Lack of sustained regression of c-MYC-induced mammary adenocarcinomas following brief or prolonged MYC inactivation

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Summary

Recent studies of oncogene dependence in conditional transgenic mice have suggested the exciting possibility that transient or prolonged MYC inactivation may be sufficient for sustained reversal of the tumorigenic process. In contrast, we report here that following oncogene downregulation, the majority of c-MYC-induced mammary adenocarcinomas grow in the absence of MYC overexpression. In addition, residual neoplastic cells persist from virtually all tumors that do regress to a nonpalpable state and these residual cells rapidly recover their malignant properties following MYC reactivation or spontaneously recur in a MYC-independent manner. Thus, MYC-induced mammary tumor cells subjected to either brief or prolonged MYC inactivation remain exquisitely sensitive to its oncogenic effects and characteristically progress to a state in which growth is MYC-independent.

Introduction

Human cancers arise through a multistep process involving the sequential activation of oncogenes and inactivation of tumor suppressor genes. Consequently, novel therapeutic approaches are increasingly being devised to reverse the effects of individual genetic alterations by specifically targeting oncogenic pathways that become activated within particular tumor types. With the advent of molecularly targeted therapies for cancer, the question of whether invasive tumors remain dependent upon a single oncogenic pathway for maintenance and growth has become a critical issue in cancer therapeutics. In principle, reversing a single genetic lesion that activates a dominant oncogenic pathway within a tumor could lead to sustained tumor regression. Alternately, oncogenic pathways critical for tumor induction could become dispensable for maintenance of the neoplastic state once tumors have formed. Similarly, tumors that initially exhibit dependence upon an oncogenic pathway may subsequently progress to a state in which growth becomes resistant to pathway inhibition, as is commonly observed during the treatment of human cancers.

To determine experimentally whether invasive tumors remain dependent upon individual oncogenic pathways for their maintenance, several transgenic mouse models for cancer have recently been developed based upon the conditional overexpression of oncogenes such as c-*MYC*, *Kras2*, *Hras*, and *BCR-ABL* within a variety of cell types. Each of these studies has demonstrated that inactivation of a single oncogene within a primary tumor can be sufficient to induce dramatic tumor regression (Chin et al., 1999; D'Cruz et al., 2001; Felsher and Bishop, 1999a; Fisher et al., 2001; Gunther et al., 2003; Huettner et al., 2000; Jain et al., 2002; Moody et al., 2002; Pelengaris et al., 1999, 2002). Moreover, oncogene dependence also occurs in tumors in which a second, independent oncogenic pathway has been activated or a tumor suppressor pathway has been inactivated (Fisher et al., 2001; Gunther et al., 2003; Pelengaris et al., 2002). Surprisingly, even advanced stages of malignancy, such as metastases, can remain dependent upon an initiating oncogenic event for maintenance and growth (Moody et al., 2002, Gunther et al., 2003). In aggregate, conditional transgenic mouse models analyzed to date support the concept that tar-

SIGNIFICANCE

Molecular therapeutics that target specific oncogenic pathways hold considerable promise. The success of such approaches, however, depends on the extent to which malignant cells remain dependent upon individual oncogenic mutations. Since c-*MYC* overexpression occurs commonly in human breast cancers, the question of whether MYC inactivation represents an effective treatment for this disease has considerable clinical importance. In contrast to other cell types, neither brief nor prolonged MYC inactivation in mammary adenocarcinomas results in sustained tumor regression or protection against MYC's oncogenic effects. Our studies emphasize that clinically important properties of tumors arise from small, yet biologically critical, subsets of cells and suggest that secondary pathways of tumor escape represent a significant obstacle to the efficacy of targeted therapeutics for cancer.

geted inactivation of a single, dominant oncogenic pathway—the so-called "Achilles heel" of cancers—may lead to the sustained regression of tumors harboring multiple genetic and epigenetic alterations (Weinstein, 2002). The relevance of this concept to at least some human cancers has been illustrated by the dramatic clinical responses observed in patients with chronic myelogenous leukemia (CML) treated with the tyrosine kinase inhibitor, imatinib (Druker et al., 2001; Kantarjian et al., 2002).

The finding that even advanced stages of tumorigenesis can remain sensitive to molecularly targeted interventions is an encouraging proof-of-principle that such targeted agents can be effective. Nevertheless, clinical experience with common epithelial malignancies has been far less encouraging. Indeed, the natural history of most human cancers entails the progressive selection and outgrowth of malignant subsets of tumor cells that possess increasingly aggressive properties. These include the development of therapeutic resistance, occurrence of distant metastasis, establishment and prolonged survival of residual neoplastic cells within the host and, ultimately, tumor recurrence. Together, these aspects of tumor progression are responsible for the vast majority of cancer deaths. Thus, in contrast to patients with CML treated with imatinib, patients with metastatic cancers of the breast, lung, colon, prostate, and pancreas—which together account for nearly two-thirds of all cancers in Western countries—are rarely if ever cured by combination therapy, much less therapy with a single agent. Rather, clinical observations repeatedly indicate that subsets of cells within genetically heterogeneous tumors become resistant to therapy by escaping the selective pressures that therapeutic interventions represent. Viewed from this perspective, lessons from mouse models—while encouraging—would seem at odds with lessons from the clinic.

Perhaps nowhere is this discrepancy more apparent than in studies of conditional transgenic models for c-MYC-induced tumorigenesis. The c-MYC transcription factor has been intensively studied due to its demonstrated role in multiple human cancers as well as in cellular processes central to cancer, such as cell growth, proliferation, differentiation, and apoptosis (Pelengaris and Khan, 2003a, 2003b). Inducible transgenic models for c-MYC have been described for hematopoietic, epidermal, pancreatic islet, and osteogenic sarcoma cells, among others (Felsher and Bishop, 1999a; Jain et al., 2002; Pelengaris et al., 1999, 2002). Based upon these models, it has been proposed that c-MYC-induced neoplasms remain dependent upon *c-MYC* transgene expression for maintenance of the malignant state, and that sustained MYC downregulation leads to sustained tumor regression and elimination of neoplastic cells from the host (Weinstein, 2002).

More provocative still has been the surprising recent report that even brief periods of c-MYC inactivation in osteogenic sarcoma cells result in sustained tumor regression (Jain et al., 2002). Surprisingly, reactivation of MYC in residual tumor cells in this system failed to restore their malignant properties and instead induced apoptotic cell death. These findings have been taken to support the exciting possibility that brief inactivation of MYC may not only be therapeutic, but may in addition exert a protective effect that renders previously neoplastic cells insensitive to the tumorigenic properties of this oncogene. Based upon these observations, it has been proposed that transient inactivation of an oncogenic pathway may represent an effective—or perhaps even preferable—treatment for human cancers.

In light of this proposal, we wished to consider whether the above findings could be extrapolated to a common epithelial cancer in which c-MYC has been implicated. *c-MYC* amplification occurs in 5%–15% percent of human breast cancers, and this oncogene is overexpressed in the majority of breast cancers in women (Liao and Dickson, 2000). Moreover, while osteogenic sarcoma is rare, breast cancer is the most common malignancy diagnosed among women in the United States and is the leading cause of death from disease among women aged 25 to 54. As such, whether brief or prolonged inactivation of c-MYC might represent an effective treatment for breast cancer is a question of considerable clinical importance.

To investigate this issue, we have developed a transgenic mouse model in which c-MYC can be conditionally overexpressed in the mammary glands of mice treated with tetracycline derivatives (D'Cruz et al., 2001). We report here that the majority of c-MYC-induced primary mammary adenocarcinomas that arise in mice are already able to grow in the absence of MYC overexpression, that the majority of tumors that do fully regress following MYC downregulation recur spontaneously, that most if not all animals bearing fully regressed tumors harbor residual neoplastic cells, and that reactivation of MYC in these residual cells results in the rapid and full recovery of their malignant properties. Furthermore, successive cycles of *MYC* transgene expression resulted in the progression of nearly all MYC-induced mammary tumors to a doxycycline-independent state. As such, rather than exerting a protective effect on neoplastic mammary epithelial cells, c-MYC-induced tumors cells subjected to either brief or prolonged MYC inactivation remain exquisitely sensitive to its oncogenic effects and characteristically progress to a state in which MYC overexpression is dispensable for growth. These findings emphasize the context-dependent nature of oncogene dependence as well as the ability of mouse models to faithfully recapitulate the natural history of human cancers.

Results

The majority of MYC-induced primary mammary tumors are MYC-independent

We have previously reported the generation and initial characterization of a bitransgenic system for the conditional expression of c-MYC in the mammary gland utilizing the tetracycline regulatory system (D'Cruz et al., 2001). In this model, the reverse tetracycline-dependent transcriptional activator, rtTA, is expressed from the mouse mammary tumor virus (MMTV) promoter and, in the presence of doxycycline, induces expression of a *c-MYC* transgene fused to a tetracycline-dependent promoter. Chronically induced MMTV-rtTA/TetO-MYC (MTB/TOM) bitransgenic animals develop solitary mammary adenocarcinomas with a mean latency of 22 weeks in a manner that is absolutely dependent upon transgene induction by doxycycline.

Following the removal of doxycycline from the drinking water of tumor-bearing bitransgenic animals, 31 of 62 deinduced mammary adenocarcinomas failed to regress to a nonpalpable state (Figure 1A). The extent of regression among incompletely regressing tumors was variable and formed a continuous range from $>$90% regression (e.g., tumors 32–42) to $<$10% regression (e.g., tumors 61 and 62). Northern hybridization analysis of incompletely regressing tumors before and after doxycycline with-

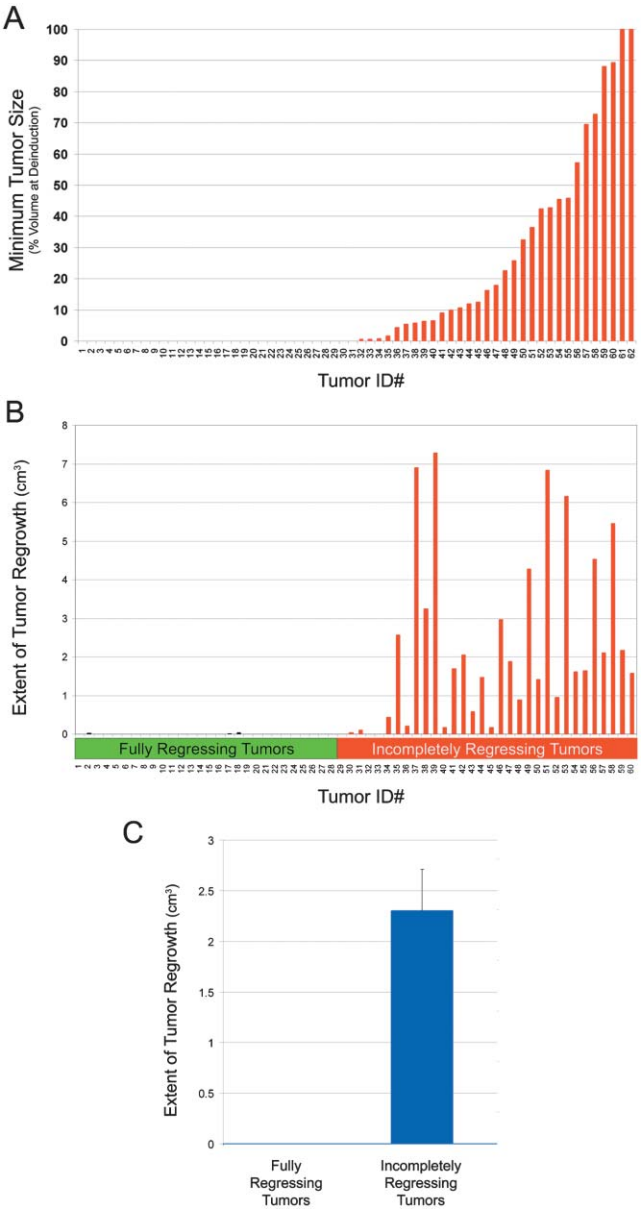

Figure 1. MYC-induced mammary adenocarcinomas resume growth following MYC downregulation

A: Sixty-two c-MYC-induced tumors were monitored for tumor regression following doxycycline withdrawal. Extent of tumor regression was calculated as a percentage by dividing the minimum tumor volume reached following doxycycline withdrawal by tumor size at deinduction. Note that 50% of the tumors fail to regress fully following c-*MYC* transgene deinduction.
B: Extent of tumor regrowth off doxycycline was measured in the 62 c-MYC-induced mammary adenocarcinomas in **A**. Tumor growth is expressed as the difference between tumor volume at 40 days postdeinduction and the minimum size reached following deinduction. Note that nearly all incompletely regressing tumors resumed growth by 40 days, whereas fully regressing tumors did not.
C: The mean growth of deinduced tumors at 40 days postdeinduction was calculated for incompletely regressing and fully regressing c-MYC-induced mammary adenocarcinoma subsets (student's t test, $p = 6.88 \times 10^{-7}$).

drawal confirmed the sustained downregulation of c-*MYC* transgene expression and MYC target genes, and the absence of overexpression of endogenous *c-myc* (D'Cruz et al., 2001 and data not shown). Thus, in contrast to findings in other cell types in which c-MYC inactivation leads almost invariably to the complete reversal of malignant lesions, half of c-MYC-induced primary mammary adenocarcinomas fail to regress fully following *c-MYC* transgene downregulation.

Given differences in the observed oncogene dependence of c-MYC-induced mammary adenocarcinomas compared to c-MYC-induced neoplasms in other tissues, we considered the possibility that failure to regress to a nonpalpable lesion might not represent a clinically significant endpoint; that is, despite their failure to regress fully, some or all incompletely regressing tumors may nevertheless have lost their capacity for malignant growth following the abrogation of *c-MYC* transgene expression. To investigate this possibility, tumor-bearing mice were followed for extended periods of time following doxycycline withdrawal. This analysis revealed that, in addition to their failure to regress fully following MYC inactivation, incompletely regressing tumors rapidly reinitiated growth in the absence of *MYC* transgene expression (Figure 1B). Specifically, within 40 days following doxycycline withdrawal, nearly all tumors classified as incompletely regressing had resumed growth, increasing in size an average of 2.3 cm^3 over this period compared to their minimum size following transgene deinduction (Figure 1C). In contrast, none of the tumors classified as fully regressing exhibited any substantial regrowth over this period (p value for incompletely regressing versus fully regressing tumors $= 6.9 \times 10^{-7}$, Figure 1C). These findings demonstrate that the majority of primary mammary tumors that arise as a consequence of MYC activation have already acquired the ability to grow in the absence of MYC overexpression. This, in turn, suggests that prolonged c-MYC inactivation has markedly different consequences in mammary tumors compared to MYC-induced neoplasms of other tissues.

Fully regressed MYC-induced mammary tumors recur spontaneously

Although half of MYC-induced primary mammary tumors that have reached 1 cm^3 in size have already acquired the ability to grow in the absence of c-MYC overexpression, it remained possible that those tumors that did regress to a nonpalpable state following transgene deinduction might exhibit sustained regression similar to that described for MYC-induced neoplasms of other tissues (Felsher and Bishop, 1999a; Jain et al., 2002; Pelengaris et al., 2002). To determine whether tumors that were initially dependent on *c-MYC* transgene expression would remain so, bitransgenic animals harboring fully regressed mammary adenocarcinomas were monitored for spontaneous tumor recurrences off doxycycline. Within one year following doxycycline withdrawal, greater than 50% of fully regressed tumors had recurred at the site of the original primary tumor (Figure 2A). The median time to recurrence was 80 days following doxycycline withdrawal (range 45–330 days; $n = 16$). All recurrent tumors exhibited a high rate of growth regardless of their time to recurrence (Figure 2B).

Four lines of evidence suggest that these tumors represented bona fide recurrences rather than de novo neoplasms arising off doxycycline. First, recurrent tumors always arose at the site of the original primary tumor; second, no tumors arose

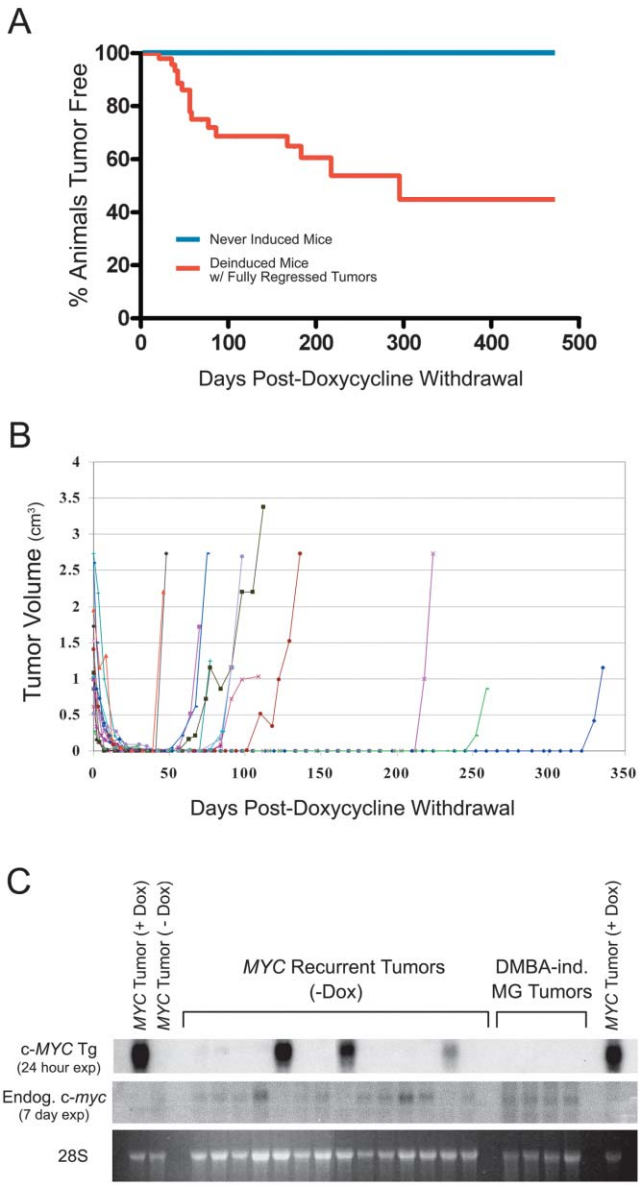

Figure 2. Fully regressed c-MYC-induced mammary tumors spontaneously recur

A: Tumor-free survival curve following doxycycline withdrawal for MTB/TOM mice bearing fully regressed mammary adenocarcinomas (n = 51). Age-matched uninduced MTB/TOM mice are shown as controls (n = 24).
B: Growth chart showing the timing of recurrent c-MYC-induced tumor growth. Tumor volumes were measured following doxycycline withdrawal.
C: Northern analysis performed on total RNA from fully regressed c-MYC-induced mammary adenocarcinomas that recurred in the absence of doxycycline. The blot was probed with a radiolabeled *c-MYC* fragment that recognizes both the *c-MYC* transgene and endogenous *c-myc* (top panel). Three of fourteen recurrent tumors demonstrated reactivation of the c-MYC transgene in the absence of doxycycline. A duplicate Northern blot was probed with a radiolabeled fragment from exon 1 of *c-myc* that recognizes endogenous *c-myc*, but not the *c-MYC* transgene (middle panel). Note that expression of *c-myc* in the eleven recurrent mammary adenocarcinomas without reactivation of the *MYC* transgene is comparable to that seen in nontransgenic tumors induced by the carcinogen, DMBA, whereas endogenous *c-myc* expression is suppressed in the three recurrent mammary adenocarcinomas that had reactivated the *MYC* transgene in the absence of doxycycline. Paired samples from a c-MYC-induced tumor harvested on doxycycline and 7 days after doxycycline withdrawal during the regression phase are shown as controls. Ethidium bromide stained 28S rRNA bands are shown as loading controls (bottom panel).

in bitransgenic animals in the absence of doxycycline treatment; third, tumors that were grafted into syngeneic hosts, allowed to grow on doxycycline, and then deinduced were also found to recur spontaneously (data not shown); and fourth, for those primary tumors biopsied prior to doxcycline withdrawal in which *Nras* mutations were identified, the identical *Nras* mutation was also found in the recurrent tumor (data not shown). Together, these findings strongly suggest that tumors reappearing at the site of a fully regressed tumor in mice maintained off doxycycline represent genuine recurrences. Furthermore, the observation that most fully regressed MYC tumors recur spontaneously indicates that the regression of primary tumors following oncogene downregulation—though dramatic—is incomplete, leaving viable residual neoplastic cells at the site of the original tumor.

MYC-induced tumors recur by MYC-dependent and MYC-independent mechanisms

To determine whether tumor recurrences occur as a result of *MYC* overexpression or by a pathway independent of MYC overexpression, we performed Northern hybridization on RNA isolated from recurrent tumors using a radiolabeled fragment of c-*MYC* exon 2 that detects expression of both the *MYC* transgene and endogenous c-*myc*. Overexpression of *MYC* was absent in 13 of 16 recurrent tumors (Figure 2C, upper panel, and data not shown). To determine whether elevated c-*MYC* transcript levels resulted from upregulation of endogenous c-*myc* or from reactivation of the *MYC* transgene in the absence of doxycycline treatment, Northern blots were probed with a cDNA fragment specific for endogenous c-*myc* (Figure 2C, middle panel). Consistent with myc's ability to repress its own transcription, expression of endogenous *c-myc* was suppressed in all three recurrent tumors exhibiting high levels of c-*MYC* expression. This finding confirms that these tumors had reactivated the *MYC* transgene in a doxycycline-independent manner rather than upregulating endogenous *c-myc*. Consistent with this, the remaining 13 recurrent tumors exhibited endogenous c-*myc* levels comparable to those found in carcinogen-induced mammary adenocarcinomas (Figure 2C, middle panel). Together, these results suggest that the majority of MYC-induced tumors recur by a MYC-independent mechanism and that residual neoplastic cells persist following the regression of MYC-induced tumors and can eventually circumvent their requirement for MYC overexpression for neoplastic growth.

It has previously been reported that osteogenic sarcoma cells in which the *c–MYC* transgene has been inactivated are protected against the malignant properties of this oncogene following *MYC* transgene reactivation (Jain et al., 2002). However, we noted that 3 of 16 recurrent mammary tumors expressed the *MYC* transgene at levels comparable to those found in tumor-bearing animals maintained on doxycycline (Figure 2C upper panel). Thus, in contrast to osteogenic sarcoma cells, the spontaneous reactivation of *c-MYC* transgene expression in a subset of recurrent c-MYC-induced adenocarcinomas suggests not only that residual mammary tumor cells are not protected from c-MYC's tumorigenic effects, but that reactivation of MYC within residual tumor cells is actually selected for as a means of mammary tumor recurrence.

Residual neoplastic tumor cells remain susceptible to c-MYC

As noted above, even brief inactivation of c-MYC has been reported to protect osteogenic sarcoma cells from MYC's onco-

genic properties due to MYC's ability to induce apoptosis in residual tumor cells following its reactivation (Jain et al., 2002). Since c-MYC expression is required for the proliferation of normal cells, these findings have important clinical implications, as they argue that long-term inactivation of c-MYC may not be required for effective cancer therapy and that transient MYC inactivation may actually confer greater antitumor effects than sustained MYC inactivation. In contrast, the spontaneous reactivation of *c-MYC* transgene expression that we observed in recurrent mammary adenocarcinomas suggests that residual tumor cells remain susceptible to this oncogene.

To further address whether residual mammary tumor cells are protected from MYC's oncogenic properties, doxycycline was readministered to eight bitransgenic animals bearing fully regressed mammary adenocarcinomas. In all cases, tumors reappeared at the site of the original tumor with an average latency of six days, far shorter than the 22-week latency required for primary tumor formation (Figures 3A and 3B). Furthermore, in bitransgenic animals in which mammary adenocarcinomas had remained nonpalpable for at least six months, reactivation of c-MYC resulted in the rapid recurrence of tumors in five out of six animals (data not shown). These observations strongly suggest that residual neoplastic cells remain viable for long periods of time at the site of the regressed primary tumor and are only one step away from reacquiring their full malignant potential. Our findings indicate that virtually all animals bearing fully regressed mammary tumors harbor residual neoplastic cells that persist at the site of the original tumor, and that these latent mammary tumor cells remain highly susceptible to the malignant properties of MYC following either brief or sustained periods of c-MYC inactivation. Thus, unlike osteogenic sarcoma cells, transient inactivation of c-MYC is not sufficient to protect mammary epithelial cells from the oncogenic consequences of its reactivation.

The protection against MYC's oncogenic effects observed in osteogenic sarcoma cells has been attributed to MYC's ability to induce apoptosis in cells in which its expression had been reactivated (Jain et al., 2002). Thus, one possible explanation for the lack of protection seen in residual mammary tumor cells is that these cells are no longer susceptible to c-MYC-induced apoptosis. However, TUNEL analysis of recurrent mammary adenocarcinomas arising in reinduced bitransgenic animals revealed levels of apoptosis comparable to those observed in primary c-MYC-induced mammary adenocarcinomas (Figure 3C). This suggests that the lack of protection against MYC's malignant properties observed among residual mammary tumor cells is not due to their inability to undergo apoptosis.

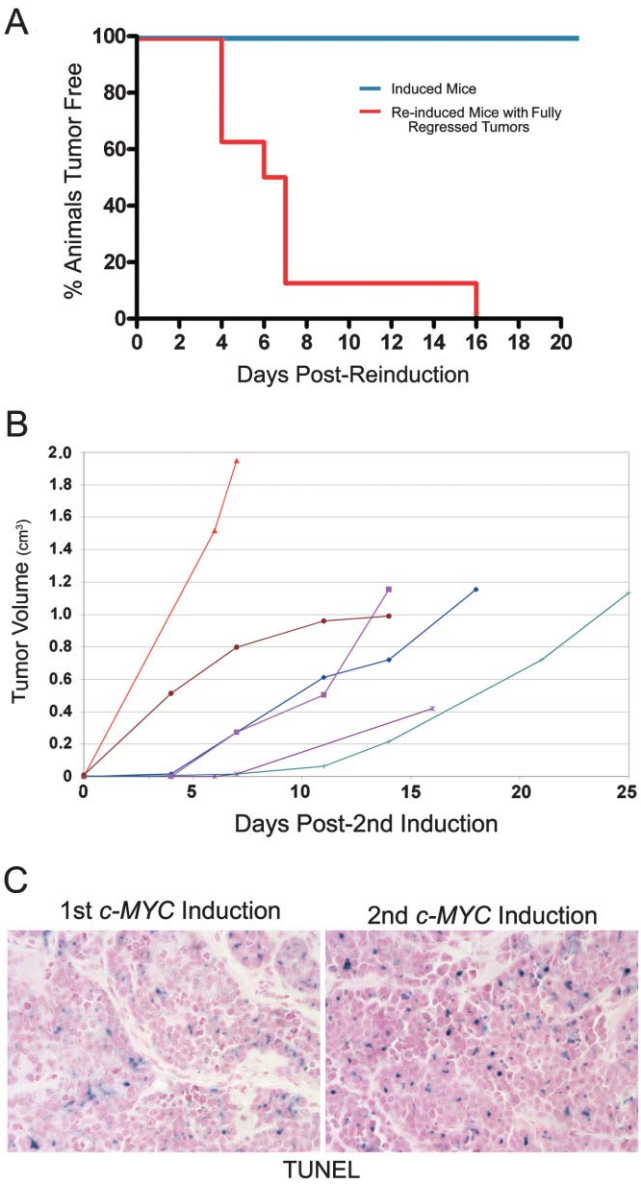

Figure 3. Residual neoplastic cells remain susceptible to MYC

A: Tumor-free survival curve for MTB/TOM animals bearing fully regressed tumors that were readministered doxycycline (n = 8). MTB/TOM mice, administered doxycycline for the first time, are shown as controls (n = 15).
B: Increase in tumor volume for representative tumors following *c-MYC* transgene reactivation (n = 6).
C: Apoptosis rates remain high in *c-MYC*-induced mammary adenocarcinomas following *MYC* transgene reactivation. Note comparable numbers of TUNEL-positive cells in representative sections from a c-MYC-induced mammary tumor sequentially biopsied during the initial induction of c-MYC transgene (left) and following reactivation of the *MYC* transgene subsequent to a period of *MYC* transgene inactivation (right).

MYC-induced tumors progress following repeated exposure to c-MYC

Tumors in inducible transgenic systems have been reported to retain their dependence on an initiating oncogene even after serial passage in syngeneic hosts, suggesting that oncogene dependence is a stable characteristic of tumors (Felsher and Bishop, 1999a). Since mammary epithelial cells are not protected from c-MYC's malignant properties following MYC inactivation, we considered whether mammary tumor cells subjected to repeated rounds of exposure to MYC would progress to a state in which tumor growth became independent of *MYC* transgene overexpression. To address this question, we evaluated whether tumors that were initially dependent upon c-MYC overexpression (i.e., had regressed to a nonpalpable state following doxycycline withdrawal) would progress to a *MYC* transgene-independent state following additional periods of exposure to MYC (Figure 4A). We anticipated that this protocol would approximate the situation in which patients are exposed to multiple courses of antineoplastic therapy as typically occurs during the course of cancer treatment.

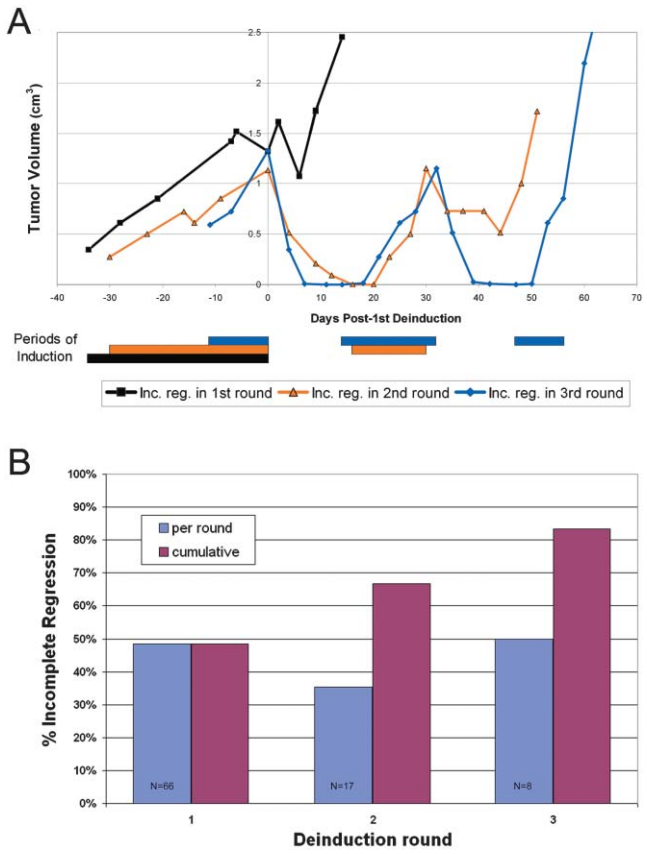

Figure 4. Mammary tumors progress to a *MYC*-independent state following repeated cycles of *MYC* expression

A: Representative tumor growth curves for c-MYC-induced mammary adenocarcinomas that acquire the ability to grow in a MYC-independent manner following one (black), two (orange), or three (blue) cycles of *MYC* transgene induction and deinduction.

B: Chart demonstrating the per-cycle and cumulative percentage of c-MYC-induced mammary adenocarcinomas that become MYC-independent during each round of *MYC* transgene induction and deinduction. Blue bars represent the percentage of tumors that grew independently of doxycycline treatment in each deinduction cycle; maroon bars represent the cumulative percentage of tumors that grew independently of doxycycline treatment by that cycle of induction/deinduction.

Bitransgenic animals were induced with doxycycline until mammary tumors developed and were deinduced when tumors reached 1 cm^3 in size. In those mice in which tumors fully regressed, *MYC* expression was reinduced. When doxycycline-induced tumor recurrences reached 1 cm^3 in size, transgene expression was again abrogated by doxycycline withdrawal. This process of sequential MYC activation and inactivation was repeated for three successive cycles. Incompletely regressing tumors from each round were assayed by Northern hybridization for expression of the *c-MYC* transgene, endogenous *c-myc*, and the MYC target gene, *Shmt1*, to determine whether doxycycline independence correlated with downregulation of the c-MYC pathway.

As in prior studies, following a single round of transgene induction and deinduction, 31 of 65 (49%) c-MYC-induced tumors resumed growth in the absence of doxycycline (Figure 4B). Transgene expression was then reinduced in the remaining 51% of mice in which tumors had regressed to a nonpalpable state following the first cycle of transgene induction and deinduction. Consistent with our previous finding that residual tumor cells remain sensitive to the oncogenic effects of MYC, all tumors rapidly regrew at their original sites following MYC reactivation (Figure 4A). When reinduced recurrent tumors reached 1 cm^3 in size, transgene expression was again abrogated by doxycycline withdrawal. Following this second round of *c-MYC* transgene induction and deinduction, 35% of tumors that had fully regressed following the first round of exposure to MYC failed to regress fully in response to the second period of MYC downregulation (Figure 4B).

To determine whether the remaining MYC-dependent tumors would progress to a doxycyline-independent state, the process of *c-MYC* transgene induction and deinduction was repeated for a third time in mice whose tumors had fully regressed for two consecutive cycles. Fifty percent of tumors that had fully regressed during the first two rounds of deinduction failed to regress fully following the third round of doxycycline withdrawal (Figure 4B). Thus, after three cycles of *c-MYC* transgene induction and deinduction, 83% of c-MYC-induced mammary adenocarcinomas had acquired the ability to grow in the absence of doxycycline. Northern hybridization analysis for the *c-MYC* transgene, endogenous *c-myc*, and the MYC target gene, *Shmt1*, demonstrated that only a single tumor in each round of deinduction had reactivated the Myc pathway in the absence of doxycycline and that all remaining tumors exhibited reduced activity of the Myc pathway (data not shown). The doxycycline-independent reactivation of *MYC* transgene expression in occasional tumors further highlights the fact that residual tumor cells remain exquisitely sensitive to the malignant properties of c-MYC despite single or multiple periods of c-MYC inactivation. Moreover, our findings also indicate that the vast majority of c-MYC-induced mammary adenocarcinomas do not remain dependent upon *c-MYC* transgene expression following repeated exposures to c-MYC, and instead progress to a transgene-independent state.

Kras2 activation contributes to MYC-independent growth in a subset of tumors

We have previously demonstrated that the presence of an activating point mutation in *Kras2* tightly correlates with the inability of primary MYC-induced mammary adenocarcinomas to regress fully following *MYC* transgene deinduction (D'Cruz et al., 2001). Thus, we considered the possibility that the progression of tumors that were initially MYC-dependent to a MYC-independent state could be due to the outgrowth of cells harboring activating *Kras2* mutations. As such, we evaluated the contribution of *Kras2* mutations to the loss of MYC dependence in MYC-induced tumors in two different experimental contexts. First, we determined *Kras2* mutation status in primary tumors that were MYC-independent at the first time of doxycycline withdrawal, as well as in MYC-independent recurrences that arose from primary tumors that had regressed to a nonpalpable state in mice maintained off doxycycline (Figure 2 and Table 1). Exons 1 and 2 of *Kras2* were amplified and sequenced to look for activating point mutations at codons 12, 13, and 61. Consistent with our previous findings, *Kras2* mutations were identified in 67% (18 of 27) of incompletely regressing primary tumors (Table 1). In contrast, only 25% (3 of 12) of spontaneous recurrences were found to have activating point mutations in *Kras2* (χ^2 =

Table 1. *Kras2* mutation status of c-MYC independent tumors

Experiment 1: Spontaneous tumor recurrence	*Kras2* mutation status
Primary, incompletely regressing tumors	67% (18/27)
Spontaneous tumor recurrences	25% (3/12)
Experiment 2: Repeated MYC induction/deinduction	***Kras2* mutation status**
Incompletely regressing tumors (1st round of deinduction)	50% (8/16)
Incompletely regressing tumors (2nd or 3rd round of deinduction)	29% (2/7)

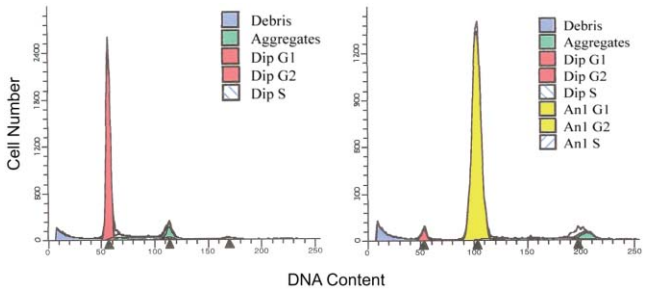

Figure 5. Transgene-independent MYC-induced mammary tumors remain diploid

The ploidy of c-MYC-induced mammary adenocarcinomas was determined by flow cytometric analysis of propidium iodide-stained nuclei. No aneuploidy was detected from nuclei of 38 tumors, 16 of which were dependent on c-MYC transgene expression for maintenance of the oncogenic state and 22 of which exhibited c-MYC transgene-independent growth. A representative DNA histogram for a c-MYC transgene independent tumor is shown (left). A tetraploid mammary tumor with known *p53* loss-of-heterozygosity was evaluated as a positive control (right).

4.25, $p = 0.04$). Thus, while acquisition of *Kras2* mutations appears to account for the progression to MYC independence in two-thirds of primary MYC-induced tumors, a significantly smaller fraction of recurrent mammary tumors achieves MYC independence by this mechanism. This suggests that mechanisms other than *Kras2* mutation contribute to spontaneous tumor recurrence in the majority of cases, and highlights the possibility that mechanisms of escape from MYC-dependence may differ between primary and recurrent tumors.

To investigate this issue further, we determined the *Kras2* mutation status of mammary tumors that had progressed to MYC independence following multiple rounds of exposure to MYC (Figure 4 and Table 1). Activating point mutations in *Kras2* were assayed in MYC-induced primary tumors that were MYC-independent following the first cycle of MYC induction and deinduction, and in tumors that became MYC-independent during the second or third cycles of MYC induction and deinduction. We found that while 50% of MYC-independent primary mammary tumors harbored *Kras2* mutations (8 of 16), *Kras2* mutations were identified in only 29% (2 of 7) of tumors that became MYC-independent in cycles 2 or 3. Our results suggest that while *Kras2* mutation represents one mechanism by which tumors become MYC-independent following sequential rounds of MYC exposure, the majority of tumors that acquire MYC-independence in this context do so by mechanisms other than *Kras2* mutation. In aggregate, our data argue that multiple mechanisms exist by which MYC-induced mammary tumors escape their dependence on MYC, and that *Kras2* mutation becomes a less commonly used pathway of tumor escape in recurrences—both spontaneous and induced—compared to primary tumors.

MYC-independent mammary tumors do not exhibit gross genomic instability

It has previously been suggested that MYC-induced tumors that arise as a consequence of this oncogene's ability to promote cellular proliferation and inhibit differentiation may remain dependent upon c-MYC, whereas tumors that arise as a consequence of c-MYC's ability to promote genomic instability may become MYC-independent (Jain et al., 2002). In support of this model, transient exposure of an immortalized cell line to increased c-MYC activity has been shown to induce chromosomal abnormalities and aneuploidy and to promote tumorigenesis in the absence of continued c-MYC expression (Felsher and Bishop, 1999b). Therefore, we considered whether the propensity of c-MYC-induced mammary adenocarcinomas to progress to transgene independence was a consequence of MYC's ability to induce genomic instability. Evaluation of the DNA content of nuclei from c-MYC-induced mammary adenocarcinomas by propidium iodide staining revealed that the vast majority of cells within c-MYC-induced mammary adenocarcinomas have a diploid DNA content (Figure 5 and data not shown). Of 38 MYC-induced mammary adenocarcinomas (22 of which exhibited c-MYC-independent growth), no tumor displayed evidence of gross genomic instability. Therefore, we conclude that c-MYC does not promote the progression of mammary tumors to transgene independence by destabilizing the genome.

Discussion

The natural history of human cancers is characterized by the progressive selection and outgrowth of cells that possess increasingly aggressive properties, such as loss of hormone dependence, resistance to chemotherapeutic agents, and the ability to invade tissues and metastasize. Moreover, residual cells from cancers that have responded to therapy frequently have the ability to survive and remain dormant within tissues for long periods of time and then subsequently resume growth. It is precisely these clinical manifestations of tumor progression that are responsible for the vast majority of cancer deaths.

Unlike the tendency of human cancers to progress to more aggressive states that are resistant to therapy, and despite the multistep nature of carcinogenesis, inactivation of MYC in experimental tumor systems has previously been shown to be sufficient to reverse the malignant properties of MYC-initiated lesions of epidermal, hematopoeitic, pancreatic islet, and osteogenic sarcoma cells (Felsher and Bishop, 1999a; Jain et al., 2002; Pelengaris et al., 2002). This has been taken as evidence that cancers can become "addicted" to the oncogenes that initiated them, thereby predisposing such tumors to a remarkable degree of dependence upon single oncogenic pathways. Consequently, it has been proposed that transient inactivation of dominant oncogenic pathways within tumors may represent

an effective—if not preferable—treatment for human cancers. This inference has particular significance for the clinic, since oncogene downregulation in experimental tumor systems can in some ways be seen to simulate idealized pharmacological inhibitors capable of efficiently blocking oncogenic pathways of interest. From this perspective, oncogene downregulation in conditional transgenic systems represents a genetic paradigm for the effects of molecularly targeted therapies.

In contrast to findings in hematopoeitic, pancreatic islet, and osteogenic sarcoma cells in which virtually all MYC-induced tumors demonstrate initial MYC dependence, we report here that most MYC-induced primary mammary tumors fail to regress fully despite MYC inactivation. Furthermore, while tumor relapse has been noted in only 10%–20% of c-MYC-induced lymphomas, recurrence of MYC-induced mammary tumors occurs as the rule rather than the exception (Felsher and Bishop, 1999a; Jain et al., 2002; Karlsson et al., 2003; Pelengaris et al., 2002). Notably, mammary tumor recurrences arise as a consequence of the presence of residual neoplastic cells that persist in the mammary glands of nearly all animals harboring fully regressed tumors. Moreover, reactivation of MYC in these residual cells results in their rapid resumption of neoplastic growth, indicating that these cells remain exquisitely sensitive to MYC's oncogenic effects. Thus, unlike the remarkable oncogene dependence observed for MYC-initiated hematopoietic and osteogenic sarcoma cells, the majority of MYC-induced mammary tumors progress to a state in which MYC overexpression is dispensable for growth. Our findings suggest that transient pharmacological inactivation of MYC may not be an effective treatment for human breast cancers in which this oncogene plays a dominant role, and emphasize that oncogene dependence is likely to be highly context-dependent.

Why then do MYC-induced experimental tumors of different tissues differ in the extent to which they remain dependent upon this oncogene? One possibility is that differences in oncogene dependence are due to differences in the response of different tumor types to MYC downregulation. For example, sustained tumor regression in osteogenic sarcoma cells following *MYC* transgene inactivation is accompanied by differentiation of these cells to mature osteocytes (Jain et al., 2002). In contrast, epithelial differentiation is not universally seen following MYC downregulation in mammary tumors. As such, the difference in response to c-MYC inactivation observed in osteogenic sarcoma and mammary adenocarcinoma cells may be explained by a failure of mammary tumor cells to undergo terminal differentiation following MYC downregulation.

The long latency of mammary tumor formation following MYC overexpression suggests that additional genetic changes are required for neoplastic transformation. These secondary genetic events could, in theory, contribute to tumor progression as well as tumor initiation. Specifically, such mutations could replace MYC as the dominant oncogenic pathway within the tumor and in this way contribute to tumor progression and the transition to oncogene independence. In this regard, we have previously reported that the incomplete regression of c-MYC-induced mammary adenocarcinomas correlates with the presence of spontaneous activating point mutations in *Kras2*. The occurrence of such mutations has not been reported for lymphomas, leukemias, or islet cell tumors initiated by MYC. Thus, differences in the mutations that accompany MYC-induced tumorigenesis in different tissues could explain the context-dependent nature of tumor regression following *MYC* transgene inactivation.

A particularly critical feature of human epithelial cancers is the ability of residual neoplastic cells to survive and persist in the host in a presumed quiescent state following the apparently successful treatment of the primary tumor. Ultimately, these residual neoplastic cells reemerge from their dormant state and resume growth, leading to cancer recurrence. Indeed, a significant fraction of breast cancers in women recur 10 years or more following treatment of the primary tumor. Analogous to this phenomenon, we report here that a subset of MYC-induced tumors that have regressed to a nonpalpable state following MYC downregulation recur spontaneously in the absence of transgene expression over periods of up to one year. Our observations further indicate that most, if not all, animals bearing fully regressed tumors harbor residual neoplastic disease long after the apparently complete regression of their tumors. Residual neoplastic cells and tumor recurrences have also been observed in animals bearing fully regressed Neu, Wnt1, and Ras-induced mammary tumors (Gunther et al., 2003; Moody et al., 2002; and data not shown). Thus, the behavior of tumors in these conditional mouse models parallels the natural history of human breast cancer and highlights the importance of understanding the mechanisms responsible for tumor recurrence.

In this regard, we have found that activating point mutations in *Kras2* contribute to the acquisition of MYC independence in spontaneous tumor recurrences arising from residual neoplastic cells, and in tumor cells that escape their dependence upon MYC following multiple rounds of exposure to this oncogene. However, while *Kras2* mutation contributes to the acquisition of MYC-independence in both primary and recurrent tumors, it appears to be a considerably more common mechanism in the former than the latter. That is, in most cases, mechanisms other than *Kras2* mutation account for the ability of tumor recurrence to escape their dependence on MYC, thereby highlighting the possibility that mechanisms of escape from oncogene dependence may differ between primary tumors and recurrences. Future analyses aimed at elucidating the oncogenic pathways that become dominant in recurrent tumors will help identify additional pharmacologic targets for the treatment of this deadly aspect of tumor progression.

To the extent that oncogene downregulation in conditional transgenic systems simulates the effects of molecularly targeted therapies, our findings have several implications for the use of such therapies. First, our observations that transient MYC inactivation does not result in sustained tumor regression and that residual neoplastic cells remain highly susceptible to the oncogenic effects of MYC suggest that common human epithelial cancers will require chronic rather than transient treatment with molecularly targeted agents. Second, our observation that prolonged MYC inactivation also fails to result in sustained tumor regression and that the great majority of MYC-induced mammary tumors progress to a state in which MYC overexpression is dispensable for growth suggests that treating human cancers with single molecularly targeted agents will likely fail. As such, our findings indicate that effective antineoplastic therapy will require combining agents that target dominant oncogenic pathways with agents that target secondary pathways of tumor escape, or that eliminate residual neoplastic disease. Third, the survival of residual neoplastic cells following tumor regression emphasizes that oncogene inactivation is not capa-

ble of fully reversing the oncogenic effects of MYC. Rather, a small number of cells persist that are able to survive in the absence of MYC overexpression and remain one step removed from frank malignancy.

Notably, even those partially regressing primary tumors that rapidly resume growth following MYC downregulation do so only after first exhibiting substantial regression, in most cases to less than one-third of their initial size. This indicates that the majority of cells within even MYC-independent mammary tumors are, in fact, dependent upon MYC for their survival. However, while sustained c-MYC inactivation results in the death of most cells within primary tumors, the existence within tumors of even a small number of cells that lack this requirement for MYC results in rapid treatment failure. As such, our findings emphasize that clinically important aspects of tumor behavior, such as recurrence, resistance, and relapse, are due to small—yet biologically critical—subsets of cells within tumors.

Finally, it is worth noting that reconciling the conflicting observations that have been made regarding the oncogene dependence of MYC-induced neoplasias may, in part, be a matter of perspective. On one hand, the dramatic regression of primary tumors that occurs following the abrogation of MYC expression in multiple tumor types—including many mammary tumors—represents an encouraging proof-of-principle that the vast majority of tumor cells bearing multiple genetic and epigenetic alterations may nevertheless remain dependent upon MYC for their maintenance. However, despite this remarkable degree of oncogene dependence, we demonstrate that MYC-induced mammary tumors typically progress to more aggressive states capable of growth in the absence of MYC pathway activation and thereby result in the death of the host. Balancing the encouraging extent to which complex tumors can remain dependent upon a single oncogenic mutation with the familiar clinical lesson that cancers treated with single agents typically relapse will be an important next step in determining the most efficacious use of molecularly targeted agents. Ultimately, elucidating the broad range of molecular alterations that occur during breast cancer progression will facilitate targeting of the multiple synergistic pathways that contribute to neoplastic progression and enhance the development of therapeutic strategies capable of dealing with tumor heterogeneity.

Experimental procedures

Animals and tissues

The generation of the MMTV-rtTA and TetO-MYC transgenic lines has previously been described (D'Cruz et al., 2001; Gunther et al., 2002). Transgenic mice were housed under barrier conditions with a 12 hr light/dark cycle and access to food and water ad libitum. Induced animals were administered doxycycline (0.2–2 mg/ml) (Sigma) in their drinking water, which was replaced weekly. Animals were inspected for tumors and existing tumors were measured weekly.

Tumor regression and recurrence analysis

Following doxycycline withdrawal from the drinking water of chronically induced bitransgenic mice that had developed mammary tumors, tumor measurements were taken every three days for the first 18 days and weekly thereafter. Calipers were used to measure tumor size in two dimensions. Tumor volume was calculated as volume = a × b × (a + b)/2. Extent of tumor regression was calculated as a percentage of the minimum tumor volume reached compared to tumor volume at the time of doxycycline withdrawal. Tumor regrowth (Figures 1B and 1C) was calculated as the difference between the minimum volume of deinduced tumors post-doxycycline withdrawal and the maximum volume that the same tumors reached by day 40 post-doxycycline withdrawal. Regressing tumors that were nonpalpable on at least two consecutive occasions were considered to have fully regressed.

Northern analysis

Snap-frozen tissue was homogenized in guanidine thiocyanate supplemented with 7 μl/ml 2-mercaptoethanol, and RNA isolated by centrifugation through cesium chloride as previously described (Rajan et al., 1996). Total RNA (3 μg per blot) was separated on a 1% LE agarose gel and passively transferred to Gene Screen (NEN). Northern hybridization was performed per manufacturer's instructions using PerfectHyb Plus Hybridization Buffer (Sigma) and a ^{32}P-labeled cDNA probe containing a portion of exon 2 of human *c-MYC*, or with a cDNA probe from exon 1 of murine *c-myc*.

TUNEL analysis

Mammary adenocarcinomas were harvested and fixed overnight in 4% paraformaldehyde, transferred to 70% ETOH, and embedded in paraffin. 5 μm sections on ProbeOn Plus (Fisher) slides were dewaxed in xylene, then sequentially rehydrated in 100%, 95%, and 70% ETOH, followed by phosphate-buffered saline (PBS). TUNEL analysis was performed using the Apoptag Peroxidase In Situ Apoptosis Detection Kit (Chemicon) according to manufacturer's instructions. Sections were pretreated in Proteinase K (20 μg/ml) for 15 min at RT, washed in deionized water twice for 2 min each, and returned to 1× PBS. Sections were then covered with equilibration buffer for a minimum of 2 min followed by incubation at 37°C for 1 hr with a 1:10 dilution of TdT enzyme in 1× reaction buffer. Reactions were terminated, developed using anti-digoxigenin-alkaline phosphatase fab fragments (Roche) and NBT/BCIP ready-to-use tablets (Roche) per manufacturer's instructions, and counterstained with nuclear fast red (Vector Labs). Control slides were used consisting of sections from involuting mammary glands day 2 postlactation.

Kras2 mutation analysis

cDNA was generated per manufacturer's instructions using First-Strand cDNA Synthesis Kit (Amersham Biosciences). DNA fragments from *Kras2* were PCR-amplified, purified, and sequenced as previously described (D'Cruz et al., 2001).

FACS analysis

Snap-frozen tumor tissue was lysed in hypotonic buffer containing propidium iodide according to the method of Vindelov (Vindelov, 1977). 20,000 events per tumor sample were analyzed on a FACScan flow cytometer. DNA content histograms were generated using ModFit software (Verity Software House Inc.)

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