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# The transcriptomic revolution and radiation biology

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## Abstract

**Purpose:** This article will briefly review the origins and evolution of functional genomics, first describing the experimental technology, and then some of the approaches applied to data analysis and visualization. It will emphasize application of functional genomics to radiation biology, using examples from the author's work to illustrate several key types of analysis. It concludes with a look at non-coding RNA, alternative reading of the genome, and single-cell transcriptomics, some of the innovative areas that may help to shape future research in radiation biology and oncology.

**Conclusions:** Transcriptomic approaches have provided insight into many areas of radiation biology and medicine, and innovations in technology and data analysis approaches promise continued contributions to radiation science in the future.

### Keywords

ionizing radiation; gene expression; transcriptomics

## Introduction

Exposure to ionizing radiation results in damage to all cellular components, triggering a network of signaling cascades, especially as a response to DNA damage. These signaling cascades coordinate cellular and tissue-level responses including DNA repair, cell cycle arrest, apoptosis, fibrosis, and immune and inflammatory responses. These responses are often directly mediated by post-translational protein modifications that alter protein binding, activity or sub-cellular localization, however, changes in gene expression programs are also recognized as a central component of radiation signaling and response (Amundson 2008). The study of gene expression changes has historically contributed to our understanding of the molecular mechanisms of radiation response, and evolving technologies continue to accelerate such studies.

The study of DNA damage-inducible genes developed out of the SOS response, an error-prone DNA repair system activated in *Escherichia coli* by exposure to ultraviolet radiation (UV) or alkylating agents (Radman 1975). A set of five coordinately regulated SOS genes were initially cloned in *E. coli* (Kenyon and Walker 1980), with many more such genes

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identified since. Six DNA damage responsive genes were also identified in yeast, with early estimates that as many as 80 yeast genes might be DNA damage inducible (Ruby and Szostak 1985).

Several DNA damage-inducible genes, including metallothionein Ila, urokinase-type plasminogen activator, and several keratins, were identified in mammalian cells using hybridization subtraction screening of cDNA libraries (Angel et al. 1986; Rotem et al. 1987; Kartasova et al. 1987). This procedure can only identify very abundant transcripts, however. Soon, it was shown that low-abundance transcripts could be enriched and differential expression could be identified using low-ratio hybridization subtraction screening (Fornace and Mitchell 1986). This approach was used to identify UV induction of metallothioneins I and II, and at least 18 novel sequences (Fornace et al. 1988), many of which were later shown to also respond to ionizing radiation.

In the early 1990s the introduction of RT-PCR-based differential display techniques provided another technical boost to studies of differentially expressed genes (Liang and Pardee 1992). More radiation responsive genes were reported, but because cloning and sequencing was required to determine the identity of individual hits, progress remained slow, with most studies describing only one or a few new radiation-induced genes and following a reductionist approach to their study (Gomez et al. 1996; Yan et al. 1996; Chang-Liu and Woloschak 1997; Goltry et al. 1998; Noel et al. 1998; Okamura et al. 2001). While the earlier focus had been on genes with increased expression following DNA damage, differential display experiments also started to identify genes with decreased abundance after irradiation (Woloschak et al. 1995; Paunesku et al. 2000; Watson et al. 2000; Zhou and Rigaud 2001). As an increasingly complex picture of the transcriptional response to DNA damage began to emerge, it became clear that multiple cellular processes, including apoptosis (Paunesku et al. 2000; Okamura et al. 2001), cell cycle regulation (Gomez et al. 1996; Zhou and Rigaud 2001), and cellular signal transduction pathways (Yan et al. 1996; Watson et al. 2000) could be impacted at the level of mRNA abundance.

## Experimental approaches for functional genomics

As the human genome project of the 1990s provided an increasing amount of gene sequence information, a shift in research focus from genomics to functional genomics began. Once all coding sequences had been determined and mapped, the next task would be to understand how the genome was used in a dynamic fashion to achieve cell differentiation and specialization, and to respond to environmental signals and challenges. The introduction of cDNA microarrays was to be revolutionary in many biological fields, including radiation biology.

The first cDNA microarray consisted of 45 arabidopsis genes that were robotically printed onto a glass slide (Schena et al. 1995). Two samples could be labeled with different fluorochromes and the relative expression levels of each gene on the array could be measured simultaneously. Early human cDNA arrays surveyed around 1000 genes, and were used to detect both known and novel genes responding to heat shock and phorbol ester (Schena et al. 1996) or to explore differences related to tumorigenicity (DeRisi et al. 1996).

Despite the technical demands of maintaining and annotating large cDNA libraries and producing consistently printed microarrays, as well as early informatics challenges, the microarray technique was soon applied to the study of the radiation-responsive transcriptome. In the first such study (Amundson et al. 1999), we reported 18 known and 30 new gamma-ray responsive sequences in a human myeloid cell line, and showed different patterns of response to various stress agents in different human cell lines. The radiation responsive genes newly identified in this study included *ATF3* and *FOSL1*, which were both shown to have some level of p53 dependence for their radiation response. This study was followed closely by the initial description of potential blood biomarkers for radiation exposure identified using the same cDNA microarray approach (Amundson et al. 2000).

Around the same time, photolithographic techniques were being applied to construct arrays of short oligonucleotides that did not rely on libraries of cDNA clones (Pease et al. 1994), although they were still limited to known sequences. This approach was commercialized by Affymetrix and widely adopted by many institutional core facilities, making the technology widely accessible.

Long oligonucleotide microarrays, either printed or synthesized in situ, also became commercially available (Ben-Dor et al. 2000; Ramakrishnan et al. 2002). These could be used with two-color hybridization protocols similar to cDNA arrays, but their high degree of standardization and quality control also enabled comparison between samples hybridized to different microarray chips. My group has used the Agilent long oligonucleotide microarray platform to study radiation bystander responses (Ghandhi et al. 2008; Ghandhi et al. 2010; Ghandhi et al. 2011; Ghandhi et al. 2014) and to build on our initial radiation biodosimetry work using human (Paul and Amundson 2008; Paul and Amundson 2011; Broustas et al. 2017; Ghandhi et al. 2019), mouse (Broustas et al. 2018; Mukherjee et al. 2019; Paul et al. 2019; Ghandhi et al. 2020), and non-human primate (Park et al. 2017; Ghandhi et al. 2018) models. Recent reviews (Lacombe et al. 2018; Zhao et al. 2018; Amundson 2021) demonstrate that many laboratories, using various whole-genome transcriptomic techniques, continue to contribute to what is now a considerable body of radiation biodosimetry gene expression work.

More recently, transcriptomic profiling has come full circle, with RNA-Seq, like earlier profiling methods, not requiring *a priori* knowledge of gene sequence for detection of differential expression (Wilhelm et al. 2008; Nagalakshmi et al. 2008). As sequencing costs have become more competitive with microarrays, RNA-Seq is poised to become the dominant technology for transcriptomic studies and appears to be gradually replacing microarrays (Figure 1). The ability to discover differential expression of previously unidentified genes may be particularly useful in understanding stress responses or disease states, where induced or altered transcripts may not have been represented in the libraries used to define the human genome.

## Functional genomics data analysis and visualization

From the early use of printed cDNA microarrays it was almost immediately apparent that the usefulness of whole-genome profiling would be limited mainly by our ability to process

and make sense of the huge amounts of data it had now become possible to generate from single samples. Early analyses mainly focused on detecting genes that had significantly different expression levels in different samples. Statistics-based tools, such as DeArray (Chen et al. 1997), provided an improvement over the direct visual or numeric comparison of individual probe intensity between two samples. It was also recognized that performing tens of thousands of simultaneous comparisons could result in many genes achieving apparent statistical significance by chance. Thus, analysis methods incorporating multiple comparison corrections and estimates of the False Discovery Rate (FDR), such as SAM (Significance Analysis of Microarrays) (Tusher et al. 2001) were developed specifically for the identification of genes differentially expressed between groups using microarray data.

Data visualization tools can also be extremely useful for extracting meaningful patterns from large-scale expression data. Many of these are available along with statistical analysis tools, from non-specific statistical tests such as ANOVA or t-tests to multiple comparison adjustment methods, in both commercial and open source platforms. For instance, a huge variety of analysis and visualization tools are available as Bioconductor or other R packages (Carey et al. 2007; Zhang et al. 2009; Huber et al. 2015; McDermaid et al. 2019). User-friendly interfaces are also available, such as BRB Array-Tools (Simon et al. 2007), which provides streamlined access to a curated suite of R packages for microarray data analysis.

Clustering algorithms, such as hierarchical clustering, K-means clustering, and self-organizing maps are often applied to microarray data (Do and Choi 2008; Zhu et al. 2008). These algorithms use different approaches to compare expression patterns of selected genes across all samples in an experiment to group the most similar patterns together. Early studies in yeast found that genes with similar function or shared up-stream regulators could be grouped together in this way (Eisen et al. 1998). Samples can also be clustered as well as genes, for instance to reveal similarities between different tumor types, or to reveal novel sub-types with specific gene expression signatures (Jazaeri et al. 2002). Clustered genes can be displayed as heatmaps, with a row for each gene, a column for each sample, and color intensity representing the relative level of gene expression. Two colors can be used to represent ratios of expression so that both increases and decreases can be clearly visualized, while a single color can be used to represent expression intensities. Figure 2A shows an example of an intensity heatmap visualizing K-means clustering of expression levels of blood genes that significantly responded to an $LD_{50}$ radiation dose in wild-type mice. Two clusters, representing up-regulated and down-regulated genes, are clearly visible in the wild types, and general ablation of the response can be seen in the two mutant strains. Details of the studies generating this data have been published (Rudqvist et al. 2018).

Multi-dimensional scaling (MDS) algorithms, such as principal components analysis, can be used to reduce an n-dimensional gene expression space to a 2- or 3-dimensional projection, while maintaining the relative relationships between the samples (Raychaudhuri et al. 2000). In practical terms, this enables visualization of the overall similarity of samples based on the expression of a selected set of genes. The example in Figure 2B illustrates a 72-gene signature of radiation dose (Paul and Amundson 2008) monitored in human blood samples between 6 and 48 hours after radiation exposure (Paul et al. 2013). Each point represents an individual sample, with different colors representing different radiation doses from 0 to 8

Gy, and different shapes representing 6, 24, or 48 hours after irradiation. In this example, it can be seen that the samples cluster by dose, but also separate by the time since exposure, with the effect of dose beginning to diminish after 48 hours compared with the earlier time points.

## Network analysis and upstream regulators

Genes that demonstrate patterns of co-expression may also be co-regulated by common upstream pathways. Chromatin Immunoprecipitation (ChIP) assays (Rodríguez-Ubreva and Ballestar 2014) can be used to isolate the DNA sequences bound to a specific transcription factor of interest, such as NFκB or TP53, followed by microarray or sequencing-based identification of the differentially bound genes. Integration of such ChIP-Seq results with expression profiling of the same experimental conditions (Jiang and Mortazavi 2018) can provide insight into mechanisms of transcription regulation following radiation exposure (Janus et al. 2018; Szołtysek et al. 2018; Hafner et al. 2020).

A large amount of non-radiation specific DNA-protein and protein-protein binding data is also publicly available through sources such as the Biomolecular Interaction Network Database (BIND) (Alfarano et al. 2005). In conjunction with gene expression results, such protein-binding information can be used to construct and visualize putative regulatory interaction networks. Free platforms, such as Cytoscape (Shannon et al. 2003), as well as commercial solutions, such as Ingenuity Pathway Analysis (IPA) (Krämer et al. 2014), are available to perform network analyses. We previously used BIND and Cytoscape to build a regulatory network of a strongly down-regulated cluster of TP53-independent radiation responsive genes in the cell lines of the NCI-60 panel. The network analysis indicated E2F4 as a potential upstream regulator of this response, and we were able to confirm response of E2F4 to radiation (Amundson et al. 2008).

A reanalysis of the same NCI-60 gene expression data using IPA is illustrated in Figure 2C. In this case, IPA was first used to predict putative upstream regulators of the genes in the down-regulated cluster. IPA assumes a normal distribution of gene up- or down-regulation for each potential upstream regulator-gene connection, and calculates a z-score (number of standard deviations from the mean) to determine the significant over-representation of “activated” or “inhibited” predictions. For each potential regulator, a z-score of ≥2 is taken as significantly likely to be activated, and a z-score of ≤−2 as significantly inhibited. E2F4 was again predicted to be activated by radiation, but additional transcription factors were also predicted as possible upstream regulators of the down-regulated genes. The strongest predictions were for activation of NUPR1 (z-score 3.5) and inhibition of FOXM1 (z-score −3.7). FOXM1 is a known regulator of cell cycle genes with roles in carcinogenesis (Myatt and Lam 2007), and its down-regulation may enhance radiosensitivity (Nagel et al. 2015; Xiu et al. 2018). Consistent with our prediction from the NCI-60 data, NUPR1 has been shown to be induced by multiple cellular stressors, including ionizing radiation (Gironella et al. 2009). We have also previously reported IPA-predicted activation of NUPR1 by direct irradiation with 123 keV/μm $^{4}$He ions, and inhibition in un-irradiated bystanders of the same cells (Ghandhi et al. 2014). This example illustrates the common finding that reanalysis of

transcriptomic data using different or updated approaches generally confirms older findings, but also often suggests additional directions for further investigation.

## Gene ontology

Gene ontology analyses are also commonly employed to gain insight into the biological functions likely to be affected in an experiment. The Gene Ontology (GO) consortium maintains annotations of biological processes and molecular functions of genes and gene families (The Gene Ontology Consortium 2019). Other databases, such as KEGG (the Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al. 2017), and Reactome (Jassal et al. 2020), organize genes into pathways, while databases, such as InterPro (Mitchell et al. 2019), SMART (Simple Modular Architecture Research Tool) (Letunic and Bork 2018), or PANTHER (Protein ANalysis THrough Evolutionary Relationships)(Thomas et al. 2003), classify protein functional domains or motifs and associate them to coding genes. Many freely available analysis platforms allow users to query gene lists against multiple such databases in order to look for terms that are overrepresented compared to their expected occurrence in a random gene list of the same length. These tools implement methods to correct for multiple comparison, and include DAVID (the Database for Annotation, Visualization and Integrated Discovery) (Huang et al. 2009), PANTHER Tools (Mi et al. 2019), AmiGO (Carbon et al. 2009), and ToppFun (Transcriptome, ontology, phenotype, proteome, and pharmacome annotations based gene list Functional enrichment analysis) (Chen et al. 2009). Gene Set Enrichment Analysis (GSEA) (Subramanian et al. 2005) is a related approach for revealing enriched biological processes through comparison of experimental results with pre-defined gene lists. An advantage of this approach is that in addition to GO or other annotated categories, any curated gene list can be used for comparison.

## Gene expression based classifiers

Numerous algorithms have been applied to develop predictive classifiers based on gene expression. These include such approaches as linear discriminant analysis, the Bayesian compound covariate predictor, and shrunken centroid, nearest centroid and nearest neighbor classifiers (Simon et al. 2007). Artificial intelligence approaches, including random forest classification, support vector machines, radial bias function neural networks, and multilayer perceptron neural networks can also be applied (Pirooznia et al. 2008). Such classification algorithms have been used for gene expression based prediction of the likelihood of cancer metastasis (van't Veer and Bernards 2008), and to predict radiosensitivity (Torres-Roca et al. 2005; Williams et al. 2011; Williams et al. 2017) or the risk of normal tissue damage (Nuyten and van de Vijver 2008; Lyngholm et al. 2015). They have also been applied in the radiation biodosimetry arena. For example, one early study used Bayesian regression models (West et al. 2001) with leave-one-out cross validation to classify samples from mice as controls or radiation exposed (Dressman et al. 2007). Similarly, a nearest centroid classifier with leave-one-out cross validation was used for non-binary classification of human blood samples irradiated ex vivo as either unexposed, 0.5, 2 or ≥5 Gy (Paul and Amundson 2008). In a different application, independent training and test sets were used with seven classification algorithms (linear discriminant analysis, Bayesian compound covariate predictor, nearest centroid, compound covariate predictor, 1- and 3-nearest neighbors, and

support vector machines) to demonstrate improved binary classification of irradiated versus control samples across DNA-repair deficient mouse strains when training sets included all genotypes (Rudqvist et al. 2018).

For binary classifiers, an algorithm's performance is commonly illustrated using a receiver operating characteristic (ROC) curve, such as the example in Figure 2D. The ROC curve in the example shows a diagonal linear discriminant analysis using 20 genes selected with a greedy pairs algorithm to discriminate at 1, 2, or 3 days after exposure between mice given a lethal dose of radiation (10 Gy) and mice given survivable doses from 0–6 Gy (Paul et al. 2019). By plotting sensitivity (the true positive rate) against 1-specificity (the false positive rate) it visualizes the trade off between sensitivity and specificity. The point in the extreme upper left corner of the graph represents perfect classification, and the diagonal line represents completely random classification. Classifier performance is reported using the area under the curve (AUC), which would be 1 for perfect classification, and for the case illustrated is 0.92.

## Time series analysis

As reflected in the MDS plot in Figure 2B, gene expression in response to radiation or other stresses is a highly dynamic process, and a single microarray or RNA-Seq analysis only provides a snapshot of an instant in time. Time-course data, or more complex experiments, such as a dose response as a function of time, or comparison of multiple irradiation conditions, can become unwieldy to analyze. Pattern clustering approaches, developed for the analysis of time-course data, cluster curves rather than individual points, and can provide insight from time courses or other complex data. One approach developed for transcriptomic data is the Short Time series Expression Miner (STEM) algorithm, which clusters genes into pre-defined patterns based on "units of change" as a function of time and tests significance using a permutation test (Ernst and Bar-Joseph 2006). A Feature Based PAM (Partitioning Around Medoids) Algorithm (FBPA) that incorporates biologically relevant features to summarize gene expression over time has also been developed to cluster gene expression curves without comparison to pre-defined profiles (Sinha and Markatou 2011). In a direct comparison between STEM and FBPA, we were able to extract more biologically relevant clusters from radiation bystander data using FBPA, including identification of a novel methylation pathway involved in the bystander response (Ghandhi et al. 2011). We have also found the maSigPro R package (Conesa et al. 2006) useful for analysis of complex radiation response data sets, including a study monitoring gene expression for two weeks in response to different amounts of internally deposited $^{137}$Cs (Ghandhi et al. 2020) and a dose-response study of gene expression covering the first week after external-beam gamma irradiation of mice (Paul et al. 2019). Two of the gene response patterns identified by maSigPro in the latter study are illustrated in Figure 2E. Both represent down-regulated genes, but the genes in these two clusters show very different time and dose relationships. In the first pattern, expression levels decrease during the first few days, and then begin to recover toward control values in a dose dependent manner. Only mice exposed to a lethal 10 Gy dose show no recovery of expression levels. In contrast, the second pattern shows a strong dose dependence, but little time dependence, with gene expression dropping rapidly by the first day after exposure, then remaining at a fairly consistent level throughout the study.

### Public accessibility of data

There is an increasing movement of support for open science and open data of all kinds. The wealth of functional genomic data being generated in radiation studies represents a great potential resource for the field. There are a number of repositories available to ensure its continued public availability. Some, such as the Gene Expression Omnibus (Barrett et al. 2009) or ArrayExpress (Brazma et al. 2003) are general transcriptomic repositories, in which it can be difficult to find relevant radiation experiments. Specialized gene expression databases have been developed targeting the radiation community, including Radiation Genes (Chiani et al. 2009) and the NASA GeneLab (Beheshti et al. 2018; Berrios et al. 2021), which integrates radiation datasets from transcriptomic, proteomic, and metabolomic studies. Such efforts, combined with the ongoing development of increasingly powerful analysis tools, which can be applied to existing public datasets, will help ensure that the maximum insight can be derived from all transcriptomic experiments, past and future.

## Future directions for radiation functional genomics

Since the initial introduction of cDNA microarrays, functional genomics techniques have expanded to enable an increasingly broad range of studies. Molecular biology has uncovered new levels of regulation by non-coding transcripts and alternatively spliced forms of known genes (Mortazavi et al. 2008). The increasing accessibility of whole-genome RNA-Seq and its ability to detect novel transcripts, as well as third-generation sequencing providing full transcript reads (Cruz-Garcia et al. 2020a), will likely enhance the study of these regulatory layers and their roles in radiation response and disease. Advances in both technology and informatics are also accelerating studies of the heterogeneity of radiation response on the single cell level. These promising directions are discussed briefly below.

### Non-coding RNA

When one of the original “growth arrest and DNA-damage inducible” genes, *Gadd7*, was found to exert growth arrest properties but to lack a protein product (Hollander et al. 1996), it was an intriguing curiosity, but the observation then languished for several decades. Now, however, non-coding RNAs (ncRNA) are understood as key regulatory factors, with roles in diverse processes from chromatin remodeling and gene transcription to protein translation. The importance of ncRNAs is underlined by the finding that their dis-regulation appears to be involved in many pathological states, including cancer (Schmitt and Chang 2013; Choudhari et al. 2020). Although multiple varieties of ncRNA are now known, long non-coding RNAs (lncRNA, such as *Gadd7*), micro RNAs (miRNA), and circular RNA (circRNA) in particular appear to contribute to the response to radiation, and may be useful as both biomarkers and therapeutic targets (May et al. 2021).

Investigation of miRNA contributions to radiation response was initially enabled by microarray platforms or low-density TaqMan arrays (Goulter et al. 2006). We used the TaqMan low-density array approach to develop miRNA signatures that could classify mouse blood samples as coming from unexposed, low LET exposed or heavy-ion exposed mice with high accuracy (Templin et al. 2011). We also found that accurate classification of human samples from total body irradiation patients was possible using miRNA signatures

(Templin et al. 2011). Comparison of miRNA expression in that study with global mRNA expression measured in the same patients (Paul et al. 2011) further identified a set of 37 genes that were downregulated after irradiation, and were also predicted to be targets of consistently upregulated miRNAs. Several biological processes including hematopoiesis and immune response were over-represented among these genes, suggesting a role for miRNA regulation in their response to radiation.

Studies of ncRNA in the context of radiation response continue to expand. Both miRNA and lncRNA have been found to participate in regulating DNA double-strand break repair (Thapar 2018), a critical response to ionizing radiation exposure. Specific lncRNA (Jiang et al. 2017; Hu et al. 2019; Ma et al. 2018), miRNA (Weidhaas et al. 2007; Zhang et al. 2011), and circRNA (Guan et al. 2020; Niu et al. 2020), have been associated with alteration of radio-resistance of human tumors, and may present targets for modification of radiation response. The development of normal tissue damage may also be modulated by ncRNA, with the lncRNA WWC2-AS1 found to be a regulator of radiation-induced fibrosis (Zhou et al. 2019).

The relative stability of ncRNA species, and their presence in serum and in exosomes, also makes them attractive targets for the development of radiation biodosimetry (Jacob et al. 2013; Beer et al. 2017; Aryankalayil et al. 2018; Yadav et al. 2020). miRNA may also provide tissue-specific biomarkers of radiation damage (Khan et al. 2013; Menon et al. 2016; Rogers et al. 2020), or early predictors of death following irradiation (Acharya et al. 2015; Tomasik et al. 2018).

## Variant transcripts and translation

Alternative reading of the genome, including the use of alternative transcription start sites, alternative splicing leading to different exon usage, alternative polyadenylation and alternative initiation of translation can all contribute to the functional regulation of the genome, and the broad extent of these alternative processes is being revealed through RNA-Seq (de Klerk and ‘t Hoen 2015). These mechanisms can also play a part in the response to radiation. Ionizing radiation has been shown to induce transcription using alternate promoters in *MDM2* (Barak et al. 1994), *PPM1D* (Rossi et al. 2008), *RRM2B* and *XPC* (Forrester et al. 2012), as well as a number of other genes (Sprung et al. 2011). Alternative splicing of genes following irradiation is also being increasingly reported, particularly in the context of radiation biodosimetry (Macaeva et al. 2016; Wahba et al. 2018). The use of radiation-induced exon expression normalized against intragenic control exons of genes including *MDM2*, *PPM1D*, and *FDXR* has been suggested as an attractive approach to provide more robust radiation biodosimetry (Forrester and Sprung 2014; Cruz-Garcia et al. 2020b).

The use of different promoters in irradiated cells has also been linked to different translational profiles for those genes (Barak et al. 1994; Rossi et al. 2008). More broadly, a microarray analysis of polysome-bound mRNA found that radiation exposure had a 10-fold greater impact on gene translation than on transcription (Lü et al. 2006). The translational profiles responding to radiation also appear to be tumor type specific, and to differ between tumor and normal cell lines (Kumaraswamy et al. 2008). RNA-Seq analysis of polysome-

bound RNA fractions has revealed a 3-fold enhancement of radiation-induced changes in translation of alternative transcripts compared to their transcription, further emphasizing the interconnection between these levels of functional genomic control (Wahba et al. 2018). Translational control and alternative transcription in response to radiation remain understudied areas worthy of more detailed investigation.

### Single-cell transcriptomics

The heterogeneity of the cellular response to radiation exposure has long been recognized. For instance, variation in the intensity of TP53 antibody staining was observed in different cells within a population exposed to the same radiation dose (MacCallum et al. 2001). Early attempts to look at the radiation transcriptional response at a single cell level also indicated a high degree of cell-to-cell variability (Ponnaiya et al. 2007; Ponnaiya et al. 2013) and suggested within-cell correlation of the expression of genes with common upstream regulatory factors (Shang et al. 2019). However, these studies were limited to the measurement of only one or a small number of genes. More recent advances in both sequencing technology and data analysis have enabled studies in which the transcriptome of individual cells within a sample can be studied, further defining the scope of cellular heterogeneity in the response to radiation (Gao et al. 2021). Such studies have also provided more granular molecular insight into heterogeneous radiation responses, for instance, identifying activation of different signaling pathways in subsets of T-lymphocytes (Moreno-Villanueva et al. 2019), and suggesting novel gene expression changes during the development of radiation resistance during tumor treatment (Wu et al. 2019). Reconstructing cell-type specific radiation signaling networks from single-cell sequencing data has also provided insights into the interplay between radiotherapy and immune cell activity (Formenti et al. 2019).

Further technological refinements are enabling true tissue-level systems biology (Moor and Itzkovitz 2017) and deconvolution of the tumor microenvironment (Wang et al. 2021) through retention of spatial information coupled with single-cell resolution transcriptomics (Teves and Won 2020). These spatial transcriptomic techniques have, for example, been applied to bone marrow stem cell niches to study conditions of homeostasis, carcinogenesis, and response to stresses including radiation (Al-Sabah et al. 2020). Spatial and single-cell transcriptomics hold great promise for the development of immunotherapies (Nerurkar et al. 2020; Castellanos-Rueda et al. 2021), and for our understanding of the radiation response of complex tissues, tumors, and interacting cell types. As functional genomic technologies continue to evolve, it seems certain that the field will have an ongoing impact in diverse areas of radiation biology and radiation oncology.

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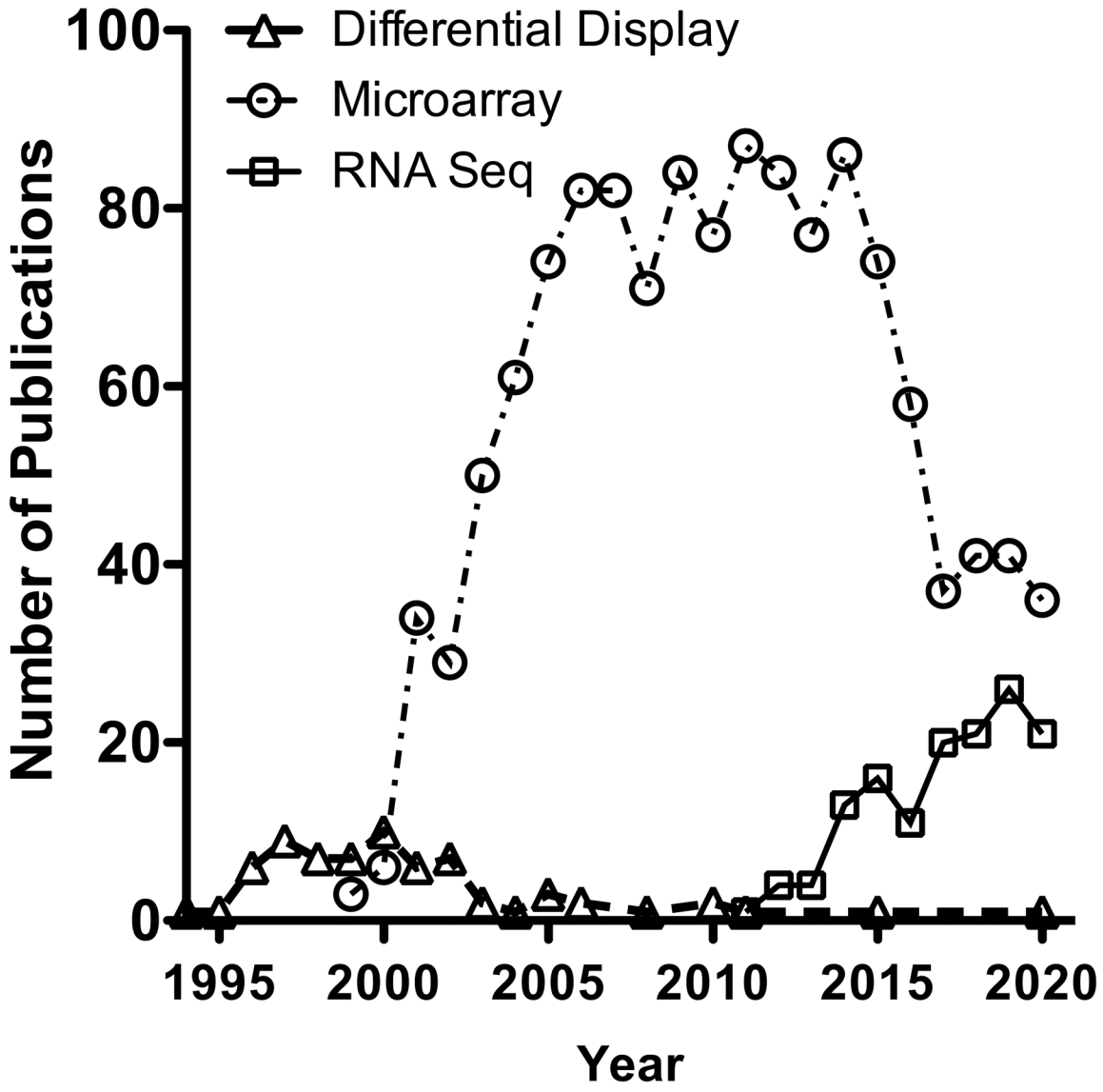


**Figure 1.**
Publications found in Pubmed by searching “ionizing radiation” combined with the terms “differential display”, “microarray”, or “RNA-Seq” plotted by year.

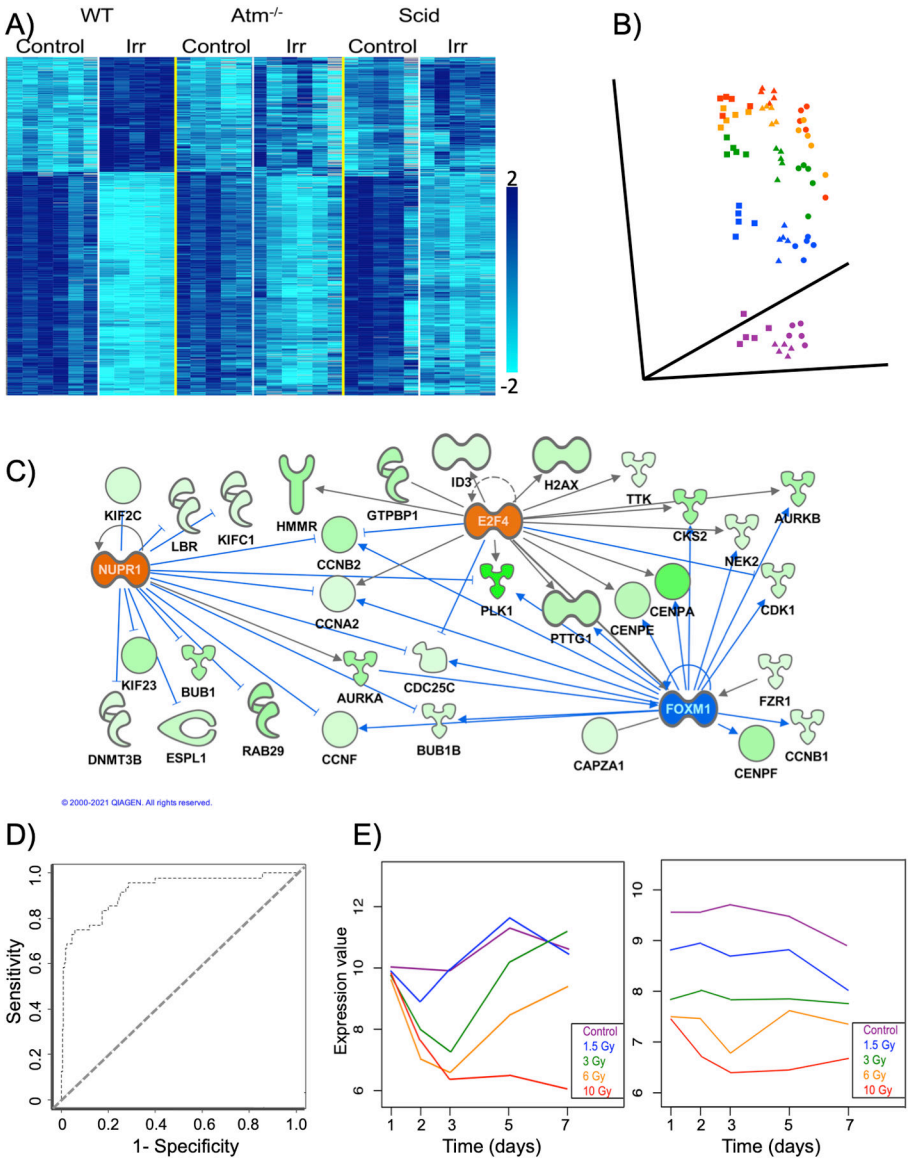


**Figure 2.**

Examples of analysis and visualization tools applied to transcriptomic data.

A) heatmap visualizing K-means clustering of gene expression levels produced using BRB-Array Tools (Simon et al. 2007). Each row represents a gene and each column an individual sample (mouse). Samples are arranged as indicated at the top of the heatmap. WT = wild type; $Atm^{-/-}$ = Atm null; Scid = $Prkdc^{scid}$; Irr = 24 h post irradiation with an $LD_{50/30}$ $^{137}Cs$ γ-ray dose. The data are from Rudqvist et al. 2018. The intensity of microarray hybridization, corresponding to the level of gene expression, is represented by the shade of blue as indicated in the key.

B) Multi-dimensional scaling plot created in BRB Array-Tools representing a 72-gene signature of radiation dose (Paul and Amundson 2008) measured in ex vivo irradiated human blood samples at 6 h (squares), 24 h (triangles), or 48 h (circles) after exposure to 0

Gy (purple), 0.5 Gy (blue), 2 Gy (green), 5 Gy (yellow), or 8 Gy (red) $^{137}$Cs γ-rays. Data from Paul et al. 2013.

C) Network generated in IPA (Krämer et al. 2014) following upstream regulator analysis of data from Figure 2 of Amundson et al. 2008. Transcription factors colored orange were predicted to be activated by radiation, and blue indicates a transcription factor predicted to be inhibited by radiation. Blue lines indicate the regulatory relationships supporting the prediction. Nodes corresponding to the down-regulated genes (green) have been colored to show the relative radiation response in HL60 cells, with darker color corresponding to a greater magnitude of decrease.

D) ROC curve summarizing performance of a diagonal linear discriminant analysis algorithm built using 20 genes in BRB Array-Tools. Mouse blood was sampled at 1, 2 or 3 days after exposure to doses from 0–10 Gy, and the samples were classified as coming from animals experiencing a survivable exposure (0–6 Gy) or a lethal exposure (10 Gy). The AUC (area under the curve) was 0.92, and the data are from Paul et al. 2019.

E) Two distinct down-regulated gene expression patterns from time-course analysis using MaSigPro (Conesa et al. 2006). Mice were exposed to 0–10 Gy γ-rays and sacrificed for gene expression measurement in blood 1–7 days later (Paul et al. 2019). Relative median expression of all genes in the pattern is plotted as a function of time, with a separate curve for each dose (color coded according to the key). The graph on the left represents 1700 genes, that on the right 98.