AD_____

Award Number: DAMD17-97-1-7110

TITLE: Cooperation of Bcl-XL and c-Myc in Mammary Tumorigenesis

PRINCIPAL INVESTIGATOR: Matthew Jamerson

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: August 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2000	Final (1 Aug 97 - 31 Jul 00)

4. TITLE AND SUBTITLE
Cooperation of Bcl-XL and c-Myc in Mammary Tumorigenesis

5. FUNDING NUMBERS
DAMD17-97-1-7110

6. AUTHOR(S)
Matthew Jamerson

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

E-MAIL: jamers1@gunet.georgetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Studies of tumor tissue taken from women with breast cancer have demonstrated that the proto-oncogene, c-*myc*, is more abundant than in normal breast (amplified in 16%, rearranged in 5%, and overexpressed in nearly 70% of all human breast cancers). Similar studies have shown that genes known to influence programmed cell death are also altered in breast tumors. Bcl-x_L, known to inhibit apoptosis, is overexpressed in some breast tumors and has been shown to be important in the regulation of apoptosis during mammary gland involution. The pro-apoptotic protein Bax has been demonstrated to be significantly reduced or altogether absent in many breast tumors and has further been shown to cooperate with tumor oncogenes in reducing the protective apoptotic effect early in mammary tumorigenesis. Our bitransgenic mouse studies with constitutive Bcl-x_L expression or loss of Bax in the context of constitutive c-Myc expression have been designed to address whether modulation of c-Myc-induced apoptotic pathways will influence mammary gland development and tumorigenesis. Current data obtained from c-*myc*/*bcl-x*$_L$ and *bax*-knockout/c-*myc* mice suggest a cooperative role for these apoptosis-modulatory genes with c-Myc in mammary tumorigenesis. Utilization of these models will aid in the dissection of the role of apoptosis in the development of breast cancer.

14. SUBJECT TERMS
Apoptosis, Bcl-XL, C-Myc, Bax, transgenic mice, tumorigenesis

15. NUMBER OF PAGES
78

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

For Final Report – Grant # DAMD17-97-1-7110
"Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis"
P.I. Matthew Hunter Jamerson

This Final Report addresses Grant # DAMD17-97-1-7110 entitled "Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis, a Pre-Doctoral Training Fellowship, covering research conducted by the principal investigator Matthew Hunter Jamerson (an M.D./Ph.D. student at the Lombardi Cancer Center, Georgetown University Medical Center) during the period from 1 August 1997 through 31 July 2000.

INTRODUCTION:

It is commonly held that oncogenesis is a multistage process with experimentation demonstrating that a minimum of two independent genetic events required in most cases to produce cellular transformation. This idea is further buoyed by the fact that multiple systems exist within cells both to control cell growth and safeguard against malignant transfomation. There is a need in the biomedical community to develop methods for studying the initiation and progression of malignancies as multistage processes with the dissection of these molecular mechanisms potentially aiding in the prevention, detection, and treatment of cancers. Genetically engineered mice (GEM) provide a highly malleable model system for evaluating the cooperation of oncogenes and tumor suppressor genes in tumorigenesis. Furthermore, the creation of promoter systems for transgene expression that allow for both temporal and tissue-specific expression increase the power of resolution in the study of these oncogenic models. The specific focus of the described studies herein was the generation of c-*myc*/*bcl-x*$_L$ and *bax*-knockout/c-*myc* bitransgenic mice and the use of these GEMs to evaluate the potential cooperative role apoptosis modulation in the initiation and progression of breast malignancies. Furthermore, these studies provide a valuable tool for assessing alterations in the mammary gland development and the normal mammary involution process.

FINAL REPORT
Grant # DAMD17-97-1-7110
"Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis"
P.I. Matthew Hunter Jamerson

BACKGROUND:

In 1982, soon-to-be Nobel Laureate, J. Michael Bishop identified the proto-oncogene c-*myc* as the normal mammalian homologue to the v-*myc* transforming gene that was responsible for avian myelocytomatosis (Vennstrom *et.al.*, 1982). c-*myc* was first identified as an human oncogenic agent when it was discovered that translocations between the myc locus on chromosome 8 and the immunoglobulin heavy chain on chromosomes 14, 22, or 2 were found in nearly all cases of Burkitt's lymphoma (Nesbit *et.al.*, 1999). Following these initial discoveries, multiple mammalian c-*myc* related genes (L-*myc*, N-*myc*) as well as differential transcriptional variants (c-Myc2, c-Myc1, c-MycS) were identified and now constitute the greater c-*myc* family of transcription factors (Dang, 1999; Liao *et.al.*, 2000; Nasi *et.al.*, 2001; Nesbit *et.al.*, 1998). While deregulated expression of L-*myc* has been found to be involved in the etiology of small cell lung cancer (SCLC) and deregulated N-*myc* expression has been identified in approximately 33% of

neuroblastomas (as well as in a small percentage of SCLCs, medullary thyroid carcinomas, retinoblastomas, alveolar rhabdomyosarcomas, and breast tumors), most attention in the fields of oncology and tumor biology is paid to the defining family member, c-*myc* (Nesbit *et.al.*, 1999). A sizable proportion of breast, lung, liver, and colon cancers, as well as some cases of melanoma, multiple myeloma, myeloid leukemia, and non-Hodgkin's lymphoma, have been attributed to aberrant c-*myc* expression and attendant functional consequences (Nesbit *et.al.*, 1999).

Deregulated expression of c-*myc*, via multiple mechanisms including translocation, proviral insertion, gene amplification, point mutation, direct transcriptional and translational effects, or post-translational modification (such as phosphorylative control of protein stability and localization) is a common feature of many human cancers and is thought to contribute to cellular proliferation and transformation when apoptosis is suppressed (Evan *et.al.*, 1992; Santoni-Rugiu *et.al.*, 1998; Dang, 1999). In 1994, Gerard Evan developed the Dual Signal Model suggesting that induction of apoptosis is an obligate function of c-*myc* expression and acts as a potent mechanism for suppression of tumorigenesis (Harrington *et.al.*, 1994); however, more recent experimentation suggests that c-*myc* may 'prime' or sensitize cells to apoptosis as a result of partial mitochondrial permeability and resultant 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). With recognition of this dualistic nature of c-Myc function, it is exciting to speculate that suppression of c-Myc-mediated apoptosis may facilitate tumorigenesis as was intimated in experiments examining the cooperation of c-Myc and knockouts of p19ARF and/or p53 in mouse embryo fibroblast models (Zindy *et.al.*, 1998). Intriguingly, recent data has suggested that c-Myc may increase genomic instability and enhance tumorigenesis, as do dominant mutator oncogenes such like MSH1 and MLH1, without absolute requirement for continued c-Myc overexpression once additional transforming genetic lesions have been generated and replicatively-affixed in the genome (Felsher *et.al.*, 1999a; Felsher *et.al.*, 1999b).

In human breast cancers, c-*myc* is amplified in approximately 16%, rearranged in roughly 5%, and overexpressed in the absence of gross locus alteration in nearly 70% of all cases, suggesting its importance in the the genesis and/or progression of these diseases (Nass *et.al.*, 1997; Deming *et.al.*, 2000). Recent data has suggested several additional mechanisms by which the expression and function of c-Myc might be altered in breast malignancies. The breast cancer-associated gene 1 (BRCA1), a tumor suppressor gene that when mutated in the germline is associated with a familial breast and ovarian cancer syndrome, has been shown to block the transcriptional activity of c-Myc; therefore, the absence of BRCA1 activity may result in a partially-unchecked c-Myc-mediated transcriptional activity resulting in a tumorigenesis (Wang *et.al.*, 1998; Deng *et.al.*, 2000). The coding region determinant-binding protein (CRD-BP), capable of binding to and stabilizing c-*myc* mRNA, is in proximal to *HER-2/neu/erbB2* on human chromosome 17 and has been found to be amplified in 12 out of 40 breast tumor and may be responsible for tumor-associated c-*myc* deregulation (Doyle *et.al.*, 2000). Hyperactivity of the mitogen-activated protein kinase (MAPK) and phosphatidyl-inositol-3 kinase (PI3K) pathways associated with HER-2/Neu/ErbB2 amplification or loss of the phosphatase and tensin homologue deleted on chromosome ten (PTEN), both common alterations in breast tumors, can result in abnormally strong and persistent Ras and Akt/protein kinase B (PKB) kinase activity (King *et.al.*, 1985; Yokota *et.al.*, 1986; Slamon *et.al.*, 1987; van de Vijver *et.al.*, 1987; Slamon *et.al.*, 1989; Li *et.al.*,

1997; Steck *et.al.*, 1997). Recently, it has been demonstrated that Ras-mediated phosphorylation of c-Myc at Serine-62 results in stabilization of the protein; furthermore, active Akt/PKB can block the kinase activity of glycogen synthase kinase 3β (GSK-3β) and thereby limit its degradation-promoting phosphorylation of c-Myc at threonine-58 (Sears *et.al.*, 2000). Therefore, it is likely that in breast tumorigenesis, specific genetic and signaling pathway lesions distinct from those alterations that occur at or near the c-*myc* locus, may arise and contribute to the aberrant expression, stabilization, or function of c-Myc.

The role of c-*myc* expression in both normal mammary development and function as well as in mammary tumorigenesis is currently a burgeoning field of inquiry. c-Myc expression is increased in the normal mammary gland during pregnancy-related proliferation, it is absent in differentiated mammary alveolar cells during lactation, and is again increased during the apoptotic mammary involution process (Strange *et.al.*, 1992). c-Myc is believed to be a nuclear mediator of mitogenic signals incident upon the cell from various receptor systems (growth factor, steroid, and contact receptors being most important in the mammary gland situation) and is contributory to, but not sufficient for, mammary epithelial cell transformation (Leder *et.al.*, 1986; Telang *et.al.*, 1990). Constitutive expression of c-*myc* has been shown to partially transform both mouse and human mammary epithelial cells (MECs), such that exhibit anchorage-independent (soft agar) growth when supplemented with epidermal growth factor (EGF) or transforming growth factor α (TGFα), and are no longer as dependent upon these factors for anchorage-dependent growth as are the parental, non-transformed cells from which they were derived (Telang *et.al.*, 1990; Valverius *et.al.*, 1990).

In addition to those studies that have been conducted *in vitro* and *ex vivo* concerning the role of c-Myc in mammary development, transformation, and tumorigenesis, four groups have independently developed transgenic mice that express the c-*myc* oncogene in a mammary-associated (MMTV-LTR-c-*myc*), mammary-specific (WAP-c-*myc*), or regulatable, mammary-associated (MMTV-LTR-tetTA / tetOP-c-*myc*) context (Stewart *et.al.*, 1984; Schoeneberger *et.al.*, 1988; Sandgren *et.al.*, 1995; D'Cruz *et.al.*, 2001). Another group has developed a mouse model, using a mammary tissue reconstitution method, in which the v-*myc* retroviral oncogene is expressed throughout the reconstituted mammae (Edwards *et.al.*, 1988). Both groups that generated WAP-c-*myc* transgenic mice reported an incidence of mammary tumors approaching 100% in multiparous animals, with all virgin females remaining tumor free over the observation period (to 14 months of age) (Schoenenberger *et.al.*, 1988; Sandgren *et.al.*, 1995). These findings are as expected owing to the temporal window for the hormone-driven activity of the whey acidic protein (WAP) gene promoter which is limited to late pregnancy (near maximal activity is achieved by day 18 of pregnancy) and throughout lactation. It remains to be determined whether c-*myc* expression in this model system is sufficient for mammary tumor development due to the confounding role of multiple pregnancy and lactation periods as both a drive for transgene and as a possible source for important survival signals that may override c-Myc-induced apoptosis. The presence of mammary adenocarcinomas was reported as 100% for multiparous transgenic mice (those with 3+ pregnancies) in which the murine mammary tumor virus long terminal repeat (MMTV-LTR) promoter/enhancer element had been placed upstream of a murine c-*myc* locus containing all three exons (Stewart *et.al.*, 1984; Leder *et.al.*, 1986). Unlike the WAP-c-*myc* model, virgin MMTV-c-*myc* females developed solitary mammary tumors in a stochastic fashion with an incidence of approximately 50% following an extended

latency of 7 to 14 months (Stewart *et.al.*, 1984). The extended latencies and solitary nature of the tumors that develop in both the WAP and MMTV-driven models coupled with the accelerative influence provided by multiparity in the MMTV model, suggest that c-*myc* is contributory but insufficient for mammary tumorigenesis in the mouse.

Two recently published studies have provided confirmation of the insufficiency of c-*myc* in mammary tumorigenesis, have indirectly demonstrated the dominant mutator effect of aberrant c-*myc* expression *in vivo*, and have presented preliminary evidence for the subsequent, apparently-patterned genetic lesions that contribute to the multistage mammary tumorigenic process. In 1999, our group, in collaboration with the National Human Genome Research Institute, used comparative genomic hybridization (CGH) and spectral karyotyping (SKY) to demonstrate that mammary tumors derived from MMTV-c-*myc* mice display distinct patterns of chromosomal aberrations (Weaver *et.al.*, 1999). The fact that these tumor-associated genomic abnormalities are similar despite having arisen in different animals suggests that specific genetic lesions cooperate with deregulated c-*myc* expression in this model and that deregulated c-*myc* alone may be causing genetic instability through a dominant mutator phenotype. Furthermore, the particular patterned chromosomal abnormalities found in the MMTV-c-*myc* mammary tumors are syntenically related to those identified in human breast tumor samples suggesting that this tumor model is valuable in recapitulating the clinically-relevant disease and that the multistage process that results in mammary tumors in mice and breast tumors in humans is likely comparable. The conditional expression of c-*myc* in the mammary glands of mice using a MMTV-LTR-driven tetracycline-responsive transgenic system has further demonstrated the insufficiency of aberrant c-*myc* expression in mammary tumorigenesis (D'Cruz *et.al.*, 2001). Mammary adenocarcinoma formation in this model was similar to that seen in the MMTV-c-*myc* transgenic system; however, elimination of transgene expression by modulation of the tetracycline response element resulted in the regression of tumors with the exception of those tumors that possessed additional genetic lesions (the majority of non-regressing tumors had activating mutations in *Kras2*) (D'Cruz *et.al.*, 2001). Furthermore, the identification of these Kras2 mutations led the investigators to evaluate the mammary tumors that arise in the simple MMTV-c-*myc* transgenics; it was determined that a similar percentage (44%) of these tumors displayed identical activating mutations in *Kras2*. These results taken together lend further support to the notion that c-*myc* can act as a primary transforming lesion; however, full tumor development and progression requires additional patterns of genetic alterations that may result from c-*myc* genomic destabilization.

The MMTV-c-*myc*/MMTV-v-*Ha-ras* cross generated in 1987 was the first mammary-directed, c-*myc*-containing bitransgenic mouse (Sinn *et.al.*, 1987). Characterization of this model demonstrated that deregulated expression of these two genes resulted in accelerated mammary tumorigenesis with an abrogation of the requirement for pregnancy (mammary tumors were observed in both virgin female as well as in male bitransgenic mice). This particular result also reflected previous work that had shown c-*myc* and *ras* as being sufficient for and capable of cooperating in the transformation of mouse embryo fibroblasts (MEFs) (Land *et.al.*, 1983; Hunter, 1991). In 1995, two mammary-directed c-*myc*/*tgfα* bitransgenic models were developed and buoyed the notion that signaling through the EGFR (ErbB1) and/or activation of Ras could

synergize with deregulated c-*myc* expression in the mammary tumorigenic process (Amundadottir *et.al.*, 1995; Sandgren *et.al.*, 1995).

TGFα is a secreted 50 amino acid glycoprotein derived from an active, membrane-bound 160 amino acid precursor. TGFα demonstrates a high level of homology (~42%) with EGF and both molecules bind the epidermal growth factor receptor (EGFR/ErbB1) with high affinity (Martinez-Lacaci *et.al.*, 1999). TGFα binding to EGFR has been demonstrated to result in receptor homodimerization as well as heterodimerization with cErbB2, c-ErbB3, and/or c-ErbB4, when these receptor family members are present in together within the cell. This receptor dimerization subsequently leads to autophosphorylation and activation of downstream signaling pathways including p42/44-MAPK, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), PI3K, phospholipase C (PLC), protein kinase A (PKA), and adenyl cyclase (AC) (Dickson *et.al.*, 1995; Siegel *et.al.*, 1998; Martinez-Lacaci *et.al.*, 1999). TGFα is expressed in the normal murine mammary gland within the basal cells of the epithelium and the terminal cells of the nascent end bud; it is also present in murine and human mammary glands during pregnancy and has been demonstrated to have similar growth promotional effects upon human and murine MECs *in vitro* (Salomon *et.al.*, 1987; Valverius *et.al.*, 1989; Bates *et.al.*, 1990; Liscia *et.al.*, 1990; Snedecker *et.al.*, 1991; Martinez-Lacaci *et.al.*, 1999). Early studies found increased TGFα expression in mammary tumors versus normal mammary gland (Derynck *et.al.*, 1987; Arteaga *et.al.*, 1988; Bates *et.al.*, 1988; Travers *et.al.*, 1988); however, the current paradigm for EGF-family growth factor participation in breast cancer involves the establishment of a pro-proliferative, anti-apoptotic, autocrine/paracrine stimulatory loop with the EGFR which is found overexpressed in approximately 50% of human breast cancers (Harris *et.al.*, 1988; Dickson *et.al.*, 1995; Dahiya *et.al.*, 1998; DeLuca *et.al.*, 1999).

Three groups independently developed transgenic mouse models in which TGFα was expressed in a metal-ion inducible, general tissue context (MT-*tgfα*) (Sandgren *et.al.*, 1990; Jhappan *et.al.*, 1990), a mammary-associated context (MMTV-*tgfα*) (Matsui *et.al.*, 1990), or a mammary-specific context (WAP-*tgfα*) (Sandgren *et.al.*, 1995). Characterization of these transgenic models suggested that constitutive *tgfα* expression accelerates mammary development, impedes apoptosis during involution, and contributes to MEC transformation by acting as both a survival and growth factor for differentiated murine MECs. Significantly, the pregnancy requirement and extended tumor latency for these *tgfα* transgenic models illustrates that aberrant TGFα expression is unlikely to be capable of serving as the sole cause of mammary cancers; rather, it is likely to be one alteration along a multistep transforming pathway. Following on this work, our laboratory and another generated transgenic mice in which both c-*myc* and *tgfα* were co-expressed in the mammary gland (Amundadottir *et.al.*, 1995; Sandgren *et.al.*, 1995). The MMTV-c-*myc*/MT-*tgfα* bitransgenic mice developed multiple mammary adenocarcinomas with a much reduced latency, as compared to the single *myc* or *tgfα* transgenics, and in the absence of any requirement for pregnancy or ovarian hormone stimulation (Amundadottir *et.al.*, 1995). The complete absence of normal mammary tissue in these bitransgenic animals and the ability of mammary tissue from 3-week old mice to form tumors in athymic mice suggest that these two important, mammary-relevant genes are capable of synergistically transforming the mammary epithelium, apparently requiring minimal, if any, additional genetic alterations. Characterization of the WAP-c-*myc*/WAP-*tgfα* bitransgenic model confirmed the potent synergy of these two

genes in promoting and accelerating mammary tumor formation when compared with the relevant single transgenic animals (Sandgren *et.al.*, 1995). Furthermore, the power of this genetic interaction is demonstrated in both models since both male and virgin female bitransgenic animals developed mammary tumors.

Subsequent work in our laboratory with single transgenic mice, c-*myc*/*tgfα* bitransgenic mice, and tumor cell lines derived from these transgenic mice, has led to the hypothesis that TGFα can cooperate with c-Myc in promoting cell cycle progression and can act to suppress c-Myc-induced apoptosis (Amundadottir *et.al.*, 1996; Nass *et.al.*, 1996; Nass *et.al*, 1998). *In situ* end-labeling of DNA fragments (terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling/TUNEL assay) in paraffin-embedded mammary tumor sections from transgenic mice indicates the presence of apoptotic MECs in c-*myc* transgenic tumors and their near absence in tumors from *tgfα* and c-*myc*/*tgfα* transgenic mice (Amundadottir *et.al.*, 1996). Observations made with tumor cell lines indicate that the overexpression of these two genes results in increased cell proliferation under both anchorage-dependent and anchorage-independent conditions, a reduced requirement for exogenous growth factor stimulation, and greatly diminished apoptosis. The cell lines derived from c-*myc* transgenic mouse mammary tumors were significantly more apoptotic than cell lines derived from *tgfα* and c-*myc*/*tgfα* mammary tumors; however, the frequency of apoptotic cells in the c-*myc* lines could be considerably suppressed *in vitro* by the addition of exogenous TGFα or EGF (Amundadottir *et.al.*, 1996). Conversely, the level of apoptosis was increased in these *myc* tumor lines when EGFR signaling was blocked by addition of PD153035, a specific, synthetic EGFR tyrosine kinase inhibitor (Amundadottir *et.al.*, 1996). Our have suggested that transformation, maintenance of the transformed phenotype, and suppression of apoptosis in c-*myc*-overexpressing mouse mammary tumor cell lines may require signaling through the p42/44-MAPK and PI3K pathways, both of which are targeted for activation by the ligand-activated EGFR (among other growth factor and cytokine receptors) (Amundadottir *et.al.*, 1998; Wang *et.al.*, 1999).

Molecular characterization of apoptosis in c-*myc*-overexpressing murine MECs derived from the MMTV-c-*myc* transgenic mice led to the recognition that Bcl-x_L, an anti-apoptotic member of the Bcl-2 family of apoptosis regulatory proteins, is a likely mediator of TGFα and EGF-directed protection against *myc*-driven apoptosis (Nass *et.al.*, 1996). Bcl-x_L mRNA and protein levels were elevated with TGFα or EGF treatment of these *myc*-expressing cell lines and expression of this anti-apoptotic molecule was significantly diminished with growth factor withdrawal, transforming growth factor β (TGFβ) treatment, or by PD153035-induced EGFR blockade. Both Bax, a pro-apoptotic Bcl-2 family member, and p53 were highly expressed and unchanged, while Bcl-2 and Bcl-x_S levels remained low or undetectable with these aforementioned treatments (Nass *et.al.*, 1996). The work in our laboratory, along with data from other studies, has led to the development of the following model explaining the cooperation between c-Myc and TGFα in enhancement of proliferation and blockade of apoptosis in the mouse mammary gland: First, deregulated c-Myc may drive cellular proliferation by activating cyclin D and E-dependent kinases (cdk4 and ckd6), promoting the transcription of both cyclin E and cyclin A, limiting the activity of cyclin-dependent kinase inhibitors p21 and p27 (transcriptional repression/protein stability and complex formation), promoting release of E2F family members from Rb pocket proteins (through transcriptional activation of Id2 as well as G1 cyclin activity),

and activating cdc25A phosphatase which is responsible for dephosphorylative activation of cyclin E/cyclin-dependent kinase-2/4 complexes (Facchini *et.al.*, 1998; Dang, 1999; Mateyak *et.al.*, 1999; Nasi *et.al.*, 2001). The combination of these effects and the induction of cyclin D1, resulting from TGFα overexpression (Liao *et.al.*, 2000), may result in deregulation of the cell cycle and abrogation of normal cell cycle checkpoint control. Second, deregulated c-*myc* expression may promote apoptosis by inducing p53 expression, both directly via transcription upregulation and indirectly by transcriptional control of $p19^{ARF}$ (responsible for p53 stabilization), and by directly or indirectly inducing the expression of Bax (Reisman *et.al.*, 1993; Miyashita *et.al.*, 1995; Packham *et.al.*, 1995; Zindy *et.al.*, 1998; Dang, 1999). Additionally, c-Myc may promote apoptosis by increasing the sensitivity of cells to death receptor (Fas and tumor necrosis factor receptor 1/TNFR1) activation as well as to mitochondrial permeability transition accompanied by the release of holocytochrome c (the physical and functional activator of the apoptotic protease activating factor-1/APAF-1-containing apoptosome complex) (Juin *et.al.*, 1999; Prendergast, 1999).

These aforementioned results, combined with those obtained from the characterization of a MMTV-c-*myc*/WAP-*bcl2* bitransgenic model (Bcl-2 expression accelerated mammary tumorigenesis and suppressed *in vivo* mammary tumor apoptosis) (Jäger *et.al.*, 1997), strongly suggests that mammary tumorigenesis is significantly enhanced when deregulated c-*myc* expression, responsible both for driving proliferation and sensitizing cells to apoptosis, is coupled with other genetic alterations that act to block the c-*myc*-mediated apoptotic pathways. Recently, a great deal of information has been published exploring the role of apoptosis regulatory proteins (with Bcl-x_L and Bax especially relevant to the projects described in this Report) in the normal development of the mammary gland as well as in the etiology of breast cancer. Bcl-x_L is expressed in the cuboidal epithelium and myoepithelium of the breast and is known to be increased during post-lactational mammary gland involution with its splice variant, Bcl-x_S, being induced more strikingly (Krajewski *et.al.*, 1994a; Li *et.al.*, 1996a). Whereas Bcl-2 levels are reduced during the early stages of mammary involution, levels of Bcl-x_L and Bax are highly upregulated with the relative levels skewed toward greater pro-apoptotic protein expression (Schorr *et.al.*, 1999). Bcl-x_L expression has been correlated with the presence of the EGFR in ER-negative breast cancer cell lines and data from our laboratory (regarding mammary tumor cell lines) confirming that EGFR signaling blockade results in decreased Bcl-x_L expression (Nass *et.al.*, 1996; Hsu *et.al.*, 1997). Bcl-x_L has been shown to block apoptosis induced by p53 in T47D and TNF/anti-Fas in MCF-7 breast cancer cell lines (Jäättelä *et.al.*, 1995; Schott *et.al.*, 1995; Srinivasan *et.al.*, 1998). With regards to the *in vivo* situation, Bcl-x_L has been shown to be overexpressed in breast tumors as compared to adjacent normal breast tissue with Bcl-x_L expression predominate to Bcl-2 expression in higher histological grade breast tumors with greater tumor cell resistance to apoptosis (Schott *et.al.*, 1995; Ogretman *et.al.*, 1996; Olopade *et.al.*, 1997; Sierra *et.al.*, 1998). Relevant to the potential influence of Bcl-x_L in cancer therapy, it has recently been published that overexpression of Bcl-x_L in a mouse mammary tumor cell line resulted in an increased resistance to chemotherapeutic killing, whereas the use of *bcl-x_L* antisense was demonstrated to induce apoptosis in a number of human breast cancer cell lines (Liu *et.al.*, 1999; Simões-Wüst *et.al.*, 2000). To date, no work has been published on the targeting of a Bcl-x_L transgene to the mammary gland of transgenic mice.

Bax is expressed in the epithelium of the normal breast (most highly in the myoepithelium and those cells that had no or limited luminal contact) and has been demonstrated to be increased during post-lactational mammary gland involution without dependence upon functionally-intact p53 protein (Krajewski *et.al.*, 1994b; Li *et.al.*, 1996b; Feuerhake *et.al.*, 2000; Shilkaitis *et.al.*, 2000). The partial or total loss of Bax in knockout mice provided evidence that the presence of Bax was likely to be unnecessary for mammary gland development and functional differentiation (though a small percentage of homozygous knockout animals did evidence some post-partum lactational incompetency); furthermore, Bax nullizygous animals exhibit reduced MEC apoptosis during the first stage of post-lactational involution (Schorr *et.al.*, 1999a; Schorr *et.al.*, 1999b). Bax was found to be weakly expressed or absent in several breast cancer cell lines and transfection of Bax into these lines resulted in increased apoptotic sensitivity and diminished tumor proliferation in athymic mice (Bargou *et.al.*, 1995; Bargou *et.al.*, 1996). Overexpression of Bax in MCF-7 breast cancer cells, a line that expresses very low levels of Bax, results in an increased sensitivity to ionizing radiation (Sakakura *et.al.*, 1996). With regard to the *in vivo* situation, Bax was found to be highly expressed in normal breast tissue and absent (or nearly so) in invasive ductal breast tumors and carcinomas (Bargou *et.al.*, 1995; Shilkaitis *et.al.*, 2000). Significant reductions in Bax expression were found in 34% of primary breast tumors in women with metastatic disease and the expression of Bax was inverse correlated with overall survival, treatment response, and metastasis (Krajewski *et.al.*, 1995; Kapranos *et.al.*, 1997). Furthermore, expression of Bax protein in metastatic breast tumors was found to be predictive of tumor response to chemotherapy independent of other predictive variables (Sjöström *et.al.*, 1998). Presently, only one study has been published in which the role of Bax loss has been correlated with murine mammary tumorigenesis (Shibata *et.al.*, 1999). A transgenic mouse designed as an *in vivo* model for prostate cancer, in which the SV40 large T antigen (*Tag*) gene was placed under the control of the C3(1) prostatein gene regulatory elements, was also discovered to be a model for mammary adenocarcinomas (Maroulakou *et.al.*, 1994). Subsequent investigation of this model led to the discovery that apoptosis, as measured by TUNEL assay, was most pronounced in preneoplastic hyperplasias and associated with an increased expression of Bax. Furthermore, apoptosis was reduced in both normal MECs and mammary adenocarcinomas with generation of crosses between the *Tag* mice and p53-nullizygous mice demonstrating that apoptosis was entirely independent of p53 status and that the absence of p53 was without influence on the expression of Bax (Shibata *et.al.*, 1996; Shibata *et.al.*, 1999). Characterization of C3(1)-*Tag*/*bax*-hemizygous and nullizygous mice resolved that partial loss of Bax resulted in reduced apoptosis in preneoplastic mammary lesions with subsequent enhancement of tumor growth rate, number, and mass. Interestingly, no alterations in apoptosis or cellular proliferation levels were discovered in mammary carcinomas in these animals; furthermore, animals in which both alleles of *bax* had been eliminated evidenced a slightly reduced number of mammary lesions, as compared to the *bax* hemizygous mice, perhaps due to mammary gland hypoplasia present in these animals (reduced field for transforming influence of *Tag*) (Shibata *et.al.*, 1999). This study lends further weight to the notion that Bax is a tumor suppressor gene and is specifically relevant to the tumorigenic processes in the mammary gland (Yin *et.al.*, 1997; Shibata *et.al.*, 1999).

Of great interest to those who study breast cancer and c-Myc is the nature of apoptosis signaling by c-Myc and its contribution to the suppression of tumorigenesis. Constitutive expression of Bcl-x_L and/or loss of *bax* are likely to disrupt the c-Myc-induced apoptotic pathways without

significant influence on c-Myc-mediated cell proliferation. The development of these combinatorial, mammary-relevant transgenic models (MMTV-c-*myc*/tetOP-*tetTA*/tetOP-*bcl-x_L* and *bax*-knockout/MMTV-c-*myc*) should provide a convincing, *in vivo* method for dissecting the role of apoptosis in c-Myc-related mammary tumorigenesis and development and may provide greater resolution of molecular pathways that might be exploited for clinical assessment and therapeutic management of breast cancer.

SUMMARY OF RESEARCH AND TRAINING ACCOMPLISHMENTS:

This final report of training and research accomplishments covers the period between 1 August 1997 and 31 July 2000 for Grant # DAMD17-97-1-7110 entitled "Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis" conducted by the principal investigator Matthew Hunter Jamerson.

Hypothesis: Constitutive expression of Bcl-x_L and c-Myc with greatly facilitate tumorigenesis in mouse mammary epithelial cells *in vivo* as a result of Bcl-x_L blocking c-Myc-induced apoptosis and not c-Myc-mediated cell cycle progression and cellular proliferation. Furthermore, constitutive expression of Bcl-x_L, in cell lines expressing the c-Myc transgene, will block apoptosis upon cell exposure to conditions of EGF deprivation and TGFβ1 addition *in vitro*.

Revised Hypotheses:
A. Constitutive expression of c-*myc* and *bcl-x_L* in a bitransgenic model will facilitate mammary tumorigenesis as a result of Bcl-x_L blockade of c-Myc-induced apoptosis and not c-Myc-mediates cell cycle progression.

B. Constitutive expression of c-*myc* in a *bax*-null background will facilitate mammary tumorigenesis due to a disruption of the c-Myc-induced apoptotic pathways.

Specific Aim #1: To determine whether constitutive overexpression of both Bcl-x_L and c-Myc will cooperate to enhance initiation and progression of mammary tumors.

A. Specific Aim #1A: To determine whether constitutive overexpression of both Bcl-x_L and c-Myc in a double transgenic mouse model will enhance mammary tumorigenesis as compared with c-Myc single transgenics.
B. Specific Aim #1B: To determine whether constitutive overexpression of Bcl-x_L via retroviral-mediated transduction into mouse mammary epithelial cells expressing the c-*myc* transgene will enhance mammary tumorigenesis upon reimplantation and regrowth in a cleared mammary fat pad of a syngenic animal.

Revised Specific Aim #1: Develop two transgenic model systems to examine the cooperation of c-Myc with *bax*-knockout and *bcl-x_L* expression in mammary tumorigenesis.

A. Revised Specific Aim #1A: Generate tetOP-*tta*/tetOP-*bcl-x_L*/MMTV-c-*myc* transgenic mice, ascertain transgene expression using tail biopsy-derived genomic DNA, and establish study groups.
B. Revised Specific Aim #1B: Generate MMTV-c-*myc*/*bax*-knockout transgenic mice, ascertain transgene expression (or lack thereof for *bax*) using tail biopsy-derived genomic DNA, and establish study groups.

Summary of Training and Research Accomplishments for Revised Specific Aim #1A:
Two MMTV-c-*myc* males on the FVB background were obtained from the Charles River Laboratories (Wilmington, MA) in September 1997 and were used to develop a breeding colony of c-*myc* transgenic mice through matings with non-transgenic female FVB mice under a current breeding license with DuPont Medical Products (Wilmington, DE). These animals were originally developed in laboratory of Philip Leder (Harvard University, Boston, MA) and find the expression of murine c-*myc* driven by the mouse mammary tumor virus long terminal repeat promoter/enhancer elements (MMTV-LTR) (Stewart *et.al.*, 1984). This particular breeding strategy is dictated by the fact that c-*myc* females are often incapable of nursing their young and subsequently their pups succumb to starvation and/or cannabalism. Ascertainment of the transgene status of offspring was conducted using a convenient polymerase chain reaction (PCR)-based strategy. Genomic DNA was obtained from tail biopsy material and was utilized in a PCR reaction with two MMTV-c-*myc* transgene-specific primers: MMTV-Myc5' primer as [5'-*CCC AAG GCT TAA GTA AGT TTT TGG*-3'] and MMTV-Myc3' primer as [5'-*GGG CAT AAG CAC AGA TAA AAC ACT*-3']. The constituents of each c-*myc* PCR reaction were as follows: 28μL Platinum PCR Supermix (Gibco BRL, Rockville, MD), 2μL of genomic DNA, and 1μL of mixed primers (stock as 100ng/μL). The c-*myc* PCR reaction conditions were as follows: 42 cycles of 60 seconds @ 95°C for denaturation, 60 seconds @ 52°C for annealing, and 75 seconds @ 72°C for extension. Transgenic animals were identified by the resolution of a single band of approximately 880bp on an ethidium bromide-stained 1.0% agarose gel (representative example given as **FIGURE 1**).

Four breeding pairs of tetOP-*bcl-x_L* transgenic mice were obtained from the laboratory of Priscilla A. Furth (University of Maryland Medical School, Baltimore, MD) in April 1998 and were subsequently used to develop a breeding colony of *bcl-x_L* transgenic mice through interbreeding on the C57BL/6 background. These animals were originally developed in the laboratory of Gabriel Nuñez (University of Michigan Medical School, Ann Arbor, MI) and find the expression of *bcl-x_L* under the control of tetracycline operon system (tetOP). Ascertainment of the transgene status of offspring was conducted using a convenient PCR-based strategy. Genomic DNA was obtained from tail biopsy material and was utilized in a PCR reaction with two tetOP-*bcl-x_L* transgene specific primers: BCLTG3' primer as [5'-*CTG AAG AGT GAG CCC AGC AGA ACC*-3'] and BCLTG5' primer as [5'-*GCA TTC AGT GAC CTG ACA TC*-3']. The constituents of each *bcl-x_L* PCR reaction were as follows: 27μL Platinum PCR Supermix, 2μL of genomic DNA, and 1μL of mixed primers (stock as 100ng/μL). The *bcl-x_L* reaction conditions were as follows: 30 cycles of 60 seconds @ 95°C for denaturation, 60 seconds @ 58°C for annealing, and 180 seconds @ 72°C for extension. Transgenic animals were identified by the resolution of a single band of approximately 450bp on an ethidium bromide-stained 1.0% agarose gel (representative example given as **FIGURE 2**).

In the absence of reliability confirmation and with the delayed availability of the MMTV-*tta* transgenic mice, we chose to use the tetOP-*tta* transgenic mouse as the basis for controlling the tetOP-*bcl-x*$_L$ transgene expression. These animals were originally developed in the laboratory of David Schatz (Yale University Medical School, New Haven, CT) to acts as a self-inducing tetracycline-regulatable system wherein the tetracycline transactivator protein gene (*tta*) and the luciferase gene (*luc*) are expressed under the control of a minimal human cytomegalovirus (hCMV) promoter and a series of seven tandemly-repeated tetracycline responsive operons (tetOP) (Shockett *et.al.*, 1995). It should be noted that this system is a tet-OFF system; therefore, in the absence of the antibiotic tetracycline (or derivative doxycycline), constitutive expression of the tetracycline transactivator protein (tTA) drives the expression of transgenes possessing tetOP elements. Two breeding pairs of tetOP-*tta* mice were obtained from the Jackson Laboratories (Bar Harbor, ME) in July 1998 and were subsequently used to develop a breeding colony of *tta* transgenic mice through interbreeding on the C57BL/6J **x** C3HeB/FeJLe-a mixed background. Ascertainment of transgene status of offspring was conducted using a convenient PCR-based strategy. Genomic DNA was obtained from tail biopsy material and was utilized in a PCR reaction with two tetOP-*tta* transgene specific primers: CMVF1 primer as [5'-*TGA CCT CCA TAG AAG ACA CC*-3'] and TTAREV1 primer as [5'-*ATC TCA ATG GCT AAG GCG TC*-3']. The constituents of each *tta* PCR reaction were as follows: 28μL Platinum PCR Supermix, 2μL of genomic DNA, and 1μL of mixed primers (stock as 50μM). The *tta* reaction conditions were as follows: 30 cycles of 45 seconds @ 94°C for denaturation, 45 seconds @ 52°C for annealing, and 90 seconds @ 72°C for extension. Transgenic animals were identified by the resolution of a single band of approximately 290bp on an ethidium bromide-stained 1.0% agarose gel (representative example given as **FIGURE 3**).

Following the establishment of these breeding colonies, tetOP-*bcl-x*$_L$ transgenic mice were mated with tetOP-*tta* transgenic mice. The resultant F_1 animals should find the *bcl-x*$_L$ transgene constitutively activated in nearly all tissues in the absence of animal tetracycline dosing. Confirmation of Bcl-x_L expression was achieved by Western blot analysis of mammary tissue lysates prepared in RIPA buffer (1X PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and proteases/phosphatase inhibitors), electrophoresed on a 10% polyacrylamide gel (under reducing conditions), transferred to a Amersham-Pharmacia (Buckinghamshire, England) Hybond-N membrane, probed with a Transduction Laboratories (San Diego, CA) rabbit anti-human/mouse Bcl-x_L primary antibody (B22630), a New England Biolabs / Cell Signaling (Beverly, MA) horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody, and resolved using the enhanced chemiluminescence (ECL) Super Signal reagant from Pierce (Rockford, IL) (**FIGURE 4**). Finally, female tetOP-*tta*/tetOP-*bcl-x*$_L$ bitransgenic mice were mated with male MMTV-c-*myc* transgenic mice to yield the F_1 study population where transgene ascertainment for each of the three transgenes was conducted as described above (representative example given as **FIGURE 5**).

For the *tta*/*bcl-x*$_L$/*myc* tumor studies, females were recruited into one of three groups: the virgin tumor group, the parous tumor group, and the developmental/involution group. In the virgin tumor group, female mice were recruited into four major genotypic subgroups (*tta*/*bcl-x*$_L$/*myc*, *tta*/*bcl-x*$_L$/wt, *tta*/wt/*myc*, *tta*/wt/wt) {n=14-15 mice/subgroup} and four minor genotypic subgroups (wt/*bcl-x*$_L$/*myc*, wt/*bcl-x*$_L$/wt, wt/wt/*myc*, wt/wt/wt) {n=4-5 mice/subgroup}. In the parous tumor group, female mice were recruited into four major subgroups (*tta*/*bcl-x*$_L$/*myc*,

tta/bcl-x$_L$/wt, *tta*/wt/*myc*, *tta*/wt/wt) {n=3-10 mice/subgroup} and were cohoused/continuously bred with a single male mouse commencing when the study female mice reached 10 weeks of age. **FIGURE 6** displays the completed recruitment for the virgin and parous tumor groups.

In the developmental/involution study, female mice were recruited into five major genotypic subgroups (*tta/bcl-x$_L$/myc*, *tta/bcl-x$_L$*/wt, *tta*/wt/*myc*, *tta*/wt/wt, wt/wt/*myc*) which were further subdivided into three endpoints (1 day, 3 days, and 10 days post-weaning). Seven-week old female mice (termed 'early-parous' in this discussion) in this study were bred with a single male mouse. The male mouse was separated from the female mouse when pregnancy was grossly observable (typically between 12-15 day post-coitus) to prevent the male from inseminating the female during the immediate post-partum estrus period. One day post-partum, the female mouse and her pups were separated (forced weaning) to trigger the involution process; this procedure was made necessary to standardize among the study subgroups owing to the fact that certain genotypes exhibited post-natal pup death / litter loss. **FIGURE 7** displays the current recruitment (and total planned recruitment) for this developmental/involution study.

Summary of Training and Research Accomplishments for Revised Specific Aim #1B:
Two female and four male *bax*-knockout mice were obtained from the laboratory of Priscilla A. Furth (University of Maryland Medical School, Baltimore, MD) in March 1998 and were subsequently used to develop a breeding colony of bax-normizygous, bax-hemizygous, and *bax*-nullizygous animals through interbreeding on the C57BL/6J x 129/SvJ mixed background. These animals were originally developed and characterized in the laboratory of Stanley J. Korsmeyer (Washington University School of Medicine, Saint Louis, MO) and find the expression of the *bax* gene eliminated by the neomycin cassette-mediated disruption of *bax* exons 2-5 (Knudson *et.al.*, 1995). Ascertainment of the transgene status of offspring was conducted using a convenient PCR-based strategy. Genomic DNA was obtained from tail biopsy material and was utilized in a PCR reaction with three primers: BPR2 primer as [5'-*GTT GAC CAG AGT GGC GTA GG*-3'], MK1 primer as [5'-*GAG CTG ATC AGA ACC ATC ATG*-3'], and NPR2 primer as [5'-*CCG CTT CCA TTG CTC AGC GG*-3']. The constituents of each *bax* PCR reaction were as follows: 31μL Platinum PCR Supermix, 2μL of genomic DNA, and 1μL of mixed primers (stock as 41.6μM for BPR2 and NPR2, 6.25μM for MK1). The *bax* PCR reaction conditions were as follows: 35 cycles of 45 seconds @ 94°C for denaturation, 90 seconds @ 55°C for annealing, and 120 seconds @ 72°C for extension. *bax*-normizygous animals were identified by the resolution of a single band of approximately 320bp, *bax*-nullizygous animals were identified by the resolution of a single band of approximately 600bp, and *bax*-hemizygous animals were identified by the presence of both bands (indicative of the presence of a wild-type *bax* allele and a disrupted *bax* allele) on ethidium bromide-stained 1.0% agarose gels (representative example given as **FIGURE 8**).

Since *bax*-nullizygous males are infertile due to a blockade of the spermatogenic process and an accumulation of premeiotic germ cells and therefore are not useful as breeders (Knudson *et.al.*, 1995) and these knockout mice are on a C57BL/6J x 129/SvJ mixed background, a two-tiered breeding strategy was employed to generate transgenic animals possessing c-*myc* in the presence and/or absence of *bax* (**FIGURE 9**). First, MMTV-c-*myc* males were mated with *bax*-nullizygous females to generate the F_1 generation of *bax*-hemizygous/MMTV-c-*myc* breeder males. Subsequently, these F_1 breeder males were mated to *bax*-nullizygous female mice to

yield the F_2 study population where transgene ascertainment for c-*myc* and *bax* were conducted as described above (representative example given as **FIGURE 10**).

For the *bax*-knockout/c-*myc* tumor studies, females were recruited into one of three groups: the virgin tumor group, the parous tumor group, and the developmental/involution group. In the virgin tumor group, female mice were recruited into four major genotypic subgroups (*bax-/- myc*, *bax+/- myc*, *bax+/+ myc*, *bax-/-* wt) {n=11-14 mice/subgroup} and two minor genotypic subgroups (*bax+/-* wt, *bax+/+* wt) {n=5 mice/subgroup}. In the parous tumor group, female mice were recruited into four major subgroups (*bax-/- myc*, *bax+/- myc*, *bax+/+ myc*, *bax-/-* wt) {n=8-10 mice/subgroup} and were cohoused/continuously bred with a single male mouse commencing when the study female mice reached 10 weeks of age. **FIGURE 11** displays the completed recruitment for the virgin and parous tumor groups.

In the developmental/involution study, female mice were recruited into five major genotypic subgroups (*bax-/- myc*, *bax+/- myc*, *bax+/+ myc*, *bax-/-* wt, *bax+/+* wt) which were further subdivided into three endpoints (1 day, 3 days, and 10 days post-weaning). Ten-week old female mice in this study were bred with a single male mouse. The male mouse was separated from the female mouse when pregnancy was grossly observable (typically between 12-15 days post-coitus) to prevent the male from inseminating the female during the immediate post-partum estrus period. One day post-partum, the female mouse and her pups were separated (forced weaning) to trigger the involution process; this procedure was made necessary to standardize among the study subgroups owing to the fact that certain genotypes exhibited post-natal pup death / litter loss. **FIGURE 12** displays the current recruitment (and total planned recruitment) for this developmental/involution study.

Specific Aim #2: To characterize the expression of Bcl-x_L and c-Myc expression in mammary tissues and correlate specific expression with histopathology and apoptosis *in situ*.

Revised Specific Aim #2: Evaluate alterations in mammary tumorigenesis resulting from the cooperation of c-Myc and Bcl-x_L and c-Myc and *bax*-knockout. Evaluate transgene expression (or lack thereof for *bax*), apoptosis and proliferation indices, and histology from transgenic animal tumors and normal mammary tissues and correlate molecular findings with histopathology.

A. Revised Specific Aim #2A: Follow F_2 generation study animals (c-*myc/tta/bcl-x_L* cross) to determine tumor latency, incidence, multiplicity, growth kinetics, metastasis, and parity dependence.
B. Revised Specific Aim #2B: Follow F_2 generation study animals (*bax*-knockout/c-*myc* cross) to determine tumor latency, incidence, multiplicity, growth kinetics, metastasis, and parity dependence.
C. Revised Specific Aim #2C: Evaluate, in c-*myc/bcl-x_L* bitransgenic mice, transgene expression, apoptosis and proliferation indices, and histology from transgenic animal tumors and normal mammary tissues and correlate molecular findings with histopathology.
D. Revised Specific Aim #2D: Evaluate, in *bax*-knockout/c-*myc* transgenic mice,

transgene expression, apoptosis and proliferation indices, and histology from transgenic animal tumors and normal mammary tissues and correlate molecular findings with histopathology.

Summary of Training and Research Accomplishments for Revised Specific Aim #2A:
Following recruitment of F_2 generation study animals (from *tta/bcl-x$_L$*/c-*myc* crosses) to the virgin and parous tumor study subgroups, female mice were examined three times a week for the development of mammary masses and/or other grossly observable morbidities. At the time of this report, all virgin study females in all genotypic subgroups have been sacrificed. At the time of this report, all parous study females in all genotypic subgroups have been sacrificed (with the exception of 3 *tta/bcl-x$_L$*/wt females that will be sacrificed by 1 June 2001 if no mammary masses are observed prior to that date). In the absence of mammary masses and/or other grossly observable morbidities, virgin tumor study female mice were sacrificed at an average age of 391.79 days (range = 298-435 days). **FIGURE 13** provides a chart of the virgin tumor study female mice and their age at sacrifice. In the absence of mammary masses and/or other grossly observable morbidities, parous tumor study female mice were sacrificed at an average age of 375.25 days (range = 194-435 days). **FIGURE 14** provides a chart of the parous tumor study female mice, parity number, and their age at sacrifice.

Among all females maintained for this virgin tumor study, only two mice developed grossly observable pathologies requiring sacrifice prior to study termination. Mouse 18i, a wt/*bcl-x$_L$/myc* virgin female, was found to have 5 ventrally located masses coincident with her mammary glands (axillary and inguinal glands were affected) at an age of 361 days. This animal also evidenced splenomegaly upon autopsy and dissection (warranting fixation of both spleen and liver for future histopathological examination). Mammary gland tissue and masses were harvested at the time of sacrifice, divided, and were either fixed in 10% neutral-buffered formalin and embedded in paraffin, snap-frozen in liquid nitrogen, or whole-mounted (all procedures will be described in Aim #2C). Mouse 57j, a *tta*/wt/*myc* virgin female, was found to have a perivaginal mass at an age of 345 days. The mammary glands of this mouse, at the time of sacrifice, evidenced a high adipose content without obvious mass lesions. The perivaginal mass was divided for fixation in 10% neutral-buffered formalin and snap-freezing in liquid nitrogen, the mammary glands were divided for fixation, snap-freezing, and whole mounting, and the spleen and liver were also collected and fixed.

Among all females maintained for this parous tumor study, only two mice developed grossly observable pathologies requiring sacrifice prior to study termination. Mouse 8w, a *tta/bcl-x$_L$/myc* multiparous female (five pregnancies), was found to have 3 masses (left shoulder region, left 3rd gland region, right axillary region) at an age of 165 days. Mammary tissues and mass lesions were divided for fixation and snap-freezing (as well as whole-mounting). Mouse 63u, a *tta*/wt/*myc* multiparous female (six pregnancies), was found to have 2 masses (right 2nd gland region, left 1st gland region) at an age of 183 days. Mammary tissues and mass lesions were divided for fixation and snap-freezing (as well as whole-mounting).

Histopathological examination of mammary tissues obtained from both virgin and parous tumor study female mice is currently underway and should provide additional information concerning the influence of c-*myc* and *bcl-x$_L$* in mammary development and tumorigenesis. Assessment of

tumor latency, incidence, multiplicity, and parity dependence will follow quickly from ascertainment of the mass lesions taken from the 4 animals described above; whereas, information concerning metastasis will require additional histopathological evaluations (including examination of liver, spleen, and lung tissues).

Summary of Training and Research Accomplishments for Revised Specific Aim #2B:
Following recruitment of F_2 generation study animals (from *bax*-knockout/c-*myc* crosses) to the virgin and parous tumor study subgroups, female mice were examined three times a week for the development of mammary masses and/or other grossly observable morbidities. At the time of this report, all virgin study females in all genotypic subgroups have been sacrificed. At the time of this report, all parous study females in all genotypic subgroups have been sacrificed. In the absence of mammary masses and/or other grossly observable morbidities, virgin tumor study female mice were sacrificed at an average age of 406.81 days (range = 317-436 days). **FIGURE 15** provides a chare of the virgin tumor study female mice and their age at sacrifice. In the absence of mammary masses and/or other grossly observable morbidities, parous tumor study female mice were sacrificed at an average age of 378.76 days (range = 164-483 days). **FIGURE 16** provides a chart of the parous tumor study female mice, parity number, and their age at sacrifice.

Among all females maintained for this virgin tumor study, only three mice developed grossly observable pathologies requiring sacrifice prior to study termination. Mouse 29j, a c-*myc bax-/-* virgin female, was found to have 6 ventrally located masses coincident with her mammary glands (left and right nuchal regions, left and right 2nd gland regions, left and right 4th/5th gland regions) at an age of 341 days. This animal also evidenced hepatosplenomegaly at the time of autopsy and dissection (warranting fixation of both spleen and liver for further histopathological examination). Mammary gland tissues and mass lesions were harvested at the time of sacrifice, divided, and either fixed in 10% neutral-buffered formalin and embedded in paraffin, snap-frozen in liquid nitrogen, or whole mounted (all procedures will be described in Aim #2C). Mouse 19j, a c-*myc bax+/-* virgin female, was found to have 3 masses (right cranial shoulder region, midline thoracic region, left 2nd gland region) at an age of 383 days. Mammary tissues and mass lesions were divided for fixation and snap-freezing (as well as whole-mounting). Mouse 73n, a c-*myc bax+/+* female, was found to have one mass (right cranial shoulder region) at an age of 297 days. Mammary tissues and this mass lesion were harvested and divided for fixation and snap-freezing (note that liver, spleen, and lung tissues were also harvested and formalin-fixed for all of these study animals).

To date, the most striking grossly observable pathologies have been in evidence in the parous tumor study group for the c-*myc* / *bax*-knockout cross. Among all females maintained for this parous tumor study group, eight mice developed mass lesions requiring sacrifice prior to study termination. Mouse 71p, a c-*myc bax-/-* multiparous female (four pregnancies), was found to have one mass (right 3rd gland region) at an age of 236 days. Mouse 72p, a c-*myc bax-/-* multiparous female (9 pregnancies), was found to have one mass (right 3rd gland region) at an age of 240 days. Mouse 76n, a c-*myc bax+/-* multiparous female (five pregnancies), was found to have three masses (left 1st/2nd gland region, right 3rd gland region, right 4th/5th gland region) at an age of 233 days. Mouse 73p, a c-*myc bax+/-* multiparous female (6 pregnancies), was found to have two masses (left 2nd gland region, left 3rd gland and shoulder regions) at an age of 193

days. Mouse 79p, a c-*myc bax*+/- multiparous female (7 pregnancies), was found to have four masses (left 3rd gland region, left 4th gland region, right 3rd gland region, right 4th/5th gland region) at an age of 206 days. Mouse 65q, a c-*myc bax*+/- multiparous female (7 pregnancies), was found to have 2 masses (left 3rd gland region, left 4th/5th gland region) at an age of 252 days. Mouse 67q, a c-*myc bax*+/+ multiparous female (7 pregnancies), was found to have one mass (right 2nd gland region) at an age of 220 days. Mouse 4s, a c-*myc bax*+/+ multiparous female (5 pregnancies), was found to have one mass (left 4th/5th gland region) at an age of 183 days. Mammary tissues and mass lesions were divided for fixation and snap-freezing (as well as whole-mounting). A summary of the mass lesion latency, incidence, and multiplicity for this parous tumor study is presented in a **FIGURE 17**.

Histopathological examination of mammary tissues obtained from bot virgin and parous tumor study female mice is currently underway and should provide additional information concerning the influence of *bax*-knockout and c-*myc* expression in mammary development and tumorigenesis. Assessment of tumor latency, incidence, multiplicity, and parity dependence will follow quickly from ascertainment of the mass lesions taken from the 11 animals described above; whereas, information concerning metastasis will require additional histopathological evaluations (including examination of liver, spleen, and lung tissues).

Summary of Training and Research Accomplishments for Revised Specific Aim #2C:
Pathohistological, immunohistochemical, and *in situ* histological studies are being conducted on 10% neutral-buffered formalin-fixed, paraffin-embedded mammary and mass lesion tissues (as well as liver, spleen, and lung tissues as required). To date, all female mice from both the virgin and parous tumor studies (with the exception of three parous study animals) have been sacrificed with their mammary glands, liver, lungs, spleen, and any mass lesions harvested at the time of sacrifice. The liver, lungs, and spleen have all been fixed in 10% neutral-buffered formalin. All mass lesions have been divided at the time of sacrifice/harvest with one portion being fixed in 10% neutral-buffered formalin, while the remainder has been snap-frozen in liquid nitrogen for future protein and RNA studies. Finally, the mammary glands of all study animals have been divided at the time of sacrifice/harvest with the tissue being split between formalin fixation, snap-freezing, and mammary gland whole-mounting. Mice were sacrificed by CO_2 asphyxiation using a desiccator into which the flow of gas can be strictly controlled by a gas-flow regulator. Tissues and mass lesions were rapidly dissected from the mouse and are placed into 10% neutral-buffered formalin, liquid nitrogen, or mammary whole mount fixative (Carnoy's Fixative). What follows is a brief description of the procedures used in processing all study-related materials.

For mammary whole mounts, briefly: the mammary tissue is stretched out onto a Superfrost Plus slide (Fisher Scientific, Pittsburgh, PA), allowed to air dry, then fixed overnight in Carnoy's fixative made up as 1 part glacial acetic acid (EM Science, Gibbstown, NJ) to 3 parts ethanol (Warner-Graham Company, Cockeysville, MD). After fixation, the whole mount is rinsed in distilled water, dehydrated in a series of ethanols (70%, 95%, 100%), and then cleared in toluene from 1 to 3 days (depending on the adipose content of the glands). Finally, the whole mounts are mounted with Permount (Fisher), cover-slipped (Corning Glass, Corning, NY), and allowed to dry.

For all tissues to be formalin-fixed, briefly: tissues, masses, and organs are fixed overnight @ 4°C in a solution of 10% neutral-buffered formalin made up from formalin (EM Science) and 1X phosphate-buffered saline (Gibco BRL, Rockville, MD). Fixed tissues are then dehydrated through a series of ethanols (70%, 95%, 100%), cleared in xylene, and paraffinized using a Shandon HyperCenter XP tissue processor (Shandon, Pittsburgh, PA). Tissues are then embedded in paraffin and stored @ 4°C prior to microtome sectioning. Prior to use for histology, immunohistochemistry, or tissue *in situ* histochemistry, paraffin-embedded tissues are section at 5 microns, floated onto Superfrost Plus slides, and dried in an air oven overnight @ 40°C.

For hematoxylin/eosin staining of all tissues sections, briefly: slides (with tissue) are deparaffinized through successive washes in xylene, rehydrated through an ethanol series (100%, 95%, 70%), and rinsed in distilled water. Slides are then stained in Harris' hematoxylin (Fisher), rinsed in running tap water and distilled water, dehydrated in 70% ethanol, and rapidly counterstained with eosin solution. Specimens are then dehydrated through an ethanol series (70%, 95%, 100%), cleared in xylene, cover-slipped and mounted using Permount (Fisher).

For evaluation of transgene expression and proliferation indices by immunohistochemistry, briefly: slides (with tissue) are deparaffinized and rehydrated through successive washes in xylene and an ethanol series (100%, 95%). Slides are then exposed to trypsinization or microwave antigen retrieval (depending upon conditions optimized for each antibody used) and are serum blocked. Slides are then exposed to the primary antibody solution, secondary antibody solution, Avidin-Biotin Complex (ABC) Elite reagent (Vector Labs, Burlingame, CA), 3,3'-diaminobenzidine (DAB) staining substrate (Sigma, Saint Louis, MO), Gill's hematoxylin (Fisher), and saturated lithium carbonate (blueing agent). Slides are then dehydrated in an ethanol series (95%, 100%), cleared in xylene, cover-slipped and mounted using Permount (Fisher).

c-*myc* and *bcl-x_L* transgene expression will be assessed by immunohistochemistry using the following primary antibodies: polyclonal rabbit anti-human/mouse c-Myc (06-340; Upstate Biotechnology, Lake Placid, NY) and polyclonal rabbit anti-human/mouse Bcl-x_L (H-62; Santa Cruz Biotechnology, Santa Cruz, CA). The Vectastain ABC Rabbit Elite kit will be used for secondary antibody detection via immunohistochemistry of the aforementioned targets. The *in situ* proliferation index is generated using immunohistochemistry for proliferating cell nuclear antigen (PCNA) as was described in the transgenic studies conducted in laboratory of Jeffrey Green (Shibata *et.al.*, 1999). The primary antibody used for detection of PCNA is monoclonal mouse anti-human/mouse PCNA (PC-10; Dako, Carpinteria, CA). Since this antibody is a mouse monoclonal that will be used on mouse tissues, the secondary antibody detection system employed will be the Animal Research Kit (Dako) which reduces non-specific secondary antibody binding to tissue endogenous antibodies. The *in situ* apoptotic index is generated using the ApopTag kit (Oncor, Gaithersburg, MD) as was utilized in transgenic studies in the laboratories of Priscilla Furth and Jeffrey Green (Li *et.al.*, 1996a; Shibata *et.al.*, 1999).

Summary of Training and Research Accomplishments for Revised Specific Aim #2D:
Pathohistological, immunohistochemical, and *in situ* histological studies are being conducted on 10% neutral-buffered formalin-fixed, paraffin-embedded mammary and mass lesion tissues (as

well as liver, spleen, and lung tissues as required). To date, all female mice from both the virgin and parous tumor studies have been sacrificed with their mammary glands, liver, lungs, spleens, and any mass lesions harvested at the time of sacrifice. The liver, lungs, and spleen have all been fixed in 10% neutral-buffered formalin. All mass lesions have been divided at the time of sacrifice/harvest with one portion being fixed in 10% neutral-buffered formalin, while the remainder has been snap-frozen in liquid nitrogen for future protein and RNA studies. Finally, the mammary glands of all study animals have been divided at the time of sacrifice/harvest with the tissue being split between formalin fixation, snap-freezing, and mammary gland whole-mounting. Mice were sacrificed by CO_2 asphyxiation using a desiccator into which the flow of gas can be strictly controlled by a gas-flow regulator. Tissues and mass lesions were rapidly dissected from the mouse and are placed into 10% neutral-buffered formalin, liquid nitrogen, or mammary whole mount fixative (Carnoy's Fixative). The procedures used in processing of all study-related materials are as described in Specific Aim #2C with the exception of the immunohistochemical assessment of the expression (or lack thereof) for *bax* which is assessed using the following primary antibodies: polyclonal rabbit anti-human/mouse Bax (N-20; Santa Cruz) and polyclonal rabbit anti-human/mouse Bax (I-19; Santa Cruz).

Specific Aim #3: To determine whether cell lines derived from study animal mammary tissues exhibit different levels of growth factor / growth inhibitor independence and resistance to apoptosis.

A. Specific Aim #3A: To determine whether cell lines derived from the single and double transgenic mice exhibit different levels of growth factor/ growth inhibitor independence and resistance to apoptosis.
B. Specific Aim #3B: To determine whether cell lines derived from c-*myc* transgene expressing mammary tissues transduced with control and Bcl-x_L-expressing retroviral vectors exhibit different levels of growth factor / growth inhibitor independence and resistance to apoptosis.

Revised Specific Aim #3: Evaluate the growth factor dependence, growth inhibitor sensitivity, and apoptotic resistance of cell lines derived from transgenic murine mammary tumors and mammary tissues.

A. Revised Specific Aim #3A: Evaluate the growth factor dependence, growth inhibitor sensitivity, and apoptotic resistance of cell lines derived from c-*myc*/*tta*/*bcl-*x_L transgenic murine mammary tumors and mammary tissues.
B. Revised Specific Aim #3B: Evaluate the growth factor dependence, growth inhibitor sensitivity, and apoptotic resistance of cell lines derived from *bax*-knockout/c-*myc* transgenic murine mammary tumors and mammary tissues.

Summary of Training and Research Accomplishments for Revised Specific Aim #3A:
Cell lines will be developed from tumors and/on non-tumorous mammary tissues from the parous tumor study animals by means employed in Amundadottir *et.al.* (1996). Briefly, animals are sacrificed using CO_2 asphyxiation and tumors are harvested, divided, and digested in DMEM media (Biofluids, Rockville, MD) enriched with fetal calf serum (FCS; Biofluids), EGF (Upstate

Biotechnology), insulin (Biofluids), and 0.01 mg% collagenase IA (Sigma). Cultures are enriched for epithelial content over a period of one to two months by differential trypsinization. Resultant cell lines will be evaluated for *in vitro* proliferative and apoptotic responses to culture in the presence and absence of previously-identified, mammary-relevant growth factors (TGFα, EGF, basic fibroblast growth factor{bFGF}, insulin-like growth factor 1{IGF1}) and growth inhibitors (TGFβ). Anchorage-dependent and independent growth assays as well as apoptosis detection assays will be conducted as previously described (Amundadottir *et.al.*, 1996).

Summary of Training and Research Accomplishments for Revised Specific Aim #3B:
Evaluation of growth factor dependence, growth inhibitor sensitivity, and apoptosis resistance of cell lines derived from *bax*-knockout/c-*myc* transgenic mammary tumors and tissues will be conducted as described in Specific Aim #3A.

Specific Aim #4: To resolve the *in vivo* tumorigenic potential of the aforementioned cell lines via reimplantation in athymic mice.

Revised Specific Aim #4: Evaluate the tumorigenicity of cell lines derived from transgenic murine mammary tumor and mammary tissues in athymic mice.

A. Revised Specific Aim #4A: Evaluate the tumorigenicity of cell lines derived from c-*myc*/*tta*/*bcl-x*$_L$ transgenic murine mammary tumor and mammary tissues in athymic mice.
B. Revised Specific Aim #4B: Evaluate the tumorigenicity of cell lines derived from *bax*-knockout/c-*myc* transgenic murine mammary tumor and mammary tissues in athymic mice.

Summary of Training and Research Accomplishments for Revised Specific Aim #4A:
The tumorigenicity of the cell lines developed in Specific Aim #3A will be assessed by subcutaneous injection of each cell line into female, athymic mice with approximately 10^6 cells injected at each of four sites on the recipient animals (Amundadottir *et.al.*, 1996). All surgical procedures will be performed using sterile equipment, techniques, and cell lines, on recipient animals maintained under anesthesia as per approved Animal Care and Use Guidelines. All injection sites will be monitored three times a week with growth of mass lesions assessed by caliper measurements.

Summary of Training and Research Accomplishments for Revised Specific Aim #4B:
The tumorigenicity of the cell lines developed in Specific Aim #3B will be assessed as described in Specific Aim #4A.

KEY RESEARCH ACCOMPLISHMENTS

For Final Report – Grant # DAMD17-97-1-7110

"Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis"

P.I. Matthew Hunter Jamerson

- ✳ Development of effective breeding strategies for the generation of the two transgenic models utilized in this work (*tta*/*bcl-x*$_L$/c-*myc* and *bax*-knockout/c-*myc*)
- ✳ Development and optimization of PCR-based assays for c-*myc*, *bcl-x*$_L$, *tta* transgenic and *bax*-knockout mouse genotyping
- ✳ Confirmation of Bcl-x_L expression in mammary gland whole cell lysates in tetOP-*tta*/tetOP-*bcl-x*$_L$ bitransgenic mice
- ✳ Optimization of mammary gland whole-mounting procedure for assessment of transgene-induced alterations in mammary gland development
- ✳ Recruitment of all virgin and parous tumor study animals, both major and minor genotypic subgroups, for both transgenic murine mammary tumor models
- ✳ Sacrifice and tissue/organ harvest from all virgin tumor study animals for both transgenic murine mammary tumor models
- ✳ Sacrifice and tissue/organ harvest from nearly all parous tumor study animals for both transgenic murine mammary tumor models
- ✣ Recruitment of approximately 50% of all developmental/involution study animals for both transgenic murine mammary tumor models
- ✣ Current optimization of immunohistochemical procedures for resolution of transgene expression, proliferative index assessment, and *in situ* apoptosis detection
- ✣ Current optimization of procedures for cell line development and tumor and cell line transplantation studies in athymic mice

REPORTABLE OUTCOMES

For Final Report – Grant # DAMD17-97-1-7110

"Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis"

P.I. Matthew Hunter Jamerson

Manuscripts:

1. **Jamerson MH,** Johnson MD and Dickson RB. (2000). Dual Regulation of Proliferation and Apoptosis: c-*myc* in Bitransgenic Murine Mammary Tumor Models. *Oncogene* **19**: 1065-1071.

2. Liao DJ, Natarajan G, Deming SL, **Jamerson MH**, Johnson MD, Chepko G and Dickson RB. (2000). Cell Cycle Basis for the Onset and Progression of c-Myc-Induced, TGFα-Enhanced Mouse Mammary Gland Carcinogenesis. *Oncogene* **19**: 1307-1317.

Abstracts and Poster Presentations:

1. **Jamerson MH**, Johnson MD and Dickson RB. Cooperation of c-Myc, Bcl-x_L, and Bax-Knockout in Mammary Tumorigenesis. Lombardi Cancer Center Research Days, Lombardi Cancer Center, Washington, DC. February 1999.

2. **Jamerson MH**, Johnson MD and Dickson RB. Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Atlanta, Georgia. June 8-11, 2000. Abstract #455.

3. **Jamerson MH**, Johnson MD, Furth PA, Korsmeyer SJ, Nuñez G, and Dickson RB. Gain of Bcl-x_L and Loss of Bax Cooperate in c-Myc-Mediated Mammary Tumorigenesis. Keystone Symposium on Molecular Mechanisms of Apoptosis, Keystone, Colorado. January 16-22, 2001. Abstract #239.

Degrees Obtained:

1. Work supported by this Grant will contribute to the completion of the requirements for a Ph.D. in Tumor Biology for the Principal Investigator, Matthew Hunter Jamerson, as part of the course of study for the combined M.D./Ph.D. program

Informatics – Animal Models:

1. Generated triple transgenic murine model: tetOP-*tta* / tetOP-*bcl-x_L* / MMTV-c-*myc*

2. Generated transgenic/knockout murine model: *bax*-Knockout / MMTV-c-*myc*

Informatics – Cell Lines:

1. Mammary tumor and/or normal tissue cells lines are being developed from tetOP-*tta* / tetOP-*bcl-x*$_L$ / MMTV-c-*myc* Parous Study Animals

2. Mammary tumor and/or normal tissue cells lines are being developed *bax*-Knockout / MMTV-c-*myc* Parous Study Animals

CONCLUSIONS

For Final Report – Grant # DAMD17-97-1-7110
"Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis"
P.I. Matthew Hunter Jamerson

This Final Report addresses Grant # DAMD17-97-1-7110 entitled "Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis, a Pre-Doctoral Training Fellowship, covering research conducted by the principal investigator Matthew Hunter Jamerson (an M.D./Ph.D. student at the Lombardi Cancer Center, Georgetown University Medical Center) during the period from 1 August 1997 through 31 July 2000. Work, on the studies proposed in this Grant, is still ongoing and it is anticipated that all proposed Specific Aims will be addressed during the course of the Principal Investigator's Ph.D. thesis research. All future publications, lectures, and poster abstracts concerning any work resulting from the studies addressed by this Grant will acknowledge the support and funding of the Department of Defense Breast Cancer Research Program and the United States Army Medical Research and Materiel Command.

The principal investigator, Matthew Hunter Jamerson, was involved in the completion of graduate coursework during the period stretching from August 1997 through May 1998 while commencing the research described in the Specific Aims of this Grant. It should be further noted that the delayed receipt of the transgenic animals required for the proposed experimentation slowed work on the Specific Aims outlined in the Grant. The initial transgenic animals, for establishment of breeding colonies and subsequent crossings, were not received until the following dates: MMTV-c-*myc* in September 1997, *bax*-knockout in March 1998, tetOP-*bcl-x_L* in April 1998, and tetOP-*tta* in July 1998. As a result of these delays, as well as setbacks involving animal fecundity/sterility and breeding scheme complexity, the majority of the work on the Specific Aims covered in this Grant were commenced during the second year of the award (July-August 1998). The Department of Defense Breast Cancer Research Program and United States Army Medical Research and Materiel Command should be assured that the proposed studies are ongoing/maturing and that proper acknowledgement shall be given for their support.

It should also be quickly summarized that the principal investigator on this Pre-Doctoral Training Fellowship, Matthew Hunter Jamerson, is currently training as an M.D./Ph.D. student at Georgetown University and that in addition to the study results gained from the conduct of this research, the Grant also has further supported the training of the principal investigator as a future Physician-Scientist in biomedical research, cancer research, and specifically Breast Cancer research. The principal investigator covered by this Grant is currently working on research with the intention of achieving a Ph.D. in Tumor Biology; furthermore, the principal investigator has completed two years of M.D. training and will conclude the final two years of M.D. training, at the Lombardi Cancer Center and Georgetown University Medical Center, upon defense of his Ph.D. thesis. It should be noted that this Pre-Doctoral Traineeship has not only funded the research conducted but also has contributed to the foundation of the principal investigator's training as a future oncologist. Following the completion of the M.D./Ph.D. training program, the principal investigator intends to conduct a Residency in Internal Medicine specializing in Hematology/Oncology and a Fellowship in Oncology. Finally, the principal investigator intends to pursue a career in Academic Medicine working on cancer research from both the basic science and clinical research perspectives.

Work on the Specific Aims outlined in the Grant Proposal and subsequent Grant Annual Summaries and Final Report has proceeded to the point where all necessary animals have been recruited, sacrificed, and processed for the long-term virgin and parous tumor studies for the *tta/bcl-x$_L$*/c-*myc* and *bax*-knockout/c-*myc* crosses. Recruitment and tissue harvesting is greater than 60% completed for the short-term, developmental/involution study for the two transgenic experiments and should be completely recruited and processed by June 2001. Results to date, absent any microscopic histological and pathohistological examinations (which are currently commencing for both virgin and parous tumor studies), suggest a possible cooperative role in tumorigenesis between c-*myc* and *bax*-knockout in one of the transgenic models (as was identified for SV40 large T antigen and *bax*-knockout by the laboratory of Jeffrey Green) but have failed to identify a strong cooperative role between c-*myc* and *bcl-x$_L$* in our other transgenic model. As far as is the case for the *bax*-knockout/c-*myc* parous tumor study, it appears that *bax* haploinsufficiency may contribute to both tumor incidence and tumor multiplicty; however, additional microscopic and molecular evaluations are warranted and currently underway.

Gross pathological examinations of virgin and parous tumor study animals for the *bcl-x$_L$*/c-*myc* crossing has failed to yet identify any significant cooperative role in mammary tumorigenesis. The absence of c-*myc*-mediated mammary tumorigenesis in these F_2 generation virgin and parous tumor study animals suggest, perhaps, that the mixed genetic background of the study animals may be suppressing mammary tumorigenesis by altering the influence of c-*myc* on distinct transforming pathways (including cell proliferation, apoptosis, and genetic instability). The potential confounding nature of mouse hybrid backgrounds on mammary tumorigenesis is further supported by the weight of evidence from the *bax*-knockout/c-*myc* F_2 study animals (a hybrid background that is distinct from that of the *bcl-x$_L$*/c-*myc* cross but certainly not an inbred strain, nonetheless) where c-*myc*-mediated mammary tumorigenesis was less penetrant than the 100% expected from the c-*myc* transgenic studies conducted on the FVB and C57BL/6J inbred backgrounds alone. One potential change that could be pursued in the future examination of the cooperation between c-*myc* and these two apoptosis-regulatory genes would find tumorigenesis studies conducted on an inbred background strain to eliminate the confounding and difficult to identify genetic and epigenetic variables that arise from studies conducted on hybrid murine strains. Of course, the contrary situation could also be appreciated in the fact that humans are not inbred animals and that data gleaned from the use of hybrid mice might more closely model tumorigenesis that occurs in people.

It will be important (as has been proposed in this Grant) to identify the molecular character of transgene expression (or lack thereof for *bax*) in the F_2 generation study animal mammary glands. A tumorigenesis result is grossly manifest for the *bax*-knockout/c-*myc* study where c-*myc* is certainly expressed in the mammary epithelium (due to the influence of the MMTV-LTR promotional elements) absent one or both copies of *bax* (since the knockout of *bax* is present within every cell of these mice). The fact that a tumorigenesis result is not grossly evident in the *bcl-x$_L$*/c-*myc* study may reflect a more basic issue of transgene expression patterning. Owing to the fact that the MMTV-LTR-*tta* mouse was unavailable for conduct of these studies, tetOP-*tta* animals were obtained and bred with tetOP-*bcl-x$_L$* mice to yield a means for eliciting the expression of bcl-x_L in the animals. Preliminary protein expression studies conducted on whole mammary gland lysates suggest that Bcl-x_L protein is abundantly expressed in the mammary

glands of *tta/bcl-x_L* bitransgenic mice; however, it is possible that the expression of Bcl-x_L is limited to a mammary gland compartment (e.g. adipocyte, myoepithelium, stromal) in which the gene is incapable of interacting with the *myc* transgene to influence either tumorigenesis of mammary development. The ongoing histological and immunohistochemical studies on the mammary gland tissues obtained from the *tta/bcl-x_L*/c-*myc* study animals should provide a more clear answer as to whether these two transgenes were expressed in a fashion that could allow for a phenotypic manifestation of their cooperation (or potential lack thereof) in mammary tumorigenesis. Any recapitulation of this particular tumorigenesis study would benefit from the use of the MMTV-*tta* transgenic mouse to drive the expression of the tetOP-*bcl-x_L* in the same cells as the c-*myc* transgene. All ongoing, aforementioned studies contribute to our molecular understanding of the development of breast cancer and therefore contribute to the Department of Defense Breast Cancer Research Programmatic goal of funding basic science research and future clinical translations.

REFERENCES

Amundadottir LT, Johnson MD, Merlino G, Smith GH and Dickson RB. (1995). Synergistic Interaction of Transforming Growth Factor α and c-*myc* in Mouse Mammary and Salivary Gland Tumorigenesis. *Cell Growth Diff.* **6**: 737-748.

Amundadottir LT, Nass SJ, Berchem GJ, Johnson MD and Dickson RB. (1996). Cooperation of TGFα and c-Myc in Mouse Mammary Tumorigenesis: Coordinated Stimulation of Growth and Suppression of Apoptosis. *Oncogene* **13**: 757-765.

Amundadottir LT and Leder P. (1998). Signal Transduction Pathways Activated and Required for Mammary Carcinogenesis in Response to Specific Oncogenes. *Oncogene* **16**: 737-746.

Arteaga CL, Hanauske AR, Clark Gm, Osborne K, Hazarika P, Pardue RL, Tio F and Von Hoff DD. (1988). Immunoreactive Alpha Transforming Growth Factor Activity in Effusions from Cancer Patients as a Marker of Tumor Burden and Patient Prognosis. *Cancer Res.* **48**: 5023-5028.

Bargou RC, Daniel PT, Mapara MY, Bommert K, Wagener C, Kallinich B, Royer HD and Dörken B. (1995). Expression of the *bcl-2* Gene Family in Normal and Malignant Breast Tissue: Low *bax-α* Expression in Tumor Cells Correlates with Resistance Towards Apoptosis. *Int. J. Cancer* **60**: 854-859.

Bargou RC, Wagener C, Bommert K, Mapara MY, Daniel PT, Arnold W, Dietel M, Guski H, Feller A, Royer HD and Dörken B. (1996). Overexpression of the Death-Promoting Gene bax-α Which is Downregulated in Breast Cancer Restores Sensitivity to Different Apoptotic Stimuli and Reduced Tumor Growth in SCID Mice. *J. Clin. Invest.* **97**: 2651-2659.

Bates SE, Davidson NE, Valverius EM, Freter CE, Dickson RB, Tam JP, Kudlow JE, Lippman ME and Salomon DS. (1988). Expression of Transforming Growth Factor Alpha and Its Messenger Ribonucleic Acid in Human Breast Cancer: Its Regulation by Estrogen and Its Possible Functional Significance. *Mol. Endocrinol.* **2**: 543-555.

Bates SE, Valverius EM, Ennis BW, Bronzert DA, Sheridan JP, Stampfer MR, Mendelsohn J, Lippman ME and Dickson RB. (1990). Expression of the Transforming Growth Factor-α/Epidermal Growth Factor Receptor Pathway in Normal Human Breast Epithelial Cells. *Endocrinology.* **126**: 596-607.

D'Cruz CM, Gunther EJ, Boxer RB, Hartman JL, Sintasath L, Moody SE, Cox JD, Ha SI, Belka GK, Golant A, Cardiff RD and Chodosh LA. (2001). c-Myc Induces Mammary Tumorigenesis by Means of a Preferred Pathway Involving Spontaneous *Kras2* Mutations. *Nat. Med.* **7**: 235-239.

Dahiya R and Deng G. (1998). Molecular Prognostic Markers in Breast Cancer. *Breast Cancer*

Res. Treat. **52**: 185-200.

Dang CV. (1999). c-Myc Target Genes Involved in Cell Growth, Apoptosis and Metabolism. *Mol. Cell. Biol.* **19**: 1-11.

Deming SL, Nass SJ, Dickson RB and Trock BJ. (2000). c-Myc Amplification in Breast Cancer: A Meta-Analysis of Its Occurrence and Prognostic Relevance. *Br. J. Cancer* **83**: 1688-1695.

Deng CX and Scott F. (2000). Role of the Tumor Suppressor Gene BRCA1 in Genetic Stability and Mammary Gland Tumor Formation. *Oncogene* **19**: 1059-1064.

De Luca A, Casamassimi A, Selvam MP, Losito S, Ciardiello F, Agrawal S, Salomon DS and Normanno N. (1999). EGF-Related Peptides are Involved in the Proliferation and Survival of MDA-MB-468 Human Breast Carcinoma Cells. *Int. J. Cancer* **80**: 589-594.

Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS and Berger WH. (1987). Synthesis of Messenger RNAs for Transforming Growth Factors α and β and the Epidermal Growth Factor Receptor by Human Tumors. *Cancer Res.* **47**: 707-712.

Dickson RB and Lippman ME. (1995). Growth Factors in Breast Cancer. *Endocrine Rev.* **16**: 559-589.

Doyle GA, Bourdeau-Heller JM, Coulthard S, Meisner LF and Ross J. (2000). Amplification in Human Breast Cancer of a Gene Encoding a c-*myc* mRNA-Binding Protein. *Cancer Res.* **60**: 2756-2759.

Edwards PAW, Abram CL and Bradbury JM. (1996). Genetic Manipulation of Mammary Epithelium by Transplantation. *J. Mammary Gland Biol. Neoplasia* **1**: 75-89.

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC. (1992). Induction of Apoptosis in Fibroblasts by c-myc Protein. *Cell* **69**: 119-128.

Facchini LM and Penn LZ. (1998). The Molecular Role of Myc in Growth and Transformation: Recent Discoveries Lead to New Insights. *FASEB J.* **12**: 633-651.

Felsher DW and Bishop JM. (1999a). Transient Excess of *MYC* Activity Can Elicit Genomic Instability and Tumorigenesis. *Proc. Natl. Acad. Sci. USA* **96**: 3940-3944.

Felsher DW and Bishop JM. (1999b). Reversible Tumorigenesis by *Myc* in Hematopoietic Lineages. *Mol. Cell* **4**: 199-207.

Feuerhake F, Sigg W, Höfter EA, Dimpfl T and Welsch U. (2000). Immunohistochemical

Analysis of Bcl-2 and Bax Expression in Relation to Cell Turnover and Epithelial Differentiation Markers in the Non-Lactating Human Mammary Gland Epithelium. *Cell Tissue Res.* **299**: 47-58.

Harrington EA, Bennett MR, Fanidi A and Evan GI. (1994). c-Myc-Induced Apoptosis in Fibroblasts is Inhibited by Specific Cytokines. *EMBO J.* **13**: 3286-3295.

Harris AL and Nicholson S. (1988). In: *Breast Cancer: Cellular and Molecular Biology*, Lippman ME and Dickson RB (eds). Kluwer Press: Boston, MA. Vol **1**: 93-118.

Hsu CKA, Rishi AK, Li XS, Dawson MI, Reichert U, Shroot B and Fontana JA. (1997). Bcl-x_L Expression and Its Downregulation by a Novel Retinoid in Breast Carcinoma Cells. *Exp. Cell Res.* **232**: 17-24.

Hunter T. (1991). Cooperation Between Oncogenes. *Cell* **64**: 249-270.

Jäättelä M, Benedict M, Tewari M, Shayman JA and Dixit VM. (1995). Bcl-x and Bcl-2 Inhibit TNF and Fas-Induced Apoptosis and Activation of Phospholipase A_2 in Breast Carcinoma Cells. *Oncogene* **10**: 2297-2305.

Jäger R, Herzer U, Schenkel J and Weiher H. (1997). Overexpression of Bcl-2 Inhibits Alveolar Cell Apoptosis During Involution and Accelerates c-*myc*-Induced Tumorigenesis of the Mammary Gland in Transgenic Mice. *Oncogene* **15**: 1787-1795.

Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH and Merlino GT. (1990). TGFα Overexpression in Transgenic Mice Induces Liver Neoplasia and Abnormal Development of the Mammary Gland and Pancreas. *Cell* **61**: 1137-1146.

Juin P, Hueber AO, Littlewood T and Evan GI. (1999). c-Myc-Induced Sensitization to Apoptosis is Mediated Through Cytochrome *c* Release. *Genes Dev.* **13**: 1367-1381.

Kapranos N, Karaiosifidi H, Valavanis C, Kouri E and Vasilaros S. (1997). Prognostic Significance of Apoptosis Related Proteins Bcl-2 and Bax in Node-Negative Breast Cancer Patients. *Anticancer Res.* **17**: 2499-2506.

King CR, Kraus MH and Aaronson SA. (1985). Amplification of a Novel v-erbB-Related Gene in a Human Mammary Carcinoma. ***Science*** **229**: 974-976.

Knudson CM, Tung KSK, Tourtellotte WG, Brown GAJ, and Korsmeyer SJ. (1995). Bax-Deficient Mice with Lymphoid Hyperplasia and Male Germ Cell Death. *Science* **270**: 96-99.

Krajewski S, Krajewska M, Shabaik A, Wang HG, Irie S, Fong L and Reed JC. (1994a). Immunohistochemical Analysis of *in Vivo* Patterns of Bcl-X Expression. *Cancer Res.* **54**: 5501-5507.

Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG and Reed JC. (1994b). Immunohistochemical Determination of *In Vivo* Distribution of Bax, a Dominant Inhibitor of Bcl-2. *Am. J. Pathol.* **145**: 1323-1336.

Krajewski S, Blomqvist C, Franssila K, Krajewska M, Wasenius VM, Niskanen E, Nordling S and Reed JC. (1995). Reduced Expression of Proapoptotic Gene *BAX* is Associated with Poor Response Rates to Combination Chemotherapy and Shorter Survival in Women with Metastatic Breast Adenocarcinoma. *Cancer Res.* **55**: 4471-4478.

Land H, Parada LF and Weinberg RA. (1983). Tumorigenic Conversion of Primary Embryo Fibroblasts Requires at Least Two Cooperating Oncogenes. *Nature* **304**: 596-602.

Leder A, Pattengale PK, Kuo A, Stewart TA and Leder P. (1986). Consequences of Widespread Deregulation of the c-*myc* Gene in Transgenic Mice: Multiple Neoplasms and Normal Development. *Cell* **45**: 485-495.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH and Parsons R. (1997). *PTEN*, A Putative Protein Tyrosine Phosphatase Gene Mutated in Human Brain, Breast, and Prostate Cancer. *Science* **275**: 1943-1947.

Li M, Hu J, Heermeier K, Hennighausen L and Furth PA. (1996a). Expression of a Viral Oncoprotein During Mammary Gland Development Alters Cell Fate and Function: Induction of p53-Independent Apoptosis is Followed by Impaired Milk Protein Production in Surviving Cells. *Cell Growth Diff.* **7**: 3-11.

Li M, Hu J, Heermeier K, Hennighausen L and Furth PA. (1996b). Apoptosis and Remodeling of Mammary Gland Tissue During Involution Proceeds through p53-Independent Pathways. *Cell Growth Diff.* **7**: 13-20.

Liao DJ and Dickson RB. (2000). c-*Myc* in Breast Cancer. *Endocrine-Rel. Cancer* **7**: 143-164.

Liscia DS, Merlo G, Ciardiello F, Kim N, Smith GH, Callahan R and Salomon DS. (1990). Transforming Growth Factor-α Messenger RNA Localization in the Developing Adult Rat and Human Mammary Gland by *in Situ* Hybridization. *Dev. Biol.* **140**: 123-131.

Liu R, Page C, Beidler DR, Wicha MS and Nuñez G. (1999). Overexpression of Bcl-x_L Promotes Chemotherapy Resistance of Mammary Tumors in a Syngeneic Mouse Model. *Am. J. Pathol.* **155**: 1861-1867.

Maroulakou IG, Anver M, Garrett L and Green JE. (1994). Prostate and Mammary Adenocarcinoma in Transgenic Mice Carrying a Rat C3(1) Simian Virus 40 Large Tumor Antigen Fusion Gene. *Proc. Natl. Acad. Sci. USA* **91**: 11236-11240.

Martinez-Lacaci I, Bianco C, De Santis M and Salomon DS. (1999). Epidermal Growth Factor-

Related Peptides and Their Cognate Receptors in Breast Cancer. In: *Breast Cancer: Molecular Genetics, Pathogenesis, and Therapeutics.* Bowcock AM (Ed.), Humana Press, Totowa, NJ. **1**: 31-57.

Mateyak MK, Obaya AJ and Sedivy JM. (1999). c-Myc Regulates Cyclin D-Cdk4 and –Cdk6 Activity But Affects Cell Cycle Progression at Multiple Independent Points. *Mol. Cell. Biol.* **19**: 4672-4683.

Matsui Y, Halter SA, Holt JT, Hogan BLM and Coffey RJ. (1990). Development of Mammary Hyperplasia and Neoplasia in MMTV-*TGFα* Transgenic Mice. *Cell* **61**: 1147-1155.

Miyashita T and Reed JC. (1995). Tumor Suppressor p53 Is a Direct Transcriptional Activator of the Human *bax* Gene. *Cell* **80**: 293-299.

Nasi S, Ciarapica R, Jucker R, Rosati J and Soucek L. (2001). Making Decisions Through Myc. *FEBS Lett.* **490**: 153-162.

Nass SJ, Li M, Amundadottir LT, Furth PA and Dickson RB. (1996). Role for Bcl-x_L in the Regulation of Apoptosis by EGF and TGFβ1 in c-*myc* Overexpressing Mammary Epithelial Cells. *Biochem. Biophys. Res. Comm.* **227**: 248-256.

Nass SJ and Dickson RB. (1997). Defining a Role for c-Myc in Breast Tumorigenesis. *Breast Cancer Res. Treat.* **44**: 1-22.

Nass SJ and Dickson RB. (1998). Epidermal Growth Factor-Dependent Cell Cycle Progression Is Altered in Mammary Epithelial Cells that Overexpress c-*myc*. *Clin. Cancer Res.* **4**: 1813-1822.

Nesbit CE, Grove LE, Yin X and Prochownik EV. (1998). Differential Apoptotic Behaviors of c-*myc*, N-*myc*, and L-*myc* Oncoproteins. *Cell Growth Diff.* **9**: 731-741.

Nesbit CE, Tersak JM and Prochownik EV. (1999). *Myc* Oncogenes and Human Neoplastic Disease. *Oncogene* **18**: 3004-3016.

Ogretman B and Safa AR. (1996). Down-Regulation of Apoptosis-Related Bcl-2 But Not Bcl-x_L or Bax Proteins in Multidrug-Resistant MCF-7/Adr Human Breast Cancer Cells. *Int. J. Cancer* **67**: 608-614.

Olopade OI, Adeyanju MO, Safa AR, Hagos F, Mick R, Thompson CB and Recant WM. (1997). Overexpression of BCL-x Protein In Primary Breast Cancer is Associated with High Tumor Grade and Nodal Metastases. *Cancer J. Sci. Am.* **3**: 230-237.

Packham G and Cleveland JL. (1995). c-Myc and Apoptosis. *Biochim. Biophys. Acta.* **1242**: 11-28.

Prendergast GC. (1999). Mechanisms of Apoptosis by c-Myc. *Oncogene* **18**: 2967-2987.

Reisman D, Elkind NB, Roy B, Beamon J and Rotter V. (1993). c-Myc *Trans*-Activates the p53 Promoter Through a Required Downstream CACGTG Motif. *Cell Growth Diff.* **4**: 57-65.

Sakakura C, Sweeney EA, Shirahama T, Igarashi Y, Hakomori S, Nakatani H, Tsujimoto H, Imanishi T, Ohgaki M, Ohyama T, Yamazaki J, Hagiwara A, Yamaguchi T, Sawai K and Takahashi T. (1996). Overexpression of *bax* Sensitizes Human Breast Cancer MCF-7 Cells to Radiation-Induced Apoptosis. *Int. J. Cancer* **67**: 101-105.

Salomon DS, Perroteau I, Kidwell WR, Tam J and Derynck R. (1987). Loss of Growth Responsiveness to Epidermal Growth Factor and Enhanced Production of Alpha-Transforming Growth Factors in *ras*-Transformed Mouse Mammary Epithelial Cells. *J. Cell. Physiol.* **130**: 397-409.

Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL and Lee DC. (1990). Overexpression of TGFα in Transgenic Mice: Induction of Epithelial Hyperplasia, Pancreatic Metaplasia, and Carcinoma of the Breast. *Cell* **61**: 1121-1135.

Sandgren EP, Schroeder JA, Qui TH, Palmiter RD, Brinster RL and Lee DC. (1995). Inhibition of Mammary Gland Involution is Associated with Transforming Growth Factor α but Not c-*myc*-Induced Tumorigenesis in Transgenic Mice. *Cancer Res.* **55**: 3915-3927.

Santoni-Rugiu E, Jensen MR and Thorgeirsson SS. (1998). Disruption of the pRb/E2F Pathway and Inhibition of Apoptosis are Major Oncogenic Events in Liver Constitutively Expressing c-*myc* and Transforming Growth Factor α. *Cancer Res.* **58**: 123-134.

Schoenenberger CA, Andres AC, Gorner B, van der Valk M, LeMeur M and Gerlinger P. (1988). Targeted c-*myc* Gene Expression in Mammary Glands of Transgenic Mice Induces Mammary Tumors with Constitutive Milk Protein Gene Transcription. *EMBO J.* **7**: 169-175.

Schorr K, Li M, Krajewski S, Reed JC and Furth PA. (1999a). Bcl-2 Gene Family and Related Proteins in Mammary Gland Involution and Breast Cancer. *J. Mammary Gland Biol. Neoplasia* **4**: 153-164.

Schorr K, Li M, Bar-Peled U, Lewis A, Heredia A, Lewis B, Knudson CM, Korsmeyer SJ, Jäger R, Weiher H and Furth PA. (1999b). Gain of Bcl-2 is More Potent than Bax Loss in Regulating Mammary Epithelial Cell Survival. *Cancer Res.* **59**: 2541-2545.

Schott AF, Apel IJ, Nuñez G and Clarke MF. (1995). Bcl-x_L Protects Cancer Cells from p53-Mediated Apoptosis. *Oncogene* **11**: 1389-1394.

Sears R, Nuckolls F, Haura E, Taya Y, Tamai K and Nevins JR. (2000). Multiple Ras-Dependent Phosphorylation Pathways Regulate Myc Protein Stability. *Genes Dev.* **14**: 2501-2514.

Shibata MA, Maroulakou IG, Jorcyk CL, Gold LG, Ward JM and Green JE. (1996). p53-Independent Apoptosis During Mammary Tumor Progression in C3(1)/SV40 Large T Antigen Transgenic Mice: Suppression of Apoptosis During Transition from Preneoplasia to Carcinoma. *Cancer Res.* **56**: 2998-3003.

Shibata MA, Liu ML, Knudson MC, Shibata E, Yoshidome K, Bandey T, Korsmeyer SJ and Green JE. (1999). Haploid Loss of *bax* Leads to Accelerated Mammary Tumor Development in C3(1)/SV40-Tag Transgenic Mice: Reduction in Protective Apoptotic Response at the Preneoplastic Stage. *EMBO J.* **18**: 2692-2701.

Shilkaitis A, Graves J, Mehta RR, Hu L, You M, Lubet R, Steele V, Kelloff G and Christov K. (2000). Bcl-2 and Bax Are Differentially Expressed in Hyperplastic, Premalignant and Malignant Lesions of Mammary Carcinogenesis. *Cell Growth Diff.* **11**: 437-445.

Siegel PM and Muller WJ. (1998). Tyrosine Kinases and Signal Transduction in Mouse Mammary Tumorigenesis. In: *Hormones and Growth Factors in Development and Neoplasia.* Dickson RB and Salomon DS (Eds.), Wiley-Liss, Inc., New York, NY. **1**: 397-419.

Sierra A, Castellsague X, Coll T, Mañas S, Escobedo A, Moreno A and Fabra A. (1998). Expression of Death-Related Genes and Their Relationship to Loss of Apoptosis in T_1 Ductal Breast Carcinomas. *Int. J. Cancer* **79**: 103-110.

Simões-Wüst AP, Olie RA, Gautschi O, Leech SH, Häner R, Hall J, Fabbro D, Stahel RA and Wittke-Zangemeister U. *Bcl-x_L* Antisense Treatment Induces Apoptosis in Breast Carcinoma Cells. *Int. J. Cancer* **87**: 582-590.

Sinn E, Muller WJ, Pattengale P, Tepler I, Wallace R and Leder P. (1987). Coexpression of MMTV/v-Ha-*ras* and MMTV/c-*myc* Genes in Transgenic Mice: Synergistic Action of Oncogenes In Vivo. *Cell* **49**: 465-475.

Sjöström J, Krajewski S, Franssila K, Niskanen E, Wasenius VM, Nordling S, Reed JC and Blomqvist C. (1998). A Multivariate Analysis of Tumour Biological Factors Predicting Response to Cytotoxic Treatment in Advanced Breast Cancer. *Br. J. Cancer* **78**: 812-815.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL. (1987). Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene. ***Science*** **235**: 177-182.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A and Press MF. (1989). Studies of the HER-2/neu proto-oncogene in Human Breast and Ovarian Cancer. *Science* **244**: 707-712.

Snedeker SM, Brown CF and DiAugustine RP. (1991). Expression and Functional Properties of

Transforming Growth Factor α and Epidermal Growth Factor During Mouse Mammary Gland Ductal Morphogenesis. *Proc. Natl. Acad. Sci. USA* **88**: 276-280.

Srinivasan A, Li F, Wong A, Kodandapani L, Smidt Jr. R, Krebs JF, Fritz LC, Wu JC and Tomaselli KJ. (1998). Bcl-x_L Functions Downstream of Caspase-8 to Inhibit Fas- and Tumor Necrosis Factor Receptor 1-Induced Apoptosis of MCF7 Breast Carcinoma Cells. *J. Biol. Chem.* **273**: 4523-4529.

Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon A, Langford LA, Baungard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH and Tavtigian SV. (1997). Identification of a Candidate Tumor Suppressor Gene, *MMAC1*, at Chromosome 10q23.3 That is Mutated in Multiple Advanced Cancers. *Nat. Genet.* **15**: 356-362.

Stewart TA, Pattengale PK and Leder P. (1984). Spontaneous Mammary Adenocarcinomas in Transgenic Mice That Carry and Express MTV/*myc* Fusion Genes. *Cell* **38**: 627-637.

Strange R, Li F, Saurer S, Burkhardt A and Friis RR. (1992). Apoptotic Cell Death and Tissue Remodeling During Mouse Mammary Gland Involution. *Development* **115**: 49-58.

Telang NT, Osborne MP, Sweterlitsch LA and Narayanan R. (1990). Neoplastic Transformation of Mouse Mammary Epithelial Cells by Deregulated *myc* Expression. *Cell Regul.* **1**: 863-872.

Travers MT, Barrett-Lee PJ, Berger U, Luqmani YA, Gazet JC, Powles TJ and Coombes RC. (1988). Growth Factor Expression in Normal, Benign, and Malignant Breast Tissue. *Br. Med. J.* **296**: 1621-1624.

Valverius EM, Bates SE, Stampfer MR, Clark R, McCormick F, Salomon DS, Lippman ME and Dickson RB. (1989). Transforming Growth Factor α Production and Epidermal Growth Factor Receptor Expression in Normal and Oncogene Transformed Human Mammary Epithelial Cells. *Mol. Endocrinology.* **3**: 203-214.

Valverius EM, Ciardiello F, Heldin NE, Blondel B, Merlo G, Smith GH, Stampfer MR, Lippman ME, Dickson RB and Salomon DS. (1990). Stromal Influences on Transformation of Human Mammary Epithelial Cells Overexpressing c-*myc* and SV40T. *J. Cell. Physiol.* **145**: 207-216.

van de Vijver MJ, van de Bersselaar R, Devilee P, Cornelisse C, Peterse J and Nusse R. (1987). Amplification of the neu (c-erbB-2) Oncogene in Human Mammary Tumors is Relatively Frequent and Is Often Accompanied by Amplification of the Linked c-erbA Oncogene. ***Mol. Cell.*** *Biol.* **7**: 2019-2023.

Vennstrom B, Sheiness D, Zabielski J and Bishop JM. (1982). Isolation and Characterization of c-*myc*, a Cellular Homolog of the Oncogene (v-*myc*) of Avian Myelocytomatosis Virus Strain MC29. *J. Virol.* **42**: 773-779.

Wang JK, Johnson MD, Rosfjord EC, Jamerson MH and Dickson RB. (1999). EGF-Dependent Survival Signaling Pathways in c-Myc-Overexpressing Mammary Tumor Cell Lines: Roles of Erk1/Erk2 and PI3K Pathways. *Proceedings of the 90th Annual Meeting of the American Association for Cancer Research*, Philadelphia, PA. April 10-14, 1999. Abstract #1093.

Wang Q, Zhang H, Kajino K and Greene MI. (1998). BRCA1 Binds c-Myc and Inhibits Its Transcriptional and Transforming Activity in Cells. *Oncogene* **17**: 1939-1948.

Weaver ZA, McCormack SJ, Liyanage M, du Manoir S, Coleman A, Schröck E, Dickson RB and Ried T. (1999). A Recurring Pattern of Chromosomal Aberrations in Mammary Gland Tumors of MMTV-c-*myc* Transgenic Mice. *Genes, Chromosomes & Cancer* **25**: 251-260.

Yin C, Knudson CM, Korsmeyer SJ and Van Dyke T. (1997). Bax Suppresses Tumorigenesis and Stimulates Apoptosis *in vivo*. *Nature* **385**: 637-640.

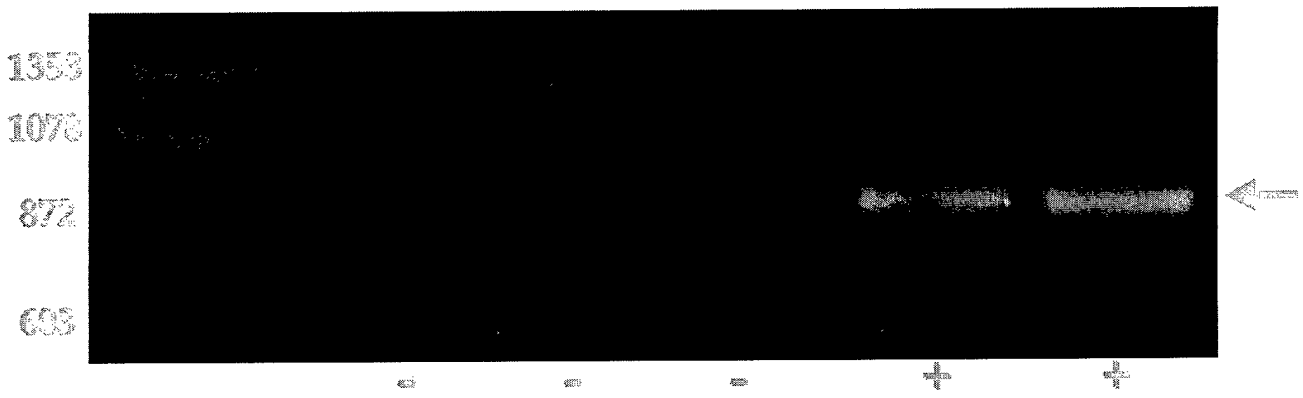
Yokota J, Toyoshima K, Sugimura T, Yamamoto T, Terada M, Battifora H and Cline MJ. (1986). Amplification of c-erbB-2 Oncogene in Human Adenocarcinomas *in vivo*. *Lancet* **1**: 765-767.

Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ and Roussel MF. (1998). Myc Signaling via the ARF Tumor Suppressor Regulates p53-Dependent Apoptosis and Immortalization. *Genes Dev.* **12**: 2424-2433.

APPENDIX A
Figures, Charts, and Tables

FIGURE 1

PCR Assessment of Mouse Genotype: MMTV-c-*myc*



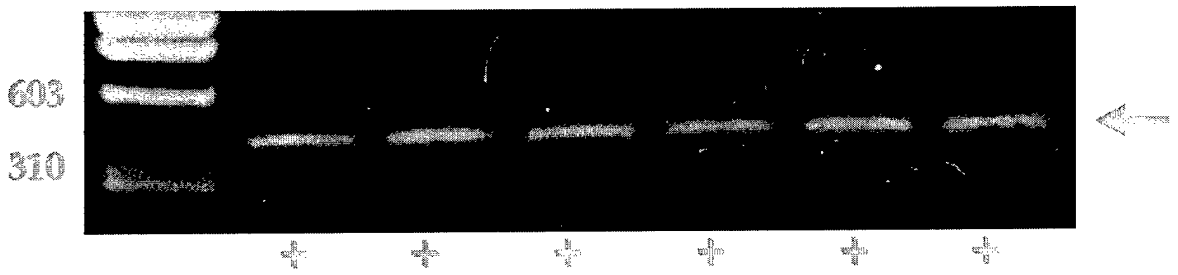
Template: Mouse Tail DNA – STE/Prot.K digestion - P/C extraction

Primers: Myc3' = 5'-ggg cat aag cac aga taa aac act-3'
Myc5' = 5'-ccc aag gct taa gta agt ttt tgg-3'

PCR: 60s @ 95° / 60s @ 52° / 75s @ 72° x 42 cycles

FIGURE 2

PCR Assessment of Mouse Genotype: tetOP-*bcl-x*$_L$



Template: Mouse Tail DNA -- STE/Prot.K digestion - P/C extraction

Primers: BclTg3′ = 5′-ctg aag agt gag ccc agc aga acc-3′
BclTg5′ = 5′-gca tic agt gac ctg aca tc-3′

PCR: 60s @ 95° / 60s @ 58° / 180s @ 72° x 30 cycles

FIGURE 3

PCR Assessment of Mouse Genotype: tetOP-*tta*

Template: Mouse Tail DNA – STE/Prot.K digestion - P/C extraction

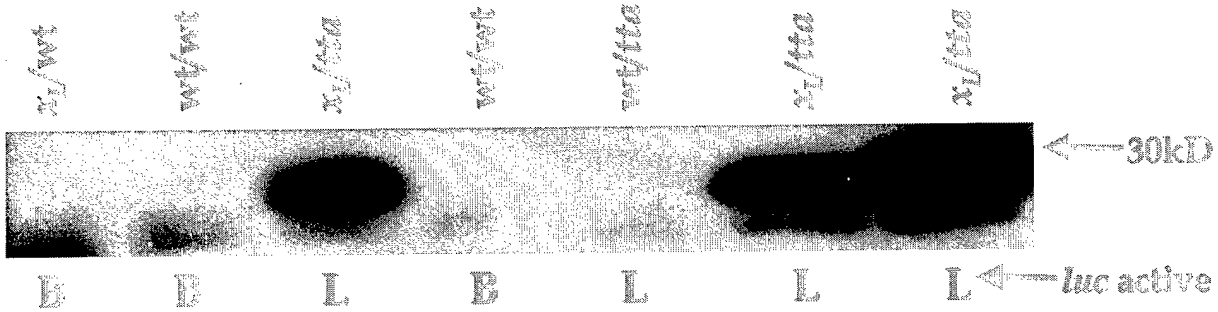
Primers: CMVF1 = 5'-tga cct cca tag aag aca cc-3'
TTAREV1 = 5'-atc tca atg gct aag gcg tc-3'

PCR: 45s @ 94° / 45s @ 52° / 90s @ 72° x 30 cycles

FIGURE 4

Confirmation of tetOP System Activity in Mammary Gland

- tetOP-*tta*(*luc*) Mouse = 7 tetOP + hCMVP.
  - tetOP-*tetracycline transactivator protein*
  - tetOP-*luciferase*
  - system ON in absence of tetracycline
- tetOP-*bcl-x*$_L$ Mouse = 7 tetOP + hCMVP + h*bcl-x*$_L$



- All virgin females without tetracycline treatment

FIGURE 5

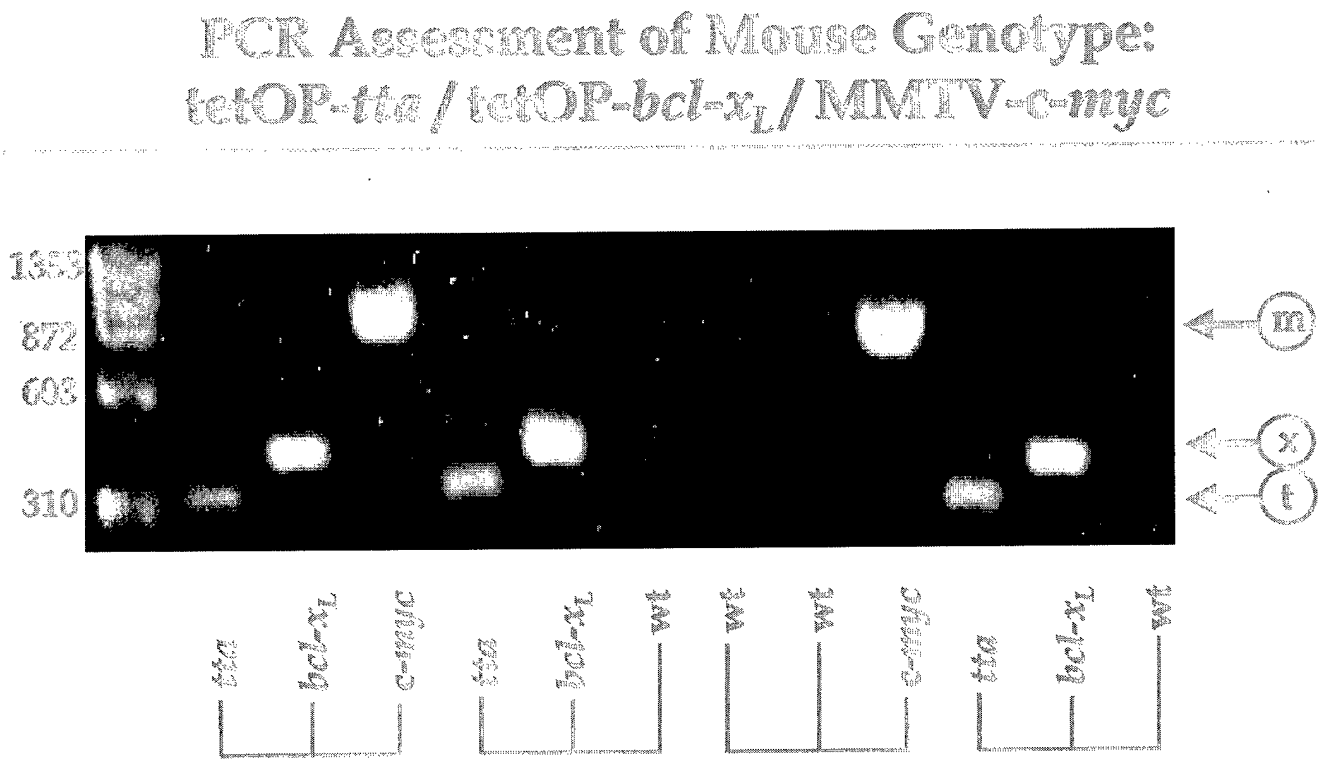

PCR Assessment of Mouse Genotype:
tetOP-tta / tetOP-bcl-x$_L$ / MMTV-c-myc
1353
872
603
310
m
x
t
tta
bcl-x$_L$
c-myc
tta
bcl-x$_L$
wt
wt
wt
c-myc
tta
bcl-x$_L$
wt

FIGURE 6

tta/bcl-x$_L$/c-myc Study Animals: Virgin and Parous Group Recruitment

tTA/xL/myc	V	*95h*	*10i*	*12i*	*13i*	*9l*	*99l*	*18m*	*22m*	*34n*	*37n*	*46q*	*47q*	*21s*	*19t*
tTA/xL/myc	P	*62t*	*44u*	*52u*	*56u*	*69u*	*93u*	*95u*	**47v**	*55v*	*8w*				
tTA/xL/wt	V	*99h*	*1i*	*14i*	*50i*	*58i*	*55j*	*63k*	*65k*	*19l*	*89m*	*63o*	*64o*	*13r*	*18r*	*19r*
tTA/xL/wt	P	*33s*	*36u*	*43u*	*46u*	*48u*	**90v**	**45w**	**74w**							
tTA/wt/myc	V	*11i*	*52i*	*57j*	*66k*	*58o*	*20p*	*23p*	*33p*	*58p*	*62p*	*62r*	*64r*	*20s*	*25t*	
tTA/wt/myc	P	*45u*	*63u*	*52v*												
tTA/wt/wt	V	*84h*	*97h*	*7i*	*8i*	*51i*	*50j*	*53j*	*74j*	*86j*	*98j*	*15l*	*29m*	*8n*	*76o*	*80o*
tTA/wt/wt	P	*52q*	*16r*	*10t*	*11t*	*16t*	*30t*	*34t*	*60t*	*40u*	*54u*					
wt/xL/myc	V	*18i*	*47i*	*64j*	*79j*	*19p*										
wt/xL/wt	V	*3i*	*6i*	*48i*	*53i*	*60p*										
wt/wt/myc	V	*88h*	*9i*	*69k*	*71k*											
wt/wt/wt	V	*94h*	*96h*	*2i*	*56i*	*25p*										

V = Virgin Tumor Study Mice
P = Parous Tumor Study Mice

ID's in **bold** = living
ID's in *italics* = sacrificed

FIGURE 7

tta/bcl-x$_L$/c-*myc* Study Animals: Developmental/Involution Study Recruitment

	t/x/m	t/x/-	t/-/m	t/-/-	-/-/m
Inv.d1	*91ac* **48af**	*77ae* **52af** **55af**	*15ae* *56ad*	*20ae* *95ad*	*61ab* *77ab* *6ac*
Inv.d3	*14ab* *22ab*	*13ae* *50ae*	*90ac* *74ad*	*73ad* *21ae*	*41ab* *59ab* *58ae*
Inv.d10	*92aa* *1ab*	*98aa* *30ab*	*16ab* *93ad* *96ad*	*23ab* *75ad*	**44af** **51af**

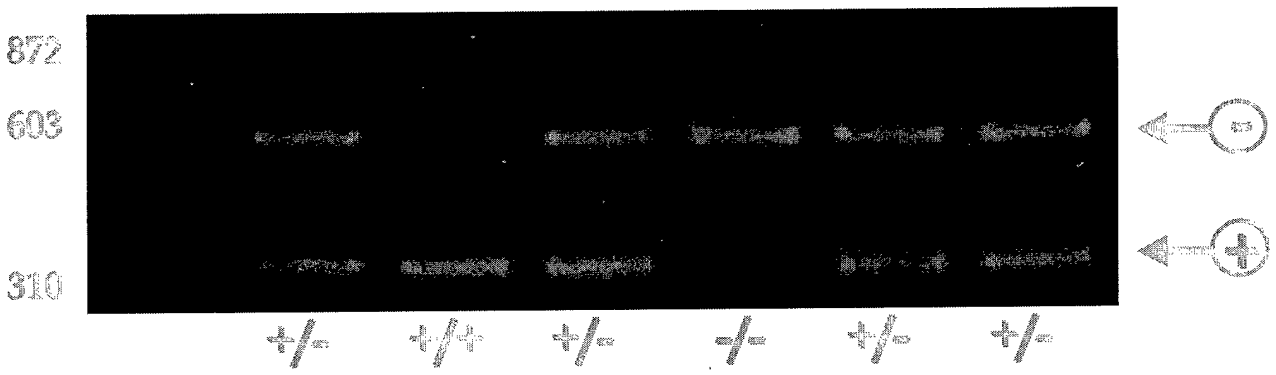
Seven-week old female mice are bred with single male mice.
Litters are removed at day 1 post-partum and mothers are sacrificed at days 1, 3, and 10 post-'weaning'.
n = 2-3 mothers per genotypic and involution phase subgroup

* Mammary glands are divided for formalin-fixation and whole-mounting.

Animal ID's in **bold** = living animals
Animal ID's in *italics* = sacrificed

FIGURE 8

PCR Assessment of Mouse Genotype: *bax*-K/O



Template: Mouse Tail DNA – STE/Prot.K digestion – P/C extraction

Primers: BPR2 = 5'-gtt gac cag agt ggc gta gg-3'
MK1 = 5'-gag ctg atc aga acc atc atg-3'
NPR2 = 5'-ccg ctt cca ttg ctc agc gg-3'

PCR: 45s @ 94° / 90s @ 55° / 120s @ 72° x 35 cycles

Breeding Strategies: MMTV-c-*myc* / *bax*-Knockout

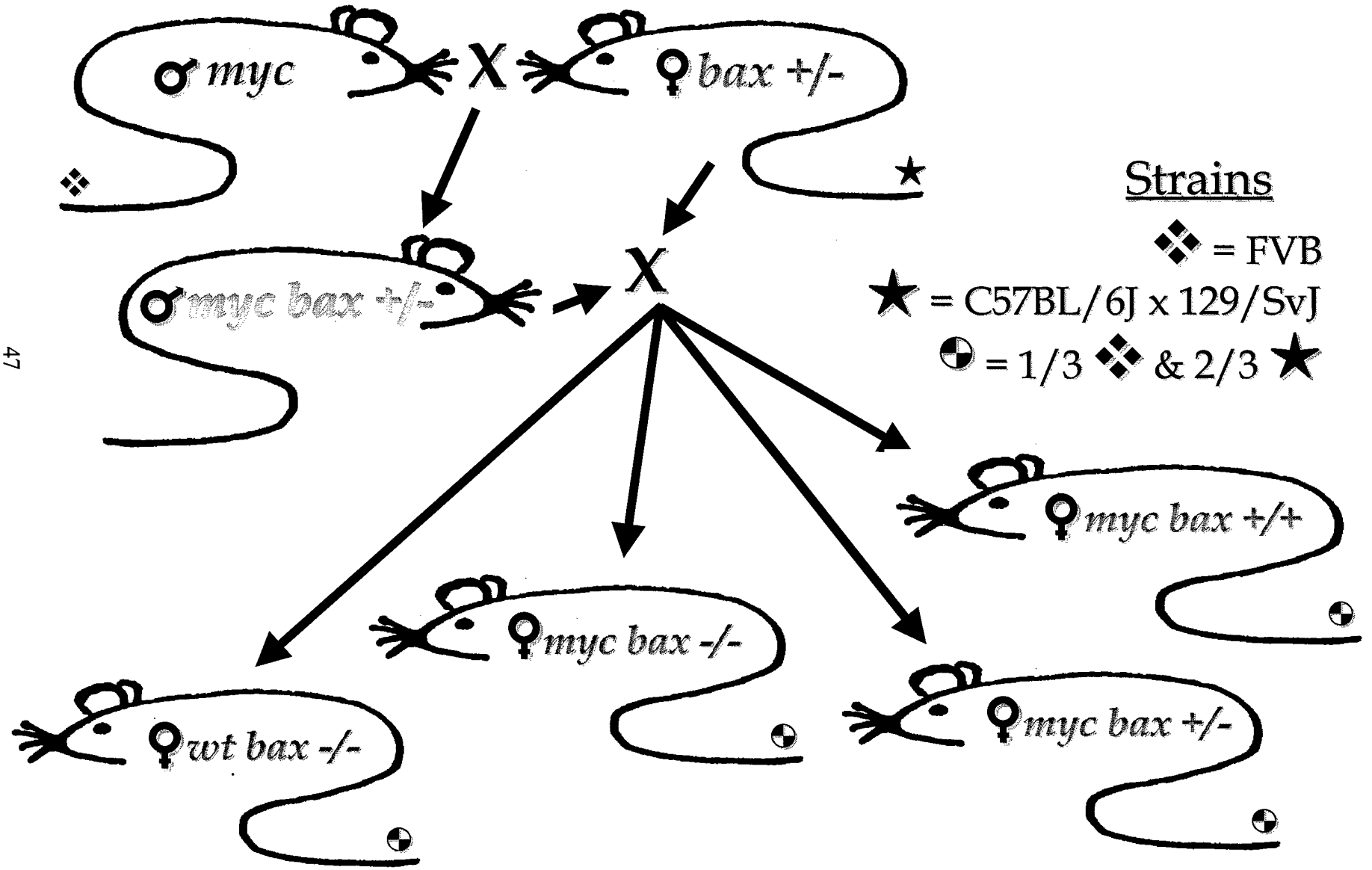


FIGURE 9

FIGURE 10

PCR Assessment of Mouse Genotype: MMTV-c-*myc* / *bax*-Knockout

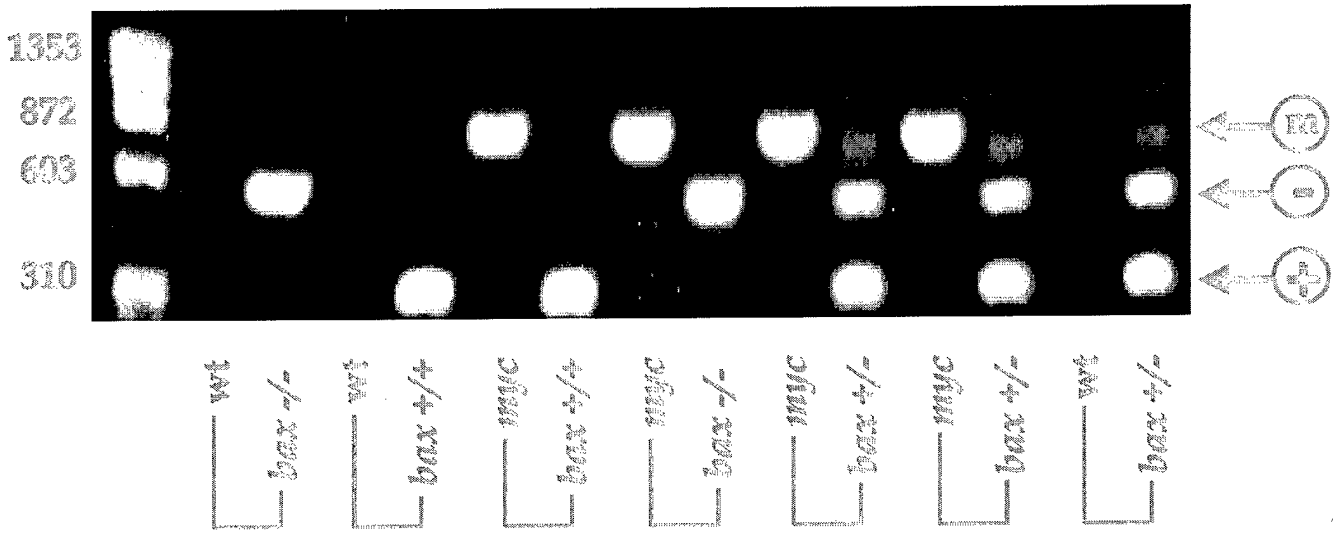


FIGURE 11

c-*myc* / *bax*-Knockout Study Animals: Virgin and Parous Group Recruitment

myc bax -/-	V	*1j*	*12j*	*20j*	*29j*	*37k*	*38k*	*97k*	*43l*	*64m*	*89n*	*10o*		
myc bax -/-	P	*45n*	*96o*	*71p*	*72p*	*81p*	*88p*	*27r*	*43r*	*54r*	*81r*			
myc bax +/-	V	*24i*	*69i*	*82i*	*84i*	*93i*	*94i*	*2j*	*19j*	*35j*	*36j*	*61j*	*6k*	*43k*	*47k*
myc bax +/-	P	*47n*	*54n*	*76n*	*73p*	*79p*	*83p*	*90p*	*22q*	*65q*					
myc bax +/+	V	*15j*	*34j*	*37j*	*38j*	*83k*	*4l*	*58m*	*55n*	*73n*	*81n*	*35o*	*90o*	*12p*	*92p*
myc bax +/+	P	*21q*	*67q*	*52r*	*53r*	*4s*	*43s*	*46s*	*64s*	*83s*	*97s*				
wt bax -/-	V	*90i*	*91i*	*21j*	*31j*	*81k*	*27l*	*44l*	*11p*	*42r*	*51r*	*58r*	*81s*	*94s*	
wt bax -/-	P	*22u*	*23u*	*65u*	*79u*	*89u*	*26v*	*62v*	*2w*						
wt bax +/-	V	*23i*	*28i*	*30i*	*5j*	*47p*									
wt bax +/+	V	*27i*	*31i*	*77i*	*96i*	*24j*									

V = Virgin Study Mice
P = Parous Study Mice

ID's in *italics* = sacrificed

FIGURE 12

c-*myc* / *bax*-Knockout Study Animals: Developmental/Involution Study Recruitment

	mb-/-	mb+/-	mb+/+	wt b-/-	wt b+/+
Inv.d1		*F99ac* *F6ad*	**F11af** **F22af** **F86af**		*F45ae* **F83ae** **F95ae**
Inv.d3		**F16af** **F87af**	**F8af** **F10af** **F84af**		*F13ad* *F5ae*
Inv.d10		*F59ad* *F63ad*	**F78ae** **F80ae** **F76af**	**F32af** **F33af**	*F58ad* *F65ad* **F97ae**

Ten-week old female mice are bred with single male mice.
Litters are removed at day 1 post-partum and mothers are sacrificed at days 1, 3, and 10 post-'weaning'.
n = 2-3 mothers per genotypic and involution phase subgroup

* Mammary glands are divided for formalin-fixation and whole-mounting.

Animal ID's in **bold** = living animals
Animal ID's in *italics* = sacrificed

FIGURE 13

tta/bcl-x$_L$/c-myc Virgin Tumor Study: Age at Sacrifice

MAJOR GENOTYPES - VIRGIN TUMOR STUDY

tTA/xL/myc V	*95h*	*10i*	*12i*	*13i*	*9l*	*99l*	*18m*	*22m*	*34n*	*37n*	*46q*	*47q*	*21s*	*19t*
age @ Sac (d)	*435*	*434*	*433*	*433*	*298*	*387*	*387*	*404*	*386*	*386*	*345*	*345*	*321*	*309*
tTA/xL/wt V	*99h*	*1i*	*14i*	*50i*	*58i*	*55j*	*63k*	*65k*	*19l*	*89m*	*63o*	*64o*	*13r*	*18r*	*19r*
age @ Sac (d)	*435*	*435*	*433*	*420*	*420*	*417*	*417*	*417*	*407*	*396*	*364*	*364*	*334*	*332*	*332*
tTA/wt/myc V	*11i*	*52i*	***57j***	*66k*	*58o*	*20p*	*23p*	*33p*	*58p*	*62p*	*62r*	*64r*	*20s*	*25t*
age @ Sac (d)	*434*	*420*	***348***	*417*	*365*	*358*	*358*	*358*	*351*	*351*	*323*	*323*	*321*	*309*
tTA/wt/wt V	*84h*	*97h*	*7i*	*8i*	*51i*	*50j*	*53j*	*74j*	*86j*	*98j*	*15l*	*29m*	*8n*	*76o*	*80o*
age @ Sac (d)	*435*	*435*	*434*	*434*	*420*	*407*	*407*	*391*	*389*	*389*	*407*	*404*	*393*	*361*	*361*

MINOR GENOTYPES - VIRGIN TUMOR STUDY

wt/xL/myc V	***18i***	*47i*	*64j*	*79j*	*19p*
age @ Sac (d)	***374***	*420*	*391*	*389*	*358*
wt/xL/wt V	*3i*	*6i*	*48i*	*53i*	*60p*
age @ Sac (d)	*435*	*435*	*420*	*420*	*351*
wt/wt/myc V	*88h*	*9i*	*69k*	*71k*	
age @ Sac (d)	*435*	*434*	*417*	*417*	
wt/wt/wt V	*94h*	*96h*	*2i*	*56i*	*25p*
age @ Sac (d)	*435*	*435*	*435*	*418*	*358*

Genotype	Avg. age @ Sac
tTA/xL/myc	379 days
tTA/xL/wt	395 days
tTA/wt/myc	360 days
tTA/wt/wt	405 days
wt/xL/myc	386 days
wt/xL/wt	412 days
wt/wt/myc	426 days
wt/wt/wt	416 days

ID's in **bold** = animals sacrificed w/ grossly observable mass lesions

FIGURE 14

tta/bcl-x$_L$/c-myc Parous Tumor Study: Parity Number and Age at Sacrifice

MAJOR GENOTYPES - PAROUS TUMOR STUDY

tTA/xL/myc	P	*62t*	*44u*	*52u*	*56u*	*69u*	*93u*	*95u*	47v	*55v*	***8w***
parity #		*13*	*10*	*10*	*10*	*7*	*11*	*11*	*8*	*1*	***4***
age @ Sac (d)		*428*	*399*	*398*	*398*	*396*	*396*	*413*	**liv**	*194*	***199***
tTA/xL/wt	P	*33s*	*36u*	*43u*	*46u*	90v	45w	74w			
parity #		*5*	*10*	*8*	*12*	11	7	4			
age @ Sac (d)		*401*	*400*	*399*	*399*	**liv**	**liv**	**liv**			
tTA/wt/myc	P	*45u*	***63u***	*52v*							
parity #		*12*	***5***	*0*							
age @ Sac (d)		*399*	***196***	*194*							
tTA/wt/wt	P	*52q*	*16r*	*10t*	*11t*	*16t*	*30t*	*34t*	*60t*	*40u*	*54u*
parity #		*9*	*9*	*1*	*13*	*9*	*10*	*10*	*13*	*8*	*12*
age @ Sac (d)		*435*	*420*	*271*	*405*	*405*	*404*	*402*	*418*	*399*	*413*

Genotype	Avg. age @ Sac
tTA/xL/myc	358 days
tTA/xL/wt	400 days
tTA/wt/myc	263 days
tTA/wt/wt	397 days

ID's in **bold** = animals sacrificed w/ grossly observable mass lesions
liv = alive @ time of reporting

FIGURE 15

c-*myc* / *bax*-Knockout Virgin Tumor Study: Age at Sacrifice

MAJOR GENOTYPES - VIRGIN TUMOR STUDY

myc bax -/- V	*1j*	*12j*	*20j*	*21j*	***29j***	*37k*	*38k*	*97k*	*43l*	*64m*	*89n*	*10o*	
age @ Sac (d)	*418*	*412*	*412*	*412*	***345***	*412*	*419*	*412*	*404*	*384*	*385*	*385*	
myc bax +/- V	*24i*	*69i*	*82i*	*84i*	*93i*	*94i*	*2j*	***19j***	*35j*	*36j*	*61j*	*6k*	*43k*	*47k*
age @ Sac (d)	*427*	*427*	*417*	*417*	*417*	*417*	*418*	***400***	*412*	*412*	*436*	*427*	*419*	*419*
myc bax +/+ V	*15j*	*34j*	*37j*	*38j*	*83k*	*4l*	*58m*	*55n*	***73n***	*81n*	*35o*	*90o*	*12p*	*92p*
age @ Sac (d)	*412*	*412*	*412*	*412*	*407*	*407*	*379*	*387*	***320***	*386*	*386*	*363*	*362*	*348*
wt bax -/- V	*90i*	*91i*	*31j*	*81k*	*27l*	*44l*	*11p*	*42r*	*51r*	*58r*	*81s*	*94s*		
age @ Sac (d)	*417*	*417*	*412*	*434*	*409*	*404*	*362*	*333*	*333*	*327*	*317*	*317*		

MINOR GENOTYPES - VIRGIN TUMOR STUDY

wt bax +/- V	*23i*	*28i*	*30i*	*5j*	*47p*
age @ Sac (d)	*423*	*423*	*423*	*412*	*354*
wt bax +/+ V	*27i*	*31i*	*77i*	*96i*	*24j*
age @ Sac (d)	*423*	*423*	*417*	*417*	*412*

Genotype	Avg. age @ Sac
myc bax -/-	400 days
myc bax +/-	419 days
myc bax +/+	385 days
wt bax -/-	401 days
wt bax +/-	407 days
wt bax +/+	418 days

ID's in **bold** = animals sacrificed w/ grossly observable mass lesions

FIGURE 16

c-*myc* / *bax*-Knockout Parous Tumor Study: Parity Number and Age at Sacrifice

MAJOR GENOTYPES - PAROUS TUMOR STUDY

myc bax -/-	P	*45n*	*96o*	***71p***	***72p***	*81p*	*88p*	*27r*	*43r*	*54r*	*81r*
parity #		*3*	*7*	***4***	***8***	*2*	*0*	*4*	*5*	*0*	*11*
age @ Sac (d)		*251*	*454*	***263***	***263***	*445*	*316*	*427*	*425*	*285*	*440*
myc bax +/-	P	*47n*	*54n*	***76n***	***73p***	***79p***	*83p*	*90p*	*22q*	***65q***	
parity #		*8*	*12*	***5***	***5***	*7*	*2*	*9*	*6*	*7*	
age @ Sac (d)		*261*	*483*	***232***	***193***	***239***	*445*	*444*	*442*	***273***	
myc bax +/+	P	*21q*	***67q***	*52r*	*53r*	***4s***	*43s*	*46s*	*64s*	*83s*	*97s*
parity #		*6*	***6***	*9*	*0*	***4***	*10*	*2*	*8*	*7*	*3*
age @ Sac (d)		*442*	***273***	*425*	*291*	***184***	*433*	*432*	*431*	*428*	*428*
wt bax -/-	P	*22u*	*23u*	*65u*	*79u*	*89u*	*26v*	*62v*	*2w*		
parity #		*3*	*3*	*0*	*7*	*2*	*5*	*1*	*0*		
age @ Sac (d)		*403*	*403*	*230*	*400*	*398*	*369*	*189*	*164*		

Genotype	Avg. age @ Sac
myc bax -/-	357 days
myc bax +/-	335 days
myc bax +/+	377 days
wt bax -/-	320 days

ID's in **bold** = animals sacrificed w/ grossly observable mass lesions

FIGURE 17

c-*myc* / *bax*-Knockout Parous Tumor Study: Summary

SUMMARY OF PAROUS TUMOR STUDY GROSS OBSERVABLE MASS LESIONS

Genotype		ID	Latency (d)	Multiplicity	Parity
myc bax -/-	P	*71p*	236	1	4
myc bax -/-	P	*72p*	240	1	9
myc bax +/-	P	*76n*	233	3	5
myc bax +/-	P	*73p*	193	2	6
myc bax +/-	P	*79p*	206	4	7
myc bax +/-	P	*65q*	252	2	7
myc bax +/+	P	*67q*	220	1	7
myc bax +/+	P	*4s*	183	1	5

		#	Avg. Latency	Avg. Multiplicity	Avg. Parity	Incidence
myc bax -/-	P	10	238.0d +/- 5.7	1	6.5	20%
myc bax +/-	P	9	221.0d +/- 53	2.75	6.25	44.40%
myc bax +/+	P	10	201.5d +/- 52.3	1	6	20%

Latency values include 95% C.I. As calculated from variance

APPENDIX B
Manuscript Reprints

Manuscript #1: **M Hunter Jamerson**, Michael D. Johnson and Robert B. Dickson. (2000). Dual Regulation of Proliferation and Apoptosis: c-*myc* in Bitransgenic Murine Mammary Tumor Models. *Oncogene* **19**: 1065-1071.

*Manuscript #1 attached as pages 57 - 63 of Final Report.

Manuscript #2: Dezhong J Liao, Geraldine Natarajan, Sandra L Deming, **M Hunter Jamerson**, Michael Johnson, Gloria Chepko and Robert B Dickson. (2000). Cell Cycle Basis for the Onset and Progression of c-Myc-Induced, TGFα-Enhanced Mouse Mammary Gland Carcinogenesis. *Oncogene* **19**: 1307-1317.

*Manuscript #2 attached as pages 64 - 74 of Final Report.

Oncogene (2000) 19, 1065–1071

www.nature.com/onc

Dual regulation of proliferation and apoptosis: c-*myc* in bitransgenic murine mammary tumor models

M Hunter Jamerson[1], Michael D Johnson[1] and Robert B Dickson*,[1]

[1]*The Lombardi Cancer Center, Georgetown University Medical Center, Georgetown University, Washington DC 20007, USA*

Recent progress in the study of c-Myc has convincingly demonstrated that it possesses a dual role in regulating both proliferation and apoptosis; however, the manner in which c-Myc influences these cellular response pathways remains incompletely characterized. Deregulation of c-Myc expression, via many mechanisms, is a common feature of multiple cancers and is an especially prominent feature of many breast cancers. Of significant interest to those who study mammary gland development and neoplasia is the unresolved nature and contribution of apoptosis to breast tumorigenesis. Recently, the use of transgenic mice and gene-knockout mice has allowed investigators to evaluate the pathological mechanisms by which different genes influence tumor development and progression. In this review, we address two distinct c-*myc*-containing bitransgenic murine mammary tumor models and discuss the contribution and possible future directions for resolution of cancer-relevant molecular pathways influenced by c-Myc. *Oncogene* (2000) **19,** 1065–1071.

Keywords: transgenic mice; mammary gland; c-*myc*; TGFα; *p53*

Introduction

The use of transgenic mice and mice bearing targeted gene disruptions (knockout mice) has given rise to current paradigms for the mechanistic evaluation of processes relevant to both physiology (e.g., embryogenesis, growth control and differentiation, morphogenesis) and pathology (e.g., neurodegenerative disease, hypertension, rheumatoid arthritis, neoplasia). Over 20 years ago, the combination of murine embryo culture with the techniques of reimplantation, DNA microinjection, and mammalian retrovirus manipulation resulted in the generation of the first transgenic mice. These animals were produced by embryo infection and microinjection methodologies (Brinster, 1972; Jaenisch, 1976; Gordon *et al.*, 1980). Three years later, the first example of a tissue-specific transgenic animal was published (Igκ gene expression in murine spleen), thus establishing the refined capacity for examining exogenous gene expression in models with greater *in vivo* relevance (Brinster *et al.*, 1983). Then, in 1984, the first transgenic animal was generated for the purpose of evaluating the relevance of a cellular proto-oncogene, c-*myc*, to mammary development and tumorigenesis (Stewart *et al.*, 1984). Subsequently, a burgeoning field of mammary-specific transgenic murine models has been generated and characterized, greatly advancing our understanding of the molecular basis for the contribution of growth factors, oncogenes and tumor suppressor genes to the pathogenesis of breast cancer.

In this review, we will address two different c-*myc*-containing bitransgenic murine models (c-*myc*/*tgfα* and c-*myc*/*p53*$^{+/-}$) that our group (Amundadottir *et al.*, 1995; McCormack *et al.*, 1998) and two other groups (Elson *et al.*, 1995; Sandgren *et al.*, 1995) have generated. We shall also discuss the contributions these models have made to our understanding of breast cancer and of molecular pathways that are influenced by the c-*myc* oncogene.

c-myc *oncogene, the mammae and breast cancer*

c-Myc is a 439-amino acid nuclear transcription factor that interacts with DNA when heterodimerized with the Max protein. This heterodimerization is required for c-Myc-mediated cell cycle progression, transformation, and apoptosis, and is facilitated via C-terminal leucine zipper and basic helix-loop-helix motifs (Harrington *et al.*, 1994; Packham *et al.*, 1995). c-Myc has been demonstrated to contribute to a number of important cellular functions, including cell cycle progression, apoptosis and DNA anabolism. In addition, c-Myc plays a role in cellular transformation via both transcriptional upregulation and transcriptional repression of target genes. The former occurs through established E-box or other less well-defined promoter elements, while the latter is most likely mediated through initiator elements or in conjunction with other transcriptional modulators such as AP-2 and C/EBP (Facchini *et al.*, 1998; Dang, 1999). The Dual Signal model, as proposed by Gerard Evan, suggests that induction of apoptosis is an obligate function of c-*myc* expression and acts as a potent mechanism for the suppression of tumorigenesis (Evan *et al.*, 1993). c-Myc expression, coupled with any block to cellular proliferation, such as growth arrest caused by serum or growth factor deprivation, has been demonstrated in fibroblasts to result in apoptosis, independent of cell cycle phase (Evan *et al.*, 1992). However, this does not occur in benzo[a]pyrene-immortalized human mammary epithelial cells (MECs) transfected with c-*myc* and deprived of epidermal growth factor (EGF) (Nass *et al.*, 1998). Instead, these cells arrest in the G1 phase of the cell cycle and do not undergo apoptosis.

c-Myc expression is increased in the normal mammary gland during pregnancy-related prolifera-

*Correspondence: RB Dickson

tion, it is absent in differentiated mammary alveolar cells during lactation, and it is again increased during the normal apoptotic mammary involution process (Strange *et al.*, 1992). c-Myc is believed to be a nuclear mediator of mitogenic signals incident upon cells from various receptor systems and is contributory to, but not sufficient for, mammary epithelial cell transformation (Leder *et al.*, 1986; Telang *et al.*, 1990). Constitutive expression of c-*myc* has been shown to partially transform both mouse and human MECs, such that they grow in soft agar in response to EGF and transforming growth factor α (TGFα) (anchorage-independent growth), and are no longer as dependent upon these growth factors for anchorage-dependent growth as are the parental, non-transfected cells (Telang *et al.*, 1990; Valverius *et al.*, 1990). Furthermore, deregulated expression of c-*myc*, via multiple mechanisms, including translocation, proviral insertion, gene amplification, point mutation, and direct transcriptional effects, is a common feature of many human cancers (including breast, lung, liver and colon), and is thought to contribute to cellular proliferation and transformation when apoptosis is suppressed (Evan *et al.*, 1992; Santoni-Rugiu *et al.*, 1998; Dang, 1999). In human breast cancers, c-*myc* is amplified in approximately 16%, rearranged in approximately 5%, and overexpressed in the absence of gross locus alteration in nearly 70% of all cases, thus suggesting its importance in the genesis and/or progression of breast cancer (Nass *et al.*, 1997; Deming *et al.*, 1999).

Three groups have independently developed transgenic mice that express the c-*myc* oncogene in a mammary-associated (MMTV-c-*myc*) or mammary-specific (WAP-c-*myc*) context (Stewart *et al.*, 1984; Schoenenberger *et al.*, 1988; Sandgren *et al.*, 1995). In addition to these c-*myc* transgenic animals, another group has developed a mouse model, using a mammary tissue reconstitution method, in which the v-*myc* oncogene is expressed by a retrovirus throughout the reconstituted mammae (Edwards *et al.*, 1988). Both groups that have generated WAP-c-*myc* transgenic mice have reported a high incidence of mammary tumors; Schoenenberger described the tumors as adenocarcinomas, while Sandgren described them as solid carcinomas. In both cases, tumor incidence approached 100% in multiparous animals, with all virgin animals remaining tumor-free over the observation period (to 14 months of age). Additionally, both groups reported the expression of the c-*myc* transgene in both neoplastic mammary tissue as well as in mammary tissue from normal female mice during the latter part of pregnancy and throughout lactation (Schoenenberger *et al.*, 1988; Sandgren *et al.*, 1995). These findings are as expected owing to the temporal window for the hormone-driven activity of the whey acid protein (WAP) gene promoter. Stewart *et al.*, (1984) reported the presence of mammary adenocarcinomas in 100% of multiparous F1 female transgenic mice derived from founder 141-3 in which the murine mammary tumor virus long terminal repeat (MMTV-LTR) had been placed immediately upstream of the mouse c-*myc* locus containing all three exons. Interestingly, WAP-c-*myc* and MMTV-c-*myc* female transgenic mice display lengthy tumor latencies and exquisite dependence upon pregnancy for tumor development, suggestive not only of the contribution but also of the insufficiency of c-*myc* in mammary tumorigenesis.

Transforming growth factor α, the mammae and breast cancer

TGFα is a secreted, 50-amino acid glycoprotein, derived from an active, membrane-bound 160-amino acid precursor. TGFα demonstrates a high level of homology (~42%) with EGF (Martinez-Lacaci *et al.*, 1999), and both molecules bind the epidermal growth factor receptor (EGFR) with high affinity. The growth factor family to which TGFα and EGF belong is now known to contain about 15 mammalian genes (Martinez-Lacaci *et al.*, 1999). TGFα binding to EGFR (also termed c-ErbB1) has been demonstrated to result in receptor homodimerization as well as heterodimerization between c-ErbB1 and c-ErbB2, c-ErbB3 and/or c-ErbB4, when present. Receptor dimerization leads to receptor autophosphorylation and activation of downstream signalling pathways including p42/p44 MAPK, JNK/SAPK, PI3K, PLC and cAMP/PKA (Dickson and Lippman, 1995; Siegel *et al.*, 1998; Martinez-Lacaci *et al.*, 1999). TGFα is expressed in normal murine mammae within the basal cells of the epithelium and the terminal cells of the end buds (Snedeker *et al.*, 1991; Martinez-Lacaci *et al.*, 1999). It is also present in murine and human mammae during pregnancy (Liscia *et al.*, 1990) and has been demonstrated to have similar growth effects upon human and murine mammary epithelial cells *in vitro* (Salomon *et al.*, 1987; Bates *et al.*, 1990; Valverius *et al.*, 1989). Exogenous TGFα expression has also been reported to contribute to the transformation of murine MECs that have been previously immortalized, suggesting that growth factor expression can cooperate with other established genetic alterations in mammary tissue in transforming pathways (Shankar *et al.*, 1989; McGeady *et al.*, 1989). Early evidence demonstrated increased TGFα expression in mammary tumors versus normal mammary gland (Derynck *et al.*, 1987; Arteaga *et al.*, 1988; Bates *et al.*, 1988; Travers *et al.*, 1988); however, the current paradigm for EGF family growth factor participation in breast cancer also involves the establishment of a pro-survival, pro-proliferative, autocrine stimulatory loop with EGFR. The EGFR has also been found to be overexpressed with or without gene amplification in approximately 50% of breast cancers (Harris *et al.*, 1988; Dickson *et al.*, 1995; Dahiya *et al.*, 1998; Martinez-Lacaci *et al.*, 1999, De Luca *et al.*, 1999).

Three groups have independently developed transgenic mouse models in which the TGFα growth factor is expressed in a metal ion-inducible, general tissue context (MT1-*tgfα*) (Sandgren *et al.*, 1990; Jhappan *et al.*, 1990), a mammary-associated context (MMTV-*tgfα*) (Matsui *et al.*, 1990), or a mammary-specific context (WAP-*tgfα*) (Sandgren *et al.*, 1995). The two groups that generated MT1-*tgfα* transgenic mice used rat and human *tgfα* under the control of the heavy-metal inducible murine metallothionein (MT) promoter. Each group reported that TGFα expression significantly influenced mammary gland development and MEC proliferation as examined using mammary gland whole mounts. In addition, TGFα expression contributed to mammary alveolar hyperplasia and

mammary adenocarcinoma in multiparous female transgenic mice (Sandgren *et al.*, 1990; Jhappan *et al.*, 1990). MMTV-LTR-driven expression of the *tgfα* transgene was also shown to contribute to mammary alveolar hyperplasia in virgin female mice and to mammary adenocarcinoma in multiparous female mice. Furthermore, TGFα protein expression was confirmed and a TGFα/EGFR autocrine loop was suspected due to the increased presence of EGFR mRNA in areas of increased expression of the transgene (Matsui *et al.*, 1990). Finally, results from the characterization of the WAP-*tgfα* transgenic model suggest that constitutive *tgfα* expression accelerates mammary development, impedes apoptotic involution, and contributes to mammary transformation by acting as a survival factor for differentiated murine MECs (Sandgren *et al.*, 1995). Significantly, the requirement for pregnancy and the extended tumor latency for TGFα transgenic models illustrates that TGFα is likely to be incapable of serving as the sole cause of mammary cancers. Rather, TGFα overexpression is likely to be one promotional step along a multistep oncogenic pathway(s). Therefore, it is particularly interesting that the tumorigenicity of cancer cell lines (liver) has been associated with the dual overexpression of *tgfα* and c-*myc*, suggesting a possible cooperativity between these two genes (Lee *et al.*, 1991).

MMTV-c-myc/*MT*-tgfα *and WAP-c*-myc/*WAP*-tgfα *bitransgenic mice*

The MMTV-c-*myc*/MMTV-v-*Ha-ras* cross generated in 1987 was the first c-*myc*-containing bitransgenic mouse (Sinn *et al.*, 1987). Characterization of this bitransgenic mouse model demonstrated that deregulated c-*myc* expression synergized with deregulated v-*Ha-ras* expression to both accelerate mammary tumorigenesis and abrogate the requirement for pregnancy in this process. Interestingly, mammary tumors were demonstrated in both virgin female and male bitransgenic mice, despite a further delay in tumor onset in males of nearly 2 months. Eight years later, our group and another group reported the generation and characterization of mice bitransgenic for c-*myc* and *tgfα*, lending support to the notion that signalling through the EGFR and/or activation of Ras could synergize with deregulated c-*myc* expression in mammary tumorigenic processes (Amundadottir *et al.*, 1995; Sandgren *et al.*, 1995). The MMTV-c-*myc*/MT-*tgfα* bitransgenic mice from our laboratory develop multiple mammary adenocarcinomas with a much reduced latency, and do so in the absence of any requirement for pregnancy or ovarian hormone stimulation. These mammary adenocarcinomas grew without requirement for estrogen (i.e., without delayed tumor growth in ovariectomized bitransgenic female mice) despite being estrogen receptor positive, as shown by estrogen receptor ligand-binding assay. Furthermore, histological evaluation of mammary gland tissue from both female and male animals as young as 5 weeks of age evidenced both hyperplastic and neoplastic changes in areas of transgene co-expression (Amundadottir *et al.*, 1995). The complete absence of normal mammary tissue in bitransgenic animals and the ability of bitransgenic mammary tissue from 3 week-old mice to form tumors in athymic mice suggest that these two important, mammary gland-relevant genes (c-*myc* and *tgfα*) are capable of synergistically transforming the mammary epithelium, apparently requiring minimal, if any, additional genetic alterations (Amundadottir *et al.*, 1995, 1996a). These studies also demonstrated that c-*myc* and *tgfα* are capable of further cooperation to drive hyperplastic and neoplastic changes in the murine salivary glands. This was not seen in single transgenic animals carrying c-*myc* or *tgfα* (Amundadottir *et al.*, 1995). Characterization of the WAP-c-*myc*/WAP-*tgfα* bitransgenic model confirmed the potent synergy of these two genes in promoting and accelerating mammary tumor formation, when compared with the relevant single transgenic animals. Furthermore, the power of this cooperative interaction between c-*myc* and *tgfα* is demonstrated in both our model and the WAP-based model since both male and virgin female bitransgenic animals develop mammary tumors (Amundadottir *et al.*, 1995; Sandgren *et al.*, 1995). The WAP promoter utilized in the latter study to drive the expression of the c-*myc* and *tgfα* transgenes is often presumed to drive transgene expression only in the latter part of pregnancy and throughout lactation and involution. However, the presence of mammary tumors in male and virgin female bitransgenics suggests that the MMTV and WAP promoters may be slightly 'leaky', in the sense that minimal transgene expression may still occur even in the absence of ovarian hormone stimulation or that minimal promoter activity may be present during estrous in these mice.

Subsequent work in our laboratory with single transgenic mice, c-*myc*/*tgfα* bitransgenic mice, and cell lines derived from transgenic mouse mammary tumors, has led to the hypothesis that TGFα can cooperate with c-Myc in promoting cell cycle progression and can act to suppress c-Myc-induced apoptosis (Amundadottir *et al.*, 1996b; Nass *et al.*, 1996,1998). Our results, together with those from another group, have suggested that transformation, maintenance of transformation, and suppression of apoptosis in c-*myc*-overexpressing mammary tumor cell lines derived from transgenic animals may require signalling through the p42/p44 MAPK and PI3K pathways, both of which are targets of the activated EGFR (Amundadottir *et al.*, 1998; Wang *et al.*, 1999). *In situ* end labeling apoptosis assays (TUNEL staining) in paraffin-embedded mammary tumor sections from transgenic animals indicated the presence of apoptotic mammary cells in *c-myc* transgenic tumors and their near absence in tumors from the *tgfα* and c-*myc*/*tgfα* transgenic mice (Amundadottir *et al.*, 1996b). Data from our tumor cell lines indicate that coexpression of c-*myc* and *tgfα* results in increased cell proliferation under anchorage-dependent and anchorage-independent conditions, a reduced requirement for exogenous growth factor stimulation, and greatly decreased apoptosis. This protection from apoptosis is abrogated when EGFR signalling is blocked by addition of PD153035–a specific, synthetic EGFR tyrosine kinase inhibitor. Furthermore, the myc83 cell line, and an additional five other cell lines derived from mammary tumors in c-*myc* transgenic mice, were significantly more apoptotic than cell lines derived from either *tgfα* or c-*myc*/*tgfα* bitransgenic mammary tumors. The frequency of apoptotic cells could be considerably suppressed by the addition of exogenous TGFα or EGF. Conversely,

apoptosis was considerably accentuated when EGFR signalling was blocked via PD153035. This augmentation of apoptosis was sensitive to reversal by addition of the survival factor basic fibroblast growth factor (bFGF), which interacts with its own specific family of receptors and does not associate with EGFR (Amundadottir *et al.*, 1996b).

Molecular characterization of apoptosis in c-*myc*-overexpressing murine MECs derived from the MMTV-c-*myc* transgenic mice led to the recognition that Bcl-x_L, an anti-apoptotic member of the Bcl-2 family of apoptosis regulatory proteins, is a likely mediator of TGFα and EGF-directed protection against c-*myc*-driven apoptosis. Bcl-x_L mRNA and protein levels were elevated with TGFα or EGF treatment of these c-*myc*-expressing cell lines, and expression of this anti-apoptotic molecule was significantly diminished with growth factor removal, TGFβ treatment, or PD153035 treatment. In addition, levels of Bax (a pro-apoptotic Bcl-2 family member) and p53 appeared relatively high and unchanged, while Bcl-2 and Bcl-x_S (another pro-apoptotic Bcl-2 family member) levels remained low or undetectable with these aforementioned treatments (Nass *et al.*, 1996). The work in our laboratory has led to the following models for the cooperation between c-Myc and TGFα in proliferation and apoptosis in the mouse mammary gland: First, with respect to proliferation, deregulated c-Myc may drive cellular proliferation by upregulating/inducing cyclin A2, E2F1, cyclin E, cdc25A phosphatase, and CAK-activating partner cdk7, and by lowering p27 levels resulting in cdk2 activation. In contrast, TGFα overexpression leads to the induction of cyclin D1 and, subsequently, the activation of cdk4/6 (Liao *et al.*, 2000). The combination of these two effects may further deregulate the cell cycle. Second, with respect to apoptosis, deregulated c-*myc* expression may promote apoptosis by directly inducing p53 expression, and by directly or indirectly inducing Bax expression. Bax has been shown to be directly responsive to p53 and also to be a potential target for c-Myc induction because of the location of four E-boxes within the Bax promoter/5′-UTR (Miyashita *et al.*, 1995). At present, there is no published evidence that c-Myc functions through these elements to induce Bax expression. As previously mentioned, TGFα appears to activate cellular survival pathways and induce the expression of the anti-apoptotic protein Bcl-x_L (Nass *et al.*, 1996). This work, combined with results from the characterization of *MMTV*-c-*myc*/WAP-*bcl*-2 bitransgenic mice, strongly suggests that mammary tumorigenesis is significantly increased when deregulated c-*myc* expression, responsible both for driving cellular proliferation as well as increasing cellular sensitivity to apoptosis, is coupled with other genetic alterations that act as survival signals to block c-*myc*-mediated apoptotic pathways. In this latter study, *bcl-2* expression accelerated mammary tumorigenesis and suppressed *in vivo* mammary tumor apoptosis (Jäger *et al.*, 1997).

p53 *tumor suppressor gene, the mammae and breast cancer*

p53 is a 393-amino acid nuclear phosphoprotein transcription factor known to bind DNA upon stabilization induced by cell cycle checkpoint controls. p53 transactivation increases the expression of genes involved in such distinct processes as apoptosis, DNA repair, and cell cycle arrest (Evan *et al.*, 1998; El-Deiry, 1998). p53 has often been termed the 'guardian of the genome' owing to the fact that it plays such a critical role as a tumor suppressor by orchestrating cell cycle arrest and DNA repair upon recognition of certain levels of DNA damage. Cell cycle inhibitory activities are believed to be controlled by p53-dependent transcriptional activation of genes, including p21/WAF1/CIP1, 14-3-3σ, and GADD45. In addition to its role in DNA damage recognition, the p53 tumor suppressor has also been linked to the recognition of oncogene activation (c-*myc* and adenovirus E1A), subsequently resulting in apoptosis induction via a pathway that includes ARF and MDM2 (Zindy *et al.*, 1998; de Stanchina, *et al.*, 1998; Sherr, 1998). p53 is capable of promoting apoptosis upon recognition of severe, irreparable DNA damage, DNA damage in the context of other environmental conditions unfavorable for maintenance of genomic integrity, and abnormal cellular proliferation as driven by oncogene activation. Thus far, p53-dependent apoptosis has been demonstrated to result from the transcriptional activation of genes, including Bax, Fas/Apo1/CD95, and DR5 Trail receptor, and from transcriptional repression of the anti-apoptotic gene Bcl-2 (Canman *et al.*, 1997; El-Deiry, 1998).

Little information exists concerning the expression pattern for wild-type p53 during development in either human or murine mammary glands. One study indicates that p53 mRNA is expressed during pregnancy and involution, but not during lactation (Strange *et al.*, 1992). Another study, however, suggests that the complete absence of p53 expression does not alter the histological or functional development of the mammae in mice, since $p53^{-/-}$ mice remain capable of lactation (Donehower *et al.*, 1992). As regards the role of p53 in mammary apoptosis, both p53-dependent and p53-independent apoptosis have been demonstrated in cultured MECs (Merlo *et al.*, 1995). In mice, one study has indicated that post-lactational mammary involution and apoptosis proceed normally without regard for p53 status (Li *et al.*, 1996); whereas, another study has demonstrated that the first phase of mammary involution is delayed in p53-null animals (Jerry *et al.*, 1998).

The *p53* tumor suppressor is one of the most frequently altered genes in a wide variety of human cancers, including breast cancer (Donehower *et al.*, 1993). Breast cancer, along with sarcomas, brain tumors, leukemias and adrenal cortical tumors, is common among women with Li-Fraumeni Syndrome, a disorder linked to germline mutations in the *p53* locus (Eeles *et al.*, 1993). Furthermore, *p53* gene mutations have been identified in approximately 17% of all human breast cancers (Dahiya and Deng, 1998). To date, results in the mammary glands of murine *p53*-knockout animals have been somewhat discordant with expectations based on Li-Fraumeni Syndrome. Specifically, non-mammary gland tumors, such as lymphomas, rapidly arise in *p53*-knockout animals, suggesting that p53 is not of predominant importance in murine mammary tumor development (Donehower *et al.*, 1992; Harvey *et al.*, 1993; Purdie *et al.*, 1994). More recent investigations of human breast cancer-relevant *p53*

missense mutations expressed in transgenic models (Li *et al*., 1998) and *wnt1* transgenic/*p53*-knockout murine models (Donehower *et al*., 1995; Jones *et al*., 1997) indicate that p53 alteration can be contributory to mammary tumorigenesis in some circumstances. It is possible that the lack of agreement concerning the role of p53 loss in murine models of cancer and human breast cancer results from interspecies differences, from the modulation of tumorigenesis by murine strain differences, from other transgenes carried in the background, and from the particular *p53* genetic knockouts and mutations modeled in these mice. The latter difference may be most significant, since the mammary tumorigenic effects noted in the study of the p53-172R/H mutant transgenic mouse resulted from the rational modeling of a specific, human breast cancer-relevant p53 alteration (Li *et al*., 1998).

MMTV-c-myc/p53$^{+/-}$ *transgenic mice*

In 1995, two transgenic models with a mammary-targeted oncogene (MMTV-*wnt1* or MMTV-c-*myc*) and *p53* deficiency were established to determine whether or not deficiencies in the tumor suppressor *p53* could cooperate with deregulated expression of Wnt1 or c-Myc to alter tumorigenesis in mammary tissues (Donehower *et al*., 1995; Elson *et al*., 1995). A cooperative effect was indeed observed between Wnt1 and *p53* deficiency, as mammary tumors in the MMTV-*wnt1*/*p53*$^{-/-}$ mice arose sooner and had a significantly higher degree of chromosomal instability than those of MMTV-*wnt1*/*p53*$^{+/-}$ and MMTV-*wnt1*/*p53*$^{+/+}$ animals (Donehower *et al*., 1995). In the MMTV-c-*myc* model, animals with *p53* disruption rapidly developed lethal lymphomas, indicating that c-*myc* and mutant p53 had a cooperative effect in terms of increasing the incidence and accelerating the onset of T-cell lymphomas. However, *p53* disruption failed to influence the mammary adenocarcinoma phenotype of the MMTV-c-*myc* animals. In those *MMTV*-c-*myc*/*p53*$^{+/-}$ female mice that survived their lymphomas long enough to acquire mammary tumors, there was no identifiable alteration in tumor latency, histology, or dependence upon pregnancy as compared with MMTV-c-*myc*/*p53*$^{+/+}$ controls (Elson *et al*., 1995). The absence of cooperation between p53 and c-Myc in terms of mammary carcinogenesis in this model may reflect intrinsic differences between murine and human mammary tumorigenesis, the cooperation between c-*myc* and *p53* in inducing extremely aggressive lymphomas that limited the mammary observation window, or the specific manner in which the *p53* alleles were targeted. It has been demonstrated that most *p53* alterations in human breast cancers are missense mutations that may influence the activity of the *p53* gene product, rather than deletions of entire *p53* exons (as was done in both of the previously mentioned models) that are capable of completely eliminating all p53 functionality (Elson *et al*., 1995; Lozano *et al*., 1998). This particular fact suggests that a cross between the p53-172R/H mutant transgenic mouse and WAP-c-*myc* or MMTV-c-*myc* transgenic mouse might be more relevant to the study of breast cancer. Unfortunately, there is no evidence to date concerning the frequency or relevance of combined c-*myc* amplification/overexpression and *p53* mutation in human breast tumors.

Recently, our group generated transgenic mice in which the mammary-targeted c-*myc* oncogene was expressed in the presence of a targeted disruption of the *p53* tumor suppressor gene (McCormack *et al*., 1998). Although our results indicated that disruption of *p53* may contribute to alveolar hyperplastic changes in the virgin female transgenic mouse, they failed to show any cooperation between c-*myc* and *p53* disruption in mammary tumorigenesis, since no alterations in latency, histology, or apoptosis were observed between c-*myc*-induced mammary tumors in animals with or without disrupted *p53* (McCormack *et al*., 1998). To determine whether or not disruption of *p53* could influence c-*myc*-induced chromosomal instability in mammary tumors from these transgenic mice, tumor-derived cell lines were subjected to spectral karyotyping (SKY) analysis (Liyanage *et al*., 1996). This analysis demonstrated that *p53* disruption did not significantly influence ploidy or other c-*myc*-induced chromosomal alterations. Analysis of these *p53*$^{+/+}$ and *p53*$^{+/-}$ tumor cells lines using both SKY and comparative genomic hybridization (CGH) also supported the concept that c-*myc*-induced chromosomal instability is unaffected by *p53* status (McCormack *et al*., 1998; Weaver *et al*., 1999). Unfortunately, the effects of complete *p53* disruption in the presence of c-*myc* transgene expression were untestable due to rapidly arising lymphomas that forced us to limit the duration of mammary observations.

Summary and future directions

Recent progress in the study of c-Myc has convincingly demonstrated that it possesses a dual role in promoting cellular proliferation and apoptosis. Work from our group and others has confirmed this dual role of c-Myc in murine mammae and has further shown that co-expression of TGFα can synergistically accelerate mammary tumorigenesis as well as abrogate tumor reliance on estrogenic signalling. These results appear similar to those obtained for c-*myc*/v-*Ha-ras* bitransgenic mice and further suggest that signalling through the EGFR (as well as activation of Ras) may induce downstream survival-signalling pathways that impinge upon c-Myc-driven apoptosis. Currently, work is being conducted in our laboratory with mammary tumor cell lines derived from the bitransgenic mice to resolve the nature and contribution of these survival pathways. The contribution of p53 mutation to breast tumorigenesis in humans is well established. Nevertheless, several studies suggest that p53 loss does not functionally or physically alter the murine mammae. Work from our group and another group has indicated a lack of obvious cooperation between hemizygous p53 knockout and c-*myc* transgene expression in bitransgenic mice. Unfortunately, the nature of these two models precluded the examination of the effect of homozygous p53 loss on mammary tumorigenesis due to the pervasive and aggressive lymphomas that arose in these animals. As was suggested by work with the p53-172R/H mutant mouse, it would be worth examining the contribution of breast cancer-specific p53 point mutants to c-*myc*-induced mammary tumorigenesis.

Of significant interest to those who study breast cancer and c-Myc is the nature of apoptosis signalling by c-Myc and its contribution to breast tumorigenesis. Greater resolution of this apoptotic pathway could suggest additional targets for breast cancer therapies. Work described herein provides the basis for the development of other combinatorial, mammary-specific transgenic models that will further dissect the relationship between c-Myc and apoptosis.

Acknowledgements
This work was supported by NIH Grants 1R01AG1496 and 1R01CA72460 to RB Dickson and a Department of Defense Grant DAMD-17-97-1-7110 to MH Jamerson. We thank Sandra L Deming and Dr Danica Ramljak for valuable discussion and critical reading of the manuscript.

References

Amundadottir LT, Johnson MD, Merlino G, Smith GH and Dickson RB. (1995). *Cell Growth Diff.*, **6**, 737–748.
Amundadottir LT, Merlino G and Dickson RB. (1996a). *Breast Cancer Res. Treat.*, **39**, 119–135.
Amundadottir LT, Nass SJ, Berchem GJ, Johnson MD and Dickson RB. (1996b). *Oncogene*, **13**, 757–765.
Amundadottir LT and Leder P. (1998). *Oncogene*, **16**, 737–746.
Arteaga CL, Hanauske AR, Clark GM, Osborne K, Hazarika P, Pardue RL, Tio F and Von Hoff DD. (1988). *Cancer Res.*, **48**, 5023–5028.
Bates SE, Davidson NE, Valverius EM, Freter CE, Dickson RB, Tam JP, Kudlow JE, Lippman ME and Salomon DS. (1988). *Mol. Endocrinol.*, **2**, 543–555.
Bates SE, Valverius EM, Ennis BW, Bronzert DA, Sheridan JP, Stampfer MR, Mendelsohn J, Lippman ME and Dickson RB. (1990). *Endocrinology*, **126**, 596–607.
Brinster RL. (1972). In: *Growth, Nutrition and Metabolism of Cells in Culture*. Rothblat G and Cristafalo V (eds). Academic: New York, **vol 2**, pp. 251–286.
Brinster RL, Ritchie KA, Hammer RE, O'Brien RL, Arp B and Storb U. (1983). *Nature*, **306**, 332–336.
Canman CE and Kastan MB. (1997). *Adv. Pharmacol.*, **41**, 429–460.
Dahiya R and Deng G. (1998). *Breast Cancer Res. Treat.*, **52**, 185–200.
Dang CV. (1999). *Mol. Cell. Biol.*, **19**, 1–11.
De Luca A, Casamassimi A, Selvam MP, Losito S, Ciardiello F, Agrawal S, Salomon DS and Normanno N. (1999). *Int. J. Cancer*, **80**, 589–594.
Deming SL, Nass SJ, Dickson RB and Trock BJ. (1999). Abstract No. 1358, Proceedings of the Annual Meeting of the AACR, Philadelphia, PA.
Derynck R, Goeddel DV, Ullrich A, Gutterman JU, Williams RD, Bringman TS and Berger WH. (1987). *Cancer Res.*, **47**, 707–712.
De Stanchina E, McCurrach ME, Zindy F, Shieh S-Y, Ferbeyre G, Samuelson AV, Prives C, Roussel MF, Sherr CJ and Lowe SW. (1998). *Genes Dev.*, **12**, 2434–2442.
Dickson RB and Lippman ME. (1995). *Endocrine Rev.*, **16**, 559–589.
Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Butel JS and Bradley A. (1992). *Nature*, **356**, 215–221.
Donehower LA and Bradley A. (1993). *Biochim. Biophys. Acta*, **1155**, 181–205.
Donehower LA, Godley LA, Aldaz CM, Pyle R, Shi YP, Pinkel D, Gray J, Bradley A, Medina D and Varmus HE. (1995). *Genes Dev.*, **9**, 882–895.
Edwards PAW, Ward JL and Bradbury JM. (1988). *Oncogene*, **2**, 407–412.
Eeles RA, Bartkova J, Lane DP and Bartek J. (1993). *Cancer Surv.*, **18**, 57–75.
El-Deiry WS. (1998). *Sem. Cancer Biol.*, **8**, 345–357.
Elson A, Deng C, Campos-Torres J, Donehower LA and Leder P. (1995). *Oncogene*, **11**, 181–190.
Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC. (1992). *Cell*, **69**, 119–128.
Evan GI and Littlewood TD. (1993). *Curr. Opin. Genet. Dev.*, **3**, 44–49.
Evan GI and Littlewood TD. (1998). *Science*, **281**, 1317–1322.
Facchini LM and Penn LZ. (1998). *FASEB J.*, **12**, 633–651.
Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA and Ruddle FH. (1980). *Proc. Natl. Acad. Sci. USA*, **77**, 7380–7384.
Harrington EA, Bennett MR, Fanidi A and Evan GI. (1994). *EMBO J.*, **13**, 3286–3295.
Harris AL and Nicholson S. (1988). In *Breast Cancer: Cellular and Molecular Biology*. Lippman ME and Dickson RB. (eds). Boston: Kluwer Press, pp. 93–118.
Harvey M, McArthur MJ, Montgomery CA, Butel JS, Bradley A and Donehower LA. (1993). *Nature Genet.*, **5**, 225–229.
Jaenisch R. (1976). *Proc. Natl. Acad. Sci. USA*, **73**, 1260–1264.
Jäger R, Herzer U, Schenkel J and Weiher H. (1997). *Oncogene*, **15**, 1787–1795.
Jerry DJ, Kuperwasser C, Downing SR, Pinkas J, He C, Dickinson E, Marconi S and Naber SP. (1998). *Oncogene*, **17**, 2305–2312.
Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH and Merlino GT. (1990). *Cell*, **61**, 1137–1146.
Jones JM, Attardi L, Godley LA, Laucirica R, Medina D, Jacks T, Varmus HE and Donehower LA. (1997). *Cell Growth Diff.*, **8**, 829–838.
Leder A, Pattengale PK, Kuo A, Stewart TA and Leder P. (1986). *Cell*, **45**, 485–495.
Lee LW, Raymond VW, Tsao MS, Lee DC, Earp HS and Grisham JW. (1991). *Cancer Res.*, **51**, 5238–5244.
Li B, Murphy KL, Laucirica R, Kittrell F, Medina D and Rosen JM. (1998). *Oncogene*, **16**, 997–1007.
Li M, Hu J, Heermeier K, Hennighausen L and Furth PA. (1996). *Cell Growth Diff.*, **7**, 13–20.
Liao DJ, Natarajan G, Deming SL, Jamerson MH, Johnson M, Chepko G and Dickson RB. (2000). *Oncogene*, in press.
Liscia DS, Merlo G, Ciardiello F, Kim N, Smith GH, Callahan RH and Salomon DS. (1990). *Dev. Biol.*, **140**, 123–131.
Liyanage M, Coleman A, du Manoir S, Veldman T, McCormack S, Dickson RB, Barlow C, Wynshaw-Boris A, Janz S, Wienberg J, Ferguson-Smith MA, Schröck E and Ried T. (1996). *Nature Genet.*, **14**, 312–315.
Lozano G and Liu G. (1998). *Sem. Cancer Biol.*, **8**, 337–344.
Martinez-Lacaci I, Bianco C, DeSantis M and Salomon DS. (1999). In: *Breast Cancer: Molecular Genetics, Pathogenesis, and Therapeutics*, Bowcock AM, (ed.). Humana Press: Totowa NJ, **vol 1**, pp. 31–57.
Matsui Y, Halter SA, Holt JT, Hogan BLM and Coffey RJ. (1990). *Cell*, **61**, 1147–1155.
McCormack SJ, Weaver Z, Deming S, Natarajan G, Torri J, Johnson MD, Liyanage M, Ried T and Dickson RB. (1998). *Oncogene*, **16**, 2755–2766.
McGeady ML, Kerby S, Shankar V, Ciardiello F, Salomon DS and Seidman M. (1989). *Oncogene*, **4**, 1375–1382.
Merlo GR, Basolo F, Fiore L, Duboc L and Hynes NE. (1995). *J. Cell. Biol.*, **128**, 1185–1196.

Miyashita T and Reed JC. (1995). *Cell*, **80**, 293–299.
Nass SJ, Li M, Amundadottir LT, Furth PA and Dickson RB. (1996). *Biochem. Biophys. Res. Comm.*, **227**, 248–256.
Nass SJ and Dickson RB. (1997). *Breast Cancer Res. Treat.*, **44**, 1–22.
Nass SJ and Dickson RB. (1998). *Clin. Cancer Res.*, **4**, 1813–1822.
Packham G and Cleveland JL. (1995). *Biochim. Biophys. Acta*, **1242**, 11–28.
Purdie CA, Harrison DJ, Peter A, Dobbie L, White S, Howie SEM, Salter DM, Bird CC, Wyllie AH, Hooper ML and Clarke AR. (1994). *Oncogene*, **9**, 603–609.
Salomon DS, Perroteau I, Kidwell WR, Tam J and Derynck R. (1987). *J. Cell. Physiol.*, **130**, 397–409.
Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL and Lee DC. (1990). *Cell*, **61**, 1121–1135.
Sandgren EP, Schroeder JA, Qui TH, Palmiter RD, Brinster RL and Lee DC. (1995). *Cancer Res.*, **55**, 3915–3927.
Santoni-Rugiu E, Jensen MR and Thorgeirsson SS. (1998). *Cancer Res.*, **58**, 123–134.
Schoenenberger CA, Andres AC, Groner B, van der Valk M, LeMeur M and Gerlinger P. (1988). *EMBO J.*, **7**, 169–175.
Shankar V, Ciardiello F, Kim N, Derynck R, Liscia DS, Merlo G, Langton BC, Sheer D, Callahan R, Bassin RH, Lippman ME, Hynes N and Salomon DS. (1989). *Mol. Carcinogen.*, **2**, 1–11.
Sherr CJ. (1998). *Genes Dev.*, **12**, 2984–2991.
Siegel PM and Muller WJ. (1998). In: *Hormones and Growth Factors in Development and Neoplasia*. Dickson RB and Salomon DS. (eds). Wiley-Liss: New York, pp. 397–420.
Sinn E, Muller W, Pattengale P, Tepler I, Wallace R and Leder P. (1987). *Cell*, **49**, 465–475.
Snedeker SM, Brown CF and DiAugustine RP. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 276–280.
Stewart TA, Pattengale PK and Leder P. (1984). *Cell*, **38**, 627–637.
Strange R, Li F, Saurer S, Burkhardt A and Friis RR. (1992). *Development*, **115**, 49–58.
Telang NT, Osborne MP, Sweterlitsch LA and Narayanan R. (1990). *Cell Regulation*, **1**, 863–872.
Travers MT, Barrett-Lee PJ, Berger U, Luqmani YA, Gazet JC, Powler TJ and Coombes RC. (1988). *Br. Med. J.*, **296**, 1621–1624.
Valverius EM, Bates S, Stampfer MR, Clark R, McCormick F, Salomon DS, Lippman ME and Dickson RB. (1989). *Mol. Endocrinol.*, **3**, 203–214.
Valverius EM, Ciardiello F, Heldin NE, Blondel B, Merlino G, Smith GH, Stampfer MR, Lippman ME, Dickson RB and Salomon DS. (1990). *J. Cell. Physiol.*, **145**, 207–216.
Wang JK, Johnson MD, Rosfjord EC, Jamerson MH and Dickson RB. (1999). Abstract No. 1093 Proceedings of the Annual Meeting of the AACR, Philadelphia, PA.
Weaver ZA, McCormack SJ, Liyanage M, du Manoir S, Coleman A, Schröck E, Dickson RB and Ried T. (1999). *Genes Chrom. Cancer*, **25**, 251–260.
Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ and Roussel MF. (1998). *Genes Dev.*, **12**, 2424–2433.

Oncogene (2000) 19, 1307–1317

www.nature.com/onc

Cell cycle basis for the onset and progression of c-Myc-induced, TGFα-enhanced mouse mammary gland carcinogenesis

Dezhong J Liao[1], Geraldine Natarajan[1], Sandra L Deming[1], M Hunter Jamerson[1], Michael Johnson[1,2,3], Gloria Chepko[1] and Robert B Dickson*[,1,3,4]

[1]*Vincent T Lombardi Cancer Center, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20007, USA;* [2]*Department of Pathology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20007, USA;* [3]*The Department of Oncology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20007, USA;* [4]*Department of Cell Biology, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington DC 20007, USA*

Using single and double transgenic mouse models, we investigated how c-Myc modulates the mammary epithelial cell cycle to induce cancer and how TGFα enhanced the process. In c-*myc* transgenic mice, c-*myc* expression was high in the hyperplastic mammary epithelium and in the majority of tumor areas. However, the tumors displayed focal areas of low expression of c-*myc* but high rates of proliferation. In contrast to E2F1 and cyclin A2, which were induced and co-localized with c-*myc* expression, induction of cyclins D1 and E occurred only in these tumor foci. Overexpression of cyclin D1 also occurred in the hyperplastic epithelium of *tgfα*-single and *tgfα*/c-*myc*-double transgenic mice. In *tgfα*/c-*myc* tumors, cells positive for cyclins D1 and E were randomly spread, without showing a reciprocal relationship to c-*myc* expression. In contrast to c-*myc* tumors, most *tgfα*/c-*myc* tumors showed undetectable levels of retinoblastoma protein (pRB), and the loss of pRB occurred in some cases at the mRNA level. These results suggest that E2F1 and cyclin A2 may be induced by c-Myc to mediate the onset of mammary cancer, whereas overexpression of cyclins D1 and E may occur later to facilitate tumor progression. TGFα may play its synergistic role, at least in part, by inducing cyclin D1 and facilitating the loss of pRB. *Oncogene* (2000) **19,** 1307–1317.

Keywords: c-Myc; TGFα; E2F; cyclins; pRB; cell cycle

Introduction

The c-Myc protein plays a crucial role in cell proliferation, differentiation, apoptosis, and transformation (Schmidt, 1999; Facchini *et al.*, 1998; Amati *et al.*, 1998; Dang, 1999). Overexpression, amplification, or rearrangement of the c-*myc* gene has been reported in over 50% of human breast cancer cases (Nass *et al.*, 1997; Amundadottir *et al.*, 1996a). About half of the virgin female mice carrying the c-*myc* transgene under control of mouse mammary tumor virus (MMTV) long terminal repeat also develop spontaneous mammary carcinomas after 9 months of age (Stewart *et al.*, 1984; Amundadottir *et al.*, 1995, 1996b). c-Myc-induced carcinogenesis may be further promoted by additional growth stimuli such as some female sex hormones, since multiple pregnancies markedly increase its incidence and shorten its latency period (Stewart *et al.*, 1984; Amundadottir *et al.*, 1995, 1996b).

One major mechanism for c-Myc to exert its functions involves its action as a transcription factor, heterodimerizing with Max and binding to the Myc E-box elements of its target genes (Cole *et al.*, 1999). Thus, cdc25A and cyclins E and A2 have been suggested as direct, c-Myc-activated target genes (Cole *et al.*, 1999; Obaya *et al.*, 1999). In contrast, the relationship between c-Myc and cyclin D1 is still under debate in the literature (Facchini *et al.*, 1998; Dang, 1999). The 5′-flanking region of the cyclin D1 gene in mouse and human contains a c-Myc recognition site (Daksis *et al.*, 1994), and expression of cyclin D1 has been shown to be induced in some c-*myc*-expressing tumor cells (Facchini *et al.*, 1998; Dang, 1999), in liver tissue, and in liver tumors from mice carrying a c-*myc* transgene under the control of the albumin gene promoter (Santoni-Rugiu *et al.*, 1998). These data seem to suggest that cyclin D1 may be a direct target of activation by c-Myc. However, it has also been shown in other systems that c-Myc suppresses transcription of cyclin D1 (Philipp *et al.*, 1994; Jansen-Durr *et al.*, 1993; Marhin *et al.*, 1996). Still other studies suggest that cyclin D1 is not a target of c-Myc-signaling but represents a pathway parallel to c-Myc signaling for control of cell replication (Roussel, 1998; Bodrug *et al.*, 1994; Alexandrow *et al.*, 1998; Solomon *et al.*, 1995). Nevertheless, these four putative c-Myc targets (cdc25A, cyclins E, A2 and D1) can function to activate cyclin dependent kinases (cdk) 4, 6 or 2 during G1 and S phases, resulting in phosphorylation of the retinoblastoma protein (pRB). pRB-associated transcription factors, of which E2F1 is the most important, are thus released and activated (Morgan, 1995; Sherr, 1996). Free E2F1 activates transcription of genes required for S phase entry and progression (Johnson *et al.*, 1998; Lavia *et al.*, 1999).

Voluminous literature has causally connected cancer onset and progression to abnormal expression or gene structure (amplification or mutation) of cyclins D1, E, and A2, as well as cdk inhibitors p16, p27, and p21 (or its key regulator, p53) (Morgan, 1995; Sherr, 1996; Gray-Bablin *et al.*, 1996; Keyomarsi *et al.*, 1993; Steeg *et al.*, 1998; Barnes *et al.*, 1998). Each of these genes encodes a protein controlling a step(s) along the cyclin-cdk-pRB pathway, alteration in which presumably results in an increase in free, active E2F1 or other

*Correspondence: RB Dickson, Research Building, W417, 3970 Reservoir Road NW, Lombardi Cancer Center, Georgetown University Medical Center, Washington DC 20007, USA
Received 20 September 1999; revised 9 December 1999; accepted 20 December 1999

E2F family members. This implies that E2F1 plays a central role in cancer development (Johnson *et al.*, 1998). Overexpression of E2F1 is an intriguing mechanism for its activation in the context of c-Myc-induced carcinogenesis, since E2F1 expression has been shown to be induced in liver from c-*myc* transgenic mice (Santoni-Rugiu *et al.*, 1998) and in fibroblasts transfected with the c-*myc* gene (Leone *et al.*, 1997).

Transforming growth factor α (TGFα) is a strong mitogen for a variety of cell types (Lee *et al.*, 1995; Dickson *et al.*, 1995) and is overexpressed in over 50% of breast cancer cases (Auvinen *et al.*, 1996; Pilichowska *et al.*, 1997; Panico *et al.*, 1996). Virgin female mice carrying a *tgfα* transgene under control of the MMTV or metallothionein-1 (MT) promoters develop mammary epithelial hyperplasia, but not mammary cancer, unless the mice undergo multiple pregnancies (Sandgren *et al.*, 1990; Jhappan *et al.*, 1990; Matsui *et al.*, 1990). However, dual carriers of c-*myc* and *tgfα* transgenes, generated in our laboratory by mating the MMTV-c-*myc* strain to the MT-*tgfα* strain, develop mammary cancers at 100% incidence, in both females and males, soon after 2 months of age. In addition, the tumors grow much faster than those occurring in the c-*myc* single transgenic strain (Amundadottir *et al.*, 1995, 1996b). These data demonstrate that TGFα overexpression strikingly enhances c-Myc-induced carcinogenesis (Sinn *et al.*, 1987), in line with the *in vitro* studies showing that co-transfection of cells with *tgfα* and c-*myc* effectively induces transformed phenotype, in contrast to transfection of either gene alone (Amati *et al.*, 1998; Land *et al.*, 1983). The mechanisms for this synergistic influence of TGFα are not yet fully clarified. With respect to the interactions of these two proteins at the cell cycle level, one possibility is that the synergistic role of TGFα is exerted via the Ras/Raf cascade, a major TGFα signaling pathway (Lee *et al.*, 1995), since overexpression of c-Ras^H has been shown to increase the c-Myc protein levels (Kerkhoff *et al.*, 1998; Sears *et al.*, 1999). Also, co-expression of c-Myc and activated c-Ras^H, but not either gene alone, is able to transform cells in culture (Amati *et al.*, 1998; Land *et al.*, 1983). However, the Ras/Raf pathway seems to recruit cyclin D1 as a major step (Lukas *et al.*, 1996), whereas synergy between c-Ras^H and c-Myc has been shown in fibroblasts to be elicited via induction of E2F1 and activation of cyclin E-cdk2, without affecting either cyclin D1 activity or pRB phosphorylation (Leone *et al.*, 1997).

By using three transgenic mouse models, in this study we set out to explore the cell cycle regulatory mechanisms whereby c-Myc elicits mouse mammary tumors and to determine how TGFα synergistically modulates these mechanisms. We found that in c-*myc* transgenic mice, induction of cyclin A2 and E2F1 were most closely associated with expression of the c-*myc* transgene and might thus mediate tumor onset. In contrast, overexpression of cyclins D1 and E occurred as later events in morphologically distinctive, rapidly growing, poorly apoptotic foci within established c-*myc* tumors. In our synergistic, bi-transgenic model, TGFα appeared to immediately induce cyclin D1 and to cooperate with c-Myc to attenuate the levels of pRB protein. We propose that these two TGFα-mediated effects may be associated with the earlier onset and faster growth of the mammary cancer in the bi-transgenic model.

Results

Morphologic characteristics of mammary tumors

In MT-*tgfα* transgenic mice, mammary glands showed hyperplasia, but without tumor formation. As observed also by others (for review see Cardiff *et al.*, 1995), the mammary tissue contained abundant, proliferating stroma. In marked contrast, the hyperplastic mammary tissue from MMTV-c-*myc* animals did not show pronounced stromal proliferation. Stromal cells were also abundant in hyperplastic mammary tissue and mammary carcinomas from bi-transgenic *tgfα*/c-*myc* mice. The epithelial cells in non-tumor areas of the mammary glands from bi-transgenic mice usually manifested atypical hyperplastic features that were similar to the morphology of tumor cells. Thus, there was no clear-cut evidence for pre-malignant stages of this tumor type.

In c-*myc* transgenic animals, about half of the relatively larger (1 cm or larger in diameter) tumors

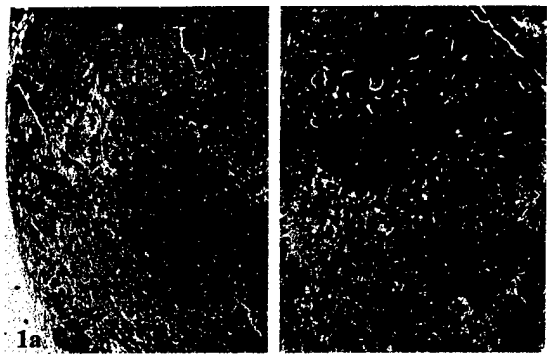


Figure 1 Hematoxylin-eosin staining of mammary tumors from two c-*myc* animals, showing three individual foci (F1, F2 and F3) within the tumors. Some areas of the foci show infiltrating growth into the adjacent tumor areas (arrow). Necrosis (N) can be discerned in focus 2

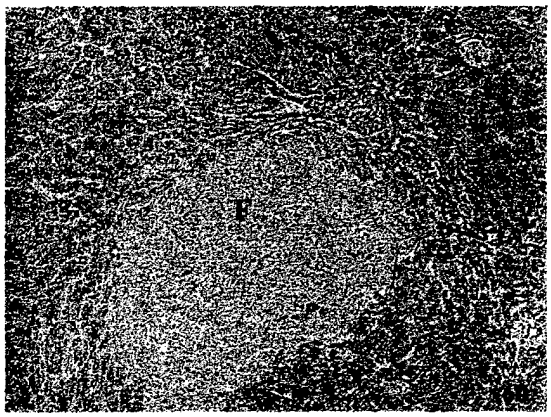


Figure 2 Tunel staining of a tumor from a c-*myc* animal, showing that apoptotic cells (dark brown staining) are distributed predominantly in the major tumor area, but rarely in the focus

contained foci that consisted of tumor cells with distinctive morphology. Specifically, tumor cells within the foci were characterized by larger nuclei and weaker staining for hematoxylin and eosin (Figure 1a,b). Although this 'tumor within a tumor' showed a clear boundary of demarcation from surrounding tumor areas, it was not encompassed by a connective tissue capsule. Usually, some portion of each focus exhibited

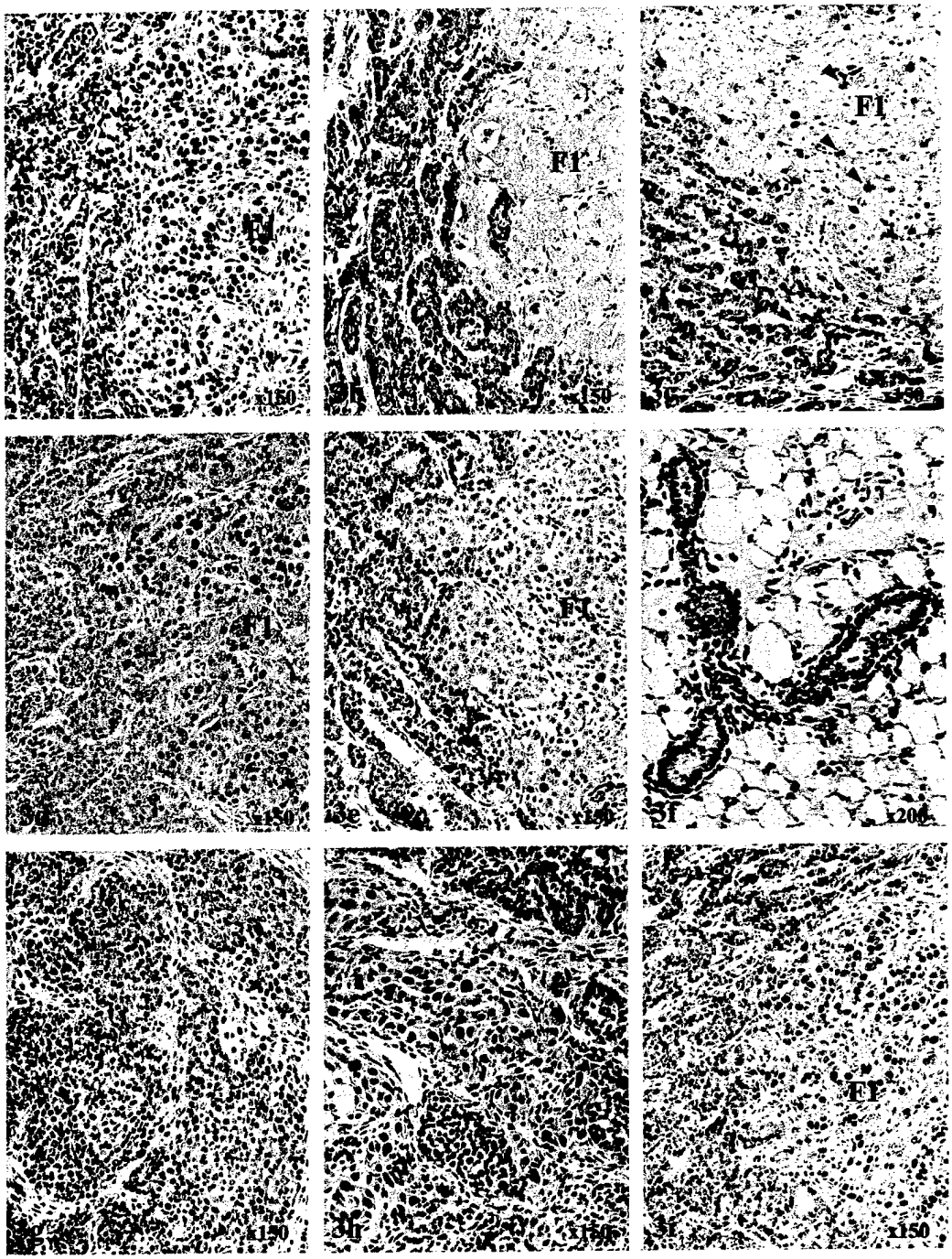


Figure 3 Immunohistochemical staining (brown color) with light hematoxylin counter-staining (blue color). Staining shown in (**a**–**e**) and (**i**) was carried out on serial sections of the same tumor and tumor focus (F1) as shown in Figure 1 (**a**). Tumor cells inside focus 1 show stronger staining for PCNA than cells outside the focus (**a**). Most tumor cells outside, but not inside, the focus exhibit strong staining for c-Myc (**b**) and cyclin A (**c**), although some stromal cells inside the focus are also positive for cyclin A (arrows). Conversely, most tumor cells inside the focus exhibit strong staining for cyclin D1 (**d**) and cyclin E (**e**), while tumor cells outside the focus are negative. Many cells in the hyperplastic mammary gland from a *tgfα* animal (**f**) and in a tumor from a *tgfα*/*c-myc* animal (**g**) also show strong cyclin D1 staining. In a *c-myc* tumor, some cyclin E-positive cells show a trend of penetrating (from up-left side) into the adjacent, cyclin E-negative area (low-right side) (**h**). Staining for cdk4 (**i**) is more intense in many tumor cells inside the focus than those in the adjacent area

infiltration into the adjacent, surrounding tumor areas (Figure 1). Necrotic areas were occasionally seen inside the foci (Figure 1b). Very strikingly, apoptotic cells within each focus were much less frequent than in the surrounding tumor areas. When foci were observed, their numbers varied between two and four in each random cross-section and their sizes varied from microscopic to about 3 mm in diameter for the animal ages of 10–12 months. The foci were not seen in tumors less than 1 cm in diameter, indicating that they might have occurred selectively at relatively advanced progression states. No such specific foci were observed in tumors from *tgfα*/c-*myc* double transgenic mice.

Assessment of cell proliferation and apoptosis

In c-*myc* tumors, PCNA staining was more intense in the specific foci than in their surrounding tumor areas (Figure 3a). The staining in *tgfα*/c-*myc* tumors was as intense as in the c-*myc* tumor foci. Moreover, the staining index for the c-*myc* tumor foci ($39.1\% \pm 3.4$) was higher than that for their surrounding tumor tissue ($20.4\% \pm 4.0$, $P<0.01$), but it was comparable to that for *tgfα*/c-*myc* tumors ($44.4\% \pm 4.2$, $P>0.05$). Hyperplastic mammary glands from all three lines of transgenic animals also showed some strongly stained cells, but the fraction was too small to allow calculation of a reliable index. PCNA-positive cells were not observed in mammary glands from the normal, non-transgenic animals.

In contrast to the PCNA staining results, the TUNEL assay for apoptotic cells showed a much higher labeling index in the major areas ($15.8\% \pm 1.8$) than in the foci ($1.0\% \pm 1.1$, $P<0.01$) of c-*myc* tumors (Figure 2). The TUNEL labeling index in the *tgfα*/c-*myc* tumors ($1.7\% \pm 1.1$) was comparable to that in the foci of c-*myc* tumors ($P>0.05$).

Expression of c-myc

Consistent with the data reported previously (Amundadottir *et al*., 1995), c-*myc* mRNA was abundantly expressed in hyperplastic mammary epithelium (Figure 4a) and in tumors (Figure 4b) from c-*myc* mice, but was undetectable in normal mammary tissue from age-matched, non-transgenic animals. A sense probe did

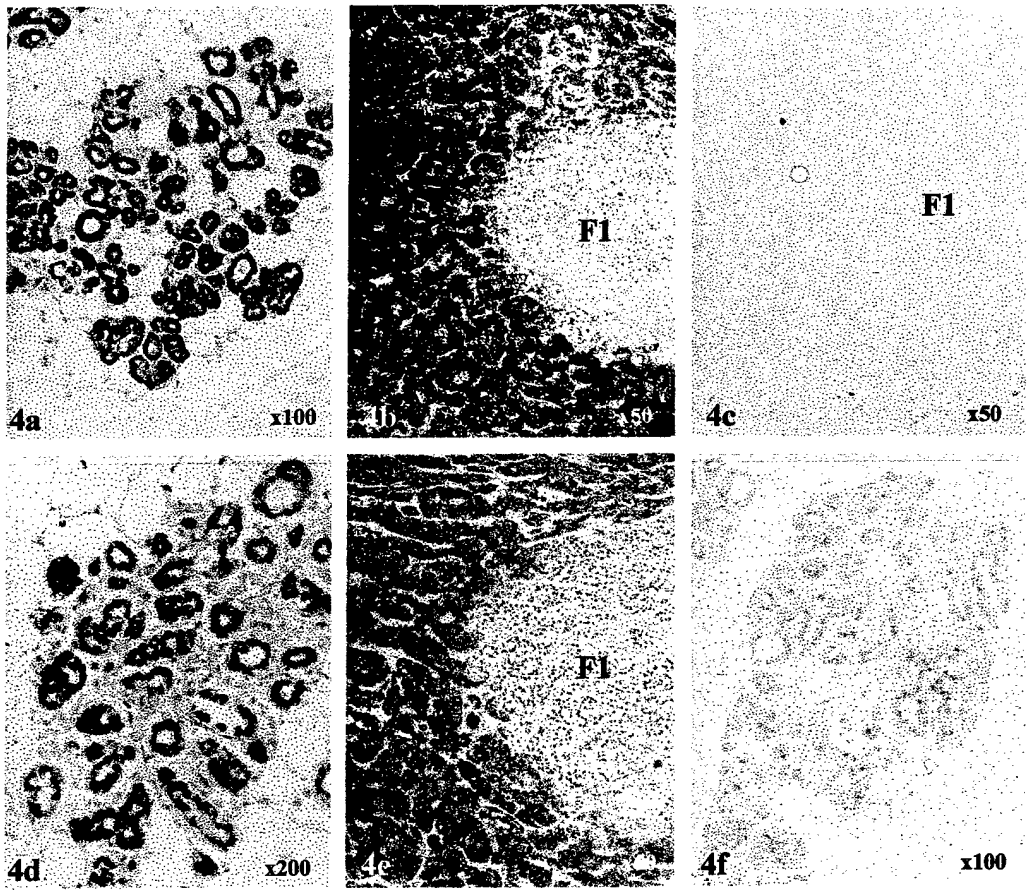


Figure 4 Nonradioactive *in situ* hybridization for c-*myc* (**a**–**c**) and *e2f1* (**d**–**f**). Hybridizations shown in (**b**), (**c**), and (**e**) were carried out on serial sections of the same tumor and tumor focus (F1) as shown in Figure 1 (**a**). In c-*myc* animals, high levels of c-*myc* mRNA were detected by antisense probe in hyperplastic mammary glands (**a**) and in the major tumor area, but not the tumor focus (**b**). No signal was detected in the same tumor area when a sense probe was used (**c**). High levels of *e2f1* mRNA expression were detected by an antisense probe (**d**) in hyperplastic mammary glands from a c-*myc* animal. The *e2f1* mRNA expression co-localizes with c-*myc* expression in the same tumor as shown in 2(**b**) (**e**). No signal was detected in the hyperplastic mammary glands from c-*myc* animal when an *e2f1* sense probe was used (**f**)

not give rise to a signal in any of these tissues (Figure 4c), demonstrating that the signal detected by the antisense probe is specific for the c-*myc* mRNA. The foci in c-*myc* tumors showed very low levels of its expression (Figure 4b), in strong contrast to their adjacent areas with high levels of c-*myc* mRNA. Immunohistochemical results also showed a much stronger positive staining in the major tumor areas than in the foci (Figure 3b). Western blot analyses revealed much higher levels of c-Myc protein in mammary tumors, compared to hyperplastic mammary tissue (Figure 5). However, this difference was due largely to the heterogeneity in cellularity, as the mammary tissues used for protein sample preparation were fat pads dominated by fat tissue. Hyperplastic epithelium and tumors from *tgfα*/c-*myc* animals also expressed high levels of c-*myc* mRNA and protein, while expression of c-*myc* was not detected in the hyperplastic epithelium from *tgfα* animals.

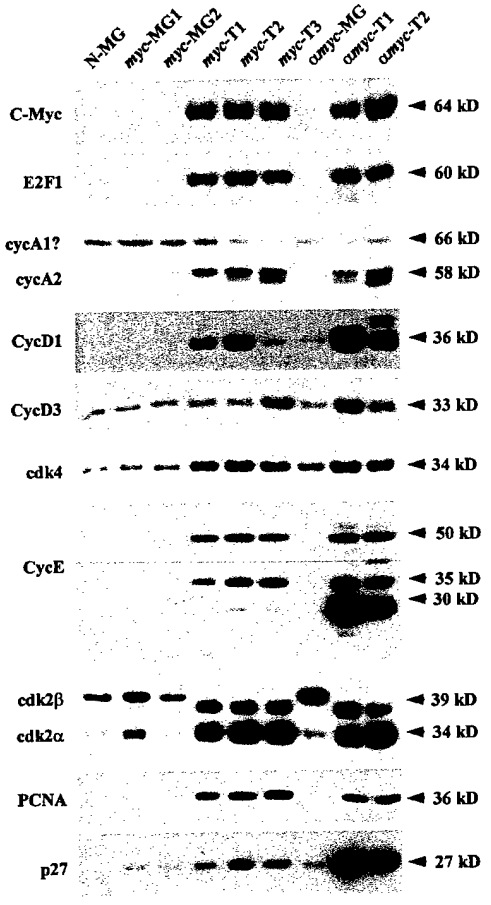


Figure 5 Western blot analyses for size comparisons of various proteins. N-MG: protein samples from normal mammary tissue pooled equally from three nontransgenic animals; *myc*-MG: hyperplastic mammary tissue from two individual c-*myc* animals; *myc*-T: three representative mammary tumors from c-*myc* animals; *αmyc*-MG: hyperplastic mammary gland tissue pooled from two individual *tgfα*/c-*myc* animals; *αmyc*-T: two representative tumors from *tgfα*/c-*myc* animals. Quantitative comparisons among different samples may not be made, as tumor tissues enriched in protein whereas non-tumor tissues were dominated by protein-poor fat tissue

Expression of E2F1

An *e2f1* antisense probe detected strong signals in the hyperplastic mammary epithelium, from both c-*myc* (Figure 4d) and *tgfα*/c-*myc* animals, but not in that from *tgfα* transgenic mice, suggesting that the induction of *e2f1* mRNA was specifically related to expression of c-Myc, but not TGFα. In tumors from c-*myc* animals, the major areas with high levels of c-*myc* mRNA and protein also expressed high levels of *e2f1*, whereas those specific tumor foci with low expression levels of c-*myc* exhibited very low levels of *e2f1* (Figure 4e), indicating that expression of *e2f1* and c-*myc* are co-localized. High levels of *e2f1* expression were also detected in *tgfα*/c-*myc* tumors (Table 1). The sense probe did not give rise to signal (Figure 4f). Northern blot analysis detected the expected two *e2f1* transcripts (Li *et al.*, 1994) in c-*myc* and *tgfα*/c-*myc* tumors (Figure 6). Western blot analysis also confirmed high levels of the E2F1 protein in these tumors (Figure 5). Immunohistochemical staining on paraffin-embedded tissues was not successful with this, nor with other antibodies.

Expression of cyclin A2

Immunohistochemistry for cyclin A2 showed that in c-*myc* tumors, positive tumor cells were localized mainly to the major areas with high levels of c-Myc (Figure 3c), indicating that expression of cyclin A2 and c-*myc* may be co-localized. Many positive cells were also discerned in the hyperplastic epithelium from c-*myc* animals, as well as in the atypical hyperplastic epithelium and tumors from *tgfα*/c-*myc* mice, but not in the epithelium

Table 1 Relationship among expression of c-Myc and of cell cycle components in c-*myc* and *tgfα*/c-*myc* tumors

	c-myc *tumors*		tgfa/c-myc
	Tumor cells with high levels of c-Myc	*Focal tumor cells with low levels of c-Myc*	*tumor with high levels of c-Myc*
E2F1	+++*	−	+++
Cyclin A	++	−	++
Cyclin D1	−	+++	+++
Cyclin E	−	+++	+++
Cdk2	++	++	++
Cdk4	+	+++	+++
Cyclin D3	+	+++	+++
PCNA	+	+++	+++
P16	+	+	+
P21	++	++	++
P27	++	++	+++

*Expression levels of the genes are subjectively grouped from three '+' to '−' in order from the strongest positive to the most negative

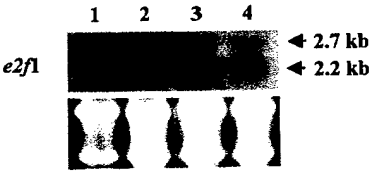


Figure 6 Northern blot analysis of the *e2f1* gene, demonstrating that *e2f1* mRNA was expressed in two randomly selected tumors from c-*myc* animals (1 and 2) and *tgfα*/c-*myc* animals (3 and 4). Loading of total RNA (10 μg per lane) was visualized by ethidium bromide staining of the gel (lower panel)

from *tgfα* animals (data not shown). On Western blot, cyclin A2 protein, at about (~) 58 kD was detected in c-*myc* and *tgfα*/c-*myc* tumors (Table 1), but not in hyperplastic mammary tissues (Figure 5). The antibody also recognized a protein at ~66 kD that, in contrast, was mainly present in normal and hyperplastic mammary tissue. This protein is likely to be cyclin A1, a newly identified member of the cyclin A family (Sweeney *et al.*, 1996; Yang *et al.*, 1997), as the peptide used for generating the antibody differs from the corresponding sequence of cyclin A1 by only a few amino acid residues. This protein might contribute to the immunohistochemical staining of some stromal cells in the major tumor areas and in the foci of c-*myc* tumors (Figure 3c, arrows), since similarly positive cells were also observed in normal mammary tissue from non-transgenic mice in which only the ~66 kD protein was detected by Western blot (Figure 5).

Expression of cyclin D1

In c-*myc* animals, cyclin D1-positive cells could be observed only in tumors, not in hyperplastic mammary epithelium. In the tumors, the cyclin D1-positive cells were exclusively localized to the specific foci with low levels of c-Myc, but not in the major areas with high levels of c-Myc (Figure 3d), suggesting that expression of cyclin D1 and c-Myc may be reciprocal. In contrast to the c-*myc* tumors, a large number of tumor cells in *tgfα*/c-*myc* animals manifested strong staining of cyclin D1 (Table 1), and they were randomly spread within the whole tumor, without forming any specific focus, nor showing reciprocal expression to c-*myc* (Figure 3g). Many cells in the hyperplastic mammary epithelium from *tgfα* (Figure 3f) and *tgfα*/c-*myc* mice also exhibited strong staining of cyclin D1, indicating that cyclin D1 expression might be induced by TGFα in the epithelium prior to tumor formation. Western blot analysis confirmed the presence of high levels of cyclin D1 protein in tumors from c-*myc* and *tgfα*/c-*myc* mice (Figure 5).

Expression of cyclin E

In c-*myc* tumors, cyclin E-positive cells were found to be co-localized with cyclin D1, exclusively in the specific focal lesions, but not the major areas (Figure 3e). Moreover, cyclin E-positive cells usually showed a trend for penetration into the adjacent areas (Figure 3h), indicating that they might have a stronger invasive potential. In *tgfα*/c-*myc* tumors, cyclin E-positive cells were randomly spread throughout the whole tumor (Table 1), without forming specific foci, similar to the distribution of cyclin D1-positive cells. However, at the subcellular level, the cyclin E staining was localized in both nucleus and cytoplasm, unlike the solely nuclear staining seen in c-*myc* tumors. Hyperplastic epithelium from *tgfα* and *tgfα*/c-*myc* mice was negative or weakly positive for cyclin E in some cells, indicating that cyclin E was not significantly induced by TGFα alone.

On Western blot (Figure 5), cyclin E proteins in c-*myc* and *tgfα*/c-*myc* tumors were present, not only as the full-length form of ~50 kD, but also as several smaller isoforms, as reported by others for breast cancer tissue and for cell lines derived from human and mouse (Gray-Bablin *et al.*, 1996; Keyomarsi *et al.*, 1993; Said *et al.*, 1995; Sgambato *et al.*, 1996). Interestingly, an ~28 kD, putative cyclin E protein was the dominant isoform in *tgfα*/c-*myc* tumors; this isoform was barely discernible in c-*myc* tumors. This cyclin E isoform may thus account for the cytoplasmic staining seen in *tgfα*/c-*myc* tumor cells.

Expression of cdk4, cyclin D3, and cdk2

Many cells in the mammary epithelium from non-transgenic mice and from c-*myc*, *tgfα*, and *tgfα*/c-*myc* animals were positive for cdk4 by immunohistochemical staining. In c-*myc* tumors, cdk4 positive cells were observed both in the cyclin D1-positive foci and in the major areas that were cyclin D1-negative, but the staining intensity was stronger in many cells within the foci (Figure 3i). No obvious differences in the staining were observed between c-*myc* tumors and *tgfα*/c-*myc* tumors (Table 1). Similar immunohistochemical data were obtained for cyclin D3 (Table 1). Western blot analyses also detected the cdk4 and cyclin D3 proteins in these tumors and in mammary tissues from non-transgenic or various transgenic animals (Figure 5).

Immunohistochemical staining for cdk2 did not reveal differences among various mammary tissues and tumors. In c-*myc* tumors, both the major areas and the specific foci showed similar staining intensity (Table 1). Western blot assay for cdk2 detected both cdk2α at ~34 kD and cdk2β at ~39 kD, respectively (Kwon *et al.*, 1998; Kotani *et al.*, 1995; Noguchi *et al.*, 1993). In mouse, rat and hamster, the cdk2β is an alternate RNA splicing form of cdk2α, the classic cdk2, with an insert of 48 amino acids between amino acids 196 and 197 of cdk2α. The cdk2α isoform occurred as a single band in normal and hyperplastic mammary tissue, as well as in tumors, and was thus likely to be the inactivated, unphosphorylated form (Gu *et al.*, 1992; Planas-Silva *et al.*, 1997). Cdk2β, on the other hand, was present mainly as the phosphorylated, activated, faster-migrating form (Gu *et al.*, 1992; Planas-Silva *et al.*, 1997) in c-*myc* and *tgfα*/c-*myc* tumors, but it occurred mainly as the inactivated, unphosphorylated slower-migrating band in normal and hyperplastic mammary tissues (Figure 5).

Expression of cdk inhibitors

Immunohistochemical staining for p16 and p21 did not show pronounced differences between c-*myc* tumors and *tgfα*/c-*myc* tumors, and between the tumor foci and their surrounding areas in the c-*myc* tumors (Table 1). Western blot analyses of these two cdk inhibitors did not detect differences between c-*myc* tumors and *tgfα*/c-*myc* tumors (data not shown). However, the levels of p27 were higher in *tgfα*/c-*myc* tumors than in c-*myc* tumors, as measured by both immunohistochemical and Western blot analyses (Table 1 and Figure 5).

Expression of pRB protein

Protein levels of pRB varied among tumor samples but they were generally higher in c-*myc* than in *tgfα*/c-*myc* tumors; pRB levels in some representative samples are shown in Figure 7. The pRB protein was detectable by Western blot in all ten c-*myc* tumors studied; however, it was present in only two of eight *tgfα*/c-*myc* tumors.

pRB was present mainly as the hypophosphorylated form (Ezhevsky *et al.*, 1997). The faster-migrating, unphosphorylated band (c-*myc* tumor samples 1 and 4 in Figure 6) and its slower-migrating, hyperphosphorylated band of pRB (c-*myc* tumor samples 1 and 2 in Figure 6) could be discerned faintly in some samples, when the autography was exposed for such a short time that signals could not be detected in other samples. Two different pRB monoclonal antibodies (pRB14001A and pRB245) gave the same results by Western blot. However, immunohistochemical staining was not successful with either of these antibodies.

RT–PCR analysis was carried out for four *tgfα*/c-*myc* tumors and for three c-*myc* tumors, where a sufficient amount of tissue was available for RNA preparation. As shown in Figure 8, the Rb cDNA was detected in all three c-*myc* tumors and in the two *tgfα*/c-*myc* tumors that also showed detectable levels of pRB protein (Figure 7, samples 2 and 5), but not in the other two *tgfα*/c-*myc* tumors. The failure of the cDNA amplification in these two tumors was not due to a technical problem, since GAPDH cDNA, included as an internal control, was amplified normally (Figure 8).

Discussion

In this study we show that in c-*myc* transgenic mice, expression of cyclin A2 and E2F1 co-localizes with that of c-*myc* in hyperplastic mammary gland and in primary mammary tumors. Thus, we propose that these two genes may be induced either directly or indirectly by c-Myc to mediate the tumor onset. In support of this hypothesis, overexpression of cyclin A2 or E2F1 has been shown to directly facilitate transformation of cultured cells and to cause tumorigenesis in animals (Desdouets *et al.*, 1995; Amati *et al.*, 1998). Overexpression of each of these proteins has been reported in the pre-malignant liver tissue and spontaneous liver tumors in c-*myc* transgenic mice (Santoni-Rugiu *et al.*, 1998). Transfection of fibroblasts with c-*myc* has also been shown to induce *e2f1*, which is independent of pRB phosphorylation (Leone *et al.*, 1997), indicating that this effect may result directly from increased E2F1 protein, a short-cut mechanism that bypasses the cyclin-cdk-pRB pathway. In addition, since in c-*myc* tumors pRB is mainly in the hypophosphorylated state and presumably binds to and inactivates a portion of increased E2F1, cyclin A2 may be a more active element than E2F1 in cell proliferation and transformation. The short-cut mechanism and the rise of cyclin A2, which acts later in the cell cycle than cyclins D1 and E, may partly explain why overexpression of cyclins D1 and E does not occur in the majority of hyperplasia and primary tumor cells.

The observation of a reciprocal expression of c-*myc* and cyclin D1 in c-*myc* tumors is the first evidence *in vivo* that favors, but does not prove, the concept that constant expression of c-Myc may suppress expression of cyclin D1. Several studies have shown that stable expression of cyclin D1, such as in mammary epithelial cells, paradoxically shortens the G1 phase and prolongs the S phase, while inhibiting growth and transformation to a malignant phenotype as the net consequence (Han *et al.*, 1995; Quelle *et al.*, 1993; Philipp *et al.*, 1994). Thus, it cannot be excluded that c-Myc suppresses expression of cyclin D1 in order to ensure a quicker completion of the cell division cycle and a more rapid onset of malignant transformation (Marhin *et al.*, 1996). However, once a tumor is formed, overexpression of cyclin D1 may be of selective value for its further progression to more aggressive phenotypes; a drop in the level of c-Myc could potentially facilitate release of repression of cyclin D1, while simultaneously decreasing c-Myc-induced apoptosis. This conjecture is supported by the decreased TUNEL labeling index in the progressed foci within primary c-*myc* tumors. This progression hypothesis (Figure 9) may explain why cyclin D1-positive cells are not seen in the hyperplastic lesions and in small tumors, but instead they occur in the foci within established c-*myc* tumors in association with enhanced staining of PCNA.

It should be noted that in human breast cancer samples, cyclin D1 overexpression is associated with immunohistochemical positivity for estrogen receptor (ER) (Barnes *et al.*, 1998). Interestingly, although ER positivity is, in general, considered a good prognostic marker, those ER positive cells that concomitantly overexpress cyclin D1 can continue to proliferate in the presence of anti-estrogens (Wilcken *et al.*, 1997). Although initially paradoxical, this is now not surprising, as cyclin D1 is known to form a direct complex with ER, allowing the complex to activate transcription without the need for estrogen (Neuman *et*

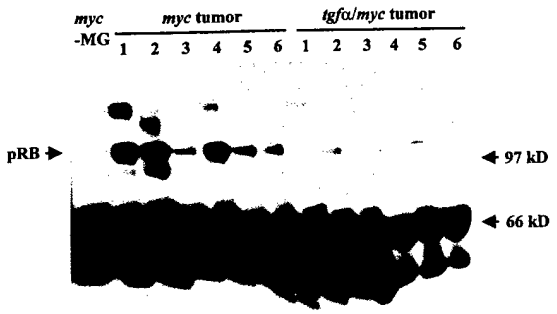


Figure 7 Western blot analysis of pRB. Eighty μg protein samples from hyperplastic mammary tissue were pooled equally from three individual c-*myc* animals (*myc*-MG) and from six individual c-*myc* tumors or *tgfα*/c-*myc* tumors and were loaded into the gel. Levels of the pRB at ~110 kD were generally higher in c-*myc* tumors than in *tgfα*/c-*myc* tumors. Two additional proteins at ~66 kD and ~55 kD were also recognized by (pRB14001A), levels of which were also slightly lower in some *tgfα*/c-*myc* tumors than in c-*myc* tumors

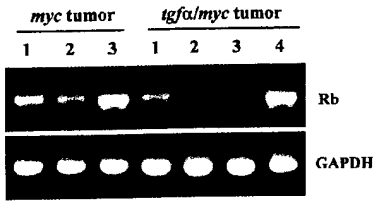


Figure 8 RT–PCR analysis of expression of Rb mRNA and GAPDH. Total RNA samples from three c-*myc* tumors and four *tgfα*/c-*myc* tumors were reverse-transcribed (RT), and the cDNA products were amplified by PCR, using the second pair of the primers described in Materials and methods. Note that two *tgfα*/c-*myc* tumors lack detectable expression of the Rb mRNA, while the GAPDH mRNA was expressed normally

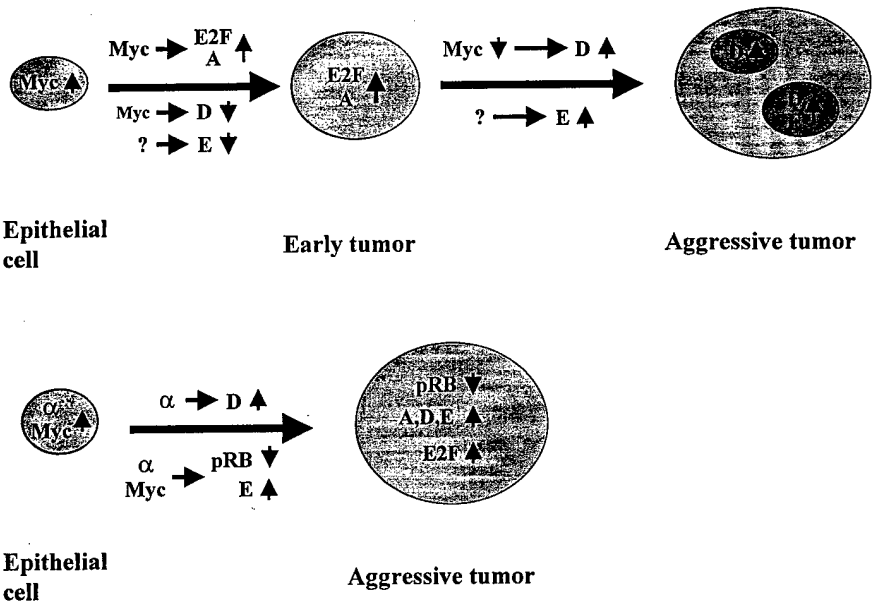


Figure 9 Illustration of hypothesis. In c-*myc* transgenic mice, constant overexpression of c-Myc protein in mammary epithelial cells directly or indirectly induces accumulation of E2F1 and cyclin A2 (A) to mediate tumor onset (upper panel). While the developing tumor continues to progress, a decrease in c-Myc expression occurs in some tumor cells, resulting in decreased apoptosis and in the overexpression of cyclin D1 (D). Cyclin E (E) overexpression is also triggered through an unknown mechanism. Each of these specific tumor cells then proliferates more aggressively to form a focus with distinct morphology. In the epithelial cells from *tgfα*/c-*myc* dual transgenic mice (lower panel), TGFα (α) induces overexpression of cyclin D1 and cooperates with c-Myc to induce overexpression of cyclin E and sporadic loss of pRB. These effects, together with the c-Myc-induced elevation of E2F1 and cyclin A2, elicit early onset of very aggressive tumor phenotypes in the bi-transgenic model

al., 1997). These observations, together with our progression hypothesis, may partly explain why about one-third of the ER-positive cases are refractory to antiestrogen therapy, why most of those who originally respond to antiestrogen later develop antiestrogen resistance (Lykkesfeldt, 1996), and why amplification of *cyclin D1* is associated with early relapse in patients with ER-positive breast cancer (Seshadri *et al.*, 1996).

In contrast to c-*myc* animals, in *tgfα* and *tgfα*/c-*myc* mice, overexpression of cyclin D1 is initially observed in the atypical hyperplastic mammary epithelium, indicating that cyclin D1 is induced by TGFα prior to the tumor onset. In *tgfα*/c-*myc* mice, this TGFα-induced cyclin D1 may have a twofold functionality (Figure 9). First, it may facilitate the early events (initiation and/or promotion) of the carcinogenic process, resulting in an earlier onset of tumors, when compared to single transgenic c-*myc* mice (Figure 9). Second, it may also contribute to the formation of a much faster-growing tumor phenotype, similar to what is discerned in the cyclin D1-positive foci within c-*myc* tumors. Moreover, in *tgfα*/c-*myc* mice the effect of TGFα on induction of cyclin D1 seems to override the suppression of cyclin D1 by c-Myc. This implies that TGFα and c-Myc may each regulate cyclin D1 as one step of their signaling pathways, and that cyclin D1 serves a pivotal role that links these two separate pathways. Cyclin D3 may not share this crucial property, as it is expressed not only in the tumor foci but also in the major tumor areas.

Expression of cyclin E in c-*myc* tumors is also reciprocal to that of c-Myc. This is surprising, as suppression of cyclin E by c-Myc has not been reported, and relevant literature suggests that c-Myc can activate expression of cyclin E *in vitro* (Amati *et al.*, 1998; Obaya *et al.*, 1999). Several studies have suggested that cyclin D1/cdk4 should be activated prior to the onset of cyclin E/cdk2 activity in order to ensure an orderly transition to S phase (Obaya *et al.*, 1999; Prall *et al.*, 1998). Thus, it is possible that the lack of a sufficient amount of cyclin D1 may hamper the expression of cyclin E. It is even possible that prevention of expression of cyclin E may facilitate the cell growth during the early stages of the carcinogenic process in c-*myc* animals, as it has been shown that stable overexpression of cyclin E, rendered by cDNA transfection, inhibits growth of mammary epithelial cells (Sgambato *et al.*, 1996). However, similar to what we have discussed for cyclin D1, once a tumor is formed, cyclin E overexpression may be required for its further progression to more aggressive phenotypes (Figure 9), as suggested by the observation that cyclin E-positive cells show a trend for more rapid proliferation and for penetration into their adjacent tumor areas. Additional support for this hypothesis is provided by the observation that the more-aggressive *tgfα*/c-*myc* tumors exhibit overexpression of cyclin E as well. The overexpression of cyclin E may result from a synergy between TGFα and c-Myc, because expression of cyclin E is not pronounced in the hyperplastic epithelium from either *tgfα* or c-*myc* animals. This hypothesis is consistent with the observation in human breast cancer, that overexpression of cyclin E is correlated with increased tumor grade (Nielsen *et al.*, 1996; Keyomarsi, *et al.*, 1994). Moreover, a well-known, but mechanistically-unclear phenomenon is that overexpression of the c-*myc* gene alone is insufficient for transformation of most types of cells either *in vitro* or *in vivo*; cooperation of c-*myc* with growth factors (like TGFα) or some oncogenes (such as

ras) greatly enhances its transforming efficacy (Valverius *et al*., 1990; Schmidt, 1999; Facchini *et al*., 1998; Amati *et al*., 1998; Dang, 1999; Nass *et al*., 1997). The reciprocal expression of c-Myc and cyclins D1 and E in c-*myc* tumors and the co-expression of these genes in *tgfα*/c-*myc* tumors raise the possibility that one role of these additional factors may be to rescue the expression of cyclin D1 and/or cyclin E. Overexpression of these cyclins may be beneficial for the transformation, but it may be hampered because of constantly high levels of c-Myc.

Our transgenic models reveal, for the first time, that cdk2*β*, but not cdk2α, occurs as the faster-migrating phosphorylated form (Gu *et al*., 1992; Planas-Silva *et al*., 1997) in a primary tumor tissue. Little is known about functions of cdk2*β*. Its expression has been shown to peak at S phase and decrease significantly at early G2 phase, in contrast to the expression of cdk2α, which usually shows little change through the entire cell cycle (Kotani *et al*., 1995). Thus, it cannot be ruled out in c-*myc* and *tgfα*/c-*myc* tumors, that the predominant partner of cyclins A2 and E during S phase may be cdk2*β*.

Levels of the pRB protein are greatly decreased in the majority of *tgfα*/c-*myc* tumors, relative to c-*myc* tumors. This may occur at the mRNA level in some cases, as shown by RT–PCR analysis. For those *tgfα*/c-*myc* tumors in which the Rb mRNA and protein are detected, it is not yet clear if the expression is contributed by the tumor cells or by the proliferating stromal cells within the tumors. Regardless of the mechanism, loss of pRB protein may be one of the major reasons why mammary tumors in double transgenic mice develop at such early ages and grow at such a rapid rate, given the fact that pRB is a potent tumor suppressor and growth inhibitor. The loss of pRB in bi-transgenic tumors may be due to a synergy between c-Myc and TGFα, rather than an effect of TGFα alone, since *tgfα* mice do not develop tumors. This implies that like cyclin D1, pRB also links the c-Myc- and TGFα signaling pathways in control of cell cycle progression. However, cooperation between c-Myc and TGFα through cyclin D1 and pRB may be mechanistically different, since TGFα antagonizes the effect of c-Myc on cyclin D1 expression but appears to promote the effect of c-Myc on the attenuation of expression of pRB, as the pRB levels in some c-*myc* tumors are also low.

In conclusion, c-Myc may induce, directly or indirectly, expression of cyclin A2 and E2F1 as primary events to mediate the onset of mammary tumors in c-*myc* transgenic mice. In contrast, overexpression of cyclins D1 and E may occur as later events to facilitate progression of focal islands within the c-*myc* tumors to more aggressive phenotypes. Similarly, by using bi-transgenic mice, we concluded that TGFα induces cyclin D1 and facilitates the loss of pRB. These TGFα-mediated effects may have a threefold consequence in the mammary carcinogenesis of *tgfα*/c-*myc* bi-transgenic animals, relative to c-*myc* mice: a much earlier tumor onset, a higher tumor frequency, and the formation of a much more aggressive tumor phenotype. Thus, during mouse mammary carcinogenesis in bi-transgenic animals, TGFα and c-*myc* cooperate to control the cell cycle progression, in particular, at the levels of cyclin D1 and pRB.

Materials and methods

Tissue collections

MT-*tgfα*, MMTV-c-*myc*, and MT-*tgfα*/MMTV-c-*myc* single or double transgenic mice were generated, housed, and genotyped as described previously (Amundadottir *et al*., 1995). Tissue materials used were from the previously reported studies (Amundadottir *et al*., 1995), with additional animal experiments carried out in the same way. Briefly, virgin female MMTV-c-*myc* or MT-*tgfα* single transgenic mice were sacrificed at ages of 10–12 months, together with age-matched, non-transgenic mice of the parental strain (FVB). MT-*tgfα*/MMTV-c-*myc* double transgenic mice were sacrificed at ages of 3–6 months, together with five age-matched, non-transgenic animals as control. Mammary or tumor tissues harvested from the animals were either stored at −80°C or fixed with 10% buffered formalin and embedded in paraffin.

TUNEL assay

The terminal deoxynuycleotidyl transferase (TdT) mediated digoxigenin-dUTP nick end labeling (TUNEL) method was carried out using a kit from Trevigen Inc., Gaithersburg, MD, USA. Paraffin sections were labeled with TdT and biotin-labeled dNTP, and were incubated with peroxidase-conjugated Streptavidin, according to the manufacturer's instructions. The signal was visualized by exposure to diaminobenzidine and H_2O_2, followed by counter-staining with hematoxylin.

In situ *hybridization assay*

Paraffin sections were hybridized overnight at 60°C with riboprobes, transcribed from the antisense or sense strands of the cDNAs and labeled with digoxigenin-conjugated UTP, as described previously (Li *et al*., 1999). The sections were then incubated with an antibody against digoxigenin, followed by incubation with a second antibody conjugated to alkaline phosphatase. The signal was visualized by color development with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. All reagents were purchased from Boehringer Mannheim, Indianapolis, IN, USA. A 1.4 kb mouse c-*myc* cDNA and a 0.9 kb mouse *e2f1* cDNA (ATCC, Manassas, VA, USA) were used for labeling of the riboprobes. To control the signal specificity, two serial sections were mounted on the same slide for hybridization with antisense and sense probes, respectively. A serial selection was also pretreated with RNase A and then post-fixed with 4% formaldehyde to denature the RNase before hybridization with antisense probe.

Northern blot assay

Ten μg of total RNA per sample were loaded and electrofractionated in an agarose gel containing formaldehyde. Roughly equal loading of lanes and RNA integrity were confirmed by staining the gel with ethidium bromide. The separated RNA was transferred to nitrocellulose membranes and hybridized with an *e2f1* antisense riboprobe, synthesized from the same cDNA as used for *in situ* hybridization, and labeled with ^{32}P-ATP (Amersham Life Science, Inc., Arlington Heights, IL, USA). After washes with SSC buffers, the membrane was subjected to autoradiography.

RT–PCR analysis

Total RNA was reverse-transcribed and then amplified using the RT–PCR kit from GIBCO/BRL, Rockville, MD, USA. The conditions for the PCR amplification were as follows: 3-min hot start at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, and 2 min at 72°C. The two pairs of forward/

reverse PCR primers for the Rb gene were 209–229 bp/1110–985 pb and 1014–1041 pb/2857–2833 pb, which overlap to span most part of the mouse Rb mRNA (Bernards *et al.*, 1989). As an internal control, mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was also amplified by PCR under the same conditions. The forward and reverse primers were 819–837 pb and 1228–1207 bp, respectively, of the mouse GAPDH cDNA sequence (Sabath *et al.*, 1990).

Western blot analysis

Methods for preparation of protein samples and for Western blotting were described previously (Liao *et al.*, 1998). Protein aliquots (20–80 μg per lane) were electro-fractionated on SDS–PAGE. Roughly equal loading was confirmed by staining the gel with Coomassie blue. One primary pRB antibody (14001A) was purchased from Pharmigen, San Diego, CA, USA and another (pRB245) was a generous gift from Dr W-H Lee (see Acknowledgements). The PCNA primary antibody (PC10) was purchased from Oncogene Research Product Inc., Cambridge, MA, USA. All other primary antibodies were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA): c-Myc (C19), E2F1 (C20 and KH95), cyclin A (C19), cyclin E (M-20), cyclin D1 (C20), cyclin D3 (C16), cdk2 (M20), cdk4 (C22), p16 (M156 and F12), p21 (M19 and F5), and p27 (C19 and N19). For all primary antibodies from Santa Cruz Biotech. Inc., where specific blocking peptides were available, in a parallel Western blot assay the antibody was incubated with fivefold excess (by weight) of the corresponding blocking peptide to neutralize the antibody before applied to the membrane. The pre-neutralized antibody sample did not give rise to the specific signals at correct molecular weights, demonstrating the specificity of the primary antibodies.

Immunohistochemical staining

A peroxidase-anti-peroxidase (PAP) method was used as described previously (Liao *et al.*, 1998). The primary antibodies were the same as used for Western blot analyses. For all primary antibodies purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA), where blocking peptides were available, in one staining with a serial section, the primary antibody was incubated with fivefold excess (by weight) of its blocking peptide for 2 h to neutralize the antibody before application to the section. The pre-neutralized primary antibody did not give rise to signal, demonstrating that the signal given by the primary antibody was specific.

Labeling index

Labeling indices for TUNEL and PCNA staining were determined for tumors from *tgfα/c-myc* mice and for specific tumor foci and their adjacent tumor areas from *c-myc* animals. Since cells in the G1 phase of the cell cycle manifest weak nuclear staining for PCNA, in strong contrast to the intense nuclear staining of cells in S phase (Eldrige *et al.*, 1993), only those cells displaying strong nuclear staining were counted. Four *tgfα/c-myc* tumors plus six foci and their adjacent tumor areas from different individual animals were counted. For each tumor or focus, three randomly selected areas, about 600 tumor cells per area, were counted. The percentage of labeled cells was calculated and presented as mean ± s.d. The χ^2 test of independence for an $r \times c$ contingency table was used for the statistical analysis.

Abbreviations

Cdk, cyclin-dependent kinase; ER, estrogen receptor; MMTV, mouse mammary tumor virus; MT, metallathionein; PCNA, proliferating cell nuclear antigen; pRB, retinoblastoma protein; TGFα, transforming growth factor α; TUNEL, terminal deoxynucleotidyl transferase nick end labeling.

Acknowledgments

We would like to express sincere gratitude to Dr W-H Lee, Department of Molecular Medicine and Institute of Biotechnology, University of Texas Health Science Center, San Antonio, TX, USA, for generously providing the antibody pRB245, to Mrs S Constable for helping in tissue processing, and to Dr E Rosfjord, J-K Wang, and Dr M-Z Dai for helping in preparing digital photos. This work was supported by NIH grants RO1 CA72460 and AG1496 to RB Dickson.

References

Alexandrow MG and Moses HL. (1998). *J. Cell Biochem.*, **70,** 528–542.

Amati B, Alevizopoulos K and Vlach J. (1998). *Front. Biosci.*, **3,** D250–D268.

Amundadottir LT, Johnson MD, Merlino G, Smith GH and Dickson RB. (1995). *Cell Growth Differ.*, **6,** 737–748.

Amundadottir LT, Merlino G and Dickson RB. (1996a). *Breast Cancer Res. Treat.*, **39,** 119–135.

Amundadottir LT, Nass SJ, Berchem GJ, Johnson MD and Dickson RB. (1996b). *Oncogene*, **13,** 757–765.

Auvinen PK, Lipponen PK, Kataja VV, Johansson RT and Syrjanen KJ. (1996). *Acta Oncol.*, **35,** 995–998.

Barnes DM and Gillett CE. (1998). *Breast Cancer Res. Treat.*, **52,** 1–15.

Bernards R, Schackleford GM, Gerber MR, Horowitz JM, Friend SH, Schartl M, Bogenmann E, Rapaport JM, McGee T and Dryja TP. (1989). *Proc. Natl. Acad. Sci. USA*, **86,** 6474–6478.

Bodrug SE, Warner BJ, Bath ML, Lindeman GJ, Harris AW and Adams JM. (1994). *EMBO J.*, **13,** 2124–2130.

Cardiff RD and Munn RJ. (1995). *Cancer Lett.*, **90,** 13–19.

Cole MD and McMahon SB. (1999). *Oncogene*, **18,** 2916–2924.

Daksis JI, Lu RY, Facchini LM, Marhin WW and Penn LJ. (1994). *Oncogene*, **9,** 3635–3645.

Dang CV. (1999). *Mol. Cell. Biol.*, **19,** 1–11.

Desdouets C, Sobczak-Thepot J, Murphy M and Brechot C. (1995). *Prog. Cell Cycle Res.*, **1,** 115–123.

Dickson RB and Lippman ME. (1995). *Endocr. Rev.*, **16,** 559–589.

Eldrige SR, Butterworth BE and Goldsworthy TL. (1993). *Environ. Health Perspect.*, **101,** Suppl 5, 211–218.

Ezhevsky SA, Nagahara H, Vocero-Akbani AM, Gius DR, Wei MC and Dowdy SF. (1997). *Proc. Natl. Acad. Sci. USA*, **94,** 10699–10704.

Facchini LM and Penn LZ. (1998). *FASEB J.*, **12,** 633–651.

Gray-Bablin J, Zalvide J, Fox MP, Knickerbocker CJ, DeCaprio JA and Keyomarsi K. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 15215–15220.

Gu Y, Rosenblatt J and Morgan DO. (1992). *EMBO J.*, **11,** 3995–4005.

Han EK, Sgambato A, Jiang W, Zhang YJ, Santella RM, Doki Y, Cacace AM, Schieren I and Weinstein IB. (1995). *Oncogene*, **10,** 953–961.

Jansen-Durr P, Meichle A, Steiner P, Pagano M, Finke K, Botz J, Wessbecher J, Draetta G and Eilers M. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 3685–3689.
Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH and Merlino GT. (1990). *Cell*, **61**, 1137–1146.
Johnson DG and Schneider-Broussard R. (1998). *Front. Biosci.*, **3**, d447–d448.
Kerkhoff E, Houben R, Loffler S, Troppmair J, Lee JE and Rapp UR. (1998). *Oncogene*, **16**, 211–216.
Keyomarsi K, O'Leary N, Molnar G, Lees E, Fingert HJ and Pardee AB. (1994). *Cancer Res.*, **54**, 380–385.
Keyomarsi K and Pardee AB. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 1112–1116.
Kotani S, Endo T, Kitagawa M, Higashi H and Onaya T. (1995). *Oncogene*, **10**, 663–669.
Kwon TK, Buchholz MA, Jun DY, Kim YH and Nordin AA. (1998). *Exp. Cell Res.*, **238**, 128–135.
Land H, Parada LF and Weinberg RA. (1983). *Nature*, **304**, 596–602.
Lavia P and Jansen-Durr P. (1999). *Bioessays*, **21**, 221–230.
Lee DC, Fenton SE, Berkowitz EA and Hissong MA. (1995). *Pharmacol. Rev.*, **47**, 51–85.
Leone G, DeGregori J, Sears R, Jakoi L and Nevins JR. (1997). *Nature*, **387**, 422–426.
Li JJ, Hou X, Banerjee SK, Liao DZ, Maggouta F, Norris JS and Li SA. (1999). *Cancer Res.*, **59**, 2340–2346.
Li Y, Slansky JE, Myers DJ, Drinkwater NR, Kaelin WG and Farnham PJ. (1994). *Mol. Cell. Biol.*, **14**, 1861–1869.
Liao DZ, Pantazis CG, Hou X and Li SA. (1998). *Carcinogenesis*, **9**, 2173–2180.
Lukas J, Bartkova J and Bartek J. (1996). *Mol. Cell. Biol.*, **16**, 6917–6925.
Lykkesfeldt AE. (1996). *Acta Oncol.*, **35** Suppl 5, 9–14.
Marhin WW, Hei YJ, Chen S, Jiang Z, Gallie BL, Phillips RA and Penn LZ. (1996). *Oncogene*, **12**, 43–52.
Matsui Y, Halter SA, Holt JT, Hogan BL and Coffey RJ. (1990). *Cell*, **61**, 1147–1155.
Morgan DO. (1995). *Nature*, **374**, 131–134.
Nass SJ and Dickson RB. (1997). *Breast Cancer Res. Treat.*, **44**, 1–22.
Neuman E, Ladha MH, Lin N, Upton TM, Miller SJ, DiRenzo J, Pestell RG, Hinds PW, Dowdy SF, Brown M and Ewen ME. (1997). *Mol. Cell. Biol.*, **17**, 5338–5347.
Nielsen NH, Arnerlov C, Emdin SO and Landberg G. (1996). *Br. J. Cancer*, **74**, 874–880.
Noguchi E, Sekiguchi T, Yamashita K and Nishimoto T. (1993). *Biochem. Biophys. Res. Commun.*, **197**, 1524–1529.
Obaya AJ, Mateyak MK and Sedivy JM. (1999). *Oncogene*, **18**, 2934–2941.
Panico L, D'Antonio A, Salvatore G, Mezza E, Tortora G, De Laurentiis M, De Placido S, Giordano T, Merino M, Salomon DS, Mullick WJ, Pettinato G, Schnitt SJ, Bianco AR and Ciardiello F. (1996). *Int. J. Cancer*, **65**, 51–56.
Philipp A, Schneider A, Vasrik I, Finke K, Xiong Y, Beach D, Alitalo K and Eilers M. (1994). *Mol. Cell. Biol.*, **14**, 4032–4043.
Pilichowska M, Kimura N, Fujiwara H and Nagura H. (1997). *Mod. Pathol.*, **10**, 969–975.
Planas-Silva MD and Weinberg RA. (1997). *Mol. Cell. Biol.*, **17**, 4059–4069.
Prall OW, Rogan EM, Musgrove EA, Watts CK and Sutherland RL. (1998). *Mol. Cell Biol.*, **18**, 4498–4508.
Quelle DE, Ashmun RA, Shurtleff SA, Kato JY, Bar-Sagi D, Roussel MF and Sherr CJ. (1993). *Genes Dev.*, **7**, 1559–1571.
Roussel MF. (1998). *Adv. Cancer Res.*, **74**, 1–24.
Sabath DE, Broome HE and Prystowsky MB. (1990). *Gene*, **91**, 185–191.
Said TK and Medina D. (1995). *Carcinogenesis*, **16**, 823–830.
Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL and Lee DC. (1990). *Cell*, **61**, 1121–1135.
Santoni-Rugiu E, Jensen MR and Thorgeirsson SS. (1998). *Cancer Res.*, **58**, 123–134.
Schmidt EV. (1999). *Oncogene*, **18**, 2988–2996.
Sears R, Leone G, DeGregori J and Nevins JR. (1999). *Mol. Cell*, **3**, 169–179.
Seshadri R, Lee CS, Hui R, McCaul K, Horsfall DJ and Sutherland RL. (1996). *Clin Cancer Res.*, **2**, 1177–1184.
Sgambato A, Han EK, Zhou P, Schieren I and Weinstein IB. (1996). *Cancer Res.*, **56**, 1389–1399.
Sherr CJ. (1996). *Science*, **274**, 1672–1677.
Sinn E, Muller W, Pattengale P, Tepler I, Wallace R and Leder P. (1987). *Cell*, **49**, 465–475.
Solomon DL, Philipp A, Land H and Eilers M. (1995). *Oncogene*, **11**, 1893–1897.
Steeg PS and Zhou Q. (1998). *Breast Cancer Res. Treat.*, **52**, 17–28.
Stewart TA, Pattengale PK and Leder P. (1984). *Cell*, **38**, 627–637.
Sweeney C, Murphy M, Kubelka M, Ravnik SE, Hawkins CF, Wolgemuth DJ and Carrington M. (1996). *Development*, **122**, 53–64.
Valverius EM, Ciardiello F, Heldin NE, Blondel B, Merlo G, Smith G, Stampfer MR, Lippman ME, Dickson RB and Salomon DS. (1990). *J. Cell Physiol.*, **145**, 207–216.
Wilcken NR, Prall OW, Musgrove EA and Sutherland RL. (1997). *Clin Cancer Res.*, **3**, 849–854.
Yang R, Morosetti R and Koeffler HP. (1997). *Cancer Res.*, **57**, 913–920.

APPENDIX C
Poster Abstracts for Scientific Meetings

Abstract #1: **Jamerson MH**, Johnson MD and Dickson RB. Cooperation of c-Myc, Bcl-x_L, and Bax-Knockout in Mammary Tumorigenesis. Lombardi Cancer Center Research Days, Lombardi Cancer Center, Washington, DC. February 1999.

c-Myc oncogene has been reported to be amplified in 25-30% of human breast cancers and overexpressed in more than 70% of human breast cancers. Analysis *in vitro* has demonstrated that c-Myc is involved in signaling for cell proliferation and apoptosis. The Bcl-x_L protein, an anti-apoptotic member of the Bcl-2 apoptosis-modulatory protein family, is known to block apoptotic cell death under a wide variety of conditions and has been shown to be overexpressed in some human breast cancers and breast cancer cell lines. The Bax protein, a pro-apoptotic member of the Bcl-2 protein family, is known to contribute to cellular vulnerability to apoptosis, has been demonstrated to possess a tumor suppressor-like function in human tumors, and has been shown to be weakly expressed or absent in human breast cancers and breast cancer cell lines.

Evidence from a c-*myc*/*tgfα* bitransgenic mouse model suggests that escape from c-Myc-induced apoptosis may be necessary for continued cell cycle progression and neoplastic development. The focus of these studies is to determine if there is a synergism between deregulated c-Myc expression and loss/diminution of apoptosis in mouse mammary tumorigenesis. We hypothesize that the constitutive expression of c-Myc and Bcl-x_L will facilitate mammary tumorigenesis as a result of Bcl-x_L blockade of c-Myc-induced apoptosis and not c-Myc-mediated cell cycle progression. It is further predicted that the constitutive expression of c-Myc in a Bax-null background will also facilitate mammary tumorigenesis due to a disruption of the c-Myc-induced apoptotic pathways.

Work to date in support of this project includes the following: the establishment and optimization of PCR-based procedures for the identification of mouse transgenic status, the establishment of breeding colonies of c-Myc, tTA-Luc, and tetOP-Bcl-x_L transgenic animals and Bax-knockout animals, the evaluation and solution of breeding and nursing problems, and the establishment of a breeding program to achieve sufficient numbers of bitransgenic and control animals for study. Recent progress and current work is focused on the generation of additional strategies for these breeding experiments and involves the production of another Bcl-x_L transgenic mouse model without tetracycline regulatory elements. Furthermore, we are pursuing the creation of c-Myc and Bcl-x_L retroviruses for use in the establishment of bitransgenic mammary glands.

This work is supported by Department of the Army Fellowship DAMD17-97-1-7110 to MHJ.

Abstract #2: **Jamerson MH**, Johnson MD and Dickson RB. Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis. Era of Hope Department of Defense Breast Cancer Research Program Meeting, Atlanta, Georgia. June 8-11, 2000. Abstract #455.

Cooperation of Bcl-x_L and c-Myc in Mammary Tumorigenesis

M. Hunter Jamerson, Michael D. Johnson PhD, and Robert B. Dickson PhD
Department of Oncology, Lombardi Cancer Center, Georgetown University
Washington, D.C. 20007-2197

E-mail: jamers1@gunet.georgetown.edu

The focus of this study is to determine whether Bcl-x_L overexpression and/or loss of Bax expression cooperate with c-Myc overexpression in facilitating mammary tumorigenesis *in vivo*. c-Myc is amplified in 16%, rearranged in 5%, and overexpressed in nearly 70% of all human breast cancers and it regulates cellular proliferation, differentiation, and apoptosis. Bcl-x_L, known to inhibit apoptosis potentially by modulating mitochondrial permeability and caspase activation, is overexpressed in some breast tumors and derivative cell lines and has been shown to be important in regulation of apoptosis during mammary gland involution. The pro-apoptotic protein Bax is known to be significantly reduced or altogether absent in many breast tumors and cell lines and has further been demonstrated, in a transgenic model, to cooperate with tumor oncogenes in reducing the protective apoptotic effect early in mammary tumorigenesis. Evidence from c-Myc/Tgf-α bitransgenic mice suggest that escape from c-Myc-induced apoptosis may be necessary for continued cell cycle progression, promotion of genetic instability, and neoplastic development.

Constitutive expression of Bcl-x_L and/or loss of Bax are likely to disrupt the c-Myc-induced apoptotic pathways without significantly influencing c-Myc-mediated proliferation. Transgenic mice overexpressing c-Myc or Bcl-x_L or nullizygous for Bax will be mated to produce offspring for evaluation of the role of apoptosis modulation on c-Myc-mediated mammary tumorigenesis and mammary gland development. To date, c-Myc transgenic/Bax-knockout and c-Myc/Bcl-x_L bitransgenic mice have been generated, genotyped, and assigned to study groups. Both virgin and multiparous study animals will be assessed for altered tumor onset, incidence, growth, and pathological/molecular characteristics once mammary tumors arise. Additional study animals will be evaluated for alterations in mammary gland development and pregnancy-associated glandular development and involution. The utilization of these model systems will aid in the dissection of the *in vivo* role of apoptosis in the development of breast cancer.

The US Army Medical Research and Materiel Command under DAMD17-97-1-7110 supported this work.

Abstract #3: **Jamerson MH**, Johnson MD, Furth PA, Korsmeyer SJ, Nuñez G, and Dickson RB. Gain of Bcl-x_L and Loss of Bax Cooperate in c-Myc-Mediated Mammary Tumorigenesis. Keystone Symposium on Molecular Mechanisms of Apoptosis, Keystone, Colorado. January 16-22, 2001. Abstract #239.

Gain of Bcl-x_L and Loss of Bax Cooperate in c-Myc-Mediated Mammary Tumorigenesis

M. Hunter Jamerson[1], Michael D. Johnson[1], Priscilla A. Furth[2], Stanley J Korsmeyer[3], Gabriel Nuñez[4], and Robert B. Dickson[1].

[1]Lombardi Cancer Center, Georgetown University, Washington, DC 20007; [2]Institute of Human Virology, Depts of Medicine & Physiology, University of Maryland Medical School, Baltimore, MD 21201; [3]Dana-Farber Cancer Institute, Harvard University, Boston, MA 02115; [4]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109.

It is commonly held that carcinogenesis is a multistage process requiring subversion of the multiple systems that exist within cells to control cell growth and safeguard against tumor formation. The use of genetically altered mice provides a highly malleable system for evaluating the cooperation of genetic events involved in the development of tumors. Evidence from c-Myc/TGF-α bitransgenic mice suggests that escape from c-Myc-induced apoptosis may be necessary for continued cell cycle progression, promotion of genetic instability, and neoplastic development.

Studies of tumor tissue taken from women with breast cancer have demonstrated that the proto-oncogene, c-Myc, is more abundant than in normal breast tissue (amplified in 16%, rearranged in 5%, and overexpressed in nearly 70% of all human breast cancers). Similar studies have shown that genes known to influence programmed cell death are also altered in breast tumors. Bcl-x_L, known to inhibit apoptosis, potentially by modulating mitochondrial permeability and caspase activation, is overexpressed in some breast tumors and has been shown to be important in the regulation of apoptosis during mammary gland involution. The pro-apoptotic protein Bax is known to be significantly reduced or altogether absent in many breast tumors and has further been demonstrated to cooperate with tumor oncogenes in reducing the protective effect early in mammary tumorigenesis.

Our bitransgenic mouse studies with constitutive expression of Bcl-x_L and/or loss of Bax are likely to disrupt the c-Myc-induced apoptotic and proliferative pathways and, therefore, modulate c-Myc-mediated mammary tumorigenesis and mammary gland development. Our pilot data in Bax-knockout/c-Myc and c-Myc/Bcl-x_L bitransgenic mice have confirmed a cooperative role for these apoptosis-modulatory genes with c-Myc in mammary tumorigenesis. The utilization of these model systems will aid the dissection of the *in vivo* role of apoptosis in the development of breast cancer. *Work supported under DAMD17-97-1-7110 to MHJ and 1R01AG1496 and 1R01CA72460 to RBD.*

APPENDIX D
List of Abbreviations

ABC	Avidin-biotin complex
AC	Adenyl cyclase
bFGF	Basic Fibroblast growth factor
BRCA1	Breast cancer-associated gene 1
CGH	Comparative genomic hybridization
CRD-BP	Coding region determinant-binding protein
DAB	3,3'-diaminobenzidine
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor (c-ErbB1)
GEM	Genetically-engineered mice
GSK-3β	Glycogen synthase kinase 3-beta
hCMVP	Human cytomegalovirus promoter
HRP	Horseradish peroxidase
IGF1	Insulin-like growth factor 1
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
MEF	Mouse embryonic fibroblast
MMTV-LTR	Mouse mammary tumor virus long terminal repeat
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI3K	Phosphatidyl-inositol-3 kinase
PKA	Protein kinase A
PKB	Protein kinase B (Akt)
PLC	Phospholipase C
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
SAPK	Stress-activated protein kinase
SCLC	Small cell lung cancer
SKY	Spectral karyotyping
Tag	SV40 Large T antigen
tetOP	Tetracycline operon system
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TNFR1	Tumor necrosis factor receptor 1
tTA	Tetracycline transactivator protein
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-digoxygenin nick end labeling
WAP	Whey acidic protein