2011

ROLE OF AUTOPHAGY IN RADIOSENSITIZATION OF BREAST TUMOR CELLS

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ROLE OF AUTOPHAGY IN RADIOSENSITIZATION OF BREAST TUMOR CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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August 2011

<u>Acknowledgements</u>

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$1,25D_3$	$1,25$ dihydroxyvitamin D_3
$25D_3$	25 hydroxyvitamin D_3
3MA	3-methyladenine
AO	Acridine Orange
ATCC	American type culture collection
ATG	Autophagy related protein
AMPK	5' adenosine monophosphate kinase
AVO	Autophagic vacuoles
BAF	bafilomycin A1
bZIP	basic leucine zipper
CDK	Cyclin dependant kinase
CKI	Cyclin dependant kinase inhibitor
CQ	chloroquine
DAPI	4'-6-diamidino-2-phenylindole
EB1089	seocalcitol or 22E,24E-Diene-24,26a,27a-trihomo-$1\alpha,25(OH)_2$-vitamin D_3; also referred to as EB
eif2α	eukaryotic translation initiation factor 2 on the alpha subunit
eIF4E	eukaryotic initiation factor 4E
ER stress	Endoplasmic reticulum stress
Gy	Gray
IR	irradiation
LC3	myosin light chain kinase 3
mTOR	mammalian target of rapamycin
p-	phospho
PI3K	phosphatidylinositol 3-kinase
PIKK	phosphoinositide 3-kinase related kinase
PTH	parathyroid hormone
RFP	Red fluorescent protein
RT	room temperature
RXR	Retinoid X receptor
S6K	p70S6 kinase
TEM	transmission electron microscopy; also referred to as EM
TK1	thymidylate kinase
TUNEL	terminal transferase dUTP nick end label
TYMS	thymidylate synthase
UPR	unfolded protein response

VDR   Vitamin D receptor
VDRE   Vitamin D response element
WT   wild type

Abstract

ROLE OF AUTOPHAGY IN RADIOSENSITIZATION OF BREAST TUMOR CELLS

By Molly Lea Bristol, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Ph.D. at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: David A. Gewirtz, Ph.D.
Professor with the Department Pharmacology and Toxicology

In MCF-7 breast tumor cells, ionizing radiation promoted autophagy that was cytoprotective; pharmacological or genetic interference with autophagy induced by radiation resulted in growth suppression and/or cell killing (primarily by apoptosis). The hormonally active form of vitamin D, $1,25D_3$, also promoted autophagy in irradiated MCF-7 cells, sensitized the cells to radiation and suppressed the proliferative recovery that occurs after radiation alone. $1,25D_3$ also enhanced radiosensitivity and promoted autophagy in MCF7 cells that overexpress Her-2/neu as well as in p53 mutant Hs578t breast tumor cells. In contrast, $1,25D_3$ failed to alter radiosensitivity or promote autophagy in the BT474 breast tumor cell line with low-level expression of the vitamin D receptor. Enhancement of MCF-7 cell sensitivity to radiation by $1,25D_3$ was not attenuated by either a pharmacological or genetic block to autophagy; this was due largely to the promotion of apoptosis via the suppression of protective autophagy that occurs in response to radiation alone. Moreover, pharmacological blockade of autophagy did not sensitize noncancerous MCF10a cells to radiation; conversely, 4T1 mouse mammary tumors were highly sensitive to pharmacological inhibition of autophagy, suggesting selective radiosensitization against cancer cell lines. The current studies are consistent with the premise that while autophagy mediates a cytoprotective function in irradiated breast tumor cells, promotion of autophagy can also confer radiosensitivity by vitamin D ($1,25D_3$).   In addition, this work highlights the technical challenge of establishing the potential cytotoxic function of autophagy in an experimental system where the cytoprotective function may be concurrently expressed.

CHAPTER ONE

Introduction and Review of the Literature

1.1 Cancer

Normal cells grow, divide, and die in an orderly fashion. Cancer cells continue to grow indefinitely and can also invade other tissues. Cancers may be caused by a number of environmental factors such as toxins and irradiation and/or may also arise because of inherited mutations in the DNA; however, the cause of many cancers remains unknown (American Cancer Society, 2010).

Solid tumors can develop in almost any organ or tissue, while some cancers, like leukemia, that involve the blood and blood-forming organs, circulate through other tissues where they can deposit and grow (American Cancer Society, 2010).

Cancer is the second most common cause of death in the US, exceeded only by heart disease. In 2010, approximately 569,490 Americans were expected to die of cancer, more than 1,500 people a day. Half of all men and one-third of all women in the US will develop cancer during their lifetimes. While these numbers remain very high, early detection and diagnosis, combined with increasing numbers of treatment options, have improved the survival rates of most cancers (American Cancer Society, 2010).

1.2 Breast Cancer and Treatment

Besides skin cancer, breast cancer is the most commonly diagnosed cancer among U.S. women. Nearly 1 in 8 women in the United States (12%) will develop invasive breast

cancer over the course of their lifetime. In 2010, an estimated 207,090 new cases of invasive breast cancer were expected to be diagnosed in women in the U.S., along with 54,010 new cases of non-invasive breast cancer; of these women, approximately 39,840 were expected to die. About 1,970 new cases of invasive breast cancer were expected to be diagnosed in men in 2010. Less than 1% of all new breast cancer cases occur in men (breastcancer.org, 2011).

There are a variety of treatments for breast cancer. Treatment will depend on the type of breast cancer and whether it has metastasized. Treatments can include surgery, radiation, chemotherapy, and hormonal therapy, alone and in various combinations. Breast cancer can be treated locally through surgical resection of only the tumor (lumpectomy) or the entire breast (mastectomy) and the tumor may be irradiated with ionizing radiation; in addition, various classes of chemotherapeutic drugs and hormone therapy are commonly used in combination with surgery or radiation. Systemic therapy that is administered before removal of the tumor is considered neoadjuvant chemotherapy. The most common reason for neoadjuvant therapy is to reduce the size of the tumor (debulking) so as to facilitate more effective surgery. The administration of chemotherapy following surgery is known as adjuvant therapy and is directed at killing cancer cells that may have been missed in the resection of the tumor. Commonly used chemotherapeutic agents for the treatment of breast cancer are cyclophosphamide, methotrexate, 5-fluorouracil, doxorubicin, epirubicin, docetaxel, and paclitaxel; trastuzumab may be given as hormonal therapy for HER2/neu positive tumors. The toxicity of these drugs is a major limiting factor in their use. Furthermore, while chemotherapy and radiation treatment may be

initially effective at suppressing breast cancer growth, disease recurrence and tumor

metastasis are major clinical problems (American Cancer Society, 2010).

1.3 Radiation Therapy

About half of all cancer patients receive some type of radiation therapy sometime

during the course of their treatment (National Cancer Institute at the National Institutes of

Health, 2011). When radiation therapy is chosen, an external beam irradiator can be used

to deliver focused irradiation to the breast tumor; internal radiation, or brachytherapy, may

also be employed, where small pellets of radioactive material are implanted at the site of

the tumor. The targeted area may include the whole breast but may also target the chest

wall and the underarm area, depending on the location of the tumor. The total amount of

radiation patients receive is measured in units called *Gray* (Gy) (American Cancer Society,

2010).

Like chemotherapy, radiation therapy is toxic not only to the tumor, but to

surrounding healthy cells. To reduce this toxicity, patients receive smaller, fractionated

doses of radiation; fractionated radiation also serves to increase the likelihood that cancer

cells are exposed to radiation at the points in the cell cycle when they are most vulnerable

to DNA damage (discussed later). Patients who receive most types of external-beam

radiation therapy may receive one dose (a single fraction), or multiple doses of radiation a

day, up to 5 days a week for several weeks. Patients generally receive a total of between

40Gy in 2Gy doses. (American Cancer Society, 2010).

Radiation has been shown to induce many types of DNA lesions that have the potential for cell killing; these can include both single and double stranded breaks in DNA. Radiation can also induce lesions via free radical-induced oxidation of DNA bases (Olive, 1998; Povirk and Steighner, 1989). Ionizing radiation (IR) is thought to produce about 1000 single-strand breaks and 25-40 double-strand breaks per diploid cell per gray administered. The cell death induced by IR is most likely the result of lack of repair or misrepair of complex lesions in DNA (Olive, 1998).

Ionizing radiation causes atoms and molecules to become ionized or excited. These excitations and ionizations can: produce free radicals, break chemical bonds, produce new chemical bonds and cross-linkage between macromolecules, and damage molecules that regulate vital cell processes (e.g. DNA, RNA, proteins)(Princeton University, 2010). The cell can undergo a number of responses to IR including: DNA repair, transcriptional responses, DNA damage checkpoint arrest, and apoptosis (Figure 1.1) (Sancar et al., 2004). When DNA damage is sensed by the cell, depending on the type of damage induced, a number of proteins are recruited to the site and are activated. This may result in a temporary arrest in which the cell will attempt to repair itself, or senescence (discussed in more detail in the following section). If the DNA damage can be repaired, it is thought that the cell is then allowed to reenter the cell cycle and resume proliferation. If the IR-induced DNA damage is too great or the repair mechanisms have been compromised, the cell may fail in its attempt to repair the lesion, consequently leading to cell death. It is also important to mention that DNA lesions induced by IR may also lead to aberrations,

allowing for unregulated cell division which can lead to cancer itself (Olive, 1998; Sancar et al., 2004).

1.4 Radiation-induced Cell Cycle Arrest

As previously stated, there are a number of proteins involved when DNA damage is sensed by the cell. Central to the cell's response to radiation is p53. The p53 gene and the protein it encodes for are known as the "guardian of the genome" and can inhibit tumorigenesis via initiation of cell cycle arrest after DNA damage to allow for repair. Levels of p53 are normally kept low within the cell via association with mdm2; following damage, levels of p53 rise dramatically through post-translational modification. Moreover, ATM phosphorylates p53, displacing mdm2, allowing for p53 stabilization and activation. Activation of p53 can allow for growth arrest or cell death depending on the downstream targets activated. p53 is the most frequently mutated gene in human cancers with the incidence rate over 50%. Defects in p53 may lead to the inability to arrest cells following DNA damage, leading to genomic instability and possibly carcinogenesis (Brady and Attardi, 2010; Lakin and Jackson, 1999; Olive, 1998).

The process of DNA replication and cell division are tightly regulated to ensure proper cell growth, transit through the cell cycle, and prevent the replication of damaged DNA. Progression through the cell cycle is regulated by cyclins, cyclin-dependant kinases (CDKs) and CDK inhibitors (CKIs). Activity of cyclins and CDKs allows for the cell to pass certain "checkpoints" in the cell cycle; CKIs prevent progression through the cell cycle promoting growth arrest (Deshpande et al., 2005). (Figure 1.2)

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Many types of cellular stress can lead to growth arrest. Cell cycle progression can be halted through growth factor withdrawal, contact inhibition, and radiation or chemotherapy-induced DNA damage. DNA damaging agents, such as radiation, can arrest cells in either the G1 or G2 phase through" checkpoints" at the transition between G_1/S and G_2/M. Cells may also exit the cell cycle and enter G_0, or quiescence. Although many cells in the G_0 phase may die, not all cells that enter the G_0 phase are destined to die; this is often simply a consequence of the cell's lacking any stimulation to re-enter in the cell cycle. Cellular senescence is distinct from quiescence because it is a state that occurs in response to DNA damage or degradation that would make a cell's progeny nonviable and is generally considered irreversible; however, there is some evidence suggesting senescence is not entirely irreversible (Chandeck and Mooi, 2010; Gewirtz et al., 2008; Lakin and Jackson, 1999; Sancar et al., 2004; Wang et al., 2011)

The G1/S checkpoint prevents cells from entering the S phase in the presence of DNA damage by inhibiting the initiation of replication. Similarly, the G2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage. If the DNA damage is double-strand breaks, such as those caused by ionizing radiation or radiomimetic agents, ATM or ATR is activated and phosphorylates many target molecules, notably p53, Chk1 and Chk2. Although the G1/S and the G2/M checkpoints are distinct, the damage sensor molecules that activate the various checkpoints appear to either be shared or to play a primary sensor role in one pathway and a back-up role in the other (Sancar et al., 2004).

Senescence is the phenomenon by which normal diploid cells lose the ability to divide; this can be due to the normal aging process, or as a response to stress or DNA damage. Senescence is typically associated with activation of p53 and p21 with subsequent inactivation of Rb and a pronounced G_1 arrest. p16, a CKI, can also be activated in senescent cells. The senescent cells are characterized by flattened and enlarged morphology, increased granularity, and expression of pH 6-dependant β-galactosidase activity (Dimri et al., 1995; Zhang, 2007).

1.5 Apoptosis

Apoptosis, or programmed cell death I, occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis can also occur as a defense mechanism when cells are damaged by disease or harmful agents. Although there are a wide variety of apoptotic stimuli, not all cells will necessarily die in response to the same stimulus. Irradiation, or drugs used for cancer chemotherapy result in DNA damage, in some cells, this can lead to apoptotic death while other cells may remain unaffected or even stimulated to grow (Elmore, 2007).

First discovered by Carl Vogt in 1842, and named in 1972 by Kerr,Wyllie, and Currie, apoptosis is a morphologically distinct form of cell death, characterized by cell shrinkage, chromatin condensation, plasma membrane blebbing, and DNA fragmentation(Elmore, 2007; Khan, 2010). Induction of apoptosis can be mediated through extrinsic or intrinsic machinery.

The extrinsic pathway is also known as receptor mediated apoptosis because this pathway

relies upon the interaction of specific death receptors with their corresponding ligands

(Figure 1.3).   These receptors are located on the cell surface and induce apoptosis via
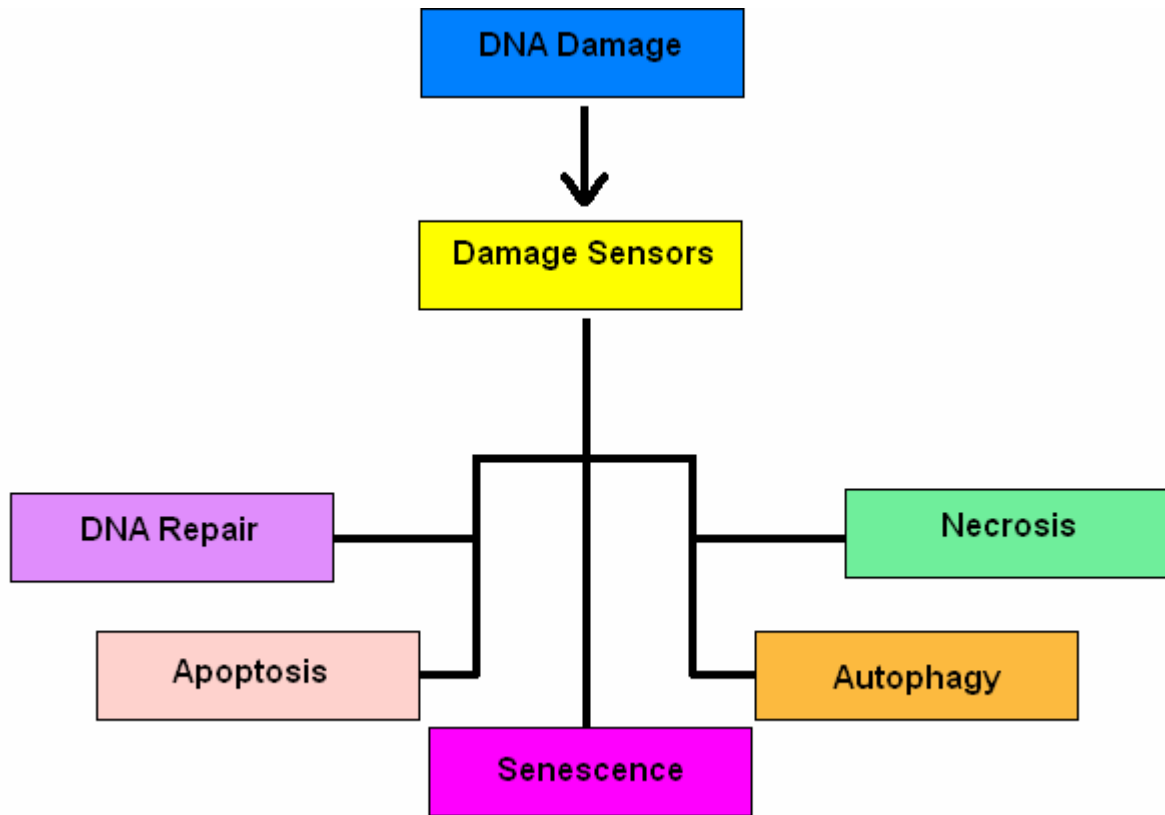
Figure 1.1 DNA damage response reactions in mammalian cells. These responses (DNA repair, apoptosis, senescence, autophagy, and necrosis) may function independently, but frequently a protein primarily involved in one response may participate in other responses.
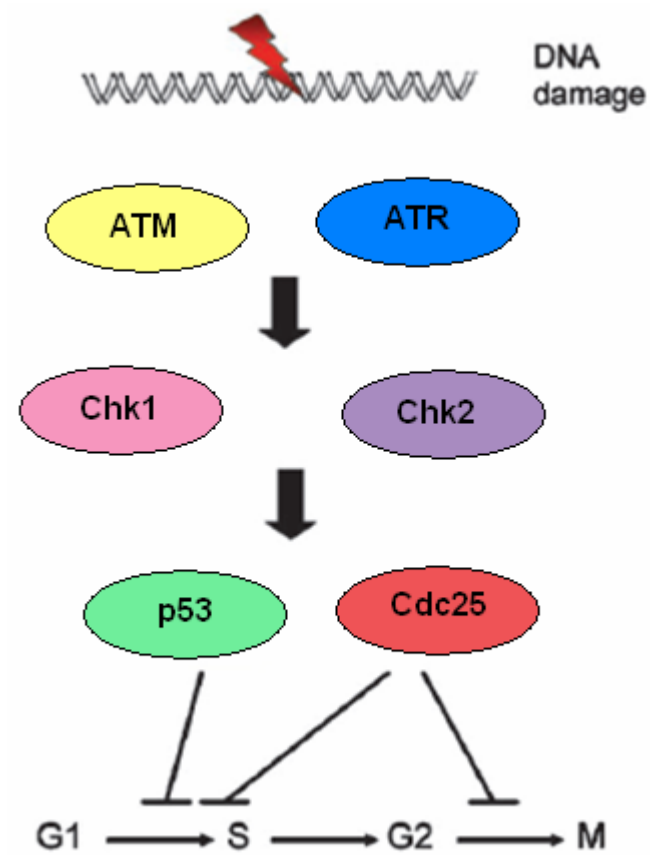
Figure 1.2 Components of DNA damage checkpoints in human cells. DNA damage is detected by sensors that signal to transducers, in turn, activating or inactivating other proteins that directly participate in inhibiting the G1/S transition, S-phase progression, or the G2/M transition.

signaling cascades initiated by their intracellular domains; the receptors are of the TNF receptor subfamily and consist of cysteine rich motifs. The most commonly extrinsic-associated receptors are Fas (APO/CD95) and TNFR (Khan, 2010); together with their ligands, Fas L and TNF, respectively, lead to the cleavage of procaspase 8 to active caspase 8, in turn cleaving procaspase 3 (or procaspases 6 or 7 into active caspase 6 or 7) into the active executioner caspase 3 which is essential for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies, resulting in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Elmore, 2007; Khan, 2010).

For the intrinsic pathway, also known as the mitochondrial pathway, signals typically originate inside the cell, but can also be activated via crosstalk with the extrinsic pathway. Signaling for the intrinsic pathway may arise from various stimuli such as DNA damage, aberrations in the cell cycle, growth factor withdrawal, hypoxia, and cytotoxic drugs; these stimuli can alter the inner mitochondrial membrane through interactions of the pro-apoptotic Bcl-2 proteins Bax and/or Bak, resulting in the permeabilization of the mitochondrial membrane (Figure 1.3). Loss of the mitochondrial transmembrane potential allows for the translocation of cytochrome c and the pro-apoptotic protein SMAC/DIABLO from the intermembrane space of the mitochondria into the cytosol. Cytochrome c binds the adaptor apoptotic protease activating factor-1 (Apaf-1), forming a large multiprotein structure known as the apoptosome. The apoptosome then mediates the
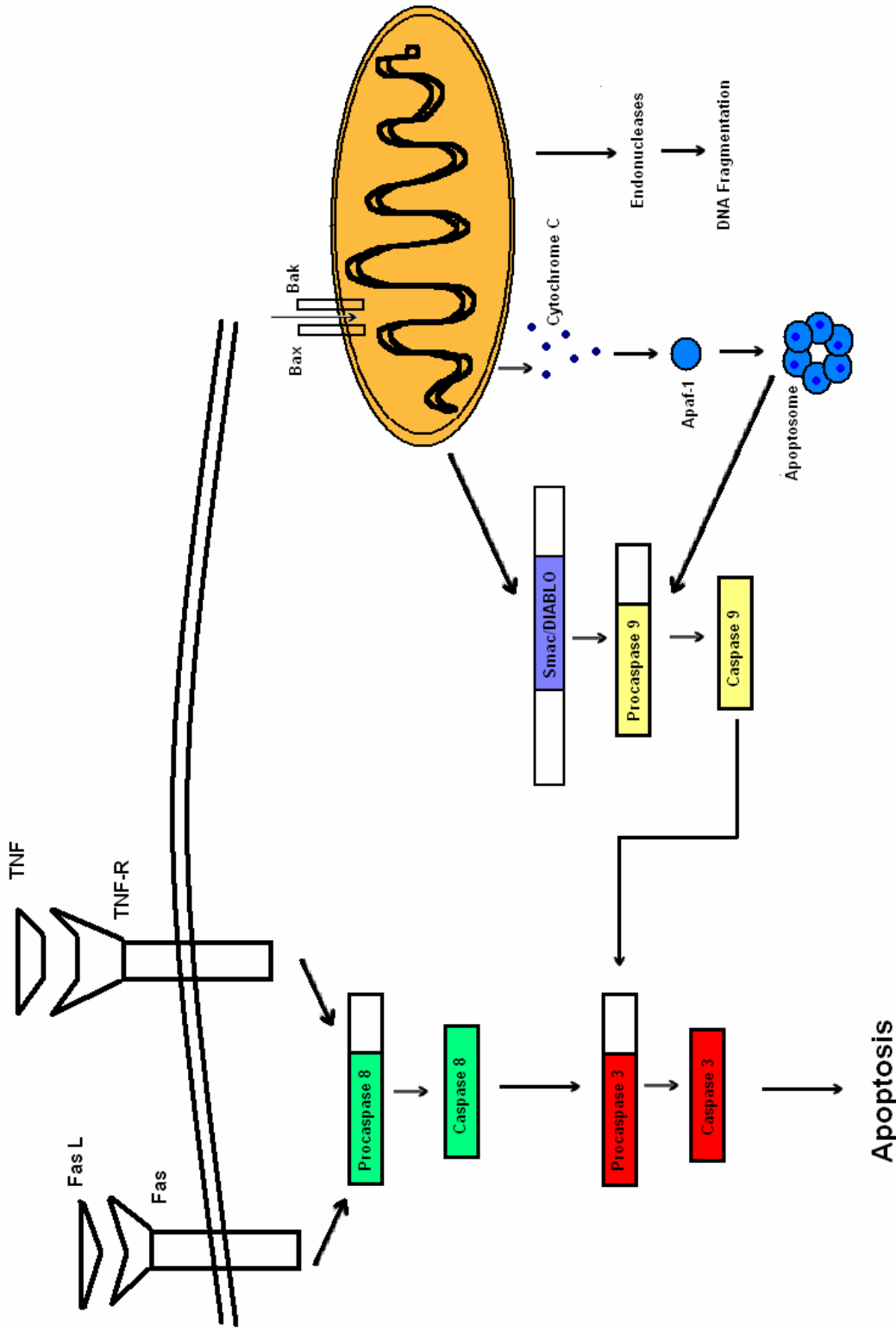
Figure 1.3. Overview of the intrinsic and extrinsic apoptotic pathways. The extrinsic pathway or receptor mediated apoptosis relies upon the interaction of specific death receptors with their corresponding ligands leading to the cleavage of the activator procaspase 8 to caspase 8, in turn cleaving procaspase 3 (or procaspases 6 or 7) into active executioner caspase 3. Alternatively, the intrinsic pathway, also known as the mitochondrial pathway, alter the inner mitochondrial membrane through interactions of the pro-apoptotic Bcl-2 proteins Bax and/or Bak, resulting in the permeabilization of the mitochondrial membrane Loss of the mitochondrial transmembrane potential allows for the translocation of cytochrome c and the pro-apoptotic protein SMAC/DIABLO from the intermembrane space of the mitochondria into the cytosol. Cytochrome c binds the adaptor apoptotic protease activating factor-1 (Apaf-1), forming a large multiprotein structure known as the apoptosome. The apoptosome then mediates the cleavage and activation of procaspase 9 into caspase 9, which in turn activates the downstream effector caspases 3, 6, and/or 7

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cleavage and activation of procaspase 9 into caspase 9, which in turn activates the downstream effector caspases 3, 6, and/or 7 (Ashkenazi, 2008; Bao and Shi, 2007; Elmore, 2007; Khan, 2010).

1.6 Autophagy

Autophagy, or programmed cell death II, is a catabolic or "self-eating" process involving the degradation of a cell's own components through the lysosome. It is a tightly-regulated process that plays an important part in cell growth, development, and homeostasis, helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products. Additionally, it is a major mechanism by which a starving cell can reallocate nutrients from unnecessary processes to more-essential processes for survival. Autophagy may help to prevent or halt the progression of some diseases such as some types of neurodegeneration and cancer, and play a protective role against infection by intracellular pathogens; conversely, in some situations, it may actually contribute to the development of a disease. Moreover, autophagy can be cytoprotective or cytotoxic; it is currently not well understood as to what may be involved in the autophagic transformation between death and survival (Klionsky et al., 2008; Mizushima et al., 1998; Mizushima and Klionsky, 2007; Mizushima et al., 2008).

Nutrient starvation, growth factor deprivation, endoplasmic reticulum (ER) stress, infection, toxic compounds, and radiation can all initiate autophagy. Signaling cascades involved in the initiation are mainly contributed to the ER stress and mammalian target of rapamycin (mTOR) pathways (discussed later); additionally, there may also be crosstalk

between the other growth arrest and cell death pathways. Autophagy occurs at a basal

level in most or all cells; as a starvation response, autophagy is subject to control by a

range of nutrients including amino acids and certain hormones such as insulin. These

receptors in turn activate downstream effectors such as the class I phosphatidylinositol 3-

kinase, and Akt/protein kinase B, resulting in the eventual activation of mTOR kinase, one

of the primary negative regulators of autophagy; however, downstream effectors of mTOR

are largely unknown. In contrast, nutrient depletion results in mTOR inhibition and

activation of autophagy. The induction process also requires a class III

phosphatidylinositol 3-kinase (Mizushima and Klionsky, 2007).

During autophagy, the cytoplasm and cytoplasmic organelles are nonspecifically

sequestered within a double-membrane cytosolic vesicle, an autophagosome, which then

fuses with the lysosome, forming an autophagolysosome; sequestered components are then

degraded via lysosomal hydrolases (Figure 1.4). The degraded products can be reused for

anabolic or catabolic reactions in the cell. The autophagic degradation process is unique in

that it can degrade whole organelles and is not limited sterically. Furthermore, autophagy

is generally considered a nonselective degradation system; however, there are many

examples of selective autophagy. In mammals, p62/SQSTM1 (p62) seems to be a

selective substrate for autophagy that is mediated by microtubule-associated protein 1 light

chain 3 (LC3) binding (Mizushima and Klionsky, 2007).
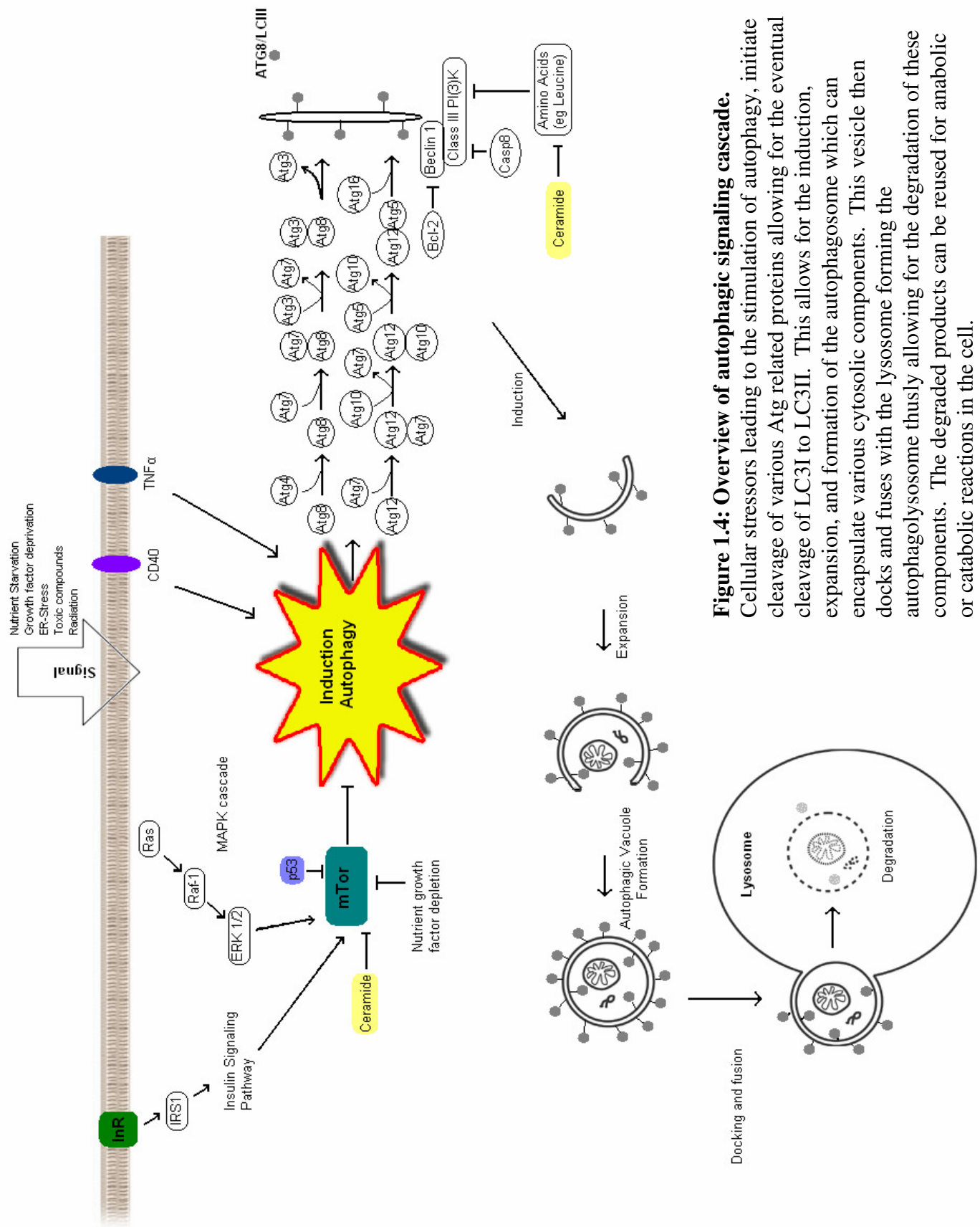
Figure 1.4: Overview of autophagic signaling cascade.
Cellular stressors leading to the stimulation of autophagy, initiate cleavage of various Atg related proteins allowing for the eventual cleavage of LC3I to LC3II. This allows for the induction, expansion, and formation of the autophagosome which can encapsulate various cytosolic components. This vesicle then docks and fuses with the lysosome forming the autophagolysosome thusly allowing for the degradation of these components. The degraded products can be reused for anabolic or catabolic reactions in the cell.

Autophagosome formation involves expansion of the cell membrane. While the process is still not fully understood, autophagy genes, and their corresponding proteins (ATG) serve to direct the membrane into a three-dimensional double membrane sphere through two interconnected conjugation processes (Figure 1.4). ATG8 (LC3 in mammals) and ATG12 are ubiquitin-like proteins, however are not ubiquitin homologues. LC3-I is processed by ATG4, exposing a glycine, whereas ATG12 is synthesized with the glycine; both proteins are activated by ATG7. The activated intermediates are transferred to ubiquitin-conjugating analogues, ATG3 and ATG10, respectively. LC3-I is then covalently attached to phosphatidylethanolamine converting it to LC3-II, causing it to become membrane-associated, whereas ATG12 covalently modifies ATG5 via an isopeptide linkage to an internal lysine. LC3 is the only ATG protein that remains associated with the completed autophagosome in mammalian cells, and thus serves as one of the few markers for autophagy. ATG5 binds ATG16 noncovalently, and the tetramerization of ATG16 results in the formation of a larger complex (Mizushima and Klionsky, 2007; Yang et al., 2005).

1.7 mTOR and Autophagy

As previously stated, there are a number of signaling complexes and pathways involved in autophagy. One key contributor is mTOR, a serine/threonine kinase involved in the majority of regulatory pathways that control the response to variations in nutrient conditions and metabolism. mTOR acts as a gate-keeper in autophagy and appears to

directly and/or indirectly affect ATG proteins, resulting in interference with the formation of autophagosomes. mTOR is active under favorable growth conditions, and promotes ribosome biogenesis, initiation of translation, and nutrient import. Under mTOR-inactivating conditions, i.e., nutrient deprivation, cells undergo autophagy (Diaz-Troya et al., 2008; Yang et al., 2005).

When nutrition is sufficient, mTOR is activated (Figure 1.5). 4E-BP1 is an inhibitor of translation and can be directly phosphorylated by mTOR. After phosphorylation, 4E-BP1 will dissociate from eukaryotic initiation factor 4E (eIF4E); free eIF4E binds RNA and promotes the progression of translation. Likewise, p70S6 kinase (S6K) is activated by mTOR; S6K is a protein kinase and its phosphorylation upregulates translation. Moreover, phosphorylation of 4E-BP1 and S6K requires the presence of amino acids, which are the final products of autophagic protein degradation, thus acting as negative feedback regulators for the process. Conversely, the amino acid/TOR signaling pathway can be inhibited by the AMP-dependent protein kinase (AMPK) (Diaz-Troya et al., 2008; Yang et al., 2005).

1.8 ER-stress and Autophagy

The endoplasmic reticulum (ER) is a crucial site for folding and quality control of proteins destined to the cell surface and intracellular organelles. Pathogen infection, nutrient deprivation inflammation, alterations in ER lumenal Ca^{2+} or redox status, genetic mutation, toxic chemicals, etc., can interfere with ER homeostasis, disrupt protein folding,
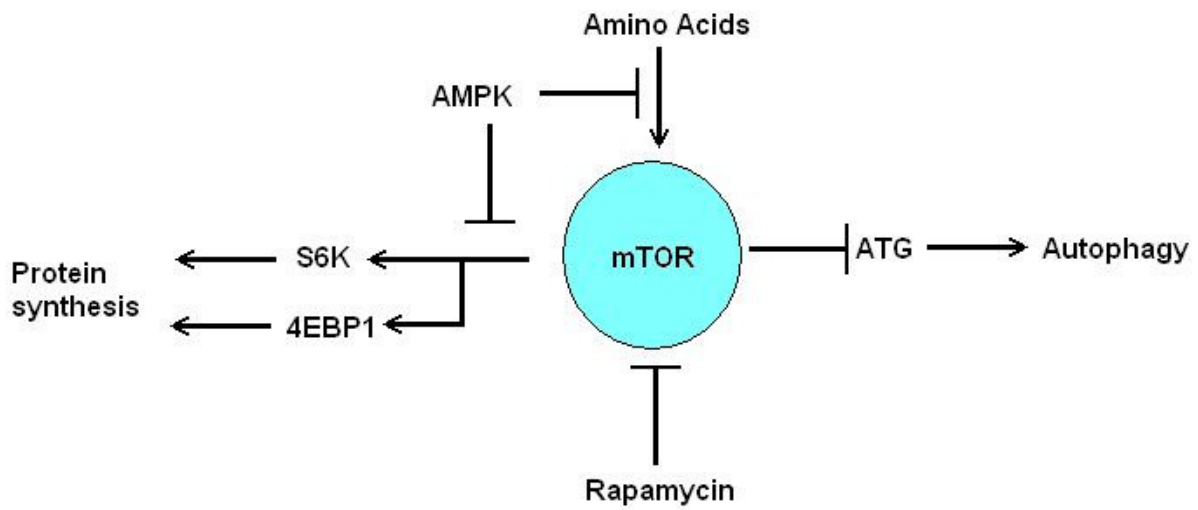
Figure 1.5: mTOR signaling overview. mTOR, a serine/threonine kinase, acts as a gate-keeper in autophagy and appears to directly and/or indirectly affect ATG proteins, resulting in interference with the formation of autophagosomes. mTOR is active under favorable growth conditions, and promotes ribosome biogenesis, initiation of translation, and nutrient import. Under mTOR-inactivating conditions, i.e., nutrient deprivation, cells undergo autophagy.
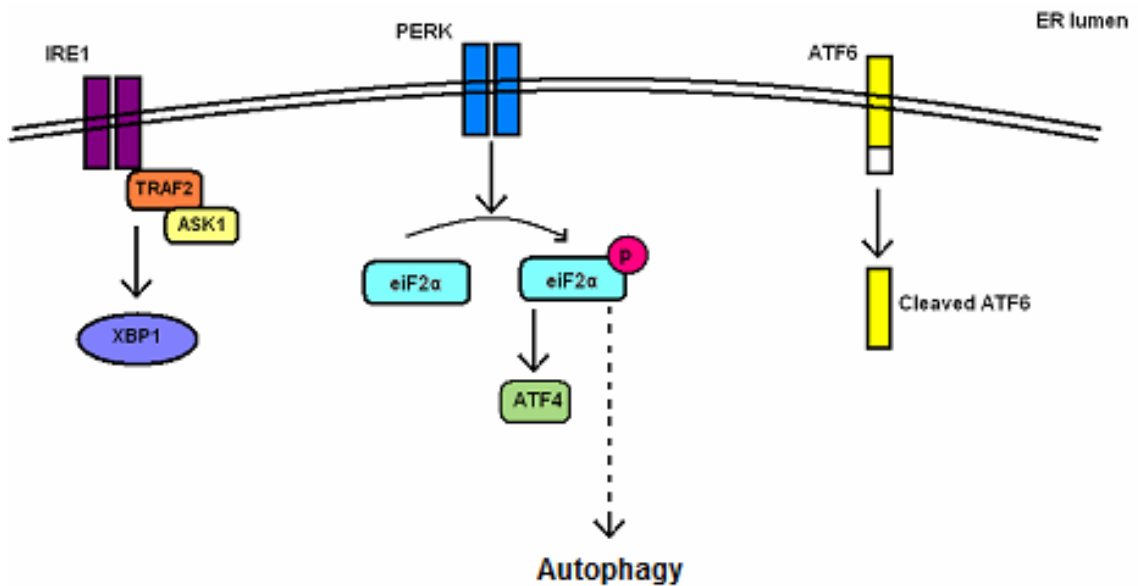
Figure 1.6: Overview of ER stress pathway: The UPR is regulated through three ER-localized transmembrane proteins (PERK, IRE1, and ATF6) that sense the accumulation of unfolded proteins.   ER stress-induced autophagy may serve an important role in the clearance of aggregated or misfolded proteins in the ER that cannot be degraded by ER-associated proteasomal degradation.

and cause the accumulation of misfolded proteins. These conditions are collectively referred to as "ER stress", and can ultimately activate an autophagic response. Autophagy serves an important role in the clearance of aggregated or misfolded proteins. It is thought that ER stress-induced autophagy evolved as a mechanism to degrade misfolded proteins in the ER that cannot be degraded by ER-associated proteasomal degradation (Sakaki and Kaufman, 2008).

Cells constantly monitor protein folding in the ER and can adjust protein synthesis, degradation, and folding capacity to prevent the accumulation of misfolded proteins via signals contributed to the unfolded protein response (UPR) pathway (Figure 1.6). The UPR is regulated through three ER-localized transmembrane proteins (PERK, IRE1, and ATF6) that sense the accumulation of these unfolded proteins (Sakaki and Kaufman, 2008).

PERK phosphorylates eukaryotic translation initiation factor 2 on the alpha subunit (eIF2α) to attenuate mRNA translation initiation. Conversely, eIF2α phosphorylation increases translation of Atf4 mRNA which encodes a transcription factor required to activate most of the UPR-induced genes. IRE1 is a bifunctional protein kinase and endoribonuclease that cleaves Xbp1 mRNA to generate a translational frame shift that produces a potent basic leucine zipper (bZIP)-containing transcription factor. Correspondingly, ATF6 contains a bZIP transcription factor domain in its cytosolic amino terminus. Upon activation of the UPR, ATF6 translocates to the Golgi complex where it is cleaved and then trafficked to the nucleus to activate transcription of genes encoding

protein chaperones and machinery for ER-associated protein degradation (Sakaki and Kaufman, 2008).

1.9 Vitamin D

Vitamin D is a steroid hormone, long associated with calcium homeostasis, but has also been shown to produce strong effects on cell growth, differentiation, and survival (Van Leeuwen and Pols, 2005). Vitamin D (no subscript) can refer to two major physiologically relevant forms of which are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_2 is a derivative of ergosterol, a membrane sterol, and is produced by some organisms of phytoplankton, invertebrates, and fungi in response to UV irradiation; D_2 is not produced by land plants or vertebrates (Mayo Clinic). All future mentions of vitamin D will refer to vitamin D_3. Vitamin D_3 is synthesized through a multistep process, starting in the skin (Figure 1.7) in which UV rays convert 7-dehydrocholesterol to pre-vitamin D; the body's internal temperature then allows for spontaneous structural conversion to the more favorable conformation: vitamin D_3 (cholecalciferol). Vitamin D_3 is further modified in the liver, predominantly through by CYP2R1, to 25α hydroxyl vitamin D_3 ($25D_3$), then 1α hydroxylated in the kidney via CYP27B1 to the hormonally active form, 1,25 dihydroxy vitamin D_3 ($1,25D_3$). Vitamin D's action is limited by its catabolism, primarily by 24-hydroxylase activity via CYP24A1; converting $1,25D_3$ to 1,24,25 trihydroxylase vitamin D_3, which is then further metabolized to excreted products such as calcitrolic acid. Vitamin D compounds are transported

7-Dehydrocholesterol
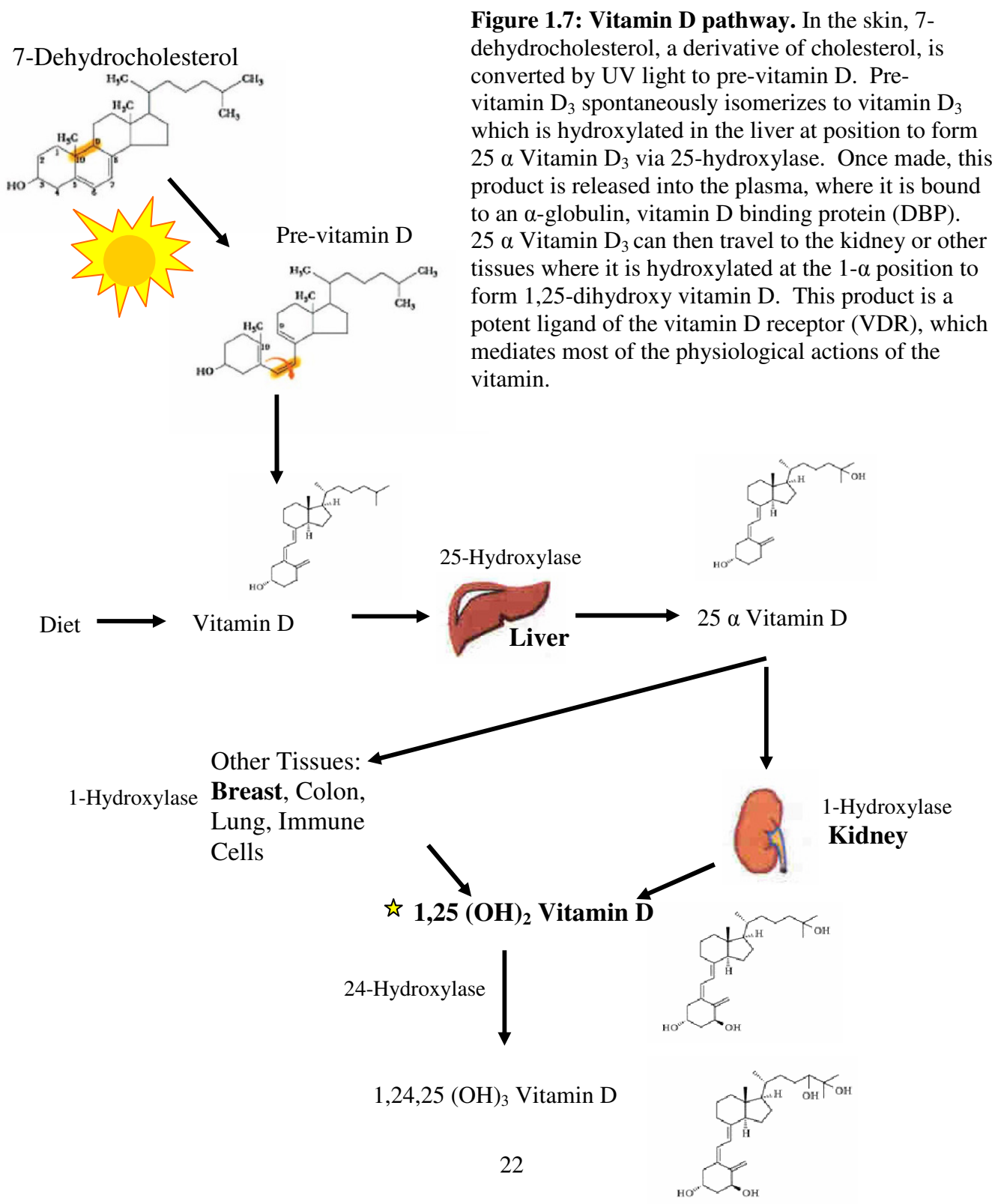
Figure 1.7: Vitamin D pathway. In the skin, 7-dehydrocholesterol, a derivative of cholesterol, is converted by UV light to pre-vitamin D. Pre-vitamin D_3 spontaneously isomerizes to vitamin D_3 which is hydroxylated in the liver at position to form 25 α Vitamin D_3 via 25-hydroxylase. Once made, this product is released into the plasma, where it is bound to an α-globulin, vitamin D binding protein (DBP). 25 α Vitamin D_3 can then travel to the kidney or other tissues where it is hydroxylated at the 1-α position to form 1,25-dihydroxy vitamin D. This product is a potent ligand of the vitamin D receptor (VDR), which mediates most of the physiological actions of the vitamin.

Pre-vitamin D

25-Hydroxylase

Diet → Vitamin D → **Liver** → 25 α Vitamin D

Other Tissues:
1-Hydroxylase **Breast**, Colon, Lung, Immune Cells

1-Hydroxylase **Kidney**

☆ **1,25 (OH)₂ Vitamin D**

24-Hydroxylase

1,24,25 (OH)₃ Vitamin D

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through the body in conjunction with its specific binding partner, the vitamin D binding protein (DBP) (Trump et al., 2010).

1.10 Vitamin D Receptor

The foremost pathway through which vitamin D mediates its biologic effects is through binding to its specific receptor, the vitamin D receptor (VDR). Vitamin D bound to VDR can form homodimers or heterodimerize with the retinoid X receptor (RXR) and its ligand (9 cis-retinoic acid) (Carlberg et al., 2005; Nishikawa et al., 1995; Trump et al., 2010). These dimers occupy specific binding sites on DNA (vitamin D response elements or VDREs). In conjunction with other transcription factors, these complexes induce transcription of vitamin D responsive genes (Evans, 1988).

Among the many genes that are transcriptionally activated by vitamin D are *CYP24A1,* and *CDKN1A* (encoding p21Waf1/Cip1), the growth arrest and DNA-damage-inducible gene, *GADD45* gene; the parathyroid hormone gene (*PTH*) is repressed by calcitriol(Silver and Naveh-Many, 2005). Vitamin D-mediated repression or activation of many protooncogenes or tumor suppressor genes has been observed in both normal and cancerous cells (Trump et al., 2010).

1.11 Vitamin D and Cancer

Recent evidence supports that the VDR is not limited to cells and tissues involved in regulation of calcium and bone metabolism but is also present in a wide variety of cells and tissues including cancer cells of various origins. This had led to a wide range of

studies on the role of vitamin D in tumor development, regulation, and treatment as well as the development of potent synthetic vitamin D analogs(Van Leeuwen and Pols, 2005). Moreover, there are significant data which indicate antitumor effects of vitamin D compounds in preclinical settings. Vitamin D compounds have been shown to inhibit growth and even kill cancer cells *in vitro* and *in vivo* and more recently have demonstrated their potential to potentiate the antitumor activity of a number of traditional cancer therapeutics (Trump et al., 2010). Additionally, clinical data supports the hypothesis that individuals with lower serum levels of vitamin D have a higher risk of a number of cancers. Unfortunately, very limited data are available to indicate whether or not vitamin D supplementation can reduce the risk of such cancers (Trump et al., 2010).

1.12 Vitamin D-induced growth arrest and cell death

Key mediators in vitamin D's antiproliferative effects appear to be linked to cell cycle perturbations. As stated previously, progression through the cell cycle is regulated by cyclins, cyclin-dependant kinases (CDKs) and CDK inhibitors (CKIs, including p21 and p27). CKIs inhibit proliferation, in part by inducing cell cycle arrest in G_0/G_1. As mentioned earlier, CDKN1A, which encodes p21, and GADD45A contain VDREs and are direct transcriptional targets of vitamin D. Vitamin D also represses TYMS, which encodes for thymidylate synthase, and TK1, which encodes for thymidine kinase, both of which are key in DNA replication (Trump et al., 2010).

Vitamin D has many indirect effects on cell cycle progression and can indirectly affect transcription via cross-talk with other signaling pathways. While tumor cells treated

with vitamin D are inhibited in progression through the cell cycle, the molecular basis for this effect can differ from one tumor cell type, to another; consequently, we have yet to determine the exact mechanism of vitamin D-mediated cell cycle perturbation (Trump et al., 2010).

In addition to its antiproliferative effects, vitamin D modulates mediators of apoptosis; it represses the expression of the pro-survival proteins Bcl-2 and Bcl-X_L and can induce the expression of pro-apoptotic proteins (Bax, Bad, and Bak). Vitamin D may also activate caspase effector molecules, and can destabilize telomerase reverse transcriptase (TERT) mRNA – inducing apoptosis through the shortening of telomeres (Trump et al., 2010).

Recent studies have linked vitamin D and autophagy and have speculated that vitamin D-induced autophagy may play a key role in the tumor-suppressing effects of vitamin D via interactions with autophagy associated genes and proteins such as beclin-1 and ATG5 (Hoyer-Hansen et al., 2005; Hoyer-Hansen et al., 2007; Hoyer-Hansen et al., 2010; Wang et al., 2008; Yuk et al., 2009). While the mechanism has yet to be elucidated, one way in which vitamin D-induced autophagy could sensitize cells is through lysosomal permeabilization; a hallmark of autophagic induction, by increasing the volume and activity of the lysosomal compartment, this allows for lysosomal destabilization. Alternatively, by inducing cytoprotective autophagy, vitamin D could also protect tumor cells against cell death triggered by nutrient deprivation and hypoxia in the tumor environment (Hoyer-Hansen et al., 2010).

1.13 Previous work from the laboratory

Our laboratory has studied the use of vitamin D_3 and two vitamin D_3 analogs, EB1089 and ILX23-7553, in combination with Adriamycin (doxorubicin, the topoisomerase II poison) and radiation. EB1089 (seocalcitol or 22E,24E-Diene-24,26a,27a-trihomo-1α,25(OH)$_2$-vitamin D_3), originally developed by Leo Pharmaceuticals in Demark, has been shown to be a more potent derivative than it parental compound in its regulation of cancer cell growth. Conversely, EB1089 has been shown to have a 50% reduction in the regulation of calcium metabolism (Hansen et al., 2001). ILX23-7553 (originally developed by Hoffman LaRoche Inc. as Ro23-7553) has also been shown to potently inhibit the growth of tumors. The structures of EB1089, ILX23-7553, and the active form of vitamin D_3, 1,25D_3, are presented in Figure 1.8.

Previous data indicated that both 1,25D_3 and EB1089 can slow the growth of MCF7 breast tumor cells and significantly decrease cell viability and colony formation after exposure to 10 Gy or 5x2 Gy IR and Adriamycin (Chaudhry et al., 2001; Demasters et al., 2006; DeMasters et al., 2004; Sundaram and Gewirtz, 1999; Sundaram et al., 2003). EB1089 has also been shown to enhance the effects of IR *in vivo* using MCF7 breast tumor xenografts in nude mice (Sundaram et al., 2003). EB1089, in combination with IR or Adriamycin *in vitro*, increased DNA fragmentation and apoptosis; however, the percentage of apoptosis observed (no more than 8%) was inconsistent with the dramatic sensitization (over 87% at 96 hours following radiation) observed with this combinational treatment (Demasters et al., 2006; DeMasters et al., 2004). Additional data further indicated that an

alternative mode of cell death, most likely autophagy, could serve as the mechanism of sensitization (Demasters et al., 2006).

ILX23-7553 was also shown to significantly sensitize MCF7 breast tumor cells to IR and Adriamycin(Chaudhry et al., 2001). Collectively, the studies with EB1089 and/or ILX23-7553 also suggested a requirement for functional p53 in the analogs' ability to sensitize tumor cells to IR or Adriamycin(Gewirtz et al., 2000; Sundaram and Gewirtz, 1999; Sundaram et al., 2000).

While our laboratory has previously established EB1089's ability to sensitize breast tumor cells to IR, more work is needed to better understand the mechanism of sensitization. Previous studies monitored apoptosis, senescence, mitotic catastrophe and alterations in DNA repair; these data were not able to account for EB1089's ability to radiosensitize MCF7 breast tumor cells. Consequently, preliminary studies indicated EB1089 in combination with IR induced an increase in autophagy.

Current studies have been expanded to include both EB1089 and $1,25D_3$, and their ability to induce autophagy in combination with IR. Studies also assess vitamin D_3's ability to sensitize radioresistant breast tumor cells. Moreover, vitamin D_3's ability to radiosensitize breast tumor cells is investigated in the presence of pharmacological and genetic inhibition of autophagy. Pharmacological inhibition with radiation alone is tested both *in vivo* and *in vitro* utilizing the mouse mammary tumor cell line, 4T1. Finally, the mechanism(s) responsible for vitamin D_3's enhancing effects on radiation are also addressed.

1,25D3
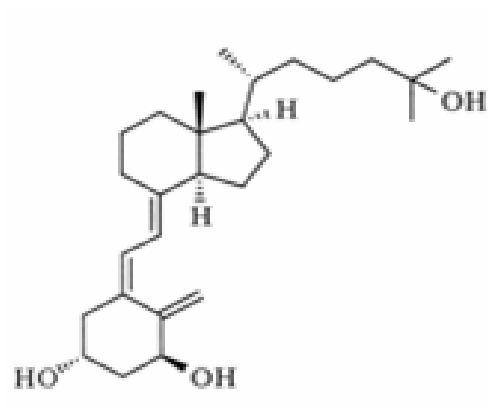
EB1089
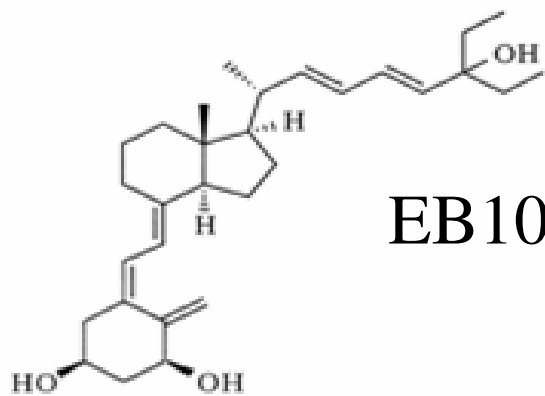
ILX23-7553
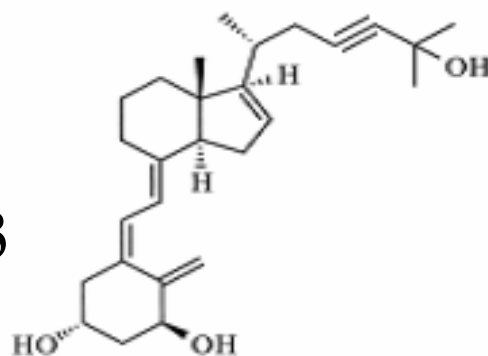
Figure 1.8: Vitamin D related structures.

CHAPTER 2

2.1 Materials

RPMI 1640 l-glutamine, trypsin-EDTA (1×; 0.05% trypsin, 0.53 mmol/L EDTA-4 Na), penicillin/streptomycin (10,000 units/mL penicillin and 10 mg/mL streptomycin), and fetal bovine serum were obtained from Life Technologies (Gaithersburg, MD). Defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT). EB 1089 was provided by Dr. Lise Binderup of Leo Pharmaceuticals (Bellarup, Denmark). Reagents used for the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (terminal transferase, reaction buffer, and fluorescein-dUTP) were purchased from Boehringer Mannheim (Indianapolis, IN). X-gal was obtained from Gold Biotechnology (St. Louis, MO). The following materials were obtained from Sigma Chemical (St. Louis, MO): 1,25 Vitamin D3, trypan blue solution, formaldehyde, acetic acid, albumin bovine (bovine serum albumin), *N*-acetyl-l-cysteine, reduced glutathione (GSH), 4′,6-diamidino-2-phenylindole (DAPI), and DMSO. Acridine orange was purchased from Invitrogen (Eugene, OR). M-PER mammalian protein extraction reagent was purchased from Thermo Scientific (Rockford, IL).

2.2 Cell Lines

The p53 wild-type (WT) MCF7 human breast tumor cell line was obtained from National Cancer Institute (Frederick, MD). BT474 and Hs578t cells as well as the non-cancerous MCF10a cell line were obtained from ATCC. Development of the MCF7/ATG5-/- cells is described below. The MCF7 RFP/LC3 construct was a generous

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gift from Dr. Keith Miskimins (Bampton et al., 2005). The MCF7/ATG7-/- cells were a

generous gift from Dr. Ameeta Kelekar (Separovic et al., 2010). The MCF7/HER2 cells

were a generous gift from Dr. Zhen Fan (Liang et al., 2003). 4T1 cells were a generous

gift from Dr. Fred Miller(Aslakson and Miller, 1992) and 4T1-luc cells were obtained from

Caliper.

2.3 Cell Culture and Treatment

All MCF7 derived cell lines were grown from frozen stocks in basal RPMI 1640

supplemented with 5% FCS, 5% BCS, 2 mmol/L l-glutamine, and penicillin/streptomycin

(0.5 mL/100 mL medium). MCF7/ATG5-/- were maintained using (200µg/ml) puromycin

(Sigma p8833) for resistance. MCF7/HER2 cells were maintained using (200µg/ml) G418

(Gibco 10131-035). BT474 cells were grown from frozen stocks in DMEM supplemented

with 10% FCS, 2 mmol/L l-glutamine, and penicillin/streptomycin (0.5 mL/100 mL

medium). Hs578t cells were grown from frozen stocks in alpha-DMEM supplemented

with 10% FCS, 2 mmol/L l-glutamine, and penicillin/streptomycin (0.5 mL/100 mL

medium). MCF10a cells were maintained in DME/F-12 1:1 media supplemented with

2.5mM Glutamine, 15mM HEPES Buffer, 10% Horse Serum, 2 mmol/L l-glutamine, and

penicillin/streptomycin (0.5 mL/100 mL medium), Insulin (10mg/ml): 500µl, CholeraToxin

(1mg/ml): 50µl, Hydrocortizone (1mg/ml): 250µl, (100 mg/ml): 100µl EGF.   All cells were

maintained at 37°C under a humidified, 5% CO_2 atmosphere. Cells were routinely

subcultured by trypsinization (0.25% trypsin, 0.03% EDTA, Gibco) upon reaching

confluence. All experiments were examined by microscope for bacterial and fungal

contamination prior to experiments. Additionally, all cell lines were determined to be free

of mycoplasma. Some cells were exposed to γ-IR using a [137]Cesium irradiator. In our

studies, some cells were exposed to 100 nmol/L EB1089 (Leo Pharmaceuticals batch no.

EB1 262160) or 1,25 Vitamin D_3 (Sigma D1530) for 72 hours before irradiation. This

sequence of exposure was based on the studies by Wang et al. (Wang et al., 2000) and our

own previous work, (Chaudhry et al., 2001; DeMasters et al., 2004; Sundaram et al., 2003)

which have shown a requirement for prolonged incubation with vitamin D_3 or its analogues

to promote sensitivity to Adriamycin and irradiation. In the cases where the radiation

doses were fractionated, five fractions of 2Gy radiation were administered on three

consecutive days (two fractions separated by 6 hours on the first 2 days followed by a fifth

dose on the 3rd day).

2.4 Cell viability

Cell viability was determined by trypan blue exclusion at various time points.

Cells were plated in triplicate at varying densities depending on the cell line and treatment

length (500 or 10,000 cells per well) in 6-well plates. After overnight incubation to allow

for surface adherence, 100nM of EB1089 or $1,25D_3$ were added for 72 hours. EB1089 or

$1,25D_3$ were then removed, the cells were washed with 1X PBS then fresh media was

added. Cells were then exposed to 5x2Gy IR. At appropriate time points, cells were

harvested using trypsin, stained with 0.4% trypan blue dye (Sigma T8154), and counted

using a hemocytometer with phase contrast microscopy or the Invitrogen Countess

automated cell counter.

2.5 Clonogenic survival

For clonogenic survival studies, cells were plated in triplicate in six well tissue culture dishes at the appropriate density for each condition (typically 100 cells per well). After 10-14 days, the cells were fixed with 100% methanol, air-dryed and stained with 0.1% crystal violet (Sigma C3886). For computing the survival fraction, groups of 50 or more cells were counted as colonies. Data were normalized relative to untreated controls, which were taken as 100% survival.

2.6 Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay for Apoptosis

The method of Gavrieli et al. (Gavrieli et al., 1992) was used as an independent assessment of apoptotic cell death in combined cytospins containing both adherent and nonadherent cells. Cells were fixed and the fragmented DNA in cells undergoing apoptosis was detected using the In situ Cell Death Detection kit (Roche 11373242910, 03333566001), where strand breaks are end labeled with fluorescein-dUTP by the enzyme terminal transferase. Cell suspensions were cytospun onto glass slides and stored at 4oC until stained. The slides were fixed by immersion in 4% formaldehyde/PBS solution for 10 minutes at RT. Following 2 washed with PBS for 5 minutes each at RT, the slides were immersed in a 1:2 dilution of glacial acetic acid to ethanol at -20°C for 5 minutes. The slides were again washed twice with PBS at RT. Using an ImmunoPen (calibiochem, San Diego, CA) a tight circle was drawn around cells to allow containment of the following solutions. The following steps were performed in a humidified chamber covered with

aluminum foil. The cells were blocked with 50µL of bovine serum albumin (BSA)/PBS (1mg/ml) solution for 30 minutes at RT; after removal of BSA, cells were again washed twice with PBS for 5 minutes each at RT. The cells were then incubated at 37°C for 1 hour with an enzyme mix consisting of 4 µL of 5X reaction buffer, 0.2µL terminal transferase, 2µL 25mM CoCl2, 0.4µL Fluorescein-12-dUTP, and 13.4µL water per sample. The enzyme mix was then removed and again washed twice with PBS for 5 minutes each at RT. The cells were then mounted using Vectashield mounting medium (Sigma D9542) containing 1:1000 DAPI. Pictures were taken using an Olympus inverted fluorescence microscope. TUNEL positive cells were quantitated by counting number of positive cells per field; three representative fields were averaged per condition. All images presented are at the same magnification.

2.7 Western Blot Analysis

(a.) Protein Isolation

Cells were typically plated at a density of 50,000-100,000 cells per 10cm culture dish. After the indicated treatments and at appropriate time points, cells were washed with cold PBS and lysed using 500-1000 μL M-PER mammalian protein extraction reagent (Thermo Scientific #78501) containing protease and phosphatase inhibitors for 5 minutes on a shaker. The lysis was then collected and centrifuged for 5 minutes at 10,000rpm. Supernatent was collected and stored at

-80°C until day of western.

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(b.) Lowry Assay for Protein Concentration

Protein concentrations were determined by the Lowry method (citation – Lowry OH et al 1951). Dilutions of isolated proteins were compared to a standard curve using various doses of BSA. Absorbance at 750um was then determined using an Ultraspec 300 UV/Visible Spectrophotometer.

(c.) Electrophoresis

Concentrations determine by the Lowry assay allowed for equal aliquots of protein (35-50 μg) were added to 5X sample buffer (60mM Tris-base (pH 6.8), 25% glycerol, 2% SDS, 14.4mM 2-mercaptoethanol, 0.1% bromophenol blue) and diluted to equal volumes (30μl) water. The samples were then loaded onto a polyacrylamide gel (8-15% - depending on the molecular weight of the protein being analyzed) and separated using SDS-PAGE running buffer for 0.5-2 hours with 60 milliamps constant current. Proteins were then transferred onto a nitrocellulose membrane electrophoretically for 1 hour at 100 volts in transfer buffer at 4°C.

(d.) Immunoblotting

The membrane was blocked in TBS-Tween 20 buffer containing 5% nonfat dry milk or Odyssey blocking buffer for one hour. After removal of the blocking buffer, membranes were immunoblotted with respective primary antibodies overnight at 4°C with an orbital shaker. Primary antibodies were removed and the blot was washed 5 times, five minutes each in blotto wash. The membranes were then incubated with the respective

horseradish peroxidase–conjugated or Odyssey secondary antibodies for 30 minutes.

Proteins were visualized using an enhanced chemiluminescence kit from Pierce (Thermo

Scientific #34080) and the light emission from the oxidation reaction exposed to

audioradiography film or via the **Odyssey** Infrared Imaging System. Primary antibodies

used were anti-p62 (SQSTM1 – Santa Cruz sc-28359), anti-ATG5 (APG5 – Biosensis R-

111-100), anti-ATG7 (APG7 – Santa Cruz sc-33211), anti-VDR (Santa Cruz sc-13133),

anti-ß actin (Santa Cruz sc-47778).

2.8 Detection of Autophagic Cells

(a.) Acridine Orange

As a marker of autophagy, the volume of the cellular acidic compartment was

visualized by acridine orange staining. (Paglin et al., 2001) Cells were seeded in six well

tissue culture dishes and treated as described earlier for the cell viability study. 24 hours

following treatment, cells were incubated with medium containing 0.4 μM acridine orange

(Invitrogen A3568) for 15 minutes; the acridine orange was then removed, cells were

washed once with PBS, fresh media was added, and fluorescent micrographs were taken

using an Olympus inverted fluorescence microscope. Again, all images presented are at the

same magnification. The number of cells with increased acidic vesicular organelles was

determined by counting at least three representative fields per treatment condition.

(b.) Transmission Electron Microscopy (TEM)

TEM services, including sample fixation, embedding, ultra-microtomy and staining were provided by the VCU Department of Anatomy and Neurobiology Microscopy Facility. Electron Microscopy Cells were harvested from culture and 1x10 5 cells were plated per plate on Permanox Petri dishes. Various drug treatments were performed until endpoint. At each time point, cells were washed with 1X PBS and then fixed with 2% paraformaldehyde/2 % glutaraldehyde in 0.1M sodium cacodylate buffer. After 1 hour at room temperature, fixative was removed and replaced with 0.1M sodium cacodylate buffer, pH 7.4. Plates were then fixed in 1% osmium tetroxide in 0.1M-cacodylate buffer for one hour, after which are they were rinsed in 0.1M cacodylate buffer for 5-10 minutes. The plates were then dehydrated in graded ethanol series: 50%, 70%, 80%, 95%; for 5-10 minutes each. Then cell plates are were dehydrated in 100% ethanol 3X, 10-15 minutes each. This was followed by propylene oxide 3X, 10–15 minutes each. Cells were then infiltrated with 50/50 mix of propylene oxide and PolyBed 812 resin mix – overnight +. Next, the cells were infiltrated with pure PolyBed 812 resin (Polysciences, Inc.)mix – + hours and overnight. The cells were placed in embedding molds can then be embeddedcontaining PolyBed 812 and placed in 60°C oven overnight to polymerize. Sectioning is done using Leica EM UC6i Ultramicrotome (Leica Microsystems). For electron microscopy, 70-90 nm thick sections were collected on copper mesh grids and stained with 5% uranyl acetate and Reynold's lead citrate. Images are taken using JEOL JEM-1230 TEM (JEOL USA, Inc.) with the Gatan Ultrascan 4000 digital camera (Gatan Inc, Pleasanton, CA). The magnification of image is indicated at the bottom of micrograph images.

2.9 RNA interference

MCF7 cells were developed to silence the expression of ATG5. ATG5 shRNA oligonucleotides were designed according to the sequence described previously (Fung et al., 2008). For each set, top- and bottom-strand oligos were synthesized separately and annealed together. The primers for siRNA corresponding to the coding region were as follows: top,

5'- gatccccGGATGAGATAACTGAAAGGttcaagagaCCTTTCAGTTATCTCATCCtttttta - 3'

and bottom,

5'- gatccccGGCATTATCCAATTGGTTTttcaagagaAAACCAATTGGATAATGCCtttttta - 3';

The BglII restriction site was included on the top strand at its 5' end; the HindIII restriction site was included at the 3' end to facilitate cloning into the pSUPER-retro-puro (OligoEngine, VEC-PRT-0002). The 2 oligos were annealed and inserted into the empty vector that has been linearized with BglII and HindIII enzymes. Positive clones were identified and sequenced and confirmed by automated DNA sequencing.

2.10 Animals.

Female BALB/c mice, 8 weeks old, were purchased from Jackson Laboratories and were housed in our animal research facility. Mice were kept in groups of four per cage and were fed with clean food and water. The animals were acclimated at least two days before use and maintained throughout at standard conditions: $24\pm2^{o}C$ temperature; $50\pm10\%$

relative humidity. All studies involving mice were approved by the Institute's Animal Care and Use Committee.

2.11 In vivo tumor experiment.

Female BALB/c mice were challenged s.c. with 5 x 10^4 4T1 mouse breast tumor cells, transfected with luciferease, in the right flank on day 0. On day 3, animals were injected with 150 mg/kg luciferin and the presence of primary tumors was determined by bioluminescence signal as imaged using an IVIS 50 (Xenogen, part of Caliper Life Sciences). Of note, the bioluminescent signal could be detected in the animal before primary tumors were palpable and could be measured with a caliper. Tumor-bearing mice were randomly assigned into groups and were injected intraperitoneally with their corresponding treatments: (a) 25 mg/kg CQ, (b) 50 mg/kg CQ, (c) 100mg/kg CQ, or (d) PBS. Additionally experiments were conducted utilizing IR with the following corresponding treatments: (e) 5 Gy IR, (f) 10 Gy IR, (g) 15 Gy IR, (h) 10Gy IR + 14 days CQ ip. Each group had 6-8 mice. Therapy started on day 3 and was repeated every day for 5 or 14 days for CQ or PBS treatment, and only on day 3 for IR treatment. The tumor growth was monitored multiple times per week by an IVIS Spectrum. After treatment ended, mice were further monitored until at least day 18 and were then sacrificed. Tumors were resected and tumor volumes were observed. Tumor growth was also evaluated by measurement of tumor diameters at various time points and the tumor volume was calculated as length x (width x 0.5)^2.

2.12 Statistical Analysis

All of the data are represented as means ± SE. Statistical differences were determined using StatView statistical software. Comparisons were made using a one-way ANOVA followed by Tukey Kramer's post-hoc test. Ps ≤ 0.05 were taken as statistically significant.

CHAPTER 3: Influence of Vitamin D3, and its analog, EB1089, on the response to

fractionated radiation in MCF7 cells

3.1 The combination of EB1089 and radiation promotes extensive autophagy.

$1,25D_3$ and vitamin D_3 analogs have previously shown to promote growth arrest (Jensen et al., 2001; Sundaram and Gewirtz, 1999; Sundaram et al., 2003; Welsh et al., 2003) or apoptosis in breast tumor cells, depending on the concentration used, and the cell line studied (Hansen and Maenpaa, 1997; Hoyer-Hansen et al., 2005; Polar et al., 2003; Welsh et al., 2003). Previous studies in our laboratory demonstrated EB1089's ability to enhance the response to IR as well as the involvement of autophagy in radiosensitization (Demasters et al., 2006; DeMasters et al., 2004; Sundaram and Gewirtz, 1999; Sundaram et al., 2003). Our current studies were designed to expand upon the previous work by focusing on the mechanism of cell death induced by the combination of EB1089 with radiation and to include studies with the active form of vitamin D_3, $1,25D_3$. As with our previous observations, the promotion of autophagy in MCF7 cells exposed to EB1089 + radiation was confirmed based on acridine orange staining of autophagosomes, redistribution of RFP-LC3 and electron microscopy evidence of autophagic vacuole formation. Quantification for each study is presented in an accompanying bar graph. Figure 3.1 shows punctate acridine orange staining, indicative of autophagy, (Paglin et al., 2001) in cells treated with EB1089 followed by radiation; acridine orange vesicle staining in MCF7 cells exposed to tamoxifen was utilized as a positive control for autophagy (Bursch et al., 2000). In contrast, untreated control cells or cells treated with EB1089

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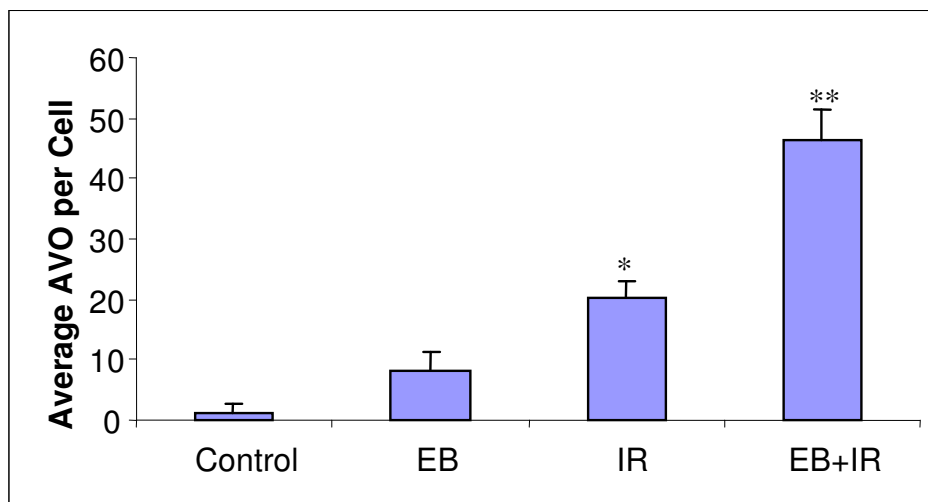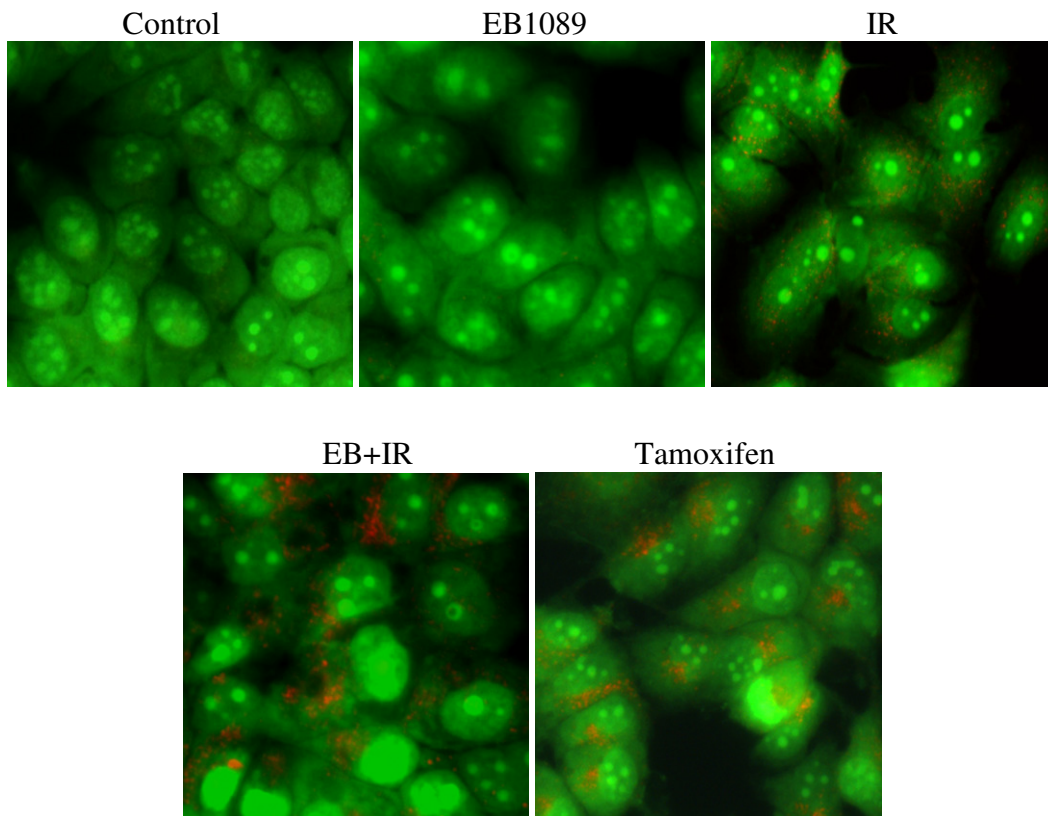
Figure 3.1: Promotion of autophagy by EB1089 + radiation in MCF-7 cells-Acridine orange staining of autophagic vesicles. Cells were exposed to 100 nM EB1089 for 72 hours alone, radiation alone (5x2Gy administered over a period of 3 days) or EB1089 for 72 hours which was then removed and followed by radiation. 2μM of tamoxifen for 72 hours was utilized as a positive control for AVO staining. AO images were taken 24 hours post-irradiation using an inverted fluorescence microscope. Average number of AVOs per cell was counted in three fields for each condition and is represented in the above graph. *p<0.05, **p<0.0001

alone exhibit little or no orange punctate staining; of note to the subsequent studies in which autophagy was blocked in irradiated cells, cells treated with radiation alone exhibit an increase in punctate acridine orange vesicle staining over that observed in controls.

In Figure 3.2, MCF7 cells stably transfected with RFP-tagged LC3 demonstrate a significant increase of red punctate fluorescence with radiation alone as well as with $1,25D_3$ followed by radiation, indicative of LC3II association with the autophagosomal membrane. (Kirisako et al., 2000) In contrast, a diffuse pattern of staining is observed in controls and in cells treated with $1,25D_3$ alone.

Electron microscopy (EM) further confirmed the induction of autophagy upon treatment of the cells with EB1089 followed by radiation; Figure 3.3 shows representative double membraned autophagosomal structures encapsulating cellular organelles for radiation alone and for EB1089 + radiation. (Boya et al., 2005; Lambert et al., 2008; Livesey et al., 2009; Wu et al., 2008) Autophagosomal vesicle formation was quite extensive with the combination treatment but relatively low with radiation alone, and was essentially absent in control cells or in cells treated with EB1089 alone.

While previous studies indicated apoptosis may also be a factor in EB1089 radiosensitization, our current studies did not indicate a substantial increase in the level of apoptosis observed in cells treated with EB1089 alone, radiation alone, or the combination of EB1089+IR (Figure 3.4), as indicated by the lack of TUNEL staining, and intact nuclei observed with DAPI; these findings as well as the absence of evidence for mitotic catastrophe (DeMasters et al., 2006) were consistent with the premise that autophagy is likely to play a major role in the cytotoxic effects of EB1089 in combination with IR.

3.2 1,25D$_3$ alters the response to ionizing radiation from growth arrest to cell death in breast tumor cells.

To confirm that 1,25D$_3$ elicited the same response as EB1089 in our system, MCF7 breast tumor cells were treated with 1,25D$_3$ alone, 5x2Gy IR alone, or 100nM 1,25D$_3$ followed by 5x2Gy, and cell viability was determined at various times post-irradiation (Figure 3.5). As with EB1089, 1,25D$_3$ initially demonstrates a reduction in cell growth; when 1,25D$_3$ is removed, cells rapidly regain proliferative capacity. Cells exposed to radiation alone continue to proliferate at a greatly reduced rate and subsequently arrest for a period of 4-6 days post-irradiation; it is of particular importance to note that the cells recover proliferative capacity following this period of arrest. Pretreatment with 1,25D$_3$ alters the cellular response to subsequent radiation, markedly attenuating cell viability by 60% from that of IR alone at day 7, and by over 84% at day 12 following radiation with no subsequent recovery. Likewise, Figure 3.6 demonstrates the impact of the treatment combining 1,25D$_3$ with ionizing radiation on clonogenic survival. While radiation alone significantly reduces colony formation (74%) in comparison to control cells, 1,25D$_3$ in combination with radiation induces an 87% reduction in colony formation and a 73% reduction in colony size. As explained previously by Demasters et al, (DeMasters et al., 2004) the clonogenic survival assay is likely to under-represent the impact of the combination treatment as compared to radiation alone since the proliferative recovery that is evident in Figure 3.5 likely does not occur under the experimental conditions of the clonogenic survival assay because of the lack of cell to cell contact; consequently, the differences between radiation alone and the combination of 1,25D$_3$ + radiation will not be

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Figure 3.2

Control	EB1089	IR	EB+IR
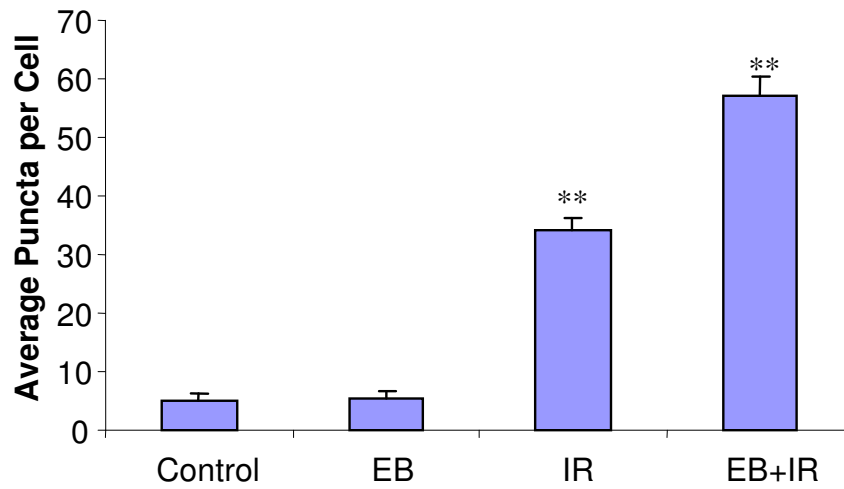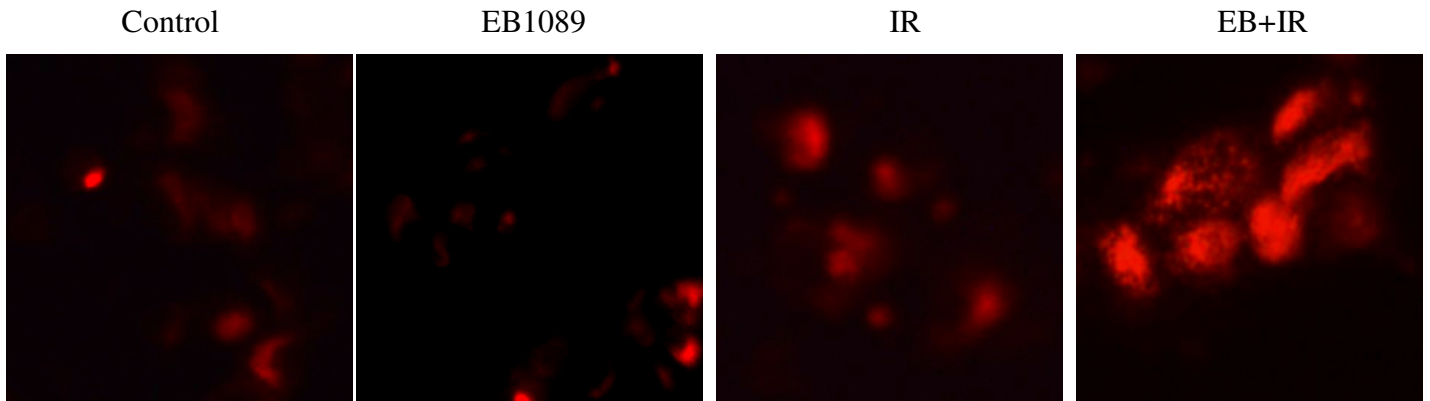
Figure 3.2: Promotion of autophagy by EB1089 + radiation in MCF-7 cells-RFP LC3 punctuate staining. MCF7 cells stably transfected with RFP-LC3 were treated as in Figure 3.1. Diffuse and punctuate staining was monitored by fluorescence microscopy; images were taken 24 hours post-irradiation. Average number of puncta per cell was counted in three fields for each condition and is represented in the above graph. **p<0.0001

Figure 3.3

Control   EB1089   IR   EB+IR

2μm   2μm   0.5μm   0.5μm
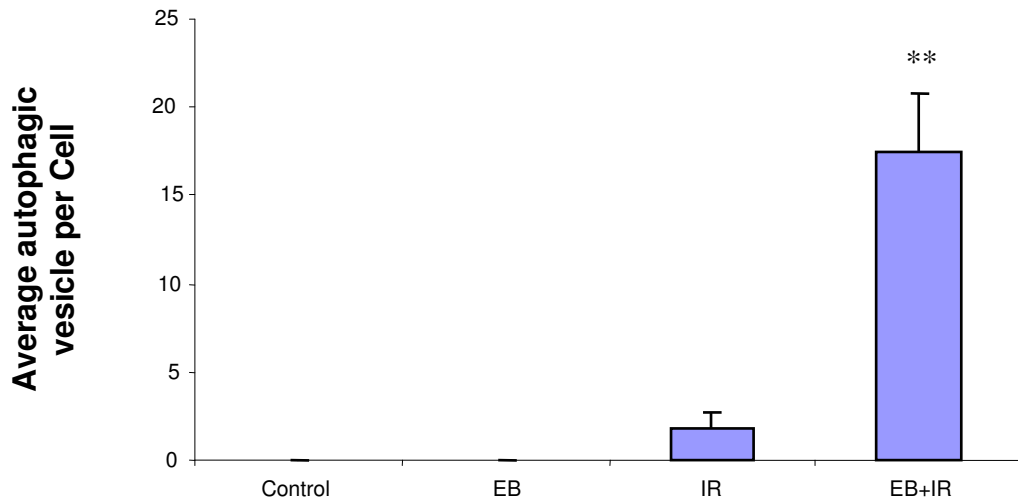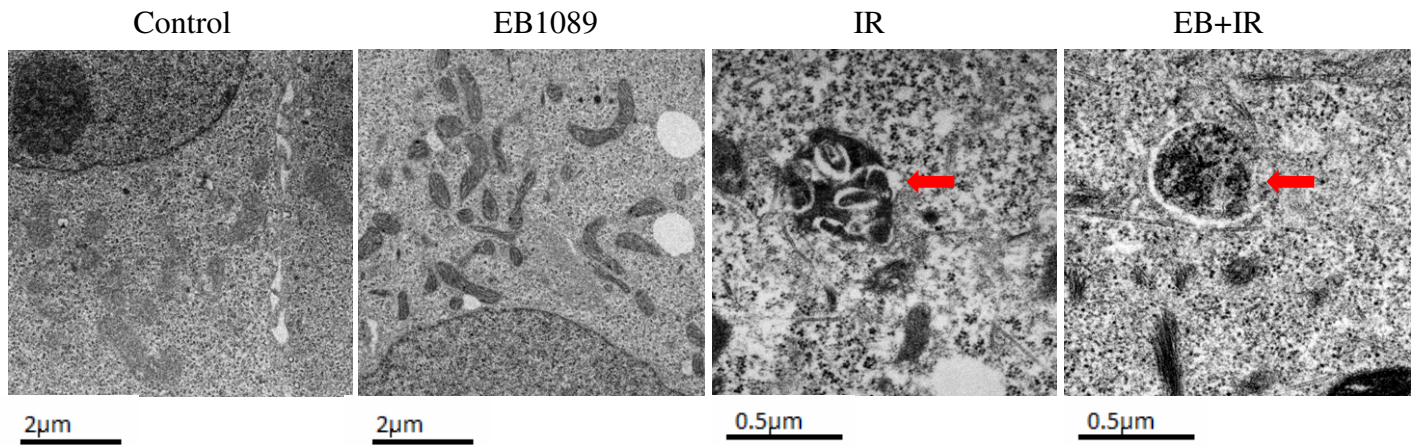
**Figure 3.3: Promotion of autophagy by EB1089 + radiation in MCF-7 cells-
Electron Microscopy of autophagic vesicles.** MCF7 cells were treated as in A. and
were fixed and subjected to electron microscopy. Autophagic vacuoles are indicated by
the red arrows. Scale bars indicate magnification. Average number of autophagic
vesicles per cell was counted in three fields for each condition and is represented in the
above graph. **$p<0.0001$
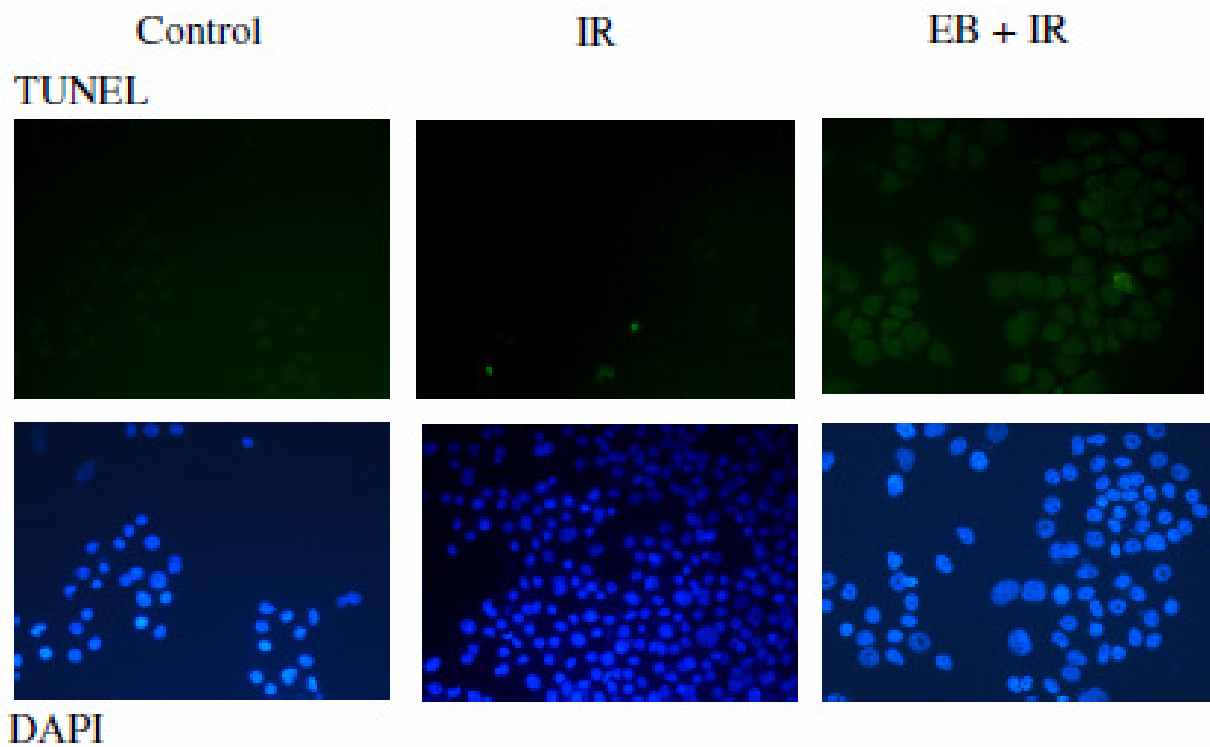
Figure 3.4: Staining for apoptosis with radiation or EB1089 + radiation. MCF7 cells were treated with IR or with EB1089 + radiation. Apoptosis was analyzed by the TUNEL assay and DAPI staining 3 days post-irradiation. Taxol treatment was used as a positive control for apoptosis (not shown).

Figure 3.5
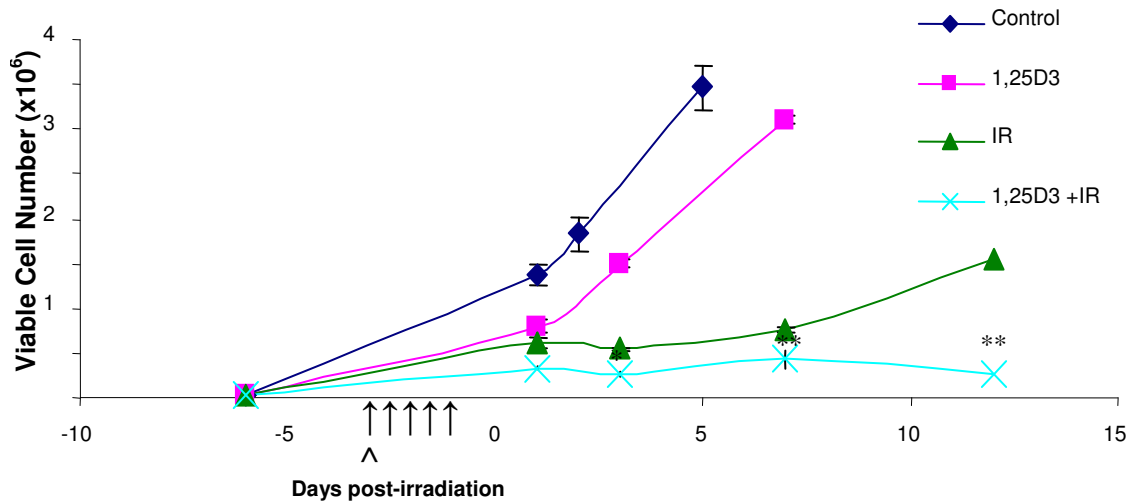
Figure 3.5 Influence of 1,25 D_3 on the temporal response to fractionated radiation in MCF7 cells. MCF7 cells were exposed to radiation alone (5x2Gy), or 100 nM 1,25D_3 prior to irradiation (5x2Gy). Viable cell number was determined by exclusion of trypan blue at the indicated days following the initiation of radiation exposure. ** $p < .0001$ between corresponding days. ↑ Indicates days when irradiation occurred. ^ Indicates when 1,25D_3 was removed.

Figure 3.6

MCF7 Colony Number

MCF7 Colony Size
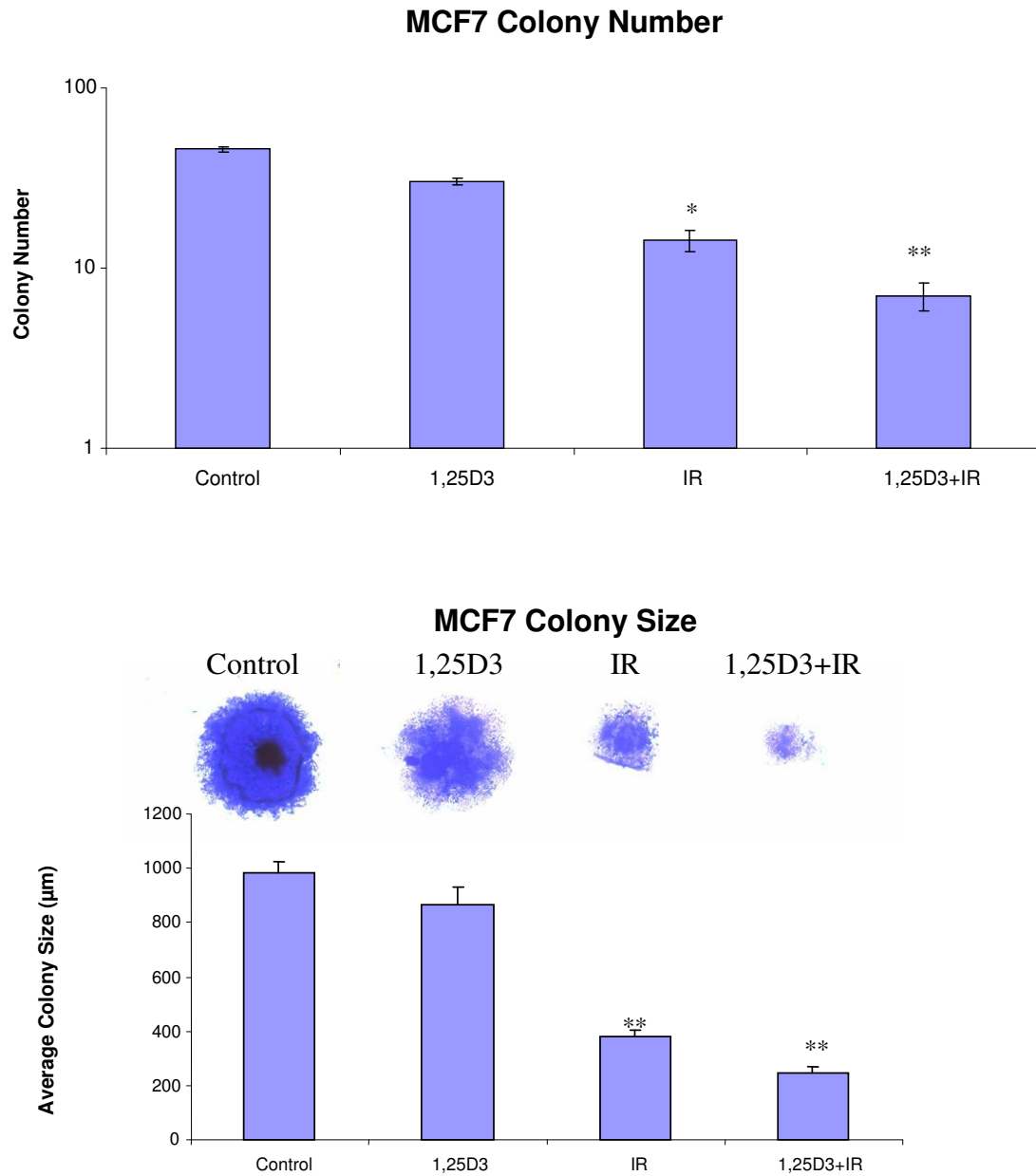
Figure 3.6: **Influence of 1,25 D$_3$ on colony formation in response to fractionated radiation in MCF7 cells.** Clonogenic survival was assessed after 14 days post-treatment. Values shown are from a representative experiment with triplicate samples for each condition. Average colony size was also determined for each condition via phase contrast microscopy and measured utilizing QCapturePro. * p<.05 and ** p < .0001 from control

as pronounced as for the studies presented in Figure 3.5.

Confirmation that $1,25D_3$ radiosensitization is also through the promotion of autophagy was based on acridine orange staining of autophagosomes, redistribution of RFP-LC3 and electron microscopy evidence of autophagic vacuole formation together with western blotting for the degradation of p62 as a measure of autophagic flux. Quantification for each study is shown in an accompanying bar graph. Figure 3.7 shows punctate acridine orange staining, indicative of autophagy, (Paglin et al., 2001) in cells treated with $1,25D_3$ followed by radiation. In contrast, untreated control cells or cells treated with $1,25D_3$ alone exhibit little or no orange punctate staining; again cells treated with radiation alone showed a modest increase in AO staining in comparison to levels observed with the combination.

In Figure 3.8, MCF7 cells stably transfected with RFP-tagged LC3, a significant increase of red punctate fluorescence is evident upon treatment with radiation or $1,25D_3$ followed by radiation, indicative of LC3 association with the autophagosomal membrane; (Kirisako et al., 2000) in contrast, a diffuse pattern of staining is observed in controls and in cells treated with $1,25D_3$.

Electron microscopy (EM) further confirmed the induction of autophagy upon treatment of the cells with $1,25D_3$ followed by IR; Figure 3.9 shows representative double membraned autophagosomal structures encapsulating cellular organelles for radiation alone and for $1,25D_3$+ radiation.(Boya et al., 2005; Lambert et al., 2008; Livesey et al., 2009; Wu et al., 2008) Autophagosomal vesicle formation was quite extensive with the

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combination treatment but relatively low with radiation alone, and was essentially absent in control cells or in cells treated with $1,25D_3$ alone.

It is now recognized that although autophagosome formation is a necessary component of the autophagic process, autophagosome formation can occur without completion of autophagy and degradation of the autophagosomal content; consequently, it becomes necessary to also assess autophagic flux. (Pankiv et al., 2007) To evaluate autophagic flux, levels of the p62 protein were monitored by Western blotting (Figure 3.10 with accompanying bar graph). As a positive control, a decline in p62 levels was evident when the cells were serum starved. Treatment with $1,25D_3$ alone did not reduce and in fact appeared to moderately increase p62 levels. The combination of $1,25D_3$ and radiation clearly promoted robust autophagic flux, as indicated by the decline in p62 levels. Furthermore, radiation alone promoted a decline in p62 levels. While autophagic flux by radiation alone is generally consistent with the data generated in other assays, the fact that suppression of p62 was similar for radiation alone and the combination of $1,25D_3$ with radiation was unexpected and will be addressed in the Discussion.

3.3 Molecular signals involved in the initiation of autophagy in response to vitamin D_3 and radiation

There are a variety of molecular pathways that may initiate autophagy. Phosphorylation and activation of the molecular target of rapamycin (mTOR), a member of the phosphoinositide 3-kinase related kinase (PIKK) family and a central modulator of cell growth, may serve as the main negative regulator of autophagy in cancer cells(Wang et al.,

2011). Furthermore, various physiological and pathological conditions may cause discrepancy among ER protein folding load and capacity, which can lead to the accumulation of unfolded proteins in the ER lumen, a condition referred to as "ER stress". ER stress can then initiate the signal transduction pathway known as the Unfolded Protein Response (UPR). This pathway aims to reduce the protein load in the ER by organizing the temporal shut down of protein translation along with an increase of ER folding capacity gene. If severe ER stress persists, this can initiate apoptosis or autophagy (Kouroku et al., 2007; Ogata et al., 2006; Szegezdi et al., 2006). The molecular links between autophagy, mTOR, and ER stress, and how these stress pathways influence therapeutic outcome, remain largely undefined, making this topic a very important area for future research in cancer therapy.

To elucidate the mechanisms involved in the initiation of autophagy following administration of radiation in the presence or absence of 1,25D$_3$, western blot analysis was conducted for proteins involved in the ER stress and mTOR pathways. To better allow for a time course following treatment, the dose of radiation administered was 5 Gy for these studies.

Upon the induction of ER stress, eif2α becomes phosphorylated. MCF7 cells received 5 Gy IR ± 72 preincubation with 1,25D$_3$ and levels of both eif2α and p-eif2α were monitored at various time points following radiation (Figure 3.11). Radiation alone did not appear to influence eif2α or p-eif2α; conversely, 1,25D$_3$+IR induced significant accumulation of p-eif2α at one hour post-irradiation which was quickly diminished. This suggests 5 Gy radiation alone is not sufficient to allow for the induction of substantial ER

stress; however, $1,25D_3$ + IR allows for phosphorylation of eif2α suggesting stimulation of ER stress. The increase in ER stress via the combination may elicit the mechanism behind $1,25D_3$+IR's ability to convert radiation induced autophagy from cytoprotective to a cytotoxic. There is clearly a need for additional components to be studied in this pathway to assess the roles of ER stress and the UPR in radiosensitization and experiments are currently being conducted.

mTOR can serve as a negative regulator of autophagy. To determine if this pathway is also involved in the differential responses with radiation and the combination, MCF7 cells were treated with 5 Gy IR ± 72 hr preincubation with $1,25D_3$ and levels of both mTOR and p-mTOR were monitored at various time points following radiation (utilizing the same conditions as presented with Figure 3.11). No bands were observed with either mTOR or p-mTOR in any of the treatments (data not shown), suggesting this pathway may not be involved. Accordingly, additional components in this pathway are being probed to better clarify mTOR's involvement, if any in radiosensitization by $1,25D_3$.

Figure 3.7

Control   1,25D3   IR   1,25D3+IR
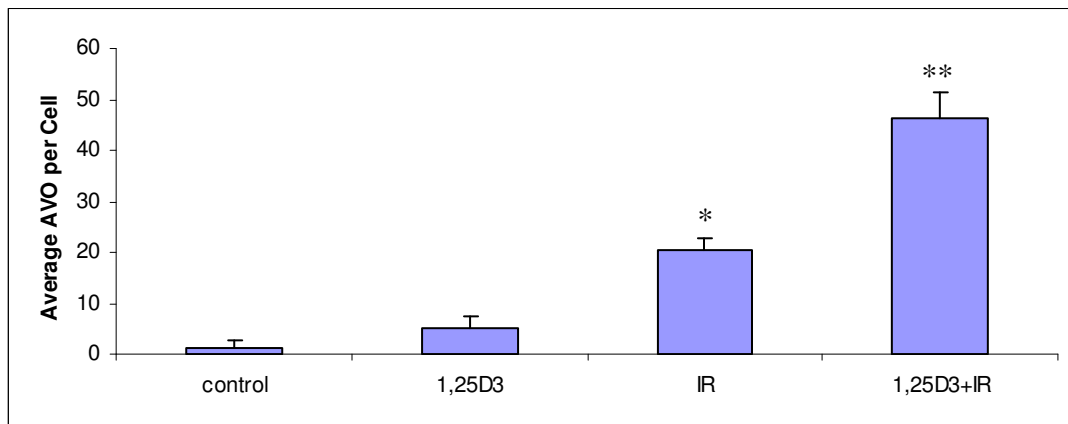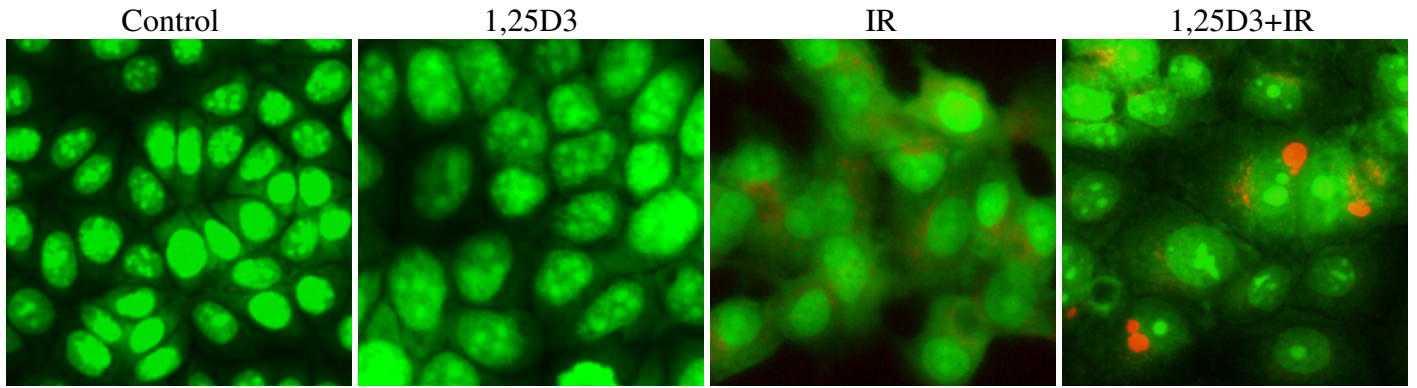
Figure 3.7: Influence of 1,25 D$_3$ on AVO formation in response to fractionated radiation in MCF7 cells. Autophagy was monitored based on acridine orange staining 24 hours post-irradiation. Average vesicle per cell was quantitated and is represented in the lower panel graph. * p<.05 and ** p < .0001 from control

Figure 3.8
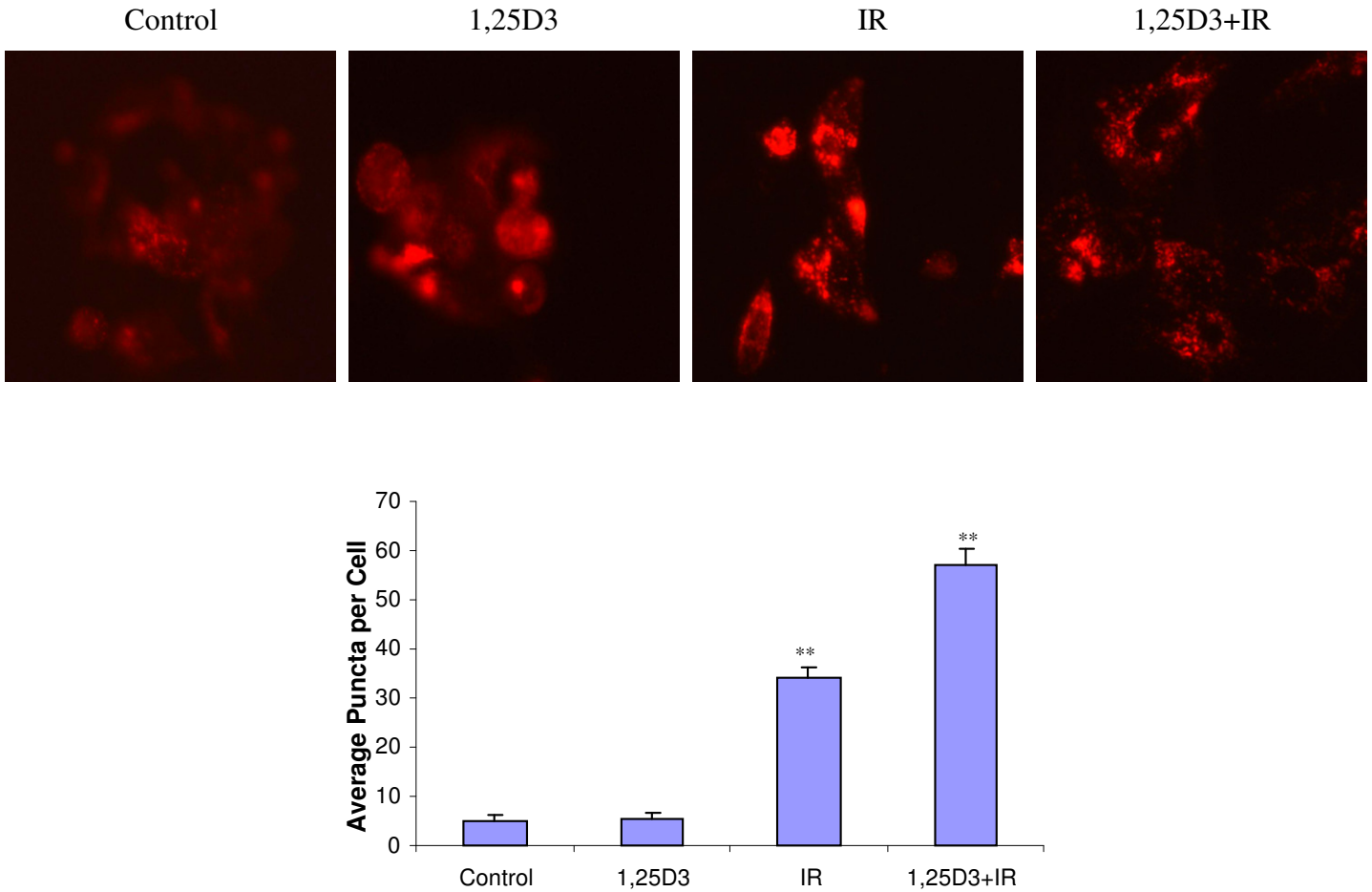
Figure 3.8: Influence of 1,25 D₃ on RFP-LC3 distribution in response to fractionated radiation in MCF7 cells. MCF7 cells stably transfected with RFP-LC3 diffuse and punctate staining was monitored by fluorescence microscopy. Again, images were taken 24 hours post-irradiation. Average number of puncta per cell were counted in three fields for each condition and are represented in the bottom panel graph. ** p < .0001 from control

Figure 3.9
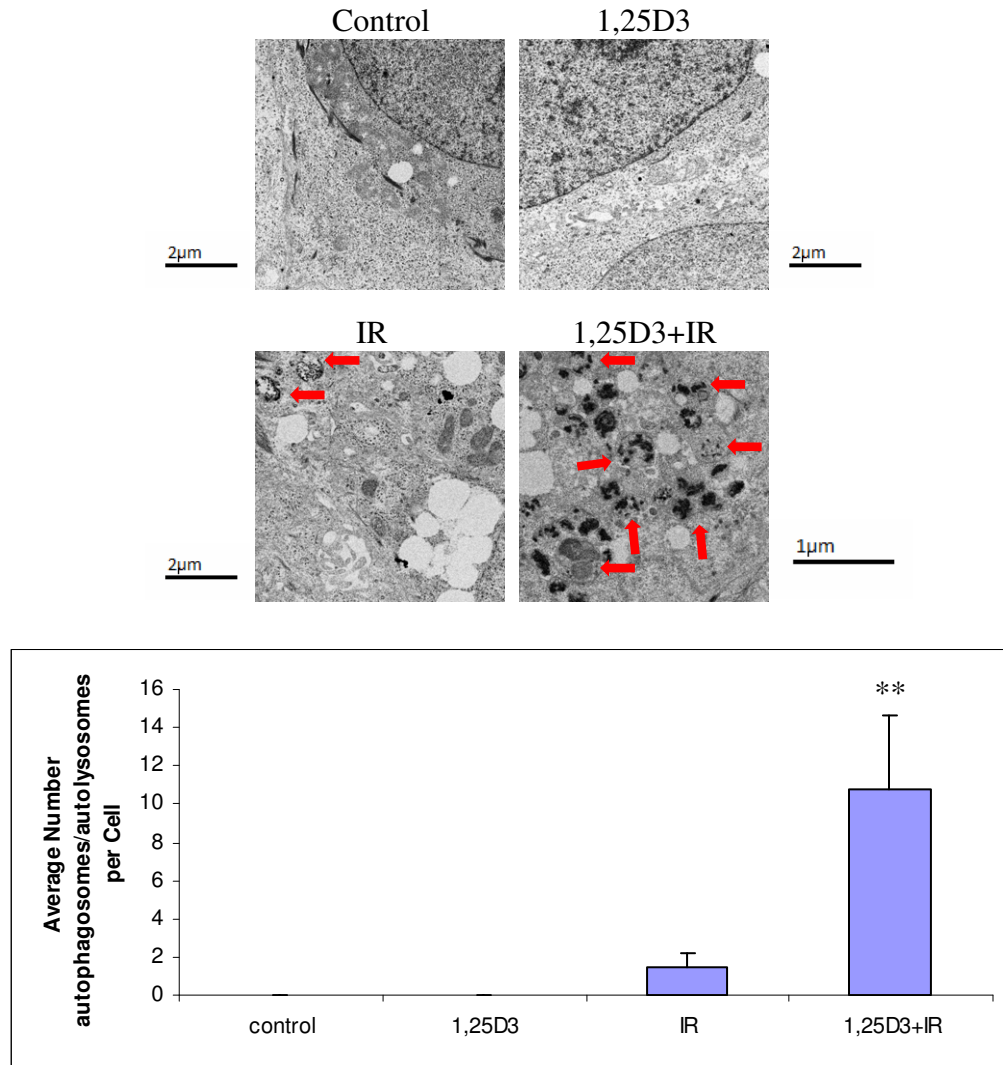
Figure 3.9: **Electron Microscopy of autophagic vesicles.** MCF7 cells were treated, fixed and subjected to electron microscopy. Autophagic vacuoles are indicated by the red arrows. Scale bars indicate magnification. Average numbers of autophagic vacuoles per cell were counted and are represented in the bottom panel graph. ** p < .0001 from control

Figure 3.10
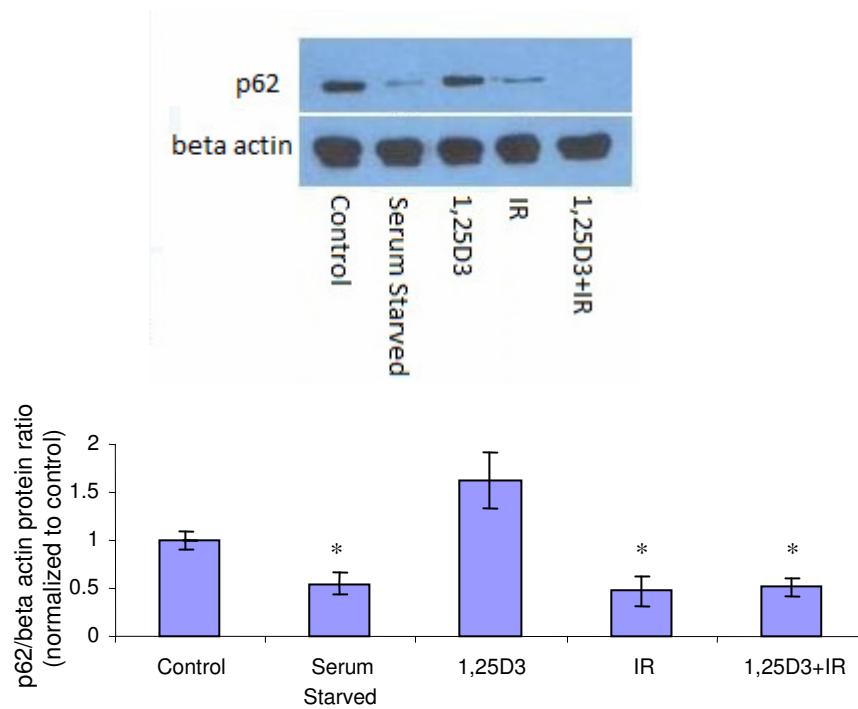
Figure 3.10. Influence of 1,25 D$_3$ on p62 degradation in response to fractionated radiation in MCF7 cells. Autophagic flux was based on the decline in p62 levels monitored by Western blotting 24 hours post-irradiation. Actin was utilized as a loading control. Serum starvation was used as a positive control for autophagic flux. The accompanying bar graph presents densitometry from three separate westerns.
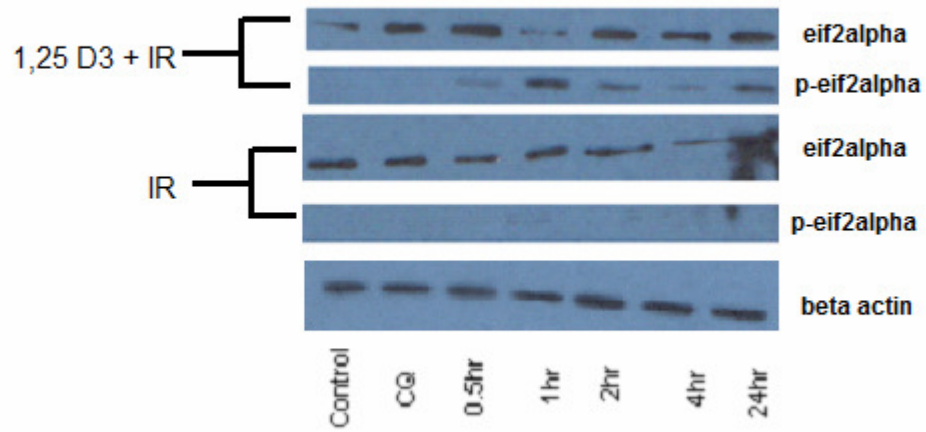
Figure 3.11: **Western Blot analysis of ER stress proteins.** MCF7 cells received 5 Gy IR ± 72 preincubation with 1,25D$_3$ and levels of both eif2α and p-eif2α were monitored at various time points following radiation.

Chapter 4: Inhibition of autophagy

4.1 Impact of autophagy inhibition on sensitivity to treatment.

Given the ongoing controversy as to the differential impact of autophagy in various experimental systems, where autophagy is either cytoprotective or putatively the mode of cell death, (Gewirtz et al., 2009) studies were designed to test the premise that interference with autophagy would influence sensitivity to the treatment with $1,25D_3$ + radiation. Three commonly used inhibitors of autophagy were initially evaluated both alone and in combination with tamoxifen (Figure 4.1). These included 3-methyladenine (10 mM), which is a PI3 kinase inhibitor, (Bursch et al., 1996) chloroquine (CQ) (25 µM), which prevents acidification and fusion between the autophagosome and the lysosome, (Solomon and Lee, 2009) and bafilomycin (100 nM), which is an inhibitor of the vacuolar-type H+-ATPase (V-ATPase). (Shacka et al., 2006) Due in large part to the toxicity of 3-methyladenine and bafilomycin, chloroquine was chosen as the pharmacological inhibitor of autophagy in our experimental system.

Figure 4.2 indicates that exposure to CQ alone produced an increase in formation of yellow autophagosomal vesicles, consistent with its ability to prevent the completion of autophagy by blocking the acidification step. Essentially identical images were generated in studies by Zhao et al using chloroquine as an autophagy inhibitor in MDA-MB231 cells. (Zhao et al., 2005)   Figure 4.2 also confirms that chloroquine interfered with the acidification step of autophagy in response to treatment with $1,25D_3$ + radiation, based on

Figure 4.1

Tamoxifen

Tamoxifen+ 3MA

Tamoxifen+ BAF
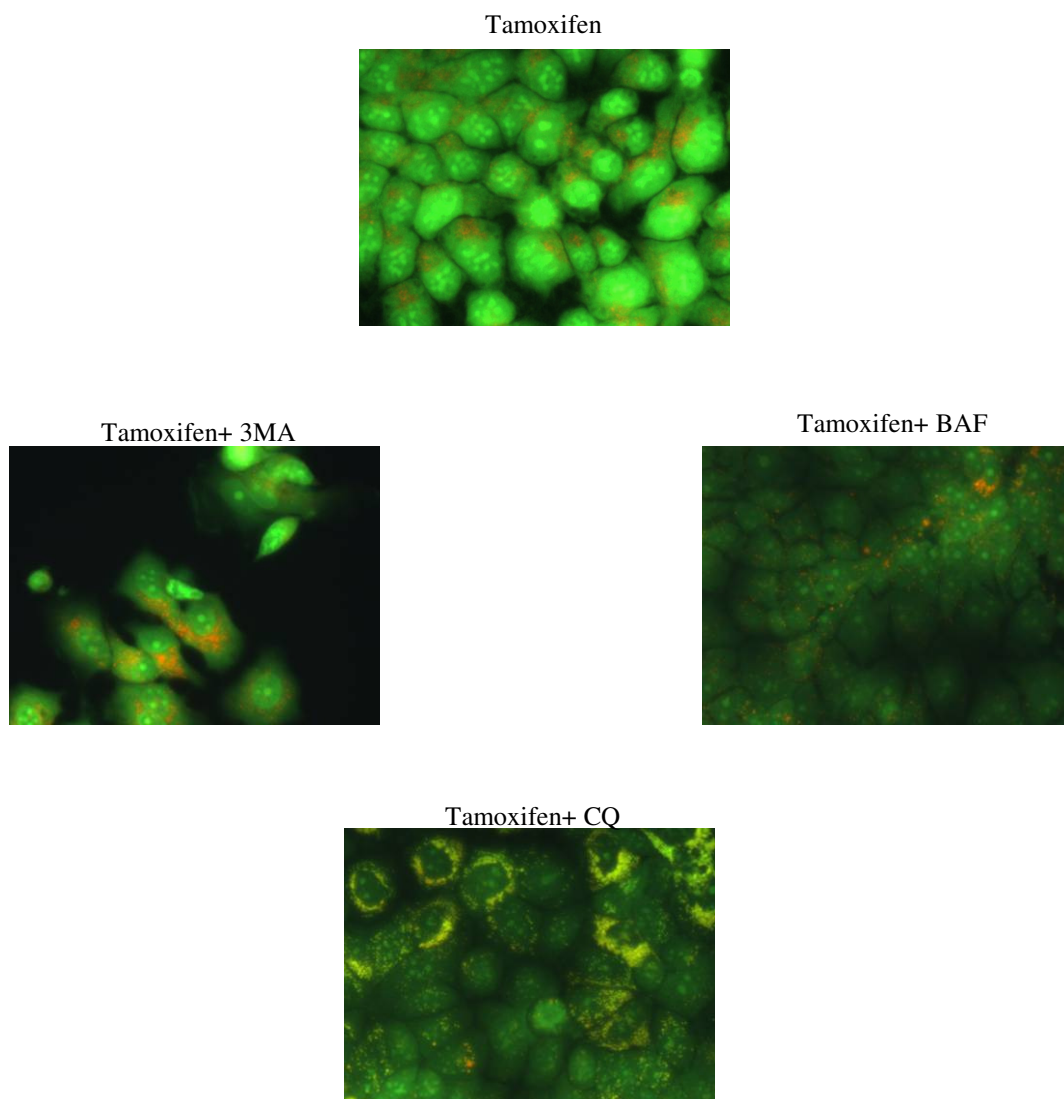
Tamoxifen+ CQ

Figure 4.1: Influence of autophagic inhibition on AVO formation in response to tamoxifen in MCF7 cells. Autophagy was monitored based on acridine orange staining 24 hours following 72 hour incubation with tamoxifen.

Figure 4.2
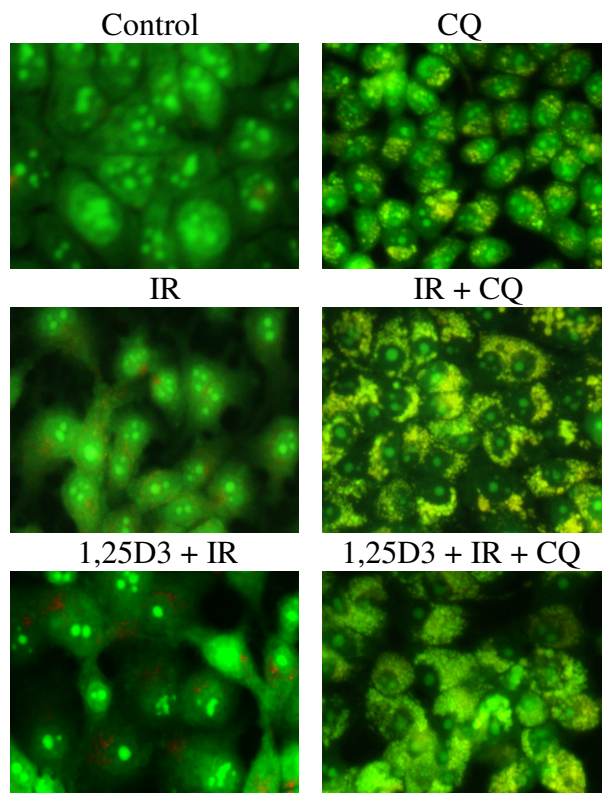
Figure 4.2: Effect of autophagy inhibition on the response to radiation or 1,25D$_3$ + radiation. MCF7 cells were treated with radiation alone or with 1,25D$_3$ + radiation with or without concurrent treatment of 25 µM CQ. Autophagy was monitored by acridine orange staining 24 hours post-irradiation.

4.3
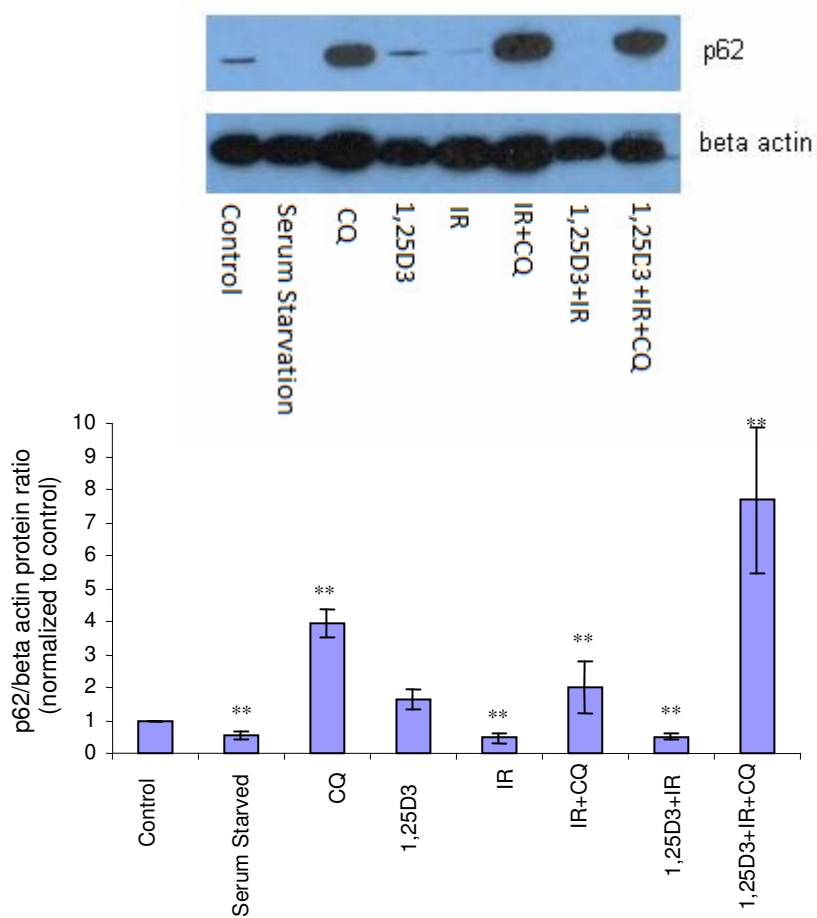
Figure 4.3a: Effect of autophagy inhibition on the response to radiation or 1,25D$_3$ + radiation. Autophagic flux was based on the decline in p62 levels monitored by Western blotting 24 hours post-irradiation. Actin was utilized as a loading control. Serum starvation was used as a positive control for autophagic flux. Densitometry from three separate westerns is presented in the lower panel.
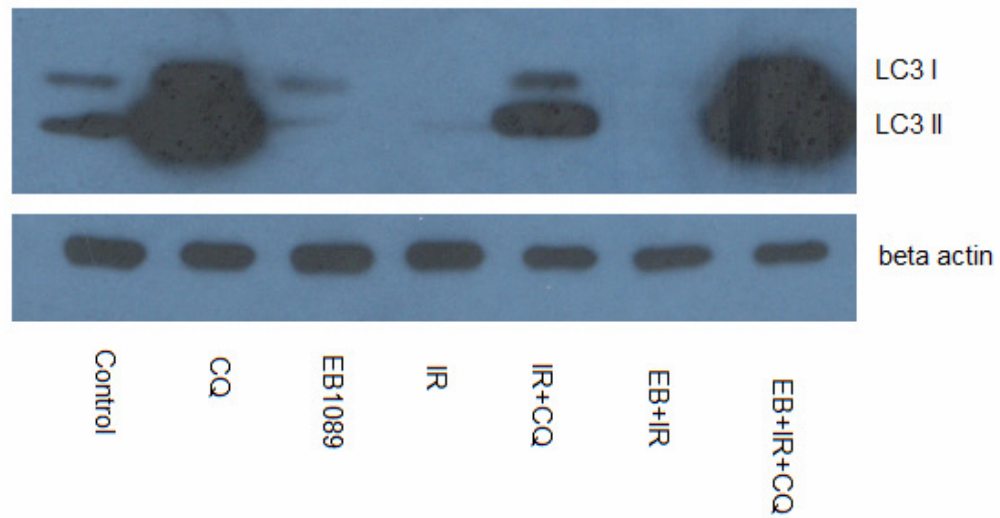
Figure 4.3b: Effect of autophagy inhibition on the response to radiation or 1,25D$_3$ + radiation.
The autophagic marker, LC3 was monitored by Western blotting 24 hours post-irradiation. Actin was utilized as a loading control. Serum starvation was used as a positive control for autophagic flux.

acridine orange staining; (Solomon and Lee, 2009) interference with tamoxifen-induced autophagy was used as a positive control (Figure 4.1). Radiation in the presence or absence of $1,25D_3$, significantly reduced p62; this is consistent with previous AO, EM, and RFP-LC3 observations. The autophagic flux induced by radiation or $1,25D_3$ + radiation is eliminated in the presence of chloroquine; evidence of this blockade of is presented in the p62 Western shown in Figure 4.3a with the accompanying bar graph. Levels of p62 were markedly elevated for the combined treatment of chloroquine with $1,25D_3$ + radiation; enhanced p62 levels in the presence of autophagic inhibitors can also be an indication of significant autophagic flux.

Another useful marker for the induction of autophagy is the cleavage of LC3I to LC3II. Upon induction of autophagy, LC3I is covalently attached to phosphatidylethanolamine converting it to LC3-II, causing it to become membrane-associated (Mizushima and Klionsky, 2007). Moreover, LC3 remains associated with the completed autophagosome via association with p62 (Mizushima and Klionsky, 2007; Yang et al., 2005).   In Figure 4.3b, MCF7 control cells have modest levels of LC3II; EB1089 decreased LC3II levels, which is consistent with the reduced autophagy as demonstrated by the increase in p62 levels induced by $1,25D_3$ in Figure 4.3a. Conversely, IR and EB+IR completely abrogate LC3; this corresponds to the abolishment of p62 levels, an LC3 binding partner, observed with these treatments in Figure 4.3a. Moreover, CQ alone or in combination with IR or EB+IR induced massive accumulation of LC3 indicative of CQ's inhibition of autophagic flux.

To confirm the role of autophagy in 1,25D$_3$ radiosensitization, the next series of studies evaluated the temporal response of MCF7 cells to radiation or 1,25D$_3$ + radiation when autophagy was suppressed by chloroquine. Figure 4.4 indicates that, as observed in our previous studies, cells treated with radiation alone undergo transient arrest that is followed by recovery of proliferative capacity. In dramatic contrast, cells treated with 1,25D$_3$ + radiation, even with a blockade to autophagy by CQ, appear to die and do not regain proliferative capacity; 1,25D$_3$ or CQ alone resulted in only a modest and transient inhibition of growth.

Although one expectation might have been that inhibition of autophagy would reduce the effect of the combination treatment, the potency of the 1,25D$_3$ + radiation treatment was not suppressed by chloroquine; in fact, the impact of the combination treatment was moderately increased with chloroquine treatment (Figure 4.4). This observation does not, however, refute the conclusion that autophagy mediates the cytotoxic actions of the 1,25D$_3$ + radiation combination treatment. As shown in Figure 4.5, blocking autophagy induced by EB1089 + radiation with chloroquine resulted in the promotion of apoptosis, based on an increase in TUNEL staining and altered nuclear morphology as evident with DAPI staining. Consequently, while autophagy appears to be the primary response to EB1089 + radiation, when autophagy is blocked the cells will die by the alternative pathway of apoptosis.

An entirely unexpected finding was that in cells exposed to radiation where autophagy was suppressed by CQ, the temporal profile was similar to that of the combination treatment; that is, cell death was evident with an apparent complete

suppression of proliferative recovery. As shown by the TUNEL and DAPI staining images in Figure 4.5, the radiation treatment in autophagy-compromised cells resulted in extensive apoptosis. These observations raised the possibility that autophagy was also having a cytoprotective action in the irradiated cells, as previously suggested in studies by Apel et al. (Apel et al., 2008) and further addressed later in this chapter.

4.2 Impact of autophagy inhibition on the sensitivity to irradiation in MCF10a cells.

A fundamental limitation in chemotherapy is the toxicity to normal surrounding cells. Previous data indicated vitamin D did not radiosensitize non-malignant MCF10A cells. (Polar et al., 2003) We therefore sought to determine if the inhibition of autophagy would sensitize non-malignant MCF10a cells, an immortalized cell line derived from normal breast epithelial cells.(Soule et al., 1990) MCF10a cells were treated with 2Gy IR in the presence or absence of 25µM CQ, and cell viability was determined at a single time-point post-irradiation (Figure 4.6). Cells exposed to CQ alone exhibited a 22% reduction in cell number 7 days post-irradiation. With radiation alone, MCF10a cells demonstrated a 44% reduction in cell number at this same time point. Finally, cells treated with the combination exhibited a 54% reduction in cell number suggesting a less than additive effect.

In order to confirm that radiation was able to promote autophagy in MCF10a cells, acridine orange was used to stain for autophagosomes. Figure 4.7 shows punctate acridine orange staining, indicative of autophagy, (Paglin et al., 2001) in cells treated with radiation alone, similar to levels seen in MCF7 cells. In contrast, untreated control cells exhibit little

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or no orange punctate staining; again cells treated with CQ with or without radiation

exhibited characteristic yellow punctate formation indicating CQ's ability to reduce

autophagosome acidification.

4.3 Impact of silencing ATG related genes on susceptibility to $1,25D_3$ and irradiation.

Pharmacological inhibitors can induce non-specific effects. To extend the findings

generated by pharmacological inhibition of autophagy, we developed an MCF7 cell line

(MCF7/ATG5-) in which ATG5, one of the critical autophagy associated genes, (Bampton

et al., 2005) was silenced. The western blot in Figure 4.8 indicates a significant reduction

in the signal for ATG5 in these cells, although ATG5 expression is not completely

silenced. Acridine orange staining in Figure 4.9 (and quantification in the accompanying

bar graph) confirms that the response of MCF7 vector control cells was similar to that of

the parental MCF7 cells (Figure 3.7). Acridine orange staining in Figure 4.10 indicates

that the ATG5 knockdown cells exhibited modest intrinsic levels of acridine orange

staining in the treatments with $1,25D_3$, radiation or $1,25D_3$ + radiation; however, none of

the conditions significantly increased acridine orange staining. The electron microscopy

images in Figure 4.11 further confirm that the autophagy that is otherwise induced by

either radiation alone or the $1,25D_3$ + radiation combination is significantly suppressed in

the ATG5 knockdown cells.

In further studies to address both the putative cytoprotective actions of autophagy

with radiation alone and the putative cytotoxic functions of autophagy for the combination

treatment, the MCF7/ATG5- cells were exposed to either radiation alone or $1,25D_3$
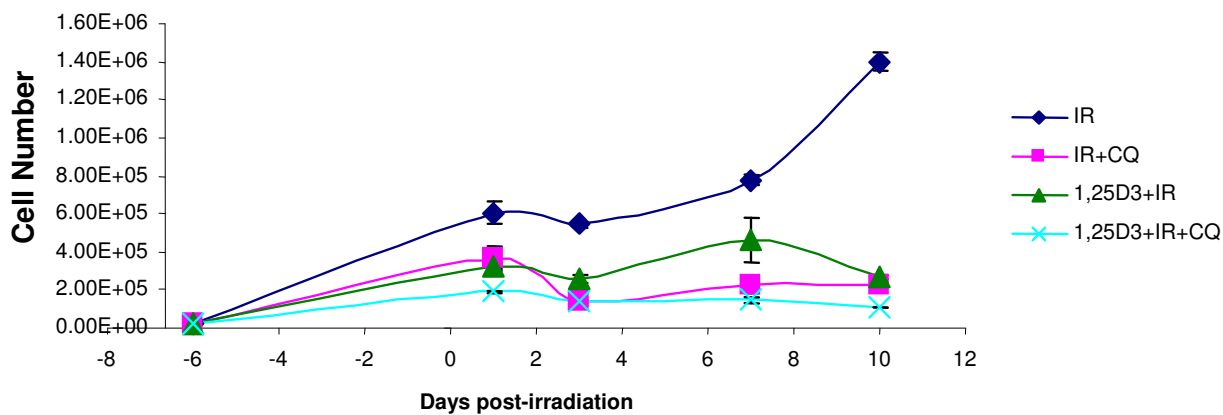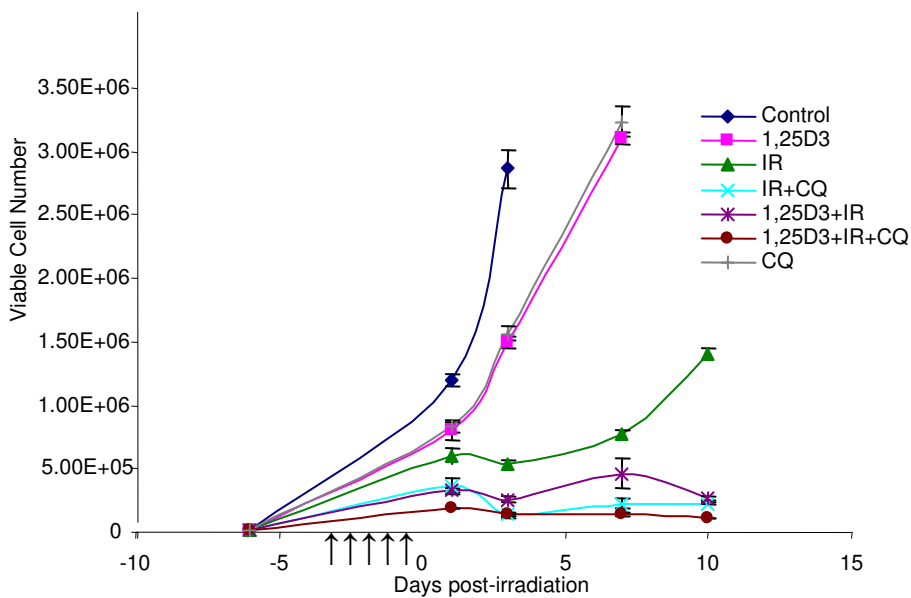
Figure 4.4: Effect of autophagy inhibition on the response to radiation or 1,25D₃ + radiation. Viable cell number was determined by trypan blue exclusion on the indicated days following treatment. ↑ Indicates dose of irradiation given. Lower graph is same data as above, plotted on a different scale.
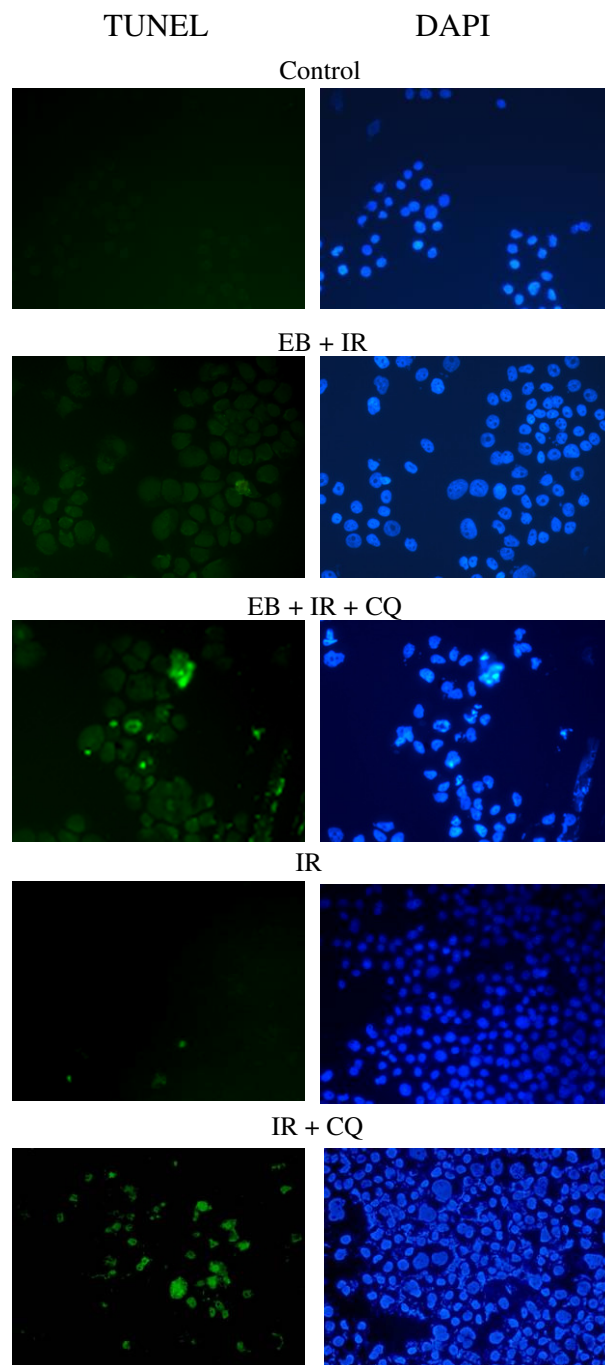
Figure 4.5: Effect of autophagy inhibition on the response to radiation or 1,25D$_3$ + radiation.
Effect of autophagy inhibition on promotion of apoptosis. MCF7 cells were treated with IR or with 1,25D$_3$ + radiation with or without concurrent treatment of 25μM CQ. Apoptosis was detected by the TUNEL assay and DAPI staining (based on the integrity of cell nuclei) 3 days post-irradiation. Taxol treatment was used as a positive control for apoptosis (not shown).

followed by radiation, and viable cell number was monitored for a period of 12 days after the last dose of radiation (Upper panel of Figure 4.12). It should be noted that the MCF7 vector control cells responded to these treatments in the same manner as parental MCF-7 cells (Lower panel of Figure 4.12); moreover there was no evidence for apoptosis as measured by TUNEL/DAPI in the MCF7 vector control cells (data not shown). These experiments in large part recapitulated the CQ findings presented in Figure 4.4 in that the prolonged growth arrest and proliferative recovery that occurs with radiation treatment was abrogated both for cells treated with radiation alone and those treated with $1,25D_3$ + radiation; moreover, these observations support the conclusion that interference with autophagy can also result in sensitization to radiation *in the same experimental system* where the promotion of autophagy appears to mediate increased sensitivity to radiation by either EB1089 or $1,25D_3$. Figure 4.13 (TUNEL and DAPI staining) indicates that cell death in the MCF7/ATG5- cells occurred largely through apoptosis, again similar to the CQ data presented in Figure 4.5; moreover there was no evidence for apoptosis as measured by TUNEL/DAPI in the MCF7 vector control cells (data not shown). The ATG5 knockdown did not appear to detectably interfere with the suppression of autophagic flux (p62 degradation) induced by either serum starvation or the $1,25D_3$+radiation treatment, although that induced by radiation alone was diminished (Figure 4.14 and accompanying bar graph).   This suggests the knockdown of ATG5 did not completely interfere with autophagic flux (p62 degradation), this observation is discussed in more detail later.

Additionally, we obtained MCF7/ATG7- cells in which ATG7, another critical autophagy associated gene, (Bampton et al., 2005; Mizushima et al., 1998) was silenced; Figure 4.15 confirms the knockdown of ATG7. Figure 4.16 indicates the MCF7/ATG7- cells have a relatively high basal level of autophagy, again based on AO staining; however, none of the treatments significantly increased the level of AO puncta. The published literature would lead us to expect that Atg7 drives autophagy by converting inactive LC3-I into active LC3-II. Alternatively, the knockdown of Atg7 appears to increase AVO levels. This observation has also been confirmed in CryABR120G Cardiomyocytes; ATG7 knockdown induced the accumulation of misfolded proteins and aggregates while p62 remained unaffected (Pattison et al., 2011). Figure 4.17 presents a time course response to treatment in the ATG7 knockdown cells that confirms increased sensitivity to radiation (sustained arrest and lack of proliferative recovery) as well as maintenance of sensitivity to the combination treatment of $1,25D_3$+radiation.

Due to the high basal AO punctate staining, we also evaluated autophagic flux in the MCF7/ATG7- cells based on degradation of p62 by Western blotting. Figure 4.18 indicates that none of the treatments significantly altered p62 levels, confirming the fact that in these cells, radiation induced stress fails to promote autophagy.

It is of interest that both serum starvation and the $1,25D_3$+radiation combination promote p62 degradation in the ATG5 knockdown cells but no degradation is observed with the knockdown of ATG7, although this may have the trivial explanation that the blockade to autophagy is incomplete with ATG5. Additionally, ATG5 is only associated with one arm of the ATG conjugation cascade, (Nedelsky et al., 2008) and therefore it is

possible cells can still undergo autophagy by utilizing the conjugation of ATG4, ATG7, and/or ATG8; since ATG7 is involved in both arms of the cascade silencing of ATG7 is likely to be more effective in suppressing the autophagic response. Furthermore, as p62 function is not limited solely to autophagy but is also associated with ubiquitin mediated protein degradation (Ding and Yin, 2008; Pankiv et al., 2007) it remains possible that our findings may reflect alternative and perhaps as yet undefined functions of p62.

Figure 4.6
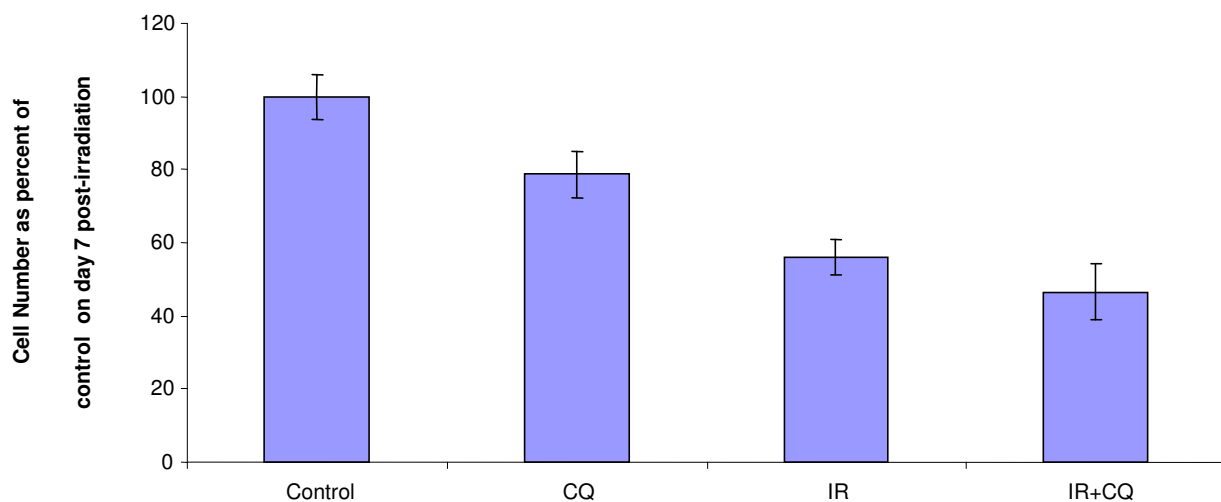
Figure 4.6: Influence of autophagy inhibition on viability of "normal" MCF10A breast epithelial cells in response to radiation. MCF10a cells were exposed to radiation alone (2Gy) in the presence or absence of 25μM CQ. Viable cell number was determined by exclusion of trypan blue at the indicated days following the initiation of radiation exposure.

Figure 4.7
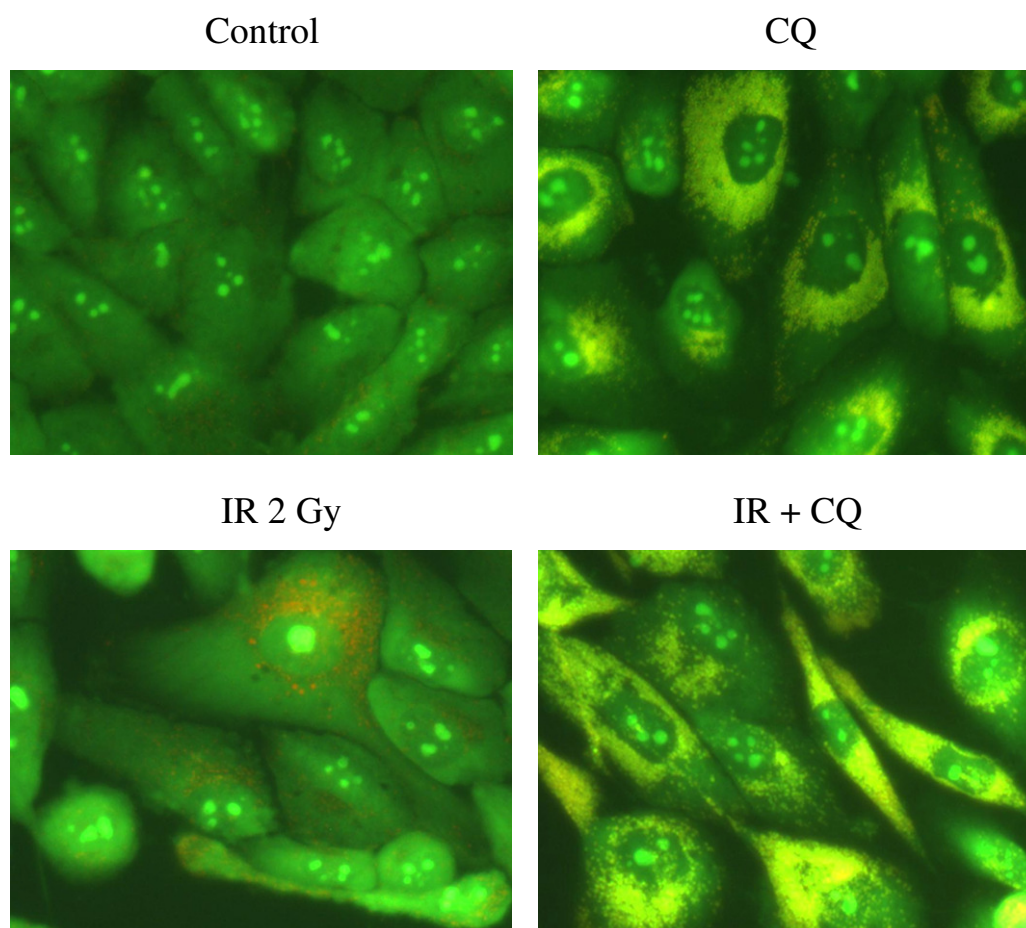
Figure 4.7. Chloroquine inhibits AVO formation in MCF10a cells. MCF10a cells were treated with 2 Gy IR in the presence or absence of CQ and stained with AO 24 hours post irradiation.

Figure 4.8

A.
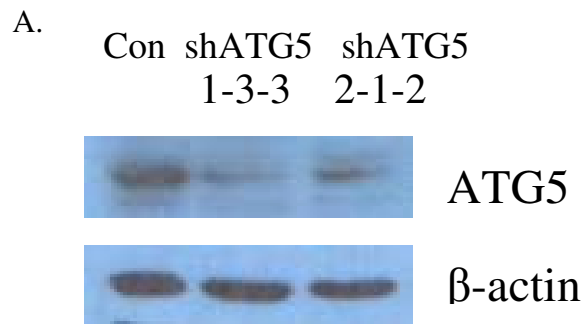
Figure 4.8: **Western blotting to determine level of ATG5 knockdown.** MCF7 cells transfected with siRNA scramble (Con) or 2 different vectors for the silencing of ATG5 were monitored for the level of ATG5 protein. Beta actin is utilized as a loading control.
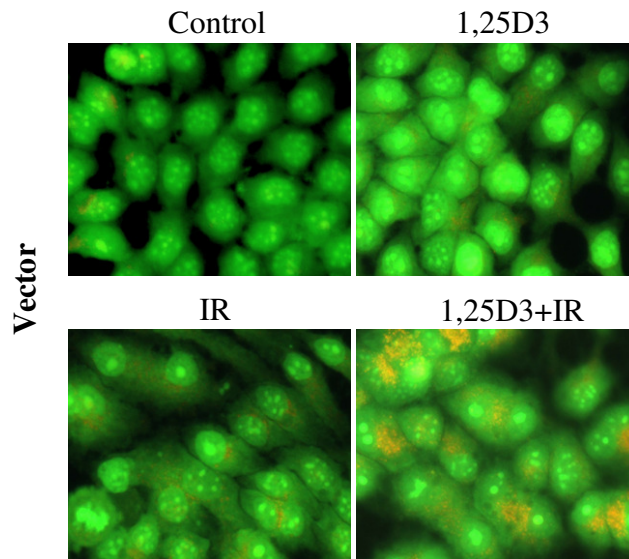
Figure 4.9: **AVO formation in vector control cells.** MCF7 cells transfected with the control vector were treated with IR in the presence or absence of 1,25D$_3$ and the formation of AVO was monitored

Control – MCF7/ATG5-   1,25D3

IR   1,25D3+IR

Figure 4.10: **AVO formation in ATG5 knockdown cells.** MCF7/ATG5- cells were treated with IR in the presence or absence of 1,25D$_3$ and the formation of AVO were monitored. Quantification of AVO is presented in the lower panel graph
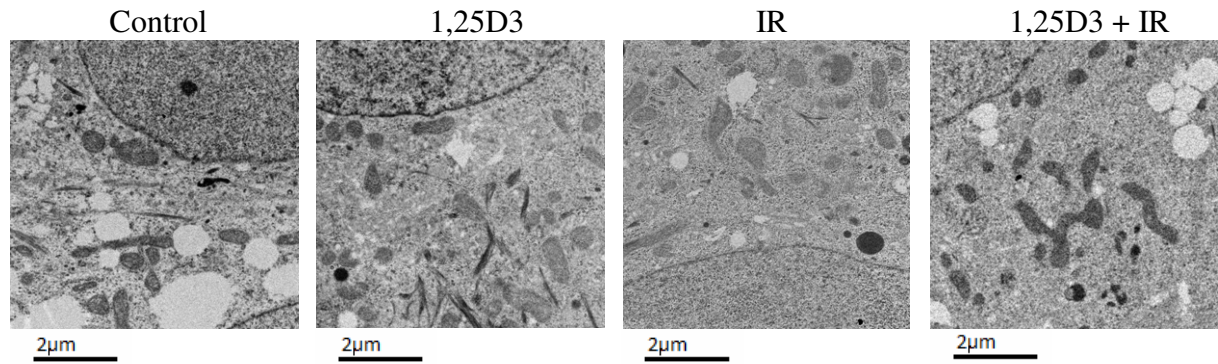
Figure 4.11. Silencing of ATG5 in MCF7 cells inhibits autophagic vesicle formation. MCF7 cells were stably transfected with either an empty vector or with a plasmid for the silencing of ATG5. Electron Microscopic imaging does not indicate the formation of autophagosomes. Scale bars indicate magnification.
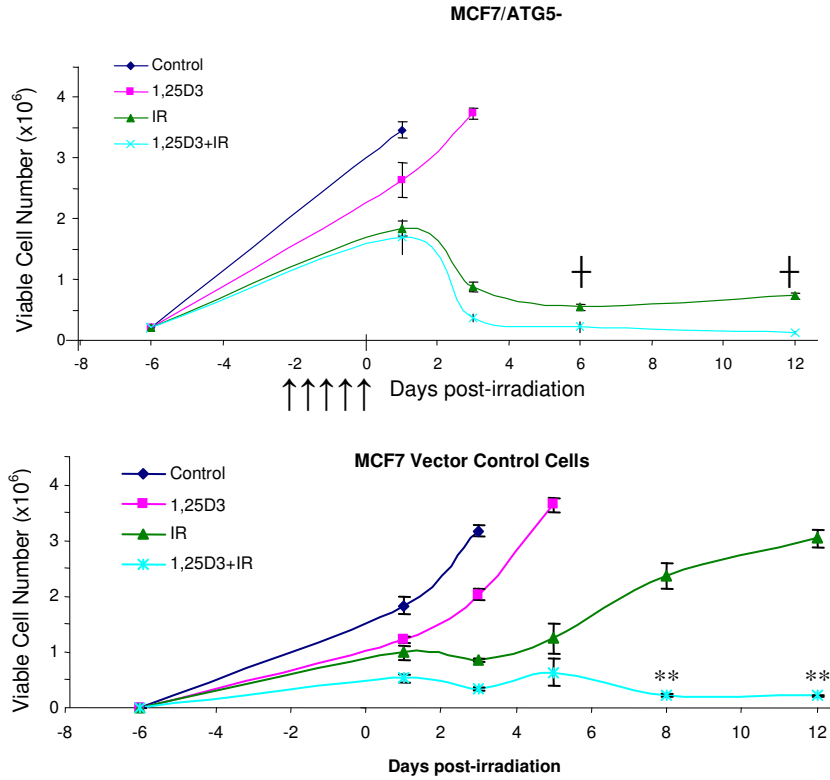
Figure 4.12: **Influence of 1,25D₃ on the response to fractionated radiation in MCF7/ATG5- cells.** ATG5-cells (top panel) or MCF7 vector control cells (bottom panel) were exposed to radiation alone (5x2 Gy), or 1,25D₃ prior to irradiation and viable cell number was determined by exclusion of trypan blue at the indicated days following the initiation of radiation exposure. ↑ Indicates when irradiation occurred. ** p<0.0001 from IR on corresponding day, ✝ p<0.0001 comparing corresponding time points in MCF7/ATG5- cells and vector control cells.

TUNEL DAPI

Control

1,25D3

IR

1.25D3+ IR

Figure 4.13. Influence of 1,25D₃ on Apoptosis in response to fractionated radiation in MCF7/ATG5- cells. MCF7/ATG5-cells were exposed to radiation alone (5x2 Gy), or 1,25D₃ prior to irradiation and apoptosis was monitored by the TUNEL assay and DAPI staining 3 days post-irradiation.

Figure 4.14. Influence of 1,25D$_3$ on Autophagy in response to fractionated radiation in MCF7/ATG5- cells. Cells were exposed to radiation alone, or 1,25D$_3$ prior to irradiation and autophagic flux was based on the decline in p62 levels monitored by Western blotting 24 hours post-irradation. Actin was utilized as a loading control. Serum starvation was used as a positive control for autophagic flux. Densitometry for p62 levels normalized for actin is presented in the lower panel. **p<0.0001 from control
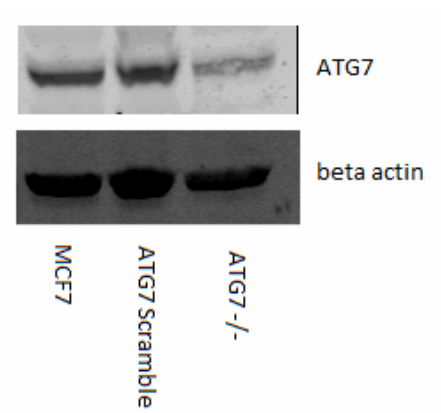
Figure 4.15. Western blot analysis to determine level of ATG7 knockdown. MCF7 cells transfected with siRNA scramble or ATG7- vectors for the silencing of ATG7 were monitored for the level of ATG7 protein in comparison to parental MCF7 cells. Beta actin is utilized as a loading control.
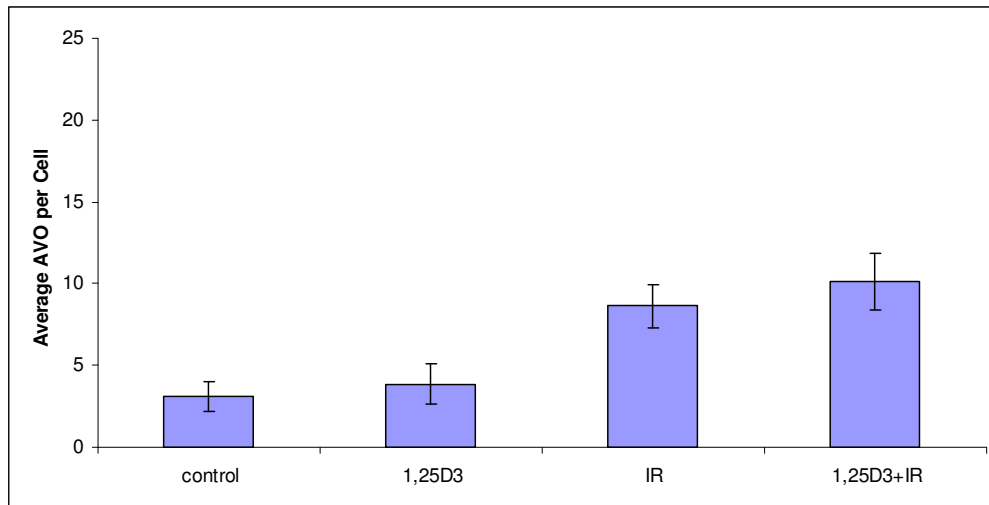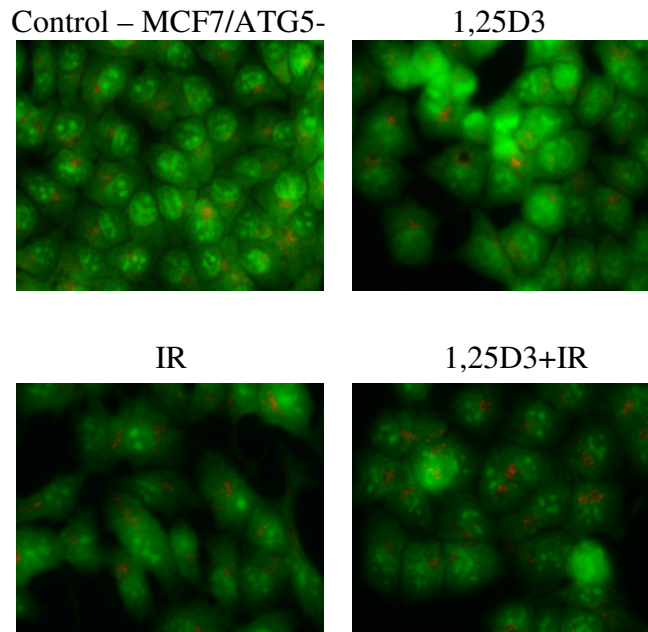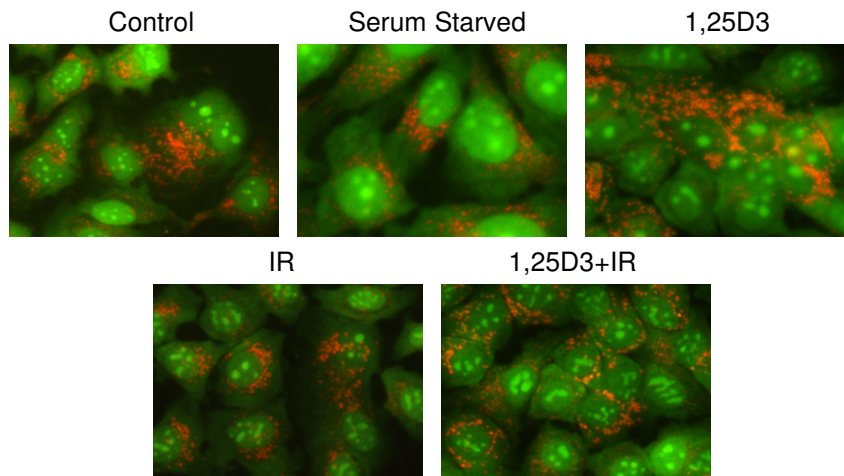
Figure 4.16. AVO formation in ATG7 knockdown cells. MCF7/ATG7- cells were treated with IR in the presence or absence of 1,25D$_3$ and the formation of AVO were monitored.
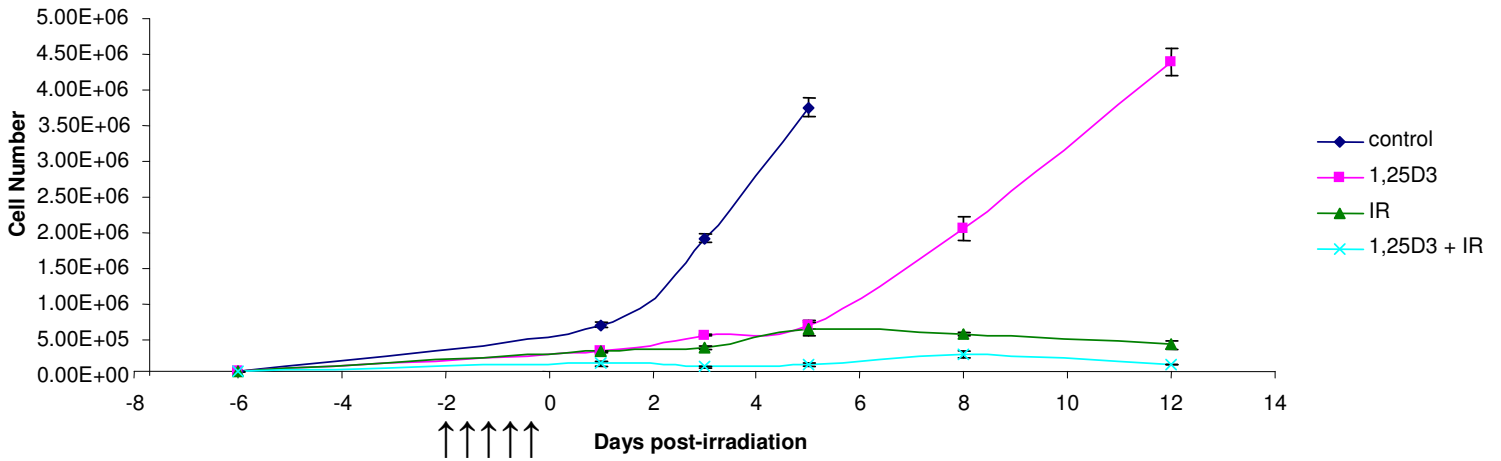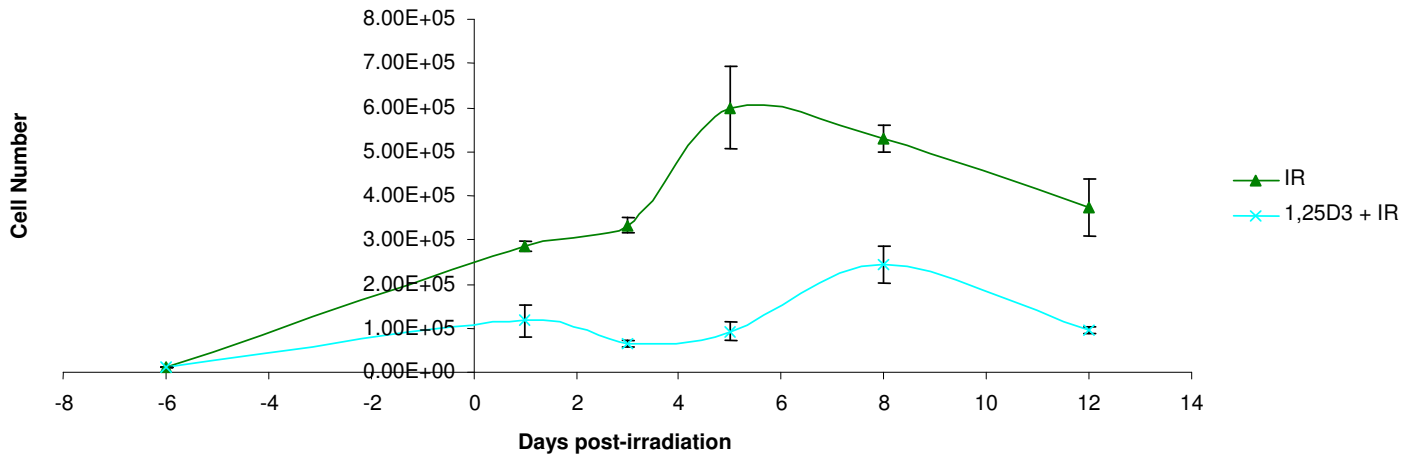
Figure 4.17: **Influence of 1,25D$_3$ on the response to fractionated radiation in MCF7/ATG7- cells.** MCF7/ATG7-cells were exposed to radiation alone (5x2 Gy), or 1,25D$_3$ prior to irradiation and viable cell number was determined by exclusion of trypan blue at the indicated days following the initiation of radiation exposure. ↑ Indicates when irradiation occurred. IR and 1,25D3+IR data is replotted in lower graph with adjusted scale.

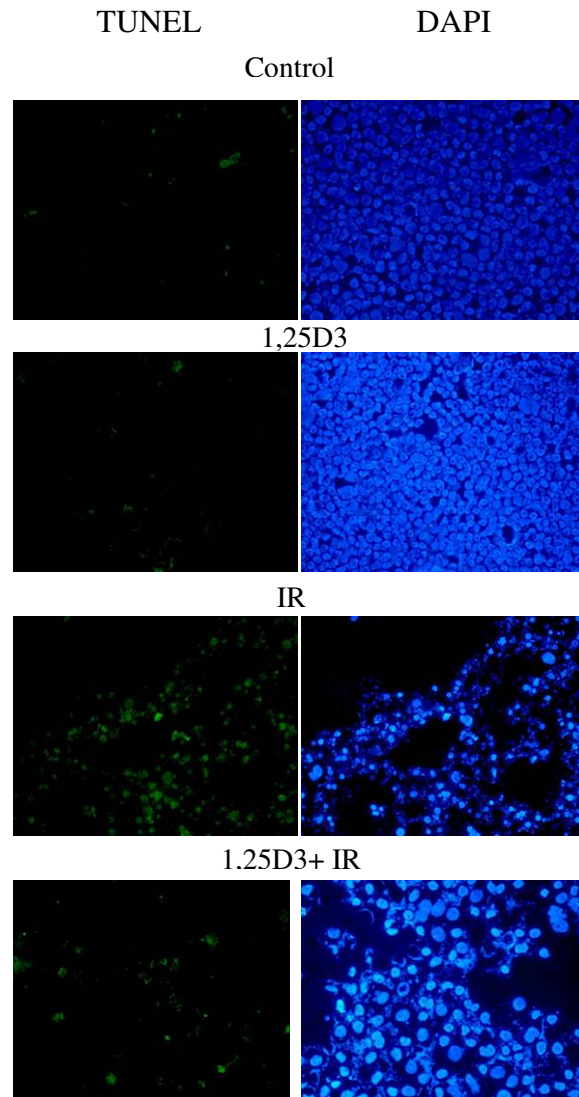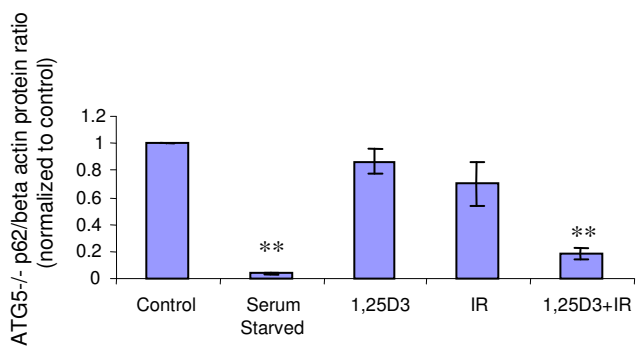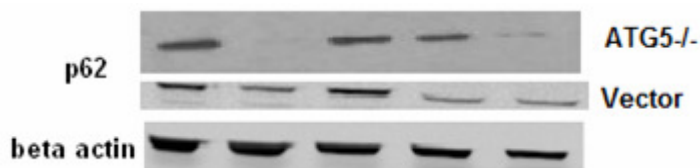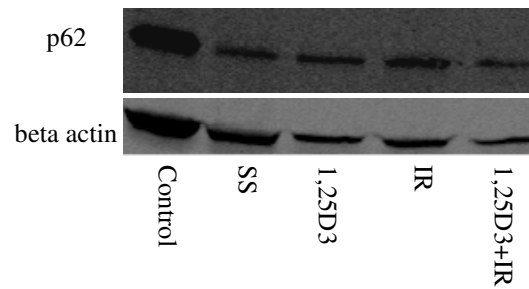**Figure 4.18. Influence of 1,25D$_3$ on Autophagy in response to fractionated radiation in MCF7/ATG7-cells.** Cells were exposed to radiation alone, or 1,25D$_3$ prior to irradiation and autophagic flux was based on the decline in p62 levels monitored by Western blotting 24 hours post-irradation. Actin was utilized as a loading control. Serum starvation was used as a positive control for autophagic flux.

Chapter 5: Influence of Vitamin D3, and its analog, EB1089, on the response to fractionated radiation in radiation resistant cell lines.

5.1 Sensitization to radiation in MCF7/HER2 breast tumor cells.

Given that primary breast cancer cells are generally considered to be relatively sensitive to radiation treatment, we sought to determine if treatment of breast tumor cells that are intrinsically resistant to radiation through the over expression of HER2/neu (Liang et al., 2003) would also demonstrate sensitization to radiation via the promotion of autophagy. Figure 5.1 confirms the over expression of HER2 in this cell line while Figure 5.2 confirms the expression of the vitamin D receptor. Figure 5.3 indicates that MCF7HER2 cells constitutively demonstrated low levels of autophagy staining (a single large vacuole is observed in control cells; in cells exposed to EB1089 there is no increase in AVO staining. Treatment with radiation or EB1089 + radiation resulted in more widely dispersed punctate staining. This is similar to our previous results in wild type MCF7 cells (and in separate studies by Eden Wilson in our laboratory, utilizing ZR-75 breast tumor cells). Figure 5.4 demonstrates that the combination of $1,25D_3$ + radiation results in a more pronounced effect in terms of reducing viable cell number than radiation alone, although this appears to be due in large part to high sensitivity to the effects of the $1,25D_3$; sensitization was also observed with EB1089 + radiation (data not shown). In these studies, we did not follow radiation effects to the point where proliferative recovery might have been evident. While other factors may play a role, the increased sensitivity to vitamin D_3 alone is likely due to upregulation in VDR expression (Figure 5.2).

5.2 Sensitization of Hs578t breast tumor cells.

A recent paper reported the radiosensitization by $1,25D_3$ of Hs578t breast tumor cells, (Mineva et al., 2009) a breast tumor cell line that is p53 mutant and intrinsically radioresistant. (Mineva et al., 2009; Tian et al., 2007) The clonogenic survival data presented in Figure 5.5 confirm that Hs578t cells are significantly less radiosensitive than MCF7 cells. Figure 5.6 further confirms the findings of Mineva et al. (Mineva et al., 2009) that $1,25D_3$ enhances radiation sensitivity (1.7 fold reduction in colony formation with combination in comparison to radiation alone). Figure 5.7 indicates that the combination of $1,25D_3$ with radiation resulted in significant autophagy in comparison to control and $1,25D_3$ treated cells (with modest autophagy induced by radiation alone), based on acridine orange staining, findings similar to those with MCF7 and MCF7HER2 cells. Studies of p62 degradation shown in Figure 5.8 confirmed that exposure to $1,25D_3$ + radiation also promoted autophagic flux. It is further of importance to emphasize that radiation alone did not appear to promote p62 degradation in this cell line, *which supports the premise that increased autophagic flux is likely to be responsible for radiation sensitization by $1,25D_3$.*

To asses whether radiation alone or in combination with $1,25D_3$ elicited apoptosis, TUNEL and DAPI staining were conducted (Figure 5.9 with corresponding quantification); while there was a significant increase in the level of apoptosis observed in cells treated with $1,25D_3$ in combination with radiation, levels observed were only that of 15%. This would indicate that apoptosis may play a role in $1,25D_3$ radiosensitization of Hs578t cells.

5.3 Lack of sensitization of BT474 breast tumor cells.

To further test whether EB1089 was able to sensitize intrinsically radioresistant cell lines, we also examined HER2 over expressing, p53 mutant, BT474 breast cancer cells. (Monazzam et al., 2009) Figure 5.10 indicates the temporal differences in viable cell number between control cells and cells that were treated with either radiation alone or EB1089 followed by radiation. The BT474 cells were clearly quite resistant to radiation treatment. Since EB1089 was unable to radiosensitize these cells and upon further examination the combination was unable to elicit an autophagic response as indicated by the lack of change in acridine orange staining in Figure 5.11.   Although the literature indicates that BT474 cells express the VDR and are responsive to vitamin D, (Mehta et al., 2003) Figure 5.2 revealed that BT474 cells actually have minimally detectable levels of the VDR, which supports the necessity for having a relatively robust VDR mediated signaling response pathway for radiosensitization by vitamin D_3 or its analogs. Interestingly, Costa et al. have suggested that the Vitamin D receptor may be unnecessary for its antiproliferative actions, which could indicate that effects on tumor cell growth and radiation sensitivity are dissociable. (Costa el at., 2009)

Figure 5.1. Western blot analysis to determine level of HER2. MCF7/HER2 cells transfected for the over expression with HER2 were monitored for the level of HER2 protein in comparison to parental MCF7 cells. Beta actin is utilized as a loading control.
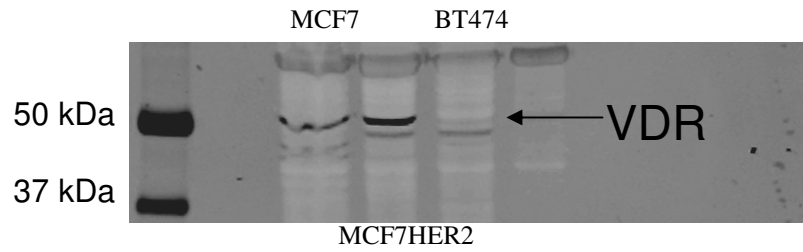
Figure 5.2. Western blot analysis of VDR expression levels. Vitamin D receptor (VDR) levels were monitored via Western blotting in MCF7 parental cells, MCF7HER2 cells, and BT474 cells.
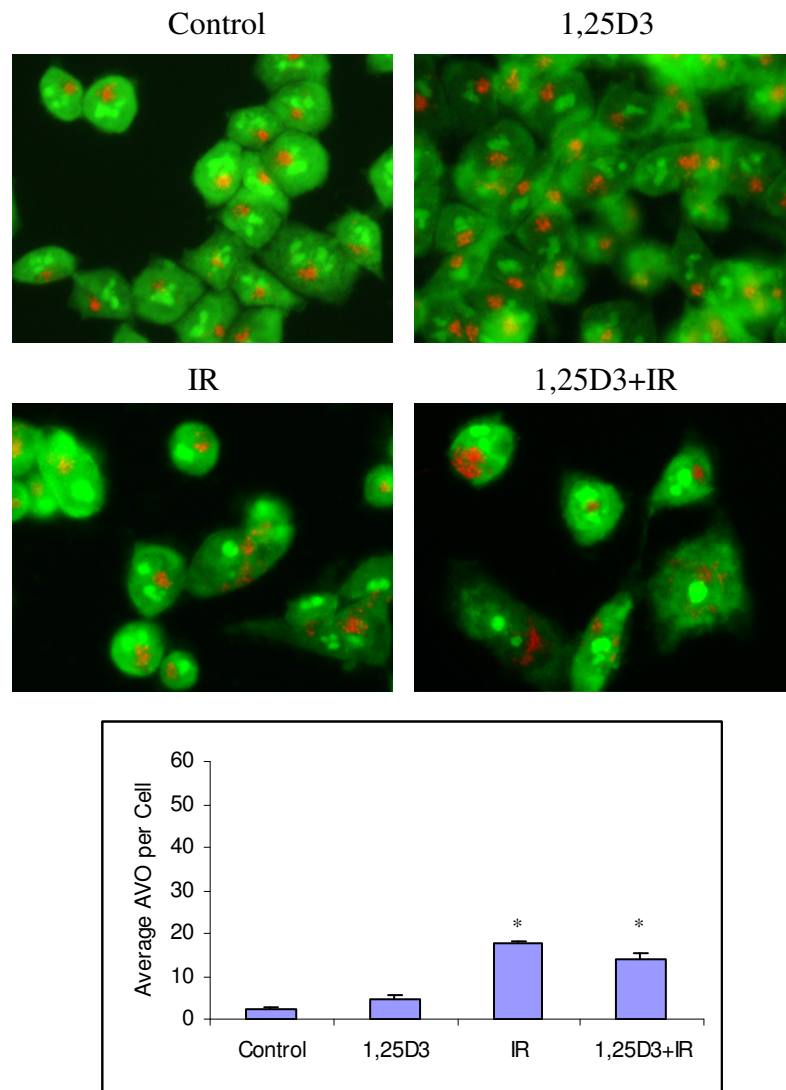
Figure 5.3. AVO formation in MCF7/HER2 cells. MCF7/HER2 Cells were exposed to radiation alone (5x2 Gy), 1,25D3 alone, or $1,25D_3$ + irradiation and autophagy was monitored based on acridine orange staining 24 hours post-irradiation; for the accompanying bar graph, the average number of AVOs per cell were counted in three fields for each condition.
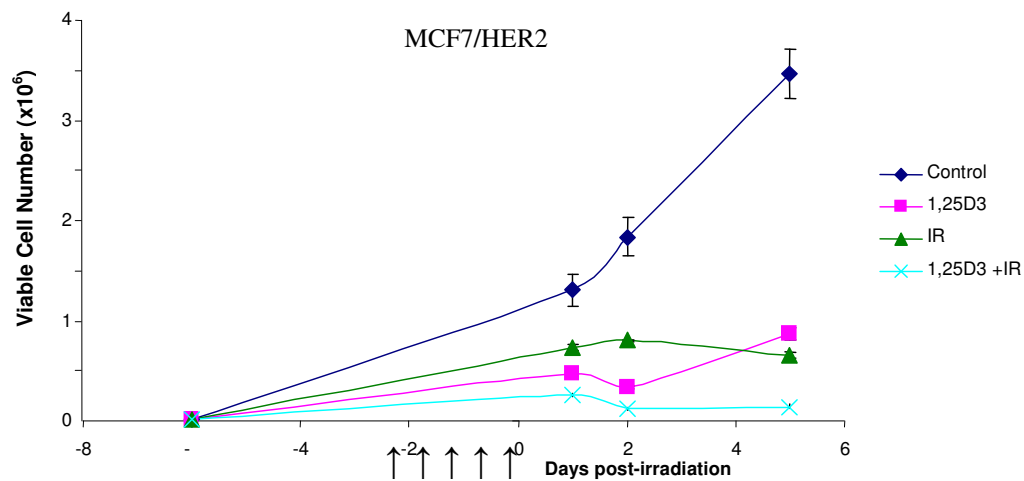
Figure 5.4. Temporal response to 1,25D₃ and radiation in MCF7/HER2 cells.
MCF7/HER2 cells were treated as in the previous figure and viable cell number was
determined by exclusion of trypan blue at the indicated days following the initiation of
radiation exposure. ↑ Indicates days when irradiation occurred.
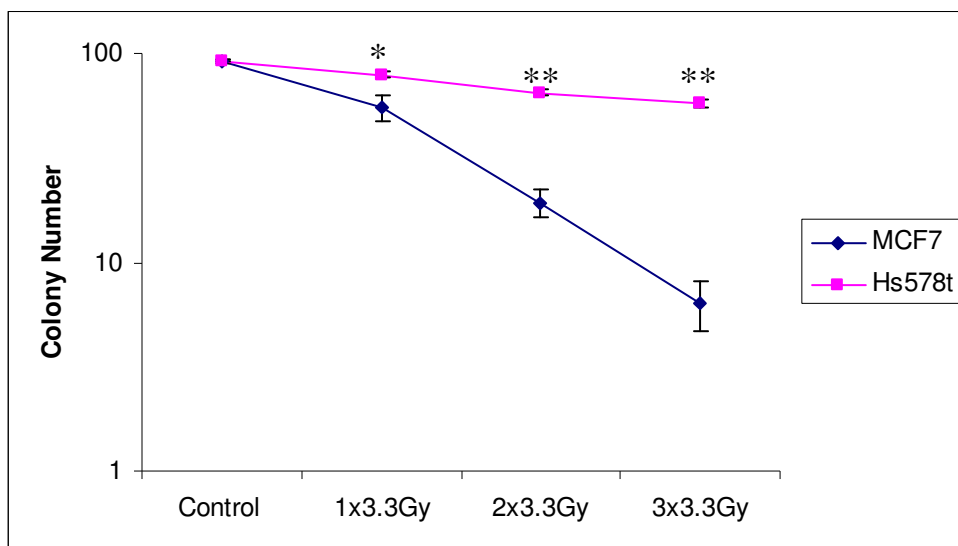
Figure 5.5. Radiation sensitivity of Hs578t breast tumor cells in comparison to MCF7 cells. MCF7 and Hs578t cell were treated with varying doses of radiation and colony formation was assessed. *p<0.05, **p<0.0001.
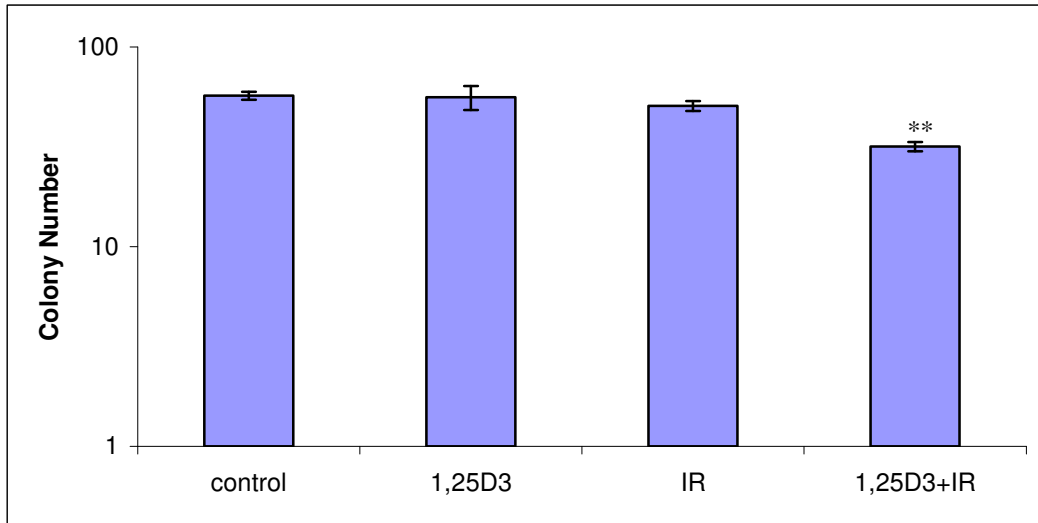
Figure 5.6. Influence of 1,25D3 on radiation sensitivity of Hs578t breast tumor cells. Hs578t cells were exposed to $1,25D_3$ alone, radiation alone (5x2 Gy), or $1,25D_3$ prior to irradiation and colony formation was assessed. **p<0.0001.

Control   1,25D3

IR   1,25D3+IR
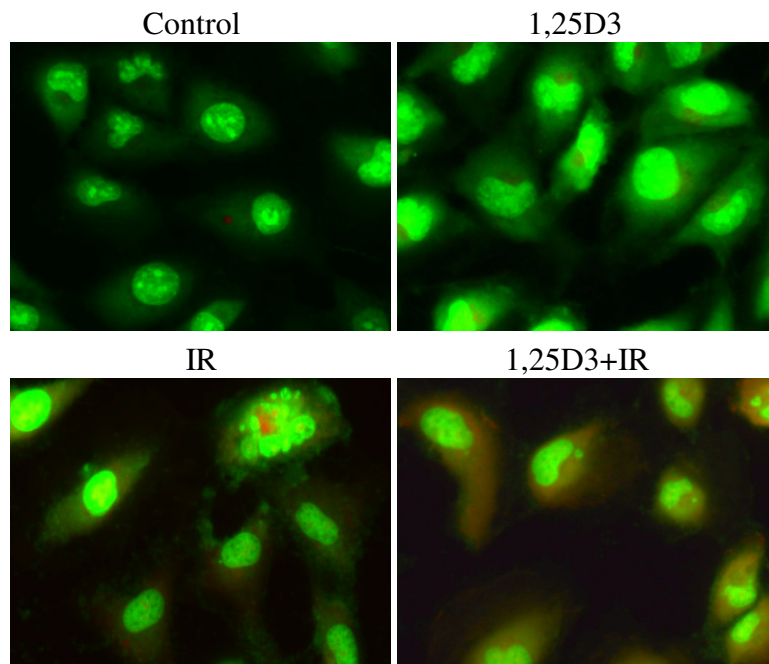
Figure 5.7. AVO formation in Hs578t breast tumor cells. Hs578t cells were exposed to 1,25D$_3$ alone, radiation alone (5x2 Gy), or 1,25D$_3$ prior to irradiation and stained with acridine orange for the presence of AVO.
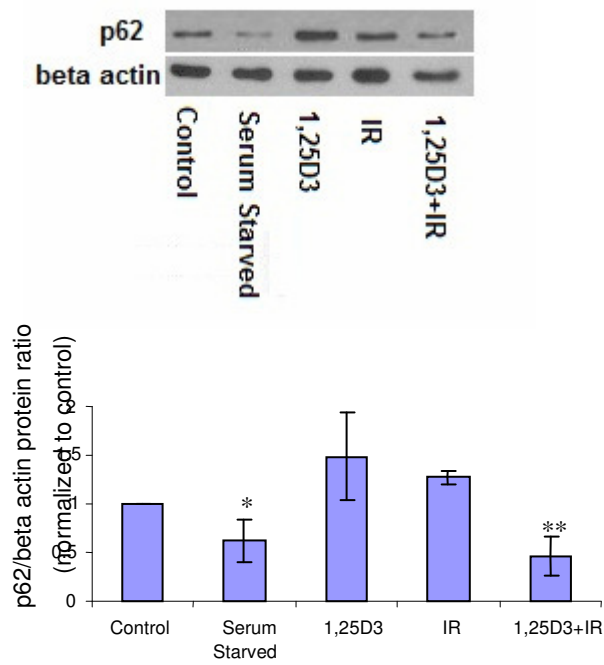
Figure 5.8. Influence of 1,25D₃ and radiation on autophagic flux in Hs578t breast tumor cells. Autophagic flux was based on the decline in p62 levels monitored by Western blotting 24 hours post-irradiation. Actin was utilized as a loading control. Serum starvation was used as a positive control for autophagic flux. Densitometry of the p62/actin ratio from three separate westerns is presented in the lower panel.
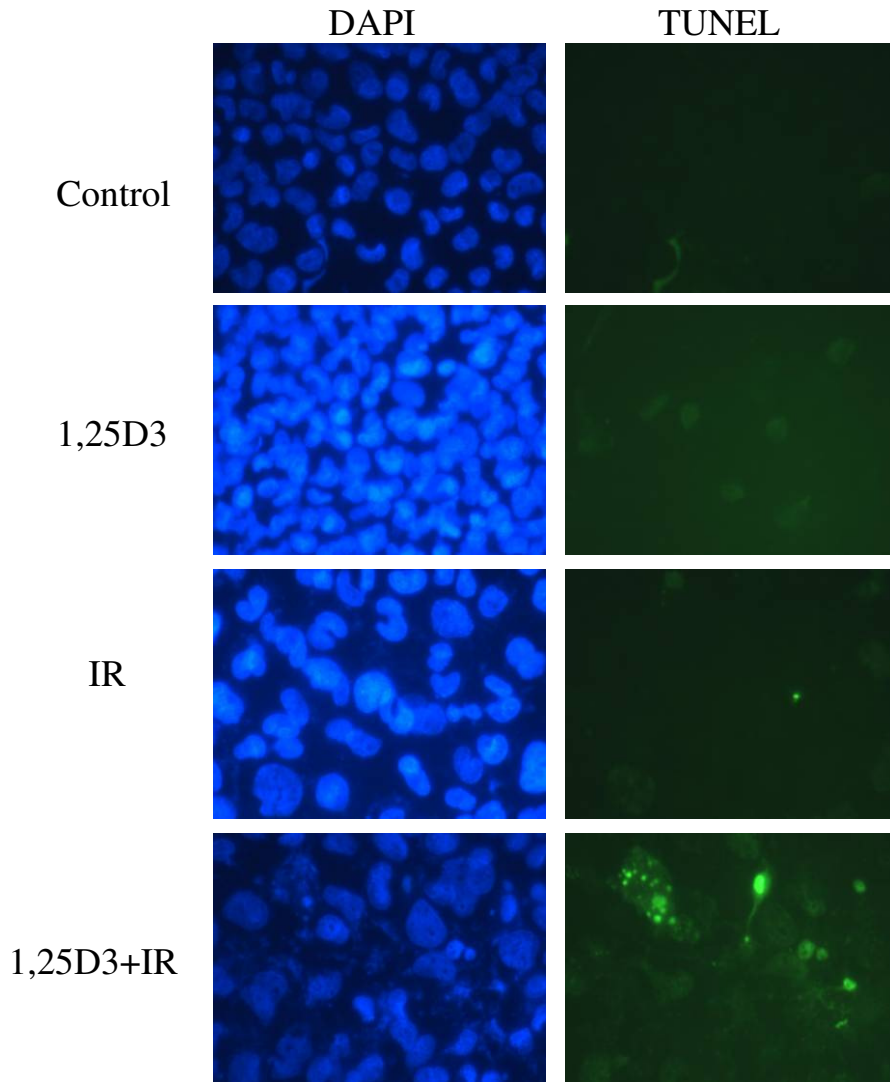
Figure 5.9. Apoptosis in Hs578t breast tumor cells. Hs578t cells were exposed to $1,25D_3$ alone, radiation alone (5x2 Gy), or $1,25D_3$ prior to irradiation and imaged with TUNEL and DAPI for the presence of apoptosis. Quantification is presented in the lower graph. **p<0.0001
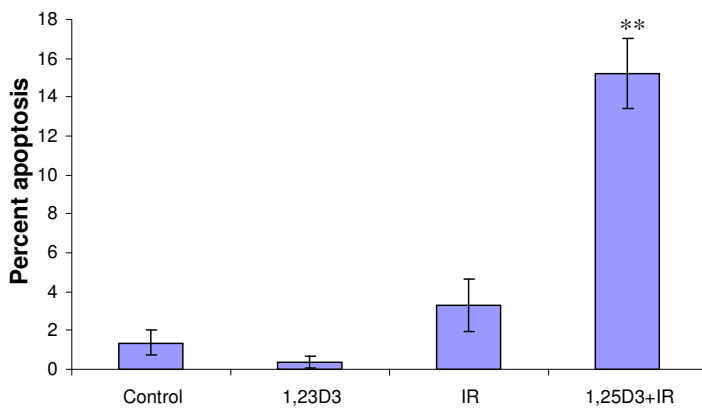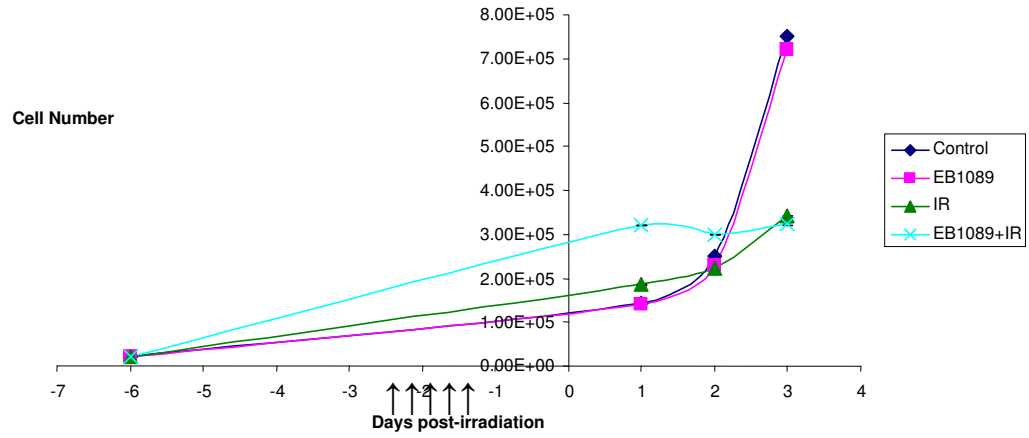
Figure 5.10. Temporal response to 1,25D3 and radiation in BT474 cells. BT474 cells were exposed to $1,25D_3$ alone, radiation alone (5x2 Gy), or $1,25D_3$ prior to irradiation and viable cell number was determined by exclusion of trypan blue at the indicated days following the initiation of radiation exposure. ↑ Indicates days when irradiation occurred.
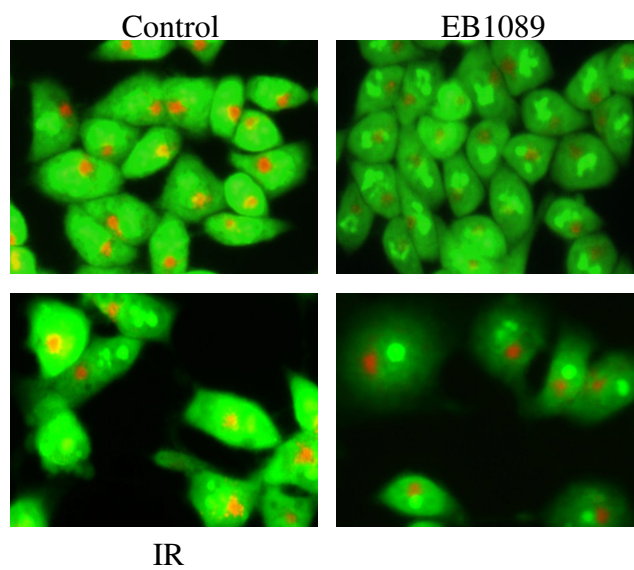
Figure 5.11. Autophagy is not altered with 1,25D$_3$ and radiation in BT474 breast tumor cells. BT474 Cells were exposed to radiation alone, or 1,25D$_3$ prior to irradiation and Autophagy was monitored based on acridine orange staining 24 hours post-irradiation.

Chapter 6: Influence of autophagic inhibition on the response to radiation in 4T1 mouse mammary tumor cells.

6.1 Chloroquine, but not 1,25D$_3$+IR, sensitizes 4T1 cells.

In vivo and *in vitro* studies of antitumor activity of cancer therapeutics can often result in differential responses. It was therefore vital to determine if 1,25D$_3$ promoted radiosensitivity *in vivo*. Because the immune system can play an important role in both autophagy (Harris, J et al., 2009; Mizushima N, et al., 2008) and the response to chemotherapy *in vivo* (Zitvogel et al., 2008), we sought to utilize a mouse mammary tumor that could be used with immune competent mice. We first chose to confirm that the mammary fat pad derived, highly metastatic, p53-null 4T1 cells (obtained from Fred Miller whom first isolated this cell line (Aslakson and Miller, 1992)), responded similarly to MCF7 cells. 4T1 cells were treated with 1,25D$_3$ alone, 5x2Gy IR alone, or 100nM 1,25D$_3$ followed by 5x2Gy, and cell viability was determined at various times post-irradiation (Figure 6.1). In contrast to the growth delay and proliferative recovery observed with MCF7 cells in Figure 3.5, 4T1 cells treated with IR alone demonstrate a growth delay followed by cell death. Because of this response, there is not a dramatic difference between viability of 4T1 cells treated with radiation alone or 1,25D$_3$ + radiation. Similarly, colony formation post-irradiation (Figure 6.2) in the presence or absence of 1,25D$_3$ is not significantly different.

4T1 cells stably transfected with luciferase (4T1-luc), to be utilized *in vivo*, were also obtained and cell viability was analyzed with 1,25D$_3$ alone, 5x2Gy IR alone, or
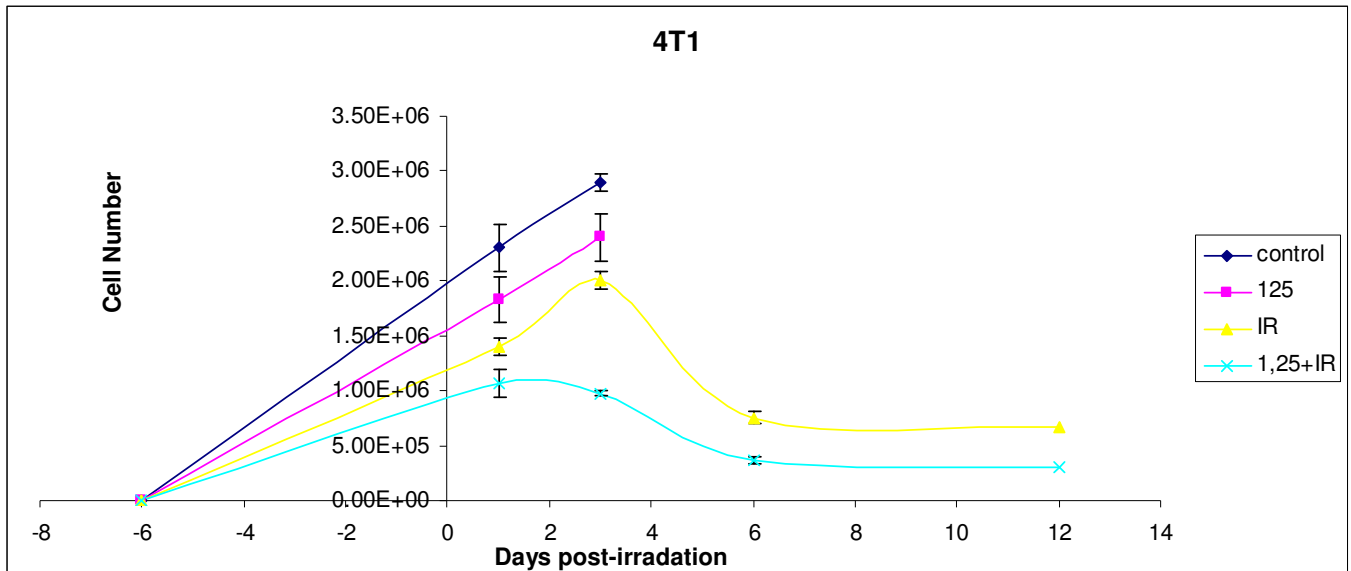
Figure 6.1: **Influence of 1,25 D$_3$ on the temporal response to fractionated radiation in 4T1 cells.** 4T1 cells were exposed to radiation alone (5x2Gy), or 100 nM 1,25D$_3$ prior to irradiation (5x2Gy). Viable cell number was determined by exclusion of trypan blue at the indicated days following the initiation of radiation exposure.
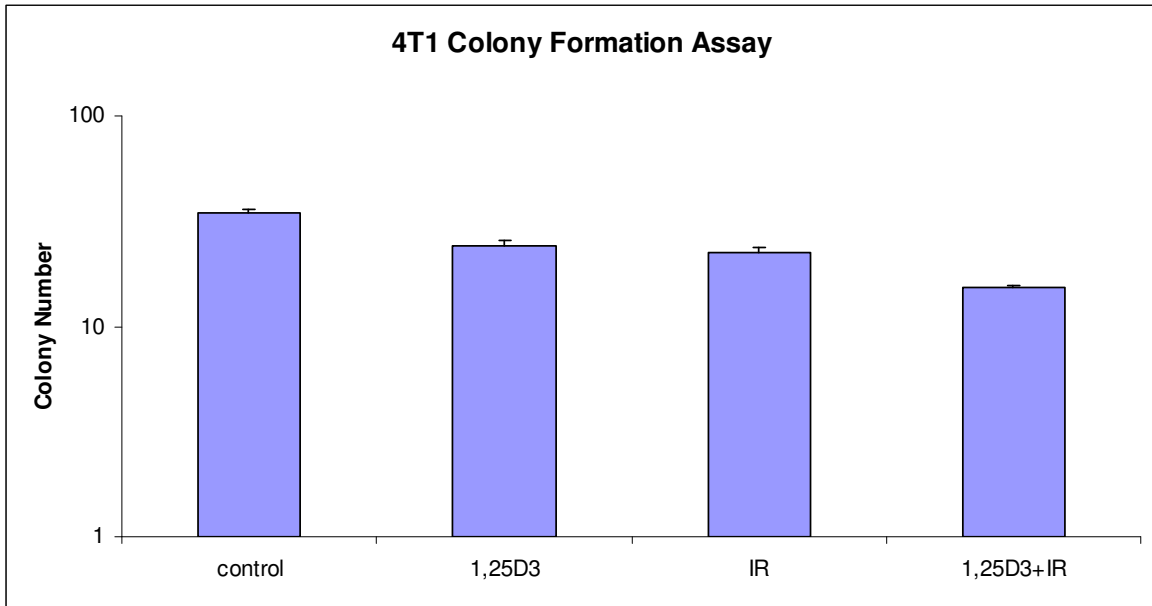
Figure 6.2: Influence of 1,25 D$_3$ on colony formation in response to fractionated radiation in 4T1 cells. Clonogenic survival was assessed after 10 days post-treatment. Values shown are from a representative experiment with triplicate samples for each condition.
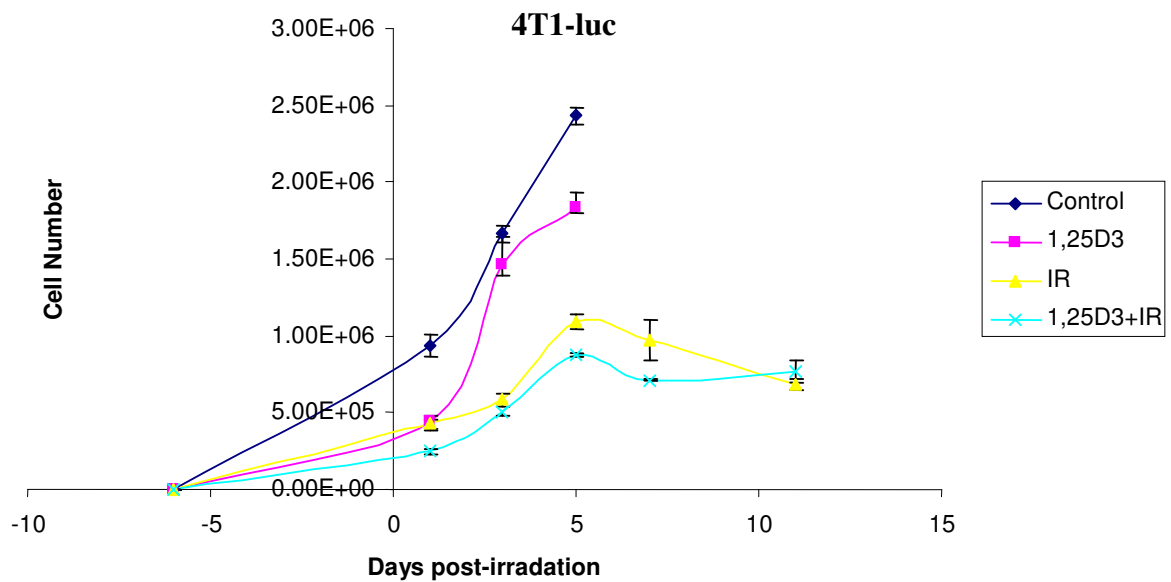
Figure 6.3: **Influence of 1,25 D$_3$ on the temporal response to fractionated radiation in 4T1-luc cells.** 4T1 cells were exposed to radiation alone (5x2Gy), or 100 nM 1,25D$_3$ prior to irradiation (5x2Gy). Viable cell number was determined by exclusion of trypan blue at the indicated days following the initiation of radiation exposure.

100nM 1,25D$_3$ followed by 5x2Gy at various times post-irradiation (Figure 6.3). Similar

to the parental cells, 4T1-luc cells were not apparently radiosensitized with 1,25D$_3$

treatment. To determine if autophagy played a role in response to radiation in this system,

cells were treated as in Figure 6.3 in the presence or absence of 25μM CQ (Figure 6.4) and

stained with acridine orange. 4T1-luc control cells exhibited high levels of basal

autophagy that were unaltered in response to 1,25D$_3$, IR, or 1,25D$_3$. Moreover, the

presence of CQ dramatically decreased cell viability (note minimal cells observed per field

of view in Figure 6.4, cell count data not shown). Due to the high basal level of autophagy

observed, an MTT assay was conducted to analyze the effects of various doses of CQ in

these cells. All doses except for 1μM were highly toxic to 4T1-luc cells (Figure 6.5). In

addition, a number of other autophagic inhibitors were analyzed via the MTT assay and

demonstrated similar toxicity (data not shown), suggesting that the 4T1 cells rely on

autophagy for viability and that inhibition of autophagy is highly detrimental to the cells.

6.2 Chloroquine in combination with radiation *in vivo*

Expanding upon the observation that CQ was toxic to 4T1-luc cells *in vitro*, female

BALB/c mice were challenged s.c. with 4T1-luc mouse breast tumor cells in the right flank

and tumor-bearing mice were randomly assigned into groups of 6-8. To determine which

doses of CQ and IR to use, a dose response for each was conducted. Experiments were

conducted utilizing IR (administered on day 3 post-challenge) at the following doses: (a) 5

Gy, (b) 10 Gy, (c) 15 Gy. Utilizing data obtained in 4T1 cells treated with CQ alone
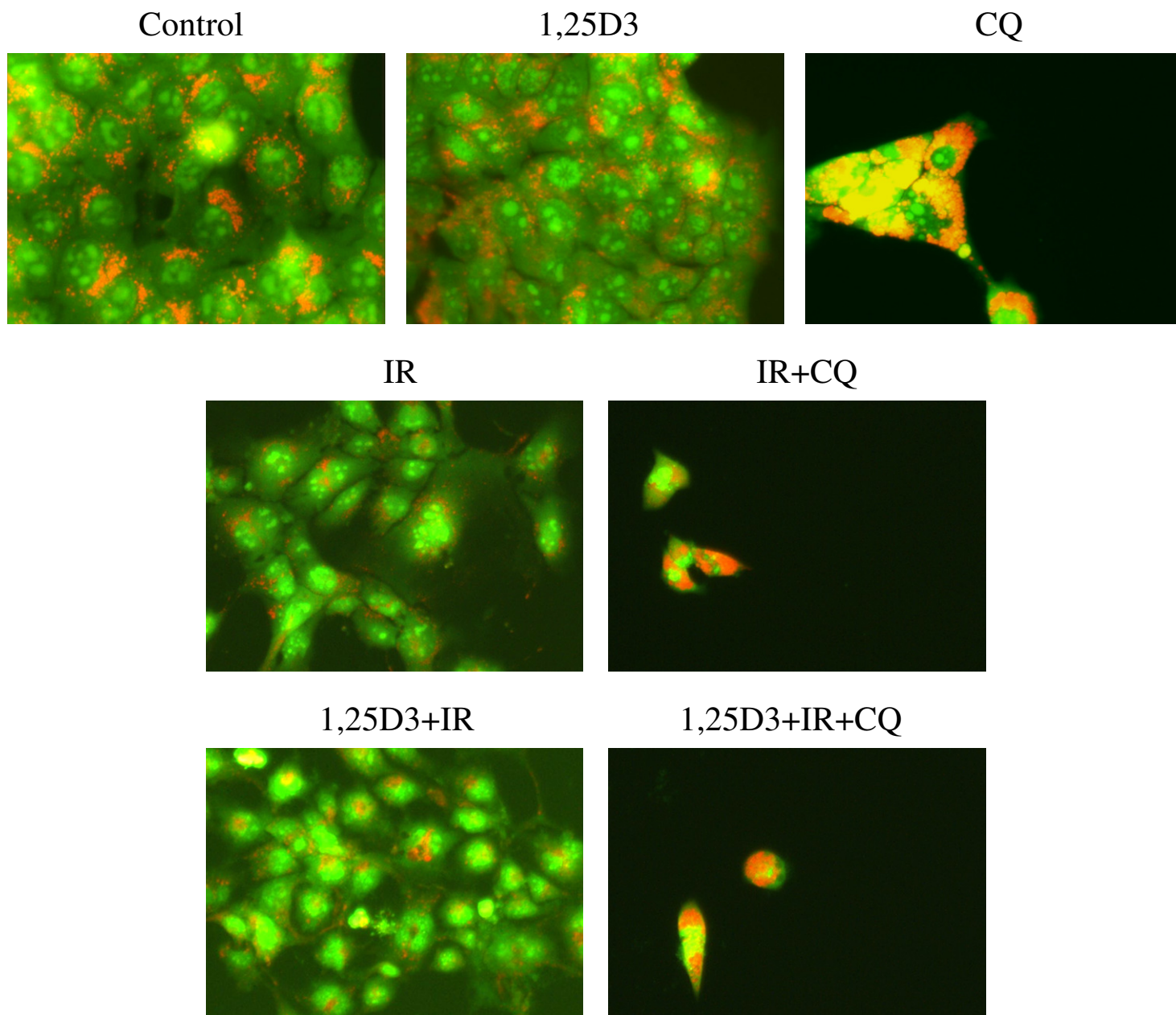
Figure 6.4. AVO formation in 4T1 cells. 4T1 cells were exposed to CQ or 1,25D$_3$ alone, radiation alone (5x2 Gy) ±CQ, or 1,25D$_3$ prior to irradiation ±CQ and stained with acridine orange for the presence of AVO.
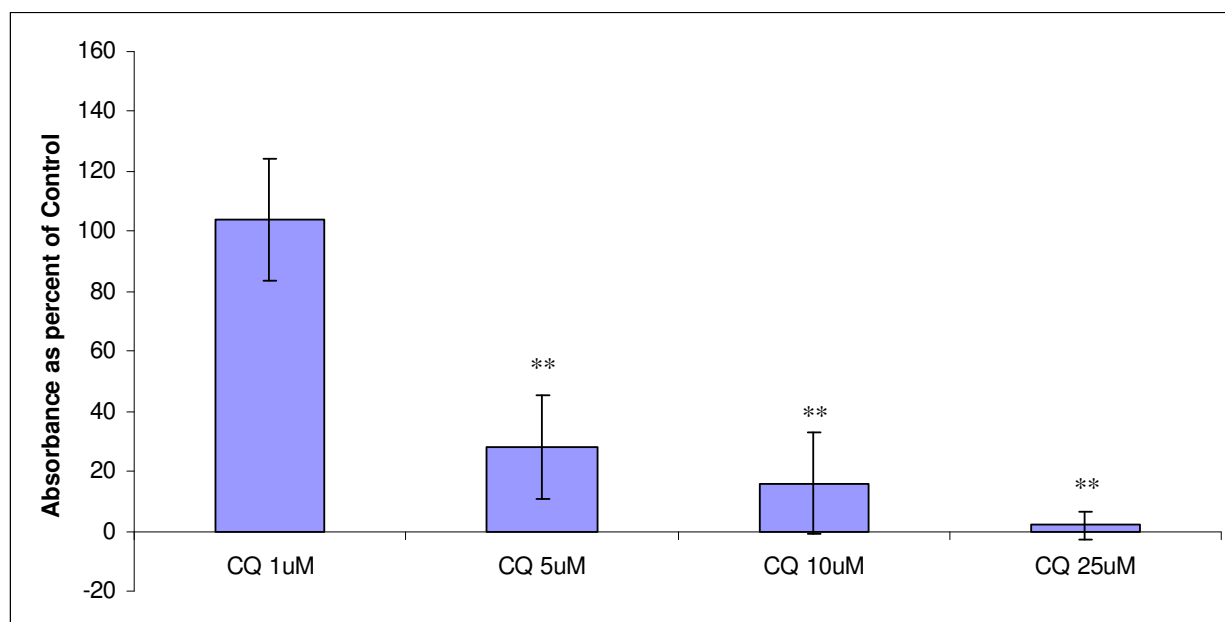
Figure 6.5: MTT indicates CQ is toxic to 4T1 cells. 4T1 cells were treated with various doses of CQ and toxicity was determined via MTT.

(Jiang et al., 2010) animals were injected intraperitoneally with their corresponding treatments: (a) 25 mg/kg CQ, (b) 50 mg/kg CQ, (c) 100mg/kg CQ, or (d) PBS; therapy started on day 3 post-challenge and was repeated every day for 5 days. The tumor growth was monitored multiple times per week by an IVIS Spectrum. After treatment ended, mice were further monitored until at least day 18 and were then sacrificed. Tumors were resected and tumor volumes were observed. Tumor growth was also evaluated by measurement of tumor diameters at various time points and the tumor volume was calculated as length x (width x 0.5)^2.

At various time points post-challenge, mice were weighed to determine if any of the treatments reduced body weight. As observed in Figure 6.6, none of the treatments altered body weight. Tumor growth was monitored throughout the studies utilizing the IVIS 50 (Xenogen, part of Caliper Life Sciences) and calipers. Of note, the bioluminescent signal could be detected in the animal before primary tumors were palpable and could be measured with a caliper. Figure 6.7 and 6.8 present caliper and luminescence data, respectively. Caliper data indicated that all CQ and IR treatments, by day 19, significantly reduced tumor volume in comparison to vehicle (Figure 6.7). On day 19, tumor necrosis and over-saturation of luminescence confounded vehicle data (Figure 6.8). However, with trends observed on prior days, it appears that all doses of radiation inhibited tumor growth; conversely, CQ did not significantly alter tumor development as measured by bioluminescence. Discrepancy between CQ caliper and bioluminescence data are probably due to the fact that calipers only measure the length and width of the tumor, but not the depth. Tumor resection in Figure 6.9 confirms the observations in Figure 6.8.

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Figure 6.6: CQ alone and IR alone do not influence animal weight. Animal body weight was monitored at various time points post-challenge; CQ alone and IR alone are compared with vehicle treatment.

Figure 6.7. Influence of CQ alone and IR alone on tumor volume. Tumor volume was monitored via caliper at various time points post-challenge; CQ alone and IR alone are compared with vehicle treatment.

CQ

IR

Figure 6.8. Influence of CQ alone and IR alone on bioluminescence. Animals were injected with luciferin and bioluminescence was monitored at various time points post-challenge; CQ alone and IR alone are compared with vehicle treatment.
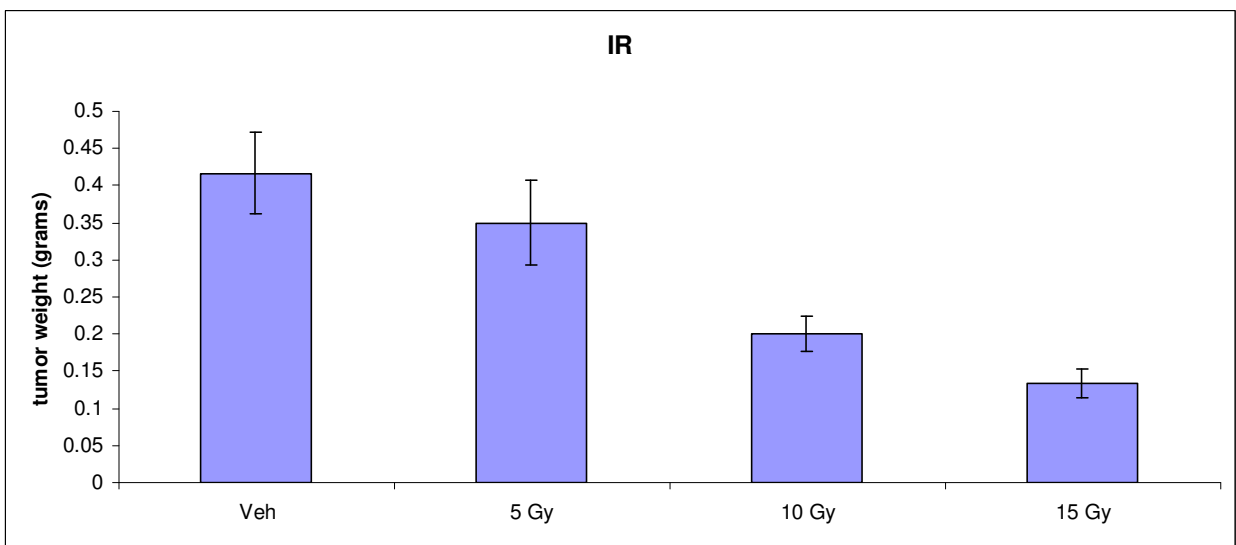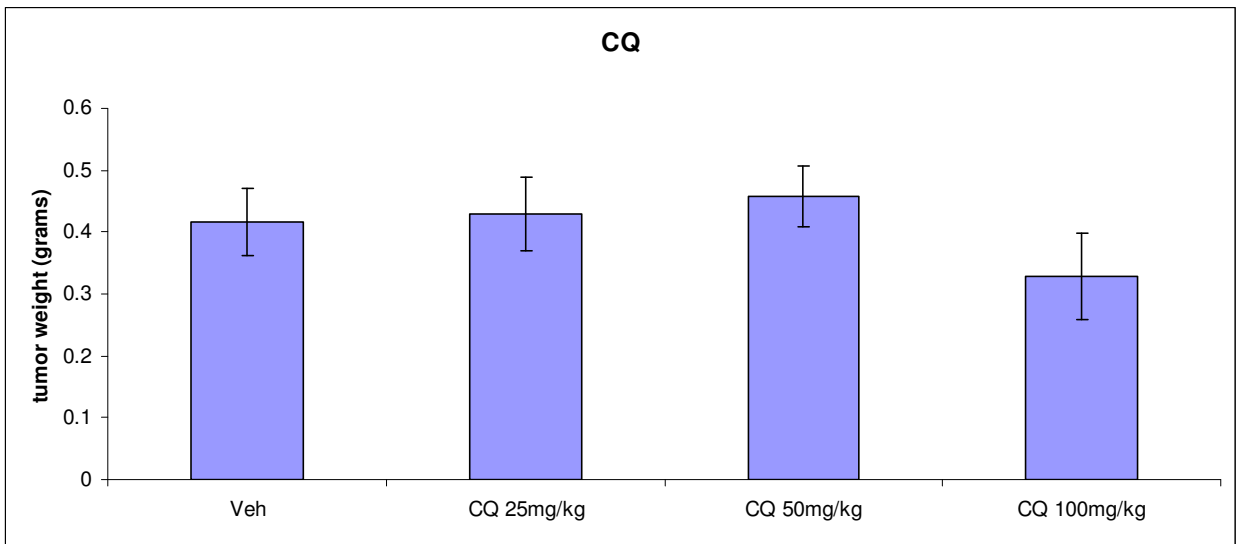
Figure 6.9. Influence of CQ alone and IR alone on tumor weight. Animals were sacrificed and tumors were resected and weighed; CQ alone and IR alone are compared with vehicle treatment.

Given that 5 days of CQ did not appear to significantly alter tumor growth at any of the

doses, a longer period of injections, 14 days, was chosen. As in Figure 6.6, 14 days of CQ

injections did not alter body weight (Figure 6.10). Disappointingly, the longer CQ

treatment also did not have significant effects on tumor development. Figure 6.11 and 6.12

indicates that the caliper and luminescence data, respectively, for CQ is not significantly

different than vehicle. Moreover, tumor resection in Figure 6.13 confirms these

observations.

While *in vitro* data presented above and *in vivo* data in the literature (Jiang et al.,

2010) indicated that CQ treatment alone should significantly reduce 4T1 tumor

development, this was not our observation. This could be due to a variety of factors that

are further elaborated upon in the discussion. Nevertheless, we sought to determine if CQ

could radiosensitize 4T1 tumor development. BALB/c mice were challenged with 4T1

tumors as before, received the following treatments: (a) 50mg/kg CQ for 14 days, (b) 10

Gy IR, (c) IR + CQ, (d) PBS (vehicle), and monitored at various time points. As observed

in Figure 6.14, radiation, CQ or the combination did not significantly alter body weight as

compared to vehicle. Figure 6.15 and 6.16 present caliper and luminescence data,

respectively. Caliper data indicates that IR and IR+CQ significantly reduced tumor

volume by day 8; additionally, CQ, IR, and IR+CQ treatments, by day 13, significantly

reduced tumor volume in comparison to vehicle (Figure 6.15). Luminescence data

presented in Figure 6.16 indicates that IR significantly reduced luminescence from vehicle

on days 8, 13, and 18. Conversely, IR+CQ was not significant from vehicle at any time

point, and by day 18, CQ luminescence is significantly increased in comparison to vehicle

(Figure 6.16).   At day 18, mice were sacrificed and tumors were resected. Figures 6.17

and 6.18 presents tumor weight and images, respectively.   Radiation, in the presence or

absence of CQ, significantly reduced tumor weight in comparison to vehicle; however, due

to variability of tumor weight, IR+CQ did not significantly reduce tumor weight in

comparison with IR alone (Figure 6.17). Moreover, radiation alone promoted

approximately a 50% reduction in tumor development at our final time points. This *in vivo*

radiation time course is highly correlative with the *in vitro* radiation time course; in that,

initially tumors develop at a reduced rate, followed by a period of growth arrest.

Furthermore, in contrast to our original expectations, our data suggests that IR+CQ is not

able to significantly reduce tumor formation over that of radiation alone. The implications

of this data are further discussed in Chapter 7.2.
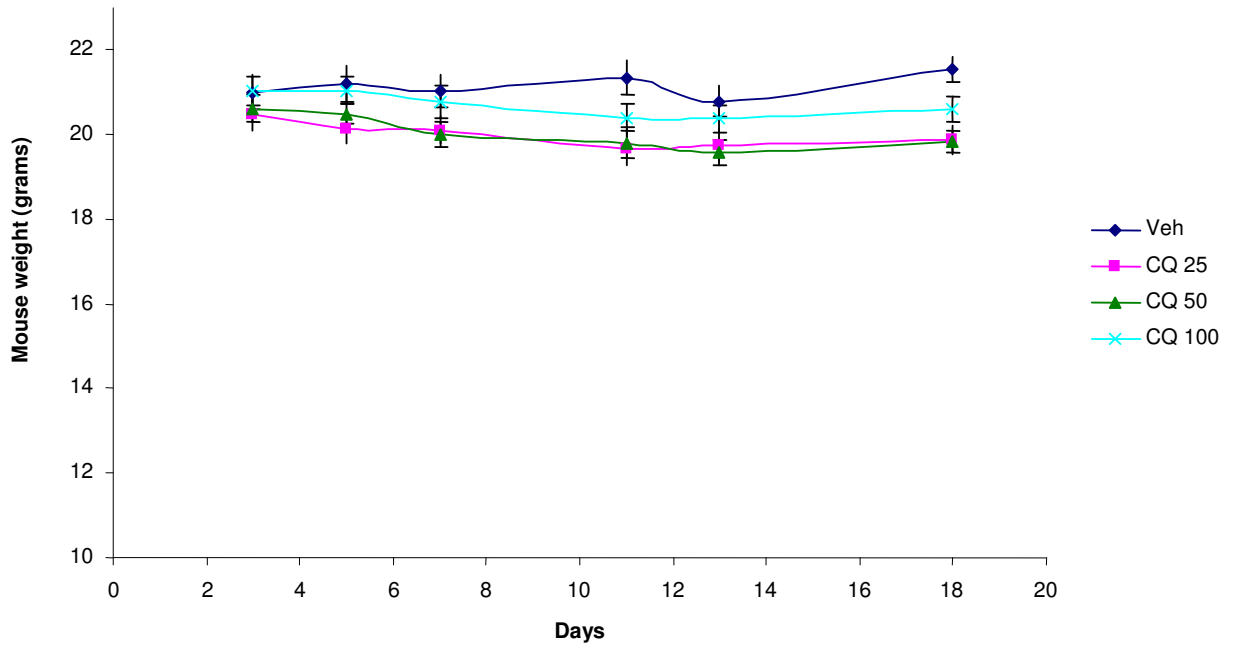
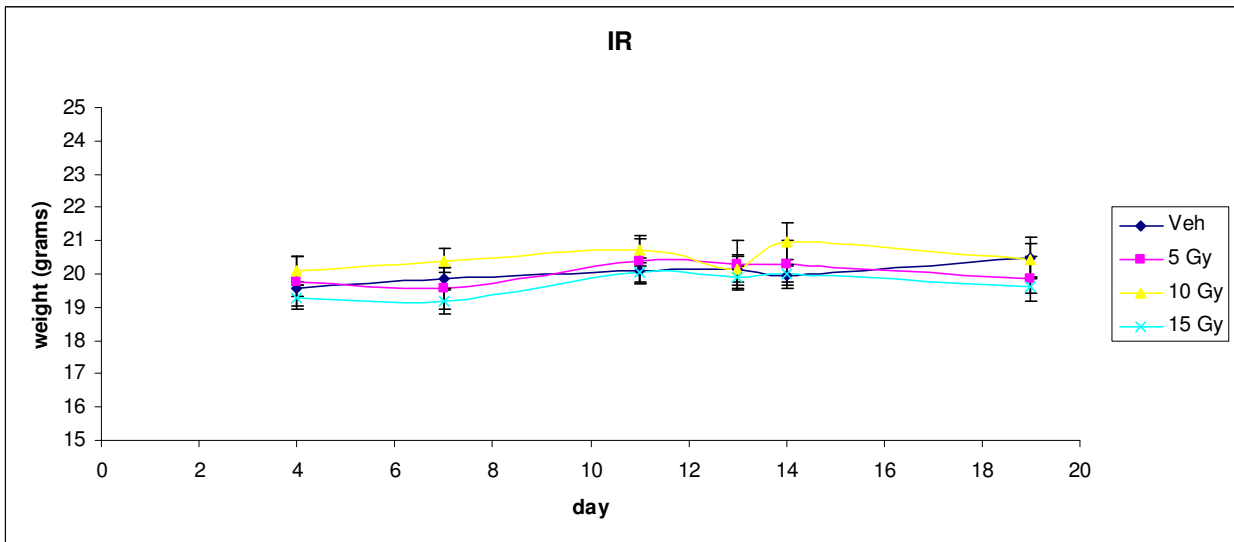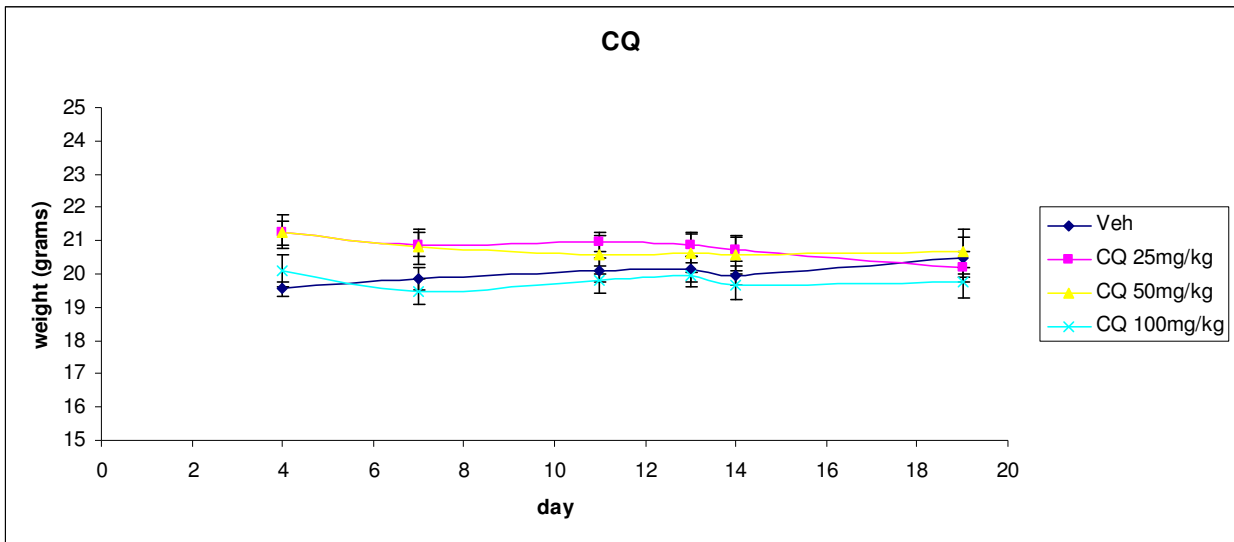**Figure 6.10: CQ for 14 days did not influence animal weight.** Animal body weight was monitored at various time points post-challenge; CQ is compared with vehicle treatment.
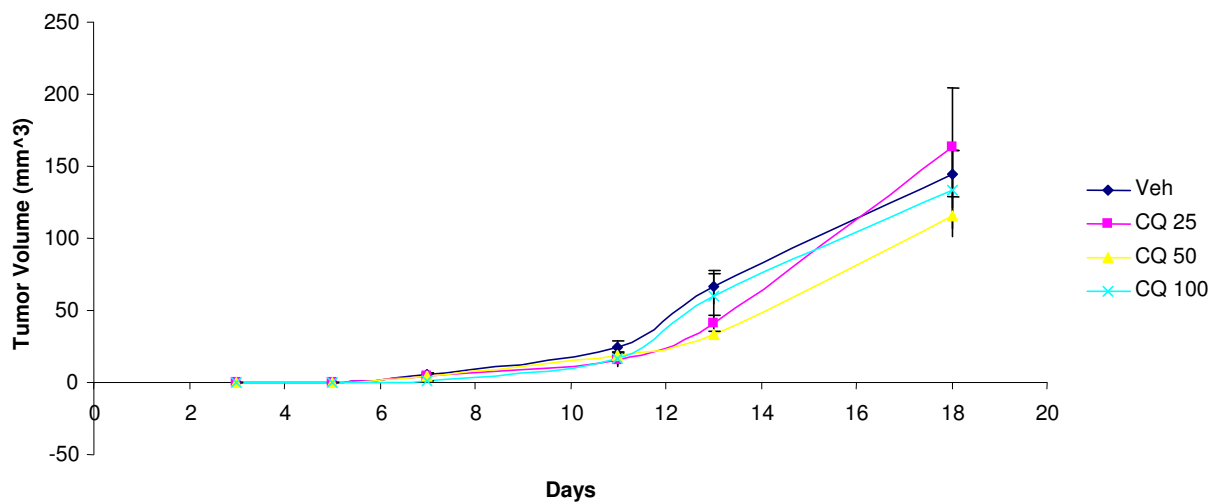
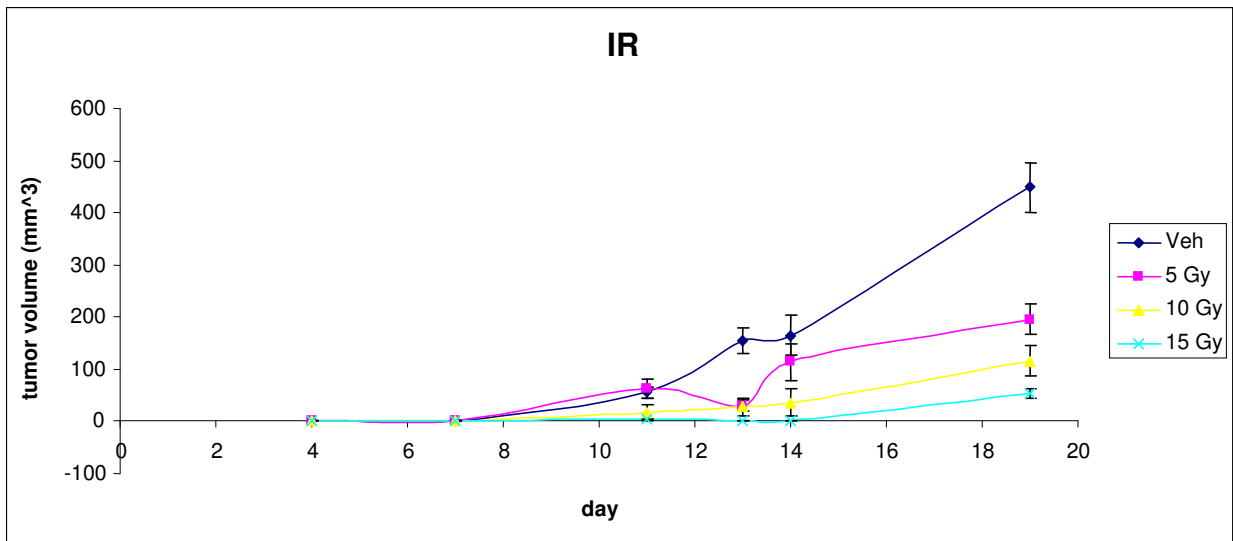**Figure 6.11. Influence of CQ for 14 days on tumor volume.** Tumor volume was monitored via caliper at various time points post-challenge; CQ is compared with vehicle treatment.
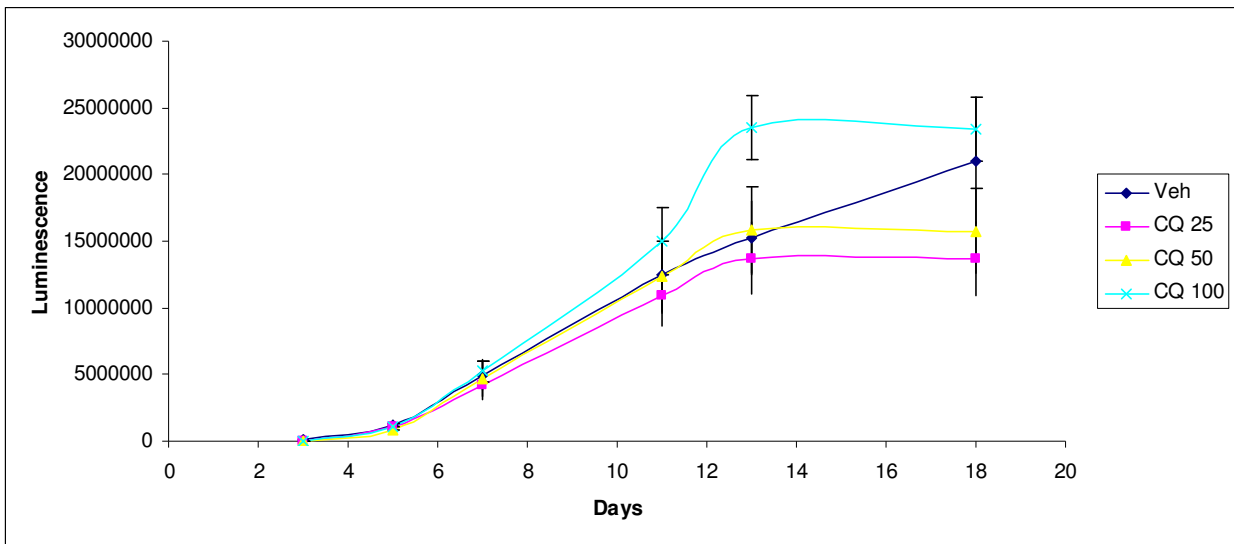
Figure 6.12: Influence of CQ for 14 days on bioluminescence. Animals were injected with luciferin and bioluminescence was monitored at various time points post-challenge; CQ is compared with vehicle treatment.
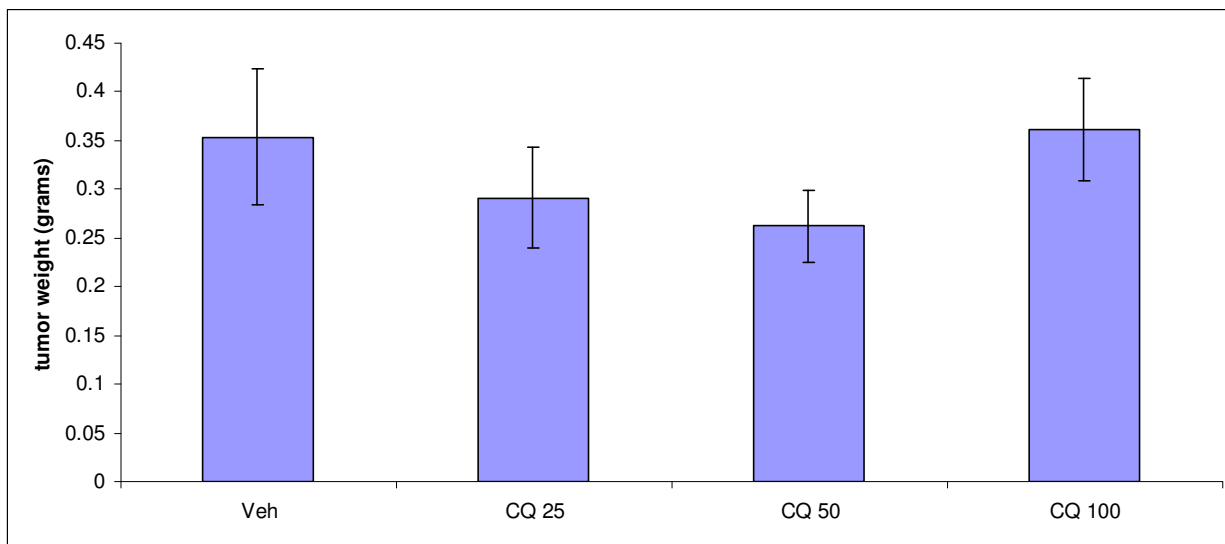
Figure 6.13. Influence of CQ for 14 days on tumor weight. Animals were sacrificed and tumors were resected and weighed; CQ is compared with vehicle treatment.
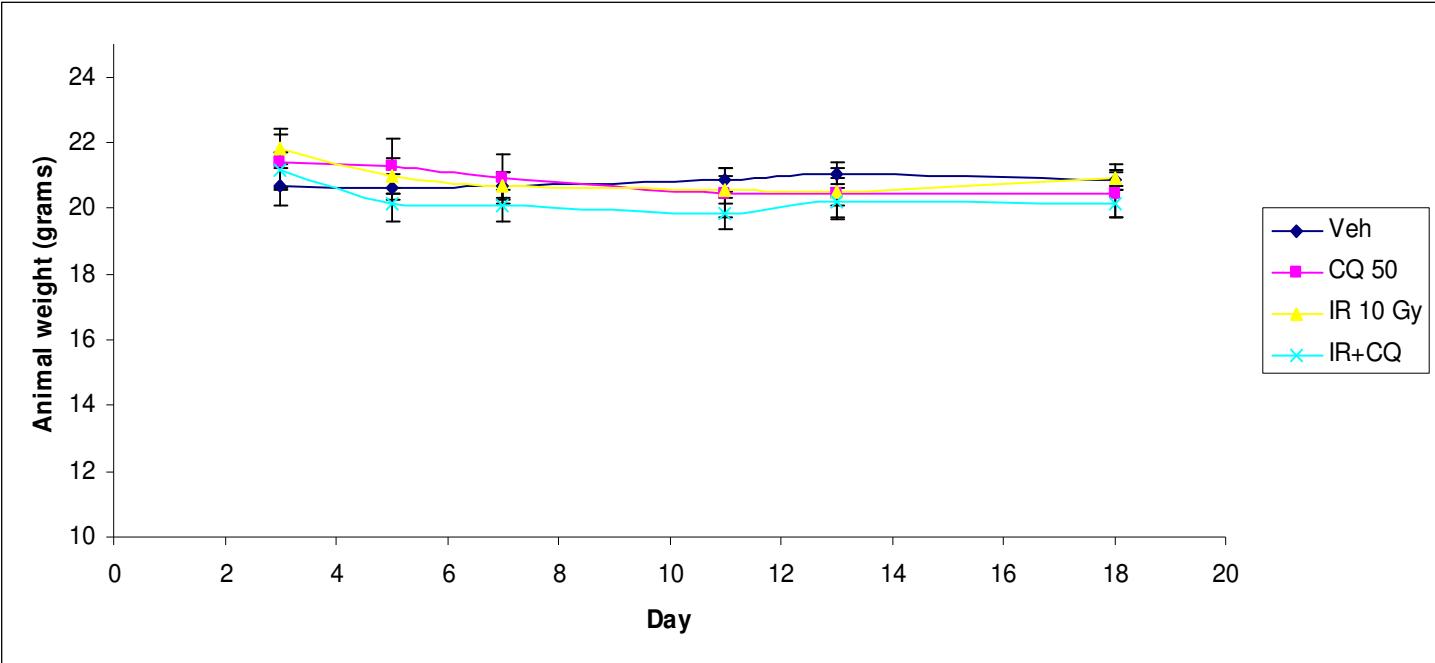
Figure 6.14: CQ, IR and IR+CQ do not influence animal weight. Animal body weight was monitored at various time points post-challenge.
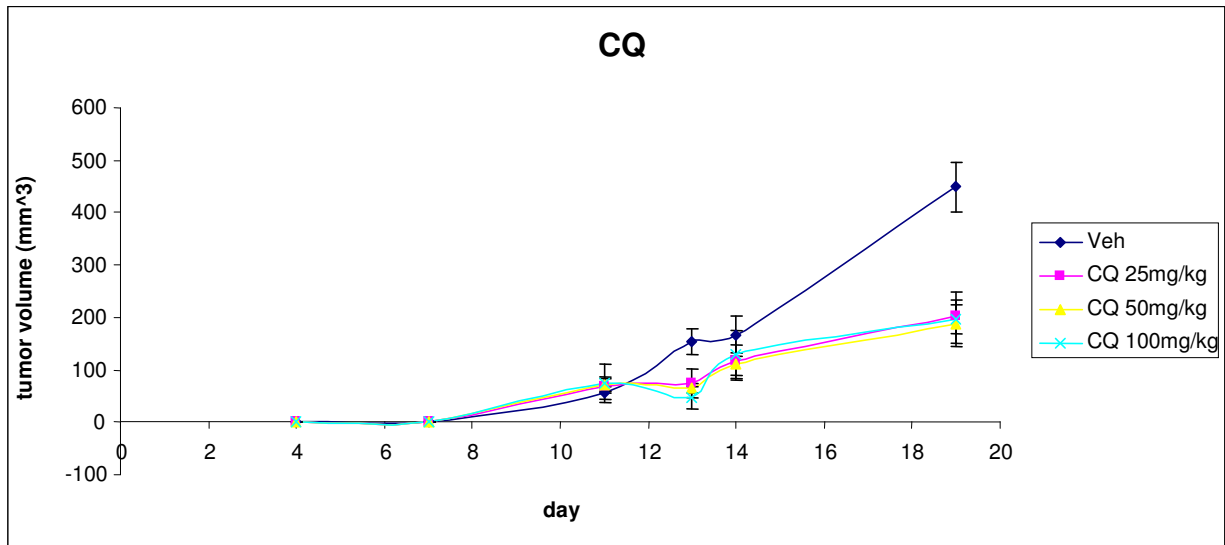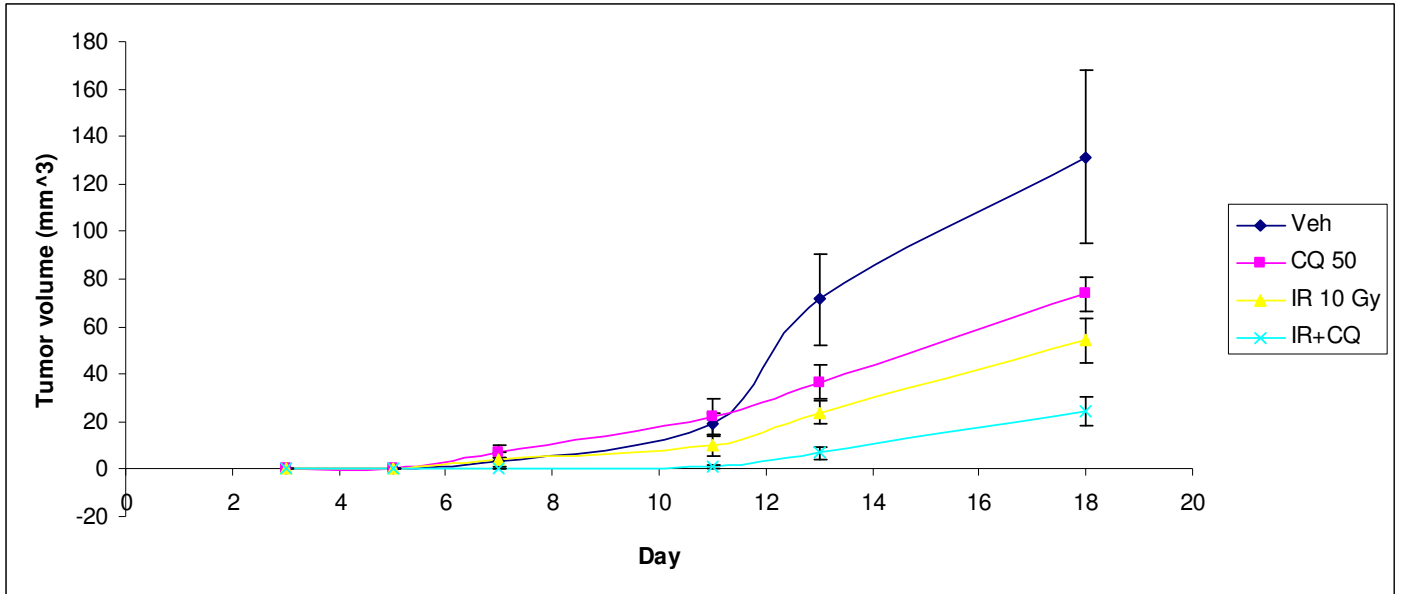
Figure 6.15: Influence of CQ, IR and IR+CQ on tumor volume. Tumor volume was monitored via caliper at various time points post-challenge. *IR and IR+CQ p<0.05 from Veh on day 9. **CQ, IR, and IR+CQ p<0.0001 from Veh on days 13 and 18.
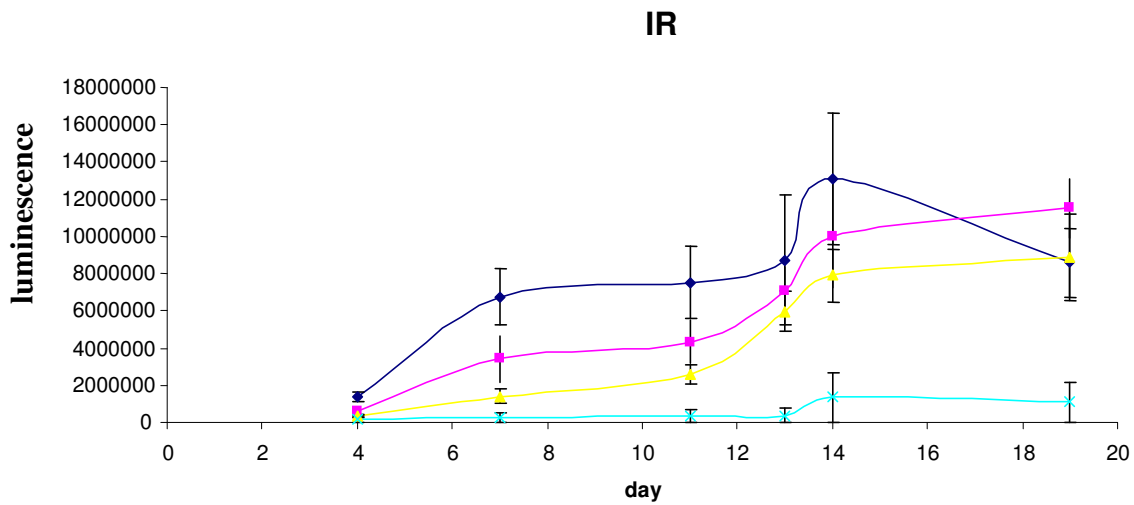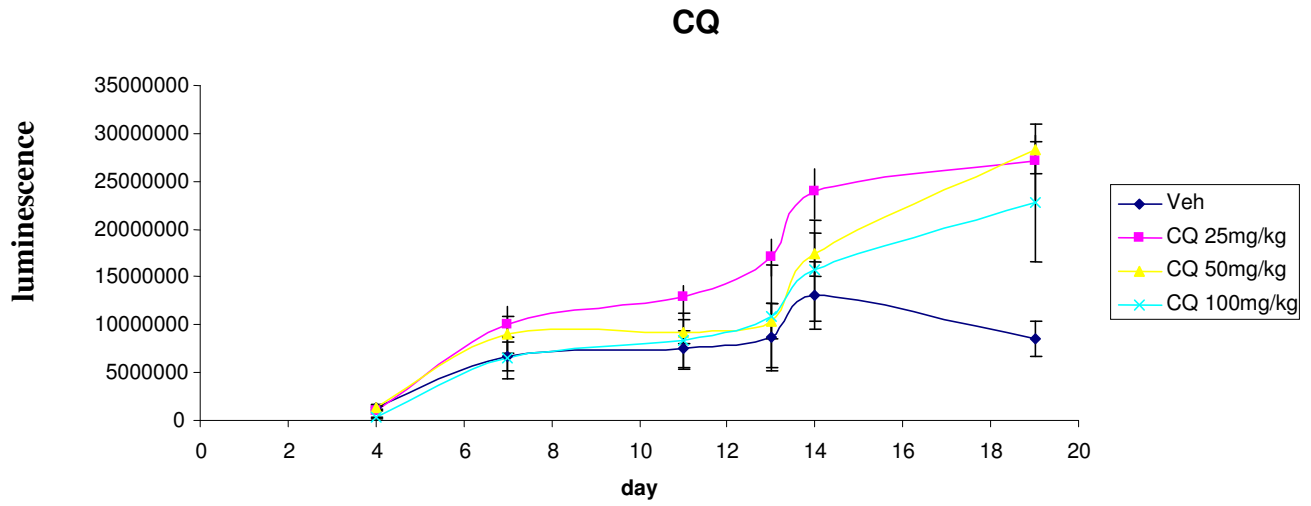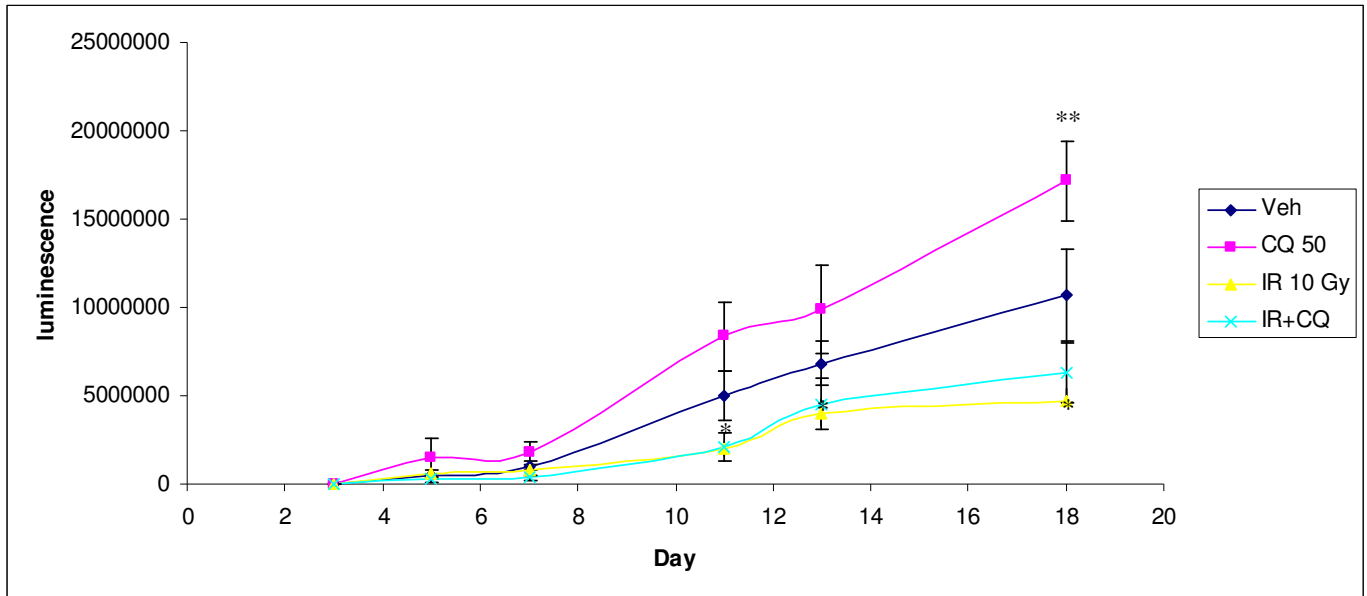
Figure 6.16: Influence of CQ, IR and IR+CQ on bioluminescence. Animals were injected with luciferin and bioluminescence was monitored at various time points post-challenge. *p<0.05 and **p<0.0001
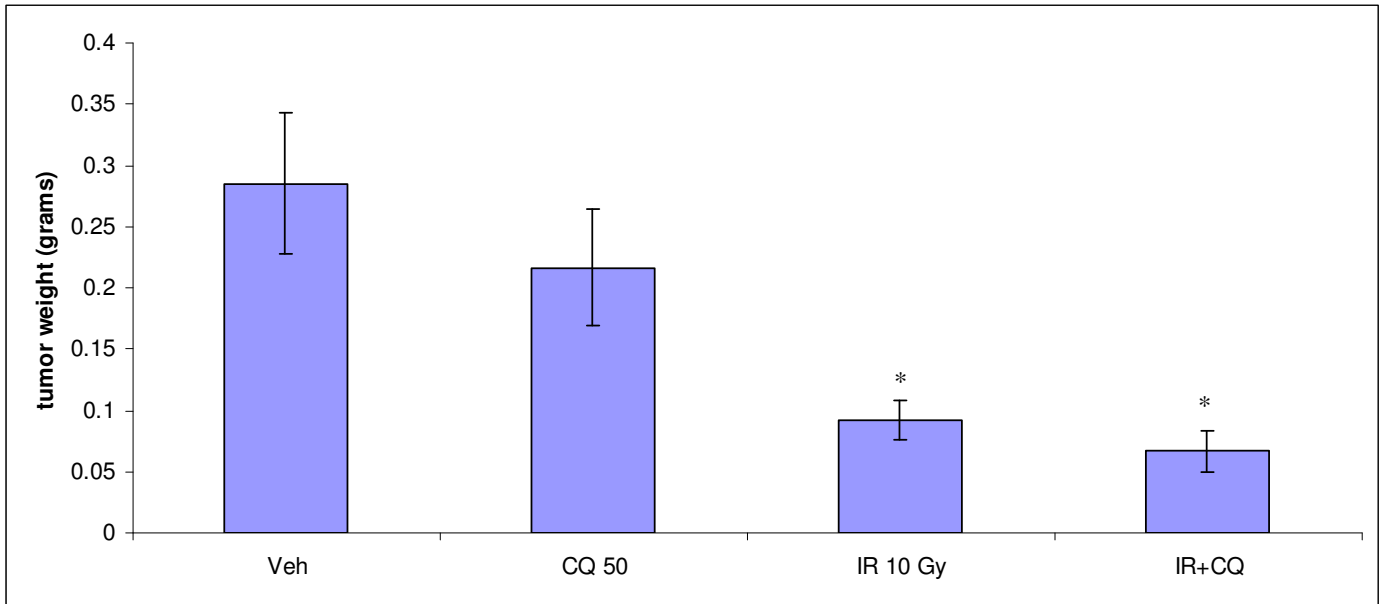
Figure 6.17: Influence of CQ, IR and IR+CQ on tumor weight. Animals were sacrificed and tumors were resected and weighed. *p<0.0001 from vehicle.
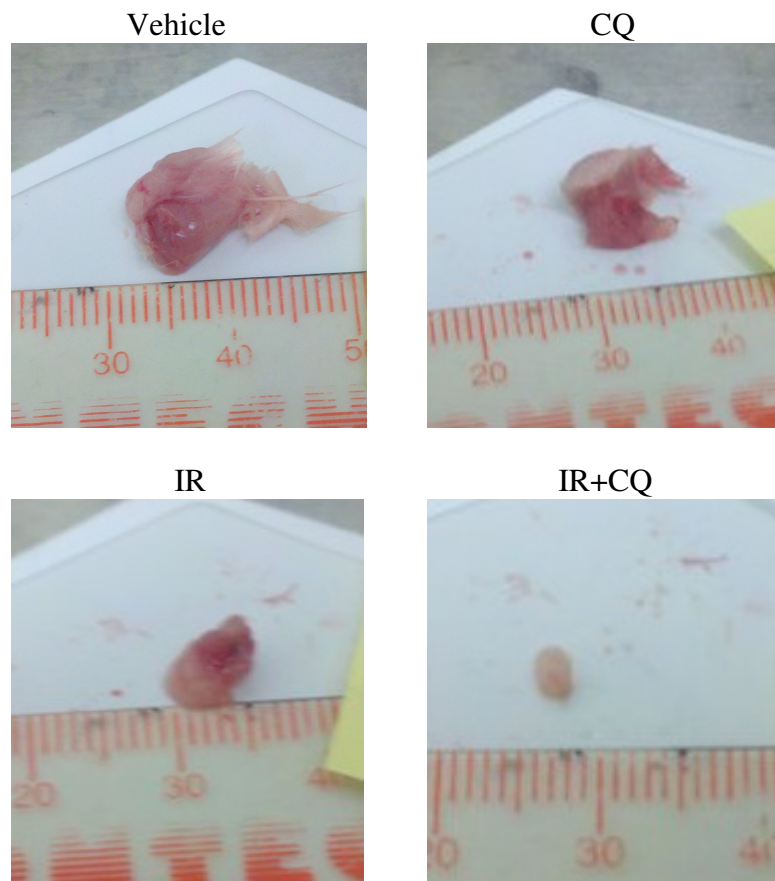
Figure 6.18: Images of resected tumors. Animals were sacrificed and tumors were resected. Representative tumor images are presented.

Chapter 7: Discussion

7.1 In vitro data.

The utility of combining with $1,25D_3$ or Vitamin D_3 analogs with conventional chemotherapeutic drugs such as tamoxifen, platinum compounds, and Adriamycin against a variety of tumor cell lines is supported by multiple studies. (Hershberger et al., 2001; Light et al., 1997; Ravid et al., 1999; Vink-van Wijngaarden et al., 1994; Wang et al., 2000) In addition, phase I and II clinical trials have suggested that high dose intermittent therapy with $1,25D_3$ itself is potentially safe. (Trump et al., 2006) Our previous work (Chaudhry et al., 2001; Demasters et al., 2006; DeMasters et al., 2004; Sundaram et al., 2003) has focused on utilizing $1,25D_3$ and its analogs to confer sensitivity to radiation in the breast tumor cell, apparently through the promotion of autophagy. (Demasters et al., 2006; DeMasters et al., 2004) Previous studies suggested that apoptosis and mitotic catastrophe did not play a vital role in vitamin D induced radiosensitivity; moreover, vitamin D in combination with radiation did not appear to increase DNA damage, alter DNA repair, or modify the generation of reactive oxygen species. (Demasters et al., 2006; DeMasters et al., 2004)

The current work is consistent with the premise in a number of studies that radiation promotes cytoprotective autophagy and that radiation sensitization can be mediated through pharmacological or genetic suppression of autophagy. However, our studies are also consistent with the conclusion that autophagy is the basis for enhanced sensitivity and cell death in response to irradiation when irradiation is preceded by either $1,25D_3$ or the vitamin D analog, EB1089; this conclusion is based on evidence for

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autophagy via multiple assays that include autophagic vesicle formation, RFP-LC3 redistribution and punctuation, electron microscopy and degradation of the p62 protein, as well as lack of evidence for other modes of cell death. Consequently, in this experimental model, it appears that autophagy may have the capacity to play dual roles, both of which can be exploited to enhance the response to radiation.

One evident limitation to confirming the cytotoxic functions of autophagy in this experimental system is that neither pharmacological nor genetic inhibition of autophagy reduce sensitivity to the combination treatment due to the fact that the irradiated cells die by an alternative pathway, that of apoptosis, which is a frequent observation when autophagy is blocked in tumor cells exposed to stress. (Amaravadi et al., 2007; Boya et al., 2005; Gonzalez-Polo et al., 2007) However, in support of our conclusions relating to cytotoxic autophagy by the combination treatment of 1,25 D_3 + radiation, we have reported that in ZR-75 breast tumor cells, *a cell line where cytoprotective autophagy is relatively weak*, the cells are unequivocally protected from the combination treatment by both pharmacologic and genetic autophagy inhibition (manuscript accepted upon revision). Moreover, cytotoxic autophagy by the combination treatment of 1,25 D_3 + radiation can also be observed in the Hs578t cells; the lack of p62 degradation with radiation alone in Hs578t cells also suggests a lack of cytoprotective autophagy and studies are currently being conducted to determine the effects of autophagic inhibition in this system.

An additional complication is that p62 degradation in MCF-7 cells is essentially identical for the cytoprotective response to radiation alone and the cytotoxic action of the 1,25D_3+ radiation combination treatment. This may be indicative of the fact that both the

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cytoprotective autophagy and cytotoxic autophagy are mediated by similar pathways. Moreover, $1,25D_3$'s capacity alone to increase p62 levels may lessen the capacity of the combination treatment to completely abrogate p62. In contrast, only the $1,25D_3$+ radiation combination but not radiation alone promotes p62 degradation in the Hs578t cells, further supporting the premise of increased autophagic flux associated with radiosensitization by $1,25D_3$. In this context, it should be noted that there is virtually no information in the literature relating to the impact of radiation on p62 stability. As p62 function is not limited solely to autophagy but is also associated with ubiquitin mediated protein degradation (Pankiv et al., 2007; Ding et al., 2008) it remains possible that our findings may reflect alternative and perhaps as yet undefined functions of p62.

While there is some evidence that EB1089 or $1,25D_3$ alone can induce autophagosome formation and autophagic flux,(Hoyer-Hansen et al., 2005; Hoyer-Hansen et al., 2007; Tavera-Mendoza et al., 2006; Wang et al., 2008; Yuk et al., 2009) under the experimental conditions of the current studies, no autophagy is observed with EB1089 or 1,25D3 alone. Our current studies with EB1089 and $1,25D_3$ demonstrate comparable temporal responses to both agents alone and in combination with irradiation, indicating equivalent utilities for both drugs. We also show that as with EB1089, $1,25D_3$ suppresses the proliferative recovery that occurs after radiation alone. This component of the response to radiation is potentially of clinical significance since the senescence that otherwise occurs with radiation alone could prove to reflect tumor dormancy that is ultimately succeeded by disease recurrence. (Gewirtz et al., 2009)(Beausejour et al., 2003)

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Our studies also indicate that sensitization can occur even in cells that are intrinsically resistant to radiation through over expression of Her-2/neu. Unexpectedly, MCF7HER2 cells were extremely sensitive to the antiproliferative effects of the $1,25D_3$. Conversely, an apparent deficiency of the Vitamin D receptor in the BT474 cells is likely to be the explanation for their lack of radiosensitization with vitamin D, and the absence of autophagy. Interestingly, Costa et al have suggested that the Vitamin D receptor may be unnecessary for its antiproliferative actions, which could indicate that effects on tumor cell growth and radiation sensitivity are dissociable. (Costa et al., 2009) With regard to the Hs578t cells, we have confirmed the report of radiation resistance and sensitization by $1,25D_3$; however, similar to the findings by Mineva et al., (Mineva et al., 2009) the extent of sensitization was somewhat lower than for MCF7 cells. This may be related to the fact that these cells have mutant p53, as we have reported previously that MCF7/E6 cells (that are essentially null for p53) are relatively refractory to sensitization; (Sundaram et al., 2003) however, other factors such as levels of the metabolic enzymes, 24 hydroxylase and alpha hydroxylase may also prove to influence the response.

Previous studies showed vitamin D did not radiosensitize noncancerous MCF10a cells. (Polar et al., 2003) Likewise, pharmacological blockade of autophagy did not sensitize noncancerous MCF10a cells to radiation suggesting selective radiosensitization against cancer cell lines. Conversely, 4T1 cells, which were not radiosensitized via $1,25D_3$, were signifantly responsive to CQ alone or in combination with radiation *in vitro*.

In summary, these *in vitro* studies are consistent with the possibility that both cytoprotective and cytotoxic autophagy can occur in breast tumor cells exposed to

radiation and furthermore that both forms of autophagy could potentially be exploited for the purpose of radiosensitization.

7.2 In vivo data.

While *in vitro* data and previous studies conducted with 4T1 cells *in vivo* (Jiang et al., 2010) indicated that CQ alone sensitizes female BALB/c mice implanted with 4T1 cells, this was not observed in our animals. A variety of factors may be involved in these differential responses and is discussed below. 4T1 cells rapidly proliferate; palpable tumors quickly become necrotic and ulcerated preventing long term observational studies. The studies by Jiang et al. injected $1x10^6$ cells s.c. per flank (double the amount of cells injected in the studies presented in Chapter 6) and animals were injected with 50mg/kg once a day for 28 days, allowing for palpable tumors to reach over 2000mm^3, which our IACUC protocol will not allow. Moreover, CQ treatment did not significantly alter tumor growth until 37 days post-challenge; rapid ulceration confounds our ability to allow for this late a time point.

A number of studies have been published indicating chloroquine can enhance the response to radiation both *in vitro* and *in vivo*.(Chaachouay et al., 2011; Gaudin and Yielding, 1969; Jiang et al., 2010; Kim et al., 1973; Livesey et al., 2009; Pazmino and Yuhas, 1974; Solomon and Lee, 2009; Zhao et al., 2005) In contrast, our studies indicate that while 4T1 cells were highly responsive to CQ alone or in combination with radiation *in vitro*, CQ did not appear to sensitize 4T1 cells *in vivo*. This may be due to a wide variety of factors; most notably the pharmacokinetic properties of chloroquine in our

system may contribute to this vast disparity. 25μM Chloroquine added *in vitro* is not

subject to rapid catabolism; therefore it is likely that a steady state is maintained

throughout the 72 hour incubation. However, daily 50mg/kg chloroquine given i.p. is

subject to absorption, distribution, metabolism, and excretion and may not allow for

consistent levels to fall within the effective dose range for this system.

7.3 Future studies.

The clinical implications and utility and of vitamin D has considerably expanded

over the last decade. A number of case studies have suggested that vitamin D deficiency

can contribute to an increased incidence, recurrence, and mortality rates in relation to

breast, colorectal, and prostate cancers (Abbas et al., 2008; Bertone-Johnson et al., 2005;

Goodwin et al., 2009; Lowe et al., 2005; Porojnicu et al., 2007; Porojnicu et al., 2008);

moreover, vitamin D_3, and its analogs, have shown promising efficacy when in used in

combination with a number of chemotherapeutics(Van Leeuwen and Pols, 2005). The

observations implicating the inverse relationship of vitamin D_3 levels and cancer needs

further elucidation into the mechanism behind this and the systemic levels of vitamin D_3

needed for these effects.

Primary breast cancers often respond quite well to initial treatment. Regrettably, a

subset of patients experience disease recurrence or metastatic spread of the disease

resulting in an increase in morbidity and mortality. The impact of current and future

studies lies in the prospect that vitamin D_3 in combination with radio- or chemotherapy,

may prevent proliferative recovery and disease recurrence. Furthermore, vitamin D_3 may

serve to sensitize cancers that might not be as initially responsive to treatment; our laboratory has now expanded vitamin D_3 combinational studies to include lung, pancreatic, ovarian cancers.

While vitamin D_3 clearly radiosensitizes breast tumor cells through autophagy, the mechanism by which has still yet to be elucidated. In several breast cancer cell lines, $1,25D_3$ can decrease bcl-2 expression (Van Leeuwen and Pols, 2005). The dissociation of beclin-1 from bcl-2 is essential for its autophagic activity; additionally bcl-2 only inhibits autophagy when it is present in the endoplasmic reticulum (ER) (Maiuri et al., 2010). Current studies suggest that an increased incidence of ER stress is observed with $1,25D_3$ and radiation in comparison to radiation alone. It is therefore possible that $1,25D_3$'s ability to reduce bcl-2 expression, allows for the uncoupling of beclin-1, which may prime breast tumor cells for ER stress-induced autophagy upon additional insult. Additional studies are currently being conducted to further clarify these pathways.

Complementary to our $1,25D_3$ studies, our data also indicates CQ may have radiosensitizing properties. This may be of particular use in tumor cell lines which utilize autophagy as a protective mechanism, and in those lacking the vitamin D receptor. Moreover, CQ is already FDA approved for the treatment of malaria and is readily available for use in the clinic. Additionally, because of minimal toxicities with use, CQ may have greater clinical utility than $1,25D_3$. With these indications, our data has led to the initiation of a phase I clinical trial that will utilize CQ in combination with radiotherapeutics in all cancer subtypes.

7.4 Additional Points.

Though autophagy appeared to play a role in $1,25D_3$ radiosensitization of Hs578t cells breast tumor cells, apoptosis was also found to be significantly increased with the combination. Additionally, the level of radiosensitization observed in Hs578t cells was not as dramatic as observed with MCF7 cells. While a variety of factors may contribute to this differential response, it may be due in part to the mutant status of p53 in the Hs578t cells. Previous data in our laboratory found that in MCF7/E6 cells, with attenuated p53 function, there was no detectable difference in viability with radiation alone or in combination with EB1089 (DeMasters et al. 2006). Correspondingly, 4T1 cells that are null in p53 responded like MCF7/E6 cells in that there was no detectable difference in viability with radiation alone or in combination with $1,25D_3$; suggesting p53 functionality plays a role in the sensitizing effects of vitamin D in response to radiation.

While it was our expectation that inhibition of autophagy in ZR-75 cells with functional caspase 3 would induce apoptosis, similar to the observation in MCF7 cells, these cells appear to be relatively refractory to apoptosis. In support of our conclusions relating to cytotoxic autophagy by the combination treatment of $1,25 D_3$ + radiation, data developed by Eden Wilson indicates that in ZR-75 breast tumor cells, *a cell line where cytoprotective autophagy is relatively weak*, the cells are unequivocally protected from the combination treatment by both pharmacologic and genetic autophagy inhibition (manuscript accepted upon revision). Additionally, utilizing a lower dose of CQ in combination with a single dose of 4 Gy IR in MCF7 cells, Wilson et al. was able to reduce blockade of cytoprotective autophagy. Employing this treatment paradigm,

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pharmacological inhibition of autophagy in MCF7 cells treated with radiation combination with 1,25D$_3$, allowed for recovery of proliferative capacity. This data further supports our contention that autophagy is the main mode of cell death induced by vitamin D radiosensitization.

Controversy in the current literature debates whether autophagy can be both cytoprotective or cytotoxic. Moreover, it is currently not well understood as to what may be involved in the autophagic transformation between death and survival (Klionsky et al., 2008; Mizushima et al., 1998; Mizushima and Klionsky, 2007; Mizushima et al., 2008). Our laboratory's current strategy is to determine the differences between cytoprotective and cytotoxic autophagy. Studies will utilize mass spectrometry to determine if there are any observable alterations in protein levels between radiation in the presence or absence of 1,25D$_3$. Moreover, gene arrays are currently being analyzed to elucidate alterations in gene expression between 1,25D$_3$, radiation, and the combination.

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Molly Lea (Hilliker) Bristol was born on October 15,1984 in Dunkirk, New York

and is a citizen of the United States of America. Molly grew up in the town of Forestville,

New York and graduated in 2002 from Forestville Central High School. Molly graduated

with a Bachelor of Science Degree in Chemistry with a minor in Biology from Virginia

Commonwealth University in May of 2006. In August of 2006, Molly began the PhD

program in the department of Pharmacology and Toxicology at Virginia Commonwealth

University, Medical College of Virginia Campus.

Research and Professional Experience
Positions and Employment:

2006-2011	Graduate research, Gewirtz Lab, Department of Pharmacology and Toxicology VCU
2005-2006	Undergraduate research, Li Lab, Department of Pharmacology and Toxicology VCU

Research Skills and Qualifications:

Cell culture:

Cell lines used – MCF-7 (parental, RFP-LC3 transfected, ATG5 -/-, and ATG7-/-), MCF-10a, BT474, Hs578T, 4T1 (parental and luciferase transfected)
Development of novel knock down cell line from MCF-7 cells (ATG5-/-)

Cell Biology techniques:

Cell Viability Assay with Trypan Blue exclusion, Crystal Violet cell growth assay, MTT Cell Viability assay, Clonogenic Survival Assay

Microscopy and imaging:

β-Galactosidase staining senescence assay, TUNEL staining for apoptosis, DAPI nuclear staining, Acridine Orange staining for autophagy, Use of Inverted Fluorescent Microscope, Use of Transmission Electron Microscope

Molecular Techniques:

Bradford and Lowry assays for protein analysis, Western blot analysis (Using both licor and chemiluminescence), primer design, HPLC

<u>Other Certifications:</u>

Use of the ^{137}Cesium irradiator for *in vivo* and *in vitro* studies

<u>Histology and tissue preparation:</u>

Dissection and harvesting of epithelial cells from bovine coronary arteries

<u>Animal handling (rodents):</u>

In Vivo drug administration: Injections (I.P., Subcutaneous, I.C.V.) In Vivo Tumor Growth Techniques: basic tumor histology analysis, Xenograft implantation and animal monitoring (using 4T1 cells), Xenogen measurement of tumor growth using luciferin

<u>Behavioral assays:</u>

Rotorod, Hot Plate, Tail Flick, Body Temperature measurement, Catalepsy

<u>Proficient in the use of:</u>

Microsoft Word, Excel, Power Point, QCapturePro, SigmaPlot, Internet

Grants and Fellowships:

10/01/08-09/30/11   DOD Predoctoral Traineeship Award; BC083070 - "Modulation of Radiation-Induced Senescence and Proliferative Recovery in the Breast Tumor Cell by Vitamin D through Promotion of Autophagy" Score 1.5 (1.5, 1.5); $95,013.

Presentations, Posters and Scientific Abstracts:

2011   Bristol ML, Di X, Gewirtz DA. "Dual cytoprotective and cytotoxic functions of autophagy in the response of breast tumor cells to radiation and in radiosensitization by vitamin D3". American Association of Cancer Research 101st Annual Meeting 2011 in Orlando Flordia. Abstract and Poster.

2010   Bristol ML, Henderson S, Gewirtz DA. "Autophagic Potentiation of Radiosensitization by Vitamin D3 and the Analog, EB1089". 2010 VCU Massey Cancer Center Research Retreat. Abstract and Poster.

2010	Department of Pharmacology and Toxicology – Seminar Series. "The autophagic response of breast tumor cells to radiation and in radiosensitization by vitamin D3." Presentation.
2010	Bristol ML, Henderson S, Gewirtz DA. "Autophagic Potentiation of Radiosensitization by Vitamin D3 and the Analog, EB1089". American Association of Cancer Research 100th Annual Meeting 2010 in Washington DC. Abstract and Poster.
2010	Hilliker ML*, Di X, Henderson S, Gewirtz DA. "Autophagic Potentiation of Radiosensitization by Vitamin D3 and the Analog, EB1089". IWH- Women's Health Research Day Annual Meeting 2010. Abstract and Poster.
2009	Gewirtz DA, Hilliker ML*, Wilson E. "Autophagy as a mechanism for tumor radiosensitization". 11th International Wolfsberg Meeting on Molecular Radiation Biology 2009. Abstract.
2009	Department of Pharmacology and Toxicology – Seminar Series. "Potentiation of the Response to Ionizing Radiation by Vitamin D3 and Vitamin D3 Analogs". Presentation.
2008	Hilliker ML*, Gewirtz DA. "Potentiation of the response to ionizing radiation in breast tumor cells by the vitamin D3 analog, EB 1089". American Association of Cancer Research 100th Annual Meeting 2009 in Denver, CO. Abstract and Poster.
2008	Department of Pharmacology and Toxicology – Seminar Series. "Potentiation of the Response to Ionizing Radiation by Vitamin D3 and Vitamin D3 Analogs" Poster and Presentation.
2007	Department of Pharmacology and Toxicology – Seminar Series. "EB1089-Induced Radiosensitization in MCF-7 Breast Cancer Cells". Poster and Presentation.

Publications:

Bristol ML, Di X , Beckman M , Wilson E, Henderson S, Maiti A, Fan Z, Gewirtz DA. Dual cytoprotective and cytotoxic functions of autophagy in the response of breast tumor cells to radiation and in radiosensitization by vitamin D3. *Currently under review*

Wilson EN, Bristol ML, Di X, Maltese WA, Koterba K, Beckman MJ, Gewirtz DA. A switch between cytoprotective and cytotoxic autophagy in the radio sensitization of breast tumor cells by chloroquine and vitamin D. Hormones and Cancer. *Currently accepted upon minor revisions.*

Di X, Bristol ML, Henderson SC, Valerie K, Hawkins A, and Gewirtz DA. Independence of autophagy and senescence responses to chemotherapy-induced DNA damage. *Currently under review*

Accepted invitation for review article on the role of autophagy in mediating antitumor effects of select agents for the journal, Frontiers in Pharmacology – to be submitted July 2011.

Gewirtz DA, Bristol ML, Yalowich JC. Toxicity issues in cancer drug development. Current Opinion in Investigational Drugs 2010

Gewirtz DA, Hilliker ML*, Wilson EN. Promotion of Autophagy as a Mechanism for Radiation Sensitization of Breast Tumor Cells. Radiotherapy and Oncology 2009. 92(3):323-8.

Li N, Yi F. Sundy CM, Chen L, Hilliker ML*, Donley DK, Muldoon DB, Li PL. Expression and actions of HIF prolyl-4-hydroxylase in the rat kidneys. Am J Physiol Renal Physiol 2007; 292 (1) F207-16.

*Hilliker to Bristol name change

Awards, Honors, and Elected Positions:

2011-Present	ADHOC Journal Reviewer – *Autophagy*
2010-Present	American Association for Cancer Research: Associate member
2008-2009	Pharmacology and Toxicology Student Organization (PTSO): Treasurer
2002-2006	VCU: Deans Scholarship
2002-2006	VCU: Supplemental Merit Award
2003-2006	VCU: Dean's List
2003-2006	Pathobiology of Pagets Disease Scholarship
2006	VCU B.S. Chemistry – Cum Laude
2002	Forestville Central School, Forestville, NY, valedictorian