Published in final edited form as:

Exp Dermatol. 2012 May ; 21(5): 352–357. doi:10.1111/j.1600-0625.2012.01470.x.

Quantitative phosphoproteomics identifies filaggrin and other targets of ionizing radiation in a human skin model

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Abstract

Our objective here was to perform a quantitative phosphoproteomic study on a reconstituted human skin tissue to identify low and high dose ionizing radiation dependent signaling in a complex 3-dimensional setting. Application of an isobaric labeling strategy using sham and 3 radiation doses (3, 10, 200 cGy) resulted in the identification of 1052 unique phosphopeptides. Statistical analyses identified 176 phosphopeptides showing significant changes in response to radiation and radiation dose. Proteins responsible for maintaining skin structural integrity including keratins and desmosomal proteins (desmoglein, desmoplakin, plakophilin 1, 2 and 3) had altered phosphorylation levels following exposure to both low and high doses of radiation. Altered phosphorylation of multiple sites in profilaggrin linker domains coincided with altered profilaggrin processing suggesting a role for linker phosphorylation in human profilaggrin regulation. These studies demonstrate that the reconstituted human skin system undergoes a coordinated response to both low and high doses of ionizing radiation involving multiple layers of the stratified epithelium that serve to maintain tissue integrity and mitigate effects of radiation exposure.

Keywords

Ionizing Radiation; Skin; Phosphorylation

Introduction

While the effects of high doses of radiation on biological systems and on human health are well documented [1], the health consequences of low dose exposures (10 cGy) are still being debated [2]. In many cases, the potential health effects of low dose exposures are extrapolated from high dose data due to difficulties in acquiring statistically significant cancer risk data at low doses [3] and these risks may be overestimated [4]. Since humans are continuously exposed to low doses of ionizing radiation from the environment, medical procedures, and in certain occupations, it is important that any health consequences are thoroughly understood. In addition, low and high dose exposures due to nuclear power accidents and terrorist attacks are plausible scenarios. A key goal of our research is to

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Author contributions: FY, MBS, CVN, LMM, RMW, MAG, and DLS performed the research. FY and DLS designed the research study. RZ, DGC, and RDS contributed essential tools. FY, KMW, BJWR, JTA and DLS analyzed the data. FY and DLS wrote the paper

understand how biological systems respond to low and high doses of ionizing radiation and determine if these responses are linear to the dose received.

Proteomic labeling strategies using isobaric tags for relative and absolute quantitation (iTRAQ) allow multiple samples to be combined within the same multiplexed analysis to quantify the effects of treatment conditions on biological systems [5-7]. The iTRAQ labels have the same mass, but after fragmentation in the mass spectrometer they release reporter groups as low m/z singly charged ions with distinct masses which can be used to determine the relative abundance of the parent peptide in each sample. While there are many methods to quantify proteomic data, the multiplexing capabilities of the iTRAQ enable higher throughput analysis of multiple samples. In addition, since the labeling is performed at the post-tryptic peptide level, this method can be applied to analyze proteins present in tissues.

We recently reported a method which facilitates the quantification of iTRAQ-labeled phosphopeptides [8]. Here, we applied this strategy to identify the effects of radiation dose on the phosphoproteomes present in a reconstituted human skin model. Reconstituted skin tissues, which are metabolically active and recapitulate many skin functions [9] have been used to study radiation responses in a 3D tissue setting [10, 11, 12] [Mezentsev, 2011 #46]. Here we exposed these reconstituted tissues to sham and 3 doses (3, 10, and 200 cGy) of ionizing radiation to assess radiation dose effects on global signaling networks. The doses used here are in the range received during common medical procedures such as CT scans (3 cGy) and cancer radiotherapy (200 cGy). From this screen we identified 1052 unique phosphopeptides and obtained quantitative information indicating 176 phosphopeptides are significantly affected by the radiation exposure. Our results suggest that even very low doses of ionizing radiation activate cell signaling pathways resulting in altered protein phosphorylation and possibly altered protein function.

Methods

Tissue Culture and Treatments

EpidermFT human skin tissues (Mattek; Ashland, MA) were irradiated with 0, 3, 10 or 200 cGy low Linear Energy Transfer (LET) radiation generated using a Pantek XRAD 320 irradiator (GE Inspection Technologies; Hurth, Germany). Cell fractionation was performed using a subcellular fractionation kit (Thermo Fisher Scientific, Rockford, IL). Western blots used antibodies to keratin 10 (Ab-2; Thermo Fisher Scientific), and filaggrin (clone 15C10; Vector Labs, Burlingame, CA).

Labeling and enrichment of phosphopeptides

One hour post irradiation, tissue lysates were prepared by homogenization in 1 ml of freshly made 8 M urea in 50 mM ammonium bicarbonate (pH 8.0) containing a 1× concentration of phosphatase inhibitor (Thermo Fisher Scientific). After incubation on ice for 30 minutes, insoluble matter was removed by centrifugation at 16,000 x g for 20 minutes. Proteins were reduced, alkylated, digested with trypsin and desalted using solid-phase extraction. Peptides (~200 Pg) from one biological replicate of each condition (0, 3, 10 and 200 cGy) were separately methyl esterified [13], labeled with two units of a different 4-plexed iTRAQ reagent (Applied Biosystems, Foster City) according to the manufacturers recommendations. After iTRAQ labeling, samples were mixed, desalted, followed by immobilized metal-ion (Fe^{3+}) affinity chromatography (IMAC) enrichment for phosphopeptides [13]. Four sets of iTRAQ experiments were performed for four biological replicates. The four iTRAQ labels were rotated in each case to minimize potential isotope bias of the iTRAQ labels [7].

Liquid chromatography-mass spectrometry analysis

All samples were separated using an automated dual-column phosphoproteome nano-HPLC platform assembled in-house [13] and analyzed using an LTQ-Orbitrap MS instrument (Thermo Fisher Scientific). This high resolution, dual column system extends the dynamic range of detection and reduces multiplexing issues (i.e. adjacent, or overlapping, precursor m/z values selected for fragmentation simultaneously) and potential manufacturing impurities associated with the iTRAQ method [7]. Pulsed-Q dissociation (PQD) with optimized normalized collision energy of 22 was used for label quantification and sequential PQD and collision activated dissociation (PQD-CAD) or sequential PQD and multistage activation dissociation (PQD-MSA) [8, 14] for the three most abundant species from each MS scan. Two MS technical replicate analyses (one with PQD-CAD, the other with PQD-MSA) were performed for each replicate. All MS/MS spectra were searched against a human protein database to identify phosphopeptides using SEQUEST and filtered for a false discovery rate of < 1%. The updated in-house developed MASIC software program [15] was applied to extract the maximum peak intensity within ± 0.5 Da of each expected iTRAQ reporter ion (PQD: 114-117 m/z) from each fragmentation spectrum using the correction factors provided by Applied Biosystems. As a result, identified peptides from either CAD or MSA spectra were able to be quantified by PQD.

Statistical Processing and Analysis

A simple algorithm was implemented to select the best peptide candidate scan. If a peptide was only seen once within a single technical replicate that data was considered. When a peptide was observed more than once within one or more technical replicates then the algorithm worked by selecting all scans associated with a single unique peptide across all biological and technical replicates (8 total = 4 biological replicates x 2 technical replicates). The two scans with the highest correlation that were not from the same group of the 8 were selected for inclusion. If groups remained that retained data for the peptide under consideration, then the next scan that correlated the highest with either of the scans already selected was chosen for inclusion. This process was repeated until no groups remained for which data is available and for which a single scan had already been selected. Any of the 8 groups that did not have a representative scan after this process were represented as missing values in the data matrix ('NaN'). In addition, if multiple scans existed for a single group and any had complete data (observed in all 4 treatments) then those were considered and the scans with missing data were discarded. Scans with only a single identification were also removed as these had no metric of variance.

All the data was consistent except for the third biological replicate, for which the 3 cGy treatment displayed outlier intensity values for both technical replicates, and thus was excluded from further analysis. In the cases where technical replicates were well correlated ($R > 0.8$), we used the average of the two technical replicates otherwise we selected the technical replicate that correlated best with all the data present for the peptide under consideration. For each peptide we used a t-test to determine if the log ratio was significantly different than 0, which is equivalent to a paired t-test in that the difference between the raw log intensity values is equal to 0. The paired test was used to directly compare treatment doses and given the small sample sizes, a multiple test correction was not implemented.

Immunohistochemical staining

Tissues were fixed in 4% paraformaldehyde, dehydrated, paraffin embedded, and cut into 5 μ m sections using a rotary microtome (Leica RM 2155, Buffalo Grove, IL). Deparaffinized and rehydrated tissues were heated in 10 mM citrate buffer for 20 min (pH 6.0, microwave) to unmask antigens. The slides were washed in water and endogenous peroxidases were

inactivated by incubation in 1 % H₂O₂ for 10 min. The samples were washed three times in PBS and nonspecific binding was blocked with 3% BSA in PBS for 1 hour. Sections were incubated with a primary antibody against filaggrin (4 ng/ ml, Abcam, San Francisco, CA) overnight at 4°C. For detection of filaggrin expression the LSABTM-HRP-DAB+ kit (Dako, North America, Inc. Carpinteria, CA) was used following the manufacturer's protocol. The slides were counterstained with Harris hematoxylin (Fisher scientific, Pittsburgh, PA). Stained tissue sections were imaged using an Olympus BX 40 microscope (Olympus America Inc.) and a Moticam 2300, 3.0M pixel camera controlled by Motic Images plus 2.0 software (Motic, Richmond, Canada).

Results

The reconstituted skin tissues used here are fully differentiated into a fibroblast derived dermis and a keratinocyte derived epidermis (Figure 1A). The epidermis consists of both proliferating keratinocytes in the basal layers and terminally differentiated keratinocytes in the granular and cornified layers. To demonstrate differentiation of these tissues, we stained tissue sections with the known differentiation marker, filaggrin, which localizes to the granular layer (Figure 1B). Basically, each skin tissue was irradiated with 0, 3, 10 or 200 cGy and processed for mass spectrometry after 1 h incubation. The tryptic peptides from each sample were labeled with different isobaric tags that generate masses of 114, 115, 116, or 117 upon fragmentation (Figure 1C). Phosphopeptides were then enriched using IMAC followed by high resolution LC-MS/MS. This experimental design was repeated 4 times in which we rotated the tags in the (unlikely) event that different tags would bias the experiment to have 4 biological replicates of the 4 radiation doses.

Phosphopeptide identification and quantitation

A representative example for the phosphopeptide identification and quantification using adjacent PQD and MSA scans is shown in Supplementary Figure 1 for the filaggrin phosphopeptide SGpSFLYQVSTHEQSESAHGR. The MSA spectrum shows almost complete complementary *b* and *y* ion series for the phosphopeptide, allowing confident identification of both the peptide sequence and phosphorylation site. In contrast, the corresponding PQD spectrum displays fewer ions that can be matched to theoretical *b* and *y* ions for this peptide, and provides an incorrect phosphorylation site to the peptide as SGSFLpYQVSTHEQSESAHGR. However, this PQD spectrum provides quantitative information for this phosphopeptide, shown by the iTRAQ reagent reporter ions resulting from different cell conditions in the zoomed view. This filaggrin phosphopeptide was detected in 3 of the 4 biological replicates and showed increased phosphorylation at each radiation dose.

Altered filaggrin phosphorylation and processing

Filaggrin is a protein important for maintaining skin barrier function and hydration and exists as a large 435 kD profilaggrin form that gets processed into filaggrin monomers that bind to and crosslink keratin fibers in the stratum corneum of skin and provide the protein degradation products that act as natural moisturizing factors (NMFs) [16]. The profilaggrin phosphopeptides are present in multiple copies in the linker regions between the filaggrin monomers (Figure 2A) where proteolytic processing of profilaggrin occurs [17]. Alignment of the phosphopeptide sequence with the profilaggrin sequence revealed that there are four copies of this phosphopeptide, while the remaining linkers exhibit varying degrees of conservation of this sequence (Figure 2B). A fifth peptide containing serine at position 1116 in which isoleucine replaces leucine at the +2 position may also be phosphorylated as these peptides are indistinguishable by mass spectrometry. The phosphorylated serine residue

located at positions S1116, S1764, S2413, S3385 and S3709 is present in all 11 linker regions of human profilaggrin.

Since the profilaggrin phosphorylation sites are located in regions that contain proteolytic cleavage sites, we ascertained if radiation impacts this processing at low (10 cGy) to very high doses (1000 cGy). As shown in Figure 3C, increasing radiation dose resulted in an increase in filaggrin antibody reactive protein bands of intermediate size within 3 hours of treatment. These intermediate sized bands had a ladder appearance indicating they may represent proteins comprised of different numbers of filaggrin monomers. While all doses examined resulted in differences in these intermediate bands compared to the sham irradiated tissues, there may be dose dependent effects as substantial differences could also be observed in the relative intensity of intermediate bands present in the 10 and 200 cGy samples. Given that there are multiple linkers exhibiting differential phosphorylation, the patterns of cleavage products may be very complex at different radiation doses. In addition to affecting phosphorylation, radiation could also induce alterations in the multiple proteases responsible for filaggrin degradation {Ovaere, 2009 #57}. The same blots were reprobed with a K10 antibody as a loading control. Analysis of 4 separate samples indicated increased presence of these intermediate bands in all radiation treated samples and a decrease in filaggrin monomers at 200 cGy. This effect remained at 8 h but disappeared by 24 h (data not shown).

Statistical analysis of iTRAQ data

A total of 1052 unique phosphopeptides were identified in this analysis from the 4 doses and 4 biological replicates. A complete list of the identified phosphopeptides can be found in Supplementary Table I. Adequate data for statistical analysis using a standard paired t-test was available for a subset of these (Supplementary Table II), which enabled identification of 176 significantly changed phosphopeptides. Of these 92 showed differences between sham irradiated and at least one dose, while the remaining phosphopeptides showed statistically significant differences between doses.

Shown in Table I are phosphorylation sites undergoing a two-fold or greater change in comparison to sham treated tissues for the 200, 10 and 3 cGy samples. While few phosphopeptides were significantly altered across all three doses as judged by the statistical analysis, proteins generally showed consistent up-regulation or down-regulation at 2 or more doses. This suggests that many additional lower abundant and potentially regulated phosphopeptides may not have passed the significance filter due to the sparseness of the data. Due to the under-sampling issues associated with LC-MS/MS for complex peptide mixtures [18], low abundance phosphopeptides are often not observed in different sample runs, making it more difficult to obtain sufficient data for comprehensive statistical analyses. In Supplementary table III, we list phosphopeptides showing consistent changes in abundance (>2 fold) across two or more biological replicates. This less stringent method identified 39, 30, and 41 phosphopeptides showing 2 fold or greater changes at 3, 10 and 200 cGy respectively.

Altered phosphorylation of skin structural proteins

A large number of the phosphopeptides identified in the total data set and the statistically significant data set corresponded to skin structural proteins, which is not surprising given that these proteins make up a large percentage of the total skin proteins. For example, 36 altered phosphopeptides were derived from multiple keratin isoforms (1, 2, 5, 6a, 6b, 10, 14, 15, 16, 17 and 19). Radiation induced both up- and down-regulation of phosphorylation of multiple sites, most of which localized to keratin head domains. Keratin phosphorylation was affected by both low and high doses of ionizing radiation with 11, 11, and 17

phosphorylation sites showing significant changes from control levels at 3, 10 and 200 cGy, respectively. While little is known about the function of specific keratin phosphorylation sites, keratin head domain phosphorylation in general is implicated in keratin filament assembly, keratin solubility and keratin/protein interactions [19, 20]. Several sites on the desmosomal plaque protein, plakophilin 1 (PKP1), were identified in our screen with S155 being a highly changed phosphorylation site detected in the 3 cGy sample. Additional proteins present in desmosomal plaques including PKP 1 and 2, desmoglein, desmoplakin, plectin 1 and multiple keratins underwent altered phosphorylation following radiation exposure (Table 1 and Supplementary Table II) suggesting that this structure is a major target of radiation induced signaling pathways. Furthermore, all of these proteins with the exception of desmoglein were affected by low doses of ionizing radiation.

Bioinformatics analysis

With the large number of structural proteins present, the small size of the datasets is too small to run extensive bioinformatics analysis with enrichment statistics. However, bioinformatics tools can be used to identify potential radiation affected functions and networks as well as identify potential functions of specific radiation affected proteins and phosphorylation sites. To identify potential functions and pathways affected by each dose of ionizing radiation, data were analyzed through the use of IPA (Ingenuity Systems, www.ingenuity.com). For all doses, the top radiation affected functions identified were related to skin disease and skin tissue development (data not shown), due in large part to the presence of the proteins noted above (filaggrin, keratins, desmosomal plaque proteins) in each dataset and the established role of these proteins in these functions.

Combining data from supplementary tables 2 and 3 enabled identification of canonical pathways potentially regulated at each dose (Table 2). Several pathways showing overlap between doses include ILK signaling and RhoA signaling pathways although proteins affected at each dose were significantly different. Other pathways such as integrin signaling (200 cGy), chemokine signaling (10 cGy) and tight junction signaling (3 cGy) indicated potential dose dependence in the regulation of some pathways. In the case of 10 cGy, myosin phosphatase target subunit 1 (MYPT1), the product of the PPP1R12A gene, was highlighted in multiple pathways. MYPT1 forms a complex with the catalytic subunit of type I phosphatase and regulates the phosphorylation of multiple substrates including myosin II [21]. The phosphorylation site identified here located at T696 regulates its binding to the plasma membrane [22]. Interestingly, the rho-associated kinase, ROCK2, which potentially regulates this phosphorylation [23], was also affected by 10 cGy radiation treatment.

Additional radiation affected proteins

This screen also identified multiple proteins with potential roles in radiation responses. These include phosphorylation of promyelocytic leukemia protein (PML) at 200 cGy (3.3 fold) with possible regulation at 3 (2.4 fold) and 10 cGy (3.7 fold). Tankyrase-1 binding protein 1 (TNKS1BP1) was a highly changed protein at 10 cGy and was also significantly affected by 200 cGy as well. While little is known about the function of TNKS1BP1, tankyrase-1 is a telomere-associated poly[ADP]-ribose polymerase (PARP) that has recently been shown to play a role in DNA damage repair in part through its PARP activity on the DNA damage kinase, DNA-PKcs [24].

Discussion

Our quantitative mass spectrometry approach identified multiple proteins and phosphorylation sites affected by ionizing radiation exposure. The total dataset of 1052

unique phosphopeptides (Supplementary Table I) can serve as a useful database of phosphorylation sites in human skin model while our quantitative data (Supplementary Table II) and semi-quantitative data (Supplementary Table III) can be used to assess proteins and molecular pathways affected by radiation. While this undoubtedly represents an incomplete representation of the phosphorylation sites present in the system, this study identified multiple radiation sensitive proteins with both known and previously unreported roles in radiation responses.

Prominent among these is profilaggrin which undergoes increased phosphorylation at multiple sites within the linker domains and the sites of proteolytic cleavage [17, 25]. Sequence alignment of the rat, mouse and human linker regions demonstrate that the amino acids comprising the phosphorylation site identified here are unique to human profilaggrin [26] and may present a novel mechanism for humans to regulate profilaggrin processing in response to ionizing radiation and possibly other types of skin stress. Our findings that increased phosphorylation of this site occurs in parallel with altered processing supports a role for this phosphorylation in profilaggrin regulation. Profilaggrin has long been known to be a heavily phosphorylated protein making it highly insoluble and a general decrease in phosphorylation is required prior to its proteolysis [27]. However, to our knowledge, this is the first description of these phosphorylation sites in the human linker regions that have the potential to impact profilaggrin processing.

Major proteins comprising the desmosome were also affected at the level of phosphorylation by low and high doses of ionizing radiation. Desmosomes have long been known to act as anchors between adjacent cells and are extremely important for maintaining skin tissue structural integrity due to their ability to link keratin filament networks of adjacent keratinocytes. More recently, attention has been given to the idea that desmosomes can be dynamic allowing cells to respond to their external environment through desmosomal remodeling important for wound healing and differentiation [28] or through intracellular signaling mechanisms that regulate transcription and other intracellular processes including DNA damage repair [29, 30]. Given that ionizing radiation can induce both tissue and DNA damage, the altered desmosomal phosphorylation observed here could play an important role in wound healing and/or DNA repair processes.

An important issue addressed by this data is whether terminally differentiated and dividing cells in a tissue all respond to ionizing radiation. Recent studies indicate that the radiation induced DNA damage response commonly observed in cell monocultures was confined mostly to the basal layers while the terminally differentiated cells in the outer layers exhibited no DNA damage response [11, 31]. In our data, we detected radiation induced alterations in proteins dispersed throughout the entire skin tissue including the fibroblast dermal (vimentin), the keratinocyte basal (keratins 5, 14 and 15), suprabasal (keratins 1 and 10), spinous (Keratin 2), granular (profilaggrin) and cornified (Cornifin A and B) layers and the extracellular matrix (collagens 6A3 and 17A1). This suggests that the skin as a whole responds to the radiation as judged by the altered phosphorylation status of proteins present in each layer. Moreover, different epithelial layers may have different roles to maintain skin structural integrity and genomic integrity following exposure to a tissue stress such as ionizing radiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the U.S. Department of Energy (DOE) Office of Biological and Environmental Research Low Dose Radiation Research Program and the Defense Threat Reduction Agency. This research also used capabilities developed by the NIH/NCRR Proteomics Center at PNNL (RR18522). Experiments and data analyses were performed in the Environmental Molecular Sciences Laboratory, a DOE national scientific user facility located at the Pacific Northwest National Laboratory (PNNL) in Richland, Washington. PNNL is a multiprogram national laboratory operated by Battelle for the DOE under Contract DE-AC05-76RLO 1830.

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Figure 1. Experimental design of quantitative iTRAQ phosphoproteomics analysis

A. Cartoon illustration of the full thickness reconstituted human skin used. Shown are the stratified layers of the epidermis and the dermal layer consisting of fibroblast cells embedded in an extracellular matrix. Each layer is not necessarily drawn to scale. **B.** Shown is a hematoxylin stained tissue section from the reconstituted human skin tissue used in this study. The granular layer is stained using a filaggrin antibody (dark brown) to demonstrate the differentiation status of this tissue. **C.** One hour post-exposure, lysates were prepared from each skin sample treated with a different radiation dose (0, 3, 10 and 200 cGy), digested with trypsin, and labeled with a different iTRAQ tag. The samples were then recombined, phosphopeptides enriched with IMAC, and analyzed by LC-MS/MS. Four biological replicates were performed in which the iTRAQ labels were rotated for each radiation dose.

Figure 2. Altered profilaggrin phosphorylation and processing after ionizing radiation
A. Domain structure of profilaggrin showing filaggrin monomers connected by linker regions. **B.** Sequence alignment of 11 linkers. The underlined regions correspond to the linker domain, the bolded regions correspond to the altered tryptic peptides identified here and the asterisks mark positions of all phosphorylation sites present in our dataset. The phosphopeptide containing S1116 is also marked as the isoleucine at position 1118 makes it indistinguishable by mass spectrometry from the other phosphopeptides containing leucine at the corresponding position. **C.** Western blots showing accumulation of intermediate filaggrin antibody reactive bands at 3 h following the radiation treatment (0, 10 200, 1000 cGy). The same blot was reprobed with an antibody against keratin 10 (K10). Arrows mark bands showing differences between control and radiation treated samples.

Table 1
Top phosphorylation sites for each dose

GENE ID	Site	3 cGy Avg	10 cGy Avg	200 cGy Avg
200 cGy				
SEMA4F	S2; T5	1.12	3.67	4.25
PML	S403	2.43	3.66	3.32
TNKS1BP1	S672	1.86	3.33	3.11
PTPLAD1	S114	1.82	1.34	2.95
KRT14	S434	1.44	3.13	2.86
VIM	S325	2.29	2.54	2.78
CYBRD1	S284	3.73	1.54	2.63
DSP	S176	1.77	0.73	2.52
AHNAK2	Multiple	2.10	1.56	2.47
PLEC1	S4626	1.76	1.32	2.30
PLEC1	S21	1.50	1.30	2.03
COL6A3	S1783	1.78	2.03	2.03
KRT6B	S71	0.98	0.59	0.49
CCDC88A		0.50	0.45	0.49
LIMA1	T487	0.47	0.57	0.46
KRT1	S530	0.39	0.47	0.45
10 cGy				
TNKS1BP1	S672	1.86	3.33	3.11
KRT14	S434	1.44	3.13	2.86
LMNA	S390; S395	1.51	2.58	1.35
KRT10	S61	1.90	2.11	1.82
AHNAK2	S280	1.35	2.03	1.52
POF1B	S56	1.04	0.49	0.66
KRT6A	S37	1.03	0.48	0.76
PKP1	S191	0.94	0.47	1.05
KRT5	S38	0.28	0.42	0.62
LMNA	S404; S407	0.56	0.41	0.70
VIM	S73	1.04	0.38	1.95
KRT5	Y66	0.81	0.35	0.73
3 cGy				
FTH1	S179	4.31	1.75	6.30
PKP1	S155	3.42	2.61	2.04
KRT17	S13	3.41	1.16	1.43
SEPT2	S218	2.07	2.13	2.16

GENE ID	Site	3 cGy Avg	10 cGy Avg	200 cGy Avg
KRT10	S42	2.06	1.00	1.48
KRT6B	S44	0.43	0.72	0.79

Shown are the statistically significant peptides showing the greatest fold change at each radiation dose.

Table 2

Canonical pathway analysis of radiation affected proteins.

2000 cGy	Gene ID
ILK Signaling	MYL9,VIM,ITGB4,DSP
Integrin Signaling	MYL9,ZYX,ITGB4,CTTN
Caveolar-mediated Endocytosis Signaling	ITGB4,PTRF
Glycolysis/Gluconeogenesis	PGM1,TPI1
Corticotropin Releasing Hormone Signaling	PRKAR2A,KRT1
RhoA Signaling	MYL9,SEPT2
10 cGy	Molecules
Chemokine Signaling	ROCK2,PPP1R12A
RhoA Signaling	ROCK2,PPP1R12A
14-3-3-mediated Signaling	VIM,AKT1S1
ERK/MAPK Signaling	PPP1R12A,HSPB1
ILK Signaling	PPP1R12A,VIM
3 cGy	Molecules
Tight Junction Signaling	TJP2,PRKAR2A,CTNNB1
ILK Signaling	VIM,CTNNB1,DSP
Phospholipase C Signaling	AHNAK,HDAC7,MARCKS
Corticotropin Releasing Hormone Signaling	PRKAR2A,KRT1
RhoA Signaling	BAIAP2,SEPT2