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# mRNA Expression Profiles for Prostate Cancer following Fractionated Irradiation Are Influenced by p53 Status[1,2]

**Charles B. Simone II*,†, Molykutty John-Aryankalayil†, Sanjeewani T. Palayoor†, Adeola Y. Makinde†, David Cerna†, Michael T. Falduto‡, Scott R. Magnuson‡ and C. Norman Coleman†**

*Department of Radiation Oncology, Hospital of the University of Pennsylvania, Philadelphia, PA; †Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD; ‡GenUs BioSystems Inc, Northbrook, IL

## Abstract

We assessed changes in cell lines of varying p53 status after various fractionation regimens to determine if p53 influences gene expression and if multifractionated (MF) irradiation can induce molecular pathway changes. LNCaP (p53 wild-type), PC3 (p53 null), and DU145 (p53 mutant) prostate carcinoma cells received 5 and 10 Gy as single-dose (SD) or MF (0.5 Gy × 10, 1 Gy × 10, and 2 Gy × 5) irradiation to simulate hypofractionated and conventionally fractionated prostate radiotherapies, respectively. mRNA analysis revealed 978 LNCaP genes differentially expressed (greater than two-fold change, $P < .05$) after irradiation. Most were altered with SD (69%) and downregulated (75%). Fewer PC3 (343) and DU145 (116) genes were induced, with most upregulated (87%, 89%) and altered with MF irradiation. Gene ontology revealed immune response and interferon genes most prominently expressed after irradiation in PC3 and DU145. Cell cycle regulatory ($P = 9.23 \times 10^{-73}$, 14.2% of altered genes, nearly universally downregulated) and DNA replication/repair ($P = 6.86 \times 10^{-30}$) genes were most prominent in LNCaP. Stress response and proliferation genes were altered in all cell lines. p53-activated genes were only induced in LNCaP. Differences in gene expression exist between cell lines and after varying irradiation regimens that are p53 dependent. As the duration of changes is ≥24 hours, it may be possible to use radiation-inducible targeted therapy to enhance the efficacy of molecular targeted agents.

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## Introduction

Prostate cancer is the most commonly diagnosed noncutaneous malignancy in the United States [1]. Radiation therapy is the most commonly employed treatment modality for prostate cancer in North America [2]. It is typically administered in daily fractions for approximately 8 weeks to allow for normal tissue repair and repopulation between fractions, as well as tumor reoxygenation and reassortment, but newer hypofractionation regimens using fewer large daily doses deliver definitive prostate radiotherapy in as short as 1 week.

Exposing mammalian cells to ionizing radiation results in DNA damage and cellular responses, including cell cycle arrest, DNA repair, and cell death [3]. These biologic effects, however, differ following exposure to lower *versus* higher doses of irradiation administered in a single fraction [3–6]. Ding et al. demonstrated that in contrast to higher doses of 4 Gy, genes induced by lower doses of 0.02 Gy generally regulate signal transduction, cell-to-cell signaling, homeostasis, and cellular defenses [3]. In contrast, genes controlling cell proliferation and apoptosis are more commonly induced by higher irradiation doses above 0.5 Gy [3,5].

Address all correspondence to: Charles B. Simone, II, MD, Department of Radiation Oncology, Perelman School of Medicine at the University of Pennsylvania, Hospital of the University of Pennsylvania, 3400 Civic Center Blvd, TRC 2 West, Philadelphia, PA 19104. E-mail: csimone@alumni.upenn.edu

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[2]This article refers to supplementary materials, which are designated by Tables W1 to W4 and are available online at www.transonc.com.

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The tumor suppressor protein p53 functions as a transcription factor and is a major regulator of cellular responses to DNA-damaging agents such as ionizing radiation. p53 regulates cell cycle control and checkpoints, cell differentiation, apoptotic pathways, cellular senescence, and angiogenesis [7–10]. Following more limited DNA damage from ionizing radiation, p53 can facilitate cellular repair through cell cycle arrest and blocking in $G_1$. For cells receiving more significant radiation-induced damage, p53 can promote apoptosis through cell cycle checkpoints [11–13].

Mutations of the *p53* gene are found in approximately half of all human cancers [14] and can result in nuclear accumulation of p53 protein, loss of p53 binding sites, and changes in the global conformation of p53 [10,15]. Abnormal p53 function can permit mitosis or replication to proceed before radiation-induced DNA damage is repaired [16,17]. This can increase the rate of radiation-induced mutations, particularly after higher doses of radiation [16]. The function of the *p53* gene, therefore, may in part determine the sensitivity to damage induced by radiation therapy or systemic therapy [11–13].

Cells that survive hypoxic or otherwise stress environments undergo numerous molecular changes [18–21]. Repeated fractions of external beam radiation therapy, as are administered for prostate cancer, represent such a stress and cause surviving cells to have altered phenotypes that may differ in susceptibility and treatment response to subsequent molecular targeted therapy. Additionally, fractionated irradiation makes varying tumor cells more phenotypically similar [22]. As such, fractionated irradiation may be able to induce potential molecular therapeutic targets in irradiated cells. Therefore, in addition to the current roles in prostate cancer of definitive, adjuvant, salvage, or palliative radiotherapy, radiation therapy may allow for the induction of a target for molecular targeted therapy rather than depending on the presence of a mutation or the nontargeted use of small molecules and monoclonal antibodies [18]. The induction of genes by ionizing radiation is dependent on the cell type, radiation dose, and time after irradiation [3,23–26]. Selected effects of irradiation on PC3 and DU145 cells following varying fractionation regimens have previously been reported by the authors and are now expanded and compared to the results for LNCaP cells [6].

More limited data describing how tumor p53 status influences the tumor cell response to radiation therapy by varying radiation fractionation regimens exist. To investigate the effects of irradiation-induced p53-dependent or p53-independent pathways in response to ionizing radiation, three prostate carcinoma cell lines with varying p53 status were selected: LNCaP is wild type for p53, PC3 is p53 null, and DU145 is p53 mutant. The following three radiation doses were used: 1) single high-dose irradiation of 5 and 10 Gy to compare with the multifractionated (MF) regimens and which are consistent with the large doses used in hypofractionated radiation therapy, 2) 1-Gy fractional low doses to understand the impact of multifraction radiation (2 Gy had been compared to 1 Gy per fraction in previous prostate experiments, as discussed in Materials and Methods section), and 3) 0.5-Gy fractional low doses that produces very little cell killing to simulate the dose that some tissues receive daily with intensity-modulated radiotherapy (IMRT). Using gene ontology classification and molecular pathway analysis, we assess changes in prostate cancer cell lines of varying p53 status after various irradiation fractionation regimens to determine if p53 status influences gene expression and if MF irradiation can induce molecular pathway changes.

## Materials and Methods

### *Cells*

LNCaP (p53 wild-type, androgen-dependent, highly differentiated), PC3 [p53 null, androgen-independent, poorly differentiated, hemizygous for chromosome 17p, mutation at codon 138 (C nucleotide deletion→frame shift with a new in-frame stop at codon 169)], and DU145 [p53 mutant, androgen-independent, moderately differentiated, mutations at codons 223 (Pro→Leu) and 274 (Val→Phe)] human prostate carcinoma cells [27] were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in ATCC RPMI 1640 medium that was supplemented with antibiotics for all cell lines and glutamine and 10% ATCC FBS for LNCaP cells. For microarray analysis, cells from passages P4, P7, and P11 were used. For confirmation and protein extraction, cells from passages P2 to P11 were used. Some data from PC3 and DU145 cells have been previously reported [6] and were selectively used in this manuscript for comparative purposes.

### *Radiation*

Cells were plated onto T175 cell culture flasks (BD Falcon 175-$cm^2$ flasks) [single-dose (SD) regimens: $2.0 \times 10^6$ cells for LNCaP and $1.6 \times 10^6$ cells for PC3 and DU145; MF regimens: $1.0 \times 10^6$ cells for LNCaP and $0.8 \times 10^6$ cells for PC3 and DU145]. The number of cells plated for each cell line was experimentally designed to allow for cell confluence of approximately 50% at the time of first irradiation exposure while also maintaining a confluence of under 70% in control flasks and limiting varying nutrient conditions at the time of the last irradiation exposure across varying fractionation regimens. After 24 hours, cells were exposed to single or fractionated irradiation in an irradiator using an Eldorado 8 $^{60}$Co teletherapy unit (Theratronics International Ltd, Ontario, Canada) housed in the Radiation Oncology Branch of the National Cancer Institute, National Institutes of Health (Bethesda, MD) that delivered dose rates between 150 and 180 cGy per minute. Fractionation regimens were designed to allow for an assessment of differential gene expression and the phenotype of surviving cells across a variety of irradiation doses and fractionation schemas.

LNCaP cells were exposed to 5- or 10-Gy irradiation. Cells receiving 5 Gy were irradiated either as an SD (5 Gy × 1) or as an MF course of 0.5 Gy twice daily for 5 days (0.5 Gy × 10). Cells receiving 10 Gy were irradiated either as an SD (10 Gy × 1) or as an MF course of 1 Gy twice daily for 5 days (1 Gy × 10). PC3 and DU145 cells had been irradiated as an additional MF course of 2 Gy once daily for 5 days [6]. However, since few genes were uniquely differentially expressed for the 2 Gy × 5 compared to 1 Gy × 10 regimens in PC3 and DU145 cells [6] (and also based on the cost of these experiments), the 2 Gy × 5 fractionation regimen was not administered to LNCaP cells. For all MF regimens, fractions were administered at least 6 hours apart. Unirradiated separate controls were maintained for all SD and MF irradiation protocols.

### *Microarray Analysis*

Microarray analysis and confirmation studies have previously been described [6,28]. For LNCaP cells, total RNA was extracted and collected at 24 hours after the final irradiation fraction using QIAshredder Spin Columns (Catalog No. 79654; Qiagen, Valencia, CA). For PC3 and DU145 cells, total RNA was extracted and collected at multiple time points 2, 6, and 24 hours after the final irradiation doses [6]. All extracted RNA was purified with an RNeasy Mini

Kit (Qiagen). The total RNA concentration was measured by spectrophotometry at $A_{260/280}$. Agilent Bioanalyzer with the RNA6000 Nano Lab Chip (Agilent Technologies, San Francisco, CA) was used to assess the quality of RNA samples. RNA isolates collected at 24 hours from irradiated and unirradiated samples from three distinct biologic replicates for all dose and fractionation regimens for LNCaP, PC3, and DU145 cells were used to assess differences in mRNA microarray analysis.

mRNA microarray analysis was performed using CodeLink Whole Genome Bioarrays representing 55,000 probes [28]. These 30-mer probes were designed to conserved exons across the transcripts of targeted genes, and each probe represented well-annotated, full-length, and partial human gene sequences from major public databases. Scanned images from arrays (gridding and feature intensity) were processed using CodeLink Expression Analysis software (GE Healthcare, Buckinghamshire, United Kingdom). Data for each feature on the array were analyzed with GeneSpring GX Software (Agilent Technologies).

The raw intensity data for each gene on each array were normalized to the median intensity of the raw values from that array. This is a global normalization procedure that divides raw intensity values from array by the 50th percentile value within each array. All data were filtered for intensity values that were above background in at least two of any set of three replicates for each time point and each cell line within each irradiation fractionation regimen. Unsupervised hierarchical clustering of all probes significantly above background detection levels in at least one condition (irradiated or control) from all three replicates defined expression profiles that were clustered by biologic replicate treatments and not by technical conditions of the experimental design. Analysis of variance was used to compare the means of each condition ($n = 3$) to ensure reliable gene measurements. Confirmation of microarray data was performed by real-time polymerase chain reaction (PCR) and Western blot analysis, with all confirmation samples also run in distinct biologic triplicates.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series Accession No. GSE36720 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36720).

### *Real-Time PCR*

Alterations in genes that exhibited significant differential expression patterns (greater than two-fold change and $P < .05$) following the different irradiation fractionation regimens or between different cell lines were confirmed by real-time PCR using TaqMan Gene Expression Assays, with the ABI PRISM 7500 Sequence Detection System equipped with Sequence Detection System version 1.4.0 software (Applied Biosystems by Life Technologies, Carlsbad, CA). All forward and reverse primers and probes were designed and produced by Applied Biosystems. Representative probes that were used included *OASL* (Hs00984390_m1), *IFI27* (Hs00271467_m1), and *UBE1L* (Hs00163295_m1). Reagents for real-time PCR analysis were obtained from Applied Biosystems. cDNA was generated from 1 μg of RNA, and PCR was performed using TaqMan Universal PCR Master Mix (Part No. 4324018). Controls were processed using the same conditions without an RNA template.

All numerical values were normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) as an internal control gene and listed as the average relative change in target genes of irradiated cells relative to unirradiated control cells from three biologically distinct experiments. Baseline mRNA levels of selected genes in control LNCaP, PC3, and DU145 cells were determined, and relative differences were calculated using the $\Delta\Delta C_T$ method [28].

### *Ingenuity Pathway Analysis*

Ingenuity Pathway Analysis (IPA, version 8.8; Ingenuity Systems, Redwood City, CA) was used to assess the functional significance of differentially expressed genes altered by irradiation. GenBank IDs of all genes with at least a two-fold change and $P < .05$ were imputed for network generation and pathway analyses and mapped to the functional networks available in the Ingenuity Pathway Knowledge Base, as previously described [28]. A score was determined for each network, reflecting the negative logarithm of the $P$ value based on the chance that the focus genes were grouped in the network by random chance. A score of 2 corresponds with a 1 in 100 chance that the focus genes are grouped together by random chance. As such, scores of 2 or higher indicate at least a 99% confidence that a true molecular relationship exists. Functional IPA was corroborated by an independent gene ontology analysis, with enrichment of individual functional gene categories determined by hypergeometric distribution $P$ values obtained from comparison of the number of genes differentially expressed to the number of annotated genes in each category.

### *Data Analysis*

Each data point represents the average ± SEM of three biologically distinct experiments. Differences between the groups were evaluated statistically by a two-tailed paired $t$ test, and statistical significance was defined as $P < .05$. Analysis of variance was used to compare the means of each condition. For mRNA microarray analysis, a greater than two-fold change (cutoff ratios of greater than 2.0 times or less than 0.5 times) and a $P$ value of <.05 relative to the respective control were selected for this study.

## Results

### *Global Gene Expression*

Among all single-fraction and MF irradiation regimens, mRNA microarray analysis revealed that 978 genes in LNCaP cells were differentially expressed (greater than two-fold change and $P < .05$) 24 hours following irradiation. Most of these genes (75% across all fractionation regimens at 24 hours) were downregulated. Downregulation occurred in 90% of all genes differentially expressed

**Table 1.** Number of Differentially Expressed Genes* in LNCaP Cells after SD and MF Irradiation.

| Dose Comparison | 10 Gy SD† | 5 Gy SD | 10 Gy MF‡ | 5 Gy MF | Genes in Common§ |
|---|---|---|---|---|---|
| 10 Gy × 1<br>5 Gy × 1 | 581 | 491 | – | – | 393 |
| 1 Gy × 10<br>0.5 Gy × 10 | – | – | 462 | 128 | 117 |
| 10 Gy × 1<br>1 Gy × 10 | 581 | – | 462 | – | 166 |
| 5 Gy × 1<br>0.5 Gy × 10 | – | 491 | – | 128 | 23 |

*Differentially expressed genes are greater than two-fold change and $P < .05$.
†SD, SD irradiation, either as 10 Gy × 1 or 5 Gy × 1.
‡MF, MF irradiation in 10 fractions, either as 1 Gy × 10 or 0.5 Gy × 10.
§Genes mutually upregulated or downregulated among both irradiation fractionation comparisons are listed as Genes in Common.

following SD irradiation, with down-regulation occurring in 88% of differentially expressed genes after 10 Gy × 1 and 92% after 5 Gy × 1. In contrast, up-regulation occurred in 53% of differentially expressed genes across MF regimens, including 46% after 1 Gy × 10 and 76% after 0.5 Gy × 10. Furthermore, more genes were differentially expressed following SD irradiation (69%) than MF irradiation (31%), with differential expression in 581 genes (10 Gy) and 491 genes (5 Gy) administered in a single fraction, compared with 462 genes (1 Gy × 10) and 128 genes (0.5 Gy × 10) in MF regimens (Table 1).

Somewhat more genes were differentially expressed with 10 Gy × 1 (581 genes) than 5 Gy × 1 (491 genes), and most of these genes (393 genes) were mutually induced following both single-fraction regimens (Table 1). While 462 genes were differentially expressed following 1 Gy × 10 fractionation, significantly fewer genes were induced with 0.5 Gy × 10 (128 genes). Nearly all genes (117/128, 91%) induced following 0.5 Gy × 10 were similarly induced following 1 Gy × 10. Including significantly upregulated (16 genes, 9.6%) and downregulated (150 genes, 90.4%) genes, 166 genes in LNCaP cells were mutually induced among the 10-Gy regimens (10 Gy × 1 and 1 Gy × 10). However, only 23 genes, including 6 upregulated (26.1%) and 17 downregulated (73.9%) genes, were commonly differentially expressed among the 5-Gy regimens (5 Gy × 1 and 0.5 Gy × 10; Table 1).

In contrast to LNCaP, fewer total genes were induced in PC3 (343 genes) and DU145 (116 genes) cells among all irradiation fractionation regimens at 24 hours. In PC3, 87 genes were induced following 10 Gy × 1 (67 upregulated, 20 downregulated), 136 following 2 Gy × 5 (129 upregulated, 7 downregulated), and 217 following 1 Gy × 10 (196 upregulated, 21 downregulated). In DU145, 63 genes were induced following 10 Gy × 1 (55 upregulated, 8 downregulated), 29 following 2 Gy × 5 (26 upregulated, 3 downregulated), and 40 following 1 Gy × 10 (38 upregulated, 2 downregulated). Of differentially expressed genes, up-regulation occurred in 86% of PC3 genes and 89% of DU145 genes.

Unlike with LNCaP cells, where only 31% of differentially expressed genes were induced by 1 Gy × 10 or 0.5 Gy × 10 multifractionation regimens compared with 10 Gy × 1 and 5 Gy × 1 single-fraction regimens, a higher proportion of differentially expressed genes were induced following 2 Gy × 5 or 1 Gy × 10 MF irradiation regimens than 10 Gy × 1 or 5 Gy × 1 single-fraction regimens in PC3 (75%) and DU145 (47%) cells. Fewer genes were mutually induced within cell lines by single-fraction and MF regimens in both PC3 and DU145 cells than in LNCaP cells. This is demonstrated for 10 Gy administered as an SD or MF regimen in Figure 1.

### *Significant Functional Categories*

All genes found to be differentially expressed (greater than two-fold change and $P < .05$) after any irradiation fractionation protocol at 24 hours for LNCaP, PC3, and DU145 cells were classified into functional categories (Table 2). Enrichment of individual functional gene categories was determined by hypergeometric distribution *P* values obtained from the comparison of the number of genes differentially expressed to the number of annotated genes in each category. A complete list of genes from the significant functional categories assessed by gene ontology that were differentially expressed by greater than two-fold change and $P < .05$ is depicted in Table W1.

Gene ontology classification of genes differentially expressed at 24 hours after irradiation revealed that cell cycle regulatory genes were most prominently differentially expressed in LNCaP cells but less significantly altered or unaffected in PC3 and DU145 cells. DNA replication (10 Gy × 1: $P = 3.01 \times 10^{-52}$; 1 Gy × 10: $P = 3.98 \times 10^{-10}$) and DNA repair (10 Gy × 1: $P = 8.43 \times 10^{-35}$; 1 Gy × 10: $P = 6.54 \times 10^{-6}$) genes were among the next most prominently altered functional categories in LNCaP cells at 24 hours but were not significantly altered in DU145 and PC3 cells. Immune response (PC3—10 Gy × 1: $P = 9.00 \times 10^{-4}$; 1 Gy × 10: $P = 5.19 \times 10^{-20}$; DU145—10 Gy × 1: $P = 2.46 \times 10^{-5}$; 1 Gy × 10: $P = 1.55 \times 10^{-3}$), interferon (PC3—10 Gy × 1: $P = 2.05 \times 10^{-3}$; 1 Gy × 10: $P = 2.06 \times 10^{-21}$; DU145—10 Gy × 1: $P = 9.79 \times 10^{-4}$;

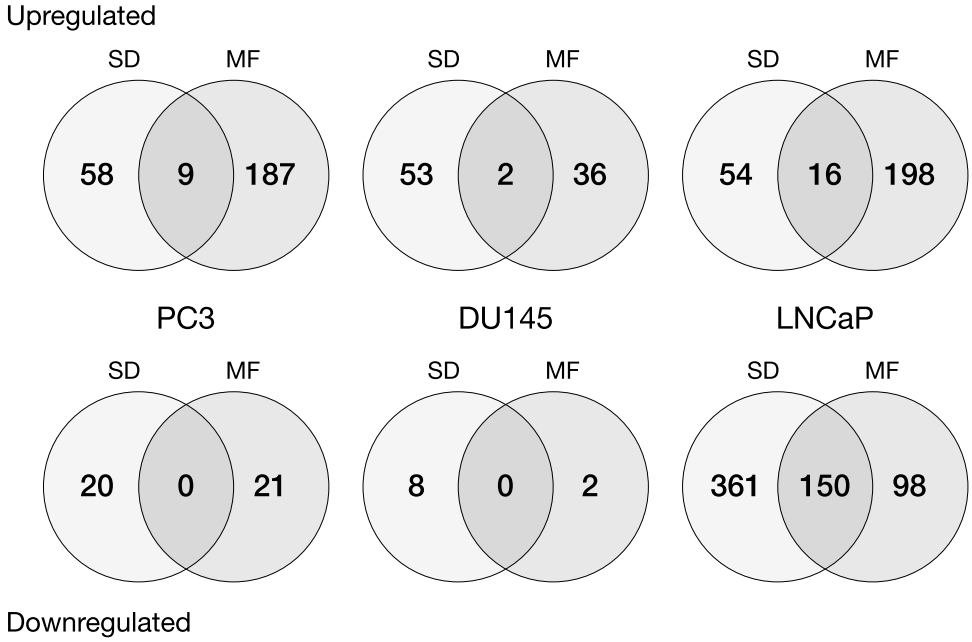


**Figure 1.** Venn diagrams depicting the number of upregulated and downregulated differentially expressed genes (greater than two-fold change and $P < .05$) at 24 hours following exposure to 10 Gy as SD or MF (1 Gy × 10) irradiation regimens.

**Table 2.** Gene Ontology Classification* among Differentially Expressed Genes for Various Prostate Cancer Cell Lines.

| Category | LNCaP | | | | PC3 | | | | DU145 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 Gy × 1 | | 1 Gy × 10 | | 10 Gy × 1 | | 1 Gy × 10 | | 10 Gy × 1 | | 1 Gy × 10 | |
| | Genes | *P* Value | Genes | *P* Value | Genes | *P* Value | Genes | *P* Value | Genes | *P* Value | Genes | *P* Value |
| Cell cycle | 128 | **3.75E–91**† | 65 | **5.61E–34** | 5 | .02 | 9 | .03 | 6 | 9.74E–04 | 1 | NS |
| DNA replication | 55 | **3.01E–52** | 17 | **3.98E–10** | 1 | NS | 3 | NS | 1 | NS | 0 | NS |
| DNA repair | 42 | ***8.43E–35*** | 12 | 6.54E–06 | 0 | NS | 0 | NS | 1 | NS | 0 | NS |
| DNA binding | 105 | 1.59E–26 | 59 | 3.68E–09 | 9 | .02 | 26 | 1.11E–04 | 9 | 3.26E–03 | 5 | .03 |
| Cyclin | 25 | 1.96E–22 | 13 | ***6.76E–10*** | 1 | NS | 1 | NS | 2 | .01 | 0 | NS |
| Response to stress‡ | 45 | 3.15E–14 | 23 | 8.97E–05 | 5 | .01 | 25 | ***1.38E–12*** | 7 | ***1.20E–04*** | 5 | ***4.34E–04*** |
| Proliferation‡ | 41 | 5.36E–14 | 28 | 1.50E–08 | 4 | .03 | 14 | 1.84E–05 | 5 | 2.61E–03 | 4 | 2.17E–03 |
| Histone | 23 | 2.36E–12 | 16 | 3.51E–08 | 4 | **9.57E–04** | 3 | NS | 3 | 4.66E–03 | 0 | NS |
| DNA damage | 11 | 6.35E–08 | 5 | 3.43E–03 | 0 | NS | 3 | .01 | 0 | NS | 2 | 2.21E–03 |
| Ubiquitin | 18 | 4.25E–04 | 6 | NS | 3 | NS | 10 | 3.04E–04 | 2 | NS | 2 | NS |
| Transcription factor | 29 | 6.70E–03 | 22 | .03 | 3 | NS | 11 | .05 | 6 | 6.28E–03 | 1 | NS |
| Apoptosis | 18 | .01 | 12 | NS | 2 | NS | 13 | 6.54E–05 | 3 | NS | 5 | **1.93E–04** |
| Signal transduction | 28 | NS§ | 20 | NS | 7 | .03 | 17 | 8.22E–03 | 3 | NS | 4 | .05 |
| Protease | 4 | NS | 6 | NS | 2 | NS | 4 | .04 | 5 | **2.22E–05** | 0 | NS |
| Interferon | 2 | NS | 5 | .02 | 3 | ***2.05E–03*** | 19 | **2.06E–21** | 3 | 9.79E–04 | 3 | **1.96E–04** |
| Immune response | 8 | NS | 13 | .03 | 6 | **9.00E–04** | 30 | **5.19E–20** | 7 | **2.46E–05** | 4 | 1.55E–03 |
| Inflammatory response | 1 | NS | 3 | NS | 1 | NS | 10 | 1.28E–08 | 2 | .02 | 0 | NS |

*Enrichment of individual functional gene categories was determined by hypergeometric distribution *P* values obtained from comparison of the number of genes differentially expressed to the number of annotated genes in each category. Selected gene categories are listed in order of descending statistical significance at 24 hours for the featured SD irradiation fractionation regimen (10 Gy × 1) for LNCaP cells.

†The most significant functional category for each cell line and each featured fractionation regimen are depicted in gray shading and in bold. The second most significant are in bold, whereas the third most significant are in bold italics.

‡Stress response and proliferation genes were among the most universally differentially expressed genes across all cell lines and all fraction regimens.

§NS, not significant ($P \geq .05$).

1 Gy × 10: $P = 1.96 \times 10^{-4}$), and apoptosis (PC3—10 Gy × 1: $P = .099$; 1 Gy × 10: $P = 6.54 \times 10^{-5}$; DU145—10 Gy × 1: $P = .067$; 1 Gy × 10: $P = 1.93 \times 10^{-4}$) genes were most prominently expressed after irradiation in PC3 and DU145 cells but were less significantly altered in LNCaP cells. Inflammatory response genes were only highly expressed in MF regimens in PC3 cells.

At 24 hours following irradiation, stress response (LNCaP—10 Gy × 1: $P = 3.15 \times 10^{-14}$; 1 Gy × 10: $P = 8.97 \times 10^{-5}$; PC3—10 Gy × 1: $P = .01$; 1 Gy × 10: $P = 1.38 \times 10^{-12}$; DU145—10 Gy × 1: $P = 1.20 \times 10^{-4}$; 1 Gy × 10: $P = 4.34 \times 10^{-4}$) and proliferation (LNCaP—10 Gy × 1: $P = 5.36 \times 10^{-14}$; 1 Gy × 10: $P = 1.50 \times 10^{-8}$; 0.5 Gy × 10: $P = 1.80 \times 10^{-3}$; PC3—10 Gy × 1: $P = .03$; 1 Gy × 10: $P = 1.84 \times 10^{-5}$; DU145—10 Gy × 1: $P = 2.61 \times 10^{-3}$; 1 Gy × 10: $P = 2.17 \times 10^{-3}$) genes were among the most universally differentially expressed genes across all cell lines and all fractionation regimens (Table 2). The several most significant functional categories at 24 hours following irradiation for each cell line and each featured fractionation regimen were similar for all assessed time points before 24 hours (data not shown).

Relative changes in individual genes from several selected functional categories for multiple irradiation fractionation regimens at 24 hours for LNCaP, PC3, and DU145 cells were color coded to demonstrate the expression patterns of those genes within each category. The heat maps demonstrate that more cell cycle regularly genes (Figure 2*A*) were differentially expressed in LNCaP cells than in PC3 or DU145 cells, whereas immune response genes (Figure 2*B*) were more significantly altered in PC3 and DU145 cells than in LNCaP cells. Furthermore, the heat maps demonstrate that most differentially expressed genes were upregulated in PC3 and DU145 cells but downregulated in LNCaP cells. Qualitatively, these heat maps also demonstrate greater changes in genes following SD irradiation for LNCaP cells (Figure 3), similar changes with SD or MF irradiation for DU145 cells, and greater changes with MF irradiation for PC3 cells (Figure 2).

Figure 3 depicts heat maps for three gene ontology categories, namely, apoptosis, signal transduction, and DNA repair (Figure 3, *A–C*, respectively), for LNCaP cells (the 0.5 Gy per fraction regimen was only performed for LNCaP cells) at 24 hours. Although very similar patterns were demonstrated following 5 and 10 Gy when administered in a single fraction, patterns demonstrated after MF regimens were significantly different from each other and from SD regimens, particularly for 0.5 Gy × 10.

## p53 *Genes*

p53-activated proapoptotic genes, including *TP53I3* and *ANGPT2*, were upregulated in LNCaP cells following all irradiation fractionation regimens, whereas no up-regulation was observed in these genes following any irradiation fractionation regimen at any time point for either PC3 or DU145 cells. Twelve of the 102 p53 regulatory genes ($P = 2.95 \times 10^{-7}$) assessed in the microarray analysis for LNCaP were differentially expressed (greater than two-fold change and $P < .05$) following irradiation (Table W2). Western blot analysis demonstrated that transcriptional activation of both *TP53I3* and *ANGPT2* were seen at the protein level for LNCaP cells (data not shown).

## *Immune Response Genes*

Immune response genes at 24 hours were generally upregulated following irradiation, particularly in PC3 cells, and particularly with MF regimens [6]. Overall, heat maps demonstrate that immune response genes at 24 hours were more significantly altered in PC3 and DU145 cells than LNCaP cells (Figure 2). Among immune response genes, *IFI27*, *OASL*, *IFIT1*, and *IFIT3* were upregulated in all cell lines. *IFI27* was highly significantly upregulated following all MF regimens in PC3 cells (fold change range of the various MF regimens, 29.5-63.5 times). *IFI27* was also upregulated in MF regimens for DU145 and LNCaP cells, but no up-regulation was seen in any

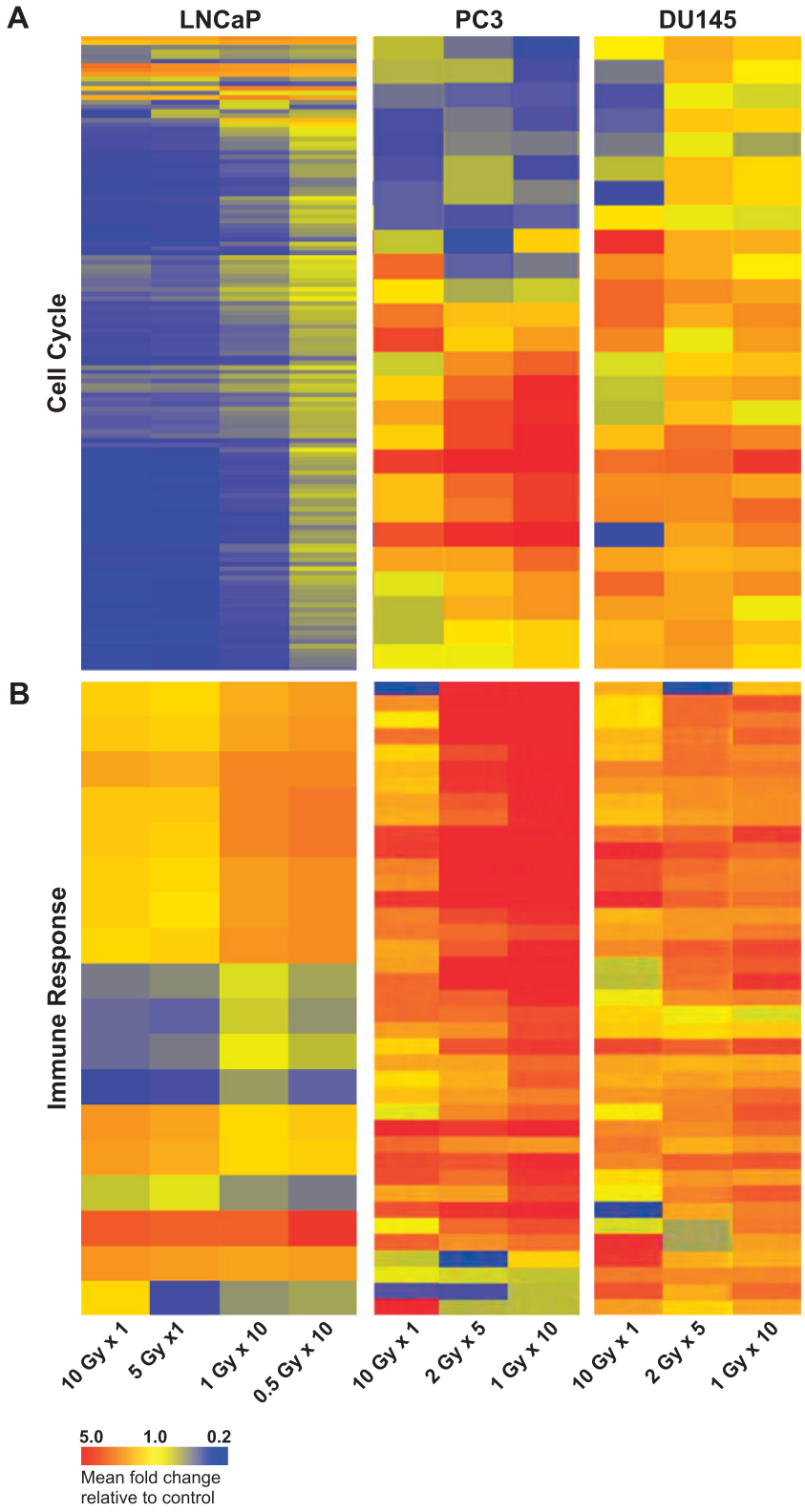


**Figure 2.** Heat maps depicting the expression patterns for (A) cell cycle regulatory genes and (B) immune response genes at 24 hours following exposure to irradiation. LNCaP cells were exposed to 10 Gy × 1 (lane 1) and 5 Gy × 1 (lane 2) SD irradiation and 1 Gy × 10 (lane 3) and 0.5 Gy × 10 (lane 4) MF irradiation. PC3 and DU145 cells were exposed to 10 Gy × 1 (lane 1) SD irradiation and 2 Gy × 5 (lane 2) and 1 Gy × 10 (lane 3) MF irradiation. Orange to red indicates upregulated genes, and blue indicates downregulated genes. Heat maps for PC3 and DU145 cells were adapted in part from John-Aryankalayil et al. [6].

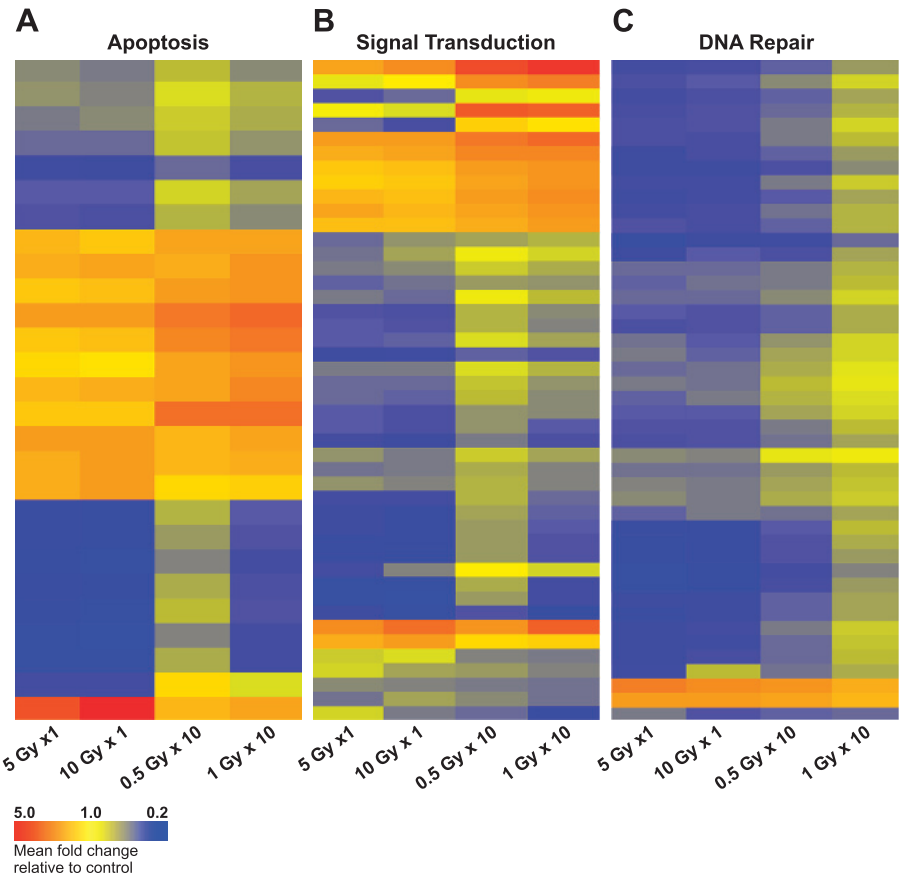


**Figure 3.** Heat maps depicting the expression patterns for (A) apoptosis genes, (B) signal transduction genes, and (C) DNA repair genes for LNCaP cells at 24 hours following exposure to SD 5 Gy × 1 (lane 1) and 10 Gy × 1 (lane 2) irradiation or MF 0.5 Gy × 10 (lane 3) and 1 Gy × 10 (lane 4) irradiation. Orange to red indicates upregulated genes, and blue indicates downregulated genes.

of the three cell lines following SD irradiation. Similarly, *OASL* was highly significantly upregulated following all irradiation regimens in PC3 cells, particularly among MF regimens (range, 7.2-12.3 times). *OASL* was also upregulated in all SD regimens for DU145 cells and all MF regimens for LNCaP cells. Both *IFIT1* and *IFIT3* were upregulated in MF regimens for LNCaP and PC3 cells but only in SD regimens in DU145 cells.

### *Cell Cycle Genes*

Overall, 14.2% (139/978) of all differentially expressed genes in LNCaP cells following irradiation were cell cycle regulatory genes, the functional category with the most significant enrichment factor. More cell cycle genes at 24 hours were differentially expressed in LNCaP cells following SD regimens (128 genes for 10 Gy × 1, $P = 3.75 \times 10^{-91}$) than MF regimens (65 genes for 1 Gy × 10,

**Table 3.** Comparison of Differentially Expressed Representative Cell Cycle Regulatory Genes at 24 Hours among Various Prostate Cancer Cell Lines.

| Symbol* | Gene Name | LNCaP | | PC3 | | DU145 | |
|---|---|---|---|---|---|---|---|
| | | 10 Gy × 1 | 1 Gy × 10 | 10 Gy × 1 | 1 Gy × 10 | 10 Gy × 1 | 1 Gy × 10 |
| | | Fold Change | Fold Change | Fold Change | Fold Change | Fold Change | Fold Change |
| *CDC20* | *Cell division cycle 20 homolog* | 0.04†, $P = 2.21 \times 10^{-5}$ | 0.23, $P = .039$ | 0.81, $P = .014$ | 1.50, $P = .735$ | 1.37, $P = .083$ | 0.99, $P = .972$ |
| *KIF20A* | *Kinesin family member 20A* | 0.04, $P = 2.70 \times 10^{-3}$ | 0.30, $P = .121$ | 0.79, $P = .205$ | 1.09, $P = .951$ | 1.22, $P = .036$ | 1.15, $P = .673$ |
| *E2F2* | *E2F transcription factor 2* | 0.08, $P = 3.43 \times 10^{-3}$ | 0.42, $P = .032$ | 0.49, $P = .023$ | 0.43, $P = .436$ | 0.79, $P = .039$ | 1.06, $P = .843$ |
| *GINS2* | *GINS complex subunit 2* | 0.15, $P = 1.13 \times 10^{-3}$ | 0.31, $P = .037$ | 0.59, $P = 1.79 \times 10^{-3}$ | 0.51, $P = .343$ | 0.49, $P = 2.83 \times 10^{-3}$ | 0.88, $P = .692$ |
| *PIF1*‡ | *PIF1 5′-to-3′ DNA helicase homolog* | 0.16, $P = .031$ | 0.35, $P = .081$ | 0.59, $P = .059$ | 1.22, $P = .800$ | 1.11, $P = .521$ | 0.94, $P = .821$ |
| *PIF1* | *PIF1 5′-to-3′ DNA helicase homolog* | 0.03, $P = 1.03 \times 10^{-4}$ | 0.17, $P = .015$ | 0.63, $P = .033$ | 1.04, $P = .968$ | 1.23, $P = .144$ | 0.87, $P = .647$ |
| *CCNB1* | *Cyclin B1* | 0.17, $P = 5.60 \times 10^{-4}$ | 0.33, $P = .015$ | 1.00, $P = .975$ | 1.09, $P = .931$ | 1.27, $P = .039$ | 1.06, $P = .837$ |
| *CCNE2* | *Cyclin E2* | 0.20, $P = 6.70 \times 10^{-3}$ | 0.48, $P = .024$ | 0.55, $P = .042$ | 0.62, $P = .333$ | 0.38, $P = 3.15 \times 10^{-3}$ | 1.05, $P = .856$ |

*All cell cycle regulatory genes differentially expressed (greater than two-fold change and $P < .05$) following every SD and MF irradiation regimen in LNCaP cells that were also differentially expressed in any PC3 or DU145 fractionation regimen at 2, 6, or 24 hours following irradiation are included.

†Gray shading indicates genes significantly downregulated (ratio < 0.5 times and $P < .05$).

‡Two separate probes were used to assess PIF1 in the mRNA microarray analysis with the CodeLink Whole Genome Bioarrays, with values for each probe depicted. Good correlation was seen between the probes. *PIF1* is one of the rare genes in which multiple probes were used for its assessment.

$P = 5.61 \times 10^{-34}$). In LNCaP cells, *AURKA*, *AURKB*, *CCNB1*, *CCNB2*, *CCNE2*, *CDC20*, *CDKN2C*, *CDKN3*, *E2F2*, *E2F8*, *GINS2*, *KIF20A*, *PIF1*, *PLK4*, and others were all significantly downregulated after 10 Gy × 1, 5 Gy × 1, and 1 Gy × 10 irradiation regimens, with the fold changes of selected cell cycle genes at 24 hours following 10 Gy × 1 and 1 Gy × 10 listed for all three cell lines depicted in Table 3.

Cell cycle genes were much less significantly induced for SD regimens for DU145 cells ($P = 9.74 \times 10^{-4}$) and were not significantly induced for MF regimens ($P = .490$), whereas cell cycle gene induction in PC3 cells was modestly significant for SD ($P = .016$) and MF ($P = .031$) regimens. Figure 2 depicts heat maps for cell cycle regulatory genes in LNCaP (10 Gy × 1 and 5 Gy × 1; 1 Gy × 10 and 0.5 Gy × 10) and PC3 and DU145 cells (10 Gy × 1; 2 Gy × 5 and 1 Gy × 10) 24 hours following SD or MF irradiation regimens.

Although 730 different genes were downregulated at any time point following irradiation regimens in LNCaP cells, significantly fewer genes were downregulated following irradiation in PC3 and

**Table 4.** Networks and Associated Functional Categories Identified by IPA for Various Prostate Cancer Cell Lines 24 Hours after Exposure to 10-Gy Irradiation Administered as an SD or MF Regimen (1 Gy × 10).

| Radiation Regimen | IPA Score | Focus Molecules | Function |
|---|---|---|---|
| LNCaP | | | |
| 10 Gy × 1 | 56* | 32† | Cell cycle, cellular assembly/organization, DNA replication/recombination/repair |
| | 47 | 29 | DNA replication/recombination/repair, cell cycle, cellular assembly/organization |
| | 47 | 29 | Cancer, gastrointestinal disease, genetic disorder |
| | 41 | 27 | DNA replication/recombination/repair, cell cycle, cancer |
| | 36 | 25 | Cell cycle, cancer, gastrointestinal disease |
| | 35 | 26 | Cellular assembly/organization, DNA replication/recombination/repair, cell cycle |
| | 33 | 23 | Nucleic acid metabolism, small molecule biochemistry, cancer |
| | 32 | 22 | Cell cycle, DNA replication/recombination/repair, cell signaling |
| | 32 | 22 | Cancer, genetic disorder, respiratory disease |
| | 31 | 23 | Cell cycle, cellular assembly/organization, DNA replication/recombination/repair |
| | 28 | 21 | Cell cycle, cellular movement, DNA replication/recombination/repair |
| | 28 | 23 | DNA replication/recombination/repair, infection mechanism, cell cycle |
| | 23 | 18 | Cell cycle, cellular development, embryonic development |
| | 22 | 17 | Developmental disorder, genetic disorder, carbohydrate metabolism |
| | 22 | 17 | Organismal injury/abnormalities, cell signaling, carbohydrate metabolism |
| | 22 | 17 | Immunologic disease, inflammatory disease, neurologic disease |
| | 20 | 16 | Organ morphology, gene expression, inflammatory response |
| | 17 | 14 | Cancer, dermatological diseases/conditions, neurologic disease |
| | 15 | 13 | Cell cycle, embryonic development, tissue development |
| | 14 | 13 | Genetic disorder, metabolic disease, DNA replication/recombination/repair |
| 1 Gy × 10 | 55 | 30 | Cellular assembly/organization, cell cycle, cancer |
| | 44 | 27 | Cell cycle, cardiovascular system development/function, organismal development |
| | 37 | 23 | Cardiovascular disease, developmental disorder, cellular development |
| | 36 | 23 | Gene expression, developmental disorder, genetic disorder |
| | 35 | 24 | Cancer, genetic disorder, respiratory disease |
| | 29 | 19 | Cell signaling/interaction, cellular assembly/organization, cellular function/maintenance |
| | 28 | 19 | Cell cycle, cell death, drug metabolism |
| | 27 | 19 | Cancer, endocrine system disorders, genetic disorder |
| | 27 | 19 | Cancer, genetic disorder, respiratory disease |
| | 24 | 17 | Cell morphology, cellular assembly/organization, nervous system development/function |
| | 23 | 18 | Cellular development, reproductive system development/function, cell cycle |
| | 21 | 15 | Cell cycle, DNA replication/recombination/repair, gene expression |
| | 17 | 13 | Cell signaling, carbohydrate metabolism, small molecule biochemistry |
| | 17 | 13 | Cardiac arrythmia, cardiovascular disease, organismal injury/abnormalities |
| | 15 | 13 | Lipid metabolism, molecular transport, small molecule biochemistry |
| DU145‡ | | | |
| 10 Gy × 1 | None | – | – |
| 1 Gy × 10 | 32 | 13 | Cancer, cellular movement, reproductive system development/function |
| | 10 | 5 | Cancer, cell morphology, cell signaling |
| PC3‡ | | | |
| 10 Gy × 1 | 45 | 19 | Cell-to-cell signaling/interaction, dermatological diseases/conditions, lipid metabolism |
| | 30 | 14 | Antigen presentation, cancer, cardiovascular disease |
| | 20 | 10 | Cancer, cellular movement, cardiovascular system development/function |
| 1 Gy × 10 | 57 | 28 | Antigen presentation, antimicrobial response, cell-mediated immune response |
| | 36 | 20 | Carbohydrate metabolism, small molecule biochemistry, cardiovascular disease |
| | 32 | 18 | Cellular development, genetic disorder, inflammatory disease |
| | 32 | 18 | Carbohydrate metabolism, hepatic system development, small molecule biochemistry |
| | 27 | 16 | Cellular development, hematological system development/function, hematopoiesis |
| | 23 | 14 | Genetic disorder, hematological disease, metabolic disease |
| | 19 | 13 | Cell morphology, cellular development, neurologic disease |
| | 17 | 11 | Cell signaling, molecular transport, vitamin/mineral metabolism |
| | 11 | 8 | Hematological disease, cancer, cell death |

*IPA score refers to statistical significance, with all genes with at least a two-fold change and a $P < .05$ imputed for network generation and pathway analyses and mapped to the functional networks available in the Ingenuity Pathway Knowledge Base. A score was determined for each network reflecting the negative logarithm of the $P$ value based on the chance that the focus genes were grouped in the network by random chance. Only scores $\geq 10$ are depicted.

†Focus molecules indicate the number of genes that could be mapped to molecules out of a possible 35 molecules in each network.

‡Adapted in part from John-Aryankalayil et al. [6].

**Table 5.** Validation of Microarray Data*.

| Gene | Relative Gene Expression | LNCaP | | PC3† | | DU145† | |
|---|---|---|---|---|---|---|---|
| | | 10 Gy × 1 | 1 Gy × 10 | 10 Gy × 1 | 1 Gy × 10 | 10 Gy × 1 | 1 Gy × 10 |
| *OASL* | Microarray | 1.4 ± 0.1 | 3.4 ± 0.3 | 3.1 ± 0.3 | 12.3 ± 2.4 | 3.7 ± 0.3 | 2.1 ± 0.5 |
| | PCR | 2.1 ± 0.2 | 5.1 ± 1.1 | 4.4 ± 0.9 | 43.1 ± 2.7 | 5.7 ± 0.4 | 4.1 ± 0.5 |
| *IFI27* | Microarray | 1.4 ± 0.1 | 3.7 ± 0.6 | 1.4 ± 0.2 | 63.5 ± 22.3 | 0.9 ± 0.2 | 2.5 ± 0.8 |
| | PCR | 1.5 ± 0.4 | 6.9 ± 0.9 | 2.1 ± 0.3 | 544.8 ± 10.5 | 1.5 ± 0.3 | 2.0 ± 0.3 |
| *UBE1L* | Microarray | 1.6 ± 0.3 | 3.5 ± 0.5 | 2.4 ± 0.2 | 3.3 ± 0.8 | 2.1 ± 0.3 | 7.9 ± 5.3 |
| | PCR | 1.7 ± 0.3 | 4.3 ± 0.8 | 1.7 ± 0.1 | 6.3 ± 0.8 | 2.5 ± 0.4 | 4.0 ± 0.6 |

*Relative expression of selected genes identified by microarray analysis were validated by real-time PCR. LNCaP, PC3, and DU145 cells were irradiated with SD (10 Gy × 1) and MF (1 Gy × 10) irradiation. Each data point represents the average ± SEM of three biologically distinct experiments.
†Adapted in part from John-Aryankalayil et al. [6].

DU145 cells. As such, only three genes were mutually down-regulated among all three cell lines. *PIF1* (5′-to-3′ DNA helicase that negatively regulates telomerase, a reverse transcriptase that maintains telomere length), *KIF20A* (microtubule-dependent molecular motor that plays important roles in intracellular transport and cell division, is potentially involved in mitosis, and facilitates myosin and kinesin-mediated processes), and *CCNB1* (encodes a regulatory protein involved in mitosis, with a gene product complex expressed predominantly during $G_2$/M phase of the cell cycle) were downregulated in all SD irradiation regimens for all three cell lines but only in LNCaP cells following MF irradiation. While the down-regulation of *PIF1*, *KIF20A*, and *CCNB1* following irradiation persisted at 24 hours for LNCaP cells, the down-regulation seen at 2 and 6 hours following irradiation for PC3 and DU145 cells did not remain significant at 24 hours. Furthermore, the magnitude of down-regulation for LNCaP cells was significantly greater for SD than MF regimens, particularly for *KIF20A* (fold change range of various SD regimens, 0.04-0.06 times).

### *Ingenuity Pathway Analysis*

Genes significantly altered following irradiation (greater than two-fold change and $P < .05$) were mapped to the functional networks in the IPA database and ranked by score to further assess the effects of varying irradiation fractionation regimens at 24 hours following the completion of irradiation for each cell line of varying p53 status (Table 4). Overall, IPA revealed significant differences between SD and MF irradiation regimens. When assessing all MF regimens, pathways in common between all three cell lines included cancer, cell signaling, antigen presentation, and cell morphology.

However, other notable differences were seen across cell lines with varying p53 status. In LNCaP cells, cell cycle and cellular assemble and organization functions predominated following SD and MF irradiation. No appreciable differences in networks were observed between the 5- and 10-Gy SD regimens. However, fewer networks with an IPA score of greater than 10 were identified following MF regimens in LNCaP cells, particularly following 0.5 Gy × 10, which was found to have networks functionally more uniquely associated with cellular movement, connective tissue development and function, hepatic system disease, and gastrointestinal disease (Table W3). In contrast, a greater number of networks were observed with MF irradiation in PC3 cells. Networks functionally related to immune response, including antigen presentation, cell-mediated immune response, antimicrobial response, and infection mechanism, were observed after all fractionation regimens in PC3 cells, whereas cell-to-cell signaling was unique to SD irradiation. Few networks with an IPA score of greater than 10 were identified following any fractionation regimen in DU145 cells. Similar finding with the miRNA expression patterns in DU145 cells after SD and fractionated irradiation in comparison to LNCaP and PC3 cells were also identified [29].

### *Confirmation of Microarray Data*

The relative expressions of selected genes following irradiation for LNCaP, PC3, and DU145 cells identified by microarray analysis were confirmed by real-time PCR and/or Western blot analysis. An extensive demonstration of real-time PCR confirmation for PC3 and DU145 cells for multiple fractionation regimens has previously been reported [6]. Similar confirmation studies were conducted for LNCaP, with selected real-time PCR data reported in Table 5 representing the average ± SEM of three biologically distinct experiments. Among genes selected for confirmation by real-time PCR (14 genes), 100% correlation was observed with microarray data for all three cell lines and for all irradiation fractionation regimens. Western blot analysis confirmed that the up-regulation or down-regulation of selected representative genes was also confirmed at the protein level (data not shown).

## Discussion

This study demonstrated that the manner in which irradiation is delivered, either as an SD or MF regimen, can dramatically influence the gene expression pattern induced by ionizing radiation. The phenotype of malignant cells surviving MF irradiation regimens differs from the starting cell population and from cells exposed to a single higher dose of irradiation. Among the three MF regimens investigated, 1 Gy × 10 induced more genes and had greater magnitudes of gene expression changes than either 0.5 Gy × 10 or 2 Gy × 5. However, gene induction was demonstrated even after irradiation fractions as low as 0.5 Gy (Table W4), a fraction size in which much of the cell population survives each fraction. Changes demonstrated in this study following 0.5-Gy fractions, therefore, may in part be attributable to an adaptive response or stress response [30].

This study also demonstrated that gene expression patterns significantly differed between cell lines of varying p53 status, as is in keeping with the findings of prior studies [23,30,31]. Although the regulation of p53-dependent genes is multifactorial, this study demonstrated that p53-activated genes were induced only in p53 wild-type LNCaP cells, whereas DU145 and PC3 cells did not result in the expression of p53-related genes following any irradiation fractionation regimen, an expected finding in tumor cells with mutated and null p53, respectively. This study also demonstrated that the number of genes differentially expressed and the proportion of genes

upregulated or downregulated following irradiation differed greatly among cell lines of varying p53 status. Following irradiation, LNCaP cells demonstrated the greatest number of genes differentially expressed (or altered), particularly for cell cycle regulatory genes. Genes were also more likely to be downregulated and altered by SD regimens in LNCaP cells than in the p53 null and mutant cell lines. p53 mutant DU145 cells showed the fewest number of genes altered following irradiation. This may be due, in part, to the higher surviving fractions for DU145 cells following SD and MF irradiation regimens. The majority of genes differentially expressed in p53 null PC3 cells, like DU145 cells, were upregulated. Genes induced in PC3 cells were proportionately the most likely to regulate immune response and be altered following MF regimens. In fact, immune response genes across MF regimens at 24 hours were the most significantly altered functional category in PC3 cells ($P = 6.72 \times 10^{-11}$), and 30 immune response genes were differentially expressed (greater than two-fold change and $P < .05$) of the 865 immune response genes assessed on mRNA microarray analysis.

When assessing changes following irradiation across cell lines, a higher proportion of genes were downregulated following SD regimens than MF regimens. This was particularly true for LNCaP cells, in which down-regulation occurred in 90% of all genes differentially expressed following SD irradiation. This is in keeping with previous finding that higher biologically equivalent doses result in more gene down-regulation than up-regulation [30,31].

Cell cycle regulatory genes at 24 hours were most prominently differentially expressed in LNCaP cells (SD and MF aggregate $P = 9.23 \times 10^{-73}$) but were not significant when aggregated across all fractionated regimens in PC3 and DU145 cells ($P \geq .05$ for both). Radiation-induced DNA damage induces numerous cellular responses, including cell cycle arrest. Irradiation of cells that express wild-type p53, such as LNCaP cells, results in an accumulation of cells in $G_1$ [13]. In contrast, we have previously demonstrated that PC3 and DU145 cells show significant $G_2$/M arrest in response to SD but not MF irradiation, with appreciable decrease in $G_1$ [6]. After cell cycle regulatory genes, DNA replication and repair genes were most prominently differentially expressed in LNCaP cells (aggregate $P = 6.86 \times 10^{-30}$). Previous studies have suggested that regulation of DNA repair may be induced at early time points following irradiation [32].

It is possible that irradiation caused a suppression of cell cycle and repair mechanisms in LNCaP cells. As more cell cycle genes were differentially expressed following irradiation in LNCaP cells than PC3 or DU145 cells, this could account for the higher proportion of genes differentially downregulated in LNCaP cells. Although the effects of irradiation were demonstrated to vary across cell lines of varying p53 status, homeostatic regulation allowed for some cell repair and propagation across the varying fractionation regimens and cell lines. A major target of radiation-induced type I interferon is the hematopoietic compartment. Shared mechanisms of homeostasis across cell lines may be through hormonal release or diffusion. Furthermore, an emerging theory of radiation homeostasis hypothesizes that low doses of ionizing radiation can be beneficial by stimulating the activation of repair mechanisms that protect against cell death that are not activated in absence of ionizing radiation. Should this theory prove accurate and refute the linear no threshold model, such effects could occur following our 0.5-Gy MF regimen.

A minority of differentially expressed genes were similarly upregulated or downregulated following irradiation in all cell lines of varying p53 status when stringent parameters for significance were applied to our microarray results (greater than two-fold and $P < .05$). Several immune response genes were mutually upregulated in all cell lines, including *IFI27*, *OASL*, *IFIT1*, and *IFIT3*, whereas few cell cycle regulatory genes were mutually downregulated, including *PIF1*, *KIF20A*, and *CCNB1*. Additionally, *UBE1L*, which is involved in apoptosis and cell growth regulation, and *HIST1H2BD*, which is a regulator of nucleosome structure, were upregulated in all cell lines. *UBE1L* and *HIST1H2BD* were upregulated following SD and MF regimens in PC3 cells, SD regimens in DU145 cells, and MF regimens in LNCaP cells. Such genes that have been demonstrated in this study to be commonly differentially expressed across cell lines of varying p53 status following MF irradiation regimens may serve as potential ideal targets for molecular therapy and warrant more investigation, particularly upregulated genes highly significantly altered, including *IFI27*, *OASL*, and *UBE1L*. It is possible that the very highly significant up-regulation seen in *IFI27* in multifraction regimens in PC3 cells is related to our findings that *STAT1* was most prominently altered in PC3 cells after MF irradiation and not after SD irradiation or in LNCaP or DU145 cells, as *STAT1* is upstream in the interferon signaling pathway and known to physically associate with the *IFI27* promoter. Further assessment of this association is needed if *IFI27* is used for molecular therapy development.

Among PC3 and DU145 cells, immune response genes were most significantly altered following irradiation. This is in keeping with previous reports demonstrating that the efficacy of radiation therapy is influenced by innate and adaptive immunity and may be significantly dependent on the type I interferon family of cytokines. Furthermore, irradiation can result in an increase in intratumoral production of interferon-β, increase production of inflammatory cytokines, and enhance tumor-specific immune responses [33]. Numerous immune response genes were mutually differentially expressed between PC3 and DU145 cells [6], and nearly half of the genes mutually altered in all three cell lines were immune response genes. Most of these genes were more influenced by MF than SD irradiation regimens, as has been shown in previous studies [6,22]. As with the other functional categories, many more immune response genes would have been found to be mutually differentially expressed between PC3 and DU145 cells if a greater than two-fold cutoff had been used as the sole stratification criteria, irrespective of *P* value, particularly with MF regimens. When using more stringent criteria of both a greater than two-fold cutoff and a *P* value < .05, fewer genes were identified as mutually differentially expressed due to intragroup variation among biologic triplicates that increased the *P* value to ≥.05 in several genes that were differentially expressed by greater than two-fold.

This study suggests that MF irradiation can elicit a common gene response across cell lines with varying p53 phenotypes, particularly for immune response, stress response, and proliferation genes. This is of significance since such radiation-induced expression of immune response genes that was demonstrated in this study may enhance tumor-specific immune responses and render tumor cells more susceptible to molecular targeted therapy through endogenous priming mechanisms and potential increased efficacy of immune-related therapy [33]. As such, radiation may act synergistically with immunotherapy to enhance immune response, inhibit immune suppression, or alter tumor cell phenotypes, making tumors more susceptible to immune-mediated killing [33,34]. Additionally, stress response and proliferation genes were among the most universally differentially expressed genes in this study. Although to a lesser extent following 0.5 Gy × 10 irradiation (stress response: $P = .026$; proliferation:

$P$ = .002), these genes were highly significantly altered following both SD and MF irradiation regimens in LNCaP cells. These genes were similarly differentially expressed following SD and MF regimens in DU145 cells, and they were altered to a much greater extent following MF regimens in PC3 cells. Many of the stress response and proliferation genes differentially expressed following 1 Gy × 10 irradiation in PC3 cells were mutually altered following the same regimen in LNCaP cells.

Fraction sizes of 0.5 and 1 Gy that were tested in this study reflect cellular response when there is repeated irradiation delivered with little cell killing. Pronounced gene expression was still achieved following 1-Gy MF regimens in LNCaP cells that largely mirrored changes observed for single-fraction regimens. However, a dramatically different expression profile was observed in LNCaP cells following repeated administration of 0.5-Gy irradiation that was most similar to the profiles observed following MF regimens in PC3 and DU145 cells, with immune response, interferon, and proliferation genes most affected. These changes may, in part, be attributable to an adaptive or stress response [30]. Furthermore, with these low-dose fractionated regimens, exposure to repeated doses of sublethal irradiation could induce an adaptive phenotype change that is preserved across generations from both irradiated and nonirradiated bystander cells [35].

Little clinical data exist using low fraction sizes below conventional 1.8- to 2.0-Gy fraction doses to treat prostate carcinoma. Regional normal tissues, tumor cells that have regionally metastasized beyond the radiation therapy planning target volume or field edge, and tumor cells that are located in a relative low-dose region within the clinical target volume may receive radiation doses of significantly less than 1.8 to 2.0 Gy daily, as were delivered in this study, particularly with widespread current use of IMRT to treat prostate cancer. Indeed, it is conceivable that the *field-within-a-field* approach with IMRT may induce tumor cell heterogeneity [35].

Administration of low fraction sizes for therapeutic effect, however, has been assessed with hyperfractionated radiotherapy and for chemotherapy sensitization. Compared with standard fractionation, hyperfractionation to an isoeffective tumoricidal dose may lower the rates of acute and late genitourinary and gastrointestinal toxicities without compromising treatment efficacy [36,37]. However, clinical applications of hyperfractionation have been limited due to the calculated low $\alpha/\beta$ ratio of prostate adenocarcinoma that would favor hypofractionated radiotherapy [37]. Low-dose fractionated radiation therapy has also been tested as a chemotherapy sensitizer in limited clinical studies. The first clinical application of this concept involved the administration of 80-cGy fractions on the first and second days of chemotherapy cycles with paclitaxel and carboplatin in patients with locally advanced squamous cell carcinoma of the head and neck [38]. Low-dose fractionated radiation therapy has also been evaluated clinically with promising results using 60- to 70-cGy fractions with gemcitabine for pancreatic cancer [39] and 60-cGy fractions with docetaxel for ovarian cancer [40]. Akin to its use as a chemopotentiator, this work serves as a basis for pursuing fractionated radiotherapy to induce targets for molecular targeted therapy. Further investigation, however, is needed to determine if the dose per fraction can be tailored to achieve a desired radiobiologic effect.

It is of no surprise that gene expression following irradiation was found in this study to be dependent on p53 status. *p53* gene mutations and nuclear accumulation of p53 protein have also been shown to result in more aggressive tumor phenotypes in prostate, bladder, lung, colorectal, brain, cervical, breast, and head and neck malignancies [41–44]. Multiple studies have demonstrated inherent radiobiologic differences or poorer outcomes in patients undergoing definitive radiation therapy for prostate cancer with *p53* gene mutations. p53 mutations are associated with increased prostate-specific antigen (PSA) serum level, advance clinical stage, higher tumor grade, decreased response to hormonal therapy, decreased response to radiation therapy, increased rates of biochemical and clinical failures following definitive radiation therapy, increased risk of metastasis, and decreased survival [45–49].

The multi-institutional Radiation Therapy Oncology Group 8610 trial of 471 patients assessed p53 status and prognosis in prostate cancer, and 18% of patients had abnormal p53 protein expression [50]. Independent of Gleason score and clinical stage, abnormal p53 expression was associated with decreased progression-free survival ($P$ = .03), decreased overall survival ($P$ = .02), and increased distant metastases ($P$ = .04), particularly among patients receiving both radiation therapy and hormonal therapy ($P$ = .001), potentially suggesting that the apoptotic effects of radiation therapy and/or hormonal therapy are blocked in patients with p53 mutations.

The multi-institutional Cancer and Leukemia Group B 9682 trial demonstrated abnormal p53 expression correlated with higher rates of PSA failure at 5 years following radiation therapy (33% *vs* 18%, $P$ = .008) in 180 patients with prostate carcinoma, even after adjusting for tumor grade, PSA level, stage, and hormonal therapy [51]. Radiation Therapy Oncology Group 9202 enrolled 777 patients with clinically localized prostate carcinoma, of which 21.6% had abnormal p53 expression [52]. Patients with abnormal expression had nearly twice the rates of cause-specific mortality [hazard ratio (HR) = 1.89, P = .014] and distant metastasis (HR = 1.72, $P$ = .013). The survival detriment with p53 overexpression was greatest for patients receiving radiation therapy with short-term *versus* long-term hormonal therapy (HR = 2.43, $P$ = .0044). p53 mutations have also been correlated with poorer surgical outcomes [53] and chemoresistance or decreased responses to chemotherapy [54,55].

The current study suggests that apoptosis may contribute to prostate cancer cell death, with the degree of this contribution differing across cell lines of varying p53 status. Apoptosis is one mechanism through which radiation therapy, with or without hormonal therapy, is believed to function to eradicate prostate cancer cells [56]. As has been demonstrated with other genitourinary malignancies, the ability of ionizing radiation to induce p53-dependent apoptosis in malignant cells is impaired in tumors with mutated p53 [57]. To date, novel molecular treatment strategies aiming to reconstitute wild-type p53 function in mutant p53-expressing prostate tumors to improve radiation sensitivity, such as through the use of adenovirus vectors, have had mixed results [58,59].

Therefore, the use of novel targeted agents represents one potential method of circumventing this interruption in the apoptotic pathway. Cell survival analysis across cell lines to determine the influence of p53 status on cell survival following irradiation has demonstrated the greatest radiosensitivity among LNCaP cells and the least radiosensitivity among DU145 cells [29]. Further study modulating p53 is needed to assess the contribution of apoptosis toward cellular death in these cell lines following irradiation.

To identify gene expression patterns and changes in molecular pathways, further study is underway to assess radiation-induced differential gene expression through miRNA, mRNA, protein array, and phosphorylation changes at the proteomic level with additional

cancer cell lines exhibiting both normal and dysfunctional p53. As p53 null PC3 cells have previously been reported to have similar survival to cells with a normal *p53* gene following SD irradiation but decreased survival following MF irradiation [45], cell cycle studies across cell lines with varying p53 status are currently underway. Molecular targeted drug regimens in combination with reconstituting wild-type p53 function will also be a focus of our laboratory. The prominence of differentially expressed immune response genes following multifraction irradiation regimens for p53 defective and null cells suggests that prostate tumors with mutant p53 may be amenable to MF radiotherapy plus immunotherapy, which is worthy of further study. We will also assess additional irradiation fractionation patterns, particularly with lower doses per fraction and potentially lower dose rates, as the fractionation regimen employed may be chosen to optimize induction of certain pathways amenable to molecular targeted therapy. This ongoing work will further advance our investigation of the novel approach of inducing drug susceptibility in cells that survive repeated stresses from MF irradiation.

Although the increasing use of IMRT and image-guided radiation therapy for prostate cancer have reduced the high-dose irradiation damage to adjacent normal tissues, thus allowing for radiation dose escalation and improved biochemical relapse-free survival rates, IMRT also results in the exposure of more normal tissue to any irradiation dose. In conjunction with the studies on prostate cancer cells presented here, we are currently evaluating the effects of SD and MF irradiation exposure on gene expression and miRNAs in human coronary artery endothelial cells (Palayoor et al., in preparation). More akin to that seen in PC3 cells than LNCaP cells, fractionated irradiation exposure results in more robust gene and miRNA changes in human coronary artery endothelial cells compared with SD irradiation, suggesting differences in response between wild-type p53-harboring normal cells and tumor cells.

This study and our prior investigations [6,22] demonstrate that the timing of irradiation, in addition to the dose per fraction, can be selected to optimize desired molecular phenotypic changes. It is also likely that MF irradiation regimens alter upstream or downstream molecules in the signaling pathway of known target molecules. Our previous [6,22] and present studies demonstrate that cells surviving a repeated irradiation stress are phenotypically altered in a manner that is dependent on the fractionation regimen employed. The use of MF irradiation regimens, therefore, may be suitable for focused biology with combined modality therapy by altering phenotypes of cells surviving such MF regimens, thus enhancing tumor cell killing and reducing normal tissue toxicity [34,60].

While previous studies have assessed gene expression following shorter time points after irradiation, the phenotypic changes demonstrated in this study were durable, with continued differential gene expression evident at least 24 hours following irradiation. Therefore, radiation-induced changes may be sufficiently stable to allow for molecular targeting of these phenotypic changes and can logistically be more easily integrated into existing molecular targeted drug regimens. Although further study is needed to determine if a radiation-inducible pathway in the absence of a mutated pathway is truly stable and druggable, it may be possible to use radiation as a stress response to induce targets for molecular targeted therapy and enhance immunotherapy rather than only depending on the presence of mutations, thus enhancing efficacy of targeted agents and exploiting the synthetic lethal concept [61] and non-oncogene addiction approaches to cancer treatment [62].

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