# MECHANISMS OF IONIZING RADIATION-INDUCED CELL DEATH IN PRIMARY LUNG CELLS

by

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# DEDICATION

*To my family, mentors, and friends.*

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# ABSTRACT

**Mechanisms of Ionizing Radiation-induced Cell Death in Primary Lung Cells**

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Exposure to high doses of ionizing radiation (IR) causes serious biological damage that can lead to death. During the course of radiotherapy, the use of IR for the treatment of thoracic cancers is limited by IR-induced cell death to the underlying normal lung tissue potentially leading to pneumonitis and/or fibrotic remodeling of the lung. Studies on the mechanisms of IR-induced cell death have been exponentially increasing, however, most of these studies are conducted on immortalized cancer cell lines that are not lung-derived and therefore do not represent the biological status of normal lung cells. We investigated the mechanisms of IR-induced cell death in low passage cultures of primary pulmonary artery endothelial cells (PAEC) exposed to varying doses of X-rays. We observed that irradiated PAEC undergo accelerated senescence as the primary mode of cell death at doses examined. The cells displayed markers of senescence including cell cycle arrest, upregulation of cell cycle checkpoint proteins *e.g.,* p21/waf1, morphological alterations, and increased senesecence-associated β-galactosidase (SA-β-gal) activity. Increasing the dose shifted the mode to apoptosis as shown by neutral comet assay. The apoptotic cell death was also confirmed by caspase-3, 8, and 9 activation assays. We found that no necrosis is induced by all doses of X-rays examined. Reactive oxygen species (ROS) have been demonstrated to cause endoplasmic reticulum (ER) stress. We thus explored the presence and contribution of ER stress in mediating cell death in

irradiated PAEC. We found that ER stress was activated as shown by increased eIF2α phosphorylation and increased mRNA expressions of GRP78, CHOP, and GADD34. Experiments using salubrinal, an ER stress inhibitor, revealed that ER stress-mediated cell death was upstream of apoptosis but not accelerated senescence. Recent investigations have also shown that accelerated senescence is a state of continuous proliferative signaling in the presence of cell cycle blockade. We used pharmacological inhibitors of IGF-1R, PI3K, and mTOR which are known proteins that function in cell growth and proliferation to interrogate the roles of these proteins in IR-induced accelerated senescent phenotype. Blocking the activity of these proteins using AG1024 (IGF-1R inhibitor), Ly294002 (PI3K inhibitor) and rapamycin (mTOR inhibitor) attenuated the increase in SA-β-gal activity, inhibited p21/waf1 upregulation, and prevented morphological alterations in irradiated PAEC. We also found a modest increase in the phosphorylation of IGF-1R at early time-point post-irradiation and upon treatment with 10 μM $H_20_2$ suggesting that the early activation of IGF-1R is mediated by ROS, possibly through deactivation of IGF-1R phosphatases. The IGF-1R-mediated accelerated senescence also required intact mTOR but not necessarily PI3K/Akt activation as AG1024 treatment leads to decreased mTOR activity shown by decreased levels of pS6. Finally, we found that IGF-1R is not involved in modulating apoptosis in irradiated PAEC as shown by absence of modulation of caspase-3 activation in the presence of AG1024 and after increasing the concentration of IGF-1 in the cell culture. Our findings reveal mechanisms of IR-induced cell death in PAEC that may be helpful in designing more effective therapeutic strategies for limiting the effects of IR during the course of radiotherapy or after exposure to accidental irradiation.

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# CHAPTER 1: Dissertation Introduction

## IONIZING RADIATION-INDUCED CELL DEATH

Exposure to ionizing radiation (IR) can cause severe to lethal consequences. During the course of radiotherapy, the use of IR for the treatment of cancers is limited by radiation-induced cell death to the underlying normal tissue. Such damage can lead to inflammation and/or fibrotic remodeling. For example, radiation-induced pneumonitis and pulmonary remodeling are relatively frequent adverse effects in patients receiving IR for the treatment of thoracic cancers (129; 198). Although studies of IR-induced damage have been exponentially increasing, the mechanisms of IR-induced cell death in primary, untransformed cells are still not well understood. This contributes to the delay in the search for effective countermeasures for the prevention of lethal effects of IR from clinical radiotherapy or from accidental radiation exposures.

Several modes of cell death following application of cellular stress have been described. These include necrosis, apoptosis, and cellular senescence. Necrosis, a form of cell death often described as a passive process, is characterized by early plasma membrane rupture and typically results from a high magnitude of stress relative to apoptosis and cellular senescence-like cell death (67; 173). A number of studies have described induction of necrosis following exposure to IR both *in vitro* and *in vivo* (3; 41; 89; 123).

## APOPTOSIS

Apoptosis refers to the distinct biological mechanism induced by various stimuli that ultimately leads to programmed cell death (96). Often viewed as an active process of

cellular destruction that occurs under physiological (e.g. during embryogenesis and organogenesis) and pathological conditions (e.g. during exposure to various insults), apoptosis is characterized by chromatin condensation, DNA fragmentation, cell shrinkage, and formation of apoptotic bodies which is a consequence of separation of membrane-bound fragments containing cytosolic or nuclear contents (96; 206). The molecular mechanisms of radiation-induced apoptosis remain to be established. Multiple interacting pathways have been proposed based on independent studies mostly performed using immortalized cell lines. The active process of programmed cell death suggests that apoptosis is tightly regulated. Two distinct yet overlapping pathways of apoptosis have been proposed – the extrinsic and the intrinsic pathways. Which pathway is utilized by the cell ultimately depends on the nature and origin of the death signal (7; 159).

The extrinsic pathway is triggered by death stimuli originating outside the cell or in the plasma membrane. It is mediated by the death-inducing signaling complex (DISC), an oligomeric protein complex formed when specific death ligands bind to their corresponding transmembrane death receptors (7; 159). The known death receptors - CD95/Fas, TNF-R1, and TRAIL receptors- are members of the TNF superfamily that contain a cysteine-rich extracellular domain and an intracellular "death domain" (30). When death ligands bind to death receptors, the death domains interact with each other, leading to clustering of the receptors. This clustering leads to recruitment of other key players in the initiation phase of the extrinsic pathway such as the Fas-associated death domain (FADD) protein and the initiator caspase-8 which bind together through their corresponding death domains to form the death-inducing signaling complex (DISC) (7).

The intrinsic pathway, by contrast, results from signaling that initiates from inside the cell (e.g. IR-induced DNA damage). Unlike the DISC, the signaling platform of the intrinsic pathway is a non-canonical transmembrane signaling receptor known as the apoptosome (159). In this pathway, the cascade of events leading to cellular dismantling is initiated when cytochrome *c* is released from the mitochondria following mitochondrial outer membrane permeabilization (MOMP) (66; 159). Considered as the "point of no return" for apoptotic cell death, MOMP is induced by pro-apoptotic proteins belonging to the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins (94; 175). Once in the cytosol, cytochrome *c* interacts with Apaf-1 which triggers the latter's oligomerization. In the presence of dATP as the source of energy, Apaf-1, together with cytochrome *c*, forms the apoptosome (66; 159). The apoptosome then activates and recruits the initiator caspase-9 in an allosteric fashion (66). This signaling pathway is considered as the canonical intrinsic (mitochondrial) pathway of apoptosis (194). Recently, a novel non-mitochondrial intrinsic pathway leading to programmed cell death has been proposed. In this new model, caspase 9 may be activated through a mechanism that does not require the involvement of Apaf-1 and cytochrome *c* (59; 155) such as during caspase-12-induced caspase-9 activation in ER stress-mediated apoptosis (155).

### ACCELERATED SENESCENCE

While the significance of radiation-induced necrotic and apoptotic cell deaths have been extensively studied, that of radiation-induced accelerated senescence has not started to gain recognition until fairly recently. The concept of cellular senescence dates back to the works of Hayflick and Moorhead in the 1960's, that established that untransformed cells grown *in vitro* lose their capacity to replicate after a certain number

of passages. This finite number was termed the “Hayflick limit” and cells that had reached this state were viewed as senescent cells (78; 79). Today, cellular senescence is widely recognized as a state of cell cycle arrest characterized by altered cell morphology, aberrant expressions of cell cycle regulatory proteins (e.g. urpregulation of p53, p21/waf1, and p16INK4a), development of senescence-associated secretory phenotype (e.g. increased secretion of proinflammatory molecules), and increased senescence-associated β-galactosidase activity (139). Over the years, various cellular stresses including DNA damaging agents and oxidative stress have been demonstrated to cause this phenotype independent of their passage number or *in vitro* age (139) This phenomenon is now commonly referred to as stress-induced accelerated senescence.

Multiple pathways have been described for the induction of accelerated senescence. One of the best described signaling pathways is the p53/p21/waf1 pathway. The cyclin-dependent kinase inhibitor p21/waf1 is upregulated to induce cell cycle arrest, one of the salient features of the senescent state (139). Recent studies suggest that, in addition to cell cycle arrest signal, senescent cells also receive continuous proliferative signaling (6; 17; 18; 43). Central to this idea is the mammalian target of rapamycin (mTOR), a cytoplasmic kinase known to be an integration point of many signals for growth, proliferation, and homeostasis in the cell (108). Inhibition of mTOR signaling attenuates the increase in senescence-associated β-galactosidase activity in several models of cellular senescence (6; 17; 18; 43). The cell cycle arrest and the development of the other markers of senescence including the alteration in cellular morphology and the increase in senescence-associated β-galactosidase activity have been thought of as uncoupled responses (139).

## DNA DAMAGE SIGNALING

Multiple interacting signaling pathways are believed to be upregulated in response to DNA damage induced by lethal doses of IR (e.g. the SAPK/JNK, p53-dependent, and NFκB pathways) (201). Ataxia telangiectasia mutated protein (ATM) plays a major role in radiation-induced apoptosis by acting as a sensor of DNA damage upstream of p53 (191). Upon detecting DNA damage, ATM phosphorylates and stabilizes p53 (22; 119; 191). p53 is a tumor-suppressor protein involved in cellular response to DNA damage and has been referred to as the "guardian of the genome" because of this role (7; 30). The status of p53 can affect the cellular response to a certain stimulus and has been shown to contribute largely to the cell's decision to undergo apoptosis or not. For example, overexpression of wild-type p53 increased apoptosis in a p53-lacking murine leukemic cell line and human colon tumor-derived cell line (170; 204). In addition, thymocytes lacking p53 were shown to be more resistant to apoptosis-inducing agents that induce DNA double-strand breaks than those having wild-type p53 (31; 121). In the context of apoptotic signaling, activated p53 is thought to regulate the expression of Bcl-2 family members by neutralizing anti-apoptotic proteins (e.g. Bcl-2 and Bcl-$x_L$) and by activating pro-apoptotic proteins (e.g. Bax) at the same time which leads to activation of the initiator caspase-9 (119; 191). In a p53-independent manner, caspase-2, the only known caspase found in the nucleus, has also been proposed to be necessary for DNA damage response, having both initiator and effector roles (25; 212). Caspase-2 may be activated directly by DNA damage suggesting that its signaling leading to the mitochondrial pathway occurs via a p53-independent pathway (66).

## PROTEIN OXIDATION-MEDIATED ER STRESS RESPONSE

An accumulating body of evidence implicates protein oxidation as a trigger of endoplasmic reticulum (ER) stress-mediated apoptosis. Besides being a site of calcium sequestration, protein folding and maturation, the ER plays a significant role as a sensor of cellular homeostasis, triggering the unfolded protein response (UPR, also known as ER stress response and referred to as such throughout the thesis) in response to cellular stress such as the accumulation of oxidized proteins (137). Oxidized proteins tend to form protein aggregates which are far more difficult to eliminate (125; 134). In a report by Yakagawa *et al.* (178), the authors suggested that ER stress is likely to be caused by generation and accumulation of ROS-induced oxidized proteins in this organelle. Accumulation of oxidatively modified, abnormal proteins in the ER may also result from ROS-induced damage to specific ER resident proteins such as foldases and/or chaperones whose functional perturbation prevents them from properly folding the newly-synthesized proteins and refolding the abnormal proteins (25; 178). Independent studies have demonstrated this preferential oxidation of ER resident proteins (e.g. Grp78, calreticulin, Hsp70, Hsp90, disulfide isomerase, etc) by ROS and ROS-generating substances through the use of modern proteomic tools (53; 124; 165; 178; 186). When abnormally high levels of oxidized proteins reach a critical threshold that the ER cannot handle, caspase-mediated apoptosis may be induced (99).

The ER stress response can be divided into three phases – the initiation phase, the commitment phase and the execution phase. The initiation phase is mediated by signaling through the ER receptors: protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), transmembrane

receptors which are all associated with GRP78, an ER-resident chaperone whose dissociation from these receptors leads to activation of the UPR (177). PERK mediates the initial response by phosphorylating eIF2α that attenuates protein translation, a response that is better related to survival than apoptosis (137; 177). On the other hand, activated ATF6 induces the expression of XBP1 mRNA which is spliced by IRE1 to yield sXBP1 mRNA (205). The spliced form of XBP1 has a more potent transcriptional activity and its sustained activation has been shown to induce endothelial apoptosis (205; 209). The initiation phase is followed by the commitment phase which involves the activation of downstream pro-apoptotic molecules including C/EBP homologous protein (CHOP) (99; 177; 178). When the stress is too much to handle (e.g. persistent protein aggregation), the response results in the execution phase where different caspases are activated (177). Several groups have demonstrated a role for ER stress response in the activation of mitochondrial dependent and independent caspases (4; 91; 142; 143). Caspase-12, which is localized in ER, has been proposed as an initiator caspase of UPR that is activated by calpain in response to $Ca^{2+}$ release from ER lumen (28; 66; 137; 176). Caspase-12 in turn activates caspase-9 without the need for activating cytochrome *c* or Apaf-1 suggesting a non-mitochondrial pathway of ER stress-mediated apoptosis (155; 177). Caspase-12 is known to be expressed only in rodents and caspase-4 is thought to fulfill the role of caspase-12 in humans (177). The link between ER stress and senescence is not as well studied as the link between ER stress and apoptosis. ER stress has been shown to induce PERK-dependent induction of p53/47 mRNA translation, an isoform of p53, thereby causing arrest at the G2 phase (21; 182). In a study conducted by Minami et al (133), the authors showed that GADD34, a downstream target of CHOP, regulates

p21/waf1 expression and induces cellular senescence which then causes loss of proliferation in fibroblasts. This suggests another role for GADD34 and that ER stress response may lead to cellular senescence.

Direct evidence that IR causes ER stress-mediated apoptosis is lacking in the literature. However, ROS such as hydrogen peroxide (132) and superoxide (53) and certain cytotoxic drugs (e.g. doxorubicin and etoposide), cadmium, sodium selenite, and cigarette smoke (53; 70; 178; 203) that generate ROS have all been demonstrated to induce ER stress that leads to apoptosis. Radiation therapy has also been reported to result in the accumulation of misfolded proteins in the ER that triggers UPR (137). Recently, He *et al.* (80) demonstrated that IR elicits the induction of ER stress in fibroblasts post-irradiation via splicing of the XBP1 mRNA, a key mediator of ER stress.

### INTRODUCTION CONCLUSIONS

In this dissertation, we address the gaps of knowledge about IR-induced cell death in normal lung endothelial cells. We found that lung endothelial cells respond to varying doses of radiation with differential modes of cell death that involve multiple signaling mechanisms. The results presented herein may be useful in designing more effective therapeutic strategies for the prevention of adverse effects of exposure to radiation during the course of radiotherapy or accidental irradiation.

# CHAPTER 2: X Irradiation Induces ER Stress, Apoptosis, and Senescence in Pulmonary Artery Endothelial Cells

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**ABSTRACT**

Purpose: The use of clinical radiation for cancer treatment is limited by damage to underlying normal tissue including to the vascular endothelium. We investigated the mechanisms of X-ray-induced cell damage to endothelial cells. Methods: We evaluated necrosis, apoptosis, cellular senescence, and the contribution of endoplasmic reticulum (ER) stress in pulmonary artery endothelial cells (PAEC) irradiated with X-rays (2 – 50 Gray). Results: Clonogenic assays showed that 10 Gy induced ~99.9% loss of cell viability. No necrosis was detected using lactate dehydrogenase assays, but a low population underwent extrinsic and intrinsic apoptosis, as indicated by the activation of caspases 3, 8 and 9 as well as by neutral comet assay. A majority of PAEC underwent accelerated senescence, as indicated by morphological changes, increased 21 kD cyclin-dependent kinase inhibitor (p21/waf1), decreased sirtuin 1 (SIRT1), and elevated senescence-associated β-galactosidase (SA-β-gal). ER stress was detected by assays for glucose-regulated protein 78 (GRP78), CCAAT/enhancer-binding protein homologous protein (CHOP), and growth arrest and DNA damage-inducible protein 34 (GADD34) mRNA, and transient phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α). The ER stress inhibitor salubrinal blocked ~50% of apoptosis with no effect on senescence. Conclusions: X-rays primarily induced cellular senescence with limited levels of apoptosis in endothelial cells. ER stress contributed to apoptosis but not to senescence.

**INTRODUCTION**

Ionizing radiation (IR) is used as a principal therapeutic modality for cancer treatment (9; 105). Radiation in the thoracic region is usually applied in fractionated

doses (often ~2 Gray/fraction) to total doses of ~30-70 Gy for the treatment of primary lung and breast cancers as well as metastatic tumors (127; 150; 200). Recent studies indicate that single doses of high radiation may provide superior results in terms of cancer cell eradication in comparison to fractionated doses (32; 83). The use of three-dimensional conformal radiotherapy to improve target delivery and minimize normal tissue exposure allows an increase of the total dose to 78-90 Gy, but radiation is still fractionated (~2 Gy/fraction) (131). However, in patients with non-small cell lung cancer, higher fractionated doses (5-24 Gy) to reach total doses of over 110 Gy were shown to provide higher patient survival rates than lower fractionated doses with lower total doses (51). In patients with thoracic cancers, stereotactic single-dose radiation, using 20-30 Gy, has been shown to be effective for both primary lung cancers and lung metastases (62). Thus, higher single doses of radiation are becoming available with improved clinical techniques.

A major limitation to the use of high dose radiation is injury to the surrounding normal tissues. The use of radiation in thoracic cancer treatment is limited by lung damage, pneumonitis and/or irreversible fibrotic remodeling (35; 163). Although much research has focused on epithelial cell apoptosis in lung injury and fibrosis, there is substantial evidence for the importance of alterations to endothelial cell function in precipitating pulmonary injury following radiation exposure. Endothelial cell damage is an early effect of radiation (65; 95; 147; 207), resulting in vascular leak, edema, and increased inflammation (35; 69; 172; 207). It is postulated that vascular leakage to the extravascular space may play a precipitating role in the subsequent development of pneumonitis and fibrosis (69; 102; 109).

Radiation can kill cells through either necrotic or apoptotic cell death and can induce accelerated cellular senescence. Necrotic cell death is characterized by early plasma membrane rupture, dilatation of cytoplasmic organelles, and uncontrolled release of cytoplasmic contents (45; 67). In cell culture, necrosis can be detected by assays of the cellular medium for released enzymes, such as lactate dehydrogenase (LDH) (45). In contrast, apoptotic cell death, also referred to as programmed cell death, involves a series of regulated events leading to destruction of the cell. The events that occur during apoptotic cell death include: the rapid externalization of phophatidylserine; permeabilization of the outer mitochondrial membrane, which may be mediated by pro-apoptotic members of the anti- B-cell lymphoma 2 (Bcl-2) protein family, allowing the release of cytochrome *c*; the activation of caspases and formation of apoptosomes; and internucleosomal DNA cleavage (45; 199). Independent measures of apoptosis in cell culture include cytochrome c release in the medium, caspase activation by activity assays or western blotting for the activated, truncated forms of the caspases, or detection of DNA laddering, by neutral comet assay or DNA gel electrophoresis (199; 202).

Cellular senescence is a phenomenon by which cells lose the capacity to divide or replicate (139). Accelerated senescence can occur as a result of environmental stress or DNA damage (139). Senescence can be detected in cell culture by a variety of markers including: the increased expression of senescence-associated beta-galactosidase (SA-β-gal); cell cycle arrest and inhibition of proliferation; increased expression of cell growth regulatory proteins including cycle checkpoint proteins; downregulation of sirtuin 1 (SIRT1); and by altered expression of anti-apoptotic proteins such as anti-apoptotic members of the Bcl-2 family; and morphological changes such as increased cell size and

flattening (139).

Specific damage to biological molecules by radiation is believed to lead to cell death and/or senescence. Recent investigations suggest that in addition to DNA, proteins may be initial and important targets of ionizing radiation in both prokaryotic and eukaryotic systems (42; 50). In eukaryotic cells the endoplasmic reticulum (ER) acts as a mediator of signals in response to damaged proteins. Besides being a site of calcium sequestration, protein folding and maturation, the ER is a sensor of cellular homeostasis. An accumulating body of evidence implicates oxidized, damaged proteins as triggers of the ER stress pathway (also known as unfolded protein response) (98; 185). Studies involving reactive oxygen species (ROS, (132) and ROS-generating substances including cytotoxic drugs (*e.g.,* doxorubicin and etoposide) (53), cadmium (203), sodium selenite (70), and cigarette smoke (178) have shown induction of ER stress that leads to cell death. Radiation has also been shown to elicit the induction of ER stress in immortalized cell lines (80). However, direct evidence demonstrating that radiation causes ER stress-mediated cell death in primary endothelial cells is lacking in the literature. As IR generates ROS inside the cell, it is possible that ER stress can be elicited leading to cell death in irradiated cells.

The mechanisms of death and/or the induction of accelerated senescence induced by radiation depend on a number of factors including radiation dosage, the cell type, and the transformed status of the cell (93; 179). Many cancer cells, including lung, prostate, immortalized keratinocytes, and colon cancer cells, have been demonstrated to primarily undergo apoptosis in response to radiation from 1 to 20 Gy (74; 107; 153; 161). High radiation exposures (≥50 Gy) have been demonstrated to induce necrosis in leukemia

cells (190). Non-immortalized chondrocytes exposed to 10 Gy radiation undergo senescence (82). Keratinocytes and bone marrow-derived mesenchymal stem cells exposed to 20 Gy radiation exhibit growth arrest and senescence but no detectable levels of apoptosis (34; 153). In contrast, smooth muscle cells exposed to 50 Gy undergo both growth arrest and apoptosis (32).

Several studies have demonstrated the lethal effects of radiation on endothelial cells. Aortic endothelial cells and human umbilical vein endothelial cells have been shown to exhibit a senescence-like phenotype upon exposure to ionizing radiation (2-15 Gy; (87; 145). Using pulmonary artery endothelial cells (PAEC), Stitt-Fischer et al. (2010) suggested that there is an absence of accelerated senescence as well as negligible levels of apoptosis after 0.5-10 Gy X-ray exposure, although there was an observation of inceased levels of tumor protein 53 (p53) and the 21 kD cyclin-dependent kinase inhibitor p21/waf1, which are markers of apoptosis and senescence. Thus, the mode of cell death induced by radiation on pulmonary endothelial cells and the underlying mechanisms remain unclear.

Here we have investigated the effects of X-ray irradiation on the pulmonary endothelium using low passage cultures of untransformed, non-immortalized bovine PAEC, which have been extensively used as a model of endothelial injury (57; 84; 135; 164; 174; 193). We first hypothesized that ionizing radiation may induce multiple modes of loss of cell viability, possibly including necrosis or apoptosis, and/or accelerated senescence. Our data indicate that accelerated senescence is the primary response to X-ray irradiation from 2-50 Gy. Apoptosis occurs at higher doses of radiation (>10 Gy) but in a minority of the cell population. No necrosis was observed at any level of radiation.

Next, we investigated the contribution of ER stress in radiation-induced apoptosis and senescence at 50 Gy. This level of radiation was utilized to provide a maximal apoptotic response in the cell population. We found that ER stress response was triggered upstream of apoptosis but not upstream of cellular senescence in PAEC. Our findings suggest that cellular senescence triggered in response to clinically relevant doses of X radiation may be a driving force behind radiation-induced vascular leak and related pathology in the lung.

## MATERIALS AND METHODS

*Cell culture and irradiation.* Bovine PAEC were purchased from American Type Culture Collection (**Manassas, VA, USA).** Passage 3-8 cells were used for all experiments. PAEC were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 (Gibco Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gemini Bio-Products, Sacremento, CA, USA), 1% penicillin/streptomycin and 0.5% fungizone (Gibco Life Technologies). Cells were grown in 5% $CO_2$ at 37$^{o}$C in a humidified atmosphere cell culture incubator. Drugs and inhibitors for culture were: bleomycin (Hospira, Lake Forest, IL, USA); caspase 3-inhibitor benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (zDEVD; R&D Systems, Minneapolis, MN, USA) and salubrinal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Control cells were treated for the same time with the same amount of dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO, USA) used to prepare drug/inhibitor solutions. Unless otherwise specified, PAEC were either irradiated or sham-irradiated at subconfluence (70-90%). Irradiation was conducted using a RS2000 Biological Irradiator (Rad Source Technologies, Alpharetta, GA, USA) at a dose rate of 2.4 Gy/min (160kV, 25mA). Cells

were first placed in cold phosphate buffered saline (PBS; 2.68 mM KCl, 1.47 mM $KH_2PO_4$, 137 mM NaCl, 8.1 mM $Na_2HPO_4$; Gibco Life Technologies) on ice for transport to the irradiator facility (5 minutes). Treatment was performed at room temperature. After irradiation fresh regular medium was added and cells were incubated at $37^0$C.

*Clonogenic assay*. The clonogenic assay was performed based on a protocol for primary cells with modifications for PAEC (60). In comparison to transformed cell lines that proliferate easily, we found that PAEC were susceptible to poor cell growth and detachment if seeded at very low densities, and subsequent X-ray exposure at such densities may significantly affect the outcome of clonogenic assay. To resolve this issue, we performed delayed plating where PAEC were first cultured to 70-90% confluence, exposed to varying doses of X-rays, incubated, and seeded then for clonogenic assays. Irradiated or sham irradiated PAEC were incubated at $37^0$C, 5% $CO_2$ for 16-24 h. Cells were then trypsinized, counted using a haemocytometer, and plated on 60 mm dishes coated with 0.5% gelatin (modification to allow attachment of PAEC). The cells were incubated for 14 days. The medium was carefully replaced every 3-4 days throughout the incubation period; cells did not detach during the change in medium. At the end of the incubation period, the colonies were stained with crystal violet. Colonies were defined as groups consisting of $\geq$50 cells. Surviving fractions were calculated as a function of plating efficiency of non-irradiated controls. The plating efficiency (PE) was defined as the percentage of the number of colonies that formed for every cell that was seeded: PE = # of colonies/# of cells seeded × 100%). The surviving fraction (SF) was then expressed

as the number of colonies formed after IR for every cell that was seeded multiplied by the PE: SF = # of colonies/# of cells seeded × PE (60).

*LDH assay*. Necrotic death of irradiated and sham irradiated PAEC was determined using the LDH Cytotoxicity Detection Kit (Clontech, Mountain View, CA, USA). The medium from irradiated or sham irradiated cells was assayed according to the manufacturer's instructions at specific time points postirradiation. Cells did not receive any additional treatments. As a positive control, equal numbers of PAEC were treated with 1% Triton X-100 to provide 100% cell lysis. Cell death is expressed as the percentage of the LDH measured in the medium of the incubated cells divided by the total LDH released in the medium of cells treated with 1% Triton X-100.

*Neutral comet assay.* The neutral comet measures double-stranded DNA breaks that occur in late stage apoptotic cells. PAEC were irradiated or sham irradiation or treated with 4.5 mU/ml bleomycin for 16 h as a positive control. Cells were embedded in 1% low-melting point agarose (Sigma-Aldrich) and placed on a comet slide according to the manufacturer's protocol (Trevigen Inc., Gaithersburg, MD, USA). Slides were placed in lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris base, 1% Na-lauryl sarcosinate, 1% Triton X-100, pH 9.9) for 20 min, washed by immersion in Tris/Borate/EDTA (TBE) buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0; chemicals from Sigma-Aldrich). The nuclei were electrophoresed for 10 min, 6 mA in a horizontal mini-electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA) with 1×TBE buffer. Cells were fixed with 70% ethanol for 10 min

and air-dried overnight. Cells were stained with 1× Sybr® Green (Molecular Probes, Eugene, OR, USA) and visualized with an Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA, USA) using 10× magnification at 478 nm excitation and 507 nm emission wavelengths. Approximately 100 cells were randomly selected and microscopically scored according to tail length. Comets were defined as apoptotic cells as previously described (104).

*Caspase activity assays.* Caspase activation occurs during early apoptotic signaling. Caspase-3/7, 8, and 9 activity assays were performed on irradiated and sham irradiated PAEC using Caspase-Glo$^{TM}$ Assays (Promega, Madison, WI, USA). At the indicated times postirradiation, PAEC were washed with cold PBS, lysed in PBS containing 1% NP-40 and 0.1% sodium dodecyl sulfate (SDS; Sigma-Aldrich). Lysates were transferred to microcentrifuge tubes, vortexed for 5 sec, incubated on ice for 20 min, and then centrifuged at 14,000 x *g* at 4$^{o}$C for 15 min. Ten μg of 50 μl lysis buffer was mixed with 50 μl of equilibrated Caspase-Glo$^{TM}$ reagent (5). After 1 h incubation at room temperature, luminescent signals were measured using a Dynex MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA, USA).

*Cell proliferation assay.* PAEC were seeded at 2 x $10^5$ in 35 mm dishes and incubated for 24 h. After incubation, cells were at about 70-90% confluence. Irradiated or sham irradiated cells were counted manually using haemocytometer at indicated postirradiation time-points.

*SA-β-gal activity assay.* As an indicator of cellular senescence, we performed the SA-β-gal assay according to established protocols with minor modifications (10; 46). To avoid density-dependent false positive expression of SA-β-gal, PAEC were seeded at 0.8-1.0 × $10^4$ cells per 35 mm dish containing coverslips (about 50-60% confluence) and incubated overnight prior to irradiation or sham irradiation. At indicated time-points postirradiation, cells were washed twice in ice cold PBS, fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min at room temperature. Cells were washed again twice in ice-cold PBS. 2 ml of freshly prepared X-gal staining solution (1 mg/ml of 5-bromo-4-chloro-3-indolyl P3-D-galactoside in 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 50 mM NaCl, 2 mM $MgCl_2$; all chemicals from Sigma-Aldrich) was then added to the culture dishes and incubated for 12-20 h at 37$^o$C (without $CO_2$). Subsequently, cells were washed twice in ice-cold PBS, once in methanol and allowed to dry. Coverslips were mounted on microscope slides for analysis. Entire cell population in 3 random microscopic fields (at least 100 cells) were analyzed for perinuclear blue staining indicative of SA-β-gal activity.

*Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR).* Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Genomic DNA was removed using the RNase-Free DNAse Set (Qiagen). RNA was quantified spectroscopically (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE, USA). RNA (1.0 μg) was subjected to reverse transcription with GeneAmp® RNA PCR kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Complementary DNA (cDNA) was diluted 10-fold with water. Diluted cDNA (2

μl) was used in a 20 μl qPCR reaction. PCR was performed in triplicates using 6 μM of each primer and 10 μl of SybrGreen® PCR master mix (Applied Biosystems). Primers for qRT-PCR were designed using NCBI /Primer-BLAST design tool. Primers were purchased from Integrated DNA Technologies (Coralville, IA, USA): glucose-regulated protein 78 (GRP78) forward 5'-GCC GAC GGC GCT GGA AAG AT-3', reverse 5'-GCC GAT GAG TCG CTT GGC GT-3'; CCAAT/enhancer-binding protein homologous protein (CHOP) forward 5'-GGT GGC AGC GAC AGA GCC AA-3' reverse 5'-CAT GCG TTG CTT GCC AGC CC-3'; growth arrest and DNA damage-inducible protein 34 (GADD34) forward 5'-AGC GGC TGA GGA GGA CCG AG-3' reverse 5'-CGG GCG GCA CAC CCT AAC AG-3'. As an internal control, mRNA levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were determined using primers: forward 5'-GAA GCT CGT CAT CAA TGG AAA-3' and reverse 5'-CCA CTT GAT GTT GGC AGG AT-3'. For quantification, the comparative threshold cycle (Ct) method was used to assess relative changes in mRNA levels between the untreated (control) and the irradiated samples.

*Western blotting.* Whole cell lysates from PAEC were prepared in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitors (Sigma-Aldrich) and 1mM phenylmethylsulfonyl fluoride (PMSF), 2mM $Na_3VO_4$ or Halt phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA). Samples were vortexed and incubated for 15-20 min at 4$^{o}$C. DNA was sheared using an ultra sonicator (Heat Systems-Ultrasonics Inc., Plainview, NY,

USA) for 10 sec at 4°C. Samples were centrifuged at 14,000 x *g* for 15 min and supernatant was collected.

Protein concentrations from whole cell extracts were determined using the bicinchoninic acid (BCA)® Protein Assay Kit (Thermo Scientific). Equal amounts of proteins were separated in SDS-PAGE and transferred to nitrocellulose membrane. Blots were blocked in either 5% milk in Tris buffered saline with Tween-20 (TBST; 20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) or 5% bovine serum albumin (BSA) in TBST (chemicals from Sigma-Aldrich). Antibodies used were: anti-active caspase-3, anti-caspase 3, and Bcl-2 (Cell Signaling Technology, Danvers, MA, USA); anti-phosphorylated-eukaryotic translation initiation factor 2 alpha (eIF2α; Millipore, Billerica, MA, USA, and Santa Cruz Biotechnology); anti-eIF2α, anti-p21/waf1, and anti-β-actin (Santa Cruz Biotechnology); anti-SIRT1 (Abcam, Cambridge, MA, USA). Proteins were detected with horseradish peroxidase-linked secondary antibodies and ***SuperSignal*** West Pico Chemiluminescent Substrate (Thermo Scientific/ Pierce Biotechnology, Inc., Rockford, IL, USA). Wright Cell Imaging Facility ImageJ software (http://www.uhnresearch.ca/facilities/wcif/ index.htm) was used for densitometry analysis.

*PCR for the detection of X-box binding protein 1 (XBP1) splicing.* Total RNA was isolated and quantified as described above RNA (500 ng) was subjected to reverse transcription with GeneAmp® RNA PCR kit according to the manufacturer's protocol (Applied Biosystems). Complementary DNA was diluted 10-fold with water, and then 5 µl of cDNA was used for 20 µl PCR reaction. PCR products were amplified with

annealing temperature at $53^0$C for 40 cycles and separated by electrophoresis on a 2.5% agarose gel. Primers for XBP1 were: forward 5'- CCT TGT AGT TGA GAA TCA GG-3', and reverse 5'-GGG GCT TTG TAT ATA CGT GA-3'. Tunicamycin (10 μg/ml) to a positive control induce ER stress was purchased from Sigma-Aldrich.

*Statistics.* Means ± standard deviations (SD) or error of the mean (SEM) were calculated, and statistically significant differences between two groups were determined by the Student's *t* test; $p < 0.05$ was considered statistically significant.

## RESULTS

### Clonogenic Survival Assay of PAEC

The clonogenic assay determines cellular replicative ability and measures all forms of cell death as well as cellular senescence (162). The dose required to reduce the surviving fraction by 63% ($D_0$) (188) was 1.2 Gy for PAEC exposed to X-rays (Figure 1). After exposure to 8 Gy ~99.9% of PAEC lost proliferative capacity (Figure 1) in close agreement with findings from a previous study (174). After exposure to 50 Gy no colonies were formed (data not shown), but individual cells that did not form colonies were visible with crystal violet staining. This suggested that 50 Gy inhibited cellular proliferation but did not abolish cellular attachment and survival.

### X-rays induce apoptosis but not necrosis in PAEC

We next investigated whether different modes of cell death may account for the loss of replicative ability identified in the clonogenic assay. We investigated the full range of radiation from 2-50 Gy (Figure 2). Release of LDH is a defining feature of cells

undergoing necrosis, including secondary necrosis in late-stage apoptotic cells. At 24 and 72 h postirradiation no significant release of LDH was detected, suggesting that X-irradiation from 2-50 Gy does not cause necrosis in PAEC.

The neutral comet assay detects internucleosomal DNA fragmentation that occurs in late apoptosis. Bleomycin, which we previously determined induces ~100% apoptosis in PAEC, was used as a positive control (140). No significant apoptosis was observed after exposure to 4 Gy (Figure 3A). Exposures of 10-30 Gy caused an 8-18% increase in apoptosis compared to sham-irradiated controls at 24-48 h postirradiation. 50 Gy X-rays also resulted in ~15-28% increase in apoptosis compared to sham irradiation at 24-48 h postirradiation. We next investigated the time course of caspase-3 activation in irradiated PAEC, a biochemical hallmark of apoptotic signaling. Caspase-3 activity, measured by the Caspase-Glo$^{TM}$ Assay, was maximally induced ~5-fold, from 0.66 ± 0.04 to 3.31 ± 0.5 ($p<0.05$) at 6 h and ~3.4-fold, from 0.72 ± 0.08 to 2.49 ± 0.34 ($p<0.05$) at 12 h postirradiation (Figure 3B). Immunoblot analysis of lysates from irradiated PAEC detected the presence of the cleaved, active form of caspase-3 at 6 and 12 h postirradiation (Figure 3C), coinciding with the increase in caspase-3 activity. To confirm that the DNA fragmentation observed in the neutral comet assay was indeed due to apoptotic signaling, we treated cells with the caspase-3 inhibitor zDEVD-FMK (Figure 3D). zDEVD-FMK contains the peptide DEVD that is recognized by caspase-3 and a FMK (fluoromethylketone) moiety that binds covalently to caspase-3 active site (14; 169) . It has been demonstrated that zDEVD-FMK has cross-reactivity towards other non-target caspases (14) but for the purpose of investigating whether apoptosis occurs in irradiated PAEC, the use of zDEVD-FMK is sufficient to address the question. zDEVD

treatment resulted in ~60% inhibition, from 20.6 ± 4.84 to 13.6 ± 2.11 $p<0.05$, of radiation-induced apoptosis as determined by the neutral comet assay. This confirmed that the observed DNA fragmentation occurred as the result of apoptotic signaling in response to X-irradiation in PAEC.

To determine whether radiation-induced apoptosis activates the extrinsic (i.e., death receptor mediated) or intrinsic (i.e., mitochondrial) apoptotic pathways, we measured the activities of initiator caspases 8 and 9, respectively. Activation of either of these caspases leads to caspase-3 cleavage. The activity of either caspase 8 or 9 was maximal (~2.5-fold increase each compared with control) at 6 h postirradiation and gradually declined in a similar manner to caspase-3 (Figure 4A and B). Taken together, these results indicate both extrinsic and intrinsic apoptotic pathways contribute to death of a fraction of PAEC exposed to high-dose X-irradiation.

**X-rays induce accelerated senescence in the majority of PAEC population**

Our data indicated that there was no significant necrosis, and that apoptosis occurred only in a limited percentage of PAEC irradiated with >10 Gy. However, the clonogenic assay indicated that at 8 Gy, over 99.9% of cells were not competent to form colonies. This discrepancy suggested the possibility of accelerated senescence in PAEC following X-ray exposure. A proliferation assay showed that irradiated PAEC do not proliferate compared to non-irradiated controls (Figure 5A). PAEC exposed to 50 Gy of X-rays produced blue staining surrounding the nucleus, indicative of SA-β-gal activity, the most widely used assay for cellular senescence (Figure 5B). Dimri et. al. (1995) first described the assay which detects SA-β−gal activity at a suboptimal pH of 6 in senescent human cell cultures

and in aging skin cells in vivo (46). SA-β-gal has since been considered as a biomarker of senescent cells that reflects an increase in lysosomal mass during replicative aging (106). SA-β-gal staining was observed in the majority of cells at 72 h postirradiation (control 8.2% ± 2.02, 50 Gy 75.2% ± 11.45, $p<0.05$) and at 120 h postirradiation (control 13.2% ± 4.21, 50 Gy 86.9% ± 5.89, $p<0.05$) (Figure 5B). Within 24 h postirradiation, the irradiated PAEC underwent morphological changes including increased cell size and flattened cytoplasmic appearance that are characteristic of senescent cells (Figure 5C).

Previous studies showed that radiation-induced accumulation of cells in $G_2$/M phase is associated with increased levels of expression of p21/waf1 (211), and that p21/waf1 accumulation is often observed in senescent cells (139). We found that irradiation of PAEC increased expression of p21/waf1 within 6 h, sustained for 48 h (Figure 5D). Recent findings indicated that loss of expression of the $NAD^+$-dependent protein deacetylase SIRTs is also associated with cellular senescence (139). We found an almost complete loss of SIRT1 expression in PAEC within 72 h postirradiation (Figure 5E), providing further evidence of senescence in PAEC. The predicted molecular weight of *Bos taurus* SIRT1 is about 80 kda. In our western blot, however, we detected a single band migrating at around ~60 kda which could be a splicing variant of the *Bos taurus* SIRT1. At the time of the dissertation, no known splicing variant has been reported in Pubmed database for *Bos taurus*, possibly due to lack of research on the *Bos taurus* SIRT1 gene, however, splicing variants for Homo sapiens and *Mus musculus* were recorded The *Homo sapiens* spliced variant is predicted to have a molecular weight of ~50 kda while that of *Mus musculus* is predicted to have a molecular weight of ~64 kda (Pubmed).

Senescent cells have been demonstrated to express higher levels of Bcl-2 (192), which correlates with their altered response to apoptotic signaling (139). Western blots of Bcl-2 protein showed a slight (not significant) decrease of Bcl-2 levels at ~3 h postirradiation, which later increased ~2-fold at 24 h postirradiation, correlating with the early onset of senescence (Figure 5F). These data suggest the onset of senescence by 6 h postirradiation.

**X-rays promote ER stress leading to apoptosis in PAEC**

Although DNA damage is considered a primary target of radiation, protein oxidation has recently been shown to be an important regulator of radiation-induced cell death (42). A critical increase in the level of unfolded proteins is detected by the ER, and the resulting ER stress can lead to apoptosis or accelerated senescence (132; 210). Protein unfolding can activate any of three ER-localized sensors: PERK; inositol-requiring enzyme 1 (IRE1); and activating transcription factor 6 (ATF6) (185). PERK phosphorylates eIF2α, leading to the inhibition of protein synthesis to reduce traffic of newly-synthesized, unfolded polypeptides to the ER (23; 76). ATF6 increases the expression of target genes including GRP78 and XBP1. GRP78 is an ER chaperone that aids in the refolding of abnormal proteins (13). XBP1 is a transcription factor that regulates a number of ER chaperones, transcription factors, ER associated protein degradation components, and proteins involved in the secretory pathway (71). Both eIF2a and ATF6 up-regulate CHOP (24; 185). IRE1 is a Ser/Thr protein kinase and endoribonuclease that initiates unconventional splicing of XBP1 mRNA to induce its active form. In a later stage of ER stress, CHOP targets expression of the GADD34 (185).

We examined signal transduction pathways activated by the three primary ER stress sensors, PERK, ATF6, and IRE1. Downstream of ATF6 activation GRP78 mRNA was increased 1.8-fold (± 0.03) at 24 h postirradiation (Figure 6A). At 24 h postirradiation we detected similar increases in gene expression for CHOP and GADD34 (1.6-fold and 2.6-fold, respectively) (Figure 6A). We next investigated eIF2α phosphorylation. Increased phosphorylation of eIF2α was transiently detected between 3 h and 6 h postirradiation (Figure 6B). We also observed the appearance of a lower molecular weight fragment of eIF2α at the 6 h and 12 h time points. The appearance of this fragment is consistent with findings from previous studies that demonstrated that eIF2α and phosphorylated eIF2α can be cleaved following the activation of caspase 3 or 8 in apoptotic cells (122; 128). To detect IRE1 activation we investigated XBP1 mRNA splicing. We did not observe splicing of XBP1 at any time points from 0 to 48 h following 50 Gy X-ray exposure (Figure 6C). As a positive control, we used tunicamycin (TM) which induced XBP1 splicing at 6 h post-treatment.

We next investigated whether ER stress signaling occurs upstream of radiation-induced apoptosis and/or accelerated senescence. PAEC were treated with salubrinal, a selective inhibitor of eIF2α dephosphorylation that was shown to disrupt the GADD34-PP1 phosphatase complex (22; 168). In the presence of salubrinal, radiation-induced apoptosis was reduced by ~50% (50 Gy 26.5 ± 2.45, 50 Gy/Sal 15.8 ± 2.23, $p<0.05$) as shown by the neutral comet assay (Figure 7A). Treatment with salubrinal also reduced the level of radiation-induced activated caspase-3 (Figure 7B). However, salubrinal did not block radiation-induced accelerated senescence (Figure 7C). Together, these results

suggest that ER stress response is activated by X-irradiation in primary PAEC and mediates a portion of X-ray-induced apoptosis.

## DISCUSSION

Clinical radiation is an important cancer therapy, but the use of this technique is limited by damage to underlying normal tissue. We investigated the effects of X-ray irradiation on primary lung endothelial cells using a combination of dose-response and time-course assays utilized to provide a comprehensive evaluation of the fate of irradiated normal lung cells. Interestingly, the loss of cell viability in PAEC appears to be independent of significant necrosis, even at very high doses of radiation. Our data instead suggest that accelerated cellular senescence is the primary response of PAEC. Our data also indicate that a limited proportion of irradiated PAEC underwent apoptosis, even after exposures as high as 50 Gy.

In order to counteract the effects of radiation on normal tissues, it is critical to understand the type of cell death induced by radiotoxicity, particularly during radiotherapy. Stress-induced cytotoxicity induces a spectrum of cell death programs that are triggered by a cascade of molecular responses. Although the mechanisms that underlie the biological selection for a specific mode of cell death remain unclear, it seems likely that the fate depends on the intensity of the stress. A recent study of chemotherapy-induced cytotoxicity of cisplatin showed that low concentrations of the drug cause mainly apoptosis while higher concentrations induce necrosis (167). Findings from another study of doxorubicin cytotoxicity revealed that low doses of the drug cause mainly a senescence-like phenotype while high doses trigger apoptosis (54). Similar to chemotherapeutic agent, radiation causes several modes of cell death (162).

Our study revealed that accelerated cellular senescence is the primary cellular fate of PAEC following exposure to a wide range of doses of X-ray irradiation. In a recent comprehensive review of cellular senescence the following criteria are given as markers of senescence: 1) cell cycle arrest/cell growth arrest; 2) altered gene expression of cell growth-regulatory proteins such as p21/waf-1; 3) morphologic transformations; 4) increased SA-β-gal activity; and 5) altered response to apoptotic stimuli through altered expression of anti-apoptotic proteins (139). We demonstrated that irradiated PAEC exhibited all of these markers for cellular senescence. We also observed decreased SIRT1 expression, which is widely accepted to be involved in a signaling pathway leading to cellular senescence (139). In contrast with our findings, a recent study conducted by Stitt-Fischer *et al.* (174) on primary PAEC exposed to 10 Gy X-rays reported the absence of significant apoptosis or cellular senescence postirradiation. We hypothesize that the differences between the findings of our present study and those of Stitt-Fischer *et al.* are due to differences in the time courses examined as well as in some assay conditions. For example, we observed caspase-3 activation by both caspase-activity assay and western blot analysis at early time-points (6 h and 12 h postirradiation); these time points were not included in the study of Stitt-Fischer. Similarly, the occurrence of SA-β-gal phenotype was demonstrated at 72 h and 120 h postirradiation in our system but not at 24 h, which was the only time point used in the Stitt-Fischer study. In addition, the apparent decrease in SA-β-gal activity in the sham-irradiated controls relative to irradiated PAEC in the Stitt-Fischer study was not observed in our system and may be due to density-induced false positive SA-β-gal staining (46) and which we were able to avoid by seeding at optimized densities.

We detected limited amounts of apoptosis in PAEC in response to higher levels of radiation exposure (>10 Gy). Our data indicate that radiation activates the intrinsic as well as the extrinsic apoptotic pathways in PAEC. Although signal transduction leading from DNA damage is primarily believed to activate the intrinsic pathway (63), radiation has also been demonstrated to activate the extrinsic apoptotic pathway through the activation of p53 and Fas (52). Our laboratory recently demonstrated that bleomycin, a chemotherapeutic agent that causes ROS-induced DNA strand breaks and is sometimes utilized as a radiomimetic in signal transduction studies (171), activated the extrinsic pathway of apoptosis in PAEC by inducing the expression of tumor necrosis factor (TNF) and members of the TNF receptor family of proteins (140). We suspect that radiation-induced activation of the extrinsic apoptosis pathway may follow a similar mechanism.

A widely held belief concerning radiotoxicity is that the most critical biological effects of radiation are the result of ROS-induced DNA damage (72; 86). Recent studies have challenged this classical view by demonstrating that proteins may be initial and important targets of radiation in both prokaryotic and eukaryotic systems (42; 50). The activation of both extrinsic and intrinsic apoptotic pathways in our system suggests that DNA damage may not be the sole signal responsible for radiation-induced apoptosis in normal lung cells. Our data provide evidence that X-rays activate two ER stress pathways in primary lung cells. Although the initial ER stress response is transient and cytoprotective (23; 76), prolonged activation of the ER stress pathway causes cell death (2; 23). Our results indicate that the eIF2α-dependent arm of ER stress is upstream of a significant proportion of X-ray-induced apoptosis, but not X-ray-induced accelerated senescence.

Endothelial cell damage is an early event following clinical radiation that induces vascular leakage (35; 69; 172; 207). It is believed that vascular leakage allowing increased inflammatory processes is a key event preceding pneumonitis and the development of fibrotic remodeling (102; 109). Several stages of vascular leak have been observed in animal models of thoracic radiation injury. Early increases in capillary permeability (minutes to several hours after radiation exposure) are believed to be caused by the activation of proteolytic enzymes (92). A later increase in vascular permeability (days after radiation exposure) cannot be inhibited by protease inhibitors, and are believed to be due to another mechanism (109; 138). While some of the early changes in vascular permeability are likely related to the effects of radiation on the endothelial cytoskeleton (95; 207), it is possible that the delayed effects are also due to cellular survival. A delayed increase in vascular permeability was demonstrated starting at 7 days post-irradiation of mouse right hemithorax (109) which correlates with increased apoptosis and cellular senescence detected in our lung radiation model of pulmonary fibrosis (maximal at 7 days post-irradiation). Increased understanding of the mechanisms leading to radiation-induced lung cell death or senescence may be of use for mitigating IR-induced damage to normal lung tissue during the treatment of thoracic malignancies or accidental irradiation.

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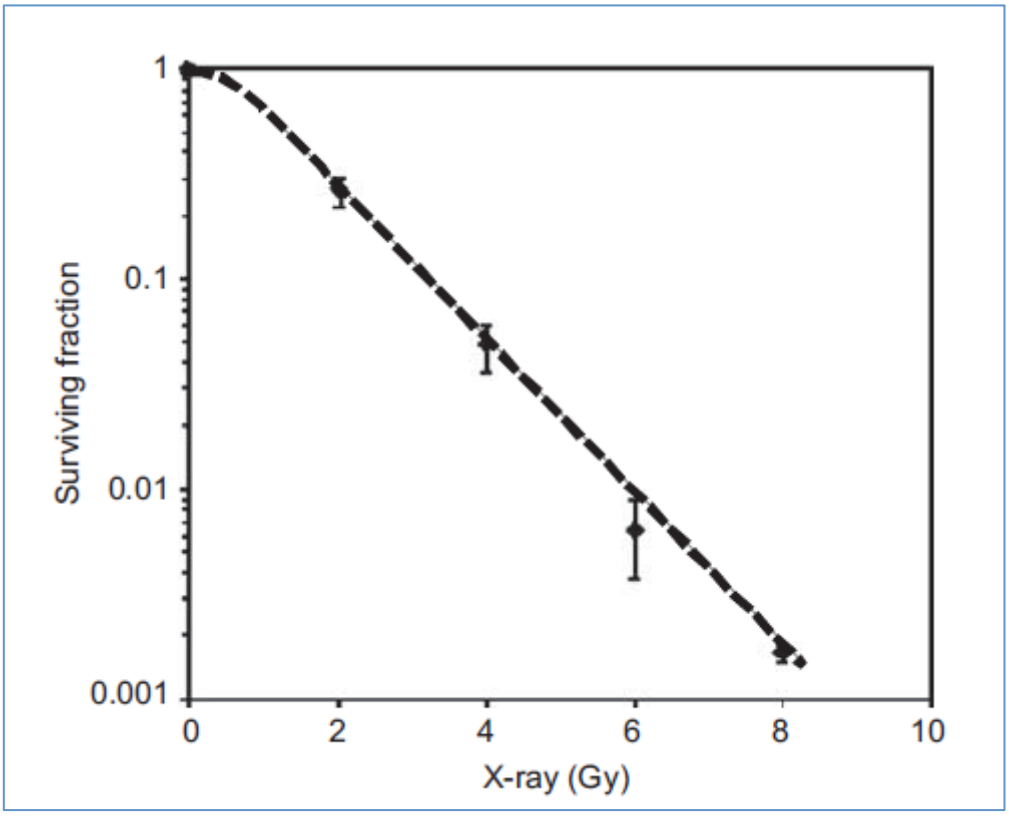
**Declaration of interests:**

The authors have no conflicts of interests to report.

## LIST OF FIGURES

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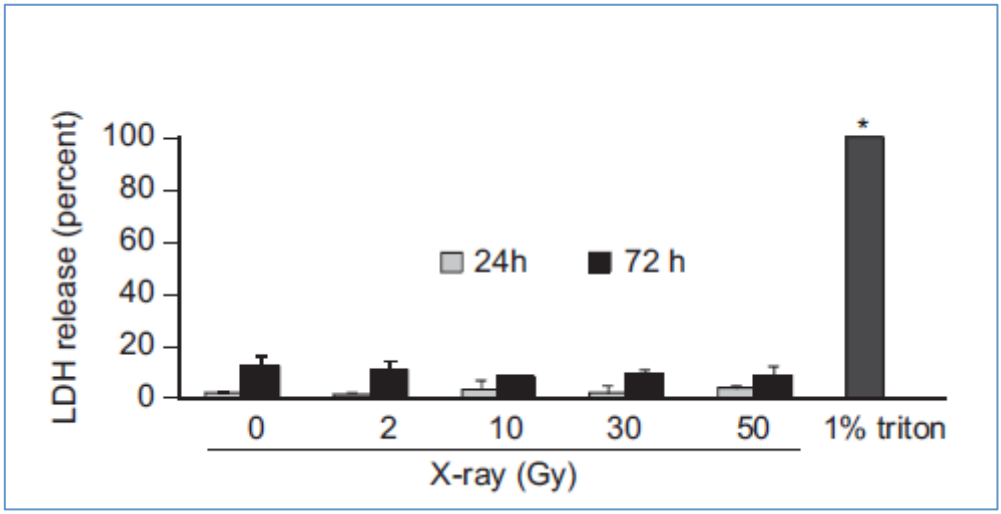


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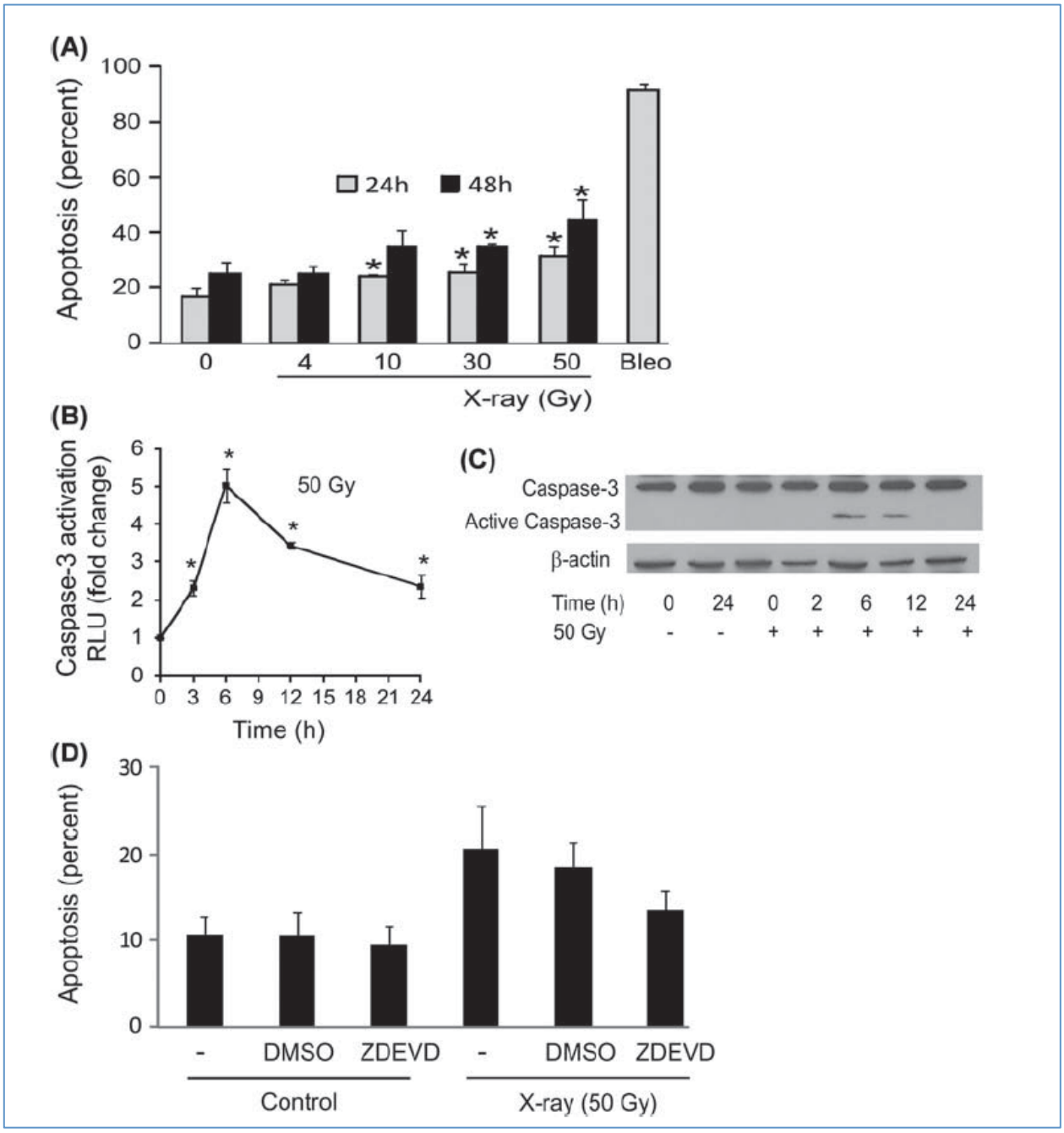


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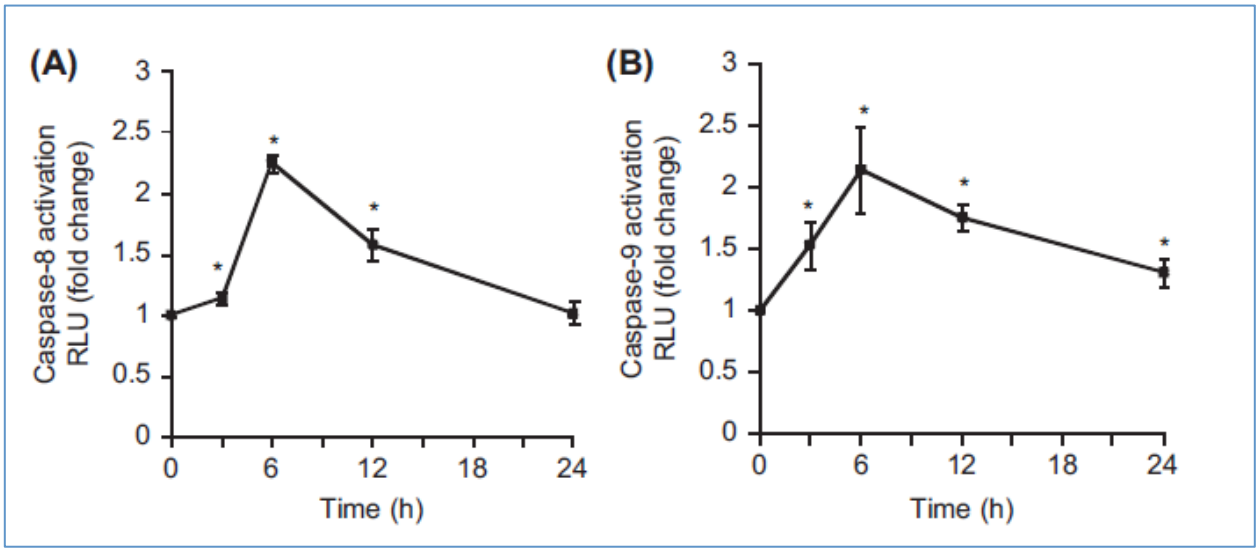


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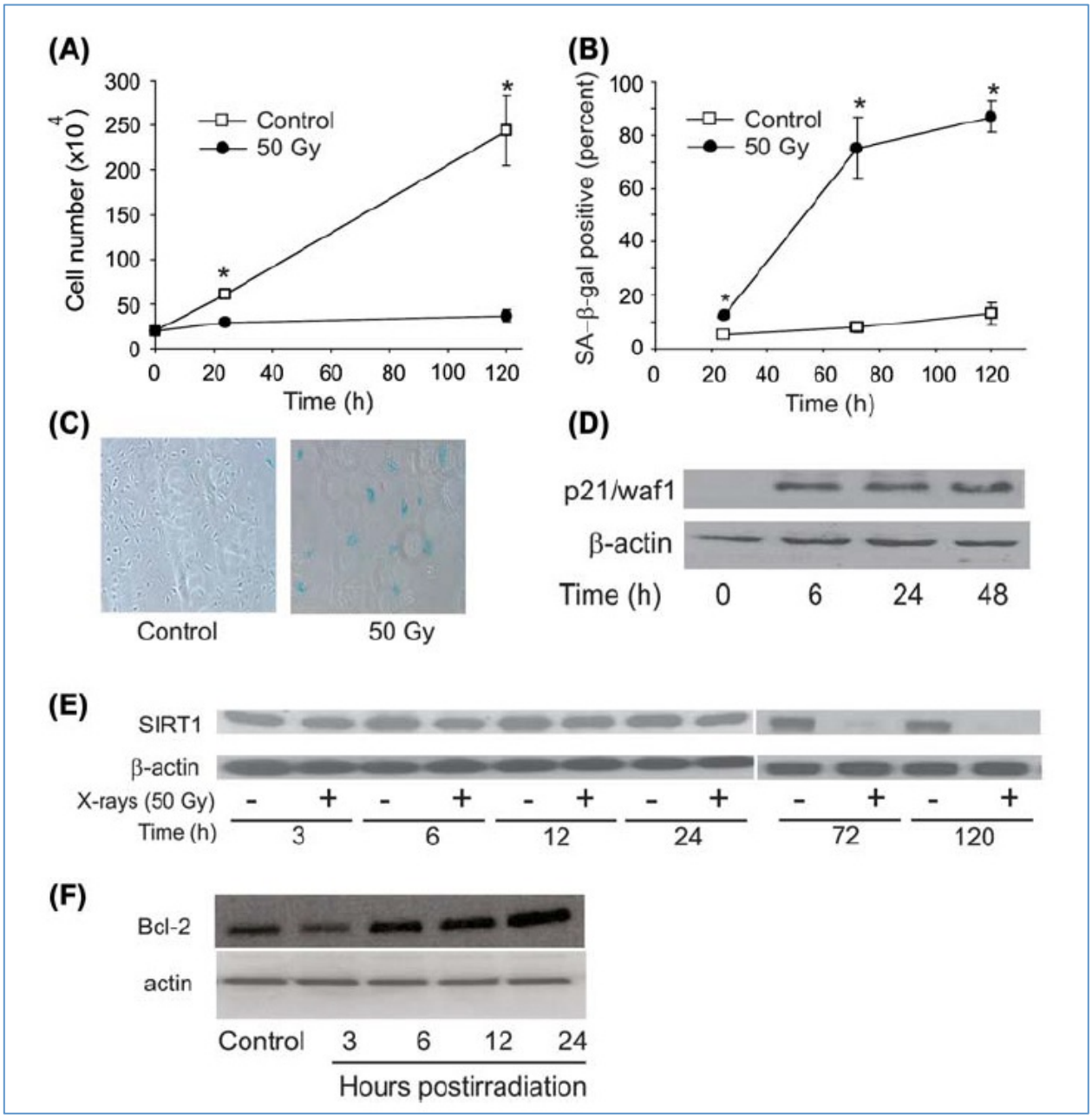


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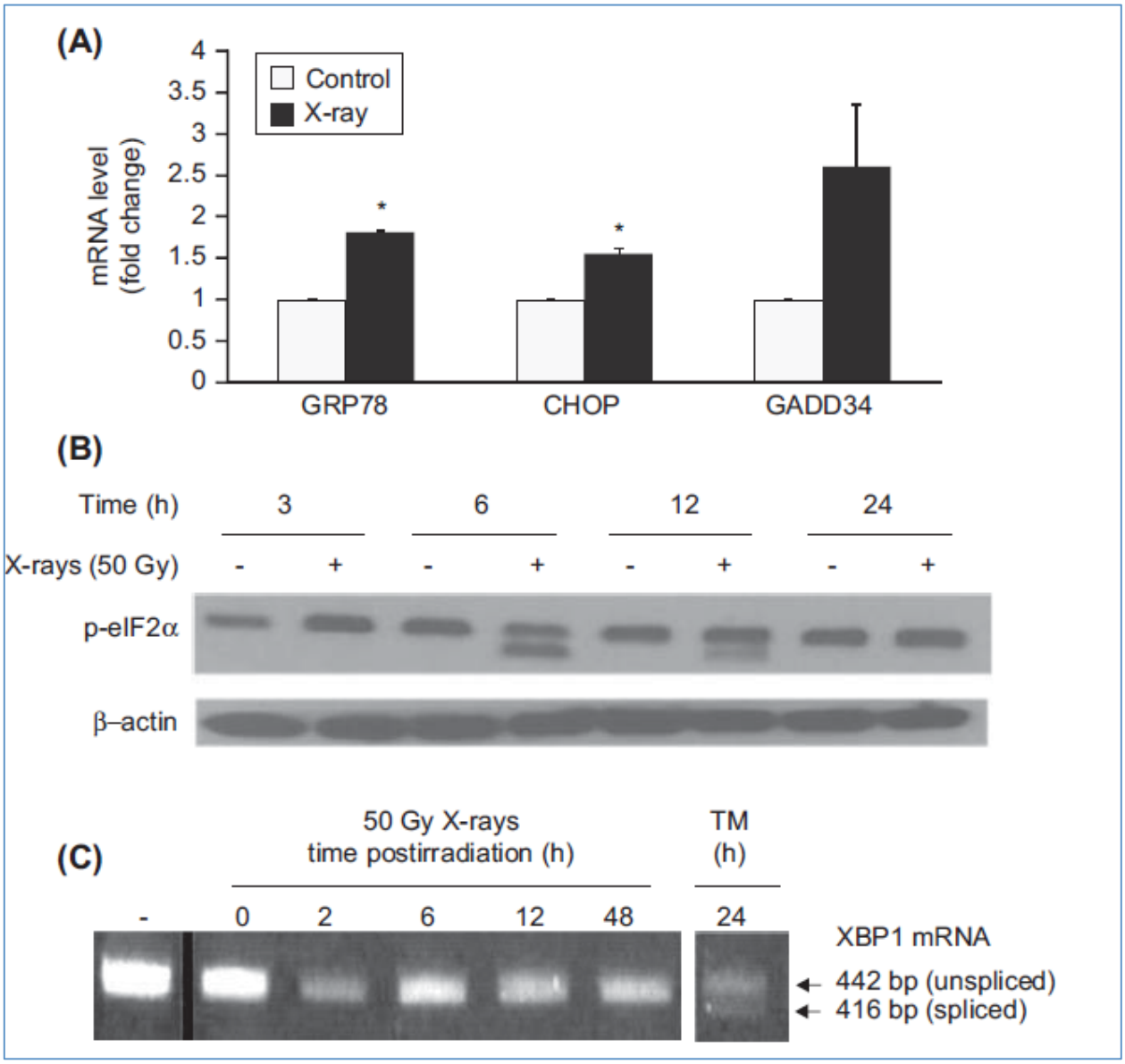


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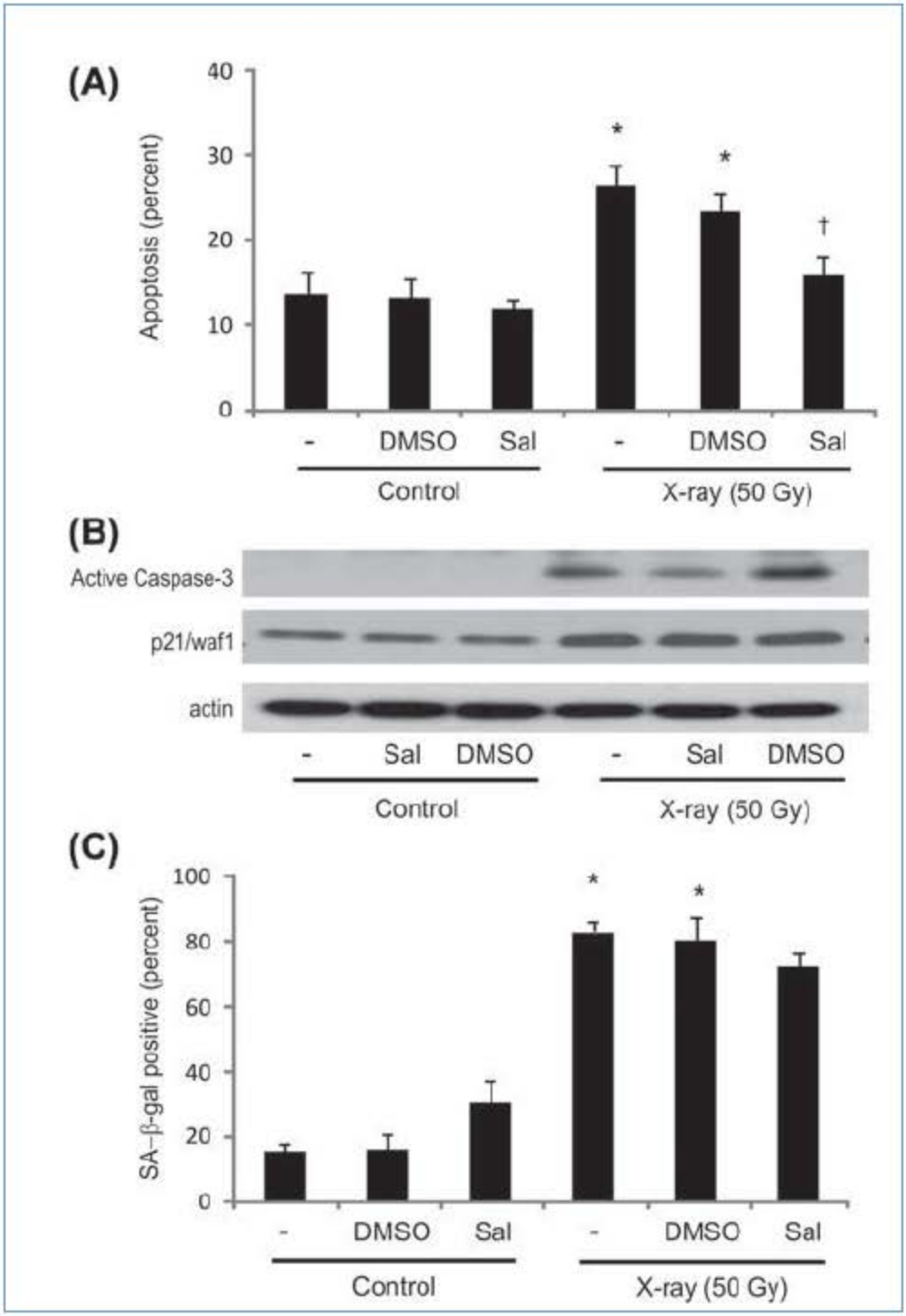


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**Figure Legends**

**Figure 1.** ***Clonogenic assay of irradiated PAEC.*** PAEC were sham irradiated (0) or X-ray irradiated at the indicated doses for clonogenic assays. Graph indicates mean ± SEM, number of samples (n) = 4.

**Figure 2.** ***X-rays do not induce necrosis on PAEC.*** PAEC were either sham irradiated or X-ray irradiated at the indicated doses. Following incubation at indicated times postirradiation, the level of necrosis was detected by measuring LDH released into the medium. Cell death is expressed as the percentage of the LDH measured in the medium divided by the total LDH release after treatment with 1% Triton X-100. Bars indicate mean ± SD with n = 3. * indicates statistical significance from control, $p < 0.05$.

**Figure 3.** ***X-rays induce caspase-3-dependent apoptosis in PAEC***. PAEC were either sham irradiated or irradiated with X-rays at indicated doses. A. Neutral comet assays were performed at 24 h and 48 h postirradiation. Bleomycin (bleo, 5 mU/ml) was used as positive control. B. Caspase-3 activity assay using Caspase-Glo® 3 substrate at the indicated time points. Graph indicates means ± SD, n = 4. * indicates statistical significance from control, $p < 0.05$. C. Equal protein amounts of whole cell lysates were immunoblotted for the cleaved form of caspase-3. β-actin was used as loading control. Representative results are shown. D. Caspase-3 inhibitor ZDVED (15 μM, in DMSO) was added to PAEC 1 h prior to X-ray exposure; a DMSO treated control was included. Neutral comet assays were performed 24 h postirradiation. Graph indicates means ± SD,

n = 3. * indicates statistical significance from control, $p < 0.05$. All experiments were repeated at least 3 times; representative data are shown.

**Figure 4.** ***X-rays activate the extrinsic and intrinsic apoptotic pathways in PAEC***. PAEC were either sham irradiated or irradiated with 50 Gy X-rays. Cell lysates were prepared at indicated times postirradiation. Caspase-8 (A) and -9 (B) enzymatic activity assays were performed using Caspase-Glo® 8 and 9 substrate, respectively. Graph represents means ± SD, n = 4. * indicates statistical significance from control, $p < 0.05$.

**Figure 5.** ***X-rays induce accelerated senescence in PAEC.*** PAEC were either sham irradiated (control) or irradiated with 50 Gy X-rays under identical conditions. A, Cell proliferation assay. PAEC were seeded at $2 \times 10^5$, cultured for 24 h prior to irradiation. Cells were counted at indicated time points using a haemocytometer. Graphs indicate means ± SD, n=3. * indicates statistical significance from control, $p < 0.05$. B. SA-β-gal activity assay was performed at 24, 72 and 120 h postirradiation. All cells were counted in 3 random fields (at least 100 cells). The graph indicates means ± SD, n = 9. * indicates statistical significance from control, $p < 0.05$. C. Representative SA-β gal activity assay (blue staining) at 120 h postirradiation. D-F. Equal protein amounts of whole cell lysates were immunoblotted for p21/waf1 (D), Sirt1 (E), or Bcl-2 (F) at the indicated times postirradiation. Blots were stripped and probed for β-actin as a loading control. Representative western blots are shown.

**Figure 6.** ***X-rays promote ER stress in PAEC*** PAEC were either sham-irradiated (control) or irradiated with 50 Gy X-rays. A. qPCR was performed to determine the levels of GRP78, CHOP, and GADD34 mRNA 24 h postirradiation. The mRNA levels were normalized to GAPDH. Bars indicate mean ± SD, n=3. * indicates statistical significance from control, $p < 0.05$. B. Whole cell lysates were prepared at indicated time points and the phosphorylation status of eIF2α was detected by western blot analysis. C. Total cellular mRNA was prepared and used for RT-PCR to detect XBP1 splicing. Cells were either control (non-irradiated) or exposed to 50 Gy irradiation for the indicated time points postirradiation. Tunicamycin (TM, 10 μ/ml) was used as a positive control. PCR reactions were run on one agarose gel, and intervening lanes were removed for publication. Experiments were repeated at least 3 times; representative results are shown.

**Figure 7.** ***Salubrinal treatment blocks IR-induced apoptosis but not accelerated senescence***. PAEC were either untreated or treated with 22.5 μM salubrinal or DMSO (vehicle) for 1 h, prior to sham irradiation (control) or exposure to 50 Gy X-rays. A. Neutral comet assay was performed 24 h postirradiation. Bars indicate means ± SD, n = 4. * indicates statistical significance from control, $p < 0.05$. † indicates statistical significance from radiation + DMSO, $p < 0.05$. B. Representative immunoblot analysis of active caspase-3 and p21/waf1 in the presence of salubrinal. C. SA-β-gal assay was performed 72 h postirradiation. Bars indicate means ± SD, n = 3. * indicates statistical significance from control, $p < 0.05$. Experiments were repeated at least 3 times; representative data are shown.

# CHAPTER 3: Inhibition of IGF-1R Prevents Ionizing Radiation-Induced Primary Endothelial Cell Senescence

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**ABSTRACT**

Accelerated senescence has been shown to occur as a primary response to cellular stresses including DNA damaging agents (*e.g.,* ionizing radiation) and is widely believed to be caused by continuous proliferative signaling in the presence of cell cycle arrest. Studies on signal transduction pathways leading to accelerated senescence have revealed that inhibition of mammalian target of rapamycin (mTOR) by rapamycin rescues cells from senescence; however, the molecular mechanisms upstream of mTOR following ionizing radiation (IR) are not well defined. Here, we investigated signal transduction leading to IR-induced accelerated senescence in cultured human pulmonary artery endothelial cells (HPAEC). Exposure of HPAEC to X-rays (10 Gy, 2.4 Gy/min) upregulated senescence markers including p53, p21/waf1, and senescence associated beta galactosidase (SA-β-gal). Treatment with Ly294002 (a phosphatidylinositol-3-kinase [PI3K] inhibitor) or rapamycin (an mTOR inhibitor) blocked the increase in cellular senescence markers suggesting roles for PI3K and mTOR. Pathway directed microarrays revealed increased expression of insulin-like growth factor I (IGF-1), a known modulator of cell growth and proliferation upstream of mTOR. qRT-PCR was used to determine that both IGF-1 and IGF-2 mRNA were increased in response to X-rays and ELISA showed increased secretion of IGF-1 protein into the medium of irradiated HPAEC. Consistent with upregulation of these ligands, we found that X-ray exposure led to hyperphosphorylation of the IGF-1 and -2 receptor, IGF-1R. Treatment with AG1024, an IGF-1R inhibitor, suppressed IR-induced upregulation of p53, p21/waf1, and SA-β-gal. Together these findings suggest that IGF-1R is a key regulatory pathway in IR-induced

accelerated senescence in a pathway that requires intact mTOR activity upstream of both p53 and p21/waf1.

## INTRODUCTION

Accelerated senescence is a well-recognized cellular response to environmental stresses that damage biological molecules especially DNA. It is characterized by loss of replicative capacity, abnormal gene expression of cell cycle regulators, altered responsiveness to apoptotic stimuli, alterations in cellular morphology, induction of senescence associated secretory proteins, and increased senescence-associated beta-galactosidase (SA-β-gal) activity (139). Accumulating body of evidence implicates a role for cellular senescence in aging, suppression of tumorigenesis, and overall tissue and organ dysfunction possibly through depletion of functional cells required for organ homeostasis and though induction of inflammation associated with the secretory phenotype (26; 90; 139).

Recent studies suggest that accelerated senescence occurs as the result of proliferative signaling in the presence of a cell cycle checkpoint blockade, often p21/waf1 (17; 43). The mammalian target of rapamycin (mTOR), a cytoplasmic kinase that is widely regarded as a central integration point for a number of cell signaling pathways including cell proliferation and homeostasis (108), has been identified as a central molecular target for the inhibition of replicative senescence as well as stress-induced cellular senescence (6; 17; 18; 43). Treatment with rapamycin, an mTOR inhibitor, prevents accelerated senescence in cells exposed to DNA-damaging agents (43; 114). Similarly, paradoxically, both mitogen activated protein kinase (MAPK) p42/p44 and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways which play roles in cell

survival and proliferation have also been shown to positively regulate the development of senescence (20; 136; 144). Akt deficiency causes resistance to replicative- and stress-induced premature senescence while its activation induces premature senescence via increased production of reactive oxygen species (ROS) (144). On the other hand, MAPK p42/p44 mediates thrombopoeitin-induced senescence during megarkaryocitic maturation (15). Both signaling pathways appear to require increased expression of the cell cycle checkpoint protein p21/waf1 for the induction of cellular senescence (8; 15).

Investigation into receptor signaling pathways that contribute to aging-associated cellular senescence has revealed the possible involvement of the insulin like growth factor-1 receptor (IGF-1R) (81; 118). IGF-1R is a single transmembrane tyrosine kinase receptor whose ligands include IGF-1 and IGF-2 (189). The activation of IGF-1R involves autophosphorylation of its intracellular domain, followed by recruitment of docking intermediates including the insulin-receptor substrate-1 (IRS-1), which in many cell types leads to activation of PI3K/Akt, MAPK, and mTOR (116; 117; 146; 158). As a growth factor receptor, IGF-1R is known to play a role in cell growth and proliferation under normal conditions and is widely expressed in most transformed cells, conferring pro-survival properties upon stress application (47; 48; 146; 197). In agreement with the hypothesis that IGF-1R acts as a mediator of cell survival and proliferation, a number of studies showed a positive correlation between activation of IGF-1R and radiation resistance in some cells (37; 58; 154; 187). However, most of these studies were focused only on the contribution of apoptosis and it is likely that IGF-1R operates on other modes of radiation-induced cellular response depending on the cellular context. Moreover, the anti-apoptotic activity of IGF-1R appears to be dispensable in the induction of radiation

resistance in a variety of tumor cells suggesting the possibility of an unidentified mechanism (180). Although the IGF-1,-2/IGF-1R signaling axis is known to promote cell proliferation and survival under most circumstances, IGF-1R was recently implicated in several models of senescence. IGF-1R expression levels increased during the development of *in vitro* replicative senescence in primary cortical neurons (38). UVB-induced premature senescence was found to require functional IGF-1R in human keratinocytes (118). IGF-1 also enhanced senescence in primary cell cultures via a mechanism that involved increased reactive ROS generation leading to induction of the p53/p21 pathway (75). In mouse embryonic fibroblasts, treatment with IGF-1 inhibited the DNA deacetylase activity of Sirtuin 1 (SIRT1) and promoted stability of p53, ultimately leading to induction of senescence (183).

In our previous studies, we determined that accelerated senescence is the primary response of normal PAEC to X-ray exposure (149). We now provide evidence for the involvement of IGF-1R in the development of IR-induced accelerated senescent phenotype in primary lung endothelial cells. Our results suggest that IGF-1R signaling is required for X-ray-induced accelerated senescence in endothelial cells.

## MATERIALS AND METHODS

*Cell Culture and Reagents.* Human pulmonary artery endothelial cells (HPAEC) were obtained from Cell Applications, Inc. (San Diego, CA) and cultured in EBM-2 basal medium containing supplements and growth factors as indicated in the manufacturer's protocol (Lonza, Walkersville, MD)**.** Cells were maintained at 37$^{o}$C in a humidified atmosphere containing 5% $CO_2$. Subconfluent HPAEC at passages 4-8 were used for all experiments. The following chemical inhibitors and their final concentrations were used:

AG1024 (5 μM, Calbiochem, EMD Millipore, Billerica, MA), Ly294002 (20 μM, Calbiochem), rapamycin (500 nM, Calbiochem), U0126 (10 μM, Cell Signaling Technology, Danvers, MA). Each inhibitor was dissolved in DMSO and added to cell cultures so that the final concentration of the solvent did not exceed 0.1%. N-acetyl cysteine (NAC, 20mM) Calbiochem) was dissolved in distilled $H_20$.

*Cell Irradiation:* HPAEC were either irradiated or sham-irradiated at subconfluence (70-90%). Irradiations were conducted using RS2000 Biological Irradiator (Rad Source Technologies, Alpharetta, GA) with 0.3 mm Cu shielding at a dose rate of 2.4 Gy/min (160kV, 25mA) at room temperature.

*Western blotting.* Whole cell extracts were prepared in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors (Sigma-Aldrich, St. Louis, Montana), 1mM PMSF, 2 mM $Na_3VO_4$ and/or Halt phosphatase inhibitors (Thermo Scientific, Rockford, IL) or SDS-laemmli buffer (Bio-Rad, Hercules, CA) containing 50 mM DTT. Samples were vortexed, incubated for 10 min at 4$^o$C, and subjected to sonication (Heat Systems-Ultrasonics Inc., Plainview, NY) for 5 sec at 4$^o$C. Samples were then centrifuged at 14,000 x *g* for 10 min and supernatant was collected.

Protein concentrations from whole cell lysates were determined using Protein® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Equal amounts of proteins were separated in SDS-PAGE and transferred to PVDF membranes (Thermo Scientific, Rockford, IL). For western blotting, the following antibodies were used: anti-phospho-

IGF1R, total IGF-1Rα, anti-p53, anti-p21/waf1, and anti-β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-total IGF-1Rβ anti-phospho-S6, anti-phospho-Akt and total Akt (Cell Signaling Technology, Danvers, MA). Proteins were detected with horseradish peroxidase-linked secondary antibodies and ***SuperSignal*** West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

*Quantitative real-time reverse transcription polymerase chain reaction (qPCR).* Total RNA was isolated from cultured cells using the RNeasy Kit (Qiagen, Valencia, CA). RNA was quantified spectroscopically (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE). RNA (400 ng) was subjected to reverse transcription in a total volume of 20 μl. Following dilution 5-fold with water, 2 μl of cDNA was used for 20 μl qPCR reaction. PCR was performed in duplicates using 6 μM of each primer and 10 μl of SybrGreen® PCR master mix (Applied Biosystems, Foster City, CA). The following primers were used for detection of IGF-1: forward 5'- TGC CCA AGA CCC AGA AGT -3' and reverse 5'- CTC CTG TCC CCT CCT TCT GTT – 3' and IGF-2: forward 5'- ACA CCC TCC AGT TCG TCT GT- 3'and 5'- and GAAACAGCACTCCTCAACGA-3'. As an internal control, mRNA levels of GAPDH were determined using primers: forward 5'-GAA GCT CGT CAT CAA TGG AAA-3' and reverse 5'-CCA CTT GAT GTT GGC AGG AT-3'. For quantification, the comparative threshold cycle (Ct) method was used to assess relative changes in mRNA levels between the untreated (control) and the irradiated samples.

*Senescence-associated β-galactosidase (SA-β-gal) Assay.* SA-β-gal assay was performed according to established protocols with minor modifications (10; 46). To avoid density-dependent false positive expression of SA-β-gal, HPAEC were seeded at a density of 1.0-2.0 x $10^4$ on 12-well or 6-well dishes and allowed to reach 50-70% confluence prior to treatment. At indicated time-points postirradiation, cells were washed twice in ice cold PBS, fixed in 2% formaldehyde/ 0.2% glutaraldehyde in PBS for 5 min at room temperature and washed again twice in ice-cold PBS. 1.5-2 ml of freshly prepared X-gal staining solution (1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside in 40 mM citric acid/sodium phosphate, pH 6.0 (made by mixing 36.85 parts 0.1 M citric acid solution with 63.15 parts 0.2 M sodium phosphate solution and then verifying pH to be 6.0, 0.1M citric acid was added to adjust pH when necessary), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$) was then added to the culture dishes and the dishes were incubated for 12-20 h at 37$^o$C (without $CO_2$). After incubation, cells were washed twice in ice-cold PBS and once in methanol and allowed to dry. Cells were examined for perinuclear blue staining indicative of SA-β-gal activity in at least 3 randomly selected fields.

*Statistics.* Means ± standard deviations (SD) or error of the mean (SEM) were calculated, and statistically significant differences between two groups were determined by the Student's *t* test. $p < 0.05$ was considered statistically significant.

## RESULTS

**HPAEC undergo accelerated senescence post-irradiation.** We previously determined in bovine PAEC that accelerated senescence is the primary cellular response to exposure

to 10 Gy X-rays with very limited apoptosis and no detectable necrosis (149). Two salient features of senescence were identified in X-ray-induced senescence in bovine PAEC – upregulation of p21/waf1 and increased SA-β-gal activity. p21/waf1, also known as cyclin-dependent kinase inhibitor 1, is a cell cycle checkpoint protein and contributes to cell cycle arrest, a necessary component of cellular senescence. SA-β-gal is a widely used marker for cellular senescence that was first described by Dimri et al. (46) and has routinely been used over the years to detect senescent cells *in vitro* and *in vivo* (61). We investigated the X-ray effects on the development of senescent phenotype in human PAEC (HPAEC). Our data indicated that HPAEC underwent cellular senescence upon exposure to 10 Gy X-rays as determined by upregulation of p21/waf1 within 3h post-irradiation. Increased SA-β-gal activity, detected cytochemically as blue perinuclear staining, was detected optimally at 4 days post-irradiation (Figure 1B). X-ray-exposed HPAEC also exhibited changes in cell morphology, displaying unusually large cell size and flattened cytoplasmic appearance compared to sham-irradiated controls (Figure 1B). This was consistent with our findings using bovine derived PAEC (149).

**X-rays activate the IGF-1R signaling cascade.** Exposure to X-rays causes alterations in global gene expression. As we had previously determined that the primary response causing loss of cell viability in pulmonary artery endothelial cells is accelerated senescence, we used a pathway-focused senescence array to examine mRNA changes over time after 10 Gy exposure in HPAEC. Among the genes observed to be altered in expression, IGF-1 mRNA level was increased ~2 fold within 24 hours post-irradiation and ~ 15 fold within 72 hours post-irradiation (data not shown). The IGF-1 mRNA results were confirmed using qRT-PCR (~2 fold at 24 hours post-irradiation and ~6 fold

at 72 hours post-irradiation, $p<0.05$, Figure 2A). Similarly, IGF-2 mRNA was increased (~ 1.7 fold at 24 hours post-irradiation and ~1.5 fold at 72 hours post-irradiation, Figure 2B). IGF-1 can act in paracrine and/or autocrine manner (33) and in cultured cells it is secreted in the medium once produced (1; 97). ELISA assays indicated that IGF-1 was increased in the medium 72 hour post-irradiation (~3.2 fold, $p<0.05$, Figure 2C). These increases in the secretion of IGF-1 and in the gene expressions of both IGF-1 and IGF-2, two known activating ligands of IGF-1R, led us to examine the activation of its receptor IGF-1R. We detected increase in IGF-1R phosphorylation which is essential for kinase activation (12; 120). As shown in Figure 2D, IGF-1R hyperphosphorylation (left panel) was detected within 3 hours post-irradiation along with Akt hyperphosphorylation at Ser 473, which is required for full activation of Akt in a PI3K-dependent manner (152). Interestingly, the increase in phosphorylation of IGF-1R and Akt occurred concurrently with p21/waf1 upregulation.

**AG1024 blocks radiation-induced accelerated senescence.** As X-rays induced IGF-1R phosphorylation, IGF-1 and IGF-2 upregulation as well as accelerated senescence in HPAEC, we hypothesized that IGF-1R activation may function in the development of accelerated senescence post-irradiation. To test this hypothesis, we exposed HPAEC to 10 Gy X-rays in the presence of AG1024 (an IGF-1R inhibitor) and examined cellular senescence. Blocking IGF-1R activation significantly reduced cellular senescence as shown by western blotting for p53 and p21/waf1, and by SA-β-gal activity assay (Figure 3C and 3B). Treatment with AG1024 also rescued the irradiated HPAEC from undergoing senescence-associated morphological changes that were notable in DMSO-treated irradiated HPAEC (Figure 3A).

**Inhibition of mTOR and PI3K, but not p42/p44 MAPK, blocks radiation-induced accelerated senescence.** Recent studies demonstrated that mTOR is required for stress-induced senescence. Treatment with rapamycin prevented the increase in SA-β-gal activity in cells exposed to DNA-damaging agents (43; 113; 115) and protected mice from radiation-induced mucositis (88). We tested whether inhibition of mTOR would result in attenuation of IR-induced accelerated senescence. As shown in Figure 4, inhibition of mTOR activity using 500 nM rapamycin blocked IR-induced cellular senescence in a similar manner conferred by AG1024 on irradiated HPAEC. Activation of IGF-1R was demonstrated to cause activation of a signaling cascade via the PI3K/Akt and MAPK pathways which can converge at mTOR activation (29; 166) (160). We therefore tested whether blocking PI3K/AKT using Ly294002 or MAPK using U0126 would result in reduction of cellular senescence in a manner similar to that conferred by IGF-1R inhibition. As shown in Figure 4, inhibition of PI3K using Ly294002 also attenuated radiation-induced cellular senescence. On the other hand, treatment with U0126, a MAPK inhibitor, did not rescue cells from undergoing accelerated senescence even though early time-point increase in p42/44 MAPK was observed (data not shown).

To determine whether IGF-1R is upstream of mTOR, we examined the phosphorylation of S6 ribosomal protein at Ser 235/236, a known target of S6 kinase which is downstream of activated mTOR (56). We also examined phosphor.-Akt with the treatment of AG1024 to determine whether IGF-1R is upstream of Akt in irradiated PAEC. AG1024 treatment blocked decreased levels of phosho-S6 significantly.

However, we were unable to detect significant inhibition of phosphorylated Akt (Figure 5).

**Radiation-induced reactive oxygen species are required for early IGF-1R phosphorylation.** Our results indicated that within 3 hours, we were not able to detect a significant increase in the secretion of IGF-1 per cell. However, our experiments indicated that IGF-1R phosphorylation could be detected at time points that preceded the increased production of ligands. We therefore investigated alternative mechanism of early IGF-1R activation. Exposure to IR is known to cause accumulation of ROS via radiolysis of intracellular $H_2O$ and through subsequent production of ROS inside irradiated cells (72; 110). ROS have also been shown to cause phosphorylation of cellular receptors, including IGF-1R in vascular smooth muscle cells (19; 195). To determine whether ROS alone would be sufficient for the induction of IGF-1R phosphorylation, we treated HPAEC with $H_2O_2$ (1 μM and 10 μM) for 20 minutes. As shown in Figure 6, $H_2O_2$ alone was sufficient to induce IGF-1R phosphorylation. We then determined whether this ROS-induced phosphorylation is ligand-dependent by treating the cells with $H_2O_2$ in the presence of AG1024 and blotting for phospho-IGF-1R. As shown in Figure 6, ROS-induced IGF-1R phosphorylation was not inhibited by AG1024. Finally, to determine whether IR-induced IGF-1R phosphorylation is mediated by ROS, we treated HPAEC with 20 mM N-acetyl-L-cysteine (NAC) for 1 hour and exposed the cells to 10 Gy X-rays. Western blotting shows that IGF-1R phosphorylation was attenuated in the presence of NAC.

**Treatment with AG1024 or increasing the concentration of IGF-1 has no effect on radiation-induced caspase-3 activation.** A number of studies have shown that

bypassing or blocking cellular senescence sensitizes cells to apoptosis following cellular stress (49; 156). In order to determine whether IGF-1R may play a role in this dynamics, we treated HPAEC with AG1024 (IGF-1R inhibitor) and exogenous IGF-1 (IGF-1R activator) and then exposed to X-rays. For this purpose, we used 50 Gy X-rays as we previously determined that this dose was effective in detecting caspase-3 activation in bovine PAEC (149). As shown in Figure 6, treatment with either AG1024 or exogenous IGF-1 did not alter the levels of active caspase-3 suggesting that IGF-1R does not regulate radiation-induced apoptosis.

## DISCUSSION

Exposure to various stresses, especially to DNA damaging agents, triggers complex cellular responses that result in either cell survival or cell death. It is thought that if damage is resolved, cells survive to continue to divide and proliferate. Irreparable damage, on the other hand, is generally considered a trigger for cell death via necrosis, apoptosis, or both (64). However, in most cases, cellular stresses may also give rise to accelerated senescence, a form of irreversible cell cycle arrest with complex phenotype that includes extensive morphological alterations, secretion of senescence-associated proteins, and development of β-galactosidase activity (139). We previously demonstrated that accelerated senescence is the primary mode of radiation-induced loss of cell viability in non-immortalized, non-cancer endothelial cells (149). In the current study, we demonstrate in primary PAEC that IGF-1R is activated in response to irradiation, initially via radiation-induced ROS generation and later in response to autocrine production of IGF-1 and IGF-2. We provide evidence that IGF-1R activation is required for IR-induced accelerated senescence, as inhibition of IGF-1R suppresses downstream activation of p53

and p21/waf1. Cells treated with IGF-1R inhibitor maintain normal morphology and do not express SA-β-gal. This is the first demonstration of the requirement of IGF-1R for accelerated senescence by ionizing radiation.

An accumulating body of evidence suggests that cellular senescence is a state of continuous cell growth activity in the presence of cell cycle blockade (17). In support of this idea, mTOR has recently been suggested as the critical effector of the continuous cell growth response component of cellular senescence. Consistent with recent findings on the effects of blocking mTOR activity on the development of cellular senescence, our results suggest that radiation-induced cellular senescence involves mTOR as shown by attenuation of SA-β-gal activity in the presence of rapamycin. Interestingly, we also find that inhibition of mTOR results in attenuation of radiation-induced p21/waf1 upregulation suggesting that mTOR is possibly upstream of p21/waf1 and thus, may contribute to cell cycle arrest as well. Indeed, a number of studies have demonstrated crosstalk between p53/p21/waf1 and mTOR pathways via regulations of MDM2 and SIRT1, which both modulate p53 accumulation (100; 111; 184).

Our current findings in HPAEC indicate that IGF-1R activation does not inhibit radiation-induced apoptosis. Paradoxically, a number of studies have demonstrated positive effects of activated IGF-1R and IGF-1 in conferring radiation resistance, mostly through prevention of apoptosis in cancer cells. For example, increased IGF-1R phosphorylation after γ-irradiation was demonstrated even in the absence of detectable IGF-1 or IGF-2 ligands, triggering anti-apoptotic activity in non-small cell lung cancer cell line (37). The presence of IGF-1 conferred radioprotection from apoptosis in hematopoietic progenitor cells (58) and in crypt and intestinal stem cells (154). Elevated

levels of IGF-1R were also found to induce radiation resistance in fibroblasts and were highly correlated with local breast cancer recurrence after radiotherapy (187). However, these studies investigated only the correlation of activated IGF-1R with resistance to radiation-induced apoptosis. Surprisingly, the anti-apoptotic activity of IGF-1R appears to be dispensable for the induction of radiation resistance, suggesting that the underlying mechanism(s) remain unresolved (180). Indeed, most previous studies did not investigate cellular senescence which could have been misinterpreted as a mechanism of radiation resistance in cell culture, since stress-induced premature senescent cells can remain metabolically active for months (208). Our finding of the requirement of IGF-1R for radiation-induced accelerated senescence is consistent with the findings of a previous study on the requirement of functional IGF-1R for the initiation of UVB-induced premature senescence in human keratinocytes (118). Furthermore, IGF-1R does not appear to modulate radiation-induced apoptosis as AG1024 did not increase caspase-3 activation yet still prevented p21/waf1 increase in 50 Gy induction of apoptosis in HPAEC.

X-rays can kill cells primarily through the radiolysis of intracellular $H_2O$ which generates reactive oxygen species that cause macromolecular damage (72). ROS can induce post-translational modifications in cellular receptors including IGF-1R (195) possibly by phosphatase inhibition (157) and it has also been shown that subsequent reactions and signaling cascades within the irradiated cells cause further ROS accumulation (Leach, Van Tuyle et al. 2001. Our results indicate that ROS, primarily through $H_2O_2$, mediate IGF-1R activation as shown by increased IGF-1R phosphorylation following exposure to $H_2O_2$ which was not inhibited by AG1024

treatment. This ROS-induced increase in IGF-1R phosphorylation is possibly due to inactivation of protein-tyrosine phosphatases that dephosphorylate IGF-1R in the same manner of ROS-mediated inactivation of protein-tyrosine phosphatases in other systems (11; 112; 181). This suggests that ROS generated by X-rays may be sufficient for the downstream signaling induced by X-rays in HPAEC. This is further supported by our data showing that antioxidants can relieve the phosphorylation of IGF-1R post-irradiation and implies the contribution of a ligand-independent mechanism. Our findings also indicate that ligand-dependent mechanism contributes to IGF-1R-mediated IR-induced senescence as revealed by the increase in both IGF-1 mRNA and secreted protein per cell and by attenuation of IR-induced p21/waf1 upregulation and SA-β-gal acivity. The continuous increase in IGF-1 levels along with an upregulation of IGF-2 over time may contribute to a vicious cycle of autocrine and paracrine signaling in the presence of the cell cycle inhibitor p21/waf1 which ultimately leads to the development of the complex senescent phenotype. It has been demonstrated the radiation can cause increase in production and secretion of IGF-I at a later time-point post-irradiation (40). Our data shows that IGF-I level in the medium normalized to the number of cells is increased in irradiated cells relative to non-irradiated controls at 72 hours post-irradiation. This appears not to be a direct physical effect of radiation since the timing occurs at a later time-point (72 hours post-irradiation). However, whether radiation causes release of IGF-1 from IGF-1 binding proteins (IGFBPs) allowing IGF-1 to bind to IGF-1R was not examined in this study. At least one IGF-1 binding protein, IGFBP-6, was shown to delay senescence and when downregulated, can lead to premature entry into senescent state (130).

Our findings also show that radiation-induced IGF-1R-mediated accelerated senescence requires the presence of intact mTOR. How does IGF-1R act through mTOR then? Two alternative pathways most commonly activated by IGF-1R are the PI3K/Akt and MAPK pathways. Both pathways are activated post-irradiation in HPAEC; however, only the inhibition of PI3K/Akt attenuates accelerated senescence suggesting that IGF-1R links mTOR via this pathway. Although PI3K/Akt is known to regulate cell proliferation and may act upstream of mTOR, it has been reported in several studies to be capable of causing senescence as well (8; 136; 148). Thus, collectively, our findings recapitulate the seemingly paradoxic idea that senescence is a state of continuous cell growth and proliferative signaling in the presence of a cell cycle blockade. The increase in cell size suggests the continuous cell growth and proliferative signaling while the absence of increase in cell number reflects the presence of cell cycle inhibitors. Interestingly, however, our results also suggest that the IGF-1R/PI3K/mTOR pathway may mediate the development of senescence by modulating the cell cycle arrest component as well.

We observed that blocking IGF-1R using AG1024 leads to an unexpected increase in the phosphorylation of Akt at Ser473 in both sham-irradiated and irradiated PAEC. This suggests that PI3k/Akt acts through a parallel mechanism that is not necessarily part of the IGF-1R pathway. PI3k/Akt has been shown to positively regulate cellular senescence (144). Another possible explanation of the apparent increase in Akt phosphorylation with AG1024 treatment is the inhibition of a negative feedback loop that modulates Akt phosphorylation. S6K, which is a downstream target of mTOR, has been shown to block further activation of Akt through inhibition of insulin receptor substrate 1 (IRS-1) (85; 126). IRS-1 is an immediate downstream target of IGF-1R and is critical for

the propagation of IGF-1R signaling (85). Since S6K appears to be downregulated as suggested by the decrease in the phosphorylation of its substrate, S6, the negative feedback loop on IRS-1 was possibly relieved which then caused the apparent increase in Akt phosphorylation. To further investigate this possible scenario, phosphorylation of IRS-1 may be assessed over time post-irradiation in the presence of AG1024 as well as the effect of inhibition of IRS-1 in IR-induced accelerated senescence.

Unlike apoptosis and necrosis, the importance of accelerated senescence in radiation-induced damage has just recently been increasingly recognized with the discovery that mTOR mediates radiation-induced cellular senescence during exposure to radiation in vivo (88). Yet, the mechanisms of radiation-induced cellular senescence remain incompletely understood. Although senescent cells are metabolically active, they are considered to be non-viable as they are incapable of replicating, and experiments have demonstrated that they eventually die through programmed cell death (68; 73). Additionally, and of clinical importance, radiation-induced cellular senescence may deplete the pool of proliferative cells which can contribute to the adverse effects of radiation therapy (37). Here, we have provided evidence that the IGF-1R intrinsic tyrosine kinase activity and signaling contribute to radiation-induced cellular senescence that can be blocked by pharmacological inhibitors. The identification of specific pathways and mechanisms for radiation-induced cellular senescence could provide novel targets for the prevention of severe effects of ionizing radiation during thoracic radiation therapy or from accidental radiation exposures.

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**LIST OF FIGURES**

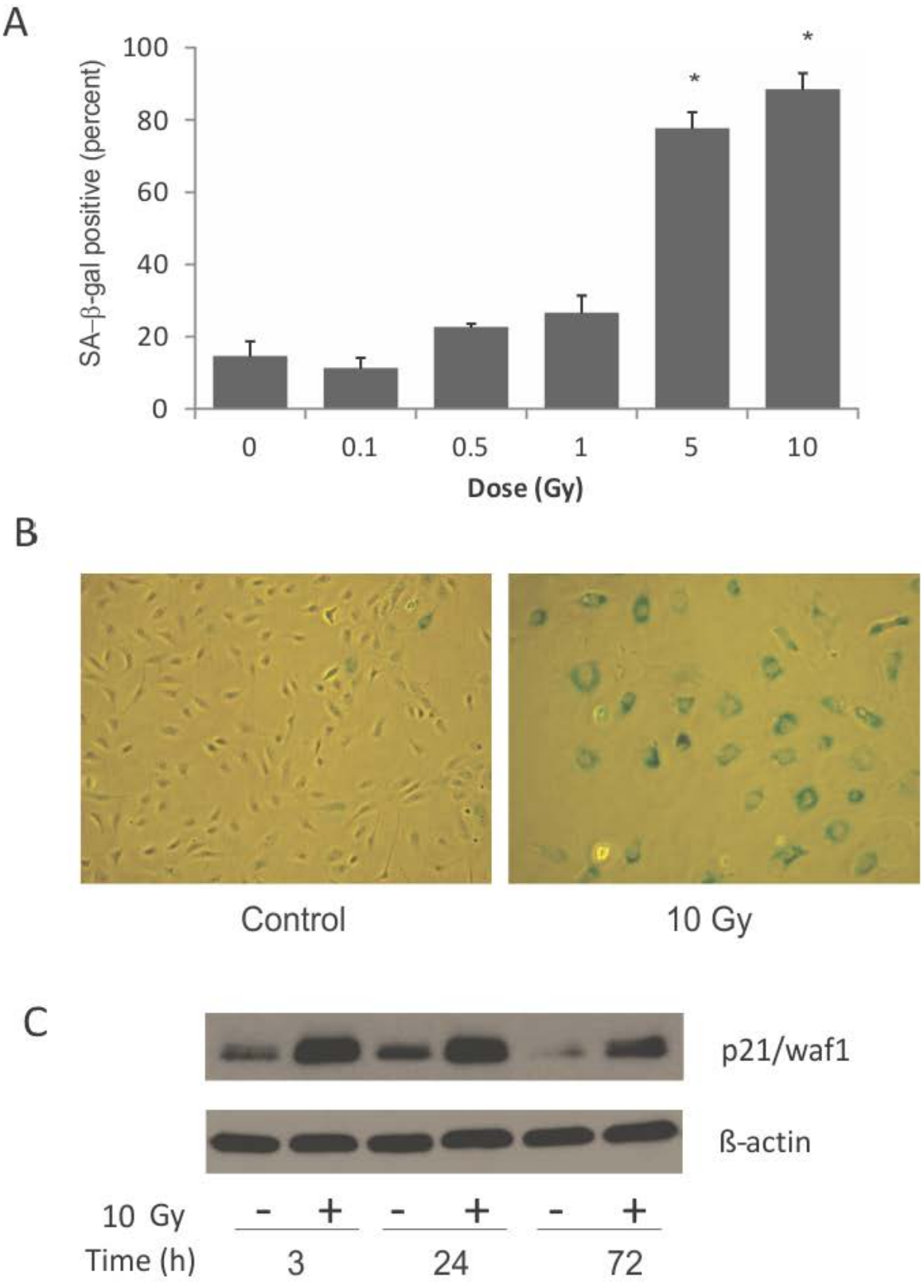


Figure 1

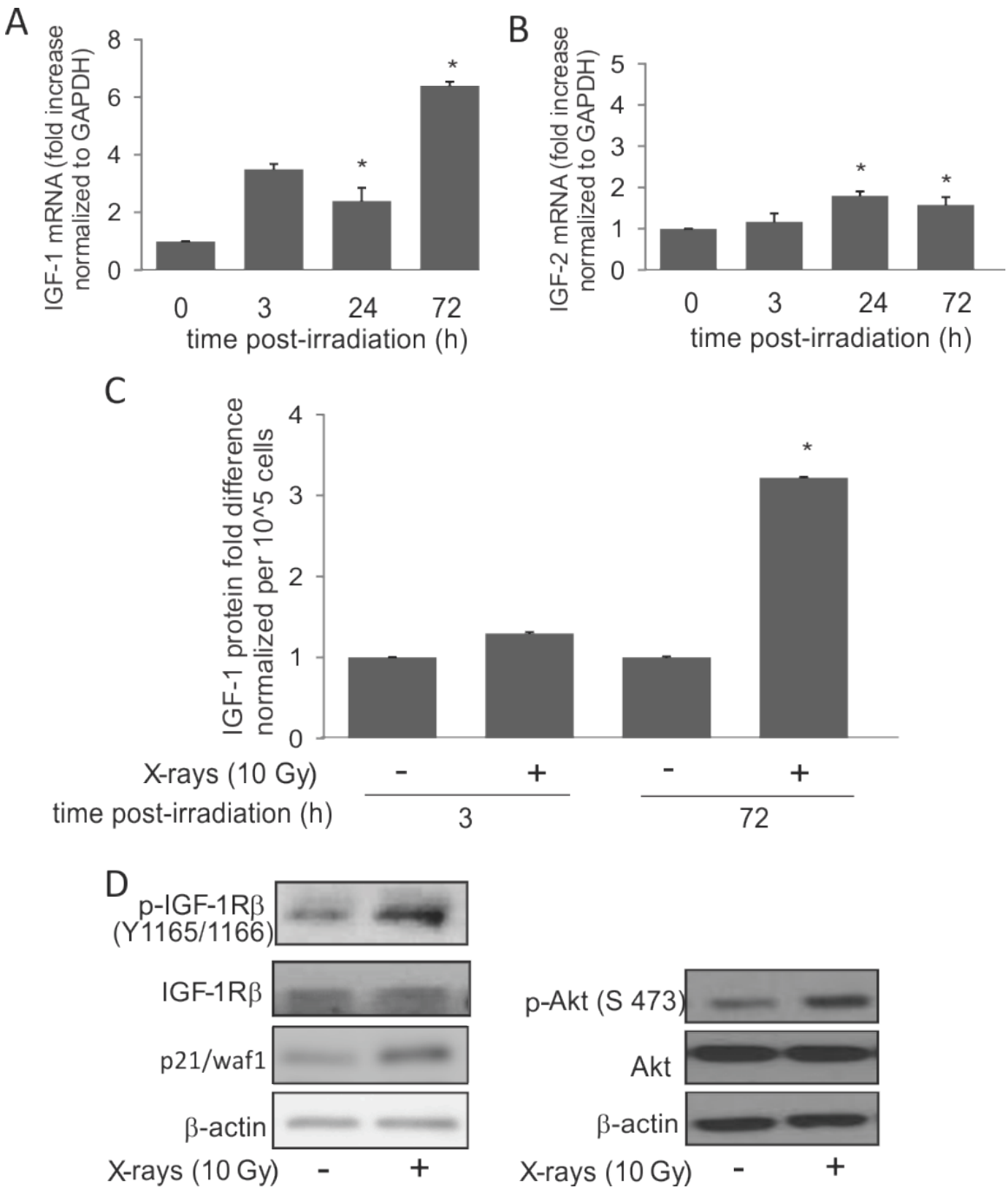



Figure 2

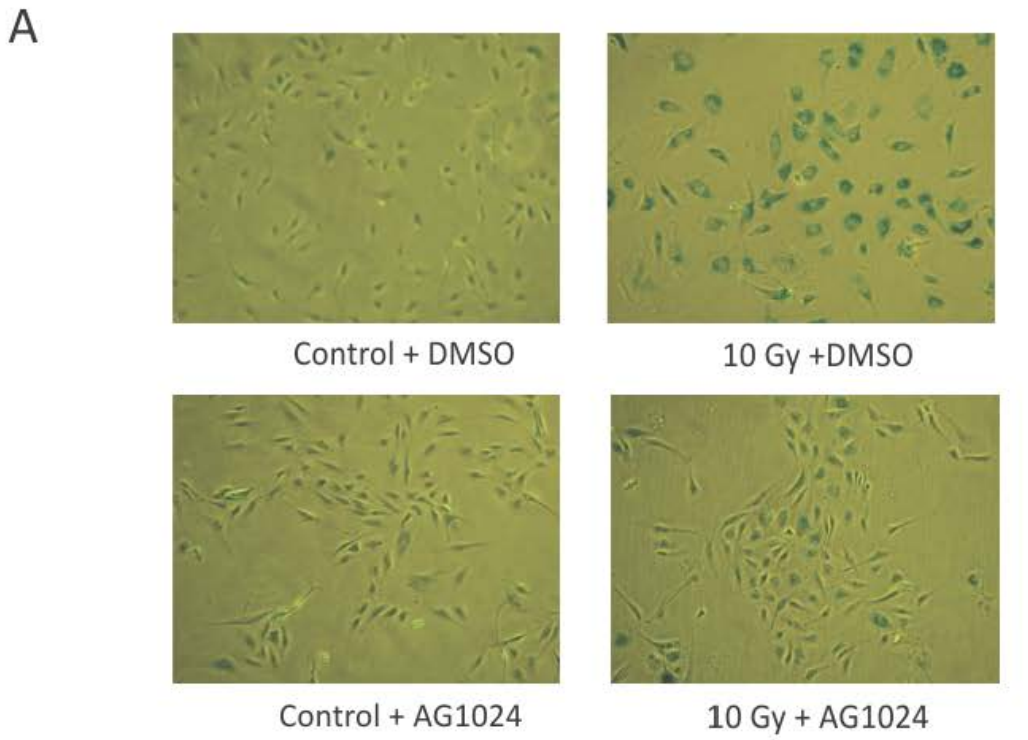
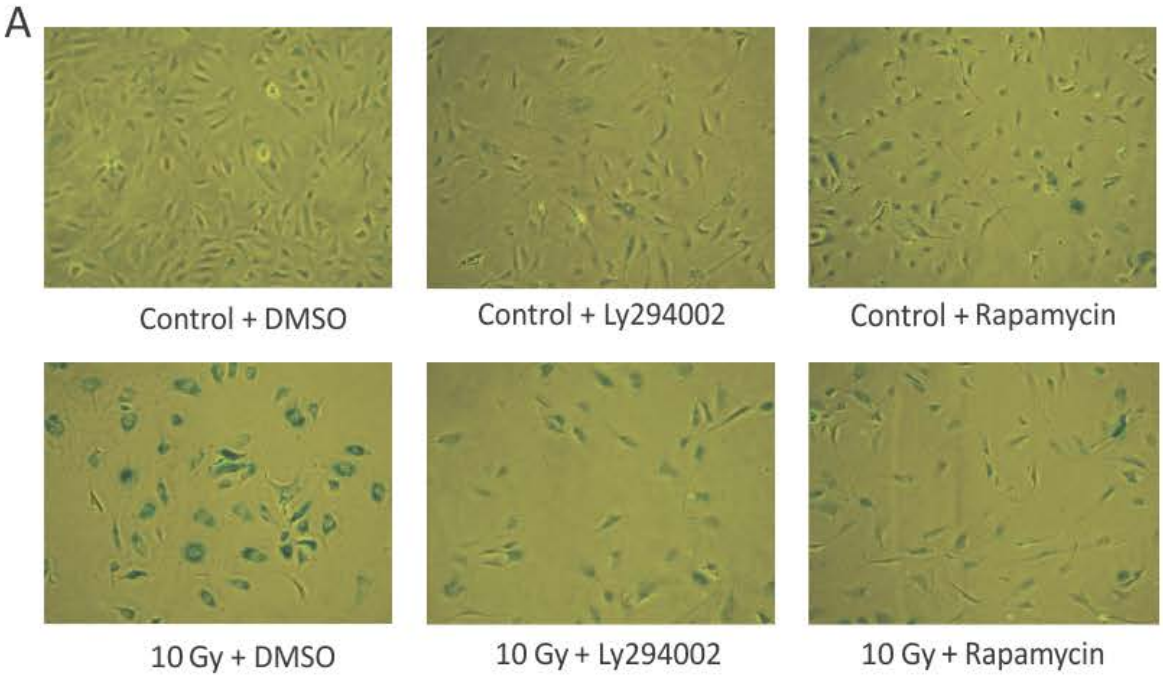
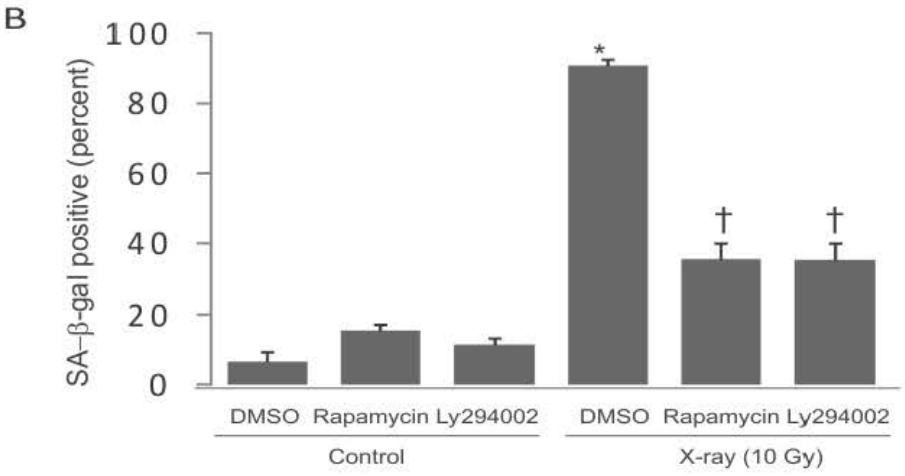

A
Control + DMSO
10 Gy +DMSO
Control + AG1024
10 Gy + AG1024
B
SA–β-gal positive (percent)
100
80
60
40
20
0
*
†
DMSO
AG1024
DMSO
AG1024
Control
X-ray (10 Gy)
C
6h
24h
p53
p21
actin
X-rays (10 Gy) - - + + - - + +
AG1024 - + - + - + - +

Figure 3



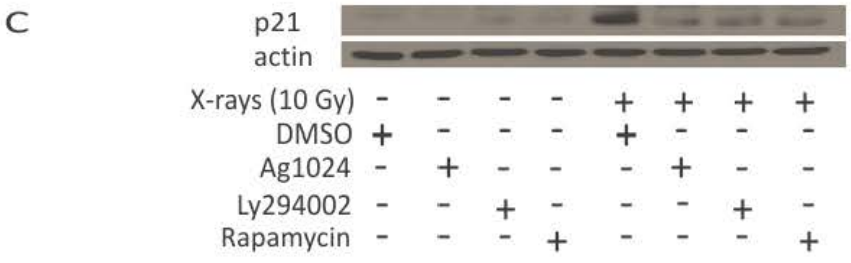
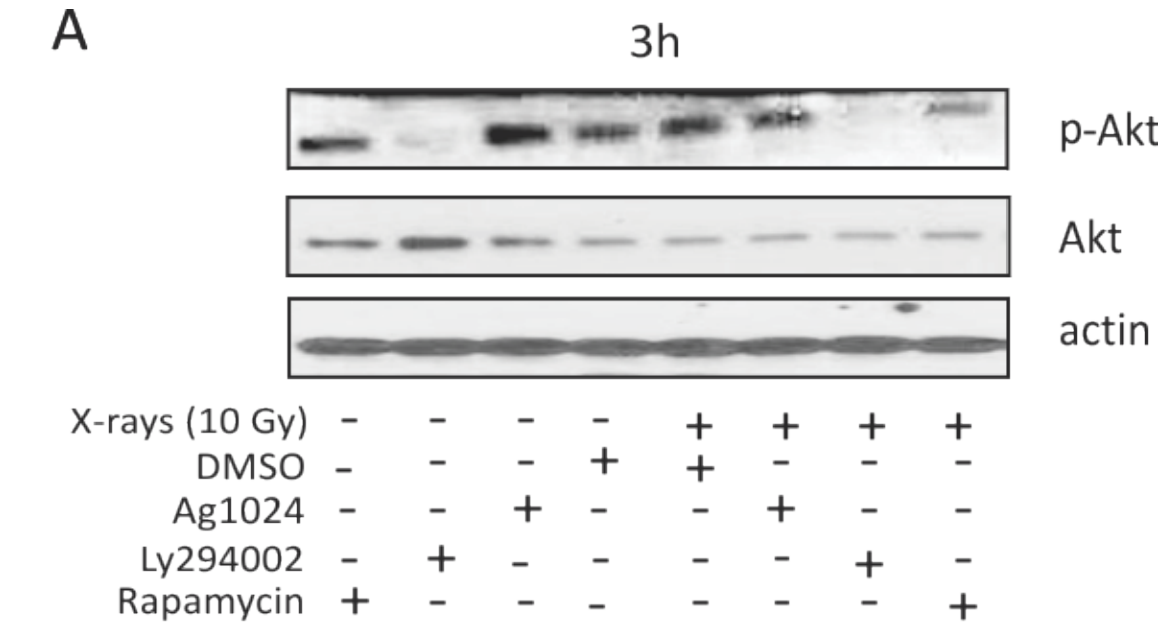




Figure 4


A
3h
p-Akt
Akt
actin
X-rays (10 Gy) - - - - + + + +
DMSO - - - + + - - -
Ag1024 - - + - - + - -
Ly294002 - + - - - - + -
Rapamycin + - - - - - - +

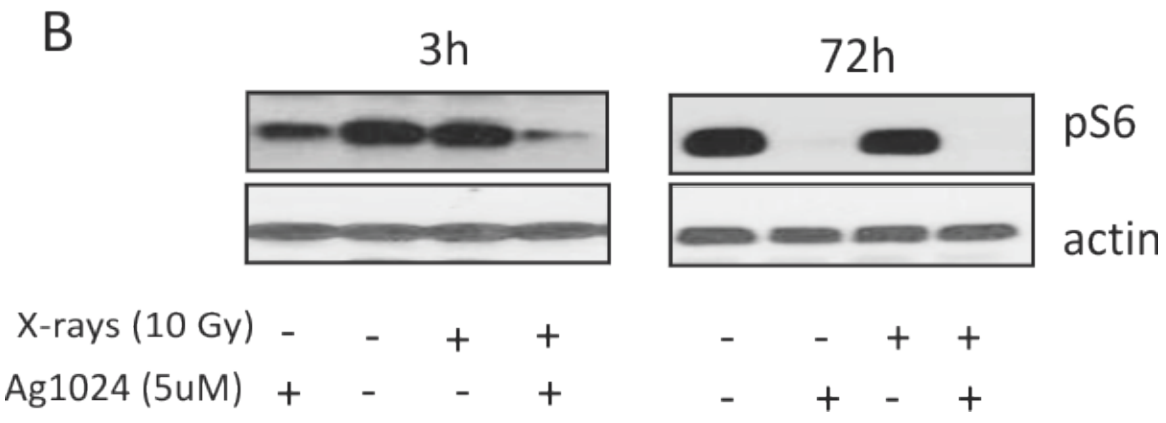

B
3h
72h
pS6
actin
X-rays (10 Gy) - - + + - - + +
Ag1024 (5uM) + - - + - + - +

Figure 5

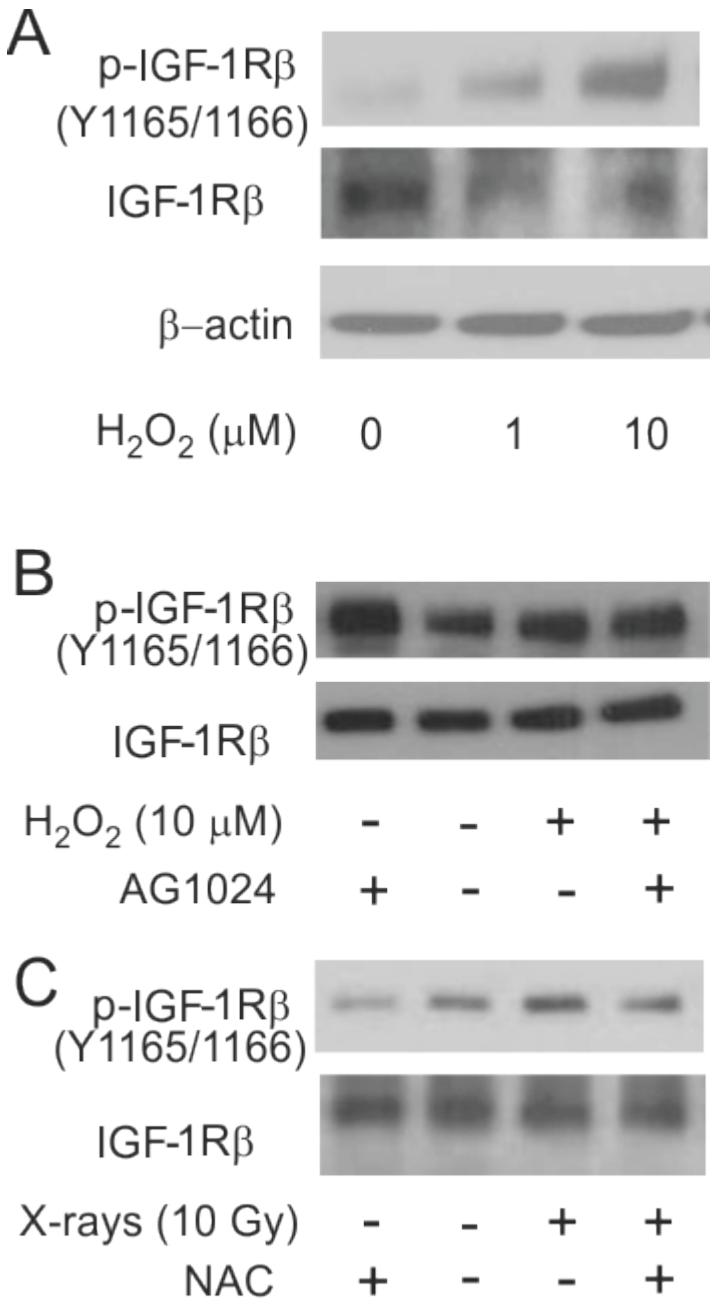

A
p-IGF-1Rβ
(Y1165/1166)
IGF-1Rβ
β–actin
H2O2 (μM) 0 1 10
B
p-IGF-1Rβ
(Y1165/1166)
IGF-1Rβ
H2O2 (10 μM) - - + +
AG1024 + - - +
C
p-IGF-1Rβ
(Y1165/1166)
IGF-1Rβ
X-rays (10 Gy) - - + +
NAC + - - +

Figure 6

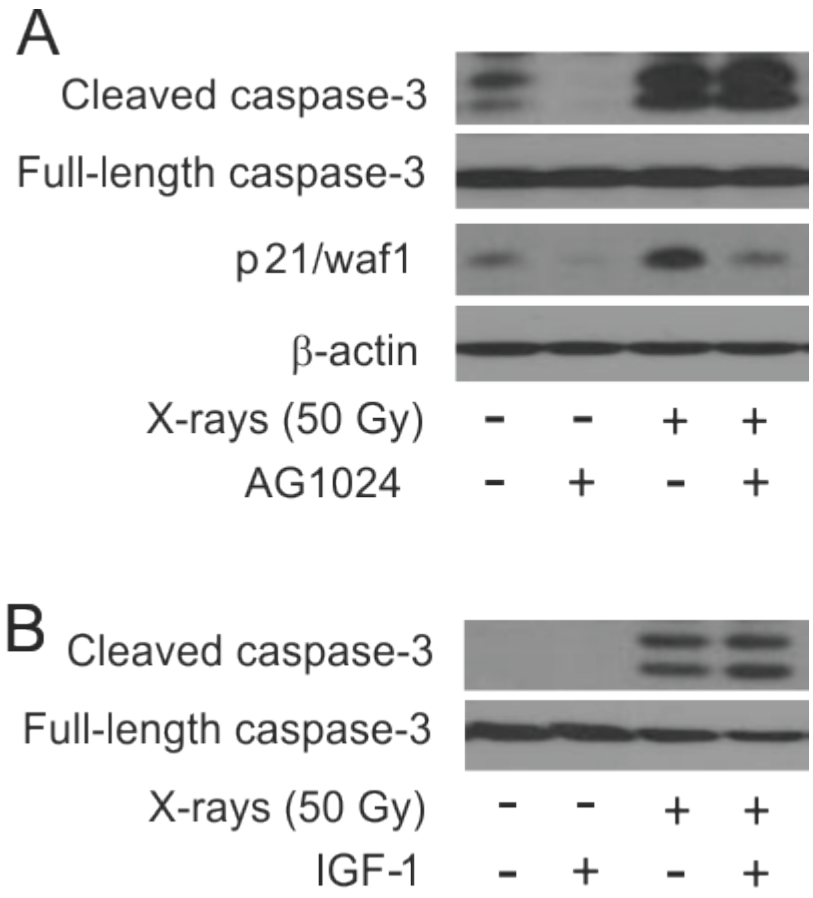

A
Cleaved caspase-3
Full-length caspase-3
p21/waf1
β-actin
X-rays (50 Gy) - - + +
AG1024 - + - +
B
Cleaved caspase-3
Full-length caspase-3
X-rays (50 Gy) - - + +
IGF-1 - + - +

Figure 7

**Figure 1. X-rays induce accelerated senescence in HPAEC.** Subconfluent HPAEC were exposed to indicated doses of X-rays and then incubated until time of assay. (A) Dose-response effect of increasing X-ray doses in HPAEC. At 4 days post-irradation, irradiated and sham-irradiated HPAEC were assayed for SA-β-gal activity. Percentage of SA-β-gal positive cells was counted in at least 3 random fields. Graph represents means ± SEM, n = 3. * indicates statistical significance from controls, $p<0.05$. (B) SA-β-gal staining sham-irradiated and irradiated (10 Gy) (C) Time-course of p21/waf1 upregulation. Western blotting for p21/waf1 expression at indicated time-points post-irradiation.

**Figure 2. Induction of IGF-1R signaling in irradiated HPAEC.** (A and B) IGF-1 and IGF-2 mRNA levels in irradiated HPAEC were assessed by qPCR at indicated time-points post-irradiation. The mRNA levels were normalized to GAPDH. Graph represents means ± SD, n = 3. * indicates statistical significance from controls, $p<0.05$. (C) IGF-1 levels in secreted medium were measured by ELISA at indicated time-points post-irrradiation. Graph represents means ± SD, n = 3. * indicates statistical significance from controls, $p<0.05$. (D) Western blotting for expressions of proteins involved in IGF-1R hyperphosphorylation (left panel) and Akt hyperphosphorylation (Ser 473, right panel) at 3 hours post-irradiation.

**Figure 3. Attenuation of radiation-induced accelerated senescence by AG1024.** Subconfluent HPAEC were pretreated with 5 μM AG1024 or vehicle (DMSO) for 30 minutes, exposed to 10 Gy X-rays and then incubated until time of assay. (A)

Representaive pictures of SA-β-gal staining at 4 days post-irradiation (B) Percentage of SA-β-gal positive cells was counted in at least 3 random fields. Graph represents means ± SEM, n = 6. * indicates statistical significance from controls, $p<0.05$. (C) Representative western blotting for p53 and p21/waf1 expression at indicated time-points post-irradiation in the presence of AG1024 or DMSO.

**Figure 4. Attenuation of radiation-induced accelerated senescence by rapamycin and Ly294002.** Subconfluent HPAEC were pretreated with 500 nM rapamycin, 20 uM Ly294002, or vehicle (DMSO) for 30 minutes, exposed to 10 Gy X-rays and then incubated until time of assay. (A) Representative pictures of SA-β-gal staining at 4 days post-irradiation (B) Percentage of SA- β-gal positive cells was counted in at least 3 random fields. Graph represents means ± SEM, n = 6. * indicates statistical significance from controls, $p<0.05$. (C) Representative western blotting for p21/waf1 expression at indicated time-points post-irradiation in the presence of AG1024, rapamycin, Ly294002 or DMSO.

**Figure 5. IR-induced IGF-1R-mediated accelerated senescence requires intact mTOR.** Subconfluent HPAEC were treated with 5 μM AG1024, 20 μM Ly294002, 500 nM mTOR or DMSO for 30 minutes and subjected to 10 Gy X-rays. (A) At 3 hours post-irradiation, whole cell lysates were prepared and western blotting for phospho-Akt (Ser 473) was performed. (B) At 3 and 72 hours post-irradiation, whole cell lysates were prepared and western blotting for phospho-S6 (Ser 235/236) was performed.

**Figure 6. $H_2O_2$ induces phosphorylation of IGF-1R.** (A) Subconfluent HPAEC were

treated with 1 uM and 10 uM $H_2O_2$ and incubated in a $CO_2$ incubator. After 20 minutes, whole cell lysates were prepared and western blotting was performed to determine the phosphorylation status of IGF-1R. (B) HPAEC were treated with 5 μM AG1024 for 30 minutes and then with 10 uM $H_2O_2$ for 20 minutes. Western blotting was then performed to determine phosphorylation status of IGF-1R. (C) HPAEC were treated with 20 mM NAC and subjected to 10 Gy X-rays. Western blotting was then performed 3 hours post-irradiation to determine phosphorylation status of IGF-1R.

**Figure 7. Treatment with AG1024 or addition of IGF-1 does not regulate IR-induced apoptosis.** (A) Subconfluent HPAEC were treated with 5 μM AG1024 or DMSO for 30 minutes and subjected to 50 Gy X-rays. At 6 hours post-irradiation, whole cell lysates were prepared and western blotting for cleaved, caspase-3 was performed. (B) Subcofluent cultures of HPAEC were added with 10x IGF-1 concentration (50 ng/ml) for 18 hours and exposed to 10 Gy X-rays. At 6 hours post-irradiation, whole cell lysates were prepared and western blotting for cleaved, caspase-3 was performed.

# CHAPTER 4: Dissertation Discussion

Studies on the biological effects of IR have been exponentially increasing since the discovery of X-rays by Wilhelm Roentgen in 1895. However, effective and safe radiation countermeasures are still largely unavailable. The lack of radiation countermeasures is in part due to the lack of understanding of the complex mechanisms of IR-induced cell death in normal cell types. Radiation-induced damage at the cellular level continues to be a limiting factor for the use of clinical radiotherapy, for example, during the course of treatment for thoracic cancers, where IR-induced cell death in the normal lung tissue leads to pneumonitis, pulmonary remodeling, and eventually death (35). Although much research has focused on IR-induced cell death, most of these studies have been geared toward understanding the mechanisms of increased radiation resistance of cancer cells and have thus been conducted on immortalized cancer cell lines that do not represent the biological status of normal lung cells. Thus, there is a pressing need to investigate the molecular mechanisms of IR-induced cell death in normal lung cells.

In the first part of this dissertation, we investigated the modes of cell death following exposure of PAEC to varying doses of X-rays. We used the traditional clonogenic assay over a range of single doses used in the clinical setting in order to determine the doses required to kill the majority of PAEC population. We note that it is essential to obtain a homogeneous population of cells undergoing the same fate in order to limit the background signals during signal transduction studies. We found that a dose of 10 Gy abolished the clonogenic potential of approximately 99.9% of the PAEC population. Similarly, a much higher dose of 50 Gy caused loss of clonogenic potential

but did not abolish the ability of PAEC to remain attached on cell culture plates throughout the duration of the clonogenic assay (14 days).

IR can kill cells through a variety of modes of cell death including necrosis, apoptosis, and accelerated senescence. We examined the presence of each of the modes of cell death using biochemical assays commonly used for characterizing each mode. We found absence of necrosis on all doses examined using lactate dehydrogenase (LDH) release assay, even at a dose of 50 Gy which was found to cause necrosis in leukemia cells (190). We found this not surprising as different cell types respond differently to various doses of radiation. The results of the LDH assay also revealed that 50 Gy X-rays does not cause significant membrane rupture, a characteristic of necrosis (103), and together with the clonogenic assay on this dose suggest that PAEC still remain metabolically active after such relatively high radiation dose.

Having ruled out necrosis, we then addressed the presence of apoptosis using neutral comet assay which detects double strand breaks representing internucleosomal DNA fragmentation that occurs in late stage apoptosis (140). We found that apoptosis was detectable beginning at 4 Gy and its presence became increasingly significant as the dose was raised. However, even after 50 Gy X-rays, the proportion of cells undergoing apoptosis was still limited. The activation of caspase-3 observed using caspase-3 activity assay and western blotting for cleaved caspase-3, together with the caspase-3 inhibition experiment confirmed the presence of apoptosis post-irradiation. Furthermore, we determined that IR-induced apoptosis involves activation of both intrinsic and extrinsic pathways of apoptosis. Although the intrinsic pathway of apoptosis in the context of radiation was well studied by a number of groups (16; 39; 55) in some cell types, the

extrinsic pathway has also been shown to occur following exposure to radiation through mechanisms that may involve activation of the death receptors, *e.g.* Fas (52)

The absence of necrosis and the limited amount of apoptosis suggest the presence of another mechanism accounting for the loss of cell viability in irradiated PAEC. We determined that cellular senescence is the primary mode of cell death accounting for the loss of clonogenic potential of irradiated PAEC. The irradiated PAEC display markers of senescence including 1) large flat cellular morphology; 2) upregulation of p21/waf1; 3) cell proliferation arrest; and 4) increased SA-β-gal activity. This is consistent with a set of criteria for cellular senescence including 1) remarkable morphologic transformations; 2) altered gene expression of cell growth-regulatory proteins; 3) cell cycle arrest; 4) increased SA-β-gal activity; and 5) altered response to apoptotic stimuli (139). We use the term accelerated senescence, as others have used it in other research, to refer to this senescent phenotype to distinguish it from replicative senescence that occurs following repeated passages of cells in culture (139). Senescent cells are still metabolically active although they have lost the capacity to proliferate (162) and how they die remains incompletely understood. In our study, whether irradiated PAEC that have undergone senescence have altered response to apoptotic stimuli is not investigated since irradiation of PAEC yields both apoptotic and senescent phenotypes. Although apoptosis has long been considered to cause the loss of clonogenic ability of cells exposed to various cytotoxic agents, treatment-induced senescence has been thought to also contribute significantly to this loss of replicative capacity (162). Our results suggest that at lower doses, accelerated senescence is the predominant mode of loss of cell viability in PAEC.

We hypothesize that IR-induced loss of PAEC viability may then contribute to increased vascular permeability and subsequent pulmonary remodeling and fibrosis.

In the present investigation, we employed a combination of dose-response and time course assays to provide a comprehensive evaluation of the fate of irradiated cells. We found that our results are inconsistent with the findings of another study conducted by Stitt-Fischer 2010 on X-rays irradiated PAEC where the authors concluded that neither apoptosis nor accelerated senescence was induced following exposure of PAEC to X-rays (174). We hypothesized that the differences between the findings and interpretations of the Stitt-Fischer study and our present investigation are due to differences in the time courses examined, as well as in the assay conditions performed during respective investigations. For example, we show through a full time course study that caspase-3 is activated at 6 and 12 hours post-irradiation which disappears at later time-points. This is confirmed by the results of the time-course assays for activation of caspases-3, 8, and 9 similarly done over a 24-hour time course. In contrast, the study by Stitt-Fischer examined the activation of caspase-3 at one time-point (174), the 24-hour time-point where caspase-3 activation is no longer detectable by either western blotting or biochemical assay.

The collective data in the first part of the dissertation reveals the fate of irradiated normal lung cells. In theory, exposure of cells to ionizing radiation results in two main consequences – survival or death. At lower doses of X-rays, a fraction of PAEC still survived; these are the cells that have undergone successful repair and remained viable. Increasing doses of X-rays increased the incidence of loss of clonogenic ability and switched the mode from accelerated senescence to apoptosis. It is likely that further

increasing the dose beyond 50 Gy would induce necrosis in some cells of the irradiated PAEC population, possibly in a similar trend observed in a study on leukemia cells (190). Our results are consistent with theories that the magnitude of stress switches cell deaths from senescence to apoptosis to necrosis (173). Indeed, findings from other investigations have shown that this happens across a variety of cytotoxic agents including cisplatin-induced cytotoxicity in human proximal tubule and lymphoma cell lines (167) and doxorubicin-treated human hepatoma cell lines (54). In the context of radiotoxicity, particularly during radiotherapy, the knowledge of the type of cell death of normal cells may be useful in designing a more precise therapeutic strategy as one or several modes of cell death may be induced by a specific dose with different signaling pathways being triggered for each mode.

In the second part of this dissertation, we examined the activation and contribution of ER stress in IR-induced cell death. A number of studies have demonstrated that ROS and agents that generate ROS induces ER stress in cells (53; 132; 178; 203). For the purposes of this investigation, we used a dose of 50 Gy to examine ER stress as this dose causes apoptosis in addition to accelerated senescence, allowing us to study ER stress especially in the context of apoptosis in irradiated PAEC. We determined that markers of ER stress are induced post-irradiation. This suggests that PAEC deal with protein misfolding, likely a result of protein oxidation, a well-established consequence of IR (42; 50). GRP78, a known chaperone for misfolded protein (13), was activated post-irradiation suggesting the induction of the ATF6 arm of the ER stress response. On the other hand, the transient increase in eIF2α phosphorylation in irradiated PAEC is indicative of the ER stress response via the PERK pathway. Moreover, the

increase in CHOP and GADD34 expressions validates the activation of the two pathways. Our results confirm previous studies showing that radiation may cause induction of ER stress (80) and we extend our investigation to clarify whether ER stress activation upon exposure to IR mediates cell death. Since salubrinal, a specific inhibitor of eIF2α dephosphorylation (22), rescues the irradiated PAEC from undergoing apoptosis as observed in neutral comet assay, the ER stress response post-irradiation in PAEC appears to mediate the induction of apoptosis. Furthermore, western blotting for cleaved caspase-3 in the presence of salubrinal suggests that IR-induced ER stress-mediated apoptosis is caspase-3-dependent. Although the activation of ER stress contributes to IR-induced apoptosis, the salubrinal experiments suggest that ER stress is not upstream of IR-induced accelerated senesence. As such, inhibition of IR-induced ER stress might alleviate the adverse side effects of IR-induced apoptosis and may increase the replicative ability of irradiated cells.

In the third part of the dissertation, we investigated the mechanisms of IR-induced accelerated senescence. For this part, we used 10 Gy of X-ray dose to eliminate the background signals that could potentially come from apoptosis if 50 Gy were used, or from fractions of surviving PAEC if lower doses were used. As 10 Gy induces loss of cell viability in the majority of the PAEC population through accelerated senescence, the use of this amount of dose suited our purpose.

We addressed the question of whether accelerated senescence induced by IR involves continuous proliferative signals in the presence of cell cycle arrest. This is a recent view on cellular senescence coming from research on inhibition of mTOR by rapamcycin demonstrating that rapamycin delays and attenuates cellular senescence (17;

27; 43). Irradiation of PAEC in the presence of rapamycin rescues the development of the senescent phenotype including p21/waf accumulation and the increase in SA-β-gal activity consistent with the findings of studies on other agents of accelerated senescence (Demidenko and Blagosklonny 2008; Blagosklonny 2011; Cao, Graziotto et al. 2011(101). The IR-induced senescent phenotype is also attenuated by Ly294002, an inhibitor of the PI3K pathway, in a similar manner conferred by rapamycin. mTOR has been considered as an integration point for various growth and proliferative signals in cells and PI3K has been shown to feed growth signals to mTOR through Akt. Although Akt has been known as a mediator of survival and growth signals, several studies have demonstrated that Akt is also involved in the development of cellular senescence and regulates the replicative life span of cells at least in vitro (8; 144). In the case of our study, the attenuation of IR-induced cellular senescence conferred by inhibiting PI3K suggests that this pathway may be involved in IR-induced accelerated senescence.

We then investigated the possibility that IGF-1R serves as a mediator of IR-induced accelerated senescence. Under normal conditions, IGF-1R is involved in cell growth and proliferation and feeds signals to mTOR (141; 151). Because of this connection, we hypothesized that IGF-1R inhibition would attenuate the development of IR-induced phenotype in a similar manner as the rapamycin effect. We used the IGF-1R tyrphostin inhibitor, AG1024, a pharmacological inhibitor acting on the IGF-1R kinase activity (196). In the presence of AG1024, IR-induced accelerated senescence in PAEC was attenuated. Those cells which were negative for SA-β-gal staining did not exhibit the characteristic large flat morphology of senescent cells; instead, they resembled the morphology of non-irradiated cells. Upregulation of p21/waf1 was also reduced in

irradiated cells. Our data is consistent with another study conducted by Lewis et al (2008) on keratinocytes, where the authors showed that UVB-induced premature senescence requires the presence of functional IGF-1R and p53. Taken together, our results recapitulate the idea that senescence is a state of continuous proliferative and growth signaling in the presence of a cell cycle blockade (118).

We also examined the possible activation of IGF-1R post-irradiation to look deeper at the mechanisms of IR-induced cellular senescence. IGF-1R was phosphorylated in irradiated PAEC at an early time-point suggesting at least transient activation of the receptor. We then examined the expression of IGF-1 and IGF-2, two common ligands capable of activating IGF-1R. The mRNA expressions for the two ligands were increased, however, the significant increase occured at a much later time-point. The same is true for the secretion of IGF-1 into the medium. This suggests that activation of IGF-1R occur through a ligand-independent manner prompting us to examine whether reactive oxygen species alone can induce phosphorylation of IGF-1R. Treatment with $H_2O_2$ alone at a relatively low concentration of 10 uM induced phosphorylation of IGF-1R with a fast kinetics, after 20 minutes of treatment which did not induce significant levels of p21/waf1. This fast kinetics was not likely due to synthesis and release of new proteins and suggests that ROS, which is generated during irradiation and through subsequent cellular reactions, contributed to the early time-point activation of IGF-1R.

How does IGF-1R inhibition relate to attenuation of IR-induced accelerated senescence by rapamycin? The IGF-1R inhibitor used in our study also blocked mTOR as shown by decreased phosphorylation of S6 ribosomal protein, a downstream target and a commonly used surrogate marker for mTOR (77) It is likely that the development of IR-

induced accelerated senescence required intact mTOR as we did not find increase in mTOR activity in irradiated PAEC. This finding is similar with the results of the previous studies discussed above (44; 88). We also found that, although inhibition of Akt rescued irradiated PAEC from undergoing senescence, the mechanism appeared to be parallel and Akt was not necessarily involved in IGF-1R-mediated accelerated senescence.

We also found that inhibition of IGF-1R and mTOR prevented cellular senescence by acting on the p53/p21 pathway. The cell cycle arrest and the induction of the other markers of senescence *e.g.* increased SA-β-gal activity and large flat morphology have been considered uncoupled responses (139). Our results show that inhibition of IGF-1R also attenuates the p21/waf1 upregulation post-irradiation suggesting that in addition to relieving the continuous proliferative signaling, it may also relieve cell cycle arrest.

Since IGF-1R is a growth factor known to promote cell proliferation under normal conditions, a number of studies have explored the role of the IGF-1R signaling pathway in the protection of cells from IR-induced apoptosis. In most transformed cells, IGF-1R is widely expressed and confers pro-survival properties following cellular stress (47; 48; 146; 197). A positive correlation has also been shown between IGF-1R activation and radiation resistance in some tumor cells (37; 58; 154; 187). Most of these studies were focused only on the contribution of apoptosis. However, the anti-apoptotic activity of IGF-1R appears to be dispensable in the induction of radiation resistance in a variety of tumor cells suggesting the possibility of an unidentified mechanism (180) IGF-1R was recently implicated in several models of senescence including *in vitro* replicative senescence in primary cortical neurons (38) and in UVB-induced premature senescence

in human keratinocytes (118). Its main ligand, IGF-1, has been shown to enhance and promote senescence in primary cell cultures (75; 183). We find that IGF-1R is not a regulator of IR-induced apoptosis as neither AG1024 treatment nor IGF-1 administration significantly attenuates caspase-3 activation.

Investigations into the mechanisms of IR-induced accelerated senescence have lagged far behind studies on IR-induced apoptosis. Although accelerated senescence has been regarded as deleterious to cells in culture, it is only recently that inhibition of accelerated senescence has been shown to mitigate IR-induced mucositis *in vivo* mouse model (88). In their study, the authors showed that inhibition of mTOR by rapamycin rescues the loss of replicative ability of the pool of stem cells in irradiated mice, thereby preserving the function of the affected organ (88). Given that inhibition of IGF-1R also blocks mTOR and attenuates IR-induced accelerated senescence in PAEC, one recommendation for future studies is to examine the potential of IG-1R inhibitors as radiation countermeasure and/or mitigator in murine models of thoracic-irradiation induced pulmonary pneumonitis and fibrosis. Various IGF-1R inhibitors are already under clinical trial for the treatment of certain cancers which will make it more feasible for use in the future. However investigating whether they can actually block IR-induced biological effects and qualify as radiation countermeasures is still a necessary requirement.

Although much research has focused on epithelial cell death in IR-induced pulmonary injury and fibrosis, accumulating evidence reveals the significance of endothelial cell injury in precipitating IR-induced pulmonary pathologies. Studies have shown that endothelial cell damage is an early consequence of lung exposure to radiation,

triggering vascular leak and inflammation (65; 95; 147; 207) that are postulated to be involved in the development of pneumonitis and pulmonary fibrosis (69; 102; 109). Senescent cells also exhibit senescence-associated secretory phenotype which is characterized by increased secretion of inflammatory factors (36). This ties the importance of IR-induced endothelial cell damage to the overall consequence of lung exposure to radiation.

This dissertation explores the mechanisms of IR-induced cell death in primary lung cells particularly endothelial cells. Although the effects of radiation varies among cell types, we expect that most of the findings in this study will be found to be consistent for the effects of radiation on a variety other normal untransformed cells in the body, including endothelial cells and epithelial-like cells of other tissues. It will still be important, however, to demonstrate our findings in other cell types and origin in order to come up with a unifying theme across all systems. It will also be interesting to confirm whether our findings will be similar for other types of ionizing radiation. Together, these will be important in designing effective and safe countermeasures during the course of radiotherapy or from exposure to accidental irradiations.

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