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Chronic low dose-rate radiation down-regulates transcription related to mitosis and chromosomal movement similar to acute high dose in prostate cells

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Abstract

Purpose: **Despite concerns over risks from exposure to low-dose ionizing radiations encountered in the environment and workplace, the molecular consequences of these exposures, particularly at representative doses and dose-rates, remains poorly understood.**

Materials and methods: **Using a novel flood source construct, we performed a direct comparison of genome-wide gene expression regulations resulting from exposure of primary human prostate fibroblast cultures to acute (10 cGy and 200 cGy) and longer-term chronic (1.0–2.45 cGy cumulative over 24 h) exposures.**

Results: **Expression profiling showed significant differential regulation of 396 genes with no measureable changes in the acute 10 cGy dose. However, there were 106 genes in common between samples given an acute 200 cGy dose compared to those given chronic doses, most of which were decreased and related to cell cycle or chromosomal movement in M-phase. Biological pathway analysis showed decreases in cell cycle, chromosomal movement, cell survival and DNA replication, recombination and repair as well as a predicted activation of transcriptional regulators TP53, RB1 and CDKN2A. In agreement with these results, prostate epithelial cells given 200 cGy or chronic doses displayed functional decreases in proliferation and mitotic cells.**

Conclusions: **In summary, we showed a contrast to the common observation of constant or reduced effect per unit dose as the dose (acute) was diminished, that even very low total doses delivered chronically could rival the perturbing effect of acute doses 100 times as intense. Underscored is the importance of the means of dose delivery, shown to be as important as dose size when considering biologic effect.**

Keywords: Low-dose effects, low dose-rate, risk assessment, ionizing radiation, prostate, gene expression

Introduction

During the average lifetime, individuals are continuously exposed to naturally occurring ionizing radiation at extremely low dose-rates, and may also accrue additional exposures through specific occupations or diagnostic and medical procedures (Brenner and Hall 2007, Zielinski et al. 2008, Fazel et al. 2009, Daniels and Schubauer-Berigan 2011). Recently, there has been particular concern over the consequences of low dose exposure from accidental events, notably at Fukushima Daiichi and Chernobyl (Baker et al. 2011, Kamada et al. 2012), and from potential terrorist attacks. The International Commission on Radiological Protection and the National Research Council of the U.S. National Academy of Sciences recommends the use of a linear no-threshold (LNT) model, for estimating the risk of exposure to ionizing radiation (Valentin 2005, National Research Council [NRC], Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation 2006). As the name suggests, the model infers risks at lower doses, where risks have been harder to gauge, by assuming the risk determined at higher doses reduces in linear proportion to dose. Consequently, this implicitly assumes there is no level of radiation exposure, no matter how low the dose, without associated risk. However, the validity of this formalism, in light of current understandings, is under debate (Brenner et al. 2003). Due to the availability of modern molecular tools, including transcriptome studies, one can now very accurately measure physiological perturbations by low dose, shedding light on this critical issue. To date, experimental findings at low doses ($\leq$10 cGy) suggest a number of biological responses and mechanistic processes modulated by ionizing radiation may differ for low doses and dose-rates, as

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compared to higher doses, but the data remain sparse and it remains uncertain how any such differential in response would impact health.

Radiobiological response to high doses of ionizing radiation ($\geq$ 200 cGy) delivered acutely or in a protracted fashion (e.g., by dose fractionation to tissues) have been extensively studied with an eye toward any subsequent detrimental heath consequences and the potential to therapeutically exploit such response (Brush et al. 2007, Fowler 2010). Exposure to high doses of ionizing radiation is known to induce signaling regulation across biological processes, particularly as cells sense radiation-induced DNA damage and attempt to repair that damage (Criswell et al. 2003, Valerie et al. 2007). With regard to cellular fate, the integration of multiple signaling pathways altered by the radiation insult such as MAPK, PI3K, NFκB, EGFR, P53 have been demonstrated to dictate cell viability or death (Schmidt-Ullrich 2003). In fact, modulation of the biological effects to radiation exposure may be exploited by altering the total dose delivery by hypofractionation to exploit beneficial therapeutic outcomes (Stuschke and Pottgen 2010, Aneja et al. 2012, Freedman et al. 2013).

The biological consequences of irradiation and the transcriptional response of the cell to control cell cycle and effect DNA damage repair have a profound influence on the fate of that cell. A number of previous studies have examined similarities and differences in gene regulation between low and high doses of ionizing radiation, delivered as single acute doses. The cell types examined under acute low-dose exposure include: Fibroblasts (Ding et al. 2005), human umbilical vein endothelial cells (Lanza et al. 2005), lymphocytes (Amundson et al. 2000, Fachin et al. 2007), lymphoblastoid cells (Wyrobek et al. 2011), mesenchymal stem cells (Jin et al. 2008), myeloid cells (Amundson et al. 2003), skin cells (Goldberg et al. 2006) and keratinocytes (Franco et al. 2005). However, these studies largely focus on acute delivery of low-dose radiation with exposure completed over seconds to minutes.

In this study, we have examined the response of primary normal prostate fibroblast cell cultures derived from several individuals to compare the differential transcriptional regulation at 24 h after the start of an acute or protracted irradiation period. That is 24 h after acute 10 cGy or 200 cGy exposures (48 cGy per minute) versus immediately after an accumulated 24-h chronic 1.0–2.45 cGy low dose-rate exposure (7–17 μGy per min). The results indicate no statistically significant perturbation in the gene expression network after an acute low-dose exposure (10 cGy). In contrast, the transcriptional changes after the chronic low dose-rate exposure (cumulative 1–2.45 cGy) resulted in a number of genes displaying a 'transcriptional radiation signature' similar to that of the acute high-dose exposure (200 cGy) in respect to critical pathways such as cell cycle regulation, chromosomal movement and RNA processing, as well as unique perturbations in angiogenesis and mitochondrial-related genes in the chronic treatment.

Materials and methods

Primary prostate tissue and cell culture

In accordance with our institutional review board approved protocol, gross identification of a normal prostate tissue sample (1 cm^3) was obtained from consented patients undergoing robotic-assisted laparoscopic radical prostatectomy. Sections of adjacent tissue used for culture were stained with hematoxylin and eosin (H&E) and evaluated by a clinical pathologist to confirm normal tissue histology (Supplementary Figure 1A, to be found online at http://informahealthcare.com/doi/abs/10.3109/09553002.2014.877175). Briefly, minced samples were collagenase digested, plated in RPMI-1640 plus 10% fetal bovine serum (Lonza, Hopkinton, MA, USA) and maintained in a humidified 37°C incubator with 5% CO_2 (Kabalin et al. 1989, Peehl 2002). Following fibroblast growth, samples were expanded and cryogenically frozen. As a confirmation of normal fibroblast growth, the cultures stained negative for E-cadherin and α-smooth muscle actin and positive for vimentin and F-actin as well as displayed a clear visual fibroblast phenotype (Supplementary Figure 1B, to be found online at http://informahealthcare.com/doi/abs/10.3109/09553002.2014.877175). The RWPE-1, prostate epithelial cell line, obtained from ATCC (ATCC, Bethesda, MD, USA), was grown in keratinocyte basal media supplemented with 0.05 mg per ml bovine pituitary extract and 5 ng per ml epithelial growth factor.

Irradiation

Cells were irradiated acutely using a Cesium-137 (Cs-137) Mark I irradiator with a dose-rate of 0.48 Gy per min. In parallel with acute exposures, chronic low dose-rate exposure used a sealed flat uniform sheet of Cobalt-57 (Eckert & Ziegler Isotope Products, Valencia, CA, USA). Samples were in a dedicated incubator directly above the Cobalt-57 source while control and acutely irradiated samples were placed in an incubator with identical conditions without an irradiation source. Acute samples were harvested after the indicated time point from the time of irradiation. Chronically irradiated samples were harvested immediately after the indicated time point and had received constant low dose-rate irradiation during that time. To measure the Cobalt-57 chronic dose-rate, four independent measurements with Luxel OSL dosimeters (Landauer, Glenwood, IL, USA) were used for two different exposure times of 7,000 min ($n = 2$) or 1,500 min ($n = 2$). The results indicated the decay corrected dose-rate at the start of the first experiment was 17.0 ± 0.8 μGy per min (2.45 ± 0.12 cGy per day). Decay corrected dose-rates were then calculated for each individual experiment.

Clonogenic assay

Cells were harvested, counted and resuspended at the appropriate concentration in complete media. Immediately following acute irradiation, they were plated incubated for 12–14 days. Colonies were fixed in 70% ethanol plus 0.4% crystal violet. The survival fraction was given as colonies

scored divided by the number of cells plated times the plating efficiency at 0 cGy.

Gene expression microarrays and analysis

Total RNA was extracted after treatment using TRIzol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. RNA quality and quantity was assessed using the 2100 Bioanalyzer (Agilent Tech., Santa Clara, CA, USA). Briefly, 500 ng of total RNA was amplified using the TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA) and 750 ng of the product was loaded onto the gene array chips. Following hybridization at 55°C, the chips were scanned using the Illumina iScan (Illumina, San Diego, CA, USA). HumanHT-12v4 expression bead chips with more than 47,000 probes covering RefSeq and UniGene annotated genes were used (Illumina, San Diego, CA, USA). A total of four independent experimental replicates were used for each sample resulting in (2 individuals $\times$ 4 experimental conditions $\times$ 4 experimental replicates) 32 microarrays.

Data files were opened with GenomeStudio software (Illumina, San Diego, CA, USA). Several Illumina specified quality control parameters were found to be consistent among all samples and met the recommended criteria for obtaining good quality data. To account for observed variations in the signal intensity ranges among samples, non-normalized, non-background corrected signal intensity values for each sample were normalized to the mean-of-means for seven housekeeping genes (TUBB2A, UBC, EEF1A1, TXN, GAPDH, ACTB, RPS9). Samples were uploaded to GenePattern, $\log_2$ transformed, quantile normalized and processed with the ComBat module to remove non-biological batch effects (Gentleman 2005, Reich et al. 2006, Johnson et al. 2007). Statistical analysis was performed on $>31{,}000$ unique genes with a one-way ANOVA on four groups (0, 10 cGy, 200 cGy or Chronic) with eight samples per group yielding 3,975 genes ($p<0.05$). Correction for Benjamini-Hochberg multiple hypothesis testing resulted in 741 genes (false discovery rate, FDR $<10\%$). Microarray data was uploaded to the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE52918.

IPA analysis

Significantly expressed genes relative to the non-irradiated control were analyzed with the IPA core analysis software (Ingenuity® Systems, www.ingenuity.com). Samples with greater than a ± 1.2-fold-change resulted in zero genes for 10 cGy, 187 genes for 200 cGy and 264 genes for chronically irradiated samples were used for analysis.

Gene Set Enrichment Analysis (GSEA)

Characterization of the preprocessed list of $>31{,}000$ genes for each experimental condition relative to the non-irradiated control was performed using GSEA. Samples were compared to the Gene Ontology C5 collection in the Molecular Signatures Database containing more than 1400 gene sets. The minimum and maximum gene set size was set to 15 and 500, respectively. Enrichment scores were calculated based on gene expression lists ranked by signal-to-noise with a gene-set permutation algorithm. Leading edge analysis was performed on gene sets with a FDR q-value of less than 0.05. Visualization of related gene sets was performed using the Enrichment Map software in Cytoscape (http://www.cytoscape.org).

Gene expression validation

Following cDNA synthesis, quantitative PCR was performed on the 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). After normalization to a multiplexed 18S housekeeping probe, gene expression for the target was calculated using the $2^{-\Delta\Delta Ct}$ method relative to the non-irradiated control. Probes were purchased from Applied Biosystems.

Flow cytometry

Flow cytometry was performed by harvesting cells and fixing single cell suspension with 2% paraformaldehyde for HMOX-1 protein expression or 70% ethanol for phospho-S10 Histone H3 analysis. Cells were indirectly labeled with a HMOX-1 antibody (Enzo Life Sciences, Farmingdale, NY, USA) or Histone H3 (Abcam, Cambridge, MA, USA) followed by an Alexa-fluor 488 secondary antibody (Invitrogen, Grand Island, NY, USA). Histone H3 cells were dual stained with propidium iodide and samples were run on the FC-500 flow cytometer (Beckman Coulter, Brea, CA, USA).

Results

Radiosensitivity of primary prostatic fibroblast cell cultures

We have derived human primary prostate fibroblast cell cultures from multiple individuals. Use of primary cells, at early passage, is preferable to use of immortalized cell lines that have adapted to long-term growth in vitro and/or have been engineered to ignore or bypass natural programmed responses (e.g., cycle arrest). The cells were irradiated at passage 3–6 to avoid the slow growth rates and cellular senescence common after longer culturing. To test primary fibroblast sensitivity to low doses of ionizing radiation, long-term clonogenic assays were used to examine the response of four individual cultures to acute doses of 2–200 cGy. No significant decrease in clonogenic survival was found at 2 or 10 cGy for these samples while average survival of the four individual cultures at 200 cGy decreased to $24\pm11\%$ (Supplementary Figure 2, to be found online at http://informahealthcare.com/doi/abs/10.3109/09553002.2014.877175). These measurements were comparable to previous studies using fibroblasts derived from skin biopsies (Geara et al. 1992). Two individual cultures from separate individuals (p1541 and p1617) with varying sensitivities to 200 cGy (14% and 32%, respectively) were chosen for further studies.

Gene expression microarray analysis of low and high dose exposure

Two individual normal prostate fibroblast cultures (p1617 and p1541) were derived from Caucasian donors aged 54

and 66 years old. Cells either received acute doses of 10 cGy or 200 cGy at 48 cGy per min, or were chronically irradiated at 7–17 μGy per min resulting in a total dose of 1.0–2.45 cGy. Differential gene expression analysis resulted in 741 statistically significant genes (FDR < 10%) of which 396 genes had a greater than ±1.2-fold change in at least one group compared to control (Figure 1A and Supplementary Table I, to be found online at http://informahealthcare.com/doi/abs/10.3109/09553002.2014.877175). There were no statistically significant changes found between the 10 cGy exposure and control (fold-change range, −1.16 to +1.12; Figure 1B). On the contrary, the 200 cGy acutely exposed sample displayed 208 genes with 12 up- and 196 down-regulated genes (fold-change range, −2.94 to +1.41) while the chronically exposed sample displayed a more balanced expression with 294 significant genes with 109 up- and 185 down-regulated genes (fold-change range, −1.68 to +2.42). Interestingly, there were 106 genes in common between the acute 200 cGy and chronic exposure of which the majority (98 genes) were down-regulated (Figure 1C–E). It is striking to find such a large overlapping gene signature between an acute high dose that in the long-term results in significant cell death and a chronic low dose-rate exposure.

In support of our findings, the study by Zhou et al. examined the transcriptional response of three normal human fibroblasts derived from neonatal foreskins. Asynchronous cell cultures were treated with a 1.5 Gy dose that caused 40–45% inhibition in colony formation (Zhou et al. 2007). Similar to our results with 200 cGy, the majority of genes were down-regulated (134 of 150 genes). Differential expression of 128 genes at 24 h after irradiation shared 59 genes in common with our 396-gene list. Furthermore, 42 of these genes overlap with the 106 genes in common between the 200 cGy and chronic exposures (Figure 1C). This compelling similarity in fibroblasts derived from two tissue types in several individuals of dramatically different ages suggests a conserved molecular response to high acute irradiation in this gene set.

Gene and protein expression validation

Validation of gene expression results by quantitative PCR were made for six genes. Cyclin A2 (CCNA2) is related to cell cycle progression in G1/S and G2/M transitions, and cyclin B2 (CCNB2) is associated with mitosis (Gong and Ferrell 2010). Ubiquitin-conjugating enzyme E2C (UBE2C) is also involved in cell cycle progression promoting exit from

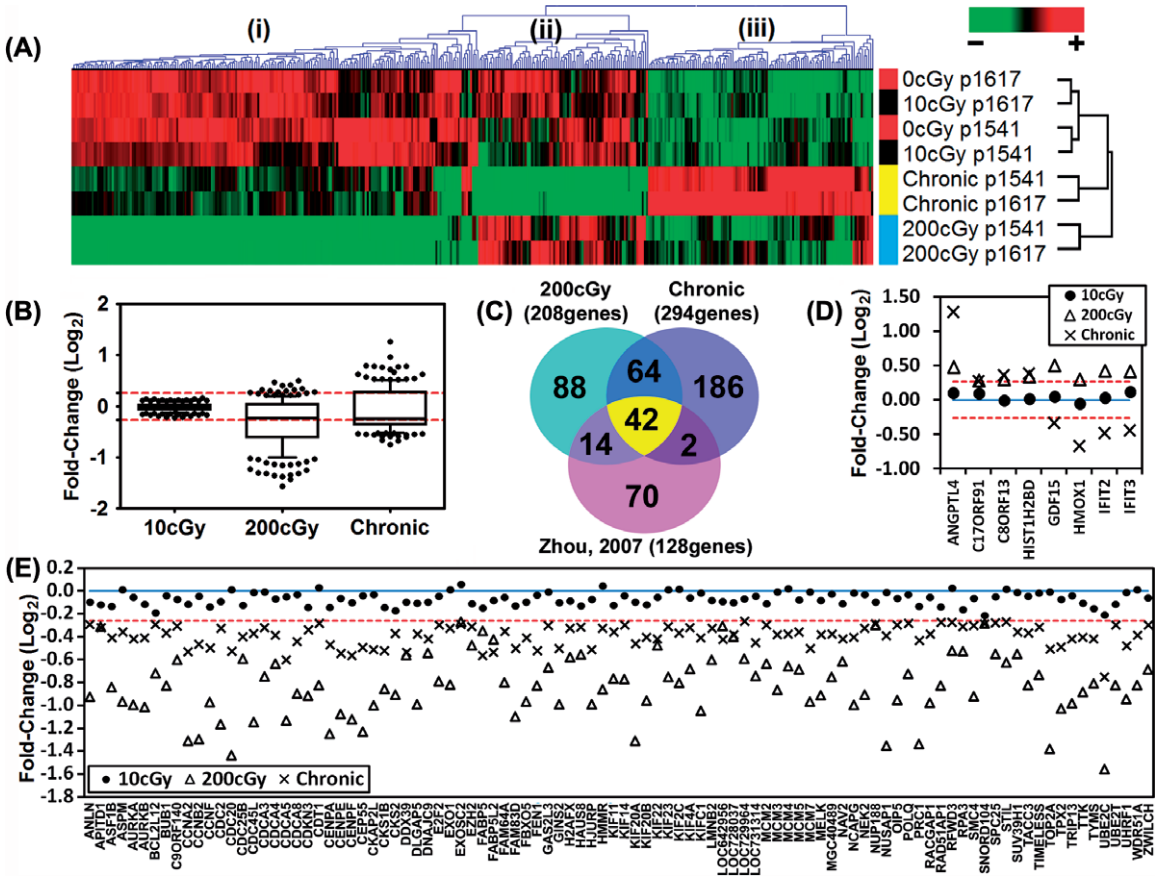

Figure 1. Microarray results for 396 significantly expressed genes. (A) Heat map of hierarchical clustering using a Euclidean algorithm for the mean signal for individual cultures p1541 or p1617. (B) Mean average signal fold-change for each radiation group compared to non-irradiated controls. Whiskers show the 5 to 95th percentile with outliers (solid circles). Red dashed lines, ± 1.2-fold change. (C) Venn diagram in this study (200 cGy or Chronic) with the 128 genes published from Zhou et al. (D) and (E) Mean average signal fold-change for p1541 and p1617 grouped by radiation exposure compared to all non-irradiated controls. (D) Eight of the 106 genes in common between 200 cGy and chronic samples either up-regulated in both or with opposing regulation. (E) Strikingly, 98 of the 106 genes in common between 200 cGy and chronic samples are down-regulated in both. Individual average signal fold-changes for p1541 and p1617 are available in Supplementary Table I, to be found online at http://informahealthcare.com/doi/abs/10.3109/09553002.2014.877175.

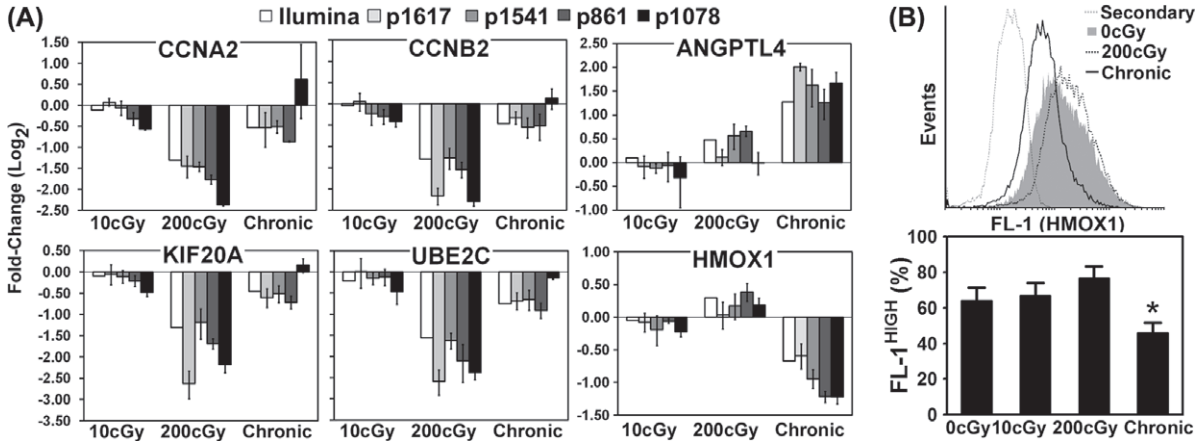

Figure 2. Validation of gene regulation. (A) Quantitative PCR comparison with microarray results for each individual sample p1617 and p1541 ($n = 4$ per culture) compared to non-irradiated control. Two additional primary fibroblast cell cultures p861 and p1078 ($n = 2$ per culture). (B) HMOX1 protein expression. Representative flow cytometry from sample p1541 at 24 hours after acute 200 cGy exposure or immediately after 24 hours of chronic accumulation to 0.9 cGy (top). Quantification of $FL1^{HIGH}$ expression levels in HMOX1 for p1541, p1617 and p1343 (bottom; $n = 2$ per culture). Error bars, SD; $^{*}p < 0.02$.

mitosis by degradation of cyclins (Townsley et al. 1997), while kinesin-like protein 20A (KIF20A) is essential for cytokinesis (Fontijn et al. 2001). Angiopoietin-like 4 (ANGPTL4) and heme oxygenase 1 (HMOX1) are both important in combating oxidative stress and promoting cellular survival (Poss and Tonegawa 1997, Zhu et al. 2011). In samples used for transcriptional analysis (p1541 and p1617), expression levels for these six genes exhibited the predicted fold-change from the Illumina microarray results (Figure 2A).

Next, the gene expression profiles of these six genes from two individual primary fibroblast cell cultures (p861 and p1078) that were not used for genome-wide gene expression profiling were also examined. Sample p861 matched the p1541 and p1617 gene expression fold-changes for all six genes. However, for p1078, the cell cycle related probes (CCNA2, CCNB2, UBE2C and KIF20A) only matched the gene expression results for the 200 cGy but did not display the similar trends for chronic irradiation seen in the three other patient samples. On the contrary, p1078 did display the same gene expression patterns for ANGPTL4 and HMOX1 genes in the chronic irradiation samples. These results suggest a largely conserved response between genetically dissimilar fibroblast cell culture samples for larger doses of ionizing radiation while the more subtle changes in chronically irradiated samples may be less conserved and more variable from person to person. However, these results require more samples from multiple individuals to make a quantitative assessment.

To validate that gene expression results in changes in protein levels, we analyzed HMOX-1 protein expression in p1541, p1617 and an additional individual sample p1343 (Figure 2B). As mentioned previously, this cytoprotective protein protects from oxidative stress and we have previously reported on the ability of high dose exposure to increase expression in this protein via the Nrf2-antioxidant response element pathway (McDonald et al. 2010). However, as predicted by the gene expression data, there was a significant decrease in HMOX-1 protein expression in the chronically irradiated samples ($p < 0.02$) and also a slight but not statically significant up-regulation after 200 cGy. During the constant oxidative stress resulting from a chronic low-dose radiation insult, it is interesting to see a decreasing expression in HMOX-1. These changes in expression uniquely dysregulated by chronic but not acute radiation exposure in addition to the striking similarities highlight the complexity of a chronic low-dose radiation exposure that requires further study.

Cell cycle suppression in response to high and chronic low dose-rate exposure

To better understand the biological significance in the altered gene expression, Ingenuity's IPA software was used. Again, the acute 10 cGy sample was not included in this analysis due to a lack of differential gene expression relative to the control. The top three canonical pathways were the same in both the acute high dose 200 cGy and low dose chronically irradiated samples as identified by the IPA library. These involved cell cycle control of chromosomal replication, mitotic division and the G2/M cell cycle checkpoint (Table I). Next, the molecular and cellular biological functions predicted to be significantly activated or inhibited were compared (Table II). Again, there was substantial overlap between the acute 200 cGy and chronic dose samples in functional categories involving an inhibition in cell cycle regulation of M phase and cytokinesis, cellular assembly and organization related to chromosomal alignment and movement, cellular proliferation and DNA replication, recombination and repair. This is in contrast to a predicted inhibition of cell survival and viability only in the 200 cGy sample that is not represented in the chronic. A unique functional category was activated in the chronic sample involving blood vessel development and angiogenesis. These results again point to a functional similarity in the molecular response to the acute 200 cGy dose and the chronic irradiation.

Finally, the predicted upstream transcriptional regulators were examined (Supplementary Table II, to be found online at http://informahealthcare.com/doi/abs/10.3109/09553002.2014.877175). A number of targets were again common

Table I. Top three canonical pathways using IPA with significantly expressed genes.

IPA canonical pathways	*p*-value*		Genes in dataset		
	200 cGy	Chronic	200 cGy	Chronic	Shared
Cell cycle control of chromosomal replication	7.94E-15	4.17E-10	MCM6, RPA1, CDK2	ORC5	MCM5, MCM3, CDC45, RPA3, MCM2, CDT1, MCM4, MCM7
Mitotic roles of polo-like kinase	5.01E-12	7.24E-06	CDC25C, PLK4, PLK1, CCNB1	–	CDC25B, KIF23, CDC20, PRC1, CCNB2, FBXO5, CDK1, KIF11
Cell cycle: G2/M DNA damage checkpoint regulation	7.94E-11	7.94E-06	CDC25C, PLK1, BRCA1, CDK1	GADD45A	CDC25B, CKS2, CKS1B, TOP2A, CCNB2, CDK1

*Significant association between the experimental datasets and IPA canonical pathways were measured in two ways: (i) The percentage of molecules that map to the canonical pathway from the total number of molecules in the pathway shown at the top of each bar, or (ii) a Fischer's exact test to calculate a *p*-value for determining the probability of that association between the genes in the dataset and the canonical pathway is explained by chance alone.

between the two experimental conditions. The strongest predictions were increases in TP53, RB1 and CDKN2A as well as a number of similar family members E2F1 and E2F1 or FOXM1 and FOXO1. It is of note that Zhou et al. also predicted the enrichment of transcription factor binding sites corresponding to E2F and NF-Y (Zhou et al. 2007).

Gene Set Enrichment Analysis (GSEA)

GSEA was used as an independent method to further characterize biological pathway significance (Subramanian et al. 2005). This analysis is less reliant on the conventional statistical analysis testing single expression levels and uses predefined sets of genes to find more coordinated changes in a molecular phenotype. Results found a number of gene sets with a FDR q-value less than 0.05 were significantly up- or down-regulated (Supplementary Tables III and IV, to be found online at http://informahealthcare.com/doi/abs/10.3109/09553002.2014.877175; number of gene sets: 10 cGy, 0 up and 7 down; 200 cGy, 1 up and 155 down; chronic, 44 up and 123 down). Similar to the gene-based overlap in the previous analysis, there were 83 down-regulated gene sets in common between the 200 cGy and chronic samples and seven down-regulated in common between all conditions. To better visualize and interpret the results, gene-sets were converted into nodes and network clusters were created. The resulting enrichment map of 200 cGy and chronic samples again displayed the similar down-regulation of categories related to mitotic cell cycle regulation, chromosome assembly, and microtubule/cytoskeleton organization (Figure 3). Also found was a DNA damage response signature in the chronic as well as 200 cGy samples. Finally, this analysis uncovered additional biological pathways for further analysis, such as RNA processing, that were down-regulated in the chronic and 200 cGy samples

Table II. IPA functional analysis identified the molecular and cellular biological functions that were most significant to the datasets.

IPA functional category	Functions annotation	Activation z-score*		*p*-value**		Number of genes in dataset		
		200cGy	Chronic	200cGy	Chronic	200cGy	Chronic	Both
Cancer	incidence of tumor	2.29	–	8.80E-04	–	11	–	0
	Lymphohematopoietic cancer	–	2.02	–	7.45E-03	–	27	0
	lung tumor	–	2.04	–	3.76E-07	–	31	0
Cardiovascular system devel. & function	development of blood vessel	–	2.54	–	5.62E-03	–	25	0
	development of cardiovascular system	–	2.58	–	9.92E-03	–	30	0
	angiogenesis	–	2.69	–	4.40E-03	–	22	0
Cell cycle	M phase	**−2.77**	**−2.05**	**4.00E-26**	**2.98E-16**	**32**	**27**	**24**
	cytokinesis	**−2.75**	**−2.27**	**3.94E-15**	**1.87E-11**	**19**	**18**	**14**
	cytokinesis of tumor cell lines	**−2.14**	**−2.14**	**3.90E-09**	**1.03E-06**	**9**	**8**	**8**
	delay in mitosis of tumor cell lines	**2.18**	**2.40**	**5.83E-17**	**6.60E-08**	**10**	**6**	**6**
	M phase of tumor cell lines	**−2.01**	**−2.16**	**1.69E-13**	**4.02E-07**	**14**	**10**	**10**
	cycling of centrosome	−2.43	–	3.31E-10	–	10	–	0
	S phase	−2.07	–	3.87E-14	–	24	–	0
	delay in mitosis of cervical cancer cell lines	2.20	–	1.20E-11	–	7	–	0
Cell death and survival	cell survival	−2.50	–	1.00E-02	–	27	–	0
	cell viability myeloma cell lines	−2.45	–	3.67E-04	–	6	–	0
	cell viability of tumor cell lines	−2.44	–	2.61E-03	–	18	–	0
Cellular assembly and organization	chromosomal congression of chromosomes	**−2.24**	**−2.24**	**3.23E-08**	**1.74E-07**	**5**	**5**	**5**
	alignment of chromosomes	**−2.14**	**−2.36**	**1.84E-21**	**6.46E-14**	**14**	**11**	**11**
	association of chromosome components	−2.21	–	4.63E-06	–	5	–	0
Cellular growth and proliferation	proliferation of cells	**−5.48**	**−2.44**	**4.36E-08**	**3.15E-10**	**81**	**112**	**53**
	proliferation of tumor cell lines	−3.62	–	9.62E-06	–	38	–	0
Cellular movement	cytokinesis	**−2.75**	**−2.27**	**3.94E-15**	**1.87E-11**	**19**	**18**	**15**
	cytokinesis of tumor cell lines	**−2.14**	**−2.14**	**3.90E-09**	**1.03E-06**	**9**	**8**	**8**
DNA replication, recombination and repair	repair of DNA	**−2.67**	**−2.02**	**3.47E-11**	**1.44E-03**	**20**	**12**	**9**
	metabolism of DNA	−2.45	–	2.99E-11	–	26	–	0
	DNA damage	2.02	–	1.50E-06	–	11	–	0
Embryonic devel.	size of embryo	**−3.39**	–	1.63E-03	–	12	–	0

*Functional categories with a significant z-score (>2.0, activated or below <-2.0, inhibited) for sample are shown. Bold indicates the pathway is significantly regulated in both samples. **Right-tailed Fisher's exact test was used to calculate a *p*-value determining the probability that each biological function assigned to the dataset is due to chance alone.

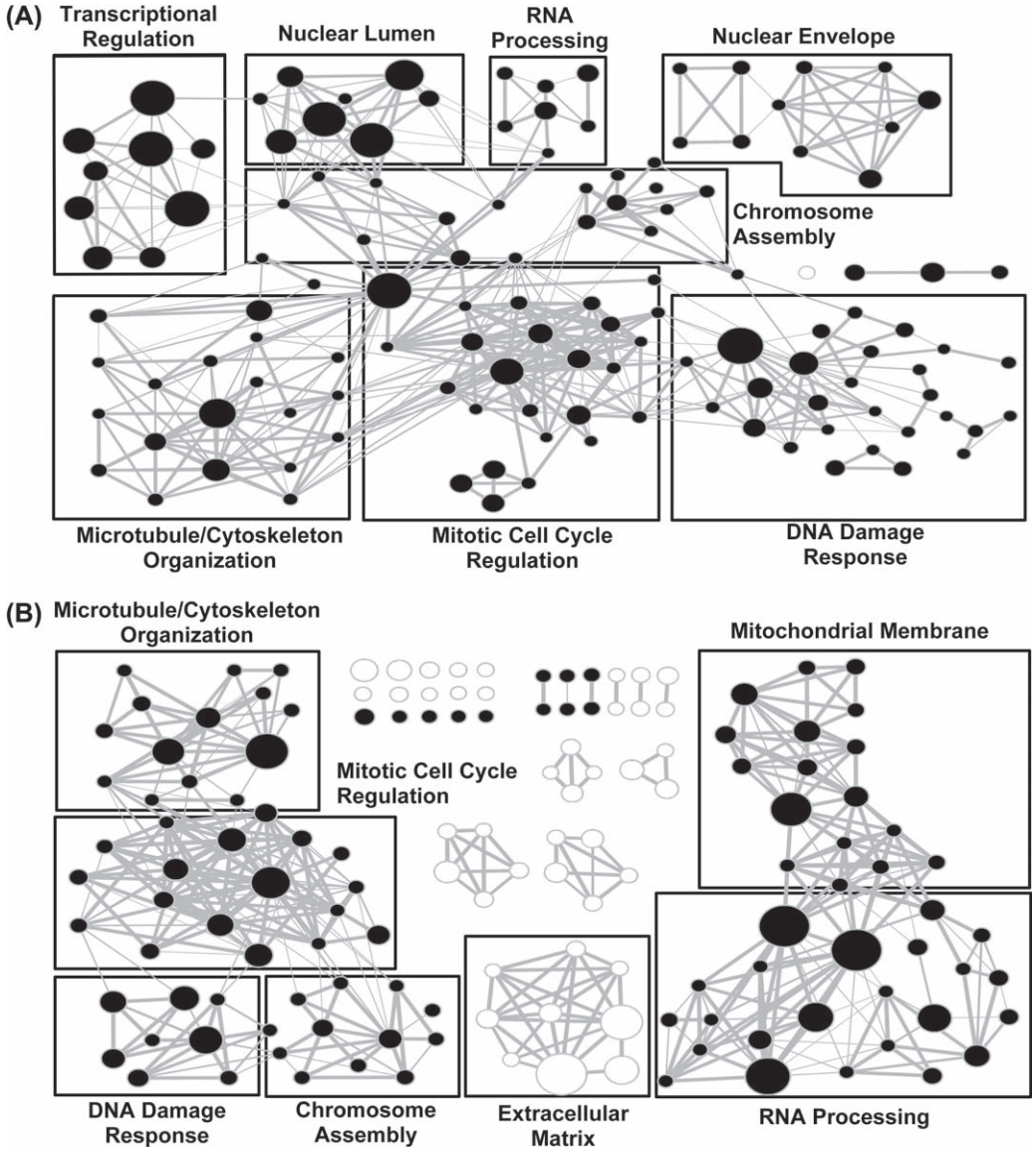

Figure 3. Enrichment map network of statistically significant GSEA gene sets. Nodes represent gene sets with an FDR q-value less than 5% up-regulated (white) or down-regulated (black) for (A) 200 cGy or (B) chronic samples relative to non-irradiated control. Node size is reflective of the number of genes in the set and edge thickness is proportional to the overlap between gene sets.

as well as the unique decrease in mitochondrial related function and increase in extracellular matrix elements found only in the chronic sample.

Chronic low dose (< 1 cGy) and acute 200 cGy exposures decrease proliferation and fraction of cells in mitosis

To test if the gene expression results had a functional impact on cell growth, cell number was measured at 24 h in the normal prostate epithelial cell line RWPE-1. The 24-h chronic low dose-rate exposure resulted in a marked decrease in proliferation that was similar to an acute 200 cGy exposure (Figure 4A). There were no measurable effects with an acute 10 cGy exposure. Finally, the phospho-S10 specific antibody for Histone-H3, a marker for cells in mitosis, was independently quantified by flow cytometry in the RWPE-1 cells at 1 or 24 h (Figure 4B). There was a significant decrease in M-phase cells at 1 hour after an acute 200 cGy or 10 cGy exposure, but not for a chronic 1-h exposure. However, at 24 h, the chronic dose did result in a significant decrease in mitotic cells whereas suppression in the 10 cGy and 200 cGy exposures had subsided.

Discussion

To better understand the potential impact of low dose and low dose-rate, we examined genome-wide gene expression profiling after 24 h of an acute 10 cGy low dose or an acute 200 cGy high dose of ionizing radiation compared to immediately after 24 h of chronic low dose-rate irradiation resulting in a total low dose exposure of 1.0–2.45 cGy. There were a total of 396 statistically significant genes differentially expressed by the high and the chronic doses compared to non-irradiated controls, but no significant differences were found between the acute low dose and the controls. Hierarchical clustering broadly demonstrated three unique expression patterns: (i) Similar suppression of

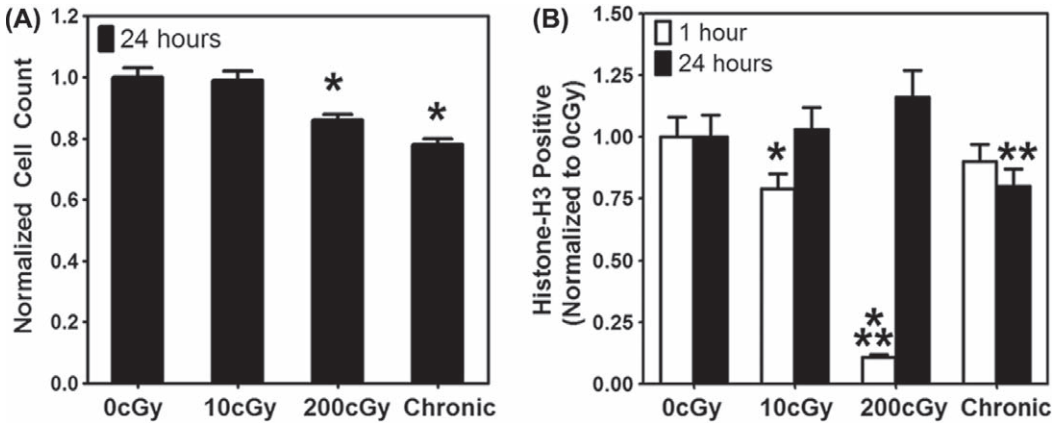

Figure 4. Suppressed proliferation in the RWPE-1 normal prostate epithelial cell line. (A) Cell count at 24 hours after acute 10 cGy or 200 cGy or immediately after a chronic 24-hour low dose-rate exposure ($n = 3$). (B) Phospho-Histone H3 analysis by flow cytometry was used to detect cells in mitosis at 1 or 24 hours ($n = 3$). Error bars, SEM; $^{*}p < 0.05$, $^{**}p < 0.02$, $^{***}p < 0.001$.

gene expression in chronic low and acute high dose, (ii) suppression in chronic only, or (iii) up-regulation in chronic only. Examination of the first pattern showed a common down-regulation in 98 genes that included cell cycle-related gene families such as cyclins CCNA2, CCNB2 and CCNF; cell division cycle genes CDC2, CDC20, CDC25B, and CDC45-like; cell division cycle associated genes CDCA3, CDCA4, CDCA5, and CDCA8; and cylin-dependent kinase inhibitor CDKN3. Related to these families were genes in the minichromosome maintenance complex components MCM2, MCM3, MCM4, MCM5 and MCM7 that together with MCM6 make a key element of the pre-replication complex. Indeed, pathway analysis predicted activation of upstream regulators TP53, RB1 and CDKN2A, all of which inhibit cell cycle. Many more genes in this common list play important roles in spindle formation and chromosomal condensation, alignment and movement. Both IPA and GSEA showed inhibition of gene sets in both samples related to mitosis, G2/M cell cycle transition, microtubule formation, and chromosomal movement and organization. These similarities in comparison are striking given that the total dose received by the chronic sample was 100- to 200-fold lower than the acute 200 cGy sample. Furthermore, the chronic sample dose was 5- to 10-fold lower than the acute 10 cGy sample, which failed to show a significant response after irradiation.

Unlike the acute high and chronic low doses, an acute 10 cGy low dose was not statistically different from the non-irradiated samples in gene expression studies; however, GSEA analysis did find seven gene sets related to mitosis that were significantly down-regulated as well as in common with 200 cGy and chronic irradiation. The ability to induce DNA damage after several milligrays of exposure has been previously demonstrated (Rothkamm and Löbrich 2003, Suzuki et al. 2006), but long-term clonogenic survival assays here show no decrease in cell death resulting from this dose. Thus, it may be concluded that the biological damage of a low acute dose is repaired and has no lasting effect on the cells, highlighting the importance of a better understanding of acute versus chronically accumulated low dose exposures. There was a measurable decrease in mitotic cells at 1 h after the 10 cGy exposure and a substantial decrease for the acute 200 cGy exposure. Interestingly, only the 200 cGy and chronic exposures were able to decrease cell proliferation over a 24-h period. In a recent study of high dose single, fractionated or continuous low dose-rate exposure in colorectal cancer cells, it was found chronic exposure was more biological ly effective in the induction of apoptosis and cell cycle arrest than acute high dose-rates (Wang et al. 2013). The time-dependent manner in which the chronic low dose exposure is delivered and accumulates results in an unintuitive measurement and possible clues to adaptation in the molecular response.

This study is unlike the previous low-dose transcriptional studies that have largely focused on short times after acute dosing. Though several long-term continuous low dose-rate exposures in vitro as well as in vivo have been examined, these generally result in the accumulation of a large total dose (>10 cGy) (Amundson et al. 1999, 2000, 2003, Goldberg et al. 2004, 2006, Coleman et al. 2005, Ding et al. 2005, Lanza et al. 2005, Fachin et al. 2007, Long et al. 2007, Jin et al. 2008, Wyrobek et al. 2011). At higher doses of ionizing radiation, it is well accepted that fractionation or decreased dose-rates of an acute exposure reduces the biological effectiveness and is interpreted by increased repair of cellular damage. This has led to the dose and dose-rate effectiveness reduction factor (DDREF) which attempts to compensate for the reduced effects in biological endpoints (Valentin 2005, NRC 2006). Given the results of our findings, a chronic dose-rate may result in an increased differential gene expression that has been largely missed by focusing on acute exposures or the accumulation of large doses at low dose-rates.

Several long-term studies with chronic low dose irradiation similar to the dose-rate used here have found significant changes on measurable biological endpoints (Sorensen et al. 2000, Olipitz et al. 2012). Uehara et al. also studied the long-term effects of three low dose-rates of approximately 2, 0.1 or 0.005 cGy per day for a total of 400 days in C57BL/6J mice for a total dose of 800, 40 or 2 cGy, respectively (Uehara et al. 2010). Gene expression microarray analysis of the liver revealed only three common

genes (Usp2, Dbp, and S3gal5) modulated between all individual animals and dose-rates while 20 and 11 genes were more than 1.5-fold change at approximately 2 and 0.1 cGy per day, respectively. The results indicated very few genes were regulated by low dose-rate irradiation in the long term. However, like many other studies, the long time scale used for exposure and/or high accumulated doses may be missing a window of opportunity to observe early cellular responses when the accumulated dose is still low.

The worldwide annual effective dose of chronically occurring natural background is estimated to be 2.4 mSv (United Nations Scientific Committee on the Effects of Atomic Radiation 2010), but additional acute and chronic doses may be accumulated through medical, occupational or accidental exposures. The infamous radiation accident at Chernobyl caused high levels of radiation exposures in humans and widespread and long-term contamination of the surrounding area (Saenko et al. 2011). The more recent Fukushima Daiichi Nuclear Power Plant accident resulted in much lower exposures in power plant workers, but also resulted in public evacuations of large areas of land for over 320,000 people to minimize risk posed by ionizing radiation exposure (Ohnishi 2012, Tsubokura et al. 2012). As found in this study, there are striking similarities, as well as subtle differences between gene regulations following an acute high dose and a low dose at a low dose-rate, that need further investigation to understand the physiological implications. Future studies of the temporal dynamics of response to chronic low dose-rate exposure, in contrast to acute low-dose exposure, will continue to reveal biological pathways underlying the impact of ionizing radiation for assessing risk to real-world exposures in human populations.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Figures 1, 2 and Tables I–IV.