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UNDERSTANDING THE EFFECT OF 1,3-BUTADIENE ON HUMAN LUNG FIBROBLASTS:
Does Exposure to 1,3-Butadiene Induce Senescence?

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UNDERSTANDING THE EFFECT OF 1,3-BUTADIENE ON HUMAN LUNG FIBROBLASTS:

Does Exposure to 1,3-Butadiene Induce Senescence?

by

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Dedication

In loving memory of my grandmother, Lucile Tyler Putman, and to my mother Laura Kathleen Palmer, who has always believed in me.

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ABSTRACT: Cellular senescence is a state of irreversible growth arrest induced by either telomere shortening (replicative senescence) or telomere-independent signals (stress-induced premature senescence or SIPS). Recent studies have shown that cigarette smoke extract induces senescence in lung fibroblasts and alveolar epithelial cells. 1,2,3,4-diexpoxybutane, or diepoxybutane (DEB), is the most reactive metabolite of 1,3-butadiene, an important hazardous air pollutant and potent genotoxic agent found in cigarette smoke. In this study we examined the effect of DEB on the proliferative capacity of human lung cells. We hypothesized that exposure of human lung fibroblasts (HLF) to DEB induces stress-induced premature senescence (SIPS). Cell culture experiments demonstrated that exposure of HLF to DEB induced persistent DNA damage and senescence. This senescence was characterized by a dose-dependent increase in senescence-associated β-galactosidase activity, senescence-associated alterations in morphology, formation of DNA damage foci, activation of the ATM-p53-p21 pathway, and irreversible growth arrest. These observations suggest that DEB induces senescence and provide the first evidence for senescence induced by an environmental toxicant. As a component of cigarette smoke and as a hazardous air pollutant, DEB-induced senescence could contribute to the pathogenesis of lung diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and cancer, by inhibiting normal lung fibroblast function and repair.

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ABBREVIATIONS

ATM	Ataxia telangiectasia mutated kinase
BD	1,3-Butadiene
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitors
COPD	Chronic Obstructive Pulmonary Disease
CSE	Cigarette smoke extract
d	Day(s)
DAPI	4'6-diamidino-2 phenylindole dihydrochloride
DEB	Diepoxybutane (butadiene diepoxide)
DEX	Dexamethasone
DPBS	Dulbecco's phosphate buffered saline
DSB	Double strand break(s)
DTT	1,4-Dithiothreitol
E2F	E2F transcription factor (Elongation factor 2)
ECM	Extracellular matrix
EGF	Epithelial growth factor
EMEM	Eagle's minimal essential medium
EPA	Environmental Protection Agency
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H2AX	Histone H2A
γH2AX	Phosphorylated H2A (serine 139)
HAPS	Hazardous air pollutants

HLF	Human lung fibroblasts
h	Hour(s)
IHC	Immunohistochemistry
IGF-1	Insulin-like growth factor 1
min	Minutes
mtDNA	Mitochondrial DNA
NAC	N-acetyl-cysteine
NIEHS	National Institutes of Environmental Health Sciences
nDNA	Nuclear DNA
PDGF	Platelet-derived growth factor
pRB	Retinoblastoma tumor suppressor protein
ROS	Reactive oxygen species
SA β-Gal	Senescence-associated Beta-Galactosidase
SIPS	Stress-induced premature senescence
TGF-β	Transforming growth factor β
WB	Western Blot

PREFACE

My research group has a long-term interest in the effects of 1,2,3,4-diexpoxybutane, or diepoxybutane (DEB), on human lung fibroblasts (HLF). DEB is the most reactive metabolite of 1,3-butadiene, an important hazardous air pollutant and potent genotoxic agent. They had investigated DEB-treated HLF effects on cell cycle progression, nuclear localization of cell cycle arrest mediators, mitotic index, and cellular proliferation. Results showed that an acute exposure of these lung fibroblasts to DEB resulted in prolonged cell cycle arrest without any increase in apoptosis or necrosis. Observed effects on cell cycle progression were concentration-dependent, evident at concentrations as low as 3.3 μM exposure for 1 hour (h), were accompanied by increases in nuclear levels of the cell cycle mediators, p53 and p21, and persisted for at least 4 days (d) (Schmiederer *et al*., 2005).

During my laboratory rotation with this group, my goal was to start probing mechanisms for these observations. Specifically, I began investigating why DEB-treated cells remained in cell cycle arrest instead of undergoing apoptosis despite the well-known capacity of butadiene and its active metabolites to damage DNA (Albertini *et al*., 2010). Initially, my research focused on the intrinsic apoptotic pathway to determine if this pathway was activated after DEB treatment and then blocked. Two predominate pathways of apoptosis are known, the intrinsic pathway and extrinsic pathway (Adrain *et al*., 2002). The extrinsic pathway is triggered on the outside of the cell via cell death receptors [FAS receptors (FasR) or tumor necrosis factor receptors (TNFR)] when

extracellular conditions, such as natural killer cells of the immune system, determine the cell's fate. On the contrary, the intrinsic pathway in activated when an injury occurs inside of the cell due to cellular stresses such as DNA damage or heat shock (Elmore, 2007). Both pathways involve cleavage and activation of proteases called caspases: however, the intrinsic pathway seemed more likely since it is induced by DNA damage, a known effect of DEB (Adrain *et al.*, 2002). The intrinsic pathway involves mitochondrial events, notably release of mitochondrial apoptotic factors such as cytochrome c from injured mitochondrial membranes and opening of the mitochondrial transition pore. However, experiments indicated that the integrity of the mitochondrial membrane was not affected after DEB treatment, neither was cytochrome c released from the mitochondrial membrane based on immunofluorescence microscopy and mitochondrial fractionation. Furthermore, apoptosis was not triggered following treatment, based on apparent non-activation of two key effector caspases of the intrinsic apoptotic pathway, caspase-9 and caspase-3, according to both Western blotting and caspase activity assays.

Since the profound ability of DEB to arrest the cell cycle of HLF could detrimentally impact critical roles of lung fibroblasts in lung repair and maintenance of lung homeostasis, I decided to further explore alternative mechanisms behind DEB-induced cell cycle arrest. My research in this area was facilitated by my receiving a pre-doctoral fellowship from our NIEHS Toxicology Training Grant (T-32 ES007254) and seed money from the Sealy Center for Environmental Health and Medicine. Insight into senescence as a mechanism occurred during one of my departmental research update seminars when someone suggested that senescence could account for the prolonged cell

cycle arrest in our cells. Based on the literature, stress-induced premature senescence (SIPS) seemed feasible since SIPS can result from DNA damage and/or oxidative stress (Serrano & Blasco, 2001; Lou & Chen, 2006). Moreover, the previously observed increases in nuclear p53-p21 (Campisi, 2005).

I began searching the senescence literature and asking others about precedents where other environmental toxicants had been linked to senescence. A paper "Cigarette Smoke Induces Cellular Senescence" (Nyunoya *et al*., 2006) reported that cigarette smoke extract (CSE) induces cellular senescence in lung fibroblasts with upregulation of the p53 and p16 pathways. Another paper about the effects of CSE on alveolar epithelial cells demonstrated similar findings (Tsuji *et al*., 2004). These seminal reports linked cigarette smoke to senescence. Given that 1) butadiene is an important component of cigarette smoke; 2) DEB is the most reactive metabolite of the potent genotoxic agent butadiene; and 3) the hallmark of senescence is an inability of cells to replicate their DNA following stimulation with growth factors: I decided to modify my research goals to test the hypothesis that the prolonged cell cycle arrest seen after DEB treatment was due to senescence.

Preliminary experiments were very promising since both a single, high-dose treatment and multiple, low-dose treatments of HLF with DEB resulted in an enlarged, flattened morphology and increased SA β-gal activity which are consistent with senescence. Therefore, based on these preliminary findings, my research group's findings, and the precedent of cigarette smoke-induced senescence by Nyunoya *et al*. and Tsuji *et al*., I hypothesized that DEB treatment induces stress-induced premature

senescence (SIPS) via persistent DNA damage, phosphorylation of ATM, upregulation of the p53/p21 pathway, and subsequent events leading to the development of a senescent fibroblast. My dissertation project evolved to address the following two research aims:

AIM I: To test the hypothesis that treatment of human lung fibroblasts to DEB results in stress-induced premature senescence.

AIM II: To determine if the ATM-p53-p21 pathway is involved in the prolonged cell cycle arrest and senescence induced by DEB treatment.

CHAPTER 1

INTRODUCTION

Environmental Toxicants

According to the National Institute of Environmental Health Sciences (NIEHS), a toxicant is "any chemical or mixture of chemicals that presents a risk of death, disease, injury, or birth defects in organisms that ingest or absorb it." An important category of toxicant is the man-made compound. Of the millions of man-made chemical compounds that have been synthesized throughout the past century, more than 85,000 synthetic chemicals have been registered for use in the United States (US) since World War II and another 2,000 are estimated to be added each year (US EPA, 2002). These chemical compounds are used in virtually all sectors of society. While some of these products, such as pharmaceuticals to treat illness and fertilizers to increase food production, have bestowed great benefits worldwide, many man-made compounds are recognized toxicants with known hazards to human health.

Furthermore, with the advent of technology, the magnitude and range of exposures to toxicants have increased drastically. People are exposed in their environments primarily via ingesting contaminated food, drinking contaminated water, or breathing contaminated air. The US Environmental Protection Agency (EPA) classifies air pollutants that can cause serious health and environmental hazards as "hazardous air pollutants" or air toxics. Scientific evidence has increasingly indicated relationships

between numerous environmental toxicant exposures and human health problems, such as asthma, cancer, chronic obstructive pulmonary disease (COPD), and other chronic diseases (Costa & Schelegle, 1999; WHO, 2006). Detrimental effects caused by exposure to toxic air pollutants can range from unseen physiological changes at the cellular level to subtle changes in breathing, such as wheezing or coughing, to more drastic changes resulting in disease caused by damage to various systems, such as the immune, respiratory, reproductive, or nervous systems (Costa & Schelegle, 1999; Kampa & Castanas, 2007).

1, 3- Butadiene: An Important Environmental Toxicant

PHYSICAL PROPERTIES

1,3-Butadiene (CH_2=CH_CH=CH_2, CAS No. 106-99-0) (BD), also known as α,γ-butadiene, biethylene, butadiene, divinyl, erythrene, and vinylethylene, is a highly volatile colorless gas with slight gasoline-like odor (Albertini *et al.,* 2010). BD is noncorrosive, has a molecular weight of 54.09 g/mol, boiling point of 4.4° C (Weast, 1986), vapor pressure of 1,790 mm Hg at 20° C (Santodonato, 1985), is readily liquefied and soluble in ethanol, diethyl ether, and organic solvents (Verschueren, 1983; Budavari, 1989), and only slightly soluble in water. The physical property of BD most relevant to its industrial utility is its reactivity as a monomer or co-monomer. The half-life of BD in soil or water is limited; however, based on its physical properties, BD is not thought to

adsorb significantly into soil or sediment. Instead, BD is expected to volatilize quickly into the atmosphere (National Toxicology Program, 1992).

PRODUCTION AND INDUSTRIAL USES OF 1,3-BUTADIENE

BD was initially produced in 1886 thru the decomposition of petroleum hydrocarbons via the addition of heat (Kirshenbaum, 1978). Commercial production of BD began in the 1930's when Germany's I. G. Farben Industrie synthesized the first styrene-butadiene rubber known as Buna S. Lack of access to natural rubber plantations in Southeast Asia during World War II fueled development of the synthetic rubber industry, and hence the demand for BD production. In 1940, the U.S. Government established the Rubber Reserve Company in an effort to stockpile natural rubber and to create a synthetic rubber program. Production of BD increased to 2.7 billion pounds in 1965 as new plants were established to meet the auto industry's demand for synthetic rubber needed for tire production (Chemical Market Reporter, 2006).

Since the 1960's, BD production has risen and fluctuated based on demands of the auto industry, which accounts for 60 percent of the overall consumption of BD (Chemical Market Reporter, 2006). In 1998, approximately 4.5 billion pounds were manufactured in the US (Grub & Loser, 2005). In 2001, worldwide, an estimated 18.5 billion pounds of BD was produced with production projected to increase yearly by 4 percent (National Toxicology Program, 2002). Current US production volumes are not available, but yearly US production capacity was estimated to be approximately 6 billion pounds by 2008 (Chemical Week, 2008).

BD is a crucial building block in polymers because of its high reactivity and facile structure. Thus, BD is used extensively as a cross-linker in the manufacture of various polymers, elastomers, and chemicals. BD's main use is in the production of styrene-butadiene rubber or synthetic rubber and thermoplastic resins (Fajen *et al.,* 1993). Automobile tires are the main end-use product of BD (Chemical Marker Report, 2006). Elastomers of BD are used in the manufacture of resins; acrylonitrile-butadiene-styrene plastics used in cars, luggage, and computers; and synthetic rubber products, including tires, hoses, and pipes (US EPA, 2002). Also, BD is used as an intermediate in the manufacture of diverse chemicals, including fungicides and nylon (EINECS, 2002).

1,3-Butadiene Carcinogenicity and Biotransformation

In 1990, BD was identified as one of the 189 hazardous air pollutants in the US EPA's Clean Air Act Amendments. In 1997, mounting evidence demonstrating carcinogenic effects of BD prompted a reduction in the permissible exposure limit to 1 ppm. BD was subsequently classified by the US Department of Health and Human Service's (DHHS) National Toxicology Program as a known human carcinogen in the Ninth Report on Human Carcinogens (IARC, 1999). This carcinogenic effect on humans is not attributed to BD directly, but instead to the epoxide metabolites that are formed by its biotransformation (Jackson *et al.,* 2000). As shown in Figure 5, cellular enzymes oxidize BD sequentially to 1,2-epoxy-3 butadiene (BMO) and 1,2,3,4-diepoxybutane or diepoxybutane (DEB) by cytochrome P450 2A6 and 2E1.

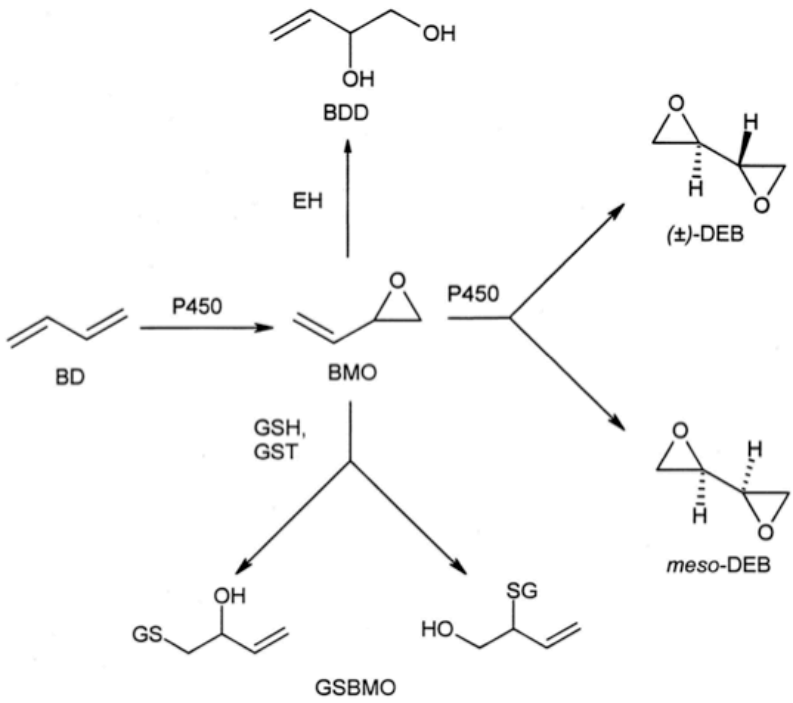

Figure 1: Scheme of 1,3-butadiene (BD) metabolism by cytochrome P450 (P450), glutathione s-transferases (GST), and epoxide hydrolases (EH). Other abbreviations are DEB, diepoxybutane; BDD, 3-butene-1,2-diol; GSEB-I, S-(2-hy- droxy-3-buten-1-yl)glutathione; GSEB-II, S-(1-hydroxy-3-buten-2-yl) glutathione (Kemper *et al*., 2001).

MUTAGENICITY AND DNA DAMAGE

An array of mutagenic responses to BD and its metabolites has been reported in over 600 publications in a variety of biological systems, including humans (US EPA, 2002; Albertini *et al*., 2010). DEB has been shown to react with DNA in ways

characteristic of bifunctional alkylating agents which cause interstrand DNA crosslinks (Lawley & Brookes, 1967). Exposure of cultured cells and animals to BD or its metabolites results in protein and DNA adducts and multiple types of genetic damage, including specific locus mutation, sister chromatid exchange, and chromosome aberrations (Jackson *et al*., 2000; Catallo *et al*., 2001; Albertini *et al*., 2010). A study evaluating exposures to BD to employees of a monomer production plant demonstrated that exposure to BD at 1-3 ppm induced a higher frequency of mutations in lymphocytes (Ammenheuser *et al*., 2001). While all three epoxide metabolites are mutagenic *in vitro* and *in vivo*, DEB is the most reactive metabolite (Elfarra *et al*., 2001; Albertini *et al*., 2010). DEB is mutagenic at concentrations 100-fold less than those observed for BMO (Cochrane & Skopek, 1994). Thus, DEB treatment of HLF provided a relevant way to investigate consequences of BD exposure in my dissertation project.

Human Exposure to 1,3-Butadiene

OCCUPATIONAL EXPOSURE

Intensive human exposure to BD occurs primarily through occupational exposure (by inhalation) in industries involved in rubber and latex production, petroleum refining, secondary lead smelting, water treatment, production of agricultural fungicides, production of adiponitrile (a main ingredient in the production of nylon), and fossil fuels (US EPA, 2009). In the US alone, each year an estimated 65,000 workers are exposed to BD (Fajen *et al.*, 1993).

ENVIRONMENTAL EXPOSURE

Environmental BD exposures occur via polluted air and water near chemical, rubber, or plastic manufacturing facilities; and ingestion of foods contaminated from plastic or rubber containers (US EPA, 2009). Common sources for BD exposure involve incomplete combustion of fuels and biomass combustion such as forest fires (Penn *et al*., 2007). Approximately 770,000 tons of BD are annually released into the atmosphere due to biomass combustion in the US (Ward & Hao, 1992). Moreover, an estimated 3,607 to 26,966 tons of BD are released from forest fires in Canada each year according to the Canadian Petroleum Products Institute (CPPI, 1997; Curren *et al.,* 2006). Automobile exhaust is a continuous source of BD in the environment, especially in urban settings, and thus a likely factor in the BD detected in ambient air of metropolitan areas (US EPA, 2009).

Cigarette smoking is a substantial source of repeated exposure to BD. Main-stream or direct cigarette smoke is estimated to contain 16 to 77 µg of BD per cigarette. Significantly higher levels of BD are found in side-stream smoke or environmental tobacco smoke (ETS) ranging from 205 to 400 µg of BD per cigarette (Brunneman *et al.,* 1990; Smith *et al.*, 2000). Smokers are thus regularly exposed to considerable amounts of BD since they inhale both direct and ETS.

Health Effects of 1, 3-Butadiene Exposure

Butadiene is absorbed via the lungs and is distributed throughout the body (EINECS, 2002). Acute, low level exposure to BD causes eye, nose, and throat irritation whereas exposure to high levels of BD can cause drunkenness-like symptoms, unconsciousness, or death (US EPA, 2002). Prolonged contact with low levels of BD causes tumors of the hematopoietic system, lung, heart, kidney, and liver in experimental animals and is associated with leukemias, reticulosarcomas, and lymphosarcomas in humans (Cochrane & Skopek, 1994; Kligerman *et al.*, 1999; Hong *et al.*, 2000). Epidemiological studies have shown excess mortality from lymphatic and hematopoietic cancers in workers with occupational exposures to BD (Delzell *et al.*, 1995; Divine & Hartman, 1996; Delzell *et al.,* 2001; Divine & Hartman, 2001). For example, a study evaluating the association between exposure to butadiene and increased mortality from cancer among 16,579 workers in the synthetic rubber industry followed over a 55-year period showed a positive association between BD exposure and leukemia (Graff *et al*., 2005).

1,3-Butadiene and the Human Lung

Role of Fibroblasts in Repair of Injured Lung

Tissue repair after an acute injury to the lung- whether the injury is chemical, physical, cytotoxic, or immunological- is characterized by extensive migration, proliferation, and differentiation of fibroblasts, the primary cell of the extracellular matrix

(ECM) (Raghow, 1994; Sacco *et al*., 2004). Lung fibroblasts play a critical role in lung repair and maintenance of lung homeostasis (Gurtner, 2008). After migrating to sites of injury, fibroblasts begin proliferating and synthesizing new collagen, fibrin, and proteoglycans to provide a provisional ECM that will seal the wound and enable granulation tissue to form (Raghow, 1994; Gurtner, 2008). Contrary to normal lung tissue where fibroblasts are fairly scarce and quiescent, fibroblasts are numerous and very active in granulation tissue. During the final phase of tissue repair, fibroblasts remodel the newly deposited matrix and granulation tissue through several processes including remodeling and contraction (Grinnell, 1994; Desmouliere *et al*., 2003). During this final stage, fibroblasts replace the provisional ECM with a more organized, permanent one. After tissue repair is completed, numerous fibroblasts are no longer needed and most undergo apoptosis. Studies by Greenhalgh *et al*. (1998) have shown that apoptosis is the primary means for decreasing fibroblast cellularity during all stages of tissue healing. Apoptosis enables populations of fibroblasts to be reduced without damage to the tissue or induction of an inflammatory response after tissue repair is complete.

Inhibition of Fibroblast Function and Development of Disease

Inadequate tissue repair is thought to play as crucial of a role in disease pathogenesis as tissue damage itself (Raghow, 1994; Ramirez & Rifkin, 2003). Inadequate repair can contribute to structural changes in the lung characteristic of pulmonary diseases associated with decreased airflow, such as COPD and pulmonary fibrosis (Husain, 2009). Inadequate tissue repair can occur when any normal functions of

fibroblasts are inhibited. For example, cigarette smoke exposure was reported to contribute to the development of emphysema by inhibiting tissue repair by Carnevalli *et al*., 1998. Specifically, this study demonstrated that cigarette smoke inhibited fibroblast-mediated collagen gel contraction by decreasing fibroblast production of fibronectin. Moreover, Wang *et al*. (2003) showed that cigarette smoke and its volatile components inhibited the proliferation and chemotaxis of fibroblasts as well as the ability of fibroblasts to remodel the ECM. Dysregulation of apoptosis in fibroblasts can result in pathologic forms of healing such as excessive scarring and fibrosis (Greenhalgh, 1998). Effects of BD or its metabolites on normal fibroblast function and wound repair have not been previously described.

The only report of BD metabolite effects on fibroblast capacity for proliferation or death published in the literature is the study by Schmiederer *et al*., 2005. One other report by Yadavilli and Muganda (2004) described the cellular effects of DEB, however, in a different cell type. They showed that DEB induced a p53 and caspase-3 mediated apoptosis in human lymphoblasts (Yadavilli & Muganda, 2004). While cells are capable of both apoptosis and senescence, both cell type and degree of damage are important determinants of ultimate cell fate. Damaged fibroblasts and epithelial cells tend to senesce, whereas lymphocytes tend to undergo apoptosis (Campisi & d' Adda di Fagagna, 2007), which helps explain the different cell fates described in these two reports.

1,3-Butadiene and the Human Lung

Research on exposure to BD and its metabolites has focused on genotoxicity and has used identified metabolites and molecular changes as biomarkers of exposure and biological effect (Cochrane & Skopek, 1994; Jackson *et al*., 2000). Moreover, most research on BD has centered on cancer as the final endpoint although epidemiological studies have not shown as association between BD exposure and lung cancer (Elfarra *et al*., 1996; Hayes *et al*., 2001).

Several consequences of exposure to BD which could significantly impact human health have not been considered. At the cellular level, detrimental effects of exposure can include dysregulation of fibroblast functions, failure of damaged cells to undergo apoptosis, and stress-induced premature senescence. These kinds of detrimental cellular effects are postulated to contribute to the systemic consequences of chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) (Kampa & Castanas, 2007).

Cigarette smoking is the most significant risk factor for the development of COPD and fibrosis (Yoshida & Tuder, 2007). Two seminal studies by Tsuji *et al*. 2004 and Nyunoya *et al*. (2006) demonstrated that cigarette smoke induces cellular senescence in human alveolar epithelial cells and human lung fibroblasts, respectively. The study by Nyunoya *et al*. compared a single exposure of cigarette smoke extract (CSE) to multiple exposures over an extended time period and examined exposed human fibroblasts for classic markers of senescence. They found that a single exposure to CSE resulted in inhibition of cell growth accompanied with a significant increase in ATM, p53, and p21

activity. In addition, multiple CSE exposures led to a profound cell cycle arrest of the fibroblasts, increased levels of p16 at later time points, an enlarged, flattened morphology, and increased SA β-gal activity consistent with the classic senescence phenotype. The identities of the specific entities among the 4000 components in cigarette smoke which are capable of inducing senescence in human lung fibroblasts remains to be determined.

Cellular Senescence

Multicellular organisms have evolved multiple mechanisms to control replication of cells at risk of oncogenic transformation due to damage by therapeutic, environmental, infectious, or endogenous insults (Campisi, 2007). For decades, damaged cells were thought to either undergo repair or to die by necrosis or apoptosis when damage was too severe for repair. However, cellular senescence has come to the forefront as another crucial mechanism for preventing damaged cells from proliferating (Collado & Serrano, 2010). Whereas apoptosis eliminates damaged cells, senescence irreversibly arrests the growth of injured cells. Why the same stimulus can induce either senescence or apoptosis hasn't been fully elucidated. Moreover, the mechanisms that determine apoptosis over senescence, and vice versa, are also not yet understood (Collado *et al.*, 2006).

CELLULAR QUIESCENCE VERSUS CELLULAR SENESCENCE

When cells exit the replicative cell cycle, they enter the resting state called G_0, also termed quiescence (Gray *et al*., 2004). Most eukaryotic cells spend the greater part of their natural lives in quiescence (Lewis & Gattie, 1991). Quiescence was originally thought to be a passive process. However, Liu *et al*. demonstrated that quiescence is an active process which activates numerous genes, which function to stop the cell cycle and maintain tissue homeostasis (2007). Cell cycle progression and entrance into quiescence intersect at the early G_1 phase of the cell cycle. In the presence of sufficient nutrients, a cell can enter late G_1 and S phase and progress through the cell cycle. However, in the absence of nutrient supplies, a cell in G_1 will enter quiescence.

A cell is able to exit quiescence when environmental conditions change, such as by the addition of nutrients. Importantly, quiescent cells can quickly exit quiescence in response to injury. For example, when an injury occurs in the lung, fibroblasts begin proliferating and initiate tissue repair. When tissue repair is completed, fibroblasts exit the cell cycle and re-enter quiescence or are eliminated by apoptosis. The reversibility of quiescence is a key characteristic that distinguishes this state from other nonproliferative states, including senescence and terminal differentiation (Coller *et al*., 2006).

In contrast, senescent cells cannot reenter the cell cycle in the presence of growth factors even though mitogen-activated pathways continue to promote the transport of nutrients into the cell, metabolism, and produce an increase in cellular mass. Senescent cells, therefore, exhibit a characteristic phenotype with increased size, active metabolism,

and secretion of mitogens and components of the extracellular matrix. Hypertrophy, therefore, is an essential characteristic of senescent cells (Blagosklonny, 2006).

Factors in serum provide central mitogenic signals in wound healing and tissue homeostasis (Liu *et al*., 2007). Serum factors initiate progression through the cell cycle and induce a transcriptional program that triggers many components of tissue repair. Senescent cells, however, are not responsive to stimulation by serum or other growth factors but remain viable for extended periods of time. Addition of serum factors to the culture medium (containing 10% fetal bovine serum (FBS)) has long been known to increase population doubling level (PDL) in young cultures but not senescent cultures (Cristafalo, 1972). Additionally, Phillips *et al*. (1984) reported a complete loss of a proliferative response in senescent WI-38 cells to platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, transferrin, and dexamethasone (DEX).

Senescent cells can, however, be pushed back into the cell cycle via infection with tumor viruses such as SV40 (Ide *et al*., 1983), human cytomegalovirus (Ide *et al*., 1984), or by fusion with transformed cells (Norwood *et al*., 1975). Furthermore, senescence can be overcome when the cell cycle is reactivated by inactivating key players which block the cell cycle including: p16, p21, p53, and pRb (Beausejour *et al*., 2003; Gire & Wynford-Thomas, 1998). Table 1 summarizes key differences between properties of senescent cells versus quiescent cells.

Table 1: Comparison of key properties of senescence versus quiescence cells.

	Senescence	Quiescence
Stability	Permanent	Reversible
Induced by	Telomere shortening Prolonged DNA damage Oxidative stress Oncogene activation	Serum starvation Growth at high density Transient DNA damage
SA β-gal expression	Present	Absent
DNA damage foci (γH2AX)	ARF p16 p53 p21 pRb	p53 p21 p27 pRb

REPLICATIVE SENESCENCE

Cellular senescence is defined as the complete and irreversible loss of the replicative ability of primary somatic cells (Campisi & d' Adda di Fagagna, 2007). Senescence was first described in the 1960s as a process that prohibits normal human fibroblasts from growing indefinitely in culture (Hayflick & Moorhead, 1961; Hayflick, 1965). This type of senescence is referred to as "replicative senescence" (Serrano &

Blasco, 2001). Usually, normal human diploid fibroblasts are capable of dividing 60 to 70 times in culture before they stop proliferating and exhibit a classic senescence phenotype including a flattened and enlarged cell morphology, resistance to apoptosis, and an increase in senescence-associated beta-galactosidase activity (Dimri *et al.,* 1995; Wang *et al.,* 1995; Marcotti *et al.,* 2004; Campisi, 2005). Additionally, senescent cells show significant changes in gene expression, especially of cell-cycle inhibitors and activators in the p53 and/or pRb pathway (Satyanarayana *et al.,* 2004; Campisi, 2005), or both. (Shelton *et al.*, 1999; Jackson & Pereira-Smith, 2006). The cyclin-dependent kinase inhibitors (CDKIs) p21 and p16 are frequently expressed by senescent cells. Moreover, senescent fibroblasts overexpress proteins involved in remodeling of the extracellular matrix (ECM), such as matrix metalloproteases, and inflammatory mediators (Campisi & d'Adda di Fagagna, 2007).

STRESS-INDUCED PREMATURE SENESCENCE (SIPS)

The concept of senescence, however, is also applied to the irreversible arrest of cells caused by a variety of stresses, including DNA damage and oxidative damage (Serrano & Blasco, 2001; Lou & Chen, 2006). This type of stress-induced cellular senescence is termed stress-induced premature senescence. Known exposures that result in SIPS include DNA damaging agents, such as ionizing irradiation (Kastan *et al.,* 1991; Di Leonardo *et al.,* 1994), hydrogen peroxide (Chen *et al.,* 1994), many chemotherapy agents including etoposide, campthothecin, bleomycin and actinomycin D (Robles & Adami, 1998), TGF-β (Yoon *et al.*, 2005), cigarette smoke extract (Tsuji *et al.,* 2004;

Nyunoya *et al*., 2006) and prolonged β-interferon treatment (Moiseeva *et al*., 2006). Stress-induced premature senescent fibroblasts are similar morphologically to replicative senescent fibroblasts and exhibit the same senescence phenotype (Di Leonardo *et al*., 1994; Stein & Dulic, 1995).

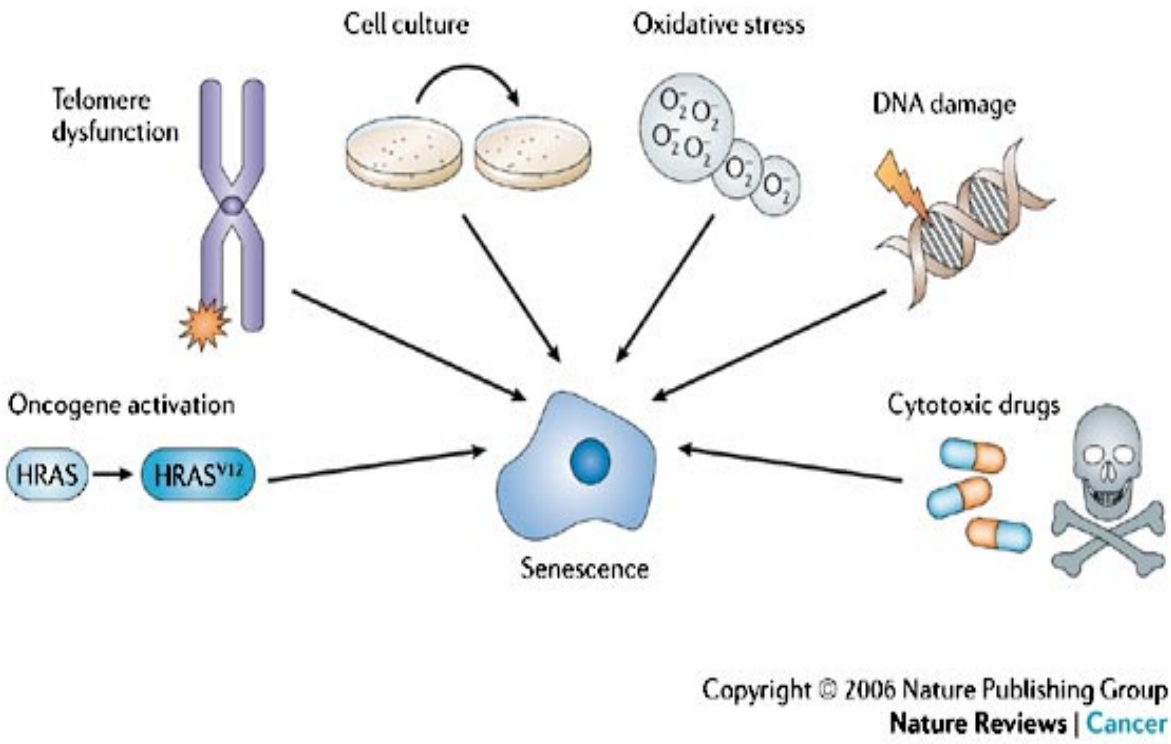

Figure 2: Many stimuli cause senescence. Diverse stimuli can engage a common program, which ends with establishment of an irreversible proliferative arrest known as senescence. Each of these stimuli evokes stressful conditions for the cell and, interestingly, many of these factors are present in the tumor environment (Collado & Serrano, 2006).

CELLULAR MECHANISMS OF SENESCENCE

Many studies have demonstrated that a cause of replicative senescence, first observed in cell culture by Hayflick, is telomere shortening (Harley *et al*., 1990; Cong, 2002). Telomeres are repetitive DNA sequences and specialized proteins that cap the ends of linear chromosomes and protect the integrity of the chromosome. Telomeres shorten with each cell cycle because the enzyme required for synthesizing telomeric DNA de novo is not expressed by most human cells (Chiu, 1996; Campisi, 2001). The exposed DNA of shortened telomeres is recognized as DNA double strand breaks (DSBs) by the DNA damage response machinery which, in turn, signals DNA damage checkpoints (d' Adda di Fagagna, 2003). To prevent shortened telomeres from compromising chromosomal integrity, cell growth becomes irreversibly arrested and the cell enters senescence. SIPS can be induced through either the p53 pathway or pRb pathway, or both (Campisi, 2005). A common cellular mechanism shared by replicative senescence triggered by telomere shortening and by SIPS is the DNA damage response pathway. Ataxia telangiectasia mutated (ATM) kinase is the apical kinase of the DNA damage pathway and is responsible for activating numerous downstream targets (Lundberg *et al*., 2000; Lou & Chen, 2006).

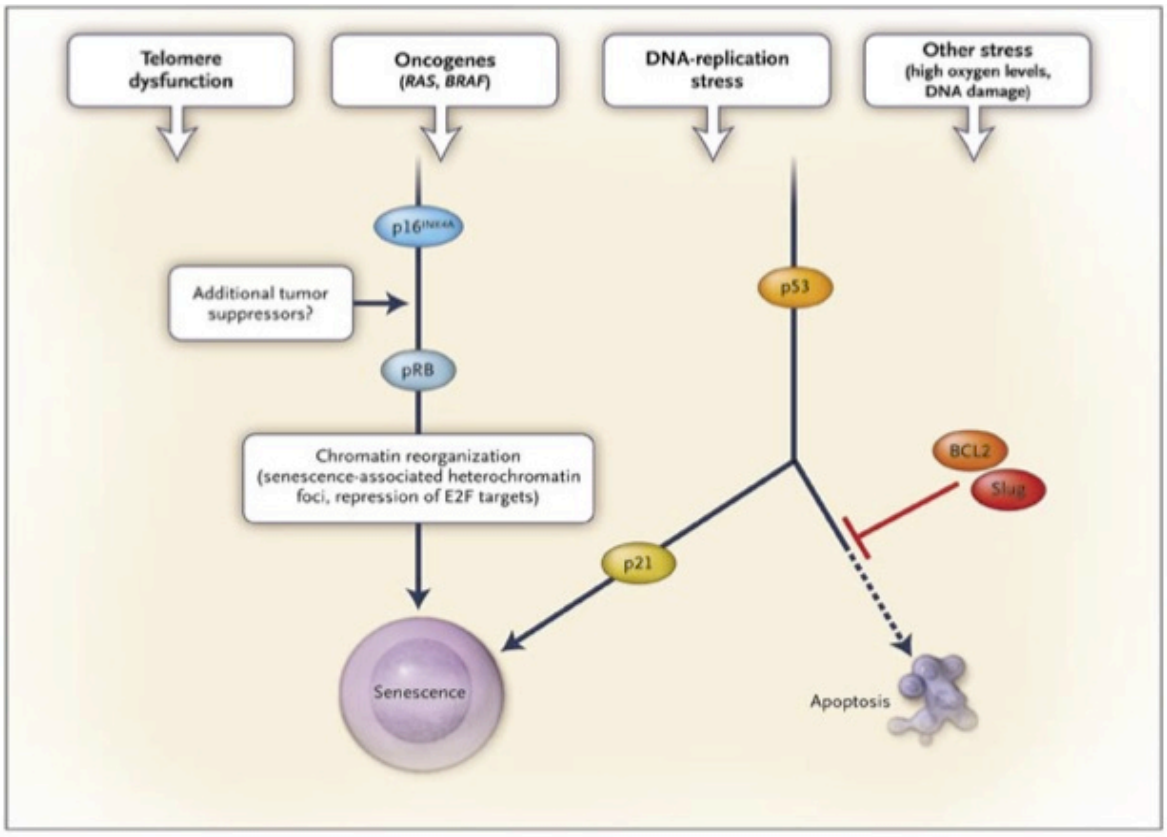

Figure 3: Types of stress which activate tumor-suppressor networks mediated senescence. Other established causes of senescence lead to activation of the two major established tumor-suppressor networks. An early event in oncogene-mediated senescence is an elevated level of the CDK inhibitor p16. As a result, the retinoblastoma tumor-suppressor protein (pRb) accumulates in its hypophosphorylated, active state. In this form, pRb binds to various downstream effectors, including E2F transcription factors, which are thus unable to activate target genes required for DNA replication. In contrast, pRb–E2F complexes repress transcription, which is associated with the nuclear accumulation of

senescence-associated heterochromatin foci. The second major tumor-suppressor pathway to senescence is characterized by activation of the p53 network. Critical targets for this transcription factor include anti-proliferative genes (such as *p21*) and various pro-apoptotic genes. The genetic context dictates the outcome of p53 activation. For example, abundant levels of the survival factors BCL2 and Slug (found in melanocytes) tip the balance in favor of senescence (Mooi & Peeper, 2006).

The pRb Pathway

The retinoblastoma tumor suppressor protein restricts cell cycle progression by guarding a late G_1 cell cycle checkpoint via association with transcription factors such as elongation factor 2 (E2F) (Sanchez *et al*., 1996). Phosphorylation of pRb by cyclin-dependent kinases (cdks), such as Cyclin-D activated cdk4 or cdk6 and Cyclin-E activated cdk2, overcomes pRb's ability to sequester transcription factors. E2F is released and the cell is able to progress into the S phase of the cell cycle. The KIP/CIP family of cyclin-dependent inhibitors (CDKI) is a family of small, inhibitory molecules that provide further control over pRb phosphorylation. Members of the KIP/CIP family of CDKIs inhibit both Cyclin-D and Cyclin-E activated kinase complexes and include p21, p27, and p57. Members of the INK family of CDKIs inhibit activation of cdk4 and cdk6 and include p16, p18, and p19 (Sherr *et al*., 1995).

p16 is a key player in the pRb tumor suppressor pathway. p16 inhibits the cyclin-dependent kinase 4/6 (cdk4/6) and keeps pRb hyperphosphorylated. In the unphosphorylated form, pRb arrests the cell cycle by preventing the transcription factor E2F from initiating transcription of the DNA. Increased levels of p16 have been shown in senescent cells (Alcorta *et al*., 1996; Hara *et al*., 1996; Robles & Adami, 1998) and overexpression of p16 has been shown to induce cellular senescence (Alcorta *et al*., 1996; Hara *et al*., 1996).

The p53 Pathway

The tumor-linked protein 53 (p53), known as the "guardian of the genome", is a crucial downstream target of the DNA damage response pathway (Harper & Elledge, 2007). Activation of the DNA damage pathway initiates the stabilization of p53 and its translocation to the nucleus. p53 thereby directly activates the upregulation of genes and the transcription of proteins responsible for cell cycle arrest or apoptosis. p21 is a key target gene of p53 and functions as the major effector protein of p53-mediated cellular senescence (Harper & Elledge, 2007).

Increased levels of p53 activity and p21 have been found in senescent cells in numerous studies (Di Leonardo *et al*., 1994; Alcorta, 1996; Yeo *et al*., 2000). The study by Robles and Adami (1998) demonstrated that drugs that induce DNA double strand breaks lead to increased levels of p16 and premature senescence of normal fibroblasts. In this study, treatment of normal human lung fibroblasts with the cancer chemotherapeutic agents, bleomycin or actinomycin D, caused DNA double strand breaks that resulted in a

permanent cell cycle arrest. This arrest was p53 dependent and resulted in the induction of p21. While protein levels of p53 and p21 were transient, the increase in p16 remained even 30 days post treatment. Results of this study suggest that while p53 and p21 play a crucial role in initiating cell cycle arrest of damaged cells, p16 could be required for maintaining premature senescence of fibroblasts. Therefore, this research focused on the p53 pathway since DEB is known to induce DNA damage (Albertini *et al*., 2010). Moreover, Western blotting analysis revealed little difference in expression of p16 between mock-treated and DEB-treated HLF.

THE ROLE OF ROS IN SENESCENCE

The mitochondrial respiratory chain complex produces energy during aerobic respiration by consuming 85 to 90% of the oxygen taken up by the cell. Reactive oxygen species (ROS) are a major byproduct of aerobic respiration. The close proximity of mitochondrial DNA (mtDNA) to the mitochondrial respiratory chain and its naked, or histoneless, structure make mtDNA extremely susceptible to oxidative damage by ROS. While the majority of research on carcinogens has focused on the interaction of toxicants with nuclear DNA (nDNA), mitochondrial damage can often be more severe and persist longer than damage to nuclear DNA (Yakes & Houten, 1997). For example, research has shown that mitochondrial DNA (mtDNA) undergoes greater modification than nDNA when exposed to the dihydrodiol-epoxide derivative of benzo(a)pyrene (Weinstein *et al*., 1976). Other studies have shown similar results for alkylating agents and for other polycyclic aromatic hydrocarbons (Wunderlich *et al*., 1970; Allen & Coombs, 1980).

While DEB is structurally different from the dihydrodiol metabolite of benzo(a)pyrene, it also contains two highly reactive epoxide moieties. However, the effects of DEB on mtDNA have not been investigated. As a result of mtDNA damage, mitochondria release more ROS. Furthermore, it has been shown that an inhibition of the respiratory chain results in increased ROS production (Benzi *et al.,* 1991). Respiratory chain dysfunction, therefore, leads to a decrease in cellular ATP and a further increase in ROS, both events which are injurious to proper cellular function (Hanna & Nelson, 1998).

ROS have been shown to play a direct role in initiation of both replicative senescence and SIPS (Ramsey & Sharpless, 2006; Passos *et al.*, 2007, Lu & Finkel, 2008). SIPS has repeatedly been associated with direct ROS-induced damage of DNA which triggers the DNA damage response (Chen *et al.*, 1995; Lu & Finkel, 2008). Other studies have shown that ROS generated by stress signaling pathways can trigger cellular senescence pathways involving p53 (Beausejour *et al.,* 2003), p21 (Macip *et al*., 2002), p16 (Beausejour *et al.,* 2003), and phosphoinositide 3 kinase (PI_3K/Akt) (Collado *et al.,* 2005). Studies about aging have shown that ROS speed up telomere shortening resulting in replicative senescence (Von Zglinicki, 2002). Passos *et al*. (2007) suggested an association between mtDNA damage and cellular senescence since excess ROS production is a major cause of telomere damage and shortening which leads to senescence. However, the role of mitochondrial metabolic ROS leakage in SIPS has not been clearly elucidated.

Yoon *et al*. (2005) described an association between mitochondrial dysfunction and TGF-β induced cellular senescence, which highlights the important role of

mitochondrial integrity in protecting the cell from entering premature cell cycle arrest. In this study, lung epithelial cells treated with TGF-β exhibited increased levels of intracellular ROS, underwent cell cycle arrest, and developed markers of senescence. Pretreatment of cells with the antioxidant N-acetyl-cysteine (NAC) prevented the cells from developing a senescent phenotype which is consistent with the proposed crucial role of ROS in long-term cell cycle arrest. A recent study by Passos *et al.* investigated the role of ROS in the establishment of both SIPS and replicative senescence. Their studies showed that a positive feedback loop between the DNA damage response and ROS production establishes and maintains senescence (Passos *et al.,* 2010).

MARKERS OF SENESCENCE

Table 2 summarizes established markers and events in senescence (Coates, 2002). Senescence-associated β-galactosidase is a well-known marker of cellular senescence which was first described by Dimri *et al*. in 1995. Expression of SA β-Gal is dependent on confluence and probably increased lysosomal content (Dimri *et al*., 1995; Kurz *et al*., 2000; Severino *et al*., 2000). Other events associated with senescence can be assessed in cells or tissues using immunohistochemical (IHC) staining to visualize increased levels of CDKIs, such as p16 and p21, and DNA damage foci. We utilized these markers to assess the hypothesized effect of DEB on senescence and HLF.

Marker	Assay	Author
SA β-Gal	Histochemistry	Dimri *et al*., 1995
p16	WB, IHC	Alcorta *et al*., 1996
p21	WB, IHC	Tahara *et al*., 1995
p53	WB, IHC	Afshari *et al*., 1993
DNA damage foci (γH2AX)	WB, IHC	d' Adda di Fagagna, 2003

Table 2: A summary of the most well-established markers of senescence currently available. SA β-Gal, Senescence-associated beta galactosidase; WB, Western blot; IHC, immunohistochemistry.

Health Implications of Lung Cell Senescence

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of mortality in North America (Cazzola *et al.,* 2007). COPD is characterized by the progressive loss of airflow over decades resulting from the destruction of lung alveolar tissue, chronic inflammation in airways, and increased mucus production. Histological analysis of lungs from patients diagnosed with pulmonary emphysema, the primary form of COPD, revealed enlarged airspaces due to alveolar structures that have been damaged or lost. Despite extensive research, many unknowns remain concerning molecular, cellular, and genetic causes of COPD pathogenesis.

Smoking and aging have both been linked to the development of emphysema. Smoking is the greatest single risk factor for COPD. In fact, 1 in 7 smokers will develop chronic obstructive pulmonary disease in their lifetime (Murray & Lopez, 1997; ATS/ERS, 2002). As a progressive disease, COPD is more prevalent among the elderly with those 65 or older having a 2.6-fold higher disease rate than those aged 45 to 64, and a 35.4-fold higher disease rate than those aged 18 to 44 (Tuder, 2006). Given this, senescence (often termed aging) has recently begun to be considered as a factor behind the development of COPD (Vogelmeier, 2007).

For over 40 years, the dominant explanation for development of emphysema has been the inflammation-protease/antiprotease imbalance hypothesis (Laurell & Erickson, 1963). This hypothesis states that emphysema results from alveolar destruction caused by inflammation triggered by cigarette smoke, environmental pollutants, or bacterial products (Shapiro, 1995). Limitations of this hypothesis raised questions, such as: "A COPD lung presents with increased numbers of neutrophils, macrophages, and lymphocytes, but are these inflammatory elements targeting directly elements of cigarette smoke or, rather, is inflammation a secondary response to cellular and molecular alterations induced by chronic cigarette smoke inhalation?" (Tuder *et al.,* 2006).

Advances in understanding of the molecular mechanisms underlying the disease pathogenesis of COPD have led to new paradigms involving senescence during the past 5 years (Shapiro & Ingenito, 2005; Karrasch *et al.,* 2008; Aoshiba & Nagai, 2009; MacNee & Tuder, 2009). Recent studies have revealed an interplay between cigarette smoke, cellular senescence, and COPD. For example, cigarette smoke-exposed human lung cells

and mouse lungs show increased markers of senescence *in vitro* and *in vivo* (Tsuji *et al*., 2004; Nakashima *et al*., 2006; Nyunoya *et al*., 2006; Aoshiba & Nagai, 2009); circulating peripheral blood cells of patients with COPD have decreased telomerase activity (Morla *et al*., 2006); and alveolar cells, lung endothelial cells, and lung fibroblasts from patients with emphysema show increased markers of senescence (Muller *et al*., 2006; Tsuji *et al*., 2006). Thus, the protease/antiprotease imbalance hypothesis has been modified to include dynamic damage to the matrix by senescence and lung cells exhibiting the proinflammatory secretory 'SASP' phenotype.

IDIOPATHIC PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is a devastating progressive fibrotic lung condition which currently lacks approved therapies. The ATS/ERS classification defines IPF as ''a specific form of chronic fibrosing interstitial pneumonia of unknown etiology, limited to the lung and associated with the histological entity of usual interstitial pneumonia'' (ATS/ERS, 2001). The prognosis for patients with IPF is poor with a median survival of three years from time of diagnosis (Maher *et al*., 2007). Similarly to COPD, unknown aspects of IPF pathogenesis impede the development of effective treatment regimens.

IPF is a progressive disease and risk factors of IPF include age, gender, genetics, and smoking (Baumgartner, 1997; Zeki *et al*., 2010). IPF is also typically considered a disease of aging, the incidence increases almost 100-fold from 3 per 100,000 in persons less than 35 years to as much as 277 per 100,000 in men and 192 per 100,000 in women

over 75 years (Raghu *et al*., 2006). Examination of the etiology underlying familial cases of IPF and an advanced understanding of the role of senescence in aging has helped shed some light on disease pathogenesis of IPF.

Telomere shortening, the cause of replicative senescence, has been linked to 8–15% of familial cases of IPF and 1–3% of sporadic IPF cases that are accompanied by detectable telomerase mutations (Armanios *et al*., 2007; Alder *et al*., 2008; Cronkite *et al*., 2008). The presence of short telomeres that lead to early replicative senescence in both familial and sporadic cases of IPF helps explain, to some extent, why this disorder is usually associated with older individuals (Tsakiri *et al*., 2007; Armanios *et al*., 2007; Alder *et al*., 2008). Moreover, CAT scans demonstrating asymptomatic structural changes in the lungs of elderly IPF patients have provided further evidence for aging and senescence as a disease mechanism (Alder *et al*., 2008). Therapeutic strategies that prevent telomere shortening and senescence may be beneficial in the treatment of IPF (Armanios *et al*., 2007).

LUNG CANCER

Recent studies have demonstrated that senescence can be both beneficial and detrimental for cancer development depending on the cell type (Rodier & Campisi, 2011). However, Rodier and Campisi suggested that cellular senescence may be antagonistically pleiotropic meaning that while senescence can be beneficial, it can also be harmful and may actually promote cancer. The evolutionary theory of antagonistic pleiotrophy specifies that a biologic process can be both and harmful, depending on the

age of the organism (Williams, 1957; Rauser *et al*., 2006). Cancer is primarily an age-related disease and the incidence of cancer drastically increases in people over the age of 50 (Balducci & Ershler, 2005). Moreover, numbers of senescence cells accumulate drastically in older people, either by increased generation (via increased oxidative stress and replicative senescence) or decreased elimination, or both (Dimri *et al*., 1995; Wang *et al*., 2009). Therefore, while senescence is a beneficial anti-cancer mechanism in young people, it might be detrimental in the aged. Furthermore, the SASP of senescence cells has been shown to promote the growth of premalignant and malignant cell both *in vitro* and *in vivo* (Krtolica *et al*., 2001; Collado *et al*., 2005; Liu & Hornsby, 2007; Collado & Serrano, 2010). Furthermore, senescent cells, including endothelial cells and fibroblasts, have been found adjacent to malignant tumors in humans and have been shown to enhance the growth rate and invasiveness of cancer cells (Charalambous *et al*., 2007; Studebaker *et al*., 2008).

Rationale

DNA damaging effects of BD and its metabolites are well known as research has focused on the genotoxicity of these compounds (See excellent current review by Albertini *et al*., 2010). However, cellular effects of DEB on humans have not been fully explored. Only three studies have been published which examined cellular effects of DEB in human cells. This study was designed to address several important questions not considered by Schmiederer *et al*. (2005), the only reported investigation of DEB effects

on fibroblasts. Does the proliferative arrest produced by DEB treatment persist after 4 days, the latest time point examined? Is this proliferative arrest preceded by DNA damage, and is this damage repaired or persistent? Can potent mitogens push DEB-arrested HLF back into a replicative cycle or have cells become irreversibly arrested? Are responses to more environmentally relevant repeated low-dose DEB treatments similar to those described for a single DEB treatment?

CHAPTER 2

MATERIALS AND METHODS

Materials and Reagents

1,3-butadiene diepoxide (DEB) and other chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). Staurosporine (*Streptomyces staurospores*) was from BD Biosciences (San Jose, CA, USA). Tissue culture plates and tissue culture flasks were obtained from Corning (Corning, NY, USA). Dulbecco's Phosphate Buffered Saline (DPBS) was from Cellgro® Mediatech, Inc. (Manassas, VA, USA). Hyclone® Minimal Essential Medium with Earle's Balanced Salt Solution (EMEM/EBSS) +2.00 mM L-Glutamine was from Thermo Scientific (Logan, Utah, USA). Trypsin EDTA (0.25%) was from Gibco (Grand Island, NY, USA).

CelLytic™ MT Mammalian Tissue Lysis/Extraction Reagent was obtained from Sigma Aldrich. Phosphatase Inhibitor Cocktail Set III was obtained from Calbiochem (La Jolla, CA, USA) and Complete-Mini EDTA free protease inhibitor cocktail tablets were obtained from Roche Diagnostics (Mannheim, Germany). BCA Protein Assay Kit was obtained from Thermo Scientific-Pierce (Rockford, IL, USA). Amersham Hybond-ECL™ nitrocellulose membranes and Amersham ECL™ Western Blotting Detection Agents were obtained from GE Healthcare (Buckinghamshire, UK). NuPage® Novex® Bis-Tris 4-12% gels, NuPage® LDS sample buffer, NuPage® antioxidant, and NuPage® MOPS SDS running buffer were obtained from Invitrogen (Carlsbad, CA, USA).

Kodak® Biomax™ XAR Film was obtained from Sigma Aldrich. Restore™ Plus Western Blot Stripping Buffer was from Thermo Scientific-Pierce. A SA β-Gal Senescence Detection Kit was obtained from Abcam (Cambridge, MA, USA). CometAssay® Kit was obtained from Trevigen Inc. and included LMAgarose, Lysis Solution, SYBR Green, EDTA, and CometSlides (Gaithersburg, MD, USA). ApoAlert™ Cell Fractionation Kit was obtained from Clontech Laboratories, Inc. (Mountainview, CA, USA). An ApoTarget™ Caspase-9/Mch6/Apaf-3 Colorimetric Protease Assay Kit was obtained from BioSource International, Inc. (Camarillo, CA, USA). Antibodies were obtained from several sources: Anti-gamma H2A.X (Ser139)(2F3) antibody from Novus Biologicals (Littleton, CO, USA); anti-phospho ATM (pS1981), anti-ATM, and anti-p21 from Epitomics (Burlingame, CA, USA); anti-actin and anti-phospho Rb from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and developing antibodies (horse-radish peroxidase conjugated anti-rabbit or anti-mouse Ig) were from Epitomics and Southern Biotech (Birmingham, AL, USA). DAPI SlowFade® Gold antifade reagent and fluorescein isothiocyanate-conjugated anti-mouse antibody were from Invitrogen Molecular probes (Eugene, OR, USA). Fluorescence Mounting Medium was from Dako (Carpinteria, CA, USA).

Cell Culture

HUMAN LUNG FIBROBLASTS

A line of human lung fibroblasts (HLF) which was developed locally with IRB approval from discarded human embryonic tissue and characterized to exhibit a normal human diploid karyotype, defined cell cycle checkpoints, and wild type p53 (Breshnahan *et al*., 1996) were used. These cells readily arrest in G_0/G_1 when cultured in media lacking serum growth factors, and exhibit contact inhibition when a saturating density is reached. HLF stocks were routinely examined for mycoplasma contamination. Cultures of HLF were prepared according to the protocol described by Albrecht and Weller (1980) and preserved in liquid nitrogen until passaged for use.

HLF were used between the 10th and 20th passage and cultured in complete growth medium (Eagles's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (referred to herein as complete medium). HLF were incubated at 37º C in 5% CO_2. The cells were trypsinized and subcultured every 5-7 days and medium was changed every 3-4 days. Cells were harvested from tissue culture flasks or dishes by washing once with Dulbecco's phosphate-buffered saline (DPBS), incubating with 0.25% Trypsin EDTA for 5 minutes (min) at 37º C, washed once in complete medium, collected by centrifugation at 800 rpm for 10 min, diluted 1:5 in 0.4% Trypan blue, and counted using a Neubauer cell chamber before reseeding (Thomas Scientific, Philadelphia, PA). Cells were counted as viable, only if Trypan blue was excluded.

An established method for arrest of cell cycle progression by serum deprivation was used (Albrecht and Weller, 1980; Breshnahan *et al*., 1996). HLF were seeded in 100 mm tissue culture dishes at 5 x 10^5 cells/mL in complete medium and incubated overnight to allow the cells to attach. After approximately 16 h, the complete medium was removed and cells were washed twice with DPBS. In order to induce a synchronized G_0/G_1 arrest, cells were incubated in serum-free EMEM at 37° C for 72 h.

DEB-TREATMENT OF HLF

BD must first undergo biotransformation to produce the reactive metabolites which damage cells (Elfarra *et al*., 1996). Therefore, HLF were treated with 1, 2, 3, 4-diepoxybutane or diepoxybutane (DEB) because this metabolite has been shown to react with protein and DNA and is the most reactive metabolite of BD (Elfarra *et al*., 1996).

Single DEB Treatment

Serum-arrested cells were treated with 100 μM DEB in serum-free medium and incubated at 37° C for 1 h. This concentration was selected because it caused maximal extent of cell cycle arrest without evidence of appreciable effects on cell necrosis or apoptosis (Schmiederer *et al*., 2005). Control cells were mock treated with serum-free medium without DEB. After treatment, the medium was removed, cells were washed 2 times with DPBS, and fresh complete medium was added to initiate proliferation. The following Figure 4A depicts the timeline of our single treatment protocol.

Multiple Low-Dose DEB Treatments

A single treatment of HLF with DEB (100 μM for 1 h) resulted in prolonged cell cycle arrest and increased levels of nuclear p53 and p21 (Schmiederer *et al*., 2005). However, since people who smoke are exposed to BD repeatedly at much lower levels, multiple exposures to DEB at lower concentrations are more physiologically relevant. Based on the protocol used by Nyunoya *et al*. (2006) to investigate human fibroblast response to repeated exposures to cigarette smoke extract, HLF were mock treated or treated with 1 μM or 10 μM DEB every second day for 2 weeks. Briefly, HLF were rinsed 2 times with DPBS, treated with DEB in serum-free EMEM for 1 h or EMEM without DEB for a negative control, washed 2 times with DPBS, and then stimulated to divide with fresh complete medium. The following Figure 4B depicts the timeline of our multiple, low-dose treatment protocol.

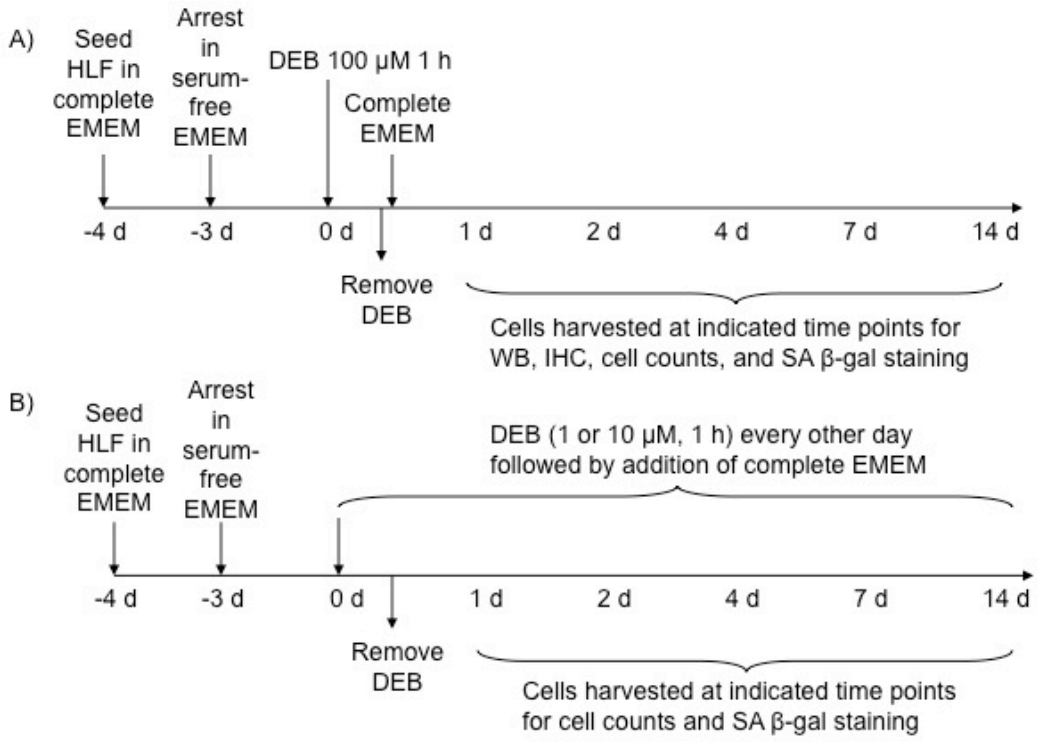

Figure 4: Experimental treatment protocols. A) Time-line of single treatment protocol depicting times for human lung fibroblast (HLF) seeding, cell cycle arrest in serum-free medium, diepoxybutane (DEB) treatment (100 µM for 1 h) and removal, addition of complete medium, and harvesting and B) multiple treatment protocol depicting times for HLF seeding, cell cycle arrest in serum-free medium, DEB treatment every other day (1 or 10 µM for 1 h) and removal, addition of complete medium, and harvesting.

APOPTOSIS ASSAYS

Cell fractionation and Western blot detection of cytochrome c were performed to confirm that apoptosis was not initiated and that mitochondrial membrane potential remained intact after DEB treatment. Moreover, caspase activation assays and Western blot analysis were performed to further confirm that the intrinsic apoptotic pathway was not activated after DEB treatment.

Mitochondria Isolations

An ApoAlert™ Cell Fractionation Kit was used according to the manufacturer's protocol to separate the mitochondrial-enriched fraction from the cytosol 24 h after treatment with DEB (100 μM for 1 h). Cells were mock treated for 1 h with EMEM without DEB to serve as a negative control. HLF were treated with 1 μM final concentration of staurosporine (*Streptomyces staurospores*), a non-selective protein kinase inhibitor, in EMEM containing 1% FBS without DEB for 6 h to serve as a control positive for apoptosis.

Specifically, HLF were collected via trypsinization and centrifuged at 600 x g for 5 min at 4° C. The supernatant was removed and the pellet was resuspended in 1 ml of ice-cold wash buffer. Cells were centrifuged at 600 x g for 5 min at 4° C, the supernatant removed, and HLF were resuspended in 0.8 ml of freshly prepared ice-cold fractionation buffer containing 2 μl of protease inhibitor cocktail mix and 1 μl of 1,4-dithiothreotal (DTT). The suspension was incubated on ice for 10 min and the cells were homogenized

by gentle pipetting up and down using a 1000 μl pipette. The homogenate was transferred to a 1.5 ml centrifuge tube and centrifuged at 700 x g for 10 min at 4° C. The supernatant was transferred to a fresh centrifuge tube and centrifuged at 10,000 x g for 25 min at 4° C. The supernatant, or cytosolic fraction, was transferred to a fresh centrifuge tube and the pellet, or mitochondrial fraction, was resuspended in 100 ml fractionation buffer.

During apoptosis, cytochrome c is released from to the mitochondria into the cytosol. In contrast, cytochrome c oxidase subunit IV (COX4), an inner mitochondrial membrane localized protein, remains in the mitochondria during apoptosis. Therefore, probing Western blots of cytosolic and mitochondrial fractions with antibodies to cytochrome c and COX4 provides a way to monitor cells for apoptosis. In control cells with intact mitochondria, both cytochrome c and COX4 should only be detected in the mitochondrial fraction. The release of cytochrome into the cytosolic fraction reflects treatment-induced apoptosis.

Confocal Microscopy

Location of cytochrome c was also visualized by confocal immunofluorescence microscopy. Specifically, cells were seeded onto sterile 22 mm x 22 mm glass coverslips in 60 mm culture dishes before serum-arrest and DEB treatment as depicted in Figure 4A. The coverslips were removed at 1, 1.5, 2, and 6 h post treatment and fixed in 4% paraformaldehyde for 10 min, rinsed twice with DPBS, and permeabilized with PBS containing 0.25% Triton X-100 for 10 min at room temperature. Coverslips were washed

3 times with PBS for 5 min each time, blocked in 1% BSA in DPBS for 30 min, and rinsed 3 times with DPBS for 5 min each time. The primary antibody (anti-cytochrome c, clone 6h.B4, Invitrogen) was diluted 1:200 in DPBS, added to the coverslips, and coverslips were incubated at 37° C for 60 min. Coverslips were washed 3 times for 10 min each time with DPBS containing 0.1% Tween (DPBST) before adding a 1:500 dilution of the secondary antibody (Alexa Flour 488 goat anti-mouse IgG, Molecular Probes) and incubating for 30 min at 37° C. Cells were washed with 3 times with DPBST for 10 min each time and coverslips were drained, dried for 2 h, and mounted on microscope slides using DAPI SlowFade® Gold antifade reagent.

Caspase Activation Assays

An ApoTarget™ Caspase-9/Mch6/Apaf-3 Colorimetric Protease Assay Kit (BioSource, Camarillo, CA, USA) was used according to the manufacturer's protocol to detect Caspase-9 activity of apoptotic cells. Briefly, cells were assayed after treatment with DEB (100 μM for 1 h) or mock treatment for 1 h with EMEM without DEB to serve as a negative control. HLF were treated with 1 μM staurosporine in 1% FBS without DEB for 6 h to serve as a positive control for apoptosis. HLF were counted 4 h after treatment and 5 x 10^6 cells were pelleted per sample and resuspended in 50 μL of chilled Cell Lysis Buffer and incubated for 10 min on ice. Cells were then centrifuged for 1 min (10,000 x g). The supernatant or cytosolic extract was transferred to a fresh microcentifuge tube and incubated on ice. Lysate protein concentration was quantified using the BCA Protein Assay Kit. Protein standards were prepared from a stock solution

of BSA. Cytosolic extract was diluted to a concentration of 100 μg protein per 50 μL Cell Lysis Buffer. Next, 50 μL of freshly prepared Reaction Buffer containing DTT was added to each sample. 5 μL of 4 mM LEHD-pNA caspase substrate was added and the samples were incubated at 37° C for 2 h in the dark. Samples were read at 405 nm in a spectrophotometer using a 100 μL micro-quartz cuvette. The fold-increase in Caspase-9 activity was determined by directly comparing increases in optical density levels in DEB-treated HLF to mock-treated controls.

Western Blot Analysis

Whole cell lysates were prepared for Western blot analysis of caspase-3. For whole cell lysates, approximately 500,000 HLF were seeded per 100 mm cell culture dish and allowed to attach overnight before being serum-arrested. The cells were then treated with DEB or mock treated according to the single exposure protocol in Fig. 4A, washed twice, and serum initiated with fresh complete medium. Mock-treated cells were trypsinized and reseeded 1 to 2 days prior to trypsinization and harvest in order to prevent the cells from becoming density-arrested. Medium was changed every 3 to 4 days. At indicated time points, HLF were washed twice with DPBS and lysed with CelLytic™ MT mammalian tissue lysis/extraction reagent supplemented with 1X Complete mini-EDTA free protease inhibitor cocktail tablets and 1X phosphatase inhibitors cocktail set III. Lysates were vortexed for 30 s every 5 min for 30 min while being incubated on ice, and centrifuged at 12,000 rpm for 20 min at 4° C. Lysate protein concentration was

quantified using the BCA Protein Assay Kit. Protein standards were prepared from a stock solution of BSA. Cell lysates were stored at -80° C until use.

Western Blot analysis of whole cell lysates was performed to detect the presence of activated caspase-3. Protein (30 μg) was electrophoresed on a 4-12% NuPAGE® Novex Bis-Tris gel in the XCell SureLock™ Mini-Cell from Invitrogen. Proteins were transferred onto nitrocellulose membranes using the XCell II™ Blot Module at 35 V for 90 min and subjected to immunoblot analysis using selected antibodies. Briefly, proteins were probed with the primary antibodies overnight at 4° C in DPBS containing 5% milk and 0.1% Tween 20. Blots were washed 4 times for 10 min each with DPBS containing 0.1% Tween 20. Secondary antibodies conjugated to horseradish peroxidase were used at a dilution of 1: 4,000 and incubated for 1 h at room temperature. The immunoblots were developed using Amersham ECL™ Western Blotting Detection Agents. Equal loading of protein samples was evaluated by stripping the blot and reprobing with anti-β actin antibodies or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. GAPDH is an enzyme, 36-40 kDA in size, that is essential for glycolysis and stably and constitutively expressed at high levels in almost all cells and tissues and, therefore, considered a housekeeping gene that can be monitored to assure Western lane loading efficiency.

ASSAYS TO DETECT DNA DAMAGE

Comet Assay

The Comet (single cell gel electrophoresis) assay is a sensitive method to estimate the extent of DNA damage at the individual cellular level. DNA damage was estimated using the CometAssay® Kit from Trevigen Inc. according to the manufacturer's instructions under alkaline conditions to detect any type of DNA damage following DEB treatment. Briefly, at indicated times, DEB and mock-treated cells were washed, trypsinized, pelleted, and resuspended in ice cold DPBS at 10^5 cells/ml. Fifty µl of the cell suspension was gently mixed with 500 µl of LMAgarose and 75 µl of the mixture was pipetted into the sample area of the CometSlides. Slides were incubated in the dark at 4° C for 30 min and then immersed in cold Lysis Solution and incubated for 30 min at 4° C. Next, excess buffer was removed and the slides were immersed in freshly prepared alkaline solution for 30 min at room temperature in the dark. Slides were transferred to a horizontal electrophoresis apparatus, Alkaline Electrophoresis Solution was added to the chamber until the slides were just covered, and run at 25 V (1 V/cm) for 30 min at 4° C.

Following electrophoresis, slides were rinsed 2 times in deionized H_2O and then immersed in 70% ethanol for 5 min. Slides were air dried at room temperature overnight and stained immediately before analysis with 50 µl of diluted SYBR® Green for 5 min at 4° C. The slides were analyzed quantitatively for tail moment and tail length using the automatic image analyzer Comet Assay IV (Perceptive Instruments, UK) attached to a fluorescence microscope. Duplicate slides were prepared for each condition, coded, and

then 50 cells were analyzed per slide without knowledge of treatment condition for tail length and tail moment as measures of damaged DNA migration. Studies have shown that increasing the number of cells per slide from 25 to 50 greatly increases the power, however, further increasing the number of cells per slide does not affect significance (Wiklund & Agurell, 2003).

Immunofluorescence Microscopy

Phosphorylated histone protein 2AX (γH2AX) promotes DNA repair and genome stability by recruiting DNA repair proteins to the sites of damage called DNA damage foci. Immunofluorescence microscopy was used to detect the formation of discrete γH2AX foci in the nucleus caused by the formation of DNA double strand breaks (Rogakou *et al*., 1998; Sedelnikova *et al*., 2002). Specifically, cells were seeded onto sterile 22 mm x 22 mm glass coverslips in 60 mm culture dishes before serum arrest and DEB treatment as depicted in Figure 4A. Coverslips were removed at indicated time points and fixed in 4% paraformaldehyde for 10 min, rinsed twice with DPBS, and cells were permeabilized with DPBS containing 0.25% Triton x for 10 min at room temperature. Coverslips were washed 3 times with DPBS for 5 min each time, blocked in 1% BSA in PBS for 30 min, and rinsed 3 times with DPBS. Primary antibody against γH2AX (Anti-gamma H2A.X (Ser139)(2F3), Novus Biologicals) was added and coverslips were incubated at 37° C for 60 min. Coverslips were washed 3 times in DPBS containing 0.1% Tween (DPBST) before adding the secondary antibody and incubating for 30 min at 37° C. Coverslips were washed 3 times in DPBST for 10 min each and 10

ng/ml of 4'6-diamidino-2-phenylindole dihydrochloride (DAPI) was added during the second wash. Coverslips were dried for 2 h before mounting with Dako mounting medium. Slides were photographed with a Photometrix CoolSNAP Fx CCD digital camera mounted on a Nikon Eclipse TE 200 fluorescent microscope. Staining patterns were analyzed and quantified using ImageJ64 Software (NIH, Bethesda, MD). Specifically, slides were coded and 10 randomly selected microscopic fields were examined on of each slide. The percentages of the total number of HLF positive for γH2AX were calculated for each slide and the mean percentage per condition was calculated from 3 independent experiments.

ASSAYS AND METHODS TO DETECT MARKERS OF SENESCENCE

Senescence-Associated β-Galactosidase Assay

SA β-gal, a well-known marker for senescence, was assessed with Senescence Detection Kit (Abcam) designed to histochemically detect SA β-gal activity characteristic in senescent cells according to the manufacture's protocol. Briefly, HLF were seeded in 25 cm^2 flasks at approximately 70% confluency and treated with the following doses of DEB: 100 μM once, and 1 and 10 μM for 1 h every other day for 2 weeks. Cells were trypsinized and plated for SA β-gal staining in 35 mm x 10 mm cell culture dishes on days 4, 7 and 14. Mock treated cells were split 1 to 2 days prior to staining to ensure that the cells did not become confluent.

Culture dishes were coded before being photographed with a Photometrix CoolSNAP Fx CCD digital camera mounted on a Nikon Eclipse TE 200 fluorescent microscope. Photographs were analyzed and quantified for SA β-gal staining using ImageJ64 Software (NIH, Bethesda, MD). Specifically, the total percentage of HLF staining positive for SA β-gal per at least 100 cells was calculated per condition. The mean percentages of HLF staining positive for SA β-gal was calculated from 3 independent experiments.

Western Blot Analysis

Western Blot analysis of whole cell lysates as described on page 58 was performed to detect the presence of key players in senescence. Both normal and phosphorylated forms of proteins were analyzed including ATM, p21, and p53.

Immunofluorescence Microscopy

In cells with DNA damage, p53 is activated and, in turn, mediates the substantial increase in transcription and expression of nuclear p21 (Bunz *et al.,* 1998). Immunofluorescence microscopy was used to analyze nuclear levels of p21 after treatment with DEB (100 μM for1 h). Specifically, cells were seeded onto sterile 22 mm x 22 mm glass coverslips in 60 mm culture dishes before serum arrest and DEB treatment as depicted in Figure 4A. The coverslips were removed at the indicated time points and fixed in 4% paraformaldehyde for 10 min, rinsed twice with PBS, and the cells were permeabilized with PBS containing 0.25% Triton x for 10 min at room temperature (RT). Coverslips were washed 3 times with PBS for 5 min each time, blocked in 1% BSA in

PBS for 30 min, and rinsed 3 times with PBS. The primary antibody was added and coverslips were incubated at 37° C for 60 min. Coverslips were washed 3 times in DPBS containing 0.1% Tween (DPBST) before adding the secondary antibody and incubating for 30 min at 37° C. The coverslips were washed 3 times in DPBST for 10 min each and 10 ng/ml of 4'6-diamidino-2-phenylindole dihydrochloride (DAPI) was added during the second wash. Coverslips were dried for 2 h before mounting on slides with Dako mounting medium. Slides were photographed with a Photometrix CoolSNAP Fx CCD digital camera mounted on a Nikon Eclipse TE 200 fluorescent microscope. The staining patterns were analyzed and quantified using ImageJ64 Software (NIH, Bethesda, MD). Nuclear p21 staining patterns were analyzed and quantified using ImageJ64 Software (NIH, Bethesda, MD). Specifically, slides were coded and at least 10 randomly selected microscopic fields were examined per slide. The percentages of HLF positive for p21 were calculated for each slide. The mean percentage of HLF positive for p21 was calculated from three independent experiments.

GROWTH FACTOR-INDUCED CELLULAR PROLIFERATION EXPERIMENT

To determine whether addition of growth factors would induce proliferation in DEB-treated HLF, cell counts after DEB treatment and stimulation with growth factors were performed. Experiments utilized the growth factor cocktail shown by Phillips *et al*. to elicit maximal growth in non-senescent lung fibroblasts and no growth in senescent fibroblasts (1984). This growth factor cocktail contains EGF-1 (25 ng/ml), IGF-1 (20 μg/ml), and DEX (55 ng/ml). Experiments followed the protocols for single treatment

and multiple low-dose treatments depicted in Figure 4 with triplicate dishes for each treatment group. Specifically, for the single treatment protocol, HLF were treated with 100 μM DEB or mock treated once, rinsed, maintained in complete medium until day 7 when medium containing the growth factor cocktail was added. For the multiple, low-dose treatment protocol, HLF were treated with 1, 10 μM DEB, or mock treated every other day for 2 weeks, rinsed, and maintained in complete medium between treatments until day 14 when medium with growth factor cocktail was added. Cell counts of DEB-treated and mock-treated HLF were determined 4 days after stimulation with growth factor cocktail.

Statistical analysis

All data are presented as the mean ± SEM. Differences between DEB-treated and mock-treated control cells for each condition were compared at various time points using an analysis of variance or by an unpaired Student's *t*-test, as appropriate. A *P* value of < 0.05 was considered to be statistically significant. All calculations were performed using SigmaPlot 12, Systat Software, Inc. (San Jose, CA, USA).

CHAPTER 3

RESULTS

Treatment of HLF with DEB Arrests Cellular Proliferation

Exposure to CSE induces cellular senescence, or irreversible loss of replicative ability, in both human lung fibroblasts and human alveolar epithelial cells (Nyunoya *et al*., 2006; Tsuji *et al*., 2004). We hypothesized that DEB, the most potent metabolite of 1,3-butadiene and a potent component of cigarette smoke, would induce long-term cell cycle arrest. To evaluate this, HLF were cell cycle synchronized by serum deprivation, treated with DEB (100 μM for 1 h) or mock treated, and incubated in complete medium. Little if any proliferation was evident in DEB-treated cells over the next 5 days as depicted in Figure 5. In contrast, mock-treated control cells proliferated with a logarithmic increase beginning around day 1 to day 3. After day 3, increased growth of control cells slowed and leveled out as the cells became contact-inhibited and density-arrested.

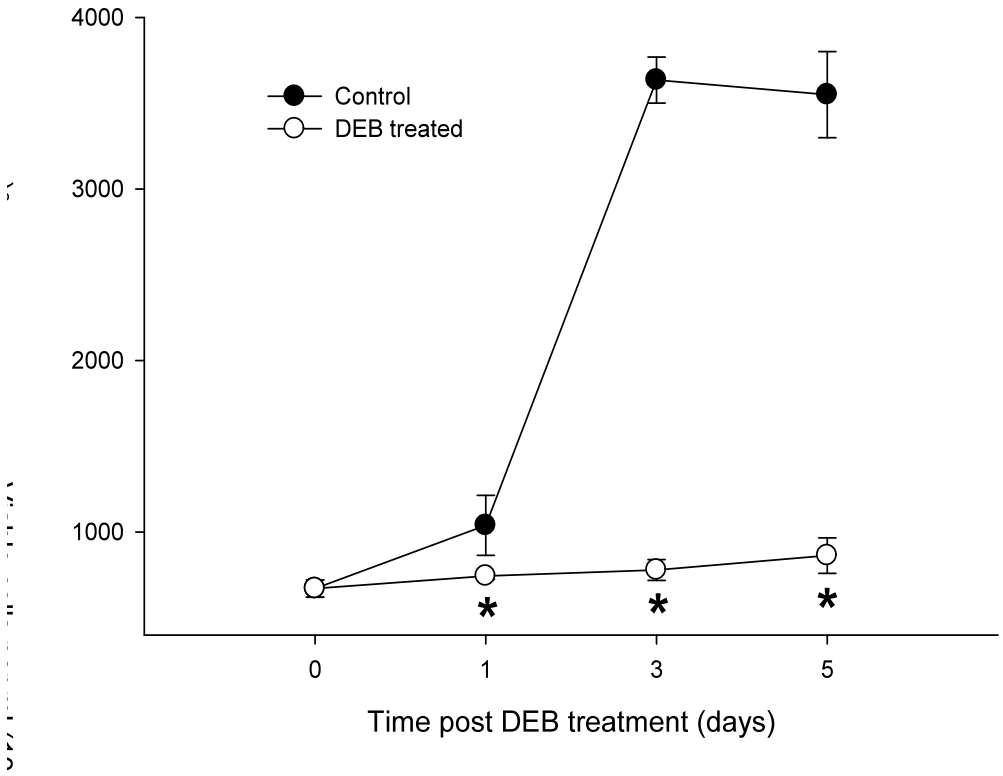

Figure 5: DEB treatment of HLF results in arrest of cellular proliferation. Growth curve for cell counts of DEB-treated HLF (100 μm for 1 h) following initiation with fresh medium containing 10% FBS. HLF were seeded in 100 mm cell culture dishes, serum-arrested, treated with DEB or mock treated, and cell counts were measured at the indicated times following serum initiation. Values are expressed as the number of viable cells/mL + SEM for three independent experiments. *$P < 0.05$ versus control cells not treated with DEB at same time point.

DEB Treatment Does Not Induce Apoptosis in HLF

To determine if the integrity of the mitochondrial membrane was affected after DEB treatment (100 μM for 1 h), we investigated whether cytochrome c was released from the mitochondrial membrane by using both confocal microscopy and Western blot comparisons of cell fractions. Visualization of cytochrome c localization via immunostaining and confocal microscopy over a time course of early times following DEB treatment (from 1 to 6 h) revealed a normal strong punctuate cytochrome c pattern (Fig. 6). Western blot comparisons of mitochondrial and cytosolic fractions confirmed that cytochrome c was not released from the mitochondrial membrane 24 h after DEB treatment (Fig. 7). Both methods demonstrated that cytochrome c was retained within the mitochondrial intermembrane space immediately following treatment and for at least 24 h, thus, indicating that the mitochondrial membrane remained intact following treatment.

Furthermore, to confirm that the intrinsic apoptotic pathway was not initiated, two key effector caspases, caspase-9 and caspase-3, located downstream from the mitochondria were analyzed via Western blotting and caspase activity assays. No activation of caspase-3 or caspase-9 was found after DEB treatment (Fig. 8 and Fig. 9). Taken together, these results indicated that treatment with DEB was not associated with either loss of mitochondrial membrane integrity or activation of the intrinsic apoptotic pathway.

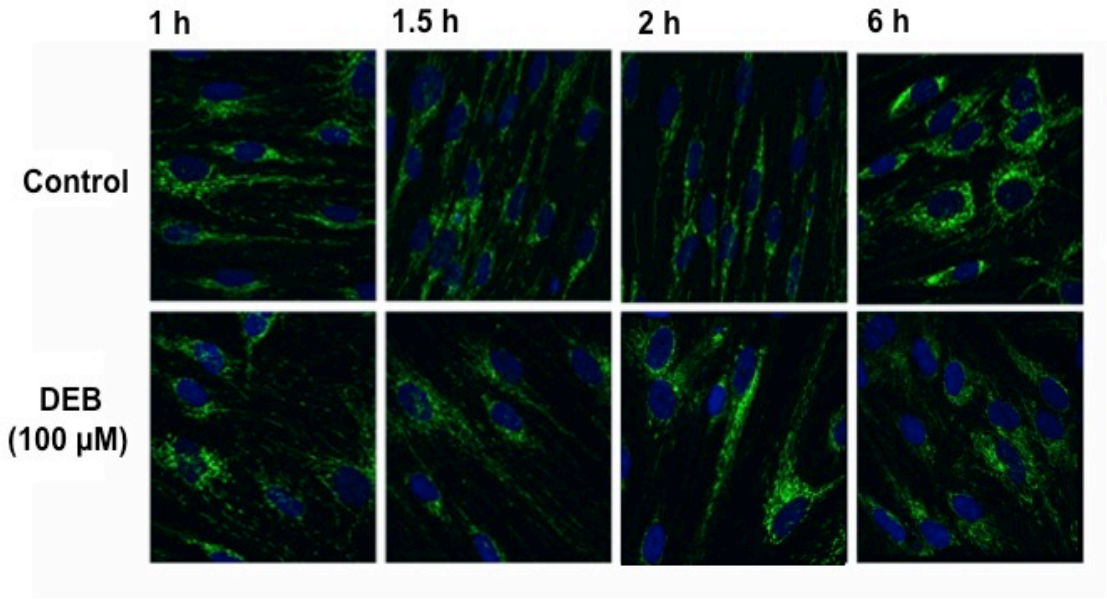

Figure 6: Localization of cytochrome c to the mitochondria in DEB-treated HLF. HLF were cultured on glass coverslips and treated with DEB (100 μM for 1 h) or mock treated (control). Cells were fixed, permeabilized, and immunostained with a cytochrome c-specific antibody (green). 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI) was used for nuclear staining (blue). Representative images for each time point are shown at 60X magnification.

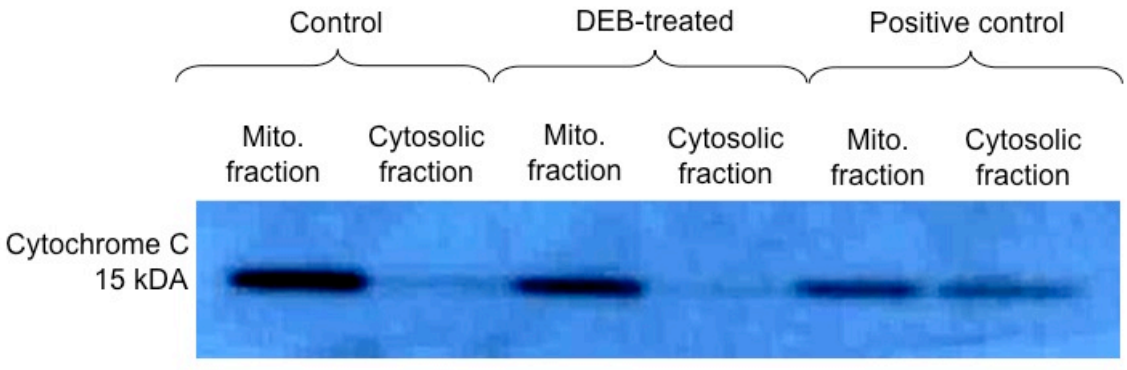

Figure 7: Analysis of cytochrome c in mitochondrial and cytosolic fractions of DEB-treated HLF. Western blot analysis of cell fractions following HLF treatment with DEB (100 μM for 1 h) or mock treatment (control). Cells were treated with staurosporine (1 μM for 6 h) to serve as a positive control. At 24 h after treatment, HLF were lysed and the ApoAlert Cell Fractionation Kit used to separate a mitochondrial-enriched fraction from the cytosol. Proteins were separated by SDS-PAGE 4-12%, transferred to a nitrocellulose membrane, and probed with anti-cytochrome c antibodies.

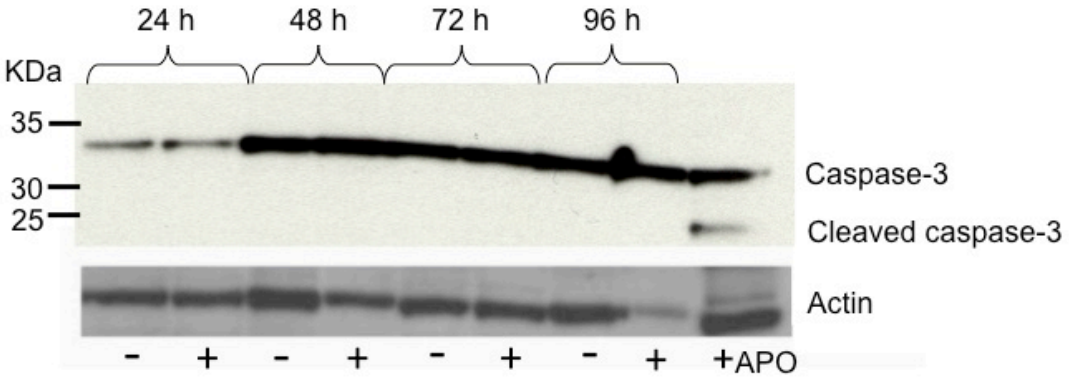

Figure 8: Caspase-3 was not induced after DEB treatment of HLF. Immunoblot analysis of caspase-3 protein in HLF treated with DEB (100 μM for 1 h) (+) or mock-treated control (-). HLF treated with staurosporine (1 μM for 6 h) served as a control positive for apoptosis (+APO). HLF were lysed at various time points and cell lysates (30 µg per lane) were analyzed by immunoblotting with an anti-caspase-3 antibody. Equal loading was verified by stripping and reprobing the blot with anti-β-actin antibodies. Western blotting results are representative of three independent experiments.

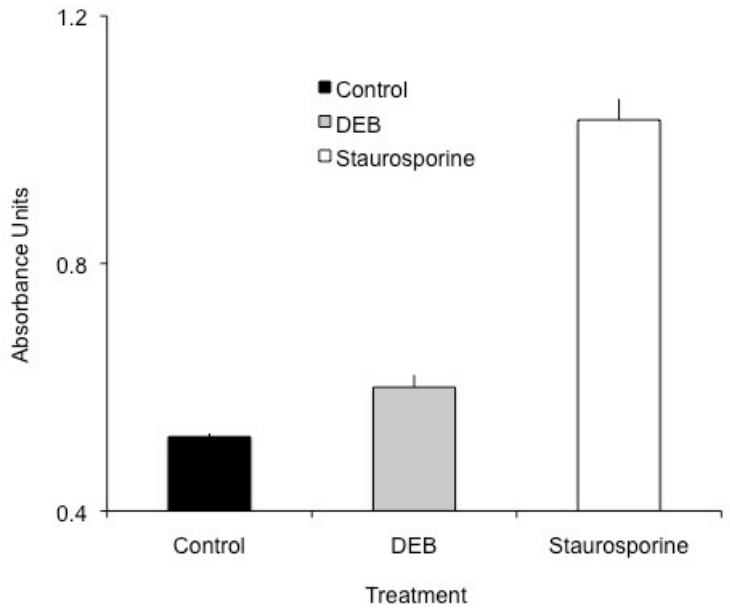

Figure 9: Treatment of HLF with DEB does not result in activation of caspase-9. Activation of caspase-9 was measured by cleavage of the caspase-9 colorimetric substrate (LEHD-pNA) following treatment of HLF with DEB (100 μM for 1 h) or mock treatment (control). HLF treated with staurosporine (1 μM for 6 h) served as a control positive for apoptosis. Following treatment, cells were incubated for 24 h, cell lysates were prepared, and the caspase-9 substrate was added. Samples were read at 405 nm to determine increases in optical density (absorbance units). Data are representative of three independent experiments.

Treatment with DEB Induces Persistent DNA Damage in HLF

1, 3-butadiene, a vapor phase component of cigarette smoke, and its metabolites cause extensive DNA damage in cultured cells and mice (Jackson *et al*., 2000; Albertini *et al*., 2010). We hypothesized that DEB will cause DNA damage in human lung fibroblasts. To analyze DNA damage, the alkaline comet assay was performed in DEB-treated and mock-treated HLF. Tail length and tail moment were selected as parameters for measuring DNA damage and image analysis was performed using the Comet Assay 4 Software (Perceptive Instruments). As shown in Fig. 10, significant increases in mean tail moment and mean tail length were evident at 6 and 48 h following DEB treatment, thus, indicating DEB-induced DNA damage in HLF.

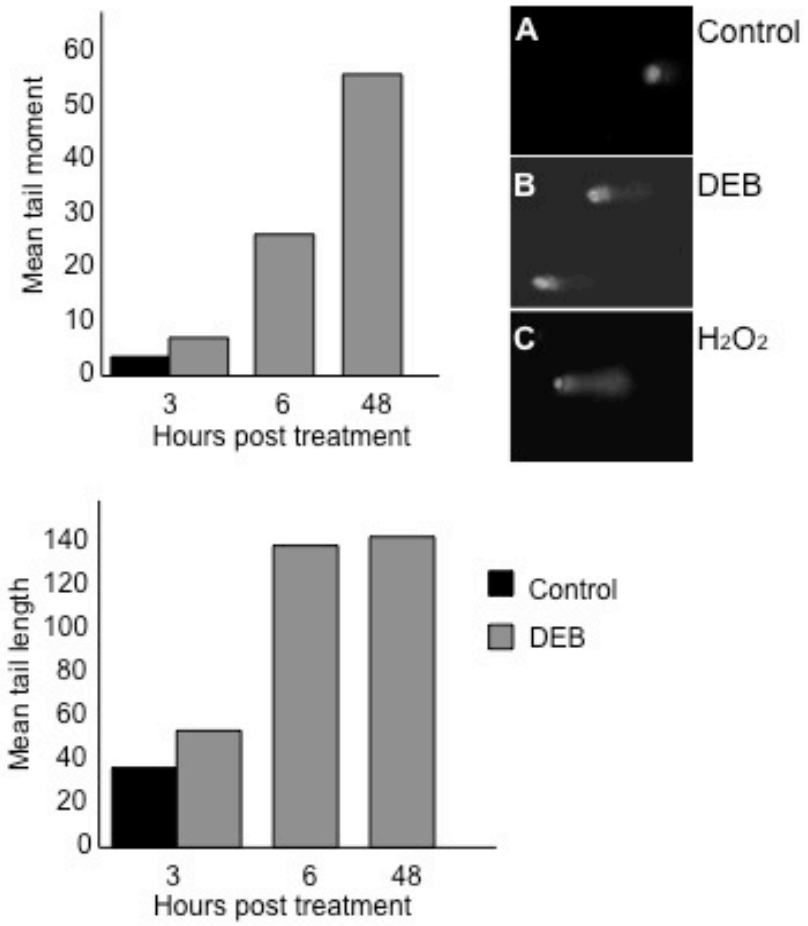

Figure 10: The Comet assay demonstrates increases in levels of DNA damage in DEB-treated HLF. HLF were DEB treated (100 μM for 1 h) or mock treated (control). HLF treated with hydrogen peroxide (200 μM for 1 h) served as a positive control. Treated cells were harvested via trypsinazation, washed twice in DPBS, mixed with LMAgarose, and placed on CometSlides. DNA was electrophoresed for 30 min and stained with SYBR® Green. Comets were analyzed using the automatic image analyzer Comet Assay IV (Perceptive Instruments, UK) attached to a fluorescence microscope. At least 50 cells per condition per duplicate slide were scored for three independent experiments. *$P < 0.05$ versus control cells not treated with DEB at same time point.

Induction of double strand breaks (DSBs) by DNA damaging agents activates ATM, which, in turn, phosphorylates the nuclear histone protein H2AX on serine 139 (Rogakou *et al*., 1999; Fernandez-Capetillo *et al*., 2004). DNA damage foci serve as important markers of DNA damage in individual cells and senescence which can be detected using an antibody specific for the phosphorylated form of H2AX (Banath & Olive, 2003; Takahashi, 2005). We hypothesized that exposure of HLF to DEB will result in the formation of persistent DNA damage foci. To evaluate this, HLF were cultured on glass coverslips with a single-exposure to 100 μM DEB for 1 h and fixed for immunofluorescence microscopy at the following time points post-treatment: 1 h, 2, 4, and 8 days. Mock-treated HLF displayed few DNA damage foci at all time points (Fig. 11), whereas, the number of cells positive for DNA damage foci increased from 55% at day 2 to 76% by day 4 (Fig. 11, Fig. 12). By day 8, the overall total number of DNA damage foci per cell appears to have decreased by day 8 but importantly 54% of cells counted remained positive for foci. These data demonstrate that DEB treatment results in DNA damage and the formation of DNA damage foci. Persistent γH2AX foci at days 4 and 8 suggest the presence of irrepairable DNA damage, which could act as a persistent signal for the DNA damage response pathway and stress-induced premature senescence (Fig. 12, Fig. 13).

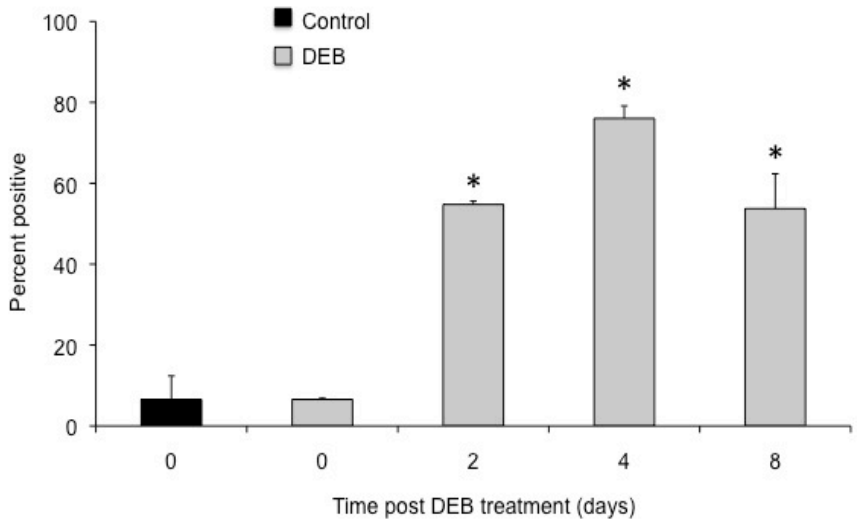

Figure 11: Increased percentage of HLF positive for DNA damage foci after DEB treatment. Values represent the mean number of nuclei positive for DNA damage foci as determined by immunocytochemistry per at least 100 cells counted ± SEM for three independent experiments. *$P < 0.05$ versus control cells not treated with DEB at same time point.

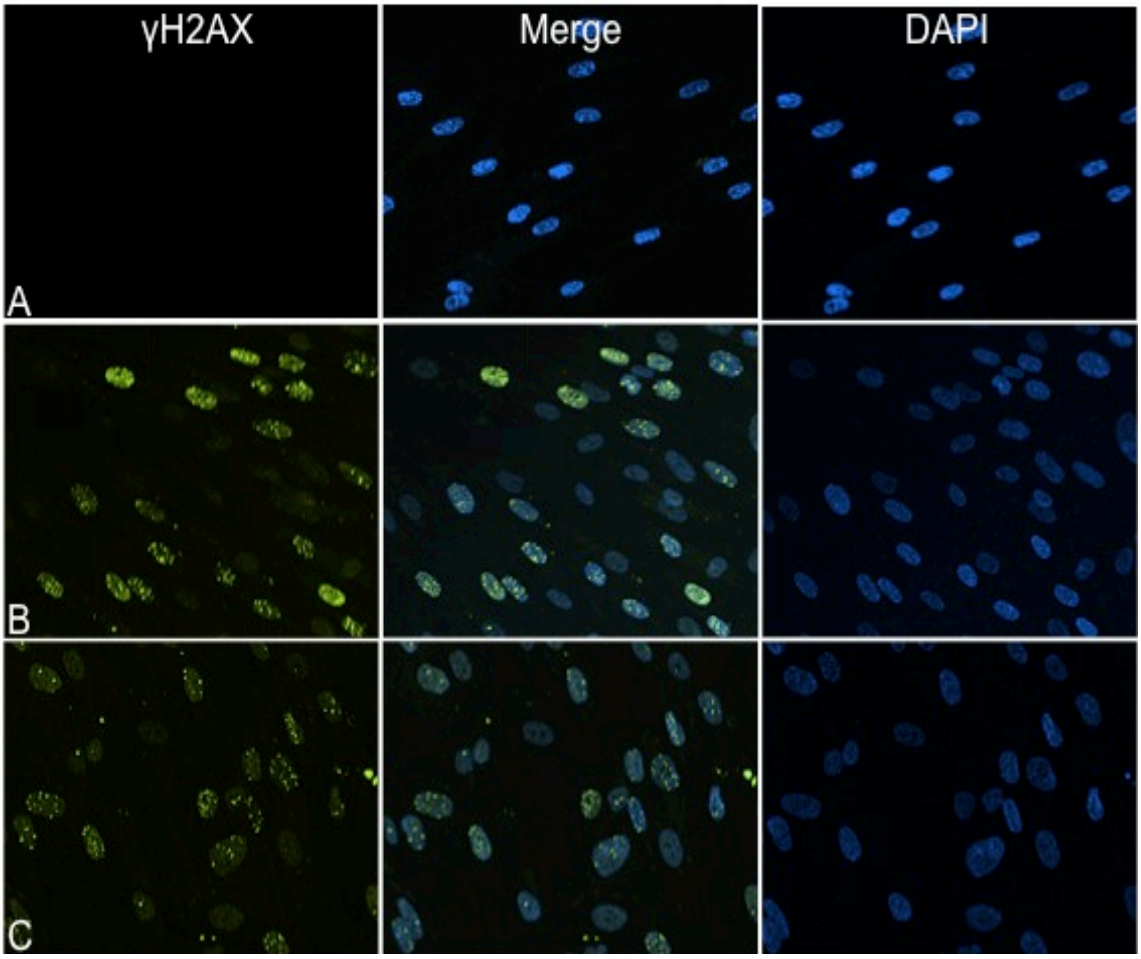

Figure 12: DNA damage foci formation following treatment of HLF with DEB. HLF cultured on glass coverslips were mock treated (A) or treated with DEB (100 μM for 1 h) and incubated in complete medium until day 2 (B) or day 4 (C). Cells were fixed, permeabilized, and stained with anti-γH2AX antibodies (green). Cell nuclei were counterstained with DAPI. Fluorescent images were captured at 60X and representative images are shown.

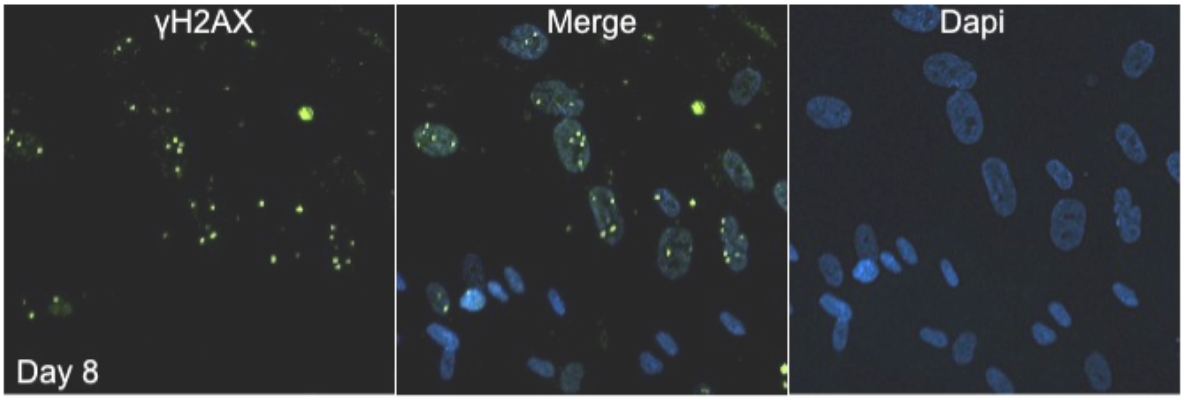

Figure 13: Persistence of DNA damage foci following DEB treatment of HLF. HLF cultured on glass coverslips were treated with DEB (100 μM for 1 h) or mock treated and incubated in complete medium until day 8. Cells were fixed, permeabilized, and stained with anti-γH2AX antibodies (green). Cell nuclei were counterstained with DAPI. Fluorescent images were captured at 60X and representative images are shown.

Treatment with DEB Induces Markers of Senescence in HLF

Two well-established characteristics of senescence cells are an enlarged, flattened morphology and increased activity of a galactosidase at pH 6.0 (Ohtani *et al*., 2004; Itahana *et al*., 2007). We hypothesized that DEB treatment would induce these characteristics of senescence in HLF. To analyze this, we examined cells for changes in morphology and increases in SA β-gal activity after single and multiple DEB treatments. A single DEB treatment (100 μM for 1 h) results in an enlarged, flattened morphology

with a striking increase in SA β-gal expression 7 days after treatment (Fig. 14). Both a single (100 μM for 1 h) and multiple treatments (1 μM or 10 μM DEB for 1 h every other day for 2 weeks) resulted in increased SA β-gal activity (pH 6.0) at day 4 following treatment(s) (Fig. 15). The mean percentages of SA β-gal positive cells were quantified for HLF treated one and treated multiple times both 4 and 7 days post treatment (Fig. 16).

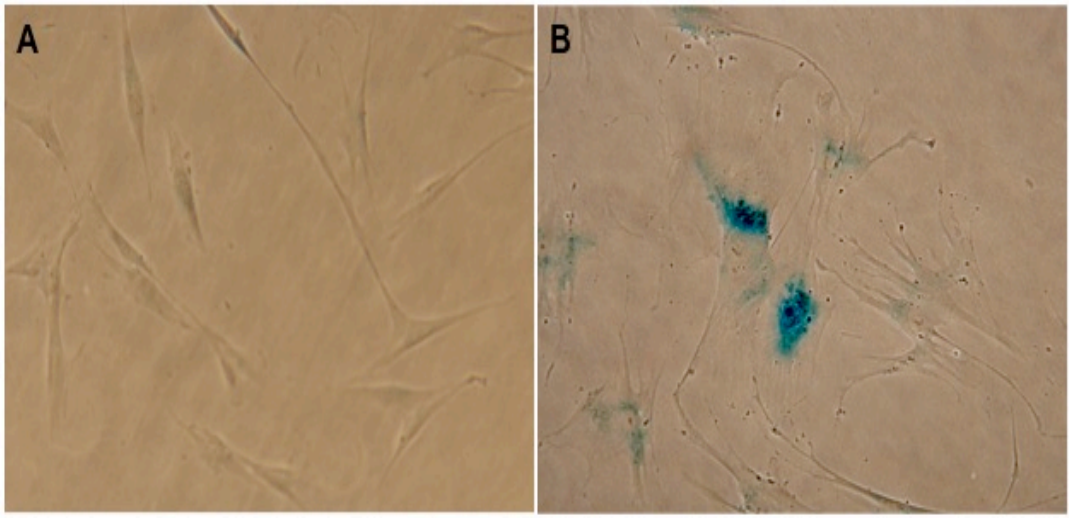

Figure 14: DEB treatment of HLF induces an enlarged, flattened morphology and SA β-Gal expression. Representative images of altered cell morphology and increased SA β-Gal activity (pH 6.0) at day 7 following mock treatment (A) or DEB treatment (B).

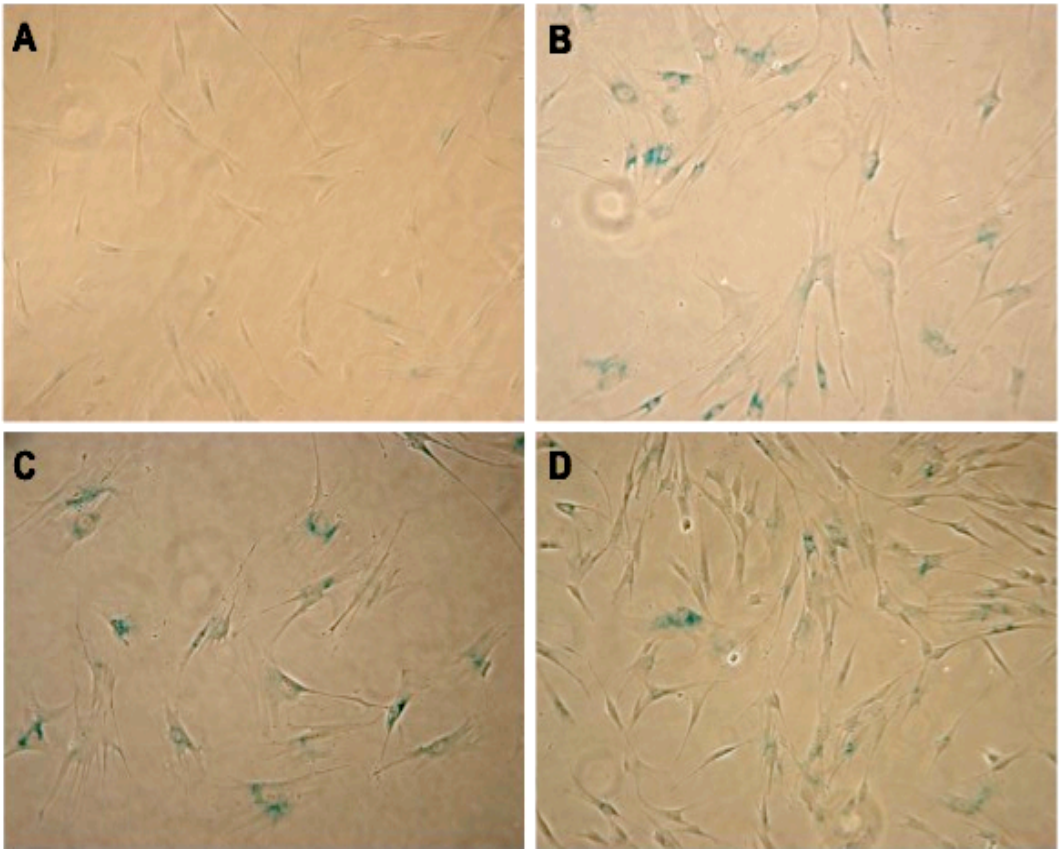

Figure 15: Treatment of HLF with various concentrations of DEB induces increased SA β-Gal expression. Representative pictures of SA β-gal activity (pH 6.0) 4 days after mock treatment (A) or a single DEB treatment (100 μM for 1 h). Parallel cultures were treated with 1 μM (C) or 10 μM (D) for 1 h every other day for 2 weeks and examined for SA β-Gal expression at day 14.

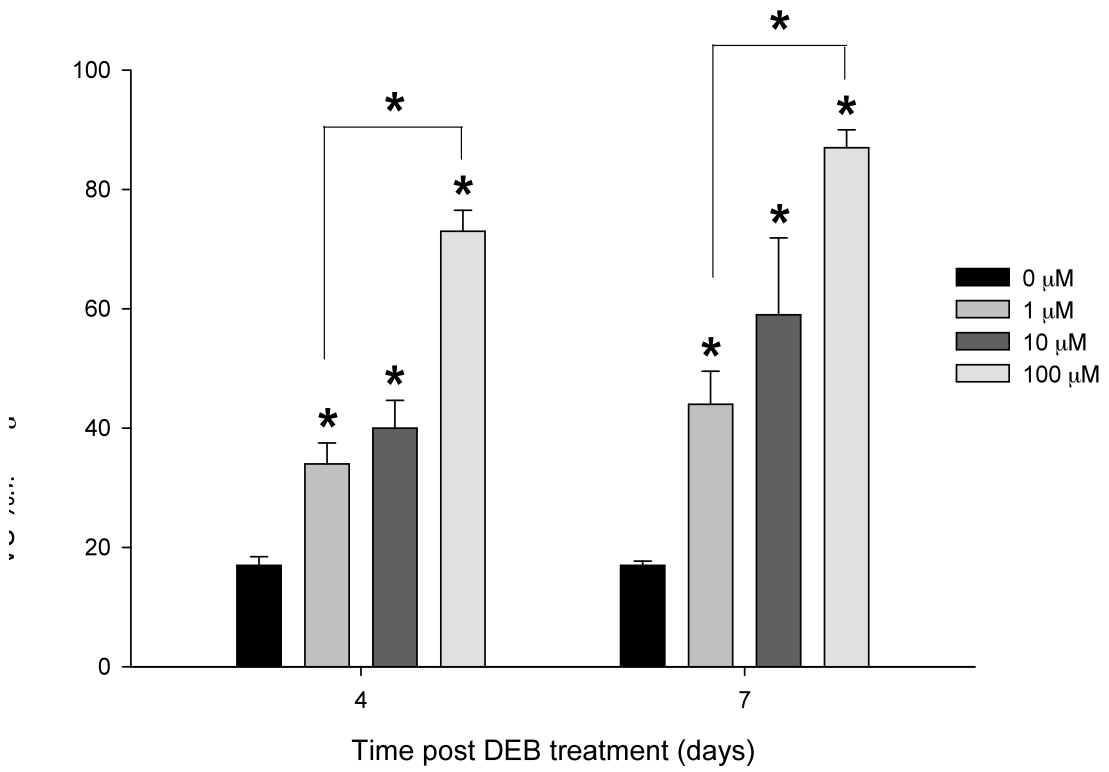

Figure 16: Increased SA β-gal activity in HLF after DEB treatment(s). Percentages of HLF stained positive for SA β-gal activity were increased in a dose-dependent manner after a single (100 μM for 1 h) or multiple, low-dose DEB treatments (1 μM or 10 μM for 1 h every other day for 2 weeks) at days 4 and 7. Values represent the mean percentages of SA β-gal positive cells per at least 100 cells counted ± SEM per treatment group for three independent experiments. *P< 0.05 versus control cells mock treated HLF or lower-dose treated HLF at same time point.

DEB Induces Activation of the ATM-p53-p21 Pathway

The formation of DNA damage foci results in the subsequent activation of the ATM-p53-p21 pathway (Von Zglinicki *et al*., 2005). We hypothesized that the observed DNA damage in HLF would result in elevated phosphorylated ATM and p21 levels characteristic of stress-induced premature senescence (d'Adda di Fagagna, 2008). To evaluate this, we examined effects of DEB on levels of ATM and p21 at 4, 8, and 12 days post treatment. Western blot analysis was performed to examine expression of phosphorylated ATM (serine 1981), unphosphorylated ATM, and p21. Exposure to DEB increased levels of phosphorylated ATM (Fig. 17) and resulted in a significant increase in p21 expression (Fig. 18).

Moreover, p21 expression was visualized via immunofluorescence microscopy at the following time points post-treatment: 1 h, 2, 4, and 8 days. Mock-treated HLF did not show nuclear p21 at any time, whereas, the number of DEB-treated HLF expressing p21 increased immediately following treatment and remained elevated at 8 days post-treatment (Fig. 19, Fig. 20, Fig. 21). Increased levels of phosphorylated ATM and p21 at days 4 and 8 correlates with the time-course assessment showing persistent DNA damage foci at days 4 and 8 (Fig. 14). These results suggest that persistent DNA damage at 4 and 8 days is associated with the persistent increase in activated ATM and p21 at these times.

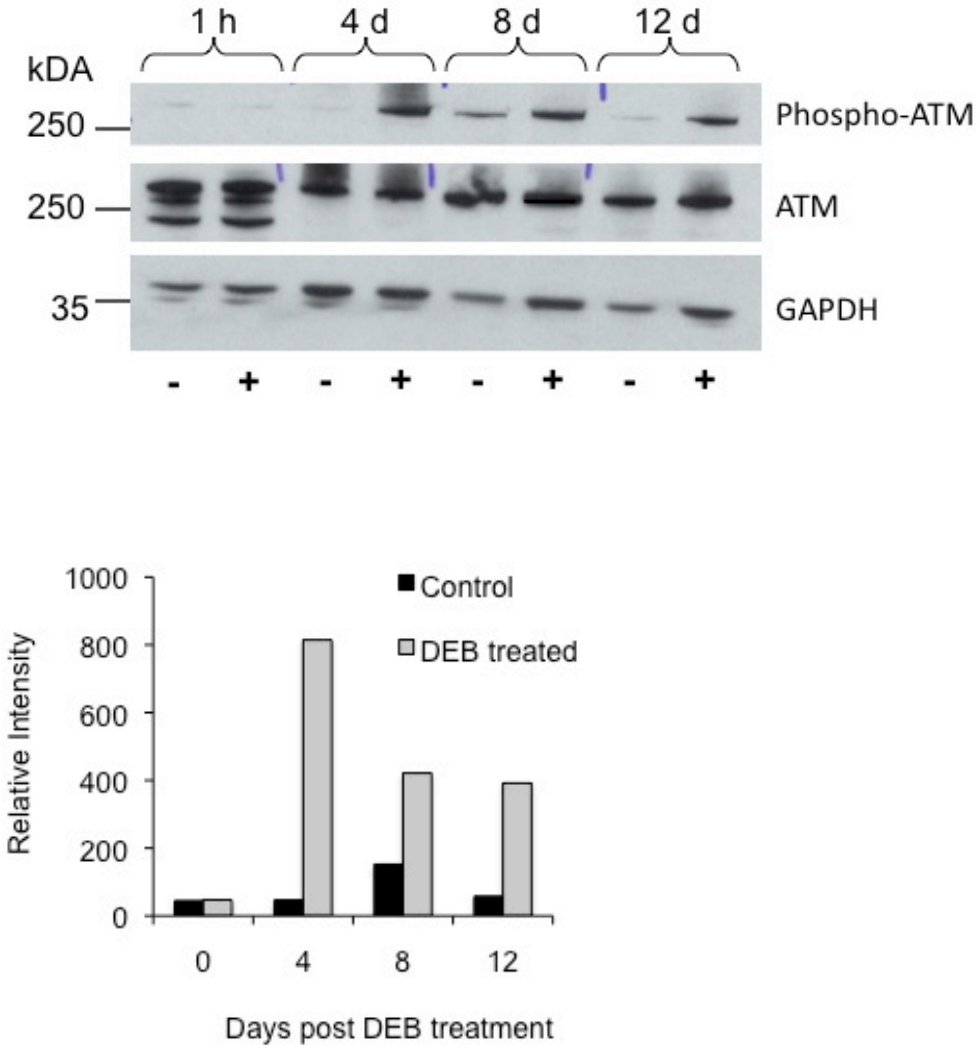

Figure 17: DEB treatment of HLF increases level of phosphorylated ATM. HLF were treated with DEB (100 μM for 1 h) or mock treated (control) and incubated in complete medium. HLF were lysed at indicated time points and separated by SDS-PAGE 4-12%. Proteins (30 μg per lane) were transferred to a nitrocellulose membrane and probed with anti-phospho ATM (pS1981) or anti-ATM antibody. Equal loading was verified by stripping and reprobing the blot with anti-GAPDH

antibodies. Western blotting data is representative of three independent experiments.

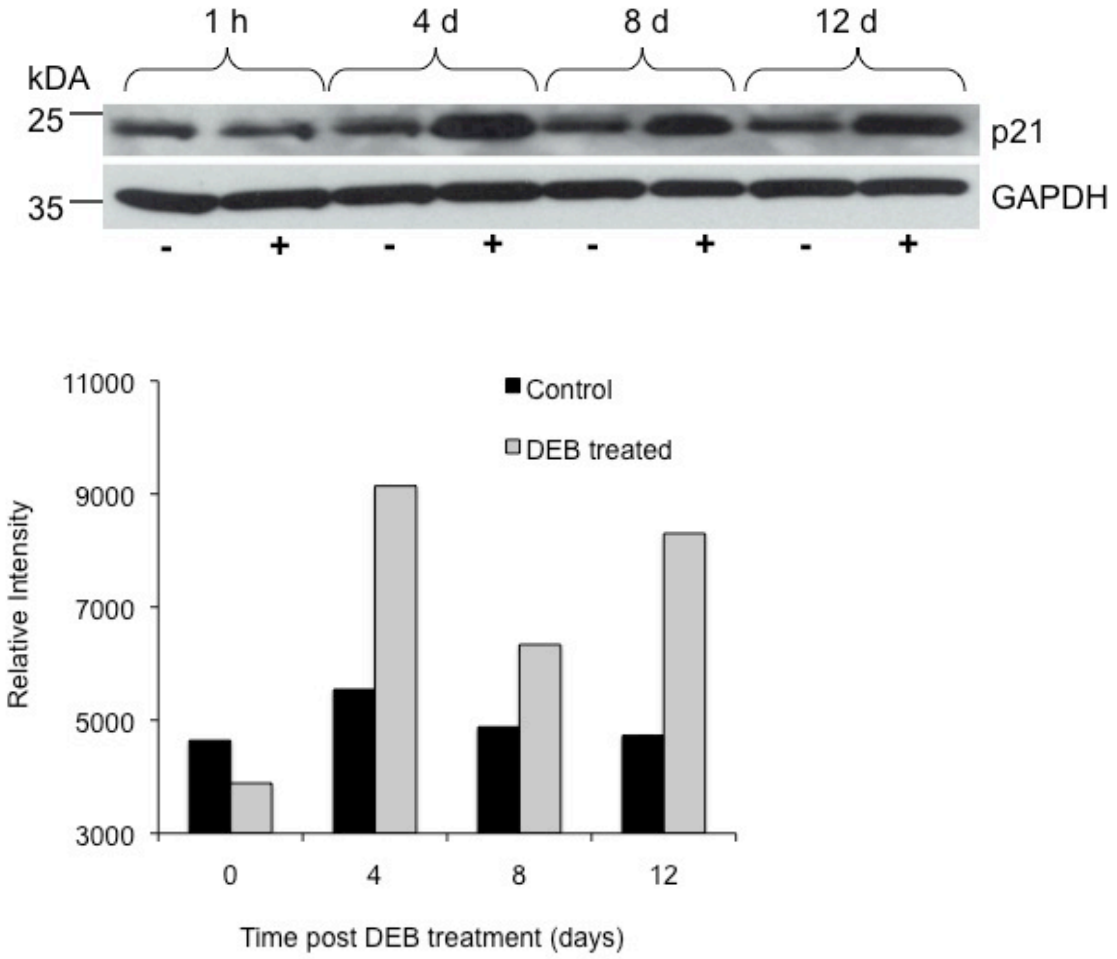

Figure 18: Increased expression of p21 in HLF after treatment with DEB. HLF were treated with DEB (100 μM for 1 h) or mock treated. Cell lysates were prepared at indicated time points and separated by SDS-PAGE 4-12%. Proteins (30 μg per lane) were transferred to a nitrocellulose membrane and probed with anti-p21 antibody. Equal loading was verified by stripping and reprobing the blot

with anti-GAPDH antibodies. Western blotting data is representative of three independent experiments.

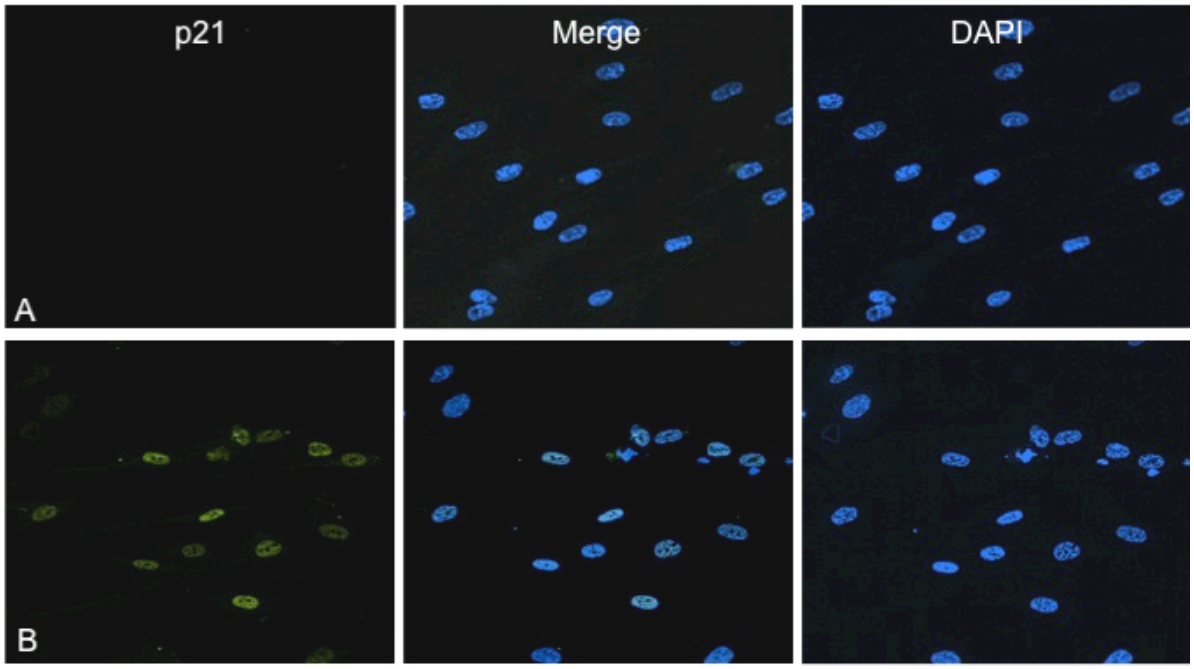

Figure 19: Increased expression of p21 in human lung fibroblasts at 1 h following treatment with diepoxybutane. HLF cultured on glass coverslips were mock treated (A) or treated with DEB (B) (100 μM for 1 h), fixed, and stained with anti-p21 antibodies (green). Cell nuclei were counterstained with DAPI (blue). Fluorescent images were captured at 60X and representative images are shown.

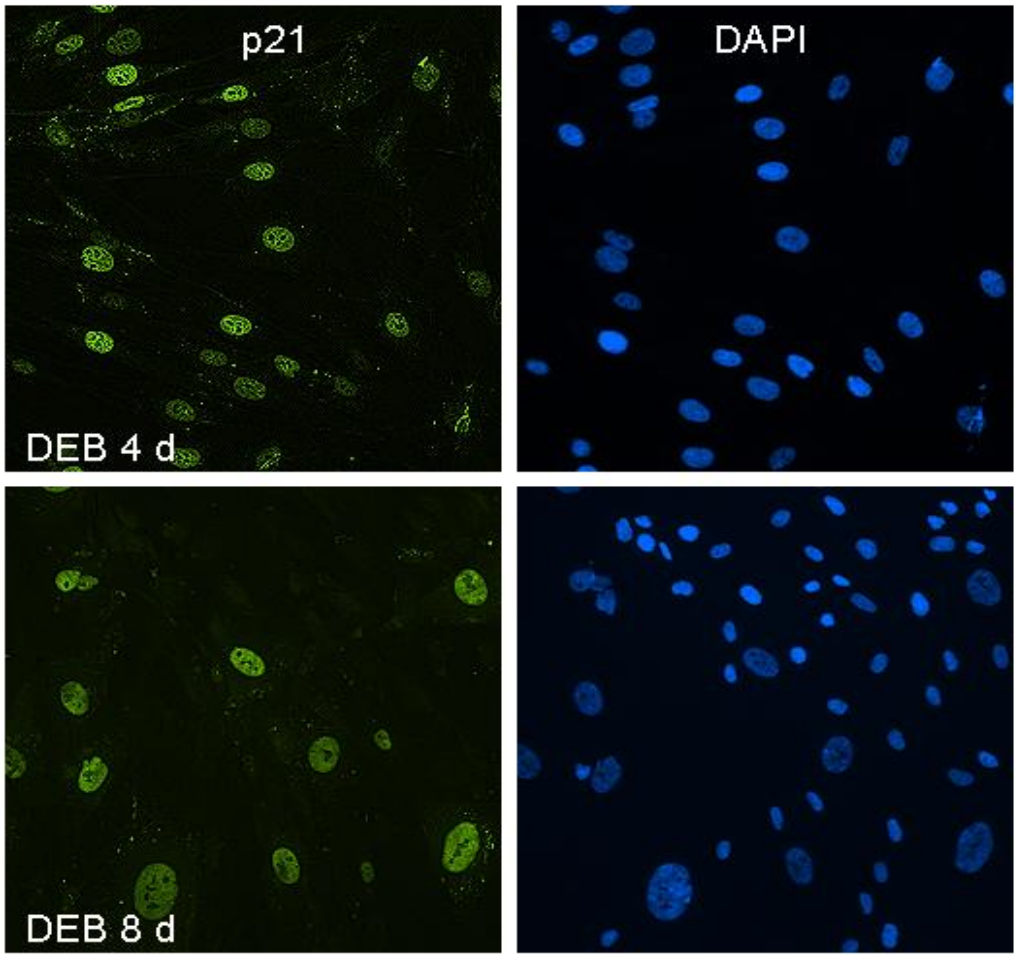

Figure 20: Persistent expression of p21 in HLF after DEB treatment. HLF cultured on glass coverslips were treated with DEB (100 μM for 1 h), fixed, and stained with anti-p21 antibodies (green) at days 4 and 8. Cell nuclei were counterstained with DAPI (blue). Fluorescent images were captured at 60X and representative images are shown.

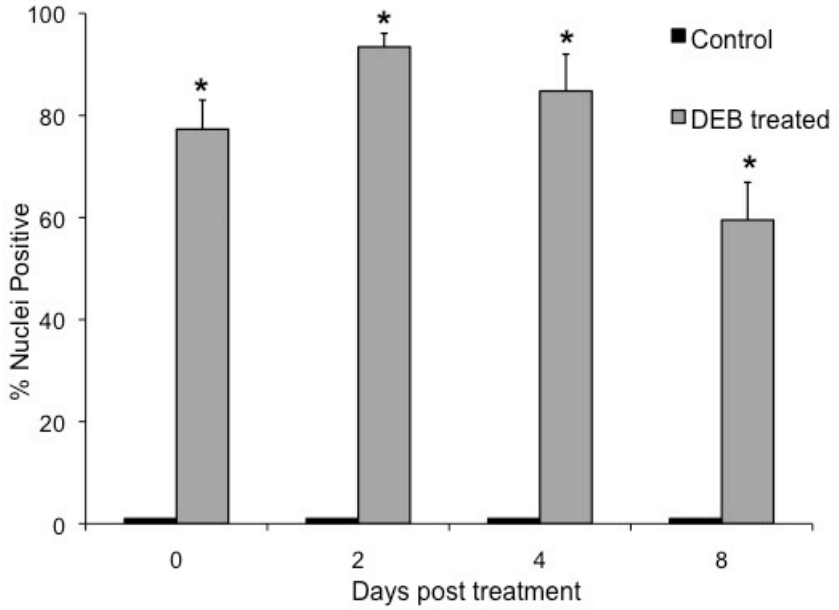

Figure 21: Increased expression of p21 in HLF following treatment with DEB. HLF cultured on glass coverslips were treated with DEB (100 μM for 1 h), fixed, and stained with anti-p21 antibodies at days 0, 2, 4 and 8. Cell nuclei were counterstained with DAPI. Digital fluorescent images were taken and staining patterns were analyzed and quantified using ImageJ64 Software (NIH, Bethesda, MD). Values represent the mean number of nuclei positive p21 per at least 100 cells counted ± SEM for three independent experiments. *$P < 0.05$ by Students' t-test compared to control at same time point.

Growth Factor Cocktail Does Not Induce Proliferation of DEB-treated HLF

To determine if DEB-treated HLF are truly senescent or if they could be stimulated to divide by a growth factor cocktail, we stimulated HLF with a cocktail of growth factors 7 days after a single DEB treatment (100 μM for 1 h). Mock-treated cells stimulated with this cocktail of growth factors underwent a 4-fold increase in total cell number whereas DEB-treated HLF did not proliferate in response to growth factors (Fig. 22).

People who smoke are exposed to BD repeatedly at much lower levels, thus, multiple DEB treatments at lower concentrations should be more physiologically relevant. Therefore, HLF were treated with 1 μM or 10 μM for 1 h every other day for 2 weeks and then stimulated with growth factors and cell counts were performed. The number of cells exposed to multiple, 1 μM DEB treatments doubled after growth factor stimulation. However, in the cells exposed repeatedly to 10 μM DEB, no appreciable increase in cells numbers was seen (Fig. 22).

However, a subpopulation of cells was able to transverse the cell cycle in response to growth factors after treatment with the lowest dose of DEB. Another subpopulation exhibited markedly decreased growth response. These results are consistent with the dose-dependent increase in SA β-Gal expression shown in Fig. 16.

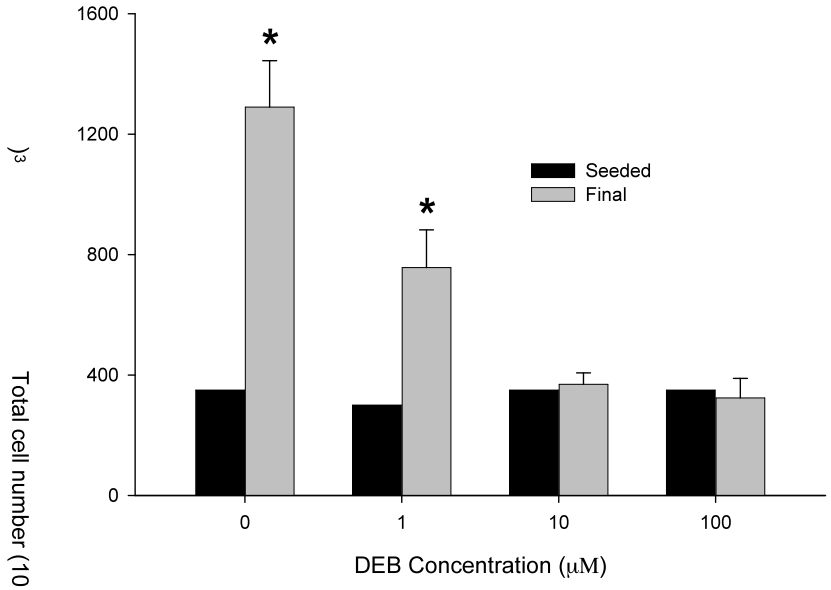

Figure 22: Lack of proliferative response of DEB-treated HLF to growth factor cocktail. DEB-treated HLF were stimulated by a growth factor cocktail [epidermal growth factor 1 (25 ng/ml), insulin-like growth facto (20 μg/ml), and dexamethasone (55 ng/ml) (Phillips *et al*., 1984). DEB-treated HLF were counted 4 days after stimulation with growth cocktail. For the single, acute treatment (100 μM, 1 h), HLF were treated, rinsed, and complete medium was added. Growth cocktail was added at day 7 and cell counts were performed 4 days later. For multiple, low-dose treatments, HLF were treated with 1 μM or 10 μM DEB for 1 h, every other day for 2 weeks, stimulated with growth cocktail, and cells counted 4 days later. Values represent cultures grown in triplicate. *$P < 0.05$ by Students' t-test compared to control at same time point.

CHAPTER 4

DISCUSSION

Senescence is defined as the irreversible growth arrest of cells that can be triggered by various mechanisms including telomere shortening, oncogenic activation, oxidative stress, and DNA damage (Campisi & d' Adda di Fagagna, 2007; d' Adda di Fagagna, 2008). DNA damaging agents in CSE and its volatile metabolites have been shown to inhibit important fibroblast tissue repair functions such as proliferation, chemotaxis, contraction, and remodeling of the ECM (Nakamura *et al.,* 1995; Carnevalli *et al*., 1998; Wang *et al*., 2001; Wang *et al*., 2003; Rennard *et al.,* 2006). These earlier studies did not examine cells for markers of senescence, however, given the findings of two seminal studies demonstrating that cigarette smoke induces cellular senescence in human alveolar epithelial cells and human lung fibroblasts (Tsuji *et al.,* 2004; Nyunoya *et al.,* 2006), it is plausible that the inhibition of fibroblasts functions were due to CSE-induced senescence. Given this precedent of cigarette smoke-induced senescence and the observation that DEB, an important component of cigarette smoke, induces persistent DNA damage and cell cycle arrest in human lung fibroblasts, we hypothesized that DEB induces senescence in HLF.

To our knowledge, based on an extensive search of the literature, this dissertation provides the first documentation of DEB-induced senescence of human lung fibroblasts as well as of human cells by acute exposure to an environmental toxicant. Our literature search did reveal the recent preliminary report by Dean *et al*. that DEB exposure induced

a senescence-like morphology in endothelial cells (Dean *et al*., 2009). Specifically, endothelial cells treated with 1 to 10 μM DEB for 3-7 days were found to exhibit complete cell cycle inhibition and a senescence-like morphology by day 4 at only 10 μM DEB. However, this meeting abstract (*FASEB J*) did not include observations about SA β-gal or other non-morphological characteristics of senescence and has not been published.

Our study used normal, diploid human lung fibroblast cells used by Schmiederer *et al*. (2005) to investigate the effects of DEB for up to 2 weeks. Compared to control cells, we observed no changes in cell numbers in DEB-treated HLF. This might be due to persistent cell cycle arrest or loss of cell viability following DEB treatment. To rule out apoptosis as the cause of the observed lack of proliferation, we investigated whether apoptosis was induced in DEB-treated cells. Two predominate pathways of apoptosis are known, the intrinsic pathway and extrinsic pathway (Adrain *et al*., 2002). While both pathways involve cleavage and activation of proteases called caspases, we investigated key players of the intrinsic pathway since it is induced by DNA damage, a known effect of DEB (Adrain *et al*., 2002; Elmore, 2007). Moreover, the intrinsic pathway involves mitochondrial events including opening of the mitochondrial transition pore and release of cytochrome c from the intermitochondrial membrane space. Experiments examining activation of key effector caspases and release of cytochrome c provided strong evidence that the intrinsic apoptotic pathway is not activated following treatment of HLF with DEB.

BD and its metabolites have been shown to cause DNA damage in cultured cells and mice (Albertini *et al*., 2010). Therefore, we analyzed HLF for DEB-induced DNA damage using the Comet assay at early times following treatment. The Comet assay is a sensitive method to estimate the extent of DNA damage at the individual cellular level by quantifying the amount of damaged DNA that migrates out of the cell during electrophoresis. Alkaline electrophoresis (12.1-12.4) facilitates the detection of single strand DNA breaks, double-stranded DNA breaks, and the majority of apurinic, apyrimidinic sites, and alkali labile DNA adducts, whereas electrophoresis at neutral pH (7-8) primarily facilitates the detection of double strand breaks and cross links (Miyamae *et al*., 1997). We chose the alkaline Comet assay because it enables detection of the broadest spectrum of DNA damage (Miyamae *et al*., 1997). One shortcoming of the Comet assay is a lack of agreement on a single appropriate parameter capable to effectively measure and describe DNA damage. Tail moment is defined as the product of DNA in the tail and the mean distance of migration in the tail (Olive *et al*., 1990). We selected tail length and tail moment as parameters for measuring DEB-induced DNA damage because they are two of the most frequently reported. We observed significant increases in both tail length and tail moment suggesting DEB induces significant DNA damage following an acute treatment.

While the Comet assay is a sensitive measure to detect DNA damage, we also evaluated HLF for persistent DNA damage at early and later time points using immunofluorescence to detect γ-H2AX. This method of DNA damage detection has become the gold standard because it is extremely sensitive and less cumbersome than the

Comet Assay (Huang *et al*., 2004). γH2AX staining was originally described solely as a marker of DNA DSB formation, however, persistent γH2AX foci are now well-described markers of senescence (Von Zglinicki *et al*., 2005). We demonstrated both rapid and persistent DEB-induced DNA damage via γH2AX staining, similar to our Comet assay results.

The common underlying etiology shared by both replicative senescence and SIPS is DNA damage. The DNA damage response (DDR) pathway is the central mediator in senescence and has to be maintained to keep cells in a senescent state (Nakamura *et al*., 2008; Rodier & Campisi, 2011). In effect, senescence can be regarded as a permanently maintained DDR state (Saretzki, 2010). The ATM protein is the apical kinase of the DDR pathway (Shiloh, 2003). Within minutes of DNA damage, ATM is robustly activated and recruited to sites of double strand breaks resulting in massive phosphorylation of histone protein H2A.X on serine 139 (Rogakou *et al*., 1999; Fernandez-Capetillo *et al*., 2004). The phosphorylated form of H2AX, referred to as γH2AX, promotes DNA repair by recruiting repair proteins to the sites of damage called DNA damage foci (Celeste *et al*., 2002; Fernandez-Capetillo *et al*., 2003; Fragkos *et al*., 2009). DNA damage foci are thought to be vital mechanism for amplifying the DDR. Recurring cycles of H2AX phosphorylation, recruitment of repair machinery, and subsequent activation of the p53-p21 pathway establishes a positive feedback loop and persistent DDR signaling that maintains senescence (Shiloh, 2003).

Senescence is established via activation of two vital tumor suppressor pathways by ATM. The p53-p21 pathway and the p16-pRb pathways can establish senescence

either individually or together (Campisi & d' Adda di Fagagna, 2007; d' Adda di Fagagna, 2008). p53 is a well-established tumor suppressor that triggers the transcriptional activation of p21, a crucial inhibitor of CDKs and mediator of cell cycle arrest. pRb functions by binding to the E2F family of transcriptional factors, thereby inhibiting downstream transcriptional cascades and progression through the cell cycle. p21 maintains cell cycle arrest by preventing the phosphorylation and inactivation of pRb (Sherr & Roberts, 1995). Our analysis of key players in the senescence pathway documented persistent activation of ATM and p21 after DEB treatment.

The p53-p21 and p16-pRb pathways can also act separately via upregulation of p16, which also controls pRb activity by inhibiting CDK complexes (Stein *et al*., 1995; Alcorta *et al*., 1996; Hara *et al*., 1996). Upregulation of p21 is believed to establish cell cycle arrest, while p16 is thought to maintain the arrest after the development of the senescence phenotype. However, the exact role of p16 in both replicative senescence and SIPS is not yet clear. While p16 has been shown to be essential for induction of SIPS in some human epithelial cells (Kiyono *et al*., 1998; Rheinwald *et al*., 2002); expression of p16 can vary significantly among different human cell lines, especially human fibroblasts (Alcorta *et al*., 1996; Stein *et al*., 1999; Itahana *et al*., 2003). Differences in p16 expression could depend on whether p53 and pRb function in a linear or parallel manner (Itahana *et al*., 2003). In cells types that do not show increased p16 expression, p53 and pRb could function in a linear pathway. On the contrary, in cells types where p16 expression is increased, p53 and pRb could work in parallel. Moreover, increases in expression of p16 have been reported to increase only weeks after human fibroblasts have

completely senesced (Stein *et al*., 1999). We did not see a significant difference in p16 expression between mock-treated HLF and DEB-treated HLF. It is possible that p53 and pRb function in a linear pathway in our cell line. However, our studies investigated the effects of DEB on HLF for only two weeks. Therefore, further studies at later times would be necessary to clarify any role of p16 in DEB-induced senescence.

Recent studies have shown that additional molecular mechanisms could contribute to DEB-induced cellular senescence (Freund *et al*., 2011). p38MAPK has been very recently reported as a novel DNA damage-independent regulator of SIPS and the development of SASP by Freund *et al*. (2011). This research group suggested that activation of the p38MAPK/NFκB pathway is a delayed molecular event required for the development of the SASP, whereas the DDR is activated immediately after DNA damage. Moreover, ROS may also play a critical role in the establishment and maintenance of senescence and the DDR. Passos *et al*. investigated the role of ROS in the establishment of both replicative senescence and SIPS. They suggested that a positive feedback loop exists between the DDR and the production of ROS in both forms of senescence. Passos *et al*. (2010) suggested that the DDR causes mitochondrial dysfunction and increased ROS production through a linear pathway involving ATM, p53, p21, p38, and TGF-β. ROS, in turn, result in persistent DNA damage foci and continuous DDR. ROS can directly damage DNA resulting in SIPS or ROS can accelerate telomere shortening inducing replicative senescence; increased levels of ROS have been demonstrated in both (Saretzi *et al*., 2003; Ramsey & Sharpless, 2006; Lu & Finkel, 2008). Furthermore, several other studies have shown the importance of ROS in

senescence and the development of positive feedback loops (Takahashi *et al*., 2006; Moiseeva *et al*., 2009).

Senescent fibroblasts with persistent DNA damage signaling remain metabolically active but experience extensive changes in gene expression, and, thus, exhibit an altered senescence-associated secretory phenotype termed "SASP" (Coppe *et al*., 2008; Coppe *et al*., 2010; Campisi *et al*., 2011). Senescent cells display an increase in the production of 40-80 factors involved in intracellular signaling and inflammation including: growth factors, proteases, cytokines, and chemokines (Coppe *et al*., 2010; Freund *et al*., 2010). Some of the most robustly secreted factors include: interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and matrix metalloproteases (Coppe *et al*., 2010; Davalos *et al*., 2010; Freund *et al*., 2010; Campisi, 2011). Therefore, senescent fibroblasts may alter the tissue microenvironment, which results in disruption of normal tissue structure and function and, thus, is thought to augment development of several lung diseases (Rennard *et al*., 2006; Freund *et al*., 2010).

We determined the extent of DEB-induced senescence by counting the percentage of cells positive for SA β-Gal activity, a well-known marker of senescence, which is readily apparent by the intense blue color of cells incubated with the artificial chromogenic substrate 5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside (X-gal). Acid β-d-galactosidase is a hydrolase found in the lysosomes of eukaryotic cells that cleaves β-linked terminal galactosyl residues from substrates such as glycoproteins and glycosaminoglycans (Suzuki *et al*., 1995). This hydrolase functions at a pH of 4.0 to 4.5 which is close to the pH of the lysosome. Dimri *et al*. (1995) was the first to describe a

β-d-galactosidase activity at pH 6.0 specific to senescent cells. This pH is vital for suppressing lysosomal beta-galactosidase activity in most nonsenescent cells. The increase in SA β-gal activity at pH 6.0 probably results from an increase in the level of β-d-galactosidase associated with the enlargement of lysosomal size in senescent cells (Kurz *et al.,* 2000). This increased lysosomal mass is attributed to an accumulation of macromolecules and organelles in autophagic vacuoles. While lysosomal SA β-Gal activity has some nonspecific activity under conditions such as increased confluency (Severino *et al*., 2000) and serum withdrawal (Yegorov *et al*., 1998), studies have demonstrated that intense staining of this marker is closely associated with the senescent phenotype (Dimri *et al*., 1995; Itahana *et al.,* 2007). Therefore, SA β-Gal activity is an effective biomarker for the detection of senescent cells both *in vitro* and *in vivo* (Mishima *et al*., 1999; Itahana *et al*., 2007; Melk *et al*., 2004), especially when combined with other markers of senescence. We demonstrated that DEB induces a flattened altered morphology characteristic of senescence and dose-dependent increases in SA β-Gal activity.

Phillips *et al*. (1984) reported a loss of a proliferative response in senescent WI-38 cells to a growth factor cocktail containing platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, transferrin, and dexamethasone. Cell cultures are comprised of a mixture of cells including rapidly and slowly growing cells and arrested cells. As cell cultures age and senesce, the ratio of growth factor responsive cells to nonresponsive cells decreases. Phillips' classical senescence study demonstrated this by comparing the responsiveness of young versus old cells to various growth factors.

Specifically, in this study, cells were serum-arrested, treated with various concentrations of growth factors, harvested via trypsinization, and counted 3 or 4 days later. The investigators found that the following concentrations of growth factors were optimal to stimulate growth of young cells: epidermal growth factor-1 (25ng/ml); insulin-like growth factor (20 μg/ml); and dexamethasone (55ng/ml). On the contrary, old, senescent cultures were virtually unresponsive to each concentration of growth factors. Moreover, effective concentrations of growth factors for responsive cells were similar regardless of age in mass cultures. Thus, sensitivity to growth factors did not decrease, instead, senescent cells became unable to respond at all. We used this same growth factor cocktail to document that DEB-treated HLF are truly senescent as indicated by a lack of proliferative response of HLF treated with 10 μM and 100 DEB. Since people who smoke cigarettes are exposed to BD repeatedly at much lower levels, our repeated low-dose (1 or 10 μM) exposure protocol served as a physiological mimic for DEB exposure via smoking. Additional experiments exposing cells more frequently over time could reveal a senescence response at lower doses of DEB and model the constant exposures that a smoker would experience during years of exposure to DEB and other components of cigarettes.

It remains unclear whether DEB-induced senescence is accompanied by shortening of telomeres. Gorbunova *et al*. (2002) found that stresses such as irradiation, and UV light, known inducers of DNA double-strand breaks, resulted in SIPS that was not accompanied by the shortening of telomeres. Moreover, Nyunoya *et al*. (2006) reported that exposure of HLF to CSE did not result in telomere attrition. Given that

HLF undergo cell cycle arrest immediately after treatment, we hypothesize that DEB-induced SIPS is not accompanied by telomere shortening. However, it is possible that DEB may directly damage telomeric DNA, therefore, telomere shortening at lower DEB concentrations should be ruled out by additional studies such as fluorescent in situ hybridization (FISH) or Real-Time PCR assay for analysis of telomere length.

These studies were limited to *in vitro* experiments in a single cell type. It remains unclear how other lung cell types, such as lung epithelial, endothelial, and alveolar cells would respond to DEB exposure. We hypothesize that similar results would be seen as other studies have demonstrated SIPS in these cell types following CSE exposure (Tsuji *et al*., 2004; Nyunoya *et al*., 2006). A mouse model to examine DEB-induced SIPS could be quite valuable in future studies. The lungs of DEB-exposed mice could be examined for markers of senescence and changes in the tissue microenvironment. In the future, the promising area of tissue engineering could enable DEB-senescence effects to be investigated in artificial lungs comprised of primary human cells.

Collectively, our observations suggest that the irreversible senescence produced by DEB in HLF occurs via the ATM-p53-p21 pathway following irreparable DNA damage. However, additional experiments examining each key player of senescence in our cell type would help to determine exact roles. For example, shRNAs, siRNAs, or small molecule inhibitors against ATM, p53, p21, p16, and pRb could be utilized to determine which cell cycle inhibitors are absolutely crucial to DEB-induced senescence. Moreover, further studies investigating the role of ROS and other signaling pathways that could be involved in DEB-induced senescence would also be useful. Additional studies

examining the enhanced production of inflammatory mediators characteristic of the SASP phenotype could provide valuable insight into the effect of lung cell senescence on fibroblasts and surrounding cells.

Conclusions

The persistent activation documented by our time-course studies of these known senescence markers suggests that DEB-induced senescence occurs via the ATM-p53-p21 pathway following irreparable DNA damage. As a component of cigarette smoke and as an environmental toxicant, DEB-induced senescence could contribute to the pathogenesis of lung diseases, such as COPD, by inhibiting normal lung fibroblast function and repair and altering the tissue microenvironment via the SASP. Furthermore, revelations that smoking and its volatile components cause senescence, recent evidence linking senescence to COPD, IPF, and Cancer (Muller *et al*., 2006; Tsuji *et al*., 2006; Tuder *et al*., 2008; Aoshiba & Nagai, 2009; Rodier & Campisi, 2011), and new insights into the inflammatory SASP of senescence cells and the role of senescence in aging could lead to the development of effective treatments for these important diseases.

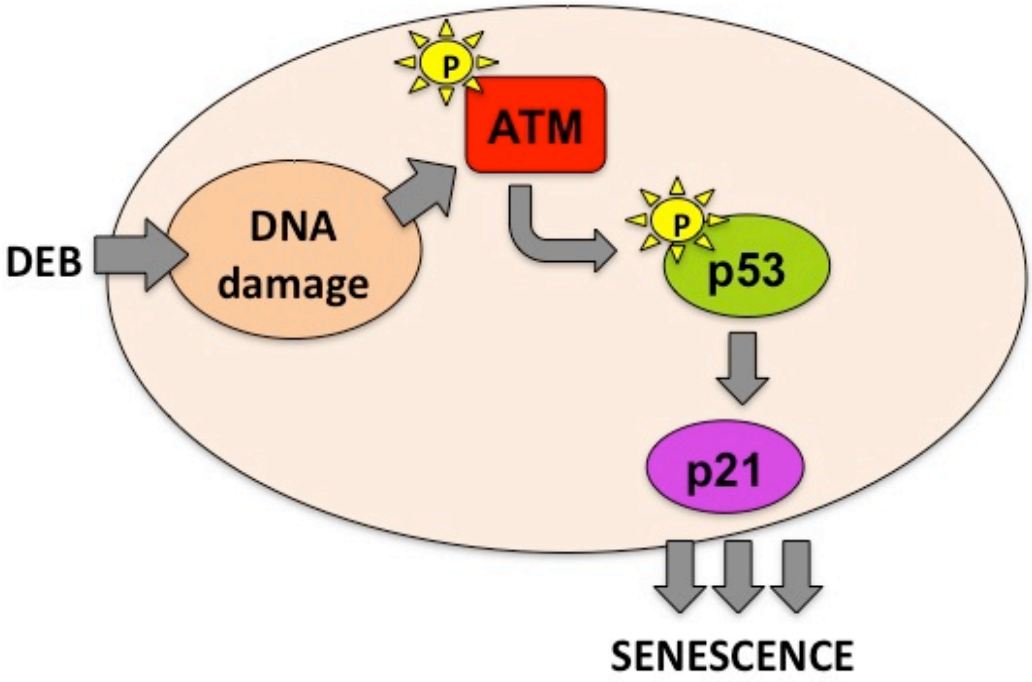

Figure 23: Proposed pathway for DEB-induced cellular senescence in HLF. Treatment of HLF with DEB induces stress-induced premature senescence (SIPS) via persistent DNA damage, phosphorylation of ATM, upregulation of the p53-p21 pathway, and subsequent events leading to development of a senescent fibroblast.

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Vita

Erica Rae Thomasson (Dahl) was born in Clinton, Iowa, on January 20, 1977, to Laura Kathleen Putman and Joseph William Dahl. She graduated from Camanche High School, Camanche, Iowa, in 1995. She graduated from Kirkwood Community College, Cedar Rapids, Iowa, in 1997 with an Associate's Degree in Science and completed her Bachelor or Arts in Biology at Wartburg College, Waverly, Iowa in 1999. She earned her Master of Public Health in Global Health and Infectious Diseases in 2002 from Rollins School of Public Health of Emory University, Atlanta, Georgia. It was Emory University's Rollins School of Public Health that opened her eyes and heart to the vision of global health. As a student in the International Health Department, coursework in international health and infectious diseases fueled these interests. Her graduate program thesis focused on the HIV/AIDS pandemic and the AIDS orphan crisis. To fulfill the department's practicum requirement, she traveled to Winterveldt, South Africa for a 10-week internship working at a community-based organization with AIDS orphans. Prior to commencing her doctoral studies at the University of Texas Medical Branch (UTMB), she worked as a Guest Researcher at the Center for Disease Control and Prevention's Division of Parasitic Diseases. Her research group focused on the molecular characterization of the parasitic protozoan, *Toxoplasma gondii.* She joined the UTMB's Department of Microbiology and Immunology in 2004 and throughout her graduate studies, she continued to develop practical laboratory skills and experience in the scientific research process to prepare her for a career in research and public health.

Education

A.S. May 1997, Kirkwood Community College, Cedar Rapids, Iowa
B.A. May 1999, Wartburg College, Waverly, Iowa
M.P.H. May 2002, Rollins School of Public Health of Emory University, Atlanta, Georgia

Publications

Dahl ER, Moslen MT, Schmiederer MW, Albrecht T. Butadiene diepoxide induces cellular senescence. For submission to *Am J Respir Cell Mol*.

Lenhart A, Eigege A, Kal D, Pam ES, Miri G, Gerlong J, Oneyka Y, Sambo J, Danboyi B, Ibraham, **Dahl E**, Kumbak D, Dakul A, Jinadu MY, Umaru J, Richards F, Lehmann T. Contributions of different mosquito species to the transmission of lymphatic filariasis in central Nigeria: Implications for monitoring infection by PCR in mosquito pools. *Filarial J* 2007; 6(14): 1-6.

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Dubey JP, Karhemere S, **Dahl E**, Sreekumar C, Diabaté A, Dabiré KR, Vianna MC, Kwok OCH, Lehmann T. First Biologic and genetic characterization of *Toxoplasma gondii* isolates from chickens from Africa (Democratic Republic of Congo, Mali, Burkina Faso, and Kenya). *J Parasitol* 2004; 91: 69-72.

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Dubey JP, Graham DH, **Dahl E**, Da Silva DS, Lehmann T, Bahia-Oliveira LMG. Isolation and molecular characterization of *Toxoplasma gondii* isolates from free ranging

chickens from Rio de Janeiro, Brazil: Mouse mortality, genotype, and oocyst shedding in cats. *J Parasitol* 2003; 89: 851-853.

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Dubey JP, Venturini M, Venturini CT, Piscopi M, Graham DH, **Dahl E**, Sreekumar C, Vianna MC, Lehmann T. Isolation and genotyping of *Toxoplasma gondii* from free ranging chickens from Argentina. *J Parasitol* 2003; 89: 1063-1064.

Summary of Dissertation

Cellular senescence is a state of irreversible growth arrest induced by either telomere shortening (replicative senescence) or telomere-independent signals (stress-induced premature senescence). Recent studies have shown that cigarette smoke extract induces cellular senescence in lung fibroblasts and alveolar epithelial cells. 1,2,3,4-diexpoxybutane, or diepoxybutane (DEB), is the most reactive metabolite of 1,3-butadiene, an important hazardous air pollutant and potent genotoxic agent found in cigarette smoke. We examined the effectS of DEB on fibroblast proliferative capacity. We hypothesized that exposure of human lung fibroblasts to DEB induces stress-induced premature senescence (SIPS). *In vitro* experiments demonstrated that exposure of fibroblasts to DEB induced persistent DNA damage that resulted in SIPS. This senescence was characterized by a dose-dependent increase in senescence-associated β-galactisidase activity, senescence-associated alterations in morphology, formation of DNA damage foci, activation of the ATM-p53-p21 pathway, and irreversible growth arrest. These observations suggest that DEB induces senescence and provide the first

evidence for senescence induced by an environmental toxicant. As a component of cigarette smoke and as an environmental toxicant, DEB-induced senescence could contribute to the pathogenesis of lung diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and cancer, by inhibiting normal lung fibroblast function and repair.

CURRICULUM VITAE

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PRESENT POSITION AND ADDRESS:

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BIOGRAPHICAL:

DOB: 01/20/1977, Clinton, Iowa, USA, Citizen of the United States

EDUCATION:

01/2004 – Present	University of Texas Medical Branch Doctoral Studies in Microbiology and Immunology Graduate School of Biomedical Sciences	Galveston, TX
08/2000 – 05/2002	Rollins School of Public Health, Emory University Master of Public Health in Global Health and Infectious Diseases	Atlanta, GA

08/1997 – 05/1999	Wartburg College Bachelor of Art in Biology	Waverly, IA
08/1995 – 05/1997	Kirkwood Community College Associate's of Science	Cedar Rapids, IA

PROFESSIONAL WORK HISTORY AND TEACHING EXPERIENCE:

(Graduate)

08/2004 – Present — Galveston, TX
Graduate Research Assistant/Doctoral Candidate
Department of Microbiology and Immunology
University of Texas Medical Branch
Doctoral Dissertation Research:

- Investigating the effects of 1,3-butadiene exposure in human lung fibroblasts.
- Research Supported by the NIEHS Pre-doctoral Toxicology Training Grant, T-32-ES007254 2007-2009.

01/2010 – 05/2010 — Galveston, TX
Graduate Teaching Assistant
Texas A&M University

01/2009 – 05/2009 — Galveston, TX
Graduate Teaching Assistant
Texas A&M University

01/2008 – 05/2008
Galveston, TX
Graduate Teaching Assistant
Texas A&M University

01/2007 – 05/2007 — Galveston, TX
Graduate Teaching Assistant
Texas A&M University

07/2002 – 07/2004 Chamblee, GA
Guest Researcher
Division of Parasitic Diseases
Entomology Branch, Vector Genetics
National Center for Infectious Diseases
Centers for Disease Control and Prevention
Job Responsibilities:

- Culture of mosquito colonies, including laboratory culture, quality control, archiving, and distribution of living and preserved malaria vectors.
- Preparing rodent blood meals, rodent handling, collecting eggs, and rearing all life stages of mosquitoes.
- Maintenance of 40 unique Anopheles mosquito colonies and 2 unique Aedes mosquito colonies.
- Morphological and genetic authentication of mosquito stocks via mosquito DNA extraction and polymerase chain reaction (PCR) for species identification.
- General insectary and laboratory maintenance.
- Molecular characterization of *Toxoplasma gondii* strains by DNA extraction of infected tissue specimens, polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP), and gene sequencing via Perkin-Elmer 377 DNA sequencer and ABI 3100 Genetic Analyzer.
- Processing, labeling, and cataloging of infected tissue samples.
- Updating databases and ordering lab supplies.

03/2002-07/2003 Atlanta, GA
Project Manager Assistant Pilot Pedometer Study
Georgia Department of Public Health
Job Responsibilities:

- Recruiting physicians to participate in Georgia Department of Public Health pedometer study.
- Organizing appointments and meeting with physicians.
- Developing data collection tools and designing study materials.

05/2001-08/2001
Public Health Intern (400 Volunteer hours) Winterveldt, South Africa
Foundation for Sustainable Development
Job Responsibilities:

- Assisting in program development at a non-residential center for

AIDS orphans.
- Implementing a Child-to Child Learning Program including lessons in nutrition, hygiene, clean water and sanitation, HIV/AIDS and other infectious diseases, and first-aide.
- Facilitating workshops for caregivers.

TEACHING EXPERIENCE

A. TEACHING RESPONSIBILITIES at UTMB:

Graduate School of Biomedical Sciences:

- MICR 6403: Fundamentals of Virology. 1 lecture: "Papillomaviridae." Fall 2006, 2007, 2008.
- BBSC 6104: Critical Reading of Scientific Literature. 1 lecture. Summer 2005, 2006, 2007.

B. TEACHING RESPONSIBILITIES AT OTHER UNIVERSITIES:

Undergraduate Course Lectures:

- MARB 414: Fundamentals of Toxicology. Texas A&M University, Galveston, TX.
  2 lectures: "Mechanisms of Tissue Injury and Repair." Spring 2007.
- MARB 414: Fundamentals of Toxicology. Texas A&M University, Galveston, TX.
  2 lectures: "Mechanisms of Tissue Injury and Repair." Spring 2008.
- MARB 414: Fundamentals of Toxicology. Texas A&M University, Galveston, TX.
  1 lecture: "Introduction to Fundamentals of Toxicology." Spring 2009.
- MARB 414: Fundamentals of Toxicology. Texas A&M University, Galveston, TX.
  1 lecture: "Introduction to Fundamentals of Toxicology." Spring 2010.

MEMBERSHIP IN SCIENTIFIC SOCIETIES/PROFESSIONAL ORGANIZATIONS:

Current

2008 – Present Society for Toxicology

Texas Public Health Association

HONORS:

1995-1997	Kirkwood Community College Dean's List
1996-1997	Phi Theta Kappa Honor Fraternity, Chapter President
1996-1997	Who's Who Among Students in America's Community and Junior Colleges
1997	KCC Math and Science Department Outstanding Student (Faculty vote)
1997	KCC Arts and Humanities Department Outstanding Student (Faculty vote)
1997	All Iowa Academic Team, All-American Scholar Nominee
1997	Wartburg College Dean's List
1997-1999	Phi Theta Kappa Scholarship
1997-1999	Beta Beta Beta Biological Honor and Service Society
2007-2008	NIEHS Pre-doctoral Toxicology Training Grant Recipient, T-32-ES007254
2008-2009	NIEHS Pre-doctoral Toxicology Training Grant Recipient, T-32-ES007254
2011	UTMB Global Health Scholarship/Field Epidemiology Course in Northern Peru

ORGANIZATIONS AND COMMITTEES:

University of Texas Medical Branch, Galveston, TX

2007 – 2009	Public Health Organization Co-President
2008 – 2009	Students Improving Global Health Together (SIGHT) Organization Co-President
2004 – 2010	Microbiology and Immunology Student Organization Member
2004 – 2006	GSBS prospective student recruitment volunteer
2005 – Present	Toxicology Journal Club Member

PUBLICATIONS:

Dahl ER, Moslen MT, Schmiederer MW, Albrecht T. Butadiene diepoxide induces cellular senescence. For submission to *Am J Respir Cell Mol Biol*.

Lenhart A, Eigege A, Kal D, Pam ES, Miri G, Gerlong J, Oneyka Y, Sambo J, Danboyi B, Ibraham, **Dahl E**, Kumbak D, Dakul A, Jinadu MY, Umaru J, Richards F, Lehmann T. Contributions of different mosquito species to the transmission of lymphatic filariasis in

central Nigeria: Implications for monitoring infection by PCR in mosquito pools. *Filarial J* 2007; 6(14): 1-6.

Lehmann T, Marcet PL, **Dahl ER**, Graham DH, Dubey JP. Globalization and the population structure of *Toxoplasma gondii*. *P Natl Acad Sci USA* 2006. 103(30): 11423-11428.

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Dubey JP, Karhemere S, **Dahl E**, Sreekumar C, Diabaté A, Dabiré KR, Vianna MC, Kwok OCH, Lehmann T. First Biologic and genetic characterization of *Toxoplasma gondii* isolates from chickens from Africa (Democratic Republic of Congo, Mali, Burkina Faso, and Kenya). *J Parasitol* 2004; 91: 69-72.

Dubey JP, Salant H, Sreekumar C, **Dahl E**, Vianna MCB, Shen SK, Kwok OCH, Spira D, Hamburger J, Lehmann T. High prevalence of *Toxoplasma gondii* in a commercial flock of chickens in Israel, and public health implications of free-range farming. *Vet Parasitol* 2004. 121: 317-322.

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Sreekumar C, Graham DH, **Dahl E**, Lehmann T, Raman M, Bhalerao DP, Vianna MCB, Dubey JP. Genotyping of *Toxoplasma gondii* isolates from chickens from India. *Vet Parasitology* 2003; 118: 187-194.

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