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Proteomic evaluation of the natural history of the acute radiation syndrome of the gastrointestinal tract in a nonhuman primate model of partial-body irradiation with minimal bone marrow sparing includes dysregulation of the retinoid pathway

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Abstract

Exposure to ionizing radiation results in injuries of hematopoietic, gastrointestinal and respiratory systems which are the leading causes responsible for morbidity and mortality. Gastrointestinal injury occurs as an acute radiation syndrome. To help inform on the natural history of the radiation-induced injury of the partial body irradiation model, we quantitatively profiled the proteome of jejunum from non-human primates following 12 Gy partial body irradiation with 2.5% bone marrow sparing over a time period of three weeks. Jejunum was analyzed by liquid chromatography-tandem mass spectrometry and pathway and gene ontology analysis were performed. 3245 unique proteins were quantified out of more than 3700 proteins identified in this study. A total of 289 proteins of the quantified proteins showed significant and consistent responses across at least three time points post-irradiation, of which 263 proteins showed strong upregulations while 26 proteins showed downregulations. Bioinformatic analysis suggests significant pathway and upstream regulator perturbations post-high dose irradiation and shed light on underlying mechanisms of radiation damage. Canonical pathways altered by radiation included GP6 Signaling Pathway, Acute Phase Response Signaling, LXR/RXR Activation, and Intrinsic Prothrombin Activation Pathway. Additionally, we observed dysregulation of proteins of the retinoid pathway and retinoic acid, an active metabolite of vitamin A, as quantified by liquid chromatography-tandem mass spectrometry. Correlation of changes in protein abundance with a well-characterized histological endpoint, corrected crypt number, was used to evaluate biomarker potential. These data further define the natural history of the gastrointestinal acute radiation

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Conflicts of Interest:

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syndrome in a non-human primate model of partial body irradiation with minimal bone marrow sparing.

Keywords

biological indicators; radiation damage; gastrointestinal tract; radiation, ionizing; partial body irradiation

INTRODUCTION

High-dose radiation exposure results in organ-specific sequelae occurring after near total body exposure to high-dose radiation including the hematopoietic acute radiation syndrome (H-ARS) and the gastrointestinal acute radiation syndrome (GI-ARS). Delayed effects of acute radiation exposure (DEARE) follow the ARS and are characterized by multi-organ injury including pulmonary injury (MacVittie et al. 2012a). GI is one of the organs most sensitive to radiation and significant pathological changes can be observed within days to weeks post-irradiation (Conard 1956; Wiernik 1966; Potten and Booth 1997; Booth and Potten 2000; Vigneulle et al. 2002; MacVittie et al. 2012a; MacVittie et al. 2012c; Wang et al. 2015). The GI-ARS is characterized by apoptotic cell death, breakdown of the mucosal epithelial barrier wall, electrolyte imbalance, fluid loss, intestinal hemorrhage, and sepsis (MacVittie et al. 2012c). Similarly, in patients undergoing radiotherapy, radiation-induced injuries of GI are represented by apoptosis of crypt cells, leakage of epithelial barrier, and inflammation of mucosa (Stacey and Green 2014). Intestinal bleeding and diarrhea can be observed as consequences of stem cell death, epithelial atrophy and shortening of intestinal microvilli (Shadad et al. 2013). Radiation-induced injuries in animal models such as mouse and non-human primate (NHP) are similar to those seen in human with NHP being the a representative animal model for human (Singh and Olabisi 2017; Singh et al. 2018; MacVittie et al. 2019b).

The partial body irradiation (PBI) with minimal, 2.5% or 5%, bone marrow (BM) sparing NHP model was developed to mimic intentional or accidental radiation exposures in humans. Such exposures are likely to include bone marrow sparing and to permit the concurrent analysis of coincident short- and long-term damage to organ systems in a time and dose dependent manner (MacVittie et al. 2012a). Previous publications by our team have described this PBI model, the analysis of resulting tissue injury, as well as its use for medical countermeasure development (MacVittie et al. 2012a; Jones et al. 2015a; MacVittie et al. 2015; Shea-Donohue et al. 2016; Cohen et al. 2017; Prado et al. 2017; Carter et al. 2019; Cohen et al. 2019; Farese et al. 2019; MacVittie et al. 2019a; MacVittie et al. 2019b; Parker et al. 2019a; Parker et al. 2019b; Parker et al. 2019c). Here we sought to further characterize the natural history of radiation-induced injury in NHP via determination of differential protein expression in jejunum. A more in-depth characterization of the natural history of radiation-induced damage in NHPs exposed to PBI with minimal BM sparing increases the usefulness of the model for medical countermeasure development as understanding of the natural history of an injury is a general expectation of drug development under the Food and Drug Administration (FDA) Animal Rule (FDA 2015).

Currently, there is no drug approved for the GI-ARS by the FDA (Rios et al. 2014), however treatment with fluids and antibiotics have been reported to mitigate GI injury (Fish et al. 2016).

A previous natural history characterization of the GI-ARS in a mouse model includes proteomic analyses similar to this study (Huang et al. 2019). The proteomic profiling of the small intestine across the time course of the GI-ARS suggests that the top canonical pathways dysregulated by radiation included protein kinase A signaling, acute phase response signaling, and LXR/RXR signaling. Among the proteins from the top three canonical pathways dysregulated by radiation, there were a number of proteins that were also annotated to be associated with inflammation and retinoic acid (Huang et al. 2019). Retinoic acid (RA) is a master regulator of gene expression mainly through ligand-activated control of transcription mediated through retinoic acid receptors (RAR) in the nucleus (Germain et al. 2006a). In the GI, retinoic acid plays important roles in regulating differentiation and migration of cell populations required for immune response (Larange and Cheroutre 2016). RA is also essential for differentiation and maintenance of the crypt-villus axis (Czarnewski et al. 2017).

Proteomics is able to measure hundreds to thousands of proteins at a time and identifies a subset of proteins that changes significantly between groups. The comparative proteomic approach is a powerful tool for the discovery of mechanism(s) of injury along the natural history of radiation-induced damage. Proteomic profiling of the natural history of GI-ARS in NHP is lacking. Proteomic profiling reports have included mouse and intestinal epithelial cell models (Bo et al. 2005; Lim et al. 2011; Huang et al. 2019). More studies have focused on the complementary metabolome; metabolomic interrogation of intestine after radiation exposure has been reported for mouse, rat and NHP (Cheema et al. 2014; Jang et al. 2016; Cheema et al. 2019a; Cheema et al. 2019b; Jones et al. 2019) and Kumar, Wang, et al. in this issue. Additionally, a mass spectrometry imaging interrogation of the lipid alterations across the natural history of the PBI/BM2.5 model has been reported (Carter et al. 2019). To help inform on the natural history of the GI-ARS in the NHP PBI/BM2.5 model, we quantitatively profiled the jejunum proteome of NHPs over a time period of three weeks. Bioinformatics analysis suggests significant pathway and biological process perturbations post-irradiation, informed on underlying mechanisms of radiation damage, and contributed to the evaluation of the potential for proteins to serve as biomarkers for radiation-induced injury.

MATERIALS AND METHODS

Radiation animal model.

All animal procedures were conducted in accordance with the NIH guidelines for the care and use of laboratory animals and experiments were performed with prior approval from the University of Maryland Institutional Animal Care and Use Committee (IACUC). Tissue was collected from a similar region of the jejunum of male rhesus macaques (*Macaca mulatta*); tissues were snap frozen in liquid nitrogen and stored at -80°C until assay. Samples were provided by the laboratory of Thomas J. MacVittie, University of Maryland School of Medicine, Department of Radiation Oncology (Baltimore, MD). Description of the animal

models, including radiation exposure and dosimetry, medical management (supportive care and health monitoring), as well as collection of tissue have been previously described (MacVittie et al. 2012a). NHP were exposed to 12 Gy partial body irradiation with 2.5% bone marrow sparing (PBI/BM2.5) with 6 MV LINAC-derived photons at 0.80 Gy min⁻¹. Bone marrow sparing was accomplished with tibiae outside the beam field. Jejunum was obtained from a natural history study where timed euthanasia was planned at day 4, 8, 15, and 21 after radiation. Additional jejunum samples were obtained from animals that were euthanized according to criteria specified in the IACUC protocol. We grouped animals from both planned and for-cause euthanasia at day 4, day 8 and 9, day 11 and 12, day 15, and day 21 and 22 to increase the numbers of n at the timepoints across our study. Two non-irradiated animals were used as baseline controls. Thirty-two jejunum samples were analyzed in total with the numbers of NHP at each time point (n) as follows: day 0 (baseline), n=2; day 4, n=4; day 8-day9, n=11; day 11-day 12, n=7; day 15, n=5; day 21–22, n=3.

Proteomic sample preparation.

Jejunum tissues were homogenized in 50 mM ammonium bicarbonate buffer (pH 7.8) by bead beating using Precellys CK14 lysing kit (Bertin Corp., Rockville, MD) as described previously (Huang et al. 2019). The lysates were further washed, reduced, alkylated and trypsinolyzed in filter as described previously (Wisniewski et al. 2009; Defnet et al. 2019).

Liquid chromatography-tandem mass spectrometry data acquisition.

Tryptic peptides were separated on a nanoACQUITY UPLC analytical column (BEH130 C18, 1.7 μ m, 75 μ m x 200 mm, Waters) over a 165-minute linear acetonitrile gradient (3 – 40%) with 0.1 % formic acid on a Waters nano-ACQUITY UPLC system and analyzed on a coupled Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer as previously described (Williamson et al. 2016; Defnet et al. 2019; Kim et al. 2019). Full scans were acquired at a resolution of 240,000, and precursors were selected for fragmentation by collision induced dissociation (normalized collision energy at 35 %) for a maximum 3-second cycle.

Liquid chromatography-tandem mass spectrometry data analysis.

Tandem mass spectra were searched against a UniProt *Macaca mulatta* reference proteome using Sequest HT algorithm described previously (Eng et al. 2008) and MS Amanda algorithm developed by Dorfer *et al.* (Dorfer et al. 2014) with a maximum precursor mass error tolerance of 10 ppm. Carbamidomethylation of cysteine and deamidation of asparagine and glutamine were treated as static and dynamic modifications, respectively. Resulting hits were validated at a maximum false discovery rate of 0.01 using a semi-supervised machine learning algorithm Percolator developed by Käll *et al.* (Kall et al. 2007). Label-free quantifications were performed using Minora, an aligned AMRT (Accurate Mass and Retention Time) cluster quantification algorithm (Thermo Scientific, 2017). Protein abundance ratios between groups were measured by comparing the MS1 peak volumes of peptide ions, whose identities were confirmed by MS2 sequencing as described above.

Bioinformatic and statistical analysis.

Pathway, upstream regulator, and statistical analysis were performed as described previously (Huang et al. 2019). Proteins showing at least a 2-fold change (FC) with a false discovery rate (FDR) adjusted ANOVA p -value < 0.05 were considered significantly changed and used for further analysis. Ingenuity Pathway Analysis (IPA) analysis was used to predict canonical pathways and upstream regulators according to the proteins that were significantly different using a Benjamini-Hochberg corrected Fisher's exact test p -value < 0.01 , and z -score was used to infer the activity change direction.

Retinoid quantification.

Jejunum samples were stored at -80°C until processed. Only glass containers, pipettes, and syringes were used to handle retinoids. Extraction of retinoids was performed under yellow lights using a two-step liquid-liquid extraction that has been described in detail previously using 4,4-dimethyl-RA as an internal standard for RA and retinyl acetate as an internal standard for retinol and total retinyl ester (Kane et al. 2005; Kane et al. 2008b; Kane and Napoli 2010; Jones et al. 2015b). Briefly, for the extraction of retinoids, 77 ± 20 mg of jejunum tissue was homogenized in 2 mL 0.9 % NaCl (normal saline) and two 1000 μL aliquots were extracted as technical duplicates. To each homogenate aliquot, 3 ml of 0.025 M KOH in ethanol was added to the homogenate followed by addition of 10 ml hexane to the aqueous ethanol phase. The samples were vortexed and centrifuged for 1 to 3 min at 1000 rpm in a Dynac centrifuge (Becton Dickinson) to facilitate phase separation and pellet precipitated protein. The hexane (top) phase containing nonpolar retinoids (retinol and total retinyl esters (RE)) was removed. 4 M HCl (185 μL) was added to the remaining aqueous ethanol phase, samples were vortexed, and then polar retinoids (RA) were removed by extraction with a second 10 ml aliquot of hexane as described above. Organic hexane phases were evaporated under nitrogen while heating at approximately 30°C in a water bath (model N-EVAP 112, Organomation Associates, Berlin, MA, USA). All samples were resuspended in 60 μL acetonitrile.

Levels of RA were determined by liquid chromatography-multistage tandem mass spectrometry (LC-MRM³) which is an LC-MS/MS method utilizing two distinct fragmentation events for enhanced selectivity (Jones et al. 2015b). RA was measured using a Shimadzu Prominence UFLC XR liquid chromatography system (Shimadzu, Columbia, MD) coupled to an AB Sciex 6500+ QTRAP hybrid triple quadrupole mass spectrometer (AB Sciex, Framingham, MA) using atmospheric pressure chemical ionization (APCI) operated in positive ion mode as previously described (Jones et al. 2015b). For the LC separation, the column temperature was controlled at 25°C , the autosampler was maintained at 10°C and the injection volume was typically 20 μL . All separations were performed using an Ascentis Express RP-Amide guard cartridge column (Supelco, 50×2.1 mm, 2.7 μm) coupled to an Ascentis Express RP-Amide analytical column (Supelco, 100×2.1 mm, 2.7 μm). Mobile phase A consisted of 0.1 % formic acid in water, and mobile phase B consisted of 0.1 % formic acid in acetonitrile. Endogenously occurring retinoid isomers including all-trans-RA, 9-*cis* RA, 13-*cis* RA, and 9,13-di-*cis* RA are resolved using a gradient separation at a flow rate of 0.4 mL min^{-1} with gradient conditions described previously (Jones et al. 2015b). The APCI source conditions and MRM³ detection parameters were as previously

described where the MRM³ transition for RA was m/z 301.1 $\rightarrow$ m/z 205.1 $\rightarrow$ m/z 159.1 and for 4,4-dimethyl RA was m/z 329.2 $\rightarrow$ m/z 151.2 $\rightarrow$ m/z 100.0 (Jones et al. 2015b).

Levels of retinol and RE were quantified via HPLC-UV according to previously published methodology (Kane et al. 2008a; Kane and Napoli 2010). Retinol and RE were resolved by reverse-phase chromatography (Zorbax SB-C18, 4.6 $\times$ 100 mm, 3.5 μ m) on a Waters Acquity UPLC H-class system and were quantified by UV absorbance at 325 nm. Analytes were separated at 1 ml min⁻¹ with a gradient separation as described previously with a typical injection volume of 30 μ l.

The amount of RA, retinol, and RE was normalized per g of tissue. Data are expressed as mean $\pm$ SEM with n representing retinoid values for individual NHP. Statistical significance was assessed with a two-sided, unpaired student's t-test as compared to the baseline value using Graphpad Prism V6.07.

Histological analyses.

Corrected crypt number as an index of crypt survival was measured as described previously (MacVittie et al. 2012b). Briefly, samples of small intestine were collected at necropsy and fixed in formalin, embedded in paraffin, cut and mounted, and stained with hematoxylin and eosin (H&E). The number of crypts, including surviving and regenerating crypts, per length of intestine was scored and the average per group is calculated. The size of surviving crypts varied which influenced the likelihood of surviving crypt in cross section, so a size correction factor was applied to reduce this error based on widths of crypts in nonirradiated control NHPs and surviving crypts in irradiated NHPs. The corrected number of crypts was calculated according to the following equation: Corrected number of crypts = (mean width in control mice / mean width in irradiated mouse) $\times$ mean number of surviving crypts in the irradiated mouse. Corrected crypt number was determined for the numbers of NHP at each time point (n) as follows: day 0 (baseline), n=12; day 4, n=4; day 8-day9, n=5; day 11-day 12, n=5; day 15, n=5; day 21–22, n=3.

RESULTS

Histological characterization.

Jejunum samples were histologically interrogated at various times after radiation up to 21 days after exposure, including 4, 8/9, 11/12, 15, and 21/22. Histological analysis of the jejunum to assess the extent of GI damage was performed as part of the natural history of 12 Gy PBI/BM2.5. Corrected crypt number declined after radiation indicating a loss of crypt cells that was maintained to three weeks (Fig. 1). This same histological index was used to evaluate the potential for metabolite biomarker candidates to inform on the GI-ARS in another manuscript in this issue (Kumar, Wang, et al.).

Expression of proteins most changed after radiation.

The jejunum proteome was assayed after radiation exposure as part of a natural history study in NHP after 12 Gy PBI/BM2.5. Jejunum samples were interrogated at various times after radiation up to 21 days after exposure, including 4, 8/9, 11/12, 15, and 21/22. To identify the

jejunum proteins that are most altered after radiation, we determined the differential protein expression using mass spectrometry-based analyses. By using nano-flow ultraperformance liquid chromatography coupled to high resolution mass spectrometry, proteins whose abundance in jejunum were significantly altered were identified using tandem mass spectrometry by matching peptide fragmentation patterns. We used bioinformatic pathway analysis to further inform on mechanisms of injury that occur post-radiation. 3245 unique proteins were quantified out of more than 3700 proteins identified in this study. A total of 289 proteins of the quantified proteins showed significant and consistent responses across at least three time points post-irradiation, of which 263 proteins showed strong upregulations while 26 proteins showed downregulations (Fig. 2). We used an $FDR < 0.01$, a protein expression $FC > 2$ -fold and an FDR corrected ANOVA p -value cut-off < 0.05 to identify significant changes in the protein expressions. Majority of the differentially expressed proteins showed strong and consistent upregulations over the time period interrogated and only small number of proteins exhibited inhibitions (Fig. 2).

Canonical pathways and upstream regulators altered by radiation.

Ingenuity Pathway Analysis (IPA) analysis was used to predict canonical pathways and upstream regulators according to the proteins that were significantly different (Kramer et al. 2014). We focused on canonical pathways and upstream regulators to understand key events and signaling nodes (Table 1 and Table 2, respectively). The calculated z-score is a statistical measure of the match between expected relationship direction from published literature and observed gene expression from the experimental dataset. It was used to infer likely activation states of pathways or upstream regulators based on comparison with a model that assigns random regulation directions. We used a Benjamini-Hochberg corrected Fisher exact test $p < 0.01$ and a nonzero z-score as criteria for inclusion of canonical pathways (Table 1). We used a Benjamini-Hochberg corrected Fisher exact test $p < 0.01$ and a z-score above 2 for inclusion of inferred upstream regulators (Table 2). Four canonical pathways were significantly altered by radiation (Table 1). Eighteen inferred upstream regulators were significantly activated (15) or inhibited (3) according to the aforementioned criteria (Table 2).

Proteins that correlate with histological analyses.

For the proteins that showed significant downregulation in Fig. 2, we assessed the correlation with established histological parameter, corrected crypt number. Protein abundances of many of these proteins show a decrease in abundance similar to the decrease in the corrected crypt number after radiation (Fig. 3). We calculated the correlations of the abundances of various proteins with the corrected crypt numbers. A number of proteins strongly correlated with the corrected crypt numbers and had correlation values that range between 0.55 and 0.91 (Table 3).

Radiation-responsive proteins with agreement across species.

Proteins identified in the current natural history in NHP study show agreement across species with a previous proteomic interrogation of the small intestine (ileum) in a murine model of the GI-ARS (Huang et al. 2019). Proteins that were observed as changed by radiation in the NHP as well as in the murine small intestine (ileum) include the significantly

and consistently upregulated ACTA1, DUOX2, DNM1, COL6A3, GAL, HP, and S100A8 (Table 4). Majority of these proteins were involved in inflammatory pathways.

Proteins related to retinoic acid activity.

As part of this natural history study, we quantified jejunum RA using quantitative multistage tandem mass spectrometry (Fig. 4). RA was reduced after radiation in agreement with our previous observations in a mouse model of the GI-ARS (Huang et al. 2019). RA was decreased an initial 20% at day 4; RA continued to decline until day 21 where it was reduced 54% (Fig. 4). Retinoid pathway proteins that were changed indicate multiple points of dysregulation (Table 5). Retinal dehydrogenase ALDH1A3 was reduced with significant reduction at day 8 and day 11. ALDH1A1 trended downward and ALDH1A2 trended upward non-significantly across all points. Retinal reductase DHRS4 was reduced with significant reduction at day 4; DHRS11 trended downward non-significantly. RDH11 and RDH13 were detected with RDH13 being upregulated across all irradiated timepoints. RBP1 was increased at day 4 and RBP4 was detected at all irradiated points but not in control. BCO2, which mediates the asymmetric cleavage of beta-carotene, was increased at all irradiated timepoints. Twenty-five proteins were found to be significantly associated with retinoic acid activity by IPA upstream regulator analysis with a Benjamini-Hochberg corrected $p < 0.0001$ (Fig. 5). Moreover, several of the proteins common to our previous murine proteomic analyses and this NHP study are also associated with RA including GAL and COL6A3. YAP1 is an additional protein associated with RA that is changed after radiation.

DISCUSSION

Sampling occurred from NHP at timed euthanasia as planned at day 4, 8, 15, and 21 after radiation as well as from NHP that were euthanized for cause according to pre-defined euthanasia criteria. Previous publications have described this PBI model and the analysis of resulting tissue injury (MacVittie et al. 2012a; Jones et al. 2015a; Shea-Donohue et al. 2016; Cohen et al. 2017; Prado et al. 2017; Carter et al. 2019; Cohen et al. 2019; MacVittie et al. 2019a; MacVittie et al. 2019b; Parker et al. 2019a; Parker et al. 2019b; Parker et al. 2019c). Here we presented results that further characterize the natural history of radiation-induced injury in NHP via determination of differential protein expression in jejunum. Additionally, we considered the proteins yielded by our data towards supporting efforts to identify proteins that could have utility as biomarkers to identify exposed populations, and may be useful during drug development under the FDA Animal Rule, or inform on medical management of radiation-induced injuries post-exposure.

Histological analyses, including corrected crypt number, have been rigorously developed to assess and characterize the natural history of the GI-ARS in both mouse and NHP (Booth et al. 2012a; Booth et al. 2012b; MacVittie et al. 2012a; MacVittie et al. 2012c; MacVittie et al. 2019b). The number of crypts in the jejunum is an index of GI damage. Intestinal epithelial stem cells reside in the crypts and provide cells that differentiate to repopulate the villus epithelial cells (Potten and Grant 1998). This histological index of GI injury has also been used to evaluate biomarkers and their ability to correlate with well-characterized endpoints of

GI injury (Jones et al. 2015a; Jones et al. 2015c). We observed a decrease in corrected crypt number after radiation consistent with previous assessments of radiation-induced GI damage in NHP after PBI (MacVittie et al. 2012a).

In this study, we used liquid chromatography coupled to high resolution mass spectrometry to identify radiation-responsive proteins in jejunum and explored underlying pathway and upstream regulator changes over a time period of three weeks at 4-day, 8/9-day, 11/12-day, 15-day and 21/22-day post-irradiation. A total of 3245 unique proteins were quantified, and compared to the baseline, 289 radiation-responsive proteins were identified, of which the majority showed upregulated expression (263 proteins) and the minority showed downregulated expression (26 proteins). Upregulated proteins included those related to inflammation, nutrient transport and absorption, as well as extracellular matrix and cytoskeletal dysfunction. A number of these proteins were consistent with previously reported proteomic results in mouse including, ACTA1, DUOX2, DNMI1, COL6A3, GAL, HP, and S100A8 (Huang et al. 2019). Proteins that exhibited a downregulation after radiation had a change in their abundances that followed a similar trend as corrected crypt numbers (Fig. 3). The correlation of downregulated proteins with corrected crypt number revealed that CKAP5, OAT, and GSTA2 had the highest correlation with these three having correlation coefficients (R) that exceed 0.8 (Table 3).

Inflammation related proteins such as DUOX2, GAL, HP, S100A8, CRP and SAA1 were highly upregulated in the NHP jejunum (Fig. 2). These changes were consistent across species; changes were consistent with what was observed previously in a murine model of GI-ARS where the ileum was profiled for proteomic changes (Huang et al. 2019). DUOX2 is a membrane oxidoreductase that is able to catalyze the conversation of oxygen to hydrogen peroxide (Carvalho and Dupuy 2013). Abnormal upregulation of DUOX2 leads to continuous reactive oxygen species (ROS) production and results in oxidative injury (Amini et al. 2019). Downregulation of DUOX2 was reported to increase radio-resistance at multiple radiation doses (Nguyen et al. 2015). Increased DUOX2 expression is an epithelial response to mucosal dysbiosis that is needed for immune homeostasis; it has been proposed as a marker of perturbed mucosal homeostasis (Grasberger et al. 2015). S100A8 (S100 calcium-binding protein A8, also known as calgranulin A) is a protein that forms a heterodimer with S100A9 called calprotectin, which plays important roles in regulating inflammatory and immune responses (Fujita et al. 2018). High S100A8 expression has been reported in inflamed intestinal epithelial cells (Fujita et al. 2018). Its increase during pelvic radiotherapy was proposed as a biomarker of radiation toxicity in intestine (Varela et al. 2009). GAL (galanin) is a regulatory peptide predominantly expressed in the central nervous system and the GI system that exerts its anti-inflammatory functions through G protein-coupled receptors (Kaplan et al. 1988; Bauer et al. 2010). GAL also modulates the immune response (Koller et al. 2019). HP is an acute phase protein which is induced in inflammatory conditions to minimize tissue injury and facilitate repair. HP binds free hemoglobin and allows degrading enzymes to access hemoglobin while preventing adverse biochemical and physiologic effects of extracellular hemoglobin (Schaer et al. 2014). CRP is an acute phase inflammatory protein that increases markedly at sites of inflammation or infection (Sproston and Ashworth 2018). SAA1 is also an acute phase protein expressed in the intestinal epithelium. It has also been reported to have bactericidal effects especially with gram-

negative bacteria (Eckhardt et al. 2010). CRP and SAA1 are also elevated in circulation after radiation in multiple species and have been studied as plasma biomarkers (Ossetrova and Blakely 2009; Bazan et al. 2014; Nylund et al. 2014; Ossetrova et al. 2014a; Ossetrova et al. 2014b; Widlak et al. 2015; Ossetrova et al. 2018).

The jejunum is specialized in absorption of small nutrient molecules, such as sugar, amino acids, vitamins, and small peptides. Our results indicate dysregulation of several transporters after radiation including SLC13A2, SLC2A1, and SLC39A7 (Fig. 2). SLC13A2 is a low affinity Na⁺/dicarboxylate transporter found on the apical membrane of cells in the small intestine where it functions in the absorption of citric acid cycle intermediates from the intestinal lumen (Pajor 2006). SLC13A2 was reduced after radiation at all days with the most pronounced decreases at day 8 and day 11 likely limiting the supply of these key intermediates. SLC2A1, also known as GLUT1, facilitates the transport of glucose across the plasma membrane where SLC2A1 expression is increased by reduced glucose. It also is a major receptor for the uptake of vitamin C (Mueckler and Thorens 2013). SLC2A1 was increased after radiation from day 8 through day 21. We also saw a decrease in SLC2A2, also known as GLUT2, with significant decreases at day 8 and day 11. Decreased intestinal SLC2A2 results in glucose malabsorption (Schmitt et al. 2017). SLC39A7, also known as ZIP7, is an intracellular zinc transporter that localizes to the endoplasmic reticulum (ER) and is upregulated by ER stress. SLC39A7 is highly expressed in the intestinal crypt and essential for spatiotemporal regulation of zinc homeostasis that is essential for epithelial proliferation (Itadani et al. 2009; Rodilla et al. 2009; Ohashi et al. 2016). SLC39A7 was upregulated after radiation at all days within the study.

Proteins related to regulation of the extracellular matrix and the cytoskeleton were dysregulated after radiation including ACTA1, COL6A3, DNM1, CLDN3, and YAP1. ACTA1, skeletal muscle alpha-actin, was upregulated at all time points after radiation. ACTA1 has been reported to be upregulated in inflammatory conditions where CRP is also upregulated (Yeremenko et al. 2013). COL6A3 was upregulated after radiation at all timepoints. COL6A3 plays a role in regulating the extracellular matrix and upregulation has been reported in a number of cancers and myopathies (Joyce et al. 2009; Bushby et al. 2014; Liu et al. 2018). DNM1, dynamin 1, is a GTPase that functions in receptor-mediated endocytosis and is involved in vesicle formation from the plasma membrane (van der Blik et al. 1993). CLDN3, claudin 3, was upregulated after radiation and is a tight junction protein involved intestinal barrier function (Patel et al. 2012). YAP1 was upregulated at all timepoints after radiation. YAP1 is a downstream effector of the Hippo pathway that regulates target gene expression; Hippo signaling is crucial to tissue homeostasis and regeneration of intestinal epithelial cells (Chang et al. 2019). Constitutively active YAP1 leads to contact inhibition in epithelial cells as well as mislocalized Paneth cells and reduced intestinal stem cell numbers resulting in loss of cellular proliferation in crypts (Zhao et al. 2010; Barry et al. 2013).

The top canonical pathways significantly activated by IR included GP6 signaling, acute phase response, LXR/RXR activation and intrinsic prothrombin activation (Table 1). The significant activations of GP6 signaling, acute phase response, and intrinsic activation of prothrombin are consistent with radiation-induced tissue injury to jejunum. Glycoprotein VI

(GP6) is a collagen receptor mainly expressed in platelets, which plays a key role in the collagen-induced activation and aggregation of platelets upon injury to the endothelial lining of vessel wall (Nurden 2019). The activation of this pathway is likely due to the abundance of blood capillaries in jejunal villi for nutrient absorption.

Acute phase response signaling, the pathway most significantly activated by radiation, is related to the rapid inflammatory response to infection, tissue injury, trauma, neoplastic growth, and immunological disorders. Consistent with acute phase response, which involves significant upregulations of inflammatory cytokines, our upstream regulator analysis identified a number of inflammatory cytokines (Table 2), including interferon alpha 2 (IFNA2), interferon gamma (IFNG), interleukin 1 beta (IL1B), interleukin 6 (IL6), transforming growth factor beta 1 (TGFB1) and tumor necrosis factor (TNF). A previous characterization of mechanisms of GI damage in the NHP also identified an upregulation of pro-inflammatory mediators after radiation (Shea-Donohue et al. 2016).

LXR/RXR signaling, which was one of the top canonical pathways affected by radiation (Table 1), is related to regulation of metabolism in response to inflammatory cytokine signaling through lipid-sensing nuclear receptors such as LXR and PPAR (Venteclef et al. 2011). CYP51A1 and LPA are two key nodes of the LXR/RXR signaling pathway. CYP51A1 (lanosterol 14- α demethylase) is directly involved in the lanosterol metabolism. It participates in the synthesis of cholesterol by catalyzing the removal of the 14 α -methyl group from lanosterol and thus is responsible for the essential demethylation step in the biosynthesis of sterols, which is regarded as the initial checkpoint in the transformation of lanosterol to other sterols that are widely used within the cell (Stromstedt et al. 1996). LPA (Lipoprotein(a)) is a low-density lipoprotein variant and contributes to atherogenesis (Pan et al. 2004) and atherosclerosis (Tsimikas et al. 2005). It attracts inflammatory cells to vessel walls and leads to smooth muscle cell proliferation (Gouni-Berthold and Berthold 2011) and may be involved in tissue repair (Kostner and Bihari-Varga 1990).

We have previously reported that radiation decreases retinoic acid in jejunum in a murine model of GI-ARS (Huang et al. 2019). RA is an active metabolite of vitamin A that regulates gene transcription through the ligand-activated nuclear receptors retinoic acid receptor (RAR) and retinoid x receptor (RXR) (Germain et al. 2006a; Germain et al. 2006b). In the small intestine, RA is an important regulator of the immune response, epithelial integrity, and essential for differentiation that maintains the crypt-villus axis (Czarnewski et al. 2017). Here, we observed a decrease in jejunum RA ranging from 20 % at day 4 to 54 % decreased at day 21 as compared to baseline (Fig. 4). RA is tightly regulated and a 54 % decline represents a significant dysregulation in RA homeostasis. For comparison, we observed a similar decrease in a mouse jejunum after radiation which exhibited similar levels of GI damage as to the NHP natural history study reported here (Jones et al. 2015c; Huang et al. 2019). This degree of reduction in RA is also similar to previous studies in NHP that show a correlation between RA concentrations and damage to the intestinal mucosa following infection (Byrareddy et al. 2016). In this regard, models of radiation-induced GI injury and models of immune dysfunction share some similarities in histological damage including loss of epithelial integrity, inflammation, villus blunting, and loss of select cell populations.

Dysregulation of proteins within the retinoid pathway indicates radiation disrupts essential nutrient homeostasis (Napoli 2012; Napoli 2020). RA is biosynthesized by a two-step enzymatic conversion of diet-derived vitamin A (retinol) consisting of a rate-limiting oxidation of retinol to retinal by retinol dehydrogenases, which is counterbalanced by retinal reductases which catalyze the conversion of retinal to retinol. No retinol dehydrogenases were detected; a number of retinal reductases were changed. RDH11 and RDH13 were detected where RDH13 was not detected in control but was upregulated in all timepoints after irradiation. RDH11 and RDH13 have retinal reductase activity; RDH13 has been reported to be mitochondrial and related to microsomal RDH11 (Belyaeva et al. 2008; Napoli 2020). DHRS4 was down-regulated with significant reduction at day 15. DHRS4 has reductase activity for retinal (Endo et al. 2009; Napoli 2020). DHRS11 non-significantly trended downward and DHRS1, DHRS7, and DHRS7B were also detected but unchanged. The second, irreversible RA biosynthetic step is the oxidation of retinal to RA by retinal dehydrogenases of the ALDH1A family (ALDH1A1, ALDH1A2, ALDH1A3). ALDH1A1 trended downward non-significantly and ALDH1A2 trended upward non-significantly. Reduction in ALDH1A3 likely reflects loss of intestinal epithelial cells similar to that reported in a model of intestinal inflammation (Collins et al. 2011). In that study, loss of intestinal epithelial cell mass resulted in reduced RA that could not be overcome by inflammation-induced upregulation of ALDH1A3. Retinoids are chaperoned by retinoid-binding proteins to protect them from non-specific oxidation and facilitate their transport and metabolism (Napoli 2012). RBP1 is the main intracellular chaperone for retinol and retinal and RBP4 is the main chaperone for retinol in circulation. RBP2, the main retinol-binding protein that facilitates vitamin A uptake was not detected here. Cellular retinoic acid-binding proteins were also not detected. RBP1 was significantly upregulated at day 4; RBP4 was only detected in irradiated samples. RA can also be derived from the cleavage of beta-carotene which can be cleaved symmetrically into two molecules of retinal by BCO1 (Harrison and Kopec 2020). Carotenoid metabolism at the intestinal barrier is an important component of nutrient homeostasis (von Lintig et al. 2019). BCO1 was not detected here. BCO2 was upregulated at all timepoints after radiation; it mediates the asymmetric cleavage of beta-carotene and has expression in epithelia and endothelial cells (Raghuvanshi et al. 2015; Harrison and Kopec 2020). Asymmetric cleavage of beta-carotene yields apo-carotenals that have been reported to be upregulated in various conditions of cellular stress and able to mediate signaling and biological activity (Harrison and Quadro 2018; Harrison and Kopec 2020).

Several proteins have dysregulation consistent with the observed decrease in RA (Fig. 4). GAL contains a retinoic acid receptor response element in its promoter and is repressed by RA (Nelson et al. 2009). Decreased RA would not allow for optimal repression of GAL consistent with its observed upregulation. COL6A3 was upregulated after radiation when RA was reduced. COL6A3 was previously reported as upregulated in RA-deficient airway smooth muscle cells in both mouse and human (Chen et al. 2018). One of the targets that YAP1 regulates is DHRS3, a key enzyme in RA biosynthesis (Billings et al. 2013). YAP1 upregulation results in an upregulation of DHRS3. DHRS3 is a retinal reductase whose overexpression inhibits RA biosynthesis (Billings et al. 2013; Wang et al. 2018a; Wang et al. 2018b).

Two other key enzymes that regulate small molecules were also among the dysregulated proteins: OAT and PTGS1. OAT is an ornithine aminotransferase that is involved in the intestinal synthesis of citrulline and arginine (Lim et al. 1998). It is highly expressed in the villus epithelium and was downregulated at all timepoints after radiation consistent with the depletion of citrulline in jejunum and plasma that was also observed in this natural history study (Kumar, Wang et al. also in this issue). OAT deficiency in infants results in hypocitrullinemia, hypoargininemia, and hypoorithinemia (Dekaney et al. 2001). We also noted that PTGS1 (prostaglandin G/H synthase 1, also known as cyclooxygenase 1, COX-1) showed a strong and consistent response across all time points after IR exposure. PTGS1 plays a key role in the biosynthesis of prostaglandins and promotes the constitutive reproduction of protective mucus lining in GI. It also promotes platelet activation and aggregation, vasoconstriction, and proliferation of vascular smooth muscle cells. It was reported that radiation injury leads to upregulation of PTGS1 expression in crypt stem cells and their progeny in irradiated mice, and that prostaglandin E₂ (PGE₂) synthesized through PTGS1 promotes crypt stem cell survival and proliferation (Cohn et al. 1997). It was also observed that inhibiting PTGS1 resulted in decrease of surviving crypts in irradiated mice (Cohn et al. 1997). PGE₂ was reported to be elevated in the intestine of irradiated mice (Cheema et al. 2014).

Several limitations of this study should be acknowledged to place the results in context. We were limited with the availability of NHP in the unirradiated group (n=2). It should be noted that, although limited in biological n, the data from the two NHP at day 0 were highly reproducible supporting that these animals were representative of protein expression in unirradiated NHP. The proteomic analyses were conducted on jejunum tissue, which was homogenized and represents an average of all cell types present. Our data does not address protein expression changes in specific cell types. Future studies interrogating specific cell populations would be particularly informative on further defining the mechanism of injury. Lastly, our proteomic analysis of the natural history of GI-ARS in the 12 Gy PBI/BM 2.5 model was a high-content, quantitative screen with rigorous bioinformatics providing statistical support for the reported protein changes. We did not perform any additional or orthogonal verification of protein expression. Future validation of the results reported herein could further elucidate the natural history and mechanism of injury.

CONCLUSION

In summary, we used a mass spectrometry-based proteomics approach to interrogate the jejunum in order to characterize the natural history of the NHP PBI/BM2.5 model. Correlation with corrected crypt number was used to assess the biomarker potential of jejunum proteins that were changed. Through an untargeted system biology approach, we identified proteomic changes that inform on key molecular dysfunction after radiation exposure in an NHP model of the GI-ARS. Proteins related to inflammation, nutrient transport and absorption, as well as extracellular matrix and the cytoskeleton were dysregulated. The most significantly activated pathways included acute phase reaction, GP6 signaling, prothrombin activation and LXR/RXR activation. We also detailed dysregulation of the retinoid pathway through protein changes and changes in the active metabolite, retinoic acid. These data will be useful for a greater understanding of the natural history and

molecular mechanisms of injury of animal models of GI-ARS and may be potentially useful toward the future development of medical countermeasures. A thorough characterization of the natural history may also be useful toward identifying molecular signatures to serve as biomarkers of damage and indicators of recovery and/or therapeutic efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Histological analysis of NHP jejunum after 12 Gy PBI/BM2.5 to assess GI damage. (a) corrected crypt numbers (b) representative histology images at designated days after dose. Each dot in (a) represents a corrected crypt number for an individual animal of the same NHP cohort as assayed for proteomic changes. Arrows in (b) point to select crypts.

Figure 2. Expression of proteins most changed after radiation.

LC-MS/MS proteomic analysis of NHP jejunum after 12 Gy PBI/BM 2.5. Minimum 2-fold change of expression for at least three time points and FDR adjusted ANOVA p -value < 0.05 were criteria for inclusion.

Figure 3. Normalized abundances of proteins in jejunum decreased after radiation as a function of time.

Representative proteins that were reduced after radiation in jejunum with similar responses to corrected crypt numbers shown in Fig 1a. Correlations with corrected crypt numbers are shown in Table 3.

Figure 4. Jejunum RA is decreased after radiation.

Retinoid quantification in jejunum after 12 Gy PBI/BM 2.5. Days after radiation dose are notated. (a.) RA, (b.) retinol, (c.) total RE. Data is mean $\pm$ standard deviation, * $p < 0.05$ using student's t -test as compared to control (0).

Figure 5. Radiation responsive genes in jejunum regulated by retinoic acid.

Proteins showing a minimum 2-fold change with an FDR adjusted ANOVA test p -value < 0.05 were selected for upstream regulator inference at a cutoff of Benjamini-Hochberg corrected Fisher's exact test p -value < 0.0001. Red color indicates significant upregulation with intensity of color corresponding to magnitude of upregulation.

Table 1.
Canonical pathways altered by radiation.

Canonical pathways altered by radiation where criteria for pathway changes were a nonzero absolute activation z-score and a Benjamini-Hochberg corrected Fisher's exact test p -value < 0.01 .

Canonical Pathways	B-H corrected p-value	Activity z-score	Radiation responsive proteins involved in the pathway
GP6 Signaling Pathway	< 0.0001	2.7	COL1A2, COL4A1, COL6A3, COL12A1, COL2A1, ADAM10, FGB, FGA, LAMC2, FGG, COL3A1
Acute Phase Response Signaling	< 0.0001	3.0	HP, HPX, MAPK14, RRAS2, ORM1, ITIH4, SAA1, CRP, FGB, FGA, MAP2K1, FGG
LXR/RXR Activation	< 0.0005	0.7	HPX, LPA, ORM1, ITIH4, SAA1, S100A8, IRF3, FGA, CYP51A1
Intrinsic Prothrombin Activation Pathway	< 0.0005	2.4	COL1A2, COL2A1, FGB, FGA, FGG, COL3A1

Table 2.
Upstream regulators altered by radiation.

Criteria for upstream regulator inference were an absolute activation z-score > 2 and a Benjamini-Hochberg corrected Fisher's exact test *p*-value < 0.01.

Upstream Regulator	B-H corrected <i>p</i> -value	Activity z-score	Radiation responsive proteins regulated by the upstream regulator
Activated			
Ifn	< 0.00005	3.1	BCL6, CASP8, IDO1, IFI16, IRF3, ISG20, LCN2, OAS3, PECAM1, S100A8, SP100, TRIM21
AGT	< 0.00005	3.3	ACTA1, ADH1C, ADIPOQ, ANK3, BGN, CASP8, CDH13, COL1A2, COL3A1, COL4A1, CRP, CYBB, DAB2, GAP43, NCALD, POLE3, PTGS1, SERPINB2, SLC2A1, TH
IL6	< 0.0001	3.4	BCL6, BGN, COL2A1, COL3A1, CRP, CYBB, FGA, FGB, FGG, GAP43, GREM1, HP, HPX, IDO1, IFI16, KRT14, LCN2, LPA, MAP2K1, MPO, ORM1, PECAM1, PRNP, PRTN3, SAA1, TH
IFNG	< 0.0001	4.1	ADIPOQ, ASNS, CASP8, CDH13, CEACAM5, CMPK2, COL1A2, CYBB, DDX60, DUOX2, FGG, GBP6, HK2, IDO1, IFI16, IFI44L, IRF3, ISG20, KRT14, LAMC2, LCN2, MAP2K1, MAPK14, MARCKSL1, NCALD, OAS3, PECAM1, PRNP, S100A8, SLC2A1, SOAT1, SP100, SQSTM1, TRIM21, TRIP11, ZYX
TNF	< 0.0001	2.4	ACTA1, ADIPOQ, ALDH3A1, ANK3, ARHGEF17, BCL6, BGN, CASP8, CDH13, COL1A2, COL2A1, COL3A1, CRP, CYBB, FGG, GBP6, HK2, HP, IDO1, IFI16, LAMC2, LCN2, MAPK14, MARCKSL1, MPO, MSLN, OAS3, ORM1, PECAM1, PLIN1, PRNP, PRTN3, PTGS1, S100A8, SAA1, SERPINB2, SERPINB8, SHBG, SLC2A1, SOAT1, SQSTM1, TH, ZYX
IL1B	< 0.0005	3.2	BCL6, BGN, CMPK2, COL2A1, CRP, CYBB, DAB2, FGB, FGG, GBP6, HK2, HP, IDO1, IFI16, ISG20, LAMC2, LCN2, MAPK14, MARCKSL1, ORM1, PTGS1, S100A8, SAA1, SERPINB2, SLC2A1, UGT1A1, ZYX
STAT3	< 0.0005	2.7	ADIPOQ, BCL6, CMPK2, COL1A2, COL3A1, CRP, FGA, FGB, FGG, GAP43, GBP6, HK2, HP, IFI16, ISG20, OAS3, PECAM1, PSMB5, SAA1, SLC2A1, TRIM5
TGFB1	< 0.0005	2.5	ACTA1, ADAM10, ADIPOQ, ASNS, BCL6, BGN, CASP8, CD34, CEACAM5, COL12A1, COL1A2, COL2A1, COL3A1, COL4A1, COL6A3, CRP, CYBB, DAB2, DDX21, DMTN, ESAM, FGA, FGB, FGG, GREM1, GSDME, IFI16, IRF3, KRT14, LAMC2, LCN2, MAP2K1, MAPK14, PECAM1, PSPH, PTGS1, RFC4, SHOC2, SLC2A1, TYMS, ZYX
CEBPB	< 0.0005	2.1	ADH1C, ADIPOQ, Akr1b7, ASNS, COL1A2, CRP, DAB2, HP, HPX, IL11RA, LCN2, ORM1, PRTN3, PTGS1, SAA1, SERPINB2, ZYX
STAT1	< 0.001	2.7	BCL6, CASP8, CMPK2, DDX60, DUOX2, GBP6, GREB1, IDO1, IFI16, IFI44L, LCN2, OAS3, PECAM1, SOAT1, TRIM21
IGF1	< 0.005	3.1	ACTA1, ADIPOQ, BGN, COL2A1, COL3A1, CYBB, EIF4B, GAP43, HSD11B2, LCN2, PDLIM2, PRNP, SFN, SHBG, SLC2A1, TH
EDN1	< 0.005	2.6	ACTA1, ADIPOQ, COL1A2, COL4A1, CYBB, MAP2K1, MARCKSL1, PTGS1, SERPINB2, SLC2A1
IRF7	< 0.01	3.0	CMPK2, IDO1, IFI16, IFI44L, ISG20, OAS3, S100A8, TRIM21, TRIM5
HIF1A	< 0.01	3.0	ADAM10, ADIPOQ, ASNS, BGN, CEACAM5, ERGIC1, FHL3, HK2, HP, KRT14, NUCKS1, SLC2A1, SLC39A7, TRIM21
IFNA2	< 0.01	2.9	CMPK2, DDX60, IDO1, IFI16, IFI44L, ISG20, MAP2K1, OAS3, SP100, TRIM21
Inhibited			
AHR	< 0.00005	-2.0	ALDH3A1, CASP8, COL12A1, COL1A2, COL3A1, COL4A1, COL6A3, COX8A, CRP, HK2, HP, IDO1, SAA1, SDSL, SERPINB2, SLC2A1, TH, UGT1A1
Alpha catenin	< 0.001	-3.0	BGN, COL1A2, COL3A1, COL6A3, DIAPH1, GAP43, S100A8, SAA1, ZYX
CR1L	< 0.005	-2.2	BGN, COL1A2, COL3A1, COL4A1, COL6A3

Table 3.
Correlation with corrected crypt number.

Protein abundance correlation with corrected crypt number as well as corresponding *p*-value. Accession number and name are included.

Correlation with corrected crypt number			
Accession	Name	R	P value
H9Z1V0	CKAP5	0.91	4.18E-13
F7BGE6	OAT	0.87	3.15E-11
F7FLK3	GSTA2	0.82	6.40E-09
A0A1D5R5Y0	MEP1A	0.79	5.25E-08
F6XW38	LOC711061	0.73	1.19E-06
A0A1D5Q9E4	LOC706528	0.68	1.22E-05
F6TVV6	DPEP1	0.64	6.44E-05
A0A1D5QB60	CA13	0.62	0.00014
H9EXM3	DHDH	0.61	0.00016
A0A1D5RDA4	NAT8	0.61	0.00019
A0A1D5QYQ4	A0A1D5QYQ4	0.6	0.00024
F7HAW1	UGT2B15	0.59	0.00033
F7GW14	UGT1A1	0.55	0.00089

Table 4.
Proteins that were observed as changed by radiation in both the NHP and murine small intestine.

Murine data from Huang et al. 2019.

Genes	Mouse ileum	NHP jejunum	Protein names	Function
ACTA1	Upregulated > 2-fold	Upregulated > 100-fold	Actin, alpha skeletal muscle	muscle contraction, response to extracellular stimulus.
DUOX2	Upregulated > 4-fold	Upregulated > 100-fold	Ferric oxidoreductase domain-containing protein	thyroid hormone generation, response to oxidative stress, cytokine-mediated signaling pathway.
DNM1	Upregulated > 4-fold	Upregulated > 100-fold	Dynamin-1	mitochondrial fission, G-protein coupled receptor internalization, dynamin polymerization involved in mitochondrial fission, endocytosis.
COL6A3	Upregulated > 2-fold	Upregulated > 100-fold	Collagen, type VI, alpha 3	cell adhesion, muscle organ development, extracellular matrix organization.
GAL	Upregulated > 2-fold	Upregulated > 100-fold	Galanin peptides	inflammatory response, neuropeptide signaling pathway, protein kinase A signaling, cAMP-mediated signaling, regulation of glucocorticoid metabolic process.
HP	Upregulated > 8-fold	Upregulated > 10-fold	Haptoglobin	receptor-mediated endocytosis, acute-phase response, positive regulation of cell death, response to hydrogen peroxide, defense response to bacterium.
S100A8	Upregulated > 8-fold	Upregulated > 10-fold	S100 calcium-binding protein A8	cytokine production, acute inflammatory response, chronic inflammatory response.

Table 5.

Retinoid pathway proteins that were changed by radiation

Genes	Protein name	Day 4	Day 8–9	Day 11–12	Day 15	Day 21–22	Function
ALDH1A1	Retinal dehydrogenase 1	n.s.	↓ > 2-fold	↓ > 2-fold	↓ > 2-fold	↓ > 2-fold	Catalyzes oxidation of retinal to RA; cellular aldehyde metabolic process; ethanol oxidation; retinol metabolic process
ALDH1A2	Retinal dehydrogenase 2	n.s.	↑ > 2-fold	↑ > 2-fold	↑ > 2-fold	n.s.	Catalyzes oxidation of retinal to RA; cellular response to retinoic acid; response to estradiol; response to vitamin A; retinal metabolic process; retinoic acid biosynthetic process; retinol metabolic process; vitamin A metabolic process.
ALDH1A3	Aldehyde dehydrogenase family 1 member A3	n.s.	↓ > 20-fold	↓ > 10-fold	↓ > 2-fold	↓ > 2-fold	Catalyzes oxidation of retinal to RA; retinal metabolic process; retinoic acid biosynthetic process; retinol metabolic process.
DHRS4	Dehydrogenase/reductase SDR family member 4	n.s.	↓ > 2-fold	↓ > 4-fold	↓ > 4-fold	n.s.	Catalyzes reduction of retinal to retinol; alcohol metabolic process; cellular ketone metabolic process; oxidation-reduction process; protein targeting to peroxisome; steroid metabolic process.
DHRS11	Dehydrogenase/reductase SDR family member 11	n.s.	↓ > 2-fold	↓ > 2-fold	↓ 2-fold	n.s.	Catalyzes reduction of retinal to retinol; estrogen biosynthetic process; steroid biosynthetic process.
RDH11	Retinol dehydrogenase 11	n.q.	n.q.	n.q.	n.q.	n.q.	Catalyzes reduction of retinal to retinol; cellular detoxification of aldehyde; retinal metabolic process; retinol metabolic process.
RDH13	Retinol dehydrogenase 13	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	Catalyzes reduction of retinal to retinol; eye photoreceptor cell development; response to high light intensity; retina layer formation; retinal metabolic process.
RBP1	Retinol-binding protein 1	↑ > 2-fold	↑ > 2-fold	↑ > 2-fold	↑ > 2-fold	↑ > 2-fold	Intracellular chaperone for retinol and retinal; response to vitamin A; retinoic acid biosynthetic process; retinol metabolic process; vitamin A metabolic process.
RBP4	Retinol-binding protein 4	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	Chaperone for circulating retinol; maintenance of gastrointestinal epithelium; positive regulation of immunoglobulin secretion; response to ethanol; response to retinoic acid; retinol transport.
BCO2	Beta,beta-carotene 9',10'-oxygenase	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	↑ > 100-fold [^]	Catalyzes asymmetric cleavage of beta-carotene at the 9',10' double bond; carotene catabolic process; retinal metabolic process; retinoic acid metabolic process.

n.s. = Fold change < 2.

n.q. = not able to quantify.

[^] = only detected in irradiated, not in unirradiated.