The p53 story: layers of complexity

Antony W. Braithwaite*, Janice A. Royds and Paul Jackson1

Department of Pathology, School of Medicine, University of Otago, Dunedin, New Zealand and 1Oncology Research Centre, Prince of Wales Hospital, Randwick, NSW, Australia and School of Medicine, University of New South Wales, Kensington, NSW, Australia

*To whom correspondence should be addressed. Tel: +64 3 479 7165; Fax: + 64 3 479 7136; Email: antony.braithwaite@stonebow.otago.ac.nz

This report is a summary of the presentations at the 12th International p53 Workshop, held in November 2004, Dunedin, New Zealand. The Convenor of the organizing committee was Antony Braithwaite, University of Otago, Dunedin. Janice Royds and Paul Jackson were also members of the organizing committee. There were 61 oral presentations and 101 posters.

Introduction

In 1992, when David Lane described p53 as ‘Guardian of the Genome’ (1), he established a paradigm embodying p53 as a tumour suppressor. In essence, p53 was a DNA damage sensor that acted to prevent the accumulation of genetic lesions that might contribute to tumourigenesis. This model could not only explain the role of p53 in cell-cycle control and apoptosis but also the stabilization of p53 after exposure to UV radiation and other DNA damaging agents, and the need for p53 function to be compromised during tumourigenesis. Indeed, it seemed like the ‘p53 story’ might be solved soon after that time. The intervening years, however, have clearly demonstrated that this was a naïve expectation. We now know that the role of p53 extends to participation in a diverse array of signalling pathways associated with cellular stress and DNA damage (Figure 1), and recent studies have even raised the possibility that p53 may be involved in pathways that have little to do with cancer.

It was in this light, that on the 25th anniversary of the discovery of p53, the 12th International p53 Workshop was held in Dunedin, New Zealand, in November 2004. The take home message from this latest biennial gathering of p53 researchers could be summarized in one word, ‘complexity’. We learnt that p53 is complex on many levels—genomic structure, regulation and function. In fact, it is far more complex than any one of us could have possibly imagined. This ‘theme of complexity’ was established in the opening address by David Lane and it continued throughout the meeting. In this report, we have endeavoured to cover as many of the oral presentations as possible but inevitably a few have been omitted. In addition, we have paid particular attention to describing some highlights, areas of new investigation and on-going research that appear to be integral to the p53 field.

p53 is a part of a growing family of proteins

New isoforms

After 25 years working with a gene we thought encoded a single protein, a novel discovery that will probably have important ramifications on much of the p53 field, was the identification of several new alternatively spliced isoforms of human p53. This was reported by J.-C. Bourdon and D. Lane (Dundee, Scotland). Using a technique to ensure that only full-length, capped p53 mRNA molecules were analysed, they identified numerous start sites for transcription: two mRNA species initiated from different start sites in exon 1 (P1 and P1′), and one mRNA was derived from a promoter (P2) within intron 4. Primers based in exon 11, also identified three splice variants of exon 9. Thus, p53 now has a total of 12 possible mRNAs and 9 proteins. Although it is still the early days, it is clear that the isoforms show different patterns of expression within tissues and cell lines. A key area of investigation will be to determine how these isoforms function. Interestingly, one isoform, ΔNp53, is a dominant negative inhibitor of wild-type (wt) p53 in transactivation and apoptosis assays, as has been reported for an N-terminal truncation of p73, a relative of p53. Some of the isoforms of p53 are abundantly expressed in tumours, suggesting a possible role in carcinogenesis. Furthermore, the existence of dominant negative isoforms provides an explanation for an impaired p53 response in the absence of coding region mutations.

p63/p73

Although not a major feature of this conference, several talks discussed p63 and p73, relatives of p53. Given the similarity between the different protein isoforms encoded by these genes, and using the domain structure of p53 as a model, X. Chen (Birmingham, AL, USA) has been mapping domains in the isoforms of p63 and p73 that are critical for transcriptional activation, growth arrest and apoptosis. He showed, in particular, that the two activation domains of p53 (amino acid residues 1–42 and 43–63) are represented in the full-length and N-terminally deleted (ΔN) versions of both p63 and p73, and are critical for all three functions.

T. Stiewe (Wurzburg, Germany) showed that reduction of p73 expression was sufficient to induce telomerase activity in telomerase negative cells, suggesting that p73 could be an endogenous repressor of telomerase activity. Stiewe also showed that hTERT is downregulated by p73 but upregulated by the ectopic expression of the dominant negative ΔNp73. These data agree with the observations that p73 upregulation...
accompanies differentiation, which in turn is coupled to a loss of telomerase activity.

In the absence of functional p53, p73 is phosphorylated and acetylated after DNA damage to activate pro-apoptotic genes. As described below, Pin-1—a propyl isomerase—is an important regulator of p53. G. Del Sal (Trieste, Italy) has been examining the role of this propyl-isomerase in the activation of p73. His studies elegantly demonstrate a clear requirement for Pin-1 in regulating the stability and conformation of p73 after DNA damage, and the ability of p73 to activate gene expression and to induce apoptosis. Furthermore, he showed that c-abl regulates the Pin-1 modulation of p73 by phosphorylating amino acid residue tyr99, thus linking p73 into one of the same signalling pathways that regulates p53.

**Upstream signalling to p53**

Control of p53 activity is a major area of research activity. Essentially, control is exerted on p53 function via post-translational modifications and/or by interacting proteins. Both areas were well represented at the meeting.

Modifications to p53 are thought to be a part of the signalling process that ‘directs’ p53 to carry out a particular task, whether it be cell-cycle arrest, apoptosis, DNA repair or some other process in which p53 plays a role. However, the importance of any particular modification for regulating p53 function is unclear as there are many conflicting reports. These conflicts, which were evident at this meeting also, may well reflect the use of different assay systems, the analysis of only limited p53 target genes and in vitro versus in vivo approaches.

**Post-translational modifications—phosphorylation**

S.-Y. Shieh (Taipei, Taiwan) addressed the role of the checkpoint kinases, Chk1 and Chk2, in p53 regulation. She showed that in addition to phosphorylation of known N-terminal residues, phosphorylation on ser313, ser314, thr377 and ser378 were also found. Furthermore, downregulation of Chk1 and Chk2 not only reduced the phosphorylation of ser20 as expected but also reduced the acetylation of lys382, leading to poor induction of the CDKN1A gene encoding the cyclin inhibitory protein p21WAF1/CIP1. The data suggest that Chk1 and Chk2 may regulate p53 function in several ways and not just by N-terminal phosphorylation. Such data serve to emphasize the importance of the C-terminus in regulating p53 function, a theme that was repeated throughout the meeting. In addition, it is interesting to note that germline mutations of Chk2 have been found in some Li Fraumeni families that have wt TP53. Further evidence for the apparent complexity in Chk2 regulation of p53 function was provided by Y. Haupt (Jerusalem, Israel). Specific mutation of pro82 prevented binding of Chk2 and Chk2-dependent phosphorylation of ser20 after DNA damage. In his attempts to identify the functional relevance of this aspect, Haupt looked at the role of Pin-1 in facilitating the Chk2 modulation of p53. Previous studies had shown that activation and stabilization of p53 by Pin-1 requires specific phosphorylation of p53 on ser33, tyr81 and tyr150.
Experiments in Pin1\(^{-/-}\) mouse fibroblasts were used to show that Pin-1 is also essential for phosphorylation at ser23 in mouse p53 (equivalent of ser20 in human p53) and stabilization of p53. Haupi also showed that Pin-1 enhances formation of Chk2/p53 complexes early after DNA damage and requires pro82. He therefore proposed a model in which p53 phosphorylation at specific residues permits binding of Pin-1, which then alters the configuration of p53 to allow binding of Chk2. Chk2 then phosphorylates ser20, thus releasing p53 from Mdm-2 and stabilizing/activating p53. A similar conclusion with respect to phosphorylation and the role of Pin-1 in regulating the interaction between p53 and Mdm-2, was reached by G. Del Sal (Trieste, Italy).

However, C. Prives (New York, USA) was unable to demonstrate any N-terminal phosphorylation of mouse p53 by Chk2 after DNA damage, and downregulation of Chk1 and Chk2 by siRNA failed to affect p53 function before or after DNA damage. Further, questioning the importance of phosphorylation in regulating p53 function, L. Giono (from J. Manfredi’s group, New York, USA), using an artificial system involving tetracycline-regulated p53 expression and therefore not involving DNA damage, reported that p53 is not phosphorylated on ser6 and only poorly phosphorylated on ser15 and ser37 and yet binds DNA very efficiently, at least to the p53 response element in the CDKN1A promoter. Furthermore, using proteosome inhibitors to elevate p53 levels, she showed that DNA binding, either in vitro or in vivo was not affected by the absence of phosphorylation on ser15 and ser392, and no acetylation at lys382. So, if post-translational modifications are not required for p53 activity, what are they required for? A probable answer is that p53 may carry out many functions once it has reached a threshold concentration. It may need to be ‘told’ to activate a specific promoter by processes, such as phosphorylation or acetylation or by interacting proteins. For example, A. Braithwaite (Dundee, NZ) also failed to observe phosphorylation on N-terminal residues using hormone inducible p53, but still obtained activation of the CDKN1A and MDM-2 promoters. However, no induction of the NOXA or BAX promoters was observed.

Some support for the idea that phosphorylation on specific residues controls p53 specificity, at least in part, came from the presentation by S. Soddu (Rome, Italy). Phosphorylation on ser46 is known to be important for p53 to induce apoptosis, at least in some instances, yet the kinase responsible for ser46 phosphorylation is unknown. The data from Soddu’s work suggested that the enzyme could be HIPK2 (homeodomain-interacting kinase 2), a member of a novel ser/Thr kinase family. Her studies were initiated by an observation that thyroid papillary tumour (TPA) have wt p53 and yet high levels of the anti-apoptotic protein galectin-3, expression of which is normally repressed by wt p53. Soddu showed that these tumours have allelic loss on chr7q32–34, which encompasses the HIPK2 gene locus and that wt p53 can only repress galectin-3, when HIPK2 is present. So, HIPK2 activates an apoptotic pathway that involves phosphorylation of p53 on ser46 and inhibition of the p53-mediated repression of galectin-3.

Ubiquitination, Mdm-2 and the control of p53 stability

It seems clear from the above discussion that much of the activity of p53 is dependent on its concentration (also discussed later). Thus, it is not only post-translational modifications, but also the level of p53 that must be regulated precisely. In a comprehensive analysis of enzymes required for ubiquitination of p53, D. Lane (Dundee, Scotland) identified UbcH5B/C as an E2-like protein involved in controlling the levels of p53 and Mdm-2. Knockdown of UbcH5B/C increased levels of p53, but this p53 was transcriptionally inactive being still bound to Mdm-2, which is also modified by UbcH5B/C. Activation of the stabilized p53 thus requires the removal of bound Mdm-2. These results in conjunction with the recent discovery that both p53 and Mdm-2 can be modified by the ubiquitin-like NEDD8, a well-characterized regulator of p27Kip1 and IκBα levels, demonstrate that there are now at least two pathways resulting in ubiquitination and inactivation of p53. Both pathways involve Mdm-2 in a two-step stabilization and subsequent inactivation of p53: the NEDD pathway and UbcH5B/C that ubiquitinate both p53 and Mdm-2. Understanding of this new complexity for p53 regulation has important implications for the design and use of therapeutic modulators of the p53/Mdm-2 interaction. In addition, work presented by M. Rossi (G. Melino’s group, Leicester, UK) showed that both p63 and p73 are also subject to tight control by ubiquitin-like degradation processes. He reported on the discovery of a new NEDD-like E3 ligase called Aip4/Itch that downregulates p73 upon DNA damage.

Consistent with these and other data presented at the meeting, G. Wahl (La Jolla, USA) showed that in normal fibroblasts, DNA damage induces phosphorylation of ser15 on p53, but this occurred ~2 h prior to induction of CDKN1A or MDM-2. This means that phosphorylation of ser15 does not always equate to ‘active’ p53 protein. During this time however, p53 becomes much more stable, as Mdm-2 becomes unstable. Proteosome inhibitors (to block Mdm-2 degradation) inhibited the transactivation function of p53 in this system, suggesting that the decrease in Mdm-2 stability contributed to p53 activation. Thus, if there is sufficient DNA damage, Mdm-2 stability is reduced and p53 becomes activated without a requirement for phosphorylation. These data provide a possible explanation for how p53 can be functional in the absence of N-terminal phosphorylation and also in p53 mutants lacking conserved serine and threonine residues (see above). Thus, once again p53 level may be the critical determinant of function.

To add to the complexity of Mdm-2 regulation, M. Oren (Rehovot, Israel) described studies reporting that Mdm-2 can directly bind the core histones and Mdm-2 mono-ubiquitinates H2A and H2B in vivo. In a GAL4 DNA-binding reporter system, Mdm-2 repressed transcription from a GAL4-response promoter, suggesting that Mdm-2 might repress transcription via changes to chromatin. Using ChiP analysis, Oren also showed that Mdm-2 can complex with p53 on the CDKN1A promoter in vivo and ubiquitinate residues on H2B. The data imply that Mdm-2 may drive gene silencing in its own right via chromatin modification.

New p53-binding proteins

In common with agents that induce post-translational modifications of p53, p53-binding proteins appear important in ‘instructing’ a specific response from p53. The list of these proteins continues to expand, and several new members were discussed at the meeting.

Y. Taya (Tokyo, Japan) reported that the cytoplasmic protein, clathrin, which normally plays a role in the formation of ‘coated pits’ in vesicle transport, binds p53 and influences its function. He reported that clathrin binds the N-terminus and to the S46F mutant in particular. Interestingly, phosphorylation...
of this residue is reported to be important for the ability of p53 to induce apoptosis (see data reported by Soddu). He went on to show by transfection and reporter assays, that clathrin enhanced p53-dependent activation of apoptosis inducing protein 1 (AIP1) and other p53-dependent promoters, which was particularly marked for the S46F mutant. In addition, RNAi studies showed that downregulation of clathrin partially ablated the ability of p53 to transactivate several target genes after UV exposure.

Binding of p53 by the Y-box factor, YB1, was reported by A.Braithwaite. YB1 binds the C-terminus of p53 and like clathrin, is generally located in the cytoplasm. YB1 translocates to the nucleus in response to various stresses and regulates transcription of several tumour-associated genes. In tumours, nuclear localization and over-expression of YB1 are associated with drug resistance and poor prognosis. Braithwaite showed that YB1 requires a transcriptionally active p53 for translocation and does not appear to require direct binding. Once in the nucleus, he showed that YB1 partially inhibits p53 activity. Genes such as BAX and NOXA involved in apoptosis were inhibited by YB1, but CDKN1A and MDM-2 were not inhibited. However, knockdown of YB1 with antisense oligonucleotides increased NOXA expression in a p53-dependent manner. Braithwaite proposed that YB1 reduces the ability of p53 to bind target promoters of low affinity, such as those in the apoptotic pathway, and that this partial inhibition of p53 function may contribute to cancer formation.

A very interesting p53 binding partner discovered 2 years ago by G.Del Sal (Trieste, Italy), is Pin-1, a propyl-isomerase. Pin-1 induces a conformational change on phosphorylated p53 facilitating further post-translational modifications, particularly acetylation. In this sense it is an important regulator of p53 function and is often over-expressed in common human cancers, such as prostate and breast. Pin-1 also influences p53 regulation by Mdm-2 (see Haupt) and appears to have analogous effects on p73.

X.Lu (London, UK) expanded her work on the pro-apoptotic ASPP (so-called because they comprise Ankin repeats, SH3 domains and a Poly Proline domain) proteins and their effect on p53 function. The ankyrin repeats bind p53 within the N-terminus. It is of interest to note that the polymorphism arg72 binds ASPP to a lesser extent than pro72. When ASPP proteins bind p53, they augment the ability of p53 to transactivate pro-apoptotic genes, but have no effect on the ability of p53 to transactivate cell-cycle genes. Although data were presented showing a similar selective influence by ASPP proteins for p73 and p63, much of the talk was focused on the regulation of ASPP function. Biochemical studies showed that ASPP1 is a substrate for MAP-kinase (MAPK), and that both ASPP1 and ASPP2 are stabilized by MAPK. Interestingly, RNAi down-regulation of ras, inhibited ASPP1 and ASPP2 mediated p53-dependent apoptosis, demonstrating that ASPP proteins link p53-dependent apoptosis to a ras-signalling pathway. In studies looking at transcriptional regulation of ASPP1 and ASPP2, they found binding motifs for E2F-3 (ASPP1, 4; ASPP2, 2 common to ASPP1) in both promoters. That E2F-1 could bind these motifs was confirmed by in vivo (chromatin immunoprecipitation, ChIP) and in vitro (reporter) assays. E2F-3 was also shown to activate both promoters. However, only E2F-1 and E2F-2 can strongly activate the ASPP2 promoter. Transfection of E2F-1 was able to induce ASPP1 and ASPP2 mRNA, leading to a proposal that induction of ASPP1 and ASPP2 by E2F-1 might sensitize cells to apoptosis.

Finally, the importance of TGFβ as a regulator of p53 function was discussed by M.Barcellus-Hoff (Berkeley, USA). Using cells derived from TGFβ1 knockout mice, she found that radiation induced p53-dependent growth arrest and apoptosis were defective. The defect was associated with an absence of ser15 phosphorylation but no overall change in p53 levels was observed and there was also no direct interaction between TGFβ and p53. In addition, studies with small molecule inhibitors of TGFβ signalling led to a similar conclusion that TGFβ is a regulator of the p53 DNA damage response. Additional evidence of a link between the TGFβ and p53 pathways, was provided by M.Aggarwal (Cleveland, USA) who showed that TGFβ mediates a p53-dependent protective cell-cycle arrest in response to dNTP imbalance.

**Apoptosis**

It is becoming increasingly clear that the regulation of p53-dependent apoptosis is complex. In simple terms, the process can be divided into three parts: (i) what tells p53 to induce apoptosis, (ii) what mechanisms are involved and (iii) what the downstream effectors are. The first part has been dealt with above and often involves post-translational modifications and/or interactions with other proteins. This session of the meeting dealt principally with the latter two components.

For many years it was thought that, like induction of cell-cycle arrest, induction of apoptosis by p53 was due solely to the transactivation of pro-apoptotic genes. However, transactivation-independent apoptosis does occur in some cells. This area has been pioneered by U.Moll (New York, USA) who believes that p53 induces mitochondrial apoptosis directly. In support of this, she showed convincing evidence of p53 associated with mitochondria. This association occurred only during p53-dependent apoptosis and in radiosensitive tissues, such as spleen and thymus, and was earlier to transactivation. She also showed that p53 binds the anti-apoptotic proteins Bcl-2 and Bcl-xL. Using modelling techniques she predicted that Bcl-2 and Bcl-xL would bind p53 within the central, specific DNA-binding domain. Using the classic cytochrome C (cyt C) release assay, she showed that tumour-derived mutants of p53 are defective for binding Bcl-xL and prevent cyt C release from mitochondria. M.Murphy (Philadelphia, USA) provided independent evidence that certain p53 mutants (those that cannot oligomerize) fail to bind Bak and as a consequence are defective for p53-dependent mitochondrial apoptosis. These data suggest that tumour-derived mutants have a double-hit, i.e. they cannot induce mitochondrial death and are transcriptionally inactive too!

Further support for a direct role of p53 at the mitochondrion was provided by D.Green (San Diego, USA) who has developed a sophisticated model system using isolated outer and inner mitochondrial membrane vesicles, as well as synthetic lipid vesicles containing dextran, to examine how individual Bcl-2 family proteins function. During apoptosis, pro-apoptotic members of the Bcl-2 family act to permeabilize the outer mitochondrial membranes, without a requirement for the inner membranes or mitochondrial proteins. As a result, cyt C is released in a manner that is sudden, rapid and complete. Using this system, he looked at induction of apoptosis by p53 in intact cells and cytoplasts and concluded that the nucleus is dispensable for apoptosis! To further look at the function of endogenous p53 in apoptosis, he purified native p53 from various cell lines after UV treatment, as well as...
several p53 mutants including ΔPro (deleted the polyproline domain between residues 62 and 92) and examined their interaction with Bcl-2 family proteins and their ability to induce cyt C release. Based on these studies, he proposed a model whereby after stabilization, p53 accumulates in the cytoplasm but is held ‘inactive’ by Bcl-xL. Nuclear p53 then activates BAX and PUMA transcription. Puma accumulates in the cytoplasm and p53 is liberated from Bcl-xL by Puma. Bax can then be activated by p53, cyt C is released and cell death occurs.

A.Strasser (Melbourne, Australia) investigated the roles of PUMA and NOXA in p53-dependent apoptosis by generating specific knockout mice. Thymocytes from NOXA−/− mice died after radiation or exposure to various DNA damaging agents but similar cells from PUMA−/− mice were resistant. Fibroblasts from PUMA−/− mice were also much more resistant to apoptosis compared with NOXA−/− fibroblasts. He concluded that neither NOXA nor PUMA were required for normal development, but that PUMA and to a lesser extent, NOXA, were involved in mediating p53-dependent apoptosis, although tissue differences were noted. Interestingly, he also showed that loss of PUMA protected cells from diverse p53-independent cytotoxics, such as staurosporine, glucocorticoids, phorbol ester and cytokine withdrawal, suggesting that PUMA is not specific to p53 pathways. By crossing PUMA−/− and/or NOXA−/− mice with Eph-2/myc mice, they found that loss of PUMA accelerated tumour growth, consistent with its role as a tumour suppressor. T.Shibue (Tokyo, Japan) also generated NOXA and PUMA knockout mice with similar results to Strasser for isolated thymocytes and embryo fibroblasts.

An interesting presentation was given by R.Rahman (from K.Wiman’s group, Stockholm, Sweden). It has long been known that p53 represses hTERT, but in this talk, the tables have been turned and now hTERT antagonizes the ability of p53 to induce apoptosis, which may be of clinical relevance (see Royds).

p53 and DNA damage repair

Although not required for DNA repair, p53 facilitates nucleotide excision repair (global and transcription coupled repair) and base excision repair. C.Harris (Bethesda, MD, USA) and L.Wiesmuller (Ulm, Germany) both extended the role of p53 in DNA repair to the repair of DNA damage occurring during replication stress. Harris presented evidence that p53 is downstream of ATR kinase, Chk-1 kinase, 53BP1. BLM helicase in response to Holliday junctions (HJ) and other replication intermediates, enhances the endonuclease activity of Mus81, a candidate resolvase of HJs, and functions as a ‘gofer’ of excessive rates of homologous recombination. Wiesmuller also presented data indicating that p53 restrains Rad51-dependent DNA exchange events involving imperfectly homologous sequences. p53 may also contribute to recombinative repair, when it is recruited to specific repair complexes by molecular interactions with topoisomerase I and other binding partners. These and other studies further establish the transcription independent and dependent multifunctionality of p53 in the DNA damage and repair response.

Animal models

Tumour-derived p53 mutations are almost always missense mutations and heterozygous, with gene disruptions being very rare. In addition, the spectrum of tumours seen in p53 knockout mice is quite different to that seen in humans, including in the Li-Fraumeni syndrome. These observations combined with a considerable body of in vitro data, have led to the conclusion that p53 mutants have some ‘gain-of-function’ in tumourigenesis, a notion supported in a presentation from M.Oren (Rehovot, Israel) showing that mutant p53 can specifically regulate the expression of CD95, EGR1 and MPE, resulting in an inhibition of apoptosis. This issue of ‘gain-of-function’ is also being explored in vivo using conditional ‘knock-in’ mutations in mice. Using this approach, K.Olive from the group of T.Jacks (Cambridge, MA, USA) and T.Iwakuma from G.Lozano’s group (Houston, USA) reported the results of studies analysing the function of mR172H (hr175H; a structural mutant) and mR270H (hr275H; DNA contact mutant; Olive only). They generated mutant/wt, mutant/null and wt/null mice but saw no significant difference in overall survival. However, the tumour spectra were very different. Mice with mutant p53 had significantly more carcinomas, brain tumours and endothelial tumours (R175H only), whereas mice with both mutant p53 and R175H showed decreased haematological tumours. Importantly, there were also novel tumours in mutant/null mice. They concluded that different p53 mutants do exhibit a clear gain-of-function and different mutants can have different effects that may be tissue specific.

L.Attardi (Palo Alto, USA) used a similar approach, but rather than using tumour mutants she examined the importance of p53 transactivation function in vivo, by generating knock-in mice with a p53 gene mutated at residues 25 and 26 to abolish transactivation and Mdm-2 binding. By looking at various p53 parameters in fibroblasts derived from these mice, she confirmed that the mutant is markedly defective for gene activation under most conditions used, and also defective for cell-cycle arrest and apoptosis. However, there were some treatment-specific differences. For example, the mutation could induce BAX expression after DNA damage, but was completely defective for induction of CDKN1A or NOXA. Interestingly, she was unable to obtain homozygous mutant mice owing to embryonic lethality. These data suggest that transcriptionally competent p53 is required for normal embryogenesis, yet paradoxically, mice that are deleted for TP53 generally develop in a normal fashion, although a significant proportion of female TP53 null mice show exencephaly. Attardi’s mutant mice therefore may have some gain-of-function that has yet to be described.

G.Wahl (La Jolla, USA) also engineered a knock-in mouse in which the 7 C-terminal lysine residues were changed to arginine (7KR). As indicated elsewhere in this review, modifications to these residues are thought to be important in dictating the nature of the p53 response to DNA damage. Wahl showed however that these mice and cells derived from them, behaved essentially as cells with wt p53. Thus, once again we have this conflict as to how critical post-translational modifications are for p53 function.

An interesting variation on the use of transgenic and knock-out mice was reported by A.Blackburn (Canberra, Australia) who is looking for modifiers of mammary gland tumourigenesis. One characteristic of Li-Fraumeni families is the early onset of breast cancer (50% risk by 50 years of age). She has developed a mouse model for Li-Fraumeni (p53+/−) on a Balb/c background in which the incidence of mammary tumours is very high, whereas in a C57BL/6 background, the mice are very resistant to the formation of these tumours. By crossing these two strains of mice and carrying out linkage
analysis on second generation offspring she identified a region of chromosome 7 (SuprMam1) and Chromosome 2 (SuprMam2) which were linked to increased frequency and reduced latency of tumour onset.

D.Bulavin (Singapore) used an approach of replacing the mouse p53 gene with a human p53 (SWAP mice). These mice do not develop spontaneous tumours and live twice as long as their p53-null counterparts. Furthermore, induction of SWAP p53 after ionizing radiation did not induce apoptosis in the thymus and spleen and even more surprisingly, in crosses with Eµ-myc mice, SWAP p53 did not prevent rapid onset of tumourogenesis. Thus, although the approach is novel, as SWAP p53 behaves very differently from expectations, its merits are unclear.

Models of tumourigenesis

The role of p53 in tumourigenesis was explored in cell culture and in animal systems by several groups. Both V.Rotter (Rehovot, Israel) and T.Tlsty (San Francisco, USA) have been looking at genetic changes that occur in primary cells as they progress from initial culture, through immortalization and eventually to tumourigenesis. In Rotter’s study, primary fibroblasts were transfected with a retrovirus expressing hTERT to induce immortalization, followed by a mutant p53 and/or Ha-Ras to induce a tumourigenic phenotype. At each stage, expression microarray was performed. Based on changes in gene expression, she concluded that the initial slow-to-fast expression microarray was performed. Based on changes in Ha-Ras to induce a tumourigenic phenotype. At each stage, looking at genetic changes that occur in primary cells as they (Rehovot, Israel) and T.Tlsty (San Francisco, USA) have been

A.W.Braithwaite, J.A.Royds and P.Jackson

Models of tumourigenesis

The role of p53 in tumourigenesis was explored in cell culture and in animal systems by several groups. Both V.Rotter (Rehovot, Israel) and T.Tlsty (San Francisco, USA) have been looking at genetic changes that occur in primary cells as they progress from initial culture, through immortalization and eventually to tumourigenesis. In Rotter’s study, primary fibroblasts were transfected with a retrovirus expressing hTERT to induce immortalization, followed by a mutant p53 and/or Ha-Ras to induce a tumourigenic phenotype. At each stage, expression microarray was performed. Based on changes in gene expression, she concluded that the initial slow-to-fast growth stage was associated with the loss of INK4A expression and reflects a defect in differentiation; the loss of p53 leads to continued proliferation and genomic instability, and fully transformed cells have characteristics that permit in vivo tumour growth. The details of this will no doubt be unveiled at the next meeting!

In the study by Tlsty, molecular pathways underlying tumourigenesis in breast epithelial cells were investigated. Normal human mammary epithelial cells (HMEC) were grown until a rare variant (vHMEC) was produced which propagated beyond the Hayflick barrier. Tlsty showed that these cells have uncoupled the tight controls linking cell division and DNA replication and that this is associated with a loss of p16INK4A expression by hypermethylation of the p16INK4A gene promoter. When HMEC (normal p16INK4A) and vHMEC (silenced p16INK4A) were further examined, it was noted that the levels of p53 and p21WAF1/CIP1 were high in vHMEC, but very low in HMEC. Biochemical studies indicated that p16INK4A might be regulating p53 stability. This reciprocal relationship between p16INK4A and p53 expressions, however, was not true of fibroblasts. As silencing of p16INK4A expression occurs in islands of cells in normal breast, the suggestion is that this represents an early stage in tumorigenesis.

An elegant two-step mouse model of multistage skin carcinogenesis has been developed by Chris Kemp (Seattle, USA), in which initial treatment with DMBA results in Ha-ras gene mutation. Continuous exposure to TPA then leads to mutation of p53, loss of the remaining p53 allele and finally to amplification of the mutated H-ras gene. His group has demonstrated a clear progression from normal cells to papillomas, then to tumours and metastases in this model. Knowing that p53 is activated either by DNA damage (via ATM and ATR) or via oncogene activation (via p19ARF), he asked which activation pathway regulates p53 tumour suppressor activity. To this end, tumours were generated in ATM−/−, p19ARF−/− and p53−/− mice. All tumours, irrespective of stage, had Ha-ras mutations, confirming that this was the initiating event. Consistent with this pathway, p53 levels were elevated in papillomas from normal and ATM−/− mice, but not in tumours from p19ARF−/− mice. The absence of p53 function accelerated malignant conversion from papilloma to carcinoma in p19ARF−/− mice and p53−/− mice and was associated with metastasis, supporting the idea that induction of p53 suppressed metastasis. The importance of p19ARF to p53 induction was further shown by the fact that loss of heterozygosity (LOH) of ARF in carcinomas overcame the need for subsequent LOH of TP53. Their data support the idea that, in this system, selection for p53 mutations is not due to external stress or DNA damage (i.e. signalling via ATM), but rather, it results directly from signalling from the initiating oncogenic lesion via p19ARF. Importantly, such data provide an explanation as to why mutational alteration of p53 is often a late event in the development of human malignancies.

p53 in the clinic

The significance of polymorphisms

Although TP53 mutation in tumours is commonplace and clearly contributes to malignant development, the role of natural TP53 variants (polymorphisms) or variants in the p53 signalling pathway, is an area that is much less explored. Despite this, such polymorphisms do influence p53 function as has been clearly shown by M.Murphy (Philadelphia, USA), who demonstrated that the arg72 variant can induce apoptosis in vitro much more efficiently than the pro72 variant, and ser47 (normally pro47) appears to have altered transactivation ability. M.Resnick (Chapel Hill, USA), as well, has identified several p53 response element variants that affect the expression level of downstream target gene transcription.

Further to the importance of natural genetic variants, A.Levine (New Jersey, USA) in an outstanding talk, identified 1335 single nucleotide polymorphisms (SNPs) associated with 82 genes directly involved in the p53 pathway—the vast majority being in non-coding regions. Analysis of the MDM-2 promoter has identified an SNP within the first intron, at nucleotide 309. Within the general population 12% are G/G, 40% G/T and 48% are T/T (wt). Functional analysis predicts that having G makes a better Sp1 binding motif (which was confirmed using gel shift assays) and increases transcriptional activity by Sp1 in reporter assays. The importance of this is that in cells with Sp1, endogenous levels of Mdm-2 are higher in cells with G/G than T/T; as a consequence, these cells are more resistant to apoptotic cell death than T/T cells, because less ‘active’ p53 is available so only high affinity promoters will be activated.

At a clinical level, analysis of 115 independent lymphoblastoid cell lines (LCL) derived from Caucasian and African American patients, showed a normal distribution of SNPs in MDM-2 promoter, with the majority being in non-coding regions. Analysis of the TP53 gene with a human gene. His group has
p53 and gliomas

Glioblastoma multiforme (GBM) is the most lethal form of brain tumour and is characterized by frequent recurrence. GBM is highly invasive, resistant to apoptosis and almost completely refractory to treatment. E.Kim (Luebeck, Germany) presented an interesting talk on an interaction between p53 and ets-1 in progression of glioblastoma. Overexpression and activation of the transcription factor ets-1, which increases expression of pro-invasive genes, such as MMPs, pre-senilin and integrins in invasive GBM, has been well documented, but the underlying molecular basis is unknown. She showed that wt p53 binds ets-1 and inhibits its ability to activate pro-invasive genes. Ets-1 induction of thromboxane mRNA is inhibited by p53 (and binding of p53 to ets-1) in transient transfections. Ets-1 also complexes with several p53 mutants that enhance the ability of ets-1 to activate transcription. Her data suggest that in GBM, there is a selection against wt p53 function, the result of which is to aid in the upregulation of pro-invasive genes by ets-1. In a related talk, J.Royds (Dunedin, NZ) examined the relationship between TP53 mutations and telomere maintenance (TM) in gliomas. Two mechanisms for TM have been described in gliomas, one requiring the enzyme telomerase and the other a recombinational method designated as alternative lengthening of telomeres (ALT). This latter mechanism was described in more detail by R.Reddl (Sydney, Australia) who demonstrated that ALT is manifested by a rapid increase in telomere length and the appearance in the nuclei of ALT-associated PML bodies. Moreover, he showed that ALT is repressed by telomerase. Since ALT complex by Sp100. Royds found a high correlation between sequestration of the MRE11/RAD50/NBS1 recombination factor. In this context, it is very clear that p53 binds to specific response elements via a sliding mechanism. This was tested by looking at the binding of p53 to genomic DNA. The p53AS form binds genomic DNA much more slowly than wt p53. So perhaps, p53 having located DNA damage, then slides along the DNA to locate its binding motifs in promoters as a rapid response to stress?

Further data on the importance of the C-terminus was provided by K.Harms-McNaughton (from X.Chen’s group, Birmingham, AL, USA). She showed that p53 activation domain 2 (amino acid residues 43–63) is required for transcription of the pro-apoptotic insulin-like growth factor binding protein 3 (IGFBP3) but the C-terminal basic domain (amino acid residues 364–393) is inhibitory. Activation domain 1 (amino acid residues 1–42) controls the inhibitory action of the C-terminus. Inhibition of apoptosis is thus alleviated by deletion of both of these domains. The C-termini of p63 and p73 also inhibit induction of IGFBP3.

Transcriptional regulation by p53

As has been discussed elsewhere in this report, several lines of evidence point to multiple mechanisms by which p53 regulates transcriptional activation: there is functional variation between p53 mutants, cofactor diversity, differences in binding motifs and differences in stress-specific responses. In addition, there are differences in promoter architecture between different p53 response genes, e.g. the CDKN1A promoter possesses two p53 binding sequences upstream of TATA/Inr element; the FAS/APO1 promoter has one p53 binding motif downstream of an Inr and PTE1 has a p53 binding motif adjacent to a GC-rich sequence. It is generally believed that p53 links upstream enhancer factors to mediators and the basal transcription machinery. The advent of ChIP has allowed detailed in vivo analyses of p53-dependent activation of transcription. Using a time-course ChIP approach, B.Emerson (La Jolla, USA) presented the results of elegant studies into the relationship between core transcription factors, p53, and the responses of p53 target promoters to different types of DNA damage. Focusing on the CDKN1A promoter, prior to UV DNA damage, p53 is bound to its motifs but unphosphorylated at ser15. Histone acetyl transferase (HAT; bound to p53) and TBP (bound to the TATA-box) are also present, but there is no transcription. TAF250 is only recruited to the TBP pre-initiation complex after UV damage and when the promoter is active. In contrast, TFIIIB and RNA polymerase II are bound also a C-terminal non-specific DNA binding domain, whose function remains somewhat controversial. Furthermore, the question remains as to how p53 recognizes its specific binding motifs within the genome as a whole? Much of this recognition process appears to be dependent on the C-terminus.

Regulation of p53 function

Notwithstanding any role played by p53 in the mitochondrion, p53 undoubtedly can and does function as a transcription factor. In this context, it is very clear that p53 binds to motifs within the promoters of a selected group of target genes via its central hydrophobic domain. However, there is
Before damage, but not present after damage. This RNA polymerase II is the unphosphorylated and active but non-elongating form of the enzyme; elongation activity is activated shortly after UV damage. Histone acetylation and TAF250 recruitment occur prior to phosphorylation of ser15 on p53 and the stabilization of p53. Therefore, the CDKN1A promoter is poised for action in response to DNA damage. However, induction is transient because TFIIIB is quickly lost, so RNA polymerase II cannot rebind and transcription is not sustained. In contrast, the CDKN1A response to doxorubicin is biphasic. After exposure, there is no recruitment of TAF250 to the CDKN1A promoter but there is an increase in TFIIIB. So after the initial burst of ‘ready-to-go’ transcription and loss of RNA polymerase II, the increase in TFIIIB means that TFIIB tends to stay on the promoter, recruit more RNA polymerase II and therefore, another wave of transcription.

Interestingly, the affinity of RNA polymerase II for the CDKN1A promoter is the highest amongst the p53 target genes (along with other genes associated with growth arrest and DNA repair). These promoters have plenty of paused RNA polymerase II compared with the apoptotic response genes. Analysis of the FAS/APO1 promoter, which has very low levels of paused bound RNA polymerase II, showed that there is recruitment of TAF250 in response to UV but not doxorubicin, and low levels of bound TFIIIB and paused RNA polymerase II remain constant after damage by UV and doxorubicin. In this case, there is little pre-initiation complex present before DNA damage but it persists afterwards, and therefore, initiation of transcription by p53 is delayed compared with CDKN1A. These results provide the basis for an explanation of how p53 generates rapid cell-cycle arrest responses but more delayed apoptotic responses.

**p53-based therapies**

Given the prevalence of p53 mutations in human cancer, the ultimate goal of our investigations into the regulation and functions of p53 is to develop novel and rational therapeutics for the treatment of a wide range of malignancies. The majority of investigations are focused on two main areas: one is targeting the Mdm-2/p53 interaction (or equivalent such as the E6/p53 complex; M.Jiang, from J.Milner’s group, York, UK), and the other aims to alter the conformation of mutant p53 proteins in order to reactivate wt p53 functions. From the former perspective, nutlins, appear to be promising candidates. Nutlins (cis-imidazolines), are small molecules that inhibit the p53/Mdm-2 interaction by mimicking binding of three critical p53 amino acids (leu26, trp23 and ph19) within the p53/Mdm-2-binding domain. In an impressive presentation, L.Vassiliev (New Jersey, USA) showed that in cells with wt p53 but not mutant p53, nutlins cause a dose-dependent elevation of p53 protein and p53 transcriptional activity. The global effect of nutlin on p53 gene activation was verified by gene array profiling. Nutlins also efficiently inhibit clonal growth and induce apoptosis in cells with wt p53, in a dose-dependent fashion. The beauty of nutlins is that they do not need DNA damaging agents to activate p53 (see Signalling section). In xenograft models, nutlins have proved more effective than paclitaxel and at higher doses can completely abrogate tumour growth, with no obvious pathology. These agents appear to show great promise as new cancer therapeutics.

Several studies have attempted large-scale screening of small molecules that might reactivate mutant p53. H.-C.Ang (from A.Fersht’s group, Cambridge, UK) provided preliminary data of an NMR approach that could be used to evaluate the effects of small molecule effects on p53 structure, thus allowing the rationale design of molecules. They are now using NMR as a sensitive tool for screening novel molecules that bind weakly to p53 and might then be refined. The advantage of this system is that only compounds that bind and alter p53 structure are detected and the approach can be automated for high throughput screening. At a more advanced stage, K.Wiman (Stockholm, Sweden) reported on efforts to identify small molecules that might be used to reactivate normal p53 function in mutants. Using a high-throughput screen of small molecules able to induce growth arrest or apoptosis in cells stably expressing mutant R273H, they originally identified PRIMA-1. PRIMA-1 inhibits tumourigenesis of human xenografts and may prove useful in sensitizing cells to killing by current anticancer drugs. Biochemical studies suggest that this sensitization may result from increased levels of mutant p53 induced by low levels of drug (e.g. cisplatin), which increases the available pool of p53 for reactivation by PRIMA-1. With this in mind, he proposed that any agent capable of elevating mutant p53 levels might synergize with PRIMA-1 to kill cells. Using a similar approach, this group has also identified another small compound, MIRA-1, that can also reactivate mutant p53.

Finally, C.Drummond (Auckland, NZ) described a compound called SN28049 that upregulates the death receptors Fas and DR5, and is preferentially cytotoxic in tumour cells with wt p53 activity.

**p53—the future**

At the end of the formal presentations, one of the co-discoverers of p53, A.Levine (New Jersey), was asked to speculate on what the next chapter of p53 research might be. Commenting on areas that were under-represented at the meeting, he thought that in the next period up to the 13th International p53 Workshop in New York in 2006, we would learn a lot more about the ‘gain-of-function’ of tumour associated p53 mutant proteins, about transcriptional repression by p53 and about the role of p53 in cellular senescence. For example, which mutants have gain-of-function? Do they all behave in the same way? Does transcriptional repression have biological meaning? How does p53 induce senescence? The increased use of transgenic knock-in mutant mice will help answer many questions about ‘gain-of-function’ mutants as well as yeast based genetic screens.

However, beyond its role in tumour suppression, and having regard for the many complex signalling pathways with which p53 appears to be involved in (Figure 1), Levine proposed that p53 may be a ‘ubiquitous sensor’ of stress and as such is involved in the prevention of other, possibly aging-related disorders. These, he suggested, may include diabetes, arthritis, atherosclerosis and other inflammatory diseases, for which there is indeed some evidence already (2–4), and even psychological stresses. Thus, rather than just acting to prevent genetic lesions accumulating in somatic tissues which can lead to cancer, p53 may act to dampen many physiological stresses that may, if unchecked, lead to disorders. Such responses may often be beneficial in the short-term, but long-term dysregulation can cause damage to body organs, resulting in premature ageing. This cumulative wear and tear is known as allostatic load (5). The idea that p53 may be important in the control of allostatic load is very exciting as
it may well account for the large diversity of signals that appear to ‘activate’ p53. A handle on this might be obtained by analysing patients for polymorphisms in TP53 and in other members of the p53 pathway, such as the SNP309 discussed above, and correlating them with various ageing-related phenotypes. It will be interesting to see if anyone picks up this gauntlet and has data by the time of the next meeting, potentially opening up even more areas of investigation, for the next generation of p53 researchers.

In the final analysis, perhaps the timeless words of T.S.Eliot (6) reflect the evolving nature of p53 research:

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.

Conflict of Interest Statement: None declared.

References


Received and accepted March 29, 2005