Minireview

Measuring a cell's response to stress: the p53 pathway

D Paul Harkin* and Peter A Hall†

Addresses: *Department of Oncology, The Queens University, Belfast City Hospital, Belfast, BT9 7AB, UK. E-mail: d.harkin@qub.ac.uk
†Department of Pathology and Laboratory Medicine, King Fahad National Guard Hospital, Riyadh 11426, Kingdom of Saudi Arabia.

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Abstract

The characterization of complex cellular responses to diverse stimuli can be studied by the use of emerging chip-based technologies.

The p53 pathway is critical to maintaining the integrity of the genome in multicellular organisms. The *p53* gene is activated in response to DNA damage and encodes a transcription factor [1], which in turn activates genes that arrest cell growth and induce apoptosis, thereby preventing the propagation of genetically damaged cells. It is the most important known tumor suppressor gene: perhaps half of all human neoplasms have mutations in *p53*, and there is a remarkable concordance between oncogenic mutation and the loss of p53 transcriptional activity [2]. There is also compelling experimental evidence that loss of p53 function (by whatever means) is one of the key oncogenic steps in human cells, along with altered telomerase activity and expression of mutant *ras* [3]. So far, however, relatively few of the genes regulated by p53 have been identified and it is not even known how many binding sites there are for p53 in the genome, although an estimate based on the incidence of the canonical p53 consensus binding site (four palindromic copies of the sequence 5′-PuPuPuGA/T-3′, where Pu is either purine) in a limited region suggests there may be as many as 200 to 300, possibly representing the same number of p53-responsive genes [4]. This makes the p53 response an attractive target for the emerging techniques for global analysis of gene expression, and two recent reports [5,6] illustrate the ways in which these techniques can be used to elucidate the spectrum of genes regulated by this key transcription factor. Vogelstein and colleagues [5] have used serial analysis of gene expression (SAGE) to identify 34 genes that exhibit at least a 10-fold upregulation in response to inducible expression of p53; Tanaka *et al.* [6] have used differential display to identify p53R2, a homolog of ribonuclease reductase small subunit (R2) as a target gene, thereby for the first time implicating p53 directly in the repair of DNA damage.

Targets of p53

The SAGE technology used by Yu *et al.* [5] is based upon the comparison of gene-specific tag-abundance between two cDNA libraries ([7]; see Figure 1). The libraries were generated by a novel inducible expression system from a colorectal carcinoma cell line differing in inducible expression of either wild-type or mutant (transcriptionally dead) p53 [5], and were then interrogated by SAGE to identify a series of cDNAs whose expression depends on p53.

What is notable about the study by Yu *et al.* [5] is not that they were able to identify as many as 34 genes (there are almost certainly more), but rather the heterogeneity of the response when evaluated in different cell lines derived from the same tissue of origin. For example, of 33 genes studied (25 identified by SAGE in addition to 8 previously described p53 target genes), only 9 were induced in a panel of 5 unrelated colorectal cell lines, 17 were induced in a subset and 8 were not induced in any of the 5 cell lines examined. This is consistent with numerous *in vivo* studies [8-11] suggesting a high degree of cell- and tissue-type specificity, possibly reflecting the requirement for cell-type specific p53 transcriptional co-activators. The existence of other p53-related genes (p73 and p63) with similar DNA-binding properties [12,13] but differential abilities to transactivate target genes further complicates the issue [14-16]. Furthermore, there are multiple isoforms of p73 and p63, and numerous distinct post-

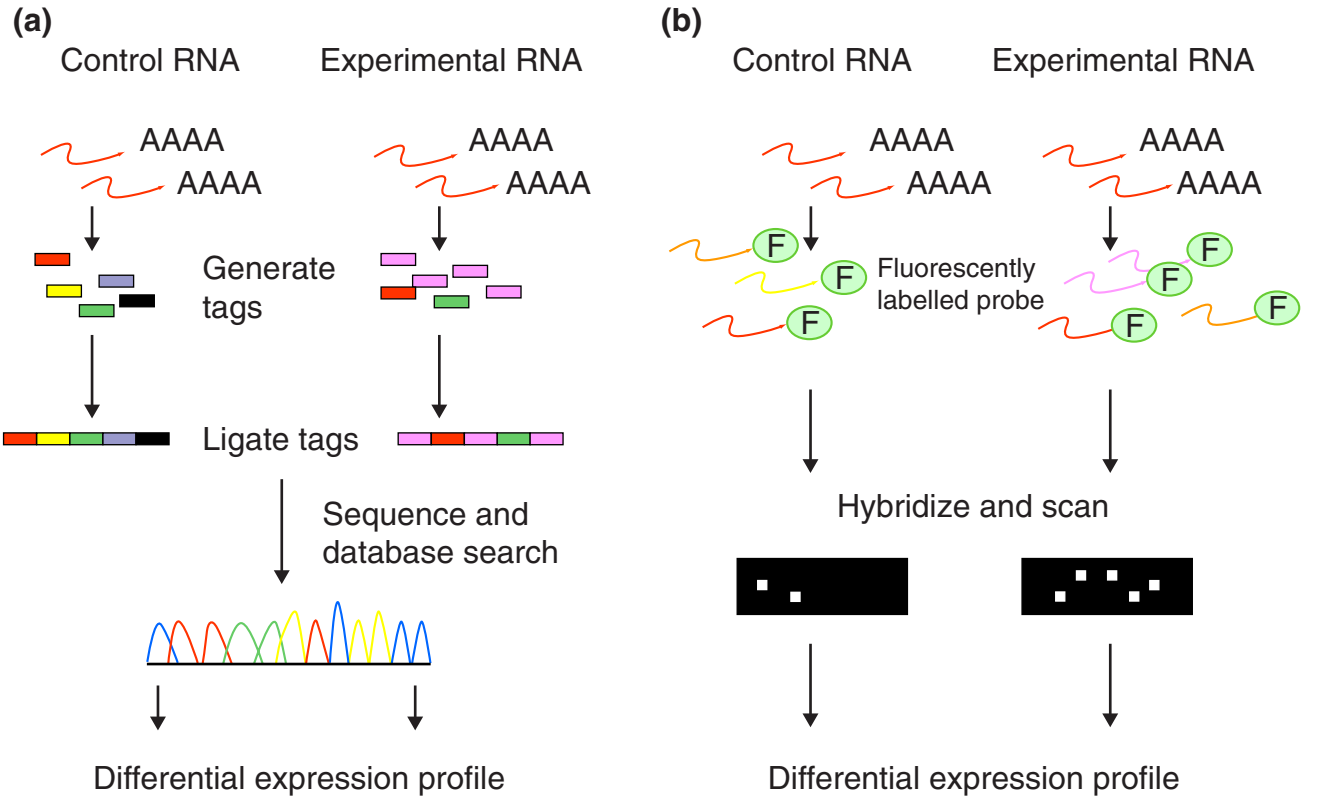

Figure 1
The basic stages involved in **(a)** serial analysis of gene expression (SAGE) and **(b)** microarray technology. (a) SAGE is a sequence-based method of identifying differentially expressed cDNAs between two experimental samples. The technique involves the generation of gene-specific tags typically 10-14 basepairs in length [28]. These tags are then ligated together to form di-tags that contain primer sites on each end to facilitate a polymerase chain reaction amplification step. The di-tags are subsequently ligated to each other to form concatamers of varying length, which are size fractionated and sequenced. The sequence of the individual 10-14 bp tag is then used to interrogate appropriate cDNA/EST (expressed sequence tag) databases to identify the specific gene in question unambiguously. (b) Microarrays, or chips, are arrays of oligonucleotides or cDNAs synthesized or spotted, respectively, onto glass or silicon slides in a predetermined spatial orientation. Total RNA is reverse transcribed, fluorescently labeled and hybridized to the microarray. The protocol for generation of probes and the type of labeling procedure varies depending on the type of array being used. Specific hybridization signals are detected by a fluorescent scanner, which facilitates the identification of the specific grid reference of the target sequence, and, therefore, target identification.

translational modifications of p53 and its homologs may have a wide range of biochemically and biologically distinct functions [1,12,13,16,17].

Further variability was seen when induction of the identified targets was assayed in response to the clinically relevant chemotherapeutic agents adriamycin and 5-fluorouracil (5-FU), DNA-damaging agents previously shown to activate the p53 response. Only six of the genes identified were induced by both agents, suggesting clear target specificity depending on the nature of the inducing signal. More surprising was the observation that - for the majority of the genes identified - p53 was not absolutely required for induction in response to adriamycin and 5-FU. This suggests that these agents do not act exclusively through p53, which further serves to emphasize the redundancy that is inherent in the majority of signaling pathways. Another important point is that, contrary to the generally held dogma, the products of the genes identified by Yu *et al.* [5] are by no means restricted to roles in growth arrest and apoptosis. A recent report from one of our own laboratories [14], in which the rate-limiting step in melanin biosynthesis, tyrosinase, is shown to be a p53-responsive gene, is a further example of this. So is the study by Tanaka *et al* [6].

To identify p53R2 as a p53 target, Tanaka *et al.* used inducible expression of p53 along with differential display. This, like SAGE, is based on the polymerase chain reaction (PCR) method and used for measuring the relative abundance of mRNAs under two different experimental conditions (in this case, induction of p53 expression in cell lines with and without a functional copy of the *p53* gene). They then demonstrated that p53R2 plays an important role in the repair of DNA damage in response to ultraviolet irradiation or exposure to adriamycin, thereby demonstrating for the first time evidence of a direct link between induction of p53 and DNA repair.

Together, these observations raise the possibility that p53 can regulate a range of homeostatic adaptive responses and not simply apoptosis and growth arrest (reviewed in [1]). The experiments of Tanaka *et al.* [6] suggest that it is possible to identify an important physiological target of p53 with this sort of approach, and those of Yu *et al.* [5] open up avenues for further exploration of the complex physiology of the p53 response. Global analyses of this kind have some limitations, however, that it is worth bearing in mind. First of all, the levels of p53 induced in these experiments are well above the physiological range; and second, there are inherent limitations to SAGE analysis, some of which also apply to other technologies for expression profiling.

Artificial induction of p53

Because natural inducers of p53 (such as adriamycin or 5-FU) also induce transcriptional changes unrelated to the action of p53, most experiments on p53 involve the use of cells transfected with constructs containing a promoter that allows the gene to be artificially induced by agents with no natural effect on the cell. In the case of Yu *et al.* [5], the p53 gene was fused to the tet operator and introduced into cells along with the tet repressor fused to the powerful viral transcriptional activator domain VP16. This chimeric regulator is activated in the presence of tetracycline to bind to promoters containing the tet operator sequence and was used to drive expression of p53. Tanaka *et al.* [6] used the lac operon and an adenoviral activator fused to LacZ to drive p53 transcription on induction by isopropylthiogalactoside (IPTG).

The p53 protein levels seen in such regulatable expression systems may be much higher than the physiological levels found *in vivo*. That the level of p53 protein can profoundly influence the resultant biological response in a cell has been elegantly shown in previous studies [18,19]. This needs to be borne in mind for much of the enormous p53 literature, which in many cases reports data based upon grossly non-physiological levels of p53 protein. Yu *et al.* [5] address this with a follow-up experiment in which they test for induction of the identified genes in response to adriamycin, a physiologically relevant stress. Most of the genes identified by SAGE following exogenous expression of p53 were found to be induced in response to adriamycin in at least one of the five cell lines tested, thereby validating these genes as physiologically relevant targets of p53. Tanaka *et al.* [6] measured the incorporation of dNTPs into DNA damaged by adriamycin before and after inhibition of p53R2 with antisense DNA.

Thus, while overexpression systems may identify large numbers of possible targets, further physiological screens will always be needed to confirm the relevance of a particular target gene. Inevitably the study of physiological systems (*in vivo*) will in the future be the desirable strategy. For example, the analysis of libraries generated from cells and tissues derived from genetically defined mouse strains treated with particular insults may generate novel insights into the transcriptional complexity *in vivo*.

The future of such experimental strategies will depend upon the ease of use, reproducibility and ultimately the cost of the assay systems. SAGE and other PCR-based methods such as differential display have many attributes but also clear disadvantages. The introduction of microarray technology based on high-density two-dimensional arrays of cDNAs or chemically synthesized oligonucleotides ([20,21]; see Figure 1) is likely ultimately to displace most other techniques for identifying differential expression patterns between samples.

SAGE versus chip technology

It is useful to consider the relative merits of array-based (chip) technologies and SAGE. Both methods measure relative message abundance (test versus control) and thus share the limitation that while this may often be a consequence of transcriptional regulation, other mechanisms can (and do) contribute (for example, message stability). (This also applies to the differential display technology used by Tanaka *et al.* [6].) SAGE allows a comparative quantitative analysis of transcript abundance in specific cell or tissue types in an approach that relies on sequencing of gene-specific tags (see Figure 1). Because these tags are derived from cDNA made from polyadenylated RNA from the cells under investigation, SAGE can identify transcripts that would be missed by techniques that rely on hybridization to genome-based arrays of known gene sequences. For example, comparison of SAGE tags against published genomic sequence can identify genes not previously identified by gene-finding programs. A major drawback of SAGE, however, is the technical complexity of generating the gene-specific di-tags as a concatamer for sequencing (see Figure 1). Another problem is the requirement for large quantities of high quality mRNA, and this may become a limiting factor for analysis of rare tissue samples. In order to address this issue, a modification of SAGE has been developed called SAGE-lite, which typically requires as little as 100 ng of total RNA per experimental sample and is proposed as a solution to the problem of limited starting material. This technique, however, requires the introduction of an additional PCR step, which may result in non-representative amplification and, therefore, bias later quantitative steps in the protocol [22].

In contrast, chip methods rely upon creating a high-density two-dimensional array of 'known' expressed cDNAs. Chip-based methods require as little as 2 μg of total RNA per hybridization, making the technique much more practical for use with limited quantities of starting material such as tissue samples. In addition, the preparation of probes for hybridization is relatively straightforward and does not require a PCR amplification step, therefore avoiding PCR bias. The approach is based on the simultaneous interrogation of target sequences and as such represents a more time-efficient approach than the sequencing-based approach used in SAGE. Currently, the

major drawback of this approach is that it is totally dependent on the state of knowledge about the genome under investigation [23]. While the completion of genomic sequencing projects will in due course eliminate this problem, it should be noted that this requires that the genome under investigation not only be fully complete (all eukaryotic genomes so far have gaps), but that all the genes have been correctly identified. This last cannot be reliably done by current eukaryotic gene-finding software and may take many months after the 'completion' of a genome sequence. On the other hand, SAGE and differential display are subject to sampling error and may also miss genes, especially those that are expressed only at low levels.

The art of the soluble

The Nobel laureate Peter Medawar once said that science is the art of the soluble. The emerging expression-profiling technologies, and in particular chip-based microarray methods, greatly extend the range of the soluble. Both SAGE and chip methods are enabling technologies that make it possible to address previously intractable biological questions. In the not-too-distant future, with the full characterization of genomes, chip-based methods will probably dominate. Further advances will depend upon miniaturization and developments in detection systems, together with economies of scale and other developments that will ease the accessibility and reduce the cost of the methods. Microarray-based approaches make possible the simultaneous analysis of tens of thousands of genes in a single experiment, thereby dramatically reducing the time frame for this type of experimental approach. For the future, physiological insight will require the examination of p53-regulated gene expression in cells and tissues in genetically defined mouse models, with and without exogenous stresses, using dedicated tissue-specific, cell-type-specific, and growth-state-specific (or many other) libraries. An essential adjunct to this will be the parallel analysis of *in situ* patterns of gene expression. Insights from mouse systems may be accelerated by the recent discovery of a p53 homologue in the fruitfly *Drosophila* [24-26], which has proved such an extraordinarily fertile model for higher animals [27]. Such approaches will then open up our understanding of complex biological systems by linking genomic information with biology.

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