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Comprehensive analysis of time- and dose-dependent patterns of gene expression in a human mesenchymal stem cell line exposed to low-dose ionizing radiation

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Abstract. We focused on the transcriptional responses induced by low and very low doses of ionizing radiation with time effect. Regardless of their importance only a few limited studies have been done. Here we applied a large-scale gene transcript profile to elucidate the genes and biological pathways. Immortalized human mesenchymal stem cells were irradiated with 0.01, 0.05, 0.2 and 1 Gy of gamma radiation and total RNA was extracted from each cell line at 1, 4, 12 and 48 h after exposure. The essential transcriptional responses were identified according to dose and time. A total of 6,016 genes showed altered expression patterns at more than one time point or dose level among the investigated 10,800 genes. Genes that showed dose-dependent expression responses were involved in signal transduction, regulation of transcription, proteolysis, peptidolysis and metabolism. Those that showed time-dependent responses were divided into two distinct groups: the up-and-down group was associated with 'cellular defense mechanisms' such as apoptosis, cell adhesion, stress response and immune response and the down-and-up group with 'fundamental cellular processes' such as DNA replication, mitosis, RNA splicing, DNA repair and translation initiation. Genes showing both dose-and time-dependent responses exhibited a mixture of both features. A highly non-linear relationship between the IR dose and the transcriptional relative response was obtained from the dose-dependent group. The time-dependent group also exhibited a non-linear relationship as the complex effect group did. Some of the early-reactive-phase (1-4 h) genes showed a differential expression response to 0.01, 0.05 and 0.2 Gy but were unresponsive to 1 Gy. Some of the late-recovery-phase (12-48 h) genes showed a differential expression to 1 Gy but were relatively unresponsive to other doses. We further characterized the gene expression patterns that could be implicated in the molecular mechanism of the cellular responses to low and very low-dose irradiation.

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Introduction

An accurate estimation of the possible health risks associated with low doses of genotoxic components in the environment is an important challenge for public health sciences (1). Current models for estimating the risk of low-dose ionizing radiation (IR) commonly involve the linear non-threshold (LNT) model. The LNT model extrapolates empirical linear fits of human high-dose radiation data collected from survivors of atomic-bomb explosions (2). In 1959, the International Commission on Radiological Protection (3) formulated regulations to protect occupationally exposed workers and the public from radiological hazards (4). Extrapolation using the LNT model may greatly overestimate the health risks of low-dose radiation and have a negative effect on public health by discouraging physicians from performing potentially useful radiological examinations such as CT scans (5). Luckey concluded that 'minute doses of IR benefited animal growth and development, fecundity, health and longevity' (6). Furthermore, debates on

the adaptive response, the bystander effect and low-dose hypersensitivity have challenged the validity of the LNT model (7).

Cellular responses to IR are known to rely on the dynamic orchestration of complex regulatory pathways including cell cycle arrest, apoptosis, DNA damage and nucleotide excision repair. There is a growing demand for the identification of radiation-inducible genes. DNA microarray technology has made it possible to measure changes in the expression levels of thousands of genes and to study multiple cellular processes simultaneously and is, therefore, a useful tool for the study of radiation-induced cellular responses (8-10).

Previous microarray studies focused on gene expression changes induced either by a large dose of IR resulting in a high degree of cytotoxicity (11-17) or by a low dose of IR resulting in transcriptional alterations (18-23). There is an increasing concern about the hazardous effects caused by a 'very-low' (<0.2 Gy) dose of IR. While there are several large-scale gene expression studies for the differential effect between the high (>2 Gy) and low (<2 Gy) dose of IR (24-27), there are fewer studies concerning the low and 'very-low' dose effects on gene expression. Moreover, most of the low or 'very-low' dose-based IR studies were performed at a single fixed time point following exposure without any consideration of the time-dependent experimental effects. To the best of our knowledge, there has been no systematic study of the differential time and dose effects of a 'very-low' dose of IR (<0.5 Gy) (28) using large scale gene expression profiling. Investigation of the differential time and dose effects of a 'very-low' dose of IR using a time-course based experimental design may help to clarify the LNT hypothesis.

In this study, we obtained global gene expression profiles of the B10 human mesenchymal stem cell (MSC) line using oligonucleotide DNA microarrays with a factorial design set at 1, 4, 12 and 48 h after exposure to 0.01, 0.05, 0.2 and 1 Gy of gamma radiation. Each experiment was triplicated to increase the reliability of the analysis. We identified and characterized genes that were induced or repressed following irradiation after different periods of time across different IR doses. Distinct patterns of gene expression were present in the early and late phase data set.

Materials and methods

Cell culture and exposure to IR. The B10 cell line used in this study is an immortalized, human bone marrow MSC line generated from human embryonic bone marrow tissues and immortalized using retroviral vectors encoding *v-myc* or the *telomerase* gene (29). B10 cells were grown in low glucose DMEM supplemented with 5% FBS and penicillin/streptomycin at 37˚C in a humidified incubator with a 5% CO_2 atmosphere. Cells were irradiated in triplicate at 0.01, 0.05, 0.2 or 1 Gy in a ^{137}CS irradiator (CIS Bio-international, dose rate 0.79 Gy/min) and harvested at 1, 4, 12 or 48 h after IR exposure.

Labeling and hybridization for microarray analysis. The isolation of the total RNA from cells was carried out using a Trizol reagent (Invitrogen Inc., USA). Labeling of the total RNA was performed using the 3DNA submicro expression array detection kit (Genisphere, USA) according to the manufacture's protocol. Briefly, total RNA was reverse transcribed using reverse transcription primers incorporating either Cy3- or Cy5-specific 3DNA capture sequences. The synthesized cDNAs were then fluorescently labeled using Cy3-3DNA or Cy5-3DNA depending on the complementary capture sequence. Changes in gene expression were assessed using the Mac Array-II Oligo-Human 10K (Macrogen, Korea) microarray chip with 10,800 probes. The hybridization of targets to specific probes was achieved by incubation in 2X formamide-based buffer (50% formamide, 8X SSC, 1% SDS, 4X Denhardt's solution) at 70˚C for 16 h. After hybridization, the arrays were washed with 2X SSC and 0.2% SDS at 40˚C for 15 min, followed by a wash with 2X SSC and another with 0.2X SSC at room temperature for 15 min each. Images were analyzed using the GenePix4000B (Axon Instruments Inc., USA). The software automatically generated flags using the default settings for poor quality and missing spots and the assay was performed in triplicate for consistency.

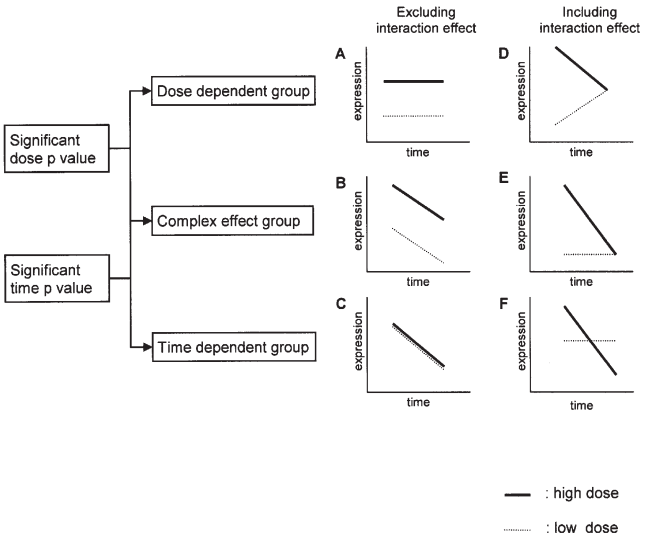


Figure 1. A schematic diagram of the analysis for the selection and assignment of genes to dose- and/or time-dependent expression groups. A multi-factorial analysis was used to divide these genes into six categories. (A) The main effect of the dose is significant. (B) The main effects of both dose and time are significant. (C) The main effect of time is significant. (D) The main effect of the dose is significant but the main effect of time is not significant. The effect of the dose depends on time. (E) The main effects of both dose and time are significant. Both of the effects depend on each other. (F) The main effect of time is significant but the main effect of the dose is not significant. The effect of time depends on the dose.

Data analysis. We adhered to the MIAME (minimum information about a microarray experiment) standard (30) for our data analysis and storage. The raw data were stored and are available at the Xperanto database (31). Data were included for further analysis if the probe signal intensity was reliably detected (scored as 'present' by the statistical expression algorithm) in at least one of the samples within a dose series of IR. For each probe, the array signal intensities were normalized using the variance stabilizing normalization method introduced by Huber *et al* (32) with the vsn package in the Bioconductor (33). After performing intensity-dependent global Lowess regression, spatial and intensity-dependent effects were managed by pin group Lowess normalization.

Number of significant IR-responsive genes in dose-time-dependent groups at different P-value cutoffs.

P-value	Dose-dependent group	Time-dependent group	Complex-effect group
0.05	214	4,966	836
1e-2	34	4,918	95
1e-6	-	2,457	-

A two-way analysis of variance (ANOVA) was applied to determine the time and dose effects and the differentially expressed sets of genes (34,35) (Fig. 1). Statistical significance was adjusted by the Benjamini-Hochberg multiple-testing correction with false discovery rate (FDR) (36). Hierarchical clustering and self-organizing maps (SOM) (37) were performed using the Avadis 4.3 software package (Strand Life Sciences, India). The biological pathway and ontology-based analyses were performed by using an ArrayXPath (http://www.snubi.org/software/ArrayXPath) (38,39). Post-hoc, two-sample Student's t-tests were also performed. This statistical analysis was performed using an R statistical package (32) and default parameters were used when unspecified. Gene sets showing significant dose and/or time effects were separated for further analysis into groups classified as time-dependent, dose-dependent or complex-effect (i.e., both time- and dose-dependent) groups (Fig. 1). These groups were then categorized into 'with' or 'without' interaction groups according to the significance of the interaction terms using a saturated linear model ('mean' plus 'dose effect' plus 'time effect' plus 'interaction').

Results

Global gene expression profiles. Table I shows the number of significant genes categorized into dose-dependent, time-dependent and complex-effect groups according to two-way ANOVA with multiple-test correction by FDR. Most genes showed a time-dependent rather than dose-dependent response.

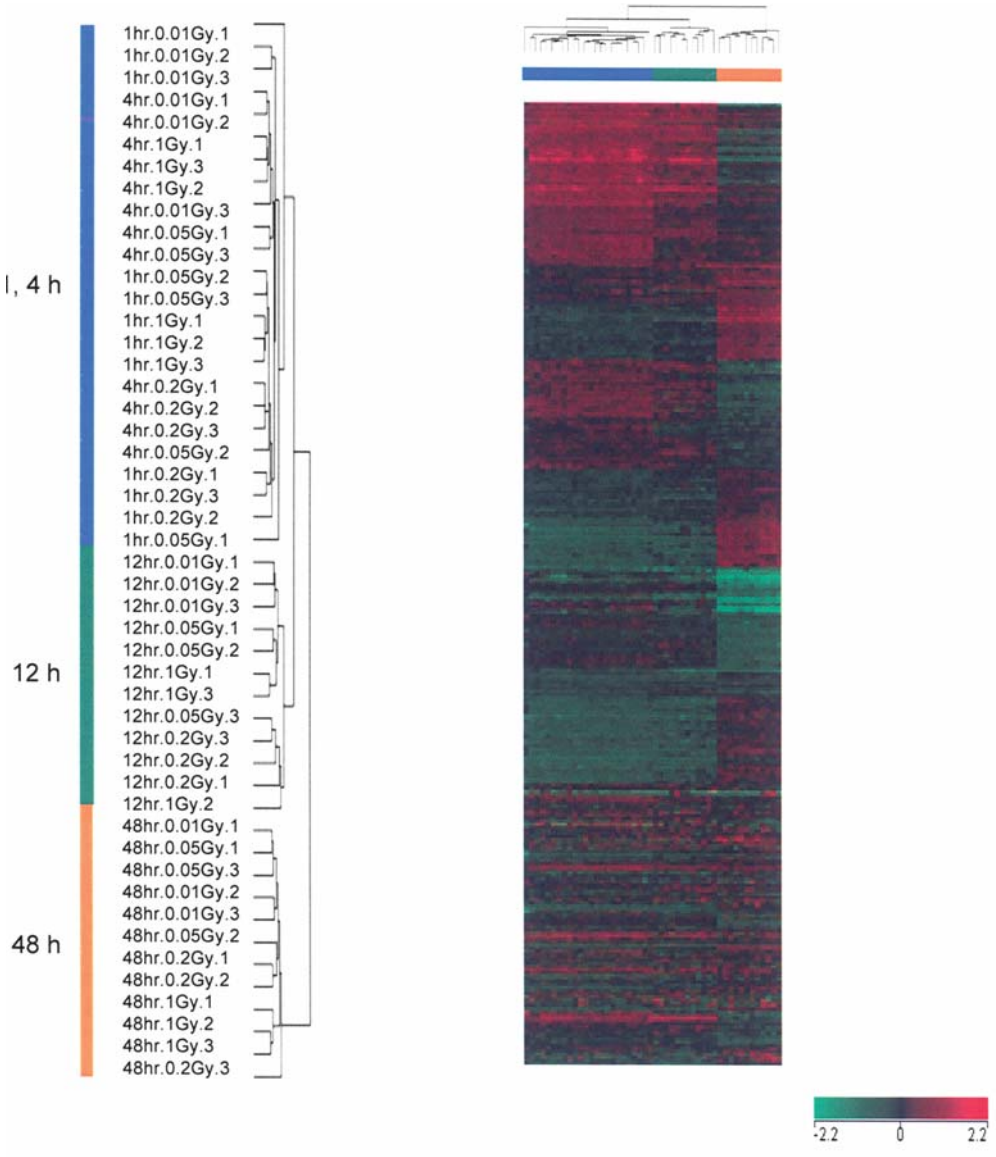
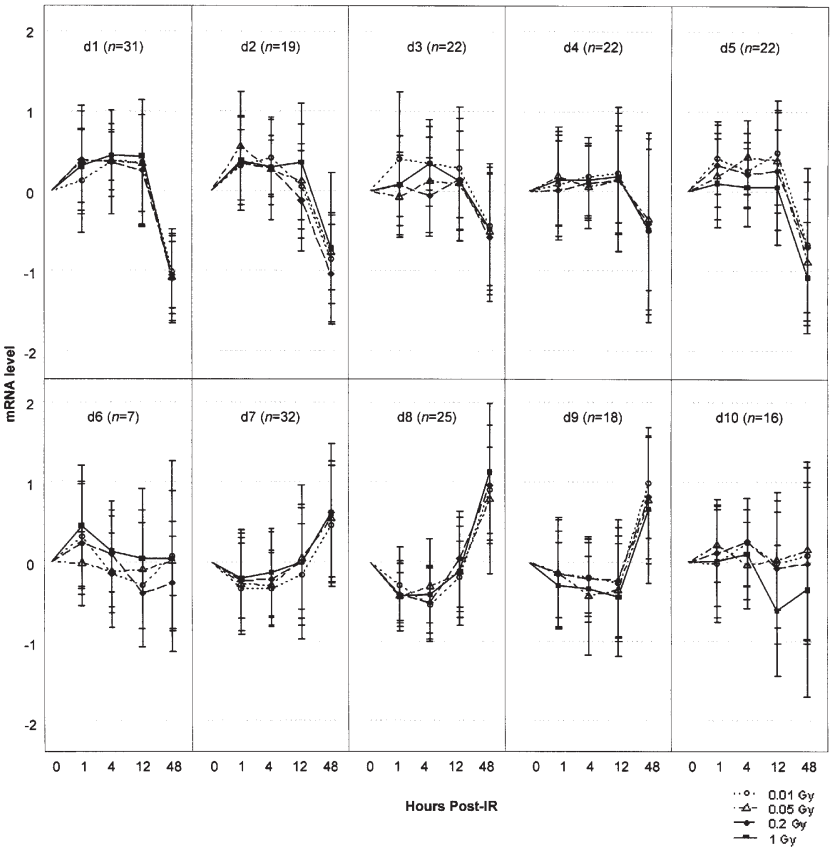


Figure 2. Hierarchical clustering analysis of the 6,016 genes by two-way ANOVA (P<0.05). The dendrogram at the top of the figure indicates the relationships between the different conditions as determined by the Avadis 4.3 software package (Strand Life Sciences, India). The red or green colors indicate a higher or lower expression, respectively, relative to the mean signal intensity. Each row represents a gene and each column a sample.

A



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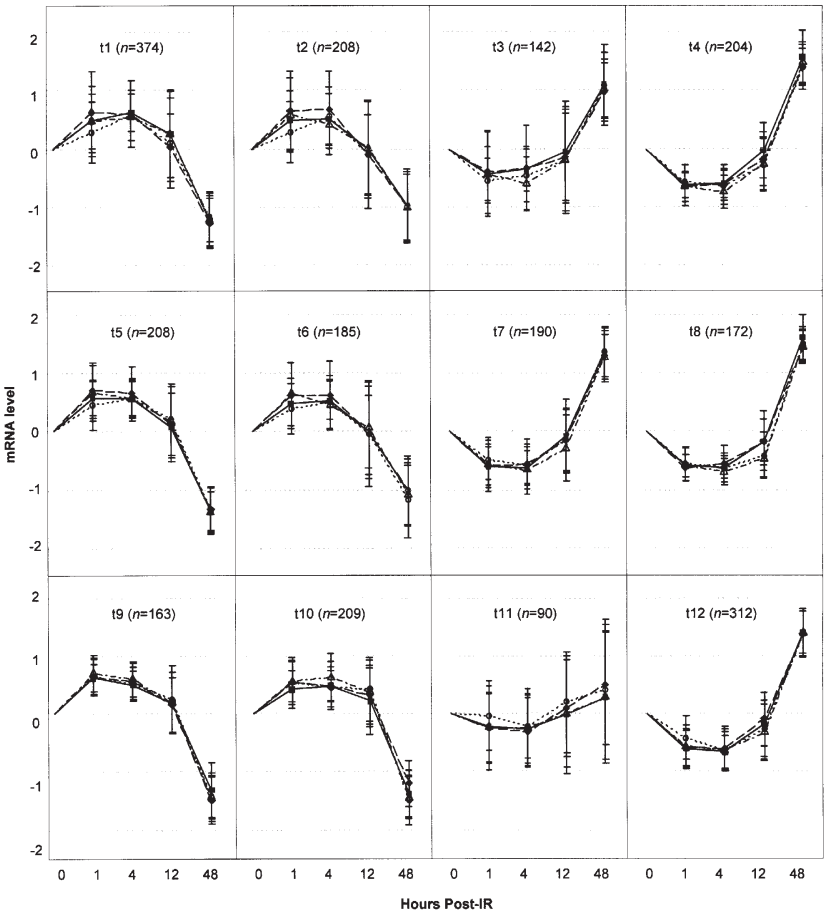

Hierarchical clustering of the genes showing a significant alteration by two-way ANOVA (P<0.05) revealed 3 specific time groups associated with the response (Fig. 2). In Fig. 2, the right group consists of the samples at 1 and 4, the middle group at 12 and the left group at 48 h after exposure to IR. Within each time group, the dose effect remained persistent, while, overall, time effect appeared to be larger than the dose effect. Based on this result, we classified the time course into

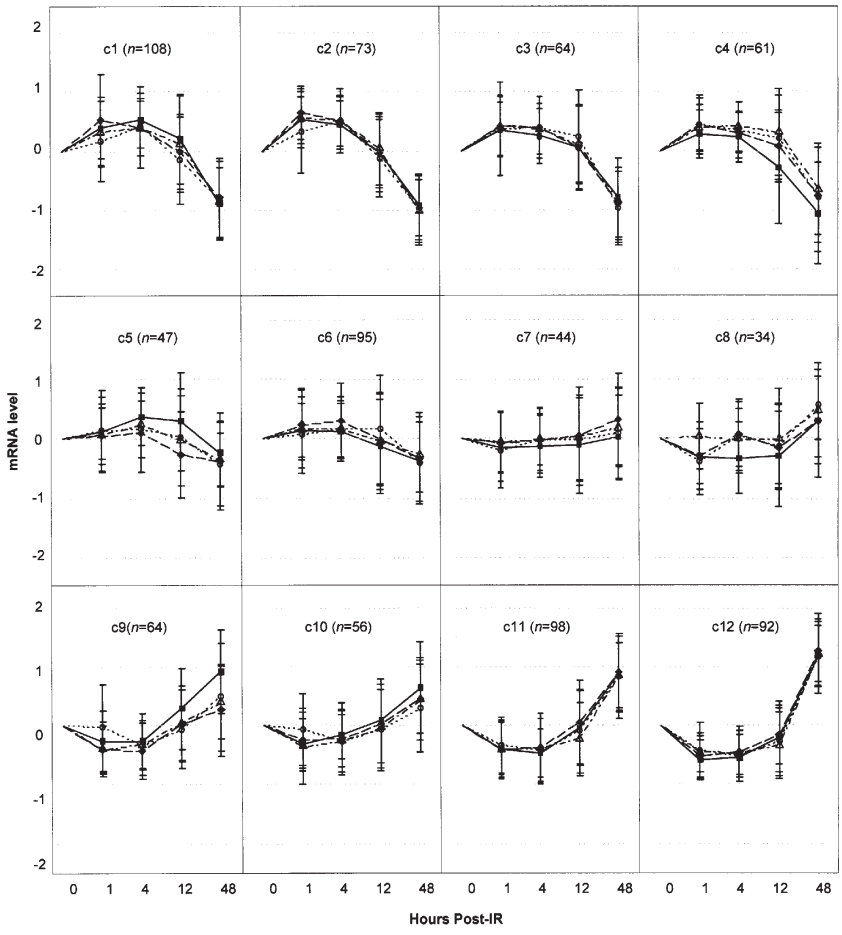

Figure 3. Gene expression patterns for dose- and/or time- dependent IR-responsive genes. Clusters were generated using self-organizing maps (Avadis 4.3). (A) Genes in the dose-dependent group were clustered into 10 clusters. (B) Genes in the time-dependent group were clustered into 12 clusters. (C) Genes in the complex-effect group were clustered into 12 clusters. The results represent the mean ± standard error.

two phases consisting of the early reactive phase (ERP, i.e., 1 and 4 h) and the late recovery phase (LRP, i.e., 12 and 48 h).

Dose-dependent gene expression patterns. Dose-dependent genes were grouped into 10 clusters by SOM (Fig. 3A) and annotated by the gene ontology (GO) terms and biological pathways. Dose-dependent genes were mainly associated with the signal transduction pathway, the regulation of transcription, proteolysis, peptidolysis and metabolism (Table II). Cluster d3 contained 22 genes that showed early up-regulation to 0.01 Gy but little response to other IR doses until 12 h. The observed response was down-regulated at 48 h after exposure (Fig. 3A). The genes in cluster d3 were associated with carbohydrate metabolism, cell adhesion, cell proliferation, DNA-dependent regulation of transcription, G-protein coupled protein signaling pathway and other intracellular signaling cascades (Table II). Cluster d10 contained 16 genes showing down-regulation after 12 h of exposure to 1 Gy but no response to other doses. These genes were functionally associated with phosphorylation, control of skeletal myogenesis and signal-dependent regulation of myogenesis by corepressor MITR.

Time-dependent gene expression patterns. Time-dependent genes were grouped into 12 clusters by SOM (Fig. 3B). Clusters were clearly divided into two distinct groups. While the clusters on the left side (i.e., clusters t1, t2, t5, t6, t9 and t10) showed up-regulation in the early phase and down-regulation in the late phase, clusters on the right side (i.e., clusters t3, t4, t7, t8, t11 and t12) showed down-regulation in the early phase and then up-regulation in the late phase. While the 'up-and-down' clusters were significantly associated with apoptosis, cell adhesion, stress response, immune response and inflammation response, the 'down-and-up' clusters were associated with DNA replication, mitosis, RNA splicing, DNA repair and translation initiation following functional enrichment analysis. It is possible that low-dose irradiation in the early phase may cause a temporary halt of fundamental cellular processes such as transcription and translation, along with an activation of cellular defense mechanisms associated with stress response. After 12 h of exposure, it is likely that cellular activities were resumed and defense systems were suppressed. For example, cluster t1 showed an association with activated cellular defense mechanisms in the early phase which included the TNF/Stress-related signaling pathway, the IL-5 signaling pathway, eosinophils in the chemokine network of allergy responses, the first multivalent nuclear factor signaling pathway and skeletal muscle hypertrophy regulated via the AKT/mTOR pathway.

Table II. Gene ontology and biological pathway-based enrichment analysis for dose-dependent group.

Cluster	Gene	Biological pathway	GO biological process
d1	GNAQ	Activation of PKC through G-protein coupled receptor	Cell adhesion
(n=31)	IL12A	IL-18 signaling pathway	Cell-cell signaling
	VIPR2	Neuropeptides VIP and PACAP inhibit the apoptosis of activated T cells	G-protein coupled receptor protein signaling pathway
		PKC-catalyzed phosphorylation of inhibitory phosphoprotein of myosin phosphatase	Intracellular signaling cascade
			Protein amino acid phosphorylation
			Proteolysis and peptidolysis
			DNA-dependent regulation of transcription
			Signal transduction
			Transport
d2	-		-
(n=19)			
d3	PAK6	Agrin in postsynaptic differentiation	Carbohydrate metabolism
(n=22)	STAT2	IFN-α signaling pathway	Cell adhesion
			Cell proliferation
			G-protein coupled receptor protein signaling pathway
			Intracellular signaling cascade
			DNA-dependent regulation of transcription
d4	MYD88	Inactivation of Gsk3 by AKT causes accumulation of ß-catenin in alveolar macrophages	Cell differentiation
(n=22)	NOTCH1	NF-κB activation by nontypeable *Hemophilus influenzae*	Cell growth and/or maintenance
		NF-κB signaling pathway	Cell surface receptor linked signal transduction
		Presenilin action in Notch and Wnt signaling	Signal transduction
		Proteolysis and signaling pathway of Notch	
		Segmentation clock	
		Signal transduction through IL-1R	
		Toll-like receptor pathway	
d5	CASP4	Caspase cascade in apoptosis	Cell adhesion
(n=22)	L1CAM	Eph kinases and ephrins support platelet aggregation	Neurogenesis
	PRNP	Prion pathway	Proteolysis and peptidolysis
			Signal transduction
			Spermatogenesis
d6	BMPR2	ALK in cardiac myocytes	Phosphorylation
(n=7)			
d7	IL12B	IL-18 signaling pathway	Cell growth and/or maintenance
(n=32)	NUMA1	NO_2-dependent IL-12 pathway in NK cells	DNA-dependent regulation of transcription
	PSMA4	PRC2 complex sets long-term gene silencing the modification of histone tails	Transport
	RING1	Proteasome complex	
		Role of Ran in mitotic spindle regulation	
		Th1/Th2 differentiation	
d8	CTBP1	Biosynthesis of neurotransmitters	Development
(n=25)	GABRA1	Cardiac protection against ROS	DNA-dependent regulation of transcription
	GAD1	NFAT and hypertrophy of the heart	Synaptic transmission
	HAND2	Sumoylation as a mechanism to modulate CtBP-dependent gene responses	
		WNT signaling pathway	
		γ-aminobutyric acid receptor life cycle	
d9	CD5	Cyclins and cell cycle regulation	
(n=18)	CDKN2D	Dendritic cells in regulating Th1 and Th2 development	DNA-dependent regulation of transcription
		G2/M checkpoint	
d10	MYOD1	Control of skeletal myogenesis by HDAC and calcium/calmodulin-dependent kinase	Phosphorylation
(n=16)		Signal-dependent regulation of myogenesis by corepressor MITR	

Complex-effect gene expression patterns. Complex-effect genes were grouped into 12 clusters by SOM (Fig. 3C). Clusters showed mixed dose-and-time-dependent expression patterns and the functional enrichment analysis reflected the same tendency. For example, cluster c4 had 61 genes that were progressively induced at 0.01, 0.05 and 0.2 Gy of IR at 1, 4 and

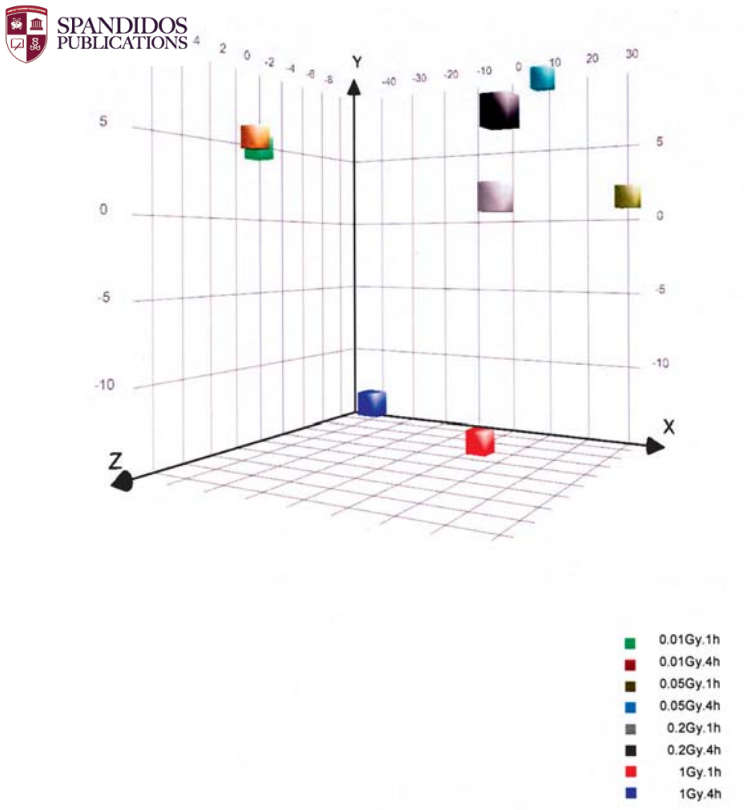

Figure 4. The principal component analysis of gene expression profiles at the early response phase (ERP) in the dose-dependent group. The gene expression pattern at each dose or at a time point is depicted as a dot in this three-dimensional graph.

12 h, while the induction declined modestly at 48 h. Induction increased at 1 Gy from 1 to 4 h then rapidly decreased after 12 h. The GO terms in the biological process category showing a significant enrichment by the hypergeometric test (P<1e-5) were signal transduction, induction of apoptosis, proteolysis, peptidolysis and electron transport. Cluster c9 contained 64 genes that were progressively repressed in response to all doses at 1 and 4 h of exposure, but were then induced modestly at 0.01, 0.05 and 0.2 Gy after 4 h of exposure up to 48 h. The same genes were rapidly induced by 1 Gy of IR. A pathway analysis revealed that the genes were associated with apoptotic DNA fragmentation, tissue homeostasis, the IFN γ signaling pathway, the IL-18 signaling pathway and regulators of bone mineralization.

Early-reactive-phase gene expression profile in the dose-dependent group. Based on the finding that dose-specific effects were different between the early and the late response phases, we split the data into early reactive phase (ERP) and late recovery phase (LRP) datasets. Both datasets were re-analyzed using the same strategy including two-way ANOVA with FDR correction, SOM clustering and GO and pathway-based enrichment studies.

The eight groups (i.e., four dose and two early time points) with the triplicated ERP dataset were subjected to a principal component analysis (PCA), revealing three major components within the total variation among the samples (Fig. 4). PCA revealed three response groups of 0.01, 0.05 and 0.2 and 1 Gy. We created 12 clusters from the dose-dependent group as determined by two-way ANOVA with FDR correction (P<0.05) for the ERP dataset (Fig. 5A). A notable finding from the visual inspection of the expression patterns of the clusters in Fig. 5A was that, for many clusters, genes seemed to respond separately to the different three-dose groups determined by PCA (Fig. 4). Specifically, for clusters e1, e2, e3, e4, e6, e10, e11 and e12, the genes tended not to respond to 1 Gy while they responded differentially to 0.01 and to 0.05 and 0.2 Gy of IR (Fig. 5A). More specifically, the genes in clusters e1, e2, e3 and e4 showed up-regulation at 0.05 and 0.2 Gy and down-regulation at 0.01 Gy. Those in clusters e6, e10, e11 and e12 showed up-regulation at 0.01 Gy and down-regulation at 0.05 and 0.2 Gy (Fig. 5A). Genes showing a dose-dependent response in the ERP group showed a tendency not to respond to 1 Gy of IR. This pattern was not found in the LRP dataset.

Late-recovery-phase gene expression profile in the dose-dependent group. The LRP dataset was analyzed using the same strategy as the ERP dataset (Fig. 5B). In contrast to ERP, most of the clusters from the dose-dependent group in LRP showed a remarkable increase or decrease, probably due to the strong time effect in LRP. The PCA results showed a distinction between 1 Gy and the other doses of IR (data not shown). The grouping of 0.05 and 0.2 Gy found in ERP was not found in LRP. Instead, many clusters including l3, l4, l5, l8 and l10 showed a differential response to 1 Gy compared to the other doses (Fig. 5B). For example, cluster l5 contained 117 genes that showed down-regulation at 1 Gy but not at the other doses. The pathway analysis revealed that the genes in cluster l5 showed a significant association with the B-cell receptor signaling pathway, Fc Epsilon receptor I signaling in mast cells, the nitric oxide signaling pathway, the T-cell receptor signaling pathway and Wnt/LRP6 signaling. The clusters l3, l4, l5, l8 and l10 showing a distinctive response pattern to 1 Gy also showed a significant association with cyclins and cell cycle regulation, cell cycle: G1/S check point, CDK regulation of DNA replication, apoptotic DNA fragmentation and tissue homeostasis. Although other clusters did not show a distinctive response pattern to 1 Gy, the pathway analysis showed that they were significantly related to the oxidative phosphorylation and electron transport chain. Oxidative phosphorylation has previously been reported to play a critical role in low-dose IR-induced adaptive responses (40). Our data suggest that genes which are responsive to a very low dose of IR are associated with adaptive responses at the later time points, while genes that are responsive to 1 Gy are associated with the cell cycle.

Discussion

We performed a systematic investigation on the gene expression patterns of a human mesenchymal stem cell line depending on dose and time in response to IR exposure using cDNA microarray technology. Our investigation focused on the transcriptional alteration induced by low and very low-dose IR exposure. We recognize significant genes that showed differential gene expression patterns in a dose-dependent manner, especially during the ERP. Exposure time is supposed

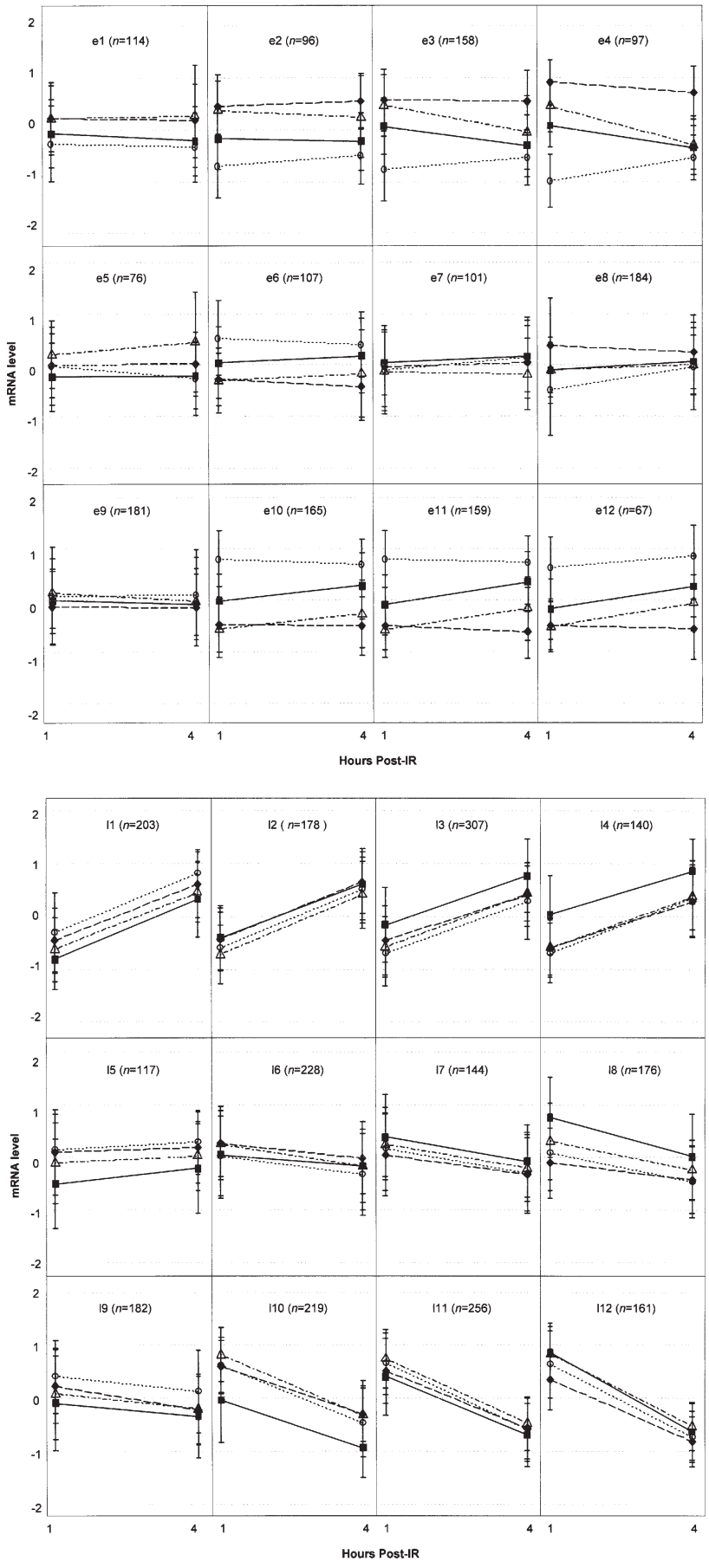

Figure 5. Gene expression patterns for dose-responsive genes at the early response phase (ERP) or late reactive phase (LRP). Clusters were generated using self-organizing maps (Avadis 4.3). (A) Cluster plot at ERP and (B) cluster plot at LRP in the dose-dependent group. The results represent the mean ± standard error.

influence on gene expression profiles during the LRP and the dose of IR delivered (Fig. 2). We expected the practical results on the dose and time effect to portray the molecular processes involved in cellular responses to gamma radiation.

The PCA analysis of the ERP showed three response groups to different doses of IR. However, we could not find the proportional relationship between the transcriptional changes and IR dose quantitatively. Moreover, genes that were up-regulated at 0.01 Gy appeared to be down-regulated at both 0.05 and 0.2 Gy and *vice versa*. Notably the genes which responded during the ERP, hardly responded to the 1 Gy dose. This significant observation suggests that non-linear pattern of cellular responses with respect to the IR dose, which requires a pertinent model to predict such a pattern.

A highly non-linear relationship between the IR dose and the transcriptional relative response is identified in the d1, d2, d3 and d8 clusters from the dose-dependent group. The time-dependent group also exhibited a non-linear relationship similar to the complex-effect group. One noteworthy pattern of altered gene expression was the nonlinear relationship, especially with the dose change (Fig. 3).

Genes that showed a time-dependent response pattern (Fig. 3B) are associated with two distinguished groups, up-and-down and down-and-up regulated genes. We speculated that the fundamental cellular processes in the ERP are seized temporarily by the external stimuli and defense mechanisms are activated as a counterpart. After 12 h of exposure to IR, we infer that cellular activities gradually resume and defense systems are down-regulated.

In irradiated mice, Yin *et al* showed that the expression levels of 1,574 out of 9,977 genes were altered in response to 0.1 and 2 Gy of IR (21). Approximately 30% of these genes were dose-responsive and 60% of them were responsive to exposure time. Here, we also found that gene expression after exposure to IR was strongly influenced by the exposure time (Table I). In a recent study, Ding *et al* reported that cyto-skeleton components, ANLN and KRT15 and cell-cell signaling genes, GRAP2 and GPR51, were responsive to low-dose radiation but not to high-dose radiation (25). They suggested that these quantitative and qualitative responses in gene expression explained the non-linear correlation of the biological effects of IR from low-dose to high-dose exposure. We found that ANLN and KRT15 showed a time-dependent response and that GRAP2 showed a complex-effect pattern. ANLN plays a role in the spatial regulation of the contractile activity of myosin II in cytokinesis during cell proliferation and KRT15 encodes keratins which are a family of structurally related proteins that form intermediate filaments in epithelial cells. Cytoskeleton components seem to be affected by IR exposure time, which may cause a malfunction of the cell proliferation or formation of the protective epidermis.

We suggest that mRNA transcripts are highly expressed in response to both low-dose (0.01-0.2 Gy) and high-dose (1 Gy) IR exposure. Combining dose response with temporal profiles of gene expression identified novel sets of coordinately regulated genes. This study provides an intuitive idea about the molecular responses to physiologically relevant doses of IR that cannot be extrapolated from high-dose studies. Specifically, we investigated a low and very-low dose range study, which showed results that are different from previous studies. More feasible and practical investigations in low and very-low doses of IR are required to construct health risk assessment. Therefore, we recognized the non-linear relationship in dose effect. However, a novel, rigorous model to find the quantitative relationship between dose and expression level will be implemented in a further study.

Acknowledgements

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