THE ROLE OF THE BCL-X GENE AS A TUMOR SUPPRESSOR

A Senior Scholars Thesis

by

FARAH EBONI MCCORVEY

Submitted to the Office of Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Approved by:

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

The Role of the Bcl-X Gene a as Tumor Suppressor (April 2007)

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Cell death, also known as apoptosis, is necessary in order to maintain a healthy tissue state. When cell death doesn't occur and cells grow uncontrollably, one pathological result is cancer. One of the genes believed to regulate cell death and act as a tumor suppressor in certain types of cancers (e.g. breast cancer) is the gene Bcl-x. I seek to determine if Bcl-x is an effective tumor suppressor in hopes that it may be used in gene targeting for treating breast cancer. The methods of my project included quantitating the Beclin levels in breast cancer cell lines, transfecting Bcl-x in to the cells. Lastly, I induced apoptosis and analyzed the susceptibility of the cells to death. Our results showed that increased Bcl-x expression did not reduce this apoptotic induction.

Acknowledgements

I would like to thank Dr. Rucker and all of the VTPP 485 and work study students that participated in this research project.

NOMENCLATURE

PCR	Polymerase Chain Reaction
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
PCD	Programmed Cell Death
cDNA	Complimentary Deoxyribonucleic acid
C-terminus	Carboxy Terminus
GADPH	Glyceraldehyde- 3 Phosphate Dehydrogenase
PBS	Phosphate- buffered Saline
ul	microliter
ml	milliliter
nm	nanometer
mg	milligram

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CHAPTER I

INTRODUCTION: PROGRAMMED CELL DEATH

Cell death is a natural and necessary process. Cells must continuously grow and die in order to maintain healthy tissue state. If too many cells are present, improper development will occur, just as if too few cells are present. One way cells maintain homeostasis is by programmed cell death (PCD). The two types of PCD are apoptosis (or cell suicide) and autophagic (or self- eating) cell death. They are two distinct mechanisms employed by cells to maintain balance within a given environment. Though they each end in cell death, their characteristics and conditions are quite distinct.

Apoptosis

Apoptosis is an essential process of multicellular development. Its role is to destroy excess, defective or potentially hazardous cells. In order for an organism to grow properly, millions of cells must die and be replaced with new cells everyday. The instrumental players in the mechanism of apoptosis are a protein family called caspases. These caspases are inactive precursor (zymogen) proteases with cysteine active-sites. Each caspase must first be activated by cleavage at two aspartic acids in its protein sequence. This activation is often carried out by previously activated caspase molecules. Once activated, the caspases are considered active enzymes. These enzymes begin apoptosis by cleaving specific target proteins within the cell. They cleave proteins that support the cytoskeleton, the nuclear membrane, and other supporting structures inside

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This thesis follows the style of Cancer Cell.

the cell. They also free a DNA-degrading enzyme known as DNAse that cuts up DNA within the nucleus. These events allow the cell to dismantle and shrink in size. The final change in an apoptotic cell occurs at the phospholipid membrane. A negatively charged molecule known as phosphatidylserine marks the cell surface so that it may be ingested by neighboring phagocytes. Phagocytosis occurs quickly to ensure that the cell does not leak. (Yu et al., 2006).

Regulation of apoptosis

Though apoptosis is the process of cell death, there are many regulators in place that control this mechanism. An example of an external factor of apoptosis is simply the failure to receive the apoptosis suppressing signals from the environment. The presence of these signals is necessary for survival in most of our cells. These "survival factors" bind to receptors on the cell surface that either block apoptosis promoters or activate apoptosis inhibitors. An internal regulator of the apoptosis mechanism is the Bcl-2 gene family. The Bcl-2 family facilitates the release of the electron carrier, cytochrome c from the mitochondrial membrane into the cytoplasm. Once in the cytoplasm, cytochrome c binds to an adaptor known as Apaf -1. This newly formed complex activates caspase-9, which leads to the surge caspase activation. This in turn speeds up the process of cell breakdown and apoptosis. (Rodriguez-Enriquez et al., 2004; Marx, 2006)

Autophagy

Autophagy is another process of cell degradation. The difference between autophagy and apoptosis is that autophagy takes place entirely from within the cell. Autophagy's

key role is to recycle cellular components in times of environmental stress. It is also important in cellular differentiation and in tissue repair. When cells experience DNA damage, hypoxia, nutrient deficiencies, or any change in environmental conditions, the process of autophagy is triggered. The process begins by sequestering organelles and cytosolic proteins in a double membrane structure called an autophagosome or autophagic vacuole. The vacuole membrane then fuses with the cell's lysosomes and releases the contents into the lumen of the lysosome. The lysosome then degrades the contents thus recycling the cellular components. The recycled components can be used as building blocks for macromolecular synthesis and a source of energy that is necessary for the cell to survive during harsh conditions. The process of autophagy can also help to eliminate damaged organelles within the cell without destroying the entire cell. For this reason, autophagy has been thought to precede apoptosis and is a "last attempt" to save the cell. When the situation exceeds autophagy's capability, apoptosis is then triggered. (Levine et al., 2004; Gozuacik and Kimichi, 2004; Marx, 2006)

Regulation of autophagy

Autophagy also has many regulatory mechanisms in place that maintain control, two of which are exhibited during cancer with Bcl- 2 and a gene called Beclin-1. Beclin-1, a homologue of a yeast autophagy gene Apg6, was discovered by Beth Levine of UT Southwestern Medical Center in Dallas. Levine's team found that when Bcl-2 is bound to products of Beclin, autophagy was inhibited. This suggests that Bcl-2 regulates autophagy activity during normal conditions so that autophagy takes place when necessary. (Yue et al., 2006) However, if Bcl-2 activity is excessive, autophagy will be

suppressed and damaged cells will continue to become malignant. Levine's team also found that Beclin-1 was either deleted or expressed at very low levels in a high number of ovarian, prostate, and breast cancers. They performed a subsequent experiment in which they knocked out a single copy of the Beclin-1 gene in mice. The results showed that mice demonstrated a decrease in autophagic activity, an increase in breast tumors and an increase in the occurrence of lymphoma and cancer of the liver and lung (Yue et al., 2003). From these observations, Beclin is presumed to be a tumor suppressor gene. Further experiments were done to test Beclin's role as a regulator of autophagy. Beclin deficient mice were generated and examined. The results showed that those mice exhibited a decrease in the formation of autophagic vacuoles (Qu et al., 2003; Yue et al., 2003). Though Beclin is not essential for autophagosome formation, its loss does cause a decreased autophagic response. Figure 1 show a schematic of the role of Beclin in the process of autophagy.

Scope of project

The purpose of this project is to determine the role of the Bcl-x gene in tumor suppression. Our goal is to introduce Bcl-x into MCF-7 breast cancer cells that express Beclin at very low levels and to chemically induce apoptosis in the cells.

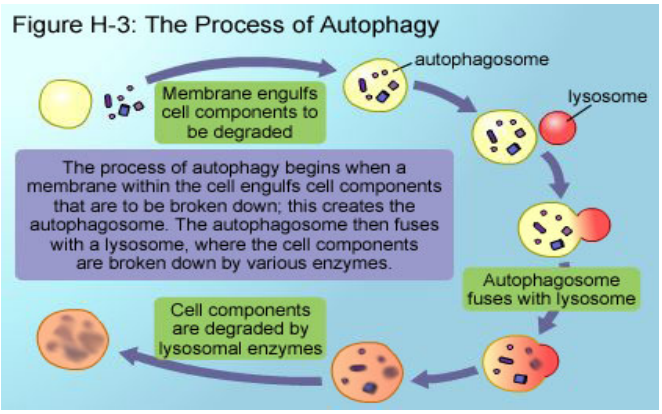

Figure 1. The process of autophagy.
Autophagy and Beclin in the turnover of damaged proteins and organelles. In autophagy the cell membrane engulfs the cell's components that need to be degraded which forms an autophagosme. Beclin-1 aides in the formation of the autophagosome. A lysosyme then fuses with the autophagosome and the cellular components are degraded by the enzymes within the lysosome (image reproduced as courtesy of HOPES).

CHAPTER II

METHODS

The first step of this project was to determine the level of Beclin expression in established MCF-7 breast cancer cell lines. We later transfected the cells with Bcl-x into the cells and induced apoptosis.

Real-time reverse transcriptase PCR

Real-Time PCR was performed on cDNA from a breast cancer cell panel using SYBR green chemistry on the ABI 7500. Beclin expression was normalized to the housekeeping gene GAPDH.

Transfection of MCF-7 cells

MCF-7 cells were transfected with the Bcl-x expression plasmid. This contained a Bcl-x-GFP (green fluorescent protein) coding sequence that encoded for a Bcl-x-GFP fusion protein. The coding sequence was controlled by a CMV (cytomegalovirus) promoter for high level expression. For antibiotic selection, this plasmid also contained a neomycin cassette to select for transfected cells that had geneticin resistance. The first step in transfection was to remove the media on the plates and replace it with 2.5 ml of fresh growth media (DMEM with 10% FBS). The transfection was done by adding 250 ul of serum free media to a clean 1.5ml eppendorf tube. Then 7.5 ul of Mirus transfection solution was added to the tube and the mixture stood for 15 minutes. Next, 2.5 ul of Beclin DNA was added to each of the cell wells. Lastly, the 44 ul of transfection

solution was added drop-wise to the cell wells. The cells were then incubated for 24 hours, after which geneticin containing medium was added (DMEM with 10% FBS and 500 ug/ml geneticin). Selection was carried out for 2 weeks to establish stable transfectants.

Cloning of transfected cells

The geneticin-resistant transfected cells were cloned using sterile cloning disks. The desired cell colonies were isolated with a marker on the bottom of the plate. The media was removed and the plate was washed with 3 ml of PBS. The cloning disks were dipped in 0.05% trypsin solution, placed on the on the plate and the plates were incubated for three minutes so that the cells would be transferred to the disk. While the plates were incubating, 0.5 ml of fresh media were added to each well of a 24 well plate. After incubation the cloning disks were removed and placed into each of the wells of the new 24 well plate. After 24 hours the disks were removed from each of the wells and the cells were trypsinized and transferred to a larger culture dish.

Apoptosis induction and detection

The apoptosis inducer set I (Chemicon) was used to trigger apoptosis in our MCF-7 and Mcf-7/Bcl-x cell lines. This set contained the following chemical inducers of apoptosis at 1000X concentrations: actinomycin D, camptothecin, etoposide, cycloheximide, and dexamethasone. Cells were treated with the specific inducer at a 1X concentration in DMEM for either 4 hours or 24 hours, and then assayed for the apoptosis. We used a

Vybrant® Apoptosis Assay Kit to detect apoptosis in our cells. A negative control was prepared by incubating the cells that did not contain the inducing agent. The cells were incubated and washed with cold PBS. The Annexin-Binding Buffer was prepared by adding 1 ml of 5X Annexin- Binding Buffer to 4 ml of deionized water. Next, a 0.1mg/ml working solution of 7-AAD dye was prepared by adding 5 ul of 7 AAD stock solution to 45 ul of Annexin- Binding Buffer. The washed cells were centrifuged and the supernatant was removed. The cells were resuspended in the 1X Annexin Binding Buffer. Cell density was determined and the cells were diluted so that a 100 ul/ ml assay was prepared. Next, 5 ul of Pacific Blue annexin V and 1 ul of AAD working solution was added to each of the 100 ul cell suspensions. The cells were then incubated for 30 minutes. Next, 400 ul of Binding Buffer solution was mixed in gently and the samples were stored on ice. Lastly, the cells were analyzed using flow cytometry and fluorescence emission was measured at 440 nm violet excitation and at 660 nm with 488 nm excitation. The cells were then separated into three groups: live cells with low level violet excitation and red fluorescence, apoptotic cells with high violet fluorescence and no red fluorescence, and necrotic cells with high level violet and high level red fluorescence.

CHAPTER III

RESULTS

RT- PCR results

A panel of cells was chosen and RT-PCR was performed in order to determine the expression levels of Beclin. Figure 2 shows the relative expression levels of Beclin of the breast cancer cell panel. MCF-7 cells were shown to have one of the lowest expression levels.

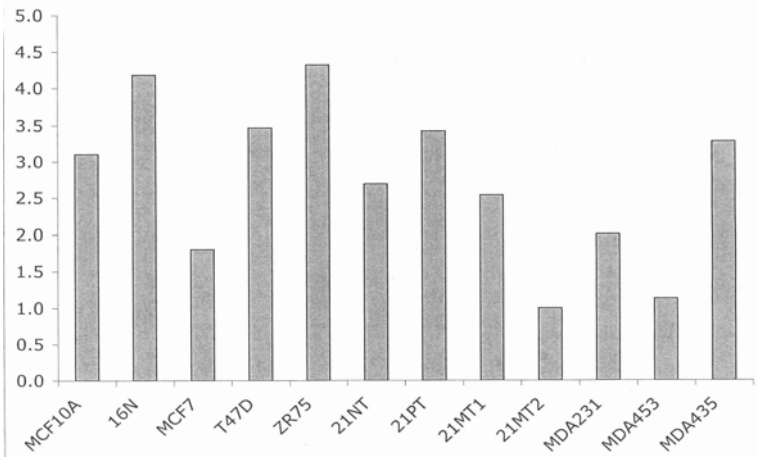

Figure 2. Expression levels of Beclin.
Relative Expression of levels of Beclin compared to GAPDH (a housekeeping gene) by real-time PCR SYBR green chemistry was used on cDNA template from a breast cancer cell panel. MCF-7, 21MT2, MDA453 cells had the lowest levels or expression.

MCF-7 cells over expressing Bcl-x-GFP

Transfected MCF-7 cells were engineered to over express a Bcl-x-GFP fusion protein. These cells are shown to express the fusion protein as shown in Figure 3. Fluorescence microscopy shows that GFP-expression localized to peri-nuclear staining in the cells, which is associated with the distribution of mitochondria. This is expected since Bcl-x interacts with the mitochondrial membrane in providing resistance to the induction of apoptosis.

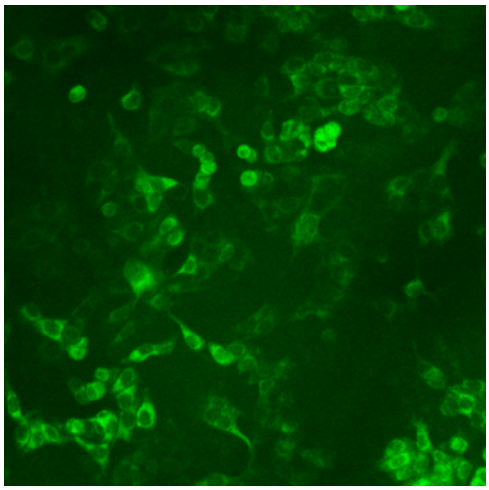

Figure 3: GFP labeled MCF-7 cells.
This is an image of the results of a Fluorescence microscopy of stable MCF-7 cells engineered with a Bcl-x-GFP transgene. This transgene expresses a Bcl-x-GFP fusion protein for tracking Bcl-x within the cells.

Apoptosis

Out of the five apoptosis inducers tested, only two were able to initiate apoptosis at the concentrations tested: camptothecin and actinomycin D. In addition, differences

between control (no inducer) and treatments were found after 24 hours but not 4 hours. Cell counts revealed a 4-fold reduction in the number of viable cells from camptothecin or actinomycin D treated cells versus controls. Camptothecin inhibits nuclear topoisomerase, thus preventing DNA replication; in contrast, actinomycin D inhibits RNA synthesis. Morphologically, treated cells contained pyknotic (condensed) nuclei and were detaching from the plates. Surprisingly, Bcl-x over expression did not reduce this apoptotic induction.

CHAPTER IV

SUMMARY AND FUTURE WORKS

Bcl-x is vital for the regulation of autophagy and apoptosis by its interaction with the tumor suppressor protein Beclin. When bcl-x is under expressed apoptosis does not occur and a damaged cell will continue to proliferate which can result in cancer. We have selected MCF-7 cells that express Bcl-x at very low levels. We transfected Bcl-x into MCF-7 cells, cloned those cells and tested their susceptibility to undergo cell death. After inducing apoptosis we found that Bcl-x over expression did not suppress apoptosis as suspected. This suggests that this type of apoptosis used did not initially engage the mitochnodria.

Future works

In the future we will focus on the specific gene Beclin-1 and determine which region of Beclin is most effective in tumor suppression. We will subclone three fragments into vectors along with an inducible tetracycline into the system that will be transfected into MCF-7 cells. Lastly, we will induce apoptosis and determine if full length Beclin or if one of the three fragments is most effective in tumor suppression. We will compare these cells' susceptibility to apoptosis. This information will allow us to better understand the link between the processes of autophagy and apoptosis, and the regions of Beclin that are necessary to provide tumor suppression; different gene targeting treatments for breast cancer can be developed from this research.

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APPENDIX

LETTER OF PERMISSION

Hi Farah,

Feel free to use any material from the site, as long as you give credit to HOPES.

Regards,

Danny Neumann

Webmaster, the HOPES project

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