Review Article

Isolation of p53-target genes and their functional analysis

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Mutations of the *p53* gene are the most common genetic alterations found in human cancers, and are known to play crucial roles in tumor development and progression. The *p53* gene encodes a protein functioning as a transcription factor, and the biological functions of p53 are manifested through the activities of its downstream genes. Identification of these downstream genes involved in the p53-signaling pathway should provide more detailed insight into the molecular mechanisms that mediate tumor-suppressor activities, as well as various responses to cellular stress. We have been attempting to isolate p53-target genes by means of various approaches, including differential display, cDNA microarray analysis, and direct cloning of the p53-binding sequences from human genomic DNA. Here I review our recent work on isolation of p53-target genes and their functional analysis. The physiological functions of p53-target genes include apoptosis (GML, p53AIP1, and STAG1), DNA repair (p53R2), inhibition of angiogenesis (BAI1), re-entry into the cell cycle (p53RFP), oxidative stress (CSR), and determination of cell fate (p53RDL1). (Cancer Sci 2004; 95: 7–11)

p53 mutations in human cancer

The *p53* gene is the most frequent target of genetic alteration so far identified in human cancers, and cells in which p53 is inactivated are known to have a selective growth advantage.[1] Many studies have indicated that p53 has multiple physiological functions through transcriptional regulation of its downstream genes by binding to specific genomic sites as a tetramer. p53 was shown to bind DNA in a sequence-specific fashion[2] and to activate transcription of genes containing a consensus binding site; these DNA sequences are likely to function as transcriptional enhancers. Normal p53 function can be lost in a variety of ways, most commonly by loss of a chromosomal region containing one allele of the gene (or of the entire chromosome 17p) and a subtle mutation involving the other allele. This scenario fits the "two-hit" model proposed by Knudson[3] for the definition of what came to be known as tumor suppressor genes. Inactivation of both alleles of p53 has been documented in a large number of human cancers, including tumors arising in the colon, brain, lung, liver, and bladder. However, in some cases a missense mutation occurring in one allele of p53 is sufficient to inactivate p53 function, when such an event increases the proportion of hetero-tetramers that contain both mutant and wild-type p53 molecules; the chimeric complexes cannot activate transcription of target genes. This "dominant-negative" effect may be exacerbated by the increased stability (and therefore higher intracellular concentration) of the mutant protein compared with that of the wild-type p53 protein. The other mechanism for inactivation is often seen in soft-tissue sarcomas, in which amplification of MDM2 creates a similar loss of functional p53 through binding and subsequent degradation of the p53 protein.[4]

Strategies for identifying p53-target genes

Several biological functions of p53 have been described, including induction of cell cycle arrest or apoptosis after DNA damage or other cellular insults, maintenance of genomic stability, and inhibition of angiogenesis. Positive and negative feedback mechanisms by downstream genes were also reported. We have adopted three strategies to identify biologically important genes that are transcriptionally regulated by p53; two of these approaches are the differential display method and cDNA microarray analysis, with which we were able to screen genes showing different expression levels between cells with or without wild-type p53. These techniques could successfully detect p53-target genes that are expressed in relative abundance. However, identifying p53-regulated genes that are expressed less abundantly or in a tissue-specific manner has proven to be difficult. To effectively isolate such genes we applied another strategy, based on the idea that p53-target genes must contain p53-binding sites in their promoter regions or within their introns. We used for this purpose a yeast system (*Saccharomyces cerevisiae*) to identify human genomic sequences, "p53-tagged sites," that activate transcription from an adjacent reporter gene in a p53-dependent manner,[5] and then isolated and characterized several genes including GML and BAI1.[6,7]

For screening p53-target genes that are expressed in relative abundance, we established a colon-cancer cell line SW480-LOWp53-1 carrying a wild-type *p53* transgene that is inducible under the control of the lactose operon. Induction of this transgene by isopropyl-1-thio-β-D-galactoside (IPTG) arrests growth of the transfected cells.[8] We applied a differential-display method to screen mRNAs isolated from this cell line, and looked for genes whose expression was activated or suppressed after induction of wild-type p53. Expression of seven known genes was significantly induced or suppressed by induction of wild-type p53 protein.[9] To identify genes that are transcriptional targets of p53 by cDNA microarray analysis, we prepared an adenovirus designed to express the wild-type p53. We used cDNA-microarray slides bearing 23,000 genes and hybridized them with cDNAs reversely-transcribed from mRNAs of p53-deficient U373MG cells infected with either Ad-p53 or Ad-LacZ. Duplicate microarrays were hybridized with a mixture of Cy5-labeled cDNA probes corresponding to Ad-p53-infected cells and Cy3-labeled cDNA probes corresponding to Ad-LacZ-infected cells. Dozens of genes showed increased signal intensities of Cy5, in a time-dependent manner.

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By applying a combination of these approaches, we have isolated and characterized more than a dozen genes of biological and medical importance, as summarized below (Fig. 1).

DNA repair; p53R2, a novel ribonucleotide reductase

The DNA-damage checkpoint plays a critical role in preventing genomic instability by regulating the cell cycle and DNA repair. Inactivation of the checkpoint may impair the DNA-repair mechanism and increase the susceptibility of cells to genotoxic agents. The *p53R2* gene contains a p53-binding sequence in intron 1 and encodes a 351-amino-acid peptide that showed striking similarity to ribonucleotide reductase small subunit (R2). Expression of the p53R2 was induced by γ- and UV-irradiation, and also by adriamycin treatment in a wild-type p53-dependent manner, while a previously known R2, that plays an important role in DNA synthesis during cell division, was not. Induction of p53R2 expression in p53-deficient cells caused G2/M arrest and prevented cell death in response to adriamycin. Inhibition of endogenous p53R2 expression in cells with an intact p53-dependent DNA damage checkpoint reduced ribonucleotide reductase (RR) activity, DNA repair, and cell survival after exposure to various genotoxins.[10)] Thus, inactivation of p53 could directly interfere with damage-induced transcription of p53R2, enhance mis-incorporation of dNTPs and increase the frequency of mutations, and thereby result in genomic instability in cancers where p53 has undergone mutation. We examined the role of this p53R2-dependent pathway for DNA synthesis in a p53-regulated cell-cycle checkpoint, comparing it to R2-dependent DNA synthesis. The elevation of DNA synthesis activity through RR in response to γ-irradiation was closely correlated with the level of expression of p53R2, but not that of R2. The p53R2 product accumulated in nuclei, while the R2 levels in cytoplasm decreased. We found a point mutation of p53R2 in the cancer-cell line HCT116, which resulted in loss of RR activity, suggesting that p53R2-dependent DNA synthesis plays a pivotal role in cell survival by repairing damaged DNA in the nucleus.[11)]

We also generated and analyzed mice lacking *p53R2*. They developed normally until weaned, but thereafter displayed growth retardation and early mortality; all mice died by the age of 14 weeks from severe renal failure. Pathological examination of these mice indicated multiple organ failure in kidney, heart, liver, skeletal muscle, lymphatic organs, and nervous system. The TUNEL staining of kidneys of *p53R2*−/− mice at the age of 8 weeks showed an increase of apoptotic cells. In kidney and liver of *p53R2*−/− mice, p53 protein was activated, leading to transcriptional induction of p53-target genes including *Bax*, *Noxa*, and *p53Dinp1*, followed by apoptosis of a large number of cells and organ failure. *p53R2*−/− embryonic fibroblasts (MEFs) showed similar rates of proliferation to *p53R2*+/+ MEFs, but they became immortal much earlier and were more susceptible to apoptosis induced by oxidative stress than *p53R2*+/+ MEFs. dNTP pools were severely attenuated in *p53R2*−/− MEFs suffering oxidative stress. Hence, p53R2 is considered to play a pivotal role in maintaining dNTP levels for DNA repair in the arresting cells, and an impairment of this pathway may activate the p53-dependent apoptotic pathway, resulting in severe renal failure, growth retardation and early mortality *in vivo*.[12)] We speculate that p53 might function as a checkpoint for damaged cells by balancing the death and survival signals.

Apoptosis-related genes; *p53AIP1, GML, p53ABC1L, STAG1, and Semaphorin3B*

Through direct cloning of p53-binding sequences from human genomic DNA, we have isolated a novel gene, designated *p53AIP1* (p53-regulated Apoptosis Inducing Protein 1), whose expression is inducible by wild-type p53.[13)] Ectopically expressed p53AIP1, which is localized at mitochondria, induced down-regulation of mitochondrial ΔΨm and release of cytochrome *c* from mitochondria in human cells. Immunoprecipitation and immunostaining experiments indicated interaction between p53AIP1 and bcl-2 proteins at mitochondria. Over-expression of bcl-2 blocked the down-regulation of mitochondrial

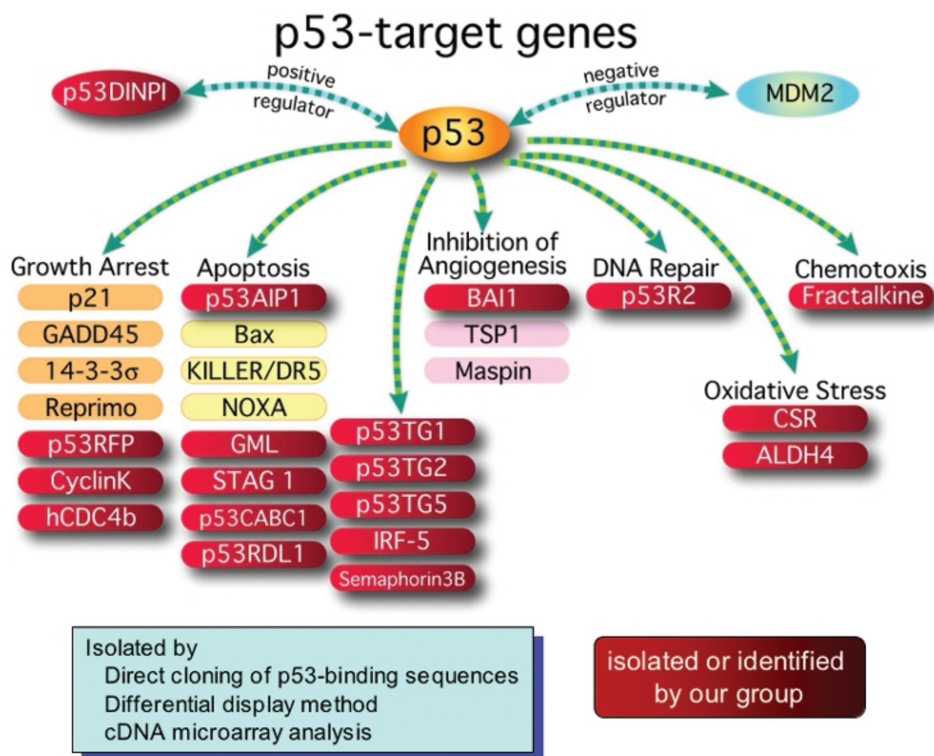

Fig. 1. p53-target genes and their physiological roles. A subset of the p53-target genes that play important biological functions, as well as genes isolated by our group, is shown.

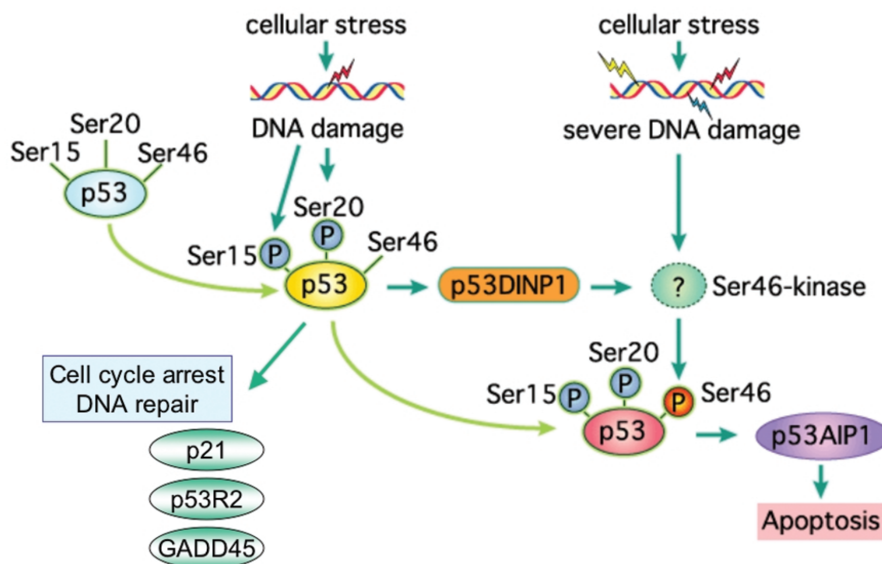

Fig. 2. Selective model for p53-target genes in cell death or survival process. When DNA damage is limited and repairable, phosphorylation of p53 occurs at certain sites (for example, Ser15 and Ser20 residues) and the modified p53 activates genes related to cell cycle arrest and DNA repair (for example, *p21*, *GADD45*, and *p53R2*). When DNA damage in cells is very severe, p53 has an additional phosphorylation at the 46th serine residue by Ser46-kinase with the co-operation of p53DINP1, and it then activates genes inducing apoptosis (for example, *p53AIP1*).

$\Delta\Psi$m and the pro-apoptotic activity of p53AIP1. We have also found that upon severe DNA damage, Ser46 on p53 is phosphorylated and apoptosis is induced. In addition, substitution of Ser46 inhibits the ability of p53 to induce apoptosis, and selectively blocks expression of p53AIP1. Our results suggest that p53AIP1 is likely to play an important role in mediating p53-dependent apoptosis, and phosphorylation of Ser46 regulates the transcriptional activation of this apoptosis-inducing gene. Considering the function of this gene, gene therapy involving p53AIP1 is expected to be useful for treatment of p53-resistant tumors (Fig. 2).[14]

Through the same approach, we isolated a *GML* (GPI-anchored molecule-like protein) gene and demonstrated an apparent correlation between its expression and the sensitivity of esophageal cancer cells to several anticancer drugs.[6] The *GML* gene product was shown to play an important role in control of the cell cycle and/or the apoptotic pathway induced by wild-type p53 after DNA damage.[15] Subsequently we introduced GML cDNA into TE10, an esophageal cancer cell line that lacks endogenous GML expression. In two resulting stable cell lines which expressed GML cDNA in the absence of wild-type p53, cell death occurred within 6 h after treatment with Taxol. TE10 parent cells or TE10 cells transfected with vector alone displayed relative resistance for 36 h. Induction of GML did not by itself affect viability. Morphological analysis confirmed that the increased chemosensitivity to Taxol conferred by GML was due to apoptosis.[16]

p53ABC1L (p53-inducible Activity of bc1 complex Like) encodes a 647-amino-acid peptide with significant sequence similarity to ABC1 (Activity of bc1 complex) in *Arabidopsis thaliana* and *Schizosaccharomyces pombe*. The *p53ABC1L* product was located in mitochondria. Inhibition of p53ABC1L expression by transfection with antisense oligonucleotide significantly reduced the apoptotic response induced by wild-type p53, suggesting that p53ABC1L plays an important role in mediating p53-inducible apoptosis through the mitochondrial pathway.[17]

A mutant version of p53 (p53-121F), in which phenylalanine replaces the 121st serine residue, can induce apoptosis more effectively than wild-type p53 (wt-p53). The *STAG1* gene was one of the transcripts showing higher expression levels in cells infected with Ad-*p53-121F* compared to those infected with Ad-wt*p53*. The encoded product appears to contain a transmembrane domain, and binding motifs for SH3 and WW. Suppression of endogenous *STAG1* using the RNA-interference method reduced the apoptotic response, whether induced by Ad-*p53-121F* or Ad-*p53*.[18]

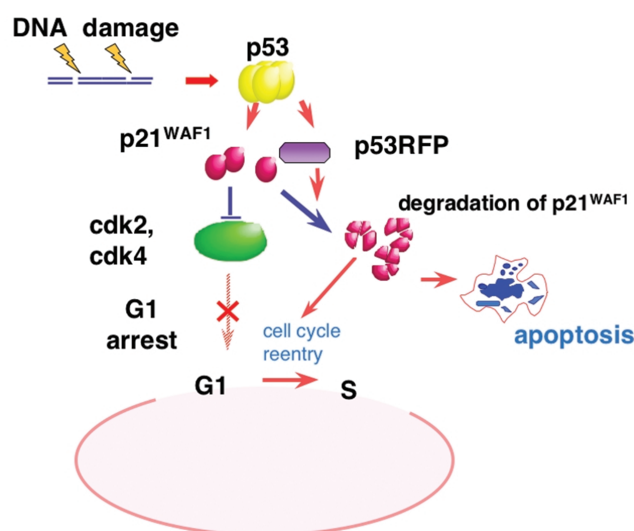

Fig. 3. Model for re-entry into the cell cycle after G1 arrest caused by $p21^{WAF1}$. p53RFP has E3 ubiquitin ligase activity and interacts with $p21^{WAF1}$ *in vitro* and *in vivo*. After cell cycle arrest and DNA repair, p53 recognizes the completion of DNA repair through an unknown mechanism and activates p53RFP for degradation of p21, resulting in re-entery of cells into the cell cycle progression. Destabilization of $p21^{WAF1}$ through transcriptional regulation of *p53RFP* represents a novel mechanism for a p53-dependent cell-cycle checkpoint.

Several groups have reported a possible role of *p53* in neuronal apoptosis; expression of p53 mRNA precedes neuronal apoptosis in neurons of injured brain and spinal cord and neuronal cells from p53–/– knock-out mice are resistant to apoptosis induced by neuronal injury, both *in vitro* and *in vivo*. These data indicate that p53 plays an important role in regulating neuronal apoptosis, probably through the transcriptional regulation of target gene(s). Semaphorin3A (Sema3A), a member of the same class as Semaphorin3B, was shown to induce neuronal apoptosis by binding to its receptor, neurophilin-1 (NRP1). Hence, Sema3B (a target molecule of p53) might be one of the mediators involved in p53-dependent neuronal apoptosis. The

information presented here should help toward a fuller understanding of the myriad physiological functions of p53.

Angiogenesis inhibition: BAI1

As normal cells progress toward malignancy, they must switch to an angiogenic phenotype to attract the nourishing vasculature that they depend on for their growth. This switch was found to coincide with loss of the wild-type allele of p53 and to be a result of reduced expression of thrombospondin-1 (TSP-1), a potent inhibitor of angiogenesis, in cultured fibroblasts from patients with Li-Fraumeni syndrome.[19] p53 stimulates the endogenous *TSP-1* gene and positively regulates TSP-1 promoter sequences; therefore, wild-type p53 in fibroblasts inhibits angiogenesis through regulation of TSP-1. Furthermore, in clinical cases genetic alterations of p53 are known to be associated with neovascularization during progression of a glioma to its more malignant form, glioblastoma. A novel p53-inducible gene, named *BAI1* (brain- specific angiogenesis inhibitor 1), encodes a 1584-amino-acid product that contains five thrombospondin type 1 (TSP-type 1) repeats and is specifically expressed in the brain.[7] We showed that a recombinant protein corresponding to the TSP-type 1 repeats present in this gene product inhibited *in vivo* neovascularization induced by bFGF in the rat cornea, and we suggested that BAI1 might play a significant role as a mediator of p53 in inhibiting angiogenesis. We also identified two novel human genes homologous to *BAI1*. Like *BAI1*, these two genes, designated *BAI2* and *BAI3*, were specifically expressed in brain, and they are likely to be expressed in the same type of cells. However, in spite of similar tissue specificity among the three *BAI* genes, only *BAI1* is transcriptionally regulated by p53.[20]

Oxidative stress: CSR and ALDH4

Oxidative stress is a pathogenic condition that causes cellular damage, and in a normally functioning cell, several transcription factors respond to this threat by modulating expression of genes whose products ameliorate the altered redox status in some way. *CSR* (Cellular Stress Response) is a novel macrophage scavenger receptor-like gene, whose transcription in normal fibroblasts was significantly elevated by exposure to UV-radiation or hydrogen peroxide, but not by DNA-damaging anti-cancer reagents, in a p53-dependent manner. Pretreatment with antioxidants prevented induction of CSR. Under conditions of oxidative stress, reactive oxygen intermediates (ROIs) were significantly depleted in CSR-overexpressing cells, indicating that the CSR product protects cells by scavenging oxidative molecules or harmful products of oxidation. Investigations into the connection of p53 as transcription factor with the regulation and function of CSR should open a way to understanding the pathogenic processes caused by oxidative stress.[21]

We also identified the aldehyde dehydrogenase 4 (*ALDH4*) gene as a direct target of p53. ALDH4 is a mitochondrial matrix NAD^+-dependent enzyme catalyzing the second step of the proline degradation pathway. The expression of *ALDH4* mRNA was induced by various cellular stresses, including hydrogen peroxide, UV-, γ-irradiation or adriamycin treatment, in a p53-dependent manner (unpublished data).

Eat-me signal: fractalkine and IRF5

Fractalkine, a CX3C-type chemokine that induces chemotaxis of monocytes and cytotoxic T-cells, was induced by ectopic expression of p53.[22] The strong induction of *fractalkine* when p53 protein was expressed by a wild-type transgene in p53-defective cells brought to light a novel role for p53; that is, potential elimination of damaged cells via the host immune-response system through transcriptional regulation of fractalkine, implying a pivotal role of p53 in immuno-surveillance to prevent cells from undergoing malignant transformation.

Expression of interferon regulatory factor 5 (*IRF5*) mRNA was also increased in the presence of exogenous p53. Expression of *IRF5* was induced in normal human dermal fibroblast (NHDF4042) cells when DNA was damaged by adriamycin or γ-irradiation, in a wild-type p53-dependent manner. Our results suggested that IRF5 might mediate the p53-dependent immune response.[23]

Cell-cycle control: cyclin K, hCDC4, and p53RFP

Cyclin K, a newly recognized member of the "transcription" cyclin family, may play a dual role by regulating CDK and transcription. We found that *cyclin K* mRNA was dramatically increased in U373MG, a glioblastoma cell line deficient in wild-type p53, by the introduction of exogenous p53.[24] A heterologous reporter assay revealed that p53BS (p53-binding site) in this gene possessed p53-dependent transcriptional activity. Colony-formation assays indicated that over-expression of cyclin K suppressed growth of T98G, U373MG, and SW480 cells, suggesting that cyclin K may play a role in regulating the cell cycle after being targeted for transcription by p53.

Expression of *hCDC4b*, which encodes one of the four subunits of the SCF (ubiquitin ligase) complex responsible for degradation of cyclin E, was dramatically up-regulated by infection with Ad-p53. An electrophoretic mobility-shift assay and a chromatin immunoprecipitation assay indicated that a potential p53-binding site (p53BS) present in exon 1b of the *hCDC4* gene was able to bind to p53. Expression of endogenous *hCDC4b*, but not the alternative transcript of this gene, *hCDC4a*, was induced in a p53-dependent manner in response to genotoxic stresses caused by UV-irradiation and adriamycin treatment. By negatively regulating cyclin E through induction of *hCDC4b*, p53 might stop cell-cycle progression at G0–G1 and may represent a novel mechanism for p53-dependent control of the cell cycle, in addition to the well-known $p21^{WAF1}$ machinery.[25]

We also isolated *p53RFP* (p53-inducible RING-finger protein), whose product has E3 ubiquitin ligase activity as a transcriptional target of p53. Inhibition of p53RFP expression using antisense oligonucleotides resulted in accumulation of $p21^{WAF1}$ protein. Furthermore, over-expression of exogenous p53RFP in the HCT116 cell line followed by exposure to γ-irradiation caused a decrease in the level of $p21^{WAF1}$ protein. The $p21^{WAF1}$ gene is a direct transcriptional target of p53, and its product inhibits cell proliferation through direct binding to cyclin-dependent kinase (CDK) or subunits of E2F transcription factor, or by interacting with proliferating-cell nuclear antigen (PCNA) to inhibit DNA replication. In DNA-damaged cells, p53 induces $p21^{WAF1}$ to arrest cells at G1. When cells have to be released from cell-cycle arrest and undergo apoptosis, $p21^{WAF1}$ is cleaved by caspase-3 or is degraded. In mammalian cells $p21^{WAF1}$ is an unstable and short-lived protein that exhibits ubiquitination *in vivo* and proteasome-dependent turnover. In humans the $p19^{SKP1}$/$p45^{SKP2}$/CUL-1 complex is likely to function as an E3 ligase that targets $p21^{WAF1}$ for ubiquitin-dependent degradation. The $p21^{WAF1}$ protein can also be degraded by the proteasome without ubiquitin attachment; 20S proteasomes are able to mediate rapid degradation of $p21^{WAF1}$ by interacting with the C-terminus of $p21^{WAF1}$ in the absence of ubiquitin conjugation. We showed that p53RFP interacts with $p21^{WAF1}$ *in vitro* and *in vivo*. Hence, p53RFP appears to represent the second known example, the first being MDM2, of an E3 ubiquitin ligase as a p53-target. Our results suggested destabilization of $p21^{WAF1}$ through transcriptional regulation of *p53RFP*, and represent a novel mechanism for a p53-dependent cell-cycle checkpoint (Fig. 3).[26]

Novel mechanism to determine cell death or survival: p53RDL1

p53RDL1 (p53-regulated Receptor for Death and Life) contains a death-domain in the cytoplasmic C-terminal region that

is highly homologous to rat Unc5H2, a dependence receptor involved in apoptosis-regulation, as well as axon guidance and migration of neural cells. It mediates p53-dependent apoptosis. However, in the presence of its ligand, Netrin-1, the p53RDL1 signaling blocked p53-dependent apoptosis through its interaction with Netrin-1, suggesting that p53 might regulate both cell death and survival of damaged cells, by balancing regulation of the p53RDL1-Netrin-1 signaling for survival and cleavage of p53RDL1 for apoptosis, thereby helping to maintain the integrity of the genome. Although the mechanism still remains to be elucidated, the possibility of applying this pathway for neuronal regeneration is interesting. In neural cells whose apoptosis is regulated by p53, Netrin-1, the ligand of p53RDL1 might be a promising drug to prevent neural cell death related to injury, neurodegenerative disorders or dementias.[27)]

Positive regulation of p53: p53DINP1

The transcription-activating function of p53 is eliminated by interaction with the *MDM2* gene product, because MDM2 promotes rapid degradation of a phosphorylated form of p53.[4)] On the other hand, p53 activates expression of the *mdm2* gene in an auto-regulatory feedback loop. This system is well known as the negative feedback system of p53. MDM2 is amplified in 30–40% of human sarcomas; this observation indicates that inhibition of p53 function by over-expression of MDM2 plays a crucial role in development of many sarcomas.

We isolated a novel p53-inducible gene, termed *p53DINP1* (p53-dependent Damage Inducible Nuclear Protein 1), that is involved in positive regulation of the p53-apoptotic pathway.[28)] Cell death induced by DSBs, as well as Ser46-phosphorylation of p53 and induction of p53AIP1, was blocked when we inhibited expression of p53DINP1 by means of an antisense oligonucleotide. Over-expression of p53DINP1 and DNA damage by DSBs synergistically enhanced Ser46-phosphorylation of p53, induction of p53AIP1 expression, and apoptotic cell death. The protein-complex interacting with p53DINP1 was shown to have a function to phosphorylate Ser46 of p53, suggesting that p53DINP1 regulates p53-dependent apoptosis through phosphorylation of p53 at Ser46, serving as a cofactor for the putative p53-Ser46-kinase.

Conclusion

We have isolated and characterized more than a dozen novel p53-target genes. We believe that our efforts have contributed to a better understanding of the physiological roles of wild-type p53, as well as the pathological effects of p53 mutations in human carcinogenesis. However, the more new information we obtain about how p53 functions, the more mysterious the physiological roles of the p53 protein become. Indeed, we are far from a full understanding of the complicated functions of the p53 protein. Although we now have almost complete information about human genome sequences, it is clear that our knowledge of human internal processes or human carcinogenesis is still very limited.

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