Chapter 10
Regulation of Programmed Cell Death by the P53 Pathway

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Abstract The p53 pathway is targeted for inactivation in most human cancers either directly or indirectly, highlighting its critical function as a tumor suppressor gene. p53 is normally activated by cellular stress and mediates a growth-suppressive response that involves cell cycle arrest and apoptosis. In the case of cell cycle arrest, p21 appears sufficient to block cell cycle progression out of G1 until repair has occurred or the cellular stress has been resolved. The p53-dependent apoptotic response is more complex and involves transcriptional activation of multiple proapoptotic target genes, tissue, and signal specificity, as well as additional events that are less well understood. In this chapter, we summarize the apoptosis pathway regulated by p53 and include some open questions in this field.

Keywords p53, apoptosis, transcription, TRAIL receptors, p53-dependent cell death.

1 Introduction

The p53 pathway is inactivated in most human tumors. It is inactivated directly as a result of mutations, with substitution mutations being common, indirectly by binding to viral or cellular proteins, or as a consequence of alterations in proteins regulating its functions (Vogelstein et al., 2000). p53 function is usually switched off, although when the cells get exposed to stress such as DNA damage induced by ionizing radiation or ultraviolet rays, activation of oncogenic signaling, hypoxia, or nucleotide depletion, p53 is accumulated in the nucleus in a tetrameric form (Bode and Dong, 2004). Upon activation, p53 mediates a growth-suppressive effect on cells by blocking the cell cycle or it can lead the cells to undergo programmed cell

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death primarily by binding to particular DNA sequences and activating transcription of specific genes (El-Deiry, 2003).

Programmed cell death, frequently referred to as apoptosis, is induced by either intracellular or extracellular stimuli. In addition to the toxic stresses mentioned earlier, serum deprivation, ligand–receptor interactions between FAS ligand (FasL)–FAS/APO1, tumor necrosis factor (TNF)–TNF receptors, and TRAIL–TRAIL receptors will also induce apoptosis (Ozoren and El-Deiry, 2003). In CD95-mediated apoptosis, there are two cell-type-specific signaling pathways, so-called type I and type II pathways (Scaffidi et al., 1998). In the type I (extrinsic) pathway, caspase-8 activation is sufficient to kill cells as a direct consequence of death receptor ligation with subsequent activation of effector caspase-3, caspase-6, and caspase-7. This death is independent of the mitochondria and is not blocked by overexpression of Bcl-2 or treatment of cells by a caspase-9 inhibitor. On the other hand, the type II (intrinsic) pathway amplifies a cell membrane-initiated death signal via the mitochondria and this form of death can be blocked by Bcl-2 or treatment of cells by a caspase-9 inhibitor.

p53 regulates these classical cell death pathways (Fig. 10.1) by either upregulating proapoptotic genes or by associating with proapoptotic genes in a transcription-independent manner. Understanding of apoptosis is very important as its dysregulation leads to variety of human diseases including cancer, autoimmune diseases, and neurodegenerative disorders. Greater insight into the pathways of apoptosis and their deregulation in disease in fundamental to understanding pathophysiology and to developing novel therapeutic agents.

2 Stabilization and Activation of P53

p53 is normally maintained at low levels in unstressed mammalian cells. The amount of p53 is determined by the rate of its degradation rather than its transcription, as blocking of its interaction with its main negative regulator Mdm2 (also known as HDM2) is sufficient to induce accumulation of the protein in cells (Michael and Oren, 2003; Vassilev et al., 2004). The primary structure of the p53 cDNA can be subdivided into three functional domains. The N-terminal region consists of a transactivation and Src homology 3-like domain, as well as a proline-rich domain. The central core consists primarily of the DNA-binding domain, where contains hot spots for various missense mutations found in human tumors. Several of the hot spots represent contact points between p53 protein and its DNA-response element. The C-terminal domain contains a nuclear localization signal, a nuclear export signal, and a tetramerization domain. The C-terminus provides a regulatory domain whose conformation and acetylation state may impact on p53 DNA binding and transactivation activity.

Mdm2 inactivates p53 by binding to its N-terminal transactivation domain to inhibit its transcriptional activity and by ligating ubiquitin at its C-terminal lysines thereby ultimately targeting p53 for proteasome-mediated degradation

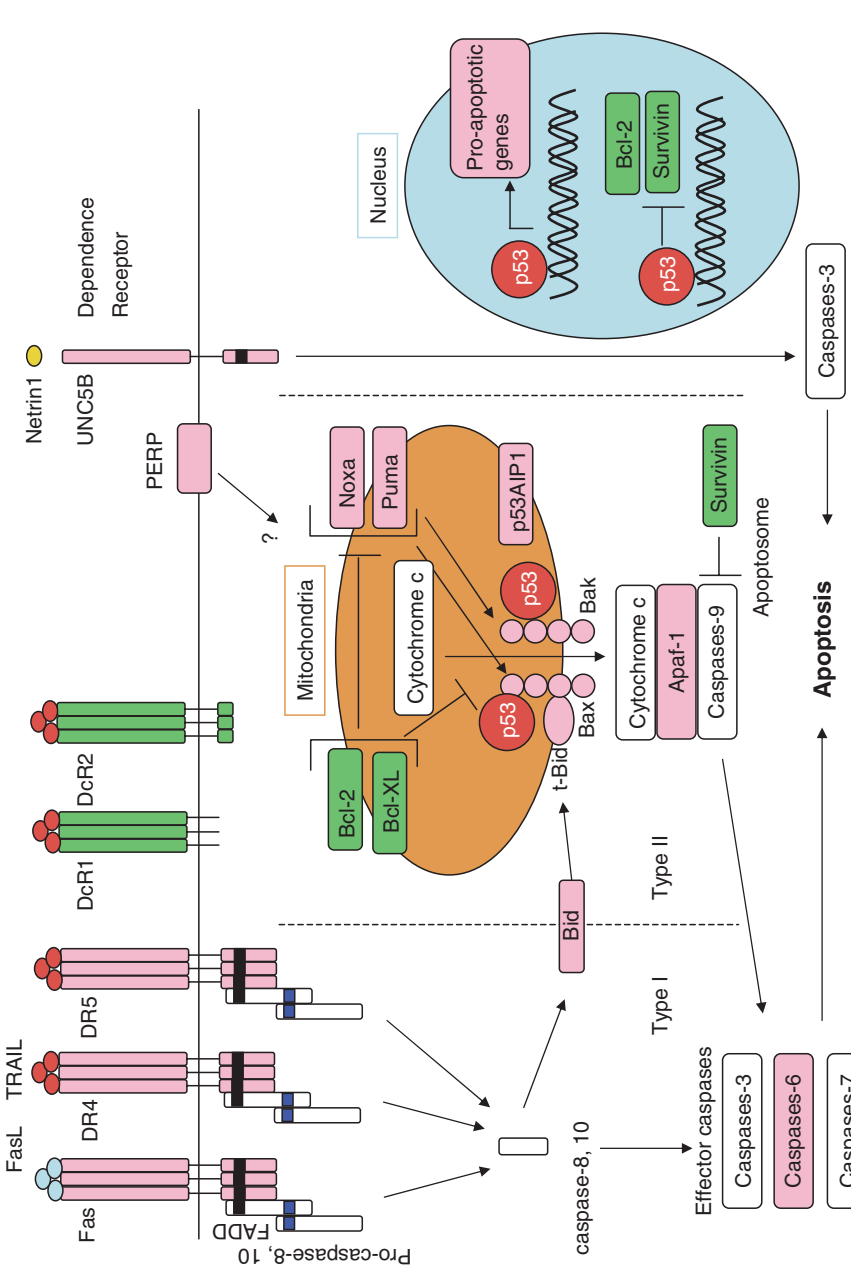

Fig. 10.1 Apoptotic pathways regulated by p53. Please see text for details of the genes, pathways, and mechanisms involved in cell death regulation and the complex signaling networks governed by p53 activity

(Rodriguez et al., 2000). Recent work by the laboratory of Wei Gu has documented monoubiquitination of p53 by Mdm2 leading to nuclear export, polyubiquitination, and degradation. As phosphorylation of N-terminal serines (particularly serine 20) blocks the interaction of p53 with Mdm2 and acetylation of C-terminal lysines prevent p53 ubiquitination by Mdm2 and subsequent degradation, these phosphorylations and acetylations can stabilize and activate p53. DNA damage induced by ionizing radiation and ultraviolet light induce p53 phosphorylation by a number of protein kinases such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), casein kinases, checkpoint kinase 1 (CHK1), checkpoint kinase 2 (CHK2), DNA-dependent protein kinase (DNA-PK), extracellular signal-related kinase (ERK), homeodomain-interacting protein kinase 2 (HIPK2), c-JUN NH_2-terminal kinase (JNK), and p38 kinase in a stimulant/kinase/phosphorylation-site-specific manner (Bode and Dong, 2004). It has also been reported and is well known that phosphorylation of serine 46 is associated with apoptosis induced by p53AIP1 (Oda et al., 2000a) and that exogenous expression of p53 mutant that has defect in serine 46 shows resistance to apoptosis (Ichwan et al., 2006). p300/CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF) acetylate lysines located at carboxyl terminus of p53. Further more, Mdm2 mediates PCAF ubiquitination and degradation (Jin et al., 2004), and can inhibit acetylation of p53 by CBP or p300 (Ito et al., 2001).

Another pathway to stabilize p53 is distinct from the first two mechanisms that involve posttranslational modification of the protein, and rather acts to inhibit the activity of the negative regulator Mdm2. This pathway is activated by oncogenic signals, for example, from Ras and Myc that in turn activate $p14^{ARF}$ leading to inactivation of Mdm2 resulting in p53 activation (Lowe and Sherr, 2003).

3 Type I Pathway

Type I pathway is initiated by ligand binding to its cognate death receptors. Overall eight receptors possessing death domains (DD) have been identified and all belong to the TNF family of receptors, including TNF-R1, Fas (CD95, APO-1), DR3, TRAIL-R1 (DR4), TRAIL-R2 (KILLER/DR5), DR6, $p75^{NTR}$, and EDAR (Ozoren and El-Deiry, 2003). Fas has a secretory decoy receptor (DcR3) that lacks a transmembrane domain. There are two decoy receptors for TRAIL, which lack a DD and these are known as TRAIL-R3 (DcR1, TRID) and TRAIL-R4 (DcR2, TRUNDD). These decoy receptors act as negative regulators of the death pathway. Till date, Fas (Muller et al., 1998), DR4 (Liu et al., 2004), KILLER/DR5 (Takimoto and El-Deiry, 2000), TRID (Ruiz de Almodovar et al., 2004), and TRUNDD (Liu et al., 2005) are reported to contain p53-specific binding sequences in intron 1 and are transcriptionally regulated by p53.

p53 target proteins Fas, DR4, and KILLER/DR5 contain cysteine-rich extracellular domains that bind their cognate ligands and intracellular portions consisting of approximately 80 amino acid DDs that transduce apoptosis-inducing signals.

As death ligands such as FasL or TRAIL exist in a homotrimeric form, binding to their respective receptors leads to receptor trimerization. The ligand/receptor interaction triggers formation of the death-inducing signaling complex (DISC) which contains Fas-activating DD (FADD) and initiator caspases, pro-caspase-8 or pro-caspase-10. FADD is the adaptor protein which links receptors and pro-caspases through its two distinct domains, a DD that binds with the DD of the receptors and a death effector domain (DED) which binds with the DED of pro-caspase-8. Association of pro-caspase-8 with the DISC generates a p20 fragment from the caspase by cleavage and further processing leads to a p10 fragment for its full activation (Medema et al., 1997). This mature caspase can cleave downstream effector caspase-3, caspase-6, and caspase-7 (Riedl and Shi, 2004). The extrinsic pathway can cross talk with the intrinsic pathway via BID. When BID is cleaved by caspase-8, truncated BID is myristoylated, translocates to mitochondria, releases proapoptotic proteins, and further activates death signaling and execution events (Zha et al., 2000).

3.1 Fas (APO-1, CD95)

The Fas receptor is a type I membrane protein expressed abundantly in various tissues. The *Fas* gene is located on human chromosome 10q24.1 and on chromosome 11 in mice (Nagata, 1999). The human and mouse *Fas* genes contain a p53 DNA-binding site in intron 1 and through this site the expression of the *Fas* gene can be transcriptionally upregulated by p53 (Muller et al., 1998; Munsch et al., 2000). Fas is also transcriptionally regulated by Sp1 and NF-κB (Chan et al., 1999; Xiao et al., 2001). The FasL–Fas interaction plays an important role in immune homeostasis, especially maintaining immune privilege in the eye and testis. As tumor cells can evade the host immune surveillance system by overexpressing FasL and can induce apoptosis in the T-cells responsible for the immune response, a phenomenon known as Fas counterattack, an understanding of the Fas-mediated apoptotic pathway is important for understanding tumor biology, It has been reported that CMT93 colon carcinoma cell downregulation of FasL has no effect on cell growth in vitro, but results in reduced tumorigenicity in vivo, possibly by the mechanism of loss of the Fas counterattack (Ryan et al., 2005).

Fas is transcriptionally upregulated by 5-FU and mediates apoptosis in a p53-dependent manner in MCF7 and HCT116 cells (Longley et al., 2004). It has also been reported that p53 relocalizes Fas to the cell surface (Bennett et al., 1998), providing a role of p53 in the Fas apoptotic pathway independent of its transcriptional activity. Wild-type p53 transduction in p53-mutant non-small-cell lung carcinoma cells induces Fas expression and the cells become susceptible to cytotoxic T lymphocyte-mediated killing (Thiery et al., 2005). There are certain p53 mutants, which can induce cell cycle arrest, but not apoptosis, so-called discriminatory mutants. Munsch et al. reported that discriminatory mutants Pro-175 and Ala-143 have activity to induce Fas transcription, but not apoptosis, suggesting upregulation of Fas is not enough to induce apoptosis in some circumstances (Munsch et al.,

2000). Furthermore, Fas does not appear to be required for p53-dependent apoptosis in response to DNA damage by irradiation (Fuchs et al., 1997).

3.2 Trail Receptors

In humans, there are four homologous TRAIL receptors including DR4, KILLER/DR5, TRID, and TRUNDD, as well as a fifth soluble receptor osteoprotegerin (Wang and El-Deiry, 2003a). The extracellular cysteine-rich domains of DR4, KILLER/DR5, TRID, and TRUNDD are 52–69% identical to each other and the DD of DR4 and KILLER/DR5 are 64% identical to each other (Ozoren and El-Deiry, 2003). As these genes are clustered on human chromosome 8p21–22, they might have arisen from a common ancestral gene (Degli-Esposti et al., 1997a). TRAIL seems to be promising for cancer therapeutics as many cancer cells are sensitive to TRAIL while normal cells are not (Wang and El-Deiry, 2003a).

3.2.1 DR4

DR4 protein is a 445 amino acid-containing type I transmembrane receptor, which is generated through the cleavage of a signal sequence of 23 amino acids from a primary protein (Pan et al., 1997a). The protein has three cysteine-rich repeats in the extracellular domain. The DD of DR4 is 30 and 19% identical with that of TNF-R1 and Fas, respectively. It has been reported that nucleotide substitutions in the extracellular domain of DR4 were correlated with increased risk of lung, and head and neck cancers (Fisher et al., 2001). Somatic mutations of DR4 have been found in non-Hodgkin's lymphoma (Lee et al., 2001), breast cancers (Shin et al., 2001), and osteosarcoma (Dechant et al., 2004). The DR4 expression level is known to be the one of the determinants to TRAIL sensitivity in many cancer cell lines (Kim et al., 2000), and homozygous deletion of the *DR4* gene has been reported in the FaDu nasopharyngeal cancer cell line and this is associated with TRAIL resistance (Ozoren et al., 2000). Approximately 20% of the normal population carries the polymorphic DR4 variant that contains adenine to guanine alteration in the DD (K441R). When a DR4 K441R expressing plasmid was transfected into human cells, it acted as a dominant negative TRAIL receptor resulting in decreased sensitivity to TRAIL (Kim et al., 2000). From these observations, DR4 seems to be a major factor determining TRAIL sensitivity. p53 overexpression by adenovirus-p53 induces upregulation of DR4 and DR5 resulting in increased apoptosis by TRAIL treatment in myeloma cells (Liu et al., 2001). As, wild-type p53 is not required for TRAIL sensitivity in many cancer cell lines (Kim et al., 2000), it is the open question that to what extent p53 is involved in DR4-mediated apoptosis.

3.2.2 KILLER/DR5

KILLER/DR5 is a 411 amino acid containing protein that includes a 51 amino acid signal peptide sequence. Other than p53, a recent study demonstrated that NF-κB can also upregulate KILLER/DR5 transcription in the presence of p53 (Shetty et al., 2005). Germline or somatic mutations of the *KILLER/DR5* gene have been reported in head and neck cancer (Pai et al., 1998), non-Hodgkin's lymphoma (Lee et al., 2001), and breast cancer (Shin et al., 2001).

Compared to DR4, many studies were conducted to elucidate the role of KILLER/DR5 in the p53 pathway, as it was first found as a DNA damage-inducible p53-regulated gene in doxorubicin-treated cell lines (Wu et al., 1997). Comparison of the apoptotic response of p53+/+ and p53−/− mice after ionizing radiation is a good in vivo model to study DNA damage-induced p53-dependent apoptosis in the context of studying p53 target gene tissue specificity. While thymus, spleen, and small intestine underwent p53-dependent apoptosis in the mouse model, among p21, E124/PIG8, Bax, Fas, and KILLER/DR5, KILLER/DR5 was the only upregulated gene after γ-irradiation in a p53-dependent manner to induce apoptosis in the spleen and small intestine, implicating a critical role of KILLER/DR5 in the radiation response (Burns et al., 2001). Recent results using DR5 knockout mice further support the importance of DR5 in the p53 pathway (Finnberg et al., 2005). DR5-null mice showed a slightly larger thymus than wild-type mice. As DR5 is the only known TRAIL receptor in mice, the results suggest that negative selection in thymocytes might be in part controlled through the DR5 receptor. In these mice, there was no evidence of spontaneous autoimmune disease as reported in TRAIL knockout mice (Lamhamedi-Cherradi et al., 2003). E1A stabilizes p53 and transactivates its target genes. The result that DR5-null mouse embryo fibroblasts (MEFs) expressing E1A did not undergo apoptosis after TRAIL treatment suggests that there are no other TRAIL receptors in mice besides DR5, which can be transactivated by p53 in mice. DR5-null tissues showed reduced amounts of apoptosis compared to wild-type thymus, spleen, Peyer's patches, and the white matter of the brain. However, because gene targeting of DR5 failed to nullify all death in these organs, it is likely that DR5 is only one of the several p53 target genes that are important in this response. In the colon, DR5 wild type and null mice showed approximately the same amount of radiation-induced cell death. However, in the human colon cancer cell line HCT116 silencing of DR5 induces accelerated growth of tumor xenografts (Wang and El-Deiry, 2004a, b) and also DR5 is required for p53-dependent TRAIL sensitivity in mismatch repair deficient Bax−/− HCT116 cells (Wang and El-Deiry, 2003b). From these observations, even in the colon, DR5 is important in apoptosis within the p53 pathway that suppresses tumor formation or progression. Taken together, DR5 seems to play a critical role in DNA damage-induced programmed cell death in the p53 pathway.

3.2.3 TRID/DCR1 and TRUNDD/DCSR2

TRID and TRUNDD contain extracellular cysteine-rich domains. TRID consists of an extracellular TRAIL-binding domain linked to the membrane through a glycosylphosphatidylinositol (GPI) anchor and completely lacks an intracellular domain, whereas TRUNDD contains an intracellular domain that has a truncated DD which can transduce NF-κB signal (Degli-Esposti et al., 1997b). Over the extracellular domain, TRID is 69 and 52% identical with DR4 and DR5, and TRUNDD is 70%, 57%, and 58% identical with TRID, DR5, and DR4, respectively. TRID mRNA is expressed in normal tissues but not in many tumor cells, giving a rationale for TRAIL on its tumor-specific apoptosis-inducing activity (Sheridan et al., 1997; Pan et al., 1997b).

Although these decoy receptors are regulated by p53, little is known about their role in the p53-regulated apoptotic pathway. TRID is overexpressed by genotoxic stress in p53 intact cells and is overexpressed in gastrointestinal tumors (Sheikh et al., 1999). TRUNDD is induced by adenovirus-p53 overexpression and TRUNDD can delay TRAIL-, p53-, and KILLER/DR5-dependent apoptosis in colon cancer cells (Meng et al., 2000). It has also been reported that silencing of TRUNDD enhances doxorubicin-induced apoptosis in HCT116 cells (Liu et al., 2005). It therefore seems that these decoy receptors are forming a negative feedback loop to dampen p53-mediated apoptotic signaling.

4 Type II Pathway

The type II death pathway is evoked through intrinsic stimuli such as DNA damage, cytotoxic drugs, hypoxia, oncogenic signaling, or even extrinsic death receptor signals in type II cells. Mitochondrial factors are crucial in the efficiency of cell death mediated by this pathway. Cytochrome c released from mitochondria assembles a cytosolic caspase-activating complex called apoptosome which consists of Apaf-1, caspase-9, and cytochrome c, while release of Smac/DIABLO and Htra2/Omi inactivate inhibitor of apoptosis proteins (IAPs), the inhibitors of caspases, enhance apoptosis (Danial and Korsmeyer, 2004). Bcl-2 family members are the key components in this process. They are categorized into three groups according to their function and numbers of Bcl-2 homology (BH) domains. The first group includes antiapoptotic members such as Bcl-2, Bcl-X_L, and MCL-1, which contains four BH domains. Their BH1–3 domains are in close spatial proximity and create a hydrophobic pocket, which can mask a BH3 domain of proapoptotic members, blocking their proapoptotic functions (Muchmore et al., 1996; Sattler et al., 1997). Multidomain proapoptotic members of the family are Bax and Bak, which are thought to form a pore in the mitochondrial membrane and release cytochrome c into the cytosol. These two molecules are thought to be required in the type II pathway as cells lacking both Bax and Bak, but not cells lacking one of them are completely resistant to tBid-induced cytochrome c release and apoptosis (Wei et al.,

2001). The last members of the family are BH3-only proapoptotic proteins, which are Bid, Noxa, Puma, Bad, Bik, and Bim. Bid provides the only known connection between the extrinsic and intrinsic pathways, while the others are thought to act upstream of Bax and Bak. Cartron et al. (2004) showed that Bid and Puma specifically bind to the first α-helix of Bax leading to its activation. Bad and Bik cannot directly activate Bax but promote apoptosis by binding to Bcl-2 to inhibit their antiapoptotic functions (Letai et al., 2002).

4.1 Bid

Bid gene is transcriptionally regulated by p53 and contains a functional p53 DNA-binding site in the first large intron (Sax et al., 2002). Bid−/− MEFs are resistant to adriamycin and 5-FU as compared to Bid+/+ MEFs, showing its role as a chemosensitivity determinant (Sax et al., 2002). Recently, Bid was shown to be a sentinel for DNA damage, and it was reported to be phosphorylated by ATM (Zinkel et al., 2005; Kamer et al., 2005). It was also shown that other than the proapoptotic function of the protein, when it is phosphorylated following exposure to low dose of ionizing radiation or the DNA-damaging agent etoposide, Bid can block the cell cycle in the G2 phase. Even though it does not induce cell cycle arrest in the G1/S phase as is brought about by p21, Bid might be one of the regulators that determines cell fate after DNA damage, i.e., whether the cells should live or die.

4.2 Puma and Noxa

Puma (bbc3) and Noxa are p53 target genes belonging to the BH-3 only proteins, contain p53 DNA-binding sequences in the first intron and can induce apoptosis by p53 overexpression or exposure to DNA-damaging stimuli (Oda et al., 2000a; Yu et al., 2001; Nakano and Vousden, 2001; Han et al., 2001). Serum starvation and glucocorticoid treatment also induce Puma and virus infection and interferon induce Noxa expression independent of p53 activation, respectively (Han et al., 2001; Sun and Leaman, 2005). In hematopoietic progenitor cells, Slug represses p53-mediated transcription of Puma in turn protecting the cells from γ-radiation-induced cell death, and it was also found that Slug itself was upregulated by p53 (Wu et al., 2005). Furthermore, it was recently demonstrated that p53 family member p73 transactivates Puma and Noxa expression independent of p53, and the other member delta p63 acts as their repressor inhibiting head and neck tumor cells from apoptosis (Rocco et al., 2006). Noxa and Puma are tightly regulated genes with redundancy in their stimulation and regulation by transcription factors, suggesting their important role in apoptosis.

These p53 targets seem to have tissue specificity. By ionizing radiation, Noxa was expressed in the red pulp, where as Puma was induced in the white pulp of the

spleen in a p53-dependent fashion (Fei et al., 2005). Puma–/– MEFs and Noxa–/– MEFs showed increased resistance in apoptosis induced by etoposide treatment or γ-irradiation. Although single gene, *Puma* or *Noxa*, knockdown could not attain the resistant level to that of p53–/– MEFs, it was suggested that Puma and Noxa have redundancy in inducing apoptosis in these cells (Villunger et al., 2003). On the other hand, Puma knockout nullified nearly all of the cell death attributed to p53 in primary hematopoietic cells and the developing central nervous system in response to γ-radiation or oncogenic signals from c-Myc, i.e., it has indispensable role in apoptosis in these tissues (Jeffers et al., 2003). Yu et al. (2003) reported that targeted deletion of *Puma* gene in HCT116 cells completely blocked apoptosis induced by p53 overexpression, adriamycin exposure, or a hypoxic environment (Yu et al., 2003). Another important notion of the study is that in the presence of p21, cellular stresses lead to cell cycle arrest, whereas deprivation of p21 by gene targeting results in enhanced apoptosis induced by the same stimuli. From such observations, it has been proposed that cell fate between cell cycle arrest and apoptosis is determined by the balance between p21 and Puma. Another study showed Noxa and Bax doubly knocked out MEFs were more resistant to apoptosis induced by adriamycin or oncogenic signals as compared to single knockouts of these genes (Shibue et al., 2003). It was suggested from the result that Noxa and Bax carry out different functions in the apoptosis pathway.

4.3 Bax and Bak

The gene-encoding BAX is a transcriptional target of p53 (Miyashita and Reed, 1995). BAK has also been reported to be upregulated by p53 (Pearson et al., 2000; Pohl et al., 1999). Bax and Bak appear to have some overlapping roles in apoptosis, as either thymocytes from Bak–/– or Bax–/– null mice do not show radiation-induced apoptosis, although thymocytes from Bak and Bax double-knockout mice show resistance to γ-radiation or etoposide treatment (Lindsten et al., 2000). Furthermore, Bak and Bax doubly deficient MEFs show resistance to multiple intrinsic death-inducing stimuli such as staurosporine, ultraviolet radiation, and growth factor deprivation (Wei et al., 2001). Bax–/– HCT116 cells are resistant to TRAIL treatment, but etoposide and camptothecin treatment of the cells restores their sensitivity to TRAIL by upregulating Bak and DR5 (LeBlanc et al., 2002). However, recent studies with DR5 or Bak knockdown suggests that this restored sensitivity relies more on DR5 upregulation and a conversion of cells from type II to type I signaling, in the case of TRAIL and chemotherapy treatment (Wang et al., 2003b).

Recent studies revealed that p53 may have a transcription-independent activity in the mitochondrial death pathway involving Bak and Bax. After DNA damage induced by irradiation or chemotherapeutic agents, p53 has been reported to translocate to the mitochondria and to activate either Bax- (Chipuk et al., 2005) or Bak- (Leu et al., 2004) dependent mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c into the cytosol. However, translocation of

p53 to the mitochondria is not sufficient to induce cell death (Essmann et al., 2005), as p53 is sequestered by Bcl-X_L at mitochondria and its activity to induce MOMP is blocked (Mihara et al., 2003; Chipuk et al., 2005). Puma has been reported to act on the complex of p53–Bcl-X_L thereby releasing p53 from Bcl-X_L to allow for the MOMP-inducing activity (Chipuk et al., 2005). However, whether the p53–Bcl-X_L or p53–Bcl2 complexes act as positive or negative regulators of cytochrome c release is still under study (Mihara et al., 2003; Chipuk et al., 2005; Tomita et al., 2006).

4.4 P53AIP1

The *p53AIP1* gene is induced following severe DNA damage associated with p53 ser-46 phosphorylation and localization of p53AIP1 at mitochondria (Oda et al., 2000b). Phosphorylation of p53 and subsequent p53AIP1 induction is also regulated by the p53-inducible protein p53DINP1 (Okamura et al., 2001). p53AIP1 has been reported to have potential to release cytochrome c from mitochondria into the cytosol and to induce apoptosis, although its precise mechanism and relation with other apoptotic factors is not clarified yet.

4.5 Apaf-1

The *Apaf-1* gene is a transcriptional target of p53 and it is also transcriptionally induced by E2F (Moroni et al., 2001). The study comparing p53−/− and wild-type mice showed that Apaf-1 expression was p53-dependent in the spleen and heart (Ho et al., 2003). Apaf-1−/− MEFs were resistant to p53-dependent cell death-induced by oncogenic Myc and Ras signaling (Soengas et al., 1999). Apaf-1 was found to be silenced in metastatic melanomas by hypermethylation and restoration of Apaf-1 expression led to efficient caspase-9 activation and adriamycin-induced cell death (Soengas et al., 2001), supporting its role as a chemosensitivity determinant.

5 Dependency Receptor Pathway

There is unique apoptotic pathway called dependency receptor pathway. In the absence of ligand, expression-dependent receptors induce apoptosis, whereas binding of cognate ligands to their receptors blocks apoptosis and this apoptotic pathway seems to be independent of mitochondria (Arakawa, 2004; Bredesen et al., 2004). Examples for the receptor/ligand are $p75^{NTR}$/neurotrophin, UNC5B (p53RDL1)/Netrin-1, and deleted in colorectal cancer (DCC)/Netrin-1. These receptors are involved in axon guidance during neuronal development and among these receptors

UNC5B was shown to be a p53 transcriptional target, which is implicated in p53-dependent apoptosis (Tanikawa et al., 2003). Loss of DCC has been reported in many cancers, and binding of Netrin-1 to UNC5B has been reported to repress the p53 target genes *Bax* and *p21*. This newly found pathway might be also involved in p53-related tumorigenesis.

6 PERP

The *PERP* gene is transcriptionally upregulated by p53 (Attardi et al., 2000) as well as p63 (Ihrie et al., 2005). It is a membrane protein involved in apoptosis induced by p53 overexpression and Bcl-2 reduces the cell death, suggesting that the mitochondria are involved in its signaling (Attardi et al., 2000). PERP localizes specifically to desmosomes, adhesion junctions important for tissue integrity. Numerous structural defects in desmosomes are observed in skin of PERP–/– mice (Ihrie et al., 2005). It was recently reported that PERP-null mice are not tumor-prone as compared to wild-type mice (Ihrie et al., 2006). As p53-null mice are tumor prone, whereas single knockout of the other p53 targets such as Puma, Bak, or Bax do not produce tumor-prone mice, the observation does not imply that PERP is not important in the p53 apoptotic pathway.

7 PIGs

The PIGs are "p53-induced genes," identified by transducing p53 into the human colorectal cancer line DLD-1 that undergoes apoptosis in response to p53 expression (Polyak et al., 1997). As many of these genes were capable of producing or responding to reactive oxygen species, the importance of reactive oxygen species in the p53 pathway was suggested. One of the PIGs, EI24/PIG8, has also been identified as the gene upregulated by etoposide treatment in murine NIH3T3 cells (Lehar et al., 1996). It was recently shown that EI24/PIG8 colocalizes at the endoplasmic reticulum with Bcl-2 and loss of EI24/PIG8 is positively related with invasiveness of breast cancers (Zhao et al., 2005).

8 Caspase-6

Caspases, the cysteine proteases that cleave after an aspartate residue in their substrate, are the central components of the apoptotic pathway. They are usually divided into two classes, the initiator caspase-2, caspase-8, caspase-9, and caspase-10, and the effectors, caspase-3, caspase-6, and caspase-7 (Riedl and Shi, 2004). Caspase-6 is a transcriptional target of p53 in the apoptotic response (MacLachlan

and El-Deiry, 2002). Caspase-1 is also a p53 transcriptional target, although it is involved in inflammatory response rather than apoptotic pathway (Gupta et al., 2001). p53 seems to have potential to activate caspase-6 and sensitize cells to chemotherapeutic drugs leading them to apoptosis by the mechanism other than its transcriptional upregulation (MacLachlan and El-Deiry, 2002). We have also previously reported that caspase-10 is directly induced by p53.

9 P53-Dependent Apoptosis Under Hypoxic Conditions

Solid tumors acquire regions of hypoxia as a result of insufficient blood supply. Cells containing wild-type but not mutant p53 undergo apoptosis in hypoxic regions (Graeber et al., 1996), leading to a powerful selection pressure to promote tumor progression and therapeutic resistance (Harris, 2002). p53 shows an altered behavior under hypoxia. Under hypoxia, p53 does accumulate in cells, although it does not upregulate most of the known p53 target genes such as *p21, Bax, GADD45, DR5*, or *Puma* (Koumenis et al., 2001; Fei et al., 2004). We have recently identified that Bnip3L is playing a role in apoptosis during hypoxia in some human tumor cell lines (Fei et al., 2004). Bnip3L was found to be a direct transcriptional target of p53 as well as hypoxia-inducible factor 1 (HIF1). p53-dependent apoptosis during hypoxia was reduced after knocking down Bnip3L. Furthermore, nontumorigenic U2OS cells were converted into a tumorigenic state in mouse xenograft experiments following stable Bnip3L knockdown.

10 Transcriptional Repression of Antiapoptotic Genes

IAPs and Bcl2 block apoptosis by inhibiting caspase activation and MOMP. In addition to transcriptional activation activity, p53 exerts its apoptosis-promoting effects by repressing antiapoptotic gene transactivation (Murphy et al., 1999; Wu et al., 2001; Hoffman et al., 2002). Its mechanism appears to involve association of p53 with histone deacetylases (HDACs) and its interaction is mediated by corepressor mSin3a. DNA damage induces the p53–mSin3a interaction and targets HDACs to the promoters of the p53-repressed genes, where HDACs deacetylate histones and create a chromatin environment that is unfavorable for transcription.

11 P53 as a Therapeutic Target

A number of strategies have been developed to target p53 in cancer therapy. For about half of human cancers, which possess wild-type p53, the Mdm2–p53 interaction could be a major target to prevent p53 from degradation. Nutlin-3 is an example of

a small molecule that specifically disrupts the p53–Mdm2 interaction. It was recently demonstrated that administration of Nutlin-3 suppressed xenograft growth in a dose-dependent manner (Tovar et al., 2006). As Mdm2 downregulation and subsequent p53 upregulation is reported to bring lymphocytopenia as a side effect in hypomorphic Mdm2 mice, further study may help to compare its benefit to disadvantage or advantages over standard chemotherapy.

Histone deacetylase inhibitors (HDACIs) have been shown to exert various antitumor effects and they are presently in clinical trials (see Chapter 13). p53 is one of the targets of HDACIs, as HDACIs inhibit deacetylation of the C-terminal lysines and induce apoptosis in gastric cancer (Terui et al., 2003) and prostate cancer cells (Roy et al., 2005). It has also been demonstrated that HDACIs enhance the tumoricidal effects of p53 adenovirally transferred gene therapy (Takimoto et al., 2005).

The status of an intact p53 pathway positively correlates with the response to the majority of chemotherapeutic drugs, most, although not all, of them being DNA-damaging agents (Weinstein et al., 1997; O'Connor et al., 1997). However, there are some clinically useful agents such as the antimitotic agent taxol, which was found to be more effective in tumor cells with mutant p53 (Weinstein et al., 1997). In this context, we have identified the Polo-like kinase family member serum-inducible kinase (Snk/Plk2) as a p53 target and its silencing by siRNA leads to mitotic catastrophe after taxol treatment, suggesting p53-dependent activation of Snk/Plk prevents mitotic catastrophe following spindle damage (Burns et al., 2003).

Much effort has been devoted to overcome mutant p53 by small molecules that can restore the wild-type functions to mutant p53. CP-31398, a strylquinazoline, was identified from a screen of the library containing more than 10,000 synthetic compounds (Foster et al., 1999). The molecule not only promotes the stability of wild-type p53, but also allows mutant p53 to maintain an active conformation, enabling transcription and subsequent tumor growth suppression. CP-31398 can cause either cell cycle arrest or cell death in tumor cell lines carrying mutant p53, and combination of CP-3198 with chemotherapy or TRAIL exhibit synergistic effects enhancing cell killing (Takimoto et al., 2002). It has been shown that stabilization of p53 by CP-31398 involves a mechanism targeting blockade of ubiquitination of p53 and its further degradation (Wang et al., 2003). Neither phosphorylation of p53 at serine 15, 20, or interaction between Mdm2 was inhibited by CP-31398, highlighting a unique mechanism by which it can activate p53. PRIMA-1 also induces apoptosis in tumor cells (Bykov et al., 2002) and it has synergistic effects with chemotherapeutic drugs (Bykov et al., 2005).

A peptide derived from the C-terminus of p53 is known to activate its specific binding to DNA including several p53 DNA contact mutants (Hupp et al., 1995). Several cationic peptides such as TAT and polyArg can penetrate into the cells through a mechanism called macropinocytosis (Wang and El-Deiry, 2004b). Utilizing this technology, Snyder et al. (2004) showed that the C-terminal peptide of p53 fused with TAT induced cell cycle arrest and apoptosis in a peritoneal carcinomatosis model and prolonged survival of the mice.

12 Future Directions

Already a quarter century has passed since the discovery of p53 and we have learned much about its important role as a tumor suppressor gene as well as its complicated network governing programmed cell death. However, there are still important problems left to be solved. There are numerous genes known to be involved in the p53 pathway, but are they all equally important? Which genes are involved in which tissues? No single gene so far can account for p53-mediated apoptosis alone, and it might be possible that there is no such gene. The principle question is that we still do not know how p53 determines cell fate. Progress towards this understanding as well as efforts to develop therapies targeting this p53 pathway and its family members represent important future directions.

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