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Functional genomics as a window on radiation stress signaling

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Exposure to ionizing radiation, as well as other stresses, results in the activation of complex signal transduction pathways, which eventually shape the response of cells and organisms. Some of the important pathways responding to radiation include the ATM/P53 pathway, MAPK cascades and NF-κB activation, as well as signaling events initiated at the cell membrane and within the cytoplasm. Alterations in gene expression play roles both as intermediaries in signaling and as downstream effector genes. Differences in cell type, interindividual genetic differences and crosstalk occurring between signaling pathways may help to channel radiation stress signals between cell cycle delay, enhanced DNA repair, and apoptosis. These differences may in turn help determine the likelihood of late effects of radiation exposure, including carcinogenesis and fibrosis. The tools of the postgenomic era enable high-throughput studies of the multiple changes resulting from the interplay of radiation signaling pathways. Gene expression profiling, in particular shows great promise, both in terms of insight into basic molecular mechanisms and for the future hope of biomarker development and individual tailoring of cancer therapy.
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Introduction

Exposure of cells to ionizing radiation induces damage in various cellular compartments and results in complex biological responses. Damage to DNA, particularly double-strand breaks, has long been considered the major initiator of cellular responses to ionizing radiation. As a single unrepaired double-strand break can be lethal to a cell (Bennett *et al*., 1996), detection and removal of these lesions is an important cellular function, and their presence rapidly results in recruitment of DNA repair machinery to sites of damage, as well as triggering multiple signaling events. DNA strand breaks can also have major effects on chromatin and nuclear structure, since a break can allow an entire region of chromatin between nuclear attachment sites to lose superhelicity with relaxation of DNA structure, decrease in density of DNA packing/stacking, and resultant local nuclear swelling. Signaling from strand-break-related damage can in turn result in cell cycle arrest, enhancement of DNA repair capacity, or activation of apoptotic pathways when damage cannot be repaired. Damage to other cellular components, including the cell membrane, mitochondria, endoplasmic reticulum, and non-DNA constituents of chromatin, may also initiate or modify stress signaling in response to ionizing radiation. Although not the only mechanism of stress response, modulation of transcription factor activity plays a major role in response to DNA damage and results in dramatic shifts in the transcription profiles of cells after exposure to ionizing radiation. The advent of the postgenomic era has ushered in new techniques offering diverse possibilities for gaining insight into the complexities of the molecular responses to ionizing radiation and other stresses.

Even before the completion of the human genome draft sequence, a number of techniques for genomic expression profiling were beginning to emerge. Differential display and serial analysis of gene expression (SAGE) do not require prior knowledge of gene sequence to identify transcripts expressed at different levels in compared samples and rely on sequencing to identify novel transcripts. Several different approaches for microarray analysis have also been developed to take advantage of the rapidly increasing availability of sequenced and annotated human expressed genes. These methods use immobilized target sequences, either oligonucleotides or spotted cDNAs, and hybridization against a complex probe consisting of the mRNA populations of the samples to be compared. More sophisticated techniques are also being refined. For instance, the chromatin immunoprecipitation (ChIP) assay has been combined with microarray analysis in yeast to provide a picture of all the binding sites in the genome for individual transcription factors (Ren *et al*., 2000). Briefly, this technique involves crosslinking proteins bound to DNA, then immunoprecipitating with a specific antibody for the transcription factor of interest. The DNA is then released from the protein and hybridized to an array containing genomic sequences. A

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similar approach is being applied to the analysis of human transcription factors using either microarrays printed with CpG island enhanced sequences (Weinmann and Farnham, 2002) or PCR products of specific promoter regions (Ren *et al*., 2002). Techniques for high-throughput analysis of cellular or serum proteins are also being developed and include antibody arrays (Sreekumar *et al*., 2001; Eickhoff *et al*., 2002) and mass spectrometry-based analyses (Ardekani *et al*., 2002; Chapman, 2002). Such techniques enable the study of global expression changes in disease states or following stresses such as ionizing radiation, and are leading to insights into the molecular mechanisms involved.

Insight into signaling pathways from genomic approaches

Since P53 is a transcription factor and the most frequently mutated gene in human cancers (Levine *et al*., 1991), it is not surprising that this pathway has become one of the most intensively studied using genomic techniques. In an early study using SAGE analysis and overexpressed P53, a number of novel P53-regulated genes were identified (Polyak *et al*., 1997). Early microarray analysis of radiation exposure to a human cell line similarly revealed several novel radiation-induced genes, including *FRA1* and *ATF3*, which encode important transcription factors and require functional P53 for radiation responsiveness (Amundson *et al*., 1999a). Although factors such as the tissue of origin, the propensity of cells to undergo rapid apoptosis, and P53 status have long been recognized to result in differential radiation modulation of specific genes (Table 1), comparison of diverse cell lines displays complex patterns of gene induction with less readily apparent underlying regulation (Table 2). Combined genetic and microarray approaches may help unravel the additional regulatory factors contributing to such patterns. Other studies have used microarray analysis to explore the effect of point mutations in P53 on radiation-induced genes (Robles *et al*., 2001; Park *et al*., 2002). The P53 family member p73a is also a transcription factor, and microarray analysis has revealed both unique and overlapping gene regulation profiles for these two genes (Fontemaggi *et al*., 2002). While such survey approaches add to our knowledge of P53 activities, more comprehensive methods may go further toward defining the entire gene regulatory

Table 1 Examples of genes differentially induced by ionizing radiation

Gene	*Apoptosis-dependent*[a]	*P53-dependent*
BAX	+	+
BCL-XL	+	+
CDKN1A	–	+
GADD45A	–	+
XPC	–	+
DDB2	–	+
ATF3	–	+
c-*JUN*	+	–
MCL-1	+	–
GADD34	+	–
GADD153	+	–
IL-8	–	–
REL-B	–	–

[a]Cell lines undergoing rapid apoptosis following ionizing radiation exposure as defined by Zhan *et al*. (1994) (many of these lines are of lymphoid or myeloid origin). '+' indicates that significant radiation induction of a gene occurs only in cell lines where the condition in question (apoptosis or P53 wild-type status) is met. '–' indicates that radiation induction of a gene can occur independently of the listed parameter. Data are summarized from Zhan *et al*. (1996, 1997) and subsequent experiments using hybridization of individual probes as described (Koch-Paiz *et al*., 2000)

Table 2 Examples of gene induction 4 h after exposure with high-dose (8 or 20 Gy) ionizing radiation in a number of specific human cell lines from different tissues of origin and differing P53 status

	Lymphoid/myeloid								*Epithelial*						
P53 status	*wt*				*Mutant*				*wt*				*Mutant*		
Gene	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
CDKN1A	+	+	+	+	–	+	–	–	+	+	+	+	–	+	-
MDM2	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–
XPC	+	+	+	+	–	–	–	–	+	–	–	+	–	–	–
DDB2	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–
PIR121	+	–	–	+	–	–	–	–	–	+	–	+	–	–	–
FRA1	+	–	–	+	–	–	–	–	–	+	–	-	–	–	–
BAX	+	+	–	+	–	–	–	–	+	+	–	-	–	–	–
IL-8	+	-	+	–	+	+	–	–	–	–	+	-	–	+	–
ATF3	+	+	+	+	+	+	–	–	–	–	+	+	–	–	–
REL-B	+	+	+	+	+	–	+	–	–	+	+	–	+	+	–
BCL-XL	+	–	+	–	–	–	–	–	–	–	–	–	–	+	–
SSAT	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–

'+' Indicates twofold or more increase in RNA levels relative to untreated controls. '–' indicates lack of or less than twofold induction. Cell lines used: (1) ML-1, (2) Molt4, (3) SR, (4) TK6, (5) CCRF-CEM, (6) HL60, (7) K562, (8) NH32, (9) A549, (10) MCF7, (11) RKO, (12) HCT116, (13) H1299, (14) T47D, (15) HCT116 P53–/–. Data are summarized from Amundson *et al*. (1999a) and subsequent experiments using hybridization of individual probes as described (Koch-Paiz *et al*., 2000)

network associated with P53. One such approach used an *in silico* analysis of the promoter and intron sequences of all human genes in parallel with microarray analysis to identify a large number of genes containing putative P53 consensus binding sequences (Wang *et al*., 2001).

Another major transcription factor activated by ionizing radiation is nuclear transcription factor-κB (NF-κB). Genes induced by NF-κB following irradiation include ICAM-1 (Hallahan *et al*., 1998), galectin-3 (Dumic *et al*., 2000), TNF-α, IL-1β, and IL-6 (Raju *et al*., 1999). Activation of NF-κB by ionizing radiation has been associated with enhanced survival in colon cancer cells (Russo *et al*., 2001) and lymphoma cells (Kawai *et al*., 1999) and enhances transcription of antiapoptotic genes, such as TRAF1, TRAF2, IAP1, IAP2 (Wang *et al*., 1998), and A20 (Krikos *et al*., 1992). This activity of NF-κB may directly oppose the proapoptotic function of P53 activation through competition for the p300/CREB-binding protein transcriptional coactivator complexes (Webster and Perkins, 1999). The reverse may be true for radiation-induced cell cycle arrest, however, where recent evidence suggests cooperation between P53 and NF-κB (Wadgaonkar *et al*., 1999; Yang *et al*., 2000). Among other efforts to disentangle the crosstalk between these two radiation signal transduction pathways, microarray analysis has been applied to a cellular system where both P53 and NF-κB function can be independently abrogated. The results indicate that a set of NF-κB regulated genes, including Cyclin B1, Cyclin D, and HIAP, may play a role in P53-independent radiation resistance (Chen *et al*., 2002).

Signaling through MAP kinase pathways can also contribute to the molecular response to radiation exposure. For instance, the ERK1/2 pathway has been implicated in the radiation induction of the EGR-1 promoter (Meyer *et al*., 2002), and of VEGF, which enhances angiogenesis in brain tumors (Mori *et al*., 2000). The MEK1/2 pathway, on the other hand, may be implicated in regulation of DNA repair, as radiation-induced increases in gene and protein levels of ERCC1 and XRCC1, as well as removal of DNA lesions and micronucleus formation, depended on MEK1/2 activity in a human cancer cell line (Yacoub *et al*., 2001). The P38 MAP kinase pathway plays a major role in the response to ultraviolet radiation (Pandey *et al*., 1996; Bulavin *et al*., 1999; Kovarik *et al*., 1999), and activation of the gamma-isoform has been reported in one publication to be involved in ionizing radiation regulation of the G2 checkpoint (Wang *et al*., 2000). As the MAP kinase cascades are clearly important signal transduction pathways that result in modification of gene transcription, integration of functional genomic studies of MAP kinase with studies of the other major radiation signal transduction pathways will ultimately be needed. As such studies of stress response become increasingly 'information-rich', increasingly complex bioinformatic approaches will be needed to synthesize data from diverse sources into a clearer understanding of signal transduction pathways.

Directing signals to modify outcomes

The complexity of signaling that occurs following exposure to ionizing radiation allows flexibility in determining the ultimate fate of a damaged cell or tissue. For instance, activation of P53 has been shown to induce cell cycle delay, senescence, DNA repair, and apoptosis. Fine-tuning of the stress signal must be needed to determine the ultimate outcome in each case. The cellular environment is one factor known to affect this decision. For instance, many lymphoid cell lines have been shown to preferentially undergo apoptosis in response to activated P53 in the absence of exogenous growth factors, but undergo cell cycle arrest if they are present (Collins *et al*., 1992; Gottlieb and Oren, 1996). Lymphoid cell lines in general are more prone to undergo apoptosis in response to radiation damage than are fibroblasts (Lowe *et al*., 1993; Di Leonardo *et al*., 1994; Radford *et al*., 1994), presumably due to endogenous signaling differences between cell types which have not yet been elucidated. Such signaling differences may be reflected in the profile of gene inductions, such as in Table 2. Interactions between P53 and its cofactors may also influence the switch between apoptosis and growth arrest. Competition between P53 and NF-κB for P300/CREB-binding proteins has been shown to sway the balance between these two mutually exclusive outcomes (Webster and Perkins, 1999), as have interactions between P53, the retinoblastoma tumor suppressor protein, c-Abl, and P73 (Urist and Prives, 2002). Functional genomics approaches are likely to be increasingly informative as more of these key switching signals are revealed.

Factors in the genetic make-up of a cell, such as mutations or polymorphisms in components of signal transduction pathways, may further modulate individual outcomes of exposure to radiation or other genotoxic agents. Such variations are likely to prove important in determining the larger consequences of radiation exposure on the level of the tissue or whole organism. For instance, if apoptosis is suppressed and excessive DNA damage is tolerated in arrested cells, this may allow additional time for misrepair, potentially leading to the accumulation of additional mutational events and increased risk of carcinogenesis. Understanding the basis for such switches in signaling and outcome may lay the foundation for future individual susceptibility profiling, leading to improved radiation protection and radiotherapy design.

One of the more common late complications of radiation therapy is tissue fibrosis, characterized by accumulation of collagen and extracellular matrix, and excessive proliferation of fibroblasts. Specific alterations in gene expression have been associated with the development of fibrosis following radiation injury and include upregulation of tenascin-C (Geffrotin *et al*., 1998) and plasminogen activator inhibitor-1 (Zhao *et al*., 2001). A recent microarray analysis of irradiated fibrosis-prone and fibrosis-resistant mice has indicated that the specific pattern of chemokine and chemokine receptor gene inductions may underlie the development

of this complication (Johnston *et al*., 2002). If such studies are borne out, they may form the basis for intervention and reduce the risks associated with high-dose radiation therapy.

Genomic approaches to radiation biomarker development

A tremendous amount of information is contained in the profiles of expressed genes or proteins, and there is much enthusiasm for exploiting the predictive potential of this information. Expression profiles already show promise for tumor classification and for predicting the response of individual tumors to specific treatment regimens. The different stages of immune cell function and physiology can be identified from gene expression signatures (Alizadeh *et al*., 2000). Human tumors can also be classified as BRCA1 or BRCA2 mutants by gene expression profiles resulting from microarray analysis (Hedenfalk *et al*., 2001). In addition to such classification, expression profiling of melanomas has identified a signature that correlates with high metastatic potential (Bittner *et al*., 2000), while profiling of diffuse large B-cell lymphoma can predict the response to treatment (Alizadeh and Staudt, 2000). As this field develops, it will hopefully lead to more effective cancer treatment. Correlations between sensitivity to various chemotherapy regimens and gene expression databases of tumors could lead to the development of tumor profiles that could be used to fine-tune therapy for the individual. Issues of individual normal tissue response could potentially also be addressed by such profiling, to limit side effects of cancer treatment. Gene expression profiles could also predict agents for modifying radiation treatment, either by sensitizing resistant tumor tissue, or by protecting normal tissue from immediate damage or the induction of second tumors.

While the idea of tailoring therapy to the individual is very attractive, gene expression profiles may also have utility in the area of molecular biomarkers to detect exposure to radiation or other toxins. Such biomarkers would be informative for epidemiological studies, as well as in exposure assessments in cases of environmental or industrial accidents. The majority of currently available biomarkers to detect radiation exposure are either highly invasive (extraction of a tooth for electron spin resonance) or require detailed analysis by skilled workers (cytogenetic-based methods). In the current environment, fears of radiological incidents, such as a so-called 'dirty bomb' attack, may inspire the search for a rapid simple method to triage potentially exposed individuals within a large population. Gene expression profiles may hold promise in this arena, although some obstacles must still be overcome.

While many of the early studies on gene expression were conducted using high, supralethal doses, much lower doses are of interest for monitoring most environmental and human exposures. In recent years, more studies have targeted this dose range of interest. For instance, we have documented the dose–response behavior of several genes following gamma-ray doses as low as 2 cGy (Amundson *et al*., 1999b). For the genes examined, such as *CDKN1A* (Figure 1), there is no obvious departure from a linear trend between 2 and

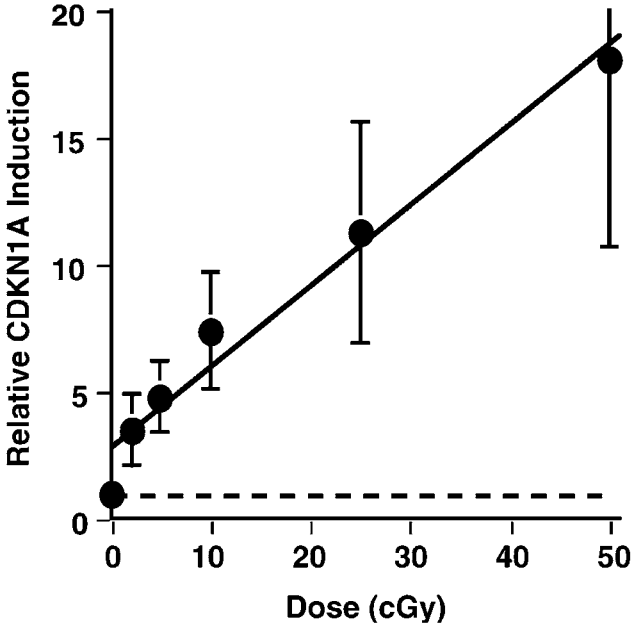

Figure 1 Dose-response for relative *CDKN1A* induction 2 h after exposure of ML-1 cells to gamma rays. Points represent the mean ± s.e.m., and are the average of four independent experiments. Linear regression through the data is also shown. The dashed line indicates the relative level of gene expression in untreated control cells

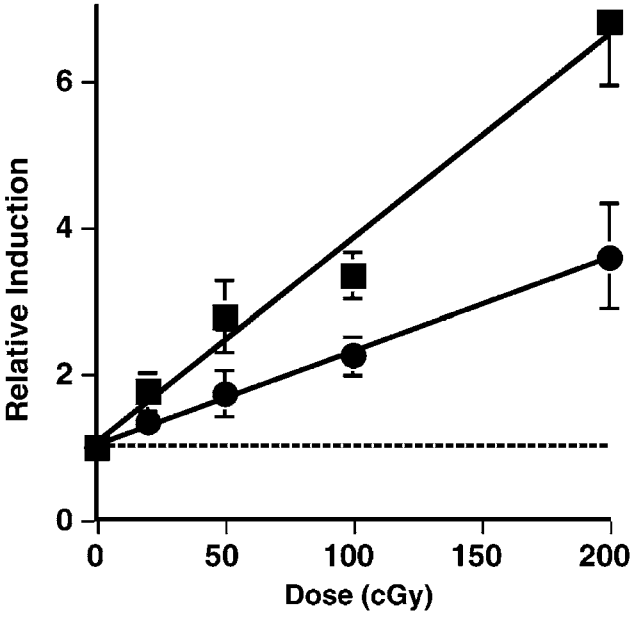

Figure 2 Induction of *DDB2* (■) and *XPC* (•) RNA in quiescent human peripheral blood lymphocytes (PBLs) measured at 24 h after exposure to gamma rays. Points represent the mean ± s.e.m. of four independent experiments with PBLs from different donors. Methods are detailed in Amundson *et al*. (2000). The dashed line indicates the relative level of gene expression in untreated control cells

50 cGy, although the response may actually be super-linear below this dose. The finding that significant gene inductions do occur at environmentally relevant doses and that the magnitude of response is related to dose is an important step toward developing the utility of expression signatures as biomarkers.

In the light of the heterogeneity of gene induction responses occurring in cultured cell lines, establishing profiles based on normal tissue responses will also be important. Since we generally have obtained the most robust responses in cell lines of lymphoid or myeloid lineage and since peripheral blood lymphocytes (PBLs) would be an easily sampled tissue, we have explored the gene expression profiles of human PBLs irradiated *ex vivo* (Amundson *et al*., 2000). Similar to the findings from low-dose irradiation of cell lines, we found a linear induction of several genes for several days after treatment with modest doses of gamma rays (Figure 2). In the same study, we also determined that the baseline expression of the inducible genes did not vary widely among unrelated donors, which would be important for any biomarker, as pre-exposure profiles would be unlikely to be available for comparison. While the stability and uniformity of these gene expression changes do need to be confirmed *in vivo,* these early studies indicate that it may be possible to develop a set of genes to discriminate exposed from unexposed populations.

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