Research Article

A Dominant-Negative c-jun Mutant Inhibits Lung Carcinogenesis in Mice

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Abstract

Lung cancer is the leading cause of cancer mortality in the United States and worldwide. The identification of key regulatory and molecular mechanisms involved in lung tumorigenesis is therefore critical to increase our understanding of this disease and could ultimately lead to targeted therapies to improve prevention and treatment. Induction of members of the activator protein-1 (AP-1) transcription factor family has been described in human non–small cell lung carcinoma. Activation of AP-1 can either stimulate or repress transcription of multiple gene targets, ultimately leading to increased cell proliferation and inhibition of apoptosis. In the present study, we show induction of AP-1 in carcinogen-induced mouse lung tumors compared with surrounding normal lung tissue. We then used a transgenic mouse model directing conditional expression of the dominant-negative c-jun mutant TAM67 in lung epithelial cells to determine the effect of AP-1 inhibition on mouse lung tumorigenesis. Consistent with low AP-1 activity in normal lung tissue, TAM67 expression had no observed effects in adult mouse lung. TAM67 decreased tumor number and overall lung tumor burden in chemically induced mouse lung tumor models. The most significant inhibitory effect was observed on carcinoma burden compared with lower-grade lesions. Our results support the concept that AP-1 is a key regulator of mouse lung tumorigenesis, and identify AP-1-dependent transcription as a potential target to prevent lung tumor progression.

Introduction

The deregulation of cell proliferation is a hallmark of cancer cell growth. The activator protein-1 (AP-1) family of transcription factors can mediate expression of many genes that play key roles in cell proliferation. The AP-1 transcription factor complex consists of homodimers or heterodimers of jun and fos family members. The jun family has three members (c-jun, junB, and junD), whereas the fos family consists of c-fos, fosB, Fra-1, and Fra-2. Whereas jun family members can either homodimerize or heterodimerize with other AP-1 proteins, fos family members are only capable of heterodimerization with members of the jun family. Dimerization is absolutely required for DNA binding and transcriptional activation. Multiple targets of AP-1 transcriptional activation have been identified, including genes involved in cell proliferation (1, 2), matrix degradation (3), and cell survival (1, 4, 5), but the exact targets of importance in lung tumorigenesis are incompletely defined.

Elevated levels of AP-1 family members are associated with several types of tumors in animal models (6–9). The majority of studies on human clinical specimens are consistent with the concept that AP-1 is elevated during lung tumorigenesis. Elevated levels of AP-1 components have been reported in human non–small cell lung carcinoma (NSCLC; refs. 10, 11), correlate with poor prognosis (12) and tumor metastasis (13), and are increased in tumors from smokers compared with nonsmokers (14). A prospective study of 216 patients with NSCLC showed decreased long-term survival in patients with elevated protein levels of c-fos and c-jun within the resected tumors (15, 16), suggesting that targeting the AP-1 pathway therapeutically could potentially lead to improved survival of NSCLC patients. The exact role of AP-1 family members in the pathogenesis of lung cancer is unclear and is likely to depend on both the cellular context and genetic composition of the cells involved.

Several agents that have shown tumor chemopreventive efficacy in animal models are known to inhibit AP-1 activity,
lending support to the hypothesis that AP-1 is an important effector of tumorigenesis. Compounds with proven efficacy in preventing tumors in animal models and known to inhibit AP-1 include glucocorticoid agonists (17, 18), tea extracts (19, 20), resveratrol (21–23), retinoids (24), and berry extracts (25, 26). Glucocorticoids and tea extracts have been shown to inhibit formation of lung adenomas in the A/J mouse model (17, 18, 20). Although inhibition of AP-1 activity is a widespread effect of known chemopreventive agents, the relative contribution of AP-1 inhibition to the overall efficacy of each of these agents is unknown and most of these agents have multiple effects on cell physiology.

TAM67 is a truncation mutant of c-jun that lacks amino acids 3 to 122 of wild-type c-jun corresponding to the transcriptional transactivation domain (27). Characterization of the transcriptional activity of this mutant revealed that it can dimerize with either jun or fos AP-1 family members (27), has similar DNA binding kinetics as wild-type c-jun (28), and functions in a dominant-negative fashion (28, 29). Transgenic expression of TAM67 in mouse epidermal keratinocytes inhibited papilloma formation in a two-stage skin tumorigenesis model (30) and also in UVB-induced skin tumor formation (31). Similarly, expression of TAM67 in breast epithelial cells inhibited mammary tumor formation (32). TAM67 inhibited the growth of nasopharyngeal (33), breast (34), lung (35), and colorectal (36) cancer cells transplanted into nude mice and osteosarcoma cell metastases following tail vein injection (37). In vitro studies showed that proliferation of NSCLC cell lines was inhibited by TAM67 via mechanisms affecting cell cycle control but not by promotion of apoptosis (35, 38). Thus, expression of a dominant-negative c-jun mutant can effectively limit cell proliferation in multiple cell types.

The goal of the current study was to determine if inhibition of AP-1 could prevent lung tumor formation and progression. Our data indicate activation of AP-1 signaling in a carcinogen-induced model of mouse lung tumorigenesis. Using transgenic mice that allow for conditional expression of the TAM67 dominant-negative mutant in lung epithelial cells, we found that induction of the TAM67 transgene, either before or after initiation of lung tumorigenesis with a chemical carcinogen, inhibited lung tumorigenesis. Transcriptional regulation by AP-1 seems to be an important mechanism mediating mouse lung tumorigenesis.

Materials and Methods

Animals and genotyping

Csp-rtta (39), Tre-Tam67 (40), and AP-1-luciferase (41) mice have been described previously. Csp-rtta and AP-1-luciferase mice were backcrossed at least seven generations onto the A/J strain. Bitransgenic Csp-rtta × Tre-Tam67 mice were generated by crossing A/J Csp-rtta mice to FVB/N Tre-Tam67 mice to generate F1 progeny for use in lung tumorigenesis studies in protocol 2. Doxycycline diet (Harlan-Teklad) containing 625 mg of doxycycline hyclate per kilogram of chow was used to induce transgene expression. The presence of transgenes was determined by PCR using DNA isolated from tail snips at weaning. PCR conditions for Csp-rtta (39) and Tre-Tam67 (40) have been previously described. PCR primers to detect firefly luciferase were used to identify the AP-1-luciferase transgene: forward primer, 5'-CCTTAGTAAACCGATGATCC-3'; reverse primer, 5'-CGGAATACCTCGAATGTCC-3'. Following an initial 5-minute denaturation at 94°C, reactions were cycled 30 times at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by a final extension at 72°C for 5 minutes. All animal procedures were carried out under Animal Studies Committee–approved protocols at Washington University in St. Louis or the University of Cincinnati.

Lung tumorigenesis protocols

Lung tumorigenesis was induced by either a single i.p. injection of benzo(a)pyrene [B(a)P] at a dose of 100 mg/kg body weight at 6 to 8 weeks of age (protocol 1) or a single i.p. injection of urethane at a dose of 1,000 mg/kg body weight at 6 to 8 weeks of age (protocol 2). Csp/Tam67 mice used in protocol 1 were on the FVB/N strain background, whereas protocol 2 used A/J × FVB/N F1 crosses. For Csp/Tam67 experiments, doxycycline diet was introduced at the times indicated in the text and continued until the termination of the experiment. At the indicated time points, mice were deeply sedated with a ketamine/xylazine cocktail followed by exsanguination. Whole lungs were removed and fixed overnight in 4% buffered formaldehyde in PBS at 4°C. Tissue was then washed with cold PBS and transferred to 70% ethanol. Lung tumor multiplicity and diameter were calculated as described previously (42). For tumorigenesis experiments with AP-1-luciferase mice, animals were injected i.p. with B(a)P (100 mg/kg) at 8 weeks of age, and lung tissue was collected 40 weeks later. Lung tissue was embedded in cold OCT embedding medium and stored at −80°C until use.

Tumor pathology

Lung tissue was fixed in 10% neutral buffered formalin overnight and then transferred to 70% ethanol. Peripheral lung lesions were counted under a dissecting microscope to determine surface multiplicity before paraffin embedding. Surface tumor diameters were measured using an ocular micrometer for calculation of tumor volumes using the equation $V = \frac{4}{3}\pi r^3$, where $r$ is tumor radius. Lung lobes were separated before paraffin embedding. Paraffin blocks were faced until tissue from all five lobes was present in a section, and this level was designated L0. Three additional 5-μm sections were obtained every 200 μm (L1, L2, and L3). Tumor histology (hyperplasia, adenoma, dysplasia, and carcinoma) of H&E-stained sections at each level was assessed by a pathologist blinded to the group identifications using previously described criteria (43). Digitized images of H&E-stained sections were obtained, and area of each tumor classification and total lung was calculated using pixel counts as previously described (44).

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**In situ hybridization**

For *in situ* hybridization, lung tissue was frozen in liquid nitrogen and embedded in Tissue-Tek Cryo-OCT embedding compound. The firefly luciferase cDNA in the pGEM7zf vector was linearized, and sense and antisense digoxigenin (DIG)–labeled riboprobes were synthesized using Sp6 and T7 RNA polymerase and a DIG RNA Labeling kit (Roche Applied Sciences). To confirm equal synthesis of probes before use, serial dilutions of DIG-labeled riboprobes were spotted onto nitrocellulose membranes and color was developed using alkaline phosphatase–conjugated anti-DIG antibodies with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as the chromogen (Roche Applied Sciences).

Frozen sections were postfixed with 4% formaldehyde in PBS for 10 minutes and washed twice for 10 minutes with PBS, once for 10 minutes with PBS + 0.3% Triton X-100, twice for 5 minutes with PBS, and then once for 5 minutes with diethyl pyrocarbonate (DEPC)–treated water. Slides were then treated with 0.3M HCl for 20 minutes, washed with DEPC-treated water for 5 minutes, and then incubated with 10 μg/mL proteinase K in PBS for 30 minutes. After two additional PBS washes, slides were postfixed with 4% formaldehyde in PBS and washed once with PBS and twice with 2× SSC [20× SSC = 3 mol/L NaCl, 0.3 mol/L sodium citrate (pH 7.0)]. After circling the tissue sections with hydrophobic marking pen, the tissue sections were prehybridized with 100 μL hybridization solution (30% deionized formamide, 10% dextran sulfate, 1× Denhardt’s solution, 4× SSC, and 500 μg/mL herring sperm DNA) at 50°C for 60 minutes. Hybridization was in the same solution with 200 ng/mL DIG-labeled riboprobe. A coveslip was carefully placed over the tissue section, and slides were incubated overnight at 50°C in a humidified chamber. Slides were then washed sequentially in 4×, 2×, 1×, 0.5×, 0.25×, and 0.125× SSC at room temperature to remove unbound probe. To detect the DIG-labeled riboprobe, sections were incubated for 5 minutes at room temperature with 150 mmol/L NaCl and 100 mmol/L Tris (pH 7.5) and then for 30 minutes with the same solution plus 10% (w/v) Blocking Reagent (Roche Applied Sciences). Sections were then incubated with 1:200 dilution of anti-DIG antibody (sheep polyclonal; Roche Applied Sciences). Sections were then incubated with 1:200 dilution of anti-DIG antibody (sheep polyclonal; Roche Applied Sciences). After circling the tissue sections with hydrophobic marking pen, the tissue sections were prehybridized with 100 μL hybridization solution (30% deionized formamide, 10% dextran sulfate, 1× Denhardt’s solution, 4× SSC, and 500 μg/mL herring sperm DNA) at 50°C for 60 minutes. Hybridization was in the same solution with 200 ng/mL DIG-labeled riboprobe. A coveslip was carefully placed over the tissue section, and slides were incubated overnight at 50°C in a humidified chamber. Slides were then washed sequentially in 4×, 2×, 1×, 0.5×, 0.25×, and 0.125× SSC at room temperature to remove unbound probe. To detect the DIG-labeled riboprobe, sections were incubated for 5 minutes at room temperature with 150 mmol/L NaCl and 100 mmol/L Tris (pH 7.5) and then for 30 minutes with the same solution plus 10% (w/v) Blocking Reagent (Roche Applied Sciences). Sections were then incubated with 1:200 dilution of anti-DIG antibody (sheep polyclonal; Roche Applied Sciences) in blocking buffer overnight at 4°C. Slides were washed twice for 5 minutes with 150 mmol/L NaCl and 100 mmol/L Tris (pH 7.5) and then once with detection buffer [50 mmol/L MgCl2, 100 mmol/L NaCl, 100 mmol/L Tris (pH 9.5)] for 10 minutes. Color was developed in detection buffer plus 200 μl/mL NBT/BCIP reagent until signal was observed, typically from 40 minutes to 2 hours. The reaction was stopped by incubating for 5 minutes in 10 mmol/L Tris (pH 8.0) and 1 mmol/L EDTA and washed once with water before counterstaining with hematoxylin. Sections were covered with aqueous mounting medium (SuperMount, BioGenex Laboratories) before microscopic examination.

**Reverse transcription-PCR**

Total tissue RNA was homogenized in TriReagent (Molecular Research Center) according to the manufacturer’s recommendations. cDNA was synthesized from 2 μg of total RNA using oligo(dT) priming and SuperScript II reverse transcriptase (Invitrogen). PCR to detect TAM67 transcript was carried out with the forward primer 5’-CCACGCTGTITTGAAGTCATAG-3’ and reverse primer 5’-GCTTCCTTTTTCGGAACCTTG-3’. Cycling conditions were an initial 5-minute denaturation step at 94°C, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final 5-minute extension at 72°C. PCR products were separated on agarose gels and visualized with ethidium bromide. Primers to detect β-actin were 5’-CCTTCTCCGACCTCCTCC-3’ and 5’-TCTCCCTGTGACACCGTTC-3’. Cycling conditions were identical but with an annealing temperature of 61°C.

**Immunoprecipitation and Western blotting**

To isolate lung proteins, lung tissue was homogenized in tissue protein extraction reagent supplemented with Halt protease inhibitor cocktail and 5 mmol/L EDTA (Thermo Scientific). Anti–Flag-M2 mouse monoclonal antibody was purchased from Sigma-Aldrich. For immunoprecipitation, 500 μg of protein lysate were incubated with 2 μg of anti–Flag-M2 antibody overnight at 4°C. A Pierce spin column loaded with 20 μL of Protein A/G Plus agarose slurry (Thermo Scientific) was used to bind immune complexes. Washes and elution were done according to the manufacturer’s recommendation. Proteins were eluted in a 50-μL volume, and 20 μL were loaded on a 4% to 12% Bis-Tris NuPage Novex gel (Invitrogen) run under reducing conditions. Gels were transferred to polyvinylidene difluoride membrane, blocked in 5% nonfat dried milk in TBS + 0.1% Tween 20 (TBST) for 4 hours, and then incubated with 1:2,500 dilution of anti-Flag antibody overnight at 4°C. After three washes with TBST, the membrane was incubated for 2 hours with 1:500 dilution of goat anti-mouse horseradish peroxidase–conjugated secondary antibody and then washed five times with TBST. Binding was visualized with SuperSignal West Femto chemiluminescent substrate (Thermo Scientific).

**Statistical analysis**

Student’s *t* test (one-tailed) was used to test the a priori hypothesis that Tam67 inhibits lung tumorigenesis. Precise *P* values are presented in the text, figure legends, or tables.

**Results**

**Induction of AP-1 during mouse lung tumorigenesis**

Because expression of individual AP-1 subunits, formation of distinct dimer pairs, and posttranscriptional modifications of subunits can contribute to overall AP-1 activity, we used a transgenic model in which a luciferase reporter gene is expressed under the control of an AP-1–dependent promoter (41) to analyze AP-1 activity in mouse lung tumors. Lung tissue of adult AP-1-*luciferase* transgenic mice was used to test the a priori hypothesis that Tam67 inhibits lung tumorigenesis. Precise *P* values are presented in the text, figure legends, or tables.
mice was previously shown to have little or no luciferase activity (45). AP-1-luciferase transgenic mice on the A/J strain background were injected i.p. with the carcinogen B(a)P at 6 to 8 weeks of age to induce lung tumorigenesis. Forty weeks after B(a)P injection, large adenomas and adenocarcinomas were apparent in mouse lung (Fig. 1A). An antisense DIG-labeled cRNA probe for luciferase was hybridized to frozen lung tissue sections followed by stringent washes, incubation with anti-DIG antibody, and color development. Hybridization signal was detected in lung tumors but not in surrounding normal lung tissue (Fig. 1B) or in lung tissue of age-matched littermates that were not injected with B(a)P (data not shown). No signal was detected on adjacent sections processed simultaneously using a sense riboprobe (Fig. 1C), showing specificity of the hybridization signal. Thus, AP-1–dependent reporter gene expression was increased in mouse lung tumors compared with normal lung tissue.

Characterization of conditional TAM67 expression in mouse lung

Because AP-1 activation was observed in mouse lung adenocarcinomas, and because TAM67 inhibits tumorigenesis in other tissues, we hypothesized that TAM67 would inhibit mouse lung tumorigenesis in vivo. Mice carrying a transgenic construct consisting of the c-jun mutant TAM67 under the control of a tetracycline-responsive promoter were described previously (40). These mice were crossed to mice expressing the reverse tetracycline-responsive transactivator (rtTA or TET-ON) under control of the lung epithelial–specific Clara cell secretory protein (CCSP) promoter (39) to obtain double-transgenic offspring. Total lung RNA from Ccsp-rtta × Tre-Tam67 double-transgenic mice (hereafter designated Ccsp/Tam67 mice) was obtained, and transgene expression was determined by reverse transcription-PCR (RT-PCR) after variable durations of doxycycline. Little or no transgene expression was detectable in the absence of doxycycline (Fig. 2A). Tam67 mRNA was variably induced after 2 days of doxycycline and further induced after 7 or 31 days (Fig. 2A). Because the Tam67 transgene carries the Flag epitope at its NH2 terminus, we used immunoprecipitation and Western blotting with anti-Flag antibody to detect the truncated c-jun protein encoded by the Tam67 transgene. Tam67 protein was undetectable in the absence of doxycycline but was induced following 7 days of doxycycline and was of the predicted size for the truncated c-jun protein (Fig. 2B). The Tam67 protein was inducible when doxycycline was administered before or after carcinogen. The induction of Tam67 was reversible, as the protein was not detected in lung tissue from mice fed doxycycline diet for 7 days followed by 6 days on normal chow (Fig. 2B). Formalin-fixed, paraffin-embedded lung tissue sections from Ccsp/Tam67 mice were examined after H&E staining and were histologically normal after 1 month of doxycycline (Fig. 2D), consistent with previous reports of low AP-1 activity in normal mouse lung tissue (45). Ccsp/Tam67 mice remain healthy when maintained on doxycycline chow for up to 40 weeks without apparent changes in lung histology (data not shown).

TAM67 inhibited lung tumor formation when induced before or after initiation

We first determined if induction of the Tam67 transgene before carcinogen injection would prevent lung
tumorigenesis. Ccsp/Tam67 mice received doxycycline diet (625 mg doxycycline hyclate/kg diet) at ~6 weeks of age and, 5 days later, received a single i.p. injection of B(a)P (100 mg/kg body weight) to induce lung tumorigenesis. Mice were maintained on doxycycline diet for 40 weeks (Fig. 3A). At this time point, lungs were fixed and lung tumor number and size were determined. Compared with control, induction of the Tam67 transgene decreased tumor multiplicity by 65% (2.62 ± 2.20 tumors/lung versus 0.92 ± 0.76; P = 0.007; Fig. 3B). Tumor load, a measure of both tumor number and tumors size, was reduced 98% compared with control (4.51 ± 6.60 mm³ versus 0.11 ± 0.18 mm³; P = 0.012; Fig. 3C) with induction of the Tam67 transgene.

Because Tam67 transgene expression was induced before carcinogen administration in protocol 1, the dominant-negative effect of Tam67 could be caused by alterations in carcinogen metabolism or clearance versus tumor formation or progression per se. Thus, in protocol 2, we initiated doxycycline 1 week after injection of the lung carcinogen.
uracnhene (postinitiation protocol). Urethane is widely used in mouse lung carcinogenesis studies and induces earlier onset and greater tumor burden compared with B(a)P. Csp/Tam67 mice on a FVB/N × A/J F1 background were injected with urethane (1,000 mg/kg body weight i.p.) at 8 weeks of age, and doxycycline treatment to induce Tam67 expression was initiated 1 week later (Fig. 4A). After 20 weeks, lungs were fixed and tumor number and size were determined. Similar to what was observed with preinitiation induction of Tam67, induction of Tam67 after carcinogen injection significantly inhibited tumor multiplicity (6.8 ± 2.9 tumors/lung versus 15.0 ± 8.4; P = 0.034; Fig. 4B). As controls, littermates with only the Csp-rtta transgene (Csp mice) were also injected with urethane and split into groups with or without doxycycline diet. Doxycycline had no effect on tumor multiplicity in Csp single-transgenic mice (Fig. 4B).

To examine the effect of Tam67 on tumor pathology, lung tissue from urethane-injected Csp/Tam67 mice was step sectioned and three lung sections separated by 200 μm were stained with H&E. Observed lesions were scored as hyperplasias, adenomas, dysplasias, or adenoacarcinomas as described previously (43). In addition, digitized images of each stained section were quantitated for total lung area and area of each of the three highest-grade lesions (adenoma, dysplasia, and carcinoma). Mice expressing the Tam67 transgene had significantly reduced carcinoma burden 20 weeks after urethane injection (2.1 ± 1.4% of total lung area versus 4.5 ± 2.1%; P = 0.039; Fig. 4C; Table 1). No significant difference in the area of adenomas or dysplasias was observed. When the sum of tumor area of all pathology grades was used (adenoma + dysplasia + adenoacarcinoma), Tam67 transgene induction also significantly reduced total tumor burden (2.5 ± 1.2% of total lung area versus 5.4 ± 2.5; P = 0.034; Fig. 4C; Table 1). Tumor number by pathology grade was also determined. Total lesions (hyperplasias + adenomas + dysplasias + adenoacarcinomas) were reduced from 32.8 ± 17.7 per lung to 17.8 ± 5.2 in Csp/Tam67 mice given doxycycline, although this effect did not reach the P = 0.05 threshold (P = 0.071; Table 1). All pathology grades except dysplasias reflected this decreasing trend.

**Discussion**

Activation of the AP-1 family of transcription factors has previously been implicated in lung tumorigenesis, but direct evidence for involvement of this pathway in vivo has been lacking. In the current study, we used B(a)P-induced lung carcinogenesis to confirm the presence of increased AP-1 activity in mouse lung tumors. Using a transgenic mouse line expressing the firefly luciferase reporter gene under control of a consensus AP-1 promoter, we detected increased luciferase mRNA in lung tumors, but not normal lung tissue, 40 weeks after injection of the lung carcinogen B(a)P. These data support the concept that AP-1 target genes are specifically activated in lung tumors during lung tumorigenesis. The use of the AP-1–luciferase reporter mice enabled a readout of AP-1 transcriptional activity using a well-established AP-1–responsive promoter. Although we cannot be certain that the AP-1 consensus promoter directing luciferase expression in this mouse is representative of all AP-1–responsive genes, the data are consistent with an important role for AP-1 activation in mouse lung tumors. Determining AP-1–luciferase reporter gene expression at additional time points will increase our understanding of the timing and extent of AP-1 activation during mouse lung tumorigenesis.

![Diagram](https://example.com/diagram.png)
Previous studies showed a tumor-inhibitory effect of the dominant-negative mutant Tam67 during mouse skin and breast tumorigenesis in vivo (30–32). Expression of Tam67 under control of an epidermal-specific promoter inhibited skin papilloma formation in a two-stage initiation/promotion model (30) and inhibited UVB-induced squamous cell carcinomas (31). Interestingly, in both cases, early epidermal hyperplasia was unaffected, although inhibition of AP-1 activity was confirmed using the AP-1-luciferase reporter mouse, suggesting that AP-1 transcriptional activation is important during the tumor promotion stage of skin tumors. In protocol 2, we observed an inhibitory effect of Tam67 on lung tumorigenesis when the transgene was turned on 10 days after carcinogen administration. This result indicates that inhibition of AP-1 during the tumor promotion stage of lung tumorigenesis is sufficient to reduce tumor number. The same Tre-Tam67 transgenic mice used in our study have also been crossed to MMTV-rtta transgenic mice, enabling doxycycline-inducible Tam67 expression in mammary epithelial cells (32). When MMTV/Tam67 mice were crossed to MMTV-ERBB2 transgenic mice that develop mammary tumors, administration of doxycycline reduced mammary tumor incidence and increased survival in trigenic mice. The present study extends to lung the tumor types that can be successfully inhibited by expression of Tam67 in vivo.

Examination of lung tumor pathology in carcinogen-treated Ccsp/Tam67 mice indicated that Tam67 expression resulted in a 56% \( (P = 0.039) \) reduction in carcinomas compared with mice in which Tam67 was not induced. There was also a trend for reduction of adenomas in the Tam67-expressing mice, whereas there was no effect on dysplasias (Table 1). These data suggest that Tam67 expression inhibited progression of lung tumors. An increase in lower-grade lesions was not observed as might be predicted if Tam67 was acting solely by inhibiting progression from lower- to higher-grade lesions. In fact, the frequency of hyperplasias was inhibited to a similar extent as carcinomas (Table 1). We interpret this result to mean that Tam67 can exert inhibitory effects throughout the process of lung tumorigenesis. It is possible that expression of the Tam67 transgene could be activated at different times in different cells during the tumorigenesis process. Expression of the transgene is ultimately determined by the 2.3-kb Ccsp promoter driving rtTA expression, and this promoter may be transiently activated or inactivated in subsets of epithelial cells within the lung. This effect may be particularly prevalent in rapidly proliferating tumor cells. In addition, the transgene promoter does not direct rtTA expression in all epithelial cells (39, 46), and thus, carcinogen-induced tumors can arise in nonexpressing cells, contributing to the partial inhibition seen. Shen et al. (32) observed that Tam67 expression was absent in breast tumors that persisted following doxycycline treatment, and a similar situation may occur in our model.

The ability of Tam67 to inhibit lung tumor formation indicates that this pathway has potential as a target of intervention in this disease. Several classes of chemopreventive agents are reported to inhibit AP-1 activity, including glucocorticoids (47) and tea catechins (19, 20), and this may mediate part of their chemopreventive effect. Because of the multiple protumorigenic effects of AP-1 signaling, it has been suggested to be an attractive target for cancer chemoprevention (48). Studies to directly address the contribution of AP-1 inhibition in the cancer chemopreventive effect of these agents are needed both to confirm the importance of this transcription factor in tumorigenesis and improve our understanding of the mechanisms used by effective chemopreventive agents. To this end, we have preliminary data indicating that a purified green tea extract, a known lung cancer chemopreventive agent, is ineffective in preventing lung tumors in induced Ccsp/Tam67 mice, although additional studies are needed to confirm this result. This approach should

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**Table 1. Lung tumor area and number by pathology grade**

<table>
<thead>
<tr>
<th>Pathology grade</th>
<th>Tumor area (% of lung area occupied by each pathology grade)</th>
<th>Tumor number (total diagnoses/lung)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Dox</td>
<td>Dox</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Adenoma</td>
<td>0.6 ± 0.6</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>0.3 ± 0.3</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>4.5 ± 2.1</td>
<td>2.0 ± 1.4</td>
</tr>
<tr>
<td>All grades</td>
<td>5.4 ± 2.5</td>
<td>2.5 ± 1.2</td>
</tr>
</tbody>
</table>

NOTE: Pathology grade of lesions was determined on H&E-stained sections as described in Materials and Methods. Values represent mean ± SD. P values were determined by one-tailed Student’s t test. Because of their diffuse boundaries, area measurements were not carried out on hyperplasias. Abbreviation: ND, not determined.

*Y. Yan et al., unpublished data.*
be useful in determining the relative contribution of AP-1 toward the efficacy of many chemopreventive agents.

The fact that Tam67 inhibited lung tumorigenesis when doxycycline was given after initiation (protocol 2) as well as before initiation (protocol 1) suggests that Tam67 has a direct effect on inhibiting lung tumor progression. However, the ability of Tam67 to affect expression of enzymes mediating carcinogen metabolism and clearance has not been explored. In the present study, protocol 1 was conducted only with B(a)P, whereas protocol 2 used urethane. Given that these two carcinogens are metabolized by distinct enzymes, it is possible that using both protocols for each carcinogen could yield carcinogen-specific results. However, both carcinogens induce Kras mutations, which are thought to be a primary oncogenic driver of these tumors. Therefore, it is likely that inhibition of AP-1 activation in developing tumors plays a key role in the inhibitory effect of Tam67.

A key unanswered question is the specific gene targets mediating the lung tumor-inhibiting effects of Tam67. Multiple studies have implicated genes involved in control of the G1-S transition of the cell cycle as targets for TAM67 inhibition, including Rb, E2F1, E2F2, cyclin A, cyclin D, and the cyclin-dependent kinase inhibitor p27 (38, 49, 50). Recent studies highlight that a subset of AP-1-responsive genes may mediate the inhibitory effects of TAM67 in a given cell type (51–53). mRNA expression profiling indicated that targets of TAM67 during inhibition of skin tumorigenesis were classified as being involved in invasion, metastasis, and inflammation to a greater extent than proliferation or cell survival (51). In addition to inhibition of AP-1, some studies have shown interactions between Tam67 and NF-κB transcription complexes (54, 55). Our results indicate that AP-1–dependent reporter gene expression is increased in B(a)P-induced lung tumors.

Our results show the inhibitory effect of Tam67 expression on mouse lung tumorigenesis. The model described here will be useful in determining the key targets mediating the lung tumor–inhibitory effects of Tam67. This in turn will enhance our understanding of the molecular alterations that occur during lung tumorigenesis and may lead to the identification of novel targets for chemopreventive and/or therapeutic intervention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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A Dominant-Negative c-jun Mutant Inhibits Lung Carcinogenesis in Mice

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