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Acute proteomic changes in lung after WTLI in a mouse model: identification of potential initiating events for delayed effects of acute radiation exposure

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Abstract

Radiation-induced lung injury is a delayed effect of acute radiation exposure resulting in pulmonary pneumonitis and fibrosis. Molecular mechanisms that lead to radiation-induced lung injury remain incompletely understood. Using a murine model of whole-thorax lung irradiation, C57BL/6J mice were irradiated at 8, 10, 12 and 14 Gy and assayed at day 1, 3, and 6 post-exposure and compared to non-irradiated (sham) controls. Tryptic digests of lung tissues were analyzed by liquid chromatography-tandem mass spectrometry on a Waters nanoLC coupled to a Thermo Scientific Q Exactive hybrid quadrupole-orbitrap mass spectrometer. Pathway and gene ontology analysis were performed with Qiagen Ingenuity, Panther GO and DAVID databases. A number of trends were identified in the proteomic data including protein changes greater than 10-fold, protein changes that were consistently upregulated or downregulated at all time points and dose levels interrogated, time- and dose-dependency of protein changes, canonical pathways affected by irradiation, changes in proteins that serve as upstream regulators, and proteins involved in key processes including inflammation, radiation and retinoic acid signaling. The proteomic profiling conducted here represents an untargeted systems biology approach to identify acute molecular events that could potentially be initiating events for radiation-induced lung injury

Keywords

biological indicators; radiation damage; lungs; rodent; radiation; ionizing

INTRODUCTION

Radiation-induced lung injury (RILI) is a delayed effect of acute radiation exposure (DEARE) where the delayed risks from radiation exposure include pulmonary pneumonitis and fibrosis (Mah and Van Dyk 1988; Mah et al. 1994). Ionizing radiation directly damages

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Conflicts of Interest:

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DNA, causing apoptosis and mitotic disorder, and subsequently disrupts cellular molecules (e.g., proteins and metabolites) and lipid membranes (Mukherjee et al. 2014). Initiation of cellular damage from irradiation (IR) exposure results in chronic oxidative stress, tissue hypoxia, inflammation, and fibro-proliferation in various tissues where lung is particularly radiosensitive to the development of fibrosis (Rodemann and Blaese 2007; Ding et al. 2013). Efforts to characterize the molecular mechanisms of RILI have included histological, cytokine, and gene expression analyses, however, the molecular mechanisms that lead to RILI remain incompletely understood (Finkelstein et al. 1994; Chen et al. 2001; Chen et al. 2002; Stone et al. 2003; Jackson et al. 2016; Jackson et al. 2017; Ghandhi et al. 2018). Additionally, whereas there are medical countermeasures in development to treat radiation-induced lung injury, there are no FDA-approved medical countermeasures (MCM) to treat RILI (DiCarlo et al. 2012; Ding et al. 2013). Drug development of MCM for RILI under the FDA Animal Rule requires the use of well-defined animal models given that human studies cannot be ethically conducted (FDA 2015). The whole thorax lung irradiation (WTLI) model has been developed and characterized extensively for both time- and dose-dependency in both mouse and NHP models for lung-DEARE (Jackson et al. 2011; Jackson et al. 2012; Garofalo et al. 2014; Jackson et al. 2014).

Several recent studies have conducted gene expression profiles in mouse lung after WTLI (Jackson et al. 2016; Jackson et al. 2017) and in peripheral blood cells after WTLI (Ghandhi et al. 2018). Radiation-induced lung inflammation and toxicity in cancer patients has been explored via proteomics (Cai et al. 2011; Oh et al. 2011). Protein profiling is complimentary to gene expression providing additional information on protein levels after mRNA is transcribed including protein translation, post-transcriptional modification, and protein degradation. Proteomic profiling using liquid chromatography-tandem mass spectrometry (LC-MS/MS) yields relative quantitation of protein abundance and allows for systems biology analyses to identify disruption of key signaling pathways and regulatory nodes (Zhang and Chen 2010; Kramer et al. 2014; Karahalil 2016). This type of proteomic approach also provides complementary information from a systems biology perspective to our metabolomic efforts to characterize radiation-induced lung injury where changes in metabolites are often the result of alterations in protein function or abundance (Jones et al. 2014). Previously, the Medical Countermeasures Against Radiological Threat (MCART) has used discovery and targeted metabolomics towards identifying potential biomarkers of radiation-induced lung injury, used mass spectrometry imaging to both identify and localize biomarker candidates and medical countermeasures in the non-human primate lung, and identified biomarker candidates with pharmacodynamic utility (Jones et al. 2014; Carter et al. 2015; Carter et al. 2016; Carter et al. 2017; Jones et al. 2017a; Jones et al. 2017b). This current study sought to use an untargeted systems biology approach to identify acute molecular events that could potentially be initiating events for radiation-induced lung injury.

MATERIALS AND METHODS

Radiation Animal Model

The animal model used in this study has been described in detail elsewhere (Jackson et al. 2012; Jackson 2017). Briefly, age matched male C57BL/6J mice that were 10-14 weeks old

on irradiation day were irradiated with WTLI at 8, 10, 12 and 14 Gy. For each dose, perfused mouse lung samples were taken from mice euthanized at day 1, 3 and 6 after exposure and compared to non-irradiated (sham) controls. Biological triplicates were collected per condition. For WTLI, animals were anesthetized with 80-100 mg/kg ketamine and 10-15 mg/kg xylazine and placed in the prone position. Animals were exposed to 0 Gy (sham radiation) or 8, 10, 12 or 14 Gy of 320 kV x-rays (1.25 Gy min^{-1} ; HVL = 1 mm Cu; XRAD320, Precision X-Ray, North Branford, CT). Sham irradiated mice are noted as “sham” and mice irradiated with WTLI are notated with the dose (in Gy) and time after dose (in days). Animals were monitored daily and euthanized according to predefined criteria (Jackson et al. 2012; Jackson 2017). All animal work adhered to the “Principles of Laboratory Animal Care” (NIH publication #85-23, revised in 1985). All experiments were performed in compliance with the animal use protocol approved by the University of Maryland Institutional Animal Care and Use Committee.

Sample preparation

Lung tissues were homogenized in phosphate buffered saline using Precellys CK14 lysing kit (Bertin Corp., Rockville, MD USA). Proteins were extracted and purified from tissue lysates by trichloroacetic acid precipitation. Protein concentration was measured by bicinchoninic acid assay. 100 ug protein was trypsinolyzed and desalted by C18 tips.

Liquid chromatography-tandem mass spectrometric data acquisition

Tryptic peptides were separated on a Waters nanoAcquity UPLC system (Waters, Milfor, MA USA) over a 95 minutes linear acetonitrile gradient (3 – 35%) with 0.1 % formic acid, and analyzed on a coupled Thermo Scientific Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo, San Jose, CA USA). Technical triplicates were performed for each biological sample. Biological triplicates were collected per condition. Full scans were acquired at a resolution of 70,000 with an automatic gain control target value of $5e^5$ and a maximum injection time of 200 milliseconds, and top 10 most abundant precursors were selected for fragmentation by higher-energy collisional dissociation (normalized collision energy at 30%) and analyzed at a resolution of 17,500 with an automatic gain control target value of $5e^4$ and a maximum injection time of 50 milliseconds. Interrogated ions were dynamically excluded from re-selection for 30 seconds.

Liquid chromatography-tandem mass spectrometric data analysis

Tandem mass spectra were searched against a UniProt *Mus musculus* reference proteome using a SEQUEST HT algorithm described by Eng *et al.* (Eng et al. 2008) with a maximum precursor mass error tolerance of 20 ppm. Carbamidomethylation of cysteines and deamidations of asparagines and glutamines were treated as static and dynamic modifications, respectively. Resulting hits were validated at a maximum false discovery rate (FDR) of 0.01 using a semi-supervised machine learning algorithm Percolator developed by Kall *et al.* (Kall et al. 2007). Abundance ratios were measured by comparing the MS1 peak volumes of peptide ions, whose identities were confirmed by MS2 sequencing as described above. Label-free quantifications were performed using Minora (Thermo Fisher Scientific), an aligned AMRT (Accurate Mass and Retention Time) cluster quantification algorithm.

Bioinformatic analysis

Pathway and gene ontology analysis were performed with Qiagen Ingenuity, Panther GO and DAVID databases, as described by Kramer *et al.*, Mi *et al.*, and Huang *et al.*, respectively (Huang da et al. 2009; Kramer et al. 2014; Mi et al. 2017). Proteins showing at least a 2-fold change (FC) with a FDR adjusted ANOVA p-value < 0.05 were considered significantly changed and used for further analysis. FDR corrected Fisher's exact p-values of < 0.05 were used in the gene ontology analyses to identify biological processes, molecular functions, and cellular components associated with observed protein changes. Ingenuity Pathway Analysis (IPA) analysis was used to predict canonical pathways and upstream regulators according to the proteins that were significantly different using an absolute activation z-score of > 2 for at least one condition with a Fisher's exact test p-value < 0.01.

RESULTS

Study design.

The lung proteome was assayed after radiation exposure to identify acute changes that could aid in the identification of the initiating events of radiation-induced lung injury (RILI), which is a delayed effect of acute radiation exposure (DEARE). Radiation doses of 8, 10, 12, and 14 Gy WTLI were assayed at 1, 3, and 6 days post-exposure. We selected the time course of the current proteomic study based upon recent publications from our team (Jackson et al. 2016; Jackson et al. 2017). One of the recent studies in the murine WTLI model reported that ultrastructural damage and gene expression profiles suggest the response to radiation within the first 24 h post-exposure determines tissue fate (Jackson et al. 2017). The other recent study interrogated gene expression changes and interpreted them in terms of pathophysiological mechanisms (Jackson et al. 2016). The study design of the proteomics study described herein was intended to assay proteomic changes on a similar timescale as these gene expression studies. The proteomic data reported here indicate that, despite mild ultrastructural changes observed in the C57BL/6 strain (Jackson et al. 2017), on day 1 post-exposure there are significant molecular changes due to irradiation. The lethal dose for 50 percent of C57BL/6J mice by day 180 (LD_{50/180}) has been reported to be 13.43 Gy (Jackson et al. 2012). Recently, the LD_{50/360} for male C57BL/6J mice was reported to be 11.24 Gy (Jackson et al. 2017). In the C57BL/6J strain, the lungs displayed scarred, retracted fibrosis over a dose range of 12.5-15 Gy after a protracted latency period (months) (Jackson et al. 2010; Jackson et al. 2011; Jackson et al. 2012; Jackson et al. 2014). Lung samples were perfused at the time of euthanasia, which was essential to reduce the contribution of circulating proteins. In a pilot experiment, the number and identity of proteins changed in response to radiation was compared between perfused and non-perfused lung samples (all radiation doses combined) (Supplementary Fig. 1). Proteins detected in non-perfused lung mostly originate from circulation and many lung proteins were not detected, even those changed over 10-fold in lung after radiation (Fig. 1, Fig. 2). Sixteen (16) proteins showed response to radiation originating from the lung regardless of the perfusion status. Four of these proteins were among the most significantly changed in perfused lung: Cyp2f2, up at all doses (Fig. 2A); Coro1a, Hnrnpa2b1, Hnrnpa3, down at all doses (Fig. 2E).

Expression of proteins most changed after radiation.

Fig. 1 shows a heatmap representing the top 200 greatest changes in protein expression after radiation, where an FDR of 0.01, a protein expression FC > 10 and the FDR corrected ANOVA p -value cut-off of $p < 0.01$ were used to identify changes in protein expression. Many proteins showed repeatable patterns, dose-dependency, and/or time-dependency over the times and dose interrogated.

Proteins showing a consistent elevation or depression in expression after radiation.

Fig. 2a and Fig. 2e show heatmaps of proteins with expression that are consistently altered across the radiation doses and days after dose studied, i.e., all observed changes were activating or inhibiting. Criteria used to identify these proteins was a FC > 2 and FDR corrected ANOVA $p < 0.05$ for at least one condition while the rest of the conditions showed changes in the same direction. Twenty-five (25) proteins were consistently activated and 22 proteins were consistently inhibited. Biological processes that were overrepresented or underrepresented in these proteins that were consistently activated or inhibited during the first 6 days after radiation exposure were then investigated (Fig. 2b and Fig. 2f, respectively). Protein FC > 2, FDR corrected ANOVA $p < 0.05$ for at least one condition. Significant biological process by FDR corrected Fisher's exact test ($p < 0.05$). Findings showed that miRNA loading onto RISC involved in gene silencing by miRNA was consistently overrepresented and that RNA splicing was consistently underrepresented. Similarly, molecular functions that were associated with the observed protein changes were analyzed (Fig. 2c and Fig. 2g). Results showed that molecular function associated with small protein activating enzyme activity were significantly elevated and that molecular function associated with poly(A) RNA binding were significantly reduced. Lastly, the association of the observed protein changes with cellular components was investigated (Fig. 2d and Fig. 2h). The extracellular exosome had proteins associated with it that were either significantly activated or significantly decreased. Other cellular components that were significantly associated with inhibited proteins after radiation included the catalytic step 2 spliceosome, intracellular ribonucleoprotein complex, nucleoplasm, and cytoplasm.

Proteins exhibiting time-dependent changes after irradiation.

Several proteins had time-dependent changes in expression (Fig. 3). Among these, some proteins displayed a progressive increase or decrease in abundance over time (highlighted in yellow frames, Fig. 3a). Biological processes associated with progressive changes in proteins over time included translation, cellular component organization or biogenesis, and blood circulation (Fig. 3b). Another population of proteins showed that their protein expression exhibits a transient increase or decrease followed by normalization of protein abundance (highlighted in green frames, Fig. 3a). Gene expression was considered recovered if difference between day 1 and day 6 is below 30%. Biological processes associated with transient changes in proteins that exhibit normalization/recovery included endocytosis, vesicle mediated transport, primary metabolic process, mRNA splicing via spliceosome, regulation of biological processes, glycolysis, and single-multicellular organism processes (Fig. 3c). Venn diagrams describing differentially expressed proteins as a function of time after dose at a given radiation dose are shown in Supplementary Fig. 2-5.

Proteins exhibiting dose-dependent changes after irradiation.

A number of proteins displayed a dose-dependency in expression (Fig. 4). Those proteins showing either a progressive increase or decrease in expression on at least one of the days are highlighted in yellow (Fig. 4a). Biological processes associated with dose-dependent changes in abundance included fatty acid biosynthetic processes, localization, protein folding, cell cycle, endocytosis, vesicle-mediated transport, and muscle contraction (Fig. 4b). Venn diagrams describing differentially expressed proteins as a function of radiation dose on a given day after dose are shown in Supplementary Fig. 6-8.

Canonical pathways and upstream regulators altered by radiation.

Ingenuity Pathway Analysis (IPA) was used to predict canonical pathways and upstream regulators according to the proteins that were significantly different (Kramer et al. 2014). Canonical pathways and upstream regulators were analyzed to understand key initiating events (Fig. 5 and Fig. 6, respectively). The calculated z-score is a statistical measure of the match between expected relationship direction from published literature and observed gene expression from 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. A z-score absolute value of >2 for at least one condition and a $p < 0.01$ was used as criteria for inclusion in Fig. 5 and Fig. 6. Twenty-two (22) canonical pathways were altered by radiation according to these criteria (Fig. 5). Forty-nine (49) upstream regulators were identified as either activated or repressed (Fig. 6).

Genes related to inflammation, retinoic acid, and radiation.

Proteins showing a minimum 2-fold change with a FDR corrected ANOVA $p < 0.05$ were selected for gene ontology analysis. Inflammation, retinoic acid, and radiation related genes were annotated according to their molecular function and biological process involved (Fig. 7). Inflammation has been reported to be an important mechanism after radiation insult that contributes to the development of RILI (Rubin et al. 1995; Mukherjee et al. 2014). Thirty-two (32) proteins were significantly associated with inflammation. Retinoic acid 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). Previous reports by our team showed that retinoic acid is reduced within 24h after irradiation and this deficit persists through 180 days (Jones et al. 2014). Here, retinoic acid was similarly reduced an average of $41 \pm 6\%$ across the dose and time range in this study (data not shown). Seven (7) proteins were significantly associated with retinoic acid including Add1, Agrn, Apoa1, Apoa2, Twf2, Yap1, and Yes1. Apoa1, which facilitates the reverse transport of cholesterol from tissue lesions to liver, was also associated with inflammation. A lesser number of proteins (four) were associated with radiation in this type of analysis: Eef1d, Rpl26, Tnks1bp1, and Yap1. Yap1, a critical downstream regulatory target of Hippo signaling pathway, was also significantly associated with retinoic acid.

DISCUSSION

A number of trends in the proteomic data were identified and they included the greatest magnitude changes (Fig. 1), protein changes that were consistently upregulated or

downregulated at all time points and dose levels interrogated here (Fig. 2), time- and dose-dependency of protein changes (Fig. 3 and Fig. 4), canonical pathways affected (Fig. 5), changes in proteins that serve as upstream regulators (Fig. 6), and proteins involved in inflammation, radiation and RA signaling (Fig. 7). As canonical pathways and upstream regulators may best give insight into mechanisms of injury, the discussion focuses on the findings in this area. Canonical pathways and upstream regulators that were dysregulated after irradiation grouped to several main functional themes: lipid metabolism and signaling, Rho family GTPases, kinase signaling, and inflammatory and immunomodulatory signaling. It is important to note that the protein changes as well as the changes in pathways and biological processes reported here, while statistically significant based upon our data, are putative and need to be further validated.

The top canonical pathway altered by radiation was LXR/RXR signaling. LXR/RXR signaling regulates lipid metabolism, inflammation, and cholesterol to bile acid catabolism (Germain et al. 2006b). Key lipid upstream regulators included RXR α , PPAR α , PPAR δ , and PPARGCA1A (PPAR γ coactivator 1a). As RXR α is a master regulator of gene expression, it is unsurprising that it regulated 35% of the identified upstream regulators in our proteomic analyses. In addition to heterodimerizing with LXR, RXR also heterodimerizes with PPARs. If both RXR and PPAR are considered, 43% of identified upstream regulators are regulated by RXR α /PPAR α /PPAR δ . Other canonical pathways related to lipid metabolism and signaling that were dysregulated include phospholipase C signaling and PEDF signaling. Phospholipase C (PLC) cleaves phospholipids to produce important second messengers that control diverse cellular processes and are substrates for synthesis of other important signaling molecules. PLC signaling has been shown to mediate endothelial cell inflammation and barrier disruption in acute lung injury (Bijli et al. 2016). PEDF (pigment epithelium-derived factor) induces PPAR γ expression which then induces p53, which impacts cell cycle regulation as well as apoptosis (Ho et al. 2007). Secreted PEDF can bind to its cell surface receptor, PEDF-R, which has phospholipase A2 activity to liberate fatty acids from glycerophospholipids (Notari et al. 2006). Activation of PLC signaling and the increase in PLA₂ activity that would result from an increase in PEDF signaling are consistent with lipidomic dysregulation observed in previous publications where a loss of phospholipids that may be related to increased cleavage rates was observed (Jones et al. 2017a; Jones et al. 2017b).

Rho family GTPase signaling is among the downstream targets of RXR. RXR activates protein kinase A (PKA) activity which negatively regulates Rho GTPases (Gambaryan et al. 2010). In addition to RXR regulation of Rho GTPases, reduction of RA levels, as observed in irradiated lung (Jones et al. 2014), would contribute to the dysregulation of Rho GTPase signaling. For example, we have recently shown that retinoic acid signaling facilitates the cytoskeletal rearrangement via a signaling cascade that involves the RhoA pathway (Wang et al. 2018). Retinoic acid also has been shown to inhibit radiation-induced changes in cell adhesion that were mediated by Rho GTPases (Nubel et al. 2004). Additionally, retinoic acid has been shown to activate phospholipase C signaling which regulates RhoA/Rho kinase signaling (Iiri et al. 1995; Kouchi et al. 2011). The reduction in lung retinoic acid may also have an effect on the inflammatory and immunomodulatory upstream regulators that were identified in this study. Retinoic acid is a high affinity ligand for RAR and has also been

shown to regulate a number of cytokines identified as upstream regulators here including IL1b, IL5, IL6, IL10, IL17A and TNF α (Gross et al. 1993; Upham et al. 2002; Germain et al. 2006a; Dawson et al. 2008; Xiao et al. 2008; Bai et al. 2009; den Hartog et al. 2013; Bakdash et al. 2015; Larange and Cheroutre 2016). Retinoic acid has also been shown to regulate receptors for some of these molecules including IL1R, IL5Ra, IL6R IL10RA, and TNFRSF1A (Upham et al. 2002; Lu et al. 2014; Larange and Cheroutre 2016). Dysregulation of RXR α signaling may contribute to the reduced retinoic acid levels as RXR α regulates ALDH1A2, a key enzyme involved in the biosynthesis of retinoic acid (Napoli 2012). An RXR α mutant mouse model displayed decreased activity of ALDH1A proteins in the liver cytosol of mice (Gyamfi et al. 2006).

Rho family GTPase-related canonical pathway dysregulation was numerous and included: signaling by Rho family GTPases, regulation of actin motility by Rho, RhoA signaling, RhoGDI signaling, Rac signaling, NGF signaling, and Cdc42 signaling. Pertinent to this study, Rho family GTPases have been shown to regulate pulmonary endothelial barrier function and pathological activation is associated with pulmonary vascular dysfunction (Storck and Wojciak-Stothard 2013; Duluc and Wojciak-Stothard 2014). Among the functions regulated by Rho family GTPases are intracellular actin dynamics, endothelial permeability, angiogenesis, nitric oxide production, smooth muscle contractility, cell proliferation, differentiation, and apoptosis. RhoA/Rock has been associated with the pathogenesis of COPD and acute lung injury. Rho GTPases mediate the effects of drugs used to treat vascular disorders, thus they may be important to consider during MCM development (Storck and Wojciak-Stothard 2013; Duluc and Wojciak-Stothard 2014).

Kinase related canonical pathways dysregulated by radiation included ILK signaling, UVA-induced MAPK signaling, ERK5 signaling, PAK signaling, and paxillin signaling. ILK (integrin-linked kinase) control the activity of serine/threonine phosphatases and has been associated with cell migration, proliferation, and adhesion (Widmaier et al. 2012). PAK proteins act as serine/threonine p21-activated kinases and serve as targets for the GTP binding proteins Cdc42 and Rac. Through this action they link Rho family GTPases to cytoskeletal reorganization and nuclear signaling (Radu et al. 2014).

Inflammatory and immunomodulatory canonical pathways included production of nitric oxide (NO) and reactive oxygen species (ROS) in macrophages, dendritic cell maturation, role of IL17F in allergic airway diseases, CXCR4 signaling, iCOS-iCOSL signaling in T helper cells, and lymphotoxin β receptor signaling. Lymphotoxin β receptor is a member of the tumor necrosis factor receptor superfamily also known as TNFRSF3. Lymphotoxin β receptor is a cell surface receptor for lymphotoxin involved in apoptosis and cytokine release that plays a role in the organization of lymphoid tissue (Crowe et al. 1994; Chang et al. 2002). iCOS-mediated signaling regulates activated T cells and effector T cell functions (Tajima et al. 2008; Takahashi et al. 2009). iCOS co-induces a variety of cytokines including IL-4, IL-5, IL-6, IL-10 and INF γ , and regulates the control of selective entry of Th1 cells into inflamed peripheral tissue (Beier et al. 2007; Clay et al. 2009; Marafioti et al. 2010). NOS2 is inducible by oxidative stress and is associated with inflammatory lung diseases (Sugiura and Ichinose 2011).

Two upstream regulators, TGF β and Ctgf (connective tissue growth factor), have been linked to fibrosis of the lung and radiation-induced lung injury (Fleckenstein et al. 2007; Leask 2008; Lipson et al. 2012; Ding et al. 2013). Targeting the TGF β 1 receptor was effective in reducing the extent of radiation-induced lung injury in a rat model (Anscher et al. 2008). Ctgf has been shown to be a mediator of tissue remodeling and fibrosis where inhibition has been shown to reverse fibrosis (Lipson et al. 2012). Additionally, an anti-Ctgf antibody has been shown to attenuate mRNA changes associated with pneumonitis and fibrosis (Bickelhaupt et al. 2017; Sternlicht et al. 2018). TNF α is a proinflammatory cytokine activated by radiation that also contributes to the late sequelae of radiation caused by dysregulated cytokine signaling (Deorukhkar and Krishnan 2010).

Several findings highlight the complementary information provided by proteomic analyses that is distinct from other -omics-based approaches, including profiling gene expression changes by mRNA (Jackson et al. 2016; Jackson et al. 2017; Ghandhi et al. 2018). Comparing to the mRNA findings of Jackson in the C57BL/6 mouse model at a similar WTLI dose (12.5 Gy), several points were noted (Jackson et al. 2016). A number of proteins are in agreement with the trends observed in mRNA including Coro1a, Ctgf, and Tgfb1. Coro1a, whose inactivation causes immuno-deficiencies, had protein expression reduced after radiation at all radiation doses consistent with mRNA reduction (Shiow et al. 2009; Moshous et al. 2013). Ctgf, a major connective tissue growth factor and Tgfb1, a known master regulator of radiosensitivity were both identified as upstream regulators in our proteomic analyses and were reduced in protein expression as well as reduced in mRNA analyses. Ephx1 represents an example of a fast responding protein change with mRNA and protein changes displayed similarly at day 1 post-IR. Rps271 represents a delayed response in protein expression with mRNA changes observed at day 1 whereas protein changes are not observed until day 6 post-IR. Il10RA is an example where mRNA and protein levels diverge. Il10RA was identified as an upstream regulator via proteomic analyses and displayed variable protein expression levels after IR, with some initial elevation followed by some reduction at later times post-exposure (day 6). mRNA levels for Il10RA were reduced day 1 post-IR whereas protein levels were elevated on day 1 post-IR. Changes in mRNA for proteins related to canonical pathways identified via proteomics were also noted including iCOS, iCOSL, Rhoh, and CXCR4. Other changes in protein levels may have an effect not detected by mRNA profiling. For example, protein levels of heterogeneous nuclear ribonucleoproteins, Hnrnpa2b1, Hnrnpa3, and Hnrnpc, were observed to be down at all doses. Since the nuclear function of HnRNPs is pertaining to gene transcription and pre-mRNA processing, the effect of their changes on target regulation would be at the protein level making it unlikely to see the mRNA changes by real-time quantitative reverse transcription PCR or microarray (He and Smith 2009).

CONCLUSION

Through an untargeted systems biology approach, we identified proteomic changes that inform on key molecular dysfunction after radiation exposure in a mouse model of radiation-induced lung injury. These data will be useful for a greater understanding of animal models of radiation-induced lung injury and may be potentially be useful toward the development of medical countermeasures. Identifying molecular mechanisms of injury may also serve useful

in the efforts to develop molecular signatures to predict who will be likely to develop delayed lung injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2. Proteins showing a consistent elevation or depression in expression after radiation.

(a.) Protein expression increased after radiation in all doses with FC > 2 for at least one condition and FDR adjusted ANOVA p-value < 0.05. (b.) Biological processes associated with protein increase. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$) (c.) Molecular function associated with protein increase. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$) (d.) Cellular component associated with protein increase. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$) (e.) Protein expression decreased after radiation in all doses with FC > 2 for at least one condition and FDR corrected Fisher's ANOVA test p-value < 0.05. (f.) Biological processes associated with protein decrease. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$) (g.) Molecular function associated with protein decrease. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$) (h.) Cellular component associated with protein decrease. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$)

Figure 3. Proteins exhibiting time-dependent changes after irradiation.

(a.) Yellow framed protein expression exhibits a progressive increase or decrease in abundance over time based on linear regression models with $R^2 > 0.81$. Green framed protein expression exhibits a transient but significant increase or decrease ($FC > 2$, FDR corrected ANOVA p-value < 0.05) followed by normalization of protein abundance. (b.) Biological processes associated with progressive changes in proteins over time. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$) (c.) Biological processes associated with transient changes in proteins that exhibit normalization/recovery. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$). Gene

expression is considered normalization/recovery if difference between day 1 and day 6 is below 30%.

Figure 4. Proteins exhibiting dose-dependent changes after irradiation. (a.) Yellow framed protein expression exhibits a progressive increase or decrease in abundance according to dose time based on linear regression models with $R^2 > 0.81$. (b.) Biological processes associated with dose-dependent changes in abundance. Significance was calculated by FDR corrected Fisher's exact test. ($p < 0.05$).

Canonical Pathway

LXR/RXR Activation
 Signaling by Rho Family GTPases
 ILK Signaling
 Phospholipase C Signaling
 Regulation of Actin-based Motility by Rho
 Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
 RhoA Signaling
 RhoGDI Signaling
 UVA-Induced MAPK Signaling
 ERK5 Signaling
 Dendritic Cell Maturation
 Rac Signaling
 NGF Signaling
 Role of IL-17F in Allergic Inflammatory Airway Diseases
 Coagulation System
 CXCR4 Signaling
 Cdc42 Signaling
 PAK Signaling
 Paxillin Signaling
 iCOS-iCOSL Signaling in T Helper Cells
 PEDF Signaling
 Lymphotoxin β Receptor Signaling

Figure 5. Canonical pathways altered by radiation.

Criteria for pathway changes was absolute activation z-score > 2 for at least one condition and Fisher's exact test p-value < 0.01 .

Figure 6. Upstream regulators altered by radiation.

Criteria for upstream regulators was absolute activation z-score > 2 for at least one condition and Fisher's exact test p-value < 0.01.

Figure 7. Genes related to inflammation, retinoic acid, and radiation. Proteins showing a minimum 2-fold change for at least one condition with a FDR adjusted ANOVA test p-value < 0.05 were selected for gene ontology clustering. Proteins annotated to have an established relationship are noted: (a) inflammation, (b) retinoic acid, and (c) radiation.