BAX and BAK mediate p53-independent suppression of tumorigenesis

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Summary

BAX and BAK are essential regulators of proapoptotic signaling, and the disruption of apoptosis is linked to the development of cancer. To investigate the role of BAX and BAK in tumorigenesis, primary baby mouse kidney epithelial cells (BMKs) from wild-type, BAX-, BAK-, or BAK- and BAK-deficient mice were transformed by adenovirus E1A and dominant-negative p53 (p53DD). In wild-type BMKs, the expression of E1A and inactivation of p53 was sufficient for transformation but not tumorigenesis. In contrast, E1A- and p53DD-transformed BAX- and BAK-deficient BMKs formed highly invasive carcinomas. Transformed BMKs deficient for either BAX or BAK were also tumorigenic, but only when heterozygous for the remaining *bax* or *bak* allele, the expression of which was lost in most resulting tumors. Thus, BAX and BAK function to suppress tumorigenesis, and their deficiency was selected for in vivo.

Introduction

Altered regulation of programmed cell death or apoptosis has long been linked to the development of cancer (Johnstone et al., 2002), and mitochondria have emerged as gatekeepers in many apoptotic signaling pathways (Wang, 2001). Members of the BCL-2 family of proteins that regulate apoptotic signaling through mitochondria are key regulators of apoptosis in mammalian development, and their deregulation is associated with disease, particularly cancer (Gross et al., 1999). There are three classes of BCL-2 family members: those that act predominantly to inhibit apoptosis (e.g. BCL-2, BCL-x_L, and adenoviral E1B 19K); those that act predominantly to promote apoptosis (e.g. BAX and BAK); and the BH3-only Bcl-2 family members (e.g. BID, PUMA, NOXA, BAD, and NBK/BIK), that contain the BH3 interaction domain that act as regulators of the anti- and proapoptotic family members (Gross et al., 1999). Signal transduction events modify the activity of BH3-only proteins that in turn interact with pro- or antiapoptotic family members to either antagonize or activate their function. Stimulation of apoptosis can therefore be achieved by antagonizing a survival activity or by activating a death activity. The biochemical events that implement death signaling, many of which occur in mitochondria, and the means by which they are deregulated in disease, still remain to be determined.

The t(14;18) translocation that results in overexpression of antiapoptotic BCL-2 is the hallmark of human B cell follicular lymphoma, and induction of BCL-2 expression is a feature of other human tumors and their progression (Gross et al., 1999; Johnstone et al., 2002). Indeed, overexpression of antiapoptotic BCL-2 family member BCL-x_L in a mouse tumorigenesis models accelerates tumor formation (Naik et al., 1996; Pelengaris et al., 2002). Mutations in proapoptotic BCL-2 family member BAX or BAK have also been observed in human cancers, particularly colon tumors that display microsatellite instability (Kondo et al., 2000; Rampino et al., 1997). Furthermore, BAX deficiency in some transgenic mouse tumor models accelerates tumor growth (Eischen et al., 2002; Shibata et al., 1999; Yin et al., 1997), yet in others, the presence of BAX has a stimulatory effect on tumorigenesis (Knudson et al., 2001). Although far from being entirely clear, these and other observations suggest that apoptosis may be disabled in some cases during human tumor development, and that it can occur by the gain of a survival activity or the loss of a death activity.

SIGNIFICANCE

The role of apoptosis in tumorigenesis has largely been ascribed to an obligatory step for rapidly dividing epithelial cells to overcome p53-mediated cell death triggered by the deregulation of the cell cycle or DNA damage. While loss of p53 function is often essential for oncogenesis, the function of other death-promoting genes, such as *bax* and *bak*, in tumorigenesis and their functional interaction with the p53 pathway has been less clear. Here we propose that a p53-independent apoptotic pathway that is regulated by BAX and BAK is involved in tumor suppression. Understanding the mechanism and the molecular events regulating this pathway may reveal novel targets for effective combinatorial therapeutic approaches to cancer treatment.

Loss of function of the retinoblastoma (RB) tumor suppressor protein is associated with deregulation of cell growth control and activation of p53-dependent apoptosis, suggesting that apoptosis may be a cellular response to the elimination of abnormal, emerging tumor progenitors (White, 1994). The p53 tumor suppressor, which is frequently mutated in human tumors (Hainaut and Hollstein, 2000), functions to activate apoptosis (Balint and Vousden, 2001). p53 regulates the transcription of genes that promote cell growth arrest (El-Deiry et al., 1993), but also activates expression of proapoptotic BH3-only Bcl-2 family members (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001) that act as upstream regulators of proapoptotic BAX and BAK in mitochondria (Cheng et al., 2001; Zong et al., 2001). Despite these indications that cancers emerge in part by defeating the capacity of cells to undergo apoptosis, clear relationships between BCL-2 family members and cancer progression, prognosis, and significance to treatment have often been obscure. Nonetheless, understanding the function of BCL-2 family members in mitochondria resides at the core of many apoptotic signaling events.

Antiapoptotic BCL-2 family members appear to function at least in part by interacting with and antagonizing proapoptotic family members, either BAX and BAK or their upstream activators, the BH3-only proteins (Gross et al., 1999). Determining the role and means by which BAX and BAK function is thus an essential component in understanding apoptotic signaling. BAX-deficient mice display lymphoid hyperplasia, perturbation of the regulation of ovarian cell death, and male germ cell hypoplasia (Knudson et al., 1995; Shindler et al., 1997). BAK-deficient mice are developmentally normal, whereas BAX- and BAK-deficient mice have numerous developmental defects associated with failure to undergo apoptosis, including accumulation of excess cells within the central nervous and hematopoietic systems, and persistence of interdigital webs (Lindsten et al., 2000). Thus, BAX and BAK are functionally redundant activators of apoptosis; however, since most of the BAX- and BAK-deficient animals die perinatally, investigation of whether they are prone to tumor formation has been limited. Furthermore, BAX-deficient mice display male sterility (Knudson et al., 1995), making it difficult to evaluate BAK and BAX deficiency in tumor-prone murine models.

Antiapoptotic BCL-2 family members such as BCL-2, BCL-x_L, or E1B 19K suppress apoptosis by regulating the function, either directly or indirectly, of proapoptotic family members BAX and BAK. BAX and BAK function in mitochondria is activated by interaction with BH3-only proteins via binding of their BH3 to BAX and/or BAK. BH3-only proteins act through activation of BAX and BAK to signal changes in mitochondria that propagate death signaling. Interaction of a BH3-only protein tBID with BAX or BAK, for example, causes BAX and BAK to undergo changes in protein conformation followed by homo- or heteroligomerzation into high molecular weight protein complexes (Desagher et al., 1999; Eskes et al., 2000; Kluck et al., 1999; Korsmeyer et al., 2000; Perez and White, 2000; Sundararajan et al., 2001; Sundararajan and White, 2001; Wei et al., 2000). BCL-2 and BCL-x_L appear to bind and inhibit BID or tBID to prevent tBID from interacting with and inducing the oligomerization of BAX and BAK (Cheng et al., 2001), whereas E1B 19K binds directly to both BAX and BAK and prevents their oligomerization (Perez and White, 2000; Sundararajan et al., 2001; Sundararajan and White, 2001). Exactly how BAX and BAK act at the biochemical level in mitochondria once activated by BH3-only proteins is the subject of much speculation, but it is linked to the release of proapoptotic signaling proteins from the mitochondrial intermembrane space into the cytoplasm (Green and Evan, 2002).

Mouse embryo fibroblasts (MEFs), transformed MEFs (Cheng et al., 2001; Wei et al., 2001; Zong et al., 2001), or transformed baby mouse kidney epithelial cell lines (BMKs) (Degenhardt et al., 2002) derived from BAX- and/or BAK-deficient mice have been useful in determining their role in apoptosis. Similar to wild-type cells, cells deficient for either BAX or BAK still release mitochondrial proteins and undergo apoptosis in response to cytotoxic agents and death receptor signaling, whereas those deficient for both BAX and BAK are profoundly defective (Degenhardt et al., 2002; Wei et al., 2001). Thus, BAX or BAK function in a redundant capacity to facilitate the release of mitochondiral proteins such as cytochrome c and SMAC/DIABLO from the intermembrane space (Degenhardt et al., 2002; Wei et al., 2001). BAX- and BAK-deficient cells are also resistant to death signaling by overexpression of BH3-only proteins, indicating that they are required downsteam components of these signaling pathways (Cheng et al., 2001; Zong et al., 2001).

Binding to and inhibition of upstream BH3-only proteins, or BAX and BAK themselves, by antiapoptotic BCL-2 family members appears to be a means by which apoptosis is inhibited. Expression of antiapoptotic Bcl-2 family members prevents both BAX and BAK oligomerization and the release of proapoptotic proteins from mitochondria (Antonsson et al., 2000; Degenhardt et al., 2002; Desagher et al., 1999; Jurgensmeier et al., 1998; Korsmeyer et al., 2000; Perez and White, 2000; Sundararajan et al., 2001; Wei et al., 2001; Wei et al., 2000). Once released, cytochrome c serves as an activator of caspase-9 in the apoptosome, whereas SMAC/DIABLO represses an inhibitor of caspase-9 activation, the inhibitor of apoptosis proteins (IAPs) (Wang, 2001). Caspase-9 in turn activates caspase-3 and the cleavage of apoptotic substrates that mediate the dismantling of the cell, morphologically recognized as cell death by apoptosis (Cryns and Yuan, 1998).

While the role of this BAX and BAK mitochondrial death signaling pathway is essential in mediating cell death by toxic stimuli and by death receptor signaling, and in development, their role in oncogenic transformation and tumorigenesis remained to be addressed. Transformation of primary BMK or baby rat kidney (BRK) epithelial cells requires expression of adenovirus E1A to drive cell proliferation, and inactivation of p53 with a dominant-negative p53 mutant or p53 diciency, to relieve p53-dependent inhibition of cell growth and induction of apoptosis (Debbas and White, 1993; Degenhardt et al., 2002). Suppression of the transforming activity of E1A is exquisitely p53-dependent, as E1A will efficiently transform primary BMK cells derived from p53-deficient mice, but not those from wild-type mice (Degenhardt et al., 2002). E1A alone does not transform primary BMK cells from BAX- and BAK-deficient mice, indicating that BAX and BAK are dispensable for p53-dependent suppression of transformation (Degenhardt et al., 2002). Since a gain-of-function of antiapoptotic BCL-2 family members and loss-of-function mutations in proapoptotic BAX have been reported in human tumors (Johnstone et al., 2002), we tested the requirement for BAX and BAK in tumorigenesis. E1A plus dominant-negative mutant p53 (p53DD) transformed wild-type BMK cell lines were

Table 1. Incidence of tumor formation by transformed wild-type or BAX- and BAK-deficient BMK cell lines in nude mice

	Genotype			Tumors/sites of injection		
Cell line	*bax*	*bak*	*p53*	10^5 cells/injection	10^6 cells/injection**	10^7 cells/injection**
W1	+/−	+/+	+/+*	0/5	0/5	0/4
W3	+/−	+/+	+/+*	0/5	0/5	0/5
D1	−/−	−/−	+/+*	1/5	5/5	4/5
D3	−/−	−/−	+/+*	1/5	5/5	5/5
W4	+/+	+/+	+/+			0/5
W5	+/+	+/+	+/+			0/5
$p53^{-/-}$-1	+/+	+/+	−/−			0/5
$p53^{-/-}$-2	+/+	+/+	−/−			0/5

*p53 is inactivated by p53DD expression (Figures 4 and 5; Degenhardt et al., 2002).
**P value is less than 0.05.

not tumorigenic when injected into nude mice, nor were p53-deficient BMK cell lines transformed by E1A alone. In contrast, E1A plus p53DD transformed BAX- and BAK-deficient BMK cell lines were profoundly tumorigenic, forming highly invasive carcinomas. Moreover, transformed BMK cell lines that were BAX-deficient and heterozygous for BAK formed tumors that lost BAK expression. Similarly, BAK deficiency and BAX heterozygosity permitted growth of tumors that had lost BAX expression with high frequency. Thus, BAX and BAK suppressed tumor formation, and their loss of function was selected for in vivo during tumor development. The role of BAX and BAK in suppressing tumor formation was p53-independent; therefore, developing the means to overcome the loss of BAX and BAK function in tumors may yield a novel approach to cancer treatment.

Results

Deficiency in both BAX and BAK promotes tumorigenesis in mouse xenografts

Stable BMK cell lines transformed with E1A plus p53DD were derived from primary kidney epithelia from wild-type (W), $bax^{-/-}$ (X), $bak^{-/-}$ (K), and $bax^{-/-}$ $bak^{-/-}$ (D) mice (Table 1; Degenhardt et al., 2002). Transformation of wild-type, BAX-, BAK-, or BAX- and BAK-deficient primary BMK cells required both expression of E1A and inactivation of p53 to select for functional transformation. Three independently cloned cell lines from each genotype were analyzed (W1, W2, W3, X1, X2, X3, K1, K2, K3, D1, D2, D3). All BMK cell lines expressed similar levels of E1A and p53DD, whereas X1, X2, and X3, and D1, D2, and D3 were deficient for BAX expression, and K1, K2, and K3, and D1, D2, and D3 were deficient for BAK expression. Wild-type and BAK-deficient BMK cell lines were derived from mice heterozygous for BAX ($bax^{-/+}$), and two BAX-deficient BMK cell lines (X2 and X3) were derived from mice heterozygous for BAK ($bak^{-/+}$), whereas X1 was homozygous wild-type for BAK ($bak^{+/+}$) (Table 1; Degenhardt et al., 2002). Cell lines of each genotype were derived from multiple animals, except for those deficient for both BAX and BAK that were independently derived E1A plus p53DD transformed clones from a single animal, since the perinatal survival of these animals was rare (Degenhardt et al., 2002; Lindsten et al., 2000). D1, D2, and D3 cells were resistant to death signaling by TNF-α in short-term and long-term clonogenic survival assays, due to the inability to release cytochrome c and activate caspase-9, whereas the apoptotic pathway remained intact in all of the W, X, and K BMK cell lines (Degenhardt et al., 2002). D1, D2, and D3 cells are also resistant to apoptosis induction by infection with proapoptotic adenoviruses (Cuconati et al., 2002), and numerous other death stimuli, including overexpression of BH3-only proteins (K.D., E.W., D. Nelson, and R. Sundararajan, unpublished data). The inability of BAX and BAK deficiency to ameliorate p53-dependent suppression of transformation and facilitate oncogenic transformation by E1A prompted us to examine their role in tumorigenesis. To test if deficiency in BAX, BAK, or BAX and BAK affected the ability of transformed BMKs to form tumors in vivo, each cell line was injected into nude mice and assessed for tumor xenograft formation and growth. W1 and W3 and D1 and D3 cells were injected subcutaneously into nude mice at concentrations of 10^5, 10^6, or 10^7 cells per injection site. Injected cells initially formed a small subcutaneous mass that began to dissipate one week postinjection of W1 and W3 cells (Figure 1). No evidence of injected transformed cells was apparent beyond two to three weeks postinjection through to the termination of the experiment at either 45 days (W3) or 58 days (W1) (Figure 1). The D1 or D3 cells also formed a subcutaneous mass upon injection; however, in contrast to the W1 and W3 cells, the size of the mass steadily increased throughout the duration of the experiment to the point at which and animals were euthanized (D3 with W3 on day 45, and D1 with W1 on day 58). Tumor formation occurred with high efficiency at 10^7 or 10^6 injected D1 and D3 cells, and was less frequent with 10^5 cells (Table 1). W1 and W3 cells did not form tumors in any of the injected mice at any injected cell number (Table 1).

The failure of transformed W1 and W2 BMKs to form tumors was not due to incomplete inactivation of p53 by the p53DD mutant, since E1A-alone transformed BMK cell lines derived from p53-deficient mice were also not tumorigenic, nor were the E1A plus p53DD transformed wild-type BMK cell lines derived in parallel (Table 1). The expression of E1A, which inactivates RB, coupled with inactivation of p53, is an extremely efficient functional combination for transforming primary rodent epithelial cells in vitro (Debbas and White, 1993). The transforming activity resulting from E1A expression and p53 inactivation is not noticeably influenced by mouse genetic background, or even rodent species, indicating that both activities are fundamentally required for the transformation of epithelial cells. These activities, however, were apparently not sufficient to confer the ability of W BMK cell lines derived from a classic rodent transformation

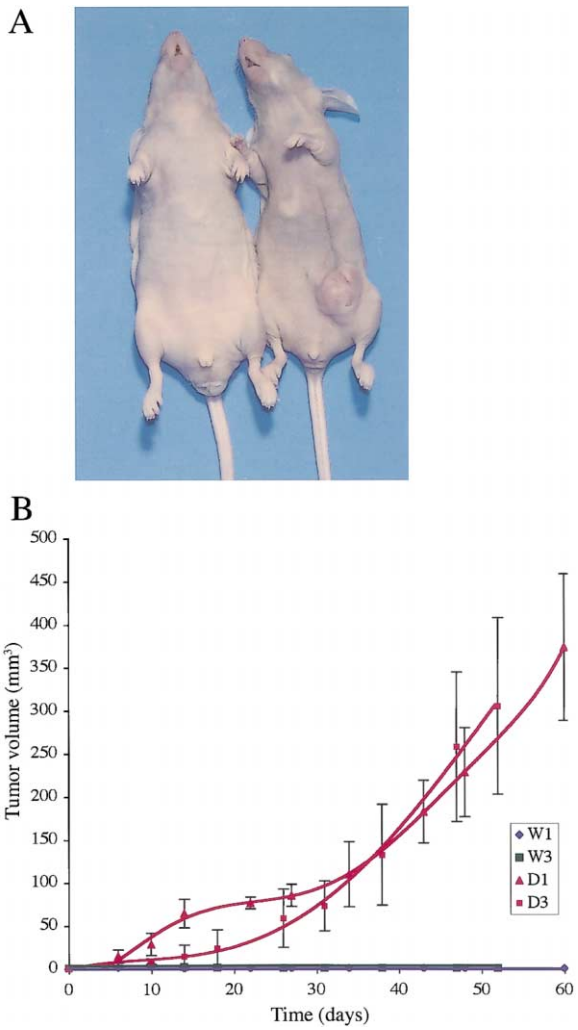


Figure 1. Tumor formation of wild-type and BAX- and BAX-deficient BMK cell lines in nude mice

10^7 cells from wild-type (W1 and W3) and BAX- and BAK-deficient (D1 and D3) transformed BMK cell lines were injected subcutaneously into nude nice and evaluated for tumor growth with time.
A: Representative animals injected with W1 (left) or D1 (right) photographed at 60 days postinjection. The D1-injected animal displays a typical tumor mass that is absent from the W1 injected animal.
B: Quantitation of tumor volume over time. The volume of the tumor mass arising from W1-, W3-, D1-, and D3-injected mice was determined at the indicated intervals. The average tumor volume for 4/5 D1- and 5/5 D3-injected, tumor-bearing animals was determined. Quantitation of the incidence of tumor formation by W1, W3, D1, and D3, for all animals at three different injected cell numbers, is presented in Table 1.

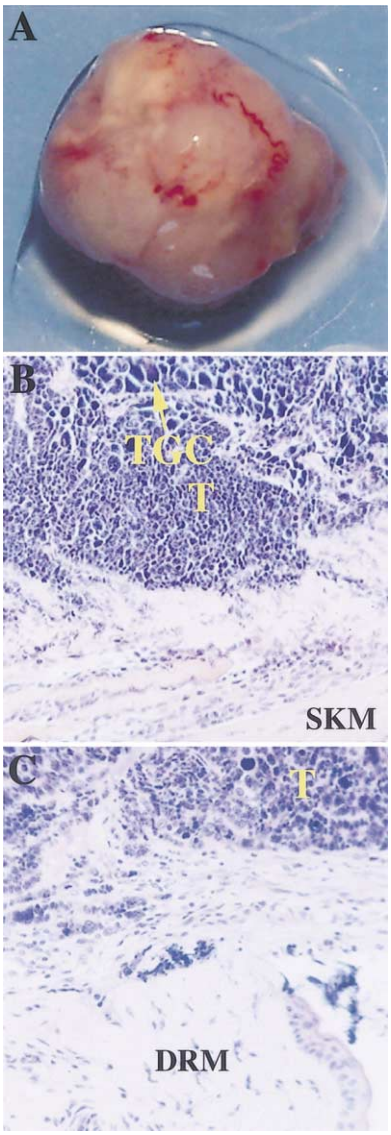


Figure 2. Morphology of tumors formed by BAX- and BAK-deficient BMK cell lines

A: Excised D1 tumor 1 cm in diameter at time of euthanasia, from the experiment described in Figure 1 and Table 1, with apparent vascularization.
B: Representative H&E staining of a thin section from a D1 tumor displaying tumor tissue (T), tumor giant cells (TGC), and invasion of skeletal muscle (SKM).
C: Representative H&E staining of a thin section from another D1 tumor (T) that arose in a different mouse displaying invasion of dermis (DRM). All tumors from D1- and D3-injected mice from Table 1 were evaluated similarly and classified as highly invasive carcinomas.

assay to establish tumors in nude mouse xenografts. In contrast, E1A plus p53DD transformed BMK cell lines derived from BAX- and BAK-deficient mice formed vigorous tumors with high frequency with a 10-fold lower amount of injected cells.

BAX- and BAK-deficient transformed BMKs form highly invasive carcinomas

Necropsy revealed large (1 cm) highly vascularized tumors at the site of injection of D1 and D3 cells (Figure 2A), and no evidence of tumors was apparent in W1 and W3 injected mice. All tumors arising from injected D1 and D3 cells (10^7 cells injected) were excised at time of euthanasia and subjected to sectioning and hematoxylin-eosin (H&E) staining to examine tumor pathology. All nine tumors were highly similar undifferentiated carcinomas displaying sheets of clustered adherent cells with large pleiomorphic nuclei and scanty cytoplasm (Figures 2B and 2C). Tumor cells had a high mitotic rate, and tumor giant cells were present. Blood vessels were also apparent throughout the tumors (data not shown). Tumor tissue was found

Table 2. BAX and BAK genotype and incidence of tumor formation in nude mice

		Genotype		Tumors/sites of injection	
	Cell line	*bax*	*bak*	Exp. 2*	Exp. 3*
$bax^{+/-}bak^{+/+} \times bax^{+/-}bak^{+/+}$	W1	+/−	+/+	0/5	
$bax^{+/-}bak^{+/+} \times bax^{+/-}bak^{+/+}$	W2	+/−	+/+	0/5	0/5
$bax^{+/-}bak^{+/+} \times bax^{+/-}bak^{+/+}$	W3	+/−	+/+	0/5	
$bax^{+/-}bak^{+/+} \times bax^{+/-}bak^{+/+}$	X1	−/−	+/+	0/5	0/5
$bax^{+/-}bak^{+/-} \times bax^{+/-}bak^{-/-}$	X2	−/−	+/−	5/5	5/5
$bax^{+/-}bak^{+/-} \times bax^{+/-}bak^{-/-}$	X3	−/−	+/−	5/5	5/5
$bax^{+/-}bak^{+/-} \times bax^{+/-}bak^{-/-}$ and $bax^{+/-}bak^{-/-} \times bax^{+/-}bak^{-/-}$	K1	+/−	−/−	5/5	5/5
$bax^{+/-}bak^{+/-} \times bax^{+/-}bak^{-/-}$ and $bax^{+/-}bak^{-/-} \times bax^{+/-}bak^{-/-}$	K2	+/−	−/−	5/5	5/5
$bax^{+/-}bak^{+/-} \times bax^{+/-}bak^{-/-}$ and $bax^{+/-}bak^{-/-} \times bax^{+/-}bak^{-/-}$	K3	+/−	−/−	5/5	5/5
$bax^{+/-}bak^{+/-} \times bax^{+/-}bak^{-/-}$ and $bax^{+/-}bak^{-/-} \times bax^{+/-}bak^{-/-}$	D1	−/−	−/−	5/5	5/5
$bax^{+/-}bak^{+/-} \times bax^{+/-}bak^{-/-}$ and $bax^{+/-}bak^{-/-} \times bax^{+/-}bak^{-/-}$	D2	−/−	−/−	4/5	4/5
$bax^{+/-}bak^{+/-} \times bax^{+/-}bak^{-/-}$ and $bax^{+/-}bak^{-/-} \times bax^{+/-}bak^{-/-}$	D3	−/−	−/−	5/5	

*P value is less than 0.05

invading skeletal muscle (Figure 2B), dermis (Figure 2C), fat, and mammary fat pad, and some tumors displayed evidence of necrosis in the center (data not shown).

BAK heterozygosity facilitates tumorigenesis in the absence of BAX

The formation of tumors by the BAX- and BAK-deficient, but not wild-type, BMK cell lines led us to investigate the individual contribution of BAX and BAK to tumor formation. W1, W2, and W3, and BAX- and BAK-deficient transformed BMK cell lines D1, D2, and D3, were injected into nude mice alongside singly BAX- (X1, X2, and X3) or BAK- (K1, K2, and K3) deficient transformed BMK cell lines in two independent experiments. All three wild-type BMK cell lines failed to form tumors in any of the 20 injected mice by seven weeks postinjection, whereas all three BAX- and BAK-deficient BMK cell lines formed tumors in 23 out of 25 mice (Table 2). Although there were minor differences (less than 2-fold) in growth rates for the W, X, K, and D cell lines, there was no correlation with tumorigenesis (data not shown). Interestingly, two of the three BAX singly deficient BMK cell lines (X2 and X3) formed tumors, whereas one (X1) did not (Table 2, Figure 3). All three BAK singly deficient BMKs (K1, K2, and K3) formed tumors (Table 2), but with delayed kinetics (Figure 3). Interestingly, tumorigenic X2 and X3, while BAX-deficient, are heterozygous for BAK ($bak^{+/-}$), whereas nontumorigenic X1 is homozygous wild-type for BAK ($bak^{+/+}$). Furthermore, all three tumorigenic BAK-deficient BMK cell lines were heterozygous for BAX ($bax^{+/-}$). These observations promoted us to examine BAX and BAK expression in tumors derived from BAX or BAK singly deficient cell lines to test for loss of expression of the only remaining BAX or BAK allele.

Tumor formation in vivo selects for BAX and BAK deficiency

X2, X3, K1, K2, and K3, along with W (BAX- and BAK-expressing) and D (BAX- and BAK-deficient) controls, were evaluated for BAX and BAK expression at the time of injection, in the resulting tumor, and in tumor derived cell lines (TDCLs) within three passages of recovery from the tumor. X2 and X3 expressed BAK but not BAX at the time of injection into nude mice as expected (Figure 4A). Similarly, K1, K2, and K3 expressed BAX but not BAK at the time of injection (Figure 4A). When the resulting tumors were evaluated for BAX and BAK expression, only traces of (X3, K1, K3) or no (X2, K2) BAX and BAK expression were found. The amount of BAK in the X3 tumor sample was similar to the amount found in K1 and K3 tumor samples (Figure 4A).

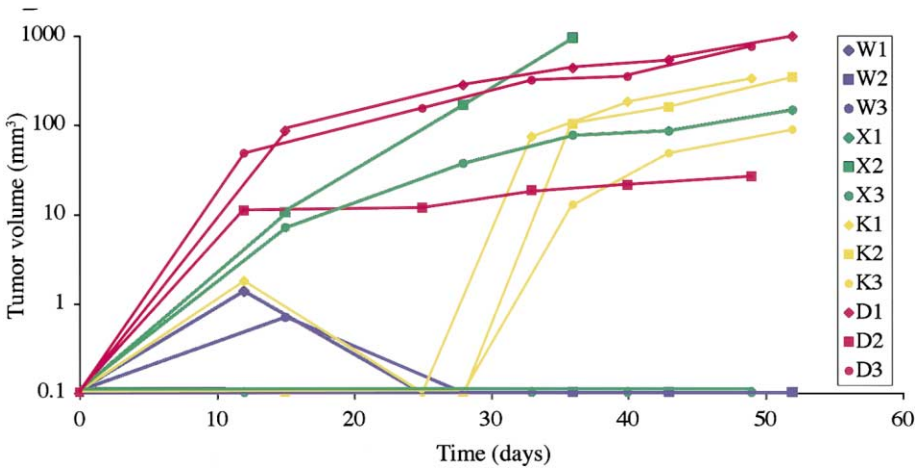
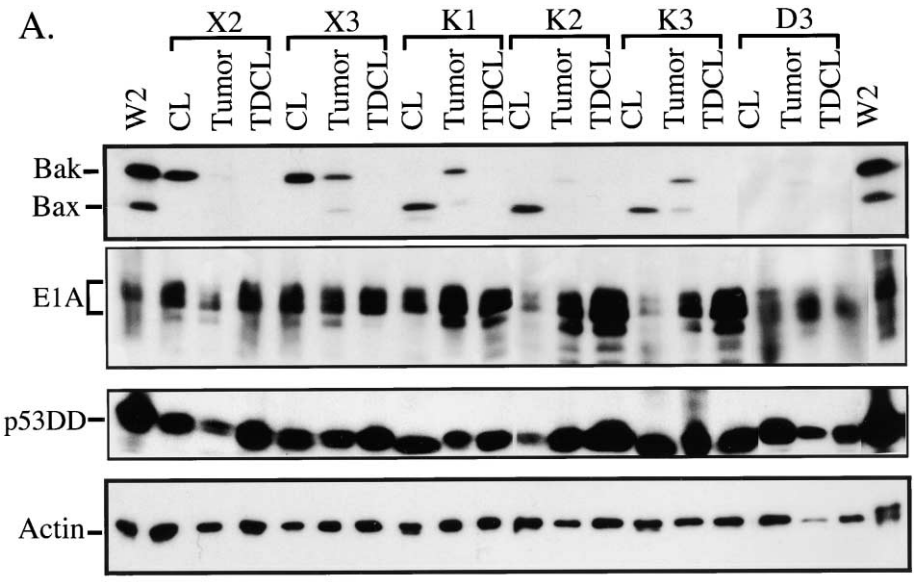


Figure 3. Tumor formation of wild-type, BAX-, BAK-, and BAX- plus BAK-deficient BMK cell lines in nude mice

10^7 cells from W1, W2, and W3, BAX-deficient (X1, X2, and X3), BAK-deficient (K1, K2, and K3), and BAX- and BAK-deficient (D1, D2, and D3) transformed BMK cell lines were injected subcutaneously into nude nice, and tumor volume was quantitated over time. The volume of the tumor mass arising from injected mice was determined at the indicated intervals. The average tumor volume for animals injected with each of the 12 transformed BMK cell lines was determined. Quantitation of the incidence of tumor formation by all 12 BMK cell lines for all animals in two independent experiments is presented in Table 2.



B. Quantitation of loss of BAX and BAK protein expression in TDCLs.

TDCLs resulting from injection with:	BAX expressing TDCLs Total number of TDCLs	BAK expressing TDCLs Total number of TDCLs
bax$^{-/-}$ *bak*$^{+/-}$ X2, X3	0/18	0/18
bax$^{+/-}$ *bak*$^{-/-}$ K1, K2, K3	2/29	0/29
bax$^{-/-}$ *bak*$^{-/-}$ D1, D2, D3	0/15	0/15

Figure 4. Tumor formation in vivo selects for loss of BAX and BAK expression in heterozygotes

A: Western blot for BAX and BAK expression in the W2 control BMK cell line, X2, X3, K1, K2, and K3 cell lines (CL) at time of injection, resulting tumor tissue (Tumor), and tumor derived cell line (TDCL) within three passages of recovery from tumor. BAX-deficient transformed BMKs X2 and X3 express only BAK at time of injection into nude mice, whereas tumors and tumor-derived cell lines resulting from X2 and X3 injection have reduced or nil BAK expression. Similarly, BAK-deficient K1, K2, and K3 express only BAX at time of injection into nude mice, whereas tumors and tumor derived cell lines resulting from K1, K2, and K3 injection have reduced or nil BAX expression. The BAX- and BAK-deficient D3 BMK cell line, resulting tumor, and tumor-derived cell line represent a negative control for BAX and BAK expression. The scant BAX and BAK expression appearing in tumor tissue likely results from contribution of normal mouse tissue vasculature to the tumor. Samples from all cell lines, tumors, and tumor-derived cell lines express E1A and p53DD by Western blotting, as expected. The actin Western blot serves as a reference for equating protein loading among different samples.

B: Quantitation of loss of BAX and BAK protein expression in TDCLs for all 8 transformed BMK cell lines that formed tumors, in two independent experiments.

K1 and K3 cell lines had no BAK when injected into the nude mice; thus, any BAK in the resulting tumor sample was contributed by the host, which likely resulted from incorporation of normal murine vasculature into tumor. This direct analysis of BAX and BAK expression in tumor tissue suggests that BAX or BAK was lost during the process of tumorigenesis of the X and K BMK cell lines retaining only a single allele of BAX or BAK.

Tumor tissue was isolated and physically dispersed, and placed in culture in vitro, from one of each of four tumors that resulted from injection with the X2, X3, K1, K2, and K3 cell lines. Isolated tumor cells survived and grew with high efficiency in culture with no apparent affect on growth or viability, and the resulting TDCLs were examined for BAX and BAK expression within three passages of isolation from tumor. As is the case with the tumors themselves, nearly all X and K TDCLs lacked expression of both BAX and BAK, even though one or the other was expressed at the time of injection (Figure 4A). This loss of BAX expression occurred in 27 of 29 TDCLs that resulted from injection of K1, K2, and K3 (Figure 4B). Loss of BAK expression occurred in 20 of 20 TDCLs that resulted from injection of X2 and X3 (Figure 4B). Since the loss of BAX and BAK expression in the isolated tumors was sustained in culture, it was not the result of suppression of expression within the tumor. Furthermore, loss of BAX or BAK expression in W, X, or K BMK cell lines has never been observed in vitro in culture, indicating that their genotype is stable. Selective pressure, such as that provided in a mouse xenograft, may be required for loss of BAX and BAK expression. All BMK cell lines, and the corresponding resulting tumors and tumor derived cell lines, expressed E1A and p53DD as expected (Figure 4A).

Loss of BAX and BAK expression in tumors results from loss of heterozygosity

Tumor formation of transformed BMKs deficient for BAX that were heterozygous for BAK and vice versa suggested that a loss of heterozygosity of the remaining *bax* or *bak* allele and the loss of both BAX and BAK expression in tumors was involved. Loss of heterozygosity through heterologous recombination would result in both alleles with same gene disruption; therefore, the same polymerase chain reaction (PCR) genotyping strategy that was used to identify the targeted disruption in mice was used to identify the loss of heterozygosity in the TDCLs. TDCLs rather than tumor tissue were used because PCR genotyping is extremely sensitive, and tumor tissue was partially composed of vasculature contributed by the wild-type xenograph host. TDCLs that arose from transformed BMKs deficient in BAX (X2 and X3) showed a loss of the remaining *bak* allele, indicated by the loss of the wild-type PCR product in 8 of 8 cases, whereas the parental cell lines were heterozygous for *bak* and expressed BAK at the time of injection into nude

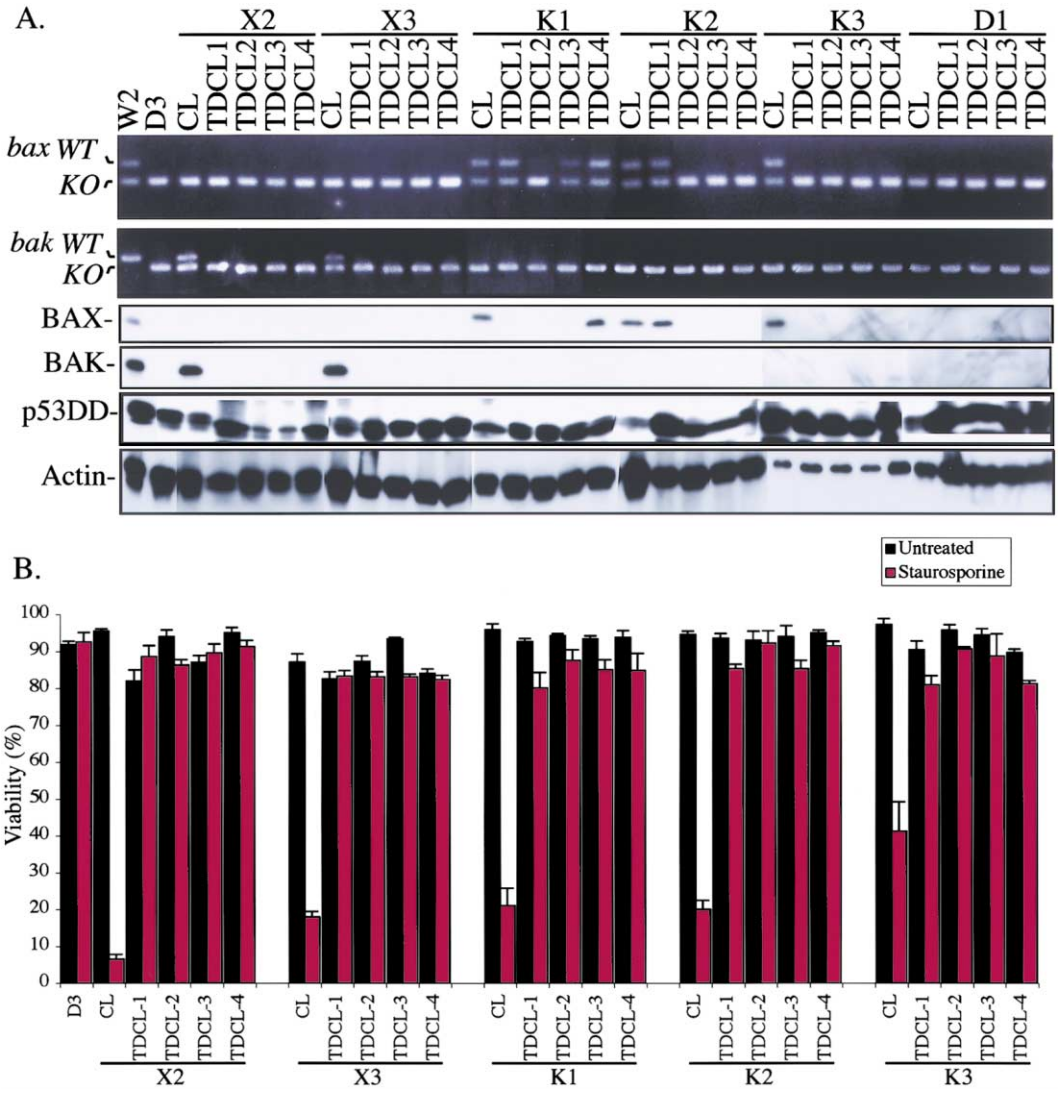


Figure 5. Tumor formation in vivo selects for loss of BAX and BAK expression by loss of heterozygosity and apoptosis resistance in vitro

A: PCR genotyping for wild-type (WT) and knockout (KO) alleles of *bax* and *bak* in W2 control, BAX- and BAK-deficient D3 control, X2, X3, K1, K2, and K3 cell lines (CL) at time of injection, and in the resulting tumor derived cell lines (TDCL) within three passages of isolation from four independent tumors derived from each of the indicated cell lines. Western blot for BAX, BAK, and p53DD expression in parallel with genotyped samples. The actin Western blot serves as a reference for equating protein loading among different samples.

B: Parental CL and TDCL viability following treatment with stuarosporine (4 μM) for 24 hr. Viability was assessed by trypan blue exclusion for BAX- and BAK-deficient D3 control, parental CL (X1, X2, K1, K2, K3) and their tumor-derived counterparts (TDCL).

mice (Figure 5A). Genotyping of the TDCLs that arose from BMKs deficient in BAK showed a loss of the remaining *bax* allele in 8 of 10 TDCLs that lost BAX protein expression, whereas the parental cell lines (K1, K2, and K3) were heterozygous for *bax* and expressed BAX at the time of injection into nude mice (Figure 5A). The apparent retention of the wild-type *bax* allele in a few TDCLs could be due to low-level contamination with wild-type tissue from the host. Alternatively, the loss of BAX protein expression in K1 TDCL1 and 3 (Figure 5A) could result from a mutation in *bax* or epigenetic effects on BAX expression. Retention of BAX protein expression in K1 TDCL4 and K2 TDCL1 may result from functional inactivation of BAX, either directly or indirectly. All BMK cell lines and their tumor-derived counterparts expressed p53DD (Figure 5A). These results demonstrated that loss of BAK during tumor formation resulted from a loss of heterozygosity in all cases, and that the loss of BAX expression predominately resulted from loss of heterozygosity.

Tumor-derived cell lines gain apoptosis resistance in vitro

To examine the apoptotic potential of the BMK cell lines and their tumor-derived counterparts, we characterized their apoptotic response to in vitro treatment with staurosporine. We have previously reported that BAX or BAK expression in transformed BMK cell lines is sufficient for induction of apoptosis in vitro, whereas deficiency in both BAX and BAK confers resistance to TNF-α and a wide variety of other apoptotic stimuli (Degenhardt et al., 2002). The parental CLs or TDCLs were untreated or

treated with staurosporine for 24 hr and analyzed for viability by trypan blue exclusion (Figure 5B). When treated with staurosporine, the viability of the BAX- or BAK-containing BMK cell lines (X13, X14, K1, K2, K3) decreased dramatically (Figure 5B). In contrast, all of the TDCLs were highly resistant to staurosporine-mediated apoptosis and were indistinguishable from the BAX- and BAK-deficient D3 BMK cell line (Figure 5B). K1 TDCL-4 and K2 TDCL-1, which retained BAX expression, were also resistant to staurosporine-induced apoptosis in vitro (Figure 5B). These results are consistent with selection during tumorigenesis for apoptotic resistance by the loss of both BAX and BAK expression and function.

Discussion

There is significant evidence that p53 acts as an upstream regulator of proapoptotic BAX and BAK. The gene encoding BAX is a transcriptional target of p53 in humans (Miyashita and Reed, 1995), although this activity is not conserved in the mouse (Schmidt et al., 1999). BAK has also been reported to be upregulated by p53 (Pearson et al., 2000; Pohl et al., 1999). Genes encoding the BH3-only proteins PUMA and NOXA are directly transcriptionally transactivated by p53 (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001), and PUMA or NOXA overexpression induces apoptosis in wild-type but not BAX- and BAK-deficient MEFs and BMKs (Cheng et al., 2001; Zong et al., 2001; D. Nelson, K.D., and E.W., unpublished data). Thus, p53 has the capacity to activate the transcription of upstream activators of BAX and BAK proapoptotic function, and possibly BAX and BAK themselves. In response to induction of wild-type p53, BAX and BAK undergo conformational changes indicative of activation, coordinate with the release of cytochrome c and SMAC/DIABLO from mitochondria, and undergo caspase-9 and -3 activation (Henry et al., 2002). Nonetheless, BAX and BAK appear to be dispensable for p53 to suppress transformation (Degenhardt et al., 2002), and BAX- and BAK-deficient mice are not overtly tumor-prone (Lindsten et al., 2000) as are p53-deficient mice (Donehower et al., 1992). Two mutually inclusive possibilities to explain these results are that p53 can suppress transformation by a nonapoptotic mechanism (El-Deiry et al., 1993), or that p53 has the ability to induce cell death by a BAX- and BAK-independent mechanism. However, once p53 inactivation has occurred, abrogation of BAX- and BAK-mediated apoptosis may be a rate-limiting step for tumorigenesis.

In support of a nonapoptotic mechanism of p53-dependent suppression of transformation, inhibitors of apoptosis such and BCL-2 or adenovirus E1B 19K facilitate transformation of primary BRK cells by E1A substantially less efficiently than direct inactivation of p53 (Rao et al., 1992; White et al., 1992). BRK cell lines transformed by E1A and a temperature-sensitive mutant of p53 undergo apoptosis when p53 reverts to the wild-type conformation (Debbas and White, 1993), and if this p53-dependent apoptosis is rescued by BCL-2 or E1B 19K expression, the cells undergo complete and sustained cell cycle arrest (Chiou et al., 1994; Sabbatini et al., 1995a). This p53-mediated cell cycle arrest is coincident with the transactivation of $p21^{WAF1}$ (Han et al., 1996; Sabbatini et al., 1995b) and is reversible upon restoration of p53 to the mutant conformation (Chiou et al., 1994; Sabbatini et al., 1995a). Thus, ameliorating p53-dependent apoptosis has no affect on induction of growth arrest by p53, and even allows the cell cycle arrest function of p53 to be revealed. Perhaps inhibition of cell proliferation and induction of apoptosis by p53 represent redundant functions capable of inhibiting oncogenic transformation. By possessing at least two mechanisms for suppressing transformation, p53 may act efficiently at a critical step to prevent the emergence of oncogenically transformed cancer cells.

Evidence that p53 may be capable of inducing apoptosis in the absence of BAX and BAK is more difficult to come by. Transformed foci initiated by E1A in both wild-type and BAX- and BAK-deficient BMK cells do regress, consistent with induction of p53-dependent and BAX- and BAK-independent cell death (Degenhardt et al., 2002). BAX and BAK deficiency does confer resistance in the short-term to DNA damage-induced cell death (Wei et al., 2001), which p53 participates in, although it is not known if this failure to undergo apoptosis is sustained in the long-term (Wei et al., 2001). If a BAX- and BAK-independent or a mitochondria-independent apoptotic pathway exists for p53-mediated cell death, as it does for death receptor signaling in some cell types (Scaffidi et al., 1999), this will be of great interest to identify.

There is substantial evidence that apoptosis plays a role in the development of human cancer, and that it may be a major mechanism of action of current anticancer drugs (Johnstone et al., 2002; Zhang et al., 2000). It is, however, not always entirely clear where or when apoptosis comes into play. This may merely be a consequence of the complex and variable nature of cancer emergence in different tissues and circumstances. These events are compounded by the multitude of mutational events, in a myriad of combinations varying over time and treatment, that are observed in human cancers. In the BMK epithelial cell model, we have established that transformation and tumorigenesis are distinct and separable functions. While inactivation of the RB pathway by E1A expression and inhibition of p53 function appear critical for the establishment of immortal, transformed epithelial cells in vitro, these cells apparently lack the ability to survive and grow efficiently in vivo in nude mouse xenografts. Inactivation of both BAX and BAK was required for tumor growth, and was selected for in vivo during tumorigenesis. Transformed cells deficient for BAX, that also possess heterozygous deficiency in *bak*, underwent selection for tumors in vivo that had lost expression of the remaining *bak* allele. The converse was true for transformed cells that are deficient in BAK and heterozygous for *bax*. In most cases, the loss of BAX and BAK expression resulted from loss of heterozygosity. In the occasional tumors that maintain expression of BAX, it is not clear if *bax* is mutated or if there are mutations elsewhere in the apoptotic pathway that could block BAX function. BAX and BAK deficiency derived in vivo during tumorigenesis, and in the rare cases of TDCLs where BAX expression was maintained in tumorigenesis, conferred apoptosis resistance in vitro. Thus, tumorigenesis in vivo selects for apoptotic resistance that in most cases can be attributed to the loss of BAX and BAK.

Similar to the molecular events that contribute to human tumorigenesis, E1A expression and p53 inactivation are both associated with induction of genetic instability that may facilitate the selection process for loss of both BAX and BAK expression. The redundant function of BAX and BAK in promoting apoptosis may, however, reduce the likelihood that apoptotic function would be abrogated as a means to promote tumorigenesis. Nonetheless, mutations in, or reduced expression of, BAX or BAK have been observed in human tumors (Johnstone et al.,

2002; Kondo et al., 2000; Rampino et al., 1997), BAX mutations confer a growth advantage during clonal tumor evolution (Ionov et al., 2000), and inhibition of apoptosis in murine models promotes late-stage tumorigenesis (Eischen et al., 2002; Naik et al., 1996; Pelengaris et al., 2002: Shibata et al., 1999; Yin et al., 1997). Furthermore, deficiency in both BAX and BAK confers resistance to apoptosis mediated by death receptors (Degenhardt et al., 2002; Wei et al., 2001), and treatment of sensitive tumor cells with death receptor ligands also selects for BAX mutations (LeBlanc et al., 2002). Thus, the process of tumorigenesis may subject genetically unstable tumor cells to selective pressure to disable apoptosis through loss-of-function in BAX and/or BAK that may confound treatment strategies.

The mechanism by which BAX and BAK suppress tumor formation is not known, but may be related to the necessity of cells from highly invasive carcinomas to survive at foreign or distant locations. Growth factor limitations may restrict survival of transformed cells beyond the initial site of emergence, limiting or preventing invasion of surrounding tissues. Tumor growth itself may restrict essential growth factors, thereby promoting apoptosis. Cell survival in the absence of normal cell-cell attachment may also enhance the tumorigenic potential of transformed epithelial cells. Finally, the resistance of BAX- and BAK-deficient transformed BMK cell lines to apoptosis mediated by death receptor signaling pathways may enhance their tumorigenic potential. Indeed, TRAIL-deficient mice are tumor prone (Cretney et al., 2002). If apoptosis mediated by BAX and BAK were responsible for these types of activities, this could account for the dramatic enhancement of tumorigenesis of mouse xenographs observed here, and may be a property of human tumorigenesis. Designing specific strategies to overcome survival in the absence of BAX and BAK function may provide novel opportunities in anticancer drug discovery. Furthermore, understanding the events regulating a p53-independent apoptotic pathway that functions in tumor suppression may reveal novel targets for effective combinatorial therapeutic approaches to cancer treatment.

Experimental procedures

Cell culture

E1A- and p53DD-transformed BMK cell lines were derived from wild-type mice and mice deficient for p53, BAX, BAK, or BAX and BAK (Degenhardt et al., 2002). W1, W2, W3, X1, X2, X3, K1, K2, K3, D1, D2, and D3 were generated from three litters of mice obtained from three matings: mice were bred by crossing $bax^{+/-}$ $bak^{-/-}$ with $bax^{+/-}$ $bak^{-/-}$; $bax^{+/-}$ $bak^{+/-}$ with $bax^{+/-}$ $bak^{-/-}$; and $bax^{+/-}$ $bak^{+/+}$ with $bax^{+/-}$ $bak^{+/+}$. Independent cell lines for each genotype were derived from at least two separate mice, except for the cell lines deficient for both BAX and BAK, which were derived from a single mouse since these mice rarely survive due to maternal neglect. W4, W5, $p53^{-/-}$-1, and $p53^{-/-}$-2 were generated from littermates of a single mating: $p53^{+/-}$ with $p53^{+/-}$. Tumor-derived cell lines were generated by simply mechanically disrupting tumor tissue that was grown in culture under the same conditions as the BMK cell lines. The BMK cell lines and TDCLs were grown in Dulbecco's modified eagle media supplemented with 5% fetal bovine serum at 38.5°C. The PC-3 cell line was grown in Dulbecco's modified eagle media supplemented with 10% fetal bovine serum and grown at 37°C.

Tumor formation in nude mice

Tumor formation in nude mice was preformed essentially as previously described (Streit et al., 1999). Briefly, cells were harvested by trypsinization and washed twice with PBS, and viable cell number was determined by trypan blue exclusion. Cells were diluted in phosphate buffered saline (PBS) and injected subcutaneously at the indicated cell numbers in the abdominal region of 6-week-old nude mice (Taconic, Germantown, NY). At the time of injection, 1×10^7 cells were pelleted and frozen for DNA preparation (see below). Tumor growth from each cell line was monitored by measurements with a 6-inch dial caliper (General Tools Mfg. Co., New York, NY) weekly. Tumor growth rates were calculated as described previously (Streit et al., 1999). Tumor-bearing mice and controls were sacrificed and the tumors were excised under sterile conditions. Tumors were sectioned and processed for histology, protein analysis, DNA preparation, and isolation of tumor derived cell lines. A 2 mm section of the tumor was fixed in Omnifix 2000 according to the manufacturer's instructions (FR Chemical Co., Mt. Vernon, NY) and sent to the Mutant Mouse HistoPathology Laboratory (U.C. Davis, Davis, CA) for H&E staining and pathology. Approximately one-fifth of the tumor was placed in tissue culture media, mechanically disrupted by pipeting, and recultured for three passages into tumor derived cell lines. Approximately one-fifth of the tumor was frozen in liquid nitrogen and processed into Western blot extracts (Geoerger et al., 2002) by homogenizing the tumor in ice cold lysis buffer (150 mM NaCl,1 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mM EDTA [pH 6.4], 1 mM dithiotretiol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A) using an Ultra Turrax T8 tissue homogenizer (IKA-Werke GMBH & Co., Staufen, Germany). The x^2 test and Fisher test for exactness to were used to calculate P values on pooled results of each genotype and compared to the BAX- and BAK-expressing controls.

Antibodies and Western blotting

The following antibodies were used for Western blotting analysis: the rabbit polyclonal antibody (NT) against amino acids 1–21 of human BAX, and the rabbit polyclonal antibody (NT) raised against amino acids 23–37 of human BAK (Upstate Biotechnology, Lake Placid, NY); the mouse monoclonal antibodies directed against p53 (pAB421); the anti-adenovirus 2 E1A (M73); and actin (Oncogene Research Products, Boston, MA). Cell extracts were analyzed by SDS-PAGE and semidry blotted as previously described (Perez and White, 2000). Proteins were detected by antibody as indicated and visualized by enhanced chemiluminescence according to the manufacturer's specifications (Amersham Pharmacia Biotechnology, Buckingshire, England).

bax and *bak* genotyping of tumor-derived cell lines

DNA was purified from cell lines using DNeasy tissue kit according to the manufacturer's specifications (Qiagen, Valencia, CA). *bax* genotyping was preformed by PCR as previously described (Shindler et al., 1997) using Advantage cDNA PCR according to the manufacturer's specifications (Clontech Laboraories Inc., Palo Alto, CA). *bak* genotyping was preformed by PCR as previously described (Lindsten et al., 2000) using Advantage-GC Genomic PCR according to the manufacturer's specifications (Clontech Laboraories Inc., Palo Alto, CA).

Staurosporine-mediated apoptosis assay

BMK cell lines were untreated or treated with staurosporine (4 μM) (Sigma, St. Louis, MO). After 24 hr of treatment, cells were harvested by trypsinization, centrifuged, and resuspended in PBS. Cells were diluted 1:100 in 0.25% trypan blue solution (Gibco BRL, Grand Island, NY) and counted in a hemocytometer to assess number of dead blue cells from the total number of cells counted.

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