

Functional genomics in radiation biology: a gateway to cellular systems-level studies

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Abstract Cells respond to ionizing radiation through an intricate network of interacting signaling cascades that are engaged in the regulation of diverse cellular functions, such as cell cycle arrest, DNA repair, and apoptosis. While changes in protein modification, activity, and sub-cellular localization may directly mediate these responses, alterations in gene expression also represent a central component of the pathways involved. Studies of altered gene expression have historically played an important role in elucidating the molecular mechanisms underlying cellular radiation response. In recent years, functional genomics approaches, such as microarray profiling, have been developed that can simultaneously monitor changes in gene expression across essentially the entire genome. However, analogous methods for global measurements of protein expression or modification have lagged behind. As global transcription profiling has become increasingly accessible, the quantity of information on gene expression responses to irradiation has increased dramatically. While many such experiments have provided improved insight into various aspects of radiation response, the diversity of experimental models and details of radiation dose, timing, and data analysis that have been employed means that no single consistent picture has emerged yet. More sophisticated methods for data analysis, data mining, and reverse engineering to reconstruct the underlying response pathways are continually being developed, and can extract additional

value from profiling studies. As methods for the global study of other biomolecules become more routine, it will be important to integrate the results of radiation response profiling across multiple biological levels, and to build from simpler experimental systems toward more complex multi-cellular and in vivo systems. The future development of “integromic” models of radiation response should add substantially to the understanding gained from gene expression studies alone.

Early studies of gene expression as a DNA damage response

Regulating the expression of specific genes is a fundamental mechanism by which cells employ the information in their DNA to achieve diverse ends, such as differentiating to perform specific cellular roles, and responding to extra-cellular stresses including ionizing radiation. While information at other levels such as DNA sequence and epigenetic modifications, protein expression, modification and localization is also important, changes in RNA levels provided an early window on dynamic responses to cellular stress. Characterization of the DNA damage inducible genes of the bacterial SOS response provided an early guide for studies of gene expression changes in mammalian cells. Interestingly, a similar paradigm of coordinately regulated genes representing a network of interacting protein products that direct or perform a physiological function is a key concept underlying much of modern systems biology.

The first DNA damage responsive genes were identified by laborious methods, and understanding the functions and regulation of the identified genes proceeded slowly in a

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reductionist manner. For instance, screening a cDNA library for UV-induced genes using low-ratio hybridization subtraction allowed cloning of 20 differentially expressed cDNA clones, only two of which matched known sequences at the time [1]. These were characterized on the basis of their response to various stresses, and by the next year, five of these genes were fully sequenced and reported as coordinately regulated by both DNA damage and growth arrest signals [2], and were made the focus of subsequent studies. It was another 3 years before one of these so called “gadd” genes, *GADD45A*, was found to be regulated by p53 following exposure to ionizing radiation [3]. Another prominent radiation response gene, *CDKN1A* (p21^{CIP1/WAF1}), was also cloned by hybridization subtraction [4] specifically as a p53 responsive gene. It was not until another 4 years later, however, that it was found that three independent clones of *CDKN1A* had been present among the 20 clones from which the “gadd” genes were originally identified [5], as these separate lines of study gradually converged. This slow accumulation and piecing together of fragmentary information came from studies centered on individual genes, pathways, or biological processes, and radiation response began to emerge as an intricate interplay between numerous signaling pathways and cellular functions. Clearly, more comprehensive methods for the study of these interactions would be needed to fully understand the molecular basis of the effects of radiation on living organisms.

Early “data-rich” studies

The concept of using large data sets containing diverse types of information to reveal connections and generate hypotheses through data-mining pre-dates the post-genomic era. An example of one such early effort in the cancer research field is the NCI anti-neoplastic drug screen (NCI-60) [6]. This set of 60 human tumor cell lines representing nine different tissues of origin was originally conceived as a tissue-specific screen for potential chemotherapy agents. Indeed, more than 100,000 compounds have been screened for toxicity in the NCI-60 cells, producing a large drug activity database, but it rapidly became apparent that addition of molecular characterization of the cell lines and development of novel analysis tools such as the COMPARE algorithm [7] were creating a very powerful model system. Integration of information from studies with the NCI-60 cell lines has allowed identification of mechanisms of drug action, advanced understanding of basic molecular interactions in cancer, and lead to the approval and use of new chemotherapy drugs such as bortezomib and oxaliplatin (reviewed in [8, 9]).

Given the high proportion of human cancers with defects in p53, this pathway was an early focus for characterization in the NCI-60. p53 sequence and activity, as well as gamma-ray induction of G1 arrest and p53 downstream genes were measured and compared with the drug activities of 123 clinically approved, anticancer agents. Defects in the p53 pathway were found to confer resistance to most chemotherapy drugs, with the exception of anti-mitotic agents [10]. In a later study, relative basal expression levels of ten genes were measured in all the cell lines of the NCI-60, and Spearman correlations between gene expression and the activity patterns of the same 123 clinical chemotherapy agents were calculated [11]. A matrix of the corresponding Spearman *P*-values revealed a strong protective effect of *BCL-X_L* (*BCL2L1*) expression across all drug mechanisms that was not confounded by doubling time or p53 status. Compared with the loss of p53 function, *BCL-X_L* expression conferred an even broader protective effect against drug killing that extended beyond the standard chemotherapy agents to a set of drugs representing an estimated 1,200 different mechanisms of action.

The first study of gene induction by ionizing radiation in the NCI-60 cells included measurement of responses of *GADD45A*, *CDKN1A*, and *MDM2* to 20 Gy gamma-rays. As all three of these genes are known to be p53 regulated, they might be expected to have similar patterns of regulation. However, a subset of the p53 wild-type cell lines induced *CDKN1A* as expected in response to radiation, but failed to induce *GADD45A* [12]. Comparison of drug activity patterns in these *GADD45A* induction-defective cell lines versus *GADD45A* induction-competent cell lines revealed that the inability to mount a *GADD45A* induction response correlated with a resistance to topoisomerase inhibitors, but not to any other class of drugs [13]. This effect could be seen clearly by plotting the distribution of Wilcoxon *P*-values for correlations between the level of *GADD45A* induction and sensitivity to killing by topoisomerase inhibitors (highly skewed toward significance) in contrast with the *P*-values for all other mechanisms of action (random distribution with all *P*-value levels represented equally). Confirmation of this lead and subsequent experiments revealed a previously unsuspected function of *GADD45A* as a chromatin accessibility factor, opening important areas of investigation.

These examples illustrate an early model system using large datasets to facilitate integration of information about genotypes, phenotypes and physiological responses, gene expression, and known mechanisms of drug action, to generate hypotheses that advanced both mechanistic understanding and clinical approaches. The utility of such model systems continues to grow as technical advances allow inclusion of global measurements of many parameters

of interest [9] and statistical and data-mining approaches become more advanced.

Transcriptional responses to radiation in the post-genomic era

Techniques for detecting differential gene expression have evolved since the early days of cDNA libraries and subtraction hybridization. Sequencing-based techniques such as differential display [14] and serial analysis of gene expression (SAGE) [15] accelerated discovery of genes involved in the DNA damage response, as did the advent of microarray hybridization [16]. Applying such transcription profiling techniques to the study of responses to various stress factors, including ionizing radiation, provides a temporal “snapshot” of a dynamic process. The kinetics of gene expression response can vary between cell lines or individuals, and even with different doses of radiation in the same model system [17], adding a layer of complexity to interpretation and comparison of results.

With the complete sequencing of the coding portions of the human genome, and commercial platforms now readily available, global gene expression profiling experiments have become commonplace. The first microarrays used spotted cDNAs and hybridization with two different samples labeled with different fluorochromes [16, 18], and monitored between a few hundred and a few thousand genes. Although long oligonucleotides are now more commonly used than cDNAs, the same basic approach is still in wide use, as is the Affymetrix platform [19], which compares individual samples hybridized to separate arrays of short oligonucleotides. Most commercial platforms now offer “complete” genome coverage for humans and several other model organisms, with features selected to represent all predicted expressed genes. While the technology for performing such highly parallel experiments has advanced rapidly, the ability to thoroughly analyze and interpret the results of these studies has lagged somewhat behind. It is on these analysis approaches that the true value of expression profiling, or any global measurement approach, will ultimately depend.

The first microarray experiment investigating response to ionizing radiation in human cells profiled 1,344 genes in a myeloid cancer cell line 4 h after 20 Gy gamma-rays [20]. It sought to provoke as large a response as possible due to uncertainties about the fledgling microarray technology, and the number of genes that could realistically be expected to show a detectable response. This initial study used the 99% confidence interval of expression ratios for a set of housekeeping genes [21] to determine significantly altered gene expression. It demonstrated good concordance between fold-change measurements

made by the microarray and quantitative single probe measurements and revealed approximately 30 previously unknown radiation responsive genes including two, FOSL1 (designated FRA1 at the time) and ATF3, which were regulated by p53. This study also provided the first opportunity to attempt reverse engineering to reconstruct biological pathways responding to ionizing radiation using a simple artificial neural network to identify sets of genes whose transcriptional levels could predict the expression level of another gene [22]. This analysis returned several known relationships among genes in the p53 pathway, as well as unknown associations. A similar microarray approach was also applied to detection of potential biomarkers in peripheral blood irradiated with doses of gamma-rays between 0.2 and 2 Gy [23], and lead to identification of XPC as yet another p53-regulated radiation response gene [24].

More recent studies have investigated lower doses of radiation and responses specific to low dose and low dose-rate. The first microarray study investigating gene expression as a function of dose rate revealed clusters of genes that responded in a human myeloid cell line to 0.5 Gy gamma-rays with either dose-rate-dependent or -independent patterns across 4 decades of dose-rate [25]. The dose-rate effect or lack thereof was confirmed for five of the genes in dose-response curves between 0.02 and 0.5 Gy. Interestingly, an effect of dose-rate was observed on induction of apoptosis in this system, but not on the magnitude or duration of cell cycle delay. Correspondingly, 46% of the genes showing dose-rate effects had known roles in apoptosis, while 67% of the genes not showing dose-rate effects had known roles in cell-cycle regulation or proliferation. In a slightly different approach, Wang et al. [26] treated a human lymphoblast cell line with a 0.5 Gy dose and identified 75 genes as possible contributors to low-dose hypersensitivity using a pre-selected 2-fold ratio cut-off. Knock-down with siRNA of one of the genes identified, *CHD6*, did produce a significant increase in survival at doses below 1 Gy, supporting the possible contribution of induction of this gene to the enhanced cell killing seen in some cell lines in the low-dose hypersensitivity range.

The radiation bystander effect is another response specific to low doses of radiation that has been investigated through the use of microarrays. The bystander effect refers to responses measured in cells in proximity to irradiated cells, but which have themselves not been irradiated. It appears that signals from irradiated cells can be transmitted both via direct cell-to-cell contact, and in the case of cultured cells, through the culture medium. The range of endpoints documented as bystander effects is similar to that seen in directly irradiated cells, and includes cell killing, apoptosis, DNA damage, cytogenetic damage, and mutation [27], but the exact nature of the signal or signals

transmitting the stress response has yet to be definitively determined. Microarray analysis of human skin fibroblasts 3 h after exposure to 0.01 Gy alpha-particles, a dose resulting in particle traversals of 6% of the cells, revealed up-regulation of connexin 43 (*GJA1*) transcript levels [28]. This was consistent with the role of connexin 43 in gap junction communication, which had already been shown to be important in bystander effect [29]. Another bystander gene expression array study used exposure of human lung fibroblasts to 0.5 Gy alpha-particles in specially designed dishes that shielded half of the cells from direct irradiation. In this study, cyclooxygenase 2 (*PTGS2*) was found to be up-regulated in the shielded bystander cells relative to non-irradiated controls [30], leading to the elucidation of a role for the arachadonic acid cascade in radiation bystander signal transduction.

The first global expression profiling of radiation bystander responses investigated the portion of the response that can be transmitted through cell culture medium [31]. Four hours after irradiation of human lung fibroblasts with 2 Gy, the medium was transferred to un-irradiated cells. Four hours after that, gene expression was compared between cells receiving medium from irradiated cells and non-irradiated control cells. Medium from irradiated cells produced a 1.5-fold or greater up-regulation of 37 genes, but no down-regulated genes. The response to the irradiated cell medium included genes with roles in extracellular signaling, growth factors, and receptors. These findings are consistent with the view that a signal from irradiated cells is transmitted through the culture medium.

The effects of low doses of radiation on directly irradiated cells have also been studied through gene expression profiling. In an effort to understand the overall relationship between responses to high and low doses, Yin et al. [32] compared the transcriptional response of mouse brain to 0.1 and 2 Gy at 0.5 and 4 h post-exposure. They used a more sophisticated analysis employing an *F*-ratio adjusted for a false discovery rate of 20%, followed by multi-factorial analysis and clustering to group the responding genes according to dose-response patterns. Significantly, this study found genes that were regulated only in response to 0.1 Gy and not in response to the higher dose, as well as genes that changed in opposite directions following high- or low-dose exposure. While it was possible that the limited time-points included in this study missed transient responses to the higher dose, the possibility of an exclusive low-dose gene expression response pattern has serious implications for using high-dose and epidemiological studies to extrapolate the risk of low-dose exposures. The concept of a transcriptional response exclusive to low-dose exposure was strengthened by a study of 0.02 and 4 Gy exposure to human skin fibroblasts that profiled gene

expression at 1, 2, 4, and 24 h after exposure. Ding et al. [33] used the significance analysis of microarrays (SAM) technique with a 5% false discovery rate (FDR) cut-off to identify 16 genes that responded significantly to the low dose but not to the high dose, as well as 47 genes responsive to both doses in a non-dose-dependent manner. They also used clustering and principal components analysis (PCA) to resolve dose- and time-dependent patterns of response. Gene ontology analysis revealed a significant enrichment of cell signaling genes responding to 0.02 Gy but not to 4 Gy, while proliferation associated genes were significantly enriched among the 4 Gy but not the 0.02 Gy responders. A similar study of gene expression responses measured between 3 and 72 h after exposure of keratinocytes to 0.01 or 2 Gy X-rays reported 140 genes that responded exclusively to the lower dose, most seen only at 48 h after exposure [34]. This re-emphasizes the importance of gene expression kinetics in such studies.

As might be expected, analysis of in vivo gene expression responses to low-dose radiation exposure in humans has proved somewhat more complicated than cell culture experiments. In a study of intact human skin 3 h after exposure to doses of 0.01, 0.1, and 1 Gy during treatment for prostate cancer, Goldberg et al. [35] found that variations in gene expression between different patients meant that standard statistical methods were inadequate to identify genes responding to low doses of ionizing radiation. They reasoned that slight variations in the timing of a response cascade might mean that at the 3-h time point surveyed, the gene expression profile of each patient may represent a “snapshot” of a slightly different point in each responding pathway. In order to address this possible diffusion of specific responses among individuals and unmask underlying biological pathway responses, Rocke et al. [36] developed an analysis method based on grouping genes by molecular class or common pathways. A standard statistical test of differential expression in each individual sample is then applied to each gene in the group, and all these *t*-statistics are then tested in aggregate to see if their values deviate from a random distribution. A positive bias would indicate the presence of the proposed diffuse response among members of the gene group in general. The significance of any such bias can then be estimated by generating empirical *P*-values from repeated re-sampling of randomly selected probe sets. This idea of looking for significant trends among large numbers of *P*-values across pre-defined sets of measurements is reminiscent of the analysis successfully applied to the early gene expression studies of the NCI-60 discussed above [11, 13]. Application of this method to the human skin irradiation data identified seven gene groups and five molecular pathways that responded to low-dose irradiation in vivo [35], revealing coherent response patterns that had been masked

by the variations between individuals, and providing a potentially valuable tool for future studies.

In a study of gene expression responses as predictors of late toxicity in radiotherapy patients, Svensson et al. [37] found a similar improvement by using defined gene groups instead of individual genes to build their predictor of response. Peripheral blood lymphocytes of patients with or without late radiation toxicity were stimulated to divide in culture, and exposed to 2 Gy X-rays. Global gene expression profiling was performed 24 h later, and the ratios of gene expression in response to 2 Gy compared to control levels for each patient were used to build and test classifiers to predict the outcome of either occurrence or absence of late toxicity. Using the expression response patterns of individual genes, this method could classify 63% of the patients correctly. Gene ontology terms were then used to define gene sets representing common molecular functions, cellular components, or biological processes. For each patient, the gene expression ratios for all the genes in a gene ontology group were then combined to obtain a single overall value for each of the defined gene sets, and these profiles were used to build and test a classifier to predict toxicity outcome as before. This new classifier using the combined behavior of functionally related genes as reflected in the “gene set” profiles increased the correct classification to 86%. This result is consistent with the conclusions of the earlier study of in vivo skin irradiation, where grouping genes by pathways resulted in a clarification of radiation response patterns that had been obscured by inter-individual variation.

Bringing the “big picture” into focus

While a variety of increasingly sophisticated techniques are revealing more subtle details of both general and low-dose-specific transcriptional responses to ionizing radiation, a consistent broad consensus picture remains elusive. A number of methods have been developed to try to reconstruct, or “reverse engineer,” the networks that link co-regulated genes identified in global expression profiling studies. One such, ARACNE, has been developed specifically to cope with the complexity of interactions within the mammalian transcriptome [38]. After identifying significantly co-regulated genes by mutual information, it removes relationships between genes that are co-regulated through intermediaries to reconstruct networks consisting mainly of direct regulatory interactions. Analysis of a large data set of B-cell gene expression profiles yielded a network with a small number of highly connected genes representing hubs, with the transcription factor MYC being identified as one of the most prominent [39]. In such network analyses, transcription factors often emerge as

“hubs” that regulate the expression of large numbers of other genes. While this sort of reverse engineering of networks solely on the basis of gene expression data can begin to build up an overall model of cell function and response, some interactions are not mediated at the transcriptional level, but rather by mechanisms like protein modification or sub-cellular localization. Such effects are known to be important in radiation response, and cannot be detected or inferred from gene expression profiling studies alone, so that integration of information from other levels of the cell will clearly be needed.

Another increasingly popular approach to reconstructing cellular networks uses information in protein interaction databases to build interaction networks onto which gene expression data can be mapped. This has the advantage of adding known protein–protein interactions or transcription factor binding to connect coordinately expressed genes through common regulatory mechanisms or functional pathways. It represents a first step toward integrating information from different cellular levels. The Cytoscape project [40] exemplifies an open source implementation of this approach, while commercial software packages such as Pathway Studio [41] are also available.

Organisms with simpler genomes, such as yeast, have provided good models for the integration of results from gene expression profiling and other global measurements. For example, the yeast galactose utilization pathway has provided a “simple” metabolic pathway that can be systematically perturbed. Integration of information from microarray hybridizations and quantitative proteomics studies with known physical interactions and specific gene knock-outs suggested mechanisms of metabolic pathway regulation and physical interactions with other metabolic pathways and cellular processes [42]. Similar approaches in yeast have also been applied to the more complex cellular perturbations in response to a DNA damaging agent, integrating global measurements of transcription factor binding profiles, targeted genetic knock-outs, global gene expression, and protein interaction data [43]. In this case, the analysis revealed DNA-damage-specific transcription factor binding motifs not previously described, while the resulting regulatory pathway map provided a unified view of the multi-level cellular response to DNA damage that could be used to generate testable hypotheses.

The relatively modest scale of the *Saccharomyces cerevisiae* genome has enabled global genomic phenotyping of yeast thanks to the availability of a complete set of single-gene deletion mutants for all non-essential genes in the genome. In this model system, each knocked-out gene has been replaced by an expression cassette encoding a unique “bar code” sequence so that the relative abundance of each knock-out strain in a pooled culture can be

measured by hybridization to an oligonucleotide array. This powerful system has been used to identify genes that confer sensitivity or resistance to a number of different stressors, including ultraviolet radiation [44], chemotherapy agents [45], and ionizing radiation [46]. Global genomic phenotype profiles of genes contributing to survival of yeast after exposure to ultraviolet or ionizing radiation, cisplatin, or hydrogen peroxide, were compared to transcriptional profiles of the genes responding to these same stresses [47], but the genes responding to a specific DNA damaging agent were found to differ from those impacting survival, highlighting again the complexity of these responses. A similar study has gone a step further using information from the *S. cerevisiae* protein interaction database to build a model of the yeast interactome, and map onto it the results of genomic phenotyping of responses to methyl methanesulfonate, 4-nitroquinoline-*N*-oxide, *tert*-butyl hydroperoxide, and ultraviolet radiation [48]. This enabled extraction of pathways and protein complexes that were most central to regulation of specific toxicological responses. These were further found to exhibit topological properties very similar to those of essential proteins. While this sort of whole-genome single gene knock-out approach may be impractical for human cells, analogous high-throughput approaches are being pioneered using libraries of small interfering RNAs [49] and may provide similar systematic genomic phenotyping capabilities.

As the availability of methods for obtaining complete “-omic” scale measurements of RNA and other biomolecules and relevant states continues to increase, more information on global responses to ionizing radiation will become available. What is learned from these measurements will likely depend on our ability to integrate the results across biological levels to build coherent models that can describe the biological basis for radiation responses. The ultimate hope might be that such models would allow prediction of organ-, tumor-, or individual-specific responses to a radiation challenge, thus enabling personalization of cancer treatment regimens or radiation risk assessment. This will most likely require integrating the already complex global measurements with the higher order structure of whole organisms [50]. While such a vision is still far from reality, building toward the required understanding through the use of simpler models, such as the integromic studies of yeast or the NCI-60 cancer cell lines, can provide both mechanistic insight, and the development of new analysis methods for information integration and data mining.

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