Bax regulates c-Myc-induced mammary tumour apoptosis but not proliferation in MMTV-c-myc transgenic mice

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The expression of the proto-oncogene c-myc is frequently deregulated, via multiple mechanisms, in human breast cancers. Deregulated expression of c-myc contributes to mammary epithelial cell transformation and is causally involved in mammary tumorigenesis in MMTV-c-myc transgenic mice. c-Myc is known to promote cellular proliferation, apoptosis, genomic instability and tumorigenesis in several distinct tissues, both in vivo and in vitro. Expression of the proapoptotic regulatory gene bax is reduced or absent in human breast cancers, and c-Myc has been shown to regulate the expression of Bax, as well as cooperate with Bax in controlling apoptosis in a fibroblast model. Additionally, loss of bax reduces c-Myc-induced apoptosis in lymphoid cells and increases c-Myc-mediated lymphomagenesis in vivo. In order to assess whether loss of bax could influence c-Myc-induced apoptosis and tumorigenesis in the mammary gland in vivo, we generated MMTV-c-myc transgenic mice in which neither, one, or both wild-type alleles of bax were eliminated. Haploid loss of bax in MMTV-c-myc transgenic mice resulted in significantly reduced mammary tumour apoptosis. As anticipated for an apoptosis-regulatory gene, loss of the wild-type bax alleles did not significantly alter cellular proliferation in either mammary adenocarcinomas or dysplastic mammary tissues. However, in contrast to c-Myc-mediated lymphomagenesis, loss of one or both alleles of bax in MMTV-c-myc transgenic mice did not significantly enhance mammary tumorigenesis, despite evidence that haploid loss of bax might modestly increase mammary tumour multiplicity. Our results demonstrate that Bax contributes significantly to c-Myc-induced apoptosis in mammary tumours. In addition, they suggest that in contrast to c-Myc-mediated lymphomagenesis, mammary tumorigenesis induced by deregulated c-myc expression requires some amount of Bax expression.

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The proto-oncogene c-myc was first identified as the mammalian homologue of the viral transforming oncogene, v-myc, responsible for avian myelocytomatosis (Vennstrom et al, 1982). c-Myc is a DNA-binding, nuclear transcription factor involved in the regulation of cell cycle progression, programmed cell death, cellular metabolism, and differentiation (Evan and Littlewood, 1993; Harrington et al, 1994; Packham and Cleveland, 1995). In 1994, the Dual Signal model proposed that induction of apoptosis, a potent mechanism for the suppression of tumorigenesis, was an obligate function of deregulated c-myc expression; however, more recent experimentation suggests that c-Myc may sensitize cells to apoptosis as a result of alterations in mitochondrial membrane permeability and movement of holocytochrome c into the cytoplasm from its typical position as a constituent of the electron transport system (Juin et al, 1999; Prendergast, 1999). Experiments examining the cooperation of c-Myc and knockouts of p19ARF and/or p53 in mouse embryo fibroblast models have suggested that suppression of c-Myc-induced apoptosis may facilitate malignant transformation and tumorigenesis (Zindy et al, 1998). c-Myc may increase genomic instability and enhance tumorigenesis without an absolute requirement for continued aberrant c-myc expression once additional transforming genetic lesions have been generated and fixed in the genome (Felsher and Bishop, 1999a, b). Deregulated or aberrant expression of c-myc, via mechanisms including translocation, proviral insertion, locus amplification, point mutation, direct transcriptional and translational effects, or post-translational modifications, is a signature of several different human tumour types and c-myc can induce tumorigenesis under conditions where programmed cell death is abrogated (Evan et al, 1992; Santoni-Rugiu et al, 1998; Dang, 1999). The relevance of aberrant c-myc expression to the pathogenesis of breast cancer is confirmed by the finding that the c-myc locus is rearranged in roughly 5%, amplified in 16%, and overexpressed in approximately 70% of human breast tumours (Nass and Dickson, 1997; Deming et al, 2000).

The role of c-myc expression in normal mammary gland development and function as well as mammary tumorigenesis is a burgeoning field of inquiry. Several in vitro studies have...
demonstrated a contributory role for c-Myc in transformation of both human and murine mammary epithelial cells (MECs) (Leder et al, 1986; Telang et al, 1990; Valverius et al, 1990). Examination of normal murine mammary development has indicated that c-Myc is expressed during pregnancy-associated proliferation and postlactational involution associated MEC apoptosis (Strange et al, 1992). To further evaluate the role of c-Myc in mammary development, function, and transformation, a transgenic mouse was generated expressing the murine c-myc gene under the control of the mouse mammary tumour virus long terminal repeat (MMTV-LTR) promontional elements (Stewart et al, 1984). MMVC-c-myc transgenic mice develop mammary adenocarcinomas in both the virgin state (50% incidence following a 7–14 month latency) and in parous mice (100% incidence in two or more pregnancies); however, the extended mammary tumour latencies and low mammary tumour multiplicities suggest that c-myc is contributory to but insufficient for mammary tumorigenesis in the mouse (Stewart et al, 1984; Leder et al, 1986).

The conditional expression of c-myc in the mammary glands of mice using an MMTV-LTR-driven tetracycline-responsive transgenic system has provided evidence for cooperative, transforming genetic alterations that may result from c-Myc-induced genomic instability (D’Cruz et al, 2001). Furthermore, the use of spectral karyotyping (SKY) and comparative genomic hybridisation (CGH) to demonstrate that MMTV-c-myc-induced mammary tumours display distinct, repeatable patterns of chromosomal alterations suggests that c-Myc may exert a dominant genomic mutator effect and that specific genetic lesions may cooperate in MEC transformation (reflecting the multistage nature of human tumorigenesis) (Weaver et al, 1999).

TGFn, a soluble growth factor of the epidermal growth factor family of ligands, is a potent survival and growth factor for human and murine MECs both in vivo and in vitro, and when overexpressed in the mammary glands of transgenic mice, induces mammary alveolar hyperplasias and occasional mammary adenocarcinomas (Bates et al, 1990; Jhappan et al, 1990; Matsui et al, 1990; Sandgren et al, 1990; Snedeker et al, 1991; Amundadottir et al, 1995). The prosurvival molecule Bcl-2 has been shown to be expressed in the normal human and murine mammary epithelium, to impede mammary gland involution when expressed as an exogenous transgene, and to suppress c-myc-induced apoptosis and cooperation with c-Myc to induce malignant appearances in transgenic mouse model (Bargou et al, 1995; Schorr et al, 1999a,b; Feuerhake et al, 2000; Eischen et al, 2001b). The importance of suppression of apoptosis in c-myc-induced murine mammary tumorigenesis has been suggested by three independently generated transgenic mouse models: MMVC-c-myc/MT-tgfs, WAP-c-myc/WAP-tgfs, and MMVC-c-myc/WAP-bcl2 (Amundadottir et al, 1995; Sandgren et al, 1995; Jäger et al, 1997). Data from these three mammary bitransgenic studies strongly suggest that mammary tumorigenesis is significantly enhanced when deregulated c-myc expression, responsible for driving both proliferation and apoptosis, is coupled with alterations that block c-myc-mediated apoptotic pathways.

Bax, a proapoptotic member of the Bcl-2 family of proteins, was first discovered in a screen of proteins that exhibited binding interactions with Bcl-2 (Oltvai et al, 1993). Bax is likely to have pore-forming activity in the mitochondrial membranes, subject to control or prevention by association with specific antiapoptotic molecules (especially Bcl-2 and Bcl-xL), related to its ability to bind to BH-3 domain-only containing Bcl-2 family member proteins, and induce the release of mitochondrial cytochrome c (Antonsson et al, 1997; Jurgesmeier et al, 1998; Desagher et al, 1999; Murphy et al, 1999; Antonsson et al, 2000; Nouraini et al, 2000; Wei et al, 2001). Bax is weakly expressed or absent in several breast cancer cell lines and transfection of bax into these lines results in increased apoptotic sensitivity and diminished tumour proliferation in athymic mice (Bargou et al, 1995, 1996; Sakakura et al, 1996). Bax is expressed in the epithelium of the normal breast and its expression is highest during postlactational mammary gland involution; furthermore, Bax expression is significantly reduced or absent in invasive ductal breast carcinomas (Krajewski et al, 1994; Bargou et al, 1995; Li et al, 1996; Feuerhake et al, 2000; Shikaitis et al, 2000). Significant reductions in Bax expression were found in 34% of primary breast tumours in women with metastatic disease, Bax expression was inversely correlated with overall survival, treatment response, and metastasis in these patients, and Bax expression was found to be predictive of tumour response to chemotherapy independent of other predictive variables (Krajewski et al, 1995; Kapranos et al, 1997; Sjöström et al, 1998).

The mechanisms by which c-Myc induces apoptosis and the mechanisms by which this apoptosis contributes to tumour suppression are largely unknown and currently being explored. Recently, Bax was determined to be transcriptionally regulated by c-Myc in a variety of human cell lines (including the SKBr3 human breast cancer cell line) and found to be critical for the induction of apoptosis by aberrant c-Myc expression in a mouse embryo fibroblast model system (Mitchell et al, 2000). Two other studies indicate that c-Myc, at least in embryo fibroblast systems, activates a proapoptotic function in Bax and induces an apoptotic program that requires Bax (or a BH3 domain peptide) to be present in the mitochondroid membrane (Souchie et al, 2001; Juin et al, 2002). In addition, bax-deficient primary pre-B cells have been shown to be resistant to proapoptotic effects of c-Myc. Furthermore, in a transgenic mouse model, loss of one or both bax allele(s) significantly accelerates c-Myc-dependent lymphomagenesis in a bax gene dosage-dependent manner (Eischen et al, 2001a). The partial or total loss of bax in knockout mice provides evidence that the presence of Bax is unlikely to be required for mammary gland development and secretory differentiation (a very small percentage of bax-nullizygous mice did evidence postpartum lactational incompetency); however, loss of bax did reduce MEC apoptosis during postlactational involution (Knudson et al, 1995; Schorr et al, 1999a,b). Loss of bax (reflecting the in vivo situation of human breast cancer patients) may disrupt c-Myc-induced apoptotic programs in mammary epithelial cells and has the potential to diminish the tumour suppressive activity of c-Myc-induced apoptosis. In this study, we have generated a combinatorial, mammary-relevant transgenic model, bax-knockout/MMTV-c-myc transgenic, to examine the influence of allelic bax loss on c-Myc-induced apoptosis and tumorigenesis in vivo.

### MATERIALS AND METHODS

#### Transgenic and knockout mice

All animal experiments were conducted in accordance with US and UK CCR guidelines (Workman et al, 1998) and in accordance with our institutionally approved protocol. MMVC-c-myc transgenic mice (FVB inbred genetic background) were obtained from Charles River Laboratories, bred under a license from DuPont Medical Products, and housed as previously described (Amundadottir et al, 1995). The MMVC-c-myc transgenic mice contain a mouse mammary tumour virus long terminal repeat promoter element driving the expression of a murine c-myc gene (Stewart et al, 1984). Mice hemizygous for bax (C57BL/6 inbred genetic background) were obtained from SJ Korsmeyer via PA Furch at Georgetown University (Knudson et al, 1995). P generation myc mice were bred to P generation bax-hemizygous mice and subsequently, their F1 generation offspring were backcrossed with P generation bax-hemizygous mice, resulting in F2 generation offspring in which the myc transgene was found in the context of no, one, or two intact wild-type bax alleles. All data reported herein were generated using F2 generation study mice on the mixed genetic background (C57BL/6 x FVB; 3:1). Parous
study mice, 10 weeks old, were housed with males and repetitively bred until euthanisation; all surviving pups were weaned at day 20 postpartum. Female parous study mice were examined bi-weekly for tumours and morbidity and euthanised if they showed signs of ill health using approved methods (Workman et al., 1998).

Genotyping

Overnight digestion of mouse tail biopsy samples (Workman et al., 1998) in STE buffer (0.1 M NaCl, 0.05 M Tris pH 8.0, 1 mM EDTA, and 1% SDS) containing 5 mg ml⁻¹ fungal proteinase K (Invitrogen, Carlsbad, CA, USA), followed by phenol/chloroform extraction and ethanol precipitation yielded genomic DNA subsequently used in genotyping of all mice utilised in this study. PCR-based genotyping was performed on a Stratagene Robocycler Gradient 40 machine using tail-derived genomic DNA, sequence-specific primers, and Platinum PCR Supermix (Invitrogen). MTVMyc5' primer (5'-CCC AAG GCT TAA GTA AGT TTT TGG-3') and MTVMyc3' primer (5'-GGG CAT AAG CAC AGA TAA ACA ACX ACG-3') were used to identify MTMV-c-myc transgenic mice (1 min denaturation at 95°C, 1 min annealing at 52°C, and 75 s elongation at 72°C for a total of 42 cycles); transgene-positive animals were identified by a resolution of a single ~880 bp band on a 1% agarose gel. BPR2 primer (5'-GTT GAC CAG AGT GGC GTA GG-3'), MK1 primer (5'-GAG CTG ATC AGA ACC ATC ATG-3'), and NR2 primer (5'-CCG CTT CCA TGG TCT AGC GG-3') were used to determine the allelic status of bax (45 s denaturation at 94°C, 90 s annealing at 55°C, and 75 s elongation at 72°C for a total of 35 cycles); animals with bax in the wild-type configuration demonstrate a single ~300 bp band, animals nullizygous for bax demonstrate a single ~506 bp band, and animals hemizygous for bax demonstrate both bands on a 1% agarose gel.

Mammary gland tumour collection, histopathology, and whole-mount preparation

Mammary gland tumours and tissues were freshly collected via routine dissection procedures and split for fixation, liquid N₂ snap-freezing (for molecular analyses), and whole-mount preparation. Mammary gland tumours and tissues were fixed in 10% neutral-buffered formalin (EM Sciences, Gibbstown, NJ, USA) in phosphate-buffered saline (PBS), embedded in paraffin, and sectioned by microtomy to 5 μm. Mammary tumour and tissue sections were stained using haematoxylin and eosin and were subjected to histopathological evaluation using light microscopy. Mammary gland tissues for whole-mount preparation, routinely taken from the inguinal glands unless otherwise tumour involved, were fixed in 75% ethanol/25% glacial acetic acid, stained in 0.2% carmine alun (Sigma, St Louis, MO, USA)/0.5% aluminium potassium sulphate solution, dehydrated through an ethanol series, cleared in toluene, mounted using Permount (Fisher, Fair Lawn, NJ, USA), and examined using dissecting stereomicroscopy.

Western blot analyses

Mammary gland tumour and tissue samples that were harvested from the study mice were immediately snap-frozen in liquid N₂, stored at ~80°C, and later thawed in a RIPA homogenisation solution (~1 PBS containing 1% NP-40, 0.5% sodium-deoxycholate, 0.1% SDS, and 10 μg ml⁻¹ PMSF). Briefly, samples were weighed, ground into a fine powder under liquid N₂, lysed for 15 min on ice in a five-fold volume of RIPA solution, and centrifuged at ~10000 g for 15 min at 4°C. Supernatants were recovered after high-speed centrifugation and subject to protein concentration quantification via BCA Protein Assay (Pierce, Rockland, IL, USA). Protein lysates were then combined with ×4 Laemmli Sample buffer (final concentration 50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 10% glycerol, 0.1% bromophenol blue, 2% SDS), boiled for 10 min, fractionated through 12% Tris-glycine gels (Invitrogen) under reducing conditions, transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA), and blocked in 1 × PBS containing 5% milk and either 0.3% Tween-20 for c-Myc detection or 0.05% Tween-20 for Bax detection. For Western analysis, blots were incubated for 1 h at room temperature with anti-c-Myc (C-19) or anti-Bax (N-20) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA), washed repeatedly, and incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (NA934 from Amersham, Buckinghamshire, UK or 611-1302 from Rockland, Gilbertsville, PA, USA for c-Myc; SC-2004 from Santa Cruz). Protein visualisation was achieved using the ECL Western Blotting Reagent Kit (Amersham) and Hyperfilm-ECL photographic film (Amersham).

Apoptosis and cell proliferation assays

Formalin-fixed, paraffin-embedded mammary tumours were sectioned by microtomy and subsequently utilised to assess the presence and extent of apoptosis in the tumours and surrounding mammary tissues. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method was used to evaluate apoptosis in these sections (ApopTag Peroxidase In situ Apoptosis Detection Kit, Serologicals, Norcross, GA, USA). Briefly, sections were cleared in xylene, rehydrated through an ethanol series, treated with Autostyle Digestion reagent (Biomed, Foster City, CA, USA), and quenched in 0.15% H₂O₂ (Sigma). The TdT reaction was allowed to proceed at room temperature, under humidified conditions, for 40 min and a peroxidase-conjugated anti-digoxigenin-antibody was used to assess digoxigenin-dUTP incorporation. A diaminobenzidine/urea chromogen substrate system (Sigma) was used to visualise the TUNEL labeling reaction. Sections were counterstained in 0.5% methyl green dye (Trevigen, Gaithersburg, MD, USA), washed in 100% butanol, and mounted using DPX mountant (Electron Microscopy Scienves). Histological assessment of apoptosis, leading to the calculation of apoptotic index values, was conducted by counting the number of TUNEL-positive apoptotic cells out of >1000 cells in contiguous high-powered (×40) fields.

Formalin-fixed, paraffin-embedded mammary tumours were sectioned by microtomy, stained with haematoxylin and, utilised to assess the presence and extent of cell proliferation in the tumours and surrounding mammary tissues. Histological assessment of cell proliferation, leading to the calculation of mitotic index values, was conducted by counting the number of mitotic figures out of >1000 cells in contiguous high-powered (×40) fields.

Statistical analyses

To evaluate the significance of differences in tumour apoptosis and proliferation between genotypes, all data were subjected to analysis of variance (ANOVA) and Scheffe post hoc testing. A Kaplan–Meier curve was generated for the tumour incidence data for the parous study mice and tumour incidence differences between genotypes were assessed using a generalised Wilcoxon test. Analysis of variance testing was utilised to evaluate the significance of tumour multiplicity differences between genotypes.

RESULTS

Loss of allelic bax alters mammary tumour multiplicity in parous MMTV-c-my c transgenic mice

In order to assess the influence of loss of allelic bax on c-Myc-induced apoptosis and tumorigenesis in the mammary gland, 10—week-old female study mice (myc bax /++, n = 10; myc bax /−++, n = 9; myc bax /−−, n = 10) were housed with male mice, bred...
repititively, and followed bi-weekly for evidence of mammary tumour development. Parous study mice were euthanised when mammary tumour burden approached 10% of animal body mass or when mice reached 1 year of age (in accordance with US and UKCCR guidelines, Workman et al, 1998). Loss of one wild-type bax allele in parous c-myc transgenic mice elevated mammary tumour multiplicity (2.75 tumours/mouse vs 1 tumour/mouse; \( P = 0.04 \) by ANOVA) as compared to parous c-myc transgenic mice in which bax was intact or completely eliminated (Figure 1A). A nonsignificant trend towards increased mammary tumour incidence was found for parous MMTV-c-myc transgenic/bax-hemizygous mice (44.4 vs 20 and 20%; \( P = 0.39 \) by Wilcoxon) as compared to parous c-myc transgenic mice in which bax was intact or completely eliminated (Figure 1B). Mammary tumour latency and parity at time of mammary tumour development were not altered by loss of allelic bax in parous MMTV-c-myc transgenic mice (data not shown).

Mammary gland whole-mount and haematoxylin and eosin-stained tissue sections were examined for evidence of mammary histopathology. Assessment of mammary gland whole-mounts demonstrated that hyperplastic alveolar nodular changes were present only in mammary glands from tumour-bearing, c-myc transgene-positive study mice and were not qualitatively different with loss of allelic bax (data not shown). Microscopic histopathological assessment of the sections indicated that the mammary tumours that developed in the parous MMTV-c-myc study mice were cribriform glandular adenocarcinomas as previously described for MMTV-c-myc mammary tumours (Cardiff et al, 2000). Furthermore, loss of allelic bax, in parous MMTV-c-myc study mice, did not alter the histopathological character of these mammary tumours nor of the peri-tumorous dysplastic mammary lesions (data not shown).

c-Myc and Bax expression in mammary tumours and tissue from parous MMTV-c-myc transgenic mice

The incidence of mammary tumours in MMTV-c-myc transgenic mice, on the FVB inbred genetic background, has been reported to be approximately 50% for virgin female mice and approaching 100% for multiparous female mice (Stewart et al, 1984; Amundadt et al, 1995). The overall mammary tumour incidence for multistrain (C57BL/6 × FVB; 3:1), multiparous, c-myc transgene-positive study mice were 27.6%, considerably lower than previously reported. Western blot analysis was utilised to determine the expression status of the MMTV-c-myc transgene in the mammary tumours and mammary gland tissue from parous study mice (c-myc transgene-negative mice were included as an assay negative control). c-Myc protein expression was only

Figure 1 Loss of bax influences mammary tumour multiplicity but not mammary tumour incidence in parous MMTV-c-myc transgenic/bax-knockout mice. (A) Mammary tumour multiplicity was increased in parous MMTV-c-myc/bax-hemizygous transgenic mice (\( * P = 0.04 \) by ANOVA). (B) Kaplan–Meier analysis by genotype demonstrates a nonsignificant trend toward decreased tumour-free incidence with bax-hemizygosity in parous MMTV-c-myc transgenic/bax-knockout mice (\( P = 0.39 \) by generalised Wilcoxon testing).
Loss of allelic \textit{bax} significantly diminishes mammary tumour apoptosis in parous MMTV-c-myc transgenic mice

Apoptotic indices were generated for both mammary adenocarcinomas and peri-tumorous dysplastic lesions by counting TUNEL-positive cell on mammary tumour and tissue sections. As shown in Figure 3, apoptosis was significantly decreased in mammary adenocarcinomas with loss of allelic \textit{bax} in parous MMTV-c-myc study mice (9.05 ± 1.12 for myc bax+/+ vs 7.38 ± 0.17 for myc bax+/− vs 5.48 ± 0.28 for myc bax−/−); furthermore, a trend toward diminished apoptosis was seen in mammary dysplastic lesions with loss of allelic \textit{bax} in parous MMTV-c-myc study mice (7.20 ± 1.30 for myc bax+/+ vs 4.17 ± 0.50 for myc bax+/− vs 2.29 ± 0.71 for myc bax−/−). In mammary adenocarcinomas, the levels of apoptosis were significantly different for each of the three evaluated genotypes (P = 0.001 for myc bax+/+ vs myc bax+/−; P = 0.033 for myc bax+/− vs myc bax−/−; and P = 0.017 for myc bax+/+/vs myc bax+/−) as well as between the adenocarcinomas and dysplastic mammary lesions in tumour-bearing, parous MMTV-c-myc/bax-hemizygous study mice (P = 0.001).

Development of mammary adenocarcinomas in intact parous study mice and c-Myc-induced mammary adenocarcinomas with loss of allelic \textit{bax} demonstrated that loss of \textit{bax} does not alter cellular proliferation in mammary tumours from parous MMTV-c-myc transgenic mice

Proliferative indices were generated for both mammary adenocarcinomas and peri-tumorous dysplastic lesions by counting mitotic figures on mammary tumour and tissue sections. As shown in Figure 4, proliferation was not significantly altered in either mammary adenocarcinomas or dysplastic mammary lesions with loss of allelic \textit{bax} in parous MMTV-c-myc study mice. Within each genotype, there was significantly more cellular proliferation in mammary adenocarcinomas as compared with peri-tumorous dysplastic mammary lesions (2.03 ± 0.58 vs 0.49 ± 0.005 for myc bax+/*, P = 0.048; 1.55 ± 0.17 vs 0.23 ± 0.05 for myc bax+/−, P = 0.024; and 1.60 ± 0.06 vs 0.20 ± 0.09 for myc bax−/−, P = 0.0001).

**DISCUSSION**

The exogenous expression of murine c-myc using the MMTV-LTR promoter system has previously been demonstrated to induce mammary tumorigenesis; furthermore, these mammary tumours are characterised by a significantly elevated apoptotic index (Stewart et al., 1984; Amundadottir et al., 1996). Results from the MMTV-c-myc/MT-tgfα transgenic mammary tumour model suggest that a diminution of \textit{in vivo} apoptosis can accentuate c-Myc-induced mammary tumour formation (Amundadottir et al., 1995). The \textit{Bax} protein is known to be a key mitochondrial regulator of apoptosis and has been shown to be a transcriptional target of c-Myc (Mitchell et al., 2000). In this latter capacity, \textit{Bax} may be responsible, in part, for apoptosis resulting from deregulated c-myc expression and its loss in human breast tumours may eliminate c-Myc’s potential tumour suppressive role. In this study, \textit{bax}-knockout and MMTV-c-myc transgenic mice were mated to generate a mammary-relevant model in which the influence of the loss of \textit{bax} on c-Myc-induced apoptosis and tumorigenesis could be investigated. Our results clearly demonstrate that loss of \textit{bax} is directly and significantly correlated with a reduction in apoptosis in mammary adenocarcinomas.
Furthermore, haploid loss of bax, in the context of MMTV-c-myc expression, results in an elevation in mammary tumour multiplicity without influencing mammary tumour latency or histopathology. However, in contradistinction to prior findings for c-Myc-mediated lymphomagenesis (Eischen et al, 2001a), complete loss of bax did not promote c-Myc-induced mammary tumorigenesis, suggesting that some amount of Bax expression is required for mammary tumorigenesis.

Previous studies have reported the incidence of mammary tumours in single strain, multiparous MMTV-c-myc mice as approaching 100%; however, no studies of MMTV-c-myc transgenic or myc-containing bitransgenic mice have reported mammary tumour multiplicity findings (Stewart et al, 1984; Jamerson et al, 1998). C57BL/6 as bearing an unknown mammary tumour transgenic mice possessing both wild-type bax alleles, the incidence of mammary tumours was 20% and the multiplicity was one tumour per mouse. This reduced mammary tumour incidence identified in our studies for c-myc transgenic mice may reflect the tumour suppressive influences of a mixed-strain background in our mouse model or may represent functional changes in the genetic control of the c-myc transgene itself. Significantly, other studies have concluded that alteration of or mixing of inbred genetic backgrounds can significantly influence transgene-induced mammary tumorigenesis (Griep et al, 1998; Lifsted et al, 1998; Rowe et al, 1998; Le Voyer et al, 2000; Rose-Hellarkant et al, 2002). The C57BL/6 X FVB cross utilised in the current study has been previously studied in this respect, implying a doubling of mammary tumour multiplicity findings (Jamerson et al, 2000) in C3H/HeN C57BL/6 F1 hybrids (Lifsted et al, 1998), consistent with the doubling of multiplicity identified in multiparous MMTV-c-myc mice with loss of one and both wild-type alleles of bax. Our studies also demonstrate that haploid loss of bax is associated with an increase in mammary tumour multiplicity in multiparous MMTV-c-myc study mice; intriguingly however, complete loss of the wild-type bax alleles results in a mammary tumour multiplicity, but not incidence identical to that for mice with intact bax. These results, of bax loss influencing mammary gland apoptosis and mammary tumour multiplicity, but not incidence, are similar, but not identical, to those seen for the C3(1)/SV40-Tag/bax knockout mice (Shibata et al, 1999). Characterisation of bax-hemizygous and bax-nullizygous mice expressing the C3(1)-Tag transgene resolved that selectively in hemizygous bax animals, apoptosis was significantly reduced in preneoplastic mammary lesions with subsequent enhancement of tumour number (Shibata et al, 1999). No such enhancement in SV40-dependent mammary tumorigenesis was observed in bax-nullizygous mice. The reductions in c-Myc-mediated mammary tumour multiplicity and incidence seen in our bax-nullizygous mice are similar to the findings reported from this previous study and may reflect mammary gland hypoplasia resulting from loss of both wild-type alleles of bax (Shibata et al, 1999). It is worth considering that Bax may have a stage-specific role in suppressing c-Myc-mediated mammary tumorigenesis (apoptosis suppression with bax loss is differentially stage-specific with respect to the inducer of apoptosis, Myc or Tag). Notably, as indicated earlier in the introduction, Bax loss strongly enhances c-Myc-dependent lymphomagenesis (Eischen et al, 2001a). It should be noted that c-Myc-dependent lymphoma- genesis in mice with all three alleles of the bax transgene resolved that selectively in hemizygous bax animals, in contrast to mammary tumorigenesis. (Elson et al, 1995, McCormack et al, 1998). Since c-Myc-dependent mammary tumours seldom contain mutated p53, in striking contrast to lymphomas, future studies could productively address the interactions between p53 and Bax in distinguishing differential, c-Myc effects on tumorigenesis in these two tissue types.

In conclusion, haploid loss of bax in multiparous MMTV-c-myc transgenic mice is associated with a significantly decreased mammary tumour apoptotic index. Loss of bax is not associated with alterations in mammary tumour proliferation. Our results indicate that bax is involved in the regulation of apoptosis in tumours of the murine mammary gland and may be, at best, a weakly negative modulator of c-Myc-mediated mammary tumorigenesis. Complete loss of bax, associated with the most significant suppression of apoptosis in mammary tumours in our model, is clearly not associated with suppression of mammary tumorigenesis, as compared with loss of one wild-type allele of bax. This finding suggests that Bax may be required for mammary tumour development at some stage, and that the contribution of other apoptotic pathways may be important to mammary tumorigenesis in our model. These results are the first published that demonstrate that haploid loss of bax, as seen in many human breast cancers, significantly reduces mammary tumour apoptosis provoked by a human breast cancer-relevant proto-oncogene, c-myc. Our results clearly show that in contrast to the role of Bax as a proapoptotic tumour suppressor in c-Myc-induced breast cancers, significantly reduces mammary tumour apoptosis provoked by a human breast cancer-relevant proto-oncogene, c-myc. Our results clearly show that in contrast to the role of Bax as a proapoptotic tumour suppressor in c-Myc-induced mammary adenocarcinomas are characterised by a cribriform phenotype that is dominantly expressed when the c-myc transgene is co-expressed with other transgenes (Cardiff et al, 2000). Therefore, our findings revealed the transforming role of the c-myc transgene in our tumour model and have suggested that expression of the cribriform tumour phenotype is not abrogated by elimination of the bax tumour suppressor gene.

As anticipated for an apoptosis-regulatory gene, loss of wild-type bax alleles did not significantly alter proliferation, in both mammary adenocarcinomas and dysplastic mammary tissue, in tumour-bearing MMTV-c-myc mice. Our results did demonstrate a significant increase in cellular proliferation between dysplastic mammary tissues and mammary adenocarcinomas, as might be expected in the progression of mammary lesions. As expected with the loss of the proapoptotic bax gene, a trend toward diminished apoptosis in dysplastic mammary tissue and a significant diminution in apoptosis in mammary adenocarcinomas was identified in tumour-bearing MMTV-c-myc mice with loss of one and both wild-type alleles of bax. Our studies also demonstrate that haploid loss of bax is associated with an increase in mammary tumour multiplicity in multiparous MMTV-c-myc study mice; intriguingly however, complete loss of the wild-type bax alleles results in a mammary tumour multiplicity, but not incidence identical to that for mice with intact bax. These results, of bax loss influencing mammary gland apoptosis and mammary tumour multiplicity, but not incidence, are similar, but not identical, to those seen for the C3(1)/SV40-Tag/bax knockout mice (Shibata et al, 1999). Characterisation of bax-hemizygous and bax-nullizygous mice expressing the C3(1)-Tag transgene resolved that selectively in hemizygous bax animals, apoptosis was significantly reduced in preneoplastic mammary lesions with subsequent enhancement of tumour number (Shibata et al, 1999). No such enhancement in SV40-dependent mammary tumorigenesis was observed in bax-nullizygous mice. The reductions in c-Myc-mediated mammary tumour multiplicity and incidence seen in our bax-nullizygous mice are similar to the findings reported from this previous study and may reflect mammary gland hypoplasia resulting from loss of both wild-type alleles of bax (Shibata et al, 1999). It is worth considering that Bax may have a stage-specific role in suppressing c-Myc-mediated mammary tumorigenesis (apoptosis suppression with bax loss is differentially stage-specific with respect to the inducer of apoptosis, Myc or Tag). Notably, as indicated earlier in the introduction, Bax loss strongly enhances c-Myc-dependent lymphomagenesis (Eischen et al, 2001a). It should be noted that c-Myc-dependent lymphoma- genesis in mice with all three alleles of the bax transgene resolved that selectively in hemizygous bax animals, in contrast to mammary tumorigenesis. (Elson et al, 1995, McCormack et al, 1998). Since c-Myc-dependent mammary tumours seldom contain mutated p53, in striking contrast to lymphomas, future studies could productively address the interactions between p53 and Bax in distinguishing differential, c-Myc effects on tumorigenesis in these two tissue types.

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lymphomagenesis (Eischen et al., 2001a), in c-Myc-dependent mammary tumorigenesis Bax is proapoptotic, but lacking in significant mammary tumour suppressive activity.

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