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Radiation-induced adaptive response: new potential for cancer treatment

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Abstract

Radiation therapy (RT) is highly effective due to its ability to physically focus the treatment to target the tumor while sparing normal tissue and its ability to be combined with systemic therapy. This systemic therapy can be utilized before RT as an adjuvant or induction treatment, during RT as a radiation "sensitizer," or following RT as a part of combined modality therapy. As part of a unique concept of using radiation as "focused biology" we investigated how tumors and normal tissues adapt to clinically relevant multi-fraction (MF) and single-dose (SD) radiation to observe whether the adaptations can induce susceptibility to cell killing by available drugs or by immune enhancement. We identified an adaptation occurring after MF (3 × 2 Gy) that induced cell killing when AKT-mTOR inhibitors were delivered following cessation of RT. Additionally, we identified inducible changes in integrin expression 2 months following cessation of RT that differ between MF (1 Gy x 10) and SD (10 Gy) that remain targetable compared to pre-RT. Adaptation is reflected across different "omics" studies, and thus the range of possible molecular targets is not only broad but also time, dose, and schedule dependent. While much remains to be studied about the radiation adaptive response, radiation should be characterized by its molecular perturbations in addition to physical dose. Consideration of the adaptive effects should result in the design of a tailored radiotherapy treatment plan that accounts for specific molecular changes to be targeted as part of precision multi-modality cancer treatment.

Introduction

The sophistication of radiation therapy (RT) technology has increased markedly in the last two decades, revolutionizing tumor targeting and normal tissue sparing. New dose and

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fraction sizes, along with the application of stereotaxis, allow safer delivery of a large single dose or a few larger fraction (hypofractionation) treatments. Additionally, the use of charged particle therapy and high dose-rate brachytherapy limits normal tissue injury and potentially results in novel radiation biology. The basic tenets of radiation biology for tumor control are traditionally the 4 R's: repopulation, redistribution, repair, and reoxygenation (1). With an increased understanding of tumor response, additional "R's" have been suggested, including "radiosensitivity" and immunological "rejection" (2–5). The mechanisms behind these concepts are known to be complicated and interact with one another during treatment (e.g. DNA damage and immune response, stem cell repopulation and radioresistance), and understanding these responses, which are likely operational for all cancer therapeutics, offers novel precision-medicine treatments.

Recognizing that data from experimental doses used in the laboratory were often not clinically relevant, we examined the impact of clinically relevant RT doses and schedules along with nonsteroidal anti-inflammatory drugs (NSAIDs) on tumors and normal tissues (6–8). Very early in the current era of immuno-oncology and check-point inhibitors, we demonstrated that fractionated radiation triggers canonical immune response pathways in both tumors and normal tissues (9). These observations led to the novel hypothesis that multi-fraction (MF) radiation induces cellular adaptations in tumors that can be targeted with drugs to which the tumors had little to no sensitivity before RT. As described in this review, the data demonstrated that single dose- (SD) and MF-induced changes depended on the underlying cell type (brain, breast and prostate tumor and endothelial cells) and occurred even at doses with little cell killing (7). Additionally, cancer stem cell repopulation can lead to acquired radioresistance (10), demonstrating another type of radioadaptive response. Thus, RT adaptation is relevant to the tumor as a whole, individual tumor cells, and surrounding normal tissue beyond the high-dose field margin.

Previous publications from our laboratories demonstrated that MF radiation (e.g. 1 Gy x 10 over 5 days) induced more differential mRNA and microRNA gene expression compared to SD radiation (e.g. 10 Gy x 1) (11,12). Herein we discuss new findings that support the applicability of radiation-induced adaptations for new therapeutic combinations (13). These adaptations can accompany the advances in physical and spatial focusing that are part of "Accurate, Precision Radiation Medicine" (14). A new paradigm established in a recent NCI workshop (13) emphasizes the importance of describing radiation in both the physical dose, Gy, and in biological perturbations. This includes the adaptations discussed in this review to potentially exploit RT-inducible targets for cancer care including developing radio-mitigators and protectants of normal tissue injury.

In essence, we consider radiation "as a drug" where the pharmacokinetics and pharmacodynamics (PK/PD) can be used in unique ways to impact both local therapy and distant metastases. The technical capability of RT allows the treatment to be focused in a wide range of targets, doses, and schedules, a concept we labeled "focused biology" (15). While we recognize the limitations of laboratory models and that there is much work to be done for these adaptations to be clinically applicable, this report provides insight into the current state of the science.

I. Different adaptations occur between MF and SD radiation.

The range and depth of “omics” analyses continually expand with studies on coding and non-coding RNAs, metabolomics, and proteomics. Our initial work on fractionation was performed *in vitro* and *in vivo* in prostate, breast, and brain tumor cell lines, and demonstrated that the tumor microenvironment influences gene expression patterns after both SD and MF (7). The pattern of gene expression differed for the different cell lines with most changes occurring in the immune response pathways (6,9,16). Both the extent and stability of changes in gene expression were greater following MF compared to SD. Additionally, findings from both our laboratory and others demonstrated that gene expression changes can vary extensively depending on whether the dose is delivered as SD or MF (7,9,17). These studies clearly demonstrated that MF exposure was shown to alter several genes, selectively providing the opportunity to explore molecular target-directed interventions to enhance the tumor response to radiation.

microRNA expression as a form of RT adaptation: dose/fractionation response—To further develop the efficacy of molecular targeted therapy following RT adaptation, we studied microRNA (miRNA) expression patterns after SD ranging from 5 to 10 Gy and MF of 10 fractions ranging from 0.5 to 1 Gy (Fig. 1A), using 3 human prostate tumor cell lines with different p53 status: LNCaP (p53 wildtype), PC3 (p53-null) and DU145 (p53-mutated). The RT-induced changes in miRNA expression pattern depend on dose size and fractionation (Fig. 1B). Despite significant changes in miRNA expression, the surviving fraction following 0.5 Gy x 10 was approximately 85%. Significant differences in the miRNA profiles of breast cancer cell line MDA-MB-361 after SD versus MF exposure have also been reported (18), signifying a dependency on dose fractionation and the presence of a radioadaptive miRNA response. We also studied the differential mRNA and miRNA expression pattern after SD (10 Gy) and MF (2Gy x 5) in normal human coronary artery endothelial cells (HCAEC) (19). The miRNA expression pattern in HCAEC was significantly altered between SD and MF at both 6h and 24h after the final RT dose (Fig. 1C). There were only 17 miRNAs altered after SD, in contrast to 103 differentially altered miRNAs detectable after MF. Among the altered miRNAs, only 5 were common to SD and MF, pointing towards the importance of dose delivery in post RT-druggable targets. Another study using a mouse model with Lewis lung carcinoma cells (LLC1) reported that gene and miRNA expression profiles are dependent upon whether the cells received SD or MF radiation (17), further demonstrating the importance of the type of dose delivery in a preclinical model. These dose delivery-dependent miRNA adaptations are important for continuation of radiotherapy and cancer treatment but could be strengthened with development of predictive miRNA biomarkers. A pretreatment signature that measures radiosensitivity of head and neck squamous cell carcinoma to SD exposure shows the potential of predictive miRNA biomarkers (20); however, more studies are needed to determine the feasibility of using such biomarkers for predicting response to MF radiation.

Early inflection point—Using prostate cancer and HCAEC cell lines, we demonstrated that cells subjected to MF promote a pro-immunogenic molecular signature, among other changes (6,9,21). To investigate the time course of the adaptation, DU145 and PC3 cells were irradiated with 1 Gy every 6 hours, 2–3 times per day, for total doses from 2–10 Gy.

The data demonstrate an inflection point starting from the 6th 1Gy fraction (Fig. 1D) such that the timing and number of fractions is relevant for the induction of a pro-immunogenic molecular signature.

Adaptation to immune checkpoint blockade—Early radiation-induced adaptations can be exploited for immunotherapy and molecular-targeted therapy (16,22–24). Dewan and Vanpouille-Box demonstrated that certain radiation doses and fractions, in combination with immune checkpoint inhibitors, were capable of inducing an immune response that produced an abscopal effect (23,25). Cytosolic DNA that accumulates as a consequence of radiation activates the cGAS/STING pathway with downstream induction of interferon type I (IFN-I) and IFN-stimulated genes. This response can be antagonized by the DNA exonuclease Trex1. IFN-I is produced during acute viral infections and plays a key role in the activation of cytotoxic CD8 T-cells that eliminate infected cells. In tumors, the acute IFN-I production triggered by radiation elicits anti-tumor CD8 T-cells (23,26); however, chronic IFN-I stimulation has been associated with therapeutic resistance (27,28), underscoring the importance of the adaptation as an evolving response that influences the type of tumor microenvironment that develops.

Given that radiation can generate anti-tumor T-cells, another type of early adaptation observed in response to fractionated radiation is the upregulation by tumor cells of immune checkpoint ligand PDL-1, which protected tumor cells from immune-mediated rejection. In this case, concomitant blockade of PDL-1 improved responses by enabling T-cells to reject the tumor, providing an example of a targetable adaptation that sensitizes the tumor to immunotherapy. PDL-1 upregulation was also implicated in radiation-induced tumor equilibrium as a chronic adaptation that led to a standstill between the tumor and the anti-tumor T-cells (29). We have recently shown that upregulation of the ectonucleotidase CD73 on breast cancer cells represents another example of early adaptation to radiation (30). CD73 generates adenosine, a pleotropic immunosuppressive mediator, preventing the infiltration of the tumor by antigen-presenting cells while increasing regulatory T-cells. The details of CD73 signaling pathways and metabolism in tumors have been previously reviewed in depth (31,32). In our recent study, antibody-mediated blockade of CD73 improved tumor response to radiation significantly.

These above findings shed light on how radiation might be used in combination therapy by modifying treatment based on biological adaptation. Effective treatment should strategically exploit adaptations rather than empirically following an initial regimen and examining changes only at the time of disease progression. Our previous work demonstrated that change in expression of a gene is not predictable based upon its initial expression (9).

II. Adaptations can be targeted and lead to cell killing

There are a range of changes that occur to enable molecular target definition (33). In proof-of-principle experiments we measured phospho-protein changes that occurred 2 hours after the last radiation dose. Using a more physiological 3-dimensional cell culture system and with the goal of targeting inducible changes post-radiation, we demonstrated targetable activation of the AKT/mTOR (Fig. 2) (34). To test the hypothesis of dose and fractionation

dependence of adaptation, a regimen of three daily 2 Gy fractions was compared to a single dose of 6 Gy. Phospho-proteomic upregulation of AKT and mTOR and increased protein-protein interaction were observed in DU145 cells at 2 h after multifractionated RT (MF, 3 × 2 Gy) but not after a single dose of 6 Gy (Fig. 2A). When drug treatment was given before and during fractionated RT there was no enhancement in cell killing (Fig. 2B); however, when given 2 hours following completion of MF radiation—at which point the activated AKT/mTOR signaling was observed—the efficacy of the AKT inhibitor was significantly increased.

Early work by Aryankalayil, indicated that adaptation can persist up to 72 hours, the latest time point tested (9). To examine the duration of the adaptation, Eke treated PC3 cells with MF (1 Gy x 10) and SD (10 Gy x 1) and cultured cells for 2 months (34). Initial cell growth was significantly slower but resumed at pre-radiation growth rate after approximately 6 weeks. Based on a long-standing interest in integrin biology and radiation enhancement with integrin-targeted therapy (35–37), the expression of integrins and the ability to target them was studied in the cells that survived SD and MF at 2 months following their last radiation treatment (Fig. 2C, D). Integrin β1 and β4 were upregulated after SD and MF compared to the mock treated controls. The ability of antibodies against β1 (AIIB2) to kill cells was enhanced following long-term adaptation after radiation when compared to unirradiated control cultures. These experiments demonstrate that post-RT adaptation persists in the surviving cells, and can be targeted long after radiation treatment (38).

Our ongoing observations from the inducible changes demonstrate there are more changes early after radiation from MF compared to SD (12,16,21,38); however, at 2 months the pattern is reversed with cells that survived SD showing more changes. These data will be the subject of future reports. As noted below, understanding and exploiting this adaptation is a key emphasis of improved molecular-targeted therapy.

III. Metabolic adaptations after radiation

Alterations in tumor metabolism, with a focus on glucose utilization, have been studied after radiation injury with the goal of more effectively destroying cancer cells (39–41). However, these studies do not necessarily take into account the speed with which these changes may occur, differences in SD versus MF schedules and to what extent these changes continue post radiation.

Preliminary metabolomic data from our laboratory indicates dynamic metabolic changes assessed by both gene expression and metabolite content at early time points between 6h, 24h and 48h after both MF (1 Gy x 10) and SD (10 Gy x 1) radiation in PC3, DU145, and LNCaP cells (Supplemental Table S1). Of interest were the changes in fatty acid oxidation after radiation treatment. Long chain fatty acids enter the cell primarily through a protein mediated system (42). Once in the cell they are bound to Coenzyme A via acyl-CoA synthases (43). Fatty acids then bind to L-carnitine via Carnitine palmitoyltransferase 1 (CPT-1), producing an acylcarnitine which is then ferried into the inner mitochondrial membrane for fuel in fatty acid oxidation (FAO) (Fig. 3A) (44). Microarray data from 24h post 10 Gy or 1 Gy x 10 radiation demonstrated downregulation of genes which regulate the long chain FAO pathway (Fig. 3B). IPA analysis (data not shown) indicates that these

changes in FAO pathway gene expression lead to significant perturbations of the FAO pathway (Fig. 3C). This was consistent with observed alterations in certain acylcarnitines in PC3 cells after MF and SD radiation compared to controls. Increases in acylcarnitines are associated with impaired FAO (45,46). Information on acylcarnitine expression is routinely obtained using blood and serum samples (47) which might serve as a biomarker of effect. Differential carnitine and acylcarnitine expression has recently been proposed as a biomarker in hepatocellular carcinoma (48).

Fatty acid metabolism is notably altered in cancer cells (49). This has recently spurred an interest in inhibitors of lipid metabolism, particularly fatty acid oxidation as novel treatments. This work is complex due to the multifaceted cell survival roles played by constituents of lipid metabolism. Some studies suggest L-carnitine itself has anti-tumorigenic effects due to its function as an HDAC inhibitor(50). Etomoxir, a CPT-1 inhibitor, has been tested as an anti-cancer agent (51). Another compound of interest, Mildronate, decreases L-carnitine entry into the cell by blocking organic cation transporter 2 (OCTN2) and inhibits endogenous L-carnitine production (52). Mildronate has been shown to decrease tumor growth in a rodent model of glioblastoma (53). Avocatin B, derived from avocados, has also been tested as a novel therapeutic in leukemia cells (54). It prevents FAO, potentially through competitive inhibition of fatty acids, accumulates in the mitochondria and increases ROS accumulation which triggers apoptosis (55). A more thorough understanding of the roles of short and medium chain fatty acids, acylcarnitines and fatty acid oxidation on cancer survival is necessary to develop effective combination therapies.

Ongoing work to characterize and utilize the potential tumor adaptation includes detailed studies of phospho-proteomics, metabolomics, DNA repair, and epigenetic changes, as well as further *in vivo* studies (16,34,56,57). Long-term studies of tumors post irradiation conducted with Citrin at NCI demonstrate long-term up regulation of integrins in PC3 tumors (38). Mitchell recently demonstrated an improvement in tumor growth delay when using a relatively standard combined chemo-radiation treatment (58). Extending drug treatment for 2 weeks post-radiation had a significantly greater effect than using the drug for 1 week only. The second week of drug treatment alone inhibited radiation-induced tumor vasculogenesis and thus delayed recurrent tumor growth. This finding is consistent with studies done by Brown et al where induction of SDF-1 is used to monitor radiation-induced vasculogenesis (59). Detailed analysis is now in progress; notably at week 1, many more transcriptional changes were observed with combined drug and radiation compared to drug or radiation alone suggesting perhaps the possibility of identifying novel targets for treatment.

While the adaptive paradigm needs more *in vivo* confirmation, the data demonstrate a) RT can induce an adaptive response that depends on dose and fractionation; b) adaptive changes occur early in treatment (days) and at the end of a week of treatment, and can persist for months, with the time interval to be studied; c) exploiting the adaptation by either taking advantage of it or interfering with it have potential for novel treatment opportunities; and d) the dose fraction size can impact immunotherapy with check-point inhibitors.

IV. Normal tissue adaptations can be exploited as biomarkers of injury and exposure

Acute and delayed normal tissue radiation damage is a dose-limiting factor for radiotherapy and a major concern of accidental radiation exposure. Our previous studies with HCAEC demonstrated that normal tissues also undergo radiation-induced molecular adaptations (6,7) (Fig. 1C) that could be exploited as biomarkers to predict and avoid or mitigate normal tissue injury, thereby improving patient outcomes. The potential of using RNA as a biomarker of tissue-specific injury and of general radiation exposure is increasing with the description of non-coding RNAs that are relatively stable in the blood, are organized in complex regulatory networks, and provide information on tissue-specific changes and identify pathways for injury mitigation.

The implementation of biomarkers for radiation exposure and damaged normal tissue requires an analytical solution similar to the time- and dose-oriented changes for cancer treatment. Such a solution will require complex analyses using various models (e.g. Random Decision Forest, Support Vector Machine) and ultimately a time- and dose-oriented Markov decision tree (60). Critical to this development is identification of stable, reproducible and readily assayable markers that have a high specificity and sensitivity for detection in blood or other body fluids. To this end, Aryankalayil used a whole-body irradiated mouse model to identify significant alterations in the expression patterns of long non-coding RNAs (lncRNAs) and of miRNAs and target mRNAs at different timepoints after various levels of exposure (61,62). Importantly, these studies demonstrated that to triage victims of a radiological incident, multiple RNA biomarkers are needed to differentiate dose at different timepoints following exposure. For example, let-7e-3p may have utility within set of biomarkers, but the varying up- and down-regulation across doses and timepoints make it insufficient as a single marker (Fig. 4A). Additionally, several other groups reported plasma- and serum-based miRNA signatures that distinguish dose, including high vs. low and lethal vs. sub-lethal doses (63–65), further validating the potential of circulating RNA biomarkers for radiation biodosimetry and the importance of multiple biomarkers.

Ongoing work continues to identify circulating RNA biomarkers for a biodosimetry decision tree using mouse, mini-pig, and non-human primate models (Fig. 4B). Tissue-specific injury markers are also being identified, which could be most useful for discerning tumor and normal tissue adaptations during radiotherapy. Additionally, studies of the microenvironment immune response to local tumor irradiation have demonstrated the role of normal tissue immune regulation on tumor recurrence, metastasis, and response to therapy (66–68). Investigation into molecular biomarkers of this immune response could therefore be informative in guiding more effective treatment regimens. Ultimately, molecular signatures for each application (e.g. biodosimetry, stromal response to radiotherapy) can be implemented into a molecular diagnostic workflow (Fig. 4C). While blood-based RNA is currently the primary molecule of interest, other sources of circulating markers, such as exosome-derived RNA, are also being evaluated.

V. Gaps to fill toward clinical implementation

Clinical application requires a more thorough understanding of the underlying biology and the development of biomarkers or imaging strategies to assess the tumor and normal tissue.

Clinical trials could then be designed with biomarkers to validate the preclinical data. Table 1 includes ongoing studies and considerations to further develop clinical targeting of RT-adaptation.

VI. Summary

Evolution in the technological delivery of RT is accompanied by rapid progress in cancer genetics and biology to decipher key molecular signaling and survival pathways and identify druggable targets. In this setting, as described in this review, the biological perturbations induced by radiation could potentially be exploited to enhance precision oncology. This includes pathways that are induced immediately after treatment and that may be sustained throughout radiation fractionation and beyond, for weeks/months after the end of radiotherapy, as a result of what we have described as RT-induced adaptation. Using a range of “omics” it is possible to interrogate the time and type of adaptation radiation induces in cancer, when compared to the pre-treatment phenotype. We speculate that different pre-treatment genotypes will have specified types of adaptation (e.g. p53 normal vs abnormal) that will help guide the initial treatment as is being studied in the match-type trials (70). A correct interpretation of these results enables rational design of multimodality therapies. Some adaptations take place within the first 5–10 or so fractions (2–4 days) and may persist for a number of days; long-term adaptations occur up to 2 months after treatment completion, and possibly longer. Early adaptation was traditionally called a “stress response” and can now be interrogated on how it is changing the tumor biological phenotype. In each model, the exact timing and how different forms of adaptation occur and how long they persist remain to be defined. Likewise, adaptation may be influenced by the irradiated tumor microenvironment, and the crosstalk between the tumor and the host. Growing evidence shows that the early changes induced by radiation in surviving cancer cells can improve the recognition of the tumor by the adaptive immune system and generate targets for immunotherapy (22,71).

While little information exists about the mechanisms of late adaptation on the tumor interaction with the immune system, some clinical papers suggest that pretreatment with radiation of chemoradiation may enhance effects of immune checkpoint blockade (72). Research in this area is warranted, particularly since novel immunotherapies are becoming rapidly available.

The PK/PD of radiation does depend on dose and fractionation and on type of radiation (e.g., photons versus particles), such that radiation can and should be considered “like a drug”, and function as a key partner within the precision medicine paradigm. In multiple models we found that the initial gene expression pattern did not predict radiation-induced gene response, indicating that investigation of adaptation mechanisms and pathways requires sequential interrogation. As outlined in Table 1, much remains to be done. Nonetheless recognizing that adaptation occurs offers the opportunity for unique approaches to cancer treatment. It is clear that post-radiation, the tumor and normal tissue “know” they have been irradiated and display persistent changes that depend on the type of radiation, dose and fractionation and whether radiation was given alone or with other modifiers, as in combination with chemotherapy, molecular-targeted therapy or immunotherapy.

Defining an individual tumor's adaptation to a radiation regimen permits testing treatments against newly emerged targets. Most importantly, radiotherapy can be used multiple times to enable testing different permutations of combinations. Indeed, drugs and/or immunological agents may become more effective after the adaptation, potentially inducing susceptibility to a drug based on cellular adaptation rather than a mutation. This could have major impact on return on investment for drug development as a drug deemed unlikely to work based on the initial tumor profile may become effective after adaptation. Complex analysis of biomarkers of both tumor and normal tissue response are warranted. The ability to utilize the focused biology (12) from RT is a potentially unique enhancement of both local and systemic therapy adding a new dimension to accurate, precision cancer care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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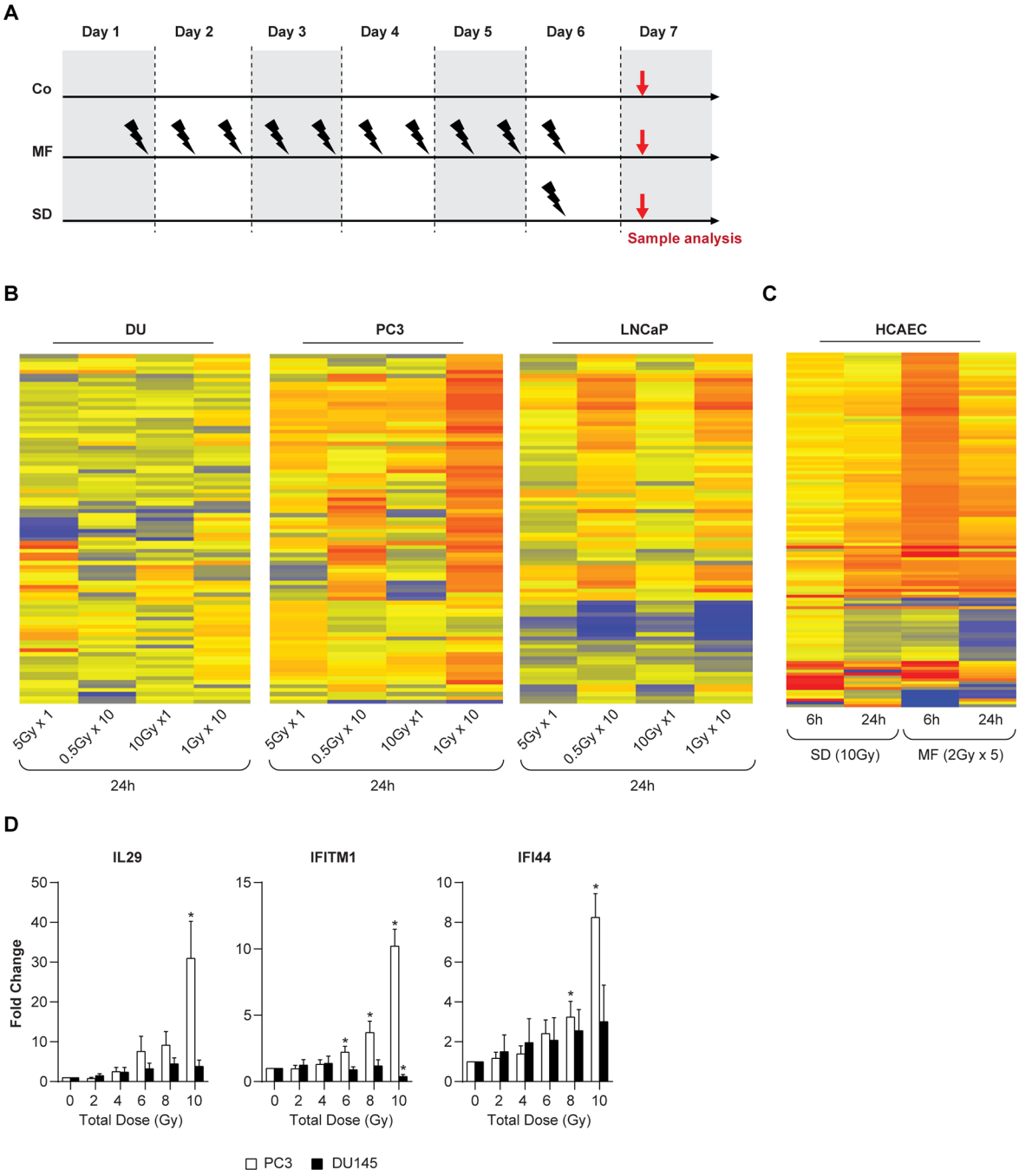


Figure 1: Adaptation and inflection point.

Schematic representation of experimental set up for Controls, along with single dose and fractionated radiation for PC3 and DU145 cells (A). Heat map of differentially expressed genes in prostate carcinoma cells (PC3and DU145) after SD (5 Gy, 10 Gy) and MF (0.5 Gy and 1 Gy x 10) radiation at 24 hours (B) Heat map of differentially expressed genes in HCAEC at 6 and 24 hours after an SD (10Gy) and 6 and 24 hours after the final dose of MF (2Gyx5) irradiation (C). Yellow to red, upregulated; blue, downregulated genes. Inflection point kinetics of immune genes for interleukin 29 (IL29), interferon induced transmembrane

protein 1 (IFITM1), and interferon induced protein 44 (IFI44) in multifractionated treated PC3 and DU145 cells as assessed by real time RT-PCR. Treatment was 1 Gy, 3x/day with at least 6 hours between fractions (D).

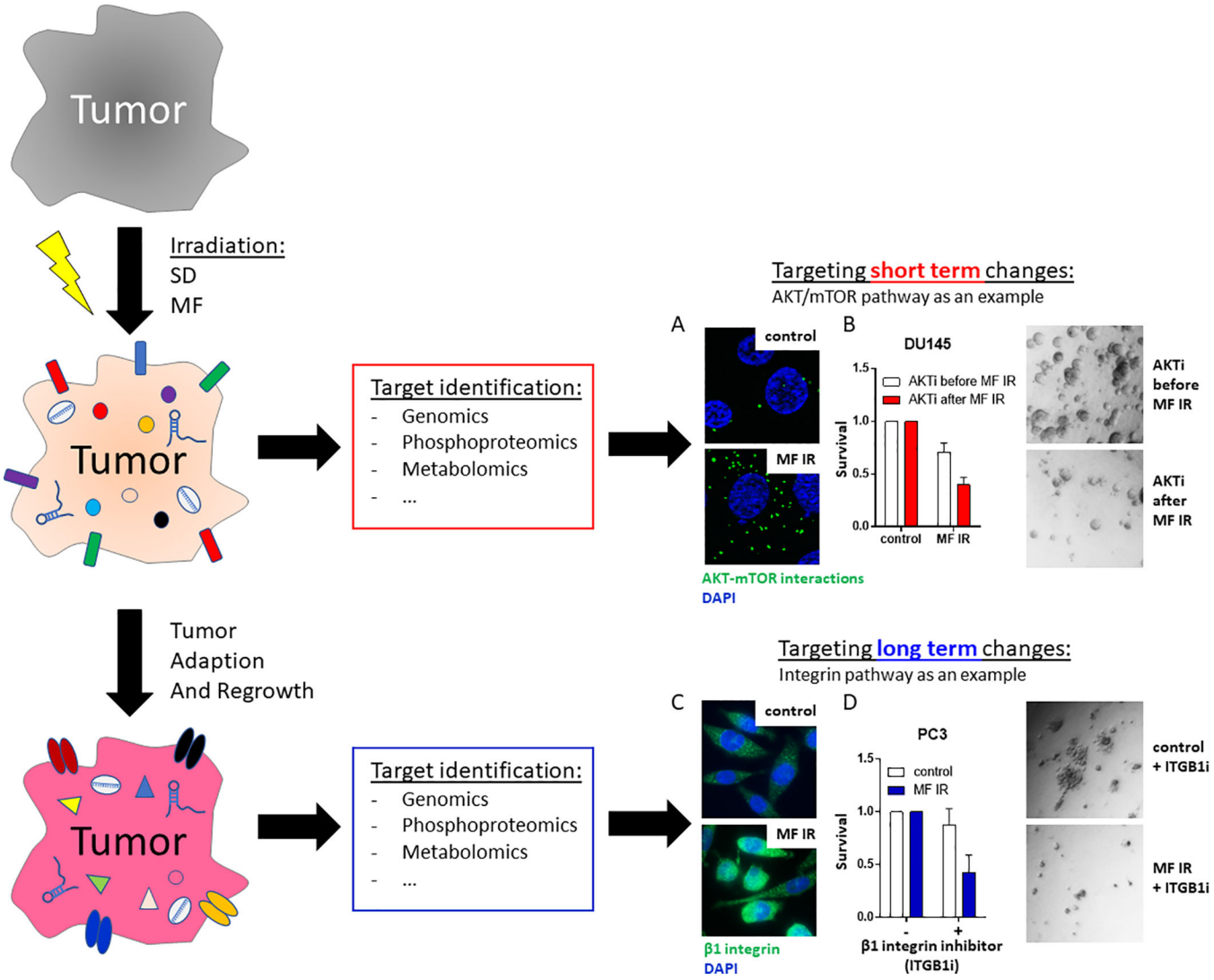
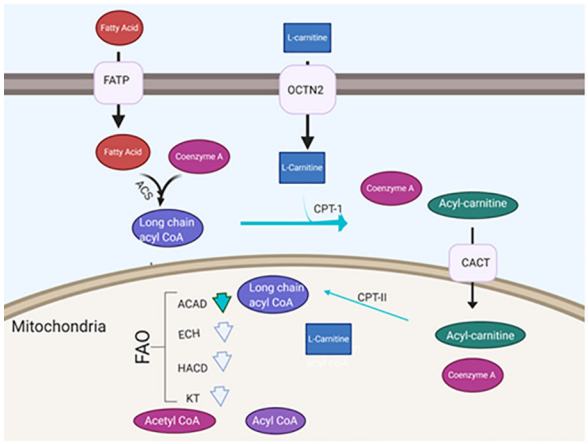


Figure 2: Target identification and inhibition for short-term and long-term cultures.

Tumors were irradiated with single dose (SD) or multifractionated (MF) irradiation. Within the first 24 h, short-term changes in mRNA, protein phosphorylation and metabolism were examined. (A) Activated AKT-mTOR signaling was (B) targeted with a small molecule AKT inhibitor (AKTi) Additionally, long-term changes in target expression were evaluated after tumor regrowth. (C) At 2 months after irradiation, β1 integrin was overexpressed in prostate cancer cells. (D) Inhibition of β1 integrin (ITGB1i) resulted in decreased clonogenic survival of MF-irradiated cells.

A



B

Gene Symbol	Gene name	10Gy/Ctrl	10x1Gy/Ctrl
CPT1A	carnitine palmitoyltransferase 1A (liver)	0.314176926	0.243277801
CPT1B	carnitine palmitoyltransferase 1B (muscle)	0.561448677	0.418338085
CPT1C	carnitine palmitoyltransferase 1C	0.431399502	0.488163966
CPT1C	carnitine palmitoyltransferase 1C	0.828339943	1.089012845
CPT2	carnitine palmitoyltransferase 2	0.088761348	0.071988255
ACAD10	acyl-CoA dehydrogenase family, member 10	0.65505268	0.555672057
ACAD10	acyl-CoA dehydrogenase family, member 10	0.374579498	0.264524479
ACAD11	acyl-CoA dehydrogenase family, member 11	1.074927398	1.071625306
ACAD8	acyl-CoA dehydrogenase family, member 8	0.223574165	0.181582839
ACAD9	acyl-CoA dehydrogenase family, member 9	0.078788005	0.065308646
ACAD9	acyl-CoA dehydrogenase family, member 9	0.175060854	0.134480647
ACADS	acyl-CoA dehydrogenase, C-2 to C-3 short chain	1.008094263	0.930438265
ACADM	acyl-CoA dehydrogenase, C-4 to C-12 straight chain	0.506712023	0.524726415
ACADSB	acyl-CoA dehydrogenase, short/branched chain	1.209918788	1.158041117
ACADSB	acyl-CoA dehydrogenase, short/branched chain	0.563608768	0.426338942
ACADVL	acyl-CoA dehydrogenase, very long chain	0.014892049	0.011754029

C

Sub Pathway	Biochemical Name	Platform	HMDB	Fold of Change - ANOVA Contrasts, Cell Line PC3					
				6h		24h		48h	
				1Gy x 10 Ctrl	10Gy x 1 Ctrl	1Gy x 10 Ctrl	10Gy x 1 Ctrl	1Gy x 10 Ctrl	10Gy x 1 Ctrl
Fatty Acid Metabolism	acetyl CoA	LC/MS neg	HMDB01206	1.87	0.26	1.81	2.22	0.67	1.33
Fatty Acid Metabolism (ak	butyrylcarnitine	LC/MS pos	HDMB02013	1.00	0.90	0.48	0.64	0.58	1.66
Fatty Acid Metabolism (ak	propionylcarnitine	LC/MS pos	HMDB00824	1.58	1.47	0.67	1.14	0.67	2.29
Fatty Acid Metabolism(Ac	acetylcarnitine	LC/MS pos	HMDB00201	2.11	1.71	0.88	1.45	0.88	2.55
Fatty Acid Metabolism(Ac	hexanoylcarnitine	LC/MS pos	HMDB00705	2.05	0.84	0.58	1.63	0.06	0.70
Fatty Acid Metabolism(Ac	octanoylcarnitine	LC/MS pos	HMDB00791	1.00	1.00	1.00	1.00	1.00	1.00
Fatty Acid Metabolism(Ac	palmitoylcarnitine	LC/MS pos	HMDB00222	2.06	3.21	0.87	2.22	0.86	3.73
Fatty Acid Metabolism(Ac	stearoylcarnitine	LC/MS pos	HMDB00848	2.59	3.06	1.08	2.15	0.72	3.06
Fatty Acid Metabolism(Ac	oleoylcarnitine	LC/MS pos	HMDB05065	2.22	3.29	0.69	1.93	0.57	3.19
Carnitine Metabolism	deoxycarnitine	LC/MS pos	HMDB01161	2.15	1.61	0.83	1.43	0.78	2.43
Carnitine Metabolism	carnitine	LC/MS pos	HMDB00062	1.89	1.66	0.86	1.30	0.80	2.79
Carnitine Metabolism	3-dehydrocarnitine*	LC/MS pos	HMDB12154	1.83	1.38	0.77	1.20	0.76	2.15

Figure 3: Perturbations in lipid metabolism after SD and MF radiation.

Figure 3A. Schematic representation of long chain fatty acid transport across cell membrane and into the mitochondria for FAO. Fatty acids utilize a protein mediated system to cross the cell membrane, for simplicity this is marked as fatty acid transport protein (FATP). Fatty acids are converted to acyl-CoAs to activate them. To enter the mitochondria, acyl-CoAs must be bound to carnitine. CPT1 converts acyl-Coas to acylcarnitines which allows the fatty acid to enter the inner mitochondrial membrane through CACT. CPTII converts acylcarnitines to acyl-CoAs. The acyl-CoA undergoes FAO. Chain length specific acyl-CoA dehydrogenase (ACAD) perform the initial step of FAO. The following three steps are catalyzed by enoyl-CoA hydratase (ECH), 3-Hydroxyacyl-CoA Dehydrogenases (HACD), and 3-Ketothiolases (KT). This produces one acetyl-CoA, one shortened acyl-CoA, NADH and FADH2, which can be fed into the TCA cycle. The shortened acyl-COA may undergo another round of FAO. Green arrows indicate decreased expression of enzymes in PC3 cells 24h post radiation as indicated in figure 3B.

Figure 3B. Microarray data from human prostate cancer cell PC3 24h after the end of SD or MF radiation exposure. Results are presented as irradiated divided by control. Green indicates that genes were statistically, significantly down regulated by paired T-test. CPT1 is considered the rate limiting enzyme for long chain fatty acid entry into the mitochondria for FAO. CPTII activates acyl-carnitines for FAO. Various members of dehydrogenase family each display specificity for fatty acid chains and are the first step of FAO.

Figure 3C. Metabolic data from PC3 cells 6h, 24h, and 48h after single dose (SD) or multifractionated (MF) radiation. Results indicate significant buildup of specific acylcarnitines at 6 and 48h after single dose(SD) radiation. Asterisk (*) indicates compounds identified based on the mass spectrometry data, but that do not currently have a reference standard to verify the identity.

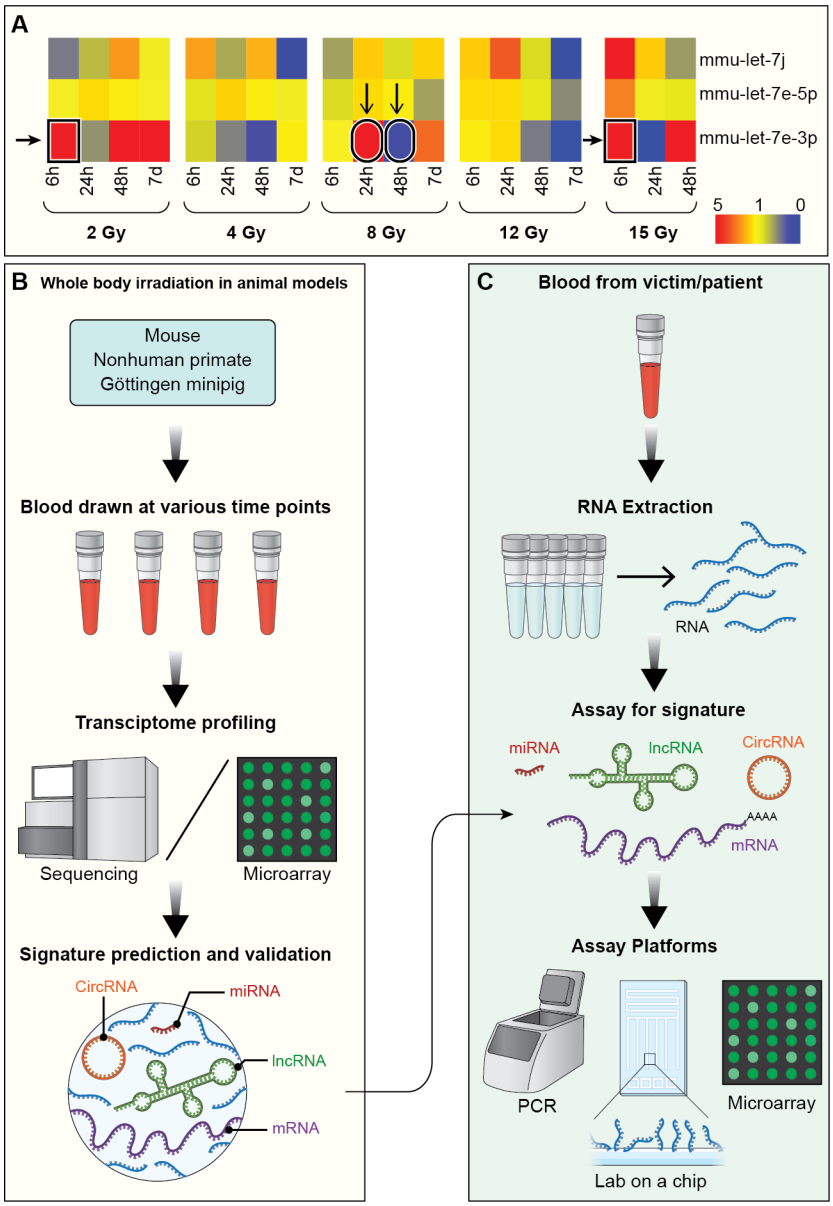


Figure 4: Predicting radiation exposure and normal tissue injury using an integrated RNA biomarker approach.

A) Heatmap displays fold change values of 3 different miRNAs of let-7 family, which share a seed sequence indicating shared targets, expressed in the blood of mice collected at various timepoints after whole body irradiation with various doses. Red signifies upregulated expression and blue signifies downregulated expression in comparison to the sham-irradiated controls. B) Schematic shows the methodology of utilizing animal models to predict dose-differentiating, RNA-based signatures from approximately 300–500μL of blood. C) Intended workflow, starting with a 500μL blood draw and using different high throughput diagnostic platforms, shows application of predicted signatures for triaging victims of accidental radiation exposure and assessment of normal tissue injury after radiotherapy.

Table 1.

Gaps and work-in-progress

Topic	Ongoing and future work	Details and hypotheses
Broadening of cell lines	Expand study of cell lines with germline mutations, with initial focus on lung cancer	Lung cancer has a dual potential for immunotherapy and molecular-targeted therapies
	Increase in spectrum of prostate cancer cell lines	Prostate cancer often treated with primary radiation, so this will enable adaptive effects to be studied
	Development of a platform to study the response to radiation in patient-derived xenografts, cell lines, and organoids, ideally from consecutive biopsies	Personalized medicine will enable more accurate precision medicine
Target determination	Identification of biomarkers of adaptation from an array of “omics” combined with proteomics or phosphor-proteomics	Likely to help define specific targets, including non-coding RNAs, e.g. miRNA (69) as potential targets
	Use of immunohistochemistry and profiling of subclones over different time points to study the proportion of cells that adapt, in addition to examining the cells that survive the post-exposure “drugging”	Adaptations are likely to be heterogeneous, possibly transient, and tumor adaptation to drugs need to be determined
Timing and transition of adaptation	Study additional tumor types and additional time points between the end of radiation and 2 months, as well as beyond 2 months	There are at least 2 general adaptation points: 1) starts during therapy, within days, whereby MF produces more changes than SD; and 2) starts by 2 months or later and SD predominates. This time course needs to be better defined
Mechanism of adaptation	Epigenetic adaptations	Preliminary data suggest that there are epigenetic changes and, if so, when does this occur and for how long does it persist?
	Ongoing in vivo studies to examine growth delay and related biological changes for radiation, drugs, and the combination	Potential collaborations with laboratories studying charged particle therapy
Normal tissue changes	Expansion of the whole-body and organ-specific radiation biomarkers	Provide biomarkers for radiation biodosimetry and normal tissue adaptations from therapy
	Pursuant to our study of endothelial changes (19) further study of radiation inducible endothelial changes from in vitro cultures, organ-on-a-chip and in vivo experiments	Understand the role of endothelial damage in radiation injury. (This is supported in a number of laboratories by NIAID[a] and BARDA[b] programs).
Clinical applicability	Through collaboration, obtain clinical samples for normal tissue biomarkers with groups studying radiation biodosimetry	Clinical samples are limited by underlying medical conditions, dose delivered, and volume of tissue irradiated
	Investigate the use of “radiation as a drug,” as part of an overall approach from the “Shades of Gy” workshop (13) toward “accurate, precision radiation medicine” (14)	Prospective intervention trials will depend on preclinical data. Some pre- and post-RT sampling may be done including pre- operative radiotherapy, intraoperative radiotherapy and brachytherapy

[a]NIAID Radiation and Nuclear Countermeasures Program. Available at: https://www.niaid.nih.gov/research/radiation-nuclear-countermeasures-program. Accessed April 24, 2020

[b]BARDA Radiological/nuclear medical countermeasures. Availalble at: https://www.medicalcountermeasures.gov/barda/cbrn/radiological-and-nuclear-countermeasures/ Accessed April 24, 2020