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LOSS OF MITOCHONDRIAL DNA CAUSES DECREASED CELL VIABILITY

A Thesis

by

ALAN M. HERRERA

Submitted to the Graduate School of
The University of Texas-Pan American
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2015

Major Subject: Biology

LOSS OF MITOCHONDRIAL DNA CAUSES DECREASE CELL VIABILITY

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August 2015

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ABSTRACT

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Mitochondria are an organellar network involved in cellular bioenergetics and apoptosis, and dynamically balance their organization between fusion and fission events. Mitochondrial DNA (mtDNA) and nuclear DNA combine to encode polypeptides for the complexes of oxidative phosphorylation (OXPHOS) in the mitochondrial inner membrane, which generate a transmembrane potential ($\Delta\psi_m$) to synthesize ATP. Loss of $\Delta\psi_m$ causes mitochondrial fission, which appears to be associated with the progression of apoptosis, a critical cell death mechanism that allows tissue homeostasis, developmental sculpturing, and the removal of unwanted cells. Using cultured human cell models of decreased $\Delta\psi_m$ (both genetic mtDNA-depleted ρ^0 and pharmacological Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-treated cell models), here we explore whether the loss of OXPHOS increases apoptosis. Based on current results, mtDNA-depleted ρ^0 cells are more basally apoptotic compared to wild-type cells, and display loss of viability when challenged with galactose-containing medium. Based on current results, future research will determine whether this loss of viability is indeed apoptosis, as well as whether the bioenergetics defect or loss of fusion mediates the effect.

DEDICATION

The completion of my master studies would not have been possible without the love and support of my family. My mother, Silvia Martinez, and my father, Hugo Herrera, wholeheartedly inspired, motivated and supported me by all means to accomplish this degree. Thank you for your love and patience.

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CHAPTER I

INTRODUCTION

During the last five decades mitochondria have become an important subject of research within multiple disciplines of biology. In 1841 Henle described an intracellular structure found in the intestine which most likely represented the mitochondria (Henle., 1841). It was not until 1890 when Altmann recognized the structure and named it “bioblasts’ and stated that it was an elementary organisms living inside the cell (Altmann, 1980). Altmann’s ideas about mitochondria were later revitalized by Lynn Margulis and the orgin of eukaryotic cells (Margulis, 1971). The name mitochondrion originated from the two Greek words “mitos”, meaning thread, and “chondros”, meaning granule, and was first introduced in 1898 referring to its microscopic appearance (Benda, 1900). During the 1900s mitochondria became a subject of study from the discovery that mitochondria contain proteins and lipids (Regaud, 1909) to the discovery that mitochondria independently synthesize proteins (Mclean et al., 1958). Historically, mitochondria are a unique organelle in cells that play in their own rules by providing ATP to the cells. Just like any other topic in science, mitochondria have also been involved in controversy, one of the earliest was proposed by Sagan with the endosymbiosis theory (Sagan, 1967), which stated that mitochondria were descended from a proteobacteria that was engulfed by a eukaryotic cell. Sagan’s theory was later confirmed by the similarity of mitochondria DNA (mtDNA) to bacterial DNA rather than human nuclear DNA. This extranuclear DNA is essential for mitochondrial ATP production (DiMauro and Schon,

2003). The human mitochondrial genome is small double-stranded circular DNA molecule composed of 16,569 base pairs. There are ~1000 copies of mitochondria per cell (eukaryotic), though this varies between cell types. DNA only contains 37 genes out of the 900 approximately gene which are encoded by the nuclear DNA (nDNA) (DiMauro and Schon, 2003). Human mtDNA encodes for 2 tRNAs, 22 rRNAs and 13 polypeptides, which are necessary for proper function and structure of the mitochondrial complexes of oxidative phosphorylation (OXPHOS) (Anderson et al., 1981). The OXPHOS complexes are located in the inner mitochondrial membrane: complexes I, II, III and IV transfer electrons and donate them to molecular oxygen, thus creating a chemiosmotic proton-motive gradient ($\Delta\psi_m$), used by complex V to synthesize ATP from ADP and P_i. In order for the complexes to function properly, the coordination of both nuclear and mitochondrial DNA-derived as transcription and translation is required, thus resulting in several genetic defects that can lead to disruption of OXPHOS complexes causing human diseases (Di Sante et al., 2015). These mutations can be located within nDNA or mtDNA causing a decrease in human health. About 99% of the mitochondrial proteins are encoded in the nucleus, deleterious and mutation in nuclear encoded factors such as thymidine kinase 2 (TK2) can cause for an increase mtDNA mutation thus expressing in neuromuscular disease (Oskoui et al., 2006). Either depletion or mutation of mtDNA can cause neuromuscular disorders. For example, Kearns-Sayre Syndrome (KSS), is caused by a large deletion in mtDNA, affecting genes encoding respiratory chain proteins (Obara-Moszynska et al., 2013). Another classical mitochondrial disease, Myoclonus Epilepsy with Lactic Acidosis and Stroke-like episodes (MELAS), is caused by point mutations in the $tRNA^{lys}$ gene in mtDNA (Sakuta, 2002). While each of the mitochondrial neuromuscular disorders is individually rare, pathogenic mtDNA mutations as a whole occur at a high frequency of 1:200 individuals (Elliott et al., 2008; Greaves

et al., 2012). The role of mtDNA in cellular metabolism and bioenergetics is critical to human health, motivating a better understanding of the elegant complexity of mtDNA's macromolecular organization and integration into both the organellar network and cell at large.

Mitochondria have been traditionally thought of as individual "football shaped" organelles, but it is now known that mitochondria exist as a network, composed of a balance between fragmented and interconnected states in response to fusion and fission events. Mitochondrial fusion is mediated by three genes: mitofusin 1 and 2 (MFN1 and MFN2) at the outer mitochondrial membrane and optic atrophy-1 (OPA1) at the inner mitochondria membrane (Olichon et al., 2003). Mitochondrial fission is mediated with three genes fission protein 1 (FIS1), dynamin-related protein 1 (DRP1) and mitochondrial fission factor (MFF) (Otera et al., 2010; Twig et al., 2008). Both pathways, fusion and fission, allow for the alteration of mitochondrial organization in response to cellular cues. Fission and fusion interaction is important for mitochondrial adaptation to different cellular processes. For example, fission is required to for apoptosis, autophagy and mitosis ((Lim et al., 2008), (Youle and Karbowski, 2005); (Twig et al., 2008)). Fusion is required in order to adapt to cellular starvation or metabolic demand (Nunnari and Suomalainen, 2012). The morphology of mitochondria is linked to its function, as a loss of mitochondrial bioenergetic capacity results in an inability to maintain the fused, networked mitochondrial organization (Santra et al., 2004). The inability to interconnect is due to the decrease of mitochondrial transmembrane potential across the inner membrane (Legros et al., 2004). Therefore, mitochondrial morphology can provide a visual representation of mitochondrial functional status, as cells with either genetic or pharmacologic mitochondrial dysfunction show an inability to maintain a united mitochondrial network (Gilkerson et al., 2008; Legros et al., 2004).

As mitochondria are central to cellular metabolism, mitochondrial dysfunction has been associated with many human diseases such as cardiovascular disease (Ballinger, 2005), neurological disorders (Oskoui et al., 2006), aging (Bender et al., 2006b), and diabetes (Ritov et al., 2010). Decreased OXPHOS activity and increased fission have been proposed as mechanisms by which mitochondrial dysfunction may cause increased apoptosis, as both have been mechanistically connected with apoptosis (Green, 2011b) (Bossy-Wetzel et al., 1998). The discovery that mitochondria participate in apoptosis prompted an explosion of interest in mitochondrial cell death mechanisms (Bossy-Wetzel et al., 1998; Collins et al., 1997; Jiang et al., 1999; Kroemer et al., 1997; Marella et al., 2007; Wang et al., 2001). Apoptosis is derived from Greek meaning "falling off" and was described by Carl Vogt in 1842. In 1972 John Kerr (Kerr et al., 1972) first referred to apoptosis as the morphological features of cell death, such as cytoplasmic and nuclear compression and breaking up of the cell into smaller fragments. Due to mitochondrial control of cell death, there has been widespread interest in potential roles of mtDNA-derived mitochondrial dysfunction in apoptosis.

A major emphasis of current mitochondrial research explores the integration of mitochondria into cell signaling networks. In recent years, the nuclear signaling pathways regulating mitochondrial biogenesis have become increasingly well characterized, centering on the PGC-1a master regulator of mitochondrial biogenesis and related factors. In these pathways, nuclear-encoded signaling factors tightly regulate mitochondrial content within the cell, modulating expression of nuclear-encoded mitochondrial proteins, as well as mtDNA copy number (via TFAM expression) and expression of mtDNA-encoded genes (Wang et al., 2001). Strikingly, however, it is also becoming clear that mitochondria can communicate with the rest of the cell via 'retrograde' signaling pathways. Accordingly, the loss of mtDNA is being

revealed to have profound effects on the rest of the cell through retrograde modulation of signaling pathways including AMP kinase, ROS-mediated NFKB, and apoptotic modulation (Tann et al., 2011). Thus, while loss of mtDNA causes the immediate bioenergetic deficit, the effects on cell signaling reverberate throughout the cell, impacting a wide variety of pathways crucial to homeostasis yet not directly involved in energy metabolism.

Despite this, it is still unknown how loss of mitochondrial bioenergetics impacts apoptosis, contributing to these widespread conditions. In order to understand this more thoroughly, we explore whether the loss of OXPHOS increase apoptosis. Using genetic and pharmacological models, our results indicate that loss of mtDNA and/or $\Delta\psi_m$ causes decreased viability, likely through increased apoptotic activity.

CHAPTER II

REVIEW OF LITERATURE

As an organellar network, mitochondria are involved in cellular energy production by oxidative phosphorylation (OXPHOS) and in the regulation of apoptosis, calcium signaling and redox homeostasis (Picard et al., 2013). Mitochondria are semi-autonomous, possessing their own genome and mechanism for replication, transcription, and protein synthesis. In the mitochondrial inner membrane Complexes I-IV are used to transfer electrons and eventually donate the electrons to molecular oxygen to create the chemiosmotic proton-motive transmembrane potential ($\Delta\psi_m$), which is then used by Complex V (Gilkerson et al., 2013). MtDNA codes for 13 polypeptides engaged in oxidative phosphorylation, and contains 2 rRNAs (12S and 16S) and 22tRNAs, which allows for translation of proteins inside the organelle (Laszczyca et al., 2004). The assembly of the OXPHOS complexes in the mitochondria requires the coordination of both nuclear and mitochondrial transcription and translation. The assembly and production of all the complexes of polypeptides derived from the chromosomal and organellar genomes. As a result the mitochondria is susceptible to mutations in either genome (Gilkerson et al., 2013). The importance of apoptosis was illustrated in the nematode *Caenorhabditis elegans,* which actively employs apoptosis during the formation of its adult stage 131 somatic cells (Horvitz, 1999). Apoptotic cell death is an essential feature and indispensable process during normal development and tissue homeostasis (Jacobson et al., 1997), and occurs in

response to a range of stimuli, including cell stress, DNA damage, deprivation of growth factors, and other stimuli (Green, 2011b), functioning as a defense mechanism towards disease or other factors that damage cells (Norbury and Hickson, 2001). Caspases are a family of genes that carry out proteolytic cleavage, causing tightly regulated cell death. Apoptotic stimuli prompt caspases to dimerize or form macromolecular complexes, resulting in a cascade of signals that lead to cell death (McIlwain et al., 2013 2013). There are two major cellular pathways known to trigger apoptosis, called the intrinsic and the extrinsic pathways (Ferri and Kroemer, 2001), both of which activate caspase-3, resulting in DNA fragmentation and cell death (Enari et al., 1998). The extrinsic pathway is activated by cell surface death receptors such as tumor necrosis factor (TNF) related family (Fas) (Peltz et al., 2004) The TNF superfamily includes TNFR1, Fas, APO-1, DR3, TRAIL-R1 and DR5. The extrinsic pathway is initiated by the binding of DR ligand which allows the procaspase-8 protein to be recruited to the death-inducing signaling complex and binding to a FAS-associated death domain (FADD). Dimerization then occurs in the procaspase-8 and caspase-8 is activated (Juo et al., 1998). The intrinsic pathway involves the release of factors, particularly cytochrome c, from the mitochondria and can be activated by DNA damage, cytoskeletal disruption and many other stimuli (Brenner and Mak, 2009). The intrinsic pathway activates caspase-9 when dimerization occurs (Shiozaki et al., 2002). When cytochrome c is released from the mitochondria due to cell stress, it binds to the protein apoptotic protease-activating factor-1 (APAF1) (Green, 2011a) inducing activation of caspase-9 (Acehan et al.).

Mitochondria are now known to be important regulators of cell death signaling events, inducing apoptosis by releasing cytochrome c from the inner membrane of mitochondrion to the cytosol (Li et al., 1997). Mitochondrial outer membrane permeabilization (MOMP) is crucial to

early-stage apoptosis, allowing Bax and Bak from the Bcl 2 family to be translocated to the outer mitochondrial membrane (Remels et al.) and concentrate into submitochondrial punctate foci (Nechushtan et al., 2001), which then allows release of cytochrome c (Liu et al., 1996). In addition to the release of cytochrome c, Smac/Diablo, apoptosis-inducing factor (AIF) and endonuclease G (Endo G) (Du et al., 2000; Li et al., 2001; Susin et al., 1999) are additional pro-apoptotic factors that are released from mitochondria during apoptosis. In the cytosol, cytochrome c then interacts with Apaf-1 to trigger formation of the apoptosome. Once the apoptosome assembles, it activates procaspase-9, which in turn is then able to activate caspase-3 and caspase-7, triggering a cascade of events leading to oligonucleosomal DNA fragmentation (Baliga and Kumar, 2003).

Recent studies have suggested that dysfunction of mitochondria can cause apoptosis. For example, transcription factor A mitochondrial (*tfam*) is a protein that play a role on mitochondria genome replication by bending mitochondrial promoter DNA to aid transcription of the mitochondrial genome. Studies in mice have demonstrated that this gene is required to regulate the mitochondrial genome copy, For example, *tfam* cardiac-specific knockout mice have mtDNA depletion and resultant mitochondrial dysfunction, and massive apoptosis (Wang et al., 2001). As an electron carrier, cytochrome c is crucial for a stable membrane potential. Thus, removal of cytochrome c from electron transport to the cytoplasm results in decreased membrane potential and ATP synthesis (Bossy-Wetzel et al., 1998). It has been shown that HeLa cells can undergo apoptosis without little or no change in the membrane potential, though DNA was fragmented. This suggests that loss of membrane potential occurs in apoptosis at a later stage, but not as a crucial early step of apoptosis (Bossy-Wetzel et al., 1998). An explanation for this is that not all cytochrome c could be participating in the electron transport chain and the release of cytochrome

c might only be due to excess. To support this, mtDNA-depleted ρ^0 cells still exhibit some membrane potential and undergo apoptosis (Jacobson et al., 1993). It was demonstrated that even without mtDNA, ρ^0 cells have a minimal membrane potential due to reversal of ATP synthase proton flux (Buchet and Godinot, 1998). OXPHOS activity appears to regulate apoptosis in a context-dependent manner: ρ^0 cells are protected against apoptosis because of the complete *absence* of electron transport, while cells with *decreased* electron transport have heightened sensitivity to apoptosis (Kwong et al., 2007).

CHAPTER III

METHODOLOGY AND FINDINGS

Methodology

Cell culture

Human 143B osteosarcoma cells were a gift from Dr. Eric A. Schon (Columbia University Medical Center). The 143B cells were maintained inactive in liquid nitrogen. In order to unfreeze the cells and grown them, cells were taken out of liquid the nitrogen tank and placed in 10 mLs of Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (FBS). 143B osteosarcoma cells were grown under standard conditions in DMEM containing 4.5 g/L D-glucose, supplemented with Antibiotic-Antimycotic (100X), uridine, and 10% fetal bovine serum (FBS). All cell culture reagents were obtained from Gibco by Life Technologies.

Confocal fluorescence microscopy

For confocal imaging, cells were grown in 6-well plates with 2 mLs of media. 10 mLs of DMEM were placed in a 15 mLs tube with 0.4 μL of MitoTracker and added to the 6-well dish and incubated for 20 minutes. The coverslips were washed with fresh DMEM two times for 7 minutes each time, followed by washes with 1X Phosphate-buffered saline (PBS) two times for 2 minutes each time. The coverslips were fixed in 4% paraformaldehyde for 30 minutes and then washed twice with 1X PBS. 100 μL of DAPI was added to each well and a cover slip added

for 5 minutes. After 5 minutes the coverslips were removed and the wells were washed with PBS 2 times for 2 minutes each. Coverslips were mounted onto slides using 50% glycerol. The slides were visualized on a Fluoview (FV10i) Olympus Confocal Microscope using 3.5% MitoTracker laser transmissivity and a wavelength of 559nm.

DNA extraction and PCR amplification

Cell plates were removed from freezer and 500 µL of equilibrated phenol was added, inverting 100 times slowly. Samples were then centrifuged for 3 minutes and the upper aqueous solution was removed to another tube, extracted again with an equal volume of phenol with chloroform and isoamyl. Again the solution was centrifuged for 3 minutes and the upper aqueous solution were then place in a new tube with 500 µL of 99% chloroform. The solution was centrifuged for 3 minutes and the upper aqueous solution was removed and precipitated with 1000 µL of Ethanol and place in -20 ºC overnight. Afterward the pellet was resuspended with 1000 µL of 70% Ethanol. The pellets were dried overnight and resuspended with 52 µL of TE buffer. The following day the sample was analyzed in a spectrophotometer (NANODrop 1000) provided by Howard Hughes Medical Institute laboratory (HHMI) in order to quantify the amount and purity of DNA in each sample.

PCR amplification

DNA sequences were amplified by using the oligonucleotide primers 4F (5’-AAATCTTACCCCGCCTGTTT-3’, bp 20) and 4R (5’-ATGAAGAATAGGGCGAAGGG-3’, bp 20). A single PCR sample containing 100 ng of template DNA was amplified in a 50 µL reaction mixture containing 5 µL 10X buffer, 1 µL dNTPS, 1 µL *Taq* polymerase, and 33 µL of water. PCRs were subjected to 30 cycles (denaturation, 1 minute at 94°C; annealing, 1 minute at 49°C; extension, 2 minutes at 72°C) and 1 final extension cycle at 72°C for 10 minutes. 10 µL of

the reaction mixture from PCR were analyzed on a 1.0% agarose/EDTA in the presence of 2.5 µL of ethidium bromide per mL and photographed (FOTODYNE) under UV illumination using a software (PC image).

Western blot

RIPA buffer was made by using 75 mLs of distilled water with 0.9 g of NaCl with a PH of 7.4. Then 1 mL of 100% NP40 was added into the solution as well with 200 µL of EDTA and maintained in ice. After removing cells from incubation, the media was removed and cells treated with 1 mL of 1X PBS for 1 minute. After 1 minute the PBS was removed and place in ice with 100 µL of RIPA buffer into each well for 5 minutes. After 5 minutes a cell lifter was used to remove the cells. All solution in each well was placed in a small 1.5 mL tube on ice for 30 minutes and vortexed every 5 minutes. The tubes were then centrifuged for 20 minutes at 4 °C. After centrifugation the liquid was then removed to a new tube and frozen. Cells were lysed in ice-cold with 8M Urea lysis buffer supplemented and protein concentration was determined using image J. Equal amount of proteins were run on 20% gradient polyacrylamide gel electrophoresis by Bio-Rad (Mini-protean TGX precast gels), and transferred to PVDF membrane (Bio-Rad, CA, USA). The membrane was blocked for 1 hr., in 1X TBST buffer with (3%) milk and incubated overnight at 4 °C, its' washed with 1XTBST and incubated with the corresponding primary antibodies overnight at 4°C. The membrane was washed 5 times for 5 minutes with 1X TBST, then incubated for 1 hr., with 1X TBST and Poly-HRP mouse secondary antibody (1:3000, Thermo Scientific, Rockford, IL, USA) and subsequently washed again 5 times for 5 minutes in 1X TBST. The membrane was then developed using Western Dura Kit (Thermo Scientific, Rockford, IL, USA) in a dark area for 5 minutes and immediately scanned for protein bands using Kodak 4000MM machine provided by Howard Hughes Medical Institute

laboratory (HHMI). The following primary antibodies were used: anti-MTCO2 (ab-110258) (Abcam, Massachusetts, USA), anti-α tubulin (T6074) (SIGMA, St. Louis, MO, USA).

Immunostaining

After treatment with MitoTracker for 20 minutes, cells were fixed with 5% formaldehyde and permeabilized with 0.01% TX-100 for 10 minutes. Immunostaining of TFAM was performed with Alexa flour 488 secondary antibodies (Life Technologies, Eugene, OR, USA).

Confocal microscopy

Imaging of fluorescence labeling for MitoTracker, DAPI and TFAM was performed using an Olympus FV10i confocal microscope (Olympus America Inc., Melville, NY). The excitation and emission wavelengths for Alexa Fluor 488 (green), DAPI (blue) and MitoTracker (red) were 488/520 nm, 350/470 nm and 579/599 nm, respectively. Composite digital images were then converted to JPG format, imported into Adobe Illustrator Artwork 16.0 (Adobe Illustrator C16; Adobe Systems Inc., San Jose, CA), and color balance was adjusted for presentation. For each image, at least 100 cells were examined. Considering the sizes and the resolution of the figure, only one to three representative cells were shown in each panel.

TMRE flow cytometry

After treatment with carbonyl cyanide m-chlorophenyl hydrazine (CCCP) for 1 hr., cells were then treated with tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE) for 20 minutes (Molecular probes by life technology, Eugene, OR. USA). Cells were trypsinized for 5 minutes and centrifuged for 3 minutes. Trypsin was remove to leave only the pellet and washed with PBS for 2 minutes and centrifuged for 3 minutes. Then re-suspended with new PBS and place in cytometry tubes. Cells were then analyzed by flow cytometry LSR Fortessa, (BD Biosciences Facscalibur, San Jose, CA. USA).

Cell viability assay

143B osteosarcoma cells were grown in 96-well culture plates. Each well contained 100 µL of DMEM with 10% FBS and the culture plates were incubated at 37 °C until the optimal density was reached. 10 µL of MTT Solution (Biotium, Hayward, CA, USA) was added to each well, incubated at 37 °C for 4 hours. Afterwards 200 µL of Dimethylsulfoxide (DMSO) was added to each well. Cellular metabolic activity was determined by measuring the absorbance of the samples with a plate reader (BIO-RAD Model 680, Hercules, CA USA) at a wavelength of 595 nm absorbance.

Statistical analysis

The Student's paired *t*-test was used to determine significant differences between means and P values below 0.05 were considered to be statistically significant. All results are expressed as the mean ± SEM.

Findings

MtDNA nucleoids are distributed throughout the mitochondrial network

First, we evaluated where mtDNA-binding TFAM found on the mitochondria network. To do so, we used 143B osteosarcoma cells and stained with MitoTracker for 20 minutes in order to label the mitochondria and then incubated with an anti-TFAM antibody to detect TFAM in the cell. TFAM was previously shown to bind to DNA; therefore it mirrors the abundance of mtDNA within the cells. We were able to demonstrate in **Fig. 1** that there is an overlap between MitoTracker and TFAM thus, suggesting that mtDNA nucleoids are distributed throughout the mitochondrial network.

Cells without mtDNA lack oxidative phosphorylation and reticular morphology

We use human 143B osteosarcoma cells WT (normal) and ρ^0 (lacking mtDNA) in order to compare them morphologically using MitoTracker and DAPI. Cells were incubated with MitoTracker for 20 minutes and DAPI for 5 minutes to stain the nuclei. By using confocal microscopy we are able to show a difference in morphology of the nuclei and mitochondria. Results obtained in **Fig. 2** show that WT cells have a more uniform nucleus that ρ^0 cells, as well as a difference in the network of mitochondria. In **Fig. 2**, looking at the detail image of the mitochondria we can see interconnected mitochondria in WT cells, compared to ρ^0 cells which mitochondria are fragmented thus, showing that the ρ^0 cells lack a reticular morphology. Early studies suggest that mitochondria fragmentation is due to a dynamin-related protein 1-mediated mitochondria division or the expression of mitofusin 1 (Mfn1) (Legros, 2004).

In order to demonstrate that ρ^0 cells in fact contain no mtDNA, we analyzed mtDNA by polymerase chain reaction (PCR) and agarose gel electrophoresis using template DNA from WT, ρ^0 and CCCP-treated WT cells. The gel electrophoresis was done by first getting PCR samples for WT, ρ^0 and WT+CCCP and then ran in a 1% agarose gel for 1 hour. As shown in **Fig. 3A** a molecular weight ladder and a control (water) was run side by side with the three main samples to show whether there was mtDNA present or no. Therefore, the result indicated that only ρ^0 cells had no band, meaning that it had no mtDNA present. Early studies have generated ρ^0 under artificial growth using ethidium bromide, interfering with the replication machinery (Kukat et al., 2011).

Thus, to investigate or to gain more evidence whether ρ^0 cells have no mtDNA, Western blotting analyzed protein expression of cytochrome c oxidase subunit 2 (MTCO2). **Fig. 3B** shows that ρ^0 cells have no MTCO2 present, which is an important component of the respiratory chain reaction by transferring electrons. Therefore, this confirms that ρ^0 cells have no mtDNA

and also lack oxidative phosphorylation. Early immunocytochemical studies have showed that MTCO2 is an mtDNA encoded subunit complex, and cells that lack mtDNA is due to either exposure to ethidium bromide or cells from patients with mtDNA depletion syndrome (Marusich et al., 1997 and MacMillan & Shoubridge, 1996).

Cells without mtDNA have increased basal apoptosis

When discussing the important of apoptosis one of the first remarks people look at is the nucleus, thus the morphology of the nucleus can tell whether the cell is stress or not. Thus, to evaluate the different stages the nucleus goes through. In **Fig. 4A** three categories have been established in order to compare WT and ρ^0 cells: Normal which is regular or healthy nucleus, Fragmented which is a nucleus that was been pinched and Late apoptosis stage which is a nucleus that was been engulf or complete fragmented. This is done through a fluorescent stain (DAPI) and confocal microscopy, using human 143B osteosarcoma 143B cells (WT and ρ^0). Statistical analysis to compare each stage **Fig. 4B** was done by using T- test to determine the significant differences between means and P values. The stages Normal and fragmented were found to have a P-value below 0.05, were considered to be statistically significant. When looking at each stage, we find that WT cell are more normal than ρ^0 with a P-value of 0.00035. Also ρ^0 were found to be more fragmented then WT cell with a P-value of 0.00025 and ρ^0 are slightly more apoptotic even though it had a P-value of 0.0936.

Previous studies have created a system for analyzing apoptosis by looking at the morphological changes that occur to the nucleus (Lazebnik et al., 1993). Apoptosis is the end form of cell death, which occurs under specific changes in the cell surface and nuclear morphology. When cells undergo cell death they reveal a unique morphological change, which

includes blebbing, nuclear and cytoplasmic condensation. Nuclear changes have been examined by using DAPI cytochemistry to demonstrate apoptotic nuclear condensation (JA Collins, 1997).

Cells without mtDNA have a lower membrane potential ($\Delta\psi_m$)

To determine $\Delta\psi_m$, cells were given media containing $\Delta\psi_m$-dependent dye tetramethyl rhodamine ester (TMRE) and assayed by flow cytometry **Fig. 5A**. Each plate was given media containing 100 nM TMRE in order to quantitate $\Delta\psi_m$. WT cells show fluorescence average of 4172 ± 172 arbitrary units (a.u), while ρ^0's show fluorescence average of 1197 ± 179 a.u and CCCP- treated cell had an average of 385 ± 26 a.u **Fig. 5B**. These finding demonstrate that either genetic or pharmacological loss of $\Delta\psi_m$ fragmentation of the mitochondrial network just as previous finding suggest (Gilkerson et al., 2008) and that ρ^0 cells maintain a modest $\Delta\psi_m$, greater than that of WT cells treated with 10 µM CCCP **Fig. 5B**. This could be due to reversal of the F_1F_0 ATP synthase (Buchet, 1998), suggesting that a minimum level of $\Delta\psi_m$ greater than that seen in either ρ^0 or CCCP-treated WT cells is required for mitochondrial fusion.

Galactose media decrease cell viability

In order to determine the viability of cell we used the MTT assay after incubating cells at 37 ºC **Fig. 6**. Using a 96 well plate, WT cells were seeded in the amounts 1, 2, 5, and 10 µL of a confluent cell suspension, and incubated with glucose media for 72 hours at 37 °C. As shown in **Fig. 6** the MTT signal increases as the number of viable cells increases. Cell viability was also determined using MTT assay with different media treatment (**Fig. 7**). 143B osteosarcoma cells WT and ρ^0 cells were treated with glucose and galactose media over 72 hours and let to grow under 37 °C. WT cells show cell proliferation over 72 hours with glucose media. When WT cells are exposed to galactose media, which make the cells undergo mitochondrial OXPHOS in order to make ATP, WT cells show a decrease of proliferation to 53% of glucose-grown WTs (**Fig.**

7A). ρ^0 cells also show growth in glucose media, but when exposed to galactose media the cell viability dramatically decreases to 9% of glucose-grown ρ^0 cells **Fig. 7B**. When cells are cultured in rich glucose media, many compounds (such as CCCP) that can affect the mitochondria specifically the oxidative phosphorylation (OXPHOS) will have a minimal cellular viability affect. Cell cultured in rich media continue to generate ATP through glycolysis, preventing cell death from occurring. These finding demonstrate that either genetic or pharmacological decreases in OXPHOS cause a decrease of cell viability when cells are cultured in galactose, the cellular metabolism remaining dependent upon mitochondrial function and compounds that will affect the OXPHOS will rapidly lead to cell death.

The loss of $\Delta\psi$ cause a decrease in cell viability

In order to confirm the MTT assay results WT, CCCP-treated WT cells, and ρ^0 143B osteosarcoma cells were treated with either galactose or glucose media in order to observe cell viability using different media over 72 hours at 37 °C. Confocal imaging of WT, WT+CCCP, and ρ^0 cells were visualized by bright field and DAPI (**Fig. 8**). When cells are cultured in rich glucose media WT, WT+CCCP and ρ^0 show a robust cell viability, which correlates with the MTT assay result in **Fig. 7**. When WT, WT+CCCP and ρ^0 cells were exposed to galactose media, WT cells retain modest proliferation under the confocal imaging, while both ρ^0 and CCCP-treated WT cells show very few cells visible. These results indicate that high glucose media allows cells to obtain enough ATP via glycolysis and when challenged with an oxidative demanding metabolic environment cell viability decreases dramatically, if OXPHOS has been inhibited genetically or pharmacologically.

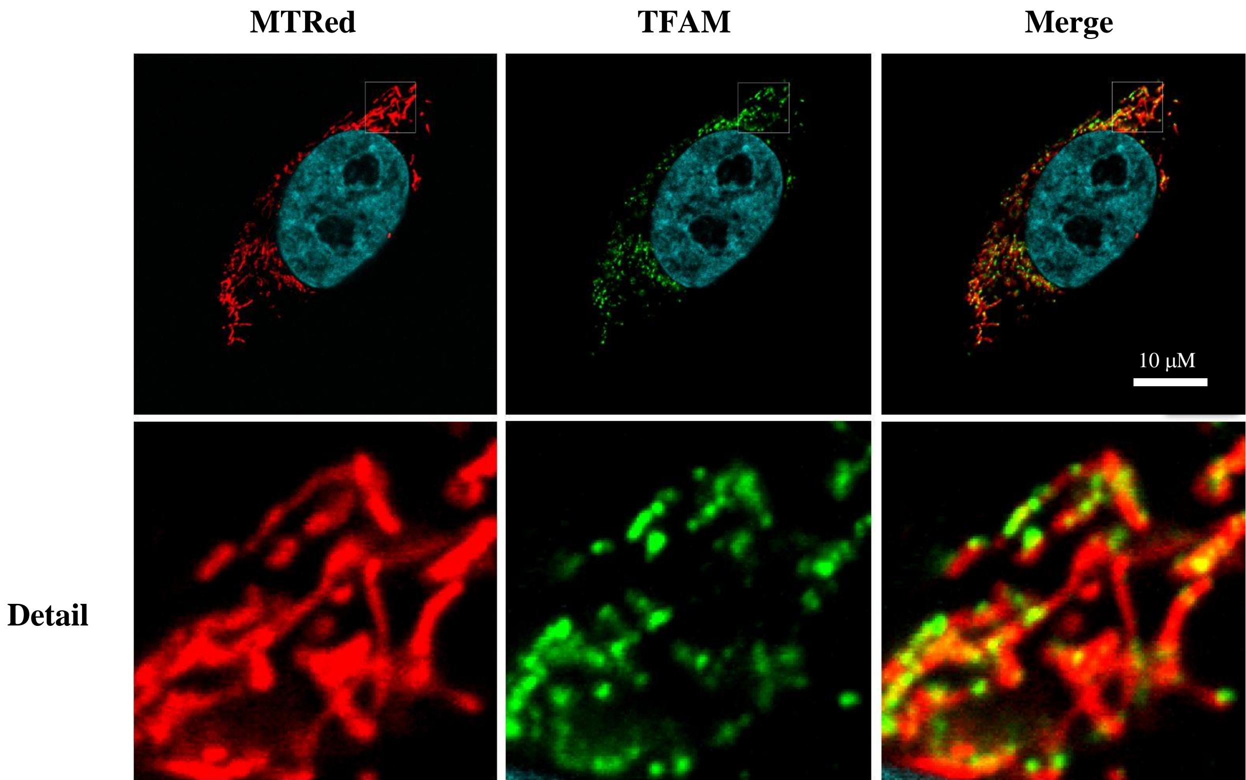


Figure 1. Confocal microscopy of mitochondrial TFAM

MtDNA nucleoids are distributed throughout the mitochondrial network. This is done by using a confocal microscopy of 143B osteosarcoma cells stained for mitochondria with MitoTracker (red) and immunolabeled for the mtDNA-binding protein TFAM (green). Nuclei were visualized with DAPI (blue). Data presented are representative of three separated experiments. Scale Bar 10 μM.

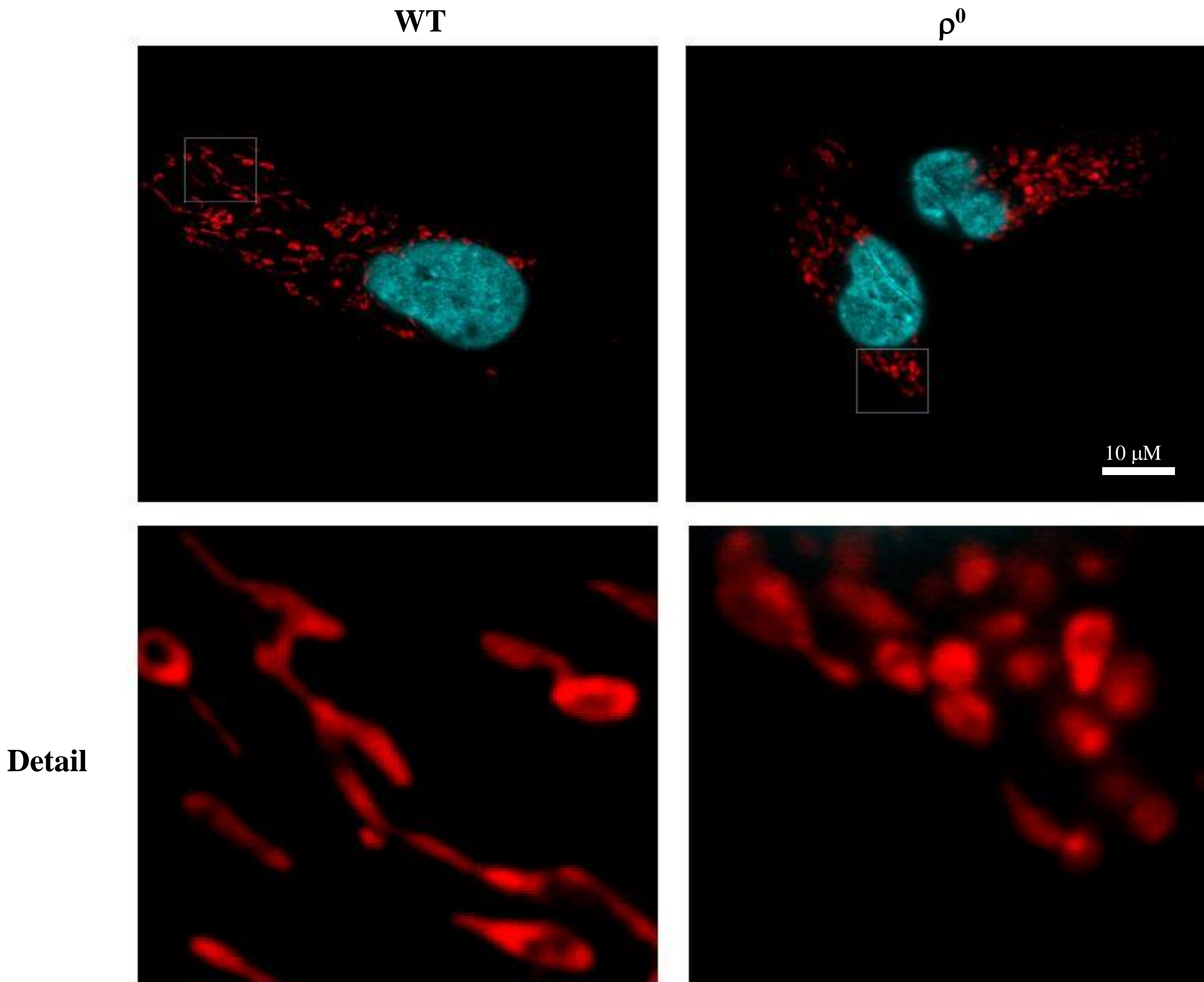


Figure 2. Confocal microscopy of WT and ρ^0 cell lines

Confocal microscopy of 143B osteosarcoma cells grown in a 6-well dish and stained with MitoTracker Red for mitochondria and DAPI (blue) for presence of nuclei. Data presented are representative of three independent experiments. Scale Bar, 10 μM.

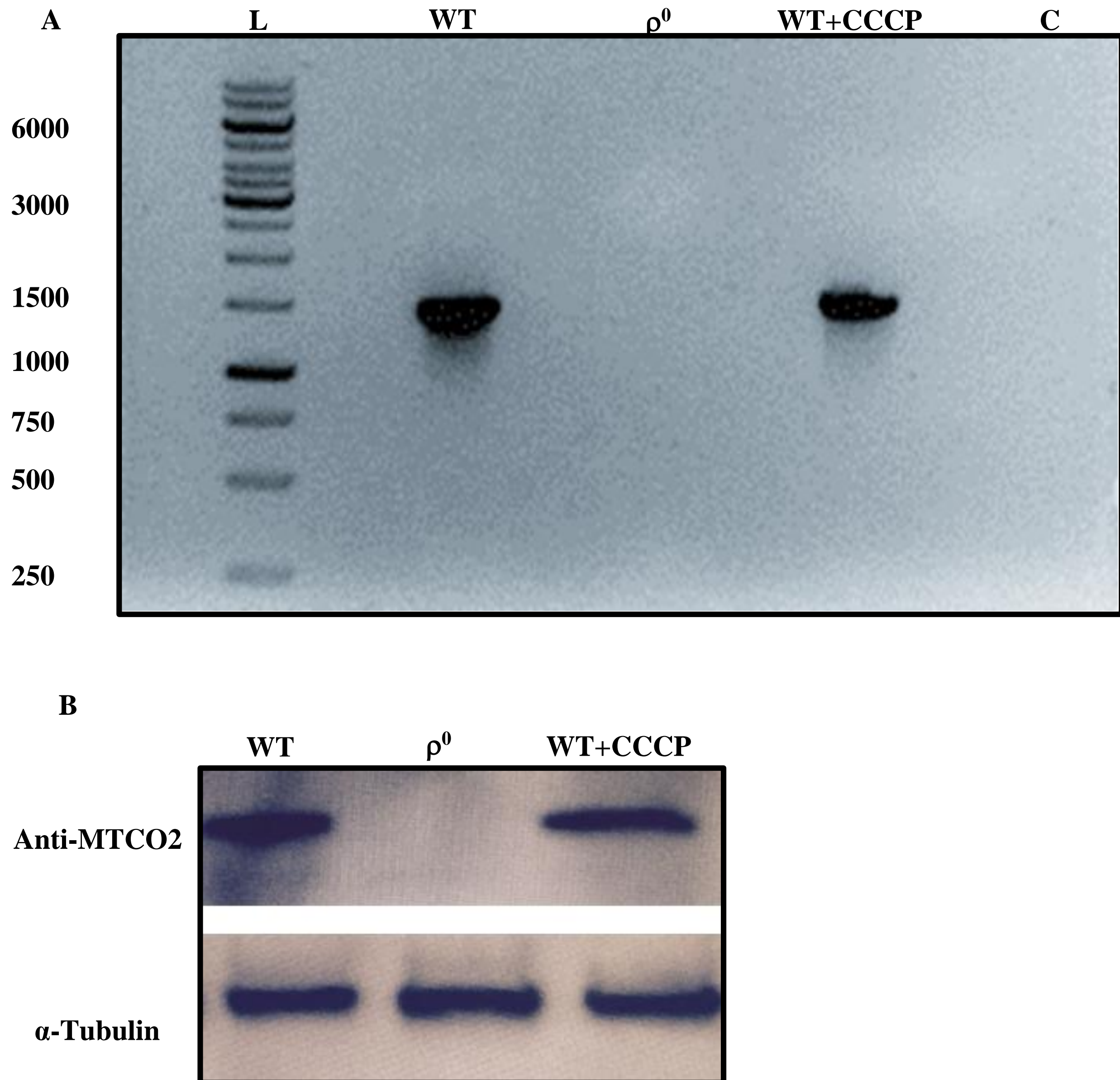
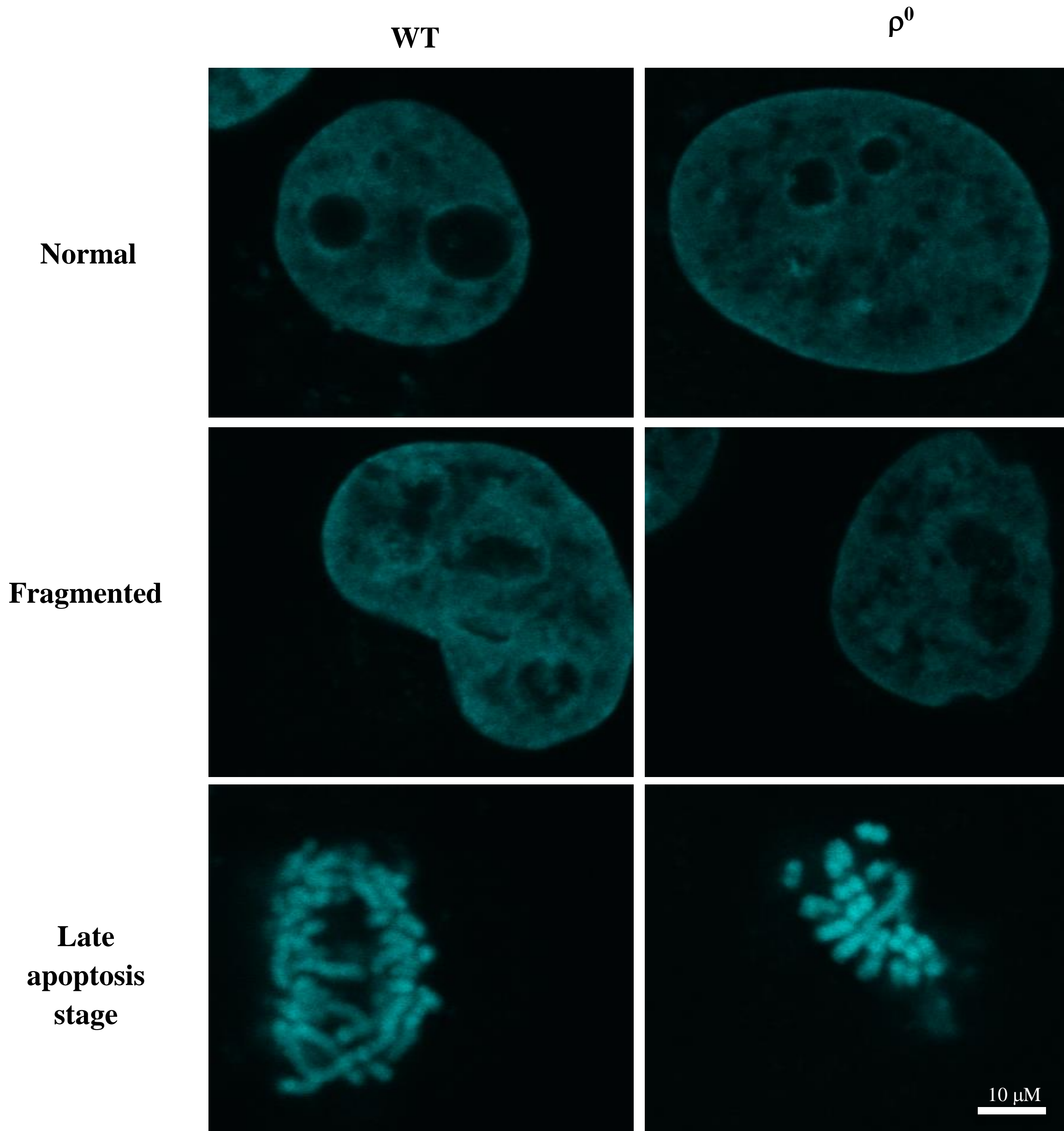


Figure 3. PCR and Western blot, analysis of WT and ρ^0 cell line

(A) Analysis of mitochondria DNA by PCR and agarose gel electrophoresis in WT, ρ^0 and WT+CCCP. Total cellular DNA was isolated and the primers at 2482-3366 were used. **(B)** Protein contents were determined by Western blot analysis, CCCP-treated cells were treated and use as a secondary control to demonstrate the present of MTCO2. Data presented are representative of three independent experiments.


A
WT
ρ0
Normal
Fragmented
Late
apoptosis
stage
10 μM

B

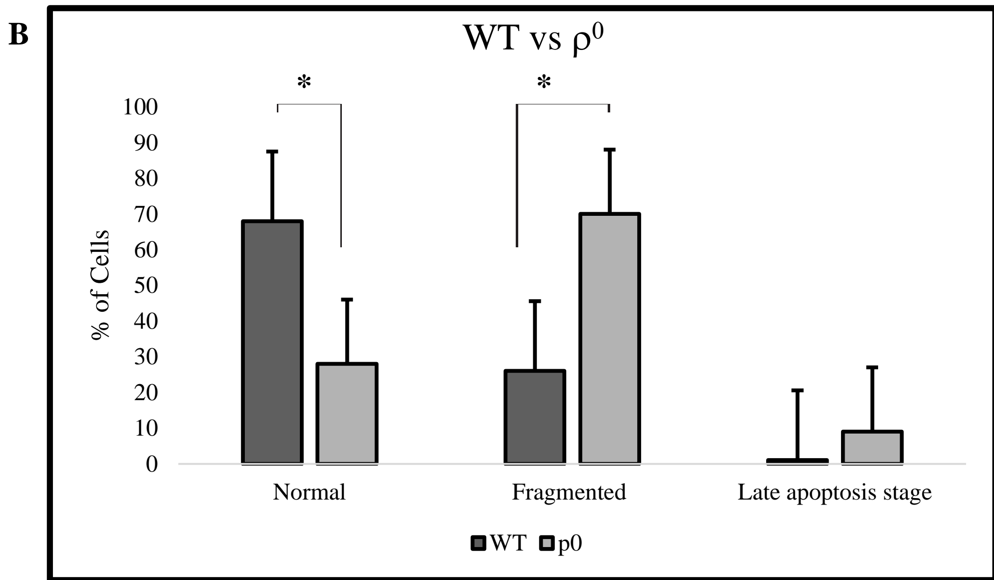


Figure 4. DAPI analysis of nuclei morphology

Analysis of the nucleus is done through a fluorescent stain (DAPI) and confocal microscopy. Using Osteosarcoma (143B) cells (WT and ρ^0) to analyze the different stage the nucleus goes through. **(A)** Three stages have been establish for comparison between WT and ρ^0; Normal which is regular or healthy nucleus, Fragmented which is a nucleus that was been pinched and Late apoptosis stage which is a nucleus that was been engulf. **(B)** Statistical overall analysis to compare ρ^0 and WT cells. Data presented are representative of three independent experiments with 100± cells counted and * denotes $p<0.05$.

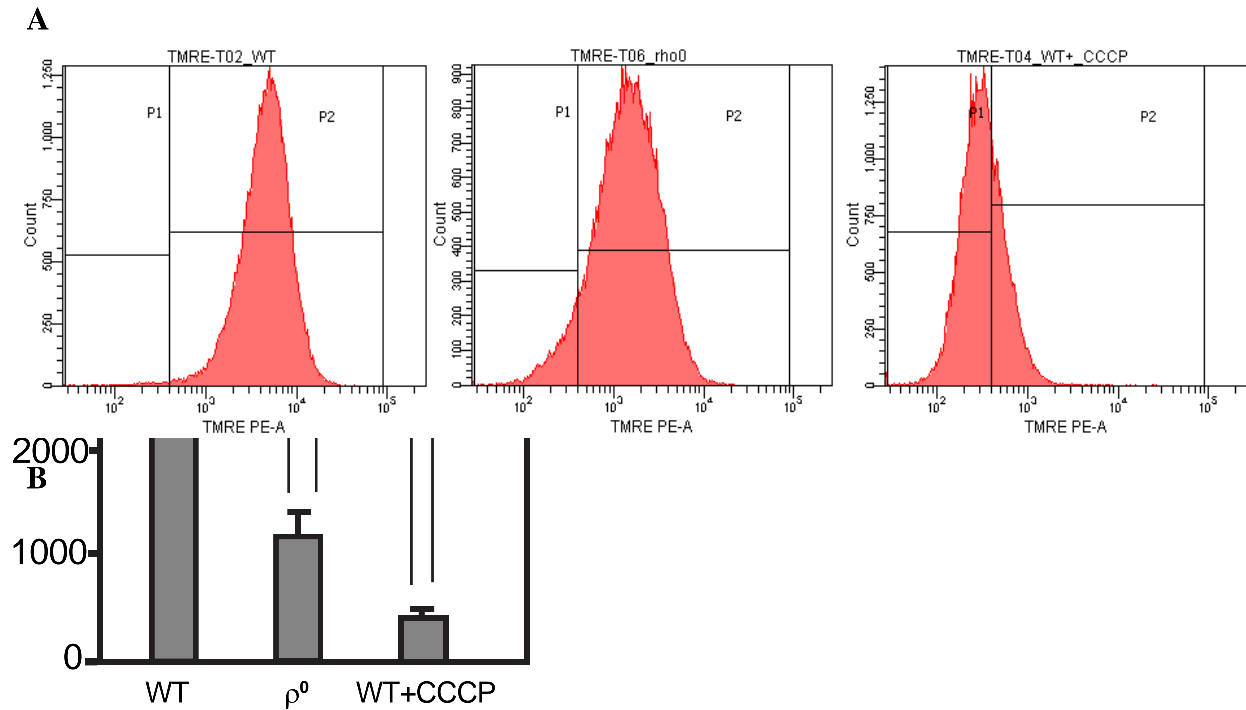


Figure 5. TMRE fluorescence: loss of Δψ in 143B osteosarcoma cells

(A) WT, ρ^0, and WT+CCCP treated cells where treated with 100 nM TMRE and assayed via flow cytometry. 50,000 cells assayed for TMRE fluorescence (X-axis) in each experiment. The Y-axis indicates the number of cells at fluorescence values expressed in arbitrary unites (a.u.). **(B)** WT, ρ^0, and WT+CCCP treated cells TMRE fluorescence average are also expressed in arbitrary units (a.u.). Each average indicated the cell line in n>3 experiments and results are expressed as the mean ± SE. statistical significance, $p<0.01$, Student's t-test.

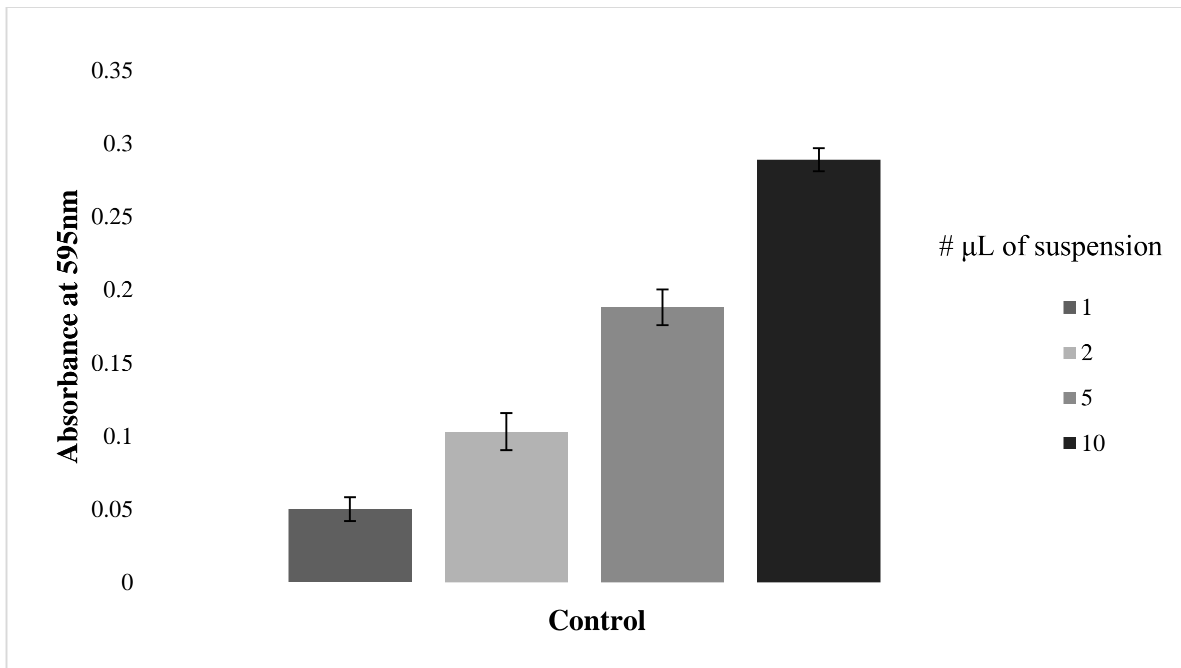
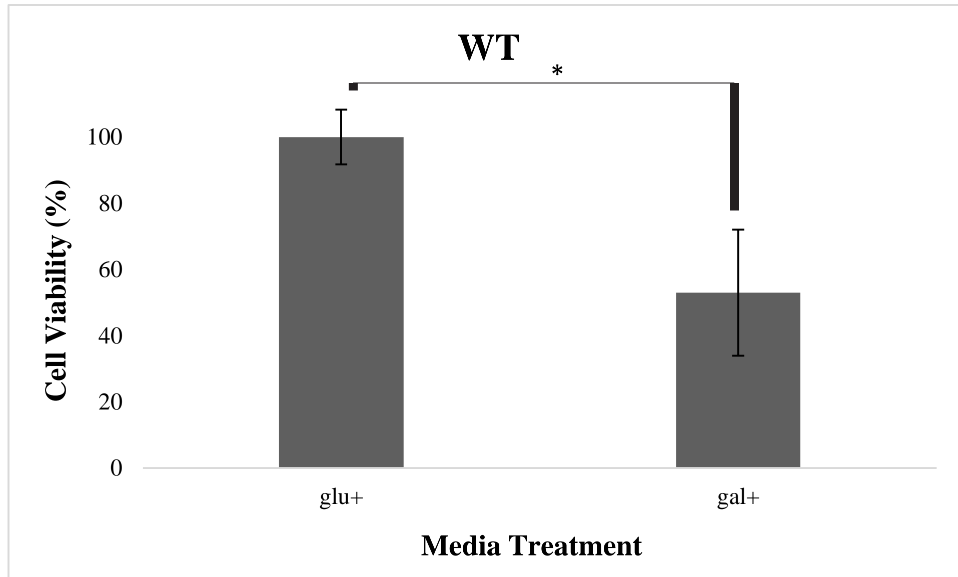


Figure 6. MTT cell assay

Different amount of cell seeded of 143B osteosarcoma cells in order to detect the amount of cell viability. Using a 96 well plate WT cells were seeded in the amounts 1, 2, 5 and 10 cells with glucose media for 72 hours for 1 hr. incubation at 37 °C. 10 µL of MTT solution is added to each well for 4hours at 37 °C. 200 µL of DMSO is added directly to each well and then cell viability is measure using a plate reader at 595nm.

A



B

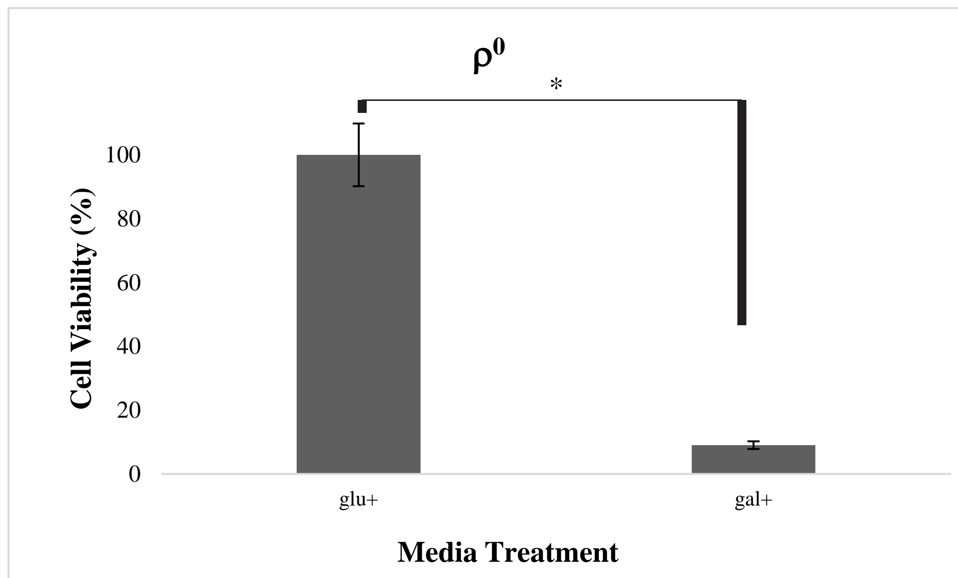


Figure 7. Cell viability in glucose versus galactose media

(A) 143B osteosarcoma cells WT cells. **(B)** ρ^0 were both treated with glucose and galactose media for 72 hours to let them grown under 37 °C. Cell viability was measured using MTT assay, 10 μL of MTT solution is added to each well for 4hours at 37 °C. 200 μL of DMSO is added directly to each well and then cell viability is measure using a plate reader at 595 nm.

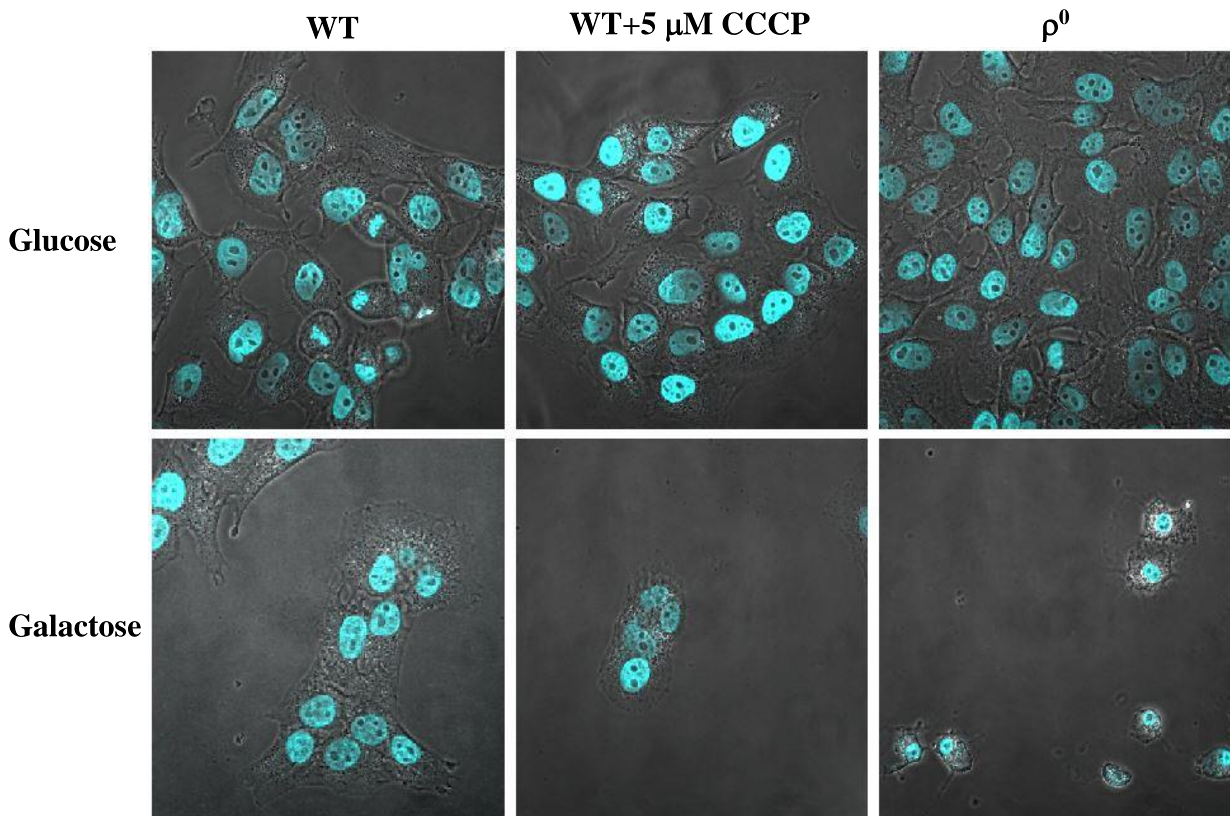


Figure 8. The loss of Δψ causes a decrease in cell viability

WT, WT+CCCP treated and ρ^0 143B osteosarcoma cells were treated with galactose or glucose media in order to observed cell viability using different media over 72 hours at 37 °C. Confocal imaging of WT, WT+CCCP ρ^0 and cells in high glucose media (top) and galactose-containing media (bottom). Cells where visualized by a bright field and DAPI. n=3 experiments.

CHAPTER IV

SUMMARY AND CONCLUSION

These findings indicate that disruption of mitochondrial structural dynamics in mtDNA-depleted cells leads to increased apoptotic sensitivity. King et al. (1989) first showed that mtDNA could be depleted from live cells in culture by treating with ethidium bromide, thus leading to decrease mitochondrial respiratory function. We first show that mtDNA nucleoids are distributed throughout the mitochondrial network (**Fig.1**) by using TFAM that previously showed to bind to DNA and mirroring the abundance of mtDNA within the cells. These mtDNA-depleted ρ^0 cells rely entirely on cytosolic glycolysis (**Fig. 6 and 7**), and can thus be passaged in cell culture. The inability to produce mtDNA-encoded polypeptides leads to incompletely assembled OXPHOS complexes (Marusich et al., 1997). This results in cells without mtDNA, lacking oxidative phosphorylation and reticular morphology **Fig. 2**. In order to demonstrate that ρ^0 cells contain no mtDNA, PCR analysis was done to show that they contain no mtDNA (**Fig. 3A),** while western blot experiments show that ρ^0 cells contain no MTCO2 proteins (**Fig. 3B**). MtDNA-depleted ρ^0 cells have increased basal apoptosis when observing the nuclei morphology (**Fig. 4**). Cells without mtDNA have decreased $\Delta\psi_m$ (**Fig. 5A**) which was previously shown (Gilkerson et al., 2012)) but ρ^0 cells do maintain a minimal $\Delta\psi_m$ and undergo apoptosis **Fig. 5B**, which is likely due to the presence of F_1F_0 ATPase, reversing H^+ transport to permit growth (Buchet and Godinot, 1998). Thus, cytochrome c̲ is available for apoptotic signaling and is not

required for electron transport in ρ^0 cells. Kwong et al. suggested that OXPHOS activity might regulate apoptosis in a context dependent manner.

Alternatively, the increased organellar fission associated with loss of mtDNA may activate increased apoptosis. Recent studies have sparked controversy whether fission is required for apoptosis. Induced by several stimuli, apoptosis fragments mitochondria via proteins such as DRP1 and FIS1 (Parone et al., 2006). Down-regulation of DRP1 has been reported to prevent fragmentation of mitochondria and release of cytochrome c. This is sufficient to inhibit Bax/Bak-dependent apoptosis, therefore demonstrating that fission is not important in Bax/Bak-dependent apoptosis (Parone et al., 2006). DRP1 was two forms an active and stable during early-apoptosis an active form of DRP1 can mediates the mitochondria and is independent of Bax/Bak which is responsible for mitochondrial permeabilization, but once Bax is recruited to the mitochondria and before the loss $\Delta\psi_m$ occurs DRP1 becomes stable (Wasiak et al., 2007). A stable form of DRP1 is dependent in Bax/Bak and leading to later events such as cristae remodeling (Germain et al., 2005) and the subsequent release of cytochrome c, eventually causing the complete loss of $\Delta\psi_m$. Staurosporine (STS) induces apoptosis by translocation of DRP1 to the outer membrane, while DRP1-negative cells can block apoptosis (Frank et al., 2001), suggesting that mitochondrial fission is necessary in order for apoptosis to occur. To determine whether the decreased viability in galactose- challenged ρ^0 cells is due to the bioenergetics defect or the loss of organellar fusion, future experiment will used mouse embryonic fibroblast deleted for the OMA1 metalloprotease. OMA1 is an ATP independent zinc metalloprotease that is located in the mitochondrial inner membrane. OMA1 was been identified as a mitochondrial quality control protease and regulator of metabolic homeostasis (Zhang et al., 2014). Previous studies have showed that OMA1 metalloprotease mediates $\Delta\psi_m$ sensitive cleavage of OPA1 (Ehses et al.,

2009), suggesting that OMA1 is a key mediator for observing $\Delta\psi_m$ threshold of mitochondrial fission/fusion balance. In order to confirm this possibility, pharmacological and genetic $\Delta\psi_m$ loss in mouse embryonic fibroblast (MEFs) either carrying OMA1+/+ or OMA1-/- for OMA1 gene (a gift from Dr. Carlos Lopez-Otin, University of Oviedo). An experiment should be carry out by using OMA1 -/- ρ^0 cells in galactose media and demonstrated if ρ^0 cells are protected or not protected against apoptosis by observing the mitochondrial interconnection.

Future work must continue in order to completely characterize apoptosis and/or decrease proliferation in mtDNA-depleted ρ^0 cells. These can be accomplished by visualizing cytochrome c release with confocal immunofluorescence microscopy and western blot. Mitochondrial cytochrome c functions as an electron carrier in the respiratory chain and then translocate to the cytosol in cells undergoing apoptosis (Bossy-Wetzel et al., 1998). When cells undergo apoptosis cytochrome c is release to the cytosol it activates cytosolic factors such as caspases. When caspases are activated, it cleaves a number of substrates that allow them to be activate or inactivate which establish the morphological and biochemical features of apoptosis (Bossy-Wetzel and Green, 1999). Previous studies have revealed that translocation of cytochrome c into the cytosol occurs in response to multiple apoptotic stimuli and can plays an important role in inducing apoptosis. In order to observed if cytochrome c is activating caspases 9 we must do a western blot of cytochrome c inside the mitochondrial and outside and compare it with amount of caspases 9 (Chen et al., 2000). To demonstrate a more accurate way of assessing cell death annexin V flow cytometry will be used. Annexin V is used to determine if cells are viable, apoptotic, or necrotic through differences in plasma membrane integrity and permeability (Lassus and Hibner, 1998). In this paper we introduce the loss of viability in galactose using

MTT assay. In the last couple of years different types of assays have been develop to more effectively test cell viability, such as CCK-8 assay (Lu et al., 2010).

Apoptosis is a terminal morphological and biochemical event of programmed cell death that is characterized by specific changes in cell surface and nuclear morphology. These finding have a directly relevance to a wide range of prevalent diseases that include mitochondrial dysfunction. Decreased OXPHOS function and disrupted fission/fusion balance is emerging as a signature of mitochondrial dysfunction in patient samples and disease models of neuromuscular disease (DiMauro and Schon, 2003), diabetes (Mootha et al., 2003) or even Parkinson's disease (Bender et al., 2006a). Our findings illustrate a basic parameter of mitochondrial participation in apoptosis by looking at the loss of OXPHOS. These finding strongly helps us understand the mitochondrial role in apoptosis, which plays a strong role in many of the most prevalent diseases facing public health.

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BIOGRAPHICAL SKETCH

The author, Alan M. Herrera, was born on May 5, 1990 in Mexico City and is now currently living in 107 W. Daffodil Ave. Pharr, TX 78577 as the eldest of Silvia Martinez, and Hugo Herrera.

He took his primary education at Sir Guy Carleton Elementary School in Vancouver Canada and Secondary education at Pharr- San Juan- Alamo North High school in Pharr, TX, were he was a consistent honor student and received the excellence in Science Award. Alan is not only academically inclined but is also good in other fields such as sports, arts and music. In the four years spent in high school, he was the co-founder for the chess club, psychology club, and history club. In 2008, he was admitted to the University of Texas Pan-American under the Bachelor of Science in Biology, with a minor in Chemistry. In 2013, he was admitted to the graduate program at the University of Texas Pan-American under the Masters of Science in Biology. Alan has earned a Masters degree in Biology from the University of Texas Pan-American in 2015.

He is a member of a cell biology research team lead by his advisor Dr. Robert Gilkerson and has published a publication at Cold Spring Harbor Perspectives in Biology in 2013. Alan has also published a first author paper in Frontiers in Bioscience in 2015 and continues to work in other manuscripts. Aside from academics, he also engaged in Community service activities. Since 2006, Alan has continued to help out in his community food bank.