MITOCHONDRIA LOCALIZED BCL-XL ACTS AS AN APOPTOSIS INHIBITOR

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ABSTRACT -- The Bcl-2 family member protein BCL-XL is located in the mitochondria of the cell. Using a green fusion protein (GFP) tag and retroviral plasmids, we were able to devise a method of viewing, under fluorescent microscope, the mitochondrial localization of GFP-BCL-XL in HeLa cells. HeLa cells over-expressing the BCL-XL protein were then treated with two pro-apoptotic stimuli; DNA damage (UV treatment) and activation of tumor necrosis factor (TRAIL treatment). Upon treatment and comparison to cells without an active expression of GFP-BCL-XL, we were able to witness the protective characteristics of the BCL-XL protein in both the intrinsic and extrinsic apoptotic pathways.

INTRODUCTION -- Programmed cell death, apoptosis, is a necessary non-inflammatory homeostatic function in the development or terminus of otherwise proliferative tissue (i.e. prevention of finger webbing, unregulated tumor growth, and deletion of toxic tissue). Apoptosis can be achieved by internal signaling of proteins activated by DNA damaging agents, faults in metabolism, or genetic defect. Two significantly known modes of apoptosis occur at the mitochondrial level upon receiving the death signal; the caspase pathway and by organelle dysfunction (Gross). The mitochondrial pathway of apoptosis

is a result of the permeabilization of the outer mitochondrial membrane and subsequent release of cytochrome C. Once released from the mitochondria, not only does Cytochrome C go on to promote caspase activation by forming the apoptosome with Apaf-1 (Figure 1), but also the electron transport chain within the mitochondria cannot be completed with insufficient Cytochrome C and ATP is failed to be produced. Subsequent protease enabled DNA cleavage and phagocytosis of the remaining cell debris is the result.

The major players in damage to the outer mitochondrial membrane are Bax and Bak, which belong to the B-Cell Lymphoma 2 (Bcl-2) family of proteins. Proteins with homologous regions (BH domain) to the Bcl-2 family proteins are responsible for the integrity of the outer mitochondrial membrane and for signaling of Bax and Bak activation. Proteins having the BH3 domain of the Bcl-2 family serve as regulators of pro-apoptotic signal containing three or four BH regions serving to protect the integrity of the mitochondrial membrane and as regulators of Bax-like proteins. Bcl-$_X$l has been chemically shown to have three of the four BH regions. Furthermore, Bcl-Xl is known to be an apoptosis prohibiting molecule. However, the exact mechanism and location of its action are not fully understood. To better characterize the action and localization of Bcl-Xl this project was designed to create a stable and inducible Bcl-Xl expressing HeLa cell line and demonstrate apoptosis inhibition viewable by fluorescent microscopy, Hoechst staining, and Western Blot assay.

METHODS -- *Plasmid Construction*--Bcl-Xl from a previous experiment was isolated from an existing GFC3-BCL-XL plasmid. The GFC3-Bcl-Xl plasmid contains a

sequence for green fluorescent protein attached to the Bcl-Xl sequence. A Polymerase chain reaction (PCR) with forward and reverse primers containing Xho1 and Not1(X/N) restriction sites, respectively was used to extract and amplify the Bcl-Xl. The multiple cloning site of the target vector contains a the Xho1 and Not1 restrictions sites. To amplify the target Bcl-$_X$l sequence, a 50 µl solution containing 1.0 µl of each primer prepared to a concentration of 100µM, 2.0 µg GFC3-Bcl-$_X$L plasmid DNA, 4.0 µl of 2.5mM solution of Deoxyribonucleotide triphosphates (dNTP's), 5.0 µL Taq buffer, 0.5 µL Taq polymerase, 0.5 µL pfu polymerase, and 36.0 µL sterile deionized distilled water (ddH20) was pipetted and thoroughly mixed in a 1.0 mL PCR tube and placed in a thermal cycler. Thermal cycling proceeded as follows; an initialization step at 94˚C for 2 minutes (m), a denaturization step at 94.0ºC for 45 seconds (s), annealing at 56.0 ºC for 45s, and an elongation step at 72.0 ºC for 1m 15s. The denaturization, annealing, elongation steps were repeated 23 times followed by a final elongation step at 72.0 ºC for 10m, and then held at 4˚C until needed for use. After thermal cycling, the PCR product was checked by gel electrophoresis by running 4µl and a 10x loading dye on a 1.2% agarose gel containing ~0.3µg/µl ethidium bromide next to a 1 kilobase pair (kbp) ladder. After running for 12m at 145V, the gel was examined under UV light to determine if the size of the PCR product corresponded to the approximate size of Bcl-Xl (1.5kbp) and a photograph taken.

A QIAgen PCR Purification kit was used to purify any excess dNTP's or reagents from the Bcl-$_X$L PCR product. 200µl of Buffer PB (binding) was directly added to the remaining 46µl of PCR product in the PCR tube, pipetted up and down several times to mix, and then the entire volume transferred to a QIAquick spin column placed inside the

provided 2ml collection tube. This solution was then centrifuged at 13,200 rpm for 1 m. The flow-through was discarded and placed column back into the collection tube.750µl of Buffer PE (wash) was added to the column and centrifuged at 13,200 rpm for 1m. The flow-through was discarded, and the column placed back into collection tube, and centrifuged at 13,200 rpm for 5m. The column was then removed from the collection tube and placed in a sterile 1.5ml microcentrifuge tube. The microcentrifuge tube was then let stand at room temperature with cap open for ~ 5 minutes to dry any remaining ethanol residue. 30µl of sterile ddH2O was added directly to the column membrane and let stand with cap closed for 5 minutes. DNA was then eluted by centrifuging at 13,200 rpm for 2m.

After purification of the GFP-Bcl-Xl PCR product an agarose gel was run using previous methods to ensure the existence of the product.

Enzymatic Digestion -- A pMIG retroviral plasmid was selected as the vector for induction of the GFP-Bcl-Xl sequence. An enzymatic digestion kit from New England Biolabs (NEB), was used to digest the pMIG retroviral plasmid and the GFP-Bcl-Xl PCR product at the Xho1 and Not restriction sites. A master mix for the reaction was prepared with 10.0µl 10x Buffer, 2.0µl 100x BSA, 2.0µl Xho1, 2.0µl Not1, and 45.0µl sterile ddH_2O. 30µl of the master mix was added in a 1.5mL microcentrifuge tube with 20.0µl of the GFP-Bcl-XL product, and 30µl of the master mix was added in a 1.5mL microcentrifuge tube with 10.0µl (1ng/µl) of the pMIG DNA and 10.0µl ddh20. The reactions were mixed well, and then incubated at 37ºC for 2 hours (h). After the DNA was digested, 10µl of 6x Loading Buffer was added directly to each 50.0µl sample. The entire 60 µl volume of each reaction was loaded into a separate well of a prepared 1.2%

agarose gel. Gel electrophoresis was run at 145V for 12 to achieve thorough separation. The gel was then immediately on a piece of Saran wrap and visualized a 365 nanometer (nm) UV lamp. A band of DNA at approximately 1.5 kbp (GFP-Bcl-Xl) a band at approximately 5.0 kbp (pMIG) were then, using a clean razor blade, carefully cut out to the smallest slice possible and the cut out slice of gel placed into a sterile 1.5ml microcentrifuge tube.

Using a QIAgen Gel Extraction kit the digested samples were purified. The gel volume was determined by weight of 0.15g to be approximately 150µl. 450µl of Buffer QG was added to the microcentrifuge tube and warmed at 50ºC for 10m to dissolve the gel completely. The gel was then gently vortexed. The tube was then returned to room temperature and 1 gel volume of Isopropanol added, mixed gently with a pipet, then the contents of each tube transfer to a QIAquick spin column placed inside the provided 2ml collection tube. Each tube was centrifuged at 13,200 rpm for ~1 minute. The flow-through was discarded and column placed back into the collection tube. 750µl of Buffer PE (wash) was added to each column, and the cap closed and let sit at room temperature for 5 minutes. Each tube was then again centrifuged at 13,200 rpm for 1 minute. The flow-through discarded, each column placed back into its collection tube, and centrifuged at 13,200 rpm for 5 minutes. Each column was removed from its collection tube and placed in a new sterile 1.5 ml microcentrifuge tube. Then each tube was let stand at room temperature with cap open for 5 minutes to dry all ethanol. 30µl sterile ddH2O was added directly to the column membrane and let stand with cap closed for 5 minutes for the Bcl-Xl DNA and 60µl sterile ddH2O was added directly to the column membrane and let stand with cap closed for 5 minutes. The DNA was then eluted by centrifuging at 13,200

rpm for 2 minutes and placed on ice. The concentration of each sample was determined by NanoDrop. The GFP-Bcl-Xl was approximately 1ng/µl and the pMIG was approximately 3 ng/µl.

2µL of each sample was assayed by Electrophoresis on a 1.2% agarose gel for 12m at 145V to verify the size of the fragments of Bcl-Xl and pMIG DNA samples. The *Ligation* -- To introduce the GFP-Bcl-$_X$l into the pMIG vector a ligation reaction was concocted in which the complementary pairing of the enzymatic restriction sites of Xho1 and Not1 would form bonds. A quick ligation kit from NEB was used to ligate the X/N digested GFP-Bcl-Xl fragment into the X/N digest pMIG plasmid. A master mix containing 1.0µl of the pMIG solution, 10.0 µL 2x Quick Ligase Buffer, 1.0µL Quick Ligase, and 7.0µL water was added to a 1.5mL eppendorf tube containing 1.5µL of the GFP-Bcl-$_X$l solution and to a control tube containing 1.5µL water. This reaction was then left at room temperature (RT) for 9m.

Transformation -- 5µl of each ligation reaction was pippeted into a new 1.5ml tube on ice. 50µl of DH5α competent *E.Coli* cells were pipetted directly to each tube, and pipetted to mix. The tubes were then placed on ice for 30 minutes. After incubating the tubes on ice, each tube was heat shocked in a 42ºC water bath for 1 m 30s. Then placed immediately back on ice for 30 seconds. The tubes were removed from ice and the entire volume pipetted from each tube into a sterile 14ml round-bottom culture tube containing 300µl of Sigma Aldrich LB Brtoh+20mM Glucose. The tubes were then shaken at 220 rpm for 1 h at 37ºC. After 1 hour of shaking, the entire volume of each tube was pipetted over the surface of separate LB agar plate containing Ampicillin. The plates were then

inverted and incubated overnight at 37˚C. After 16h, the plates were removed from the incubator and the colonies observed for each.

2 colonies were selected from each plate and placed in sterile 14ml round-bottom culture tube containing 5ml of LB with ampicillin. The tubes were shaken overnight at 220 rpm in a 37ºC incubator. After 16h the tubes were removed from the shaker. The plasmid DNA was then purified using a Wizard Plus SV Miniprep DNA Purification Kit. The shaken culture was pelleted at 2600 relative centrifugal force (rcf) for 5 minutes. ALL the LB supernatant was drained by vacuuming off the entire volume of LB, and inverting the tube over an absorbent disposable surface for 2 minutes, and then the liquid was dabbed and wiped away from the mouth and sides of the tube using a kim wipe. 250µl of Cell Resuspension Solution was added to each pellet, and the cells were resuspended and the whole volume of each tube transferred to a 1.5ml tube. 250µl of Cell Lysis Solution was added to each sample and inverted 5 times to mix then incubated 2 minutes at room temperature to ensure thorough lysis. 10µl of Alkaline Protease Solution was added to each sample and inverted until homogenous and incubated for 5 m at room temperature. 350µl of Neutralization Solution was added to each sample and inverted 5 times to mix. After a precipitate of cell debris formed on the surface of the solution each tube was centrifuged 13,200 rpm for 10 m at RT to pellet cell debris. The supernatant was pipetted into a spin column and placed inside a 2ml collection tube. Each tube was then centrifuged the samples at 13,200 rpm for 1 m. The flow-through was then discarded and the column reinserted into the collection tube. 750 µl of Wash Solution was then added to each tube and centrifuged at 13,200 rpm for 1 m. The flow-through was then discarded and the column reinserted into the collection tube. 250µl of Wash Solution was added

and the tubes were centrifuged at 13,200 rpm for 5 minutes. The spin column was then transferred directly to a sterile 1.5ml tube and let sit for 5 minutes at RT to dry any remaining ethanol. 60µl of Nuclease-Free Water was then added to the spin column and let sit for 5 minutes at room temperature to dissolve the DNA. Each tube was then centrifuged at 13,200 rpm for 2 minutes to elute the DNA.

Clone Screen -- To enzymatically digest and screen for positive transfer of clones a 2x master mix of 4.0µl 10x Buffer, 1.0 µl 100x BSA, 1.0µl Xho1, 1.0µl Not1, and 28µl sterile ddH2O was added to 3 µl of each plasmid DNA and incubated for 1 hr at 37˚C. After digestion was completed, electrophoresis on a 1.2% agarose gel was performed at 145V for 12m and viewed under 365nm UV light to determine if there was indeed successful transformation.

Viral Generation -- The transfection for viral was prepared with 40ng of the pMIG-GFP-Bcl-$_X$l vector (this was done by diluting in water), 700ng pcDNA, and Buffer EC to a75uL total volume, then adding 3uL Enhancer. The reactions were incubated at RT for 5 minutes after which, 4uL Effectene was added and incubated for 15 minutes. The reactions were each added to separate 300uL aliquots of Dulbeccos Modified Eagle Medium (DMEM) and then added dropwise to 293-GP DNA packaging cells plated 2.5 x 10^5 cells per 35mm plate which were then placed at 37˚ C. The same reaction was performed with the empty pMIG-GFP vector. Virus was harvested 48 hrs after transfection and filtered through a 0.45 µg cellulose acetate filter.

Infection -- A 1:5 harvested uncut pMIG-GFP and pMIG-GFP-Bcl-$_X$l virus to DMEM 10% in a total volume of 3 mL were introduced to separate plates of HeLa cells plated at

2.5 x 105 cells per 35mm in the presence of Polybrene (10 µg/mL) and allowed to incubate for 24h.

Treatment -- Infected HeLa cells were exposed to 200 power UV and treated with 12.5ng TNF-Related-Apoptosis-Inducing Ligand (TRAIL). The cells were counted and stained 8hrs after UV and 5hrs after TRAIL treatment.

Cell Counting -- After treatment, a 1:15000 dilution of Hoechst Stain was added to the each plate and allowed to incubate for 15 min. Duplicate images (GFP filter and Hoecsht filter) were taken in two randomly selected locations for each culture plate (Figure 7). Percent GFP-positive cells undergoing pycnotic nuclear condensation (an indicator of apoptosis) among all of the GFP-positive cells was calculated for each viewing area. Untreated cells were then harvested and assayed by Western Blot to further verify the expression of the GFP-BCL-XL protein (Figure 6).

RESULTS -- The agarose gel resulting after the PCR amplification revealed a band at 1.5 kbp. After X/N digestion of the PCR product and of the pMIG vector electrophoresis revealed bands at approximately 1.5kbp and 5.0kbp respectively. After ligation and transformation, there was no observable bacterial growth on the plates with the unligated pMIG, while there was significant bacterial growth on the pMIG-GFP-Bcl-Xl plates (Figure 3). After picking colonies and screening for clones by electrophoresis, the colonies picked from the pMIG-Bcl-Xl plates revealed a band at approximately 5.0kbp (Figure 4). After viral infection and viewing under fluorescent microscopy the pMIG-GFP did not show any localization of GFP, as the entire cell appeared green (Figure 6). The pMIG-GFP-Bcl-Xl appeared green in various regions of the cell. The mean apoptotic

cells in HeLa cells with no treatment was 6.9% (SD ± 2.5%). The mean apoptotic cells in HeLa cells treated with UV light (20 J/m2) was 93.7% (SD ±1.5%). The mean apoptotic cells in HeLa cells treated with TRAIL (12.5 µg/mL) was 80.0% (SD ± 5.0%).The mean apoptotic cells in BCL-XL HeLa cells with no treatment was 6.0% (SD ± 1.8%). The Mean apoptotic cells in BCL-XL cells treated with UV light (20J/m2) was 4.0% (SD ± 1.7%). The mean apoptotic cells in BCL-XL HeLa cells treated with TRAIL (12.5 µg/mL) 3.3% (SD ± 2.6%) (Figure 7). Western Blot analysis revealed a protein at 50KD for the harvested GFP-Bcl-Xl and a protein at approximately 26 KD for the GFP when immunoblotted for Bcl-Xl and GFP (Figure 5).

CONCLUSIONS -- Our results show we were successfully able to clone and infect HeLa cells with the Bcl-$_X$l gene. Furthermore these results show a significant difference between apoptotic cells exogenously expressing Bcl-Xl indicating that upon exposure to UV light and treatment with TRAIL, these cells are resistant to programmed death. We were able to show localization of the Bcl-Xl gene when compared to a GFP only control. However, a mitochondrial localization control or further mitochondrial analysis is needed to determine if our localization of Bcl-Xl is to the mitochondria.

DISCUSSION/CLINICAL IMPLICATION -- The Bcl-2 family of proteins is important to understanding the mitochondrial pathway of apoptosis. With viewable mitochondrial localizing Bcl-Xl, the interaction between theses molecules can be studied in more depth. The apoptotic inhibitory action of the expressed Bcl-$_X$l gene and the subsequent localization and presence of the Bcl-$_X$l protein is crucial to understanding apoptosis. As

cancer cells show reduced apoptotic activity understanding apoptotic mechanisms, specifically of Bcl-Xl can lead to more specific chemotherapeutic targeting as well as potential for genetic therapy with these molecules. There is need for further study of the entire Bcl-2 network of proteins as well other molecules, such as cytochrome C, important to the process of apoptosis. The ability to express and monitor these proteins through genetic recombination and viral integration can be further used to monitor the biological importance and function at the molecular level of cellular processing.

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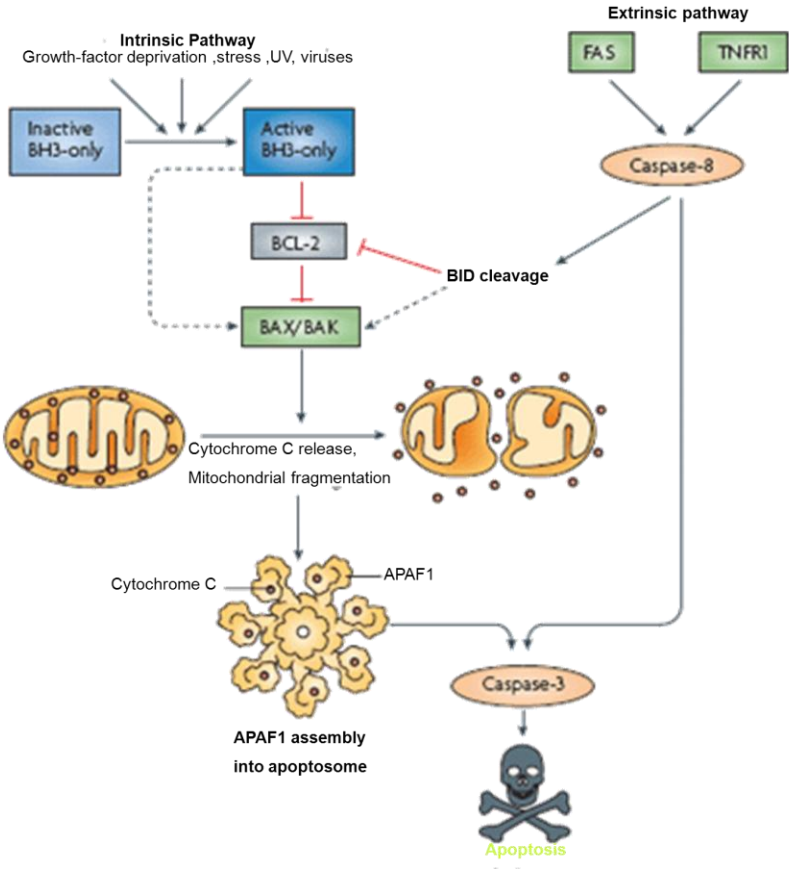

FIGURE 1. Theoretical model of Apoptotic pathways. Adapted from Youle, R.J. & Strasser, A. 2008. Scheme depicting intrinsic and extrinsic pathways of apoptosis. The BCL-2 protein family: opposing activities that mediate cell death. Nature Reviews Molecular Cell Biology.1:50.

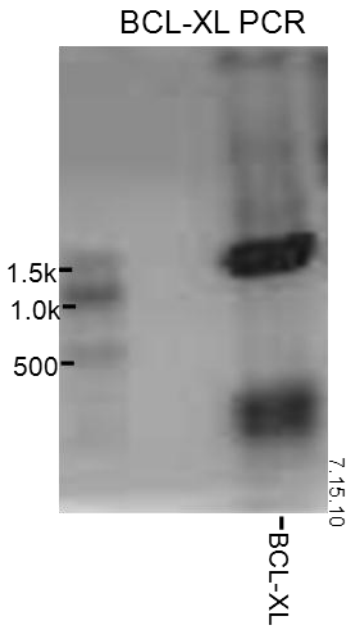

FIGURE 2. PCR for Bcl-$_X$l (~1.5kbp) with Xho1 and Not1 primers. 4 µL PCR reaction was added to 1 µL SDS loading dye and electrophoresed in a 1.2% agarose gel.

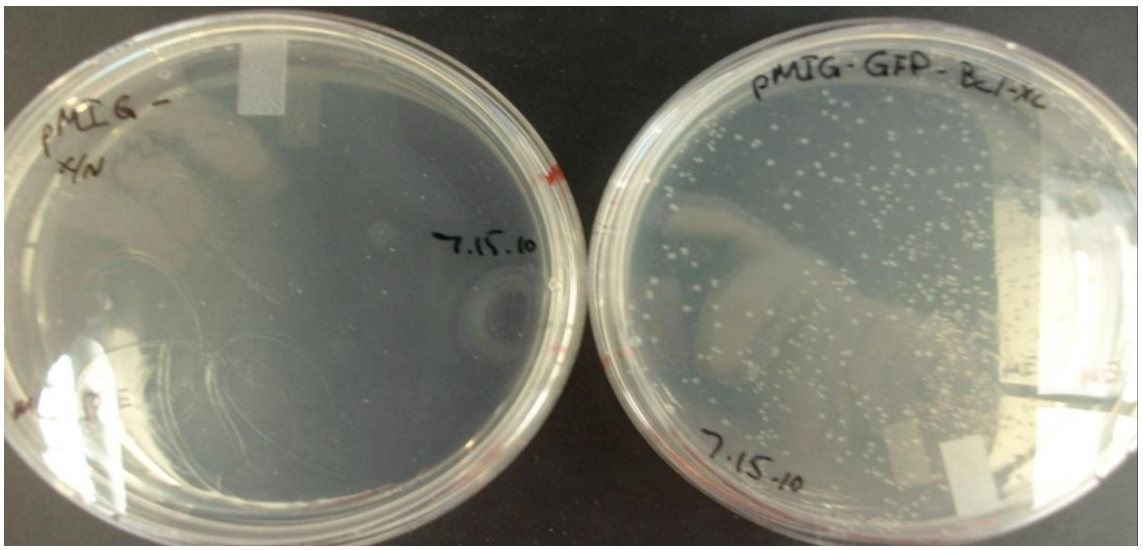

FIGURE 3. X/N pMIG (left) and pMIG-GFP-BCLXL (right) bacterial transformation growth on agar + Ampicillin culture plates.

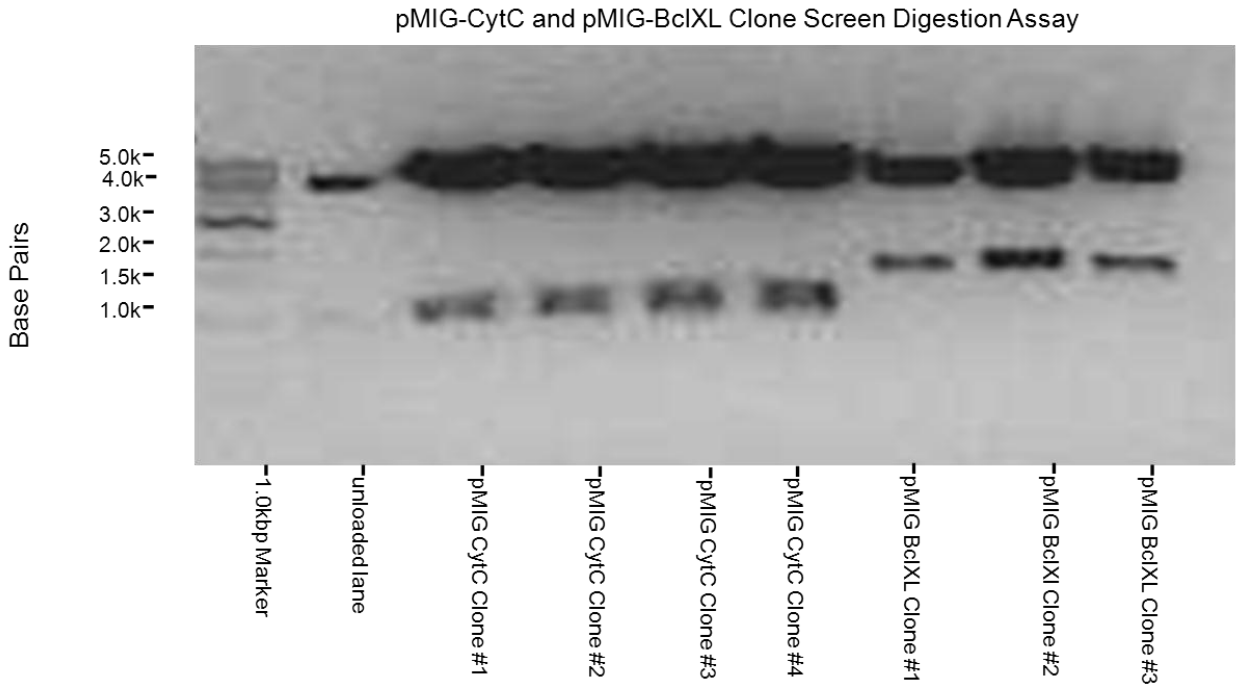

Figure 4 E/N Clone screen digestion reactions for pMIG-CytC positive for clones 1-4 and X/N for pMIG-BclXL positive for clones 1-3 in 1.2% agarose gel. *pMIG-CytC were used in another experiment.

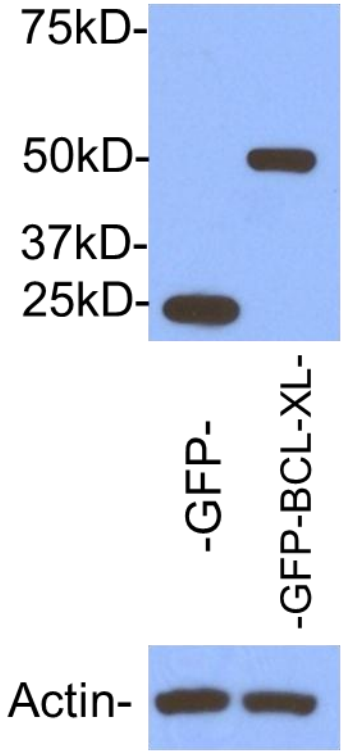

FIGURE 5. Western Blot Assay of BCL-XL expression. Harvested DNA was normalized and ran in an 8% SDS PAGE.

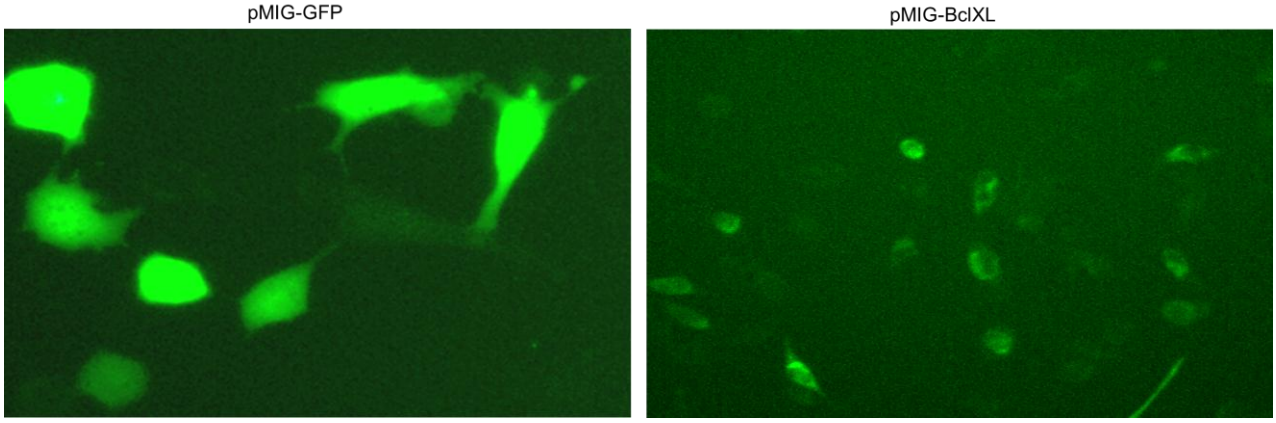

FIGURE 6. To view the mitochondrial localization of BCL-XL, pMIG-Bcl-$_X$l, and pMIG-GFP plasmid DNAs were each transfected into HeLa cells. Each transfection reaction was mixed in a total volume of 75 µL containing Buffer EC the expressing DNA and pcDNA + 3 µL Enhancer. After 10min incubation at room temperature (RT) 4 µL Effectene was added and allowed to incubate at RT for 15 min. 300 µL Dulbecco's Modified Eagle's Medium supplemented with antibiotics and 10% fetal calf serum (DMEM 10%) was added to each reaction. Each reaction was added dropwise to HeLa cells plated 2.5 x 105 cells per 35mm plate. The cells were viewed under ultraviolet microscopy 18hrs after transfection at 10x magnification.

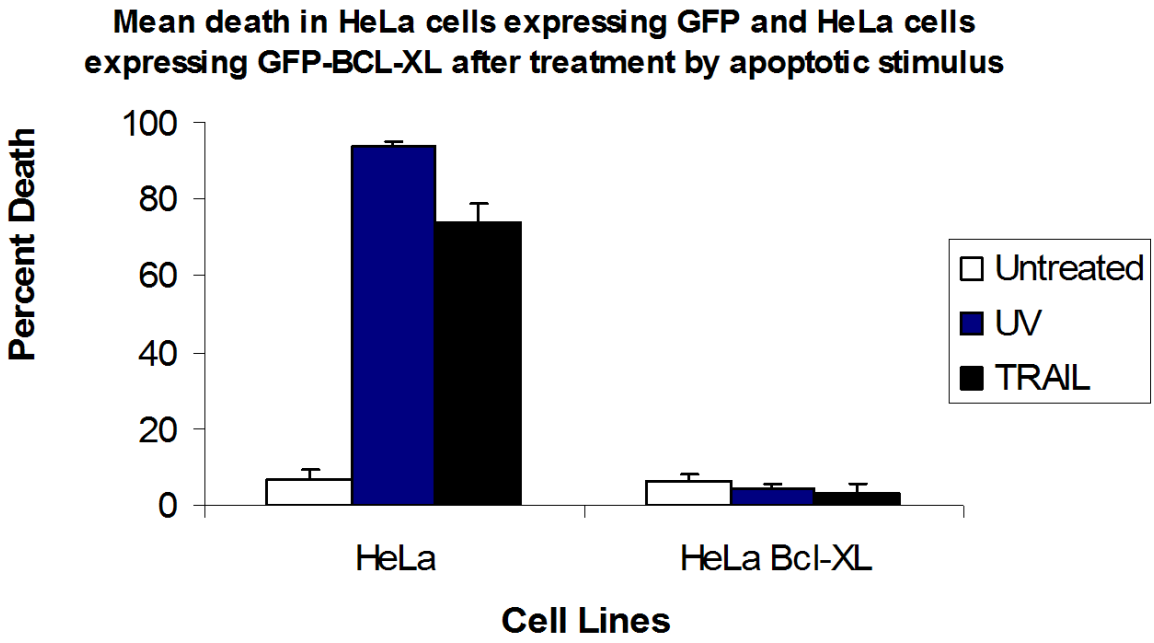

FIGURE 7. Mean 6.9% (SD ± 2.5%) death in HeLa cells with no treatment. 93.7% (SD ±1.5%) death in HeLa cells treated with UV light (20 J/m2). Mean 80.0% (SD ± 5.0%) death in HeLa cells treated with TRAIL (12.5 µg/mL). Mean 6.0% (SD ± 1.8%) death in BCL-XL HeLa cells with no treatment. Mean 4.0% (SD ± 1.7%) death in BCL-XL cells treated with UV light (20J/m2). Mean 3.3% (SD ± 2.6%) death in BCL-XL HeLa cells treated with TRAIL (12.5 µg/mL). Counted and stained 8hrs after UV and 5hrs after TRAIL treatment.

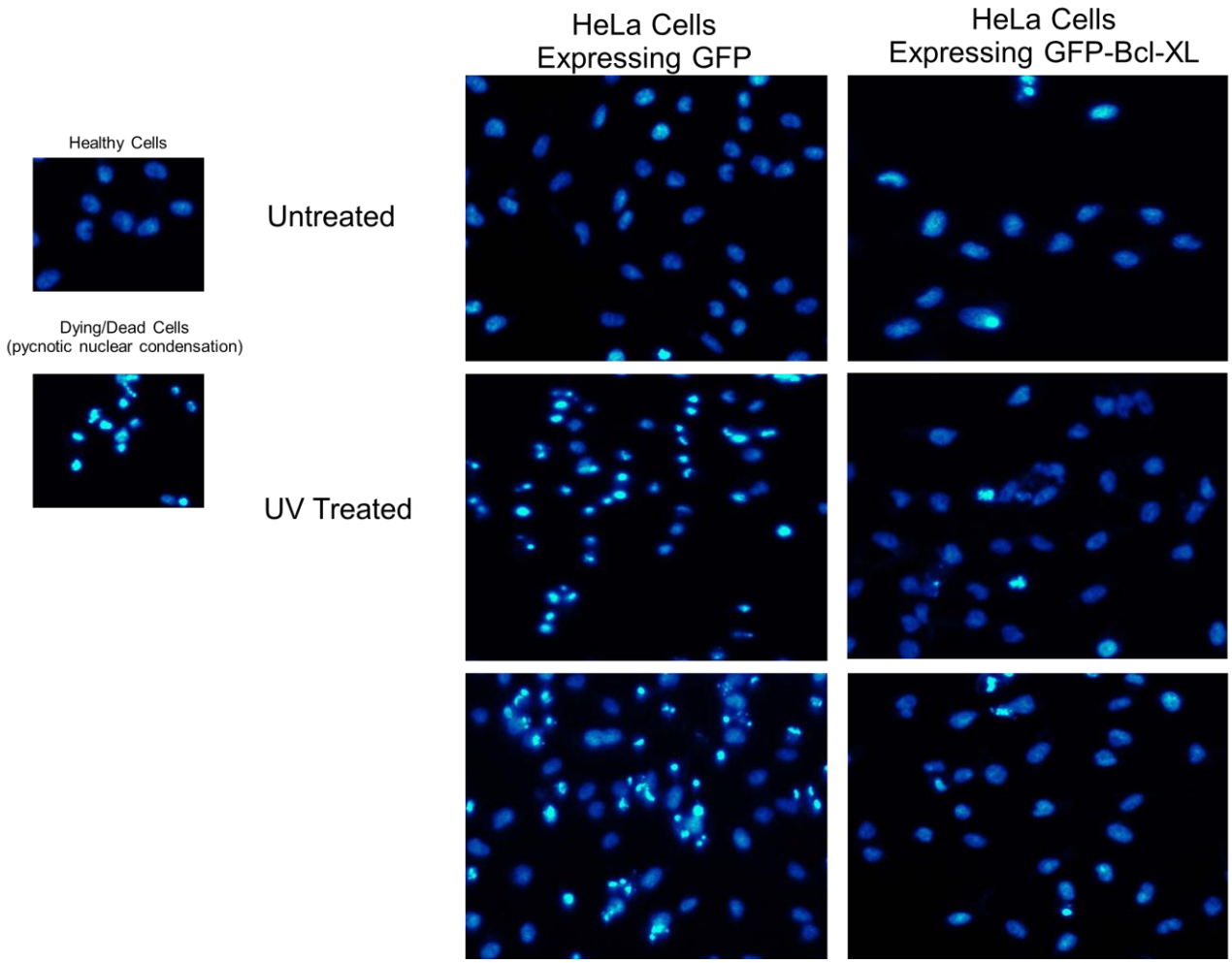

FIGURE 8. Hoechst staining showing pycnotic nuclear condensation 6 hrs after UV treatment (20J/m2) and 8 hours after treatment with TRAIL (12.5 µg/mL) in HeLa-GFP and HeLa-GFP-BCL-XL cells viewed under 10x immunofluorescent microscopy.