SENESCENT HUMAN DIPLOID FIBROBLASTS UNDERGO A CASPASE-INDEPENDENT APOPTOTIC DEATH

By

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ABSTRACT

Senescent Human Diploid Fibroblasts Undergo a Caspase-Independent Death

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Apoptosis functions to maintain a homeostatic environment within an organism and the slightest disruption in the pathway can trigger the onset of diseases such as cancer and Alzheimer's. Many researchers have made tremendous contributions to the field by deciphering the relevant components of this process. As the elderly are most susceptible to disease, understanding the cause of this vulnerability is important. To study changes associated with aging, we use human diploid fibroblasts as our model system. These fibroblasts cease division after 60-70 ± 10 population doublings (depending on cell type).

As apoptosis occurs in many experimental systems, senescent fibroblasts were considered resistant to apoptotic death. We previously provided evidence against the aforementioned finding, in that senescent WI38 fibroblasts when challenged with okadaic acid underwent apoptosis. In light of our previous results, a series of experiments were performed to determine the molecular apoptotic pathway used by senescent fibroblasts in the presence of okadaic acid.

A novel pathway has been discovered in that senescent fibroblasts, in response to okadaic acid, go through a caspase-independent death.

Interestingly, in the absence of caspase activation, we see down-regulation of a caspase substrate DNA Fragmentation Factor 45 in senescent cells challenged with okadaic acid. In a search for proteins which cause apoptosis in a caspase-independent manner, Apoptosis Inducing Factor was released from the mitochondria and translocated to the nucleus.

Upon closer examination of the mitochondria, we find they become less clustered in senescent cells 24 hours after the addition of okadaic acid. Furthermore, a mitochondrial fission protein, Drp1, binds to these fragments along with Bak. When mitochondrial membrane potential was analyzed, we see elevated levels in young cells, but the potential in senescent cells decreases as early as 6 hours after the addition of okadaic acid.

In view of our findings, it is evident that senescent cells are capable of undergoing an apoptotic death and this death is regulated in a caspase-independent manner. Our results will aide in our understanding of senescent cell death.

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INTRODUCTION

Types of Cell Death

Programmed cell death was coined by Lockshin & Williams in 1965 based on their observations in silkmoth development (Lockshin and Williams, 1965). Lockshin and Williams noticed cells of the intersegmental muscle consistently met their demise in a timely fashion due to the activation of the lysosomal enzyme cathepsin (Lockshin and Williams, 1965). Like Lockshin and Williams, Saunders also noticed similar observations during chick development (Saunders, 1966). In the early 1970's the group lead by Andrew Currie furthered the above findings by examining normal and cancerous tissues from humans and rats.

Various insults to the tissues such as adrenocorticotrophic hormone removal or obstruction of portal blood supply to liver tissue resulted in nuclear condensation and fragmentation, cell shrinkage, and engulfment by phagosomes (Kerr *et al.*, 1972). They illustrated the distinct morphology of an apoptotic cell in comparison to one undergoing a necrotic death. In light of their findings, Kerr *et al.* (1972) coined this type of programmed cell death apoptosis which is Greek for "falling off". Regardless of tissue type the apoptotic morphological characteristics were similar. Kerr *et al.* (1972) hypothesized that apoptotic death was responsible for the programmed cell death measured during development and the lack of this event was responsible for the onset of tumors. Apoptosis, they predicted, could have therapeutic implications as well (Kerr *et al.,* 1972).

Twenty years later with advancements in molecular biology, the predictions and implications of apoptotic death made by Kerr *et al*. (1972) were confirmed (Cohen, 1997; Bröker *et al.,* 2005; Li *et al*., 2003; Garrido and Kroemer 2004). Evidence suggests that the manner in which a cell dies varies depending on the signal received by the cell. To date there are five different types of cell death, these include: apoptosis (type I), necrosis (type II), autophagy, endoplasmic reticulum-mediated cell death, caspase-independent apoptosis (Lockshin and Zakeri, 2004; Kroemer and Martin, 2005; Fietta, 2006). The five types of cell death are described in detail below.

Apoptosis

Apoptosis is a genetically conserved event that occurs in many organisms from yeast to mammals (Ameisen, 2002). This type of cell death occurs as a normal function of organismal development. It also serves to sculpt the nervous and immune systems (Lockshin and Zakeri, 2001; Lee *et al*., 2000; Bouillet *et al*., 2002; Saunders, 1966). Richard Flavell's group was one of the first to grossly demonstrate the importance of apoptotic death in the nervous system using caspase-9 knockout mice (Kuida *et al*., 1996). Embryonic mice harboring mutations in caspase-9 were lethal.

The morphological characteristics associated with apoptosis include membrane blebbing, cytochrome *c* release, chromatin condensation, DNA fragmentation, destabilization of cytoskeletal proteins and the formation of apoptotic bodies that are eventually engulfed by neighboring cells or phagocytes (Kerr *et al*., 1972; Joaquin and Gollpudi, 2001; Zakeri *et al*., 1993). The phases

of apoptosis can be divided into three categories: (1) the induction phase (Joaquin and Gollapudi, 2001), (2) the effector phase (caspase activation) (Green, 2000) and (3) the degradation phase (fragmented DNA and the engulfment of apoptotic bodies) (Green and Kroemer, 1998; Krieser and White, 2002). The induction phase involves signals that can arise extrinsically or intrinsically (Ashkenazi and Dixit, 1998) (Figure 1).

Extrinsic death

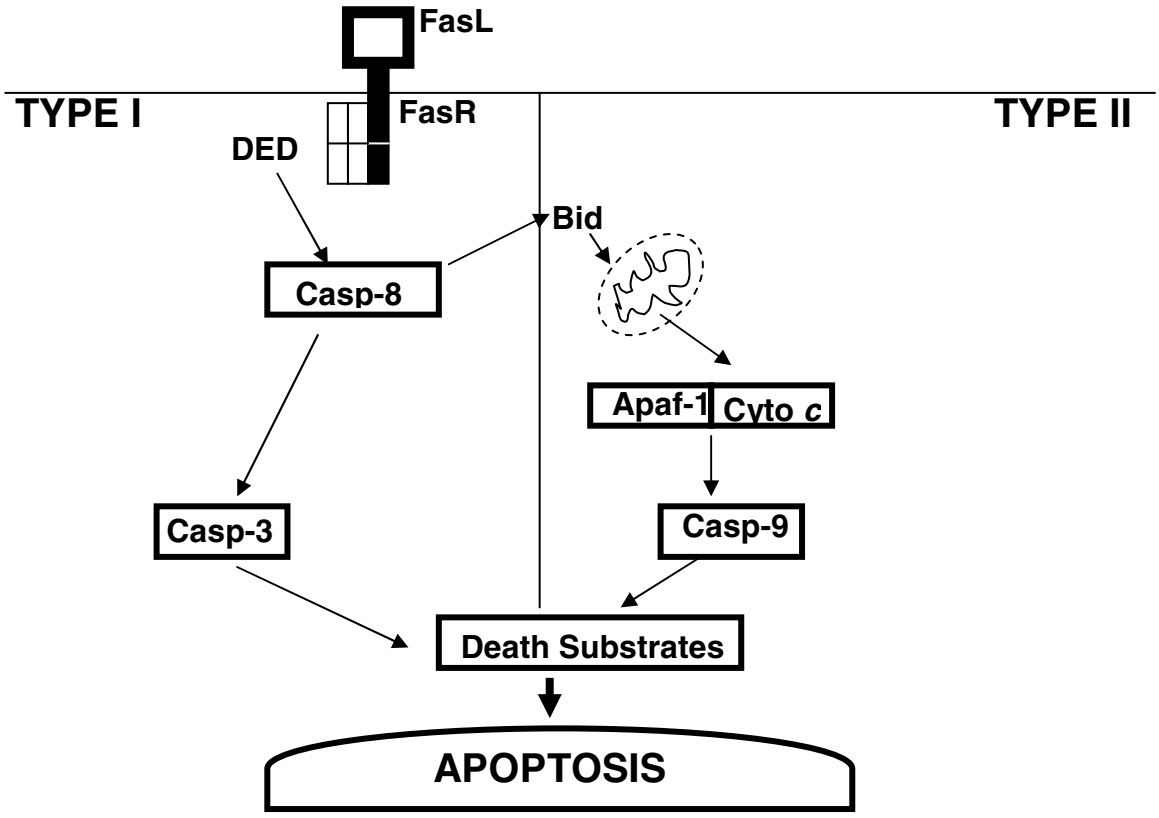
The death receptors belong to the tumor necrosis factor receptor (TNFR) gene super-family (Enari *et al*., 1995). Members of this family all contain similar cysteine-rich extracellular domains (Enari *et al*., 1995). In addition, the death receptors all contain a homologous cytoplasmic sequence termed the death domain (Enari *et al*., 1995). CD95/Fas/Apo-1 (a type I membrane receptor protein) and TNFR1 are the best-characterized death receptors (Enari *et al*., 1995). Other death receptors found on the cell surface include death receptor 3/Apo3, death receptor 4, death receptor 5/Apo2 and avian CAR1 (Ashkenazi and Dixit, 1998; Özören and El-Deiry, 2003). Receptor-ligand binding will lead to dimerization of the death effector domains (DEDs) and result in a downstream cascade of events (Green, 1998). The cell meets its demise following the activation of initiator caspases which then lead to the activation of effector caspases and inactivation of their respective substrates (Green, 1998).

Intrinsic death

Intrinsic cell death typically involves the mitochondria, although initiator caspase activation through receptor-ligand binding can also participate

Figure 1. Schematic representation of Apoptotic Pathways.

Type I involves activation of caspase-8 followed by caspase-3 activation and cleavage of death substrates. In Type II apoptosis the mitochondria most often contribute to the demise of the cell.



(Kominsky *et al.,* 2002) (Figure 1). Cytochrome *c* is released into the cytosol where it interacts with an adaptor protein Apaf-1 (Green, 2005). The cytochrome c/Apaf-1 complex will then cleave and activate initiator caspase-9 (Green, 2005). The three proteins together form the apoptosome and function to activate effector caspase-3. As in the case off extrinsic death, once caspase-3 is activated the cell is committed to die (Ranger, 2001; Hao *et al*., 2005). Intrinsic cell death is regulated mainly by the Bcl-2 family of proteins whose properties will be discussed below. Both extrinsic and intrinsic death serve to execute cell death such that the apoptotic bodies are rapidly engulfed preventing an inflammatory response (Hamon, 2002). Necrosis on the other hand does not occur in this way.

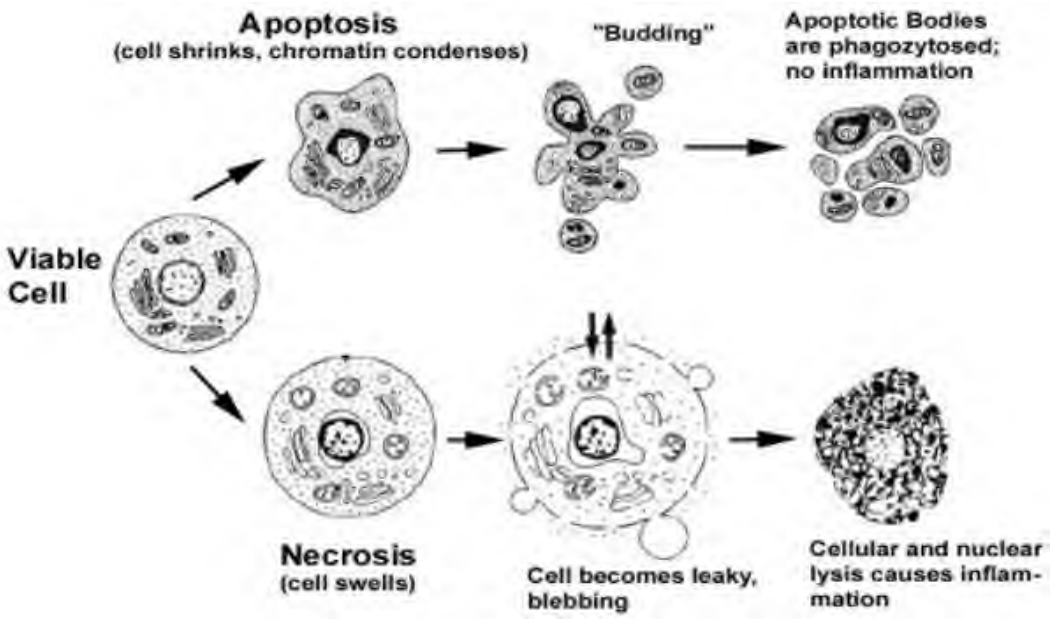
Necrosis

Necrosis is a passive form of cell death where the demise of the cell is not programmed and usually occurs in ischemic injury or oxygen deprivation (Kerr *et al*., 1972; Lockshin and Zakeri, 2004; Fietta, 2006). The phenotype of a necrotic death consists of increased cytosolic volume, swelling of the ER, mitochondrial condensation, irregular nuclear chromatin clumping and depletion of the ATP supply (Majno and Joris, 1995 and Figure 2). Cellular contents are released due to the permeabilization of the plasma membrane. An inflammatory response is elicited because necrotic cell death does not involve the engulfment of the damaged cell (Fietta, 2006).

Recent data has shed some light about the downstream effects resulting from necrotic death (Xu *et al*., 2001). Necrotic events are oftentimes the result of increased Ca^{2+} due to unregulated Ca^{2+} channels. The Driscoll group examined

Figure 2. Diagram describing the morphological differences between apoptosis and necrosis.

Apoptosis ensures careful packaging of apoptotic bodies by phagocytes or neighboring cells. A necrotic cell death will elicit an inflammatory response due to the swelling and eventual rupturing of the cell.



Van Cruchten, 2002

necrosis via a hyperactive MEC-4(d) channel in *C. elegans.* Ectopic expression of mec-4(d) under the control of a *unc-8* promoter resulted in an influx of Na^+, increased intracellular Ca^{2+} to neurons , degeneration of ventral nerve cords and ultimately paralysis (Xu *et al*., 2001). The paralyzed worms were then mutagenized with Ethyl Methanesulfonate (EMS) (Brenner, 1974; Xu *et al*., 2001). The F2 progeny with restored locomotion were then screened for suppressor mutants (Brenner, 1974; Xu *et al*., 2001). Calreticulin, a calcium binding protein found in the lumen of the ER, was identified as a strong suppressor in the paralyzed worms (Xu *et al*., 2001). Further analysis revealed calrecticulin was capable of relieving the necrotic death observed above (Xu *et al*., 2001). Regulation of Ca^{2+} stores, therefore, is essential in preventing necrotic death in *C. elegans*. Necrosis has been the cause of neuronal degeneration in mammals (Danial and Koresmeyer, 2004) and this study added further insight into neuronal loss by using a simple organism. Preventing necrotic death in mammals is important as many stroke victims tend to lose neuronal cells in a necrotic manner resulting from rapid influx of calcium (Kristián and Siesjö, 1998). The development of drugs to intervene in this neuronal loss will be a tremendous benefit.

Autophagy

Autophagy involves the engulfment of cytoplasm and organelles in double or multi-membrane vesicles (autophagosomes) and the contents face degradation by the cells own lysosomal machinery (Liu *et al*., 2004). This event

is active under conditions of starvation, in the presence of damaged organelles or during insect development (Levine and Yuan, 2005). Genetic screening has given researchers the opportunity to understand how autophagy is controlled. Type I and III phosphatidylinositol-3 (PI3) kinases have both been shown to regulate autophagic events (Edinger and Thompson, 2003; Levine and Yuan, 2005). Type I PI3 kinases can suppress autophagy through downstream signaling events with Akt and mTOR (Kim *et al*., 2006; Ogier-Denis and Codogno, 2003) alternatively, Type I PI3 kinases can be negatively regulated by the tumor suppressor PTEN (Arico *et al*., 2001). Type III PI3 kinases have been shown to be necessary for the formation of the autophagosome and its transport to the lysosome for degradation. Included in this group is the Atg (autophagosome) family (Liang *et al*., 1999).

Starvation, insect development and apoptosis are three instances where autophagy has been implicated. Under conditions of starvation the Atg genes function to help maintain cellular ATP and protein synthesis. In such instances breakdown of long-lived proteins into precursor molecules occur which can be reused by the organism (Lum *et al.,* 2005). Mutations in Atg genes are embryonic lethal phenotypes in mice due to lack of food from the placenta (Levine and Klionsky, 2004; Kuma *et al.,* 2004). Lockshin and Williams were one of the first groups to recognize autophagic death in development. They found that it functioned mainly during morphogenesis, specifically in the degradation of intersegmental muscle (Lockshin and Williams, 1965). The findings of Lockshin and Williams occurred in a non-classical apoptotic (i.e. no involvement of the

caspases or Bcl-2 family) manner. There is now emerging evidence showing a molecular connection between classical apoptosis and autophagic death.

Two groups have studied the interactions between autophagy proteins and anti-apoptotic members of the Bcl-2 family (Bcl-x_L and Bcl-2) (Yousefi *et al.*, 2006; Pattingre *et al.*, 2005). An interaction between Atg-5 and calpain, a cysteine protease was revealed using neutrophils and breast cancer cell lines (HeLa, Jurkat and MD-MA-231) challenged with death-inducing drugs. Calpain induced cleavage of Atg-5 to an active 24 kDa product (Yousefi *et al.*, 2006). The 24kDa product then translocated to the mitochondria where it colocalized with Bcl-x_L resulting in the release of cytochrome *c* and subsequent activation of effector caspase-3 (Yousefi *et al.*, 2006).

Previous data suggested a novel interaction between the anti-apoptotic Bcl-2 and Beclin-1 (Atg6), a type III PI3 kinase, but the biological significance was not known (Liang *et al.*, 1999). The Levine group sought to determine the importance this interaction under conditions of starvation (Pattingre *et al.*, 2005). The function of Bcl-2/Beclin-1 interaction in their studies served to prevent autophagosome formation. Mutant Bcl-2 proteins failed to bind Beclin-1, causing increased autophagy. In a similar fashion, Beclin-1 proteins defective in binding Bcl-2 resulted in increased autophagy. As the anti-apoptotic Bcl-2 has been extensively studied under apoptotic conditions, these studies suggest a role for Bcl-2 in autophagy as well.

Noteworthy findings have come about in the area of autophagy and diseases such as cancer, Alzheimer's and Parkinson's diseases (Liang *et al.,*

1999; Levine and Yuan, 2005; Nixon, 2006). Beclin-1 is found on human chromosome 17q21, more specifically in the tumor susceptibility locus. This locus is deleted in 40-75% of ovarian and breast cancers (Liang *et al.,* 1999). Increased autophagic vesicle formation in MCF7-*beclin1* transfectants was observed when beclin-1 was overexpressed in a human breast cancer cell line during serum starvation (Liang *et al.,* 1999). This was the first study which implicated autophagy as having a therapeutic benefit in cancer.

Alzheimer and Parkinson's diseases greatly affect the aging community. Efforts have been made to understand the mechanism by which these diseases develop. As both chaperone-mediated autophagy (CMA) and macroautophagy functions decline significantly during aging, it seemed logical to study this decline from a neurological perspective (Martinez-Vicente *et al*., 2005). Studies using mice deficient in autophagic functions show a lack of removal of mis-aggregated neuronal proteins such as amyloid β and α-synuclein which are toxic to the aging brain (Martinez-Vicente *et al*., 2005; Hara *et al*., 2006; Komatsu *et al*., 2006).

Autophagy is an important mechanism which functions to remove unwanted materials. This process is essential under starvation conditions and may prove to be beneficial to the organism. Future work on the molecular link between autophagy, apoptosis and cancer will hopefully prove fruitful with regards to therapeutic benefits.

Endoplasmic reticulum-mediated cell death

Proper folding of membrane proteins, secretory proteins, lipid and sterol synthesis and responding to calcium needs are important physiological roles of the endoplasmic reticulum (ER) (Boyce and Yuan, 2006). This organelle will respond to various alterations in its environment by either sending signals to the nucleus allowing the activation of genes for cell survival or apoptosis is induced (Rao *et al*., 2004). Misfolded proteins, expression of mutant proteins or subunits, and irregular calcium homeostasis can promote ER stress (Rao *et al*., 2004). Once the stress is recognized a series of genes are up-regulated forming what is known as the uncoupled protein response (UPR) (Kaufman, 1999). The UPR is composed of three paths: sensors, modulators and effectors. The sensors (GRP78, PERK, IRE1, ATF6) (Liu *et al*., 2000), modulators (Bcl-2 family, Gadd153) (Nutt *et al*., 2002) and effectors (caspase-12, BAP31) (Breckenridge *et al*., 2003; Nakagawa and Yuan, 2000) all have distinct functions under ER stress in that either transcription of nuclear stress genes occurs or an apoptotic response is induced as discussed below.

ER Sensors

The glucose-regulated protein-78 (GRP-78) is an ER chaperone protein located on the ER lumen which aides in protein folding (Kaufman, 1999). Oxidative stress, treatment with calcium ionophores, and inhibitors which function to disrupt ER structure have been found to induce the expression of GRP proteins (Ron, 2002); thus activation of GRP78 is used as a marker of the UPR. GRP78 binds to several proteins under unstressed conditions, these proteins include: PERK, IRE1 and ATF6 (Kaufman, 1999). GRP78 undergoes a

conformational change due to binding of altered proteins. As a result PERK, IRE1 or ATF6 are released and translocate to the nucleus. In the nucleus they function to increase transcription of ER stress genes which participate in the ER's ability to fold newly synthesized proteins or degrade abnormal proteins (Kaufman, 1999).

PERK (PKR-like ER kinase) is a protein kinase that functions to inhibit initiation of protein synthesis (Boyce and Yuan, 2006). In the presence of misfolded proteins, which bind GRP78, PERK undergoes dimerization and *trans*-autophosphorylation (Liu *et al*., 2000). Activation of the kinase domain leads to phosphorylation of the alpha subunit of eukaryotic translation initiation factor-2 alpha (eIF2α). As a consequence translation and protein synthesis are terminated (Ron, 2002).

IRE1 was first identified as an ER membrane-spanning receptor kinase required for inositol phototrophy (Cox *et al*., 1993). It was later determined that IRE1, like PERK is a sensor for unfolded/misfolded proteins in the ER lumen and was negatively regulated by GRP78 (Boyce and Yuan, 2006). PERK and IRE share similar functions such that studies in yeast and mammals show that their luminal domains are interchangeable (Liu *et al*., 2000; Bertoltti *et al*., 2000). IRE1 was first characterized in yeast and named IRE1p. It was found that this protein has unique RNA splicing ability. Under normal conditions IRE1p activity is inhibited by Kar2p, a chaperone protein (Boyce and Yuan, 2006). When unfolded proteins are present, they bind Kar2p allowing for the release of IRE1p. IRE1p becomes activated by *trans*-autophosphorylation and binds HAC1. HAC1

is normally inactive in the cell due to the presence of a translation attenuator intron (Kaufman, 1999). IRE1p will splice the attenuator intron from the HAC1 gene generating a product that encodes a bZIP transcription factor that promotes expression of the UPR genes (Kaufman, 1999).

The manner by which mammalian systems respond to ER stress is similar to that of yeast, but as expected, is more complex. The mammalian homologues of yeast IRE1p are IRE1α and IRE1β (Boyce and Yuan, 2006). IRE1α is found in all tissues, while IRE1β is only present in the intestines (Bertoltti *et al*., 2000; Tirasophon, *et al.,* 1998). The activation of IRE1 in mammals occurs in a similar fashion as in yeast. Activation of IRE1 arises when misfolded proteins bind GRP78 releasing IRE1, IRE1 then dimerizes and is *trans*-autophosphorylated thus activating its RNase function (Liu, 2000). As IRE1p was essential in yeast, its role in mammals was found not to be as crucial in regards to development (Harding, *et al.,* 2002). Mammals have the transcription factor X-box-binding protein-1 (XBP1) that is similar to that of HAC1 in yeast (Rao *et al*., 2004). Once spliced by IRE1 α, XBP1 functions to upregulate the genes necessary to respond to ER stress (Kaufman, 1999).

Activating transcription factor 6 (ATF6) is inactive during unstressed conditions due to its binding to GRP78. When misfolded proteins accumulate, ATF6 moves towards the Golgi where it is cleaved by site-1 and site-2 proteases. As a result, the free amino terminal portion of ATF6 is allowed to translocate into the nucleus where it binds ER stress elements thus activating the transcription of ER molecular chaperone proteins (Liu and Kaufman, 2003).

ER Stress-Induced cell death modulators

Cellular calcium is regulated by the ER (Xiong *et al*., 2006; Szabadkai *et al*., 2006). Disruption of Ca^{2+} homeostasis results in apoptotic death in most instances. The exchange between $[Ca^{2+}]_{ER} \rightarrow [Ca^{2+}]_{M}$ occurs through a transport tunnel known as pseudosynaptic contacts (Rizzuto *et al*., 1998). Studies have examined the biological significance of this movement and have found that the Bcl-2 family play a major role (Nutt *et al*., 2002). The Bcl-2 family (discussed below) consists of anti- and proapoptotic members. Members of the family have been shown to reside in the mitochondria as well as the ER (Thomenius *et al*., 2003) depending on tissue and cell type. Although the functions of the proteins in the mitochondria have been examined, how they regulate apoptotic death with regards to the ER was not well understood (Thomenius *et al*., 2003). Clarification of their role has been provided by performing experiments which disrupt Ca^{2+} homeostasis (Nutt *et al.,* 2002).

Overexpression of Bak and Bax using an adenoviral vector resulted in the efflux of ER Ca^{2+} stores and an influx of Ca^{2+} to the mitochondria as early as 10 hours after transfection. Cytochrome *c* release was observed and was associated with increased mitochondrial Ca^{2+} stores. This situation was caspase-independent as the pancaspase inhibitor zVAD-fmk did not disrupt cytochrome *c* release (Nutt *et al.,* 2002).

As mentioned above, like the proapoptotic members, the anti-apoptotic members of the Bcl-2 family reside in the mitochondria and ER. Their role in Ca^{2+} homeostasis was intricately defined in overexpression experiments. No

apparent change was noticed in the Ca^{2+} levels in the ER and mitochondria when Nutt and co-workers overexpressed Bcl-2 in a prostate cancer cell line followed by exposing them to staurosporine. Furthermore, the levels of cytochrome *c* release decreased in the presence of overexpressed Bcl-2. Taken together these studies on calcium fluctuations indicate a direct role for the pro- and anti-apoptotic Bcl-2 proteins in ER stress. When the mitochondria were examined more closely, the release of cytochrome *c* correlated with the abundant influx of mitochondrial Ca^{2+} (Nutt *et al.,* 2002). It is important to note that the response obtained in such experimentation is inducer- and cell type-specific.

ER Stress-Induced cell death effectors

As mentioned above, an accumulation of misfolded proteins and an imbalance of Ca^{2+} can induce ER stress. Caspase-12 and BAP31 are two proteins that function as effectors upon insult to the ER and the result is apoptotic death (Nakagawa and Yuan, 2000; Breckenridge *et al*., 2003; Groenendyk and Michalak, 2005). The ER stress sensor, IRE1, has dual roles in that it can initiate translation of transcription factors or it can act in an apoptotic manner. IRE1 has been shown to bind and activate caspase-12 and together with caspase-9 can activate downstream effectors such as caspase-3 (Groenendyk and Michalak, 2005). The caspase family will be discussed in more detail in the following sections. BAP31, an ER transmembrane protein functions to bind newly synthesized protein that is in transit from the ER and *cis*-Golgi (Kaufman, 2000). When ER stress ensues, BAP31 is cleaved by active caspase-8, releasing a BAP31 p20 fragment (Breckenridge *et al*., 2003). This fragment causes Ca^{2+}

release from the ER and Ca^{2+} entry into the mitochondria (Breckenridge *et al*., 2003). Interestingly, the cleavage of BAP31 by caspase-8 results in mitochondrial fission which will further enhance cytochrome *c* release from the mitochondria (Breckenridge *et al*., 2003).

To summarize, ER stress is an event that is triggered by misfolded proteins or a disruption of Ca^{2+} homeostasis (Groenendyk and Michalak, 2005). The ER responds to this stress in one of two manners: inhibition of protein synthesis or apoptosis (Kaufman, 1999). First the ER makes an effort to correct the problem by sending signals through ER sensors, i.e. PERK, IRE1 and ATF6 (Rao *et al*., 2004). These proteins serve to inhibit protein translation and ultimately relieve the stress. Drug-induced ER stress can result in a disproportionate amount of Ca^{2+} ($\downarrow[Ca^{2+}]_{ER} \rightarrow \uparrow[Ca^{2+}]_{M}$) in the cell and this balance is intricately regulated by the Bcl-2 family (Nutt *et al.,* 2002). This data provides a link between the ER and mitochondria in that ER stress induces apoptosis upstream of mitochondrial-induced release of cytochrome *c*.

There have been significant findings with regard to characterizing the genes responsible for the ER stress response. However, it is unclear how a cell decides to use a specific pathway, i.e., activation of ER stress response or whether apoptosis should be elicited. Furthermore, how does a cell consider when “too much” ER stress is sufficient to trigger a response. These questions remain to be answered by researchers and their findings may serve to aid in our understanding of how disease progresses particularly neuronal diseases such as

Alzheimer's and Parkinson's where misfolded proteins are thought to be responsible for the onset of disease.

Discussed above are the accepted modes of cell death. As studies in this field increase, more types of death will undoubtedly be discovered. Researchers are finding these different modes of death share common paths or that they converge at a common point. Lemasters and collegues, for instance, have developed the term "*necrapoptosis*" to point out the fact that shared paths may exist between apoptosis, necrosis and autophagy (Rodriguez-Enriquez *et al.,* 2004).

The important regulators of apoptosis include (1) caspases (Ranger *et al*., 2001), (2) the mitochondria (Olson and Kornbluth, 2001), (3) the Bcl-2 family (Kim, 2005) and (4) inhibitors of apoptosis proteins (Abraham and Shaham, 2004) are important in apoptotic functions as discussed below.

The Caspases

C. elegans predictably loses 131 out of 1090 somatic cells during development resulting in a 959-celled organism (Yuan *et al*., 1993). The simplistic nature of the nematode allowed Sydney Brenner to perform elegant genetic studies to detect genes which affected behavior in this organism (Brenner, 1974), a task that could not be performed with the more complex Drosophila at that time. These pioneering experiments showed *C. elegans* was an ideal model to study genetics and development thus paving the road for others (Ellis and Horvitz, 1986). As Brenner had done, Ellis and Horovitz made

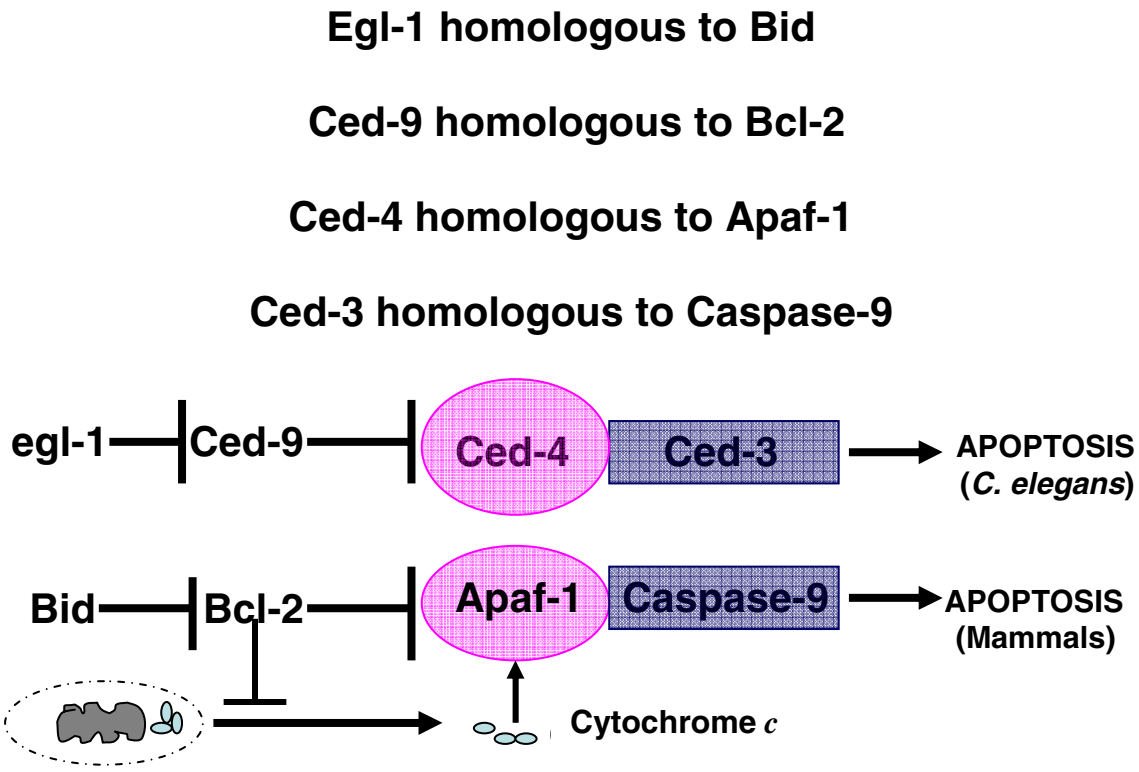
groundbreaking findings when using *C. elegans* to study apoptosis and development. They answered the fundamental question of how 131 somatic cells unfailingly die at a particular point in development (Ellis and Horvitz, 1986). The discovery of the *C. elegans* death (*ced*) genes has had a significant impact in the field of apoptosis.

The CED proteins share homology to its mammalian counterparts (Hengartner, 1998). CED-9 is related to the Bcl-2 family of anti-apoptotic proteins; CED-4 is homologous to an adaptor protein Apaf-1 and CED-3 is homologous to the effector caspases responsible for the deletion of cells in *C. elegans* (Hengartner, 1998). (Figure 3). These genes function together where CED-4 binds to CED-9 thus inactivating its function (Hengartner, 1998). Free CED-4 then causes the auto-processing of CED-3, thus inducing the cell to die by apoptosis (Hengartner, 1994). CED-3 activity can be inhibited by CED-9 (Hengartner, 1994). Conradt and Horvitz identified a protein called EGL-1, which can induce a chain of events that result in the dissociation of CED-4/CED-3 from CED-9 and ultimately the activation of CED-3 (Conradt and Horvitz, 1998).

In mammals calpains and caspases are two classes of enzymes function in the apoptotic process (Mandic *et al*., 2002; Goyal, 2001). Calpains are a family of neutral proteases that are important in cell growth and apoptosis (Squier *et al*., 1999) They behave in a similar fashion as caspases in that they cleave their substrates such as cytoskeletal proteins, membrane receptors and enzymes at specific residues (Tompa *et al*., 2004). Just recently calpains have been implicated in autophagic cell death. Calpains have been shown to cleave Atg5

Figure 3. *C. Elegans* Death proteins.

C. elegans **death (CED) proteins and their involvement in apoptotic death in the nematode in comparison to homologous mammalian apoptotic proteins.**



Adapted from Jiang and Wang, 2004

into a 24kDa fragment. This fragment translocates to the mitochondria where it interacts with Bcl-x_L an anti-apoptotic protein and release of cytochrome *c* (Yousefi, *et al.,* 2006).

Caspases are a group of cysteine proteases that are closely involved in the execution of the cell (Green and Kroemer, 1998; Nicholson, 2001). To date there are fourteen caspases identified in mammals (Green, 1998; van de Craen *et al*., 1998; Nakagawa *et al*., 2000; Danial and Korsmeyer 2004). The caspases normally exist as zymogens, or inactive enzymes consisting of a large and small subunit (Nicholson, 2001). A complex feedback mechanism exists in the caspase family, thus the activation is not a linear event (Kroemer and Martin 2005). Caspase activation can occur autocatalytically or by previously activated caspases (Abraham and Shamam, 2004 ; Kroemer and Martin, 2005). Caspases are cleaved at specific aspartic acid residues and are activated by the sequential proteolytic activation of each successive procaspase in the apoptotic cascade (Goyal, 2001). When the caspases become active, they are responsible for the cleavage of key substrates such as poly-ADP-ribose polymerase (PARP) (Koh *et al*., 2005), α-fodrin (Vanags *et al*., 1996), Lamin A (Oberhammer *et al*., 1994), and DFF45 (Sharif-Askari *et al*., 2001). Cleavage of these substrates results in the eventual death of the cell by apoptosis. (Figure 1).

As mentioned above, the caspase family is comprised of fourteen members, but not all are involved in apoptosis. Human caspases-1, 4 and 5, for instance, are involved in inflammatory response or cytokine production (Garrido and Kroemer, 2004). Caspases-2,-3-,-6,-7,-8,-9,-10,-11,-12 participate in

apoptotic death (Green, 1998; Garrido and Kroemer, 2004). The caspase family consists of initiator and effector caspases. The initiator caspases include caspase 2,-8, -9, -10 and -11, which are involved in upstream regulatory events (Green, 1998). The effector caspases are located further downstream and include caspase 3,-6 and 7 (Green and Kroemer, 1998). Caspase function has been explored in a variety of cell lines and organisms (Ellis and Horvitz, 1986; Chandler *et a*l., 1998; Zeuner *et al*., 1999; Ellerby, *et al.,* 1999; Liang *et al*., 2001; Zhivotovsky and Orrenius 2003; McCall, 2004).

The initiator caspase-9 is ubiquitously expressed in mammalian tissues, particularly in the heart (Li *et al.,* 1997). Its relevance in neuronal development was brought to the forefront in experiments by Kuida *et al.* (1998). During embryonic development, up to 50% of the neuronal cells must die (Kuida *et al*., 1998; Vaux and Korsmeyer, 1999; Zhang and Herman, 2002). If excess neuronal cells are not removed during development, the embryo dies (Kuida *et al*., 1998). Kuida *et al.* used caspase-9 double knockout mice and noticed incomplete closure of the neural tube. Incomplete closure and excess cells resulted in massive brain tissue erupting from the opening. In comparison to cells of the nervous system, cells of the heart, and lung developed normally (Kuida *et al*., 1998).

Caspases share similar domains particularly within their functional group suggesting that caspases have redundancy (Liang *et al*., 2001). Caspase-3 is critical for the eventual demise of the cell (Jänicke *et al*., 1998). There are instances where this enzyme does not function but apoptotic death can still occur

(Liang *et al.,* 2001). Caspase-3 is not expressed in MCF-7 breast cancer cells due to a 47 bp deletion within exon 3 of the caspase-3 gene (Liang *et al.,* 2001). Challenging MCF-7 cells with neocarzinostatin, a DNA-cleaving anti-mitotic agent, apoptosis to occurred (Liang *et al*., 2001). Effector caspases-6 and-7 were responsible for the eventual demise of the MCF-7 cells (Liang *et al*., 2001). These studies illustrate the redundancy within the caspase family.

Caspases have proven essential in apoptotic death particularly during development. There are instances where caspases are present, but are not active (Berg, 2001). Berg et al. found mature anucleated human red blood cells (RBCs), which express caspase-8 and caspase-3 do not undergo apoptosis (Berg *et al*., 2001). RBCs lack mitochondrial proteins Apaf-1 and cytochrome *c*, which are associated with the induction of apoptosis (Berg *et al*., 2001; Li, *et al.,* 1997). The above caspases are present in the RBCs, but they are not catalytically processed (Berg *et al*., 2001). Cell death was not observed in these erythrocytes even with the addition of staurosporine, ionizing radiation or etoposide (Berg *et al*., 2001). Interestingly, an increase in intracellular calcium was able to activate calpain, but not the caspases (Berg *et al*., 2001).

The caspase family has been at the forefront in apoptotic studies as their involvement has proven critical. The mitochondria, however, have been shown to be just as significant. The Orlandi group demonstrated the role of the mitochondria in cell death (Benedetti *et al.,* 1988). Histological examination of rat livers after five days of ethanol treatment resulted in noticeable alterations in mitochondrial structure (Benedetti *et al.,* 1988). Aside from known proteins which

function in cellular respiration, the mitochondria also houses proteins that participate in apoptosis.

Mitochondria and Apoptosis

Mitochondria have been found to have major roles in apoptosis (Susin *et al.*, 1998). There is compelling evidence that mitochondria are just as important as the caspases (Susin *et al.*, 1998; Rodriguez-Enriquez, *et al.,* 2004). Cytochrome *c*, AIF, Endonuclease G and Omi/HtrA2 reside in the mitochondrial intermembrane space and are released in response to apoptotic stimuli (Green, 2000; van Loo *et al.*, 2001; Lee *et al.*, 2005). Preceding the release of the above proteins, mitochondrial membrane potential is believed to be compromised due to the opening of the mitochondrial permeability transition pore (Kroemer and Reed, 2000; Harris and Thompson, 2000).

Changes in mitochondrial membrane potential were first examined by Hunter in 1976 (Hunter *et al.*, 1976). Obvious changes in mitochondrial structure were seen by electron microscopy when exogenous Ca^{2+} was added to isolated mitochondria (Hunter *et al.*, 1976). A transition from an aggregated (condensed) state to an orthodox state (diffuse) and a decrease in oxidative phosphorylation was observed (Hunter *et al.*, 1976). Furthermore, permeability of the inner membrane was dependent on the uncoupling of oxidative phosphorylation (Hunter *et al.*, 1976). Hunter's results laid the foundation for studying mitochondria in relation to apoptosis.

Further examination of mitochondria revealed that the mitochondrial transition pore is regulated by two transmembrane proteins, the voltage

dependent anion channel (VDAC) and adenine nucleotide translocator (ANT) (Harris and Thompson, 2000). VDAC is positioned on the outer mitochondrial membrane while ANT is found on the inner mitochondrial membrane (Harris and Thompson, 2000). Both channels serve to transport ATP and ADP and other metabolites across the inner and outer mitochondrial membranes particularly those which reside in the intermembrane space such as cytochrome *c*, AIF, and Omi/HtrA2 (Harris and Thompson, 2000).

When the cells are unstressed mitochondrial membrane potential is high due the select transport of metabolic ions across the inner and outer mitochondrial membrane (Kroemer and Reed, 2000). A transition occurs in mitochondria when apoptotic stimuli are present such that VDAC/ANT pores remain open and apotogenic factors residing in the intermembrane space are released and a decrease in mitochondrial membrane potential is observed (Hunter *et al*., 1976; Harris and Thompson, 2000; Kroemer and Reed, 2000).

Synthetic compounds such as bongkrekic acid and cyclosporine A (CsA) have been shown to have an inhibitory effect on the decrease of mitochondrial membrane potential ($\Delta\Psi_m$) (Zamzani *et al*., 1996). BALB/c mice were injected intraperitoneally with dexamethasone. Twelve hours after injection primary splenocyte cultures were made and mitochondrial membrane potential was determined using the cationic lipophilic fluorochrome $DiOC_6(3)$ (Zamzani *et al*., 1996). Mitochondrial membrane potential was found to be low in primary cells from dexamethosone-treated mice while mitochondrial membrane potential of untreated mice was high. When cells were incubated with CsA or bongkrekic

acid increases in mitochondrial membrane potential were observed. Increased membrane potential was due to the ability of CsA to promote resealing of pores that were previously opened. Bongkrekic acid on the other hand promoted increased membrane potential by affecting the conformation of ANT thus reducing the probability of PT pore opening (Zamzani *et al*., 1996). Interestingly, both CsA and bongkrekic acid were only able to rescue cells in the early stages of apoptotic death (Zamzani *et al*., 1996). In later stages of mitochondrial depolarization, superoxide anion generation increases. Increases in superoxide anions result in the conversion of hydroethidine (HE) to ethidium (Eth) (Zamzani *et al*., 1996). Neither CsA or bongkrekic acid were able to increase membrane potential once the mitochondrial respiratory chain no longer functioned (Zamzani *et al*., 1996). These results show that the proper functioning of the MPT is required to prevent apoptosis.

Inhibitors of Apoptosis

While the anti-apoptotic members of the Bcl-2 family are the most studied inhibitors of apoptosis, the baculovirus genome contains a series of proteins that have been shown to prevent apoptosis, these proteins include p35/CrmA and the Inhibitors of Apoptosis (IAP) family. Genetic analysis of the baculovirus genome led to the discovery of p35, a direct caspase inhibitor (Xu *et al*., 2001). This protein functions by forming a complex with caspase-8 through a covalent thioester bond between the catalytic C360 of caspase-8 and D87 of p35 (Xu *et al*., 2001). In addition to p35/CrmA, another inhibitor was discovered in the baculovirus family, the inhibitors of apoptosis proteins (IAP) due to their ability to

protect infected cells from death (Wang, 2001). This family contains a homologous domain called the baculoviral IAP repeat (BIR) which acts as a direct inhibitor of caspases, thus preventing the apoptotic process. These IAP domains have been conserved from yeast to mammals (Deveraux and Reed, 1999). The mammalian IAP family includes X-linked IAP (XIAP), c-IAP1, c-IAP2 and survivin. All have been shown to have anti-apoptotic function (Deveraux *et al.,* 1998). Survivin is unique in that it is the only member of the mammalian IAP family that contains single BIR domain (Deveraux and Reed 1999).

XIAP, c-IAP1 and c-IAP2 contain three BIR domains at the N-terminal portion of the molecule and one really interesting new gene (RING) finger motif at the C-terminal (Huang *et al*., 2001). XIAP functions by competitively inhibiting caspase-3, while noncompetively inhibiting caspase-7. Protein binding assays revealed XIAP needs an active site in order to effectively inhibit the function of caspase-3 (Riedl *et al*., 2001; Suzuki *et al*., 2001). Closer examination of the XIAP caspase-3/7 complex revealed binding occurs through the linker region and the BIR2 domain. Mutational analyses show that the disruption of Asp148 of caspase-3 decreased the ability of the IAPs to bind effectively to caspases (Suzuki *et al*., 2001).

Proteins found in the mitochondria function to counteract the effect of the IAPs on caspase activity (Du *et al*., 2000; Verhagen *et al*., 2000). The **s**econd **m**itochondria-derived **a**ctivator of **c**aspases (Smac)/DIABLO complex is a pro-apoptotic complex which antagonizes the anti-apoptotic effects of X-linked inhibitor-of-apoptosis protein (XIAP). This protein complex is released from the

mitochondria in the presence of apoptotic stimuli (Du *et al.*, 2000; Verhagen *et al.,* 2000). Structural studies revealed Smac/DIABLO requires the N-terminal residue to function properly (Chai *et al.*, 2000). Deng *et al.* showed the release of Smac/DIABLO from the mitochondrion is a direct result of Bax, a member of the Bcl-2 proapoptotic family (Deng *et al.*, 2002). Bax functions to disrupt the mitochondrial membrane permeability transition pore, thus causing the release of cytochrome *c* and Smac/DIABLO.

Smac/DIABLO functions such that it will bind to the BIR2 or BIR3 domain of the XIAP or cIAP, thus preventing the inhibition of caspases and consequently apoptosis (Chai *et al.*, 2000). There is no interaction between Smac/DIABLO and BIR1 although there is strong sequence homology among the three domains (Chai *et al.*, 2000). Incubations with purified Smac/DIABLO, procaspase-3 and XIAP revealed a minimum of 150nM of Smac/ DIABLO is sufficient to prevent XIAP from binding to procaspase-3 (Chai *et al.*, 2000).

B-cell Lymphoma-2 (Bcl-2) Family

Interchromosomal translocations are the major causes of various cancers (Graninger *et al.*, 1987). Translocations have been associated with B cell lymphomas particularly in follicular lymphoma (Graninger *et al.,* 1987). In follicular lymphomas, there is a t(14;18) (q32;q21) translocation in approximately 80% of individuals diagnosed with this disease (Graninger *et al.*, 1987). As with other oncogenes, Bcl-2 is silenced in resting B cells, but becomes up-regulated with B cell activation. Translocation events result in aberrant expression of the B cell lymphoma-2 gene and up-regulated protein expression of Bcl-2 (Graninger *et*

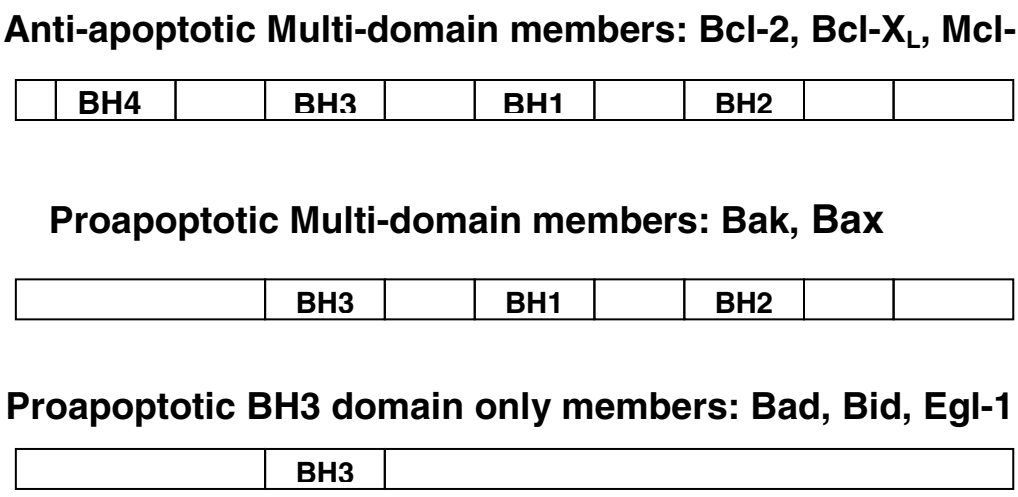
al., 1987). With regards to apoptotic death, increased levels of Bcl2 protein will most often result in a resistance to apoptotic stimuli (Reed, 2006). Since the discovery of the Bcl-2 oncogene, other homologues have been discovered. The family consists of about ten members that are either anti-apoptotic or proapoptotic.

The anti-apoptotic members include: Bcl-2, Bcl-x_L, Bcl-w and Mcl-1 (Kaufmann *et al.,* 2004; Schinzel *et al.,* 2004). The proapoptotic members include: Bad, Bak, Bax, Bik, Bim, and Bok (Reed, 2006; Huang and Strasser, 2000). The family is further divided based on their shared homology domains appropriately named Bcl-2 homology (BH) domains (Reed, 2006; Huang and Strasser, 2000) (Figure 4). The anti-apoptotic members Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 share four BH domains (BH1-BH4). The proapoptotic members can have three BH domains as is the case for Bak and Bax. The remaining members, Bad, Bid and EGL-1 contain one BH domain (Reed, 2006; Huang and Strasser, 2000).

The BH domains have been shown to play important roles in apoptotic death (Sato *et al.,* 1994). The proapoptotic Bcl-2 family members with multiple BH domains are integrated in both the outer mitochondrial membrane and the ER membrane (Schinzel *et al.*, 2004). The multidomain proapoptotic members tend to interact with VDAC to keep the mitochondrial pore open (Schinzel, *et al.,* 2004; Reed 2006). The BH3-only proteins of the proapoptotic family act to dimerize and bind the anti-apoptotic Bcl-2 family members (Huang and Strasser 2000). The anti-apoptotic members are inserted in the mitochondrial or ER membrane (Reed, 2006). The proapoptotic members (Bid and Bad) are found in the

Figure 4. Schematic representation of the Bcl-2 homology domains.

The anti-apoptotic members share four homology domains. Bak and Bax, proapoptotic members of the Bcl-2 family contain three domains. Bad, Bid and the C. elegans protein EGL have only one BH domain.



cytoplasm and can translocate to the mitochondria or ER in the presence of apoptotic stimuli (Donovan and Cotter 2004). Under non-apoptotic conditions, Bid is found at its full length of 24 kDa and is cleaved by an active caspase-8 to generate a 15 kDa fragment (tBid) (Kamer *et al.*, 2005). This truncated fragment will migrate to the mitochondria where it binds to Bcl-2 or Bcl-x_L and induces activation of Bak or Bax (Kamer *et al.,* 2005). As a result, mitochondrial pores remain open, mitochondrial membrane potential decreases and cytochrome *c* is released. In its phosphorylated state, Bad remains localized to the cytoplasm (Yu *et al.*, 2004). When it becomes dephosporylated, Bad will translocate to the mitochondria to inhibit anti-apoptotic Bcl-2 members allowing for the release of cytochrome *c* (Huang and Strasser 2000; Schinzel *et al.*, 2004).

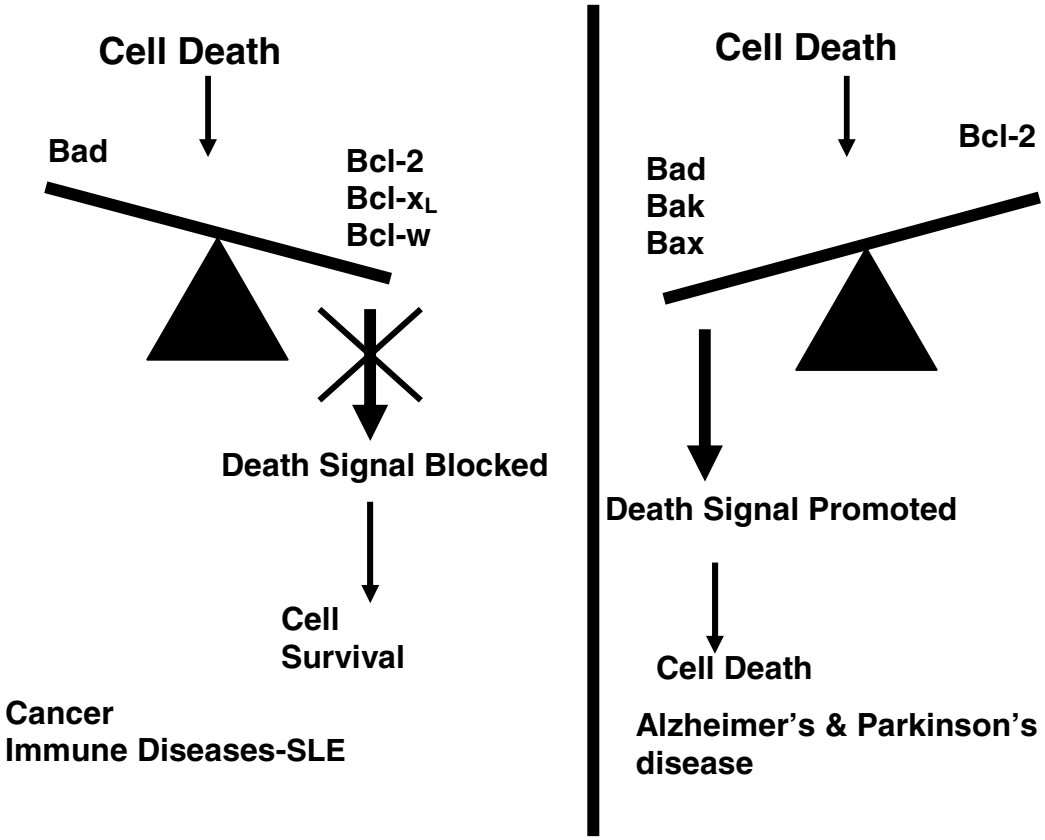
As shown in Figure 5, diseases can manifest when an imbalance exists between the anti- and proapoptotic members of the Bcl-2 family. These imbalances that may be present in certain diseases and may be of interest with regards to preventing disease (Graninger *et al.*, 1987).

Apoptosis and Disease

Aberrant functioning of proteins involved in the apoptotic process can lead to premature apoptosis and diseases such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (ALS) (Thompson, 1995; Bredesen, 2001; Rohn *et al.*, 2001; Gupta *et al.,* 2005). Increases in the expression of Fas receptors at the cell surface have been observed in the Alzheimer's brain and correlates with activation of the caspase cascade (de la Monte *et al.,* 1997; Rohn *et al.,* 2001). In addition to the immune and nervous system, cardiovascular diseases and

Figure 5. Bcl-2 family and disease.

When excess anti-apoptotic members are in abundance, the death signal is blocked resulting in cell survival. In such instances diseases such as cancer will develop. When proapoptotic Bcl-2 proteins are present in large quantitites, the death signal is promoted. Diseases such as Alzheimer's and Parkinson's may arise under these conditions.



stoke caused by ischemic injury are also associated with increased apoptosis (Phaneuf and Leeuwenburgh, 2002; Bredesen, 2001). On the other hand, when there is too little apoptosis various cancers can develop (Bredesen, 2001).

The mechanism of apoptosis is necessary to rid the body of any damaged cell(s) that will compromise the organism. Cells are oftentimes equipped with the necessary "tools" to deal with this insult (Warner *et al.,* 1997). As the organism ages the availability of resources becomes important in order to combat insults and diseases which are detrimental to the organism as a whole result (Gruen, T. and Davies, K.J.A., 2001). A strong correlation now exits between apoptosis and diseases of aged individuals (de la Monte *et al.,* 1997; Rohn *et al.,* 2001). While there is a connection between apoptosis and aging, teasing out the exact mechanism becomes a feat since cells from different tissues do not respond in the same manner. As our understanding of apoptosis in aging systems at the bench increases, clinical trials may develop in hopes of benefiting the aging community.

Aging

All organisms age. Attempts have been made to decipher the underlying mechanisms of this process using model systems such as *C. elegans, S. cerevisiae, Drosophila*, and rodents (Trifunovic *et al.,* 2004; de Magalhães, 2004). It has been found that organismal and cellular aging have been associated with increased DNA damage, telomere shortening, impaired mitochondria resulting in the release of reactive oxygen species (ROS), lipid

peroxidation, variable changes in mRNA levels, and accumulation of altered proteins (Johnson *et al.,*1999).

Gene regulation becomes defective in senescent cells. Decreases in rRNA from a variety of mammalian tissues have been observed. Consequently, inadequate amounts of ribosomes are formed to support cellular functions (Han *et al.*, 2001). In addition, mRNA transcripts have also been found to decrease or increase with age (Han *et al.*, 2001). The mRNA of Hsp70 is altered which accounts for the decrease in protein expression (Han *et al.*, 2001). There is also reduced expression of transcription factors such as c-fos, Id and AP1. These studies have furthered our understanding of this process and in hopes of ultimately intervening to hinder the formation of damaged products.

In vivo studies using mice prone to accelerated aging, SAMP1, and mice resistant to aging, SAMR1, shed light on the aging process (Hosokawa *et al.*, 2000). SAMP1 mice have a shortened life span, living less than one year of age as compared to normal mice who have a life span of more than two years. Increased single-stranded DNA breaks were observed in the liver, lung, brain, and heart tissues of SAMP1 mice. These studies confirm in an *in vivo* system, that there is an association of DNA damage and aging (Hosokawa *et al.*, 2000). Many have studied the causes of DNA damage during aging, and it has been found that oxidative damage can contribute to increases in DNA damage (Beckman and Ames, 1998).

Oxidative damage progressively increases as an individual ages (Beckman and Ames, 1998). Harmon, in 1956 devised the “Free Radial Theory

of Aging". This theory states that the generation of reactive oxygen species occurs *in vivo* as a by-product of redox reactions (Beckman and Ames, 1998). The onset of increased damage caused by free radicals can result in disease (Stadtman, 2001). Hydroxyl radicals are formed as a by-product of the mitochondrial respiratory chain, which is located in the inner mitochondrial membrane. The generation of ROS results in the onset of neurological disease due to the immature death of post-mitotic neurons (Beckman and Ames, 1998). Cells have an innate system to combat the buildup of ROS, including, superoxide dismutase (SOD), glutathione peroxidases and catalases (Hipkiss 2006). These enzymes function to scavenge reactive oxygen species (Hipkiss 2006). Deficiencies in these enzymes, however, have been linked to increase ROS, thus leading to decreased lifespan (Orr and Sohal, 2003).

Recently Yasuda, *et al.* examined the age-related changes in energy metabolism, the accumulation of altered protein and the rate of ROS, superoxide anions (O_2^-) in particular (Yasuda *et al.,* 2006). Using *C. elegans* they found an inverse correlation between oxygen consumption and age as there was a significant decrease in oxygen consumption as the animals aged (Yasuda *et al.,* 2006). They also observed changes in mitochondrial complexes I and II as well as increases in carbonylated proteins which are by-products of oxidative stress (Yasuda *et al.,* 2006). The lifespan of *C. elegans* is approximately 40 days (Brenner, 1974). The changes observed above occurred as early as 8 days in the adult population and more dramatic decreases were seen by day 12. In addition, the survival rate of the 8-12 day-old animals was over 95% (Yasuda *et*

al., 2006). These results show that the generation of ROS and protein modification are events that can occur early in the life of an organism, but the effect is not seen until later in life. Most damaging effects of ROS are ameliorated by cellular repair systems. However, as the organism ages the ability to cope with the oxidative stress decreases and hence there is more damage to various organs. The damage becomes so overwhelming such that as an organism ages, there is an increase in cancer and age-related pathologies. Significant advances have been made in the field aging using the whole organism as well as *in vitro* systems.

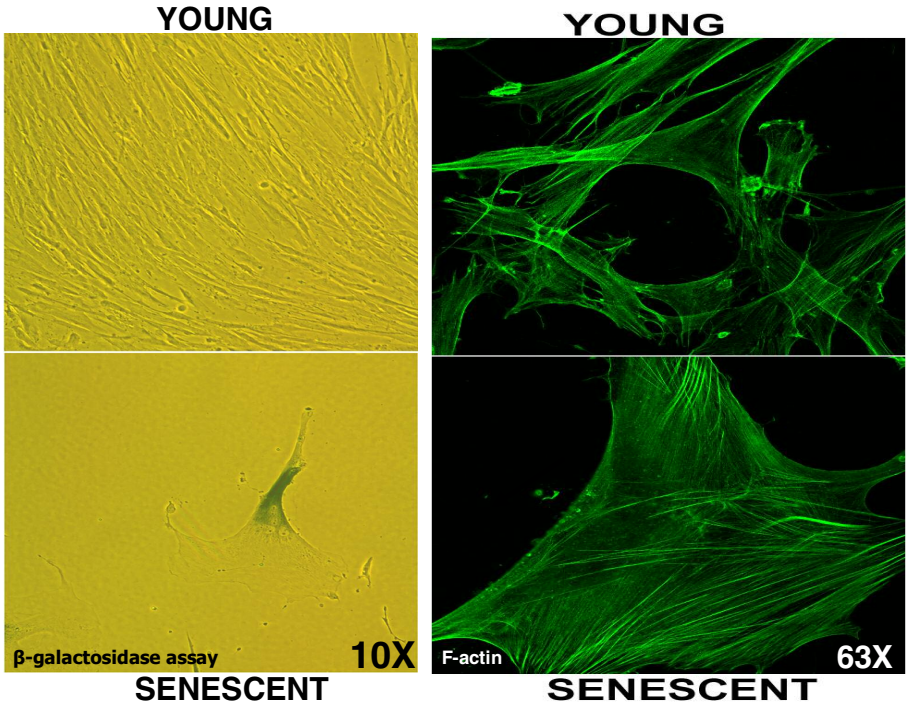
Cellular Senescence/Replicative Senescence

Senescent cells are unique in that they have the ability to cease division by irreversibly exiting the cell cycle and do not respond to stimuli such as serum (Itahana *et al.,* 2001). Replicative senescence should not be confused with the quiescent or G_0 state. Cells can be forced into the quiescent state by serum withdrawal. During the G_0 state cells are not dividing (Jha *et al.,* 1998). If serum is reintroduced to the quiescent cultures, cells will re-enter the cell cycle (Jha *et al.,* 1998).

In 1961 Hayflick and Moorehead isolated lung fibroblasts from a three month old embryo (Hayflick and Moorehead, 1961). They noticed cell division ended after approximately 60 ±10 population doublings (PDL) (Hayflick and Moorehead, 1961). Morphological observations have been made in that senescent cells become large, flattened and have an explained increased ex-

Figure 6. SA-βgalactosidase assay and F-actin staining in young and senescent WI38 fibroblasts.

Young and senescent cells were fixed with 2% paraformaldehyde and stained with β-galactosidase solution (left). Young cells (upper left) do not stain positive for β -galactosidase while senescent cells stain blue indicating β -galactosidase activity (lower left). Also seen in the lower left panel is the large flattened morphology of senescent cells. The arrow indicates mutiple nuclei typically found in a senescent cell (lower left). Young and senescent cells were fixed and stained with fluorscein isothiocyanate-conjugated-phalloidin. Young cells are devoid of stress fibers (upper right) while in senescent cells the stress fibers are abundant and overlapping (lower right).



pression of senescence-associated β-galactosidase as well as increased stress fibers (Dimri *et al.,* 1995; Hubbard and Ozer, 1999; Wei *et al.,* 2001) (Figure 6). Two widely accepted, although controversial, mechanisms of cellular senescence have been introduced; one mechanism involves the telomeres, and the other involves specific cell cycle genes (Jacobs and de Lange, 2004; Davis *et al.*, 2005; Zheng *et al.,* 2004).

James Watson recognized a problem with DNA replication in his studies using the T4 phage (Greider, 1998). He noticed that the phage had difficulties in replicating the ends of its DNA and would compensate by joining the ends of other genomic DNA before replication began (Greider, 1998). Alexei Olovnikov, independent of Watson, also noticed a problem with DNA replication. An analogy was made while watching an approaching train. The engine of the train was comparable to DNA polymerase and the tracks represented DNA. As the engine proceeded, he noticed a piece of the track (DNA) would not be replicated because it was covered by the engine (DNA polymerase). Being familiar with Hayflicks work on cellular senescence, he then reasoned that this covered region was responsible for the onset of the Hayflick limit and thus cellular senescence; this region of non-replicated DNA is now known as the telomere (Olonikov, 1996; Greider, 1998).

Early studies by Barbara McClintock showed that telomeres served to prevent chromosome fusion (McClintock 1942). This region consists of 15kb of TTAGGG tandem repeats in mammals (Fouche *et al.,* 2006). Approximately 50-150 telomeric base pairs are deleted with each round of cell division (Reddel

2003; von Zhlinicki *et al.,* 2005). Telomere erosion is more prevalent in somatic cells than germline cells because the germline expresses high levels of the enzyme telomerase (Mantell and Greider, 1994). Telomerase is a ribonucleoprotein that functions to add telomeric repeat sequences to the ends of chromosomes (Mantell and Greider, 1994). The enzyme is not essential in somatic cells, but is extremely important in maintaining the germline. Telomerase is constitutively active in 85% of all cancers and as a result cancer cells have the ability to continuously divide (Harrington 2003; Reddel 2003). As the telomere region is ablated, a crisis signal is sent to the cell cycle machinery allowing for cessation of cell division most often senescence (Hornsby, 2002). Termination of the cell cycle is associated with upregulation of proteins such as p16, p53 and the retinoblastoma protein.

The INK4A/ARF locus, located on human chromosome 9p21, encodes well known tumor suppressor proteins: $p15^{INK4b}$, $p16^{INK4A}$ and ARF (Kim and Sharpless, 2006). The third gene encoded by the INK4A/ARF locus, ARF is responsible for the upregulation of the tumor suppressor p53. Under normal conditions, the **m**urine **d**ouble **m**inute chromosome clone number **2** (MDM2) functions as an E3 ubiquitin ligase; it binds p53 and targets p53 for proteosomal degradation (Kim and Sharpless, 2006). A conformational change in MDM2 results when ARF binds to MDM2 resulting in the release of p53 (Kim and Sharpless, 2006). Liberated p53 then acts to inhibit the cell cycle, aid in DNA repair or induce apoptosis. Activated p53 initiates transcription of p21. Transient expression of p21 allows for increased expression of p16. Eventually the cell

cycle inhibitor p16 becomes constitutively expressed. $p16^{INK4a}$ will bind and inhibit the Cdk4/6 Cyclin D complex and prevent phosphorylation of the retinoblastoma protein (Rb) (Tan and Wang, 1998; Takahashi, *et al.,* 2006). Hypophosphorylated Rb will result in cessation of the cell cycle at G1 (Tan and Wang, 1998). Novel roles for the tumor suppressor p16 have recently been unveiled in haematopoietic stem cells and neuronal cells of the forebrain (Janzen *et al.,* 2006; Molofsky *et al.,* 2006). These studies suggest a connection between p16, longevity and disease.

As mammals age, the proliferative capacity of tissues, particularly those that contain stem cells decreases (Janzen *et al.,* 2006). Haematopoietic stem cells (HSC), give rise to white blood cells (WBC) and initial experiments found the levels of p16 to increase with age (Janzen *et al.,* 2006). It was then reasoned that limitations may arise when using HSC from older donors (Janzen *et al.,* 2006). HSC from bone marrow of young and old mice deficient of p16 ($p16^{-/-}$) and HSC from wild type controls were isolated to test this hypothesis. The HSCs from both groups were transplanted into gamma-irradiated female mice as gamma irradiation will ablate white blood cell production (Janzen *et al.,* 2006). HSC from young mice, both wild type and $p16^{-/-}$, had no significant difference in the number of WBC generated in the gamma irradiated animals (Janzen *et al.,* 2006). Transplanted HSC from older wildtype animals, however, gave dramatically different results in that HSC from old wild type mice resulted in limited numbers of WBCs. HSC from old $p16^{-/-}$ were similar to that of WBC from transplanted HSC from young mice (Janzen *et al.,* 2006). Janzen *et al.* also

established a connection between p16 and apoptosis in that old wild type mice were more susceptible to apoptotic death via gamma irradiation than their $p16^{-/-}$ counterparts. Taken together these data indicate that the lack of p16 promotes cellular proliferation while the higher levels of p16 make cells more susceptible to apoptosis (Janzen *et al.,* 2006).

Like Janzen *et al.,* Molofsky *et al.* found similar results with regards to proliferation and increased p16 levels. Molofsky *et al.* used neuronal cells of the subventricular zone from young (2 month) wildtype and $p16^{-/-}$ and old (2 years) wildtype and $p16^{-/-}$ mice (Molofsky *et al.,* 2006). BrdU staining showed the proliferation rate of neuronal cells from older wild type mice were significantly lower than their young counterparts (Molofsky *et al.,* 2006). The number of proliferating cells from the old p16 knockout mice was significantly higher than 2-year old wildtype mice (Molofsky *et al.,* 2006). Neuronal cells of the subventricular zone form neuroblasts that will migrate to the olfactory bulb and become olfactory neurons (Parent *et al.*, 2002). Molofsky *et al.,* found no deficiency in new olfactory neurons in young wild type, young $p16^{-/-}$ and old $p16^{-/-}$ mice but found old wildtype mice to severely lack the formation of new olfactory neurons. Taken together, these results suggest: (1) increased p16 will slow neuronal cell proliferation; (2) failure of neuronal stem cells to divide occurs independently of the p53 pathway as ARF protein levels remained unchanged; (3) as p16 levels rise with age, generation of neuronal cells necessary for proper functioning of the organism decreases.

Stem cells are necessary to replace those differentiated cells that have been lost to injury or disease (Maslov *et al.*, 2004). As an organism ages stem cells have a decreased capacity to divide due to rises in p16 levels (Janzen *et al.,* 2006; Molofsky *et al.,* 2006). The studies described above provide a possible explanation as to why the proliferative capacity of HSC from aging individuals giving insight regarding the onset of disease.

Cell lines which have a finite lifespan can be immortalization to expand our knowledge of cellular senescence (Ozer *et al.,* 1996; Hubbard and Ozer, 1999; Mawal-Dewan *et al.,* 2003). Simian virus 40 (SV40) large T antigen caused HS74 fetal bone marrow fibroblasts to surpass their limit of 60 PDL to 90 PDL (Ozer *et al.,* 1996). The mechanism by which this occurs is by binding to and inactivating p53 and pRb leading to an extension in growth (Hubbard and Ozer, 1999). In this case the cell cycle inhibitors are bypassed and the cell enters a state of 'crisis' at the end of their life span which includes a high rate of apoptosis.

Chemical treatment of WI38 fibroblasts can also extend the life span of human diploid fibroblasts (Mawal-Dewan *et al.*, 2003). Lifetime supplementation of dexamethasone to WI38 fibroblasts resulted in decreased $p21^{Waf1/Cip1Sdi1}$, p16 and p53 levels (Mawal-Dewan *et al.*, 2003).

Apoptosis and Senescence

Organ function declines as one ages. Studies using the aging heart found increased levels of cytosolic cytochrome *c* and a decrease in the amount of anti-apoptotic Bcl-2 protein (Phaneur and Leeuwenburgh, 2002), while the levels of

Apaf-1 and Bax remain the same between young and old rats (Phaneur and Leeuwenburgh, 2002). During cellular senescence a number of modifications in genes will affect PCD (Wang *et al.*, 2004). These changes include, increases in membrane Fas receptor, decreased levels of the anti-apoptotic Bcl-2 protein, decreased telomere and decreased mitochondrial function (Sugrue *et al.,* 1999; Hosokawa *et al., 2000*; Joaquin and Gollapudi, 2001). Despite the genetic changes, many have shown senescent cells are incapable of dying by apoptosis, while others have shown that these senescent cells have the ability to die by apoptosis (DeJesus *et al.,* 2002; Saski *et al.,* 2001). Like the latter, DeJesus *et al.* found senescent human fibroblasts indeed die by apoptosis, but this death is inducer-specific (DeJesus *et al.,* 2002). Taken together, these results suggest that senescent cells may be capable of dying apoptotically, but this death is cell type and inducer specific.

In light of the fact that there is a strong correlation between apoptosis and cellular senescence, we sought to determine the mechanism by which senescent human diploid WI38 fibroblasts undergo apoptosis when challenged with okadaic acid. The data presented here offers a novel manner by which these cells die.

CHAPTER 1

INVOLVEMENT OF CASPASES IN THE APOPTOTIC DEATH OF SENESCENT WI38 FIBROBLASTS

1.1 INTRODUCTION

Kerr, Wyllie and Currie performed novel experiments to examine programmed cell death others observed in development (Kerr *et al.,* 1972; Lockshin and Williams, 1965; Saunders, 1966). Specific phenotypical changes such as condensed chromatin, membrane blebbing were observed under normal and treated conditions (Kerr *et al.,* 1972). From their work, they renamed the phrase, programmed cell death, to what is now known as apoptosis (Kerr *et al.,* 1972). Many obstacles remained as they did not know what factors were involved in promoting this apoptotic death. While their experiments proved that all death was extremely regulated, the mechanism(s) were unknown. In the late 1980's and early 1990's various groups made tremendous contributions to the field of apoptosis. Groundbreaking studies by H. Robert Horvitz's group were largely responsible for laying the foundation of our current understanding of apoptosis particularly in development, disease and aging (Ellis and Horovitz, 1986).

As mentioned in the Introduction, the fate of 131 somatic cells of the nematode *C. elegans* is predictable in that without failure these cells are ablated during development. Fourteen specific *C. elegans* death genes (ced) were shown to participate in the death of these 131 somatic cells (Hedgecock *et al.,* 1983). The CED proteins, CED-3 in particular, was shown to be involved in the death of these 131 somatic cells (Ellis and Horovitz, 1986). Subsequent cloning and characterization of ced-3 by Yuan *et al.* resulted in the identification of a

mammalian homologue, interleukin 1β-converting enzyme (ICE) (Yuan *et al.,* 1993).

Interleukins (IL) which are synthesized in the monocyte are a family of proteins that function in inflammatory responses. Interleukin-1 can be divided into two subclasses: Interleukin-1α and Interleukin 1β (Thornberry and Molineaux, 1995). The two proteins are encoded on separate genes, but bind to identical receptors (Thornberry and Molineaux, 1995). Of the two subclasses, only IL-1β must be proteolytically cleaved in order to become active. The enzyme responsible for this cleavage event is interleukin-1β-converting enzyme (ICE) (Black *et al.,* 1989). This cysteine protease, unlike any other protease, discriminates in its substrate cleavage pattern in that ICE cleaves specifically at aspartic acid residues; IL-1β is cleaved at Asp^{116}-Ala^{117} by ICE and as a result the 33 kDa protein is truncated resulting in the emergence of an active 17.5 kDa fragment (Kostura *et al.,* 1989). Sleath *et al.* further demonstrated this fact by substituting the Asp residue of IL-1β with any amino acid of their choice and noticed ICE activity decreased significantly with Kcat/Km values decreasing more than 100-fold (Sleath *et al.,* 1990).

ICE was eventually cloned and characterized in 1992 by the Black group (Cerretti *et al.,* 1992). They found that this protease was different than the known proteases at the time. Consequently Yuan *et al.* found CED-3 shared sequence homology to ICE (Yuan *et al.,* 1993). ICE, along with CED-3 and NEDD-2 (murine homologue) were the first group of cysteine proteases shown to play a role in apoptosis (Yuan *et al.,* 1993). Yuan *et al.* found ICE to be 28% identical to

CED3 at the amino acid level and a region consisting of 115 amino acids shows a 48% identity (Yuan *et al.,* 1993). In this span of 115 amino acids resides a cysteine that has been shown to be essential for ICE function (Yuan *et al.,* 1993). In addition, ICE consists of two subunits, p20 and p10 which are processed from a full length 45 kDa precursor protein (Yuan *et al.,* 1993). This functional similarity lead to several conclusions: (1) The cysteine proteases, i.e. CED-3, NEDD-2 and ICE are conserved across species; (2) ICE could potentially be important in apoptotic events; (3) Analysis of other *ced* genes and hence their mammalian counterparts could further our understanding of disease as predicted by Kerr *et al.,* (Yuan *et al.,* 1993; Kerr *et al.,* 1972). One of the first experiments to help define the significance of ICE was carried out by Miura *et al*.

Miura *et al*. used a mouse thymus cDNA library to clone mouse ICE (mICE) and performed overexpression studies (Muira *et al.,* 1993). Overexpression of mICE in Rat-1 fibroblasts resulted in increased apoptosis as assayed by trypan blue exclusion (Muira *et al.,* 1993). Rat-1 fibroblasts which overexpressed bcl-2 as well as transfected with the mICE-lacZ fusion construct showed decreased in apoptosis (Muira *et al.,* 1993). Likewise overexpression of crmA, a cowpox virus gene, which had been shown to inhibit ICE activity, resulted in decreased apoptosis (Ray *et al.,* 1992; Muira *et al.,* 1993). The relationship between CED-3 and ICE were further characterized by placing *ced-3* into Rat-1 fibroblasts to determine whether it would function in the same way as in *C. elegans*. As expected, overexpression of *ced-3* in Rat-1 cells resulted in death which could be prevented when *bcl-2* or *crmA* were overexpressed (Muira

et al., 1993). These experiments provided evidence that CED-3 and ICE are indeed functionally homologous across species. Furthermore, the enzymatic activity of ICE is essential to promote apoptosis while inhibition of this enzyme impedes the apoptotic process (Muira *et al.,* 1993). From these pioneering studies the caspase family was discovered.

The study of apoptosis and senescence has recently piqued the interest of many researchers in the aging community as there is a prevalence of disease which affects the elderly population (Agrelo *et al.,* 2006; Chan, 2006; Menini *et al.,* 2006). Both *in vivo* and *in vitro* aging models have proven beneficial in furthering our understanding of diseases that manifest as a result of the aging process (Yasuda *et al.,* 2006; Janzen *et al.,* 2006). This project focuses on aging as well as we sought to examine the mechanism of apoptosis in human diploid fibroblasts.

Whether senescent cells are capable of undergoing apoptosis remains controversial (Wang 1995; DeJesus *et al.,* 2002; Campisi, 2003; Mammone *et al.,* 2006; Ohshima, 2006). Senescent cells were capable of undergoing apoptosis whether senescence was induced by H_2O_2 or serial passage (DeJesus *et al.,* 2002; Ohshima, 2006). We have found senescent cells were capable of undergoing okadaic acid-induced apoptosis. The studies mentioned in my thesis examine possible mechanisms that are involved in okadaic acid-induced apoptosis in senescent cells. Initial experiments were done to assess the contribution of caspase activation.

1.2 MATERIAL AND METHODS

Cell Culture

WI38 fibroblasts (Coriell Cell Repositories, Camden, NJ) were incubated in a humidified chamber at 37°C with 5% CO_2.(NuAire, Plymouth, MN) and cultured as described by Hubbard and Ozer (Hubbard and Ozer, 1999). Fibroblasts were grown in 100mm tissue cultures plates (Fisher Scientific, Suwanee, GA) with Dubelcco's Modified Eagles Medium (DMEM)/Ham's F10 medium, (pH 7.4) (CellGro/MediaTech, Inc, Herndon, VA). Fetal bovine serum (FBS) (Invitrogen, Grand Island, NY) was added to a final concentration of 10%. Penicillin/streptomycin (CellGro/Media Tech, Inc, Herndon, VA) at 1 unit/ml was supplemented to the media. Cells were subcultured 1:4 until they reached the appropriate population doubling (PDL). Young cells (log phase) were used in apoptosis assays at a PDL of 21-33 whereas; growth-arrested (G_0) cells were generated by withdrawing serum from log phase cells were at the same PDL. Serum was removed for 72 hours, after which the cells were deemed growth-arrested. Senescent cells were generated by serial passage until 62+ as determine by SA-β-galactosidase assay (Dimri *et al.,* 1995).

Senescence Associated beta-galactosidase (SA-β-galactosidase) Assay

The SA-β-galactosidase assay was used to determine whether WI38 fibroblasts had reached senescence. This assay has been used as a marker for senescence (Dimri *et al.,* 1995). Senescent WI38 fibroblasts were fixed in 2% paraformaldehyde for 20 minutes at room temperature. 10ml of β-galactosidase

staining solution (20mg/ml X-gal, 40mM citric acid/sodium phosphate buffer, pH 4.0; 5mM potassium ferricyanide, 5mM potassium ferrocyanide; 150mM sodium chloride; 2mM magnesium chloride) was added to plates. The plates were then incubated at 37°C for 16 hours. Plates were observed under a Leica microscope using the 10X objective.

F-actin Staining

Fluorescent phallotoxins have been used to label F-actin (stress fibers). Stress fibers tend to increase with age and has been used as a marker of senescence (Davis *et al.,* 2005). Senescent WI38 cells were cultured in 4-well chamber slides (Fisher Scientific, Suwanee, GA). Slides were washed with 1X Phosphate Buffered Saline (PBS) (11.9 mM Phosphates; 137 mM Sodium Chloride; 2.7 mM Potassium Chloride) and fixed with 2% paraformaldehyde for 15 minutes at room temperature. Slides were then washed in 1X PBS and permeabilized with 0.1% Triton X-100 (Fisher Scientific, Suwanee,GA) for 5 minutes at room temperature. Slides were washed in 1X PBS. Nonspecific background staining was eliminated by incubating cells for 20 minutes in 10% Bovine Serum Albumin (BSA) at room temperature. F-actin (Alexa Fluor®488 Phalloidin) (Molecular Probes, Eugene, OR) was added for 20 minutes at room temperature, protected from light. The slides were then mounted with SlowFade Antifade solution (Molecular Probes, Eugene, OR). Images were observed using a Zeiss LSM510 Confocal Microscope (Zeiss, Germany) with a 60X objective under an Argon laser.

Treatment with Okadaic Acid/ Protein Isolation and Quantitation

Okadaic acid (OA) (Calbiochem, La Jolla, CA) was dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific, Suwanee, GA) to a final stock concentration of 10 microM. Young, growth-arrested and senescent fibroblasts were treated with 10 microM OA at a final concentration of 10 nM and incubated at 37°C in a 5% CO_2 incubator for 18-24 hours. After the appropriate incubation time, the cells were lysed using 1X Sodium Dodecyl Sulfate (SDS) lysis buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT) or 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA). If the 1X SDS lysis buffer were used, the following protease inhibitors were added: leupeptin (1µg/ml), pepstatin (1µg/ml), phenylmethylsulphonylfluoride (PMSF) (50µg/ml). If the 1X cell lysis buffer were used, PMSF was added to a final concentration of 100 mM. Cells were then passed through a 25-gauge needle to ensure complete cell lysis and to fragment nucleic acids. Lysates were then incubated on ice for 15 minutes after which they were transferred to a Hermle refrigerated centrifuge and spun at 4°C for 10 minutes at 11,000rpm. Aliquots were removed to quantitate the isolated protein.

If the 1X SDS lysis buffer were used, to ensure complete removal of SDS, a modified Brönstead/Lowery Assay, Reagent Compatible/Detergent Compatible (RC/DC) Assay (BioRad, Hercules, CA) was performed according to the manufactures instructions. If the 1X cell lysis buffer were used, the standard BioRad assay was performed (Labie *et al.,* 1999). Protein was quantitated using

a Beckman Spectrophotometer at 750nm if the RC/DC Assay were used and 590nm if the 1X cell lysis buffer were used.

Western blotting and Detection

After obtaining the correct total protein concentration, 40µg protein was separated using 8-15% SDS-polyacrylamide (30% acrylamide; 0.8% *bis*-acrylamide) gel electorphoresis. Separated protein was then transferred at 70V for 90 minutes at 4°C onto Polyvinylidene Fluoride (PVDF) membrane (Osmonics, Minnetonka, MN). Membranes were then blocked in 5% non-fat dried milk for one hour at room temperature. Primary antibody dilutions were used at 1:1000 in 5% non-fat dried milk. The primary antibodies were allowed to incubate overnight at 4°C. The membranes were then washed three times in 1X Tris-buffered saline (10X solution consisted of: 24.2g Tris base; 80g NaCl; pH to 7.6 with HCl; used at 1X) with 0.1% Tween-20 (Fisher Scientific, Suwanee, GA) (1X TBST). Secondary anti-rabbit-horseradish peroxidase antibody or anti-mouse-horseradish peroxidase (Amersham, Piscataway, NJ) was added at a 1:1000 dilution and allowed to incubate for one hour at room temperature. The signal was detected using Enhanced Chemiluminescent (ECL)-Plus (Amersham Piscataway, NJ).

Blots were subsequently probed for actin or GAPDH (Chemicon International, Temecula, CA) which was used as a loading control. Primary antibody dilutions were at 1:1000 and incubated one hour at room temperature. Secondary anti-mouse HRP (Amersham, Piscataway, NJ) was used at 1:1000

and incubated at room temperature for one hour. The signal was detected using ECL-Plus (Amersham, Piscataway, NJ).

Antibodies Used

Caspases 9, 6, 8, alpha-fodrin, lamin A, DFF40, and DFF45 were purchase from Cell Signaling Technology Danvers, MA. Caspases 3 and 7 were purchased from BD Biosciences, Palo Alto CA. Caspase-12 was purchased from Chemicon International, Temecula, CA. PARP was purchased from Clonetech Palo Alto, CA. Anti-rabbit-horseradish peroxidase and anti-mouse-horseradish peroxidase were purchased from Amersham, Piscataway, NJ.

1.3 RESULTS

1.3.1 AN ANALYSIS OF CASPASE ACTIVITY IN WI38 HUMAN DIPLOID FIBROBLASTS

As previously shown by DeJesus *et al.* senescent fibroblasts are capable of undergoing apoptosis, but this event is inducer specific (DeJesus *et al.*, 2002). Young and senescent cells were challenged with 10μM ceramide, 10nM okadaic acid or 10ng/ml TNF-α for 24 hours. As DNA fragmentation is a hallmark of apoptosis we set out to determine the frequency of this event in the presence of the cell death inducers. Treatment with okadaic acid resulted in more than 80% TUNEL-positive senescent cells (DeJesus *et al.,* 2002). Others have also found okadaic acid to induce an apoptotic death (Li *et al.,* 2001; Rami *et al.,* 2003). We next set out to determine which initiator and effector caspases participate in this event. Total cellular protein was isolated from young, growth-arrested (G_0) and senescent fibroblasts that had been challenged with 10nM okadaic acid as described in **Materials and Methods**. Caspase protein levels were analyzed by Western blotting. Caspase-8,-9 and -12 are three initiator caspases involved at the beginning of the caspase cascade. Caspase-8 is usually associated with receptor ligand binding at the cell membrane (Green, 1998) as there are no okadaic acid receptors on the cell membrane. When caspase-8 activity was determined, we did not detect cleavage (data not shown). We went onto determine other known caspases involved in apoptosis.

Caspase-12 is most often associated with ER stress-induced apoptosis as mentioned previously (Breckenridge *et al.,* 2003). Caspase-12 was first cloned from rodent cells and has sequence homology to human caspases-1,-4 and -5

and is located on the cytoplasmic side of the ER (Breckenridge *et al.,* 2003). There is some controversy as to whether caspase-12 actually functions in humans as mutations in human caspase-12 have been observed (Fischer *et al.,* 2002). Human caspase-12 is located on chromosome 11q22.3 (Fischer *et al.,* 2002) which is also the location of human caspase-1,-4 and -5. Capase-12 mRNA in tissue has been found to have the highest level in the lung for humans as is the case for mice (Fischer *et al.,* 2002). Nine different isoforms of caspase-12 were identified in human lung cDNA samples (Fischer *et al.,* 2002). These isoforms, after further analysis, show severe deletions in exons 2, 3, 5 and 7 which makes it not functional in humans (Fischer *et al.,* 2002).

Protein from the three different growth states were fractionated and probed for human caspase-12 antibody (Figure 1.3A). Our results show control and OA-treated samples have multiple banding patterns for caspase-12 in both young and growth-arrested cells. In senescent cells, we see a single band which corresponds to full-length caspase-12 in both control and senescent-treated samples (Figure 1.3A). These results suggest, as reported by Fisher *et al.*, that caspase-12 has major mutations which may exclude it as a bona fide initiator caspase. Furthermore, while others have shown caspase-12 to play a role in a rodent Alzheimer's model, its functions in humans may not be the same or necessary for that matter. (Nakagawa *et al.,* 2000; Mehmet, 2001). We went on to determine whether caspase-9 was a participant in okadaic acid-induced cell death.

Caspase-9 is implicated in type II apoptosis as a result of insult to the mitochondria. Apoptosome formation (cytochrome *c*, Apaf-1 and active caspase-9) will cleave and activate effector caspase-3 (Green 2005). In our system when the fibroblasts at different growth states were challenged with OA, we find slight cleavage of full length initiator caspase-9 of 57 kDa to its cleaved product of 47 kDa in young and G_0 cells (Figure 1.3B). However, in senescent cells, full length caspase-9 was present but not the cleaved product (Figure 1.3B). Our data indicates that initiator caspases are not involved in the apoptotic death of senescent cells. Despite the fact that initiator caspases do not function in senescent cell apoptosis, we went on to determine whether there is any involvement of the effector caspases.

Once upstream initiators are cleaved and activated, they are responsible for cleavage of the downstream effectors (Abraham and Shamam; Green and Kroemer, 1998). Once activation of effector caspases ensues, the cell reaches a point of no return in that the cell will undoubtedly die an apoptotic death. Caspases-3,-6 and -7 are the known executioners of apoptosis and have been proven to elicit apoptotic-positive cells. When we examined caspase-7, we found that it is not cleaved into its active product of 30 kDa in young, G_0 and senescent cells challenged with OA (Figure 1.3C). Since caspase-7 was not involved, we went on to determine whether caspase-6 was cleaved. Cleavage of caspase-6 to its 15 kDa active product was observed in young and growth-arrested cells challenged with OA (Figure 1.3D). This was not the case with senescent cells as the full length was the only observed product in both control and treated (Figure

1.3D). Finally, we sought to determine whether caspase-3 was involved. Full length caspase-3 is most often cleaved into p20, p17, and p11 subunits (Saunders *et al.,* 2000). Young and growth-arrested cells, both control and treated, have abnormal caspase-3 cleavage. Interestingly, full length caspase-3 is completely absent in senescent cells, both untreated and treated (Figure 1.3E).

Caspases are not the only family of cysteine proteases proteolytic enzymes functioning in apoptosis. Calpains, calcium activated proteases, have been shown to perform similarly to the caspases (Tompa *et al.,* 2004). These calcium activated proteases have several substrates which are also involved in apoptosis such as caspase-3, PARP, and α-fodrin (McGinnis *et al.,* 1999). Calpains can be divided into two families, m-calpains and μ-calpains and become activated in the presence of millimolar and micromolar amounts of calcium respectively (Squire *et al.,* 1999). When analyzed by western blotting, we find μ-calpains were active in young and G_0 cells while m-calpain activity was present in senescent WI38 fibroblasts Figure 1.3F, 1.3G). McGinnis *et al.* observed abnormal caspase cleavage occurring in neuroblastoma cells (McGinnis *et al.,* 1999) in the presence of calpain activity which results in an abnormal caspase-3 cleavage pattern. Their results correlate with our findings. Therefore, it is questionable as to whether caspase-3 is indeed functional in young and growth-arrested cells.

With regards to the absence of caspase-3 at the protein level in senescent fibroblasts is explained by recent data from the Wang group (Marcotte *et al.,* 2004). mRNA isolated from senescent WI38 fibroblasts show a deleterious mutation in exon 3 of caspase-3 (Marcotte *et al.,* 2004) which accounts for the

absence of caspase-3 in our studies. The Wang group suggested the deletion explains why senescent WI38 fibroblasts are resistant to apoptotic death (Marcotte *et al.,* 2004). Although we do not see caspase activity in senescent cells, our previous data has shown TUNEL positive senescent cells when they are challenged with OA (DeJesus *et al.,* 2002). In light of this, we went on to determine putative death substrates cleaved in senescent cells undergoing apoptosis.

1.3.2 CLEAVAGE OF CELL DEATH SUBSTRATES IN HUMAN DIPLOID FIBROBLASTS

Some of the well-known caspase substrates include: PARP, Lamin A, alpha-fodrin and DFF. What is significant about these substrates is that they are cleaved by different effector caspases. Caspase-3 has been shown to cleave PARP, alpha-fodrin and DFF while caspase-6 and 7 both cleave Lamin A (Tewari *et al.,* 1995; Gu *et al.,* 1995; Jänicke *et al.,* 1998; Liu *et al.,* 1997; Takahashi *et al.,* 1996; Orth *et al.,* 1996). As there was no caspase activity in senescent cells, it first seemed like a futile attempt to determine putative substrates. The next series of experiments, however, resulted in very interesting results.

Poly-ADP-ribose polymerase (PARP), a 116 kDa protein, has enzymatic activity and functions to maintain DNA by recognizing DNA strand breaks. (Cherney *et al.,* 1987). PARP cleavage results in cleavage of the 116 kDa protein into an 85 kDa product which contains the automodification and NAD-binding domain (Cherney *et al.,* 1987). There is also a 25 kDa fragment released

which houses the N-terminal DNA binding domain (Gu *et al.,* 1995). When analyzed by western analysis we see partial cleavage of PARP into the 25 kDa fragment which represents the N-terminal binding domain. Interestingly, complete PARP cleavage is seen in senescent cells both control and treated samples (Figure 1.3H).

There are several implications regarding this data in that we see only partial cleavage of PARP in young and G_0 cells; if a higher concentration of OA were used we may see more activity as there is only slight caspase activation in these particular growth states. Gu *et al.* found a 50-100 fold increase in ICE activity is necessary to cleave PARP. Thus, if caspase activity had been higher we might have noticed a more significant cleavage pattern in young and G_0 cells. Regarding the senescent cells, there is an age-associated decline in gene expression as cells in culture age (Cristofalo *et al.,* 1998). PARP activity is inversely proportional to age (Salminen *et al.,* 1997). Damaged DNA increases as cells age in culture due to shortened telomeres and or increased protein oxidation (von Zglinicki *et al.,* 2005; Stadtman, 2001). Although PARP is inactive in untreated and treated samples, TUNEL positive cells are not observed in senescent control cells (DeJesus *et al.,* 2002). PARP was the first caspase substrate recognized in the apoptotic process, since then, Lamin A, alpha fodrin and DFF have come to the forefront as putative caspase substrates. Given that PARP was abnormally cleaved, we continued our search by examining Lamin A.

Nuclear lamins are responsible for maintaining the structure of the lamina. They are located underneath the nuclear inner membrane and function to

organize chromatin (Rao *et al.,* 1996). Lamin A and Lamin C are found on the same gene and are alternatively spliced. Lamin B is located on a different chromsome (Rao *et al.,* 1996). The three Lamins, however, are structurally similar. Groups have shown that these lamins can be proteolytically cleaved by caspase-6 at Asp^{230} upon insult (Rao *et al.,* 1996; Orth *et al.,* 1996; Takahashi *et al.,* 1996). After challenging fibroblasts at different stages with okadaic acid, we did not see cleavage of Lamin A in any of the three growth states (Figure 1.3I). Alpha fodrin as mentioned is another potential substrate that can be cleaved by caspase-3.

One of the hallmarks of apoptosis is the dismantling of the cytoskeleton allowing for cell shrinkage (Kerr *et al.,* 1972). The cytoskeletal protein alpha fodrin (non-erythroid spectrin) has been implicated in apoptotic death through cleavage by caspase-3 (Martin *et al.,* 1995; Jänicke *et al.,* 1998; Tahzib *et al.,* 2004). In young and growth-arrested cells, we found that alpha fodrin is cleaved from its 240 kDa form to 150 kDa. The antibody used also recognized a smaller 35 kDa product whose significance is not known presently. Full length alpha fodrin was present in control and OA-treated senescent cells as observed in Figure 1.3J. We can conclude that alpha fodrin is not a candidate substrate that is cleaved when senescent cells are challenged with OA. DNA Fragmentation Factor (DFF) has not been the focus of many studies due to the fact that active caspase-3 is most often associated with PARP cleavage. OA-treated senescent cells revealed interesting results with regards to DFF.

Studies examining the ability of caspase-3 to cleave DNA fragmentation factor (DFF) found it to exist as a heterodimer in the nucleus (Liu *et al.,* 1997; Sharif-Askari *et al.,* 2001). The heterodimer consists of a 45 and 40 kDa (DFF45/DFF40) (Liu *et al.,* 1999). The homologue in mice is known as CAD/ICAD (caspase-activated deoxynuclease (CAD)/inhibitor of CAD (ICAD) (Liu *et al.,* 1997). Incubations with purified caspase-3 resulted in complete cleavage of DFF45 into 30 and 11 kDa fragments while DFF40 remained intact (Liu *et al.,* 1997). Considering the fact that caspase activity is nonexistent in senescent cells but fragmented DNA is present when challenged with OA, we set out to determine whether DFF45 is cleaved in the fibroblasts.

After protein fractionation using polyacrylamide gels and subsequent use of an antibody which recognizes both DFF45 and its isoform DFF35, we did not observe cleavage of DFF45 in young and growth arrested cells (Figure 1.3K). In senescent cells, however, decreased levels of DFF45 were noticed (Figure 1.3K). Although DFF45 has been shown to be a substrate for caspase-3, we found DFF45 cleavage in senescent cells challenged with OA in the absence of caspase-3. This data suggests that DFF45 can be cleaved by an alternative process as there may be another caspase-3-like enzyme responsible for the cleavage shown in our studies.

1.4 DISCUSSION

Many advances have been made in the study of apoptosis due to the discovery of the *ced* genes and the subsequent discoveries of the ICE and caspase family of proteases (Kumar and Harvey, 1995; Cohen, 1997). As caspases share similar domain sequences, many theorized that caspases may share similar functions. Zheng *et al.* tested this theory by using caspase-9 and caspase-3 knockout mice (Zheng *et al.,* 2000). When mice where injected intraperitoneally with an antibody against Fas, 2, -6 and -7 were activated (Zheng *et al.,* 2000). These results show a compensatory mechanism existing within the caspase family.

Our previous data have shown that senescent fibroblasts were indeed capable of undergoing an apoptotic death when challenged with OA, but the mechanistic pathway was not known (DeJesus *et al.,* 2002). We began our studies by determining which caspases were involved in apoptotic death induced in senescent cells. The present data provide us with interesting results in that caspases, which have been shown to be crucial in the apoptotic process, do not function in senescent cells (Figure 1.3A-E). These data suggest an age-related decline in apoptotic proteins, the caspases in particular. Similarly work by the Lubec group has shown a similar decline in patients with Alzheimer's disease (Engidawork *et al.,* 2001). Using human brain postmortem tissue from Alzheimer's patients, they found decreases in caspase-3, -8 and -9. The results presented here imply that the death associated with okadaic acid is caspase-

independent. This data supports and adds to other studies showing the occurrence of caspase-independent deaths (Lorenzo 2004).

As there was no caspase activity in senescent cells, we continued our studies to determine whether known caspase substrates were involved. Once effector caspases are activated, downstream substrates such as PARP, lamin A, protein kinase C δ, hnRP C1 and C2, fodrin, DNA-PK_{CS} are targeted (Cohen, 1997). Our data shows abnormal PARP cleavage in senescent cells while this cleavage pattern is not seen in young and growth-arrested states (Figure 1.3H). This means that PARP is not active in senescent cells under control or treated conditions. In young cells, however, cleavage of the 25 kDa NAD^+ subunit is seen. The caspase activity data show slight cleavage of full length caspase-6 and to a lesser extent caspase-3. It is possible that the activity of caspase-6 may not be sufficient to generate complete cleavage of PARP (Gu *et al.,* 1995).

Caspase-3 in young and growth-arrested cells was abnormal in that various fragments were observed. Our search for other reports lead to the realization that caspase-3 can serve as a substrate for calpains (McGinnis *et al.,* 1999). As shown in figures 1.3F, 1.3G calpain activity was present in all three growth states. Our data correlates with what has been previously shown with regards to calpain activity and abnormal caspase cleavage (McGinnis *et al.,* 1999). Prior to the availability of antibodies which recognized active calpain, cleavage of non erythyroid spectrin or alpha fodrin was used to determine calpain activity (McGinnis *et al.,* 1999). Similar to what others have shown, we see that there was cleavage of alpha fodrin in young and growth arrested cells.

Cytoskeletal structure is compromised under apoptotic conditions (Jänicke *et al.,* 1998). When young and growth-arrested cells were challenged with OA, cell shrinkage was observed after the 18-24 hour incubation, but the cells were not dying. Enhanced apoptosis can occur when a combination of drugs are used (Niwa *et al.,* 1999). TNF-α for instance induces relatively low levels of apoptosis on its own. When used in conjunction with the protein synthesis inhibitor cycloheximide, apoptotic levels rise significantly (Niwa *et al.,* 1999). Using OA with another drug may enhance the ability of young and growth-arrested cells to undergo apoptosis.

DFF45 which resides in the nucleus in complex with DFF40 was the only substrate down-regulated in senescent cells challenged with OA. This result was interesting in that down-regulation occurred in the absence of caspase activity implicating a caspase-independent death. These results lead us to search for proteins which were capable of inducing a caspase-independent death and factor(s) that may be responsible for the down-regulation of DFF45 in senescent cells challenged with OA.

APPENDIX: Figures and Figure Legends for Chapter 1

Figure 1.3A Immunoblot of Caspase-12 expression in WI38 fibroblasts.

Cells were cultured, treated and lysed as described in the Materials and Methods sections. 40µg total cellular protein was separated on a 12% polyacrylamide gel by electrophoresis. Multiple bands were present when a caspase-12 antibody was used. Full length caspase-12 corresponds to 55 kD. As discussed in the text, caspase-12 may not play a role in humans as many mutations have been described.

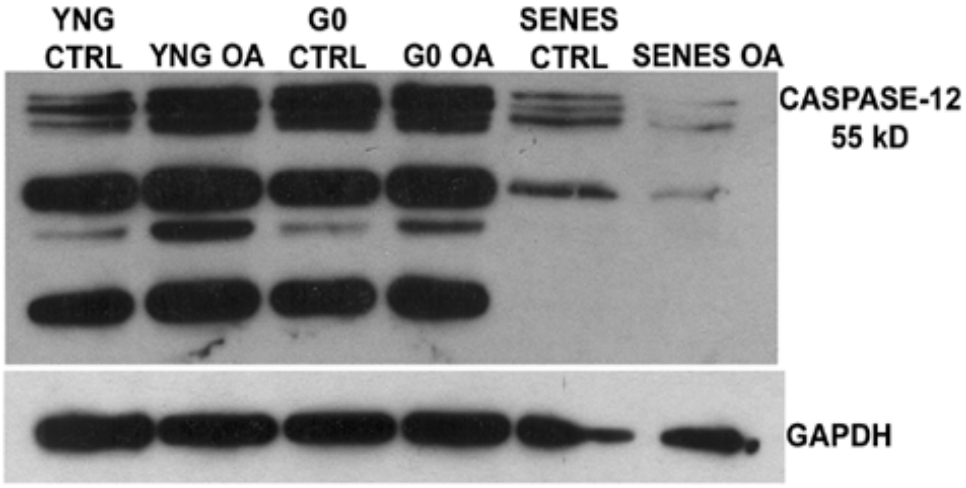


Figure 1.3B. Western analysis of caspase-9 activity in WI38 fibroblasts.

Cells were cultured, treated and lysed as described in the Materials and Methods sections. 40µg total cellular protein was separated on a 12% polyacrylamide gel and electrophoresed. Probing blots with a caspase-9 antibody, we find that caspase-9 is cleaved from its full length product of 47 kDa to a smaller 35 kDa active product in young and G_0 cells (indicated by arrow). In senescent cells, however, full length caspase-9 and not the cleaved product is seen. GAPDH was used as a loading control.

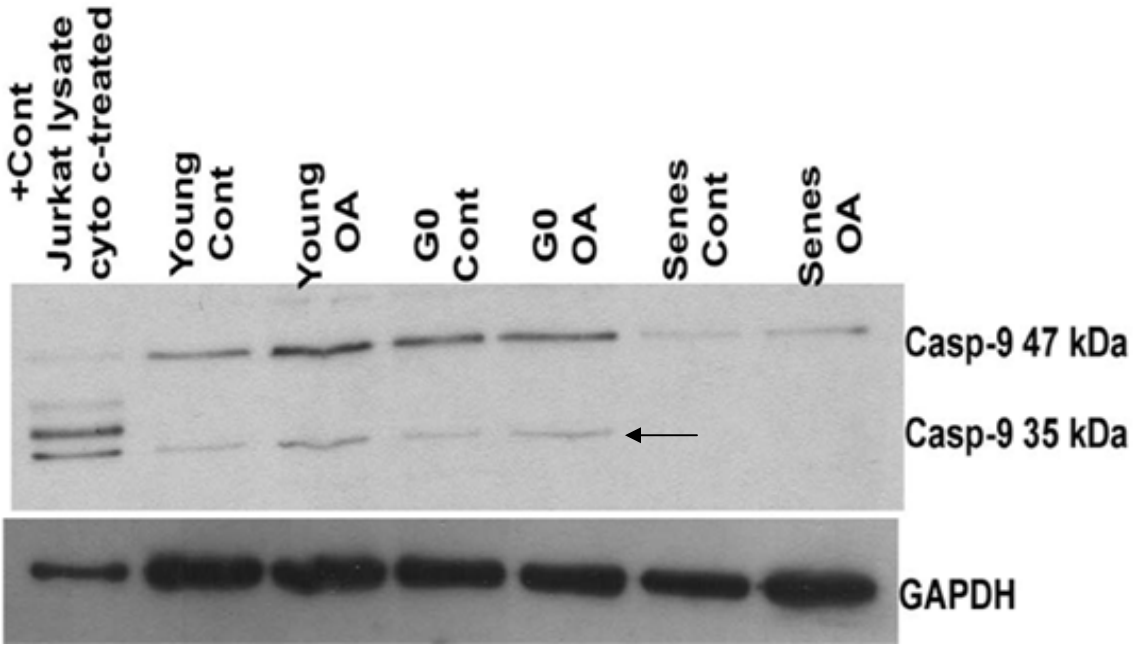


Figure 1.3C. Caspase-7 expression in WI38 fibroblasts challenged with OA.

After treatment and lysis, 40µg total cellular protein was added to a 12% polyacrylamide gel and subjected to electrophoresis. When probed for caspase-7, the full length product at 35 kDa is present. The smaller 30 kDa active form was not present. GAPDH was used as a loading control.

YC=Young Control; YOA=Young Okadaic Acid; G0C=Growth-arrested Control; G0 OA=Growth-arrested Okadaic Acid; SC=Senescent Control; S OA=Senescent Okadaic Acid.

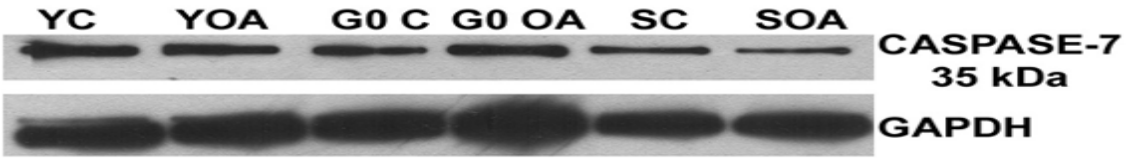


Figure 1.3D. Caspase-6 expression in WI38 fibroblasts.

Cells were challenged and total cell protein isolated as described in the Materials and Methods sections. 40µg total cellular protein was separated on 15% polyacrylamide gels and electrophoresed. Caspase-6 antibody recognized full length (35 kDa) and cleaved caspase-6 (15 kDa) in young and G0 cells as indicated by the arrow. In senescent cells however, full length caspase-6 is present at 35 kDa. The cleaved product is not seen here. GAPDH was used as a loading control.

YC=Young Control; YOA=Young Okadaic Acid; G0C=Growth-arrested Control; G0 OA=Growth-arrested Okadaic Acid; SC=Senescent Control; S OA=Senescent Okadaic Acid.

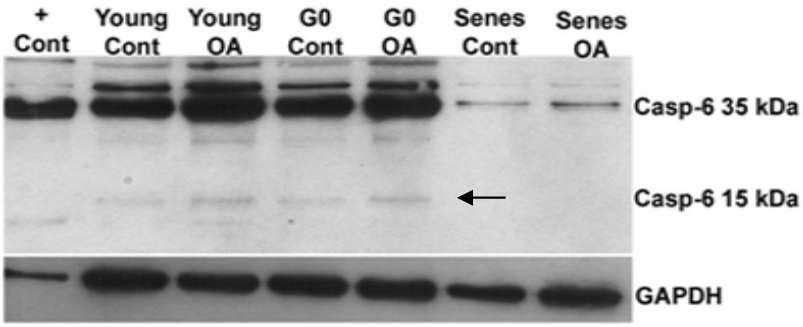


Figure 1.3E. Caspase-3 expression in WI38 fibroblasts.

Young, G_0 and senescent fibroblasts were challenged with 10 nM OA for 18 hours and total protein was isolated. 40µg total cellular protein was separated on a 15% polyacrylamide gel by electrophoresis. Results show caspase-3 is abnormally cleaved in control and treated young and G_0 samples. In senescent cells, however, caspase-3 was not observed. GAPDH was used as a loading control.

YC=Young Control; YOA=Young Okadaic Acid; G0C=Growth-arrested Control; G0 OA=Growth-arrested Okadaic Acid; SC=Senescent Control; S OA=Senescent Okadaic Acid.

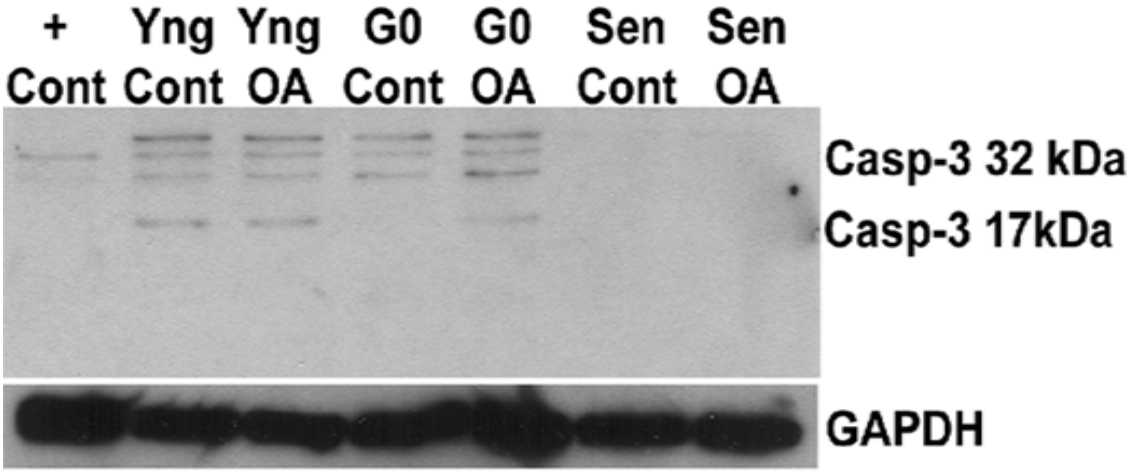


Figure 1.3F. Calpain I expression in WI38 fibroblasts.

Cells were treated and lysed as described in Materials and Methods. 40µg total protein lysates was fractionated on 10% polyacrylamide gels. Active calpain I (58 kDa) was only observed in senescent cells, control and OA-treated. GAPDH was used as a loading control.

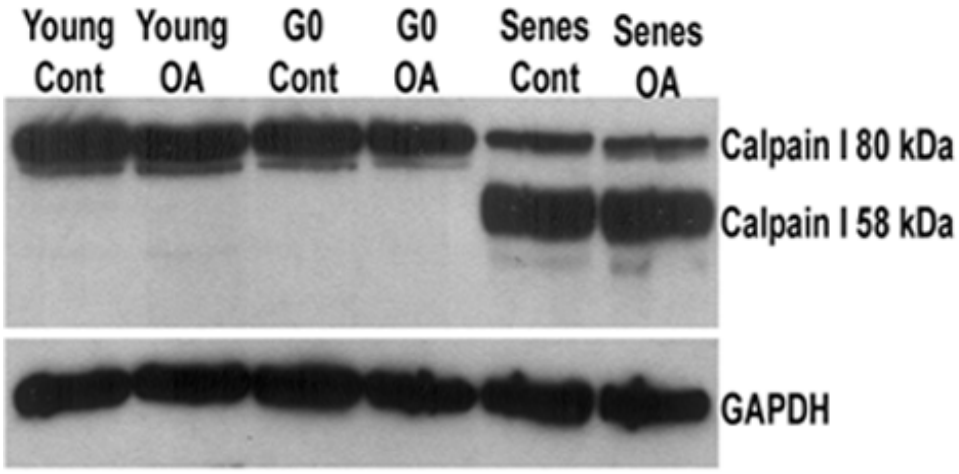


Figure 1.3G. Analysis of calpain cleavage in WI38 fibroblasts.

Cells were challenged and total protein isolated from young, G0 and senescent cells. 40µg total cell protein lysates was separated on 10% polyacrylamide gels. Antibodies specific for Calpain II were used for detection. Low levels of active calpain II (58 kDa) was present in all three growth states. GAPDH was used as a loading control.

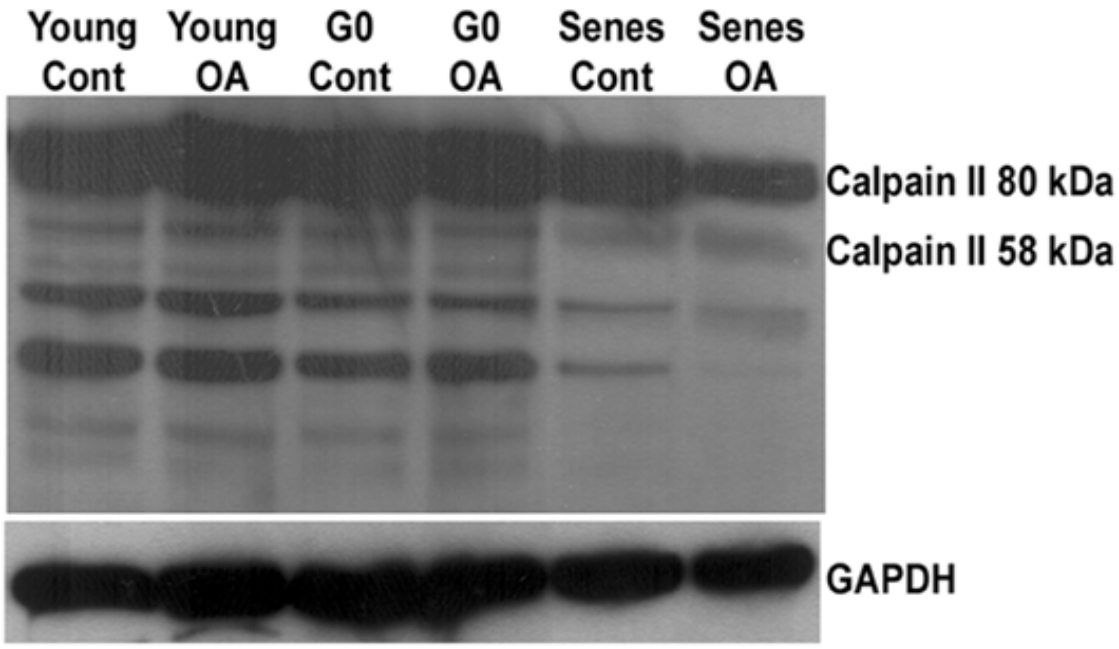


Figure 1.3H. PARP expression in WI38 fibroblasts.

Abnormal PARP cleavage was observed in senescent cells as it was completely cleaved to its 85 kDa inactive form. PARP was not cleaved in young or G0 cells as the 116 kDa full length product was present. Interestingly the PARP antibody detected a 25 kDa product in young and G0 cells. This 25 kDa product was not seen in senescent cells. 40µg total cellular protein and an 8% polyacrylamide gel was used for this experiment. Actin was used as a loading control.

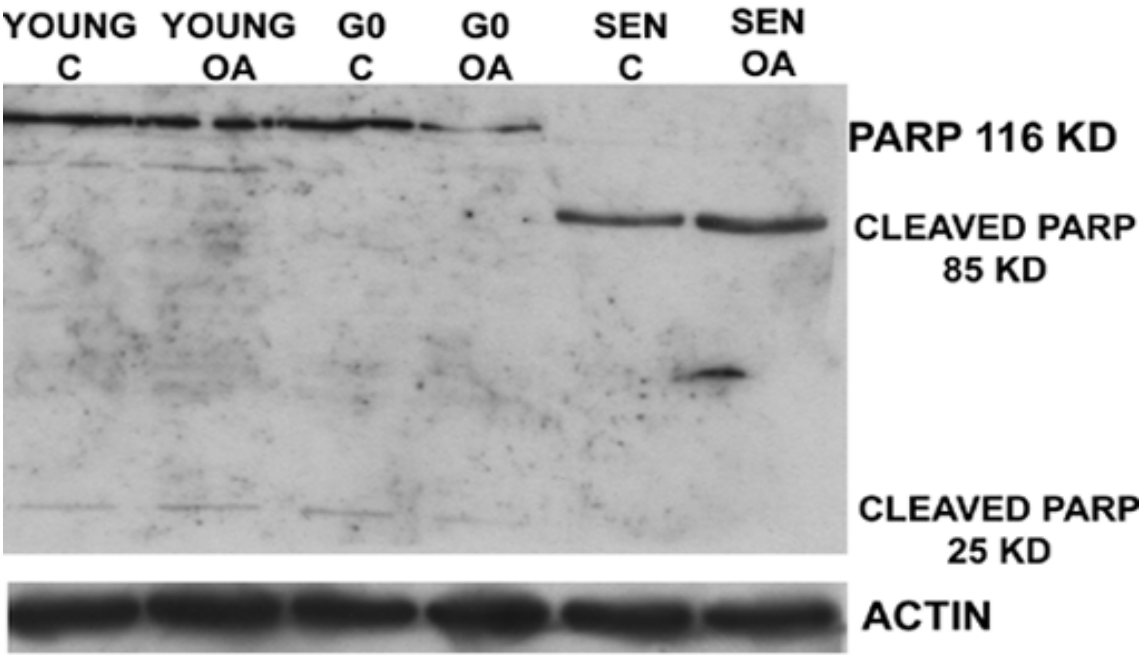


Figure 1.3l. Lamin A expression in WI38 fibroblasts.

Eighteen hours after the addition of OA, young, G0 and senescent cells were lysed and 40µg total cell lysates was separated on a 12% polyacrylamide gel. An antibody against Lamin A was used. Lamin A was present as a doublet at 70 kDa in all growth states. Cleavage of Lamin A to 35 kDa was not seen. GAPDH was used as a loading control.

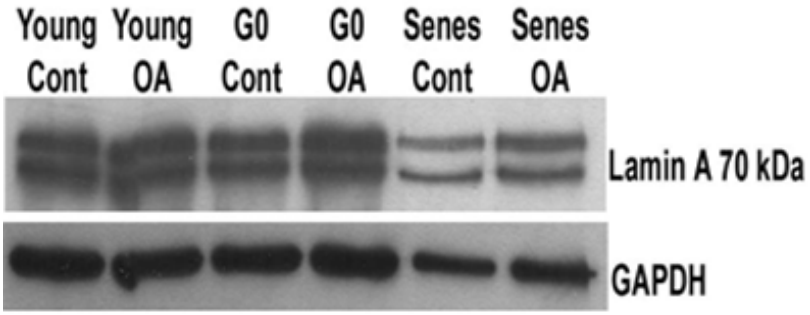


Figure1.3J. Alpha fodrin levels in WI38 fibroblasts.

Full length alpha fodrin was present in all three growth states at 240 kDa. The cleaved product of 150 kDa was only observed in young and G_0 cells. A smaller 35 kDa fragment was seen in young and G_0 cells, but not in senescent cells. 40µg total cell lysates was used for this experiment. Samples were separated on an 8% polyacrylamide gel as described in the Materials and Methods sections. GAPDH was used as a loading control.

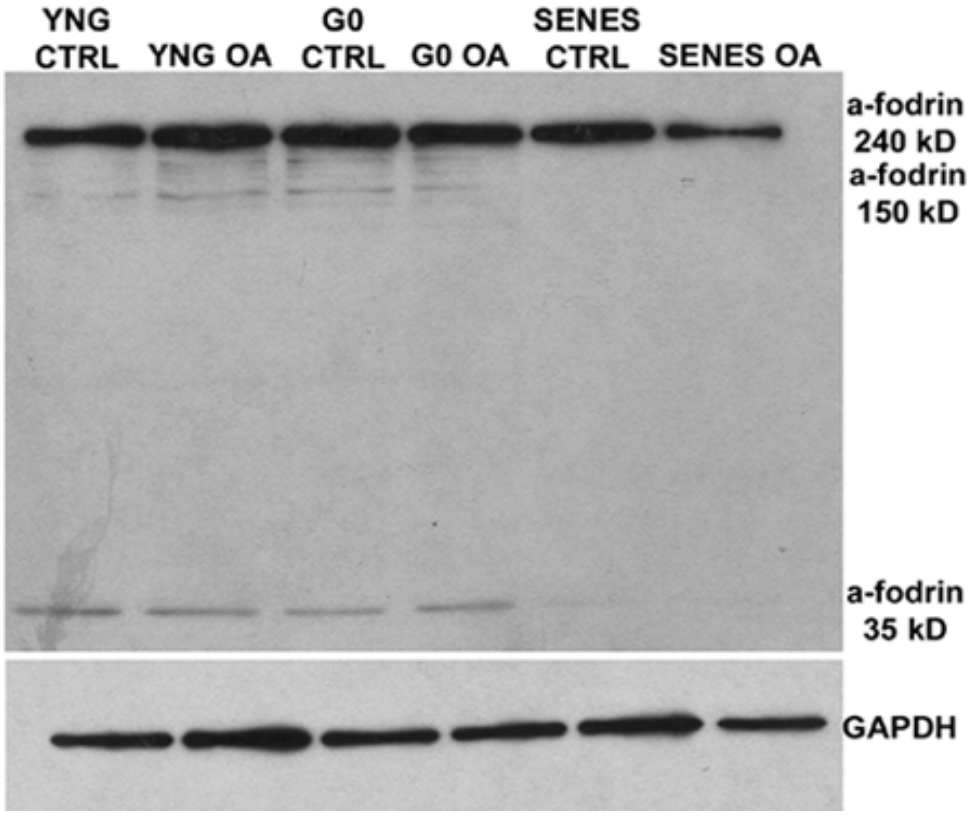
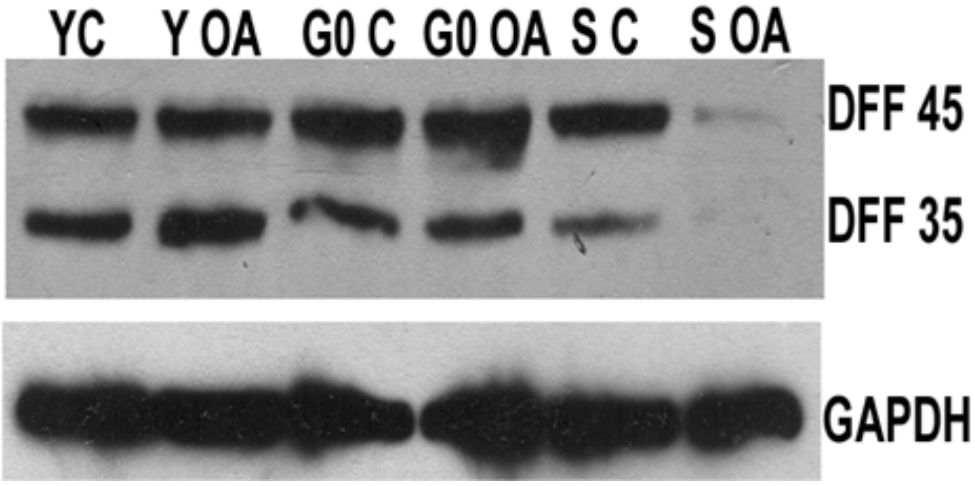


Figure 1.3K. DFF45 expression in WI38 fibroblasts.

Cells were lysed and quantitated as described in the Materials and Methods sections. 40µg total cellular protein was separated on a 12% polyacrylamide gel. DFF45 antibody was used to detect the protein. We found DFF45 levels remained consistent in young and G_0 cells whether they were treated or not. Untreated senescent cells showed full length DFF45. When challenged with OA, the levels decreased. This antibody also recognized an isoform of DFF45, DFF35. GAPDH was used as a loading control.

YC=Young Control; YOA=Young Okadaic Acid; G0C=Growth-arrested Control; G0 OA=Growth-arrested Okadaic Acid; SC=Senescent Control; S OA=Senescent Okadaic Acid.



CHAPTER 2

THE ROLE OF CASPASE-INDEPENDENT PROTEINS APOPTOSIS INDUCING FACTOR AND ENDONULCEASE G

2.1 INTRODUCTION

As discussed earlier in Chapter 1, caspases are not involved in the apoptotic death of senescent fibroblasts challenged with okadaic acid. We next sought to identify factors that could induce apoptotic death in the absence of caspase activity. Apoptosis Inducing Factor (AIF) and Endonuclease G (EndoG) are two proteins that reside in the inner mitochondrial space and have both been shown to induce apoptosis in a caspase-independent manner (Uren *et al.,* 2005).

Endonuclease G

Endonuclease G is well conserved from invertebrates (*Neurospora crassa*) to vertebrates (bovine, humans) and is found as a single copy gene on chromosome 9q34.1 (Low, 2003). As Endonuclease G resides in the mitochondrion and nucleus, both forms were purified and characterized (Low, 1988; Gerschenson *et al.,* 1995). Puriified nuclear and mitochondrial Endonuclease G could be activated by Mg^{2+} and Mn^{2+} and in some instances Co^{2+} and were able to cleave ssDNA and dsDNA (Low, 1988; Gerschenson *et al.,* 1995; Tomkinson and Linn, 1986). Most importantly, the two nucleases maintained their discriminatory pattern of cleavage in that cleavage sites occurred at G-rich sequences (Côté *et al.,* 1989; Gerschenson *et al.,* 1995). When DNA sequences of C-rich sites were incubated with the enzymes, identical nicking patterns were observed indicating the complementary cysteine residues can also be attacked by both types of endonucleases (Gerschenson *et al.,* 1995). Inhibition assays showed increased KCL concentrations could quench as much as 90% of the enzyme activity of both nucleases (Cummings *et al.,* 1987).

Mitochondrial Endonuclease G has come to the forefront in apoptosis as it has been implicated in caspase-independent apoptosis. Li, *et al*. demonstrated its role a cell-free system consisting of mitochondria and nuclei as incubations with recombinant tbid (truncated bid, a proapoptotic Bcl-2 family member previously cleaved by caspase-8) resulted in fragmented DNA (Li *et al.,* 2001). Fractionation and purification of the supernatant allowed for the identification of a 30 kDa product which correlated with the incidence of fragmented DNA (Li *et al.,* 2001). Further characterization of this product revealed it is encoded in the nucleus and transported to mitochondria via a mitochondrial localization sequence that is cleaved upon entry into the mitochondria (Li *et al.,* 2001). Immunofluorescent studies allowed Li *et al*. to definitively conclude that EndoG can induce apoptosis caspase independent manner. Mouse embryonic fibroblasts deficient in DFF45, a caspase substrate, were challenged with ultraviolet irradiation and EndoG localization was traced. Their results show movement of EndoG from the mitochondria to the nucleus while cytochrome *c* remained in the mitochondria (Li *et al.,* 2001). Endonuclease G is not the only protein released from the mitochondria to elicit apoptosis in the absence of caspases. Apoptosis Inducing Factor has also been implicated in this independent death.

Apoptosis Inducing Factor

Apoptosis Inducing Factor (AIF) is a phylogenetically conserved protein located in mitochondria. Characterization of AIF revealed it shares an overall sequence identity of 92% with mouse AIF (Susin *et al.,* 1999). The AIF gene

resides on the human X chromosome region Xq25-26 and is originally synthesized in the cytosol as a 613 amino acid, 67 kDa protein (Susin *et al.,* 1999). Located at the amino terminus is the mitochondrial localization sequence which targets the protein to this organelle. Once AIF enters mitochondria, approximately 100 amino acids are proteolytically removed and a flavin adenine dinucletide (FAD) group is attached (Susin *et al.,* 1999). AIF is then allowed to refold generating a mature 57 kDa protein which ultimately localizes to the mitochondrial intermembrane space (Susin *et al.,* 1999). Human AIF has two FAD-binding domains and one NADH-binding domains, thus the protein is also considered to have oxidoreductase functions in humans as it shares considerable homology with oxidoreductases of *Xenopus, C. elegans* and *S. pombe* (Modjtahedi *et al.,* 2006; Lorenzo *et al.,* 1999).

Additional members of this family have been discovered namely, AMID and AIFL (Modjtahedi *et al.,* 2006). These two proteins tend to localize in the cytosol because they do not contain well-defined mitochondrial localization sequences (Modjtahedi *et al.,* 2006). AMID tends to localize in the cytosol, but when its C-terminal fragment is overexpressed in cells, it will translocate to mitochondria, thus possibly suggesting an embedded MLS (Wu *et al.,* 2002). Although human AIFL, does not contain a well defined MLS, it still tends to localize to mitochondria (Xie *et al.,* 2005).

Immunocytochemical studies with AIF showed that it is released from mitochondria upon insult with *tert*-butylhydroperoxide, ceramide, serum withdrawal, staurosporine or Ca^{2+} (Susin *et al.,* 1999; Daugas *et al.,* 2000).

Microinjection of recombinant AIF, showed decreased mitochondrial membrane potential, and nuclear condensation (Susin *et al.,* 1999). Although AIF release was not prevented by z-VAD-fmk, a pan-caspase-inhibitor, overexpressing Bcl-2 had a suppressive effect (Susin *et al.,* 1999; Daugas *et al.,* 2000). DNA fragmentation is one of the well-known hallmarks of apoptosis where discrete 200bp fragments are created (Zakeri *et al.,* 1993). As AIF was observed to cause condensed chromatin, it was of interest to determine whether fragmented DNA occurred as well. Introduction of purified AIF did indeed result in fragmented DNA, however, not the normally observed 200bp fragments. They observed instead as large internucleosomal fragments of 50kbs (Daugas *et al.,* 2000). Subsequent experiments revealed AIF contains negative charges which allow it to interact with DNA, thus contributing to the above effect (Ye *et al.,* 2002). As mentioned above and below, AIF has an oxidoreductase function hence the presence of the FAD and NAD domains (Lorenzo *et al.,* 1999). It was of interest to determine whether the two domains were necessary for the apoptotic events observed with AIF. When the FAD and NAD domains were deleted cell death still occurred, suggesting they are not essential to elicit an apoptotic death (Miramar *et al.,* 2001).

As mentioned in the general introduction, there are many types of cell deaths and the line separating them is beginning to dissipate. It is now becoming accepted that necrotic cell death and programmed cell death may share a common link as evidenced by Daugas *et al*. (Daugas *et al.,* 2000). Classical type II apoptosis involves high levels of ATP as this is what drives the formation of the

apoptosome (cytochrome *c*, APAF-1 and caspase-9) (Adams and Cory, 2002). Incubating Rat-1 cells in glucose-free medium (decreases ATP production) along with staurosporine (a known apoptotic stimulant) resulted translocation of AIF from the mitochondria to the nucleus (Daugas *et al.,* 2000). These data provide further evidence that a connection indeed exits between necrosis and apoptosis.

Aside from its role in apoptosis, AIF has also been implicated in cellular response to oxidative stress. Harlequin (Hq) mice were generated to study the role of AIF in oxidative phosphorylation (Klein *et al*.; Vahsen *et al.,* 2004). Harlequin mice have a retroviral insertion into the AIF gene and as a result AIF expression is decreased 10-20% of its normal value (Klein *et al.,* 2002). Mutations in AIF resulted in severe neurodegeneration which lead to blindness due to retinal degeneration (Klein *et al.,* 2002). Interestingly, when Klein, *et al*. isolated neurons from the Hq mice, they noticed these neurons were more susceptible to oxidative stressors. Vahsen *et al.* extended these oxidative phosphorylation studies by examining AIF deficient HeLa and mouse embryonic stem cells (Vahsen *et al.,* 2004). The results obtained in these studies were important as it demonstrated AIF was necessary to allow proper functioning of the respiratory chain as its function was vital in complex I function (Vahsen *et al.,* 2004).

Since the discovery and characterization of AIF, reports have been published regarding the regulation of AIF (Ravagnan *et al.,* 2001; Stambolsky *et al.,* 2006). HSP70 contains two regions through which proteins come into contact: the ATP binding domain and the peptide binding domain (PBD) (Li *et al.,*

1992; Strub *et al.,* 2002). Single deletions of both domains reveal the ATP binding domain is indispensable, and that AIF binds to the PBD suggesting HSP70 binding of AIF can occur in the absence of ATP (Ravagnan *et al.,* 2001). The formation of the HSP70:AIF complex prevents ensuing apoptotic events in the presence of known apoptosis-inducing drugs (Ravagnan *et al.,* 2001). It is not only the interaction between AIF and HSP70 that has been studied, just recently AIF was found to be regulated by basal levels of the tumor suppressor p53 (Stambolsky *et al.,* 2006).

AIF contains a p53 response element (p53RE) located in the fourth intron on AIF and this is where binding of p53 occurs (Stambolsky *et al.,* 2006). Basal levels of p53 result in a cytoprotective effect under conditions of mild stress where AIF is destined to participate in scavenging harmful free radicals (Stambolsky *et al.,* 2006). On the other hand, conditions of severe genotoxic stress (i.e. incubation with doxorubicin or cisplatin) resulted in increased transcription of AIF by p53 and cell death in the absence of caspases (Stambolsky *et al.,* 2006).

Both Endonclease G and AIF have been implicated in caspase-independent death during heart development, hair loss in the inner ear (Bahi *et al.,* 2006; Han *et al.,* 2006) and just recently EndoG has been connected to the invasiveness of human breast cancer cells (Basnakian *et al.,* 2006). Basnakian *et al.* showed differentiated non-invasive breast cancer cells had high endonuclease activity making the cells more sensitive to apoptotic death. Differentiated invasive cancer cells, however, had lower levels of EndoG making

them less susceptible to apoptotic death. (Basnakian *et al.,* 2006). Given the fact that we observed that caspases were not involved in the death of senescent cells in the presence of okadaic acid, we sought to determine whether Endonuclease G, and AIF, either independently or together could elicit a caspase-independent death.

2.2 MATERIALS AND METHODS

Cell Culture

WI38 fibroblasts (Coriell Cell Repositories, Camden, NJ) were incubated in a humidified chamber at 37°C with 5% CO_2.(NuAire, Plymouth, MN) and cultured as described by Hubbard and Ozer (Hubbard and Ozer, 1999). Fibroblasts were grown in 100mm tissue cultures plates (Fisher Scientific, Suwanee, GA) with Dubelcco's Modified Eagles Medium (DMEM)/Ham's F10 medium, (pH 7.4) (CellGro/MediaTech, Inc, Herndon, VA). Fetal bovine serum (FBS) (Invitrogen, Grand Island, NY) was added to a final concentration of 10%. Penicillin/streptomycin (CellGro/Media Tech, Inc, Herndon, VA) at 1 unit/ml was supplemented to the media. Cells were subcultured 1:4 until they reached the appropriate population doubling (PDL). Young cells (log phase) were used in apoptosis assays at a PDL of 21-33 whereas; growth-arrested (G_0) cells were generated by withdrawing serum from log phase cells were at the same PDL. Serum was removed for 72 hours, after which the cells were deemed growth-arrested. Senescent cells were generated by serial passage until 62+ as determine by SA-β-galactosidase assay (Dimri *et al.,* 1995).

Treatment with Okadaic Acid and Protein Isolation and Quantitation

Okadaic acid (OA) (Calbiochem, La Jolla, CA) was dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific, Suwanee, GA) to a final stock concentration of 10 microM. Young, growth-arrested and senescent fibroblasts were treated with 10 microM OA at a final concentration of 10 nM and incubated at 37°C in a

5% CO_2 incubator for 18-24 hours. After the appropriate incubation time, the cells were lysed using 1X Sodium Dodecyl Sulfate (SDS) lysis buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT) or 1X cell lysis buffer (Cell Signaling Technology, Danvers, MA). If the 1X SDS lysis buffer were used, the following protease inhibitors were added: leupeptin (1µg/ml), pepstatin (1µg/ml), phenylmethylsulphonylfluoride (PMSF) (50µg/ml). If the 1X cell lysis buffer were used, PMSF was added to a final concentration of 100 mM. Cells were then passed through a 25-gauge needle to ensure complete cell lysis and to fragment nucleic acids. Lysates were then incubated on ice for 15 minutes after which they were transferred to a Hermle refrigerated centrifuge and spun at 4°C for 10 minutes at 11,000rpm. Aliquots were removed to quantitate the isolated protein.

If the 1X SDS lysis buffer were used, to ensure complete removal of SDS, a modified Brönstead/Lowery Assay, Reagent Compatible/Detergent Compatible (RC/DC) Assay (BioRad, Hercules, CA) was performed according to the manufactures instructions. If the 1X cell lysis buffer were used, the standard BioRad assay was performed (Labie *et al.,* 1999)). Protein was quantitated using a Beckman Spectrophotometer at 750nm if the RC/DC Assay were used and 590nm if the 1X cell lysis buffer were used.

Western Analysis

After obtaining the correct total protein concentration, 40µg protein was separated using 8-15% SDS-polyacrylamide (30% acrylamide; 0.8% *bis*-

acrylamide) gel electorphoresis. Separated protein was then transferred at 70V for 90 minutes at 4°C onto Polyvinylidene Fluoride (PVDF) membrane (Osmonics, Minnetonka, MN). Membranes were then blocked in 5% non-fat dried milk for 1 hour at room temperature. AIF (Santa Cruz, Santa Cruz, CA), Endonuclease G (Chemicon International, Temecula, CA), DFF40 (Cell Signaling Technology, Danvers, MA) were used at 1:1000 in 5% non-fat dried milk. The primary antibodies were allowed to incubate overnight at 4°C. The membranes were then washed three times in 1X Tris-buffered saline (10X solution consisted of: 24.2g Tris base; 80g NaCl; pH to 7.6 with HCl; used at 1X) with 0.1% Tween-20 (Fisher Scientific, Suwanee, GA) (1X TBST). Secondary anti-rabbit antibody (Amersham, Piscataway, NJ) was added at a 1:1000 dilution and allowed to incubate for 1 hour at room temperature. The signal was detected using Enhanced Chemiluminescent (ECL)-Plus (Amersham Piscataway, NJ). Blots were subsequently probed for GAPDH (Chemicon International, Temecula, CA) which was used as a loading control. Primary antibody dilutions were at 1:1000 and incubated 1 hour at room temperature. Secondary anti-mouse HRP (Amersham, Piscataway, NJ) was used at 1:1000 and incubated at room temperature for 1 hour. The signal was detected using ECL-Plus (Amersham, Piscataway, NJ).

Immunoprecipitation

Immunoprecipitations were performed using 100µg total protein lysates. AIF was immobilized using 20µl agarose bead slurry (Amersham, Piscataway, NJ).

Samples were incubated overnight at 4°C with rotation. Samples were washed four times with 1X Cell Lysis Buffer. 20µl 2X SDS Loading buffer was added and samples were boiled for 5 minutes. Samples were added to a 12% SDS polyacrlyamide gel and electophoresed. Western blotting was performed as described above.

Immunocytochemistry and Confocal Microscopy

Young WI38 fibroblasts were seeded into 4-well chamber slides (Fisher Scientific, Suwanee, GA) at a concentration of $1X10^4$/ml. As individual plates of senescent cells were usually less than 40% confluent, 2-4 plates were combined and approximately 0.5-$1X10^3$ cells/ml were seeded into 4-well chamber slides. One day after seeding, the cells were challenged with 10nM OA 18-24. Cells were washed with 1X Phosphate Buffered Saline (PBS) (Fisher Scientific, Suwanee, GA) (11.9 mM Phosphates; 137mM Sodium Chloride; 2,7 mM Potassium Chloride; pH7.4) and fixed using 2% paraformaldehyde in PBS for 20 minutes at room temperature. Cells were then permeabilized with 0.1% Triton X-100 (Fisher Scientific, Suwanee, GA) for 5 minutes. Cells were blocked 1 hour at room temperature with 10% bovine serum albumin (BSA). Primary antibodies were used at the following dilutions: AIF (Epitomics, Burlingame, CA) 1:50; Endo G (ProSci, Poway, CA) 1:50 and DFF45 (Cell Signaling Technology, Danvers, MA) 1:50 and incubated overnight at 4°C on a rotating platform. After overnight incubation, slides were washed three times with 1X PBS with 0.1% Tween-20 (1XPBST) 5 minutes each. Secondary anti-rabbit fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, OR), anti-rabbit-Cy-3 (Molecular Probes,

Eugene OR) or anti-mouse FITC (Molecular Probes, Eugene, OR) were used at 1:200 dilutions and incubated for 1 hour at room temperature. Slides were then washed with 1X PBS three times 5 minutes each. To label mitochondria, slides were incubated with 200nM Chloromethyl-X-rosamine (CMXRos) (Molecular Probes, Eugene, OR) for 15 minutes in 1XPBS at room temperature, then washed once with 1X PBS. Slides were then mounted with SlowFade AntiFade solution with 4',6-Diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene OR) and analyzed using a Zeiss LSM510 confocal microscope (Germany) using the following lasers: Diode (405-430 nm), HeNe543 (543 nm) and Argon (488 nm).

AIFGFP Transient Transfection

Young WI38 fibroblasts were cultured in 100mm plates DMEM:F10 containing 10% serum without antibiotics. For transfection studies, the cells were cultured in DMEM:F10 without serum and without antibiotics. 24µg AIFGFP plasmid DNA (kind gift of Guido Kroemer) and Lipofectamine 2000 (Invitrogen, Grand Island, NY) were used at a 1:2.5 (DNA:Lipofectamine 2000) for 5 hours at 37°C with 5%CO_2. After the 5 hour incubation period, the transfection medium was removed and fresh medium containing 10% FBS without antibiotics was added. Two days after transfection, 10 nM OA was added for an additional 18 hours. Total cell protein was isolated, quantified and analyzed by Western immunoblotting as described above.

AIFGFP Localization via Confocal Microscopy

1 X 10^4 young WI38 fibroblasts were cultured as described above for transfection experiments in 4-well chamber slides (Fisher Scientific, Suwanee, GA). They were transfected with AIFGFP (24µg DNA) or GFP (24µg DNA) alone at a ratio of 1:2.5 (DNA:Lipofectamine 2000) for 5 hours at 37°C with 5%CO_2. Transfection medium was removed and fresh medium containing 10% FBS without antibiotics was added for 48 hours. The cells were then transfected an additional 18 hours with 10 nM OA. Cells were then washed once with 1X PBS and fixed with fresh 2% paraformaldehyde for 20 minutes at room temperature. To label mitochondria, slides were incubated for 15 minutes with Chloromethyl-X-rosamine (CMXRos) (Molecular Probes, Eugene OR) at room temperature. Slides were then mounted with SlowFade-antifade solution with DAPI (Molecular Probes, Eugene, OR) and analyzed using a Zeiss LSM510 confocal microscope (Germany) using the following lasers: Diode (405-430 nm), HeNe543 (543 nm) and Argon (488 nm).

2.3 RESULTS

2.3.1 AIF and EndoG are present in WI38 fibroblasts

Chapter I showed noticeable changes in protein levels of the caspases as well as abnormal PARP cleavage as the fibroblasts aged. We also provided evidence of a caspase-independent death. We therefore wanted to determine whether AIF and EndoG were responsible for the death observed in senescent fibroblasts. Total cellular protein was isolated from young, growth-arrested and senescent cells as described in materials and methods. Protein samples were fractionated on 12% SDS-polyacrylamide gels. When we probed membranes with an AIF antibody that recognizes the amino terminus, AIF was abundant in all three growth states at 57 kDa (Figure 2.3A). Similarly EndoG was present in all three growth states at 35 kDa although the signal was somewhat lower in senescent cells(Figure 2.3B). The results indicate that the proteins were indeed present in the fibroblasts, but localization between mitochondria and the nucleus could not be determined. Immunocytochemical experiments were carried out to determine localization patterns.

We may have found a possible modification of AIF in senescent fibroblasts. AIF was immunoprecipitated from total cell lysates from young and senescent cells (untreated and treated) using an antibody which recognizes the COOH-terminus of AIF (Epitomics, Burlingame, CA). Subsequent Western analysis using the same COOH-terminus antibody resulted in the absence of an AIF signal in senescent cells while the signal was strong in young untreated and

treated cells (Figure 2.3C). When immunoprecipations were performed with a different AIF antibody (Santa Cruz, Santa Cruz, CA) which recognizes the amino-terminal portion of the protein, we observed a robust signal in both young and senescent cells (Figure 2.3D). These results suggest a modification of AIF at the COOH-terminus. It is unclear as to what putative modification has been made in AIF, but we can speculate there may be age-related change in its sequence and structure.

2.3.2 Examining AIF and EndoG translocation in young and senescent fibroblasts by immunofluorescence

Young and senescent cells were seeded into 4-well chamber slides and challenged with OA as described in Materials and Methods. Figure 2.3E-F show young and senescent WI38 fibroblasts untreated control and OA-treated images taken by a Zeiss LSM510 confocal microscope. Further analysis revealed approximately 66% and 51% EndoG/mitochondria colocalization occurred in young control and OA-treated cells, respectively (Figure 2.3E-1, Figure G). In senescent cells 57% and 100% EndoG/mitochondria colocalization occurred in senescent control and treated cells, respectively (Figure 2.3F-1, Figure 2.3G). Regarding EndoG nuclear translocation, there was no significant difference between the two groups (Figure 2.3H). These results suggest that EndoG does not translocate from the mitochondria to the nucleus in WI38 fibroblasts.

As AIF is also present in the mitochondria, and can promote caspase independent apoptosis, we sought to determine its ability to translocate to the nucleus when WI38 fibroblasts are challenged with OA. Shown in Figure 2.3I-J

we found AIF to be absent from the nuclei in untreated young cells (Figure 2.3I). In senescent cells we see slight nuclear localization in control and OA-treated cells. Analyzing colocalized AIF/mitochondrial pixels we find no significant difference between young and control and senescent control ($p=0.06$) (Figure 2.3I-1, Figure 2.3K). In the presence of 10 nM OA, however, there is a significant although smaller amount of colocalized AIF/nuclear pixels in senescent cells when compared to young OA-treated. ($p<0.05$) (Figure 2.3J-1, Figure 2.3L).

The data presented above indicate that EndoG remained localized to the mitochondria regardless of treatment in young and senescent fibroblasts. We found that AIF moves to the nucleus as others have shown (Daugas *et al.,* 2000; Polster *et al.,* 2005). In Chapter I our data show that DFF45 levels decrease when challenged with OA in senescent cells while the levels remain consistent in young and growth-arrested cells. Since caspases are not activated in senescent cells, we hypothesized that AIF, upon entering the nucleus, could target DFF45, thus leading to the release of the DFF40. DFF40, as mentioned in Chapter 1, is an endonuclease that can act on DNA inducing double-stranded or single-stranded DNA fragments. DFF40 could be the endonuclease that explains the TUNEL positive okadaic acid-treated senescent cells observed previously by us (DeJesus *et al.,* 2002). We then overexpressed AIF in the fibroblasts to determine whether: (1) There is indeed translocation of AIF to the nucleus and (2) DFF45 levels decreased when challenged with OA in this overexpression system.

2.3.3 Transient expression of AIF results in decreased expression of DFF45

Due to the fact that there was decreased expression of DFF45 in senescent cells challenged with OA, we sought to determine whether there was a potential interaction between AIF and DFF45. Our immunofluorescent data showed little, but significant AIF localization to the nucleus. We used an AIFGFP fusion plasmid, a gift from Guido Kroemer, to overexpress AIF in WI38 fibroblasts (Figure 2.3M). Young WI38 cells were transfected with 24 µg AIFGFP plasmid DNA for 48 hours followed by treatment with 10 nM OA for 18 hours. Immunofluorescent data with AIFGFP correlates with observations from the Kroemer group in that the transfected protein translocated to the nucleus (Figure 2.3N,O) When we analyzed DFF45 protein levels there was a decrease in DFF45 protein in senescent cells challenged with OA (Figure 2.3P). In controls (Figure 2.3P, lanes 1-3) DFF45 levels remained unchanged. Overexpression of AIF in the presence of OA resulted in decreased protein levels of DFF45 (Figure 2.3P, lane 6). These results are similar to what was observed in senescent cells (Figure 1.3K) and suggests that AIF and DFF45 may interact in the nucleus of senescent cells challenged with OA. However, we cannot rule out there may be other intermediary factors that elicit the decrease in DFF45 protein levels.

DNA Fragmentation Factor (DFF) exists as a heterodimer in the nucleus. DFF45 has been shown to have dual function in that it acts as a chaperone protein to ensure proper folding of DFF40, a nuclease. DFF45 also serves to inhibit DFF40 nuclease activity, thereby preventing DFF40 from cleaving DNA. In light of the fact that DFF45 cleavage occurred under normal and

overexpression of AIF, we examined the role of DFF40 in human fibroblasts. Our initial experiments showed that DFF40 could not be detected in young nor senescent cells (Figure 2.3Q).

2.4 DISCUSSION

Mitochondria contain several proteins that have been implicated in apoptosis. These proteins include cytochrome *c*, AIF, EndoG, Smac/DIABLO, Omi/HtrA2 (van Gurp *et al.,* 2003). Our studies focused on AIF and EndoG, two proteins that are associated with caspase-independent deaths. We have shown that EndoG is not involved in our system, as it remains localized to the mitochondrion in both control and OA-treated young and control cells (Figure 2.3E-F; 2.3E-1, 2.3F-1). Furthermore, statistical analysis reveals no significant difference in all treatments with regards to nuclear translocation (Figure 2.3G-H). On the other hand, AIF does migrate from mitochondria to the nucleus, but this is only evident in senescent cells (Figure 2.3J, Figure 2.3J-1). This translocation was statistically significant $p<0.05$ (Figure 2.3L) in agreement with previous observation by others (Candé *et al.,* 2002).

Given the fact that AIF translocated to the nucleus, we hypothesized that AIF could be responsible for the observed cleavage of DFF45. We overexpressed AIF. We found that transfected cells challenged with OA resulted in decreased DFF45 levels (Figure 2.3P). AIF was originally discovered as a protein that could elicit large DNA nucleosomal fragments (Candé *et al.,* 2002). Our data suggests a possible interaction between AIF and DFF45. Whether AIF has the capability of acting as a caspase-like protein is not known.

APPENDIX: Figures and Figure Legends for Chapter 2

Figure 2.3A. Western analysis of AIF localization.

Young and senescent fibroblasts were challenged and protein isolation was performed as described in the Materials and Methods sections. AIF levels are abundant in young and senescent cells.

YC= Young Control; YOA=Young Okadaic Acid; SC= Senescent Control; SOA=Senescent Okadaic Acid.

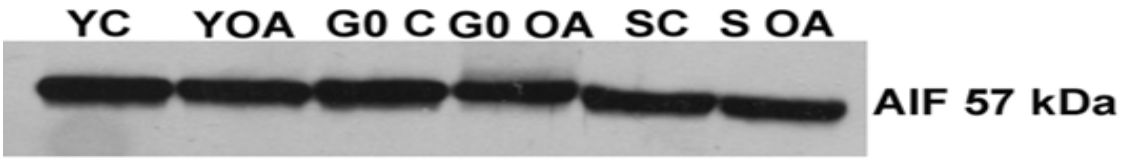


Figure 2.3B. Endonuclease G expression in young, G_0 and senescent cells.

Cells were treated and lysed as described in the Materials and Methods sections. 40µg total cellular protein was separated on 12% polyacrylamide gels and electrophoresed. Subsequent addition of EndoG antibody revealed EndoG is present in all three growth states.

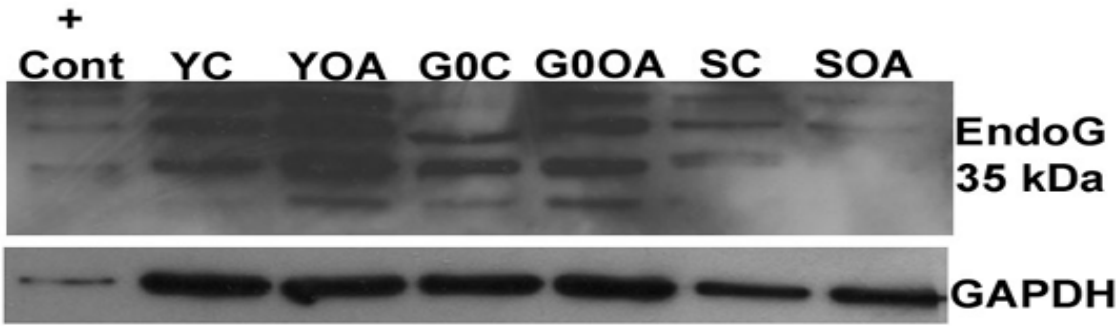


Figure 2.3C. Potential modification of AIF in senescent WI38 fibroblasts.

Young and senescent cells were challenged as described in the Materials and Methods sections. When an antibody which recognizes the carboxy-terminal of AIF was used, no apparent signal for AIF was detected in senescent cells. YC= Young Control; YOA=Young Okadaic Acid; SC= Senescent Control; SOA=Senescent Okadaic Acid.

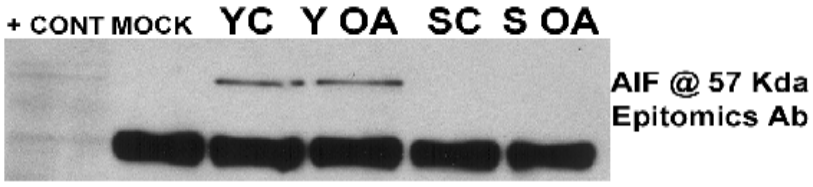


Figure 2.3D. Potential AIF Modification in senescent WI38 fibroblasts.

Young and senescent cells were challenged as described in the Materials and Methods sections. When an antibody which recognizes the amino-terminal of AIF was used, AIF was detected in all growth states. YC= Young Control; YOA=Young Okadaic Acid; SC= Senescent Control; SOA=Senescent Okadaic Acid.

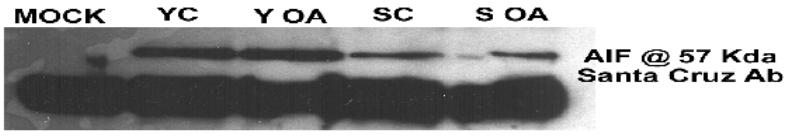


Figure 2.3E-F. Immunofluorescent images of EndoG localization in young and senescent WI38 fibroblasts.

Cells were treated with OA as described in Materials and Methods. When analyzed by confocal microscopy, we see complete mitochondrial localization in young cells as (Figure 2.3E). When senescent cells were analyzed we did not find EndoG localization in untreated control or OA-treated cells (Figure 2.3F). Total magnification= 1000X.

Figure 2.3E. Immunofluorescent image of EndoG localization in young WI38 fibroblasts.

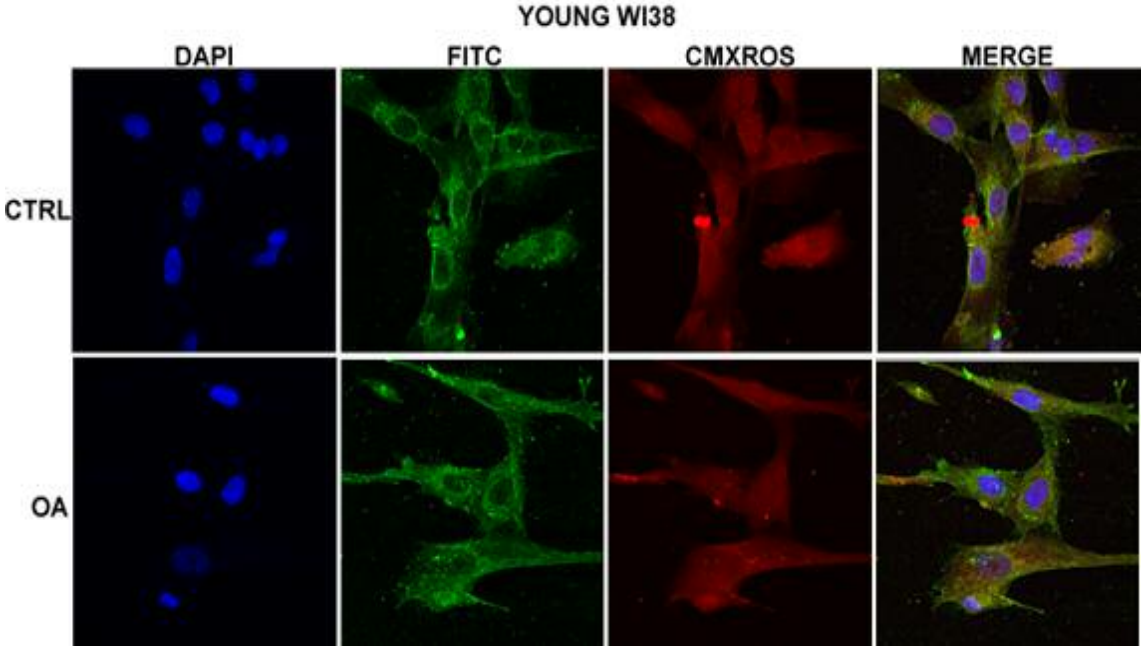


Figure 2.3F. Immunofluorescent image of EndoG localization in senescent WI38 fibroblasts.

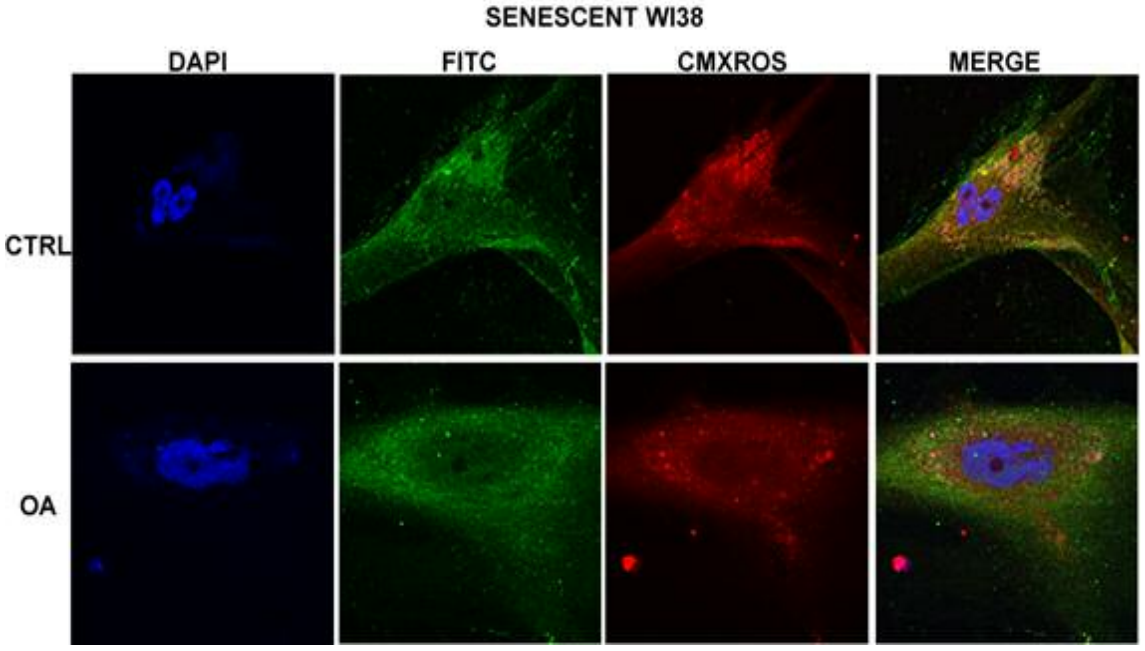


Figure 2.3E-1. Mitochondrial localization and nuclear exclusion of EndoG in young WI38 fibroblasts.

ENDOG/MITO localization indicates localization to both compartments. The software available on the LSM510 confocal microscope allowed us to separate the two populations of EndoG (mitochondrial or nuclear). When EndoG mitochondrial localization is analyzed, EndoG has a perinuclear staining pattern (65% colocalized in young control; 51% colocalized in young OA). Regarding nuclear localization we found 19% and 13% colocalized pixels in young control and young OA-treated respectively. Total magnification= 1000X.

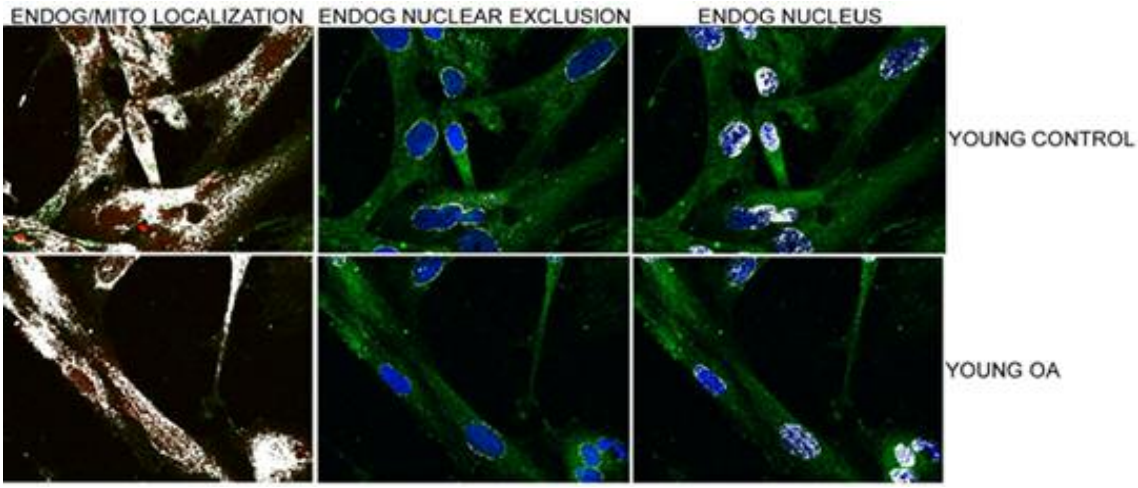


Figure 2.3F-1. Mitochondrial localization and nuclear exclusion of EndoG in senescent WI38 fibroblasts.

ENDOG/MITO localization indicates localization to both compartments. The software available on the LSM510 confocal microscope allowed us to separate the two populations of EndoG (mitochondrial or nuclear). When EndoG mitochondrial localization is analyzed, EndoG has an intense mitochondrial staining pattern in senescent control and treated (57% colocalized in senescent control; 100% colocalized in senescent OA). Regarding nuclear localization, we found 7% and 15% colocalized pixels in senescent control and senescent OA-treated respectively. Total magnification= 1000X.

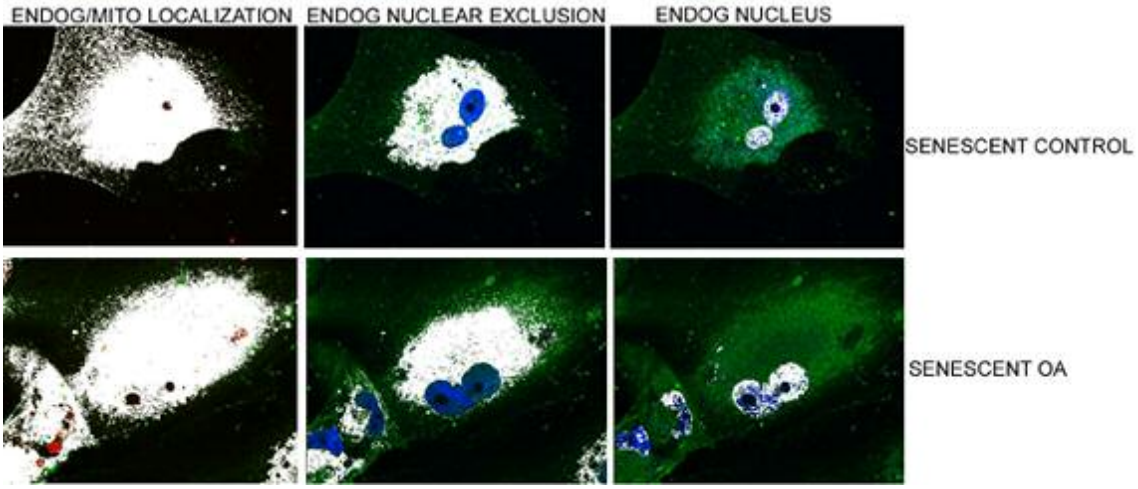


Figure 2.3G. Quantitation of Endonuclease G colocalization to the mitochondria.

EndoG mitochondrial colocalization was determined from (Figure 2.3E-F) using the colocalization program installed on the LSM510 confocal microscope. EndoG/mitochondrial colocalization in young control and OA-treated was 66% and 51%, respectively. While in senescent cells EndoG/mitochondrial colocalization was 57% and 100% in senescent control and OA-treated cells respectively.

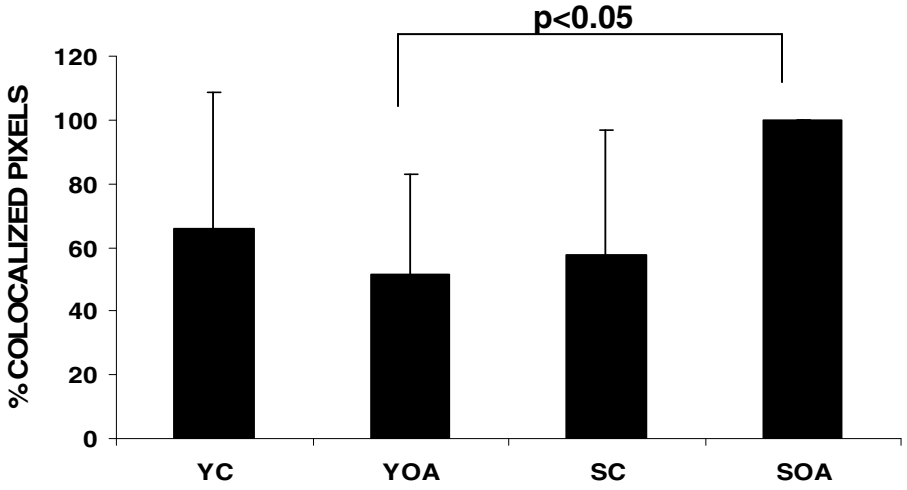


Figure 2.3H. Quantitation of Endonuclease G and nuclear colocalization.

Although there was staining of EndoG in the nucleus of young and senescent samples, there was no statistical significance p>0.05.

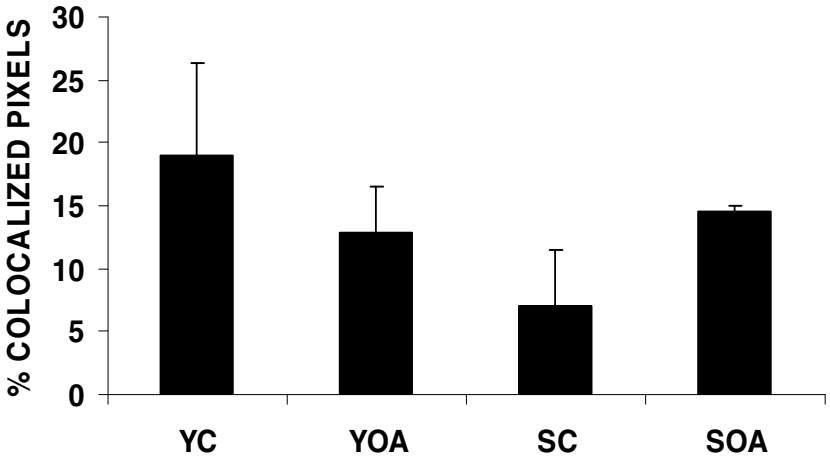


Figure 2.3I-J. Immunofluorescent images of AIF localization in young and senescent fibroblasts challenged with OA.

Young and senescent cells were challenged and stained as described in the Materials and Methods sections. The cells were analyzed by confocal microscopy. We found AIF remains localized to the mitochondria in young fibroblasts (Figure 2.3I) while translocation is evident in senescent cells (Figure 2.3J). Total magnification= 1000X.

Figure 2.3I. Immunofluorescent image of AIF localization in young WI38 fibroblasts.

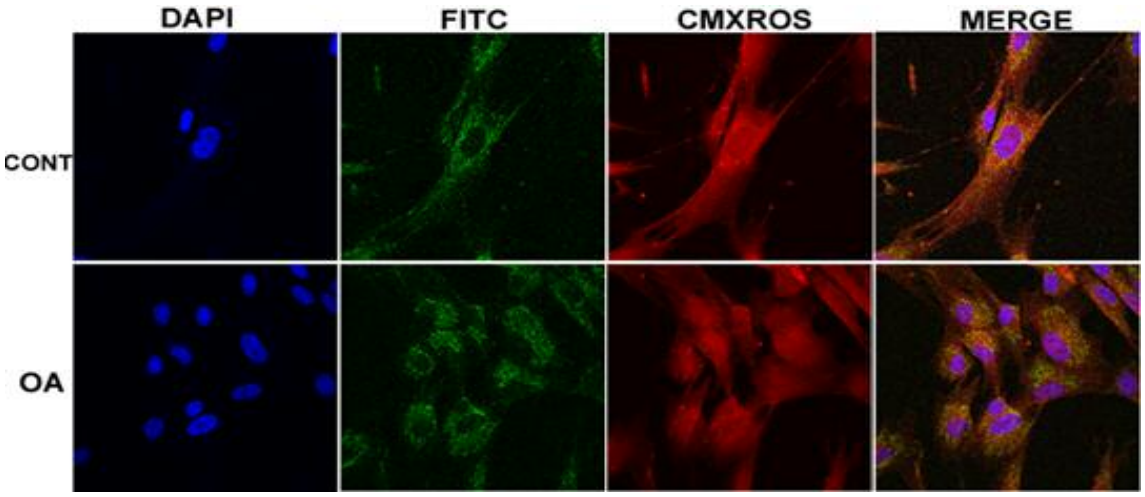


Figure 2.3J. Immunofluorescent image of AIF localization in senescent WI38 fibroblasts.

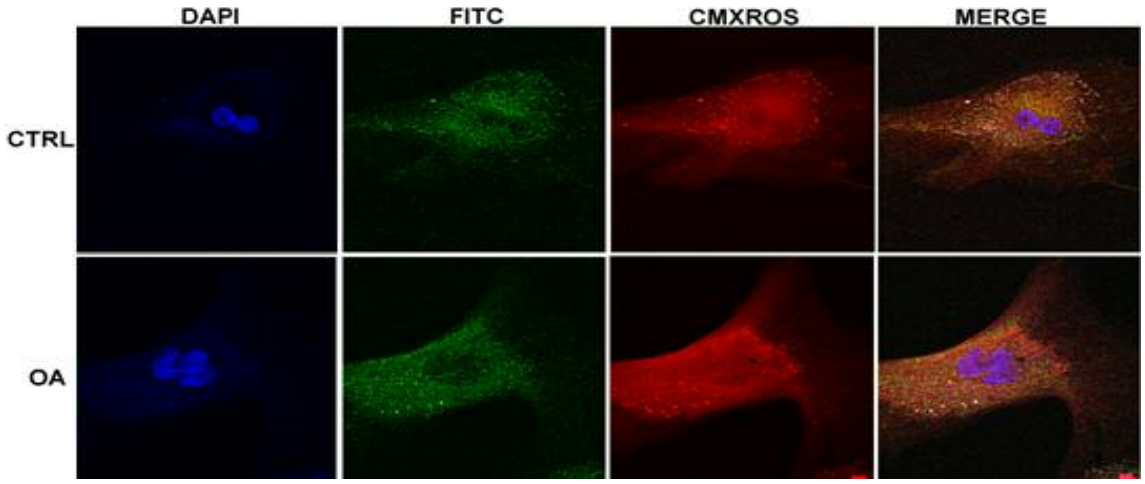


Figure 2.3l-1. Nuclear and mitochondrial localization of AIF in young fibroblasts.

Using the software from the LSM510 confocal microscope, we were able to separate AIF into mitochondrial localization and nuclear localization. When AIF/mitochondria colocalized pixels was determined we found young control and young OA 10% and 27% colocalized pixels was observed respectively. Analysis of nuclear colocalization revealed 2.3 and 1.6% colocalization in young control and treated respectively. There was no significant difference. Total magnification= 1000X.

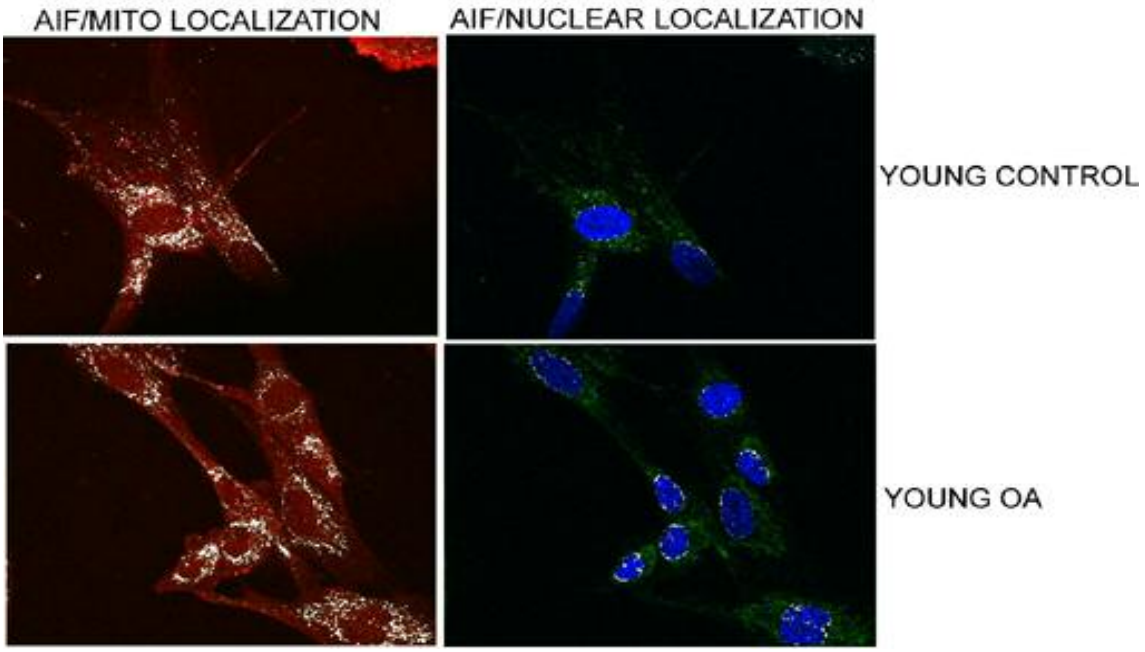


Figure 2.3J-1. Nuclear and mitochondrial localization of AIF in senescent fibroblasts.

Using the software from the LSM510 confocal microscope, we were able to separate AIF into mitochondrial localization and nuclear localization. When AIF/mitochondria colocalized pixels was determined we found senescent control and senescent OA 27% and 56% colocalized pixels was observed respectively. Analysis of nuclear colocalization revealed 2.9 and 3.5% colocalization in senescent control and treated respectively ($p<0.05$). Total magnification= 1000X.

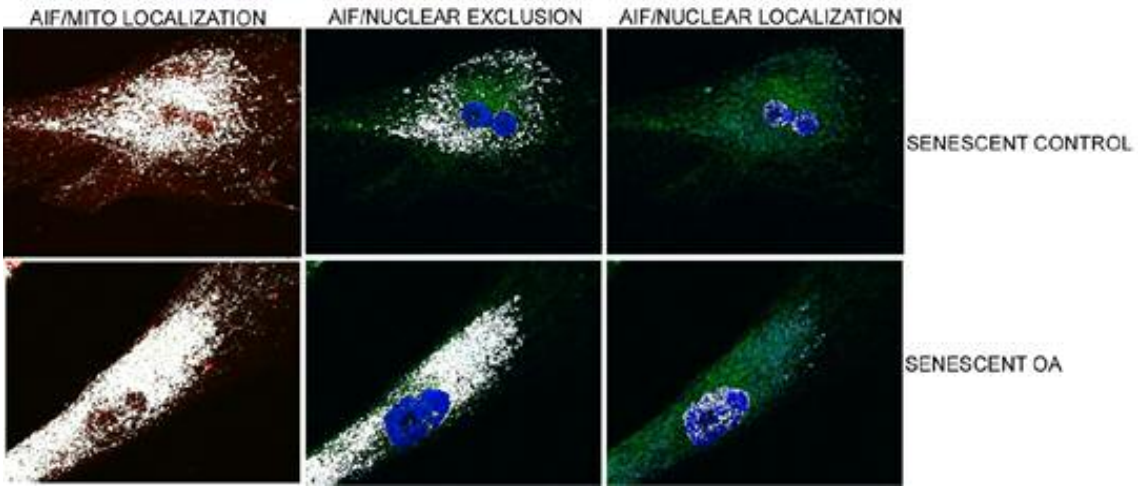


Figure 2.3K. Quantitation of AIF/mitochondrial colocalized pixels.

Quantitation of fluorescent images (Figure 2.3J-1 and 2.3J-2).

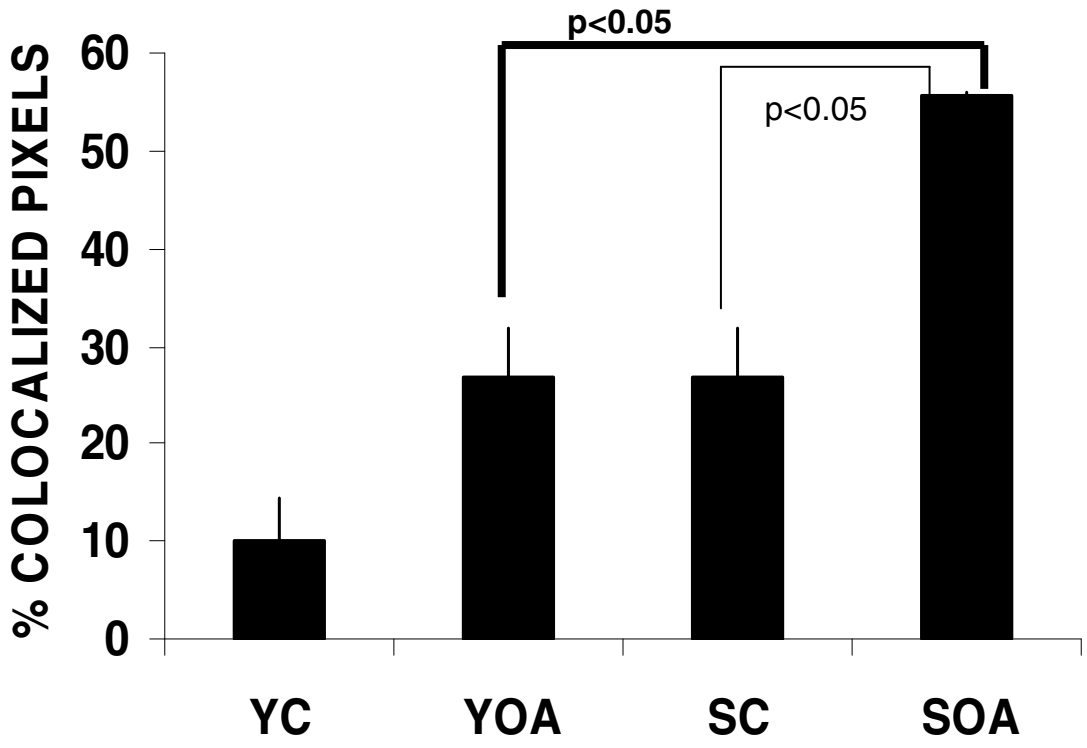


Figure 2.3L. Quantitation of AIF/nuclear colocalization in young and senescent WI38 fibroblasts.

Data was obtained from Figure 2.3I-1 and 2.3J-1. There was significane ($p<0.05$) in AIF/nuclear colocalization when young OA and senescent OA were subjected to a Student's two-tailed t-test.

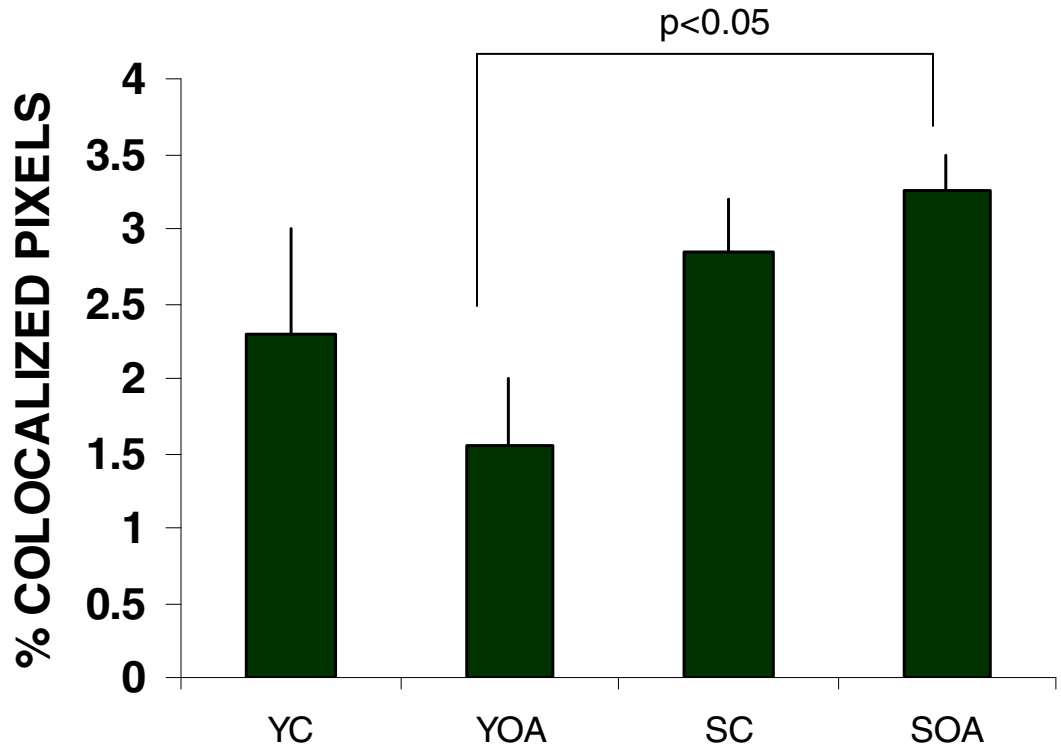


Figure 2.3M. Restriction mapping of AIFGFP plasmid.

Restriction mapping was done to confirm the presence of AIFGFP in the pcDNA plasmid. Digestion of plasmid DNA with PmeI resulted in excision of the 2.7kb AIFGFP DNA.

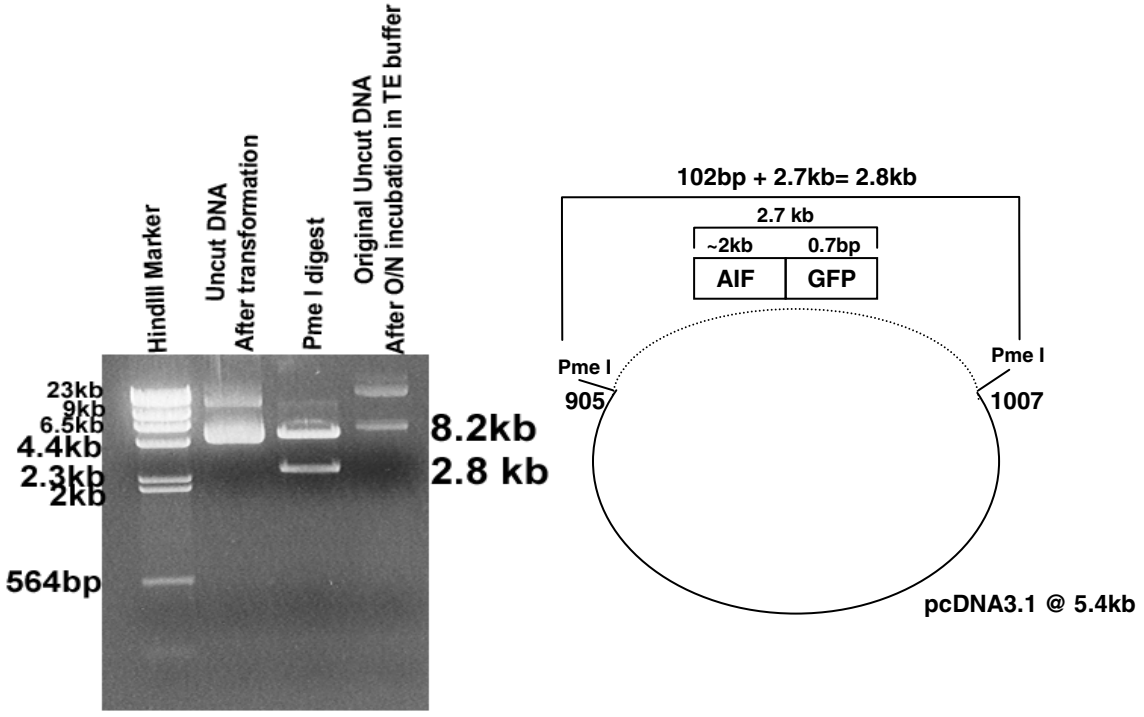


Figure 2.3N-O. AIFGFP transfection in young WI38 fibroblasts.

Young fibroblasts were transfected with 24µg DNA as described in the Materials and Methods section. After 48 hours, the transfected cells were challenged with 10 nM OA for 18 hours. AIFGFP translocation was analyzed by an LSM510 confocal microscope. AIFGFP remained localized to the mitochondria (Figure 2.3N). When challenged with OA, we see AIFGFP translocates to the nucleus (Figure 2.3N). Cells were also transfected with GFP only to confirm it remains localized to the cytoplasm (Figure 2.3O). Total magnification= 1000X.

Figure 2.3 N. AIFGFP localization after transfection.

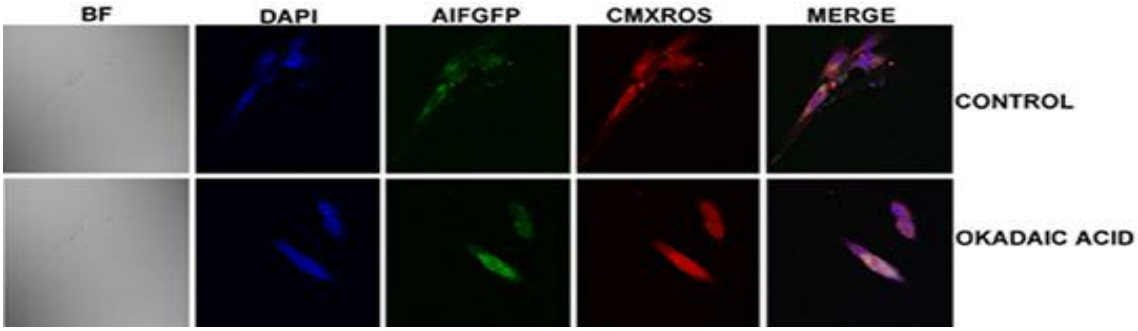


Figure 2.3O. Green Fluorescent Protein (GFP) localization after transfection.

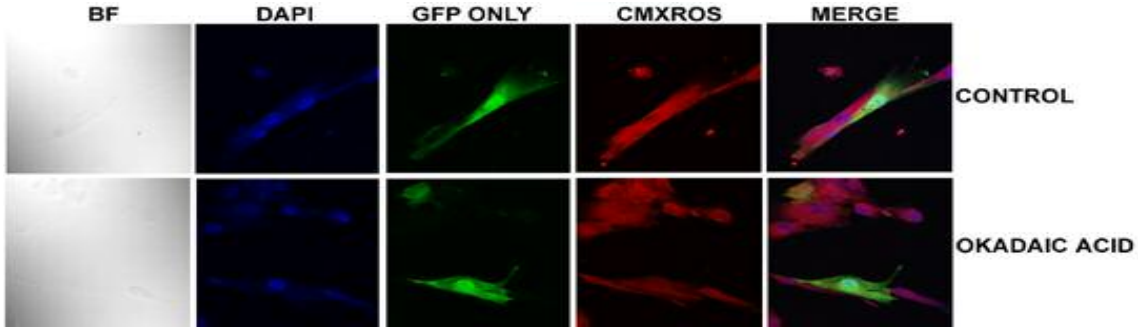


Figure 2.3P. Western analysis of DFF45 when AIF was overexpressed in WI38 fibroblasts.

Young cells were transfected with AIFGFP as described in Materials and Methods. Immunoblotting revealed DFF45 protein levels remained unchanged in controls (Lanes 1-3). When untransfected cells were challenged with OA we find DFF45 protein level did not decrease (Lane 4) Cells transfected with GFP only and OA did not yield decreased DFF45 levels (Lane 5). When AIFGFP transfected cells were treated with OA, we find DFF45 levels were lowered (Lane 6).

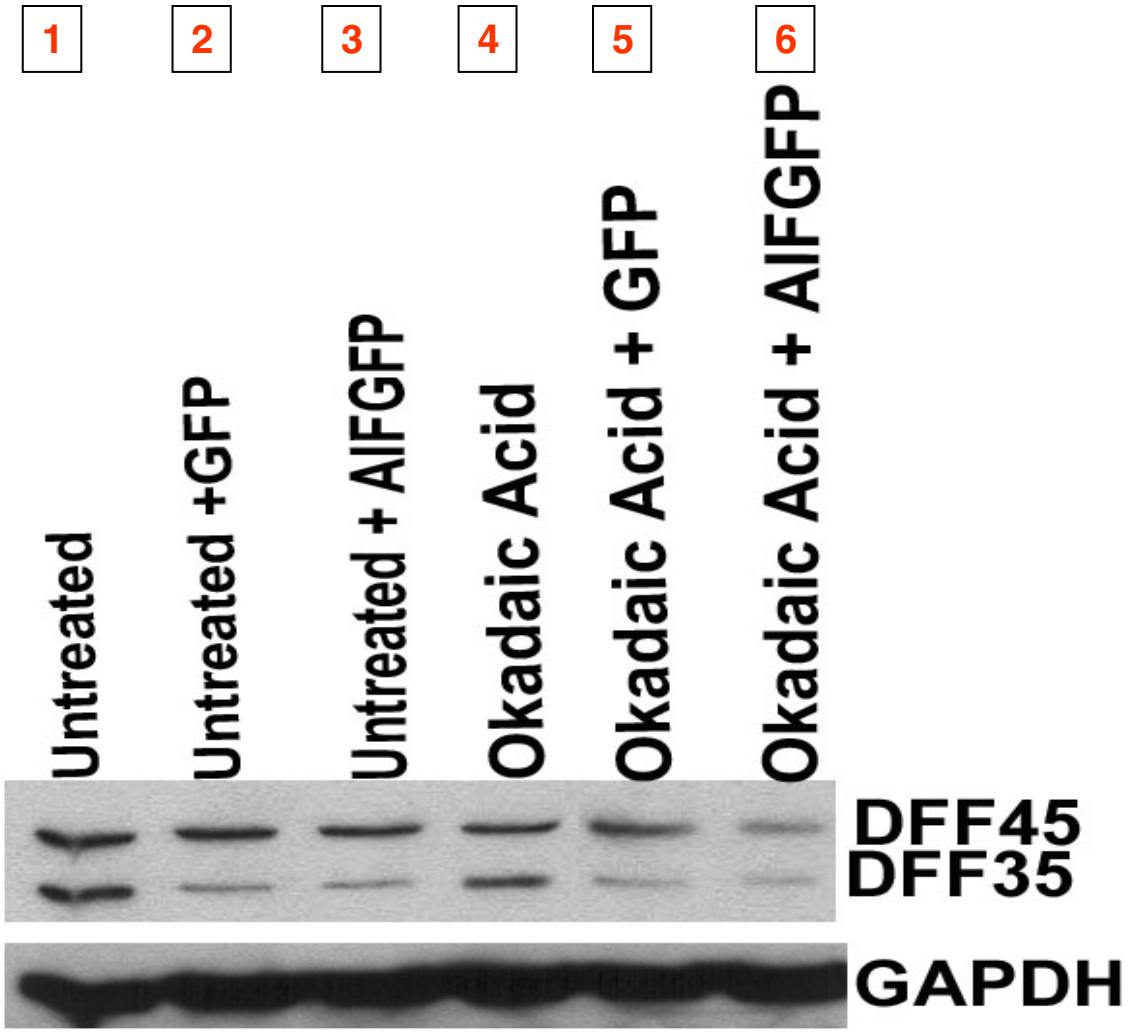
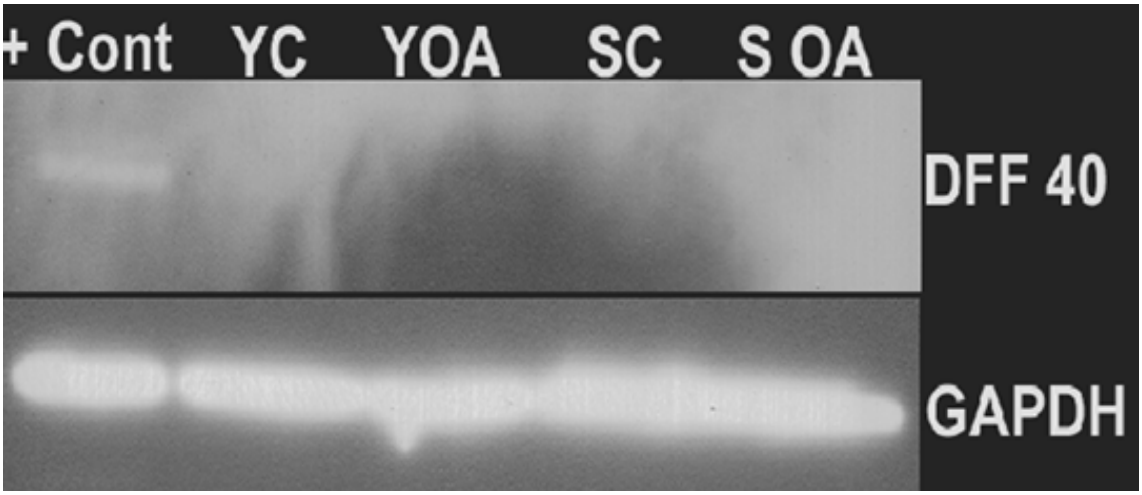


Figure 2.3Q. DFF40 protein expression in young and senescent WI38 fibroblasts.

Total cellular protein was extracted from young and senescent cells challenged with OA as described in Materials and Methods. Antibody recognizing DFF40 revealed DFF40 was not present in senescent cells challenged with OA. DFF40 was recognized in the Positive Control (+ Cont).



CHAPTER 3

AN EXAMINIATION OF MITOCHONDRIA AND THE BCL-2 FAMILY IN SENESCENT HUMAN DIPLOID FIBROBLASTS

3.1 INTRODUCTION

Mitochondria were thought to have a passive role in apoptosis (Kerr *et al.,* 1972; Martin *et al.,* 1988). When cancerous cells were induced to undergo apoptosis, Kerr *et al.* noticed little change in mitochondrial structure. Similar to this group, Martin *et al.* showed that mitochondria of neuronal cells remained intact when subjected to apoptotic stimuli (Martin *et al.,* 1988). The significance of the mitochondria in apoptosis came to the forefront in the early-mid 1990's (Cossarizza *et al.,* 1994). It is now known that mitochondria house proteins which are essential to carry out apoptosis (Green, 2000).

It has been shown that mitochondria are under strict regulation by both anti- and proapoptotic members of the Bcl-2 family (Werner *et al.,* 2002; Schinzel *et al.,* 2004; Bouillet *et al.,* 2002; Urase *et al.,* 1999). It was natural to ask how this family of proteins which share similar structural homology are regulated. JC Reed's group answered this question by using a yeast two-hybrid system (Sato *et al.,* 1994). They found interactions could occur within a group such that anti-apoptotic Bcl-2 interacted with antiapoptotic Bcl-xL (Sato *et al.,* 1994). Alternatively, interactions could occur between proapoptotic members such as Bax and anti-apoptotic members such as Mcl-1 (Sato *et al.,* 1994).

Anti-apoptotic Bcl-2 proteins (Bcl-2, Bcl-xL) are anchored in the mitochondrial outer membrane (Cheng *et al.,* 2001; Schinzel *et al.,* 2004). This insertion into the mitochondrial membrane is believed to help prevent inappropriate mitochondrial pore opening (Kroemer and Reed, 2000; Kaufmann

et al., 2004). The antiapoptotic activity of Bcl-2 can be inhibited by proapoptotic members such as Bid and Bad (Chittenden, 2002; Huang and Strasser, 2000). This interaction results in a conformational change such that Bcl-2 can no longer inhibit pore opening (Huang and Strasser, 2000).

The mitochondrion although small in size, can have a tremendous effect on the stability of a cell, tissue, and organism (Kroemer and Reed, 2000). Mitochondria are most often involved as a source of reactive oxygen species (ROS). ROS are most often formed in complex I of the mitochondrial respiratory chain (Lesnefsky and Hoppel, 2005). NADH dehydrogenase as well as iron-sulfer proteins which reside in complex I can reduce molecular oxygen to $^{\bullet}O_2^-$. Associated with aging is a decrease in enzymes which act as scavengers of these free radicals such as superoxide dismutase and glutathione transferase (Kokoszka *et al.,* 2001; Pollack and Leeuwenburgh, 2001; Balaban and Nemoto, 2005). Disruption of the mitochondrial membrane potential has been associated with the high amounts of ROS generated with age (Lieven *et al.,* 2003).

Maintenance of mitochondrial membrane potential is controlled by the Bcl-2 family of anti-apoptotic proteins. When the mitochondria were examined more closely it was found that membrane potential was regulated by the voltage dependent anion channel (VDAC) and the adenine nucleotide translocator (ANT) (Isenberg and Klaunig, 2000). ANT and VDAC are two transmembrane proteins located on the inner and outer mitochondrial membranes, respectively. Both form pores through which select mitochondrial proteins are release. The proapoptotic Bid has been shown to regulate opening of VDAC while

pharmacological agents such as bongkrekic acid and cyclosporin A have been shown to inhibit pore opening (Kroemer and Reed, 2000; Rostovtseva *et al.,* 2004).

This chapter addresses several questions regarding the role of mitochondria in senescent cell apoptosis. We first examined mitochondrial morphology to determine whether there was increased fission occurring, as this is an event which occurs upstream of any the known hallmarks of apoptosis. We also wanted address the role of the Bcl-2 family in our studies, as this family has been shown to be just as important as the caspases in other systems. And lastly, we wanted to know whether mitochondrial membrane potential was compromised in senescent cells challenged with OA. The results obtained in this section has provided new insight as to the role of mitochondria in senescent cell death.

3.2 MATERIALS AND METHODS

Determination of Mitochondrial Morphology

Young and senescent WI38 fibroblasts were cultured as described in Chapter 2. Young and senescent cells were seeded onto 4-well chamber slides (Fisher Scientific, Suwanee, GA) at a density of $1X10^4$ and $0.5\text{-}1X10^3$ cells/ml per chamber respectively. Cells were then challenged with 10 nM OA for eighteen hours. Mitochondria were stained with 400 nM Chloromethyl-X-rosamine (CMXRos) (Molecular Probes, Eugene, OR) in complete cell culture medium (DMEM:F10 with 10% FBS) for twenty minutes at 37°C with 5% CO_2. Subsequent to mitochondrial staining, slides were washed twice with complete cell culture medium. Cells were then fixed with 3.7% paraformaldehyde in complete cell culture medium for fifteen minutes at 37°C with 5% CO_2. Chambers were washed once with complete cell culture medium then twice with 1XPBS. Slides were then mounted with SlowFade AntiFade (Molecular Probes, Eugene, OR). Slides were analyzed with a Zeiss LSM510 confocal microscope (Zeiss, Germany) using 60X oil objective with a HeNe543 laser.

Electron Microscopy

Young and senescent WI38 fibroblasts were challenged with 10 nM OA for eighteen hours at 37°C in a 5% CO_2 incubator (NuAir, Plymouth, MN). Cells were then trypsinized using Trypsin-EDTA (0.5g Trypsin and 0.2g EDTA, 4 Na/L in Hanks Balanced Salt Solution) (Invitrogen, Grand Island, NY) and collected in medium. Cells were placed in a Hermle refrigerated centrifuge and spun at 1500

rpm for ten minutes. The medium was aspirated and cells were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for at least one hour at room temperature. Cells were then washed with 0.1M sodium cacodylate buffer three times five minutes each. Postfixation was carried out in 1% osmium tetroxide in 0.1M cacodylate buffer for one hour at room temperature in a chemical hood. Cells were then washed in 50 mM sodium maleate buffer (pH 5.2) three times five minutes each. 2% uranyl acetate in maleate buffer was used to stain cells for one hour at room temperature in the dark. Cells were washed three times five minutes each. Cells were then dehydrated five minutes with two changes of increasing amounts of ethanol 50%, 70%, 90. The final 100% ethanol wash was done three times five minutes each. Propylene oxide washes were done 4-5 times to remove plastic residue. Solution was replaced with 50% propylene oxide/50% Epon and allowed to rotate for two hours. Cells were placed in an eppendorf tube and cured overnight at 60°C. Cells were stained with toluidine blue for five minutes after which ultrathin sections of 60-90nm thick were collected and placed onto grids. Sections were allowed to dry overnight before analyzing.

Drp1/Mitochondrial Localization

Young and senescent WI38 fibroblasts were seeded onto 4-well chamber slides (Fisher Scientific, Suwanee, GA) at $1X10^4$ and $0.5X10^3$ cells/ml per chamber respectively. Twenty-four hours after seeding, cells were challenged with 10 nM OA for 18-24 hours. Cells were then fixed with 2% paraformaldehyde in 1XPBS

for twenty minutes at room temperature and permeabilized with 0.1% Triton X-100 (Fisher Scientific, Suwanee, GA). Cells were blocked with 10% BSA for 1 hour at room temperature. Drp1 antibody (BD Biosciences, Palo Alto, CA) was used at a 1:50 dilution in 10% BSA and incubated overnight at 4°C. Anti-mouse- Fluorescein isothiocyanate (FITC) secondary antibody (Amersham, Piscataway, NJ) was added at 1:200 dilution and incubated one hour at room temperature. Slides were then incubated for 15 minutes at room temperature with 75nM CMXRos. Slides were washed in 1X PBS and mounted with SlowFade AntiFade Solution with 4',6-Diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR). Slides were analyzed with a Zeiss LSM510 confocal microscope (Zeiss, Germany) using 60X oil objective. DAPI was visualized with a Diode (405-430 nm) laser and Drp1-FITC was visualized with an Argon (488 nm) laser.

Immunocytochemistry for Analysis of Bcl-2 Family Colocalization with Drp1

Young and senescent WI38 fibroblasts were seeded into 4-well chamber slides (Fisher Scientific, Suwanee, GA) at $1X10^4$ and $0.5X10^3$ cells/ml per chamber respectively. Twenty-four hours after seeding, cells were challenged with 10nM OA for 18-24 hours. Cells were then fixed using -20°C methanol for fifteen minutes, rinsed with 1X PBS and blocked for 1 hour with 10% BSA at room temperature. Bak (Epitomics, Burlingame, CA), Bad (Epitomics, Burlingame, CA) and Drp1 (BD Biosciences, Palo Alto, CA) primary antibodies were used at a 1:50 dilution in 10% BSA. Slides were incubated overnight at 4°C

with rotation. Cells were washed with 1X PBS with 0.1% Tween-20 (1X PBST) three times five minutes each. Secondary anti rabbit-FITC (Amersham, Piscataway, NJ) or anti-mouse-Cy3 (Amersham, Piscataway, NJ) antibodies were used at a 1:200 dilution in 1X PBS and incubated at room temperature for 1 hour. Cells were then washed with 1XPBST three times five minutes each and mounted with SlowFade Antifade with DAPI (Molecular Probes, Eugene, OR) solution. Slides were analyzed with a Zeiss LSM510 confocal microscope using a 60X objective with the following lasers: Diode (405-430 nm), HeNe543 (543 nm) and Argon (488 nm).

Mitochondrial Membrane Potential

The determination of mitochondrial membrane potential was performed using living cells. Young and senescent cells were seeded onto 4-well chamber slides (Fisher Scientific, Suwanee, GA) at $1X10^4$ and $0.5X10^3$ cells/ml respectively. Twenty four hours after seeding, cells were challenged with 10 nM OA or 5 µM valinomycin (Sigma, St. Louis, MO) for 0,1,3,6,18 and 24 hours. At the end of the indicated incubation time slides were removed, and fresh complete medium (DMEM:F10 with 10%FBS) containing 100 nM tetramethylrhodamine (TMRE) (Molecular Probes, Eugene, OR) and 1 µM Calcein AM (4'5'-bis (N, N-bis(carboxymethyl) **a**mino**m**ethyl) fluorescein) (Molecular Probes, Eugene, OR) were added and incubated for fifteen minutes at 37°C in a 5% CO_2 incubator. Staining medium was removed and cells were mounted in 1XPBS and analyzed by confocal microscopy. To inhibit mitochondrial membrane potential 10 µM

cyclosporine A (Sigma, St. Louis, MO) was added two hours before the addition of OA or valinomycin.

3.3 RESULTS

3.3.1 Effect of okadaic acid on mitochondrial morphology in WI38 fibroblasts

Mitochondria tend to fuse (fusion) and divide (fission) as a normal function to maintain a balanced environment within the cell (Smirnova *et al.,* 2001). It has been shown that apoptotic stimulants are capable of increasing the amount of unfused mitochondria and the rate of mitochondrial fusion decreases (Karbowski *et al.,* 2006). We sought to examine whether fusion and fission events differ in young and senescent cells challenged with 10 nM OA. Living cells were stained and fixed as described in the Materials and Methods sections. Analysis by confocal microscopy revealed that young untreated cells had elongated fibrous mitochondria (Figure 3.3A [a,b]). Young cells challenged with OA had mitochondria that were not tubular, but they formed an interconnected network taking on a "web-like" appearance (Figure 3.3A[c,d]). Untreated senescent cells had a similar mitochondrial morphology as young untreated cells in that they were tubular and elongated (Figure 3.3B [a,b]). When senescent cells were challenged with OA, the mitochondria lost their elongated morphology and shorter, rounded mitochondria were observed (Figure 3.3B [c,d]).

Electron micrographs of young and senescent fibroblasts challenged with OA confirmed our immunofluorescent results. Untreated young cells had elongated mitochondria, some were even seen undergoing fusion (Figure 3.3C). Young cells challenged with OA showed clusters of mitochondria (Figure 3.3D). Senescent cells however, offered very different results. Elongated mitochondria

were evident in untreated cells although there were unfused mitochondria can be seen (Figure 3.3E). When challenged with OA we see these mitochondria as short, rounded organelles (Figure 3.3F).

Our data suggests that okadaic acid does not have a severe affect on young cells as mitochondria do not become short and round. Bundles of mitochondrial networks are seen instead. Senescent cells lose there long tuberous appearance when challenged with OA. A significant amount of short, rounded mitochondria result due to increased fission events. We then sought to identify proteins that were involved in our mitochondrial fusion/fission observations. The protein Drp-1 has been implicated in mitochondrial fission (Smirnova *et al.,* 2001).

Drp-1 was first discovered in *C. elegans* and later in yeast (Misaka, *et al.,* 2002). It is related to the dynamin family of GTPases which are known to participate in coated vesicle formation and endocytosis (Schmid *et al.,* 1998). Drp-1 has been shown to participate in apoptosis by preventing mitochondrial fusion events (Karbowski *et al.,* 2002; Smirnova *et al.,* 2001). As others discovered a relationship between mitochondrial fission and Drp1, we sought to determine whether Drp1 was involved in our system.

3.3.2 Localization of Drp1 to mitochondria

Young and senescent cells were cultured and treated as described in the Materials and Methods sections. Colocalization of Drp1 with mitochondria was more prevalent in senescent cells challenged with OA (Figure 3.3H) than in treated young cells (Figure 3.3G). When we quantitated our findings, we found

11% and 15% of the pixels colocalized in young untreated and treated cells respectively (Figure 3.3I). Untreated senescent cells had colocalization values similar to that of young controls (10% colocalization, (Figure 3.3I). Senescent cells challenged with OA showed a 30% increase over that of senescent untreated cells (Figure 3.3I, $p<0.05$).

In the presence of OA there is an increase in unfused mitochondria particularly in senescent cells as they take on a short, rounded appearance as shown in Figure 3.3B and Figure 3.3F. These findings suggest Drp1 functions to maintain an unfused state in senescent cells. It has been shown that proteins which participate in mitochondrial fission associate with proapoptotic members of the Bcl-2 family, namely, Bak and Bax. The next experiment sought to determine whether an interaction between Drp1 and the proapoptotic Bcl-2 members Bak and Bad occurred.

3.3.3 Bcl-2 family and Drp1 colocalization

As previously mentioned the mitochondria are tightly regulated by the Bcl-2 family. Many have found this family of proteins can retard or promote apoptotic death (Yin, 2006; Adrain *et al.,* 2003; Postigo *et al.,* 2006). We saw increased unfused mitochondria as well as a significant increase in Drp1-bound mitochondria. Karbowski *et al.* (Karbowski *et al.,* 2002) have shown an interaction can be formed between Drp1 and Bax. We then set out to determine whether this occurs in WI38 fibroblasts.

We examined first the interaction between Bak and Bad, two pro-apoptotic proteins in young and senescent cells (Figure 3.3J-1, Figure 3.3J-2). When we measured colocalization between the Bak and Drp1 we found that colocalized pixels were highest in senescent cells challenged with OA (Figure 3.3K, $p<0.0005$). There was no statistical significance in Bak/Drp1 colocalization (Figure 3.3K). We then determined whether Bad colocalized with Drp1 (Figure 3.3L-1 and 3.3L-2). Although we see a higher percentage of colocalized pixels between Bad/Drp1, there was no significant between young and senescent cells Figure 3.3M ($p=0.2$ and $p=0.4$ respectively).

3.3.4 Mitochondrial Membrane Potential

Since there was increased unfused mitochondria fragmentation in senescent cells due to enhanced fission, we went on to explore mitochondrial membrane potential. One of the earliest papers describing mitochondrial membrane potential was by Hunter (Hunter *et al.,* 1975). Mitochondrial membrane transitions occurred with incubation of Ca^{2+}. The maintenance of mitochondrial membrane potential has proven necessary to maintain a balanced environment intercellular (Stavrovskaya and Kristal, 2005). We set out to determine whether membrane potential was affected in young and senescent cells challenged with OA.

Confocal microscopy showed young cells maintained their membrane potential even after a twenty-four-hour incubation with OA with fluorescent intensity at 99% (Figures 3.3P and 3.3Z). Senescent cells had a decreased membrane potential as early as 6 hours after addition of OA from 100% to 92.3%

(Figures 3.3Rand 3.3Z). Membrane potential senescent cells decreased to 89.7% after twenty-four hours in the presence of OA (Figures 3.3S and 3.3Z). Valinoymcin was used as a positive control as it rapidly decreases membrane potential (Verity *et al.,* 1981). As little as 5 μM valinomycin was able to diminished membrane potential as early as one hour in young and senescent cells (Figure 3.3N and Figure 3.3Q).

We next wanted to determine whether Cyclosporin A (CsA), a drug which potently inhibits mitochondrial pore opening would offer any protection to senescent cells in the presence of OA. As shown in Figures 3.3T-V, membrane potential in young cells was relatively lower after a one-hour incubation in the presence of CsA (compare Figures 3.3T and 3.3N). The addition of CsA may have had an initial disruptive effect on the mitochondria, but if allowed to recover, mitochondrial respiration will continue (Falchi *et al.,* 2005). This was indeed the case in our system. There was an increase in mitochondrial membrane potential after six hours in the presence of CsA (Figure 3.3U) indicating repolarization of the mitochondria. Mitochondrial membrane potential in senescent cells was robust even after treatment with OA for twenty-four hours (Figure 3.3V). The observed increase in mitochondrial membrane potential differed from senescent cells treated with OA without CsA (Figure 3.3N).

CsA did not rescue the effects of valinomycin as membrane potential decreased as early as one hour after treatment with valinomycin (Figure 3.3T and 3.3W). Valinomycin prevents pore closure by binding VDAC which is located on the outer mitochondrial membrane, allowing for rapid mitochondrial

membrane dissipation (Doran *et al.,* 2000). CsA inhibits cyclophilin D. Cyclophilin D prevents ANT closure (Woodfield *et al.,* 1998). We suspect valinomycin acts on VDAC and not ANT in WI38 fibroblasts, thus explaining the lack of protection from CsA.

3.4 DISCUSSION

The data presented here implicate the mitochondria in okadaic acid-induced apoptosis of senescent cells. We began by determining mitochondrial morphology when young and senescent cells were challenged with OA. The mitochondrial structure in young untreated cells had an elongated, tubular appearance. When young cells were challenged with OA, however, there was an increase in mitochondrial networking (Figure 3.3A). The formation of mitochondrial bundles indicate unbalanced fusion (van der Bliek, 2000). When senescent cells were challenged with OA, increased fission was observed (Figures 3.3B, 3.3F). Furthermore, examination of the mitochondrial fission protein, Drp1 showed increased binding of Drp1 to mitochondria in senescent cells challenged with OA (Figures 3.3H, 3.3I). Our observations suggest Drp1 functions to prevent fusion events in senescent cells.

The morphology of mitochondria in senescent cells change from tubular structures to more rounded short forms when challenged with OA (Figure 3.3B). Recent data implicate Bak and Bax in maintaining mitochondrial fusion (Karbowski *et al.,* 2006). This group used primary mouse embryonic fibroblasts which did not contain Bak or Bax. They noticed increased unfused mitochondria in these cells (Karbowski *et al.,* 2006). In addition, Drp1 has been shown to bind Bak and Bax allowing for increased fission (Karbowski *et al.,* 2006). When analyzed for Drp1/Bak colocalization, we see colocalization increasing from 0.6% in untreated senescent cells to 6.7% in OA-treated senescent cells (Figures 3.3J-2, 3.3K). One model to explain our results is that in senescent cells challenged

with OA, Drp1 interacts with Bak thus preventing it from functioning in mitochondrial fusion. This inhibition allows for increased unfused mitochondria.

When mitochondrial membrane potential was analyzed mitochondria in young cells was robust as indicated by the retention of TMRE (Figure 3.3N-P). The mitochondrial membrane potential in senescent cells began to decrease after six hours of treatment with OA (Figure 3.3R), and a further decrease after 24 hours (89.7% fluorescent intensity) was observed. Incubations with cyclosporin A did not offer protection as there was a no significant difference in those cells treated with OA in the presence of CsA (Figure 3.3Z). Interestingly, there was an initial decrease in mitochondrial membrane potential after young cells were incubated with OA for one hour (Figure 3.3T). This decrease, however, was not permanent because after six hours membrane potential increased (Figure 3.3U). There was no significant differences when fluorescent intensities were quantitated (Figure 3.3Z).

Our data show mitochondria are involved in the apoptotic death of senescent cells as there is increased fission. Associated with increased fission is a decrease in senescent cell membrane potential. Decreased membrane potential indicate the opening of mitochondrial pores. Our data suggests a possible manner by which AIF may be released and translocate to the nucleus.

APPENDIX: Figures and Figure Legends for Chapter 3

Figure 3.3A. Mitochondrial morphology in WI38 fibroblasts.

Young fibroblasts were challenged with 10 nM OA for 18 hours. The mitochondria were stained with 400 nM CMXRos as described in the Materials and Methods section. Panels a and b show untreated young fibroblasts. Panel b, an enlarged area of Panel a (yellow box), show elongated filamentous mitochondria. Panels c and d represent young cells treated with OA. Clustered mitochondria can be seen in Panel d an enlarged area of Panel c (yellow box). Total magnification= 1000X.

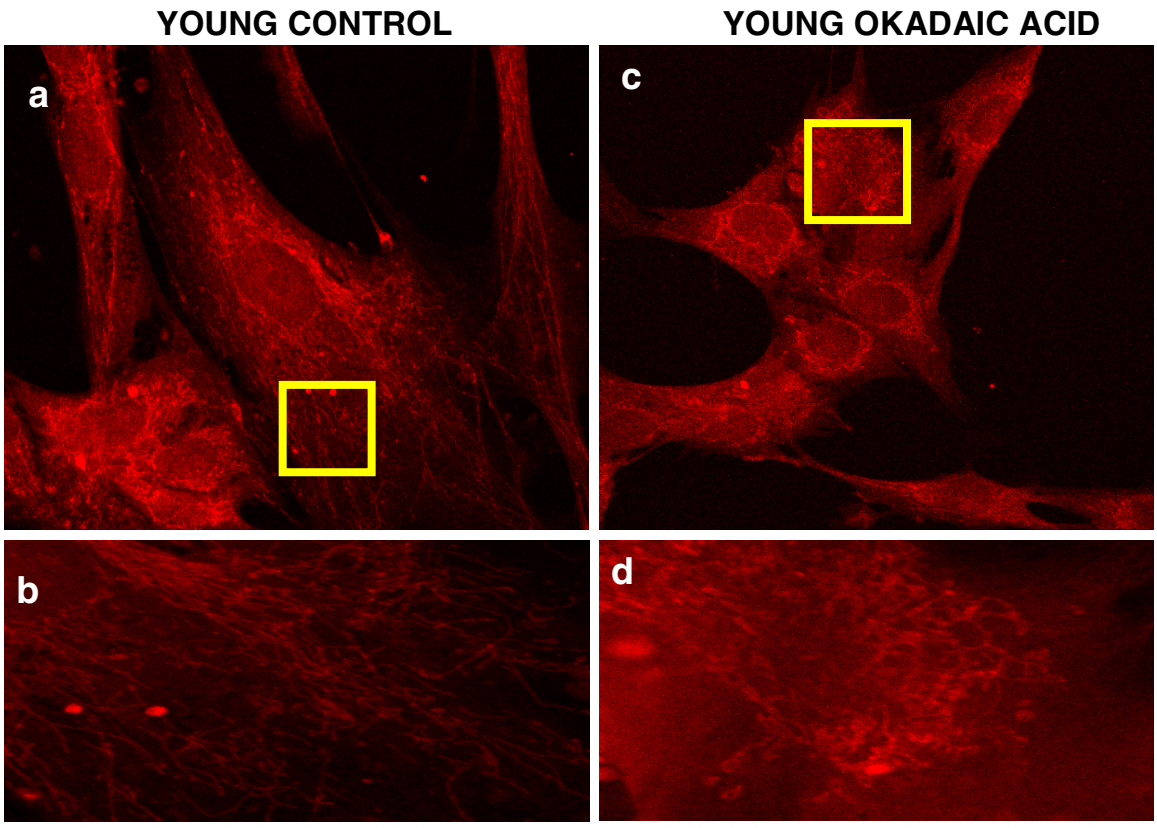


Figure 3.3B. Mitochondrial morphology in senescent WI38 fibroblasts challenged with OA.

Senescent fibroblasts were challenged with 10 nM OA for 18 hours and stained with 400 nM CMXRos as described in the Materials and Methods section. Panels a and b show untreated senescent fibroblasts with elongated mitochondria. Panel b is an enlarged area of Panel a (yellow box). Panels c and d represent senescent cells treated with OA. Panel d shows a closer inspection of mitochondria in OA-treated senescent cells. There is an abundance of unfused mitochondria in these cells. Total magnification= 1000X.

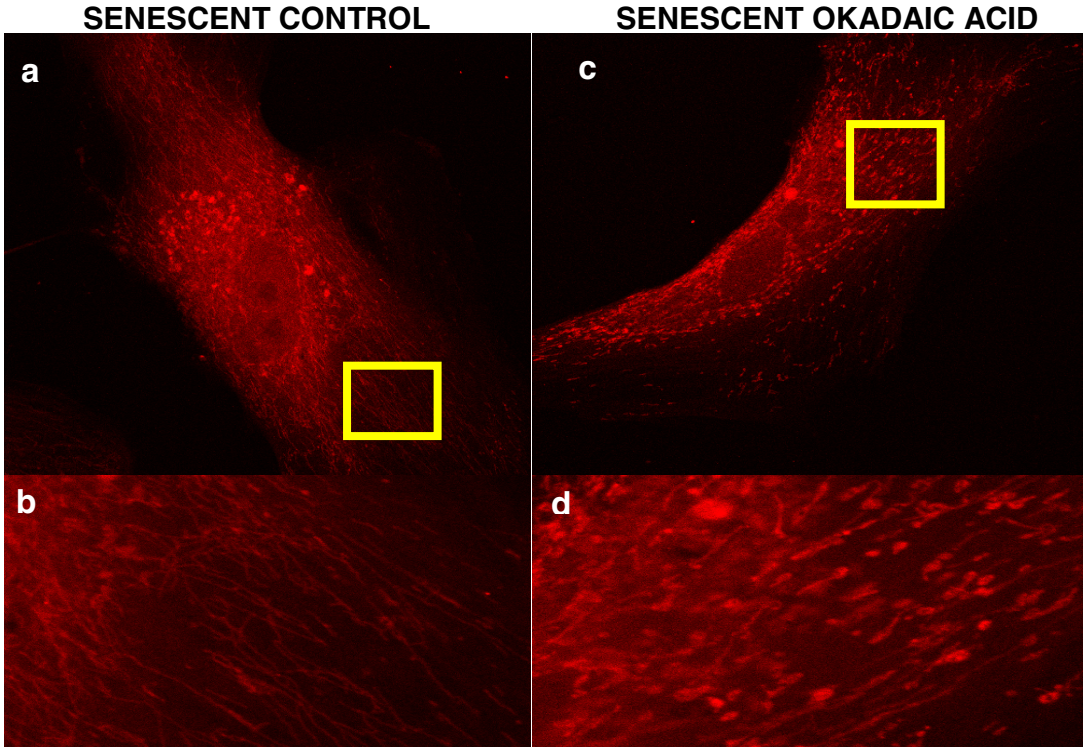


Figure 3.3C. Electron micrographs of untreated young WI38 fibroblasts.

Cells were prepared as described in the Materials and Methods section. Fused and elongated mitochondria are seen at the indicated magnifications. At 20,000X elongated mitochondria are visable at 20,000X (green arrows). Fused mitochondria are seen at 30,000X as indicated by red arrows.

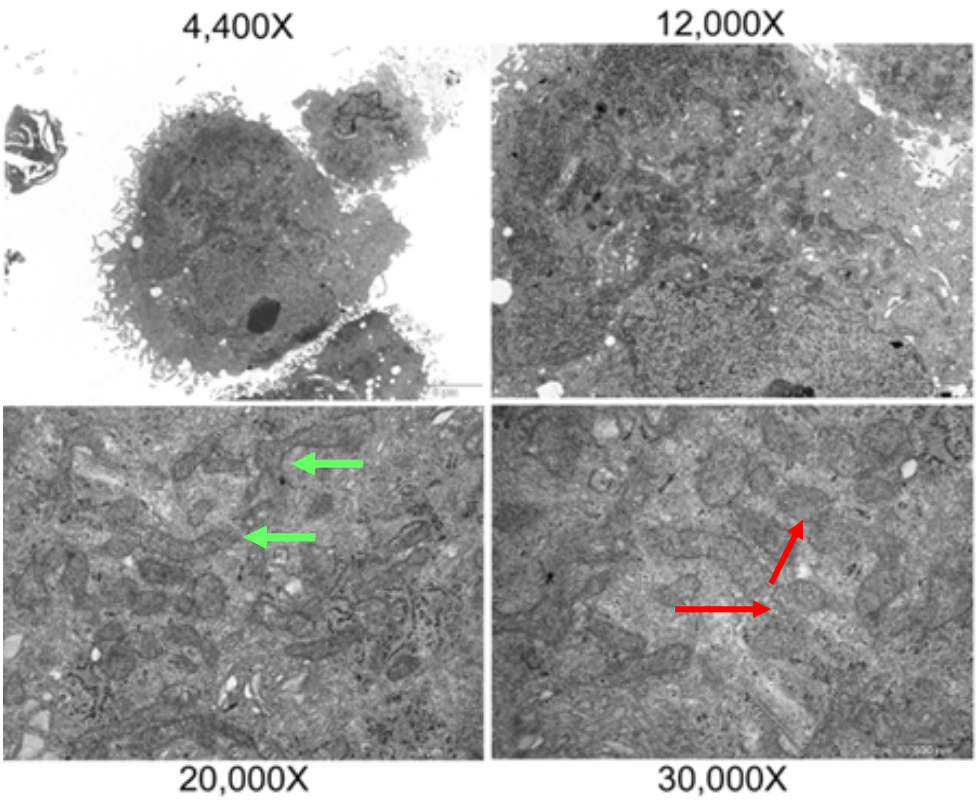


Figure 3.3D. Electron micrographs of treated young WI38 fibroblasts.

Young cells were prepared as described in the Materials and Methods sections. Images were taken at the indicated magnifications. Mitochondria of Young OA-treated fibroblasts formed clusters 4,000X and at a higher magnifications of 20,000X. Elongated mitochondria are seen at 30,000X. Shown at 85,000X is a mitochondrion undergoing fusion (green arrow).

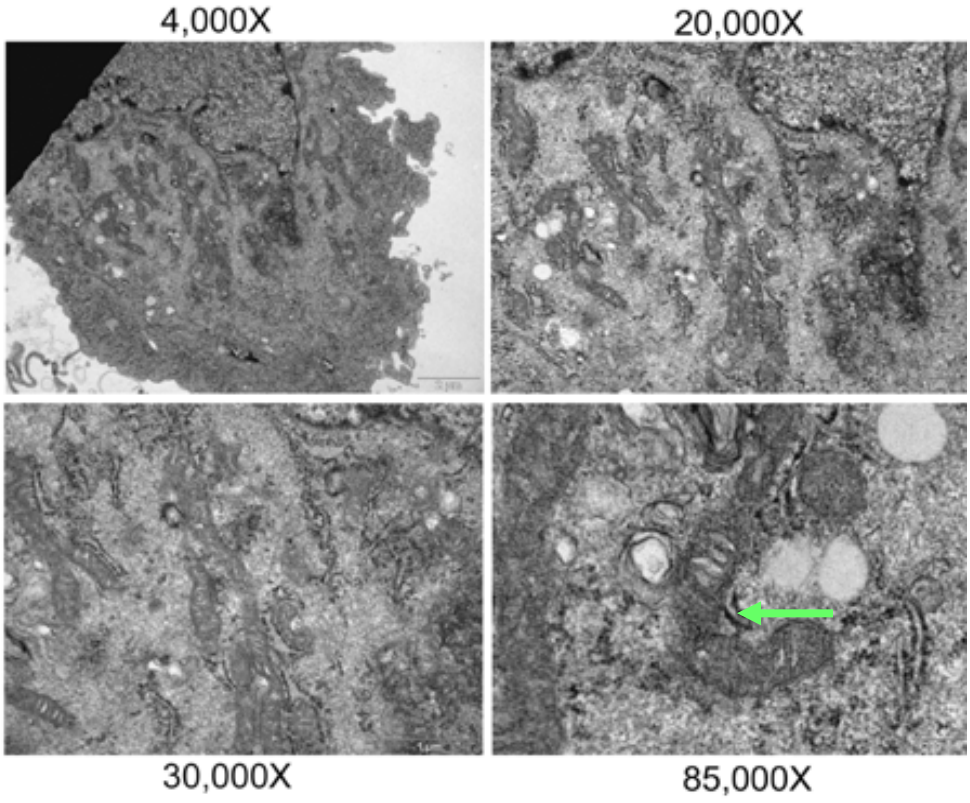


Figure 3.3E. Electron micrographs of untreated senescent cells.

Senescent cells were prepared as described in Materials and Methods. Images were taken at the indicated magnifications. At 20,000X mitochondria can be seen as elongated structures (yellow arrows). There are also unfused mitochondria present (green arrows).

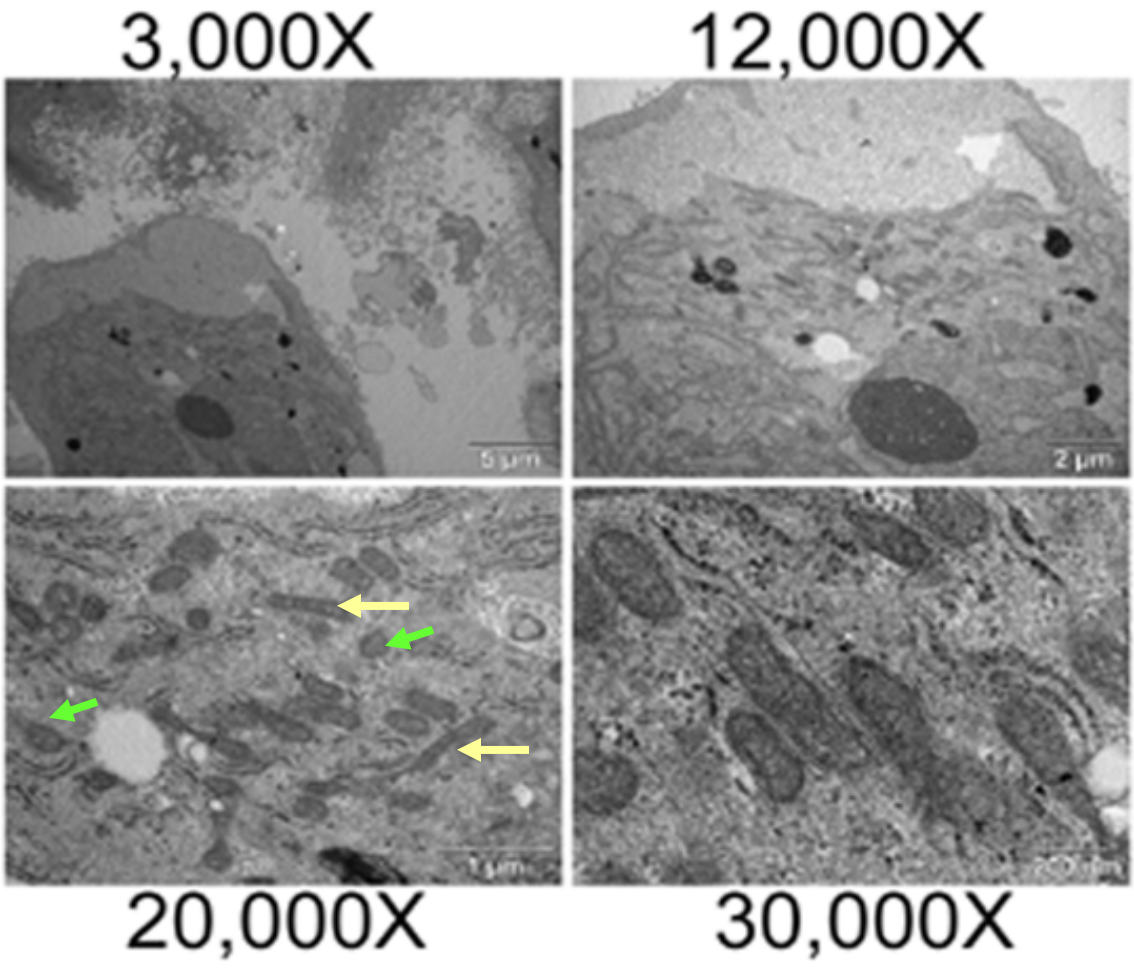


Figure 3.3F Electron micrographs of senescent cells challenged with OA.

Senescent cells were prepared as described in the Materials and Methods sections. The 20,000X images show short rounded mitochondrial structures (indicated by arrows). As shown in the 30,000X image there are fused mitochondria and elongated mitochondria present as indicated by red arrows.

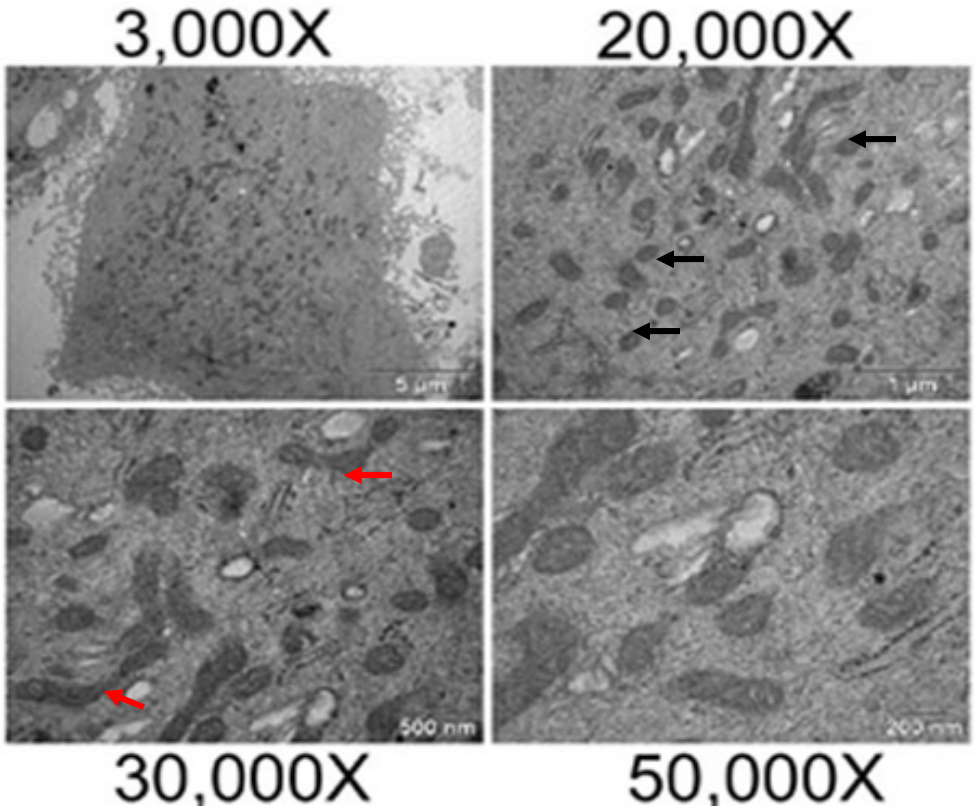


Figure 3.3G. Immunfluorescent images of Drp1 localization in young WI38 fibroblasts.

Cells were treated and stained as described in the Materials and Methods section. Challenging young cells with OA did not cause Drp1 to localize to the mitochondria. Total magnification=1000X.

BF=Bright Field; DAPI=nuclei; CMXRos=mitochondria. FITC only represent cells stained with anti-mouse FITC antibody only.

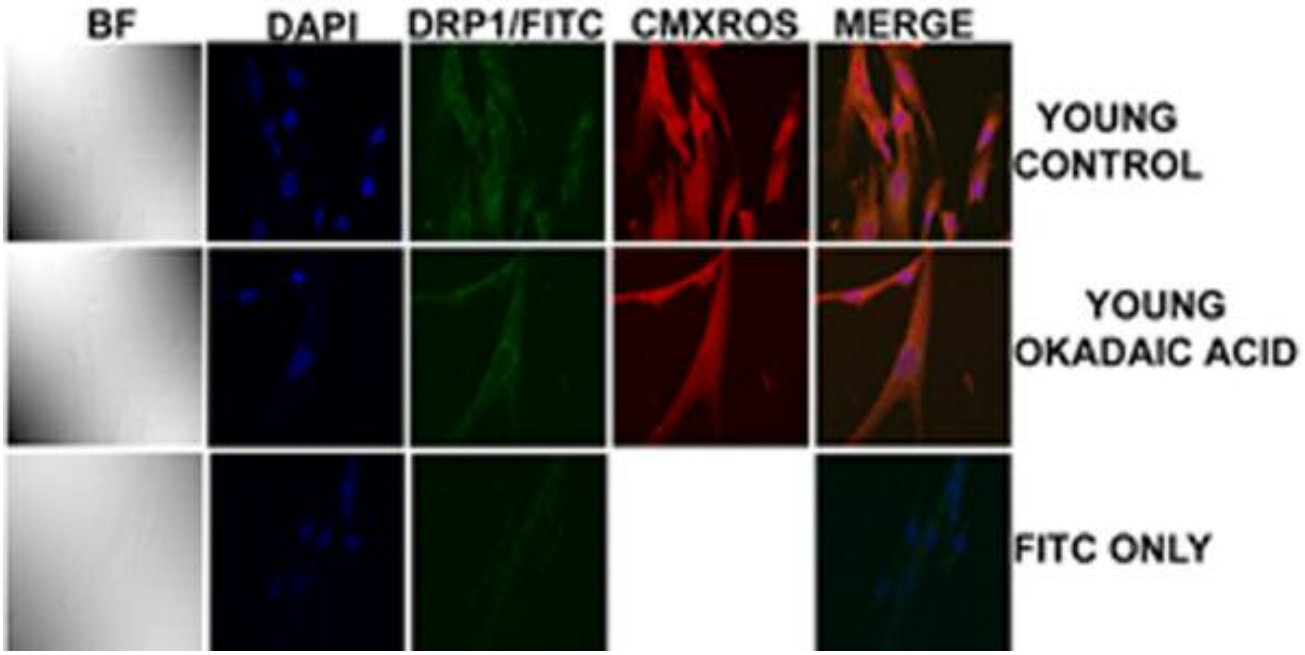


Figure 3.3H. Immunofluorscent images of Drp1 localization in senescent WI38 fibroblasts.

Cells were challenged and stained as described in the Materials and Methods section. Drp1 and the mitochondria colocalize as seen in the overlay (Drp1/FITC and CMXRos). Total magnification=1000X.

BF=Bright Field; DAPI=nuclei; CMXRos=mitochondria. FITC only represent cells stained with anti-mouse FITC antibody only.

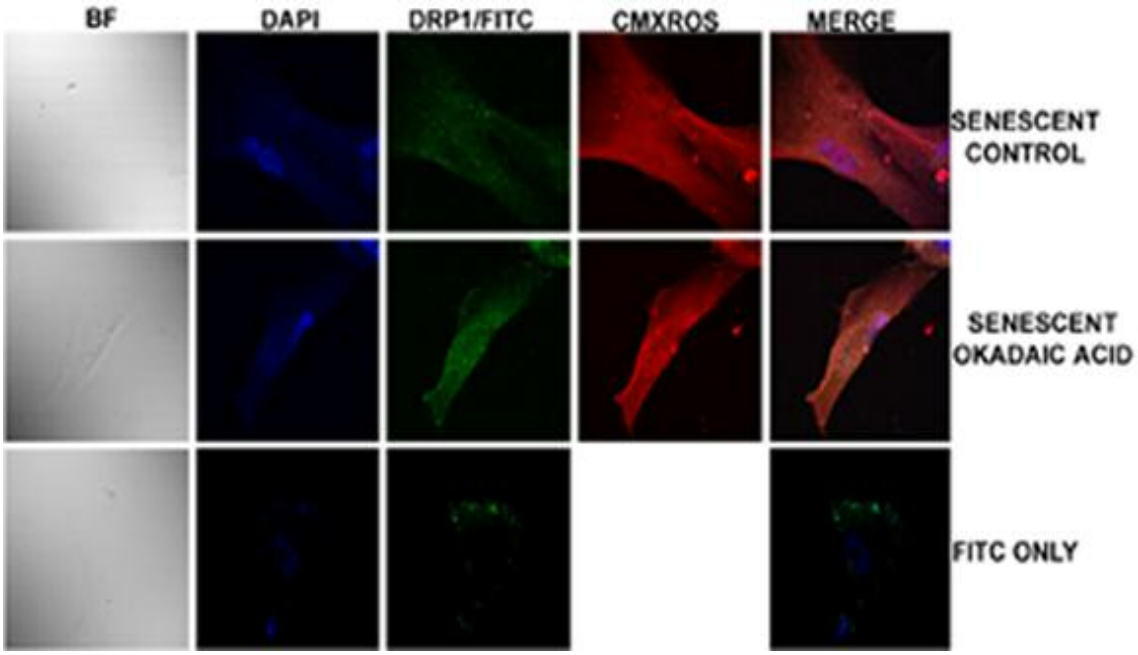


Figure 3.3I. Quantitation of Drp1 and mitochondria colocalized pixels.

Percent colocalized pixels were determined from the immunofluorescent images (Figures 3.3G-H). A comparison between young control and OA-treated cells revealed 11% and 15% colocalized pixels respectively. A comparison between senescent control and OA-treated showed 10% and 30% colocalized pixels respectively. The difference in Drp1/mitochondria Colocalization between senescent control and treated was significant as $p<0.05$.

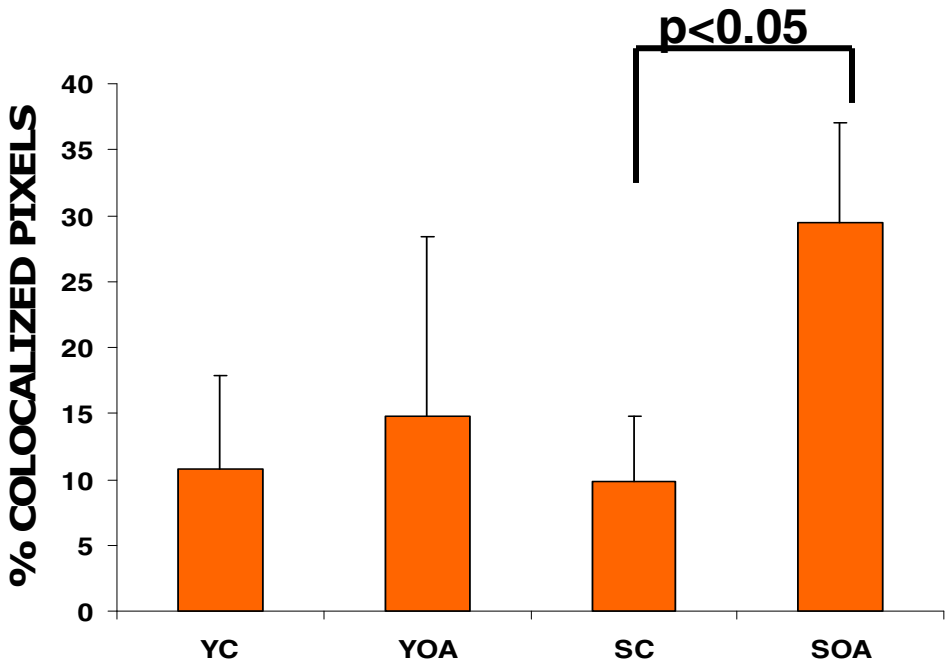


Figure 3.3J1-2. Immunofluorescent image of young and senescent WI38 fibroblasts and localization of Bak and Drp1.

Young and senescent WI38 fibroblasts were challenged and stained as described in the Materials and Methods section. Analysis by confocal microscopy revealed little colocalization between Bak and Drp1 in young cells, control and treated (Figure 3.3I-1). When senescent cells were analyzed, there was strong colocalized signal when the FITC and Cy-3(CMXRos) channels were merged (Figure 3.3I-2). Total magnification=1000X.

BF=Bright Field image. DAPI was used to indicate nuclei. FITC/Cy-3 only represents cells stained with anti-rabbit-FITC only and anti-mouse-Cy3 only.

Figure 3.3J-1. Immunofluorescent image of Bak/Drp1 localization in young WI38 fibroblats.

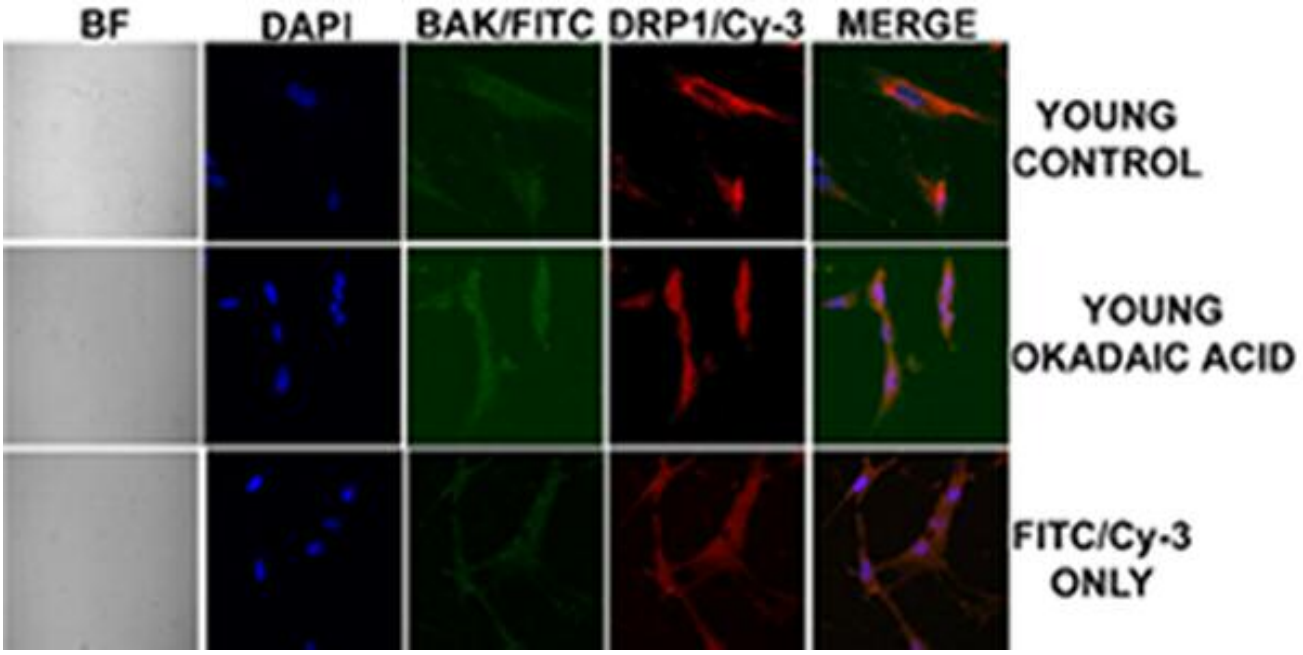


Figure 3.3J-2. Immunofluorescent image of Bak/Drp Colocalization in senescent cells.

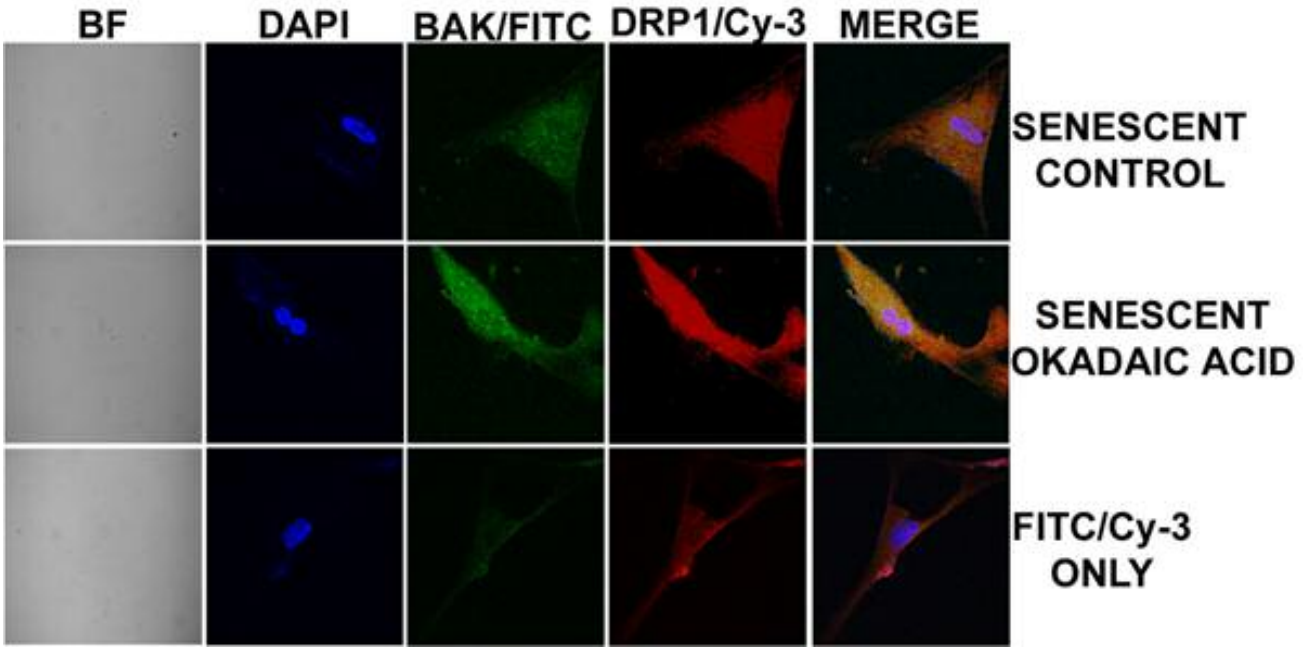


Figure 3.3K. Quantitation of Bak and Drp1 colocalized pixels.

The fluorescent images in Figures 3.3J-1, 3.3J-2 were quantitated and the percent colocalized pixels are represented in the graph below. There was no significant difference between Bak/Drp1 colocalization in young control and OA-treated cells. When senescent cells were compared, greater colocalization was observed when senescent control and OA-treated cells were compared. This was significant as p<0.0005.

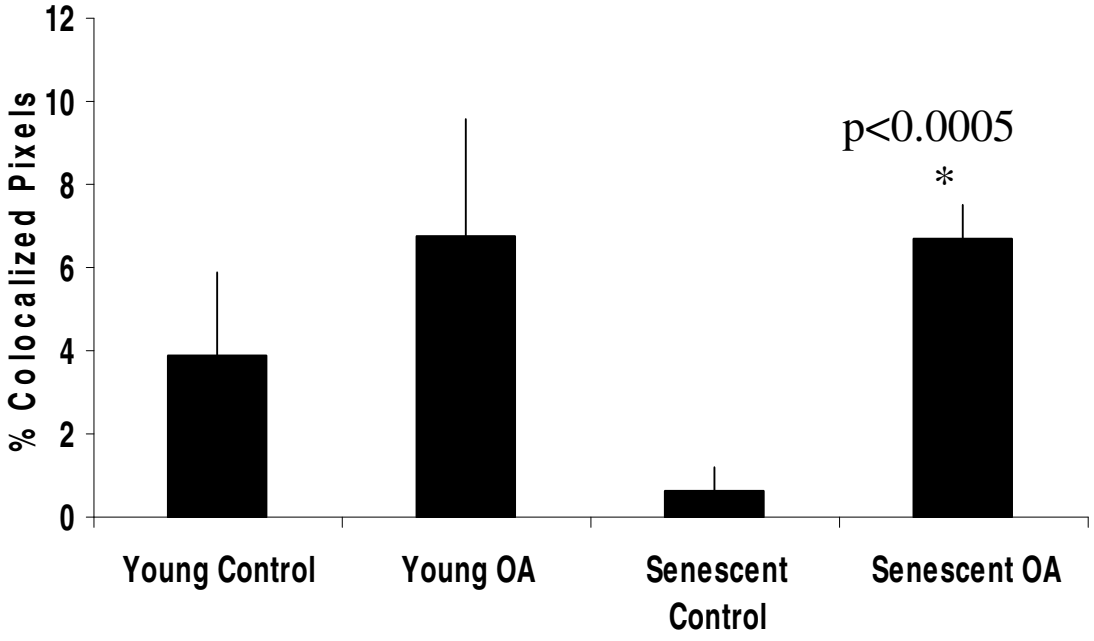


Figure 3.3L1-2. Immunofluorescent image of young and senescent WI38 fibroblasts and localization of Bad and Drp1.

Young and senescent WI38 fibroblasts were challenged and stained as described in the Materials and Methods section. Analysis by confocal microscopy revealed colocalization between Bad and Drp1 in young cells challenged with OA as seen in the Merge (Figure 3.3I-1). When senescent cells were analyzed, colocalization was also evident in OA-treated cells as seen in the Merge (Figure 3.3I-2). Total magnification=1000X.

BF=Bright Field image. DAPI was used to indicate nuclei. FITC/Cy-3 only represents cells stained with anti-rabbit-FITC only and anti-mouse-Cy3 only.

Figure 3.3L-1. Bad and Drp1 colocalization in young cells challenged with OA.

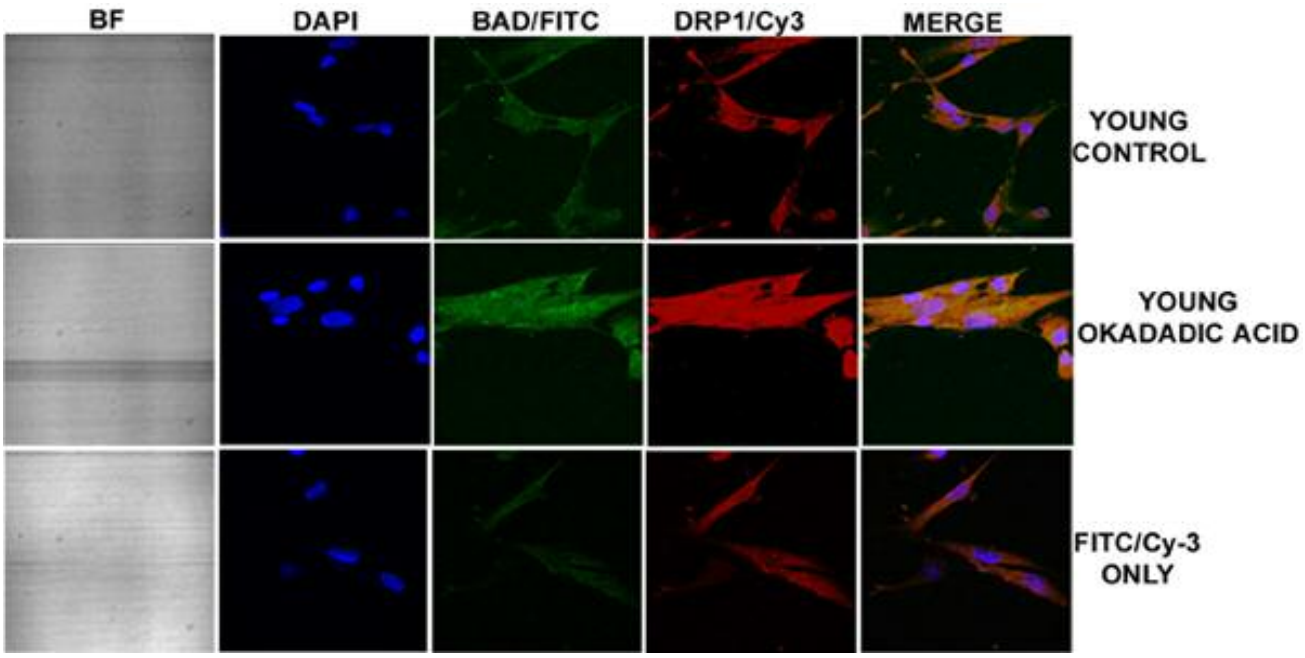


Figure 3.3L-2. Drp1 and Bad localization after treatment with 10 nM OA.

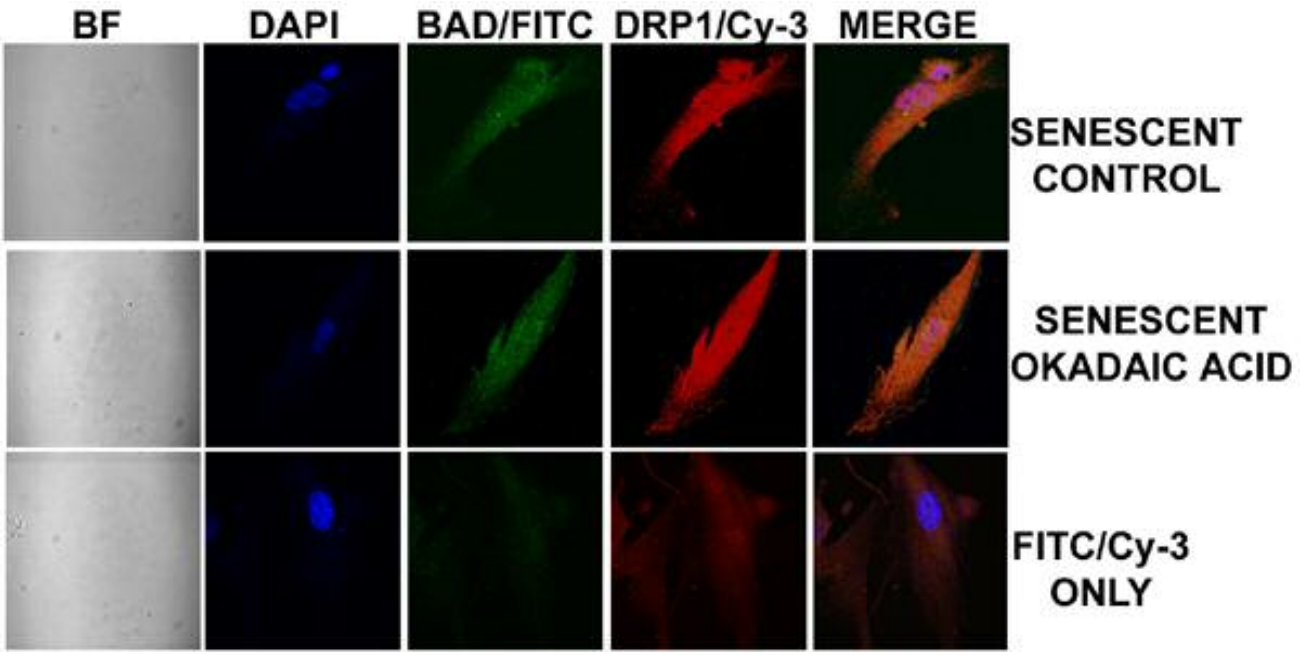


Figure 3.3M. Quantitation of colocalized Bad and Drp1 pixels.

Colocalized pixels were determined from the fluorescent images in Figure 3.3G-1 and 3.3G-2. There was no significant difference between young control and OA-treated or senescent control and OA-treated.

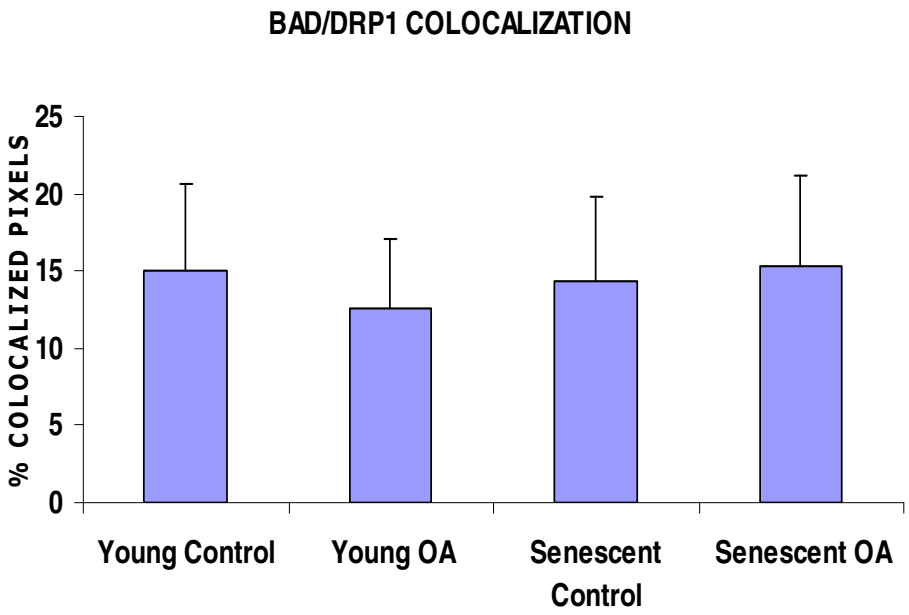


Figure 3.3N. Young WI38 fibroblasts were challenged with OA for 1 hour.

Living cells were stained and analyzed by confocal microscopy. Mitochondrial membrane potential was high as can be seen by the strong TMRE signal. Calcein AM remains cytoplasmic an indication that mitochondria are actively respiring. Mitochondrial membrane potential rapidly decreased in the presence of valinomycin which functions to keep mitochondrial pores open. Total magnification=1000X.

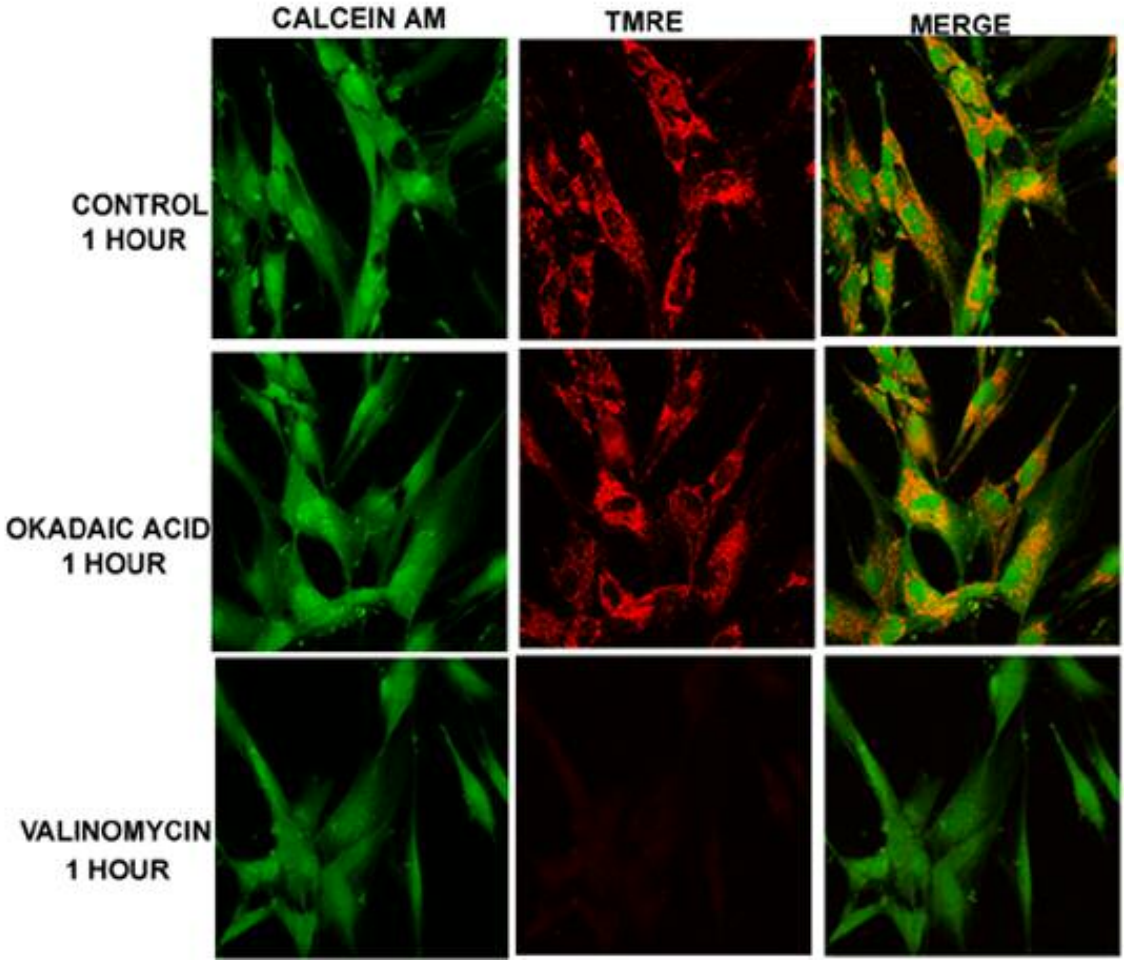


Figure 3.3O. Mitochondrial membrane potential in young cells challenged with OA for six hours.

Cells were stained and analyzed as described in the Materials and Methods sections. After 6 hours membrane potential remained relatively high. Total magnification=1000X.

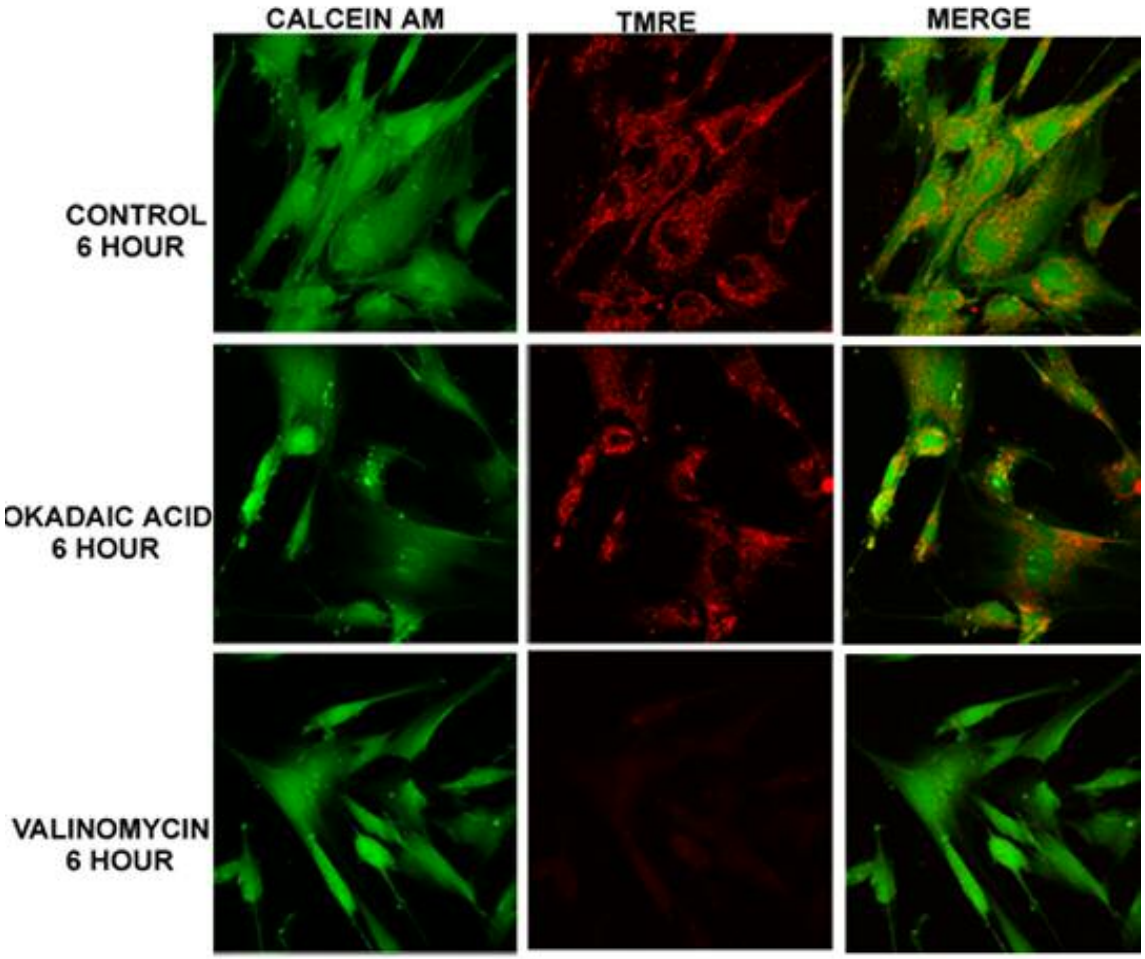


Figure 3.3P. Mitochondrial membrane potential in young cells after treatment with OA for 24 hours.

After twenty-four hours the mitochondrial membrane potential decreased when compared to treatment after 1 hour (Figure 3.3N). Total magnification=1000X.

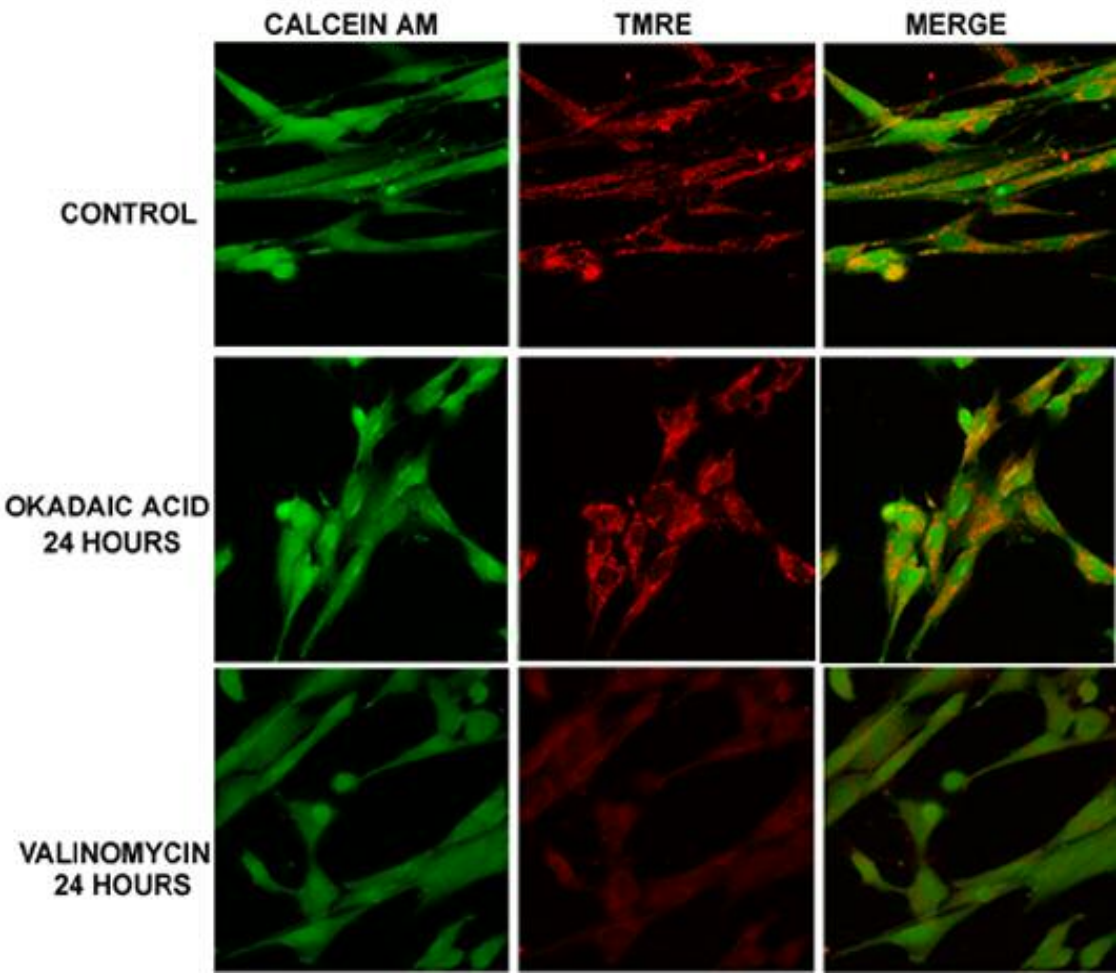


Figure 3.3Q. Mitochondrial membrane potential in senescent cells after one hour of treatment with OA.

Cells were treated and stained as described in the Materials and Methods sections. Membrane potential was lower than that of young cells after one-hour treatment with OA. Valinomycin completely dissipated membrane potential after one hour. Total magnification=1000X.

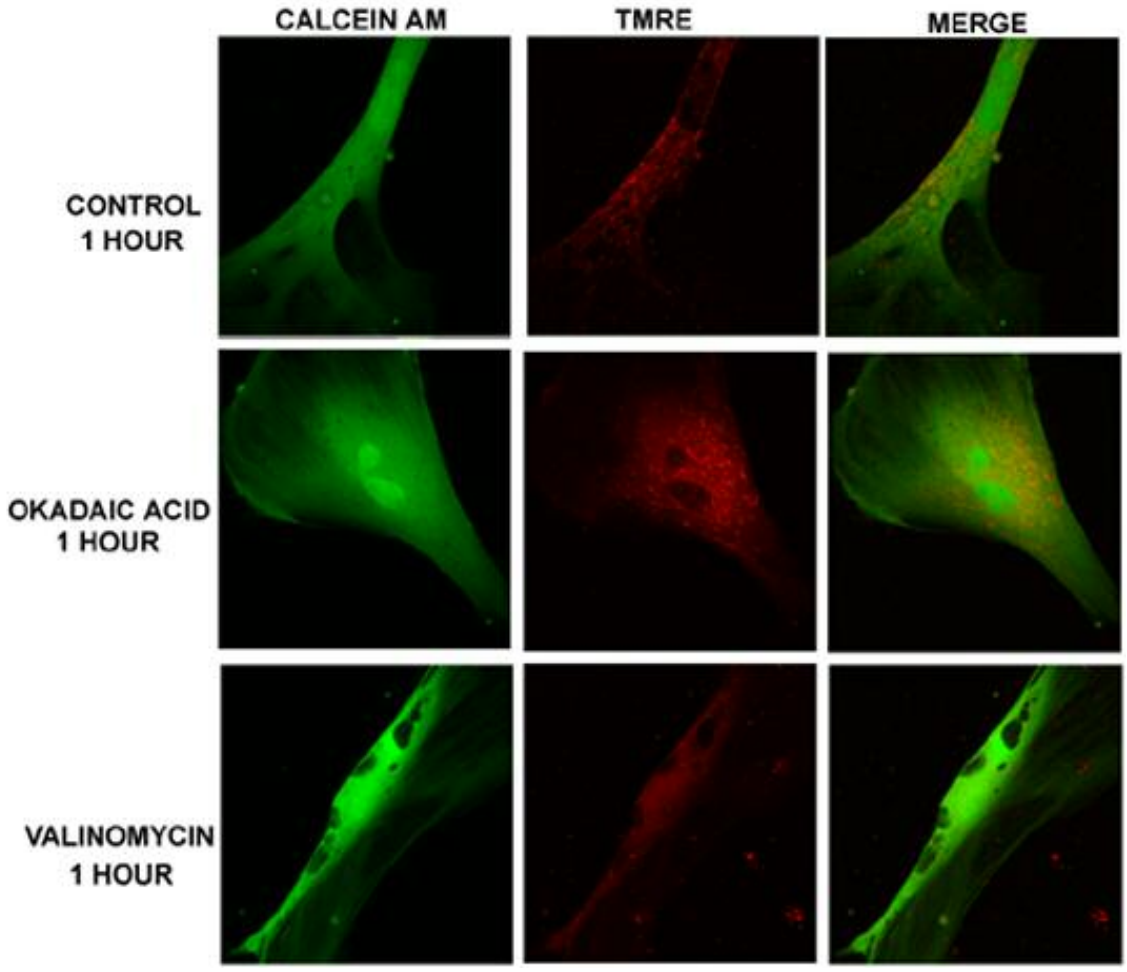


Figure 3.3R. Mitochondrial membrane potential in senescent cells after six hours treatment with OA.

Live cells were stained as described in the Materials and Methods section. After six hours, there is a decrease in membrane potential when compared to the one hour OA treatment. Calcien AM remained cytoplasmic. Total magnification=1000X.

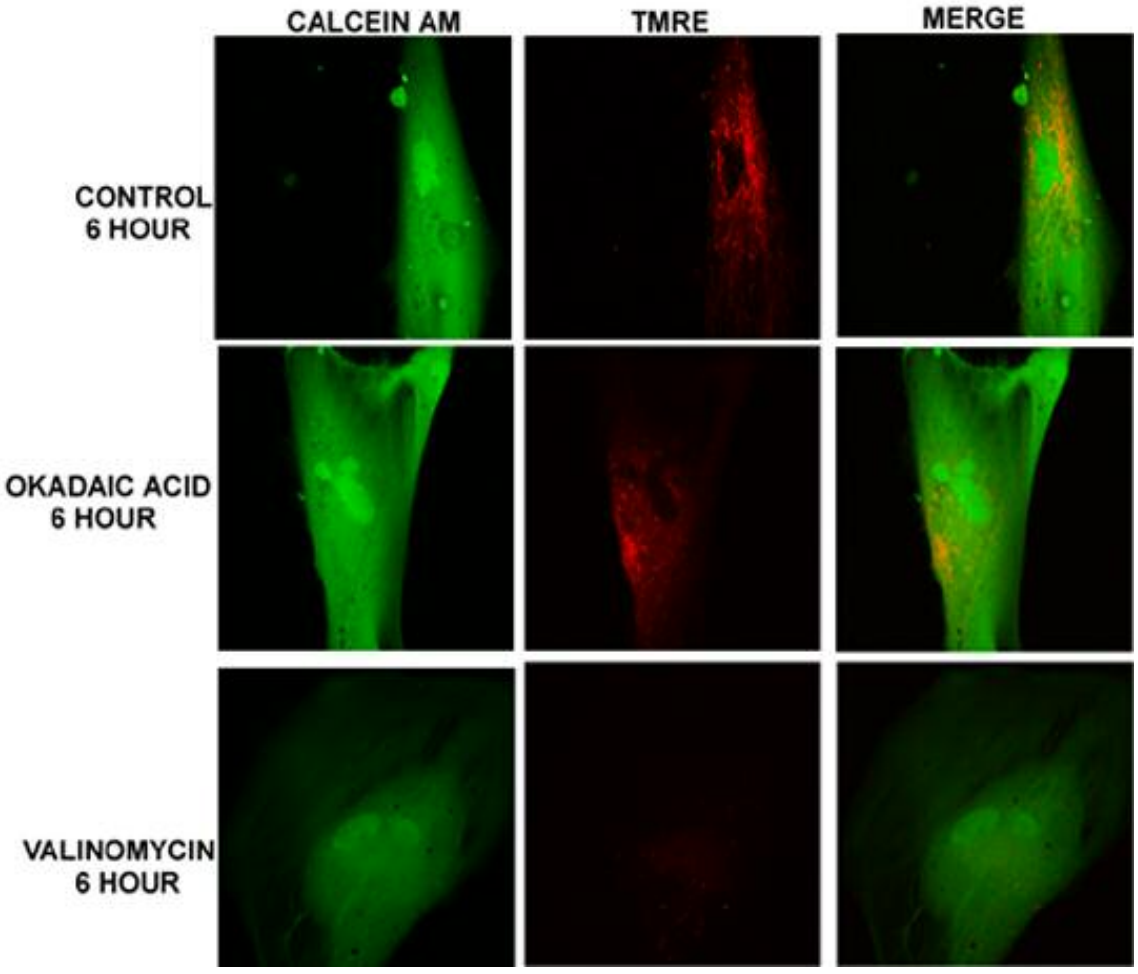


Figure 3.3S. Determination of mitochondrial membrane potential after challenging senescent cells with OA for 24 hours.

Cells were treated and stained as described in the Materials and Methods sections. Incubation with 10 nM OA for twenty-four hours resulted in a dramatic decrease in membrane potential compared to untreated samples. Calcein AM remained cytoplasmic. Total magnification=1000X.

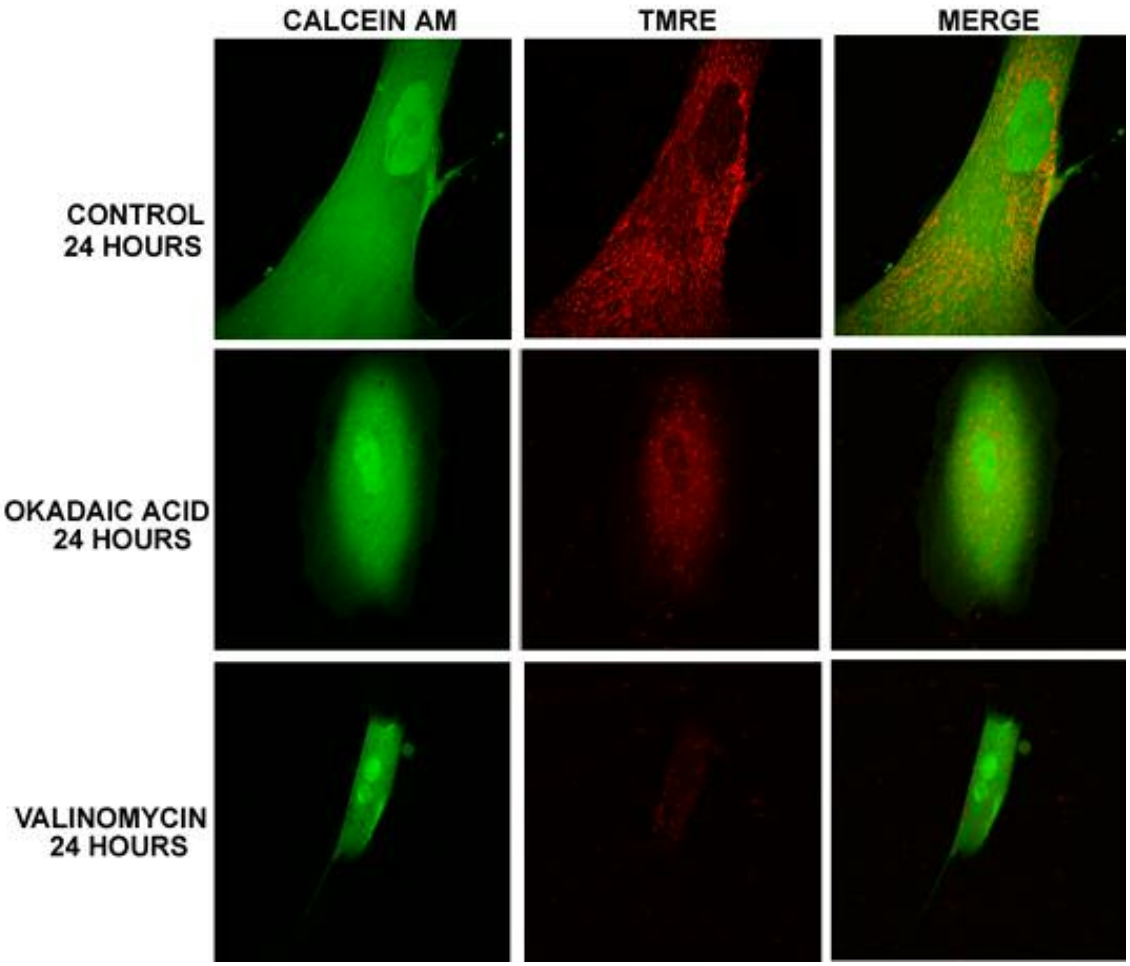


Figure 3.3T. Mitochondrial membrane potential in young cells challenged with OA for one hour in the presence of Cyclosporin A (CsA).

CsA (10 μM) was added to young cultures 2 hours before the addition of OA. Live cells were analyzed by confocal microscopy. Membrane potential was lowered after one hour treatment with OA in the presence of CsA. Total magnification=1000X.

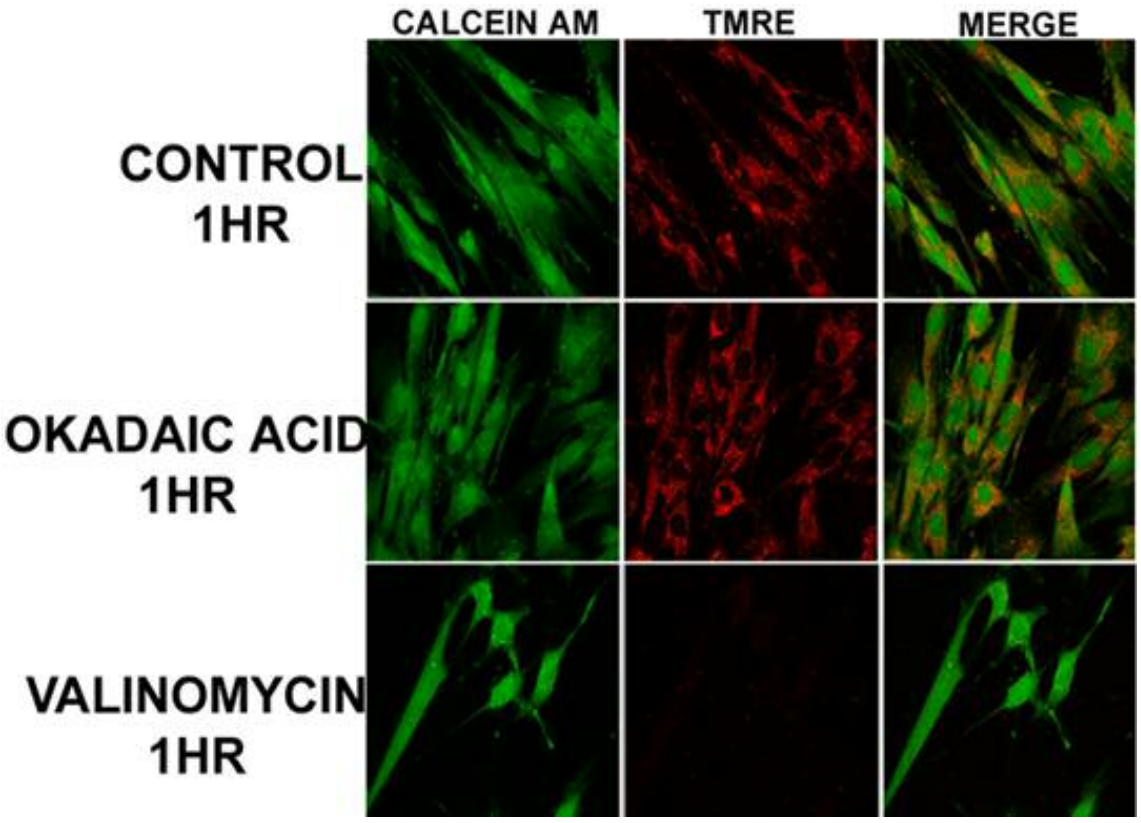


Figure 3.3U. Mitochondrial membrane potential in young cells challenged with OA for six hours in the presence of Cyclosporin A (CsA).

CsA (10 μM) was added to young cultures 2 hours before the addition of OA. Live cells were analyzed by confocal microscopy. Mitochondria seem to have recovered and respiration increased with six hours as indicated by increased TMRE signal. Total magnification=1000X.

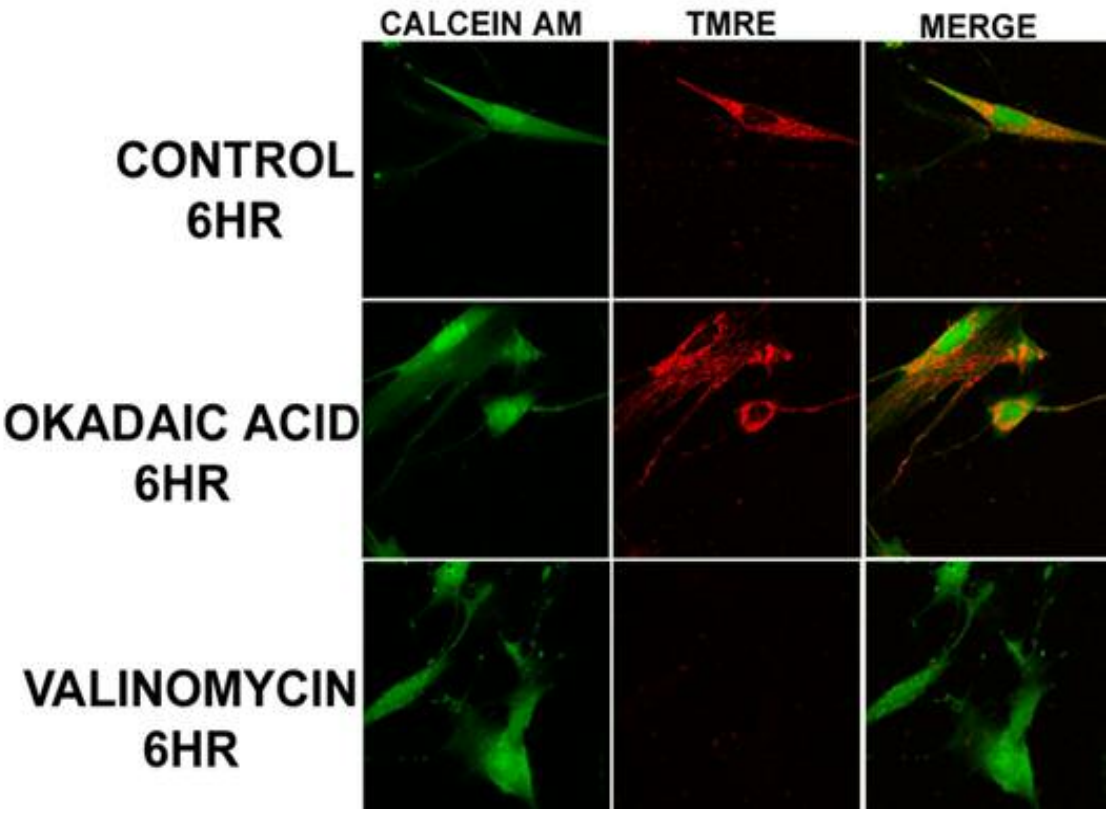


Figure 3.3V. Mitochondrial membrane potential in young cells challenged with OA for twenty-four hours in the presence of Cyclosporin A (CsA).

CsA (10 μM) was added to young cultures 2 hours before the addition of OA. Live cells were analyzed by confocal microscopy. Membrane potential was unchanged in young cells (compare with Figure 3.3P). Total magnification=1000X.

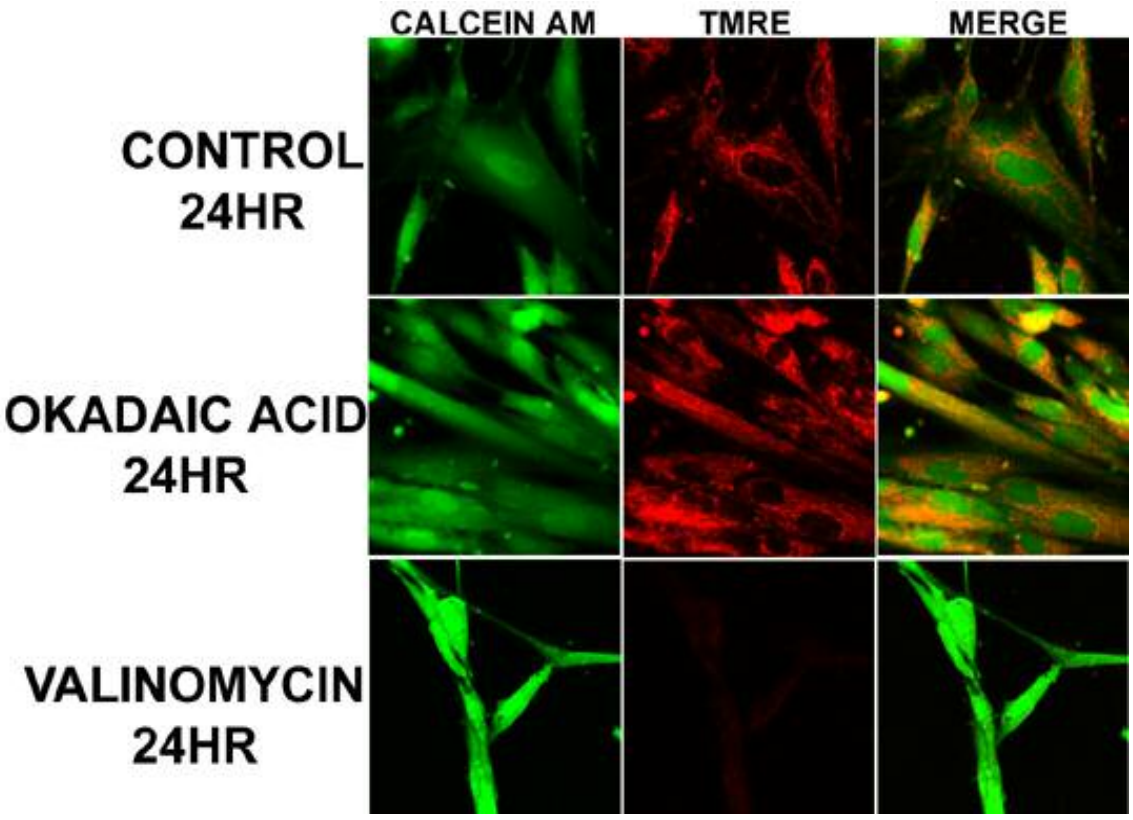


Figure 3.3W. Mitochondrial membrane potential in senescent cells challenged with OA for one hour in the presence of Cyclosporin A (CsA).

CsA (10 μM) was added to young cultures 2 hours before the addition of OA. Live cells were analyzed by confocal microscopy. One hour after the addition of OA TMRE signal was similar to the control. Total magnification=1000X.

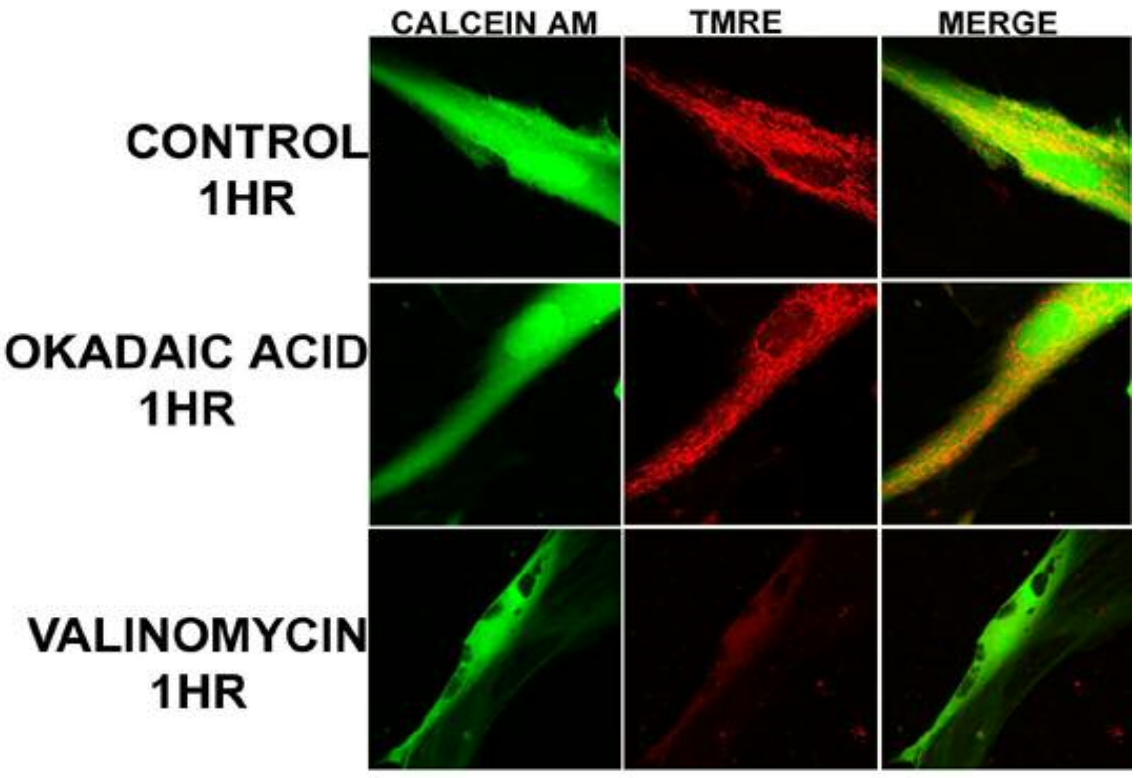


Figure 3.3X. Mitochondrial membrane potential in young cells challenged with OA for six hours in the presence of Cyclosporin A (CsA).

CsA (10 μM) was added to young cultures 2 hours before the addition of OA. Live cells were analyzed by confocal microscopy. After six hours TMRE signal show membrane potential similar to that of six-hour control. This is unlike cells challenged with OA in the absence of CsA (Figure 3.3R). Total magnification=1000X.

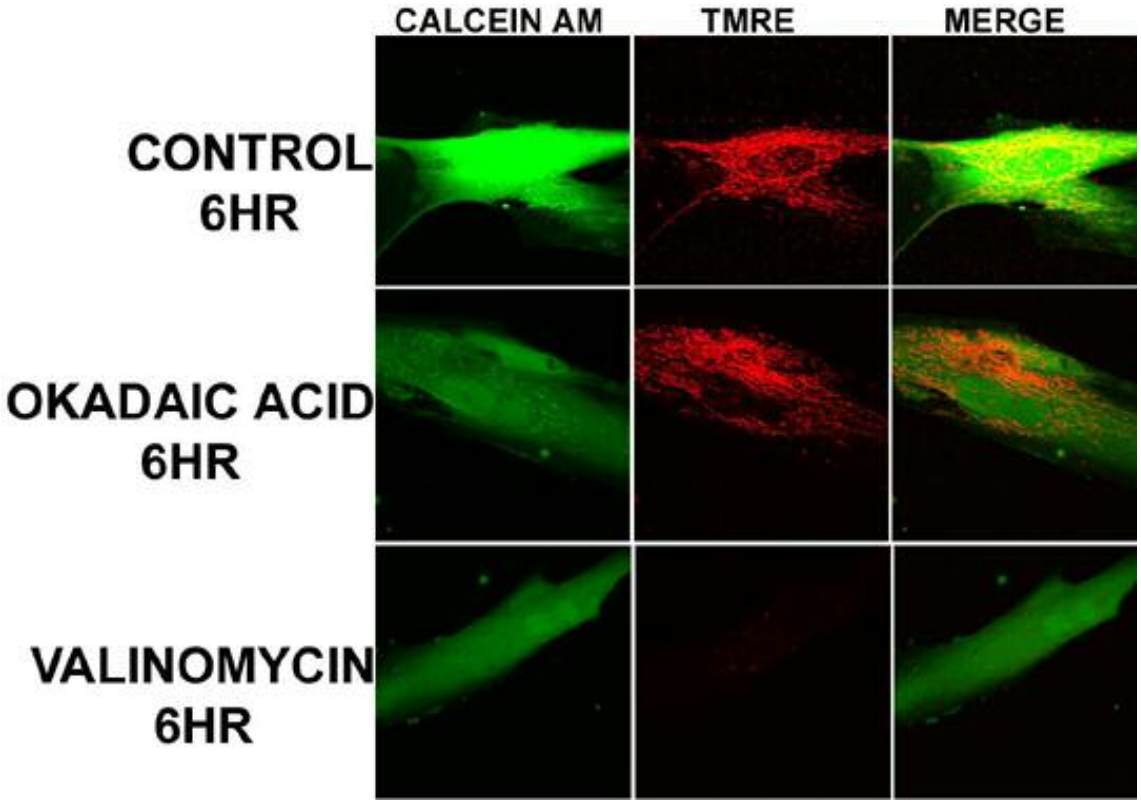


Figure 3.3Y. Mitochondrial membrane potential in young cells challenged with OA for twenty-four hours in the presence of Cyclosporin A (CsA).

CsA (10 μM) was added to young cultures 2 hours before the addition of OA. Live cells were analyzed by confocal microscopy. Membrane potential remained elevated even after twenty-four hour incubation with OA (compare Figure 3.3S). Total magnification=1000X.

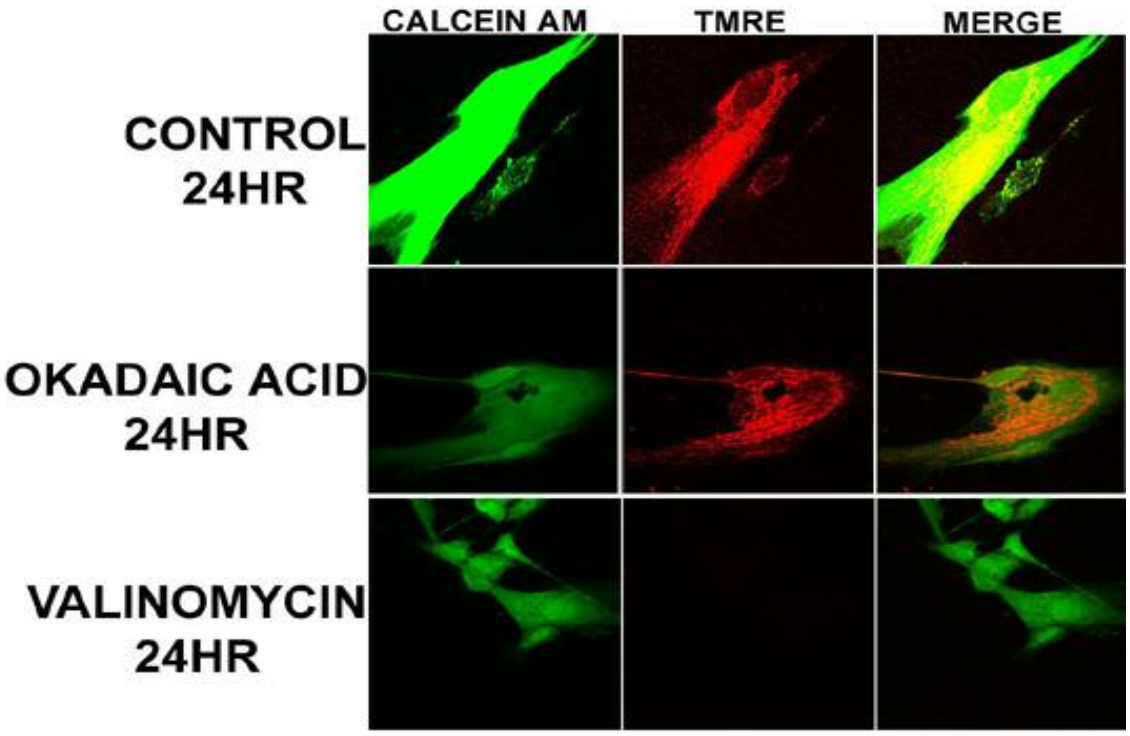
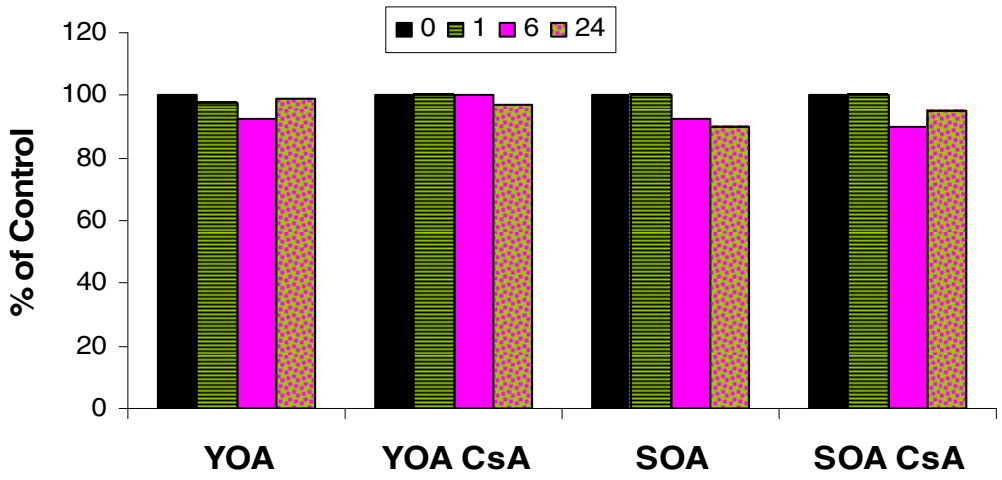


Figure 3.3Z. Quantitation of mitochondrial membrane potential.

Fluorescent intensities of mitochondrial membrane potential were quantitated using the respective controls. In the presence of Cyclosporin A (CsA) there was an increase in mitochondrial membrane potential in senescent cells challenged with OA. Measuring fluorescent intensity in OA-treated samples was 89.7% after 24-hour treatment. When CsA was present mitochondrial membrane potential increased to 95.3%. This difference however was not no significant. Data was obtained from three independent experiments.



SUMMARY AND CONLUSIONS

Apoptosis provides a stable environment throughout the life of an organism (Ameisen, 2002). Genes involved in apoptotic death are programmed to turn on or off when necessary (Daniel and Korsmeyer, 2004). Proper regulation of apoptosis therefore is necessary for the health of the organism. Aberrant expression of proteins involved in the apoptotic pathway can lead to disease such as cancer, cardiovascular diseases, diabetes and hearing loss (Bahi *et al.,* 2006; Chang *et al.,* 2006; Lee and Pervaiz, 2006). Neurological diseases such as Alzheimer's and Parkinson's emerge when neuronal cell loss occurs prematurely (Engidawork *et al.,* 2001). Reports show upregulation of caspase-8 and Fas receptors on neuronal cell surface, which lead to premature neuronal loss (Engidawork *et al.,* 2001). In addition, groups have found autophagic cell death play a role in neurological disease. Researchers show toxic proteins such as β-amyloid and α-synuclein are not properly phagocytosed leading to an accumulation of these proteins in Alzheimer's and Parkinson's patients respectively (Martinez-Vicente *et al.,* 2005). Many of the diseases mentioned affect aged individuals.

Researchers have used various systems to study aging *in vivo* (Ellis and Horvitz, 1986; Kamer *et al.,* 2005). The use of human diploid fibroblasts to study aging *in vitro* has provided a wealth of information to the aging community (Brooks-Frederich *et al.,* 1993; Venable *et al.,* 1995; DeJesus *et al.,* 2002; Gerhard *et al.,* 2002). Human diploid fibroblasts are unique because they stop dividing after a 60 $\pm$ 10 population doublings having permanently exited the cell

cycle (Hayflick, 1991). The term used to describe this state is cellular senescence. Associated with the senescent phenotype are changes in transcriptional regulation, increased DNA damage, mRNA and protein levels (Han *et al.,* 2001). As there are changes in DNA, RNA and protein synthesis, disruption of the mitochondria is also evident.

Reactive oxygen species (ROS) increase with age. This increase affects cellular respiration and ATP production (Yasuda *et al.,* 2006). A recent report using *C. elegans* has shown ROS generation is a cumulative effect (Yasuda *et al.,* 2006). ROS production occurs early in life but defenses (free radical scavengers like superoxide dismutase) are present and will act to relieve the stress. As the organism ages, however, there is a dramatic decrease in free radical scavengers and ROS increase (Yasuda *et al.,* 2006). The abundant ROS generated eventually becomes harmful to the organism such that lifespan shortens and decreases mitochondrial membrane potential (Yasuda *et al.,* 2006). Residing in the mitochondrion are proteins that participate in mitochondria-induced apoptosis (Harris and Thompson, 2000).

Cytochrome *c*, AIF, EndoG, and Smac/DIABLO all reside in the intermembrane space (Harris and Thompson, 2000). While cytochrome *c* promotes a caspase-dependent apoptosis, AIF and EndoG can induce apoptosis in a caspase-independent manner (Green, 2000). Smac/DIABLO will bind to and inhibit the Inhibitors of Apoptosis Protein (IAP) family thereby promoting apoptosis (Verhagen, 2000). As cells age/senesce an alteration in apoptotic proteins occurs such that apoptosis may or may not take place (Wang *et al.,*

2004). Our previous data reported senescent cells were capable of undergoing an apoptotic death, but the death was inducer-specific (Dejesus *et al.,* 2006). Treatment with 10 nM okadaic acid offered 85% fragmented DNA as measured by the TUNEL assay (DeJesus *et al.,* 2002). This work sought to determine the mechanism of apoptosis in senescent human diploid fibroblasts when challenged with 10 nM okadaic acid.

In Chapter 1, we wanted to determine whether caspases participated in our system. Western analysis revealed caspases are not involved in senescent cell death (Figure 1.3A-E). Although caspase activity was not evident in senescent cells a caspase-3 substrate, DNA Fragmentation Factor 45 (DFF45) levels decreased when senescent cells were challenged with OA. We found that caspases were not involved, we examined proteins that could elicit an apoptotic death in a caspase-independent manner.

Chapter 2 focused on determining the role of Apoptosis Inducing Factor, (AIF) and Endonuclease G (EndoG) in young and senescent fibroblasts. Immunofluorescent data revealed the release of AIF but not EndoG from the mitochondria to the nucleus (Figure 2.3C-J). In addition, significant difference was observed in this translocation event between young OA-treated and senescent OA-treated cells as the Student's two-tailed t-test revealed $p<0.05$ (Figure 2.3J-2). We furthered our studies by overexpressing AIF with an AIFGFP fusion plasmid. Immunofluorescent data indicate AIF translocates to the nucleus when challenged with OA (Figure 2.3L). In cells that overexpress AIF we found that the DFF45 protein decreased (Figure 2.3M).

DFF45 acts to inhibit the nuclease activity of DFF40. In light of the fact that DFF45 levels decreased, we wanted to determine whether DFF40 played a role in young and senescent WI38 fibroblasts. Surprisingly, we did not detect DFF40 by Western analysis in young nor senescent fibroblasts either untreated or treated (Figure 2.3N).

Taken together our data from Chapters 1 and 2 suggest senescent WI38 fibroblasts undergo a caspase-independent death. AIF may be involved in cell death, as we found that it translocated from the mitochondria to the nucleus. In addition, DFF45 was decreased in young and senescent fibroblasts as well as in cells that overexpressed AIF. We thought initially DFF40 was the nuclease responsible for introducing DNA-strand breaks, but DFF40 was not present in neither young nor senescent cells. Researchers have found a homologue to mammalian AIF in *C. elegans*, WAH-1 (Wang *et al.,* 2002). WAH-1 translocates the nucleus where it interacts with CPS-6 the homologue of mammalian EndoG to promote DNA degradation (Wang *et al.,* 2002). A small fraction of EndoG colocalizes with the nucleus in senescent cells (Figure 2.3E2). It would be interesting to determine whether there is an interaction.

In chapter 3, we wanted to determine the role of mitochondria in our system. We found that OA induced mitochondrial morphological changes in young cells. A transition from elongated structures to bundle-like structures was apparent in young cells challenged with OA (Figure 3.3A). Electron microscopy confirmed the immunofluorescent data (Figure 3.3C,D). The mitochondria of senescent cells a offered somewhat different result as an increase in unfused

mitochondria were evident in senescent cells challenged with OA when evaluated by immunofluorescence (Figure 3.3B). Electron micrographs showed increased unfused shortened mitochondria (Figure 3.3E,F). We went on to determine whether the fission protein Drp1 was involved in the increased fission observed in mitochondria of senescent cells. There was a significant difference in colocalized Drp1 and mitochondria in senescent cells ($p<0.05$ and Figure 3.3I). We next investigated the role of the Bcl-2 family of pro-apoptotic proteins.

Bak and Bax bind to the mitochondrial outer membrane resulting in a permanent opening of the mitochondrial pore that results in the release of apoptotic proteins as well as a decrease in mitochondrial membrane potential (Karbowski *et al.,* 2006). Furthermore, others have shown an interaction between fission proteins and Bak or Bax. When challenged with OA we find Bak and Drp1 interact at potential sites of fission (Figure 3I).

Continuing our studies with mitochondria, we sought to determine the effect of OA on mitochondrial membrane potential. There was no change in young cells challenged with OA even after 24 hours (Figure 3.3P and Figure 3.3Z). Membrane potential in senescent cells decreased after six hours of OA treatment and continued to decline up to 24 hours (Figure 3.3R,S and Figure 3.3Z). An inhibitor of mitochondrial pore opening, Cycolsporin A (CsA) offered little protection as there was no significant difference (Figure 3.3Z).

Mitochondrial fission is an early stage of apoptosis. As it can occur before cytochrome *c* release (Martinou and Youle, 2006). The data presented in Chapter 3 show mitochondrial fission events increase while fusion events

decrease in senescent cells challenged with OA. Decreased fusion indicates the mitochondria are no longer functioning to their full capacity, therefore leading to apoptotic death in senescent cells (DeJesus *et al.,* 2002). Furthermore, the increased binding of Drp1 to unfused mitochondria lends additional support to our theory on the role of the mitochondria in senescent cell apoptosis in that Drp1 prevents fusion events from occurring (Arnoult *et al.,* 2005)

Apoptotic pathways can become extremely complex as there are many proteins that participate in this type of cell death. Cells of different tissues will not respond to apoptotic agents in the same manner. Investigations that further delineate the mechanisms whereby different cell types respond to apoptotic stimulants will yield insight for potential therapeutic agents that will be rewarding particularly within the aging community.

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