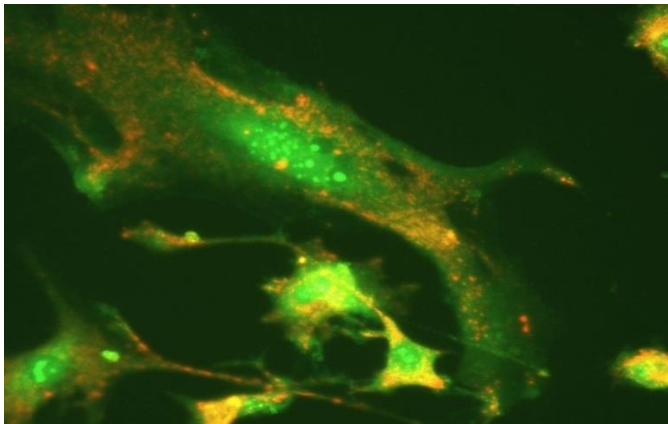
CONT Day 3 post radiation



IR Day3 post radiation



IR Day 7 post radiation



Serum starved positive control

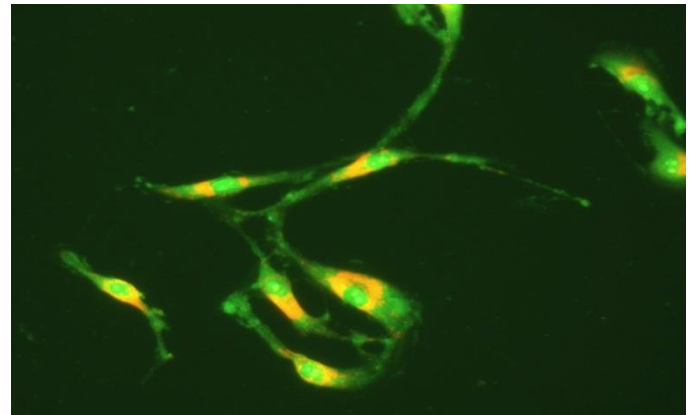


Figure 3.2: Induction of autophagy in Hs578t cells after radiation. Autophagy induction was performed using Acridine orange staining on day 1,3,5, and 7 post radiation with 5X2 Gy. Three different experiments were done to confirm the results.

3.3 Quantification of Autophagy upon Radiation Using Flow Cytometry

Another technique that is used to evaluate the autophagy is the quantitation of acidic vacuoles by Flow Cytometry. In this experiment, cells were plated, irradiated over a period of three days, and acidic vacuoles were quantified on Day 1, 3, and 5-post radiation.

In Figure 3.3, Hs578t control cells show minimum basal autophagy, while irradiated cells show the promotion of autophagy. The same experiment was repeated three times and in all three times statistical difference were observed between irradiated cells and control cells. Therefore it appears that irradiation of Hs578t breast tumor cells promotes autophagy.

FACS analysis of Autophagy

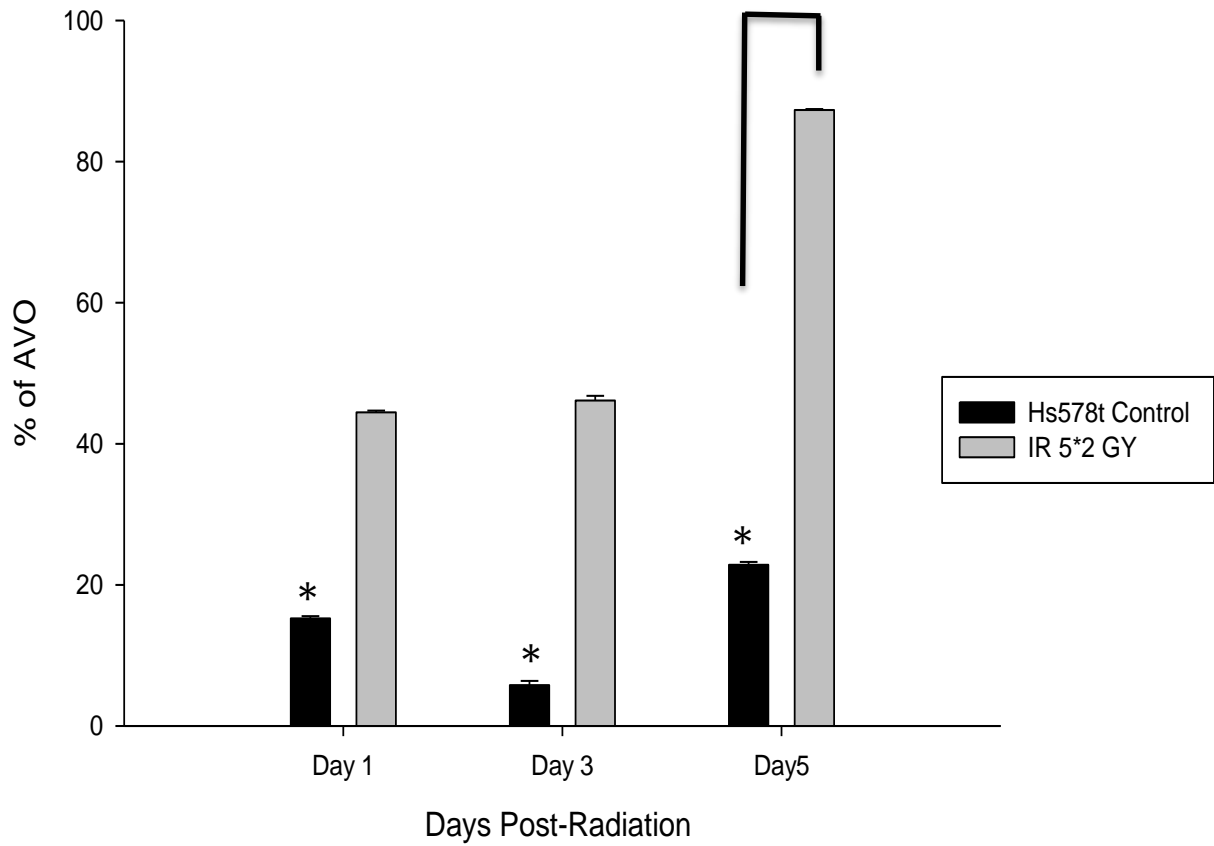


Figure 3.3 Quantification of radiation induced autophagy. Hs578t cells were plated and irradiated with fractionated radiation (5x2Gy) and then were analyzed using Flow cytometry on days 1,3, and 5 post radiation. In each experiment, triplicates were used. Three different experiments were done to confirm the results. (* p value< 0.05 compared IR to control using ANOVA followed by tukey). AVO= acidic vesicular organelles.

3.4. Induction of senescence in irradiated Hs578t cells.

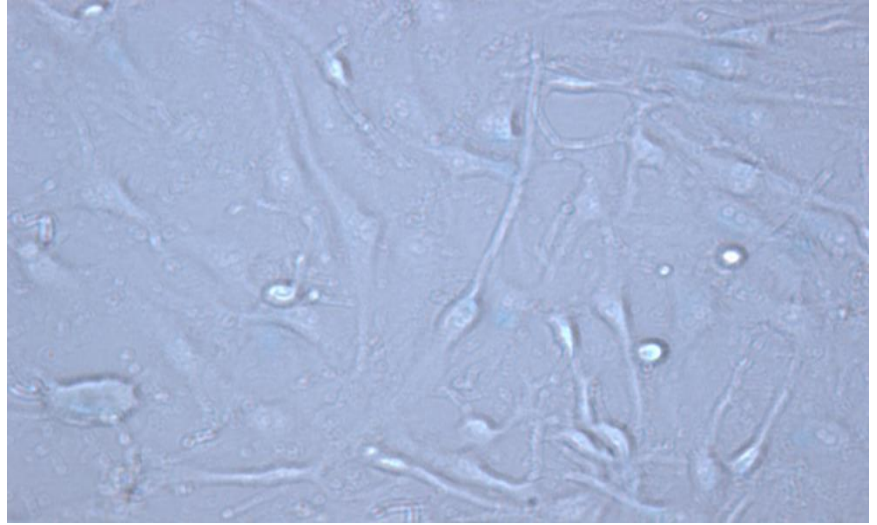
Cellular senescence is a phenomena in which a cells stop to divide, but stay metabolically active. It has been reported that ionizing radiation promotes senescence in both normal and tumor cells.^[30] Our laboratory has previously reported the induction of accelerated senescence by ionizing radiation and that this is largely p53 dependent.^[39]

The Hs578t breast cell line is mutant in the tumor suppression protein, p53.^[48] The β galactosidase-staining assay is used to distinguish proliferating and senescent cells. In senescent cells, beta galactosidase enzyme is overexpressed and upon adding x-gal which is a chromogenic substrate to the cells, - β galactosidase enzyme cleaves the x-gal and gives insoluble blue substance at pH 6.

In Figure 3.3, there is β -gal staining upon radiation of Hs578t breast cells, which suggests that induction of senescence upon radiation is not dependent on functional 53 at least in Hs578t cells.

CONTROL

Day 5 post-radiation



IR (5X2 Gy)

Day 5 post-radiation

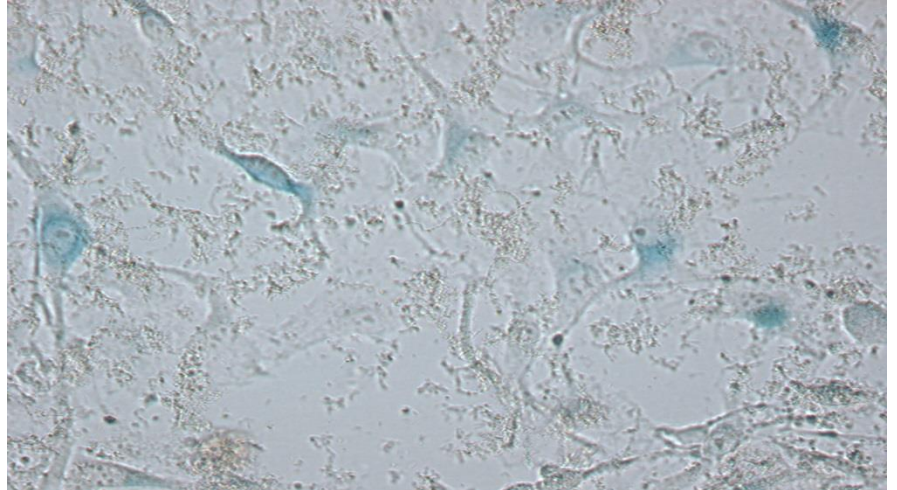


Figure 3.4: Staining of the cells using X-gal shows induction of senescence upon irradiation of Hs578t breast cell line. Three different experiments were done to confirm the results.

3.5 Quantification of Senescence upon Radiation Using Flow Cytometry

The flow cytometry method is extremely useful in quantitative analysis of cellular senescence phenomenon. This flow cytometric analysis is able to detect the hydrolysis of 5-dodecanoylaminofluorescein di- β -D-galactopyranoside (C₁₂FDG), a membrane-permeable and nonfluorescent substrate of β -galactosidase, which, upon cleavage, stays inside the cytosol and produces green fluorescence. Flow Cytometry allows estimation of the enzyme activity and also provides quantitative information on the population size of SA β -Gal-positive cells.^[51]

In figure 3.5, irradiated cells show promotion of senescence as compared to control. Same experiment was repeated three times and in all three times significant differences were observed between irradiated cells and control cells. Therefore it appears that irradiation of Hs578t breast tumor cells promotes senescence.

FACS Analysis of Senescence After Radiation

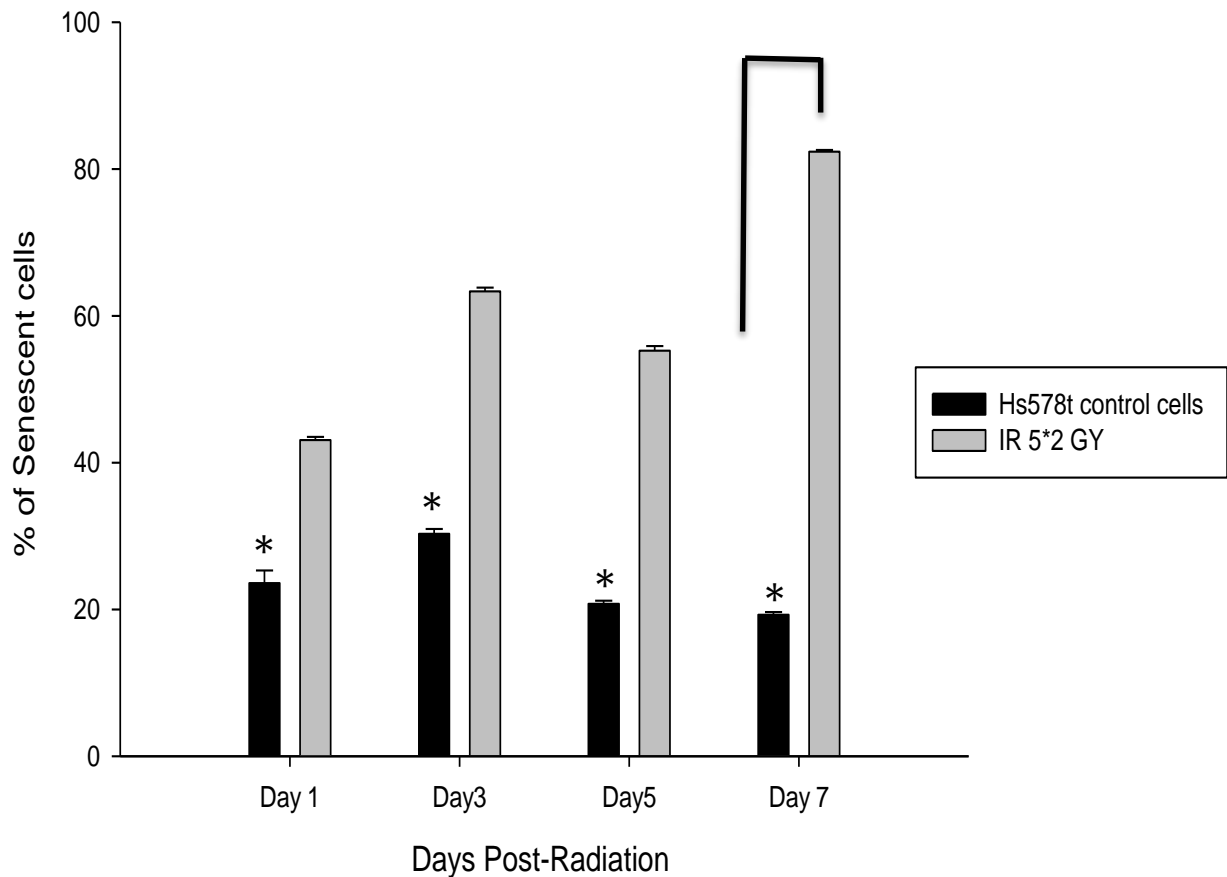


Figure 3.5 Quantification of radiation induced senescence .Hs578t cells were plated and irradiated with fractionated radiation (5x2Gy) and then were analyzed using Flow cytometry on days 1, 3, 5 and 7 post- radiation. In each experiment, triplicate samples were used. Three different experiments were done to confirm the results. (* p value< 0.05 compared IR to control on Day 7 using ANOVA followed by tukey)

3.6 Pharmacologic and Genetic Inhibition of Autophagy

3.6.1 Determination of the effective dose of Ammonium Chloride

It has been shown that autophagy might lead to cancer cell resistance to chemotherapy and radiation treatment. Inhibition of autophagy can lead to the sensitization of resistant cancer cells to anticancer treatment. ^[40]

Inhibition of autophagy can be performed genetically and pharmacologically. There are many different chemical agents that can inhibit autophagy such as chloroquine which is used clinically, 3 methyl adenine, bafilomycin and ammonium chloride. Also silencing of autophagy related proteins such as Atg5, Atg7 and beclin1 leads to inhibition of autophagy.

In our experiments, ammonium chloride has been used as an example of a pharmacological autophagic inhibitor. Ammonium chloride is an organic compound that is capable of inhibition of the fusion of autophagosome with the lysosome in the last step of degradation of the autophagosome contents. ^[41] In order to use ammonium chloride in the experiments, non-toxic doses were first determined using the MTT assay. Based on the previous literature, studies have shown that 10 mM is an effective dose that inhibits autophagy. ^[41] In figure 3.6, the 10 mM ammonium chloride showed no significant differences between control and NH₄Cl treated cells.

MTT assay for NH₄CL

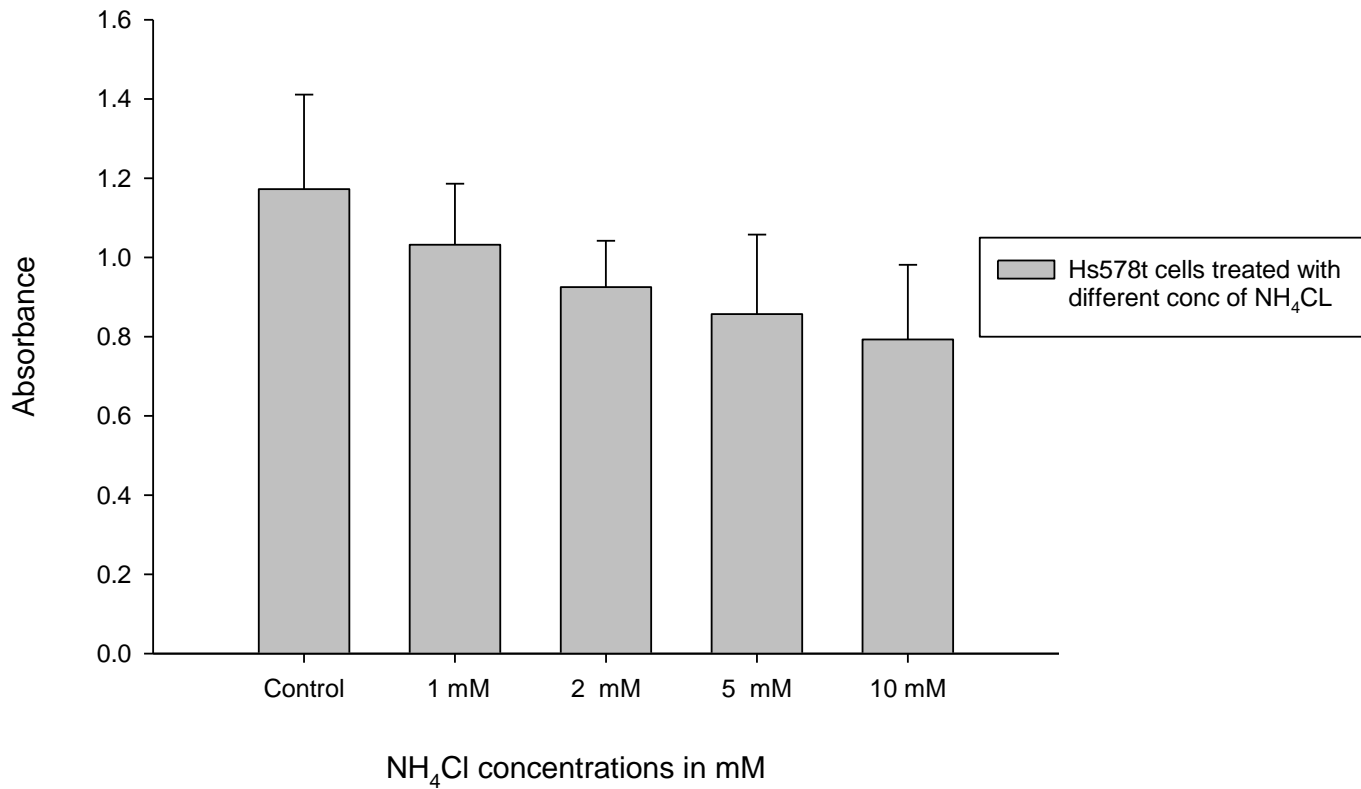


Figure 3.6. Determination of effective dose of ammonium chloride using the MTT assay. The dose of 10 mM ammonium chloride has relatively low toxicity profile. The experiment was repeated twice and the results given are the average of two experiments with standard Errors. No statistical differences were detected between control cells and cells treated with 10 mM NH₄CL.

3.6.2 Effect of the autophagy inhibitor, ammonium chloride (NH₄CL), on colony formation

Clonogenic survival assays study the ability of a single cell to form a colony. Cells were plated in triplicate in six wells tissue culture dishes at a density of 150 cells per well and allowed to grow for 10-14 days.

The clonogenic survival assay in Figure 3.7 indicates lack of sensitization of Hs578t cells to radiation even after inhibition of autophagy. Two conditions, IR (5*2 Gy) and IR + NH₄CL were compared to assess statistical significance. The experiment was repeated three times and in all three times, no statistical difference was observed between irradiated cells and cells treated with IR and NH₄Cl (10 mM) in their colony forming abilities. It can be said here that Hs578t cells treated with IR when also treated with NH₄Cl do not become sensitized which is in accordance with our previous data. Therefore it appears that Hs578t breast tumor cells may not be using autophagy as a mechanism to survive. Hence it can be said that the role of autophagy here is a non-cytoprotective role.

Clonogenic Survival Assay for NH₄CL

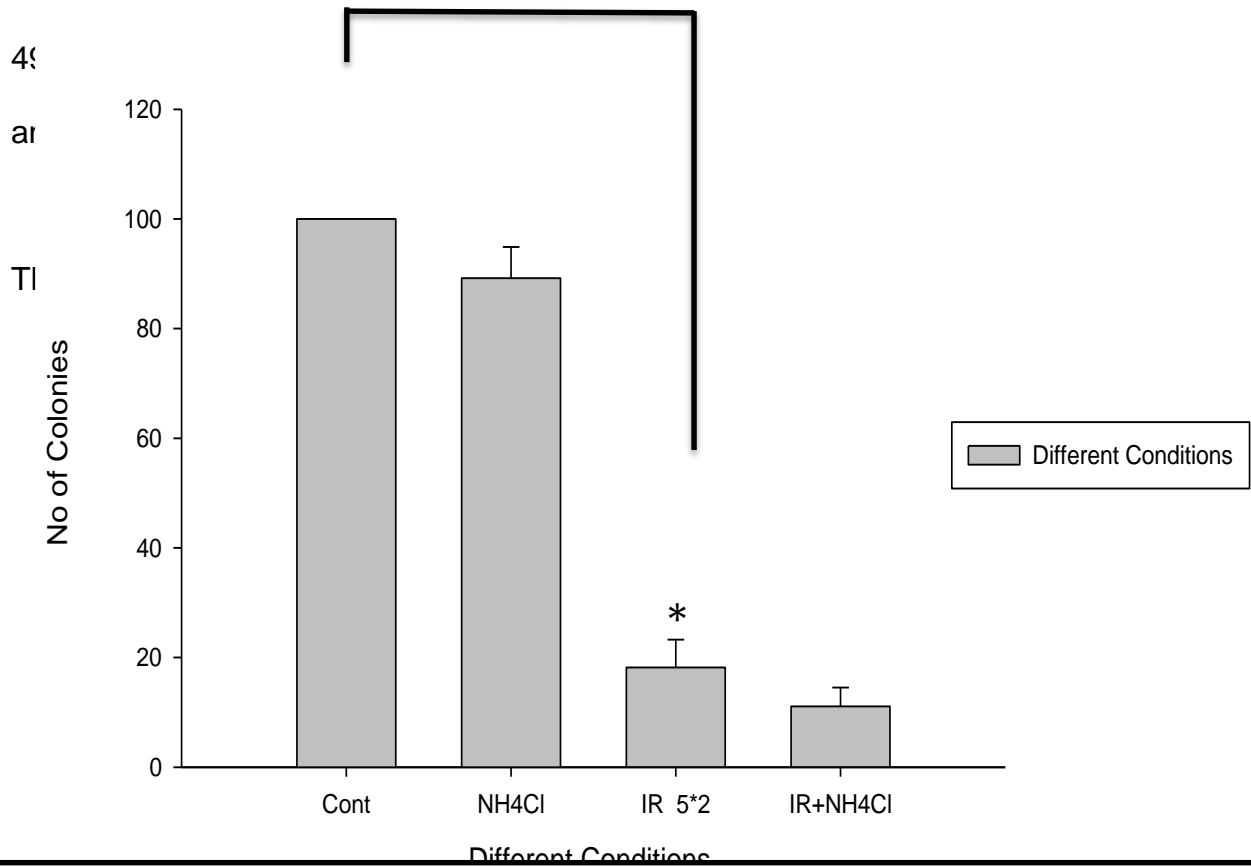


Figure 3.7 Clonogenic survival assay. Ammonium Chloride (10 mM) does not sensitize Hs578t breast tumor cells cells to radiation. In each experiment, triplicate samples were used. Three different experiments were done to confirm the results. (* p value< 0.05 IR compared to control using ANOVA followed by tukey) (No statistical differences is observed between IR treated cells alone or combination of IR and NH₄CL)

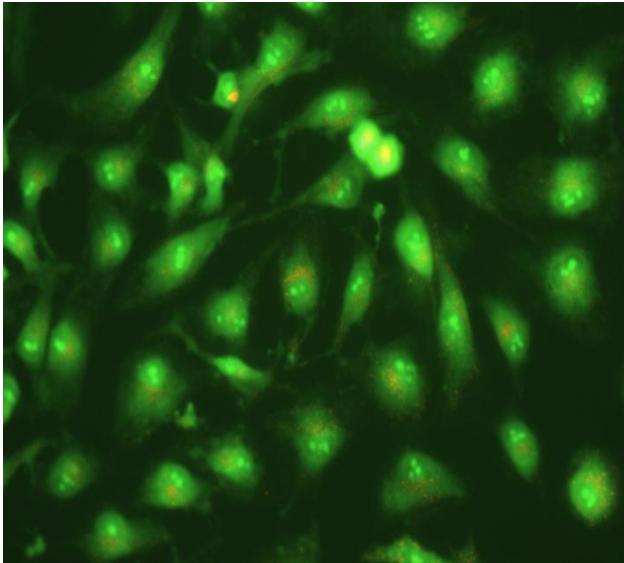
3.6.3 Ammonium Chloride was effective in inhibition of autophagy based on Acridine orange staining

The findings in the previous figure are not consistent with much of the literature relating to autophagy. It was therefore necessary to confirm that the treatment indicated actually was effective in inhibiting autophagy in the Hs578t cells.

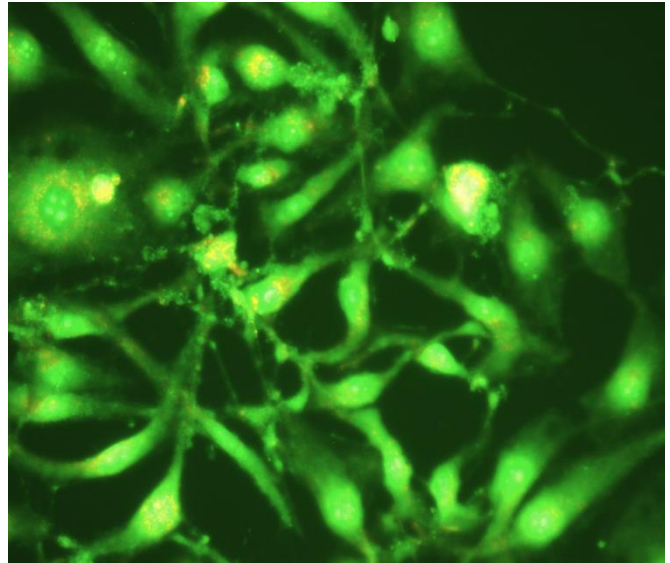
Staining of vacuoles with orange is an indication of induction of autophagy while yellowish color shows inhibition of autophagy since ammonium chloride interferes with the acidity of the lysosomes.

In Figure 3.8, control cells show a low basal level of autophagy, while irradiated cells undergo induction of autophagy. In cells treated with ammonium chloride, it is clear that autophagy has been inhibited as indicated by the yellowish color. Inhibition of autophagy by NH_4Cl occurs in the last step, this explains why there are many vacuoles that stained with the yellowish color.

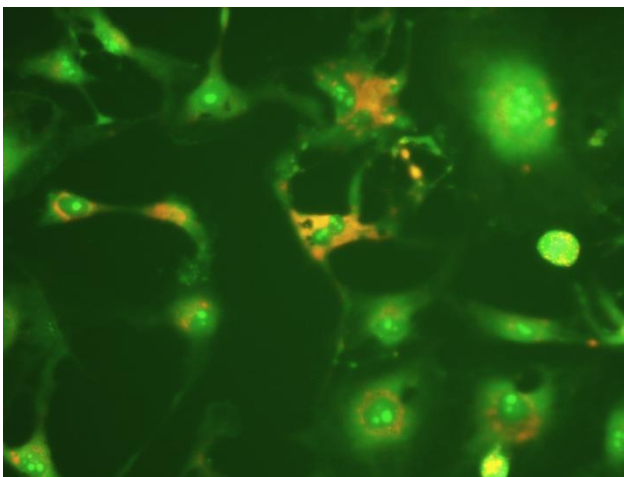
CONT Day 1 post-Radiation



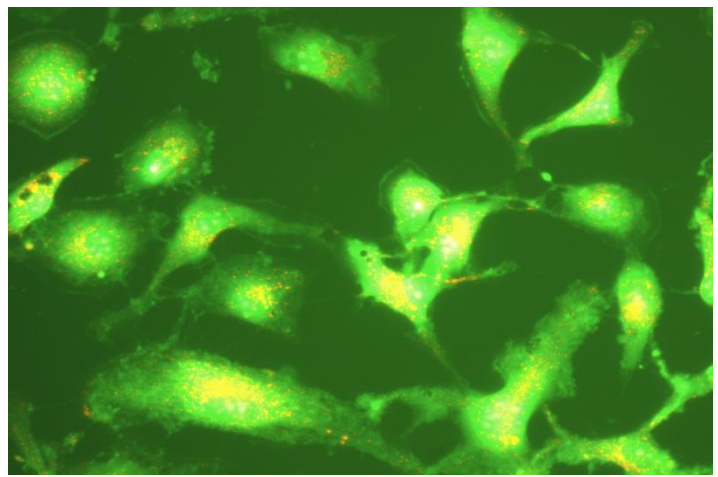
NH₄CL 10 mM Day 1 post-Radiation



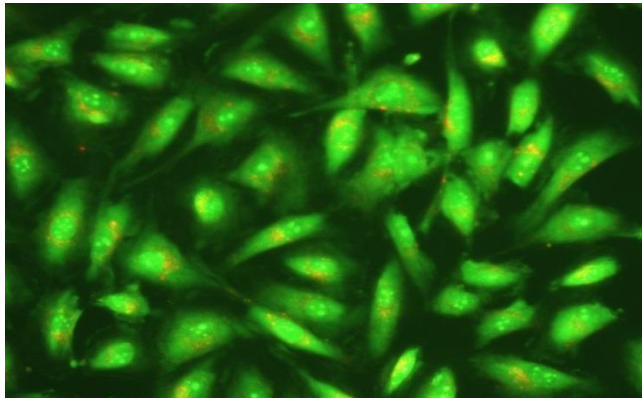
R 5 X 2 GY Day 1 post-Radiation



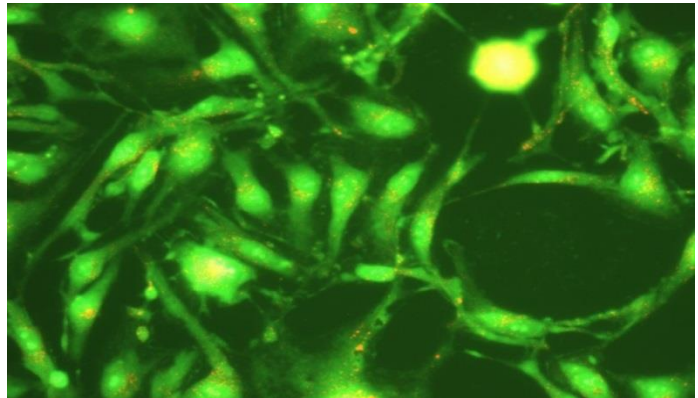
IR+NH₄CL Day 1 post-Radiation



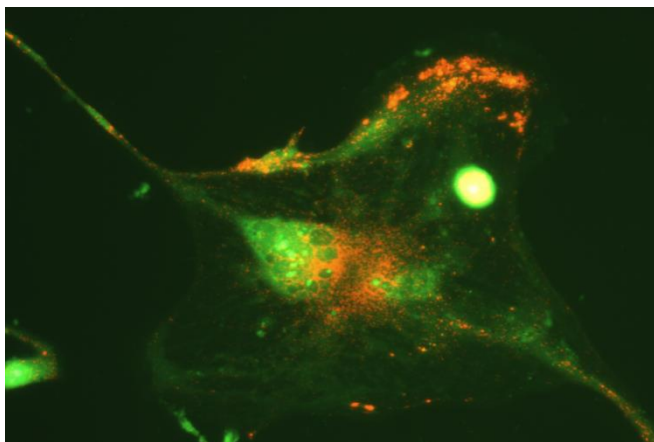
CONTROL Day 3 post-Radiation



NH₄CL 10 mM Day 3 post-Radiation



IR 5 X 2 GY Day 3 post-Radiation



IR+NH₄CL Day 3 post-Radiation

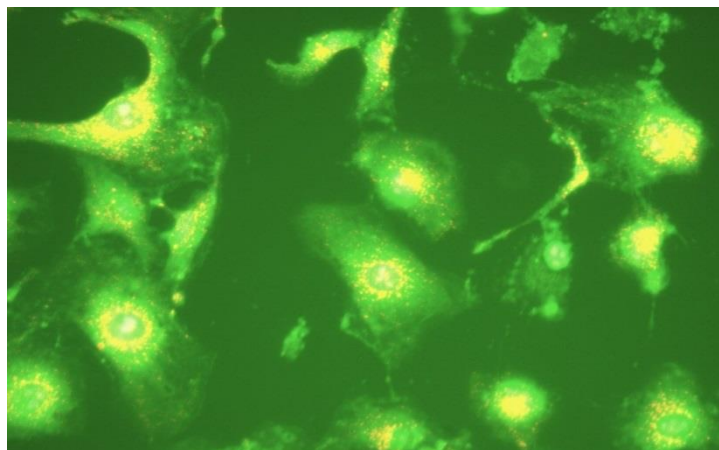


Figure 3.8 Determination of the inhibition of autophagy by 10mM Ammonium chloride using Acridine orange staining. Three different experiments were done to confirm the results. All images have the same magnification 20X.

3.6.4 Silencing of Beclin1

One classical strategy for interfering with autophagy is by silencing of autophagy related genes. Consequently, we developed an Hs578t breast cell line (Hs578t/Beclin-) in which Beclin was silenced. Beclin-1 and its binding partner class III phosphoinositide 3-kinase (PI3K), are essential for the initiation and formation of the autophagosome in autophagy. [22]

In Figure 3.9 , western blotting indicates a significant decrease in the levels of Beclin1 as compared to control.

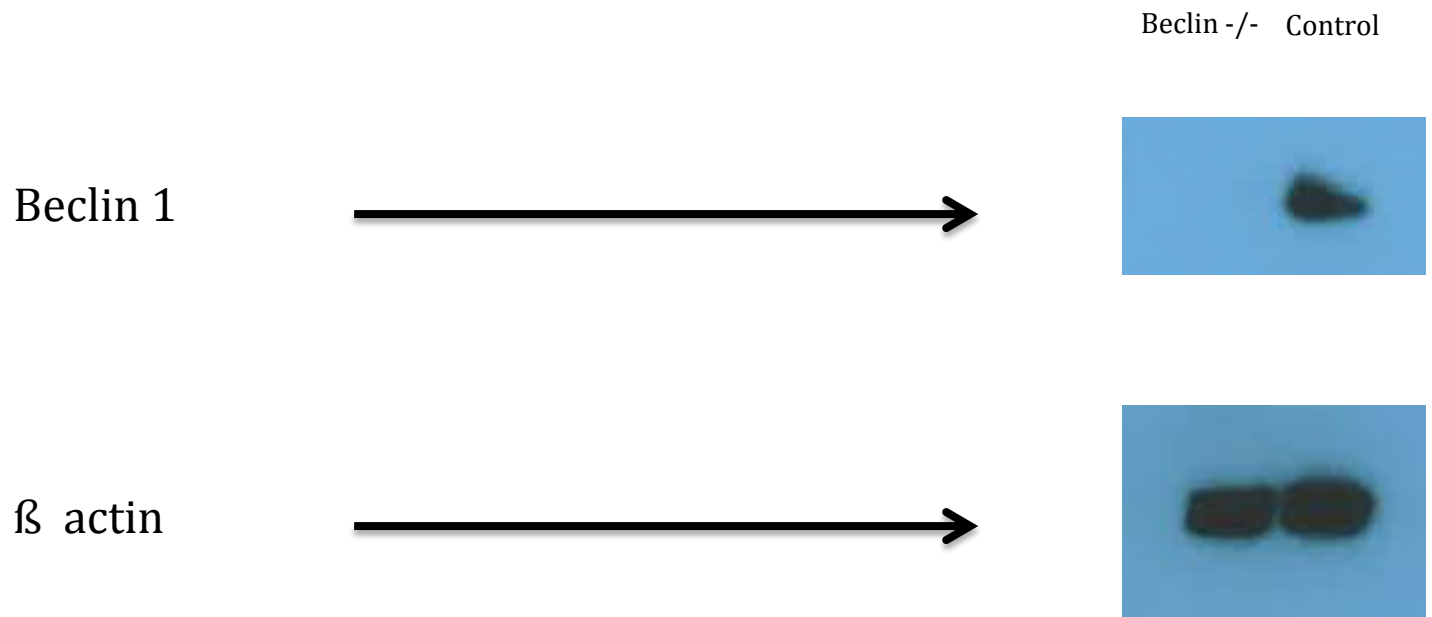


Figure 3.9 Western Immunoblotting to determine silencing level of Beclin1.

Experiment was repeated twice.

3.6.5 Effect of Beclin1 Silencing on radiation sensitivity of Hs578t breast tumor cells

To determine the effect of autophagy inhibition on sensitization of Hs578t cells to radiation, beclin 1 was silenced and both wild type and beclin-1 silenced Hs578t cells were irradiated with 5X2 Gy for three days. Then, the number of viable cells was counted. Figure 3.10 indicates that genetic inhibition of autophagy does not sensitize Hs578t cells to radiation.

Cells Viability Assay

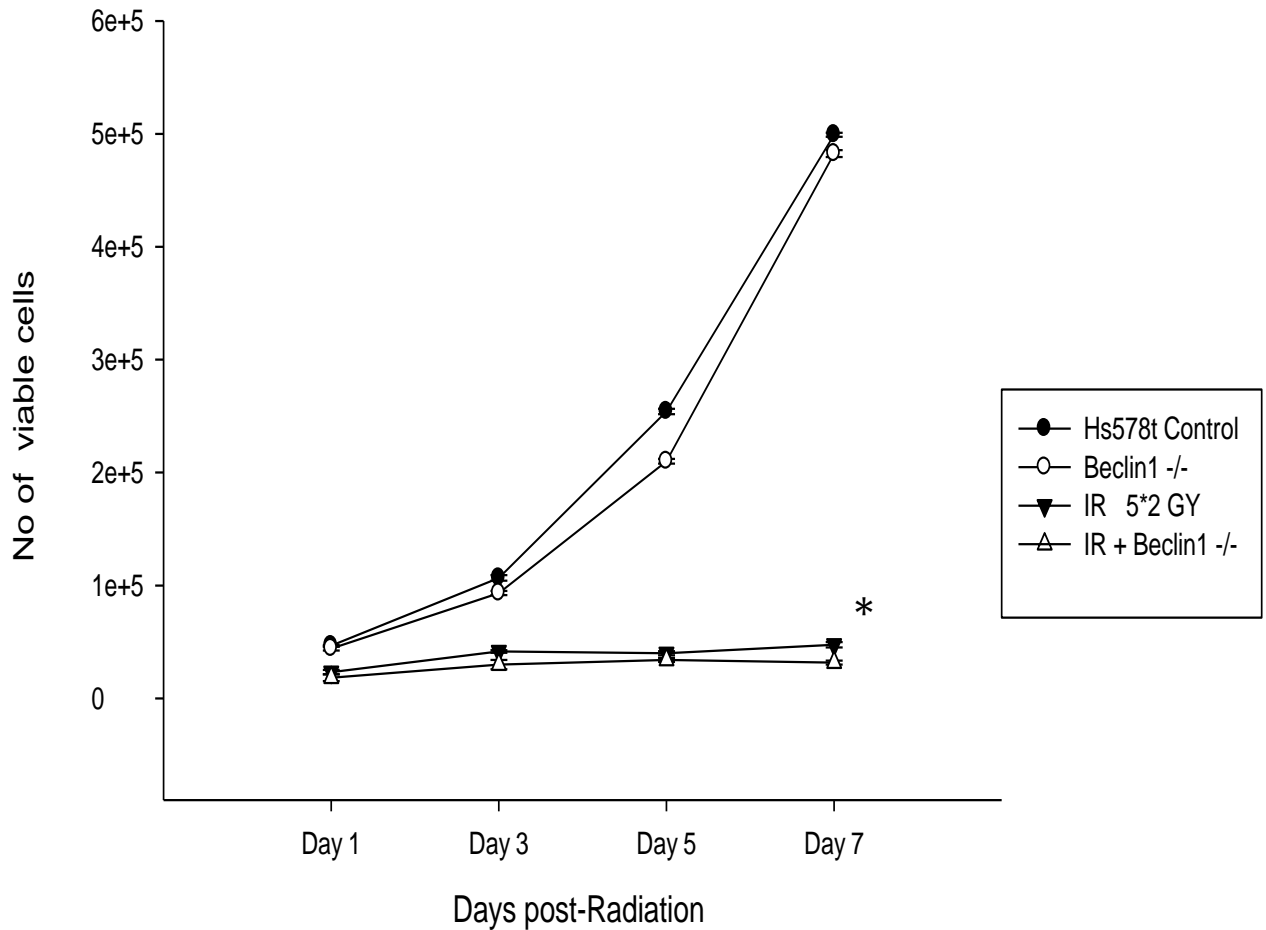


Figure 3.10 Cell viability assay shows that silencing of Beclin1 failed to sensitize Hs578t cells to radiation. In each experiment, triplicate samples were used. Two different experiments were done to confirm the results. (* p value < 0.05 compared IR to control using ANOVA followed by tukey) (No statistical differences between IR and combination IR+Beclin-/-)

3.6.6 Effect of the Beclin1 Silencing on the ability to form colonies

Wild type Hs578t breast tumor cells and Hs578t/beclin1- were plated at low density 150 cells per well, then irradiated for three days with radiation dose 5×2 Gy. Plated cells were incubated for 10-14 days. Number of colonies in each well was counted and calculated as a percentage of control. Figure 3.11 provides further confirmation that silencing of Beclin 1 does not sensitize Hs578t breast tumor cells to radiation.

Clonogenic Survival Assay of Hs578t wild type and Beclin1 silencing breast cells lines

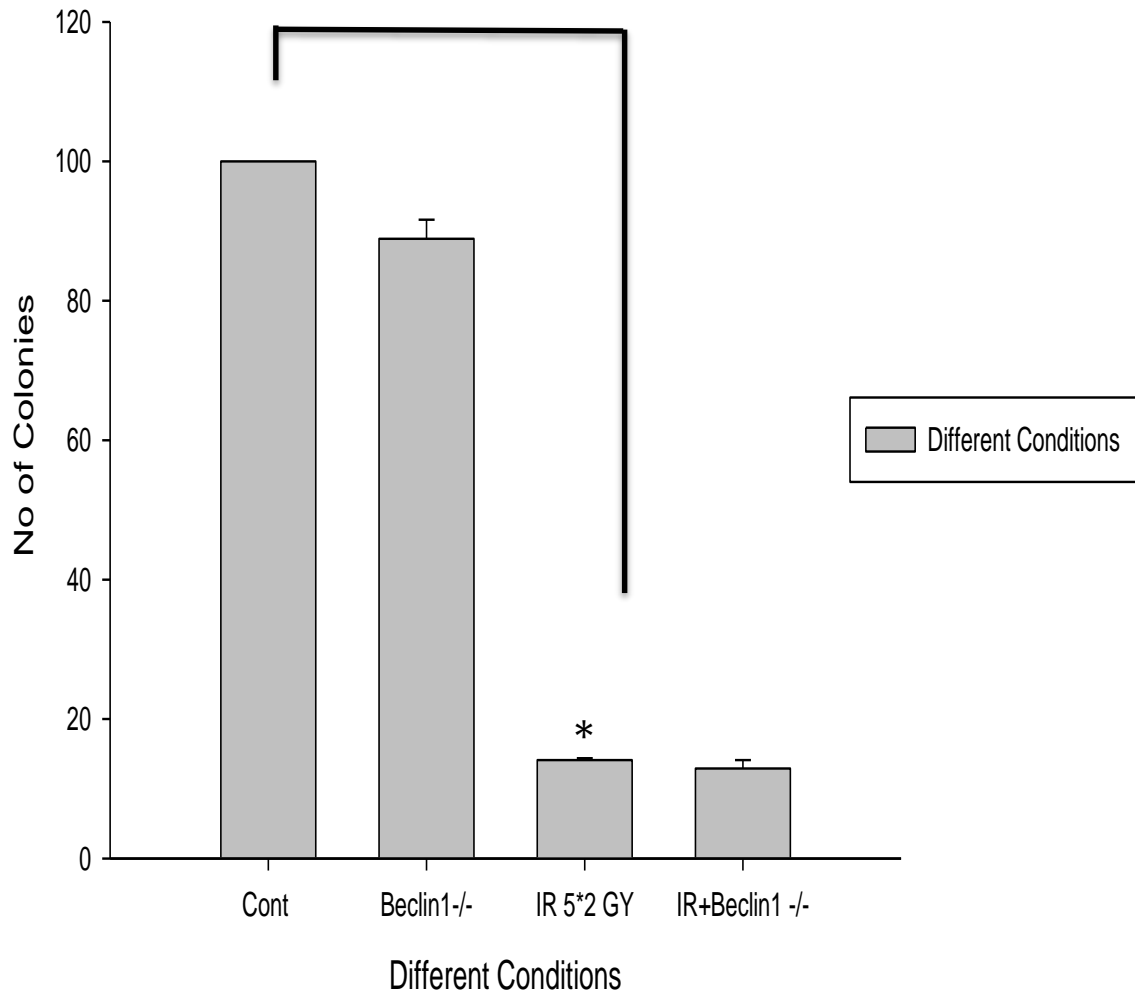
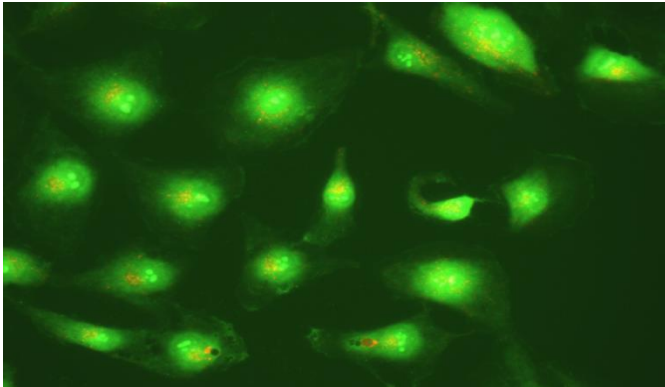


Figure 3.11 Clonogenic survival assay by silencing Beclin-1 does not sensitize Hs578t breast tumor cells to radiation. In each experiment, triplicate samples were used. Four different experiments were done to confirm the results. (* p value < 0.05 compared IR to control using ANOVA followed by tukey)

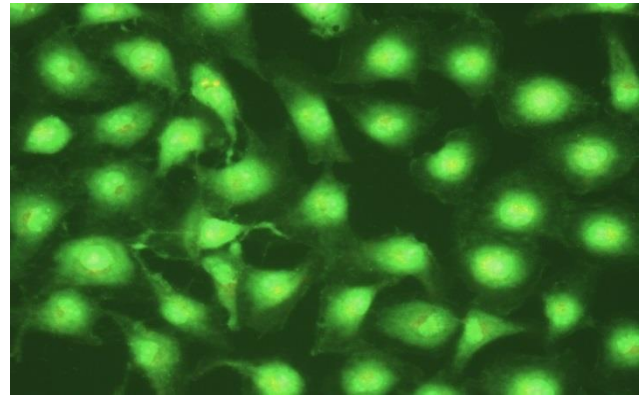
3.6.7 Beclin1 was effective in inhibition of autophagy based on Acridine orange staining

As in the studies using NH_4Cl , it was necessary to confirm that the silencing of beclin was effective in inhibition of autophagy. In Figure 3.12 where studies using control, beclin-, and combination of beclin- and irradiated cells are presented, there is minimum induction of autophagic vacuoles in the Beclin-1 silenced cells while irradiated cells have shown extensive induction of orange color as indicated by acridine orange staining.

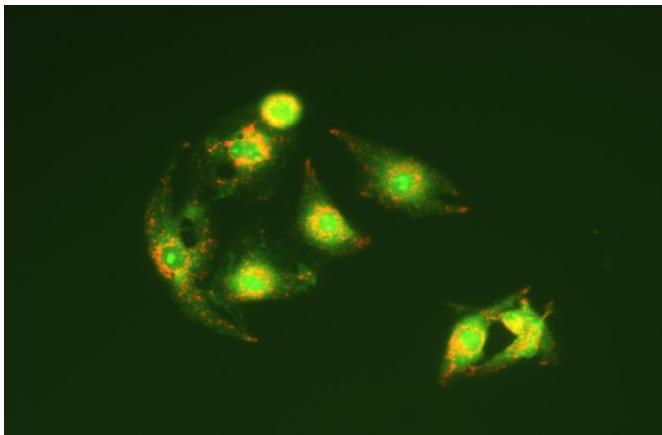
Cont Day 1 post-Radiation
Radiation



Beclin- Day 1 post-



IR 5*2Gy Day1 post-Radiation



IR+Beclin- Day 1 post-Radiation

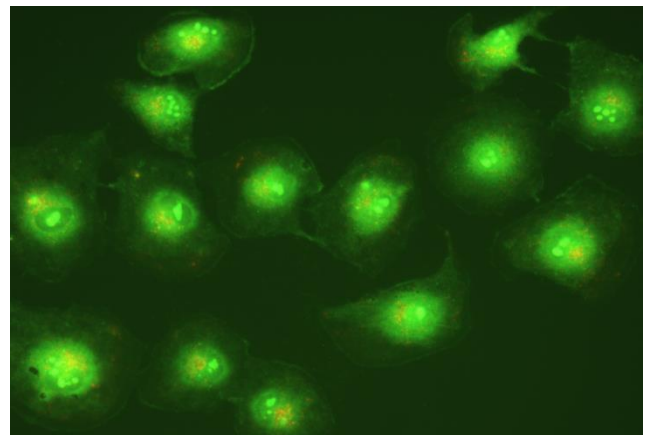


Figure 3.12 Determination of the inhibition of autophagy after silencing of beclin using Acridine orange staining. Three different experiments were done to confirm the results and a representative experiment is shown. All images have the same magnification 20X.

3.6.8 Degradation of p62 representing autophagic flux

P62 is a protein associated with the nuclear envelope. The complete process of autophagy is called autophagic flux. To confirm autophagic flux, p62 may be used as a marker. ^[27] p62 accumulates when autophagy is inhibited, and decreased levels can be observed when autophagy is induced. ^[27] LC3 is a receptor for p62. When p62 binds to LC3, autophagy degradation is promoted. LC3 II is a microtubule-linked protein, which is transformed from LC3I to LC3 II once autophagy is induced. Therefore, LC3 and p62 are together autophagy associated proteins and broadly used as markers for autophagy.

Figure 3.13 shows p62 levels in control cells, beclin1^{-/-} cells, irradiated cells and in irradiation in Hs578t cells where beclin-1 was silenced. While irradiated parental cells show degradation of p62 that indicates the induction of autophagic flux, p62 accumulates in the Beclin-1 silenced cells with radiation, indicating that autophagy has been inhibited.

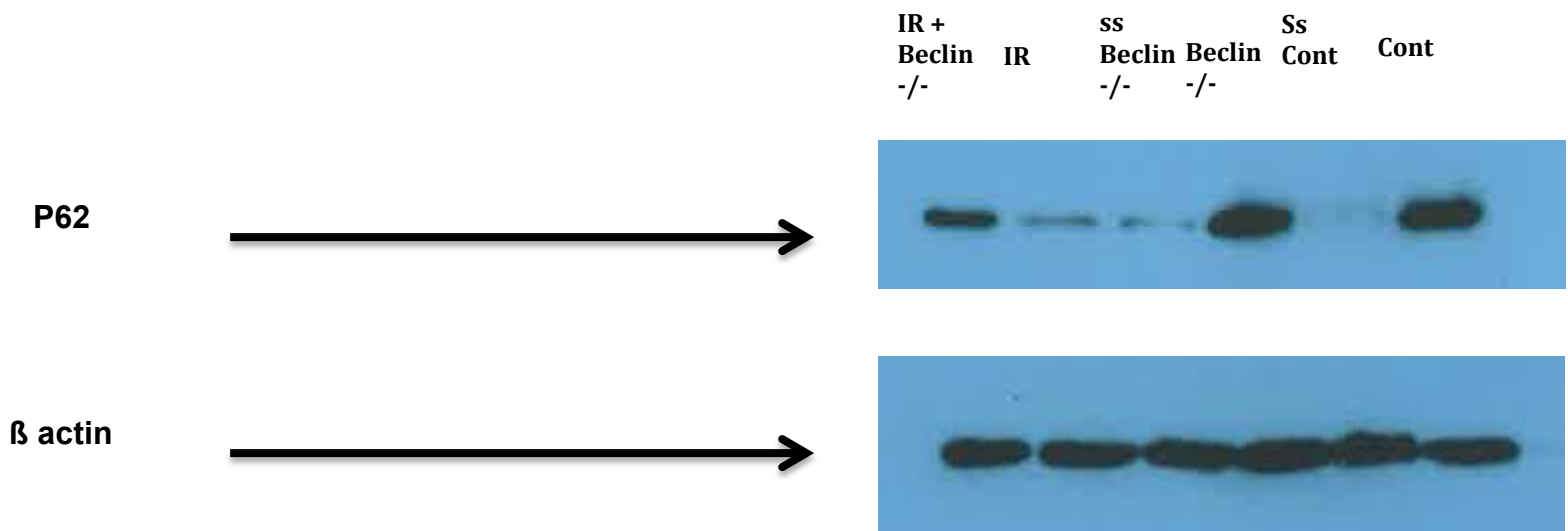


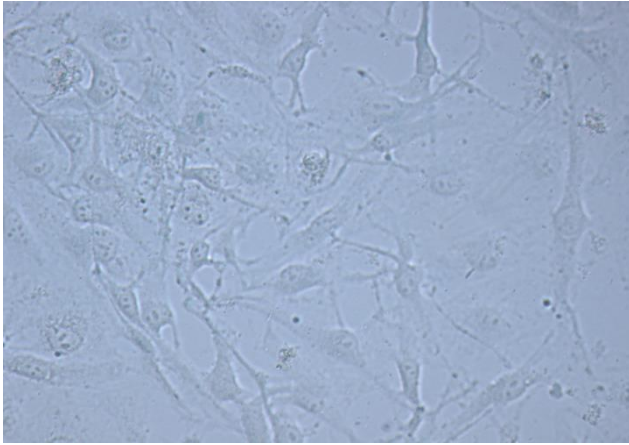
Figure 3.13 Western Immunoblotting to Assess degradation of p62. All proteins were collected on Day 5 post radiation. Serum starved cells proteins were collected after 72 hours of plating.

3.6.9 Relationship between senescence, autophagy inhibition and irradiation in Hs578t breast tumor cells using beta galactosidase staining.

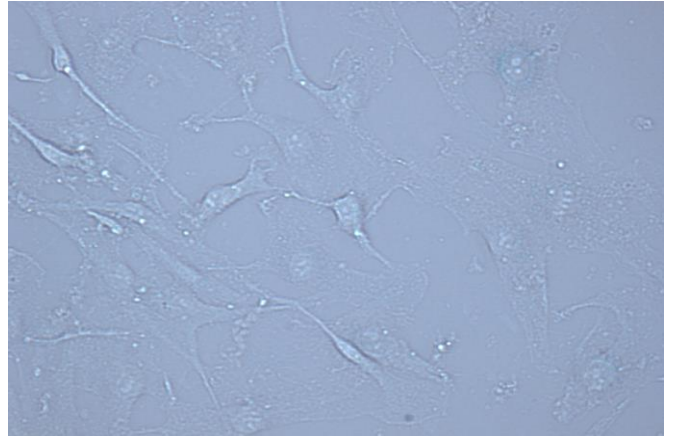
Previous studies have shown that there is an induction of autophagy and senescence upon irradiation of Hs578t cells. [45] To assess the relationship between autophagy, senescence and radiation, cells were plated and treated with different autophagy inhibitors and then irradiated with 5X2 Gy for three days. On days 1, 3, 5, and 7 post-radiation, beta galactosidase staining was performed to evaluate senescence.

Figure 3.14 confirms, as shown above, that irradiation induces senescence in the Hs578t cells and that inhibition of autophagy results in a reduction in the extent of senescence.

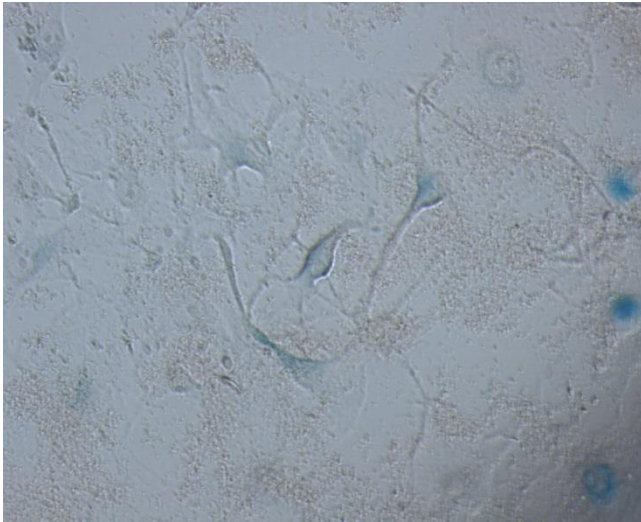
CONT Day1 Post-Radiation



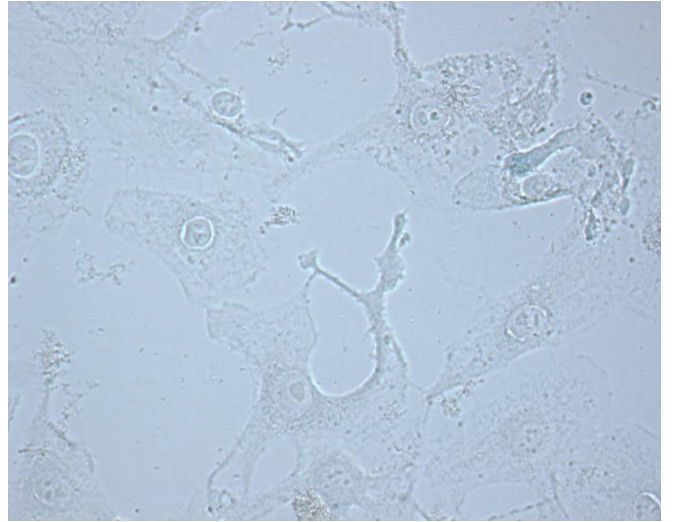
NH₄CL Day1 Post-Radiation



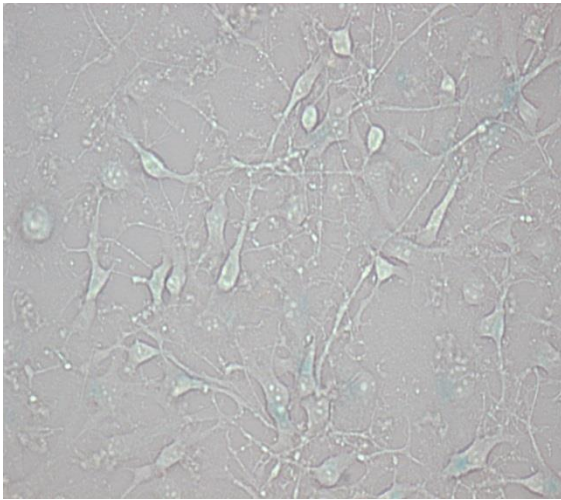
IR Day1 Post-Radiation



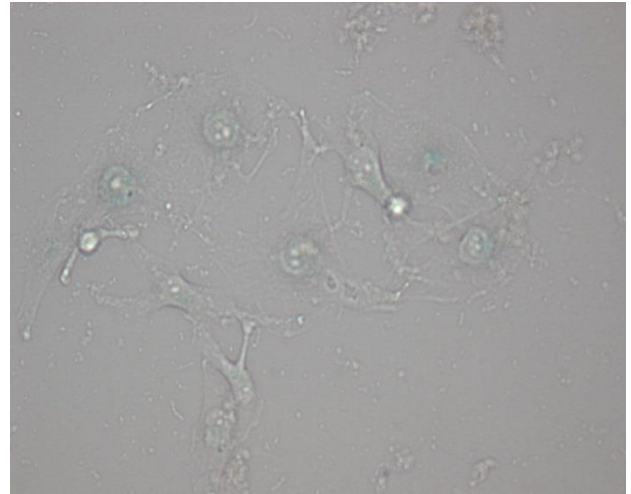
IR+NH₄CL Day1 Post-Radiation



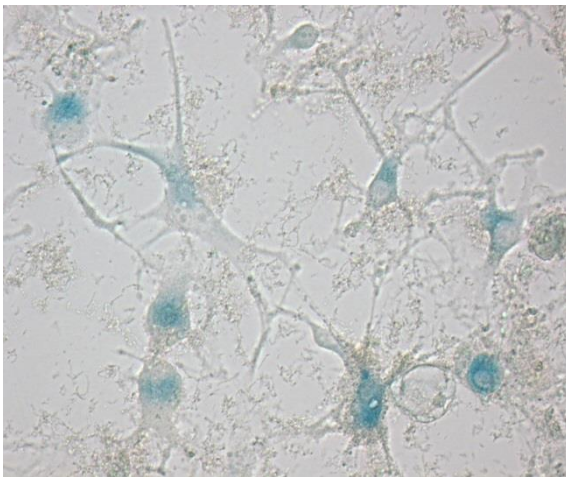
CONT Day5 Post-Radiation



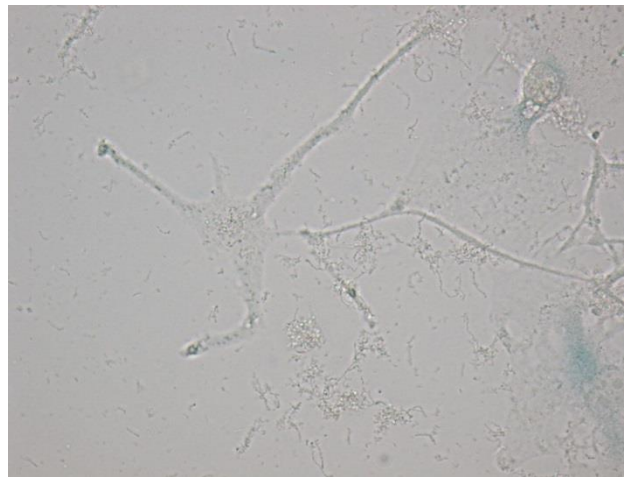
CQ Day5 Post-Radiation



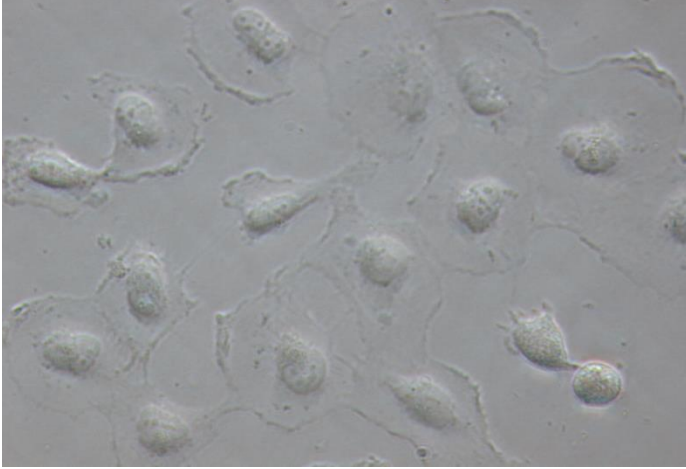
IR Day5 Post-Radiation



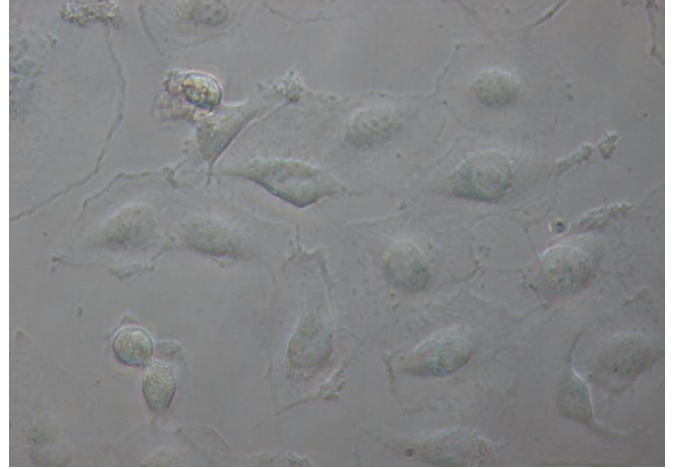
IR + CQ Day5 Post-Radiation



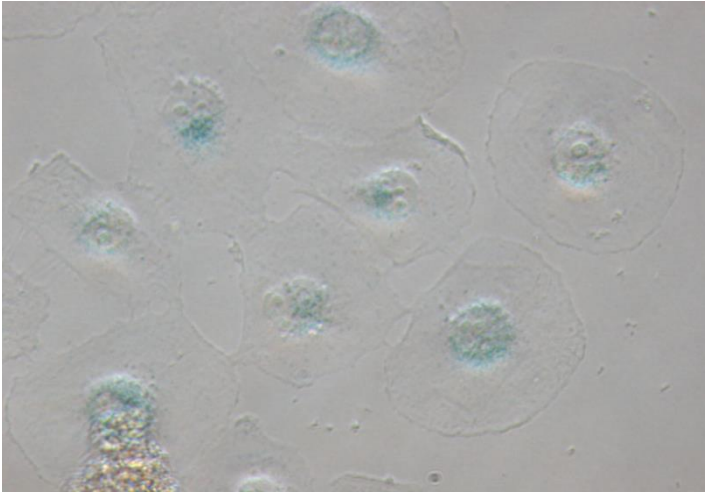
CONT Day 1 post-Radiation



Beclin-/- Day 1 post-Radiation



IR Day 1 post-Radiation



IR + Beclin -/- Day 1 post-radiation

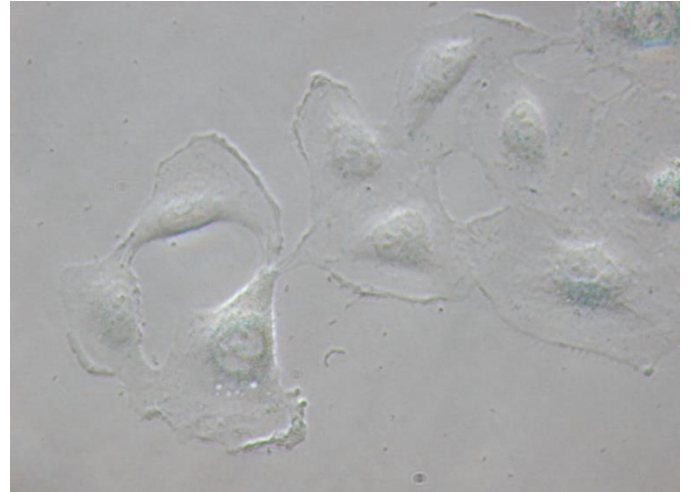


Figure 3.14 Beta Galactosidase staining that shows the reduction of senescence after using different approaches autophagy inhibitor. Three different experiments were done to confirm the results. All images have the same magnification 20X.

2.6.10 Quantification of senescence by flow cytometry for Senescence upon radiation and silencing of beclin-1

To further evaluate the relationship between senescence and autophagy inhibition upon radiation, senescence was quantified by FACS analysis in Beclin-1 silenced cells that were irradiated with 5X2 Gy for three days.

In figure 3.15, the irradiated cells show induction of senescence. With the combination Beclin-/- and irradiation, there is a reduction of the extent of senescence.

FACS Analysis of Senescence after Gene Silencing of Autophagy

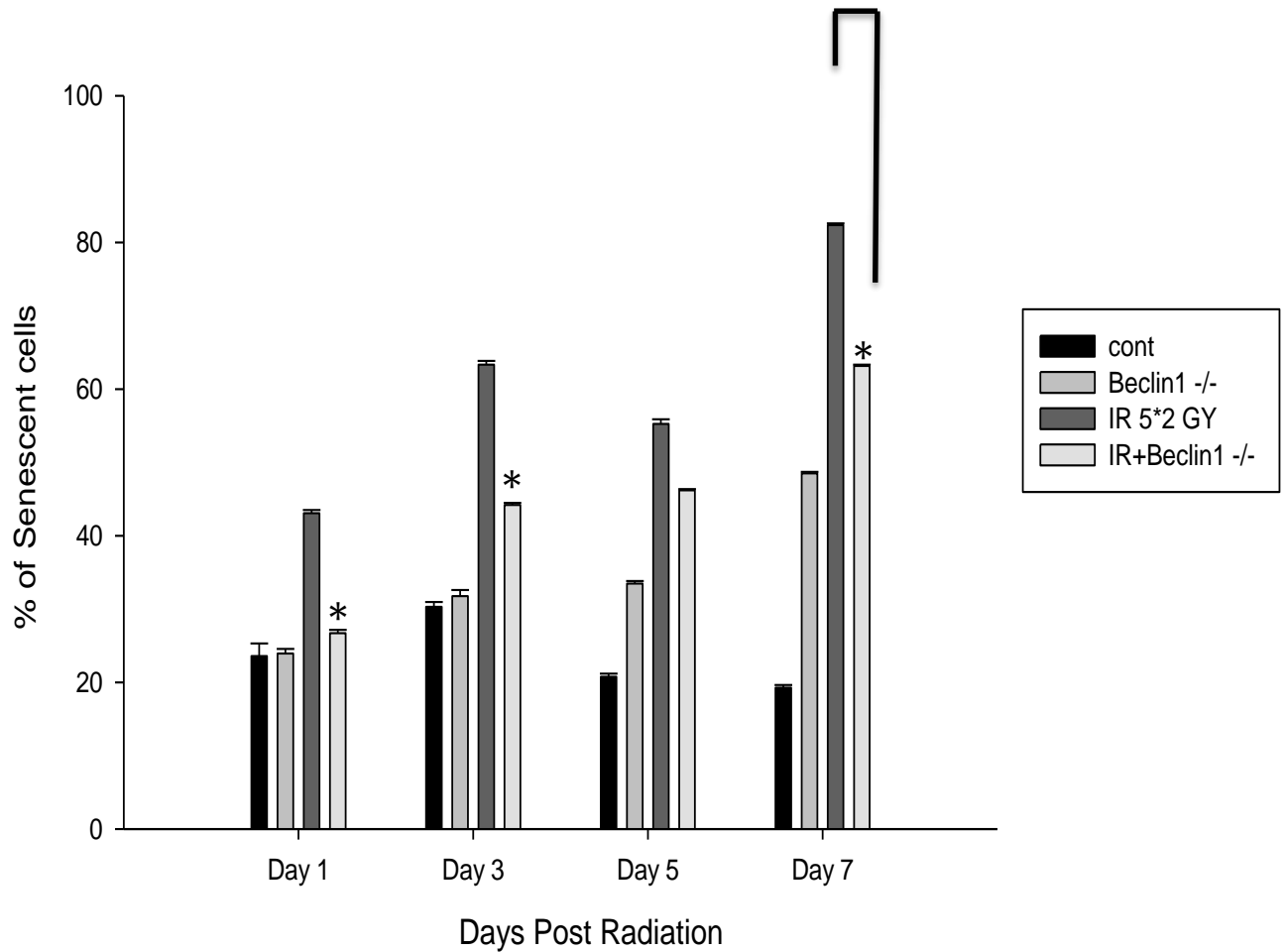


Figure 3.15 Quantification of senescence. Hs578t cells were plated and irradiated with fractionated radiation (5x2Gy) and then were analyzed using Flow cytometry on days 1,3, 5 and 7 post radiation. Silencing of beclin-1 leads to reduction of senescence upon radiation. In each experiment, triplicate samples were used. Two different experiments were done to confirm the results. (* p value< 0.05 compared IR to IR+Beclin1 -/- using ANOVA followed by tukey)

Section 4: Discussion

4.1 Overview

Radiation is essential as adjuvant therapy in the management TNBC. ^[48] One of the major limitations in radiation therapy is the development of resistance. In theory, sensitization of tumor cells to radiation can be accomplished by inhibition of cytoprotective autophagy. Inhibition of autophagy can be achieved by using the anti-malarial drug chloroquine and leads to sensitization of some breast tumor cell lines to radiation. ^[36]

Studies from our laboratory have shown that Hs578t cells are relatively resistant to irradiation compared to other breast cancer cell lines such as MCF7 cells. Also in our laboratory, MCF-7 and ZR-75 breast tumors were sensitized to radiation upon treatment with an autophagy inhibitors such as chloroquine. ^{[36][38]} However, inhibition of autophagy by chloroquine fails to sensitize Hs578t breast cell line to radiation. (Chakradeo S et al under revision). In the current work, we sought to evaluate the effect of autophagy inhibition using another pharmacological inhibitor and genetic silencing of autophagy related genes on the sensitivity of Hs578t cells to radiation.

4.2 Response of Hs578t cells to radiation

Studies in our laboratory have shown that Hs578t cells are relatively resistant to irradiation compared to other breast cancer cell lines such as MCF7 cells.^[38] Initial experiments were designed to identify the response of Hs578t breast tumor cells to radiation. When cells were irradiated with a 5x2 Gy of radiation over a period of 3 days, cell numbers declined between Day 1 to Day 3 and from Day 3 to Day 7, cells undergo an apparent growth arrest. In our laboratory MDA-MB-231 breast tumor cells upon radiation undergo apoptosis and the cells are not showing recovery.^[50] While in Hs578t cells are slightly recovered and showed sustained growth arresting which indicate the radio resistance of Hs578t compared to other cell lines.^[38]

4.3 Effect of irradiation on autophagy

Autophagy is a catabolic process in which select cellular organelles and proteins are digested by lysosomal degradation. It has been shown that the cellular response to radiation in many cell lines is by autophagy.^[40] Our studies using Hs578t cells demonstrated that the irradiated cells show a characteristic of autophagy in that there is an increase in the formation of acidic vacuoles upon irradiation.

P62 is a protein complex linked with the nuclear envelope and its degradation is used as marker of autophagy flux. [27] In this study, the degradation of P62 in irradiated cells confirms the induction of autophagic flux by irradiation.

4.4 Effect of irradiation on senescence

Several studies have shown that induction of autophagy is associated with the promotion of senescence in cancer cells. [45] Our observations indicate that irradiation induces autophagy in Hs578t breast cells line, as furthermore the time course study of cell viability demonstrated a sustained growth arrest pattern, which can be an indication of senescence. Our experiments demonstrated that the irradiated cells show morphological characteristics of cells that undergo senescence, and there is an increase in the number of cells that stained with X-gal which is a marker for senescence upon irradiation. Our observations suggest that radiation induces senescence in the Hs578t breast cells line.

4.5 Effect of autophagy inhibition on radiation sensitivity

Autophagy might be responsible for cancer resistance to chemotherapy and radiation treatment. Inhibition of autophagy can lead to sensitization.[40] In this study, several experiments were designed to determine whether autophagy inhibition could sensitize Hs578t breast tumor cells to irradiation.

Previous data from our laboratory in Hs578t cells using chloroquine and 3-methyl adenine as pharmacological inhibitors have shown lack of sensitization in irradiated cells. Use of one more pharmacological inhibitor NH₄CL also confirmed lack of sensitization. To prove that lack of sensitization is not specific to pharmacological inhibitors, genetic inhibition of radiation-induced autophagy was assessed, which confirmed lack of sensitization by genetic inhibition. The data of combination of irradiation and inhibitors of autophagy suggest that Hs578t cells do not become sensitized to irradiation. However the combination effect might suggest additive effect rather than sensitization. Since Sensitization is a state of supra additive outcome where the total effect of two agents is more than the additive influence of each of those agents separately

4.6 Relationship between autophagy and senescence

Young et al.(2009) reported that autophagy was an effector mechanism of senescence and contributed to the establishment of oncogene-induced senescence. Our laboratory has also generated data that senescence and autophagy can be dependent on each other. ^[45] Experiments were designed to study the relationship between autophagy and senescence induced by radiation in Hs578t cells. β -galactosidase staining was used to assess the relationship between senescence and autophagy using different pharmacological and genetic inhibitors of autophagy.

Data from beta galactosidase staining suggest that irradiation of Hs578t cells promote senescence, and that inhibition of autophagy leads to reduction of that senescence. Flow cytometry quantification of senescent cells confirm the same results. Thus , a conclusion that can be made that in Hs578t cells, autophagy and senescence might be closely linked responses.

In conclusion, In MCF-7 breast tumor cells, ionizing radiation stimulated autophagy that was cytoprotective; pharmacological or genetic inhibition of autophagy stimulated by radiation resulted in growth suppression and/or cell killing (mainly by apoptosis). So inhibition of the cytoprotective autophagy can be used in clinic to sensitize the MCF-7 to radiation. However, in Hs578t breast tumor cells, ionizing radiation stimulated autophagy that was non-cytoprotective. So in order to save patients time, using the pharmacological inhibitor of autophagy is useless in Hs578t treatments. In order to enhance the treatment field of Hs578t cells, more efforts should be devoted to target the proliferative pathways like mTor, AKT, MAPK, and PI3K pathways.

Section5:

Future Experiments

The current findings indicate that the autophagy that is induced by radiation in the triple negative Hs578t human breast tumor cell line appears to be non-protective in that inhibition of this autophagy does not increase sensitivity to radiation. Our findings also show that this autophagy is not cytotoxic, since its inhibition does not reduce radiation sensitivity.

To further study the role of autophagy in Hs578t cells exposed to radiation, the influence of autophagy inhibition on radiation sensitivity should be determined by silencing other autophagy related protein. Since Beclin-1 is one initiator of the autophagy pathway, silencing another protein that has a role in the late steps of autophagy, such as ATG7 or ATG5 should be performed.

Previous data from our laboratory has shown that irradiation induces minimal apoptosis in the Hs578t cells, which does not increase with autophagy inhibition. The utilization of additional genetic inhibitors of autophagy should confirm these conclusions. In addition, it would be important to further establish the relationship between autophagy and senescence, again with the silencing of additional autophagy related genes. In the long term it would be of value to identify the signaling elements that distinguish cytoprotective from non-protective autophagy.

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VITA

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