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A New Paradigm for Radiation-Induced Persistent Cellular Stress and Genomic Instability in Lung Carcinogenesis

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14. ABSTRACT
We study cellular stress responses to sublethal doses of 1 or 2 Gy X Rays that persist up to a week in cultures of an immortalized normal bronchial epithelial cell line, C3KT. We have found that p38MAPK is a driver and genomic instability and cell nsformation an outcome of these responses, thus potentially involved in the development of iogenic cancer. Further studies into these responses revealed altered levels of quitination associated to chromatin, which can be modulated by interfering with p38MAPK ivity. The goal of this project is to evaluate whether dysregulated chromatin quitination is involved in driving persistent genomic instability and is the target for MAPK regulation. The main findings of the concluding period are radiation caused erations in cell cycle progression, the potential identification of persistent DNA repair i as 53BP1 bodies, predominant usage of the homology dependent DNA repair pathway and that atment of HBEC3KT cells with a p38MAPK inhibitor reduces deubiquitinase (DUB) activity.

15. SUBJECT TERMS
genomic instability, cellular stress, cell transformation, DNA repair, ubiquitination

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Table of Contents

	Page
1. Introduction……………………………………………………….	1
2. Keywords……………………………………………………….	1
3. Accomplishments………..…………………………………………...	1
4. Impact…………………………...……………………………………	4
5. Changes/Problems...….……………………………………………	5
6. Products…………………………………….……….…………….	5
7. Participants & Other Collaborating Organizations…………..	5
8. Special Reporting Requirements……………………………………	6
9. Appendices…………………………………………………………	6

1. **Introduction**:

We study cellular stress responses to sublethal doses of 1 or 2 Gy X Rays that persist for up to a week in cultures of an immortalized normal bronchial epithelial cell line, HBEC3KT. We have found that p38MAPK is a driver and genomic instability and cell transformation an outcome of these responses, thus potentially involved in the development of radiogenic cancer. Further studies into these responses revealed altered levels of ubiquitination associated to chromatin, which can be modulated by interfering with p38MAPK activity. The goal of this project is to evaluate whether dysregulated chromatin ubiquitination is involved in driving persistent genomic instability and is the target for p38MAPK regulation. The main findings of the concluding period are radiation caused alterations in cell cycle progression, the potential identification of persistent DNA repair foci as 53BP1 bodies, predominant usage of the homology dependent repair pathway and that treatment of HBEC3KT cells with a p38MAPK inhibitor reduces deubiquitinase (DUB) activity.

2. **Keywords:**

X rays, genomic instability, protein ubiquitination, cell cycle, p38MAPK, deubiquitinase, human bronchial epithelial cell, cell transformation, DNA repair, cellular stress.

3. **Accomplishments**:

a. Major goals of the project:

Specific Aim 1: To determine whether exposure to IR disrupts chromatin associated ubiquitin signaling leading to persistent genomic instability and cellular stress.

Specific Aim 2: To evaluate whether p38MAPK mediated stress responses promote genomic instability and carcinogenesis by regulating chromatin associated ubiquitination

b. Accomplishment of tasks listed in the SOW to be completed during the first year:

For Specific Aim 1:

Major Task 1: Establish methods to evaluate outcomes potentially affected by exposure to IR and by the chromatin associated ubiquitination pathway.

Subtask 1: Development of DNA repair reporter cell lines stably expressing reporter plasmids available from Addgene. They will be validated employing specific DNA damaging agents and/or knock down of key regulating proteins. 100% completed

We obtained cells from the Stark lab that originated the reporter cell systems to monitor the repair of an enzymatically induced DSB: DRG to detect homologous recombination (HR) mediated repair, EJ5 to monitor total NHEJ repair events and EJ2 to detect alternative NHEJ repair events (*1*), thus we did not have to generate and validate reporter cell lines.

*Subtask 2: Examine DNA repair pathway choice by flow cytometry at day 7 post-irradiation employing U2OS cell lines generated in Subtask 1.1.*50% completed

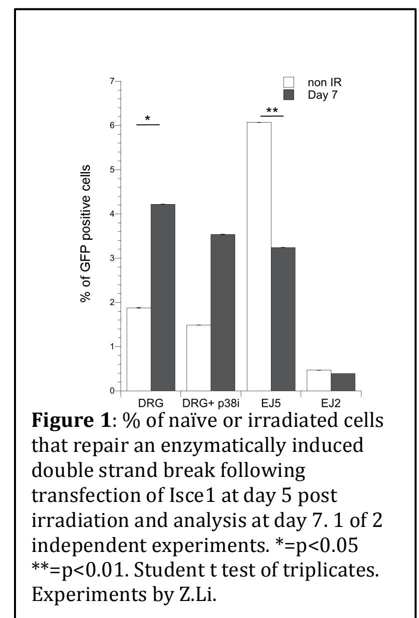

Figure 1: % of naïve or irradiated cells that repair an enzymatically induced double strand break following transfection of Isce1 at day 5 post irradiation and analysis at day 7. 1 of 2 independent experiments. *=p<0.05 **=p<0.01. Student t test of triplicates. Experiments by Z.Li.

We analyzed the efficiency in the 3 reporter cell lines by transfecting the cDNA of ISce1 at day 5 and analyzing for GFP expressing cells at day 7 following exposure to a dose of 2Gy. **Figure 1** shows that after correcting for transfection efficiency in all samples, irradiated cells exhibit a higher rate of repair via HR and lower usage of NHEJ. In contrast, we did not detect any difference in the usage of the alternative NHEJ pathway. This is an additional tool to examine the effect of various strategies to modify ubiquitination and stress responses. We performed one set of experiments in cells treated for 24h with the p38MAPK inhibitor (not shown), where we

detected a small reduction in HR mediated repair, but not statistically significant probably due to a small number of replicates. We will repeat these experiments and incorporate known modulators of HR dependent repair as a reference for the effects observed.

Subtask 3: Examine endogenous DNA repair protein and ubiquitination effector recruitment to foci and end resection activity by immunofluorescence. 10% completed

We have not had success with the detection of new effectors employing commercial antibodies used in publications in cells at day 5 or 7 post-irradiation nor in cells fixed after 2h recovery from1Gy X rays as a positive control. We will employ GFP tagged constructs that we will request to investigators in the field in addition to a different set of antibodies.

Subtask 4: Evaluate cell cycle progression by flow cytometry of non-irradiated and day 7 post-irradiation cells following a 2Gy challenge. 100% completed

We analyzed the cell cycle distribution of HBEC3KT and U2OS cells at day 5 and day 7 post exposure to a 2Gy dose and found, in two independent experiments, a smaller fraction of cells in S phase with a slightly larger fraction of cells in G2 at day 5 post exposure in both cell types (Figure 2A). This observation suggests that exposure to radiation induces an alteration in cell cycle progression. To test this hypothesis, we synchronized U2OS cells with mimosine, which halts the progression of the cell cycle at G1-entry to S phase (2). A16h incubation increased the fraction of cells in G1. Following wash out of the drug, the cells progressed to S-G2 earlier and in larger proportion in irradiated cells (Figure 2B). One explanation for this observation is that irradiated cells have an activated checkpoint that reduces the rate of progression to M and G1. We will test this hypothesis by measuring cells in mitosis (phospho H3) and checkpoint activation. In the next period, we will follow up on these findings, because adaptation to the G2/M checkpoint is a known mechanism leading to micronucleus formation (3) and the generation of 53BP1 nuclear bodies (4), which we have observed in preliminary experiments with irradiated U2OS and HBEC3KT at day 5 (Figure 3). This mechanism is dependent on ATM, which would be consistent with our previously published data, where we showed that micronucleus formation and other phenotypes persisting after radiation exposure are dependent on ATM function (5). In addition to point to a specific mechanism and pathway to follow, these findings have two additional implications: 1) adaptation to and the length of G2/M can be pharmacologically modulated and 2) dysregulation of cell cycle is expected to affect sensitivity to chemotherapeutic drugs with activity selective for different phases of the cell cycle. We have started examining PARP inhibitors, which affect cells with alterations in HR repair (6). Initial clonogenic survival assays suggest that 5 day irradiated U2OS cells are not more sensitive to the PARP inhibitor ABT888, suggesting that the HR pathway is functional and supporting an effect impinging on cell cycle progression. We will test sensitivity to other agents that exhibit cell cycle phase dependency.

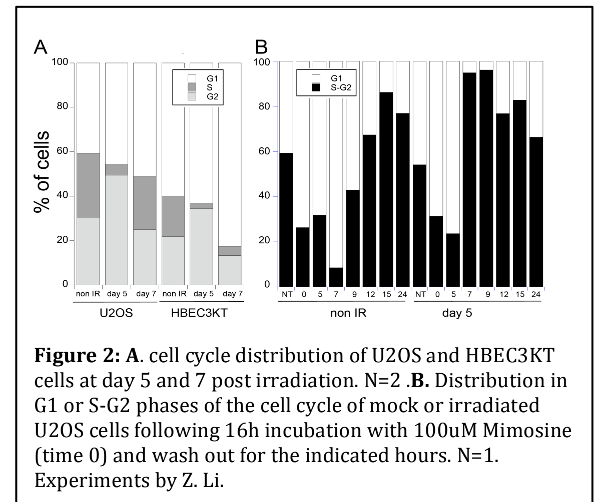

Figure 2: A. cell cycle distribution of U2OS and HBEC3KT cells at day 5 and 7 post irradiation. N=2 .**B.** Distribution in G1 or S-G2 phases of the cell cycle of mock or irradiated U2OS cells following 16h incubation with 100uM Mimosine (time 0) and wash out for the indicated hours. N=1. Experiments by Z. Li.

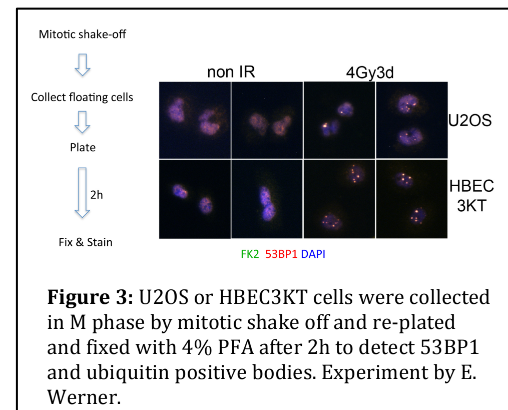

Figure 3: U2OS or HBEC3KT cells were collected in M phase by mitotic shake off and re-plated and fixed with 4% PFA after 2h to detect 53BP1 and ubiquitin positive bodies. Experiment by E. Werner.

Major Task 2: Evaluate the functional consequences of Trip12 and BRCC36 dysregulation at day 7 post-exposure to IR.

Subtask 1: Optimize transfection to increase and reduce the expression of the chromatin ubiquitination regulators Trip12 and BRCC36 by tag-cDNA and siRNA transfection. Verify overexpression by Western Blot for the tag and downregulation by RT-PCR. 10% completed

We started evaluating the effects of BRCC3 and Trip12 overexpression on micronucleus formation rates, however we have not been able to detect overexpression of either protein following transfection of the cDNA. We still have options for optimization, however an alternative approach will be to overexpress different constructs (GFP tagged). We have not started with knock-down as we need to establish a reliable method to detect reduced expression of the proteins to select the best moment for transfection.

Subtask 3: With the conditions established in Subtask 2.1, test the effect of varying Trip12 or BRCC36 expression on assays to measure genomic instability, cellular stress (ROS, cytokine release) and growth in soft agar.

Start pending upon completion of Subtask 1.

Major Task 3: For aim 1c to evaluate whether chromatin-associated ubiquitination can be altered to affect outcomes.

Subtask 1: Optimize RNF168, USP3 and USP44 overexpression by tagged cDNA transfection and anti tag western blot analysis. 70% completed

We have performed transfections with tagged USP3 and USP44 and observed over expression of USP3 so far, which increases micronuclei formation in irradiated cells (Figure 4). We will test next other USPs that are nuclear localized and have been reported to play a role in DSB repair, including USP7, USP28, USP11 (*7*).

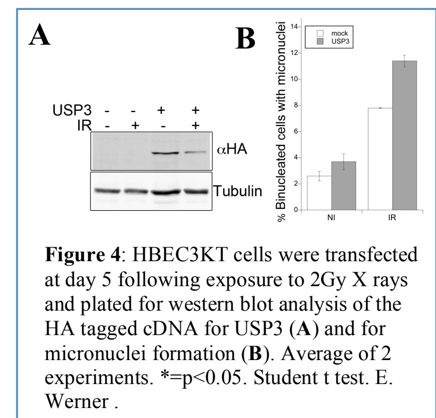

Figure 4: HBEC3KT cells were transfected at day 5 following exposure to 2Gy X rays and plated for western blot analysis of the HA tagged cDNA for USP3 (**A**) and for micronuclei formation (**B**). Average of 2 experiments. *=p<0.05. Student t test. E. Werner .

Subtask 2: Employing the conditions established in subtask 3.1, evaluate chromatin-associated ubiquitin following RNF168, USP3 or USP4 overexpression by western blot and immunofluorescence employing chain specific antibodies and FK2 antibody. 10% completed.

To accomplish this aim in the coming period, we will employ histone H2A as a reporter for RNF168 and USP3 activity at the chromatin level, because we were able to modify outcomes by overexpression of USP3.

For Specific Aim 2:

Major Task 1: Aim2a: Profiling of linkage types of ubiquitin chains accumulated in response to radiation and p38MAPK inhibitor treatment

Subtask 1: Test linkage specific antibodies by western blot.

*Subtask 2: Probe samples of day 7 post-irradiation cells treated or not with p38 MAPK inhibitor by western blot with antibodies tested in subtask 1.*1

We are still in the process of establishing protocols to detect specific linkages.

Major Task 2: Aim 2b To test whether p38MAPK regulates chromatin associated ubiquitin accumulation by modulating the efficiency of K63 linked ubiquitin addition and removal

Subtask 1: Optimize conditions for p38MAPK, MAPKAP2 and ATDC knockdown and inhibition.

We have not started with this task because we did find a phenotype of interest in Subtask 3.

Subtask 2: Examine RNF8 and downstream DNA repair proteins recruitment in inhibitor treated and knockdown cells optimized in subtask 1.

The execution of this task is pending on identifying suitable antibodies.

Subtask 3: Establish conditions for DUB labeling with HA tagged probe ubiquitin vinyl sulfone and/or the methyl ester derivative. 100% completed

We have established a protocol to label active DUBs in cell lysates. Figure 5 shows a discrete repertoire of active DUBs which does not appear to change in 7 day irradiated cells (n=3 experiments. However, we did find that an 18h treatment with SB203580, the p38MAPK inhibitor, reduces DUB labeling. Interestingly, reduced labeling is not due to interference with the assay and is consistent with our observation that p38MAK inhibition leads to increased accumulation of ubiquitinated proteins. We will evaluate other structurally and mechanistically unrelated p38MAPK inhibitors and examine next whether the subcellular localization affects DUB activity and sensitivity to p38MAPK inhibition.

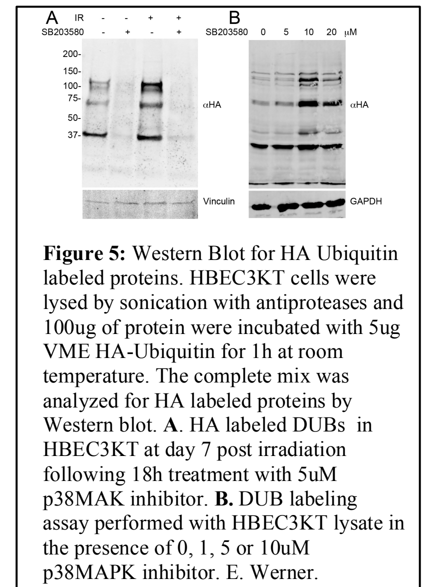

Figure 5: Western Blot for HA Ubiquitin labeled proteins. HBEC3KT cells were lysed by sonication with antiproteases and 100ug of protein were incubated with 5ug VME HA-Ubiquitin for 1h at room temperature. The complete mix was analyzed for HA labeled proteins by Western blot. **A**. HA labeled DUBs in HBEC3KT at day 7 post irradiation following 18h treatment with 5uM p38MAK inhibitor. **B.** DUB labeling assay performed with HBEC3KT lysate in the presence of 0, 1, 5 or 10uM p38MAPK inhibitor. E. Werner.

Mayor Task: Aim 2c To identify p38MAPK dependent nuclear proteins that promote genomic instability.

Subtask 1: Optimize protocol for nuclear fraction enrichment. Evaluation by western blot for markers of plasma membrane, cytoplasm, nuclear soluble and nuclear insoluble protein for nuclear integrity and purity of the fraction. 100% completed

We found that the best conditions for permeabilization of HBEC3KT cells and efficient sample recovery is the REAP method (*8*) ,employing 0.1% NP40 as a detergent (Figure 6).

We will follow up next with fractionating further soluble vs chromatin associated proteins in the nuclear fractions, but these experiments require a large amount of cells which is more challenging to obtain with HBEC3KT than cancer cell lines.

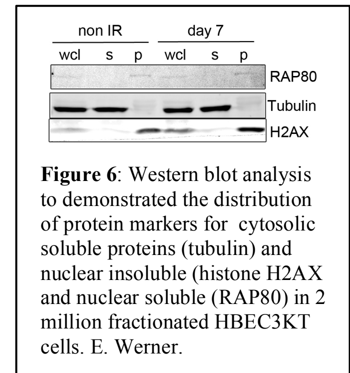

Figure 6: Western blot analysis to demonstrated the distribution of protein markers for cytosolic soluble proteins (tubulin) and nuclear insoluble (histone H2AX and nuclear soluble (RAP80) in 2 million fractionated HBEC3KT cells. E. Werner.

b. <u>Training and professional development opportunities provided by the project during the reporting period</u>: Inclusion of Dr. Zhentian Li will provide him opportunities to broaden his expertise and to co-author papers.

c. <u>Result dissemination to communities</u>: Results generated during this period were disseminated in joint lab meetings with the Yu lab and in our monthly seminars involving multiple laboratories from Emory and Georgia Tech with common research interest in nucleic acid metabolism, damage and repair. General knowledge about DNA damage and radiation was disseminated in an upper level undergraduate class at Emory: HLTH411 "Many Diseases, Few Causes" focused on the mechanisms by which inflammation and oxidative stress contribute to disease state. Dr. Erica Werner gave the lecture: "Role of oxidative stress and inflammatory responses in radiation induced damage". An abstract will be presented at the 2018 Meeting of the Radiation Research Society (included in appendix)

d. <u>Plans to accomplish the project goals in the next period</u>: we will complete the activities listed in the SOW for the ending period and continue to perform the activities listed in the SOW for the next period. To accomplish this, Dr. Werner is increasing her effort to 75% time and Dr. Li will continue contributing to the project for the full year.

4. Impact:

a. Impact on the development of the principal discipline(s) of the project:

Impact on the discipline was effected by publication of a peer reviewed article (included in the appendix) and submission of a meeting abstract.
b. Impact on other disciplines: Nothing to Report.
c. Impact on technology transfer: Nothing to Report.
d. Impact on society beyond science and technology: Nothing to Report.

5. Changes/ Problems:

a. Changes that had a significant impact on expenditures: we had to modify effort assignments in October 2017 and modify the PI in March 2018. We were not able to find a postdoc for only 50% effort. We solved this problem by hiring Dr. Zhentian Li how has experience with the techniques, cell cultures and subject area, thus no training or learning period is necessary.
b. Changes that caused delays in the activities and progress: during this period the lab moves to a new location, we had several personnel changes and X ray machine breakdowns in two opportunities. These are expected to be one-time occurrences.
c. Changes in approach: Nothing to Report.

6. Products:

a. Publications:

Werner E., Wang Y., Doetsch P.W. A single exposure to low or high LET radiation induces persistent genomic damage in mouse epithelial cells vitro and in lung tissue. Radiation Research 188 (2017): 373- 80. PMD: 28753066. Cover featured and acknowledgement of DoD support.
d. Abstract presentation for the Radiation Research Society 2018.

7. Participants and Other Collaborating Organizations:

a. Individuals working on the project during the reporting period:

Name: Paul Doetsch.
Project Role: Principal Investigator.
Nearest person month worked: 3CM (adjusted for an 8 month budget period).
Contribution to the Project: the overall scientific guidance, study design and approach evaluation. Oversight of the analysis and interpretation of data and results.
Funding Support: not relevant for the next period.

Name: Natalya Degtyareva Ph.D
Project Role: Co Investigator.
Nearest person month worked: 1CM.
Contribution to the Project: set up of flow cytometry analysis and transfcction conditions for reporter cell lines.
Funding Support: not relevant for the next period.

Name: Erica Werner Ph.D
Project Role: Co Investigator.
Nearest person month worked: 6CM .
Contribution to the Project: experimental design and planning, implementation and execution of experiments involving cell culture, transfections and outcome analysis, data analysis, interpretation of results, manuscript and report preparation as well as communication of findings at meetings.
Funding Support: no other external funds.

Name: Zhentian Li Ph.D
Project Role: Post-doctoral fellow
Nearest person month worked: 1.5CM (adjusted for a 3 month budget period)
Contribution to the Project: flow cytometry analysis of the experiments with U2OS reporter cell lines and cell cycle, data analysis and interpretation of results.
Funding Support: no other external funds.

b. Changes in active other support during the reporting period: Nothing to Report.
c. Other organizations involved: Nothing to Report.

8. Special Reporting Requirements: Nothing to Report

9. Appendices:

a. Bibliography:

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b. Meeting Abstract:

Exposure to ionizing radiation induces cholesterol biosynthesis in normal and tumor derived cell lines

Erica Werner, Andrew Alter, Eric Dammer, Ya Wang, Nick Seyfried and Paul Doetsch

While evidence supporting the notion that exposures to heavy ion radiation increase the risk for cancer and other diseases development is accumulating, the underlying biological mechanisms remain poorly understood. To identify in an unbiased manner novel phenotypes that may be related to increased disease risk and persist over time concurrently with oxidative stress and genomic instability, we performed a quantitative global proteome analysis of nearly normal bronchial epithelial cells (HBEC3KT) at day 7 post exposure to 1Gy Fe ions (600 MeV/nucleon, LET= 175 keV/μm). The analysis revealed a significant increase in the expression of 5 enzymes of the cholesterol biosynthesis pathway. Elevated expression of enzymes of the cholesterol pathway was associated with increased cholesterol levels in irradiated cells and lung tissue measured by a biochemical method based on enzymatic cholesterol oxidation and by filipin

staining of cell-associated cholesterol. Radiation-increased cholesterol levels were reduced by treatment with inhibitors affecting the activity of enzymes in the biosynthesis pathway.
While a 1Gy dose of Fe ion was sufficient to induce a robust response, a dose of 5Gy X-Rays was necessary to induce a similar cholesterol accumulation in HBEC3KT cells. To examine the implications of this finding for radiotherapy outcomes, we screened a panel of lung cancer cell lines for cholesterol levels following exposure to a X rays dose in the radiotherapy range of 2Gy. We identified a subset of cell lines that increased cholesterol levels following exposure to low LET radiation. This increase was associated with cytotoxicity as treatment with statins reduced the radiosensitivity in these cell lines.
In summary, our findings uncovered a novel radiation-induced response, which may modify radiation treatment outcomes and may contribute to risk for radiation –induced cardiovascular disease and carcinogenesis.

c. Publication:

RADIATION RESEARCH **188**, 373–380 (2017)
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DOI: 10.1667/RR14685.1

A Single Exposure to Low- or High-LET Radiation Induces Persistent Genomic Damage in Mouse Epithelial Cells *In Vitro* and in Lung Tissue

Erica Werner,[a,b,1] Ya Wang[b] and Paul W. Doetsch[a,b,c,1]

Departments of [a] Biochemistry, [b] Radiation Oncology and [c] Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia

Werner, E., Wang, Y. and Doetsch, P. A Single Exposure to Low- or High-LET Radiation Induces Persistent Genomic Damage in Mouse Epithelial Cells *In Vitro* and in Lung Tissue. *Radiat. Res.* 188, 373–380 (2017).

Exposures to low- and high-linear energy transfer (LET) radiation induce clustered damage in DNA that is difficult to repair. These lesions are manifested as DNA-associated foci positive for DNA repair proteins and have been shown to persist *in vitro* and *in vivo* for days in several cell types and tissues in response to low-LET radiation. Although in some experimental conditions these residual foci have been linked with genomic instability and chromosomal aberrations, it remains poorly understood what type of damage they represent. Because high-LET radiation induces complex DNA lesions more efficiently than low-LET radiation, we compared the efficacy of several heavy ions (oxygen, silicon and iron) in a range (17 , 70 and 175 keV/μm, respectively) of LET and X rays at a 1 Gy dose. Persistent genomic damage was measured by γ-H2AX-53BP1-positive residual foci and micronucleus levels during the first three days and up to a week after *in vitro* and *in vivo* irradiation in lung cells and tissue. We demonstrate that in an *in vitro* irradiated mouse bronchial epithelial cell line, the expression of residual foci is readily detectable at 24 h with levels declining in the following 72 h postirradiation, but still persisting elevated over background at day 7. At this time, foci numbers are low but significant and proportional to the dose and quality of the radiation. The expression of residual foci *in vitro* was mirrored by increased micronuclei generation measured in cytokinesis-blocked cells, indicating long-term, persistent effects of genomic damage in this cell type. We also tested the expression of residual foci in lung tissue of C57BL/6 mice that received whole-body X-ray or heavy-ion irradiation. We found that at day 7 postirradiation, Clara/Club cells, but not pro-SPC-positive pneumocytes, contained a subpopulation of cells expressing γ-H2AX-53BP1-positive foci in a radiation quality-dependent manner. These findings suggest that *in vivo* persistent DNA repair foci reflect the initial genotoxic damage induced by radiation and a differential vulnerability among cells in the lung. © 2017 by Radiation Research Society

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INTRODUCTION

The lung is a radiosensitive organ, susceptible to radiation-induced toxicity, which limits the tolerable dose that can be delivered during radiotherapy, as well as causing the emergence of radiotherapy-associated late injuries, including pneumonitis and fibrosis at high doses and secondary malignancies at low to moderate doses (*1, 2*). The lung is also one of the organs of highest solid tumor incidence after acute exposures in the atomic bomb survivor cohort (*3*) and patients receiving radiotherapy (*4*) and after extended low-dose occupational exposures (*5*). Thus, this is an organ of concern for patients as well as for astronauts in future exposures to space radiation, which is a combination of high-charge and energy nuclei, high-energy protons and solar particle events (*6*).

The biological effects of heavy-ion radiation remain relatively unknown compared to the effects of low-LET radiation, but distinct effects are expected as heavy ions have a unique inhomogeneous pattern of energy distribution (*7*). This type of radiation generates a track composed of high-density ionizations described by LET along the trajectory of the particle (core) and secondary ionizations (delta rays), which can account for up to 50% of the total dose occurring at variable distances and in a much larger volume from the core (*8*). *In vitro* and *in vivo* studies have demonstrated that relative to low-LET radiation, heavy ions induce biological responses with generally higher efficacy, including cell killing, cellular stress responses, inflammation and carcinogenesis (*7, 9*).

One of the factors determining increased biological effect is the induction of complex lesions in DNA-containing double-strand breaks (DSB) together with additional damage that is difficult to repair and leads to increased cell death and genomic aberrations (*10, 11*). Such DSB are manifested as DNA-associated foci containing DNA repair proteins, are repaired with distinct kinetics and may persist

long after the initial radiation exposure. Residual DSB foci persisting at 24 h postirradiation are also induced by low-LET radiation, although to a lesser magnitude, and have been observed in several tissues of irradiated animals (*12*), and to be dependent on the DNA repair status of the individual (*13*). Thus, foci have been explored as biodosimetry markers, to assess individual DNA repair capability (*14–16*) and individual radiosensitivity (*17, 18*). The energy associated with the particle is a factor influencing the persistence of foci induced by high-LET radiation (*19, 20*).

We have previously shown that residual foci positive for the DSB repair factors γ-H2AX and 53BP1 persist at least up to day 7 postirradiation to a single dose of 1 Gy X-ray irradiation or ^{56}Fe-ion irradiation in proliferating cultures of human bronchial epithelial cells (*21*). This phenotype is associated with a broader stress response involving increased mitochondrial ROS production, p38MAPK activity and increases in micronuclei formation indicating genomic instability. Residual foci as well as micronuclei are, at day 7, a cell autonomous phenotype of the irradiated cells' progeny, which is not propagated or transferred to naïve cells. However, the progeny of irradiated cells is a source of nontargeted effects, releasing pro-inflammatory cytokines, which influence, in trans, the function of neighboring cells not directly targeted by radiation (*22*). While persistent foci after irradiation have been demonstrated in several organs in mice and humans, the majority of studies have contemplated exposures to high doses of low-LET radiation and analysis at 24 h postirradiation. Detection of these markers at later times *in vivo* would implicate prolonged effects of DNA damage on vulnerable cell types and persistent stress signaling, potentially affecting other cell types less sensitive to the initial DNA damage, thereby impacting tissue and organ function.

In this work, we examined whether the induction of residual foci persists in the lungs of whole-body irradiated wild-type C57BL/6 mice, as well as in an isogenic lung epithelial cell line, and compared the effect of several ion beams of a range of LET. Employing a dose in the lower range, we found that a subpopulation of cells in the lung display residual foci, revealing a cell population that may be differentially susceptible to radiation and stress phenotypes *in vivo*.

MATERIALS AND METHODS

Irradiation Experiments

Experiments utilizing mice followed the American Association for Laboratory Animal Science policies and were approved by the Emory and Brookhaven National Laboratory Institutional Animal Care Use Committees (IACUC). Six-week-old C57BL/6 female mice (Jackson Laboratory, Bar Harbor, ME) were whole-body irradiated with 1 Gy of either X rays or 600 MeV/nucleon iron (LET = 175 keV/μm), 300 MeV/nucleon silicon (LET = 70 keV/μm) and 600 MeV/nucleon oxygen (17 keV/μm) heavy ions, each at an average 50 cGy dose rate in the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (Upton, NY). Ion energy was calibrated by the NSRL dosimetry group and LET was estimated based on the energies (*23*). Low-LET irradiation was performed at the Winship Cancer Institute of Emory University using an X-RAD 320 X-ray machine (320 kV, 10 mA Precision X-Ray Inc., North Branford, CT).

Cell Culture

The mouse bronchial epithelial cell line originated from spontaneous immortalization after continuous subculture of cells isolated from the bronchi of newborn C57BL/6 mice (*24*). Cells were plated in triplicate T25 flasks for irradiations at 50% confluence. Each flask was subcultured 1:3 on day 4 and plated for the assays at day 6 postirradiation.

Cytokinesis Blocked Micronucleus Assay

Cells were plated at a density of 20,000 cells/well on glass coverslips before irradiation or at day 6 and treated the next day for 18 h with 3 μg/ml cytochalasin B in complete media (*25*). After fixation, the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich® LLC, St. Louis, MO) and binucleated cells were counted and scored for at least 10 binucleated cells with micronucleus per sample. We counted 500–700 binucleated cells per replicate in nonirradiated samples and 200–300 after irradiation (*21*).

Immunofluorescence Microscopy

DNA repair foci were detected in cells fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton™ X-100. Antibodies used were γ-H2AX (EMD Millipore, Billerica, MA) and 53BP1 (Novus Biologicals, Littleton, CO). Antibodies for lung cell markers were anti Pro-surfactant Protein C (cat. no. ab40879; Abcam®, Cambridge, MA) and uteroglobin (cat. no. ab40873; Abcam). Nuclear DNA was stained with DAPI. Cells were imaged either in an Olympus epifluorescence inverted microscope or in a Zeiss LSM 510 META confocal microscope (Carl Zeiss Inc., Thornwood, NY) using a 20× Plan-Apo objective (NA = 0.75). Images were processed using contrast/brightness enhancement only. An average of fifty cells per image were counted and scored for number of nuclei with 0, 1 and 2, 3 or more foci positive for both markers, larger than 0.5 μm in at least five different fields.

Tissue Preparation and Staining

Lung tissue was collected from mice euthanized at day 7 postirradiation and snap frozen in liquid nitrogen to preserve molecular identity of the cells and for confocal laser microscopy imaging. The tissues embedded in optimum cutting temperature compound (Tissue-Tek®) were cut frozen into 5-μm-thick sections. Slides were fixed in acetone, rinsed with PBS and incubated for 15 min in 4% PFA, blocked in 5% goat serum, stained for the indicated antigen and imaged following the same procedure as with the cells. For quantification, total nuclei in bronchioles as well as nuclei with double-positive foci were recorded for each acquired image.

Statistical Analysis

The statistical test applied in each experiment is stated in the Figure Legends. Excel was used for paired two-tailed Student's *t* test analysis assuming equal variance of the samples. GraphPad Software version 5 (San Diego, CA) was used for analysis of variance (ANOVA) employing a Bonferroni (repeated measures) or Dunnett's (one-way) post hoc test.

RESULTS

To correlate the expression of markers for genomic instability with *in vitro* cell killing and *in vivo* lung

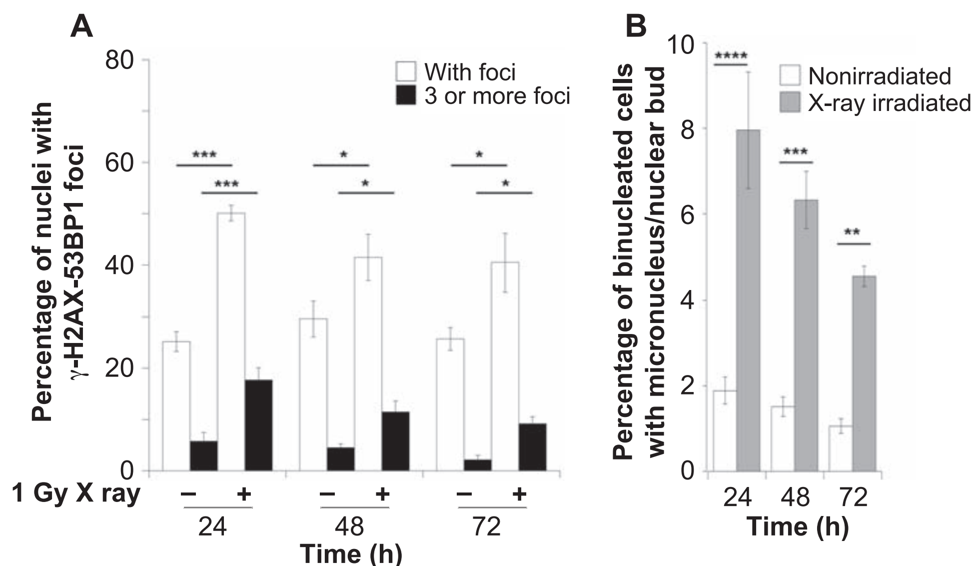


FIG. 1. Residual foci and micronucleus formation in X-ray-irradiated mouse bronchial epithelial cell cultures. Panel A: Early residual foci distribution in mouse bronchial epithelial cells exposed to 1 Gy X rays or mock irradiated, fixed and stained for γ-H2AX and 53BP1 at the indicated time postirradiation (average of 4 independent experiments is shown; error bars = SEM). Statistical significance was evaluated by a repeated measures ANOVA followed by a Bonferroni post hoc test (*$P < 0.05$ ***$P < 0.005$). Panel B: Micronuclei levels at the indicated time points after a single exposure to 1 Gy (average of 3 experiments; error bars = SEM). Statistical significance was evaluated by a repeated measures ANOVA followed by a Bonferroni post hoc test (**$P < 0.01$, ***$P < 0.005$ and ****$P < 0.001$).

carcinogenesis measurements from a previous study, we employed a 1 Gy dose and ion energies and LET, as previously described elsewhere (*24*). A dose of 1 Gy X rays has been shown to be sufficient to induce radiation-induced foci (RIF) positive for two DNA repair associated proteins, γ-H2AX and 53BP1, that persist at 24 h postirradiation (*11, 26*). Exposure of mouse bronchial epithelial cells (MBEC) to a moderate noncytotoxic dose of 1 Gy was sufficient to induce residual foci at 24 h, which declined over the next several days (Fig. 1A). Two-way repeated measures ANOVA indicated a significant effect of radiation on the number of nuclei with foci ($F = 36.36$; $P = 0.001$) and on the number of nuclei that had 3 or more foci ($F = 58.32$; $P = 0.0003$). While the test excluded interaction between time and radiation, there is an effect of time on the number of nuclei with 3 or more foci ($F = 6.7$; $P = 0.01$), suggesting that this cell population is a sensitive reporter of both variables. The same cultures displayed increased micronuclei formation levels as measured by the cytokinesis-block assay (Fig. 1B), which declined steadily over the same period of time. Two-way repeated measures ANOVA indicated a significant effect of radiation ($F = 57.2$; $P = 0.0016$) and time ($F = 7.8$; $P = 0.013$) on the number of binucleated cells with micronuclei and excluded interaction between both variables. After continuous culture and proliferation of the cells, elevated foci and micronuclei levels were still detectable at day 7 (Fig. 2A and B). At this later time point, one-way ANOVA revealed a significant effect of radiation dose on cells with foci ($F = 2.97$; $P = 0.03$) and on cells with 3 and more foci per nuclei ($F = 7.8$; $P = 0.0004$). Persistent foci induction was roughly proportional to the initial radiation dose up to 4 Gy, at which point the response reached a plateau for cells containing 3 or more foci (Fig. 2A). Similarly, micronuclei levels were still elevated in the cultures at day 7, at levels proportional to dose and reaching saturation at doses above 4 Gy (Fig. 2B). Both markers mirrored each other in the temporal course of expression and in their response to X-ray dose reaching a plateau. These results reproduce a trend/feature that has been described previously for several markers of persistent genomic instability (*27–29*).

To examine the effect of high-LET radiation, we compared the efficacy of different ion beams at a 1 Gy dose, but varying in particle size and energies. One-way ANOVA indicated that all ions induced residual foci in a significant percentage of the cells ($F = 14.89$; $P = 0.00024$) and increased the percentage of cells with 3 or more foci per cell ($F = 5.47$; $P = 0.013$) (Fig. 2C). Silicon and iron induced foci in a larger percentage of the cell population while oxygen increased the fraction of cells with a high number of foci (Fig. 2C). All ions induced foci in a greater number of cells and a larger subpopulation with 3 or more foci compared to X rays. These cell cultures also exhibited increased levels of micronuclei, in a magnitude equivalent to a 3 Gy X-ray dose at comparable levels for all ions (Fig. 2D).

We next evaluated whether exposure to low- and high-LET radiation induces the expression of residual foci *in vivo*, in lung tissue of C57BL/6 mice. Epithelial cells with nuclear foci positive for both γ-H2AX and 53BP1 were present in the lungs at day 7 after whole-body irradiation.

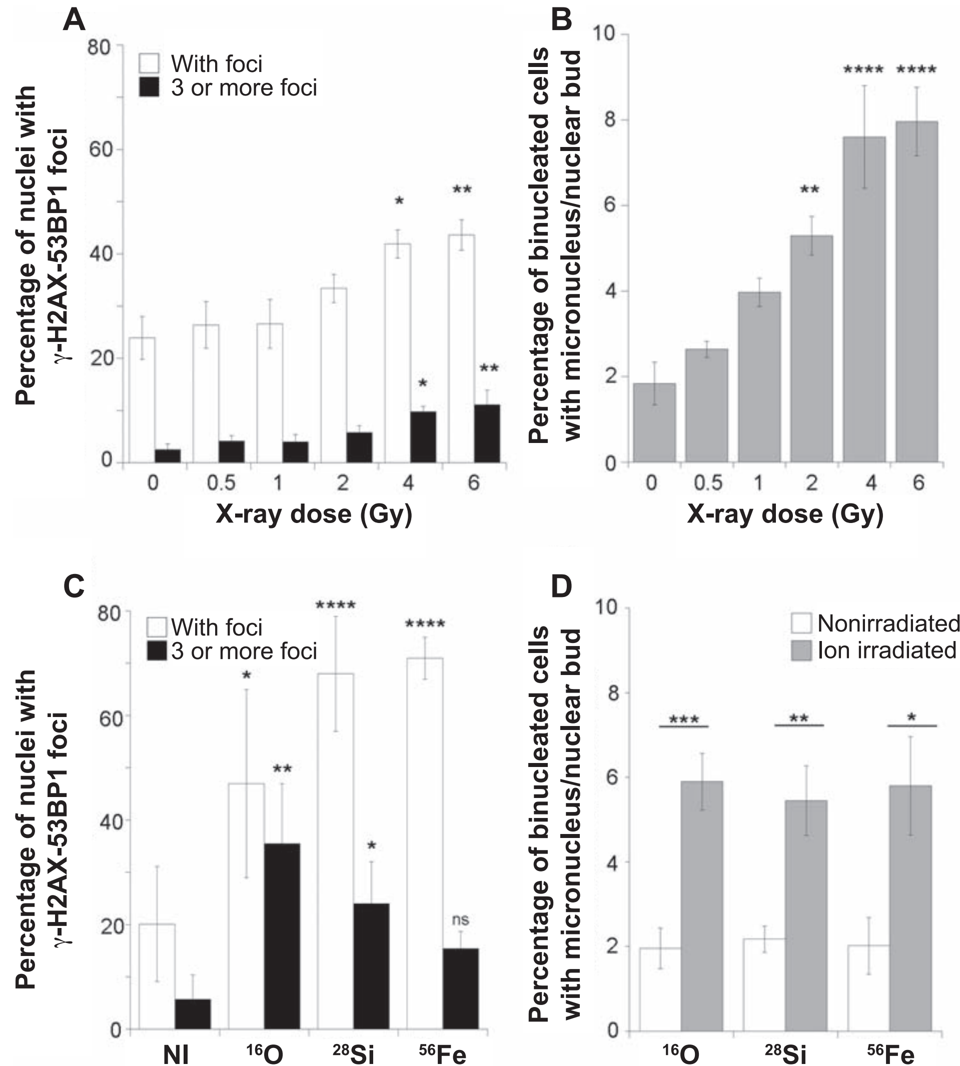
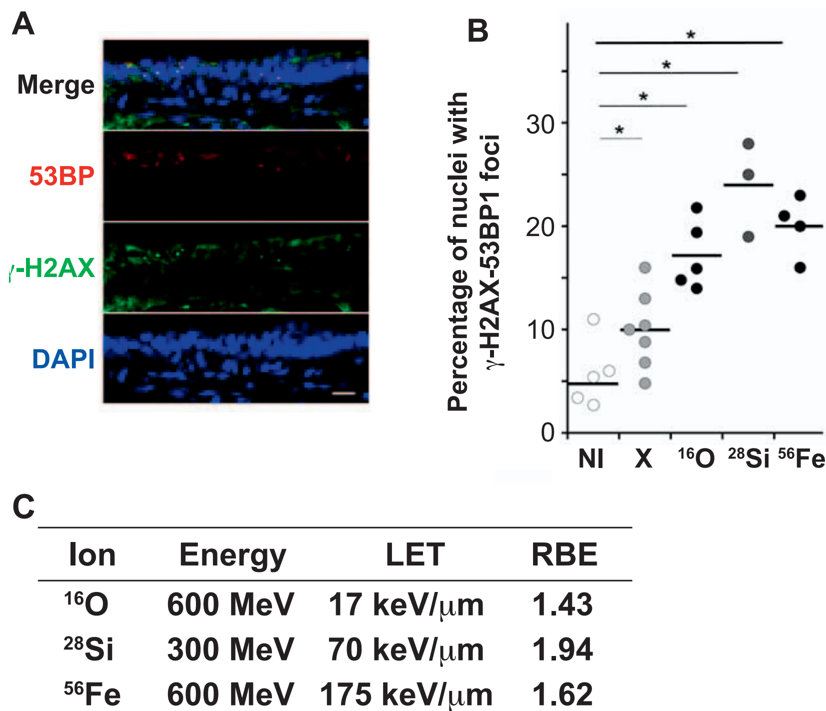


FIG. 2. Persistence of residual foci and micronucleus formation in low- and high-LET irradiated mouse bronchial epithelial cell cultures. Panel A: Residual foci distribution at day 7 in response to X-ray dose. Statistical significance was evaluated by one-way ANOVA followed by a Dunnett's multiple comparisons post hoc test (*$P < 0.05$, **$P < 0.01$). The differences between 0 and doses below 4 Gy were not statistically significant. Panel B: Micronucleus formation rates at day 7 in response to X radiation. Average of 3 experiments, error bars = SEM. Statistical significance was evaluated by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (**$P < 0.01$ and ****$P < 0.001$). The difference between 0 and doses below 2 Gy were not statistically significant. Panel C: Residual foci distribution in mouse bronchial epithelial cells mock irradiated or exposed to 1 Gy, oxygen, silicon or iron radiation, fixed at day 7 postirradiation and stained for γ-H2AX and 53BP1 (n = 1; error bars: STDEV of 4 technical replicates). Statistical significance was evaluated by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test (*$P < 0.05$, **$P < 0.01$) (ns = no statistical significance). Panel D: Micronuclei levels at day 7 after receiving 1 Gy of the indicated heavy-ion (average of 2 independent irradiations in duplicates; error bars = SEM). Statistical significance was evaluated by a paired comparison with a Student's *t* test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$).

We readily detected epithelial cells lining small airways with residual foci positive for both markers after exposure to all types of radiation (Fig. 3A), while rarely in the lamina propia (Figs. 3A and 4B) or alveolar tissue (Fig. 4B and data not shown). We quantified this effect by counting the number of cells with and without foci lining small bronchioles per imaged field in nonirradiated and irradiated mice, a measurement independent of potential changes in cell volume or sampling area. Analysis of the cells containing foci lining small airways revealed a larger effect for particle radiation than for X rays (Fig. 3B). To reflect these differences, we estimated a relative biological effect (RBE) for each ion, defined as the ratio of the value of the biological effect observed for HZE radiation and that observed for the reference radiation (X rays) at a 1 Gy dose. Among all the ions tested, the most effective was silicon (RBE = 1.94), followed by iron (1.62) and oxygen (1.43) (Fig. 3C).



Ion	Energy	LET	RBE
^{16}O	600 MeV	17 keV/μm	1.43
^{28}Si	300 MeV	70 keV/μm	1.94
^{56}Fe	600 MeV	175 keV/μm	1.62

FIG. 3. Persistence of residual foci in the small airways of mice exposed to low- and high-LET radiation. Panel A: Immunofluorescence image of a tissue section from the lung of a mouse irradiated seven days earlier with 1 Gy silicon ion stained for γ-H2AX, 53BP1 and DAPI (scale bar = 20 μm). Panel B: Dot plot of the percentage of cells with residual foci lining small airways of mice exposed to the indicated radiation. Each data point represents the percentage of nuclei with foci of an imaged portion of small airway in different tissue sections of one irradiated mouse per ion. The bar indicates the median. Statistical significance was evaluated by a paired comparison with a Student's *t* test (*$P < 0.05$). Panel C: Calculated relative biological effect of heavy-ion radiation compared to X radiation at 1 Gy on residual foci persisting in lung tissue at day 7 after whole-body irradiation.

To test whether the cells with DNA repair foci are associated with proliferating cell populations in the lung that may be involved in repair and regeneration (*30*), we double-stained lung sections for γ-H2AX and markers identifying these cell populations. Secretoglobin 1A1 (SCGB 1A1; also called uteroglobin, CCSP or CC10) is a marker for Clara/Club cells, abundant in small caliber, peripheral airways and a facultative transit amplifying cell population, which proliferates to maintain homeostatic tissue turnover and to replace injured cells (*30, 31*). We also evaluated the presence of foci in type II pneumocytes positive for pro-surfactant protein (pro-SPC), as these represent another cell population with regenerating properties in peripheral lung tissue (*32*). Immunofluorescence identified all foci-positive cells in small airways as Club cells (Fig. 4A), while no cell positive for pro-SPC contained γ-H2AX foci in the nucleus (Fig. 4B). This result suggests that a subpopulation of Club cells may be uniquely sensitive to radiation-induced DNA damage and may express DNA repair foci as part of a persistent stress response.

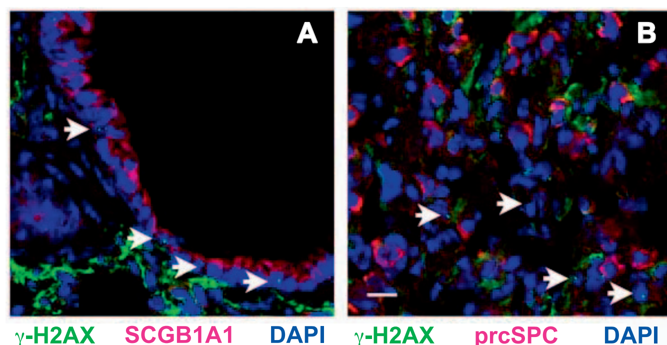


FIG. 4. Characterization of cell types expressing residual foci in the lung at day 7. Immunofluorescence image of a tissue section from the lung of a mouse irradiated seven days earlier with 1 Gy iron ions stained for nuclear DNA (DAPI), γ-H2AX and secretoglobin 1A1 (panel A) or pro-surfactant protein (panel B) (Scale bar = 20 μm).

DISCUSSION

While radiation-induced foci (RIF) are considered a bona fide marker for DSB during the first hours postirradiation (*26, 33*), it remains unclear whether at later times these represent ongoing generation of DSB (*34*). X rays and particularly high-LET radiation induce clustered damage in DNA, which is repaired with slower kinetics than the bulk of the damage (*35*). Differences in the number, size and kinetics of foci positive for other DNA repair proteins (pATF2 and pSMC1) generated by high- and low-LET

radiation have been reported, with 1,000 MeV iron exposure showing more persistent foci at 24 h (*36*). While residual foci detected at 24 h postirradiation may well reflect clustered damage, foci at later times after the occurrence of cell division may reflect other chromatin alterations, such as the degree of heterochromatin compaction or incorrect chromatin restoration (*37, 38*).

The persistence of residual foci for several days in proliferating cells might be driven by heritable mechanisms such as epigenetic modifications, altered genomic structure or altered cellular signaling. Residual foci may be passed on to the cellular progeny, as has been shown by analysis of cytochalasin B-treated cells at 24 h (*39*) and at day 7 (*21*). The generation of 53BP1-positive foci that can be passed to the cell progeny has been reported under conditions where the cells enter mitosis with DNA damage (*40*) or deficient Holliday junction resolution (*41*). Our findings that residual foci correlate with radiation quality, support their association with early DNA damage and repair events. Residual foci persisting at day 7 in the skin of mice exposed to X rays correlate better with repair competency than measurements at earlier times (*42*). While *in vitro*, the cells with residual foci may become lost by cell death or repair or diluted by proliferation of cells without these phenotypes, additional factors may affect the persistence of these cells in tissues *in vivo*. Some factors such as local hypoxia and the microenvironment may affect DNA repair competency, as well as cell turnover and differentiation dynamics in the particular tissue (*43, 44*).

We propose that residual foci are a reflection of a broader response to the initial genotoxic damage, which may affect the function of healthy cells in tissues based on the following: In our *in vitro* experiments with a mouse epithelial cell line, we discovered a correlation between residual foci and micronuclei levels *in vitro* as reflected by their time, dose and radiation quality dependence, similar to our previous findings in human bronchial epithelial cells (*21*) and reproducing features of persistent genomic instability in other biological models (*28, 29*). Several published studies have shown that markers for genomic instability plateau and this has been attributed to loss or death of the cells with increased damage or to the saturation in activity of a component of the mechanism leading to genomic instability (*27, 28*). While we did not examine whether cells in the lung *in vivo* are capable of generating micronuclei at day 7, persistent clastogenic effects of particle radiation and low-LET radiation on cells of the respiratory tract have been previously documented. When the relative sensitivity to clastogenic effects was compared among the different cell populations isolated from lungs of irradiated mice, epithelial cells from deep portions of the lung were found to exhibit higher levels (*45, 46*). Additionally, in low-LET radiation-exposed skin, the levels of residual foci at day 7 correlated with increased micronuclei levels in fibroblasts isolated from the same tissue (*42*). Both markers may be part of a more complex and broader cellular phenotype, which would include the activation of the DNA damage response leading to H2AX phosphorylation. An additional component of this phenotype could be oxidative stress, which we have described to correlate with residual foci and micronuclei at day 7 *in vitro* (*21*). *In vivo*, residual foci persisting for a year have been found in association with persistent oxidative stress in mouse intestinal cells after exposure to 1 Gy iron ions (*47*).

Heavy-ion radiation was more effective than X-ray radiation at inducing these phenotypes *in vitro* and *in vivo*, however, we did not observe a large effect from LET. The effect of the different components of the track structure on residual foci at 24 h has been examined *in vitro*. In these conditions, ions generated more residual foci at lower energies and in a dose-dependent, but LET-independent fashion (*19, 20, 36*). These studies suggested that particles of relatively lower energy, with a higher density of delta rays close to the particle trajectory and narrower track width, generate a greater level of residual foci compared to higher energy particles. While we did not test energy as a variable, silicon had the lowest energy among the ions we used, while oxygen and iron had the highest relative energies, resulting in a lower number of cells with foci *in vivo*. Interestingly, parallel studies employing identical irradiation conditions demonstrated that silicon was more effective than iron and oxygen in lung tumor induction in this mouse strain (*24*). Although residual foci and persistent micronucleus formation are markers of genomic instability, a feature of many cancer cells, they have not been directly associated with cell transformation or tumorigenesis yet.

Our analysis of lung tissue from whole-body irradiated mice revealed that at the relatively low dose of 1 Gy of low- or high-LET radiation, few cells in lung tissue express residual foci at day 7 postirradiation. We identified these cells as belonging to transit amplifying Club cells. These are functionally relevant cells for the lung, as SCGB 1A1-positive Club cells are facultative progenitor cells that can proliferate in response to injury and have been implicated in the long-term maintenance and repair of the lung airway. Their impact on alveolar epithelium is still controversial (*32, 48*). However, when we examined the status of a functional equivalent in the respiratory compartment (pro-SPC expressing cells), we did not find residual foci expression in this population. Interestingly, both of these cell populations have the potential to originate lung tumors when the promoters of these proteins are employed to express oncogenic ras (*49*).

We did find a few proSPC-negative cells in the alveolar tissue with residual foci, which could be fibroblasts. Several published studies have previously shown that fibroblasts exhibit residual foci and micronucleus formation in *in vitro* and *in vivo* experiments after low- and high-LET irradiation (*46, 50*). Persistence of cells with residual foci in small airways could be due to a slow turnover of this cell population. Employing lineage tracing and clonogenic growth approaches, it was reported that exposures to 1 Gy

X-ray or ^{56}Fe radiation do not promote excessive cell turnover in the airways, but reduce clonogenic potential, which suggests functional consequences on cells with proliferative potential (*51*). Exposure to higher doses induced clonal expansion of epithelial progenitor cells in the distal airways, which exhibit γ-H2AX foci accumulation (*52*). Interestingly, different cell populations are mobilized for repair in the lung depending on the type of injury. For example, in response to bleomycin injury, alveolar cells are maintained at steady-state levels by SPC-positive cells, but are subsequently replaced by SCGB 1A1-positive cells (*32*). Our findings suggest a differential vulnerability of Club cells to radiation. This notion is supported by the observation that viral infection of Club increases the damage inflicted by radiation (*53*). Thus, residual foci expression at late times postirradiation could be a biomarker for radiation-sensitive cell populations according to our *in vivo* findings, and reflect cells with genomic instability in accordance with our *in vitro* findings.

Residual foci have been evaluated in the clinical setting to predict radiation therapy outcomes and toxic effects (*10, 18, 54*), and while foci identify individuals with significant DNA repair deficiencies (*55*), this assay shows extensive interindividual variability. Further studies are needed to elucidate the processes associated with their persistence and their predictive value as a biomarker.

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