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Dr Al Fornace Ph.D.
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Dear Dr Fornace:
Please find enclosed manuscript entitled “Role of TNF-α and TRAIL in high dose radiation-induced bystander signaling in lung adenocarcinoma” for publication in the “Cancer Research”.
This manuscript highlights the importance of "High-dose radiation induced factors in bystander therapeutics" This study is the first direct evidence that high-dose X-ray irradiation can induce the release of factors into the cell culture to mediate bystander responses as measured by cell survival and apoptosis in lung adenocarcinoma. Based on this, we strongly believe that high dose bystander signaling mechanisms could be the underlying mechanism involved in a novel treatment modality such as high dose spatially fractionated radiation GRID therapy (SFGRT) for management of advanced cancers. In particular, the functionality of EGR1 in mediating the expression of TNF-α is a pivotal genetic factor in the bystander signaling events mediated by high dose radiation in lung adenocarcinoma cells. Thus, this study opens up avenues in understanding the genetic and epigenetic modifications and their interactions leading to differential bystander signaling could be useful in designing appropriate therapeutic approaches as adjuvants of high-dose GRID radiation therapy that is currently being utilized in the treatment of radio-resistant and bulky tumors.

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Because of the conflict of interest, we would like to EXCLUDE Dr. Mary H. Barcellos-Hoff from Berkeley and William Morgan from University of Maryland from the review of this manuscript.

Sincerely yours,

Mansoor M. Ahmed, Ph.D.

Role of TNF-α and TRAIL in high dose radiation-induced bystander signaling in lung adenocarcinoma

Running Title: TNF-α and TRAIL in radiation-induced bystander signaling

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Abstract

In the present study, ionizing radiation (IR)-induced bystander effects were investigated in two lung cancer cell lines. A549 cells were found to be more resistant to radiation conditioned medium (RCM) obtained from A549 cells exposed to 10 Gy IR when compared to the H460 cells. Significant release of TNF-α was observed in A549 cells following IR/RCM exposure and the survival reversed when the neutralizing antibody against TNF-α was added to RCM. In H460 cells, significant release of TRAIL was observed in response to IR, RCM exposure or RCM+2Gy but not TNF-α and neutralizing antibody against TRAIL diminished RCM-mediated clonogenic inhibition. Mechanistically, TNF-α present in 10 Gy RCM of A549 was found to mediate NFκB translocation to nucleus, whereas, the soluble TRAIL present in 10 Gy RCM of H460 cells mobilized the nuclear translocation of PAR-4 (a pro-apoptotic protein). Analysis of function of IR-induced EGR-1 that regulates TNF-α, demonstrated that EGR-1 was functional in A549 cells but not in H460 cells. A significant decrease in RCM mediated apoptosis was observed in both A549 cells transiently expressing siRNA EGR-1 and EGR-$1^{-/-}$ MEF cells. Thus, the high-dose IR-induced bystander responses in A549 may be dependent on the EGR-1 function and its target gene TNF-α. These findings demonstrate that the reduced bystander response in A549 cells is due to activation of NFκB signaling by TNF-α, whereas, enhanced response to IR-induced bystander signaling in H460 cells was due to release of TRAIL associated with nuclear translocation of PAR-4.

Introduction

A central radiobiological paradigm for several years contended that the biological effects of exposure to ionizing radiation (IR) occur only in directly irradiated cells (1). Over the past two decades, data has been emerging that challenge this paradigm, indicating that important biological consequences of exposure to IR may arise in cells which in themselves receive no radiation exposure. Biological effects arise in cells that receive no radiation exposure as a consequence of damage signals transmitted from neighboring cells, a phenomenon known as *radiation-induced bystander effect* (2). It suggested that irradiated cells could secrete molecules into both the culture medium and pass on to neighboring cells by gap junction (3).

Although evidence for these factors has been accumulating over past decades (2), their exact nature as well as the mechanisms by which they cause the distant bystander effects have proven elusive. One such mechanism might be through radiation-induced early genes, that function as transcriptional factors. The most common, radiation induced early gene, early growth response-1 gene (*Egr*-1), has a radiation inducible promoter (4). Induction of EGR-1 promotes the elevation of growth factors or their receptors, such as transforming growth factor-β1, TNF-α, or epidermal growth factor receptor (5). Ectopically expressed EGR-1 synergized IR-inducible tumor necrosis factor-alpha (TNF-α) expression and apoptosis (6, 7). TNF-α and TNF-related apoptosis-inducing ligand (TRAIL) are directly involved in apoptosis and are induced by ionizing radiation (6-9). To understand the molecular mechanisms involved in the effects of IR-induced bystander signaling in non small cell lung cancer cells and to identify the putative soluble clastogenic factors, we used lung carcinoma A549 and H460 cell lines that are functionally EGR-1 positive and negative respectively. In order to decipher the role of factors released by irradiated lung cancer cells into the medium, in the present study, medium transfer

experiments were employed. The findings presented here demonstrate a distinct EGR-1 dependent and independent role in conferring the cell killing effects of IR-induced bystander signaling regulated by TNF-α and the soluble form of TRAIL.

Material and Methods

Plasmids constructs and Transfections

The reporter construct, EBS-CAT, was described earlier (10). pSilencer control and pSilencer vector used for generation of EGR1 siRNA stables, were obtained from Panomics, CA, USA. Adenoviral EGR1 constructs (EGRI293F) was a gift from Dr. Jeffery Milbrandt from Washington University School of Medicine (11, 12). Transient or stable transfections were performed by effectene transfection reagent kit (Qiagen Inc., Valencia, CA).

Cell lines, culture and generation of stable clones expressing si-Egr-1

The lung cancer cell lines, A549 and H460 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). These lung cancer cell lines were cultured in RPMI-1640 media with 10% FBS, 1% penicillin/streptomycin. Primary cultures of mouse embryonic fibroblast (MEF) cells from homozygous (-/-) and heterozygous (+/-) EGR-1 knock-out mice were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO_2. To generate *Egr*-1 siRNA stable cell lines, the plasmids p*Silencer*-si-*Egr*-1 and p*Silencer* vector DNA were transfected separately in A549 cells. The stable clones were selected in medium containing 100 μg of neomycin/ml and were maintained in medium containing 50 μg of neomycin/ml.

Generation of "Radiation Conditioned Media" (RCM)

Exponentially growing cells at 80% confluency were irradiated (1-10 Gy) in freshly transferred media using a 100-kVp industrial X-ray machine (Phillips, Netherlands) at room

temperature. The medium from the irradiated cells was removed after 24 hours and centrifuged at 3000 RPM for 3 minutes. The supernatant from this spin, hereby called as "Radiation Conditioned Medium" (RCM), was used for subsequent experiments to study the bystander effect mediated through the soluble factors present in the RCM.

Colony-forming assay and quantitation of apoptosis

For clonogenic cell survival studies, sets of A-549 and H-460 cells were either left untreated or exposed to 1 to 6 Gy IR, or exposed to RCM generated from 1 to 6 Gy exposure. Subsequent studies were performed with 2 or 10 Gy derived RCM. For blocking experiments, neutralizing antibody to either TNF-α or TRAIL (R&D System, Minneapolis, MN), was added prior and post irradiation. Surviving fraction was calculated as described previously (7). To quantify apoptosis, the TUNEL labeling kit (Roche) was used as described by manufacturer and as reported previously (7). Mean and standard error were calculated from four independent experiments.

Enzyme-linked Immunosorbent Assay (ELISA) and Western blot analysis

Radiation-induced TNF-α and TRAIL levels secreted in the culture supernatants were analyzed by ELISA. Microtiter ELISA plates were coated with anti-TNF-α or anti-TRAIL monoclonal antibody (R & D Systems). These plates were used to estimate levels of TNF-α and TRAIL in RCM, as per the procedure described by us previously (13).

Total proteins isolated using Laemmli buffer were subjected to Western blot analysis using anti-EGR-1 antibody (polyclonal), (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or monoclonal anti-β-actin antibody (Sigma Chemical Co, St, Louis, MI) as an internal loading control.

Immunofluorescence

A549 or H460 cells were cultured on Lab-Tek chamber slides (Nunc Inc., Naperville, IL) and were either left untreated or exposed to 10 Gy IR or exposed to RCM from 10 Gy IR or RCM exposure followed by 2 Gy IR. After blocking with 3% BSA in PSBT (PBS, pH 7.4, with 0.25% Tween-20) slides were then incubated overnight at 4ºC in primary antibodies, mouse anti-p65 and/or rabbit p50 (Santa Cruz Biotechnology) or anti-TRAIL or anti-PAR4 diluted 1:100 with the blocking buffer. Following a series of washes in PBST, the cells were overlaid with secondary antibodies, Cy2-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG, diluted 1:1000 in blocking buffer. The slides were mounted using antifade and counterstained with DAPI (VectaSheild, Vector). Slides were visualized using a Zeis epifluorescence microscope.

Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP)

Nuclear extract were isolated from untreated and treated A549 and H460 cells and subjected to EMSA using Nushift kit for Egr-1 (Geneka Biotechnology, Montreal, Canada). The oligonucleotide consensus probe that contains Egr-1 binding site (underlined): 5'-GGATCCAGCGGGGGCGAGCGGGGGCGAACG-3' was obtained from Geneka Biotechnology (Montreal, Canada). Rabbit EGR-1 polyclonal antibody (Geneka Biotechnology,

Montreal, Canada) was used for super-shift experiments. Binding reactions were electrophoresed on a 5% polyacrylamide gel electrophoresis buffer to separate the bound and unbound probe.

ChIP assay was performed as per Orlando et al with some modifications (14) using treated or untreated 2-5 x 10^9 A549 cells and were fixed using 1% formaldehyde for 10 min. After stopping the cross-linking reaction, cell lysates were subjected to sonication and incubated with rabbit anti-EGR1 antibody for 3 hr at 4ºC. The cross links were reversed at 65ºC for 12 hrs. Proteinase K digestion of the extracts was performed at 37ºC for 7 hrs. DNA was isolated by phenol/chloroform method. ^{32}P-PCR was performed using specific primers for TNF-α promoter (5'-CCACCCAGCCTTTCCTGAG-3', 5'-CGGAAAACTTCCTTGGTGGAG-3') that yielded ≈500 base pair product. Primers for human G6PD gene were used as an internal control.

Results

Differential radiation sensitivity of A549 and H460 cells

Initially, we assessed the cell survival response to IR and RCM in A549 and H460 cells by colony forming assay. Cell survival curves showed that A549 cells were more radio-resistant (D_0= 159 cGy) when compared to H-460 (D_0= 81 cGy) that is similar to previous reports (15, 16). The surviving fraction (SF) at direct 2 Gy dose were 0.656$\pm$ 0.04 and 0.255 $\pm$ 0.02 for A-549 and H-460 respectively. When exposed to RCM from 2 Gy, A-549 and H-460 showed an SF of 1.11 $\pm$ 0.107 and 0.78 $\pm$ 0.2, respectively. These results indicate that the direct radiation responses were similar to that of the RCM responses for both cell lines (Figure 1A, Supplementary-Figure 1, 2). The degree of RCM mediated inhibition was greater in H-460 cells than A549 cells and a proportionate increase in clonogenic inhibition was observed in both the cell lines when a 2 Gy direct radiation was given after RCM exposure (Figure 1A, Supplementary-Figure 1, 2). These findings demonstrate that high dose radiation

induces soluble factors in the media that will contribute to such bystander mediated clonogenic inhibition.

TNF-α mediates the radiation-induced bystander signaling in A549 cells

In order to understand the factors responsible for differential direct and bystander responses to high-dose radiation, we investigated the release of cytokine TNF-α in response to IR, since we reasoned that TNF-α is a most common radio-inducible cytokine. A549 cells exposed to direct IR, RCM and RCM + 2 Gy IR showed significant induction of TNF-α protein in the media as assessed by the ELISA. In H-460 cells, no significant changes in the levels of TNF-α were observed in different treatments as compared to untreated control (Figure 1B). In order to ascertain the specificity of TNF-α in regulating the bystander cellular responses, experiments were designed to block the radiation-induced TNF-α function by using neutralizing antibodies against TNF-α and the effect was assessed on cell survival of A549 and H460 cells by colony-forming assays. A549 cells showed increased cell survival when the function of TNF-α was blocked by neutralizing antibody in a dose dependent manner irrespective of any treatment approach used (Figure 1C). On the other hand, H460 cells showed little or no changes in cell survival when the TNF-α was blocked in all treatment groups (Figure 1C). These results demonstrate strongly that TNF-α might play a potential role in eliciting bystander mediated clonogenic inhibition in A549 cells but not in H-460 cells.

TRAIL mediates the radiation-induced bystander responses in H460 cells

Clonogenic inhibition observed in H460 cells exposed to RCM or RCM plus 2 Gy direct radiation was found to be independent of TNF-α effects (Figure 1B,C). Therefore, we searched for the genes mediating such responses in H460 cells based on the following contentions (i) should be inducible by ionizing radiation; (ii) should be functionally involved in apoptosis; and

(iii) should be released into the medium. One of the genes that met all the above criteria was the Apo2 ligand (Apo2L) / TNF-related apoptosis-inducing ligand (TRAIL) (17, 18). To ascertain the potential involvement of TRAIL in high dose bystander responses, we analyzed the kinetics of TRAIL in response to direct and RCM treatments in both the cell lines. While significant induction of TRAIL was observed in H-460 cells exposed to direct radiation, RCM and RCM+2 Gy, as measured by ELISA, no significant induction of TRAIL was observed in A549 cells in response to any of the treatments (Figure 1D). Blocking the activity of TRAIL using neutralizing antibodies did not alter the cell survival of A549 cells (Figure 1E). However, H460 cells showed an increase in cell survival when treatment-induced TRAIL was blocked by neutralizing antibody against TRAIL, largely in the RCM and RCM+2 Gy exposure as compared to the IgG control (Figure 1E). These results demonstrate that TRAIL can be a potent bystander soluble factor in mediating high-dose radiation-induced bystander effects in H460 cells.

TNF-α causes the activation of NFκB in A549 cells

It is known that TNF-α initiates NFκB nuclear translocation by causing dissociation of the inhibitory protein IκBα from NFκB dimeric complex and thereby rapid degradation of IκBα (19). Since IR-induced TNF-α levels were elevated in A549, we reasoned that this TNF-α would mobilize the subunits of NFκB (p65 and p50) into the nucleus, leading to the activation of the NFκB signaling. To assess this activation, double immunofluorescence was performed in A549 and H460 cells exposed to either 2 Gy, 10 Gy IR, RCM and RCM + 2Gy direct IR. It was found that A549 cells but not the H460 cells showed the translocation of NFκB subunits into the nucleus (Figure 2A). Next, to ascertain if the RCM from A549 cells, which is specifically enriched in TNF-α could induce the translocation of NFκB to the nucleus of H460 cells, H460 cells were exposed to RCM obtained from A549 cells, and immunofluorescence was performed.

Results demonstrated that RCM from A549 cells caused translocation of NFκB subunits into the nucleus of H460 cells (Figure 2A). In order to confirm the specificity of this mechanism, TNF-α was blocked using a neutralizing antibody against TNF-α and the translocation of NFκB complex to the nucleus was assessed by immunoflourescence in H460 cells containing RCM from A549 cells. Anti-TNF-α neutralizing antibody against TNF-α blocked the nuclear translocation of p50 in both A549 and H460 cells exposed to RCM derived from A549 cells (Figure 2B). These results demonstrate the specificity of TNF-α present in the RCM of A549 and such event might contribute to the degree of resistance to bystander responses.

TRAIL mobilizes PAR4, a pro-apoptotic protein, into the nucleus

The *Par*4 gene is specifically induced during the process of apoptosis (20, 21, 22, 23). It was demonstrated that nuclear localization of PAR4 is essential for apoptosis by PAR4 (24). Further, ectopic expression of PAR4 in neoplastic lymphocytes augments sensitivity to TRAIL-induced cell death (25). In order to understand the molecular mechanism involved in TRAIL mediated cell death, we analyzed the PAR4 localization in cells exposed to recombinant TRAIL or soluble TRAIL in RCM from H460 cells, by immunofluorescence. We observed that the PAR4 translocated to the nucleus in both the A549 and H460 cells in response to IR although there was no evidence showing the release of TRAIL into medium of A549 cells (Figure 2C). In addition, both the cell lines showed the translocation of PAR4 to the nucleus in response to the treatment with recombinant TRAIL (Figure 2C). These results signify that TRAIL mediated effects might involve PAR4 translocation to the nucleus, that is essential to execute cell death in H460 cells.

Differential induction of membrane bound form of TRAIL in A549 and H460 cells

TRAIL was significantly elevated in RCM from H460 cells when compared to that from A549 cells (Figure 1D). However, the mobilization of PAR4 to the nucleus was observed in both the A549 and H460 cells in response to radiation as well as recombinant TRAIL (Figure 2C). Since both soluble and membrane bound form of TRAIL have been described in transformed T and B cells (26), it was possible that ELISA results demonstrated the presence of only the soluble form of TRAIL. The conversion of the membrane bound form of TRAIL to soluble form is regulated by cysteine protease. Thus, the differential release of TRAIL into the media could be due to the differences in the expression of cysteine protease regulating the expression of different forms of TRAIL. To test this hypothesis, we analyzed the TRAIL expression in the A549 and H460 cells by immunofluorescence. Our results demonstrated that there is a significant increase in the membrane bound TRAIL in response to IR in A549 cells (Figure 2C). On the other hand, there was a significant decrease in the membrane bound TRAIL in H460 cells exposed to IR (Figure 2C). To observe if RCM from A549 and H460 cells can cause the mobilization of PAR4 to the nucleus, A549 and H460 cells were exposed to the RCM from A549 and H460 cells and the localization of PAR4 was analyzed respectively. As expected, the RCM from H460 but not that from the A549 cells caused translocation of PAR4 into the nucleus in both the A549 and H460 cells (Figure 2D). Further, neutralizing antibody against TRAIL, abrogated the effects of H460 RCM on PAR4 mobilization to the nucleus (Figure 2D). These findings indicate that the differential bystander responses in these two cell lines could be due to the induction of membrane versus the soluble form of TRAIL. Hence, these findings helped to dissect the molecular mechanism of pro-apoptotic effects of bystander signaling (through TRAIL and PAR4) in the RCM of H460 cells.

RCM swapping in A549 and H460 cells reverses the clonogenic response of the cells

In order to understand if the bystander mediated cell survival responses are unique to the cell type or the soluble factors released in the RCM, we exposed the RCM from A549 and H460 cells to H460 and A549 cells respectively and assessed the surviving fraction. Iso-transfer of RCM resulted in H460 cells being more sensitive to bystander effects when compared to A549 cells. However, survival effect was reversed when RCM was swapped (hetero-transfer) in which A549 cells showed increased sensitivity when exposed to the RCM from H460, whereas, H460 cells showed reduced sensitivity when exposed to RCM from A549. These observations demonstrate the specificity of soluble factors in RCM of both A549 and H460 in mediating the bystander responses (Figure 3A,B). Similar results were observed in MEF cells with none or a single copy of Egr-1 gene when the RCM was swapped between these cells suggestive of a role for EGR-1 in high radiation dose induced bystander signaling (Supplementary Figure 4).

Radiation induces EGR1 in A549 cells

Since TNF-α is a known target of EGR1 (5), we investigated if the differences in bystander responses are due to functionality of EGR1. Western blot analysis of the EGR-1 protein expression in A549 and H460 cells exposed to direct or RCM or RCM plus 2 Gy direct IR, showed that EGR-1 protein level was significantly induced in A549 but was absent in H460 (Figure 4A). To further confirm the function of endogenous EGR1 in A549 cells in response to radiation, EMSA was performed. Maximal binding of EGR1 consensus probe was observed in RCM + 2 Gy dose as compared to RCM exposure. This kind of specific binding was absent in H460 cells (Figure 4B). The transactivation function of EGR1 as measured using EBS-CAT reporter activity was present in A549 cells in response to the above treatments but was absent in H460 (Figure 4C), indicating that A549 cells harbor functional EGR1. Previous studies from our

group and others had clearly demonstrated the role of EGR1 in regulating TNF-α expression (6-8). To ascertain if EGR1 in A549 cells regulates the expression of TNF-α, the binding of EGR1 in TNF-α promoter was assess by ChIP assay. Significant binding of EGR1 to TNF-α promoter was detected in untreated and irradiated A549 cells as compared to the absence of PCR product for HG6PD gene in immunoprecipitate (Figure 5A), confirming the specificity of EGR1 targeting TNF-α promoter. In addition, slightly higher enrichment was observed for TNF-α promoter in cells treated with 10 Gy radiation as compared to the untreated cells (Figure 5A) indicating that radiation-induced EGR1 protein is directed towards binding to TNF-α promoter. To further ascertain the role of EGR1 in regulating the expression of TNF-α, endogenous EGR1 expression in A549 cells was silenced by stable transfection using siRNA against EGR1 (Supplementary Figure 3). EGR1 siRNA caused significant decrease in expression of TNF-α in all treatment exposures (Figure 5B). These observations strongly suggest that the TNF-α induction is mediated by EGR-1 in bystander responses in A540 cell background.

EGR1 function is essential in eliciting bystander mediated apoptotic responses in fibroblast and A549 cells

Functional studies have demonstrated that EGR1 acts to increase the potency of apoptotic agents (5, 27, 28). In contrast, the studies using A-549 and H-460 cells clearly demonstrates that EGR-1 function might play an opposite role in regulating direct and bystander responses of A549 and H460 cells. Because the cell lines tested here were genetically unstable cells with complex aberrations, it was imperative to test this hypothesis in normal isogenic background with disruption of only EGR-1 gene. Therefore, to further dissect the role of EGR-1, we used genetically matched isogenic MEF cells for EGR-1 function. When compared to *Egr*-$1^{+/-}$ MEF cells, the *Egr*-$1^{-/-}$ MEF showed 50% diminished apoptosis in response to RCM or RCM plus 2

Gy IR (Figure 5C). When EGR-1 protein expression was restored in *Egr*-1$^{-/-}$ MEF cells by Ad/GFP-*Egr*-1 infection, the apoptotic response was reversed, resulting in a greater response than that observed in parental MEF cells harboring one allele of *EGR*-1 (Supplementary Figure 5). These observations strongly indicate that EGR1 function is pivotal in eliciting high-dose radiation bystander response. Having ascertained the role of EGR1 function in normal cell fibroblast background, we sought to determine the impact of EGR1 inhibition on bystander response in A549 cells. A549 cells stably expressing EGR1 siRNA showed diminished apoptosis when compared to the control siRNA expressing A549 cells in response to 2 Gy IR, RCM or RCM plus 2 Gy exposures (Figure 5D). These findings suggest that EGR1 play an important role in regulating the high-dose bystander response in both epithelial and fibroblast cell type.

Discussion

This study is the first direct evidence that high-dose X-ray irradiation can induce the release of factors into the cell culture to mediate bystander responses as measured by cell survival and apoptosis in lung adenocarcinoma. Previously, experiments using high–LET micro-beam and biophysical modeling have demonstrated that bystander effects are observed even at high doses of IR (29). While the low dose bystander effect in a biological system is of relevance to carcinogenesis and could have serious implications for radiation risk assessment, the bystander signaling induced by high dose has significant therapeutic implications. High dose bystander signaling mechanisms could be the underlying mechanism involved in a novel treatment modality such as high dose spatially fractionated radiation GRID therapy (SFGRT) for management of advanced cancers (30-34). Significant induction of LDL-enriched ceramide, secretory SMase and TNF-α in serum from patients treated with SFGRT is suggestive of involvement of bystander signaling mechanisms in this therapeutic approach (13, 35). From this

perspective of using an *in-vitro* approach involving radiation-conditioned media transfers, our study establishes first evidence in the literature that high-dose radiation induces bystander signaling that contributes to a robust cell killing effect.

It has been shown that a protective adaptive response was elicited, where bystander cells (human lung fibroblasts exposed to media from 1 cGy) that are subsequently irradiated at doses 2 or 4 Gy were more radio resistant than cells not exposed to bystander signals (36). In our studies, the RCM from 10 Gy was significantly potent to super-sensitize the effects of subsequent 2 Gy dose. Based on the findings of Iyer et al (36) that low-dose inducing factors might cause induced radiation resistance response for subsequent doses of radiation and on the contrasting side that the results of this study strongly demonstrates that high-dose inducing bystander factors contribute to hyper-radiation sensitivity response to the subsequent radiation doses.

While the existence of radiation-induced bystander effects (at low doses) has been well established, the underlying mechanisms and the nature of signals in high dose radiation-induced bystander effects are largely unknown. Using the inhibitors of ROS, including SOD and catalase, Yang et al (37) demonstrated that ROS has no effect on the survival of bystander human fibroblast cells, suggesting that irradiated cells (at 0.1 to 10 Gy) release toxic factors other than ROS into the medium (37). Several non-ROS factors that we had deciphered as bystander signals in clinical samples, such as TNF-α, SMase and Ceramide could be involved in mediating such bystander effects at high doses of radiation (13, 35). Thus, our study is the first one to report the induction of TNF-α and TRAIL as high dose IR-induced bystander pro-apoptotic factors in lung adenocarcinoma cells.

Our previous studies demonstrated the elevation of TNF-α in SFGRT patients suggestive of its role in eliciting bystander and abscopal signals (13). TNF-α was reported to regress tumors in both humans and mice in an acute and dramatic manner, hence, the name tumor necrosis factor was established. TNF-α, a key pro-inflammatory cytokine when bound to TNF receptor 1 may cause either survival or apoptosis, depending on the biochemical modification that determines the type of complex formed; one complex causes NFκB activation (pro-survival) while the other complex recruits caspases and causes apoptosis (38). In this study, we found mobilization of NFκB complex into the nucleus in A549 cells subjected to high-dose radiation direct and bystander exposures. This mobilization of NFκB complex into the nucleus could explain the differential resistance of the A549 cells where TNF-α is elevated in response to high-dose radiation bystander effects when compared to the H460 cells. These findings demonstrate that even though TNF-α renders pro-apoptotic response to high-dose bystander effects, it might at the same time cause pro-survival signaling due to increased activation of NFκB.

Activation of death domain-containing TNF-related apoptosis-inducing ligand (TRAIL) receptors presents an alternative opportunity to exploit the extrinsic apoptotic pathway to destroy cancer cells as the TRAIL induces cell death predominantly in transformed cells (39). Despite the advantages of TRAIL over TNF-α-mediated therapy, constitutive activation of NFκB dependent genes critically determines the cellular susceptibility towards apoptosis induction by TRAIL receptors R1 and R2 (40). Induction of TRAIL was observed in lymphoma patients treated with radiation therapy and similar induction was found in several cell lines suggesting that TRAIL is a potential bystander signal in mediating abscopal effects in heamatopoietic malignancies (9). Lack of TNF-α induction in H460 cells provoked a search for a different factor that could mediate the bystander response. We found that the expression of soluble TRAIL was

significantly enhanced in the H460 cells when exposed to radiation (Figure 1D). Hence, the enhanced release of TRAIL in RCM explained the increased cell death in RCM exposed H460 cells. The decrease in the cell death observed in A549 cells with the RCM from A549 cells could also be due to increased activation of NFκB cells as compared to the H460 cells. Hence, the swapping of the RCM from the A549 and H460 cells reversed the cell survival effects demonstrating a role of specific factors in mediating the cell death and cell survival signals (Figure 3A, B). Similar experiments of swapping the medium in MEF cells (EGR-1-/- and EGR-1+/-) (Supplementary Figures 4,5) show that the effects of RCM derived from EGR-1 positive MEF cells as compared to EGR-1 negative cells on cell survival shows a direct positive correlation of EGR-1 function and cell death unlike H460 and A549 cells. This could be due to differences in the genetic background of A549 and H460 cells. The decreased expression of membrane TRAIL in seen H460 cells in response to IR could be due to induction of specific cysteine proteinase which is responsible for cleaving the membrane bound form of TRAIL to produce the soluble form and hence increase soluble TRAIL in RCM was detected in H460 cells. Identification of the factor regulating the expression of cysteine protease responsible for the release of TRAIL into the medium upon IR exposure could explain the differential expression of TRAIL isoforms. In addition, the effects of membrane TRAIL on the A549 cell could possibly be blocked by the activation of NFκB (40) contributing towards increased radio-resistance. Further, it has been demonstrated that PAR4 when gets translocated into nucleus initiates programmed cell death (24). Nuclear localization of Par-4 by NLS2 domain was found to be essential for apoptosis by Par-4 (24). In this study, we found increased mobilization of PAR4 to the nucleus in response to the RCM from H460 but not A549 cells and this was due to TRAIL

(Figures 2 C, D). This may be one of the mechanism by which PAR4 expressing cells are more prone to TRAIL effects as demonstrated by Boehrer et al (25).

Conditioned medium generated by radiation exposure (10 Gy) have demonstrated a role of p53-dependent growth inhibition suggesting that the genetic phenotype is crucial in regulating the bystander effects (41). Since, in this study we found TNF-α is a crucial player in bystander response, we reasoned that an upstream gene (EGR1) that regulates TNF-α gene expression might play a role in bystander response. The functional role of Egr-1 in radiation-induced signaling is pivotal since the promoter of *Egr-1* contains radiation-inducible CArG ($CC(A/T)_6GG$)DNA sequences (42). EGR1 is regulated differentially in response to radiation in A549 and H460 cells. Although the molecular mechanism responsible for this differential regulation is not clear, it is possible that the differential epigenetic regulatory mechanisms of EGR1 transcription may be involved. While our studies clearly demonstrate a role for EGR1 in bystander mediated apoptosis both in the MEF and A549 cells, the relative contribution of EGR1 towards the differences in the radio sensitivity is difficult to assess as the cancer cells often harbor multiple genetic lesions.

The findings from our study are modeled in Figure 6. IR causes the induction of EGR1 expression in A549 cells that leads to increased expression of TNF-α protein. IR also increases the expression of TRAIL in both A549 and H460 cells through an unknown mechanism. However, the release of soluble TRAIL from H460 but not A549 cells may be due to selective induction of an uncharacterized cysteine protease in H460 cells by IR. Membrane TRAIL as well as the soluble TRAIL is capable of nuclear translocation of PAR4 in A549 and H460 cells respectively. However, activation of NFκB by TNF-α in A549 cells may lead to abrogation of

pro-apoptotic effects of both TNF-α and TRAIL explaining the differential sensitivity of these two lung adenocarcinoma cells.

In conclusion, the high dose IR-induced bystander responses like that induced by the low doses, are dependent on function of the genetic makeup of the target as well as the bystander cells. The functionality of EGR1 in mediating the expression of TNF-α is a pivotal genetic factor in the bystander signaling events mediated by high dose radiation in lung adenocarcinoma cells. Thus, understanding the genetic and epigenetic modifications and their interactions leading to differential bystander signaling could be useful in designing appropriate therapeutic approaches as adjuvants of high-dose GRID radiation therapy that is currently being utilized in the treatment of radio-resistant and bulky tumors.

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Abbreviations

RCM-	Radiation Conditioned Medium
TNF-α-	Tumor Necrosis Factor-alpha
TRAIL-	TNF-Related Apoptosis-Inducing Ligand
NFκB-	Nuclear Factor Kappa B
EGR-1-	Early Growth Response-1
PAR4-	Prostate Apoptosis Response-4

Figure Legends

Figure 1: **Differential bystander radio-responsiveness and signaling factors of H460 and A549**. (A) Cell survival of the A549 and H460 cells in response to RCM derived from 2 Gy and 10 Gy as assessed by colony forming assay. Error bars represent standard error derived from three independent experiments. (B) Exponentially growing cells were exposed to 10 Gy, RCM from 10 Gy or RCM plus 2 Gy radiation to assess cytokine expression by ELISA. Significant induction of TNF-α was observed in A549 but not in H460 cells in response to different mode treatments. Error bars represent standard error derived from three independent experiments performed with triplicate samples each time. (C) Blocking the TNF-α mediated bystander signaling by neutralizing anti- TNF-α antibody led to reversal of the cell survival responses in different radiation exposure approaches in A549 but not in H460. *One treatment of anti-TNF-α antibody*: Cells were exposed to anti-TNF-α antibody (2.5 μg/ml), then irradiated with 2 Gy or 10 Gy and the medium was transferred to unirradiated cells and colony-forming assay was performed 24 hours later. *Two treatments of anti-TNF-α antibody*: Cells were exposed to anti-TNF-α antibody (2.5 μg/ml), then irradiated with 2 Gy or 10 Gy and the medium was transferred to un-irradiated cells 24 hours later with a second treatment of anti-TNF-α antibody (1.25 μg/ml) and colony-forming assay was performed. *Three treatments of anti-TNF-α antibody*: Cells were exposed to anti-TNF-α antibody (2.5 μg/ml), then irradiated with 2 Gy or 10 Gy and the medium was transferred to un-irradiated cells 24 hours later with a second treatment of anti-TNF-α antibody (1.25 μg/ml) and colony-forming assay was performed. Twenty-four hours later, these colonies were irradiated with 2 Gy and were immediately exposed to third treatment of anti-TNF-α antibody (2.5 μg/ml). Error bars represent a mean of two experiments and represents standard error. (D) Exponentially growing cells were exposed to 10 Gy, RCM from 10 Gy or

RCM plus 2 Gy radiation to assess cytokine expression by ELISA. Significant induction of TRAIL was observed in H460 but not A549 cells in response to different modes of treatment. Error bars represent standard error derived from three independent experiments performed with triplicate samples each time. (E) Blocking the TRAIL mediated bystander signaling by neutralizing anti-TRAIL antibody led to reversal of the cell survival responses in different radiation exposure approaches in A549 but not in H460. *One treatment of anti-TRAIL antibody*: Cells were exposed to anti-TRAIL antibody (2.5 μg/ml), then irradiated with 2 Gy or 10 Gy and the medium was transferred to unirradiated cells and colony-forming assay was performed 24 hours later. *Two treatments of anti-TRAIL antibody*: Cells were exposed to anti-TRAIL antibody (2.5 μg/ml), then irradiated with 2 Gy or 10 Gy and the medium was transferred to un-irradiated cells 24 hours later with a second treatment of anti-TRAIL antibody (1.25 μg/ml) and colony-forming assay was performed. *Three treatments of anti-TRAIL antibody*: Cells were exposed to anti-TRAIL antibody (2.5 μg/ml), then irradiated with 2 Gy or 10 Gy and the medium was transferred to un-irradiated cells 24 hours later with a second treatment of anti-TRAIL antibody (1.25 μg/ml) and colony-forming assay was performed. Twenty-four hours later, these colonies were irradiated with 2 Gy and were immediately exposed to third treatment of anti-TRAIL antibody (2.5 μg/ml). Error bars represent a mean of two experiments and represents standard error. Colony-forming assay was used to analyze the cell survival of A549 and H460 cells.

Figure 2. **Differential signaling pathways induced in response to high-dose radiation mediated bystander signals.** (A) TNF-α causes the activation of NFκB in A549 cells. A549 and H460 cells were left either untreated or exposed to 2 Gy or 10 Gy direct radiation or exposed to RCM from 10 Gy IR treated cells or RCM plus 2 Gy direct IR exposure. The cells were stained for p65 and p50 by double immunofluorescence. p65 and p50 staining were merged (yellow,

merge or red and green) and shown along side the DAPI (blue) staining for nucleus. All the treatment modalities induce the activation of NFκB as assessed by the nuclear localization of p65 and p50 subunits. None or minimal staining of p65/p50 was observed in the nuclei of H460 cells. (B) Nuclear translocation of NFκB is specifically mediated by TNF-α. A549 and H460 cells were exposed to RCM derived from the 10 Gy treated A549 cells. RCM from A549 cells caused nuclear (red, psuedocolored DAPI) translocation of p50 subunit (green) of NFκB complex that was blocked using a neutralizing antibody against TNF-α. (C) Radiation-induced or recombinant TRAIL causes the translocation of PAR4, a pro-apoptotic protein to the nucleus. Radiation exposure of the A549 and H460 cells (24 hrs) caused increased nuclear (red, pseudo colored DAPI) translocation of PAR4 (green), a pro-apoptotic protein. Membrane bound form of TRAIL (green) was induced with radiation in A549 cells. However, there was a decrease in the membrane bound form of TRAIL as compared to the untreated in H460 cells indicating the release of TRAIL in the medium as a soluble form. Exposure of both the A549 and H460 cells to the recombinant TRAIL caused the translocation of PAR4 into the nucleus. (D) Nuclear translocation of PAR4 is specifically mediated by TRAIL. A549 and H460 cells were exposed to either RCM from H460 or RCM from A549 cells and the localization of PAR4 (green) was studied by immunofluorescence. RCM from H460 that had increased release of TRAIL, but not from A549 cells, induced the mobilization of PAR4 to the nucleus (red, pseudo colored DAPI). This effect was abrogated by the addition of neutralizing antibody against TRAIL in the RCM from H460 cells.

Figure 3. Reversal of cell survival effects in A549 and H460 cells by swapping RCM. A549 cells showed increased cell survival as compared to H460 cells when these cells were exposed to the iso-cell line derived RCM (A). This effect was reversed when these cells were exposed to

hetero-cell line RCM (A549 cells were exposed to RCM from H460 and vice versa) (B). Cell survival was analyzed by the colony-forming assay. Error bars represent standard error from the mean of two experiments.

Figure 4. EGR1 is non functional in H460 cells. (A) EGR1 was expressed constitutively at basal levels and was induced in response to different radiation modalities including direct exposure to 10 Gy, exposure to RCM from 10 Gy irradiated cells and 10 Gy RCM exposure with a consecutive 2 Gy direct exposure, in A549 but not H460 cells as demonstrated by Western immunoblot. β-Actin was used as a loading control. (B) Gel mobility shift assay showing the binding of EGR1 to its consensus oligos in A549 cells but not in H460 cells irrespective of the mode of radiation exposure. The specificity of this shift was demonstrated by super-shift using an antibody against EGR1. Lane B is probe only; lane C represents positive control cell extract and S is super-shift lane. (C). CAT reporter assay demonstrating the induction of EGR1 activity in response to radiation in A549 but not H460 cells.

Figure 5. EGR1 regulates the expression of TNFα and apoptosis. (A) ^{32}P-PCR showing the enrichment of TNF-α promoter in EGR1 ChIP in cells treated with 10 Gy radiation and incubated for 3 hours. There is more enrichment of TNF-α promoter in cells treated with 10 Gy IR than the untreated cells. (B) A549 cells were stably transfected with either EGR1 siRNA or a control siRNA vector. Cells were selected by neomycin. Cells extracts were analyzed for the expression of EGR1 by Western immunoblot. TNFα concentrations were assessed in A549 cells expressing stable EGR1 siRNA versus the control siRNA vectors. EGR1 siRNA caused a decrease in the TNFα concentrations assessed 3 hours after exposure to 10 Gy RCM and 10 Gy RCM exposure plus 2 Gy direct IR exposure. (C) MEF/EGR1$^{-/-}$ or MEF/EGR1$^{+/-}$ cells were exposed to 10 Gy RCM or 10 Gy RCM plus 2 Gy IR. Apoptosis was quantified by TUNEL

assay at 24 and 48 hours. To determine the percentage of cells showing apoptosis, 2000 cells were counted for each experiment. Background levels in untreated were normalized over those in treated cells. Data represents a mean of two experiments. The error bars represent standard deviation. (D) A549 cells expressing the stable vectors of EGR1 SiRNA and its control cell expressing the empty vector were either untreated or treated with 2 Gy RCM, 10 Gy RCM or a combination of 10 Gy RCM plus 2 Gy IR and were incubated for 24 hours. Approximately 2000 cells in total were scored for TUNEL positive cells in each experiment. Data shown are the percent of TUNEL-positive cells as a function of irradiation. Data represents a mean of two experiments. The error bars represent standard deviation.

Figure 6. A diagrammatic model depicting the molecular mechanism of the differential bystander signaling in A549 and H460 cells, induced by high-dose radiation. High-dose radiation induces both TNF-α (via EGR-1) and TRAIL. The TNF-α exerts an induction of NFκB activity in the bystander cell with simultaneous activation of caspases and death, which is more prevalent in A549 cells. Induced TRAIL is processed into soluble form of TRAIL by cysteine proteases and this soluble TRAIL causes nuclear translocation of PAR4 to promote cell death. This signaling is present in H460 cells. Thus, it can be speculated that both the EGR-1/TNF-α and TRAIL/PAR4 signaling might be involved in conferring high-dose radiation-induced bystander effects.

Figure 1

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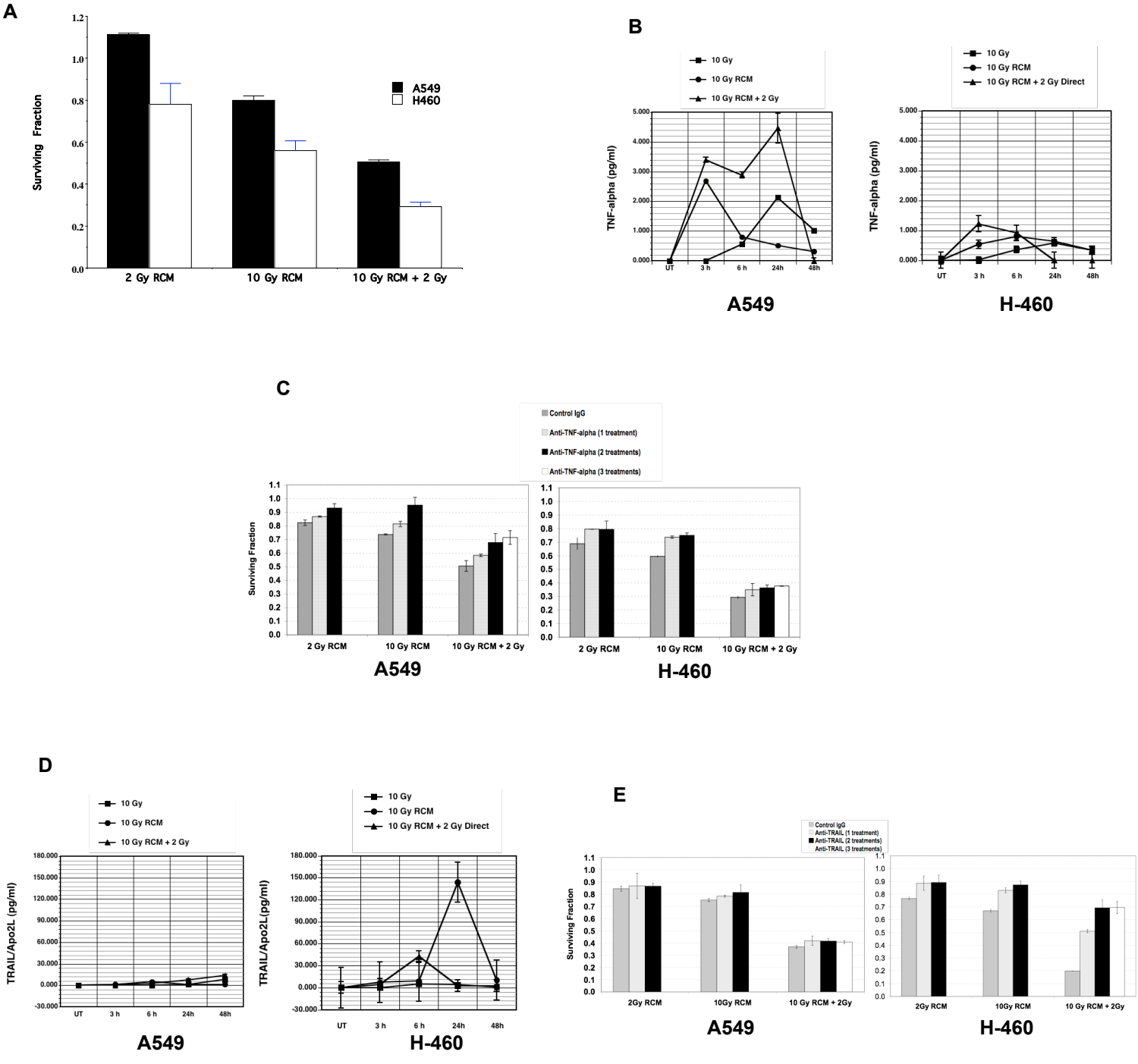


Figure 2

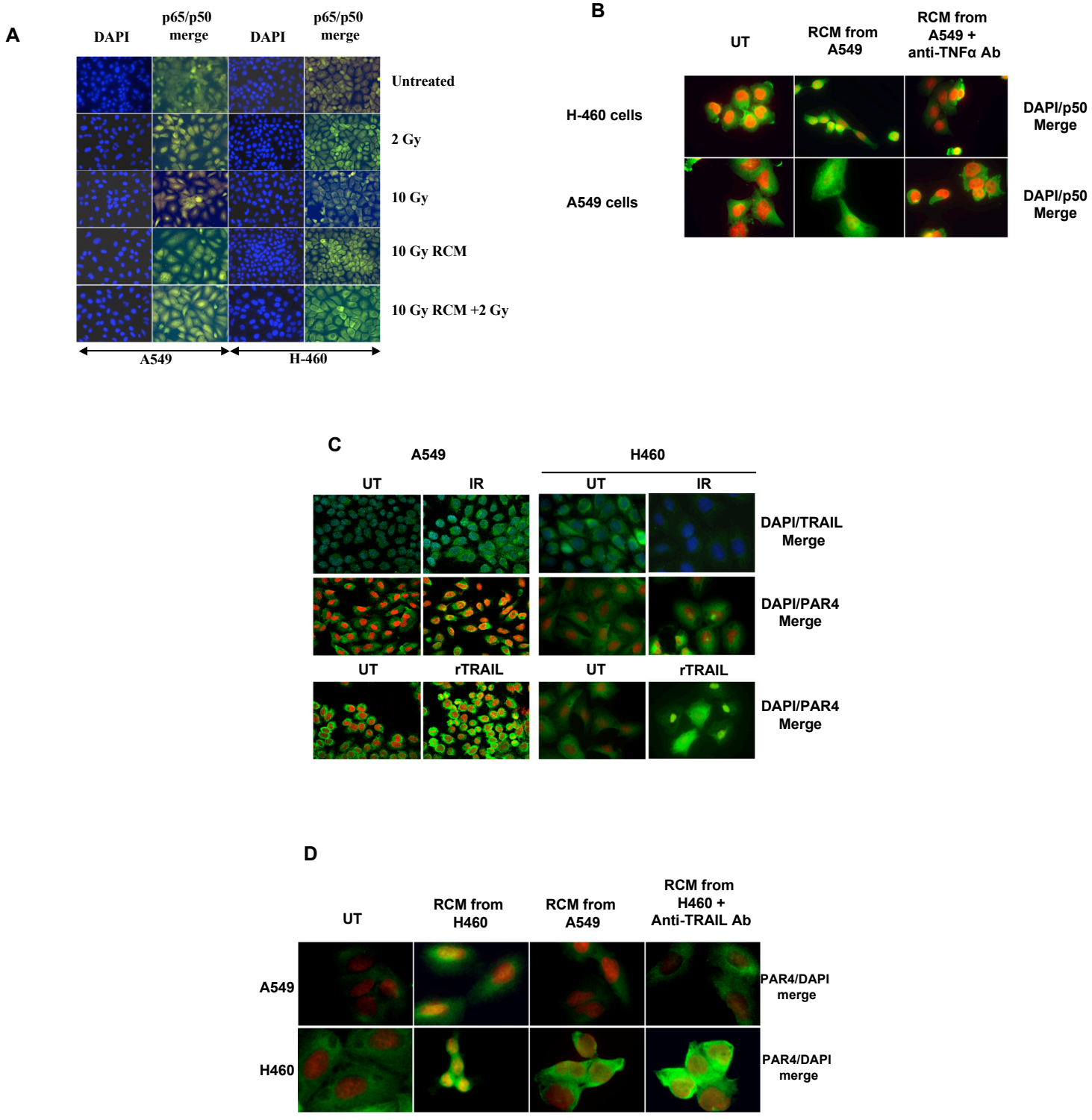

A
DAPI
p65/p50 merge
DAPI
p65/p50 merge
Untreated
2 Gy
10 Gy
10 Gy RCM
10 Gy RCM +2 Gy
A549
H-460
B
UT
RCM from A549
RCM from A549 + anti-TNFα Ab
H-460 cells
A549 cells
DAPI/p50 Merge
DAPI/p50 Merge
C
A549
H460
UT
IR
UT
IR
DAPI/TRAIL Merge
DAPI/PAR4 Merge
UT
rTRAIL
UT
rTRAIL
DAPI/PAR4 Merge
D
UT
RCM from H460
RCM from A549
RCM from H460 + Anti-TRAIL Ab
A549
H460
PAR4/DAPI merge
PAR4/DAPI merge

Figure 3

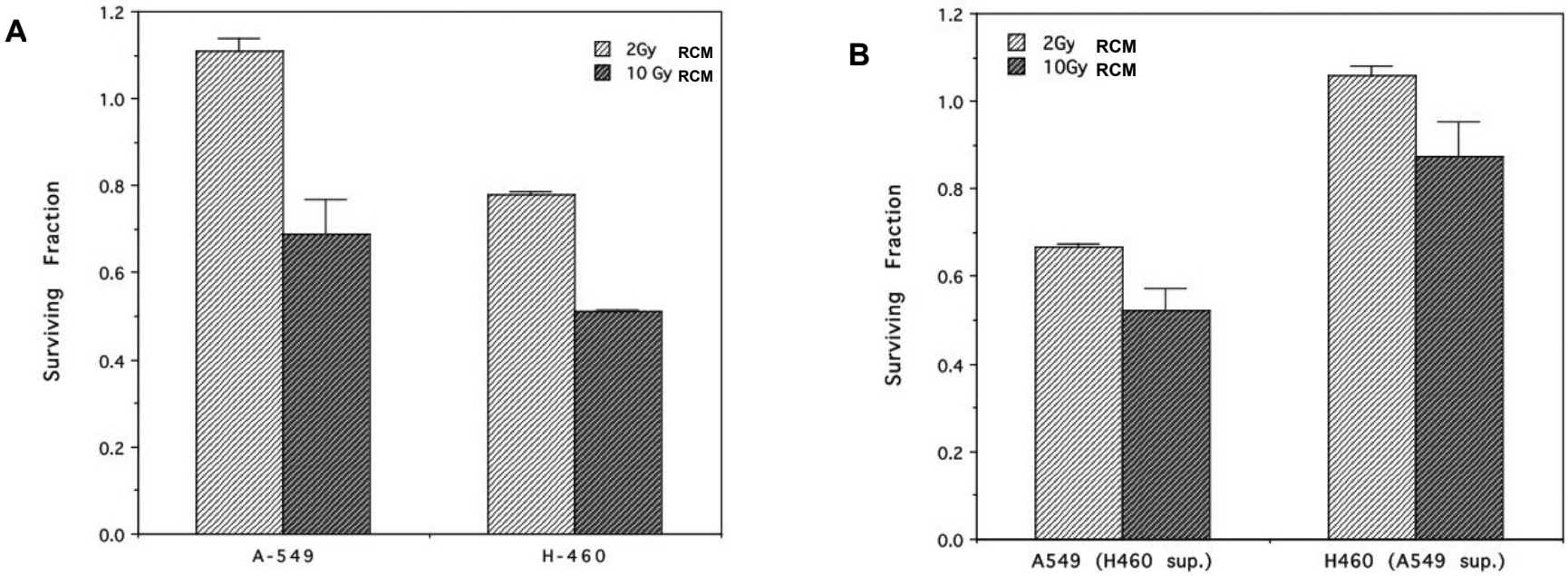

A
Surviving Fraction
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2Gy RCM
10 Gy RCM
A-549
H-460
B
Surviving Fraction
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2Gy RCM
10Gy RCM
A549 (H460 sup.)
H460 (A549 sup.)

Figure 4

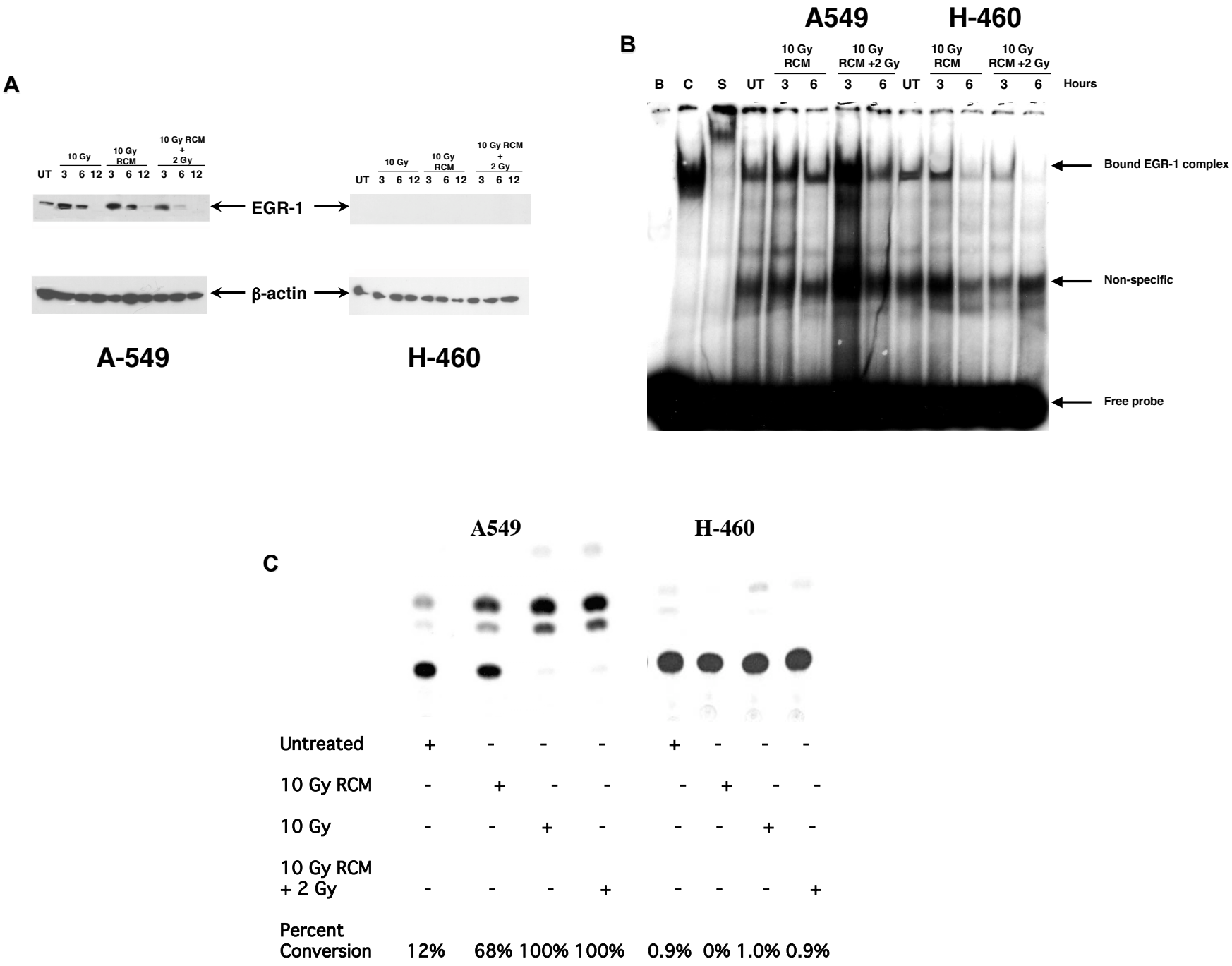
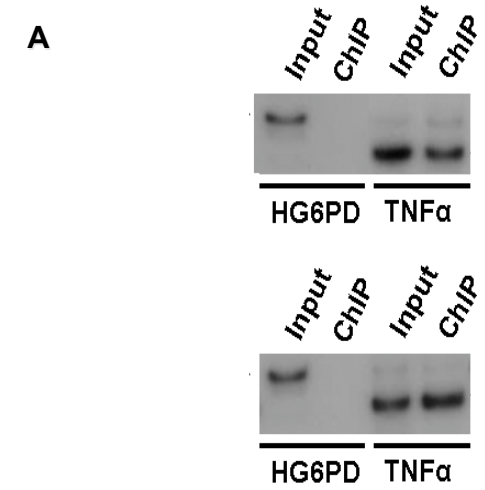
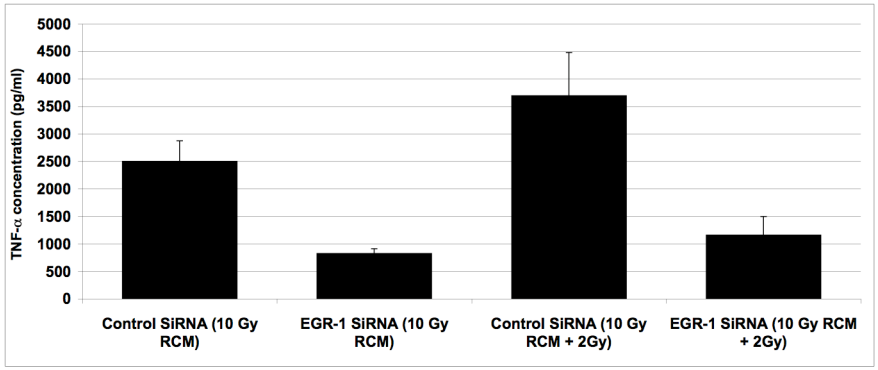
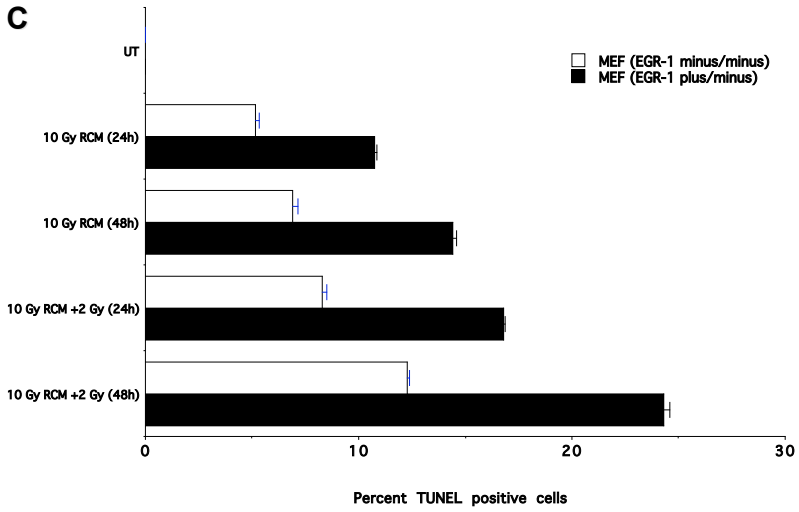

A
10 Gy
10 Gy RCM
10 Gy RCM + 2 Gy
UT 3 6 12 3 6 12 3 6 12
EGR-1
β-actin
A-549
H-460
B
A549
H-460
10 Gy RCM
10 Gy RCM +2 Gy
B C S UT 3 6 3 6 UT 3 6 3 6 Hours
Bound EGR-1 complex
Non-specific
Free probe
C
A549
H-460
Untreated + - - - + - - -
10 Gy RCM - + - - - + - -
10 Gy - - + - - - + -
10 Gy RCM + 2 Gy - - - + - - - +
Percent Conversion 12% 68% 100% 100% 0.9% 0% 1.0% 0.9%

Figure 5


A
Input
ChIP
Input
ChIP
HG6PD
TNFα
Input
ChIP
Input
ChIP
HG6PD
TNFα


B
TNF-α concentration (pg/ml)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Control SiRNA (10 Gy RCM)
EGR-1 SiRNA (10 Gy RCM)
Control SiRNA (10 Gy RCM + 2Gy)
EGR-1 SiRNA (10 Gy RCM + 2Gy)


C
MEF (EGR-1 minus/minus)
MEF (EGR-1 plus/minus)
UT
10 Gy RCM (24h)
10 Gy RCM (48h)
10 Gy RCM +2 Gy (24h)
10 Gy RCM +2 Gy (48h)
0
10
20
30
Percent TUNEL positive cells

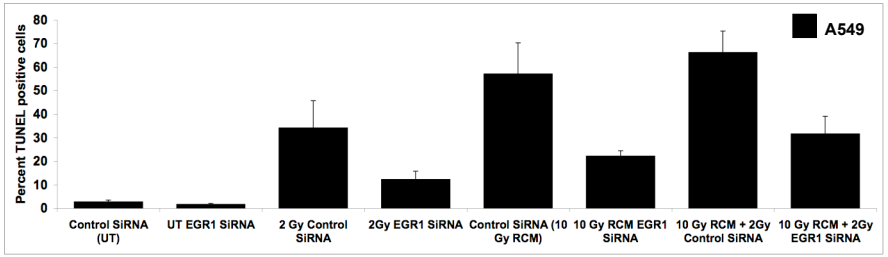
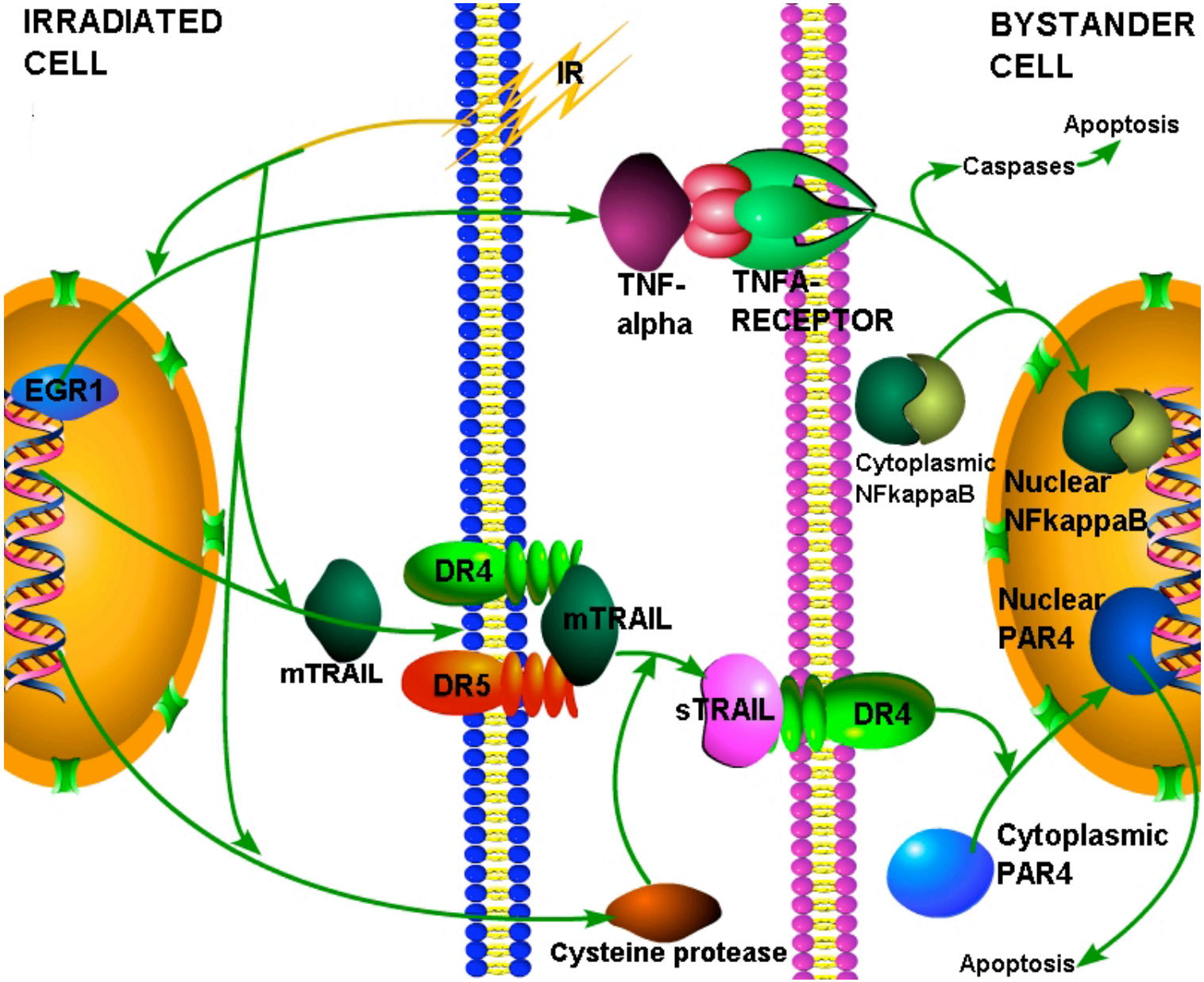

D
A549
Percent TUNEL positive cells
80
70
60
50
40
30
20
10
0
Control SiRNA (UT)
UT EGR1 SiRNA
2 Gy Control SiRNA
2Gy EGR1 SiRNA
Control SiRNA (10 Gy RCM)
10 Gy RCM EGR1 SiRNA
10 Gy RCM + 2Gy Control SiRNA
10 Gy RCM + 2Gy EGR1 SiRNA

Figure 6


IRRADIATED CELL
BYSTANDER CELL
IR
Apoptosis
Caspases
TNF-alpha
TNFA-RECEPTOR
EGR1
Cytoplasmic NFkappaB
Nuclear NFkappaB
DR4
mTRAIL
mTRAIL
DR5
Nuclear PAR4
sTRAIL
DR4
Cytoplasmic PAR4
Cysteine protease
Apoptosis