MECHANISMS OF IRF-1 INDUCED CANCER GROWTH INHIBITION

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Submitted to the Graduate Faculty of

The Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2006

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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Michaele JoAnn Armstrong, PhD

University of Pittsburgh, 2006

The tumor suppressor IRF-1 has been gaining interest as a mediator of anticancer therapies and its role in mediating apoptosis and cell cycle arrest are currently being elucidated. Through the creation of recombinant adenoviral (Ad-) IRF-1 in our lab, we are in a unique position to study the underlying mechanisms of IRF-1 mediated tumor growth inhibition.

First, we will further determine the role of IRF-1 in caspase-mediated apoptosis. Our work will examine the mechanism of IRF-1 activation of initiator caspase 8 and effector caspases 3 and 7 and the role of soluble factors. Our second course of study will delineate the role of IRF-1 mediated cell cycle effects and with a focus on G1 arrest and $p21^{waf1cip1}$ upregulation.

Our initial hypothesis that IRF-1 induces caspase 3/7 mediated apoptosis through a death receptor pathway in conjunction with the secretion of soluble factors in cancer was not supported by results obtained. We found that death ligands were not mediating IRF-1 growth inhibition; however we did find that the caspase cascade was clearly involved. Moreover, we have shown that caspase 8 activity is central in mediating IRF-1 apoptosis. While investigating the intrinsic pathway we made a novel discovery that IRF-1 localizes to the mitochondria. The significance of this finding is still under investigation.

Studies of p21 knock down confirmed that IRF-1 utilizes p21 in p53 independent G1 cell cycle arrest. We hypothesized that cell cycle arrest would "protect" the cells from apoptosis but found that p21 up regulation by IRF-1 corresponded to caspase cleavage and that apoptosis was suppressed in our p21 knock down cell lines. We also found that the inhibitor of apoptosis, survivin may account for this effect.

Finally, we show that IRF-1 growth inhibitory effects are directed to malignant and not normal breast cells. We show that this too may be linked to survivin which is commonly overexpressed in cancers and suppressed by IRF-1.

Greater understanding of the mechanisms of IRF-1 cancer growth inhibition is significant to public health because it may allow better utilization and development of IRF-1 and agents that are mediated by IRF-1 in cancer treatment.

STATEMENT OF PUBLIC HEALTH RELEVANCE

Breast cancer, as the second leading cause of cancer mortality in women in the United States, is estimated to kill 40,970 women this year. Current statistics show that a woman has a 1 in 8 chance of diagnosis with invasive breast cancer within her life (Ries et al. 2005). Improved breast cancer survival in current years is credited to better detection and treatment including novel and established therapeutics that have recently been found to be mediated by interferon (IFN) regulatory factor (IRF) – 1.

Studies of breast cancer treatments including faslodex, an anti-estrogen, and tamoxifen indicate IRF-1 as a major mediator of their apoptotic induction. Mullerian inhibiting substance (MIS) also utilizes IRF-1 in its apoptotic signaling. While IRF-1's tumor suppressor functions are widely appreciated, its roles in growth suppression are not adequately understood. Through the creation of recombinant adenoviral (Ad-) IRF-1 in our lab, we are in a unique position to study the underlying mechanisms of IRF-1 tumor growth inhibition.

Greater understanding of the mechanisms of IRF-1 cancer growth suppression may allow better utilization of novel and established agents that are mediated by IRF-1 in cancer treatment as well as development of IRF-1 and it's pathways as potential adjuvant therapies for cancer. Therefore, our objective is to further reveal the mechanisms of IRF-1 mediated cancer growth inhibition through studies of both apoptosis and cell cycle arrest. We hypothesized that IRF-1 induces caspase 3/7 mediated apoptosis through a death receptor pathway in conjunction with the secretion of soluble factors in cancer and that IRF-1 induces cell cycle arrest through p21.

Herein we show that IRF-1 does induce caspase 8 cleavage and the resulting caspase cascade that corresponds with apoptosis, however we also found that soluble factors including death ligands were not mediators of this effect. Further we found evidence that IRF-1, possibly through caspase 8, also utilizes mitochondrial death signaling i.e. cytochrome C and Smac/Diablo release during apoptosis. While carrying out experiments to analyze cellular fractionations, we made the novel discovery of IRF-1 in the mitochondrial enriched fraction when cells were stimulated with IFNγ. This line of study has yet to be developed, but may have implications in heretofore unknown mitochondrial DNA regulation by IRF-1.

We also demonstrate upregulation of p21 by IRF-1 and subsequent G1/S cell cycle arrest that appears to be retinoblastoma (Rb) independent, but involves down regulation of cyclins/cdk

complexes. Our following studies attempted to show that p21 induction and cell cycle arrest would give a measure of protection against apoptosis. To our surprise, markers of apoptosis including caspase cleavage correlated to the presence of p21, suggesting a pro-apoptotic role for p21 in the IRF-1 treatment environment.

These advances in the understanding of IRF-1, a nuclear transcription factor, tumor suppressor, and mediator of cancer therapies, may create opportunities to increase survival for women diagnosed with breast cancer.

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PREFACE

I owe a debt of gratitude to both my mentor, Dr. John H. Yim and my academic advisor, Dr. Robert E. Ferrell, who through their patient listening and explanations guided my research, academic progress, and professional development. Heartfelt appreciation for astute recommendations, consistent encouragement, and thoughtful mentorship also go to Dr. Jack C. Yalowich and Dr. Lin Zhang of my advisory committee.

This doctoral effort could not be achieved without notable others whose timely support, both scientific and personal, deserves more than acknowledgement, but I will do what I can here. My current realities and successes fully rest on the shoulders of my husband, Simon, parents, Michael L. and J. Gail Studer, sister, Diane "Rae" Taylor, research associates including Mike Stang, Ye Liu and Greg Watson, and a host of friends, family, and colleagues who are too numerous to list. Thank you; thank you so very much. The developments I have seen in myself have surprised me more than the unexpected discoveries I have made in the lab; with God's grace both achievements will be put to good use.

~ Michaele

"If one advances confidently in the direction of one's dreams, and endeavors to live the life which one has imagined, one will meet with a success unexpected in common hours." – Henry David Thoreau

"Today is a new day!" – Chicken Little (Disney 2005)

1.0 INTRODUCTION AND BACKGROUND

1.1 CELLULAR GROWTH INHIBITION

In this dissertation we consider two methods of growth inhibition employed by IRF-1: apoptosis and cell cycle arrest. Normal cells commonly undergo either type of growth inhibition in response to intra and extracellular regulatory signals. Neoplastic cell clones, through a series of genetic mutations, add to their cancerous potential by losing the ability to properly undergo apoptosis and becoming less receptive to anti-growth signals, including those involved in cell cycle arrest.

1.1.1 Apoptosis

Coined by Currie, apoptosis, or programmed cell death is a molecular method by which organisms can dispose of cells that threaten healthy functioning through overpopulation or damage. Numerous internal and external signals initiate and mediate this process that is significant in regulating homeostasis in cell populations (Hengartner 2000). Observations of specific patterns of morphological changes in various organisms, tissues, and cells suggest a conserved pathway of common mediators. Defects in these apoptotic pathways may lead to immortalization of the cell and are a major contributor to cancer formation.

Apoptosis occurs mainly through two distinct pathways that share certain molecular players: the extrinsic and the intrinsic pathways. Extrinsic refers to signaling that comes from outside the cell in the form of death ligands. Intrinsic refers to internal signaling that prompts the mitochondria to release cytochrome C and other small molecules that promote cell death. Both of these pathways converge at the caspase cascade leading to DNA fragmentation and typical

apoptotic morphology including membrane blebbing, cellular shrinkage, and nuclear condensation (Huppertz, Frank et al. 1999).

1.1.1.1 Extrinsic Pathway

Cells express death receptors across their cytoplasmic membranes and these include TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5, Fas, and TNFR-1. Of these, the Fas receptor is most well studied and characterized, and provides an example of extrinsic signaling that leads to apoptosis. (Jin and El-Deiry 2005); (Kruidering and Evan 2000); (Danial and Korsmeyer 2004) (Figure 1) When trimeric death ligands bind their specific receptors, homotrimerization of the receptors ensues. Intracellular death domains, on the cytoplasmic tails of the death receptors, aggregate and this in turn recruits a death adaptor protein, Fas-associated death domain protein (FADD), to stimulate a homotypic interaction with the FADD death domains. TRAIL-associated death domain (TRADD) is the death adaptor protein for the TRAIL receptors and functions similarly to FADD. The N-terminal Death Effector Domain (DED) of FADD recruits caspase 8 dimers (Carrington, Sandu et al. 2006), and other molecules to finish the formation of the death-inducing signaling complex (DISC). The DISC then initiates cleavage of caspase 8 simply by bringing the caspases in close proximity for autoproteolytic activation. Cleaved caspase 8 subsequently initiates the caspase cascade by cleaving downstream caspases.

Several layers of regulation prevent the extrinsic pathway from preemptively destroying the cell. Decoy receptors exist for certain ligands that will bind the ligand but not induce a death signal, for example TRAIL receptors DR1 and DR2. This may function to regulate the apoptotic stimuli. Some apoptotic stimuli require further induction of caspase 8 cleavage in order to effectively initiate apoptosis. This requires mitochondrial amplification that loops caspase 8 cleavage of Bid to apoptosome formation followed by further caspase 8 cleavage (Danial and Korsmeyer 2004). Synthesized as inert zymogens, initiator and effector caspases must be activated through one of three functions: cleavage by other caspases (i.e. caspases 3, 7 and 6), binding of caspase recruitment domains (CARD) (i.e. caspases 2 and 9) and/or DISC recruitment (i.e. caspases 8 and 10). These protective steps help to ensure that the caspase cascade is not unintentionally initiated.

Caspases are cysteine proteases that cleave and thereby activate substrates after aspartic acid residues (Thornberry, Rano et al. 1997). These substrates include nucleases, cleaved by executioner caspases 3 and 7, which in turn lead to DNA laddering by cleaving DNA between nucleosomes (Wyllie 1980), and nuclear lamin cleavage via caspase 6, that leads to deconstruction of the nucleus (Buendia, Santa-Maria et al. 1999). Caspase 8, in addition to initiating the caspase cascade, truncates Bid, a pro-apoptotic Bcl-2 family member, which may consecutively activate the intrinsic pathway of apoptosis resulting in cytochrome C release.

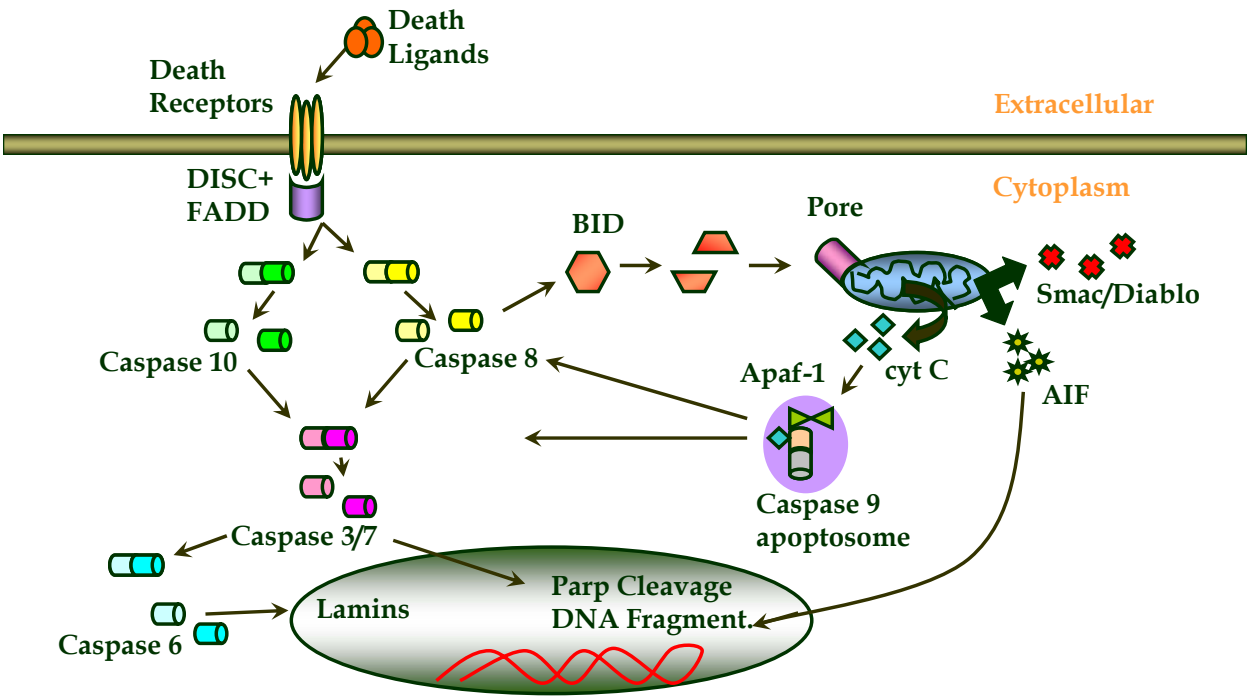

Figure 1. Extrinsic and Intrinsic Apoptotic Pathways

1.1.1.2 Intrinsic Pathway

Mitochondrial mediated apoptosis is regulated and controlled by Bcl-2 family members in a delicate balance of pro- and anti-apoptotic molecules. Pro-apoptotic Bcl-2 family members

including Bid, Bak, and Bax form homo- and hetero dimeric complexes on the mitochondrial outer membrane creating a pore or channel (Reed 1997; Kim, Emi et al. 2006). This channel causes permeabilization of the mitochondrial outer membrane inducing the release of cytochrome C, Second mitochondria-derived activator of caspase (Smac)/Direct inhibitor of apoptosis-binding protein with low pI (Diablo), and other mitochondrial intermembrane signaling molecules. Cytochrome C is an integral protein for apoptosome formation as it complexes with caspase 9 and Apaf-1 to form the apoptosome (Figure 1). The apoptosome in turn cleaves caspase 3 and 7 to initiate the caspase cascade. Smac/Diablo functions to suppress inhibitors of apoptosis that would prevent successful apoptosome formation and activation of the caspase cascade (Du, Fang et al. 2000). Both the induction of executioner caspases and the disruption of essential mitochondrial functions act to kill the cell.

Regulation of mitochondrial mediated apoptosis is derived from the relative balance of pro- and anti-apoptotic Bcl-2 members that bind and neutralize one another (Adams and Cory 1998). An overabundance of either population sensitizes the cell or makes the cell resistant to apoptosis. However, the mechanism of regulation of Bcl-2 family member via transcriptional control or inactivation in healthy cells is poorly understood.

1.1.2 Cell Cycle Arrest

A second form of growth inhibition occurs when cells suspend mitotic activity. Growth and proliferation are regulated through the cell cycle, a progression that results in the formation of two daughter cells from a parental cell. The processes of the cell cycle are divided among four main phases; G1, S, G2 and mitosis. Proliferating cells may arrest when checkpoints indicate dysfunction during growth or replication of the DNA. Many cells maintain themselves at G0, or a quiescent phase when further growth is not desired. In the process of mutation, certain cells gain a survival advantage by creating their own pro-growth signals or becoming immune to signals that would arrest growth, thus giving those cells oncogenic potential. This is commonly seen in cancers that have mutated tumor suppressors like p53, an inducer of p21 mediated cell cycle arrest, or mutated retinoblastoma (Rb) which can no longer restrict the cell from initiating S phase.

1.1.2.1 Cyclins and Cyclin Dependant Kinases

Our focus on cell cycle arrest will be the G1/S checkpoint (Figure 2). Cyclins and their enzymatic partners, cyclin dependent kinases (Cdks) are the major mediator of cell cycle progression. Cyclin expression patterns fluctuate throughout the cell cycle and regulate the activity of the Cdks through their presence or absence. Cdks 2, 4, and 6, when complexed with their corresponding cyclins, E or D, promote progression through the G1/S restriction point (Schwartz and Shah 2005) by phosphorylating retinoblastoma.

Rb, a tumor suppressor, in its active state is hypophosphorylated and tightly binds the transcription factor E2F, preventing transcription of S phase genes (Figure 2). This prevents the cell cycle from progressing until cyclin D and/or E conjoin with their appropriate Cdks to phosphorylate Rb and render it inactive. The newly released E2F then transcribes S phase genes that encourage cell cycle progression through the next phase (Malumbres and Barbacid 2001).

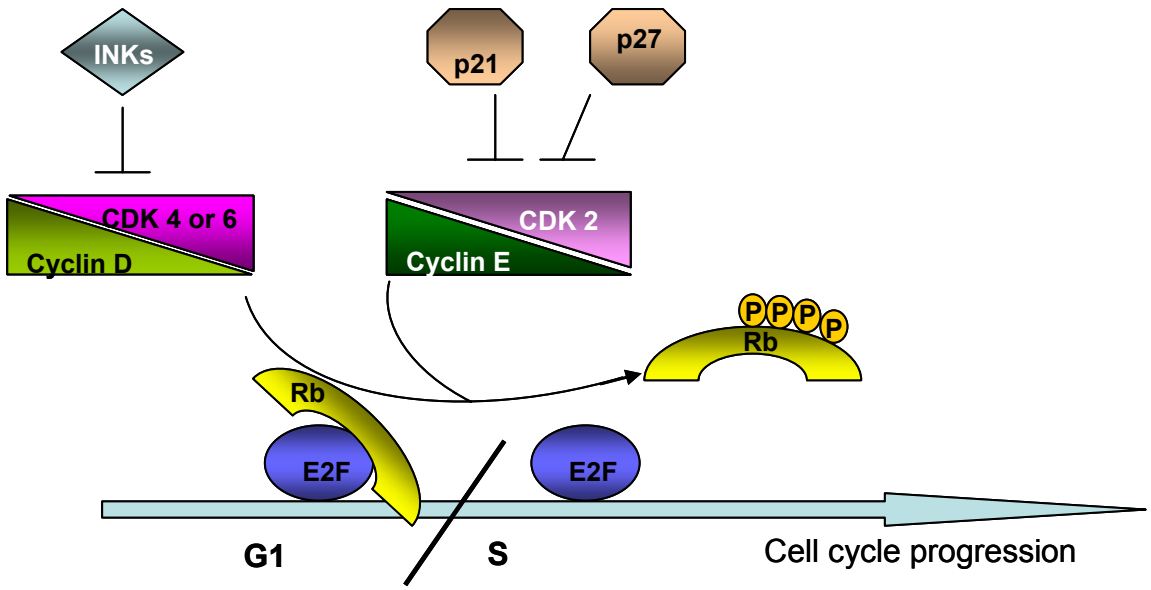

Figure 2. G1/S Cell Cycle Checkpoint

While cyclin expression is the first level of regulation of cell cycle progression, fail safe measures are employed to maintain proper growth of the cell. Inhibitors of the cyclin/Cdk complexes include kinase inhibitors, p21 and p27, as well as a family inhibitors, the INKs. While INKs are more successful suppressing cyclin D/Cdk complexes, p21 and p27 appear to effectively inhibit cyclin E/Cdk2 complexes (Sherr and Roberts 1999) through binding the cyclin and physically blocking the ATP loading groove of Cdk 2 (Russo, Jeffrey et al. 1996). Inhibition of cyclin E/Cdk2 prevents the phosphorylation of Rb and encourages G1 arrest. Normal cells will regularly arrest in G1 if the cell is not yet ready to synthesize DNA because of DNA damage checks or other indicators. Oncogenic cells however have usually overcome arrest mechanisms and proliferate despite damage or overcrowding.

1.2 INTERFERON REGULATORY FACTOR -1 (IRF-1)

As the first interferon regulatory factor family member characterized, IRF-1 was discovered as an inducer of IFN β (Fujita, Sakakibara et al. 1988) and is a transcriptional activator. The nuclear transcription factor translocates to the nucleus principally as a result of interferon (IFN) γ activation of the JAK/STAT1 pathway and subsequently alters transcriptional activity of genes involved in immunomodulation, antiviral responses and tumor suppression (Kroger, Koster et al. 2002). It's homologue, IRF-2, functions to bind the same sequences as IRF-1 and prevents upregulation of those IFN inducible genes that are typically involved in growth inhibition.

1.2.1 IRF-1 as a Tumor Suppressor

The tumor suppressor role of IRF-1 was established by the Taniguchi lab in studies of wild-type and IRF-1 -/- mouse embryonic fibroblasts (MEFs) where IRF-1 suppressed oncogenic cell transformation. A "single hit" with c-Ha-ras induced transformation in IRF-1 -/- MEFs, unlike wild type MEFs that require two or more oncogenic mutations for transformation. This suggests that a key growth regulator had been compromised in the IRF-1 -/- cells. Working with NIH 3T3

cells, they also demonstrated the ability of IRF-1 to revert cells transformed by oncogenic IRF-2 (Harada, Kitagawa et al. 1993), c-myc and fosB (Tanaka, Ishihara et al. 1994) expression, back to a nontransformed phenotype. These studies have now been confirmed in vivo and in vitro by estrogen inducible IRF-1 reversion of NIH 3T3 cells transformed with c-myc and c-Ha-ras (Kroger, Dallugge et al. 2003). The restoration of nontransformed phenotype implies that IRF-1 is an important regulator of controlled cellular growth.

Localized at 5q31.1, IRF-1 is often deleted in human leukemia (Nagai, Fujita et al. 1997; Green, Slovak et al. 1999), esophageal (Ogasawara, Tamura et al. 1996; Peralta, Casson et al. 1998), gastric (Tamura, Ogasawara et al. 1996), and renal cancers (Sugimura, Tamura et al. 1997). Since cancer is formed in part through stepwise mutations that impart losses of genes that control growth, the loss of IRF-1 in these cancers implicates IRF-1 as a tumor suppressor. Interestingly, IRF-1 is a target of the oncogenic virus that causes cervical cancer. Onco-protein HPV E7 inactivates IRF-1 protein by attenuating IRF-1's transactivation domain (Park, Kim et al. 2000).

Other studies also illustrate correlates between IRF-1, cancer formation, and growth inhibition. IRF-1 is decreased in the normal growth of mammary tissue in pregnant rats and increased during involution of the mammary glands (Hoshiya, Gupta et al. 2003). Furthermore, a study in endometrial cancer, (Giatromanolaki, Koukourakis et al. 2004) suggests that IRF-1 repression is a feature of tumor progression. IRF-1 expression has been shown to associate with the degree of differentiation of endometrial endothelium and adenocarcinoma (Kuroboshi, Okubo et al. 2003). Less differentiated cells usually have increased proliferative and oncogenic potential. In melanoma, IRF-1 positively correlated with less advanced disease morphology (Lowney, Boucher et al. 1999). Similar findings in liver exhibit decreased expression of IRF-1 in human hepatocellular carcinoma versus normal liver tissues. (Moriyama, Nishiguchi et al. 2001). All these studies indicate that IRF-1 associates with less severe disease states and more tightly regulated growth.

IRF-1 has been linked to other gene pathways coupled with inhibition of uncontrolled growth that will be discussed at greater length throughout this dissertation. IRF-1 is widely accepted as a tumor suppressor as evidenced by studies that have demonstrated reversion of transformed cells and growth inhibition functions. As such, IRF-1 is found to mediate two crucial preventions of neoplastic progression: apoptosis and cell cycle arrest.

1.2.2 The role of IRF-1 in Apoptosis

We and others have noted and published the observation that IRF-1 can induce apoptosis either through forced expression (Kim, Armstrong et al. 2004; Pizzoferrato, Liu et al. 2004; Bouker, Skaar et al. 2005; Watson, Queiroz de Oliveira et al. 2006) or as a mediator of IFNγ (Kim, Lee et al. 2002; Tomita, Bilim et al. 2003; Egwuagu, Li et al. 2006), retinoic acid (Arany, Ember et al. 2003; Wang, Peng et al. 2006), tamoxifen (Bowie, Dietze et al. 2004), faslodex (Bouker, Skaar et al. 2004), and Mullerian inhibiting substance (Hoshiya, Gupta et al. 2003). While it is well known that IRF-1 induces or mediates apoptosis, little is known about the mechanism or pathways through which IRF-1-induced apoptosis is achieved.

Several studies, including our own discussed below, have suggested downstream mediators of IRF-1 by using inducible IRF-1 systems or overexpression of IRF-1 through a viral vector that result in apoptosis. IRF-1, in conjunction with HER1-induced apoptosis that required Stat5a (Kirchhoff and Hauser 1999) increased p21, caspase 7 cleavage and decreased Bcl-2 (Sanceau, Hiscott et al. 2000). Through comparisons with dn-IRF-1, further studies suggest that caspases 3/7 and 8 may be involved (Bouker, Skaar et al. 2005). Additionally, IRF-1 upregulates caspase 1 in vascular smooth muscle cells (Kim, Lee et al. 2002) and in pancreatic cells (Detjen, Farwig et al. 2001) to induce apoptosis.

With our creation of Adenoviral (Ad-)IRF-1, we have achieved high efficiency of gene transfer and published three studies identifying IRF-1 mediated apoptosis in both mouse (Kim, Armstrong et al. 2004) and human (Pizzoferrato, Liu et al. 2004; Watson, Queiroz de Oliveira et al. 2006) cell lines in *vitro* and in *vivo*. We demonstrate that executioner caspase 3 (Kim, Armstrong et al. 2004) is activated and cleaved with IRF-1 treatment, and caspase 7 mRNA and protein are increased (Tomita, Bilim et al. 2003; Kim, Armstrong et al. 2004) and show proteolytic cleavage (Sanceau, Hiscott et al. 2000). Caspase 8 mRNA is also upregulated by IRF-1 in breast cancer (Kim, Armstrong et al. 2004; Ruiz-Ruiz, Ruiz de Almodovar et al. 2004). The activation of effector caspases 3 and 7 may take place through several intersecting cascade pathways since IRF-1 has been shown to influence or mediate the anticancer signaling of extrinsic apoptotic inducing ligands including FasL (Chow, Fang et al. 2000; Tomita, Bilim et al. 2003), TRAIL (Clarke, Jimenez-Lara et al. 2004; Park, Seol et al. 2004), and TNF (Suk, Chang et al. 2001). These studies suggest that IRF-1 may act to stimulate the extrinsic pathway of

apoptotic induction. Upregulation of Bak (Kim, Armstrong et al. 2004) as well as the suppression of anti-apoptotic Survivin expression (Pizzoferrato, Liu et al. 2004) is also shown in our studies which may implicate mitochondrial involvement in apoptosis.

IRF-1's relationship with death receptors and their ligands is currently being uncovered, but few conclusions have been drawn. Interestingly, FasL contains an IRF-1 binding site within its promoter region, suggesting a pathway for IRF-1 induced apoptosis (Kirchhoff, Sebens et al. 2002) and IFN γ induction of Fas mediated apoptosis was partially abrogated by IRF-1 anti-sense treatment (Tomita, Bilim et al. 2003). IRF-1 upregulation was required for RA induction of TRAIL (Clarke, Jimenez-Lara et al. 2004). Further, synergistic apoptosis by TRAIL in conjunction with IFN γ was abrogated when treated with anti-sense oligonucleotides specific to IRF-1 (Park, Seol et al. 2004). TNF-α-induced apoptosis also shows synergy with IFN γ treatment (Suk, Chang et al. 2001) inferring yet another convergence of IRF-1 with death receptor/ligand pathways.

1.2.3 The role of IRF-1 in Cell Cycle Arrest

While our previous studies center on the apoptotic mechanisms of IRF-1 induced growth inhibition, published data suggest that apoptosis may not account for all IRF-1 effects. We and others have found upregulation of p21 (Coccia, Del Russo et al. 1999; Miyazaki, Sakaguchi et al. 2004; Pizzoferrato, Liu et al. 2004), a key molecular mediator of cell cycle arrest, in response to IRF-1. Functional studies demonstrate that IRF-1 binds to the promoter regions of p21 (Coccia, Del Russo et al. 1999) which is highly suggestive of further growth inhibition through cell cycle regulation. IFN-γ, a key inducer of IRF-1, significantly decreased the fraction of cells in S phase (Hoshiya, Gupta et al. 2003).

Induction of cell cycle arrest by IRF-1 was first suggested by embryonic fibroblasts (EFs) from IRF-1 -/- mice that were found deficient in their ability to undergo DNA-damage-induced cell cycle arrest, a phenotype similar to that observed in EFs lacking p53 (Tanaka, Ishihara et al. 1996), a classic regulator of the cell cycle. Cell cycle arrest was also found in breast cancer and myeloid cells treated with MIS (Hoshiya, Gupta et al. 2003) or N-RAS (Passioura, Dolnikov et al. 2005) respectively, and their effects have been shown to be mediated by IRF-1. Cyclin dependant kinase (Cdk) 2, involved in G1 and S progression, was suppressed by IRF-1 (Xie,

Gupta et al. 2003) and is a target of p21 repression. IL-4 anti-proliferation in renal cell carcinoma was mediated by upregulation of IRF-1 and a corresponding increase in p21/Waf1 and a decrease in Cdk2 (Yu, Kim et al. 2004). Antisense IRF-1 partially releases cells from TGF-β induced growth arrest, and prevents IRF-1 induction of p21/Waf1 (Miyazaki, Sakaguchi et al. 2004). While these studies make a strong case for IRF-1 induced cell cycle arrest, no direct studies have been done to elucidate a pathway or to establish causality.

1.3 BREAST CANCER

1.3.1 Breast Cancer Epidemiology and Treatment

Through a step wise progression of mutations in DNA, cells gradually gain or lose functions that promote aberrant cell proliferation and cancer. Cancer of the breast will be diagnosed in nearly 213,000 women and is estimated to cause over 40,000 deaths this year in the United States (Ries LAG 2005). This makes breast cancer the second most lethal cancer for women in the United States. Only 5 - 10% of breast cancers are due to genetic susceptibility; other risk factors include age, age at first child birth, obesity, previous benign or cancerous disease, and age at menarche/menopause[1]. The lack of preventative factors within the risk list is notable and highlights the importance of research that may increase survival and prevent recurrence of previous tumor development.

Currently, four general treatments are utilized for treating breast cancer: surgery, radiation therapy, chemotherapy, and hormone therapy (including tamoxifen and faslodex). The treatment options employed depend on the stage, type of cancer and other factors including the patient's medical history[2,3].

[1] http://bmj.bmjjournals.com/cgi/content/full/321/7261/624

[2] http://www.cancer.gov/cancertopics/pdq/treatment/breast/Patient

[3] http://www.cancerlinksusa.com/breast/index.asp

1.3.2 Relationship of IRF-1 and Breast Cancer

5q deletions, which may include IRF-1, have been found in primary breast carcinoma both by loss of heterozygosity (Devilee, van den Broek et al. 1991) and comparative genomic hybridization (Loveday, Greenman et al. 2000; Cowell, LaDuca et al. 2005). Patients with the BRCA1 mutation displayed losses of 5q in 82-86% of breast tumors (Tirkkonen, Tanner et al. 1998; Wessels, van Welsem et al. 2002). These studies, while suggestive, do not specifically identify IRF-1 deletions in breast cancer. Other studies of breast cancer pathology and therapeutics mark IRF-1's importance in this growing field.

1.3.2.1 Marker of Pathology

Several studies demonstrate the role of IRF-1 and breast cancer pathology. Doherty et al. (Doherty, Boucher et al. 2001) examined patients with ductal cancer in situ (DCIS) and invasive ductal cancer and found that normal breast tissue stained for IRF-1 in 97% of samples, whereas patient samples with high nuclear grade DCIS and patients with lymph nodes positive for invasive ductal cancer showed significantly less IRF-1 staining.

Other studies (Gu, Lee et al. 2002; Bouker, Skaar et al. 2004) demonstrated that IRF-1 is down-regulated in breast cancer cell lines resistant to antiestrogens. Resistance to this treatment suggests that the cancerous cells have subdued molecules/pathways that would normally allow the treatment to kill the cells. The level of IRF-1 mRNA expression among a panel of breast cancer cell lines was inversely correlated with metastatic or invasive potential (Bouker, Skaar et al. 2005) implying that IRF-1 may be important in preventing metastasis or invasion by breast cancer. This paper also demonstrates that IRF-1 could reduce the formation of MCF-7 tumors in *vivo* while a dn-IRF-1 increased the incidence of xenograft formation, implying that IRF-1 is inversely correlated to oncogenic potential.

1.3.2.2 Mediator of Anti-cancer Therapeutics

Investigations of IRF-1 regulated faslodex induced apoptosis, an anti-estrogen that has successfully completed clinical trials (Bouker, Skaar et al. 2004). IRF-1 mRNA is upregulated by faslodex in antiestrogen sensitive cells and faslodex is resisted by IRF-1 dominant negative clones. Faslodex is not the only hormone therapy that utilizes IRF-1 signaling in its apoptotic

induction. Bowie et al demonstrated in acutely damaged, p53 suppressed, estrogen receptor poor epithelial cells, that tamoxifen's apoptotic effects including the activation of caspases 1 and 3 were blocked by siRNA against IRF-1. These cells, while not cancer, have p53 suppressed by forced expression of HPV E6, which is considered an oncogenic event, demonstrated induction of IRF-1 and apoptosis in response to tamoxifen treatment (Bowie, Dietze et al. 2004).

Investigations of these clinically utilized therapeutics have demonstrated the importance of IRF-1 as a mediator of apoptosis in breast cancer and the value of awareness of IRF-1's growth inhibition mechanisms.

1.4 STATEMENT OF HYPOTHESIS

While recent studies have established the role of IRF-1 in mediation and induction of apoptosis, the specific mechanisms of IRF-1 in apoptosis and cell cycle arrest have not been fully investigated. One factor in difficulties with studies of IRF-1, particularly in *vivo*, is that overexpression of this transcription factor creates a growth suppressive effect making it almost impossible to identify and expand stable transfectants (Kirchhoff, Schaper et al. 1993; Skaar 1999). Most studies have been limited to correlational evidence and explanations of IRF-1 mechanisms restricted to possible transcriptional mediation (Coccia, Del Russo et al. 1999; Kirchhoff, Sebens et al. 2002) with the exception of one study that suggests a novel protein-protein binding between IRF-1 and p300 (Dornan, Eckert et al. 2004).

Our hypothesis is that IRF-1 induces neoplastic growth inhibition in part through activating initiator caspase 8 and executioner caspases 3 and 7 through the extrinsic and/or intrinsic apoptotic pathway. We also hypothesize that IRF-1 induces G1/S cell cycle arrest through p21 upregulation. By over expressing IRF-1, we will test our hypotheses and illuminate a mechanistic background that will further our understanding of IRF-1; it's mediation of anti-cancer activity, and its possible development as an adjuvant cancer treatment.

While investigating the intrinsic death pathway of specific aim 1, we found a population of IRF-1 within mitochondria. We began to explore the validity of the localization and possible functions of IRF-1 in the mitochondria. We propose that mitochondrial IRF-1 acts to further regulate cellular proliferation.

Our studies also suggested a cancer specific growth inhibition that we further characterized. We hypothesize that IRF-1 suppresses neoplastic overexpression of survival molecules, particularly survivin.

2.0 THE ROLE OF SOLUBLE FACTORS AND CASPASE 8 IN IRF-1 MEDIATED APOPTOSIS

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Manuscript in preparation

2.1 INTRODUCTION

Interferon regulatory factor-1 (IRF-1) is a nuclear transcription factor that induces transcription of genes involved in immunomodulation, anti-viral effects and tumor suppression in response to IFNs (Kroger, Koster et al. 2002) and other signals. IRF-1 can contribute to an apoptotic cell death response either in conjunction with death inducing ligands, DNA damage or a part of the cellular response to interferon signaling. We have previously shown IRF-1 is able to induce apoptotic cell death through overexpression via adenoviral gene transfer (Kim, Armstrong et al. 2004; Pizzoferrato, Liu et al. 2004) in vitro and in vivo. However, the mechanism of this IRF-1 induced apoptosis has not been clearly established. In this study, the molecular mechanisms of IRF-1 mediated cell death were investigated the human breast adenocarcinoma cell line MDA-MB-468.

Previous studies have found associations between IRF-1 induced apoptosis (Kim, Lee et al. 2002) and induction of caspase transcription and cleavage. We have shown that IRF-1 activates and cleaves executioner caspase 3 (Kim, Armstrong et al. 2004). IRF-1 also upregulates caspase 7, increasing mRNA and protein (Tomita, Bilim et al. 2003; Kim, Armstrong et al. 2004) and stimulates proteolytic cleavage of caspase 7 (Sanceau, Hiscott et al. 2000). Initiator caspase 8 mRNA is also upregulated by IRF-1 in breast cancer in two separate studies (Kim, Armstrong et al. 2004; Ruiz-Ruiz, Ruiz de Almodovar et al. 2004) and in this study we show cleavage of caspase 8 for the first time in response to IRF-1 treatment. Initiation of the caspase cascade is typically associated with either prior death ligand binding of a death receptor or previous activation of the intrinsic, mitochondrial, death pathway. Thus activation of effector caspases 3 and 7 may take place through several intersecting cascade pathways and IRF-1 has notably been shown to influence or mediate the anticancer signaling of death ligands including FasL (Chow, Fang et al. 2000; Tomita, Bilim et al. 2003) TRAIL (Clarke, Jimenez-Lara et al. 2004; Park, Seol et al. 2004), and TNF(Suk, Chang et al. 2001).

IRF-1's affiliation with death receptors and their ligands is currently being revealed, but no decisive mechanisms have been reported. Interestingly FasL contains an IRF-1 binding site

within its promoter region, suggesting a pathway for IRF-1 induced apoptosis (Kirchhoff, Sebens et al. 2002) and IFNγ induction of Fas mediated apoptosis was partially abrogated by IRF-1 antisense treatment (Tomita, Bilim et al. 2003). These studies imply that IRF-1 simply induces transcription of a death ligand that then incites known death receptor apoptotic pathways. Similarly, IRF-1 upregulation was required for RA induction of TRAIL (Clarke, Jimenez-Lara et al. 2004). Further, synergistic apoptosis by TRAIL in conjunction with IFNγ is abrogated when treated with antisense oligonucleotides specific to IRF-1 (Park, Seol et al. 2004). These observations suggest that IRF-1 is important in TRAIL transcription and the interaction between IFNγ and TRAIL apoptotic pathways. TNF-α induced apoptosis also shows synergy with IFN γ treatment (Suk, Chang et al. 2001) inferring yet another convergence of IRF-1 with death receptor/ligand pathways. After consideration of these studies and our own observations, we hypothesize that IRF-1 may utilize the extrinsic pathway to achieve caspase mediated apoptosis (Figure 3).

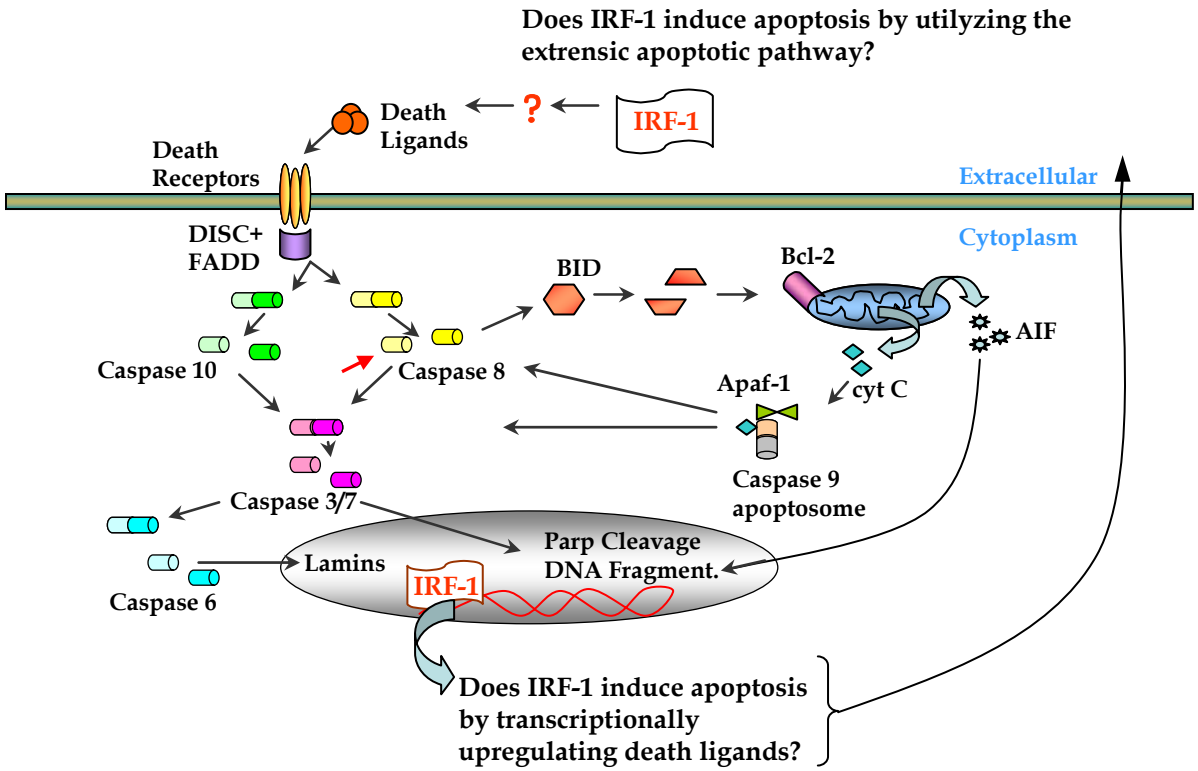

Figure 3. Hypothesized IRF-1 induction of apoptosis

Importantly, we also note other associations that suggest the possibility of multiple mechanisms of IRF-1 induced apoptosis. We have previously published the upregulation of BAK (Kim, Armstrong et al. 2004), which may lead to induction of the mitochondrial death pathway, as well as the suppression of Survivin, an inhibitor of apoptosis, expression (Pizzoferrato, Liu et al. 2004) in response to IRF-1 treatment.

2.2 MATERIALS AND METHODS

Cell Lines and Culture:

p53 mutated MDA-MB-468 was purchased from American Type Culture Collection (Manassas, VA), while p53 mutant, U373MG was generously provided by Dr. Seol. The MDA-MB-468 tumor cells were propagated in Dulbecco's modified Eagle's medium (BioWhittaker, Inc., Walkersville, MD) and Ham's F-12 (Invitrogen Life Technologies, Carlsbad, CA) media at a 1:1 ratio with 10% fetal bovine serum, L-glutamine and antibiotics. U373MG were propagated in Dulbecco's modified Eagle's medium (BioWhittaker, Inc., Walkersville, MD) with 10% fetal bovine serum, 2mM L-glutamine and antibiotics. All cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Apoptosis Assay:

Cells were harvested at various time points using 0.25% trypsin, rinsed in PBS, and stained using the Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). Apoptosis was assessed by flow cytometric analysis using a Becton Dickinson FACSort (San Jose, CA, USA). A minimum of 10,000 cells per sample was recorded, and cell debris was excluded by appropriate forward light scatter threshold setting. Data analysis using CELLQuest software (Becton Dickinson, St Louis, USA) was performed, and numbers of cells positive for Annexin V-FITC, PI, or combinations thereof, were calculated.

Immunoblotting Analysis and Antibodies:

Protein from either whole cell lysates or cytosolic and mitochondrial fractions were separated by SDS–PAGE and transferred onto a nitrocellulose membrane. Loading of equal protein amounts was assessed by staining of nitrocellulose membranes with 0.1% Ponceau S (Sigma) in 5% acetic acid. Nonspecific binding was blocked with PBS-T (14 mM sodium phosphate, monobasic, monohydrate; 88 mM dibasic sodium phosphate, anhydrous; 100 mM

NaCl; and 0.1% Tween 20) containing 5% nonfat milk for 1-h incubation with agitation at room temperature. Antibodies were purchased as follows: caspase 3, caspase 7, caspase 8, Smac/Diablo, and Bid (Cell signaling technology, Danvers, MA), Cytochrome C (BioVision, Mountainview, CA), IRF-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Assessment of soluble factors:

5 x 10^5 MDA-MB-468 cells were plated per well in a six well plate and 3 x 10^5 cells per Transwell® co-culture insert (Corning Inc., Corning, NY). After overnight incubation, cells in the six well plates were then treated with no infection, Ad-Ψ5 or Ad-IRF-1 (MOI 25) for four hours in OptiMEM. Cells were washed three times and media replenished. The co-culture inserts were then introduced to the pre-treated six well plate. Inserts have 0.4 μM pores to allow circulation of media and secreted factors. Cells from both the six well plate and co-culture inserts were harvested at 24 and 48 hours and assessed by flow cytometry and western blot.

Cellular Fractionation:

MDA-MB-468 cells were cultured at 3 x 10^6 cells per 10 cm dish and after overnight adhesion treated with either no treatment, IFNγ (1000 units/ml), Ad-Ψ5 or Ad-IRF-1 (MOI 25). 24 hours later cells were harvested with trypsin (0.25%), washed and resuspended in cytosol extraction buffer in accordance with Mitochondrial/Cytosol Fractionation kit (BioVision, Mountain View, CA) instructions. Fractionation continued by kit instructions with the exception that the mitochondrial pellet was resuspended in 50 μl of mitochondrial buffer.

Inhibition of IRF-1 induced apoptosis and caspase cleavage:

MDA-MB-468 cells were cultured and allowed to adhere overnight cells were then treated with no infection, IFN-gamma (1000 units/ml), Ad-Ψ5 or Ad-IRF-1 (MOI 25) and simultaneously with either DMSO (control, ATCC, Manassas, VA), 200 μM ZVAD (Pan-caspase inhibitor, Promega, Madison, WI), 20 μM DEVD (caspase 3/7 inhibitor), or 20 μM IETD (caspase 8 inhibitor) or Ad-cFLIP, Ad-LacZ (MOI 25). For apoptotic inhibition using IRF-1 siRNA, cells were cultured and 12 hours later transfected with IRF-1 or scrambled siRNA (20 nM in siPORT Amine (Ambion, Austin, TX) transfection reagent according to manufacturer's

directions). Cells were harvested at various time points after treatment and either analyzed for apoptosis by flow cytometry or immunoblotted for caspase cleavage.

Death ligand and neutralizing antibodies:

Cells were cultured in either 6 or 12 well plates and allowed to adhere overnight. FasL, TNFα (Biovision Inc, Mountain View, CA), crosslinked TRAIL (Axxora, San Diego, CA) or CH-11 (all at 100 ng/ml)(Upstate, Lake Placid, NY) were added to media. Cells were then harvested at 24 or 48 hours for growth inhibition or apoptosis.

For neutralizing antibody experiment, cells were treated with antibody to Fas (Immunotech, Marseille, France), TNFR-1 (Biosource International, Carlsbad, CA), DR4 and/or DR5 (R&D Systems, Minneapolis, MN) (20 ug/ml) after adhering overnight. 3 hours later cells were treated with no infection (NI), Ad-Ψ5 or Ad-IRF-1 (MOI 25) cells were harvested after 24 hours and assayed by Annexin V-FITC/PI staining for apoptosis.

2.3 RESULTS

2.3.1 IFNγ induces a caspase dependent apoptosis that is mediated by IRF-1

As the primary upstream inducer of IRF-1, IFNγ has been shown in several experimental models to, at a minimum, increase the susceptibility of tumor cells to the induction of apoptotic cellular death. Such susceptibility has been demonstrated in pharmacologic and ionizing radiation (Tamura, Ogasawara et al. 1996) provoked genotoxic stress as well as enhancing the ligand mediated death signaling of TNF (Kim, Kim et al. 2005), Fas ligand (Siegmund, Wicovsky et al. 2005) and TRAIL (Lee, Shin et al. 2006). We have observed that some *in vitro* tumor models show variable growth suppression in response to the addition of IFNγ alone during normal culture conditions (Yim, Ro et al. 2003). It was proposed at that time that this significant growth suppression was the result of apoptotic cell death. To explore this further, we performed extensive time course experiments of the human breast adenocarcinoma cell line MDA-MB-468 in the presence or absence of IFNγ under normal culture conditions

followed by labeling of exposed cell-surface phosphatidylserine by FITC conjugated Annexin V and flow cytometric analysis. We found that IFNγ induced a modest but detectable cell death as early as 20 hours and the fraction of Annexin V positive cells continued to increase and accumulate for the duration of the time course (Figure 4A). To explore the possible molecular mechanisms involved in this response, we examined the ability of IFNγ induced apoptosis to be inhibited by the pan-caspase inhibitor ZVAD. For these initial experiments, the detectable cellular death induced by IFNγ was completely blocked by caspase inhibition (Figure 4B) without a concomitant increase in cellular necrosis (not shown) lending to our conclusion that IFNγ alone is able to initiate a caspase dependent apoptotic cellular death.

IRF family members, including IRF-1, have been implicated in mediating many of IFNγ's inflammatory and growth inhibitory effects. An early study utilizing a dominant negative interferon consensus sequence binding protein (ICSBP) prevented IFNγ mediated growth inhibition and inhibited upregulation of IFNγ inducible gene expression (Thornton, Ogryzko et al. 1996). We and others have repeatedly shown IFNγ upregulation of IRF-1 and we further published that antisense oligonucleotides to IRF-1 attenuated IFNγ induced growth inhibition in two breast cancer cell lines (Yim, Ro et al. 2003). More recent studies have identified IRF-1 specifically as a mediator of IFNγ enhancement of both TRAIL (Park, Seol et al. 2004) and Fas (Ruiz-Ruiz, Ruiz de Almodovar et al. 2004) mediated apoptosis. Other studies linking IRF-1 to IFNγ include Hisamatsu's finding that IRF-1 may mediate IFNγs activation and upregulation of CARD4/NOD1 during inflammation of intestinal epithelium (Hisamatsu, Suzuki et al. 2003) and a separate study using wild type and IRF-1 -/- MEFs that implicates IRF-1 as the mediator of IFNγ attenuation of oncogenic hTERT expression and telomerase activity (Lee, Kim et al. 2003). Therefore after confirming caspase mediated IFNγ induced apoptosis, we introduced IRF-1 siRNA to our IFNγ treated cells and assayed again for changes in apoptosis. We show that IFNγ induced apoptosis was eliminated in the presence of IRF-1 siRNA (Figure 4C) but not with a scrambled siRNA, which leads us to believe that IRF-1 mediates IFNγ induced apoptosis.

We have previously described an apoptotic cell death which is induced through adenoviral transgene expression of recombinant IRF-1 (Kim, Armstrong et al. 2004; Pizzoferrato, Liu et al. 2004). Adenoviral GFP transgene expression with escalating multiplicities of infection were utilized followed by flow-cytometric analysis for fluorescent

protein expression to determine the lowest quantity of adenoviral infection needed to facilitate >95% of cell expressing the gene of interest (data not shown). To examine the potential molecular mechanisms involved in this cellular death response, we first established a time course of the apoptotic response following IRF-1 expression. Following infection with either Ad-IRF1 or Ad-Ψ5 (Null - empty adenoviral vector control), cell-surface phosphatidylserine labeling with Annexin V was used to determine the percentage apoptotic cells present at time points from 24 to 36 hours post-infection (Figure 4D). A significant apoptotic cell death response above that of baseline was able to be detected as early as 24 hours. The time dependent increase in Annexin V labeling was due to a caspase mediated apoptosis as demonstrated by the ability of the pan-caspase inhibitor ZVAD to significantly block cell death to as late as 36 hours (Figure 4E).

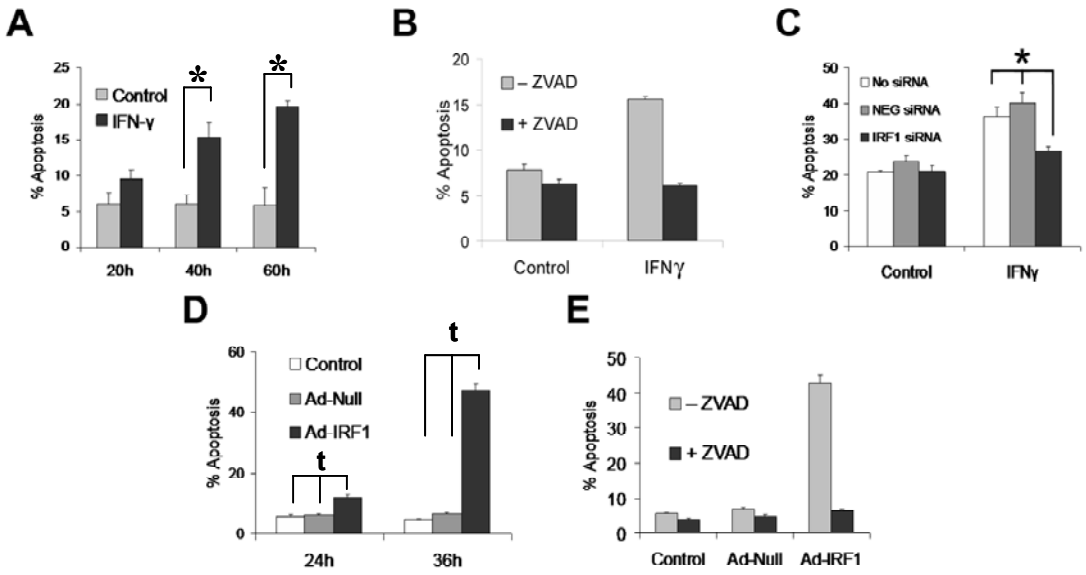

Figure 4. A. IFNγ induces apoptosis in MDA-MB-468 cells (* = p<0.01). **B.** Pan-caspase inhibitor ZVAD inhibits IFNγ induced apoptosis. **C.** siRNA to IRF-1 abrogates IFNγ induced apoptosis, p<0.05. **D.** IRF-1 induces apoptosis (t = p<0.001) and (**E.**) ZVAD also inhibits IRF-1 induced apoptotis

2.3.2 Caspase cleavage and induction of the intrinsic apoptosis pathway associated with IRF-1 induced apoptosis

Since IRF-1 induces substantial cell death in our model system and it appears from the ZVAD inhibition of IRF-1 induced apoptosis that caspase activity is linked to the cellular commitment to death, we examined the time course of caspase cleavage for initiator caspase 8, that we had previously shown was upregulated by IRF-1, and executioner caspases 3 and 7, that had also been linked to IRF-1 in our previous publications (Kim, Armstrong et al. 2004; Pizzoferrato, Liu et al. 2004). Ad-IRF-1 treatment clearly expresses IRF-1 as early as 12 hours and strong expression continues through our assay to 30 hours (Figure 5A). Caspase cleavage begins to occur by 24 hours for caspases 8 and 3. Caspase 7 cleavage is slight at 24 hours and more obvious by 30 hours. While this data supports the caspase mediation of IRF-1 induced apoptosis, the evident cleavage of caspase 8 also directs our attention to possible inclusion of the mitochondrial apoptotic pathway.

In the most fully studied connection between the extrinsic and intrinsic death pathways, caspase 8 is known to truncate and activate the pro-apoptotic Bcl-2 family member Bid, which then translocates to the mitochondria. Since IRF-1 effectively induced cleavage of caspase 8 in our time-course, we then investigated the possibility of Bid truncation in response to IRF-1 treatment (Figure 5B). Parental Bid is conspicuously absent and suggests cleavage with IRF-1 treatment even though no cleavage product was indicated by our western blot at 24 hours.

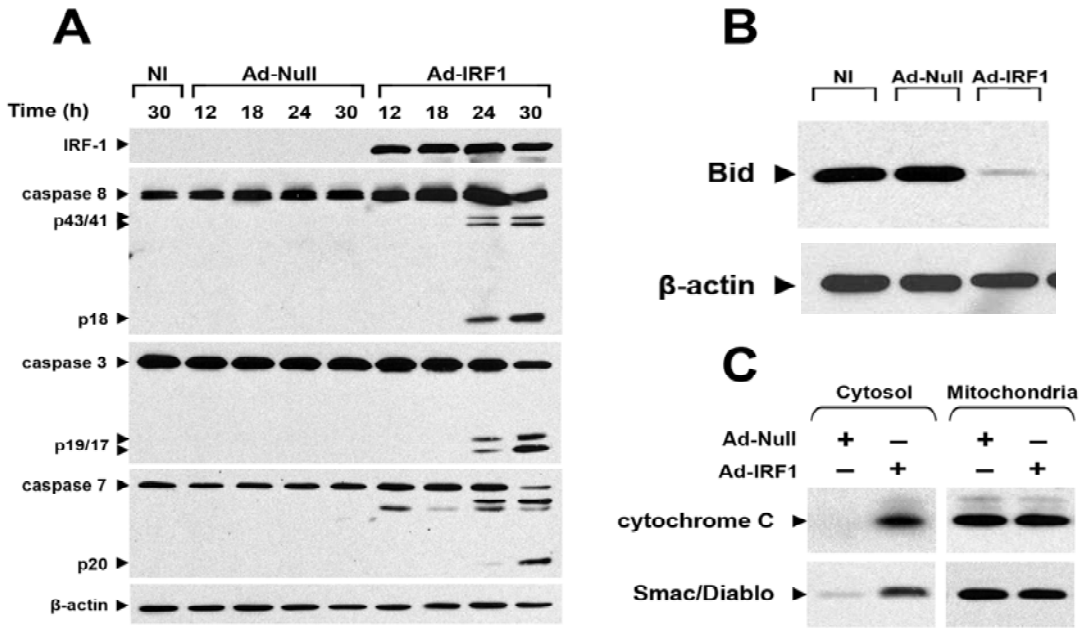

Figure 5. A. IRF-1 induces cleavage of caspases 8, 3 and 7. **B.** Parental Bid decreases with IRF-1 and (**C.**) IRF-1 induces the release of cytochrome C and Smac/Diablo from the mitochondria

Once truncated Bid translocates to the mitochondrial membrane it will form homo or heterodimeric complexes with other Bcl-2 family members that results in pore formation on the outer mitochondrial membrane and release of small intermembrane molecules. To further determine the possibility of IRF-1 induction of the intrinsic pathway, we separated the cellular lysate into cytosolic and mitochondrial fractions to analyze whether the outer mitochondrial membrane had been compromised to release cytochrome C and SMAC/Diablo. Indeed, we found evidence of mitochondrial cytochrome C and Smac/Diablo release in response to Ad-IRF-1 (Figure 5C). This result also occurred with IFNγ treatment (not shown).

2.3.3 Caspase 8 mediation of IRF-1 induced apoptosis

Having confirmed caspase 8 cleavage and suspecting that IRF-1 may induce apoptosis through the extrinsic pathway, we next set out to determine if caspase 8 was the initiator of the caspase cascade activation. By western blot analysis we could clearly determine that caspase 8 and subsequent Bid cleavage (Figure 5A and B) were evident at approximately the same time that apoptotic cellular death was detectable by flow cytometry (Figure 4D). To further determine whether caspase 8 cleavage and activation is a proximal event and responsible for the IRF-1

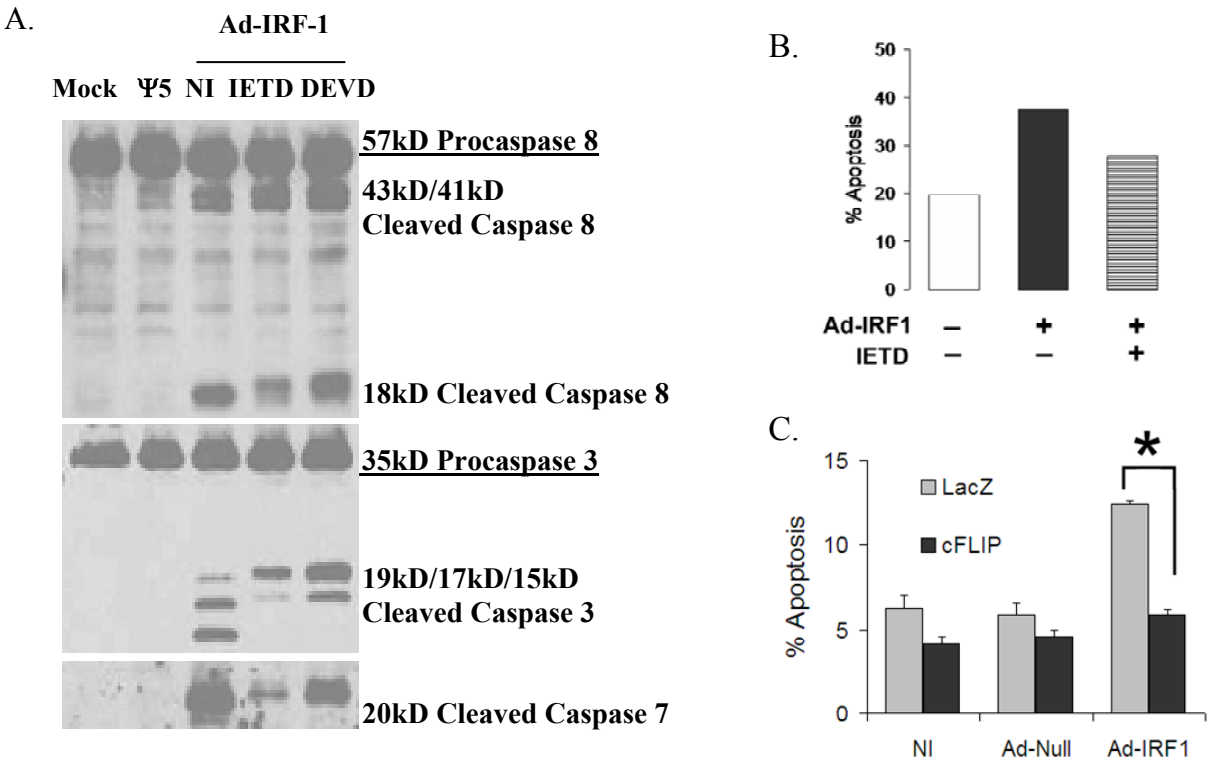

Figure 6. A. Caspase 8 inhibitor, IETD, abrogates IRF-1 induction of Caspase 3 and 7 cleavages; however Caspase 3/7 inhibitor, DVAD, does not alleviate caspase 8 cleavage. **B.** Caspase 8 inhibitor abrogates IRF-1 induced apoptosis. **C.** cFLIP, an inhibitor of caspase 8 activity prevents IRF-1 induced apoptosis, ($p<0.05$).

induced activation of downstream caspases 3 and 7 we added caspase specific inhibitors of caspase 8, IETD, and caspases 3/7, DEVD, to IRF-1 expressing MDA-MB-468 cells (Figure 6A). We found by western blot analysis that caspase 3 cleavage to the partial 17 and 12 kD species is blocked by the caspase 8 inhibitor and this suppression of cleavage is even more effective than the caspase 3/7 inhibitor DEVD. These results were mimicked in the suppression of caspase 7 cleavage by IETD. However, the cleavage of caspase 8 to the 18 kD partial species is not affected by caspase 3/7 inhibitor DVAD. Further, the initial 20 kD cleavage product of caspase 3 was increased by caspase inhibition (Figure 6A) and may represent a less apoptotically active cleavage product in the light of our apoptosis findings (not shown). Of the caspase specific inhibitors used, the caspase 8 specific inhibitor IETD was most able to inhibit caspase processing of caspases 3 and 7 to the partial product p17.

In addition, cells treated with IETD resisted IRF-1 induced apoptosis (Figure 6B), as measured by the percent of cells positive for Annexin V. The cellular FADD-like interleukin-1beta-converting enzyme-inhibitory protein, c-FLIP, is known to restrict caspase 8 and 10 function and prohibit apoptosis through the extrinsic pathway; (Day, Najafi et al. 2006) therefore we tested to see if overexpression of cFLIP would inhibit IRF-1 induced apoptosis (Figure 6C). In fact cFLIP was statistically significant in it's suppression of IRF-1 induced apoptosis compared to co-infection of IRF-1 with the LacZ control. Taken together, this data suggests that caspase 8 may serve the role as an apical caspase and is at least partially responsible for processing of the downstream effector caspase 3.

In other experiments that also implicate caspase 8 as the apical caspase and mediator of IRF-1 induced apoptosis, we infected caspase 8 and p53 deficient U373MG cells with Ad-IRF-1 (Figure 7A). The cells did not undergo apoptosis at an MOI of 25 by 48 hours despite high levels of IRF-1 production by our Ad-IRF-1 that were not seen with our vector control, Ad-Psi5 (Figure 7B).

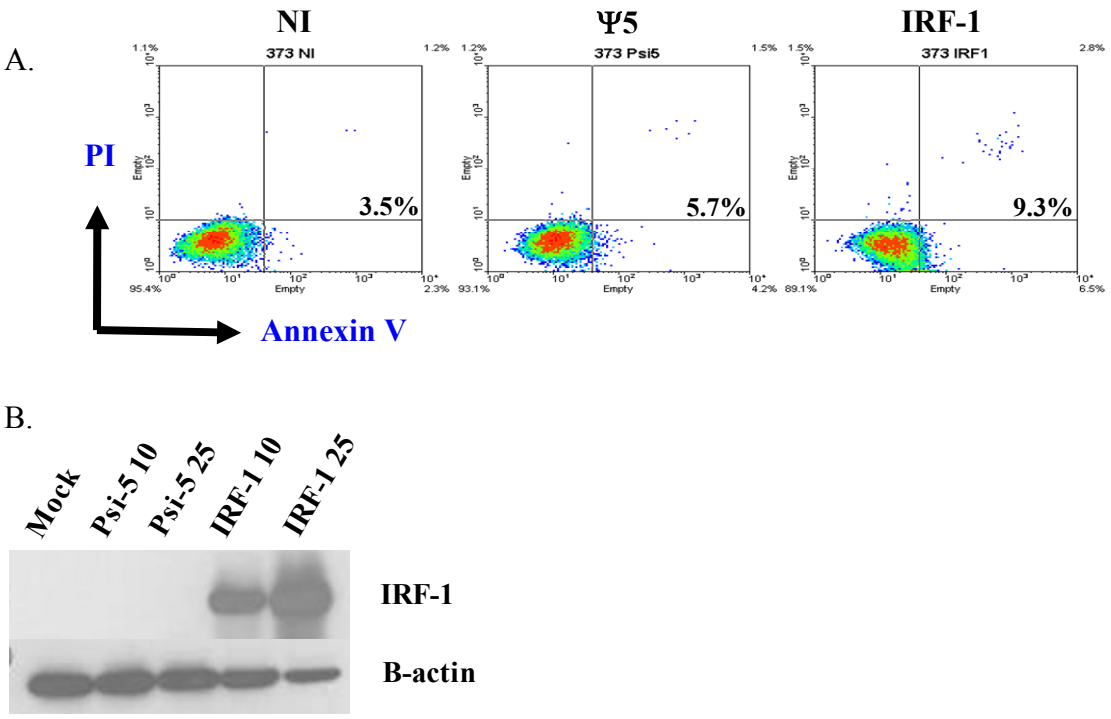

Figure 7. A. Caspase 8 deficient U373MG cells do not undergo IRF-1 induced apoptosis despite **B.** high levels of IRF-1induction

2.3.4 IRF-1 induced secretion of death ligands not implicated in IRF-1 induced apoptosis

Since IRF-1 has been shown to be an inducer of FasL (Chow, Fang et al. 2000), we investigated the possibility that IRF-1 may stimulate the production of a soluble death ligand which in turn may induce apoptosis, specifically extrinsic apoptosis through the caspase cascade with caspase 8 as an initiator (Figure 1).

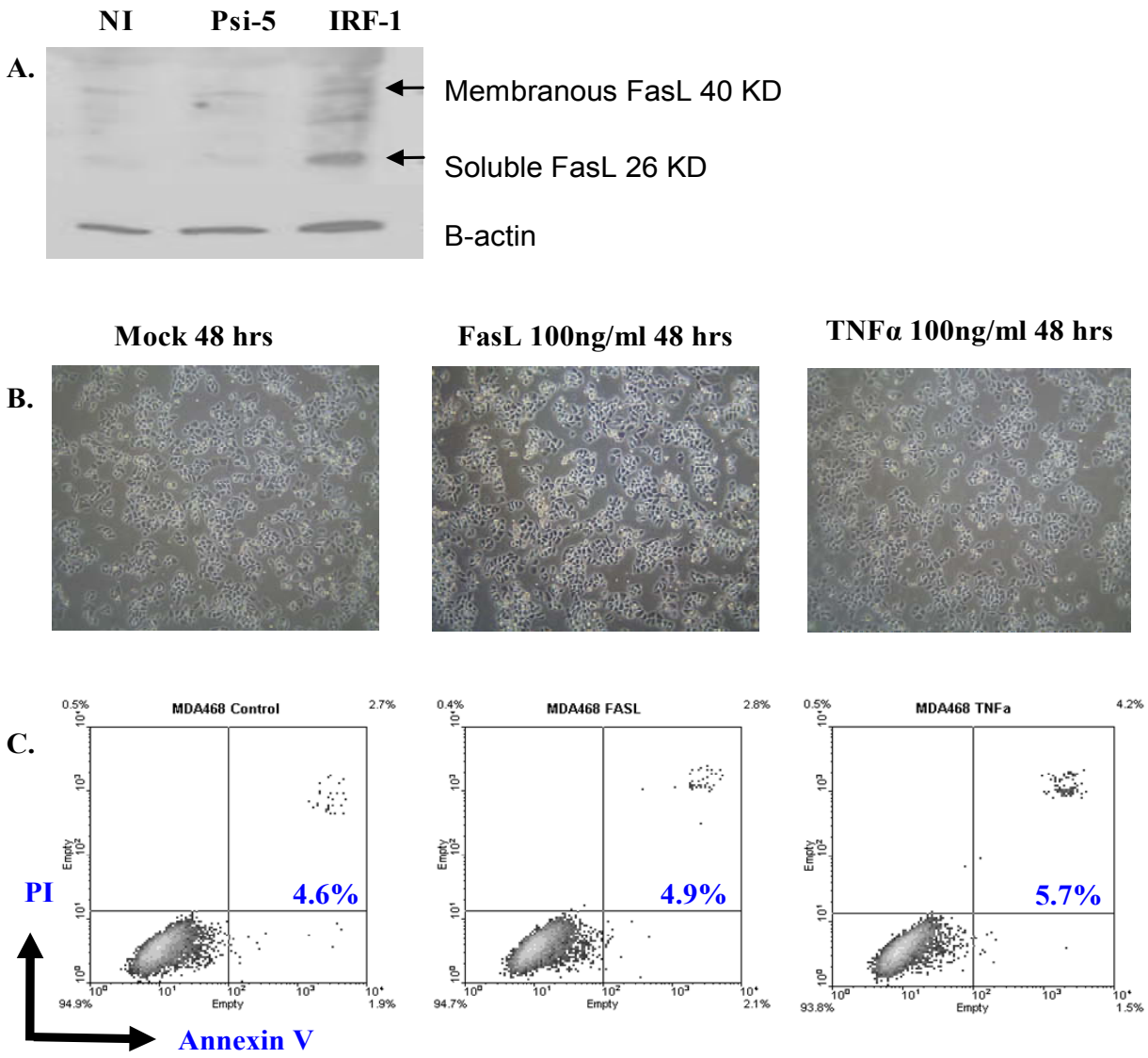

Figure 8. A. IRF-1 upregulates soluble FasL in the media of treated MDA-MB-468. **B.** Recombinant FasL and TNFα do not induce obvious growth inhibition in our model. **C.** Recombinant FasL and TNFα do not induce apoptosis in MDA-MB-468.

Treatment of MDA-MB-468 with IRF-1 led to the upregulation of soluble FasL (Figure 8A); however MDA-MB-468 displayed no growth inhibition (Figure 8B) or apoptosis (Figure 8C) when treated with 100 ng/ml recombinant FasL. We then investigated if other possible death ligands may account for IRF-1's induction of the caspase cascade and found that recombinant TNFα was also insufficient to induce growth inhibition or apoptosis. Treatment with recombinant cross-linked TRAIL (Figure 9A) alone in MDA-MB-468 cells will induce apoptosis. We also tested Fas activating antibody, CH-11 (Figure 9A) as a surrogate for recombinant FasL and confirmed that apoptosis is not induced.

During our experimentation we found that not all recombinant TRAILS were equally adept at inducing apoptosis in our model and these differences in recombinant molecules may prevent us from adequately assessing death ligands as possible mediators of IRF-1 induced apoptosis. Therefore, as a secondary experiment, we also used neutralizing antibodies to the death receptors in an attempt to block IRF-1 induced apoptosis. While the neutralizing antibodies to TRAIL receptors DR4 and DR5 were able to block TRAIL induced apoptosis (Figure 9C), IRF-1 induced apoptosis was not inhibited by neutralizing antibodies to Fas, TNFR-1, or DR4/5 (Figure 9B). This finding suggests that IRF-1 does not induce apoptosis through the known death receptor/ligand binding mechanisms that characterize the extrinsic pathway.

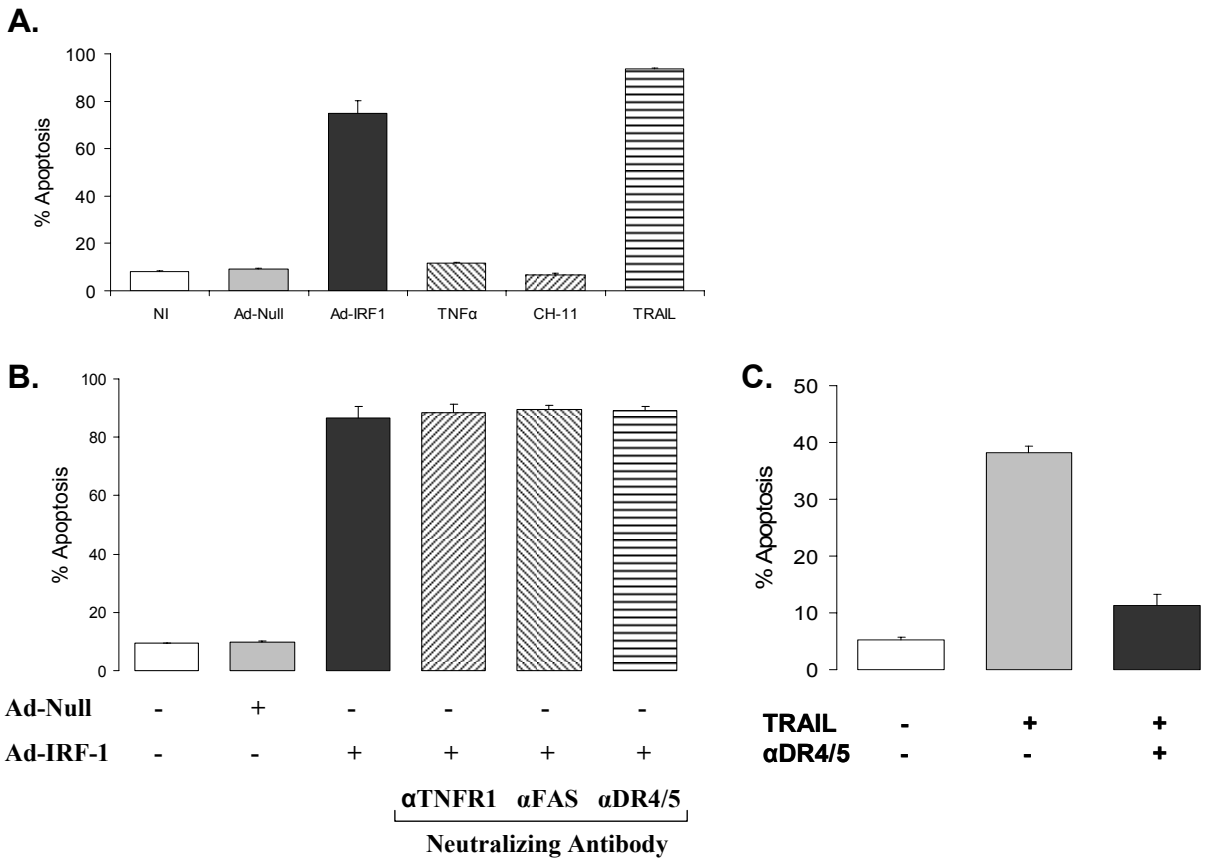

Figure 9. A. MDA-MB-468 cells are resistant to apoptotic induction by TNFα and Fas activating antibody, CH-11, but show high levels of apoptosis with IRF-1 and TRAIL. **B.** IRF-1 induced apoptosis is not decreased with neutralizing antibodies to known death receptors. **C.** TRAIL induced apoptosis is alleviated by neutralizing antibodies to TRAIL receptors, DR4/5.

2.3.5 IRF-1 induced apoptosis not mediated through soluble factors

Since no known death ligands accounted for IRF-1 induced apoptosis, we then tested whether an unknown soluble factor may mediate IRF-1's apoptosis. MDA-MB-468 cells were cultured separately in 6 well dishes and transwell inserts. The 6 well dishes, or parental wells, were treated with no infection, Ad-Ψ5, or Ad-IRF-1. After a four hour incubation period the parental wells were washed three times to prevent viral carry-over and the media was refreshed with or without recombinant TRAIL. Transwell inserts were then placed in the parental wells to determine if treated cells in the parental wells could induce a paracrine apoptotic signal.

Both samples cultured in the parental well and the transwell of the TRAIL treated media displayed increased apoptosis in comparison to no infection (Figure 10), suggesting that the transwell system appropriately allowed the flow through of soluble factors. While the cells in the parental well treated with IRF-1 underwent increased apoptosis, the cells in the corresponding transwell did not, implying that IRF-1's apoptotic induction does not rely on any soluble or secreted factor.

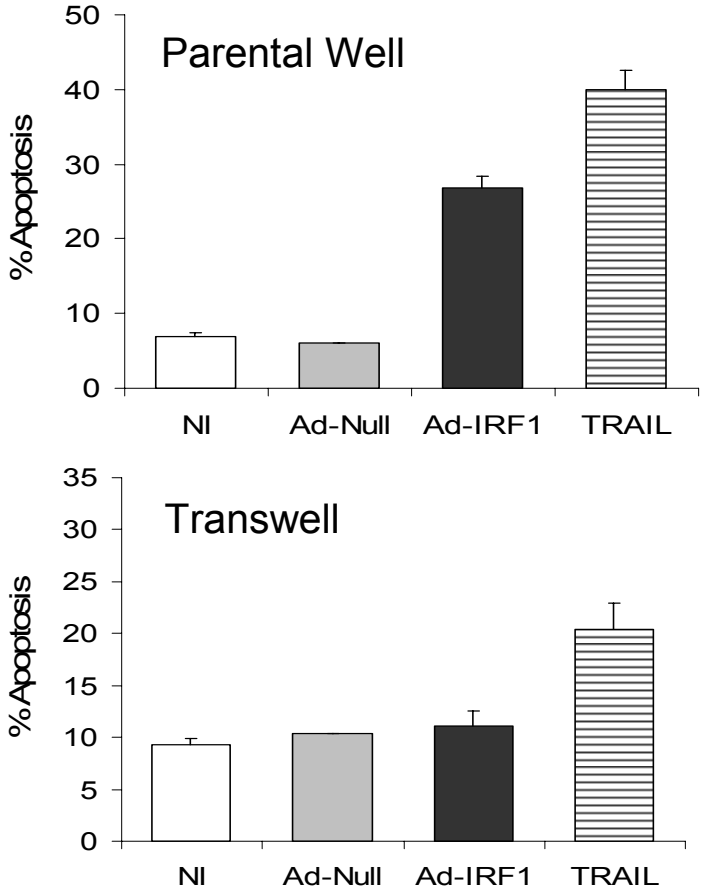

Figure 10. Parental wells treated with IRF-1 and TRAIL increased apoptosis, however no apoptosis was seen in cells sharing the same media as the IRF-1 treated cells. Dispersion of soluble factors from the parental well to the transwell is shown by the increase in apoptosis of cells sharing the same media as the TRAIL treated cells.

2.4 DISCUSSION

While IFNγ and IRF1 have been more described with respect to their ability to "sensitize" cells to apoptosis and more specifically sensitizing to death ligands, (Suk, Chang et al. 2001; Park, Seol et al. 2004) ; (Tomita, Bilim et al. 2003) our study focuses on direct induction of apoptosis by IFNγ and IRF-1. We assert that IFNγ induced apoptosis is mediated by IRF-1 and then focus our studies on the effects and mediators of IRF-1. Our claim is supported by previous studies where IRF-1 has been shown to regulate IFNγ induced apoptosis (Kano, Haruyama et al. 1999; Kim, Lee et al. 2002).

We and others showed that IRF-1 associated caspase cleavage correlated with apoptosis (Kim, Armstrong et al. 2004; Pizzoferrato, Liu et al. 2004; Bouker, Skaar et al. 2005). Also, we describe the inhibition of IRF-1 and IFNγ induced apoptosis in the presence of pan-caspase inhibitor, ZVAD. Since prior literature (Chow, Fang et al. 2000; Suk, Chang et al. 2001; Kirchhoff, Sebens et al. 2002; Clarke, Jimenez-Lara et al. 2004; Park, Seol et al. 2004) and our own studies have clearly suggested involvement of IRF-1 in death receptor/ligand mediated apoptosis, and we show dependence on caspases for IRF-1 mediated apoptosis, we hypothesized that IRF-1 was utilizing the extrinsic pathway to achieve its apoptotic induction.

Caspase 8 cleavage has known cross-talk with the intrinsic pathway of apoptosis; therefore we studied and found evidence of Bid cleavage and mitochondrial involvement in apoptosis. Caspase 8 inhibition altered the cleavage patterns of the downstream effector caspases 3 and 7; however, the upstream apical caspase 8 pattern is not altered by inhibitor to caspase 3/7. We also show that caspase 8 inhibitors, both c-FLIP and IETD, abrogate IRF-1 induced apoptosis. Collectively these studies including the cleavage of Bid and the likely involvement of mitochondrial apoptotic signaling suggest that caspase 8 is an "apical" caspase. This too fits with the hypothesized extrinsic mechanism of IRF-1 induced apoptosis and previous literature that indicates caspase 8 induction and activation via IRF-1 (Kim, Armstrong et al. 2004; Ruiz-Ruiz, Ruiz de Almodovar et al. 2004; Bouker, Skaar et al. 2005).

We then attempted to look upstream of caspase 8, at possible death receptors and ligands that may mediate the apoptotic signaling of IRF-1. Since IRF-1 is known to increase both FasL and Fas, (Chow, Fang et al. 2000; Watson, Queiroz de Oliveira et al. 2006) and because the Fas pathway is well known to induce caspase 8 activation, we began by verifying induction of FasL in our cancer model. We confirmed that cells treated with IRF-1 secreted an increased amount of FasL into the media. Assuming that secretion of FasL mediated IRF-1 induced apoptosis we then treated cells with recombinant FasL or CH-11, an agonistic Fas antibody, both at high concentrations. Surprisingly our cells displayed no apoptosis or growth inhibition. Billecke et al. also found MDA-MB-468 resistant to death receptor induced apoptosis (Billecke, Ljungman et al. 2002).

We then hypothesized that perhaps IRF-1 was utilizing a different death ligand, even though no literature has suggested IRF-1 transcriptional activity in either TRAIL or TNFα. We found that MDA-MB-468 cells treated with crosslinked TRAIL but not recombinant TNFα underwent apoptosis. To determine if the TRAIL ligand/receptor mediated IRF-1 induced apoptosis we then used neutralizing antibodies to inhibit TRAIL induced apoptosis but the same treatment did not inhibit IRF-1 induced apoptosis. Neutralizing antibodies to the other known death receptors were equally impotent in preventing IRF-1 induced apoptosis. This suggested that IRF-1 induced apoptosis is independent of known extrinsic death stimuli. We then determined through the use of transwell technology (Farberman, Hoffmann et al. 2004; Popi, Lopes et al. 2004) that IRF-1 did not utilize paracrine signaling of soluble factors to induce apoptosis. This implies that IRF-1's action of apoptotic induction rests in its ability to alter transcription of genes or through direct protein interaction.

In unpublished studies, Stang et al., has shown that DN-FADD abolishes IRF-1 induced apoptosis and that FADD and caspase 8 co-precipitate in cells treated with IRF-1. The proximal event to the activation of caspase 8 is then likely to be FADD dependent (Stang 2006). The involvement of FADD and caspase 8 signaling appears to be independent of any know death

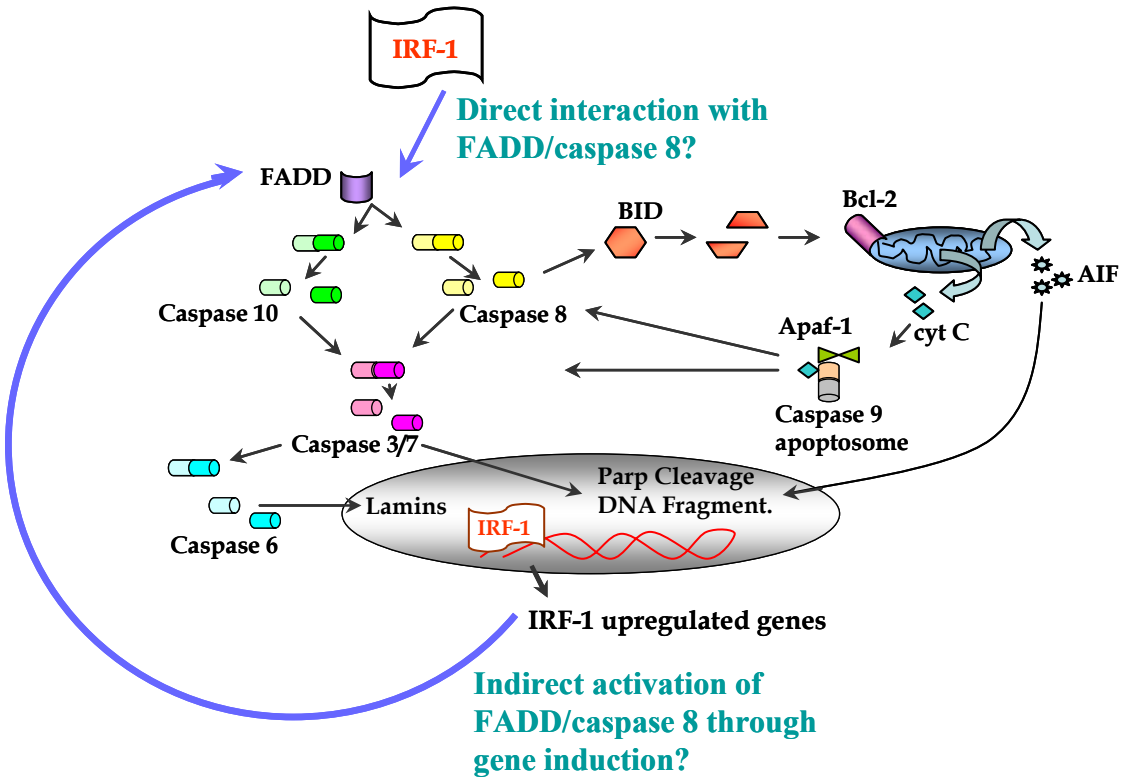

Figure 11. IRF-1 induction of apoptosis utilizes the caspase cascade beginning with caspase 8, but does not rely on known upstream death ligand/receptor binding. IRF-1 also induces Bid truncation which in turn stimulates mitochondrial release of cytochrome C and Smac/Diablo.

ligand or a more non-descript "soluble factor". This leads to obvious further study as to how IRF-1 might induce FADD/Caspase 8 complexes that appear to mediate IRF-1 induced apoptosis (Figure 11).

In conclusion, we have effectively demonstrated that IRF-1 induces a caspase mediated apoptosis that is likely mediated by caspase 8 activation. Interestingly, this induction of caspase 8 cleavage is likely FADD dependent (Stang 2006) but independent of any upstream autocrine or paracrine signaling including known death ligand/receptor complexing. Our further studies will focus on the mechanisms by which IRF-1 may induce transcriptional or protein interactions with FADD or Caspase 8 to induce autonomous activation of the caspase cascade (Figure 11).

3.0 IRF-1 LOCALIZES TO THE MITOCHONDRIA

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Unpublished

3.1 INTRODUCTION

The nuclear transcription factor, IRF-1, has been shown to regulate gene expression through both DNA binding activity and protein binding activity of coactivator protein, p300. Functional experiments have previously assumed and studied nuclear localization of IRF-1. However we have found IRF-1 in mitochondrial enriched fractions of IFN-γ stimulated MDA-MB-468 cells. While repeated experiments confirmed this novel discovery, there is currently little evidence to support a specific function.

Though commonly known to localize to the nucleus, especially when induced by retinoic acid (Niitsu, Higashihara et al. 2002; Arany, Ember et al. 2003; Wang, Peng et al. 2006) and IFNγ (Connett, Hunt et al. 2003), IRF-1 has been found in the cytoplasm in breast cells (Connett, Hunt et al. 2003), endometrium (Qiao, Prabhakar et al. 2002), and immuno related monocytes and macrophages (Kuroboshi, Okubo et al. 2003). One group found that IRF-1 may have interactions with lysosomal or mitochondrial bound proteases thereby increasing IRF-1 binding activity (Qiao, Prabhakar et al. 2002) though they did not speculate if IRF-1 was translocating to facilitate these interactions.

More than a hundred years ago mitochondria were described by early light microscopists. Their subsequent isolation by differential centrifugation and structural studies by electron microscopy gave rise to cellular biology. Later experiments recognized mitochondria as a central element of cellular metabolism, the "powerhouse" that creates ATP. The discovery of mitochondrial DNA gave rise to both disease studies of mutations in the mtDNA and evolutionary studies that probe the quandary of two genomes interacting within an organelle and a cell. Lately attention has been focusing on the non-metabolic functions of mitochondria, including its role in apoptosis.

The mitochondria are double membraned organelles that contain a loop of DNA that encodes for ribosomal and tRNAs and proteins primarily involved in ATP synthesis. These genes are compacted to the point that promoter regions may reside within the translated regions of the mitochondrial DNA in addition to being identified in the D-loop region. Currently no in vivo

procedure exists to test promoters with reporter genes in mitochondria. It is notable that over 1000 nuclear genes encode for mitochondrial proteins, for example the mitochondrial transcription factor (mtTFA) and 10 of the 13 cytochrome oxidase (COX) IV subunits are encoded in the nucleus. This implies that a large number of proteins translocate to the mitochondria in order to assist in proper mitochondrial function and includes interactions with the mitochondrial genome (Scheffler 2002).

Although no literature of IRF-1 translocation to the mitochondria exists, another nuclear transcription factor and tumor suppressor, p53, has recently been found to localize to the mitochondria (Marchenko, Zaika et al. 2000; Lomonosova, Subramanian et al. 2005) and to induce apoptosis independent of transcription (Moll, Wolff et al. 2005). The findings thus far evidence that p53 interacts with mitochondrial import proteins and Bcl-2 family members to permeabilize the outer mitochondrial membrane and induce release of cytochrome c. These recent studies of p53 may provide insight into the functions of IRF-1 in the mitochondria.

Additionally, survivin, a tumor antigen and inhibitor of apoptosis that is suppressed with IRF-1 expression, (Pizzoferrato, Liu et al. 2004) has been found in the mitochondria in tumor cell lines (Dohi, Beltrami et al. 2004) This population of survivin inhibits apoptosis through rapid expansion and release into the cytosol upon the cells reception of stress or apoptotic stimulation. The mechanism of IRF-1 downregulation of survivin has yet to be characterized and this newly discovered proximity of possible interaction within the mitochondria creates intriguing hypotheses.

3.2 MATERIALS AND METHODS

Cell Lines and Culture:

P53 mutated MDA-MB-468 was purchased from American Type Culture Collection (Manassas, VA). The MDA-MB-468 tumor cells were propagated in Dulbecco's modified Eagle's medium (BioWhittaker, Inc., Walkersville, MD) and Ham's F-12 (Invitrogen Life Technologies, Carlsbad, CA) media at a 1:1 ratio with 10% fetal bovine serum, L-glutamine and antibiotics. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Isolation of mitochondrial enriched fraction:

MDA-MB-468 cells were cultured at 3 x 10^6 cells per 10 cm dish and after overnight adhesion treated with either no treatment, IFNγ (1000 units/ml), Ad-Psi5 or Ad-IRF-1 (MOI 25). 24 hours later cells were harvested with trypsin (0.25%), washed and resuspended in cytosol extraction buffer in accordance with Mitochondrial/Cytosol Fractionation kit (BioVision, Mountain View, CA) instructions. Fractionation continued by kit instructions with the exception that the mitochondrial pellet was resuspended in 50 ul of mitochondrial buffer.

Immunoblotting Analysis and Antibodies:

Protein from either whole cell lysates or cytosolic and mitochondrial fractions were separated by SDS–PAGE and transferred onto a nitrocellulose membrane. Loading of equal protein amounts was assessed by staining of nitrocellulose membranes with 0.1% Ponceau S (Sigma) in 5% acetic acid. Nonspecific binding was blocked with PBS-T (14 mM sodium phosphate, monobasic, monohydrate; 88 mM dibasic sodium phosphate, anhydrous; 100 mM NaCl; and 0.1% Tween 20) containing 5% nonfat milk for 1-h incubation with agitation at room temperature. Antibodies were purchased as follows: Grp75, Calnexin, α-tubulin, and PCNA (Abcam, Cambridge, MA), IRF-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Immuno florescence:

MDA-MB-468 cells were cultured on cover slips in a 12 well plate. Cells were treated with mock or IFNγ (1000 units/ml) for 24 hours. Cells were then incubated with 100 nM Mitotracker Red (Cambrex) for 30 minutes. Cells were fixed in 2% paraformaldehyde and permeabolized with 0.1% Triton X in PBS for 15 minutes. After washing in PBS and 0.5% BSA, cells were blocked with 1% BSA and 5% Goat Serum (Sigma Alderich) for 45 minutes. Cells were probed with hIRF-1 ab (Santa Cruz, 1:1000 dilution) followed by incubation with a FITC labeled secondary. Cells were subsequently stained with Draq5 (BioStatus, Leicestershire, United Kingdom). Cover slips were mounted on slides with gelvatol. Several pictures (x10 magnification) of the cells were taken with an Olympus BX51 fluorescent microscope (Olympus America, Melville, NY).

Immunoelecton microscopy:

Mock and IFNγ treated mitochondrial fractions of MDA468 cells or mouse liver section (kindly provided by Dr. Alan Tsung) were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde for 20 min at room temperature and embedded in LR White resin under UV cross-linking on ice. Ultrathin sections will be labeled with mouse IgG or IRF-1 antibodies (10 µg/ml) followed by sheep anti-mouse IgG conjugated to 12 nm colloidal gold (Jackson ImmunoResearch) and viewed under a Zeiss EM 10. Secondary antibody alone will be run as control. These studies will be performed in collaboration with Dr. Donna Stolz at the Center for Biologic Imaging. MDA-MB-468 cells were labeled with a polyclonal IRF-1 Ab (Santa Cruz). Mice liver sections labeled for IRF-1 by immuno EM with mouse monoclonal Ab (BD transduction labs, cat # 612046, mouse 2nd)

Images were quantitated for the number of gold flecks identified per mitochondria in the MDA-MB-468 cells treated with Mock (n=15 mitochondria) and IFNγ (n=10 mitochondria). Averages and standard deviations were graphed. Students two-tailed T-test for unequal variance was applied.

Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences:

Available online at http://psort.hgc.jp/, PSORT II Prediction was used to analyze the IRF-1 amino sequence (NCBI sequence: P10914 – IRF-1). Briefly, PSORT II analyzes the amino sequences of animal and yeast genes by applying rules for sequence features of known protein sorting signals. Then it computes and reports the likelihood of the input protein to be localized at each candidate site.

Binding sequence homology:

IRF-1 known binding sequences were derived from the current literature (Upreti and Rath 2005) and compared against the full mitochondrial DNA sequence. Only exact matches were reported.

3.3 RESULTS

3.3.1 IRF-1 localizes to the mitochondria

While elucidating the mediation of IRF-1 induced apoptosis through the intrinsic pathway, we discovered IRF-1 in the mitochondrial enriched fraction of IFNγ treated cells. This unexpected detection of cellular IRF-1 in the mitochondrial enriched fraction was, at first, consigned to technical error. Repeated and better controlled experiments led us to believe that we were observing a previously unreported phenomenon of IRF-1 localization in mitochondrial enriched fractions of MDA-MB-468 cells treated with IFNγ by immunoblot (Figure 12). These lysates were also probed for α-tubulin (cytosolic marker), Grp75 (mitochondrial marker), PCNA (nuclear marker) and calnexin (ER marker) to determine the purity of the fractionation. While PCNA was detected in the mitochondrial fraction, this contamination was ruled out as confounding factor, since equal or greater amounts of PCNA were also found in the cytosolic sample treated with IFNγ, but no IRF-1 was detected.

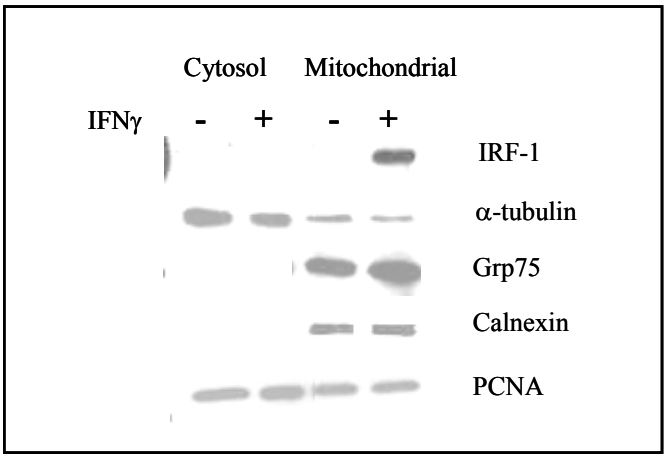

Figure 12. IFNγ treated MDA-MB-468 cells express IRF-1 in the mitochondrial enriched fraction.

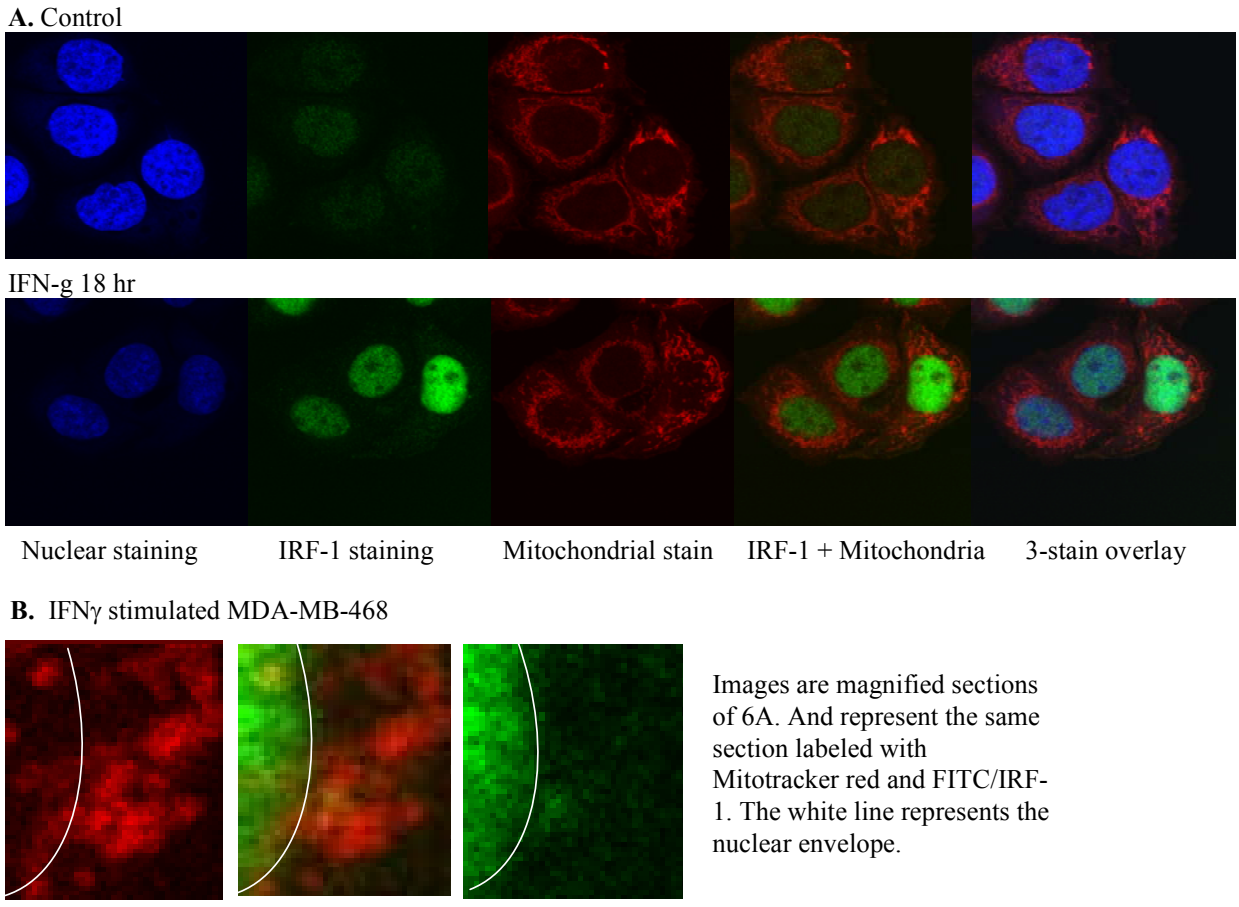

Figure 13. A. Immunoflroscence of MDA-MB-468 shows increased IRF-1 in response to IFNγ treatment; Control cells have no IFNγ. **B.** Magnification clarifies cytosolic IRF-1 and overlap of IRF-1 with mitochondria.

Calnexin associating with the mitochondrial enriched fraction by immunoblotting suggested that IRF-1 in the process of being translated or otherwise related with the ER may also explain our results. Immuno-florescent images (Figure 13A & B) verified cytoplasmic IRF-1 that overlapped with areas stained with mitotracker red, however true co-localization of IRF-1 with the mitochondria was difficult to determine and did not rule out IRF-1 association with the ER as an explanation for our results. We did note that IRF-1 was significantly upregulated in the nucleus in response to IFNγ stimulation.

Electron microscopy clearly revealed IRF-1 localizing to the mitochondria in MDA-MB-468 cells with (right) and without (left) IFN-γ treatment (Figure 14A). Surprisingly, cells treated

with IFNγ, displayed significantly less labeling than cells treated with mock (Figure 14B). This contradicts our immunoblotting data that demonstrates an increase of IRF-1 in the mitochondrial enriched fraction with IFNγ treatment and suggests that further testing is in order. Mouse liver sections labeled for IRF-1 and analyzed by electron microscopy (Figure 14C) with a mouse specific IRF-1 antibody also revealed IRF-1 localizing in the mitochondria.

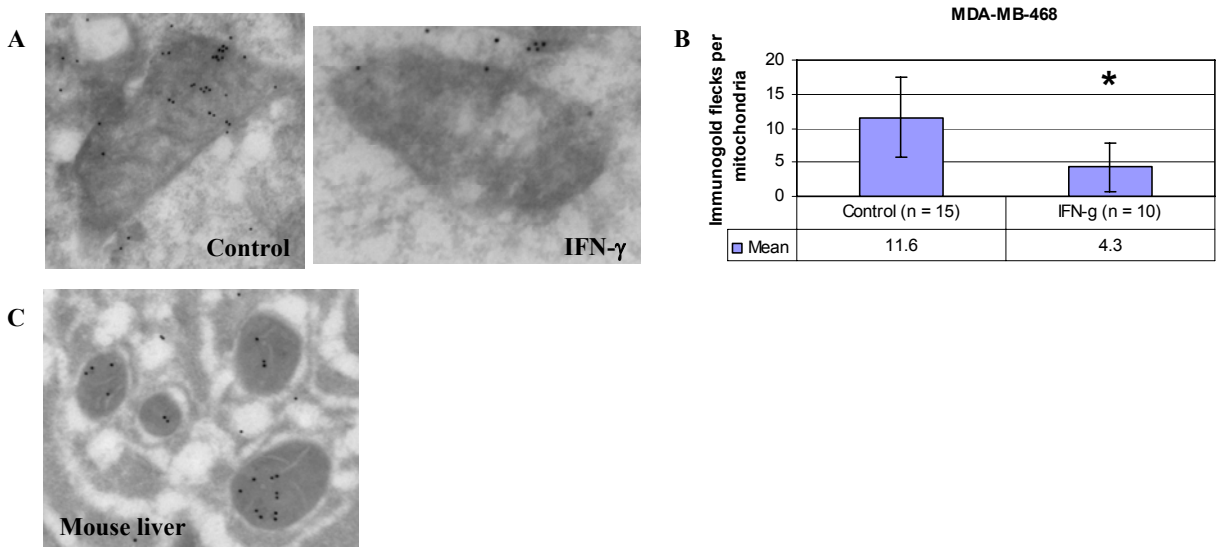

Figure 14. A. Mitochondria of untreated MDA-MB-468 (left) and with IFN-γ (right) present with IRF-1 labeling through electron microscopy. **B.** MDA-MB-468 cells treated with IFNγ display fewer immuno gold particle markers of IRF-1 than mock,(* = $p<0.001$). **C.** Mouse liver sections also reveal IRF-1 labeling in the mitochondria

In an effort to further elucidate a mechanism by which IRF-1 translocates to the mitochondria we performed an analysis that predicts protein sorting signals and localization sites in amino acid sequences (PSORT II). This analysis found that IRF-1 has nuclear localization sequences and a DNA binding motif (appendix A), as expected, and possible mitochondrial targeting sequences as well as a Gavel cleavage site that would predict mitochondrial localization. The data is summarized in Figure 15.

Input Sequence (NCBI sequence: P10914 – IRF-1)

QUERY (325 aa)
MPITRMRMRP WLEMQINSNQ IPGLIWINKE EMIFQIPWKH AAKHGWDINK DACLFRSWAI
HTGRYKAGEK EPDPKTWKAN FRCAMNSLPD IEEVKDQSRN KGSSAVRVYR MLPPLTKNQR
KERKSKSSRD AKSKAKRKSC GDSSPDTFSD GLSSSTLPDD HSSYTVPGYM QDLEVEQALT
PALSPCAVSS TLPDWHIPVE VVPDSTSDLY NFQVSPMPST SEATTDEDEE GKLPEDIMKL
LEQSEWQPTN VDGKGYLLNE PGVQPTSVYG DFSCKEEPEI DSPGGDIGLS LQRVFTDLKN
MDATWLDSLL TPVRLPSIQA IPCAP

Results of the *k*-NN Prediction

k = 9/23
82.6 %: nuclear
8.7 %: mitochondrial
4.3 %: cytoplasmic
4.3 %: cytoskeletal
>> prediction for QUERY is nuc (k=23)

Figure 15. PSORT Analysis of IRF-1 (Appendix A for full results).

3.3.2 Possible functions of IRF-1 in the mitochondria

As a nuclear transcription factor with known DNA binding sequences (Upreti and Rath 2005), we queried the mitochondrial genome for possible binding sites for IRF-1. Only exact matches were reported. Six binding sites were found, representing two sequences. Four of these sites were clustered in a 750 base pair region of the mitochondrial 12S rRNA gene (Figure 16).

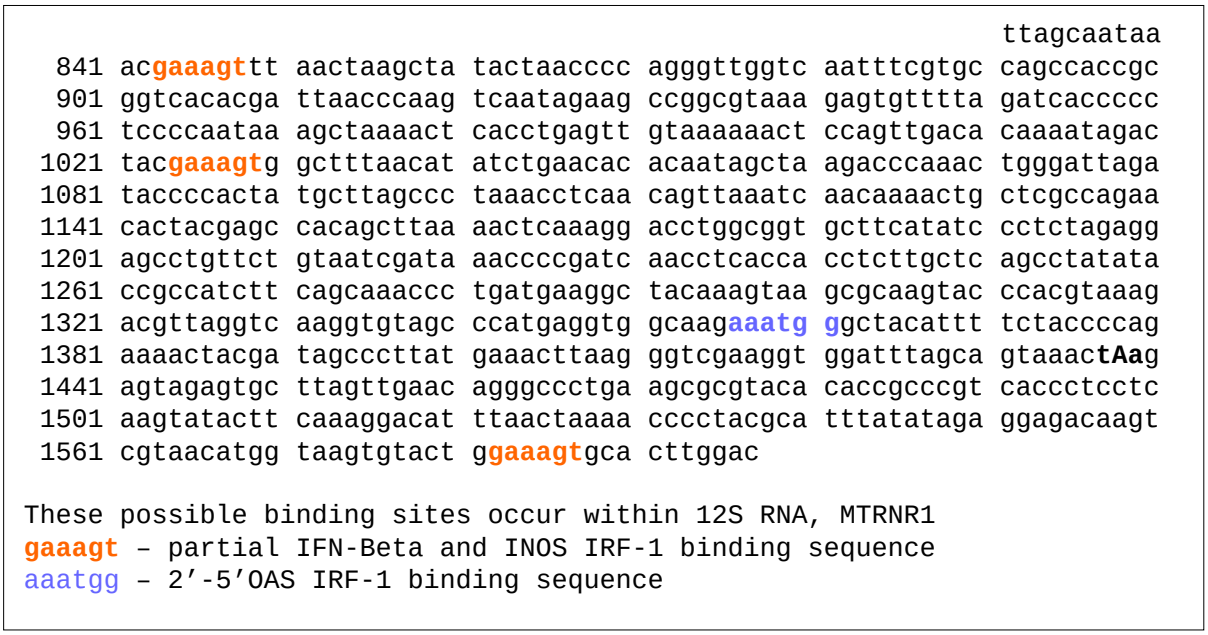

Figure 16. Putative IRF-1 binding sequences in the mtDNA.

3.4 DISCUSSION

While studies have acknowledged cytoplasmic IRF-1 as a nuclear transcription factor, IRF-1 has been assumed to be active primarily in the nucleus. Our novel observation of IRF-1 in a mitochondrial enriched fraction of cells treated with IFNγ, both by western blot and Immuno EM suggests that it may function in the mitochondria as well. It is noteworthy that endoplasmic reticulum (ER) marker, calnexin, was also found isolated to the mitochondrial enriched fraction. ER is known as an important control point for mitochondrial apoptosis because it harbors Bcl-s, Bax, and Bak (Breckenridge, Germain et al. 2003).

PSORT analysis of IRF-1’s amino sequence supports this hypothesis by predicting IRF-1 principally translocates to the nucleus, however the analysis indicates IRF-1 may have sequences and a Gavel cleavage site that encourage translocation to the mitochondria. While these positively charged amino termini mitochondrial targeting sequences lack a strictly conserved

primary structure (Roise 1997), conserved amino acid sequences within the mitochondrial targeting peptides are associated with cleavage by matrix processing proteases (Gavel and von Heijne 1990). Gavel et al. suggests that these motifs have a predictive value of proteins that localize to the mitochondria. Immunoflorescence vividly displays IRF-1 in the nucleus, especially so with IFNγ treatment, however mitochondrial localization was difficult to determine with this technique. We then pursued immunoelectron microscopy which shows IRF-1 in the mitochondria of both cultured breast cancer cells and mouse liver samples.

Further confirmation of IRF-1 in the mitochondria is necessary in light of our conflicting findings regarding IFN-γ upregulation of IRF-1 in the mitochondria by immunoblotting and yet apparent decrease with IFN-γ when immunogold flecks were counted in the immunoelectron micrographs. These experiments would rely on our adenoviral expression of IRF-1 to see if IRF-1 is increased in the mitochondria as well as siRNA to IRF-1 to look for decreases in mitochondrial IRF-1. If we see no differences with these treatments the other results may indicate non-specific binding of our antibodies.

Since IRF-1's traditional function is to bind promoter sequences and regulate transcription, we scanned the mitochondrial DNA for possible IRF-1 binding sequences. Six sites were found, four of which were clustered in a 750 bp region in the mitochondrial DNA sequence coding for 12S ribosomal RNA, designated MTRNR1.

These sequences may or may not be found in a promoter region and prompt questions of whether or not IRF-1 would bind to these sequences and if so what function would be served by that action? Also perplexing is the discovery that in cells treated with IFNγ a statistically significant decrease of IRF-1 binding immuno-gold staining was seen in the immunoelectron images. This may suggest that IRF-1 is leaving the mitochondria in response to IFNγ treatment. However this result is opposite of what was observed in the cellular fractionation data. Also interesting is the presence of IRF-1 in the mitochondria of mouse liver tissues, which may imply a normal role for IRF-1 that is independent of disease status.

These observations create obvious questions for further study. A) Where is the IRF-1 localized within the mitochondria? We performed a trial digitonin study, unfortunately, the data was inconclusive. Further advancement of the technique and subsequent trials may yield better results. B) Is the IRF-1 binding the mitochondrial DNA? Isolation and tagging of the MT-DNA as a probe for EMSA would produce evidence to answer that question. C) If binding is found

does IRF-1 serve to upregulate or suppress 12S rRNA, or does IRF-1's binding effect genes downstream/upstream of the binding site? Mitochondrial gene chip data may be enlightening as to possible MT-DNA transcriptional regulation; alternatively RT-PCR for mitochondrial genes of interest may also produce results. D) If IRF-1 is not binding MT-DNA is it interacting with other proteins in the mitochondria, like survivin that has also been found in the mitochondria? Isolation of mitochondria followed by careful immunoprecipitation of IRF-1 and western blotting for possible protein targets may be in order.

Based on the target of IRF-1 binding/interaction further questions about significance could be formed. If IRF-1 is binding and upregulating 12S rRNA transcription, it's translocation away from the mitochondria in response to IFNγ treatment may slow the production of other mitochondrial genes and possibly downstream ATP synthesis, thus working to abrogate cellular functions. Alternatively IRF-1 may be interacting with proteins, similar to its interactions with p300, to regulate growth or possibly for some other purpose or downregulating bound mitochondrial genes. As there is no other literature regarding this observation, our further speculations and questions currently stand alone and remain, so far, untested.

Thus far we have explored the role of IRF-1 in apoptosis through the extrinsic and intrinsic death pathways and followed an intriguing tangent to the novel detection of IRF-1 mitochondrial localization. We currently resume our studies of growth inhibition, this time focusing on the role of p21 induction by IRF-1.

4.0 P21 MEDIATES IRF-1 INDUCED GROWTH INHIBITION

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Manuscript in preparation

4.1 INTRODUCTION

The nuclear transcription factor IRF-1 is a known tumor suppressor that binds interferon response elements in the promoters of genes including p21 (Coccia, Del Russo et al. 1999) to induce their expression (Pizzoferrato, Liu et al. 2004). IRF-1 is also a mediator of anti-cancer therapeutics that employs both apoptotic and cell cycle arrest properties. Previously, we detailed a caspase mediated mechanism of IRF-1 induced apoptosis. In this study we began characterizing the cell cycle arrest properties of IRF-1 and reveal a relationship between IRF-1 and p21 mediated growth inhibition in cancer.

IRF-1 was first discovered in studies by the Taniguchi lab (Fujita, Sakakibara et al. 1988; Miyamoto, Fujita et al. 1988); these findings supported a relationship between p53 and IRF-1 in tumor suppression of transformed mouse embryonic fibroblasts (MEFs). Induction of cell cycle arrest by IRF-1 was primarily suggested by MEFs from IRF-1 -/- mice that were found deficient in their ability to undergo DNA-damage-induced cell cycle arrest, a phenotype similar to that observed in MEFs lacking p53 (Tanaka, Ishihara et al. 1996). Prost et al (Prost, Bellamy et al. 1998) confirmed and furthered these studies in a hepatocyte model by finding that IRF-1 null cells are deficient in both global and transcription-coupled repair. These studies imply that IRF-1 may be an important mediator of DNA repair and may regulate the corresponding cell cycle arrest. Moreover this alludes that mutation or deletion of IRF-1 may lead to dysregulated cell cycle progression that may then promote neoplastic formation.

Numerous studies since then associate IRF-1 with cell cycle arrest. IRF-1 was shown to mediate G1 arrest in cervical squamous cells when those cells were infected with measles virus. Transfection of antisense IRF-1 released the cervical squamous cells from virus induced growth arrest (Yokota, Okabayashi et al. 2004). The Stevens group (Stevens and Yu-Lee 1992; Stevens, Wang et al. 1995), in a separate studies demonstrated biphasic IRF-1 production in prolactin treated T-cells. Increases in IRF-1 expression correlated with arrests in early G1 and at the G1/S transition and were transcriptionally induced by prolactin response elements located in the IRF-1

promoter. These publications of IRF-1 and G1/S arrest support IRF-1 mediation but do not indicate a mechanism by which IRF-1 regulates the cell cycle.

In related experiments, IRF-2 was identified in cell cycle progression and DNA replication through upregulation of the cell cycle element, H4 histone activity in early G1(Vaughan, Aziz et al. 1995). This implies that as the natural antagonist to IRF-2, IRF-1 may inhibit cell cycle progression possibly through competitive binding of the same sequence. The authors of this study also note in a later publication that IRF-2 acts to promote cell cycle progression in an E2F independent manner at the G1/S phase transition (Vaughan, van der Meijden et al. 1998).

Not only has IRF-1 been linked to cell cycle arrest, specifically at the G1/S transition, but studies also reveal putative mediators of cell cycle arrest that are being associated with IRF-1. IL-4 anti-proliferative effects in renal cell carcinoma were mediated by upregulation of IRF-1 (Yu, Kim et al. 2004). There was a corresponding increase in p21/Waf1, a known cell cycle

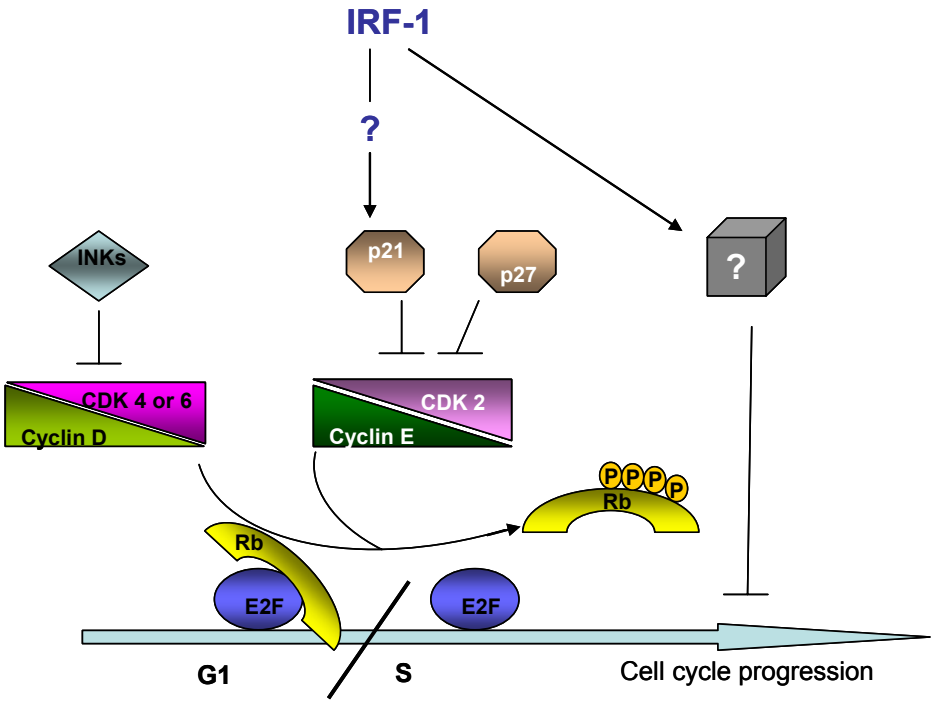


Figure 17. Hypothesized IRF-1 induction of G1/S cell cycle arrest

inhibitor, and a decrease in Cdk2 with IRF-1 upregulation in this study. Induction of p21 in response to IRF-1 is not unexpected since the finding of three IRF-1 binding sites within the mouse p21 promoter region (Coccia, Del Russo et al. 1999). p21 functions to inhibit cell cycle progression through inhibition of G1/S cyclin/Cdk complexes, including Cdk2 (Harper, Elledge et al. 1995). Cdk 2, involved in G1 and S progression through phosphorylation and inactivation of retinoblastoma, has been shown to be suppressed by IRF-1 (Xie, Gupta et al. 2003). Additionally antisense IRF-1 partially releases cells from TGF-β induced growth arrest, suggesting that IRF-1 is a mediator of TGF-β induced growth suppression. This study also showed that IRF-1 is found to induce p21/Waf1 expression (Miyazaki, Sakaguchi et al. 2004) with TGF-β treatment and established that IRF-1 mediation of cell cycle arrest was independent of secreted factors, suggesting that IRF-1 induces cell growth inhibition through its transcriptional activity. Detjen et al. recognized that IFNγ, the primary inducer of IRF-1, prevented the induction of G1 cyclins and reduced the activity of Cdk4 and Cdk2 in SK-Hep-1 cells (Detjen, Murphy et al. 2003). Interestingly they also found ***decreases*** in p21 with IFNγ treatment that mediated apoptosis in hepatocellular carcinoma cell, suggesting that the presence of p21 protects the cell from apoptosis.

Molecular studies of IRF-1 mediated cell cycle suppression are forming the foundation of more clinically relevant studies. MIS (Hoshiya, Gupta et al. 2003) and N-RAS treatments (Passioura, Dolnikov et al. 2005), whose growth inhibitory effects are mediated by IRF-1, exhibit cell cycle arrest. Oberg (Oberg 2000), reviewed literature on over 500 patients with neuroendocrine gastroenteropancreatic tumors that were clinically treated with IFNα, a known inducer of IRF-1. IFNα treatment of midgut carcinoids is associated with G1/S cell cycle arrest and induction of IRF-1, p21, and p27. These studies further bolster a potentially important clinical role of IRF-1 in cell cycle arrest. Yano et al, (Yano, Iemura et al. 1999), found that cell growth inhibition in liver cancer cell lines was proportional to the dose of IFNα and that this was due in part to G1/S arrest in 12 of the 13 cell lines studied. However, IFN therapy has adverse effects that are not seen when utilizing the downstream effectors IRF-1 and ICSBP that serve to upregulate p21 and p27 expression to inhibit lens carcinoma cells (Egwuagu, Li et al. 2006).

These studies when taken together strongly suggest that IRF-1 may mediate a G1/S cell cycle arrest through induction of p21 (Figure 17), and that this may offer a protective effect against apoptosis. We also garner from these studies that IRF-1 mediated cell cycle arrest is important in the efficacy and development of anti-cancer agents for multiple cancers.

4.2 MATERIALS AND METHODS

Cell Lines and Culture:

p53 mutated MDA-MB-468 and H1299 were purchased from American Type Culture Collection (Manassas, VA). The MDA-MB-468 tumor cells were propagated in Dulbecco's modified Eagle's medium (BioWhittaker, Inc., Walkersville, MD) and Ham's F-12 (Invitrogen Life Technologies, Carlsbad, CA) media at a 1:1 ratio with 10% fetal bovine serum, L-glutamine and antibiotics. H1299 were propagated in RPMI (BioWhittaker, Inc., Walkersville, MD) with 10% fetal bovine serum, L-glutamine and antibiotics. All cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Immunoblotting Analysis and Antibodies:

Protein from either whole cell lysates or cytosolic and mitochondrial fractions were separated by SDS–PAGE and transferred onto a nitrocellulose membrane. Loading of equal protein amounts was assessed by staining of nitrocellulose membranes with 0.1% Ponceau S (Sigma) in 5% acetic acid. Nonspecific binding was blocked with PBS-T (14 mM sodium phosphate, monobasic, monohydrate; 88 mM dibasic sodium phosphate, anhydrous; 100 mM NaCl; and 0.1% Tween 20) containing 5% nonfat milk for 1-h incubation with agitation at room temperature. Antibodies were purchased as follows: Cyclin D, Cyclin E, Cdk 2, Cdk 4, Cdk 6 (Abcam), p21 and IRF-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Cycle Analysis:

Cells were incubated with BrdU 3 hours prior to being harvested with 0.25% trypsin, rinsed in PBS, and fixed in 70% ultra cold ETOH while vortexing. Cells were then incubated with FITC-anti BrdU and stained with propidium iodide according to manufacturers directions

(BD Pharmingen). Cell cycle was assessed by flow cytometric analysis using a Becton Dickinson FACSort (San Jose, CA, USA) (Becton Dickinson, St Louis, USA).

Cells were harvested using 0.25% trypsin, rinsed in PBS, and fixed in 80% ultra cold ETOH. Cells were then washed and suspended in PBS prior to treatment with 200 ug/ml DNase free RNase A followed by 30 ug/ml propidium iodide for 30 minutes at room temperature. A minimum of 10,000 cells per sample was recorded using Coulter Epics LX-MCL (Beckman Coulter, Fullerton, CA) flow cytometer. Cellular debris and doublets were excluded. Data analysis using Modfit software was performed, and percentages of cells in each phase of cell cycle were calculated.

p21 knockdown:

In order to determine the role of p21 in IRF-1 induced cell cycle arrest, we plan to perform Ad-IRF-1 infection of MDA-MB-468 cells which are treated with CDKN1A (p21) siRNA (Ambion, Austin, TX). We will confirm p21 knockdown by western blot and will evaluate differences in growth between the MDA-MB-468 p21 knockdowns and the parental cell lines via PI staining.

shRNA plasmid vector construction

shRNA specific for human p21 was designed according to the manufacturers protocol for designing shRNA hairpins encoded by the shRNA expression vector pSilencer CMV 4.1 (Ambion, Austin, TX). The target sequence was attained from a predesigned and verified siRNA purchased again from Ambion ("pre-designed and validated siRNA ID #1621"). The 55 nt oligonucleotides encoding human p21 specific shRNA were designed by employing Ambion's online tool (http://www.ambion.com/techlib/misc/psilencer_converter.html). The sense and antisense oligo-nucleotides were annealed and ligated to the BamHI and Hind III sites of pSilencer 4.1-CMV (Ambion Co., Austin, TX) to form plasmid pSilencer 4.1-CMV-p21 shRNA. The inserts were confirmed by sequencing. pSilencer 4.1-CMV-NEG was the negative control of nonspecific shRNA provided by the company.

Stable clones were generated by transfecting the respective plasmid using FuGene (Roche, Basel, Switzerland) per the manufacturers protocol followed by selection with 600 micrograms/mL of G418. At least 10 healthy colonies were isolated using sterile cloning rings.

Following expansion, the stable cell lines were then screened for p21 knock-down by western immunoblot from human p21 following stimulation with IFN-g 1000 U/ml for 24 hours.

Apoptosis Assay:

Cells were harvested using 0.25% trypsin, rinsed in PBS, and stained using the annexin V-FITC apoptosis detection kit according to the manufacturer's instructions (BD PharMingen, San Diego, CA, USA). Apoptosis was assessed by flow cytometric analysis using a Becton Dickinson FACSort (San Jose, CA, USA). A minimum of 10,000 cells per sample was recorded, and cell debris was excluded by appropriate forward light scatter threshold setting. Data analysis using CELLQuest software (Becton Dickinson, St Louis, USA) was performed, and numbers of cells positive for Annexin V-FITC, PI, or combinations thereof, were calculated.

4.3 RESULTS

4.3.1 p21 mediates IRF-1 induced cell cycle arrest

4.3.1.1 IRF-1 induces p21 and p27

Previous studies identify IRF-1 as an inducer of p21 so we first verified p21 induction with IRF-1 overexpression through infection of Ad-IRF-1 into our breast adenocarcinoma, MDA-MB-468 (Figure 18). Western blotting for IRF-1 and p21 clearly demonstrates the effectiveness of IRF-1 gene transfer and induction of p21 that is not seen with controls of no- infection (NI) or virus alone (Ad-Ψ5). Since p27 is a homologue of p21 and fellow inhibitor of cyclin dependent kinases, induction of p27 was anticipated and also verified.

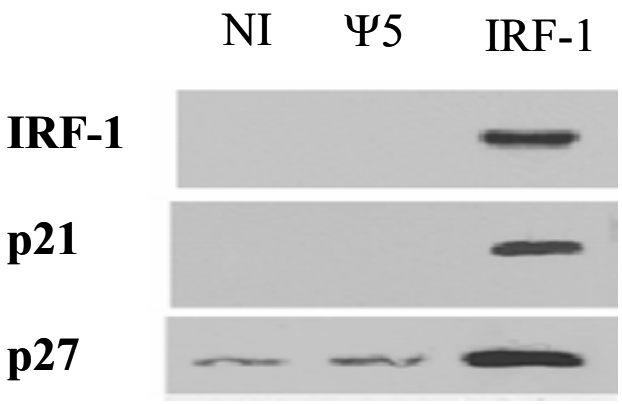


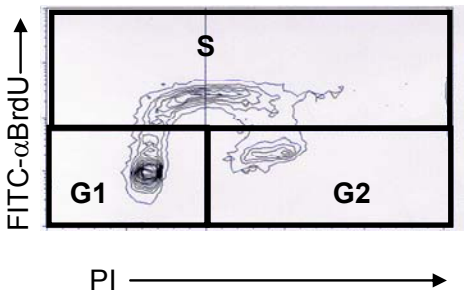
Figure 18. IRF-1 induces p21 and p27

Table 1. A. IRF-1 induces a time and dose dependent G1 arrest in MDA-MB-468 **B.** An example of bivaraite staining

A.

		Day 1			Day 2		
	MOI	G1%	S%	G2%	G1%	S%	G2%
NI	0	56	34	10	63	28	9
Psi-5	2.5	49	38	13	54	28	19
	5	46	39	15	46	29	25
	10	42	41	17	43	25	32
IRF-1	2.5	54	34	12	62	26	12
	5	57	29	14	66	22	12
	10	61	22	17	72	15	13

B.



4.3.1.2 IRF-1 induces G1 arrest

With p21 induction via IRF-1 treatment established, we then analyzed cells for cell cycle arrest through FITC-BrdU labeling and propidium iodide staining of our MDA-MB-468 cell line. We chose to do both 24 and 48 hours and a range of doses in order to determine the relationship of IRF-1 to cell cycle phases (Table 1). While virus alone suppressed the percentage of cells in G1 at 24 and 48 hours and increased the proportion of cells in S phase at 24 hours, IRF-1 gene transfer opposed the effects of virus to induce a G1 arrest that is dose dependent both at 24 and 48 hours. This is significant in suggesting that IRF-1 is opposing the effect of adenovirus in generating a G1 arrest. IRF-1 treatment extended and increased this effect at 48 hours.

4.3.1.3 Cyclin and cyclin dependent kinase regulation by IRF-1

Since IRF-1 effectively induced p21 and G1 arrest, we then evaluated the effect of IRF-1 on cyclins and Cdks associated with cell cycle progression (Figure 19). Cell cycle inhibitors p21 and p27 are known to effectively inhibit Cyclin E/Cdk2 complexes and have been shown to associate, but less effectively inhibit Cyclin D/Cdk4 or 6 complexes (Sherr and Roberts 1999). Our immunoblot indeed shows a decrease in Cdk2 and Cyclin E. We also observe decreases in Cdk1 and 4 and Cyclin A. There was no obvious change in Cyclin D2 and interesting increase in Cdk6 expression.

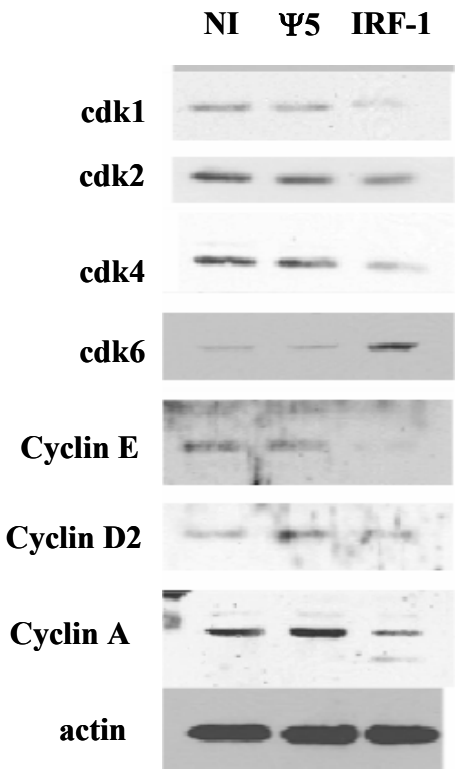


Figure 19. Effects of IRF-1 on Cyclins and Cdks in MDA-MB-468.

4.3.1.4 Knockdown of p21 prevents IRF-1 induced G1 arrest in H1299 cells

With advancement of our techniques and data, we further evaluated the cell cycle effects of IRF-1 by PI staining alone with the addition of p21 siRNA to determine if IRF-1 induced cell cycle arrest is abrogated with p21 suppression (Figure 20B, C, and D). We first confirmed that siRNA to p21 was effective in inhibiting p21 and was specific (Figure 20A). Western blotting of H1299 lysates shows upregulation of p21 by IRF-1 and p21 siRNA abrogated that effect. Scrambled siRNA was ineffective in suppressing p21 and p27 was not affected by either siRNA.

IRF-1 was successful again in inducing a G1/S arrest both in our MDA-MB-468 cell line as well as a lung cancer cell line, H1299 (Figure 20B). Then addition of CDKN1A (p21) siRNA to knockdown p21 noticeably abrogated the G1 arrest in the Rb wild type H1299 cell line but had no statistically significant decrease in the Rb deficient MDA-MB-468. When cell cycle phase was normalized to NI, it becomes apparent that p21 siRNA is increasing the percent of

cells in S phase and this may represent a releasing of G1 arrest. Since p21 itself was a potent inducer of G1 cell cycle arrest in MDA-MB-468 (not shown) we can only assume that an inhibition of p21 would logically prevent G1 arrest, unless IRF-1 utilizes more than one mechanism to induce G1 arrest in this model.

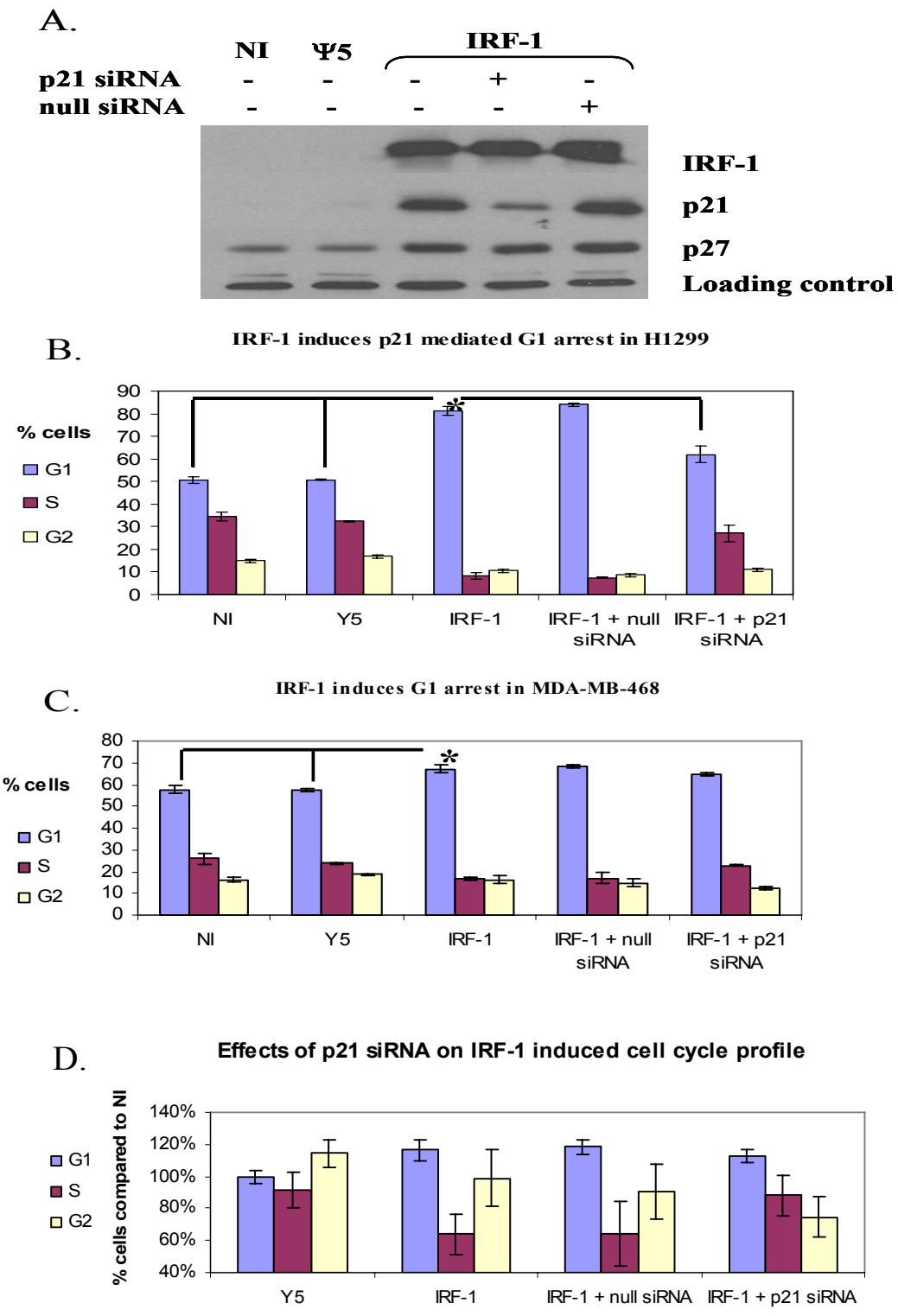


Figure 20. A. p21 siRNA suppresses IRF-1 induced p21 but not p27. **B.** p21 mediates IRF-1 induced G1 arrest in H1299 cells, ($p<0.05$). **C.** p21 does not appear to mediate G1 arrest in MDA-MB-468 cells, ($p<0.05$), but (**D.**) does release cells into S phase compared to NI.

4.3.2 IRF-1 induced apoptosis is p21 mediated in MDA-MB-468 cells

In our introduction, Detjen et al. (Detjen, Murphy et al. 2003) noted a possible protective effect against apoptosis when p21 is present. If this is true we would expect to see that IRF-1 induced apoptosis is abrogated in the presence of p21 and more effective with p21 inhibition. We created a stably transfected p21 knockdown cell line of MDA-MB-468. We then infected the cells with Ad-Ψ5 or Ad-IRF-1, or no infection and assessed caspase cleavage by western blot.

4.3.2.1 Knockdown of p21 suppresses apoptosis in MDA-MB-468

We have demonstrated that p21 is upregulated in Ad-IRF-1 transduced breast cancer cells, and this has been published previously in the literature (Pizzoferrato, Liu et al. 2004). p21 expression has been found to be sufficient for mediating G1 arrest. We hypothesize that the

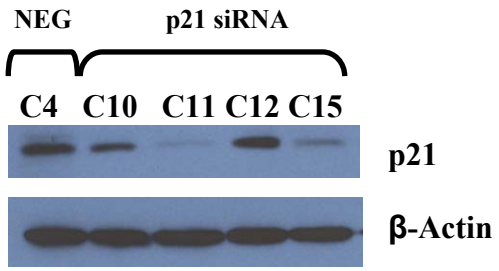


Figure 21. p21 knockdown in MDA-MB-468 stable transfectants.

mechanism of IRF-1induced G1 arrest that may protect cells from apoptosis and may be primarily through upregulation of p21. We have obtained a p21 siRNA vector and have proved suppression of p21 expression in stably transfected MDA468 cells and have expanded the best p21 "knock-down" clones (clones 11 and 15, see Figure 21). This should allow us to more easily assess the role p21 in IRF-1 induced apoptosis in breast cancer cells.

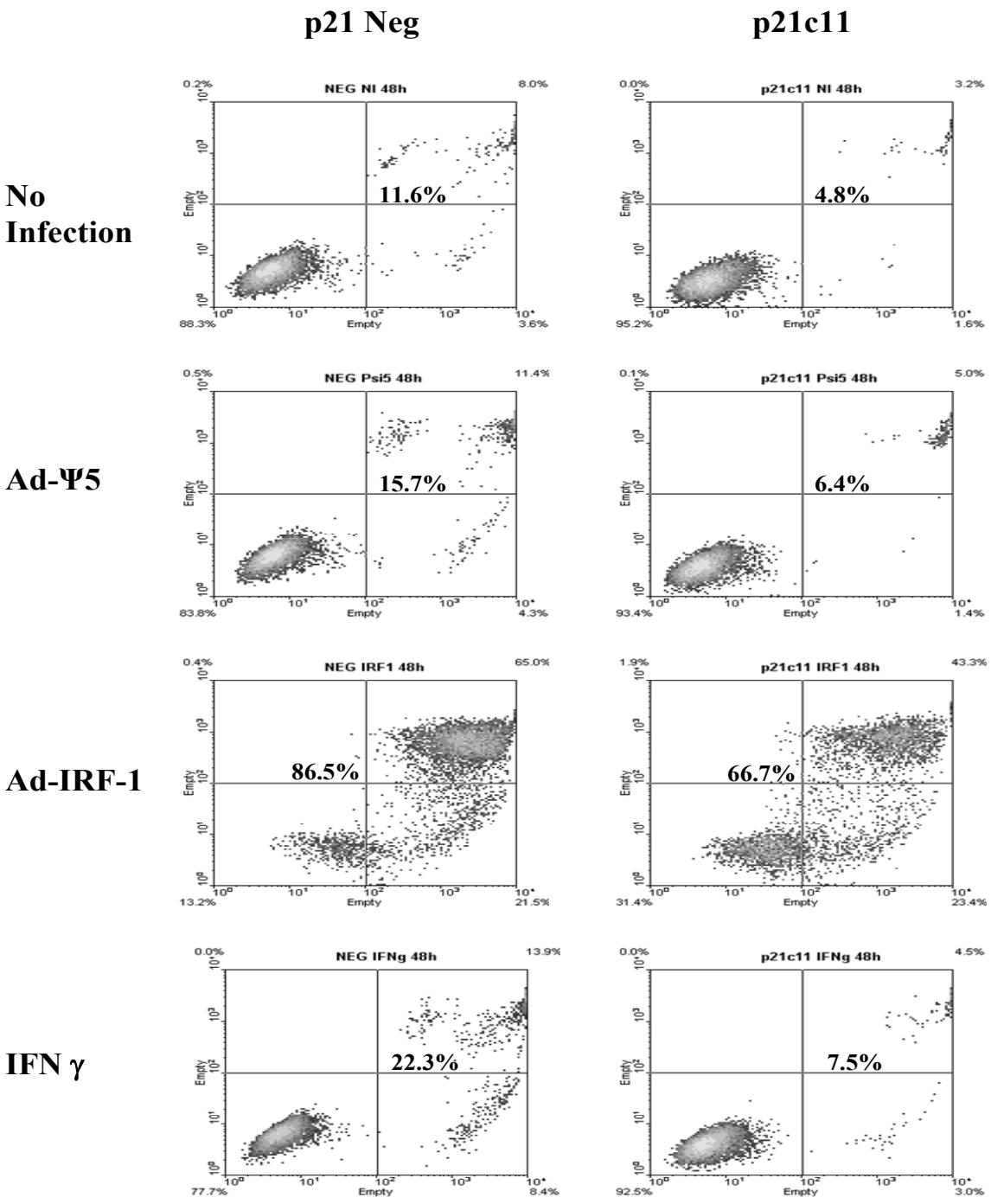


Figure 22. p21 knockdown suppresses apoptosis in MDA-MB-468

In order to determine if an increase in p21 represented a "protective" decrease in apoptosis, we performed flow cytometry on Annexin V-FITC and propidium iodide stained cells. These data show that knockdown of p21, in our p21c11 line, ***suppresses*** apoptosis in MDA-MB-468 cells (Figure 22) regardless of treatment with IFN-γ or infection with vector control (Ad-Ψ5) or Ad-IRF-1.

4.3.2.2 Knockdown of p21 abrogates IRF-1 suppression of Survivin

p21 is known to suppress Survivin expression (Lohr, Moritz et al. 2003) as is IRF-1. Since we have shown that IRF-1 induces p21 we then tested whether IRF-1's suppression of Survivin is mediated through p21. Immunoblotting of p21 knockdown cells in comparison with control MDA-MB-468 cells indicate that suppression of p21 partially alleviated IRF-1 down-regulation of Survivin with both IRF-1 and IFNγ treatments (Figure 23A and B).

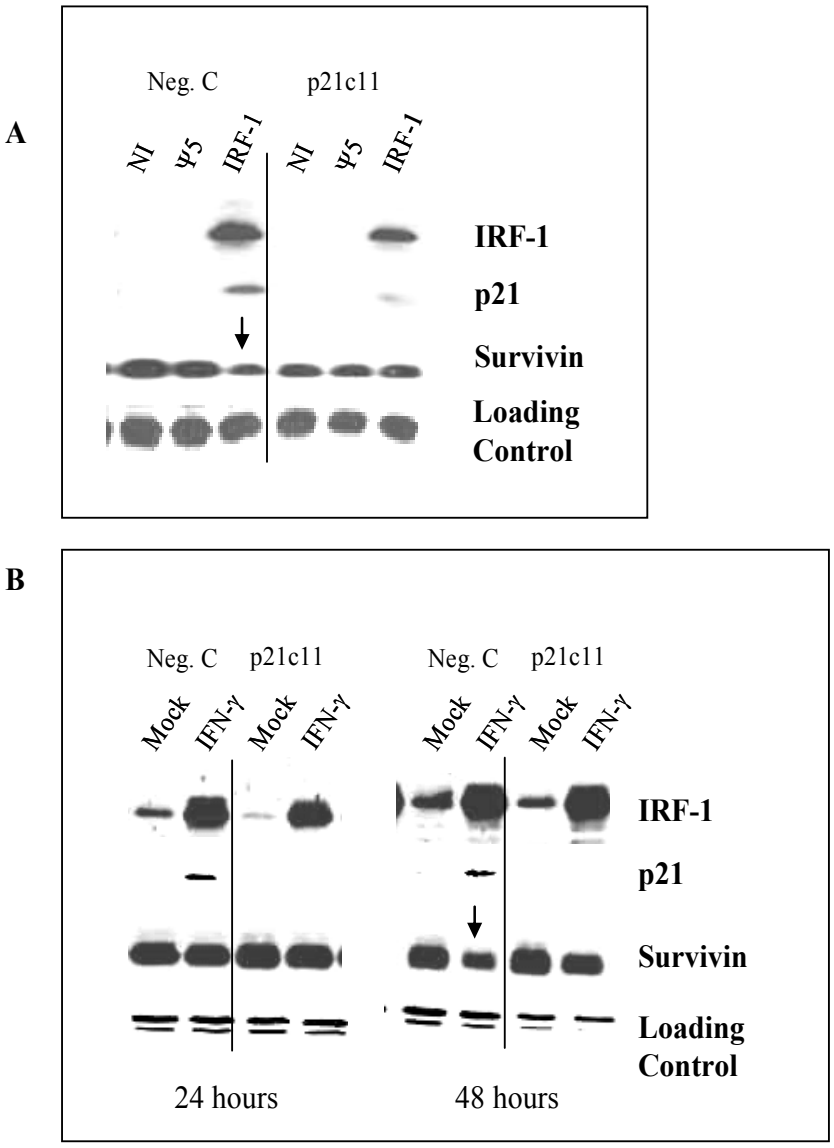


Figure 23. A. IRF-1 induced p21 suppresses survivin in control MDA-MB-468 but not in p21 knockdown cells. **B.** IFNγ induces p21 and a latent suppression of survivin in control cells but not in p21 knockdown cells

4.4 DISCUSSION

Growth inhibition by IRF-1 is not solely mediated by apoptosis alone. Studies showing IRF-1 mediation of N-ras and Mullerian inhibiting substance affirm that cell cycle arrest is part of the growth inhibition seen (Hoshiya, Gupta et al. 2003; Passioura, Dolnikov et al. 2005) with these treatments. Though previous studies did not attempt to characterize a mechanism of IRF-1 induced apoptosis, they provided observations of IRF-1 associating with upregulation of cell cycle inhibitors (Harper, Elledge et al. 1995; Coccia, Del Russo et al. 1999; Yu, Kim et al. 2004), suppression of Cdk2 (Detjen, Murphy et al. 2003; Xie, Gupta et al. 2003) and G1/S arrest (Stevens and Yu-Lee 1992; Yokota, Okabayashi et al. 2004) that when taken together create obvious pathway possibilities.

Cyclin dependent kinase inhibitor p21 is known to induce a G1 arrest and has been shown to be upregulated by IFN γ in arrested cells (Harvat and Jetten 1996). IRF-1 binding sites have been located in the promoter region of p21 (Coccia, Del Russo et al. 1999) and IRF-1 and p21 upregulation are associated in many studies of cell cycle arrest (Harper, Elledge et al. 1995; Stevens, Wang et al. 1995). Interestingly, any upregulation of p21 would be p53 independent in our model since these cell lines are p53 mutant or null. Our studies clearly confirm upregulation of p21 in MDA-MB-468 and H1299 cells and also show that p27 is induced by IRF-1.

To determine if cell cycle arrest was associated with IRF-1 treatment and p21 upregulation, we performed double staining with PI and FITC-αBrdU in BrdU pulsed MDA468 cells that were mock infected or treated with Ad-IRF-1 or Ad-Ψ5. The IRF-1 induced dose dependant G1 arrest is confirmed in MDA-MB-468 in direct opposition to S and G2 shift caused by Ad-Ψ5. That Ad-IRF-1 directly opposes the vector control further confirms the role of IRF-1 in G1 arrest.

Since p21 is a known cyclin dependent kinase inhibitor and we have confirmed its induction in response to IRF-1, we then immunoblotted for cyclins and cyclin dependent kinases. Cells treated with IRF-1 supported the observation of IRF-1 induced G1/S cell cycle arrest by

exhibiting the suppression of Cdk2 in response to IRF-1 treatment. We also observed suppression of Cyclin E, the counterpart to Cdk2.

Using siRNA specific to p21, we then abrogated IRF-1 induced G1/S arrest in Rb wild type H1299 cells. However IRF-1 induced cell cycle arrest in MDA-MB-468 cells was not obviously influenced by p21. Perhaps we need to extend this study to 48 hours where IRF-1 induced cell cycle arrest is more apparent and therefore a release due to p21 siRNA may also be more significant. This may also suggest that IRF-1 may have an alternative mechanism of cell cycle arrest that does not require p21 upregulation even though p21 is a potent inducer of G1 arrest in these cells (not shown). Interestingly, all arresting mechanisms would be independent of Rb, since our MDA-MB-468 cells are Rb deficient.

Conventional theory suggests that cell cycle arrest and p21 may protect cells from apoptosis, allowing the cells to undergo repair instead. Our data however demonstrate that in MDA-MB-468 cells p21 engages in pro-apoptotic behavior. Annexin V-FITC and PI analysis of apoptosis display decreased apoptosis in cells with p21 knock down compared to control MDA-MB-468 cells.

p21 is a known suppressor of survivin (Lohr, Moritz et al. 2003) an inhibitor of apoptosis that is commonly overexpressed in cancers. We have previously published IRF-1 induced suppression of survivin in our MDA-MB-468 model (Pizzoferrato, Liu et al. 2004). We hypothesized that the induction of p21 may suppress survivin creating a threshold that allows increased apoptosis. MDA-MB-468 control cells treated with IRF-1 displayed decreases in survivin that correlate with the increased apoptosis previously shown. However in our MDA-MB-468 p21 knock down cells prevented suppression of survivin in response to IRF-1 by 24 hours. Treating control cells with IFNγ similarly increased p21at 24 and 48 hours and had survivin suppression by 48 hours. The p21 knockdown cells did not exhibit suppression of survivin which may explain the corresponding resistance to apoptosis compared to control cells.

5.0 IRF-1 MEDIATES CANCER SPECIFIC APOPTOSIS IN BREAST CELLS

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Manuscript in preparation

5.1 INTRODUCTION

We and others have clearly demonstrated that IRF-1 induces growth inhibition of cancer that is achieved through both cell cycle arrest and apoptosis. These studies, while enlightening, ultimately are intended to assist in improving clinical outcomes. Thus, the tumor suppressor role of IRF-1 established by the Taniguchi lab has been further developed through clinical investigations of Tamoxifen (Bowie, Dietze et al. 2004) and Faslodex (Bouker, Skaar et al. 2004) as well as N-ras (Passioura, Dolnikov et al. 2005), and Mullerian inhibiting substance (MIS) (Hoshiya, Gupta et al. 2003).

Several studies demonstrate a role for IRF-1 in breast cancer pathology. Doherty et al. (Doherty, Boucher et al. 2001) examined patients with ductal cancer in situ (DCIS) and invasive ductal cancer and found that normal breast tissue stained for IRF-1 in 97% of samples, whereas patient samples with high nuclear grade DCIS and patients with lymph nodes positive for invasive ductal cancer showed significantly less IRF-1 staining. Another study by Gu et al. (Gu, Lee et al. 2002) and confirmed by Bouker et al. (Bouker, Skaar et al. 2004) demonstrated that IRF-1 is down-regulated in breast cancer cell lines resistant to antiestrogens and has shown IRF-1 to regulate apoptosis by Faslodex, a potent anti-estrogen. IRF-1 is decreased in the normal growth of mammary tissue in pregnant rats and increased during involution of the mammary glands (Hoshiya, Gupta et al. 2003). These studies support the contention that normal expression of IRF-1 is associated with healthier breast tissues, while deficiencies of IRF-1 expression may be correlated with neoplastic growth.

One study from Chapman et al. demonstrated that IRF-1 can have an *anti*-apoptotic role in mammary epithelial cells during mammary gland involution (Chapman, Duff et al. 2000). In IRF-1 knockout mice mammary glands, significantly greater numbers of apoptotic cells were found in involuting glands and the alveolar structure had collapsed when compared to control wild type IRF-1 mouse glands. This is the only study to demonstrate an anti-apoptotic role for IRF-1, and raises the possibility that IRF-1 may produce different results in normal mammary epithelial cells versus breast cancer cells.

Since IRF-1 appears to be expressed more in normal breast epithelium compared to breast cancer, we tested the effects of overexpression of IRF-1 in a normal epithelial cell line, HMEpC, as well as a non-malignant breast fibroadenoma cell line, MCF-10F, in comparison to MDA-MB-468, a malignant breast adenocarcinoma. As a mediator of apoptosis in clinically utilized breast cancer treatments, we hypothesize that IRF-1 may promote differential growth inhibitory effects on transformed breast cells versus non-transformed breast cells, inducing apoptosis in malignant cells while sparing normal cells. This has obvious clinical significance suggesting the tolerance of normal tissues to the growth inhibitory effects of IRF-1 mediated therapies that include faslodex and tamoxifen or the possibility of using expression of IRF-1 as an adjuvant therapy.

5.2 MATERIALS AND METHODS

Cell Lines and Culture:

All cell lines except HMEpC and C3-L5 were purchased from American Type Culture Collection (Manassas, VA). The MDA-MB-468 and C127I tumor cells were propagated in Dulbecco's modified Eagle's medium (BioWhittaker, Inc., Walkersville, MD) and Ham's F-12 (Invitrogen Life Technologies, Carlsbad, CA) media at a 1:1 ratio with 10% fetal bovine serum, L-glutamine and antibiotics. HMEpCs were purchased from Cell Applications and propagated in their complete HMEpC media (Cell Applications). MCF10F were propagated in DMEM (BioWhittaker, Inc., Walkersville, MD) with 10% fetal bovine serum, L-glutamine and antibiotics. All cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. HCT-116 WT and p53 -/- were generously provided by Dr. Bert Vogelstein. C3L5 were generously provided by Dr. Tim Billiar and TS/A, C3-L5 and SKBR3 were propagated in RPMI (BioWhittaker, Inc., Walkersville, MD) with 10% fetal bovine serum, L-glutamine and antibiotics. NMuMG propagated in Dulbecco's modified Eagle's medium with 4.5 g/L glucose and 10 mcg/ml insulin, 90%; fetal bovine serum, 10%.

MTT:

MTT was applied to duplicate wells 48-72 h after infection and absorbance was measured. Percent growth inhibition was calculated by %=[1-mean abs (Ad-IRF-1)/mean abs(Mock)] x 100.

Flow Cytometry:

The MDA-MB-468, mammary carcinoma cell line, and non-transformed MCF10F and Human Mammary Epithelial Cells (HMEpC) were treated with either no infection (NI), or infected with recombinant adenovirus Ad-Psi-5 (Ad-Null, vector control), or Ad-IRF-1 +/- Z-VAD (200 μM). MDA-MB-468, HMEpC, and MCF10F cells were treated with 25, 150, and 100 multiplicity of infection (MOI) respectively, which corresponds to >95% viral infection for each cell line. Cells were harvested at 48 hours for HMEpC and MCF10F and 36 hours for MDA-MB-468. Cells were then stained for Annexin V-FITC and PI and analyzed by flow cytometry.

Additionally HCT116 WT and p53 KO were treated with either no infection (NI), or infected with recombinant adenovirus Ad-Psi-5 (vector control), or Ad-IRF-1 at MOI of 100. Cells were harvested at 36 hours, stained for Annexin V/PI, and analyzed by flow cytometry.

Immunoblots:

MCF10F, MDA-MB-468, and SKBR3 were infected with Ad-IRF-1 at noted MOI's and harvested 24 hours later. Cellular lysates were separated by SDS-PAGE and blotted for IRF-1.

SKBR3 and C3L5 cells were treated with Interferon gamma (IFNγ) 1000 units/ml and then treated with either no infection (NI), or infected with recombinant adenovirus Ad-Psi-5 (Null) or Ad-Lac-Z (vector controls), or Ad-p53 at MOI's of 25 & 200 respectively. Whole cell lysates were separated by SDS-PAGE and immunoblotted for IRF-1.

MDA-MB-468 and HMEpC cells were treated with 25 and 150 MOI's non-infected control, Ad-Null or Ad-IRF1 respectively and harvested at 24 hours. Total cellular protein was separated by SDS-PAGE and blotted for caspase-3, -7, and -8 and Survivin (Cell Signaling Technology).

5.3 RESULTS

5.3.1 IRF-1 mediates growth inhibition in cancer but not non-malignant breast cells

We and others have observed IRF-1 mediated growth inhibition in breast cancer cell lines, through both overexpression of IRF-1 (Kim, Armstrong et al. 2004; Pizzoferrato, Liu et al. 2004; Watson, Queiroz de Oliveira et al. 2006), IFNγ treatment (Yim, Ro et al. 2003) and through clinically utilized anti-cancer agent, faslodex (Bouker, Skaar et al. 2004). Studies also show that normal breast epithelium express IRF-1 to a greater degree than growing cells and cancers (Doherty, Boucher et al. 2001). Taken together these studies raise interesting questions about the tolerance of non-malignant cells to overexpression of IRF-1.

We began our experiments by treating a panel of breast cancer cell lines and non-malignant breast cells with either Ad-IRF-1 or our empty vector control, Ad-Ψ5. The breast cancer cell lines represented all contain mutated or deleted p53, while our non-malignant lines are p53 wild type. As seen in Figure 24A, breast cancer cell lines have disproportionate growth inhibition compared to non-malignant cell lines when treated with IRF-1. Ad-IRF-1 infection resulted in growth inhibition in breast cancer cells ZR75-1, MDA-MB-468, SKBR3, TS/A and C3-L5 at shown MOI's but not in non-malignant breast cell lines MCF10F, C127I, or NMuMG by MTT assay. All cells demonstrated greater than 95% transfection at the MOIs used as assessed by percentage of green cells found at 24 hours after infection with Ad-EGFP in repeated experiments (data not shown), and equivalent amounts of IRF-1 protein by Ad-IRF-1 (data not shown).

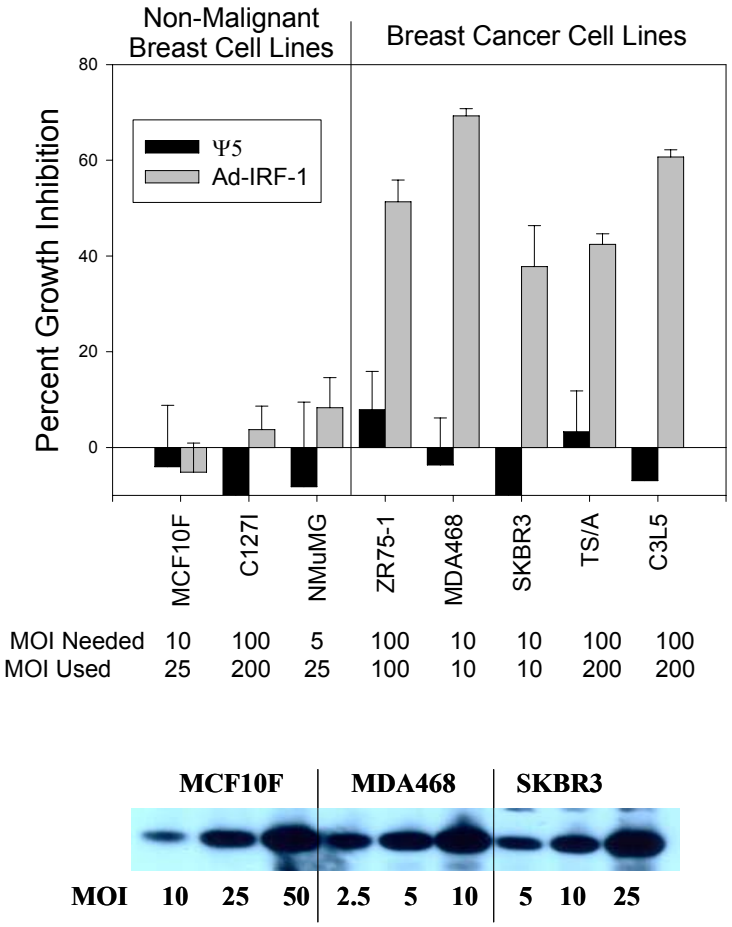

Figure 24. A. Ad-IRF-1 induces growth inhibition in cancerous but not non-malignant breast cells. **B.** A representative immunoblot showing equal IRF-1 expression across cell lines requiring differing MOI's.

Since different cell lines are more or less susceptible to viral infection, we not only determined the MOI that provided 95% or greater infection we also tried to determine that we were equally expressing IRF-1 between cell lines. This representative blot (Figure 24B) demonstrates equivalent IRF-1 protein by Ad-IRF- 1 at the MOIs used in human cell lines MCF10F, MDA468, and SKBR3. Significant growth inhibitory response was found in the breast cancer cell lines (MDA468 and SKBR3) versus the mammary epithelial cell line (MCF10F) across the MOIs shown in this immunoblot.

5.3.2 IRF-1 mediates apoptosis in cancer but not normal cells

We and others have readily shown that IRF-1 growth inhibition is due in part to IRF-1 induced apoptosis; therefore we evaluated apoptosis in non-malignant fibroadenoma, MCF10F in comparison to adenocarcinoma MDA-MB-468. While the MCF10F is not considered a cancerous cell line, we acknowledge that it is not the best model of "normal" breast cells either, so we also assayed apoptosis in p53 wild type human mammary epithelial cells (HMEpC).

MCF10F and to a greater extent MDA-MB-468 demonstrated an increase in the apoptotic fraction with IRF-1 treatment (Figure 25A) but the HMEpC demonstrated no increase in apoptosis with overexpression of IRF-1 despite using higher MOI's than necessary for >95% transduction and observing time points further out than MDA468. As we have shown before, IRF-1 induced apoptosis is opposed by pan-caspase inhibitor ZVAD treatment. Percents represent cumulative Annexin V labeling (right upper and right lower quadrants).

Interestingly, when these results are graphed (Figure 25.B.), we observe a higher baseline apoptosis in HMEpC cells in comparison to MCF10F and MDA-MB-468 which may suggests a gained survival advantage by the tumor cell lines.

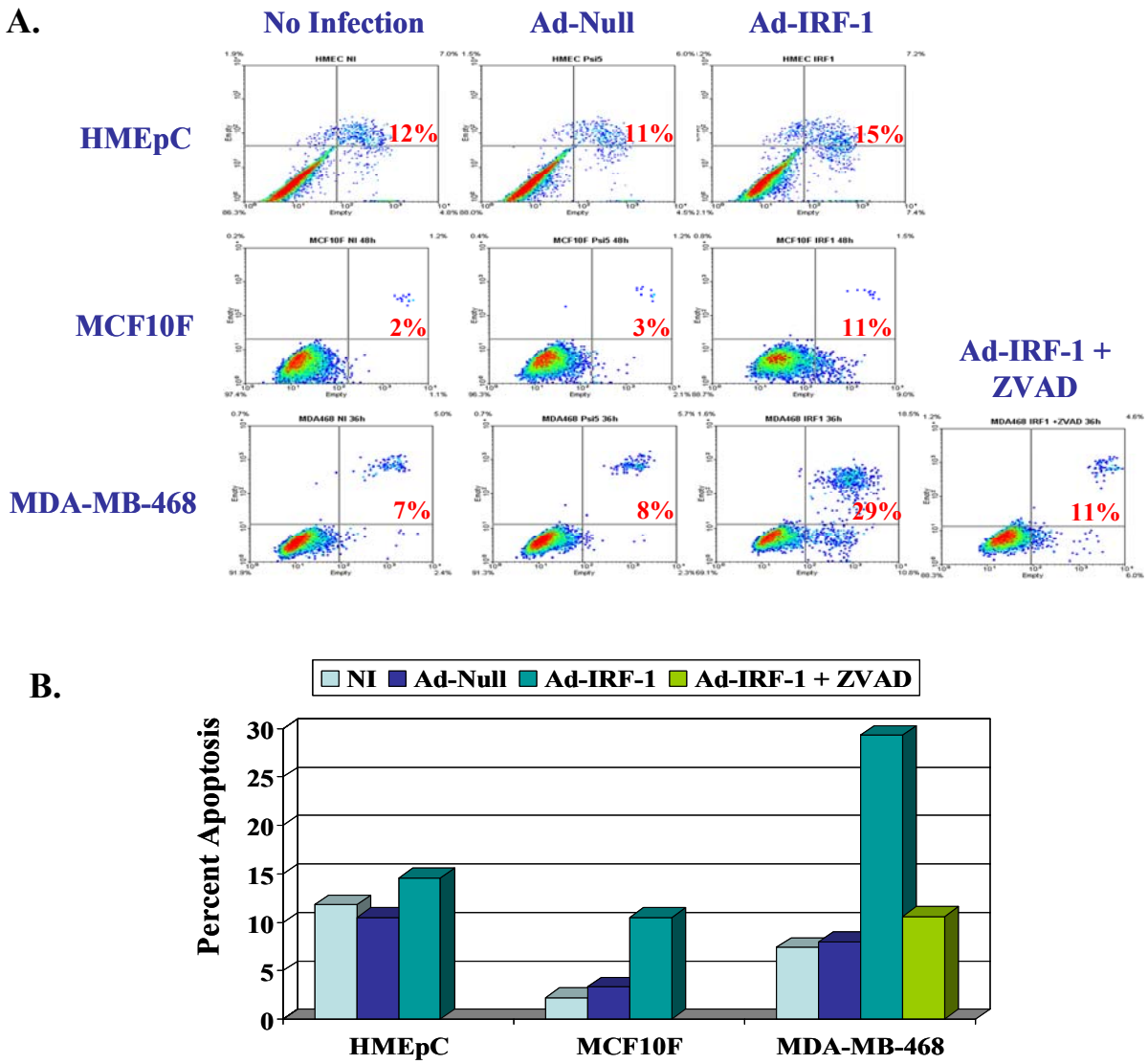

Figure 25. A. HMEpC show no increase in apoptosis when treated with IRF-1, however MCF10F and MDA-MB-468 show increases in apoptosis which are caspase mediated in the MDA-MB-468. **B.** HMEpCs have a higher base-line apoptosis compared to MCF10F and MDA-MB468

5.3.3 p53 as a mediator of IRF-1 induced apoptosis?

In our growth inhibition study we noted that the non-malignant cells were all p53 wild-type while the cancer cell lines had mutated or deficient p53. The presence or absence of p53 may explain the postulated survival advantage seen in the differences in baseline apoptosis between HMEpC (wild-type) and MDA-MB-468 (mutant) cell lines. The Taniguchi lab first suggested an overlap between IRF-1 and p53 functions in studies of apoptotic induction, though through different pathways, of T lymphocytes (Tamura, Ishihara et al. 1995). In mouse embryonic fibroblasts lacking either IRF-1 or p53, DNA-damage induced cell cycle arrest was abrogated but transient cotransfection of the tumor suppressors restored the phenotype (Tanaka, Ishihara et al. 1996). Prost also found that IRF-1 may regulate p53 expression (Prost, Bellamy et al. 1998) in hepatocytes where IRF-1 deficiency correlated with deregulated p53.

Our preliminary western blot of SKBR3 (human) and C3L5 (mouse) p53 deficient breast cancers reveals the typical induction of IRF-1 by interferon gamma (Figure 26A). This expression is suppressed when cells are treated with Ad-p53, suggesting that p53 may regulate IRF-1. However, HCT116, colon cancer lines with and without p53 show little appreciable difference in IRF-1 induced apoptosis (Figure 26B) even though Ad-IRF-1 infection induced high expression of IRF-1 in both cell lines (Figure 26C).

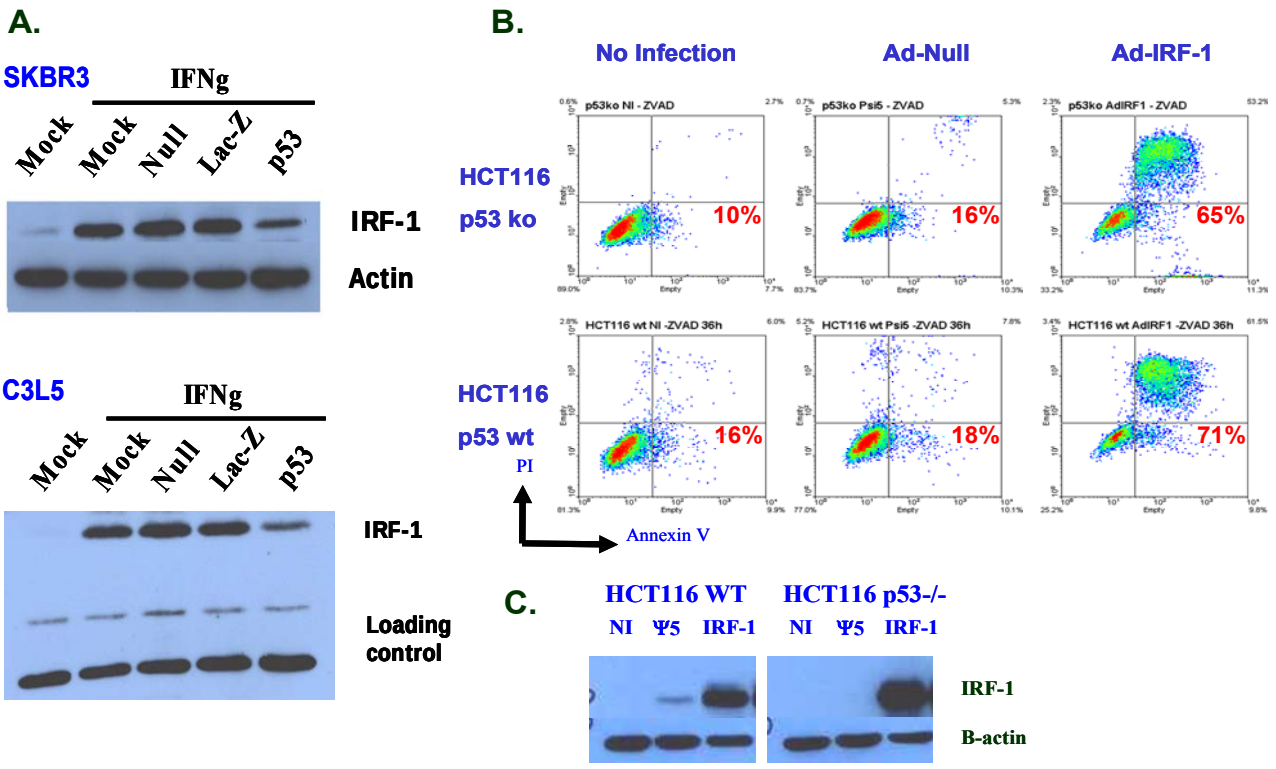

Figure 26. A. Ad-p53 suppresses IFNγ upregulation of IRF-1 in human and mouse breast cancer cell lines. **B.** p53 presence or absence does not prevent IRF-1 induced apoptosis. **C.** Immunoblotting shows high levels of IRF-1 expression in both HCT116 wild type and p53 knockout cell lines with Ad-IRF-1 infection.

5.3.4 IRF-1 suppresses constitutive over expression of Survivin in MDA-MB-468

When we observed the increased baseline apoptosis in the HMEpC line we began to hypothesize that the malignant MDA-MB-468 cells may have increases in an inhibitor of apoptosis that may impart a survival advantage. While the studies of p53 are inconclusive, our previously publication demonstrates that IRF-1 suppresses, Survivin, an inhibitor of apoptosis that is commonly found overexpressed in cancers (O'Driscoll, Linehan et al. 2003).

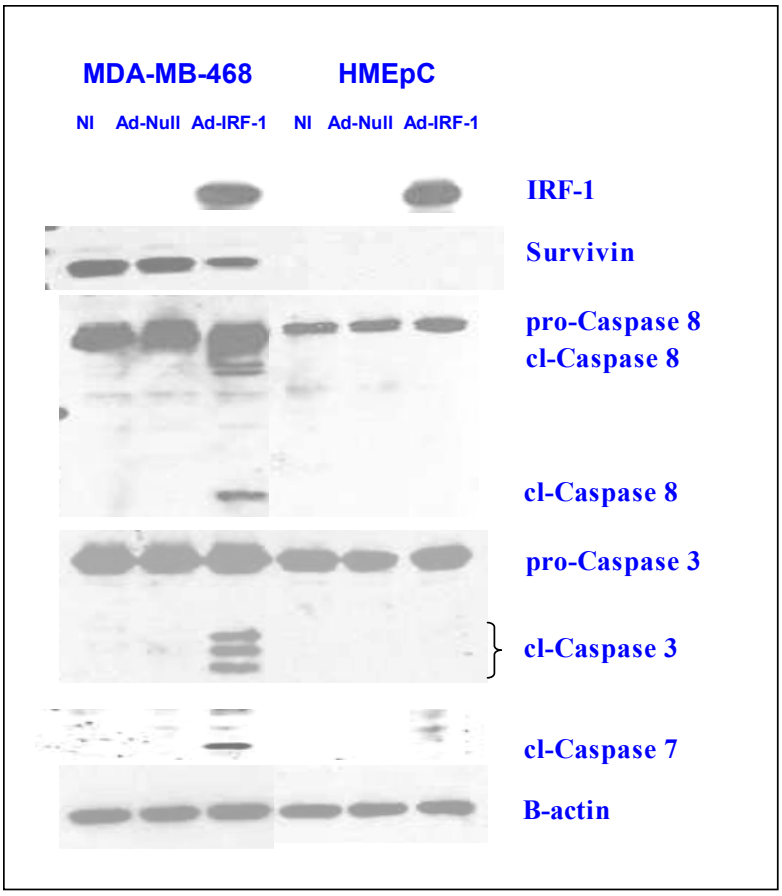

Figure 27. IRF-1 induces suppression of overexpressed Survivin and caspase cleavage in the cancer cell line MDA-MB-468 and no caspase cleavage in HMEpCs. Lysates were equally loaded on same gel and probed on the same blot.

Immunoblotting of MDA-MB-468 cells demonstrate overexpression of survivin in comparison to HMEpC (Figure 27), and that IRF-1 suppresses survivin. This suppression of survivin correlates to caspase 8, 3 and 7 cleavage in MDA-MB-468 cells. HMEpC immunoblotting lacked survivin expression and revealed little if any levels of caspase cleavage.

IRF-1 induction of apoptosis is largely caspase mediated following the observation that a pan-caspase inhibitor (ZVAD) can block the vast majority of cellular death as measured by Annexin V labeling and flow cytometry (Figure 25). Caspase mediated apoptosis is further supported by the demonstration of caspase 8, 3, and 7 cleavage in MDA-MB-468 suggestive of caspase pathway activation correlating with the induction of cellular death (Figure 27). These

markers of apoptosis are inhibited in the non-transformed HMEpC cell line, and are consistent with our apoptosis data.

5.4 DISCUSSION

Studies of IRF-1 in cancer to this point have focused on its ability to restrict growth and induce apoptosis either directly or as a mediator of anti-tumor treatments. IFNs are currently used to treat cancer clinically (Oberg 2000; Egwuagu, Li et al. 2006) as are faslodex (Bouker, Skaar et al. 2004) and tamoxifen (Bowie, Dietze et al. 2004). As a mediator of the anti-tumor effects of these treatments, we are investigating the effect of IRF-1 on "normal" human mammary epithelial cells versus breast adenocarcinoma and fibroadenoma. We would expect that "normal" cells would be more tolerant of IRF-1 than cancerous cells since breast tissue in human and mouse express IRF-1, but the levels of IRF-1 decrease in adjacent tumors or when growth occurs (Doherty, Boucher et al. 2001; Hoshiya, Gupta et al. 2003). However, we are also aware that upstream inducers of IRF-1, IFNs are currently utilized for cancer treatments and can display adverse effects (Egwuagu, Li et al. 2006) and this leads us to question how tolerant "normal" cells will be of overexpressed IRF-1.

We have found in a panel of malignant versus non-malignant cell lines that IRF-1 differentially inhibits cell growth even with comparable expression of IRF-1. The differences in growth inhibitory response in non-malignant breast cell lines versus breast cancer cell lines is due at least in part, if not in entirely, to a minimal apoptotic response in the non-malignant breast cells to IRF-1. IRF-1 treatment did not induce apoptosis in HMEpC and mildly induced MCF10F to undergo apoptosis in comparison to the apoptosis seen in MDA-MB-468 by Annexin V/PI flow cytometry.

When analyzed graphically, we see that HMEpC have a higher baseline apoptosis in comparison to MDA-MB-468. This may suggest a survival advantage garnered by the cancer cell line that prevents it from undergoing apoptosis. One known difference that logically deserves scrutiny is the absence of functional p53 in the malignant cell lines first tested for IRF-1 growth inhibition, that was not absent in the non-malignant cell lines tested. While overexpression of p53 was effective in suppressing IRF-1 in two of our breast cancer cell lines, we found no

difference in apoptosis between wild type and p53 deficient colon cancer cell lines. These conflicting data require further testing prior to drawing any meaningful conclusions.

As an inhibitor of apoptosis, survivin is found overexpressed in many cancers and assists in evasion of cell death from multiple stimuli, while normal tissues express little or no detectable survivin. The reduction in MDA-MB-468 survivin (Figure 27) and corresponding apoptosis may denote a mechanism of IRF-1's selectiveness in inhibiting cancer cell growth while sparing cells that are less dependant on such survival signals. HMEpC had little or no caspase cleavage in comparison to the apparent and repeatable caspase 8, 3 and 7 cleavages seen in MDA-MB-468.

This group of studies would indicate that IRF-1 induces growth inhibition by inducing apoptosis in cancer cells while non-malignant cells are resistant to IRF-1 induced apoptosis. The mechanism may be related to expression of surviving in cancer cells that is not expressed in non-malignant cells. The lack of apoptotic induction accounts for the majority of the resistance to IRF-1 induced apoptosis, however we have yet to analyze cell cycle effects in HMEpC treated with IRF-1.

Other further studies will obviously include suppression of p53 by siRNA in normal and breast cancer cell lines to determine if that allows IRF-1 induced apoptosis. We are also eager to investigate if suppression of survivin by siRNA creates a "threshold" by which the cell can then normally induce apoptosis or if a milieu effect in which IRF-1 suppresses survivin and then induces a caspase cascade is required for apoptosis.

6.0 COLLECTIVE DISCUSSION, CONCLUSIONS, AND FURTHER STUDIES

6.1 IRF-1 INDUCED GROWTH INHIBITION

In this dissertation we investigate two methods of growth inhibition employed by IRF-1: apoptosis and cell cycle arrest. Normal cells commonly undergo either type of growth inhibition in response to regulatory signals but neoplastic cell clones have added to their cancerous potential by avoidance mechanisms to growth inhibition. Localized at 5q31.1, IRF-1 is often found deleted in human leukemia (Nagai, Fujita et al. 1997; Green, Slovak et al. 1999), esophageal (Ogasawara, Tamura et al. 1996; Peralta, Casson et al. 1998), gastric (Tamura, Ogasawara et al. 1996), and renal cancers (Sugimura, Tamura et al. 1997). Since cancer is formed in part through stepwise mutations that impart losses of genes that control growth, the loss of IRF-1 in these cancers implicates IRF-1 as a tumor suppressor.

We and others have noted and published the observation that IRF-1 can induce apoptosis either through forced expression (Kim, Armstrong et al. 2004; Pizzoferrato, Liu et al. 2004; Bouker, Skaar et al. 2005; Watson, Queiroz de Oliveira et al. 2006) or as a mediator of IFNγ (Kim, Lee et al. 2002; Tomita, Bilim et al. 2003; Egwuagu, Li et al. 2006), retinoic acid (Arany, Ember et al. 2003; Wang, Peng et al. 2006), tamoxifen (Bowie, Dietze et al. 2004), faslodex (Bouker, Skaar et al. 2004), or Mullerian inhibiting substance (Hoshiya, Gupta et al. 2003). While it is well known that IRF-1 induces or mediates apoptosis, little is known about the mechanism or pathways through which IRF-1 induced apoptosis is achieved.

We began our illumination of IRF-1 mediated growth inhibition by studying the caspase cascade and upstream regulators. We found that IFNγ induces IRF-1-mediated and caspase dependent apoptosis. IRF-1 also induces caspase mediated apoptosis. Previous studies have found associations between IRF-1 induced apoptosis (Kim, Lee et al. 2002) and induction of caspase transcription and cleavage. We have shown that IRF-1 activates and cleaves executioner

caspase 3 (Kim, Armstrong et al. 2004). IRF-1 also upregulates caspase 7, increasing mRNA and protein (Tomita, Bilim et al. 2003; Kim, Armstrong et al. 2004) and stimulates proteolytic cleavage of caspase 7 (Sanceau, Hiscott et al. 2000). Initiator caspase 8 mRNA is also upregulated by IRF-1 in breast cancer in two separate studies (Kim, Armstrong et al. 2004; Ruiz-Ruiz, Ruiz de Almodovar et al. 2004) and in this study we show cleavage of caspase 8 for the first time in response to IRF-1 treatment. We also confirm that caspases 3 and 7 cleave in cells treated with IRF-1.

Caspase 8 cleavage appears to be a proximal event in the caspase cascade since IETD, the caspase 8 inhibitor, abrogates IRF-1 induced apoptosis and caspase 3 cleavage. IRF-1 induces Bid cleavage and Smac/Diablo release from the mitochondria.

Interestingly, FasL contains an IRF-1 binding site within its promoter region, suggesting a pathway for IRF-1 induced apoptosis (Kirchhoff, Sebens et al. 2002) and IFN γ induction of Fas mediated apoptosis was partially abrogated by IRF-1 anti-sense treatment (Tomita, Bilim et al. 2003). IRF-1 upregulation was required for RA induction of TRAIL (Clarke, Jimenez-Lara et al. 2004). Further, synergistic apoptosis by TRAIL in conjunction with IFN γ is abrogated when treated with anti-sense oligonucleotides specific to IRF-1 (Park, Seol et al. 2004). TNF-α induced apoptosis also shows synergy with IFN γ treatment (Suk, Chang et al. 2001) inferring yet another convergence of IRF-1 with death receptor/ligand pathways. We confirmed that IRF-1 upregulates soluble FasL in the media of cells, but the cells are resistant to apoptosis via treatment with recombinant FasL, which is consistent with similar published findings (Billecke, Ljungman et al. 2002). Though FADD is involved (Stang 2006), and in spite of the many associations of IRF-1 and death ligands/receptors, we find that IRF-1 does not induce apoptosis through a soluble factor or the known death receptors.

While investigating the intrinsic death pathway, we found a novel population of IRF-1 in mitochondria of MDA-MB-468 cells. We began to explore the validity of the localization and possible functions of IRF-1 in the mitochondria. Further confirmation of IRF-1 localization is necessary and if verified, we then propose experiments to elucidate the function of mitochondrial IRF-1 and hypothesize that it acts to further regulate cellular proliferation.

While our previous studies center on the apoptotic mechanisms of IRF-1 induced growth inhibition, published data suggest that apoptosis may not account for all of IRF-1's effects. We and others have found upregulation of p21 (Coccia, Del Russo et al. 1999; Miyazaki, Sakaguchi

et al. 2004; Pizzoferrato, Liu et al. 2004), a key molecular mediator of cell cycle arrest, in response to IRF-1. Functional studies demonstrate that IRF-1 binds to the promoter regions of p21 (Coccia, Del Russo et al. 1999) and is highly suggestive of further growth inhibition through cell cycle regulation. IFN-γ, a key inducer of IRF-1, significantly decreased the fraction of cells in S phase (Hoshiya, Gupta et al. 2003).

In these studies we have confirmed IRF-1 upregulation of p21 and p27. We also show that overexpression of IRF-1 induces G1/S arrest in a dose and time dependant manner and that this arrest is p21 mediated. Cyclin E and Cdk2 were suppressed in response to IRF-1 treatment.

A recent publication (Detjen, Murphy et al. 2003) suggests that p21 upregulation may protect cells from apoptosis, allowing for DNA repair instead. Our data, however, demonstrate that in MDA-MB-468 cells p21 engages in pro-apoptotic behavior. Initial immunoblotting of control versus p21 knockdown cells show that cleavage of caspases 3 and 7 correlate with increased p21. Similarly Annexin V-FITC and PI analysis of apoptosis display decreased apoptosis in cells with p21 knock down compared to control MDA-MB-468 cells. These results suggest that IRF-1 apoptosis is mediated by p21. Knowing that IRF-1 suppresses the inhibitor of apoptosis, survivin, (Pizzoferrato, Liu et al. 2004) and that p21 is a negative regulator of survivin (Lohr, Moritz et al. 2003), we investigated and found that IRF-1 suppression of survivin is mediated by p21.

IRF-1 induced growth inhibition appears to be cancer specific. p53 does not seem to regulate IRF-1 induced apoptosis in HCT116 cells, though more testing is required to determine if IRF-1 and p53 have a co-regulatory association. In comparison to HMEpCs, MDA-MB-468 overexpresses Survivin and has a lower baseline of apoptosis. IRF-1 suppresses the overexpression of survivin in MDA-MB-468.

6.2 PROSPECTUS

6.2.1 Further studies of Caspase 8 mediated IRF-1 induced apoptosis

In the study of secreted factors and caspase 8 mediation of IRF-1, a collaborative colleague found that FADD too is involved in the signal pathway. Therefore our anticipated experiments will continue to close the gap between IRF-1 and its downstream effects. Is IRF-1 transcriptionaly upregulating caspase 8 to the point that it is aggregating and autoproteolytically cleaving and does this require FADD? RT-PCR and EMSA would validate the hypotheses that IRF-1 directs transcriptional induction of caspase 8. However, answers are rarely that simple. Are there other upstream recruiters, besides the TNF family of death receptors, of FADD that are induced by IRF-1? Or is there a more improbable association of IRF-1 acting in a direct manner with FADD/caspase 8? Co-immunoprecipitation of IRF-1 with FADD or Caspase 8 may reveal a protein/protein interaction.

6.2.2 Further studies of IRF-1 localization and functions in the mitochondria

We observed IRF-1 localizing to the mitochondria and our further studies would investigate possible functions and implications of this novel finding. A) Where is the IRF-1 localized within the mitochondria? We performed a trial digitonin study, unfortunately the data was inconclusive. Further advancement of the technique and subsequent trials may yield better results. B) Is the IRF-1 binding the mitochondrial DNA? Isolation and tagging of the MT-DNA as a probe for EMSA would produce evidence to support or refute that hypothesis. C) If binding is found does IRF-1 serve to upregulate or suppress 12S rRNA, or is does IRF-1's binding effect genes downstream/upstream of the binding site? Mitochondrial gene chip data may be enlightening as to possible MT-DNA transcriptional regulation; alternatively RT-PCR for mitochondrial genes of interest may also produce results. D) If IRF-1 is not binding MT-DNA is it interacting with other proteins in the mitochondria, like survivin that has also been found in the mitochondria? Isolation of mitochondria followed by careful immunoprecipitation of IRF-1 and western blotting for possible protein targets may reveal important new information.

Based on the target of IRF-1 binding/interaction further questions about significance could be formed. If IRF-1 is binding and upregulating 12S rRNA transcription, it's translocation away from the mitochondria in response to IFNγ treatment may slow the production of other mitochondrial genes and possibly downstream ATP synthesis, thus working to abrogate cellular functions. Alternatively, IRF-1 may be interacting with proteins, similar to its interactions with p300, to regulate growth.

6.2.3 Further studies of IRF-1 induced cell cycle arrest and pro-apoptotic p21

We see that IRF-1 induced cell cycle arrest appears to be in part mediated through p21. We also note previous studies that show IRF-2 having a direct effect on cellular proliferation through H4 histone. One line of further study is to determine whether IRF-1 also binds H4 histone in order to cause an E2F independent cell cycle arrest as studies of IRF-2 may suggest(Vaughan, Aziz et al. 1995; Vaughan, van der Meijden et al. 1998). Also pertinent is the role IRF-1 may have in regulation of DNA damage repair as reported previously (Tanaka, Ishihara et al. 1996; Prost, Bellamy et al. 1998).

We also note that IRF-1 is suppressing survivin through p21 upregulation. We hypothesize that suppression of survivin may allow the cells to undergo apoptosis. Investigations utilizing siRNA to survivin will be helpful in answering these questions. We would also overexpress survivin and would expect to see inhibition of IRF-1 induced apoptosis, including suppression of caspase cleavage.

Our studies have primarily centered on breast cancer and we would like to expand our results to determine if these results can be generalized to other cancers as well. We have noted that H1299, lung cancer cells, are sensitive to IRF-1 induced growth inhibition and would use this model to duplicate the studies of apoptosis and cell cycle arrest to further investigate the mechanisms of IRF-1 induced growth inhibition.

6.2.4 Further studies of IRF-1 induced cancer specific growth inhibition

We have yet to analyze cell cycle effects in HMEpC treated with IRF-1. We would also like to suppress of p53 by siRNA in normal and cancerous cells to determine if that allows IRF-1 induced apoptosis. We are also eager to investigate if suppression of survivin by siRNA creates a "threshold" by which the cell can then normally induce apoptosis or if a milieu effect in which IRF-1 suppresses survivin and then induces a caspase cascade is required for apoptosis, this would be similar to our studies of survivin in the setting of pro-apoptotic p21.

APPENDIX A

PSORT II ANALYSIS

Input Sequence (NCBI sequence: P10914 – IRF-1)

```
QUERY (325 aa)

MPITRMRMRP WLEMQINSNQ IPGLIWINKE EMIFQIPWKH AAKHGWDINK
DACLFRSWAI HTGRYKAGEK EPDPKTWKAN FRCAMNSLPD IEEVKDQSRN
KGSSAVRVYR MLPPLTKNQR KERKSKSSRD AKSKAKRKSC GDSSPDTFSD
GLSSSTLPDD HSSYTVPGYM QDLEVEQALT PALSPCAVSS TLPDWHIPVE
VVPDSTSDLY NFQVSPMPST SEATTDEDEE GKLPEDIMKL LEQSEWQPTN
VDGKGYLLNE PGVQPTSVYG DFSCKEEPEI DSPGGDIGLS LQRVFTDLKN
MDATWLDSLL TPVRLPSIQA IPCAP
```

Results of Subprograms

```
PSG:  a new signal peptide prediction method
      N-region:  length 9;  pos.chg 3;  neg.chg 0
      H-region:  length 3;  peak value  -3.20
      PSG score:  -7.60

GvH:  von Heijne's method for signal seq. recognition
      GvH score (threshold: -2.1): -11.05
      possible cleavage site: between 59 and 60

>>> Seems to have no N-terminal signal peptide

ALOM: Klein et al's method for TM region allocation
      Init position for calculation: 1
      Tentative number of TMS(s) for the threshold  0.5:   0
      number of TMS(s) .. fixed
      PERIPHERAL  Likelihood =  5.99 (at 308)
      ALOM score:   5.99  (number of TMSs: 0)

MITDISC: discrimination of mitochondrial targeting seq
      R content:       3       Hyd Moment(75):  3.34
      Hyd Moment(95):  0.23    G content:       1
      D/E content:     2       S/T content:     2
      Score: -5.25
```

Gavel: prediction of cleavage sites for mitochondrial preseq
      R-2 motif at 19 MRP|WL

NUCDISC: discrimination of nuclear localization signals
      pat4: none
      pat7: none
      bipartite: RKERKSKSSRDAKSKAK at 120
      bipartite: RKSKSSRDAKSKAKRKS at 123
      content of basic residues: 11.7%
      NLS Score: 0.51

KDEL: ER retention motif in the C-terminus: none

ER Membrane Retention Signals:
      XXRR-like motif in the N-terminus: PITR

none

SKL: peroxisomal targeting signal in the C-terminus: none

SKL2: 2nd peroxisomal targeting signal: none

VAC: possible vacuolar targeting motif: none

RNA-binding motif: none

Actinin-type actin-binding motif:
      type 1: none
      type 2: none

NMYR: N-myristoylation pattern : none

Prenylation motif: none

memYQRL: transport motif from cell surface to Golgi: none

Tyrosines in the tail: none

Dileucine motif in the tail: none

checking 63 PROSITE DNA binding motifs:
      IRF family signature (PS00601): *** found ***
         PKTWKANFRCAMNSLPDIEEVKD at 74

checking 71 PROSITE ribosomal protein motifs: none

checking 33 PROSITE prokaryotic DNA binding motifs: none

NNCN: Reinhardt's method for Cytplasmic/Nuclear discrimination
      Prediction: nuclear
      Reliability: 94.1

COIL: Lupas's algorithm to detect coiled-coil regions
      total: 0 residues

Results of the k-NN Prediction

$k = 9/23$

```
   82.6 %: nuclear
    8.7 %: mitochondrial
    4.3 %: cytoplasmic
    4.3 %: cytoskeletal

>> Prediction for QUERY is nuc (k=23)
```

PSORT II Users' Manual:

`http://psort.ims.u-tokyo.ac.jp/helpwww2.html`

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