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The AP-1 transcription factor regulates postnatal mammary gland development

Qiang Shen [a], Yun Zhang [a], Ivan P. Uray [a], Jamal L. Hill [a], Hee-Tae Kim [a], Chunhua Lu [a], Matthew R. Young [b], Edward J. Gunther [c], Susan G. Hilsenbeck [a], Lewis A. Chodosh [c], Nancy H. Colburn [b], Powel H. Brown [a,*]

[a] Breast Center, Baylor College of Medicine, MS600, 1 Baylor Plaza, Houston, TX 77030, USA
[b] Gene Regulation Section, LCP, NCI-Frederick, Frederick, MD 21702, USA
[c] Department of Cancer Biology, Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA 19104, USA

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Abstract

The AP-1 transcription factor is activated by multiple growth factors that are critical regulators of breast cell proliferation. We previously demonstrated that AP-1 blockade inhibits breast cancer cell growth in vitro. Yet a specific role of AP-1 in normal mammary gland development has not been studied. Using a bi-transgenic mouse expressing an inducible AP-1 inhibitor (Tam67), we found that the AP-1 factor regulates postnatal proliferation of mammary epithelial cells. Mammary epithelial proliferation was significantly reduced after AP-1 blockade in adult, prepubertal, pubertal, and hormone-stimulated mammary glands. In pubertal mice, mammary cell proliferation was greatly reduced, and the cells that did proliferate failed to express Tam67. We also observed structural changes such as suppressed branching and budding, reduced gland tree size, and less fat pad occupancy in developing mammary glands after AP-1 blockade. We further demonstrated that Tam67 suppressed the expression of AP-1-dependent genes (*TIMP-1*, *vimentin*, *Fra-1*, and *fibronectin*) and the AP-1-dependent growth regulatory genes (*cyclin D1* and *c-myc*) in AP-1-blocked mammary glands. We therefore conclude that AP-1 factor is a pivotal regulator of postnatal mammary gland growth and development.
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Keywords: AP-1 transcription factor; Transcription factor blockade; Mammary epithelium; Mammary gland development

Introduction

Mammary gland development is a multi-stage and complex process involving hormone-dependent and hormone-independent stages. A better understanding of mammary gland development will provide insights into the molecular mechanisms that control normal mammary epithelial cell growth and malignant transformation. Mammary epithelial cell transformation may occur at the level of the mammary stem cells or possibly more differentiated breast cells (Al-Hajj et al., 2003; Dontu et al., 2003; Welm et al., 2002, 2003). Thus, intervention of the growth signals at both early and late stages may be required to block normal mammary gland transformation to ultimately prevent breast cancer. In this study, we have attempted to block these growth signals in the mammary gland by specific transcription factor inhibition.

The AP-1 factor is an important transcription factor that regulates multiple cellular functions such as proliferation, apoptosis, and organ development. As a key component of many signal transduction pathways, AP-1 is composed of dimers

Abbreviations: AP-1, activating protein-1; ATF, activating transcription factor; BrdU, bromodeoxyuridine; C/EBP, CCAAT/enhancer binding protein; cJunDN, cJun dominant negative mutant; Dox, doxycycline; E + P, estrogen + progesterone; ER, estrogen receptor; ERE, estrogen response element; IGF, insulin-like growth factor; IGF-IR, insulin-like growth factor I receptor; IHC, immunohistochemical staining; MG, mammary gland; NF-κB, nuclear factor κB; PR, progesterone receptor; PRL, prolactin; PRLR, prolactin receptor; TEB, terminal end buds.

* Corresponding author. Fax: +1 713 798 1642.
E-mail address: pbrown@breastcenter.tmc.edu (P.H. Brown).

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of Jun (cJun, JunB, and JunD), Fos (cFos, FosB, Fra-1, and Fra-2), or other closely related factors such as ATF proteins (Angel and Karin, 1991). The Jun members homodimerize or heterodimerize with different Fos or ATF members, while Fos members only heterodimerize with different Jun members. Differential expression and activation of Jun and Fos members allow AP-1 complexes to control a wide variety of cellular functions (Chiu et al., 1989; Jochum et al., 2001; Schutte et al., 1989; Yoshioka et al., 1995; Young et al., 2003). AP-1 is critically involved in controlling cellular proliferation (Holt et al., 1986), differentiation (Szabo et al., 1991), apoptosis (Ham et al., 1995), oncogene-induced transformation (Brown et al., 1993, 1994), and cellular invasion (Angel et al., 1987; Mackay et al., 1992; Matrisian, 1994; McDonnell et al., 1990). Its component c-Jun is a positive regulator of cellular proliferation in fibroblasts and in other cells (Behrens et al., 1999; Johnson et al., 1993; Schreiber et al., 1999; Wisdom et al., 1999). In AP-1 knockout mice, cJun deletion causes embryonic lethality at E12.5 (Eferl et al., 1999; Hilberg and Wagner, 1992; Hilberg et al., 1993; Johnson et al., 1993). We have previously shown that a cJun dominant-negative mutant, Tam67, inhibits the growth of breast cancer cells induced by peptide growth factors and estrogen (Liu et al., 2002, 2004; Ludes-Meyers et al., 2001). We have also demonstrated that many estrogen-induced growth regulatory genes are critically dependent on AP-1 (DeNardo et al., 2005).

The role of AP-1 factor in mammary gland development has not previously been examined. In this study, we hypothesized that blockade of the AP-1 factor would interfere with proliferation and growth of normal mammary gland. To test this hypothesis, we generated a bi-transgenic mouse that expresses an inducible AP-1 inhibitor, Tam67, in the mammary gland. This cJun dominant-negative mutant (cJunDN) lacks the transactivation domain of cJun, yet retains its DNA-binding and dimerization domains (Brown et al., 1993). We have used the Tet-On inducible vector to produce mice that have inducible expression of Tam67 in the mammary glands. Inducible Tam67 expression resulted in in vivo AP-1 blockade which in turn suppressed proliferation and growth in developing and hormone-stimulated mammary glands. We found that mammary cell proliferation was significantly decreased after AP-1 blockade in adult, pubertal, prepubertal, and hormone-stimulated mammary glands. AP-1 blockade also resulted in morphological alterations in the prepubertal and pubertal mammary glands with reduced gland size, branching, and budding. We further demonstrated that Tam67 downregulated the transcription of AP-1-dependent genes including two critical cell cycle regulators, *cyclin D1* and *c-myc* in vivo. These studies show that the AP-1 transcription factor is critical for postnatal normal mammary gland growth.

Materials and methods

Generation and genotyping of transgenic mice

The experimental FVB strain mouse for breeding and generation of transgenic mice was purchased from the Center for Comparative Medicine at Baylor College of Medicine, Houston, TX. The FVB strain MMTV-rtTA mouse was generated in and obtained from Dr. Chodosh's laboratory (Gunther et al., 2002). All studies involving the mice were regulated under the protocol approved by Baylor IACUC. The Tam67 transgene, regulated by a tetracycline-inducible promoter (prepared from the pUHD-Tam67 vector; Ludes-Meyers et al., 2001), was microinjected into FVB/F1 one-cell embryos at both Baylor College of Medicine Transgenic Mouse Facility and at NCI-Frederick. The pUHD-Tam67 transgenic mice were backcrossed with wild type FVB mice to produce heterozygous Tam67-positive ($Tam67^{+/-}$) and Tam67-negative mice ($Tam67^{-/-}$). The heterozygous Tam67-positive mice were backcrossed with siblings to generate homozygous Tam67 transgenic mice ($Tam67^{+/+}$). These homozygous Tam67 mice were crossed with the MMTV-rtTA mouse to produce a heterozygous pUHD-$Tam67^{+/-}$/MMTV-$rtTA^{+/-}$ bigenic mouse. Offsprings carrying both the pUHD-Tam67 and MMTV-rtTA transgenes were identified by PCR analysis of tail DNA. The pUHD-Tam67 primer sequences are: forward primer, 5′ATGGACTACAAGGACGACGA3′, and reverse primer, 5′GCGATTCTCTCCAGCTTC3′. MMTV-rtTA PCR reactions were carried out as described previously (Gunther et al., 2002). PCR products were separated on a 1.5% agarose gel, and mice found to carry both transgenes were selected for these studies.

Doxycycline (Dox) treatment

For mice treated with Dox to induce Tam67 transgene, doxycycline hyclate (Sigma-Aldrich, Cat# D9891, St. Louis, MO, USA) was supplied in drinking water at a 2 mg/ml concentration. The Dox-containing water was replaced every 3 days.

Estrogen and progesterone stimulation of mammary glands

The method was described to mimic pregnancy (Laidlaw et al., 1995). Briefly, silastic tubing was prepared by mixing dry estrogen and progesterone into the cavity of the tubing. Each transplantable tubing was approximately 1 cm long and contained approximately 5 μg of estrogen and 20 mg of progesterone. After autoclaving, the tubing was emerged in saline for 24 h before inserting subcutaneously into the back of experimental mice for the indicated period of time. Alternatively, β-estrodiol 3-benzoate (E_2) (Sigma E-8515), progesterone (Sigma P-0130), and sesame oil (Sigma S-3547) were purchased from Sigma-Aldrich Co, St. Louis MO. The mixed suspension of estrogen and progesterone (E + P) contains 10 μg of E_2 and 10 mg of progesterone dissolved in 1 ml of sesame oil. E + P suspension was injected at 0.1 ml per mouse/day subcutaneously for the indicated days. The two methods gave similar results.

In vivo BrdU incorporation

Two hours before sacrificing animals, BrdU solution (Amersham, Piscataway, NJ) was injected IP at a 0.1 ml/10 g of mouse body weight. BrdU-positive cells were detected by IHC staining using an anti-BrdU antibody, as described below. BrdU positive cells were counted in 40 fields (200×), and shown as a percentage of the total mammary epithelial cells counted.

Mammary gland whole mount analysis

The #4 inguinal mammary glands were harvested and spread onto slides, fixed with 10% formalin overnight. The glands were placed in 70% ethanol for 1 h followed by 30 min in ddH_2O before staining overnight with 0.2% carmine. The mammary glands were dehydrated sequentially in 70%, 90%, and 100% ethanol for 30 min each, and cleared in toluene to dissolve fat in the gland. The slides were maintained in methyl salicylate for analysis under light microscope. The number of primary and secondary branches, and ductal ends were counted for the entire gland. For fat pad occupancy measurement, the fat pad area was divided into 10 equal sections from the major gland duct to the farmost edge of the fat pad, each section was assigned a 10% of the total fat pad volume. The mammary gland tree occupancy was then estimated according to the sections

occupied. Data points from all mice in the same group were averaged and plotted.

Immunohistochemical (IHC) analysis

The mammary glands were collected from FVB mice carrying either no transgene or the following transgenes: Tam67$^{+/-}$, MMTV-rtTA$^{+/-}$, and Tam67$^{+/-}$/MMTV-rtTA$^{+/-}$. The samples were fixed in 4% paraformaldehyde in PBS solution overnight, and then embedded in paraffin. Tissue sections were then mounted onto slides and processed for hematoxylin and eosin staining or IHC staining as previously described (Liu et al., 2002). Briefly, 4 μm tissue sections were cut and mounted onto slides. The slides were deparaffinized, and endogenous peroxidase was blocked in 3% hydrogen peroxide buffer. The anti-Flag antibody was used as the primary antibody (1:100, M2, Sigma-Aldrich, St. Louis, MO, USA) followed by a biotinylated rabbit anti-mouse antibody (1:100). Peroxidase activity was visualized using Vector DAB or NovaRed Substrate Kit. Other primary antibodies used include anti-BrdU and anti-cleaved Caspase 3 (1:100, BD Pharmingen, San Diego, CA, USA). The slides were counterstained with hematoxylin for 1 min followed by mounting with a coverslip. Staining-positive cells were counted in 40 fields and data were expressed as a percentage of total epithelial cells counted. The above specified antibodies for Flag and BrdU were used to conduct fluorescent study on the mammary gland sections from bigenic pUHD-Tam67/MMTV-rtTA mice. The nuclei were stained with DAPI, Tam67 with FITC, and BrdU with Texas Red fluorescent dye.

Preparation of enriched mammary epithelial cell pellets

The inguinal mammary glands were aseptically removed from the experimental mice and placed in DMEM:F12 medium with antibiotics. After removing the lymph nodes, the mammary tissue was minced into pieces ≤1 mm with a sharp scalpel. The minced tissue was then digested in medium (DMEM:F12 + antibiotics + collagenase 2 mg/ml + hyaluronidase 0.1 mg/ml) and shaken at 37°C for 3 h at a speed of 125 rpm. The digested tissue was then pelleted (1000 rpm for 3 min) and the supernatant removed. Pelleted cells were washed with PBS + 5% FBS four times (Medina and Kittrell, 2000). These enriched mammary epithelial cells were then used for RNA and protein preparation.

Western blot analysis

Tissue lysates were prepared by incubating the mammary glands or MCF-7 breast cancer cells in lysis buffer. Protein concentrations in these lysates were measured by BCA analysis. Equal amounts (40 μg) of total cellular protein extract were electrophoresed on a 10% acrylamide gel and transferred onto a nitrocellulose membrane (Amersham, Arlington Heights, IL). The primary antibodies used were anti-Flag M2 (1:2000 dilution, Cat# F1804, Sigma-Aldrich, St. Louis, MO), anti-β-Actin (1:8000 dilution, Cat# A5441, Sigma-Aldrich, St. Louis, MO), anti-cyclin D1 (1:1000 dilution, Cat# RM-9101, Lab Vision, Fremont, CA), anti-c-Myc (1:500 dilution, Cat# SC-40, Santa Cruz Biotech, Santa Cruz, CA), and anti-TIMP1 (1:400 dilution, Cat# ab 4047, Abcam, Cambridge, UK). An anti-mouse or anti-rabbit antibody (1:4000 dilution, Amersham, Piscataway, NJ) was used as the secondary antibody. The Western blotted bands were visualized using the enhanced chemiluminescence (ECL) procedure (Amersham, Piscataway, NJ, USA).

Preparation of total RNA from mouse mammary tissue

Total RNA was extracted as described previously (Liu et al., 2004; Yang et al., 2001). RNA was prepared from enriched epithelial pellets from mouse mammary glands (individual mouse or pool of inguinal glands from 5–7 mice) following the protocol provided by Qiagen Inc. RNA from fixed mammary tissue in paraffin blocks was prepared by first cutting the tissue into 10 μm sections, and extracting the RNA from 10 sections using a kit from Epicenter, Inc. RNA samples were analyzed using quantitative RT-PCR as described below.

Quantitative reverse-transcriptase PCR (Q-RT-PCR)

Quantitative real-time reverse transcriptase PCR (Q-RT-PCR) assays of transcripts were carried out using gene-specific double fluor-labeled probes in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The PCR reaction mixture consisted of 300 nM each of the primers, 100 nM probe, 0.025 units/μl of Taq Polymerase, 125 μM each of dNTPs, 5 mM $MgCl_2$, ROX reference dye, and 1× Taq Polymerase buffer. Cycling conditions were 94°C for 1 min, followed by 94°C for 12 s and 60°C for 1 min for 40 cycles. All primers and probes were designed with Primer Express 1.0 software (Applied Biosystems Foster City, CA). 6-Carboxy fluorescein (FAM) was used as the 5′ fluorescent reporter while blackhole quencher 1 (BHQ1) was added to the 3′ end as quencher. Standard curves for the quantification of each transcript and β-actin were generated using the serially diluted solution of synthetic templates, and genome equivalent copies were calculated from the standard curve. For each sample, TaqMan PCR reactions were performed in triplicate, plus a non-RT control for each gene of interest, and β-actin to normalize for input cDNA. The ratio between the values obtained provides relative gene expression levels. Statistical significance was determined on triplicate samples using a student *t* test.

Statistical analysis

Statistical significance was obtained using two-sided Student's *t* test to compare proliferation, apoptosis, mammary gland developmental markers, and AP-1 regulated gene expression (QPCR) in animals treated with or without Dox, using Prism GraphPad 3.0 Software (San Diego, CA).

Results

Generation of a bigenic mouse line that expresses inducible Tam67 in the mammary glands

We previously used an inducible Tam67 vector stably transfected into MCF-7 cells to study the role of AP-1 in controlling the growth of breast cancer cells (Liu et al., 2002, 2004; Ludes-Meyers et al., 2001). Using this system, we constructed a Tet-On Flag-Tam67 transgene which was microinjected into pronuclei of fertilized mouse oocytes (Fig. 1A). Founder mice were screened by Southern blot analysis (data not shown) and PCR analysis using tail DNA samples. Two independent founder lines were established for breeding and experimental purposes that were subsequently bred to homozygousity (pUHD-Flag-Tam67$^{+/+}$). We then crossed these mice with our MMTV-rtTA transgenic mouse (Gunther et al., 2002) to generate bigenic pUHD-Tam67$^{+/-}$ | MMTV-rtTA$^{+/-}$ mice (Fig. 1B). The MMTV-rtTA transgenic mouse does not show any mammary-specific phenotype, but breeds, nourishes, and behaves normally as compared to its FVB strain counterparts (Gunther et al., 2002). Upon feeding the mice the inducer doxycycline (Dox) (which was dissolved in drinking water) for 2 weeks (Fig. 1C), Tam67 was induced in the mammary glands in adult virgin mice as shown by Western blotting and immunohistochemistry (IHC) (Figs. 1D–E). No expression was observed in salivary glands or other tissues as shown in Fig. 1D. An average of 40% of mammary epithelial cells was found to express induced Flag-Tam67 in 16-week-old adult female mice fed with Dox (Fig. 1F). No expression of Tam67 was detected in control mice that did not receive Dox. Using two

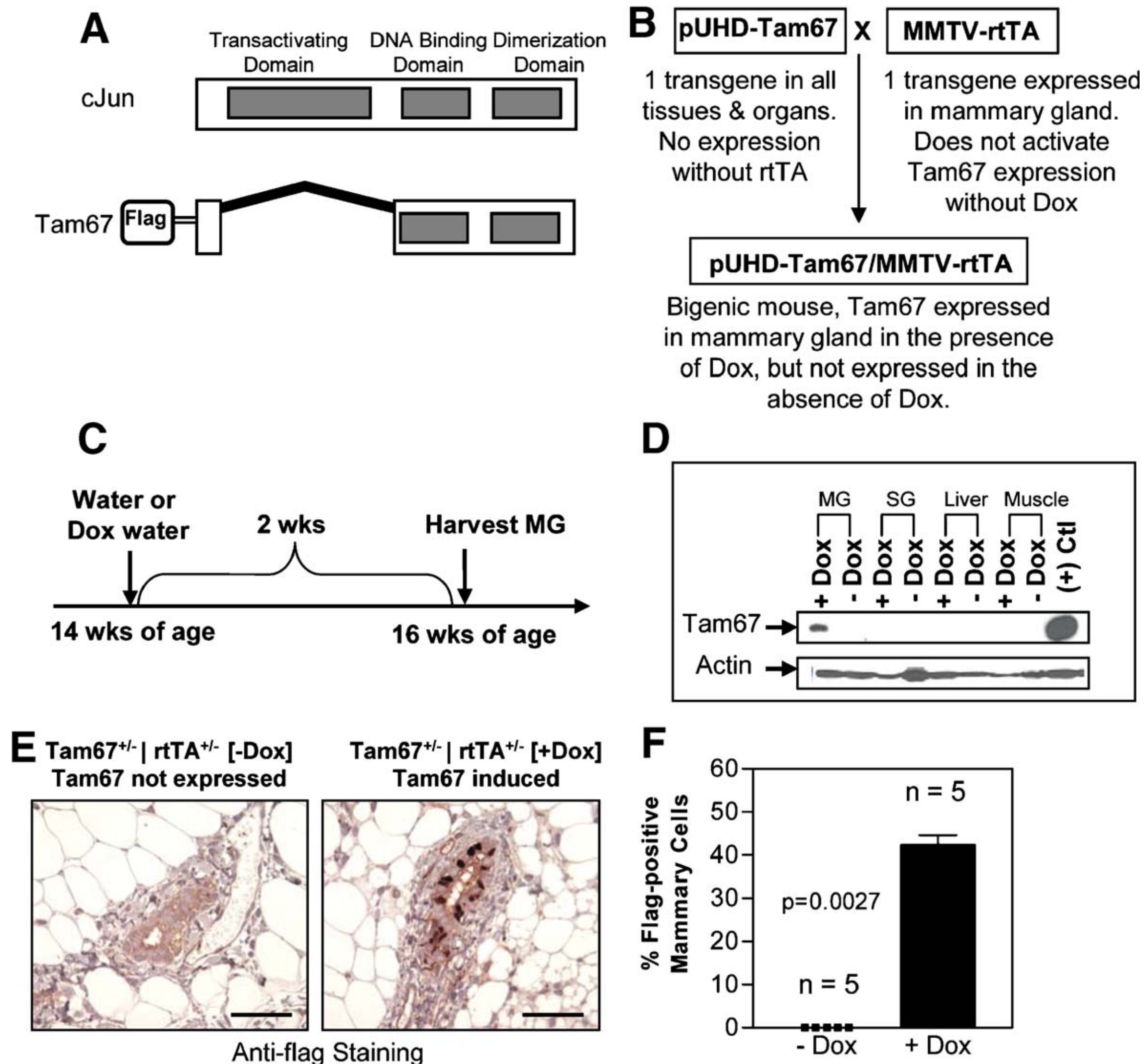


Fig. 1. Generation of bigenic pUHD-Tam67/MMTV-rtTA mice and induction of Tam67 in mammary gland. (A) Schematic drawing of the cJun mutant Tam67 in which it lacked the transactivating domain, while the DNA binding domain and dimerization domain were intact. A Flag tag was added to the N-terminal of the mutant protein. (B) Bigenic pUHD-Tam67/MMTV-rtTA mice were generated by crossing heterozygous or homozygous pUHD-Tam67 mice with MMTV-rtTA mice. Offspring were screened by PCR using tail DNA. (C) The adult bigenic mice were fed with regular water or Dox-water for 2 weeks, and then mammary glands were collected for protein and IHC studies. (D) Total tissue lysates were prepared from mammary and salivary glands, liver, and muscle from bigenic mice in panel C. Tam67 was detected in mammary glands of Dox-treated mice by Western blotting using an anti-Flag antibody. (E) Induction of Tam67 in mammary epithelial cells was confirmed by IHC using anti-Flag antibody. Original magnification 400×. Scale bar: 50 µm. (F) Data from quantifying 5 bigenic mice treated with water or Dox-water. Error bars represent SEM.

founder lines of mice, we obtained similar results in all experiments described below.

AP-1 blockade in vivo does not alter the global and histological architecture of mammary glands in adult mice

We next examined whether in vivo Tam67 expression caused morphological and/or histologic alterations in the mammary glands of adult virgin female mice (16 weeks old). We found no significant difference in AP-1-blocked or normal mammary gland whole mounts at different estrous cycle stages (Fig. 2A). Histologic sections showed no significant differences (Fig. 2B). In addition, expression of Tam67 did not affect these mice in feeding, nursing, social, and physical activities.

AP-1 blockade in vivo reduces mammary epithelial cell proliferation in adult glands

We next determined the effect of in vivo Tam67 expression on cellular proliferation in adult mouse mammary glands by counting BrdU-incorporated cells in bigenic animals (Tam67$^{+/-}$ | rtTA$^{+/-}$) fed with or without Dox. We found that the percentage of mammary cells positive for BrdU was reduced from 3.8% in control mice to 1.2% in mice fed Dox (P = 0.0011, Figs. 2C–D). We also counted the BrdU-incorporated cells in unigenic animals (either Tam67$^{+/-}$ | rtTA$^{-/-}$ or Tam67$^{-/-}$ | rtTA$^{+/-}$) fed with or without Dox, and found no significant difference when compared to the control bigenic mice (Tam67$^{+/-}$ | rtTA$^{+/-}$, no Dox) (data not shown). We next examined apoptosis in

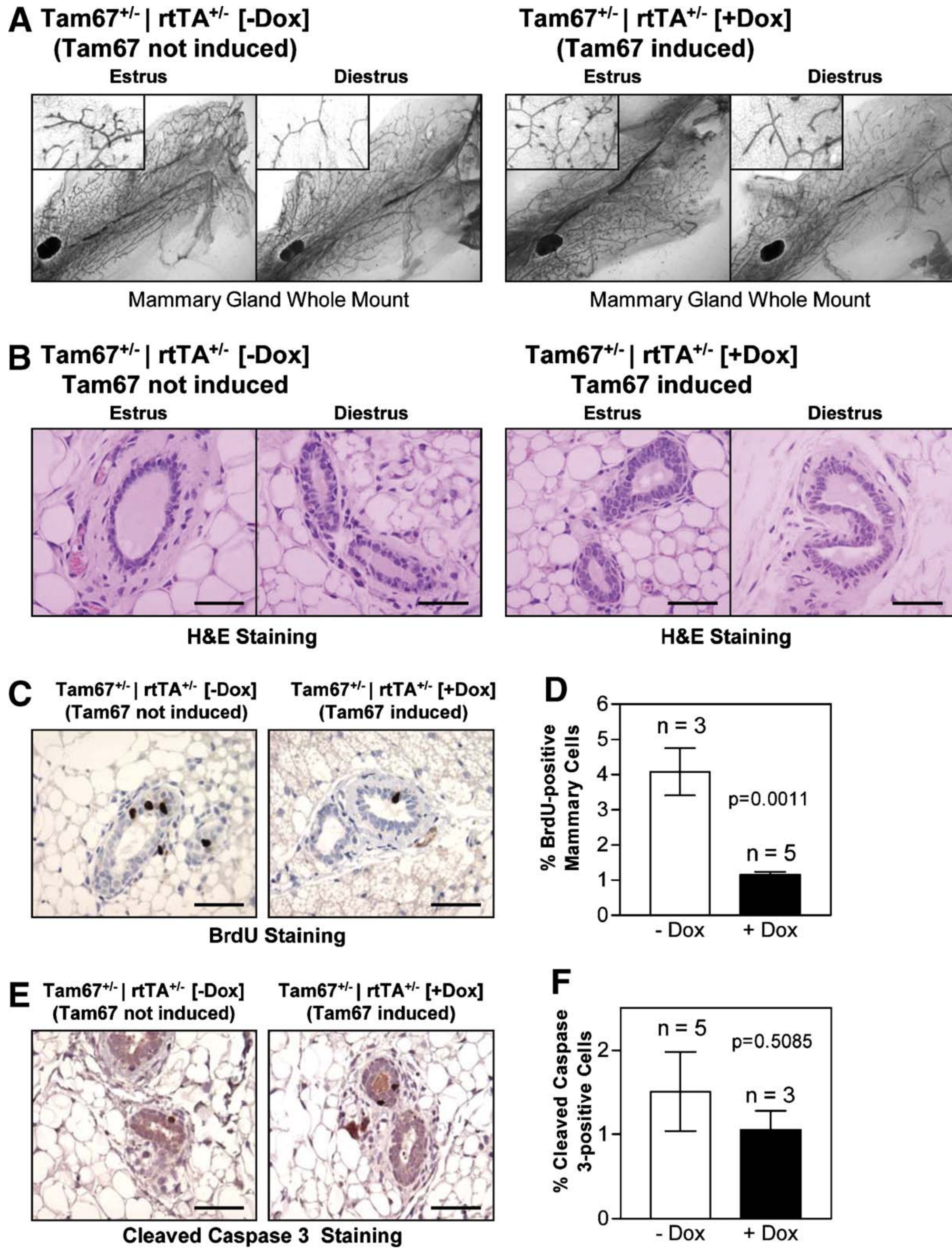


Fig. 2. Mammary gland architecture and histology were not altered, but mammary cell proliferation was reduced after AP-1 blockade. (A) Representative mammary gland whole mount (#4 inguinal gland) analysis of adult bigenic Tam67/rtTA mice fed with water or Dox-water (2× magnification). The global morphology of the mammary glands showed no significant changes in ductal tree and terminal end buds at two estrous cycle stages. (B) Representative H&E staining of mammary gland. Original magnification 400×. Scale bar: 50 μm. (C) Representative proliferation assay by IHC staining using anti-BrdU anti-body. Original magnification 400×. Scale bar: 50 μm. (D) Data from 3–5 bigenic mice treated with water or Dox-water. Error bars represent SEM. (E) Representative cleaved caspase 3 staining by IHC. Original magnification 400×. Scale bar: 50 μm. (F) Data from 3–5 bigenic mice treated with water or Dox-water. Error bars represent SEM.

these mammary epithelial cells by IHC using antibody against cleaved caspase 3. There was no significant difference between Dox-treated and control animals in the number of cleaved caspase 3-positive cells (Figs. 2E–F). These data demonstrate that AP-1 blockade suppresses mammary cell proliferation in adult virgin mice.

AP-1 blockade suppresses hormone-dependent mammary gland development in pubertal mice

We next examined the effect of in vivo AP-1 blockade on the developing mammary gland in mice at 4–6 weeks of age. We started feeding bigenic pUHD-Tam67$^{+/-}$ | MMTV-rtTA$^{+/-}$ mice with Dox at 4 weeks of age, and stopped at 6 weeks of age (Fig. 3A). The effects of AP-1 blockade by Tam67 were examined at the organ and cellular levels. We counted total numbers of primary mammary duct branches and ductal terminal ends, measured the outgrowth of the ductal trees, and estimated fat pad occupancy in the entire #4 mammary gland whole mounts from Tam67-expressing and control animals. AP-1 blockade significantly suppressed mammary gland development as shown in whole mounts (Fig. 3B). The mammary glands of Tam67-expressing mice showed significantly reduced outgrowth of the mammary gland trees (normal AP-1, 5.8 ± 1.3 mm vs. AP-1 blockade, 1.8 ± 1.4 mm, $P = 0.0009$), less occupancy of gland tree in fat pads (normal AP-1, 72 ± 4% vs. AP-1 blockade, 47 ± 5%, $P < 0.0001$), fewer primary branches (normal AP-1, 10.33 ± 1.25 vs. AP-1 blockade, 5.67 ± 0.75, $P < 0.0001$), and fewer ductal ends (normal AP-1, 277 ± 48 vs. AP-1 blockade, 130 ± 10, $P < 0.0001$), as compared to control mice (Figs. 3B–C).

AP-1 blockade in vivo reduces proliferation of mammary epithelial cells and increases apoptosis in pubertal mice

At the cellular level, approximately 34% of mammary epithelium was positive for Tam67 expression as confirmed by IHC staining using an anti-Flag antibody (Figs. 3D–E). Flag-tagged Tam67 was seen in all mammary gland branches, ducts, and ductal ends in animals fed Dox (Fig. 3D). We then examined mammary cell proliferation and apoptosis in the mammary glands. We first counted BrdU-positive cells in all bigenic animals (Tam67$^{+/-}$ | rtTA$^{+/-}$) fed with or without Dox. The percentage of cells positive for BrdU was significantly reduced in AP-1 blocked mice (5%), as compared to no Dox control mice (14% BrdU positive cells) ($P = 0.0019$, Figs. 3F–G). We also counted the BrdU-incorporated cells in unigenic animals (either Tam67$^{+/-}$ | rtTA$^{-/-}$ or Tam67$^{-/-}$ | rtTA$^{+/-}$) fed with or without Dox and found no significant difference in these unigenic mice compared to bigenic control mice (Tam67$^{+/-}$ | rtTA$^{+/-}$, no Dox) (data not shown).

We then measured apoptosis in the pubertal mammary epithelial cells by staining for cleaved caspase 3. Apoptosis was significantly increased from 1% in control mice to 2.5% in mice expressing Tam67 ($P = 0.0039$). From these data, we conclude that in vivo AP-1 blockade by Tam67 suppresses mammary gland growth in pubertal mice. These significant changes are due to decreased proliferation and increased apoptosis in the pubertal mammary glands.

Dissociation of proliferation of mammary epithelial cells and Tam67 expression

Since in vivo AP-1 blockade results in defective mammary gland development in pubertal mice, we hypothesized that Tam67 has a direct influence on the growth of cells expressing this AP-1 inhibitor. Using multi-color immunofluorescent staining, we determined whether Tam67 expression and BrdU incorporation occurred in the same cell. Using mammary gland sections from 6-week-old bigenic mice fed with Dox, we stained for Tam67 (anti-Flag antibody), proliferation (BrdU antibody), or nuclei (DAPI). Shown in Fig. 4A are transverse sections from two representative bigenic mice with or without Dox. Terminal buds from control mice show BrdU-positive cells, while buds from the glands of AP-1 blocked mice show both Tam67 and BrdU-positive cells. Most cells that express Tam67 did not stain for BrdU, demonstrating that Tam67-expressing cells were not proliferating. To quantitate the number of cells that were positive for either Tam67 or BrdU, or positive for both, we randomly selected 65 fields from six AP-1-blocked pubertal mice and counted the number of total mammary epithelial cells, Tam67-expressing cells, BrdU-positive cells, and cells positive for both Tam67 and BrdU. In a total of 11,825 mammary epithelial cells, 3340 cells were positive for Tam67 (30%), 604 cells positive for BrdU incorporation (5%), and 12 cells positive for both Tam67 and BrdU (0.1% of total cells counted, 1.99% of BrdU-positive cells) (Fig. 4B). Thus, 98.01% of BrdU-positive cells show no co-localization with Tam67. This represents a highly significant negative association between BrdU and Tam67 expression ($P < 0.0001$, the correlation coefficient is −0.14). We therefore conclude that Tam67 expression strongly prevents proliferation of mammary epithelial cells that express this AP-1 inhibitor.

AP-1 blockade in vivo suppresses mammary gland development in prepubertal mice

We also examined the effect of in vivo AP-1 blockade on prepubertal mammary glands in 4-week-old mice. To induce Tam67 expression in neonatal mice, we treated the mother (heterozygous Tam67 or rtTA mice) with Dox-water on the day of delivery, and continued for 4 weeks while the pups drank milk from their mother and Dox-water (Fig. 5A). We observed expression of Tam67 in the mammary glands of the neonatal mice by IHC with anti-Flag staining (Fig. 5B), indicating the presence of Dox in the milk from the mother. At 4 weeks of age, the mammary glands from mice expressing Tam67 show a globally smaller ductal tree, fewer primary branches and ductal ends, compared to the other seven groups of control animals that have one or no transgene (Tam67$^{+/-}$ | rtTA$^{-/-}$, Tam67$^{-/-}$ | rtTA$^{+/-}$, or wild type FVB pups) ($P < 0.0001$, compared to all control groups, Figs. 5C–E). From these data, we conclude that AP-1 blockade suppresses the development of mammary glands at

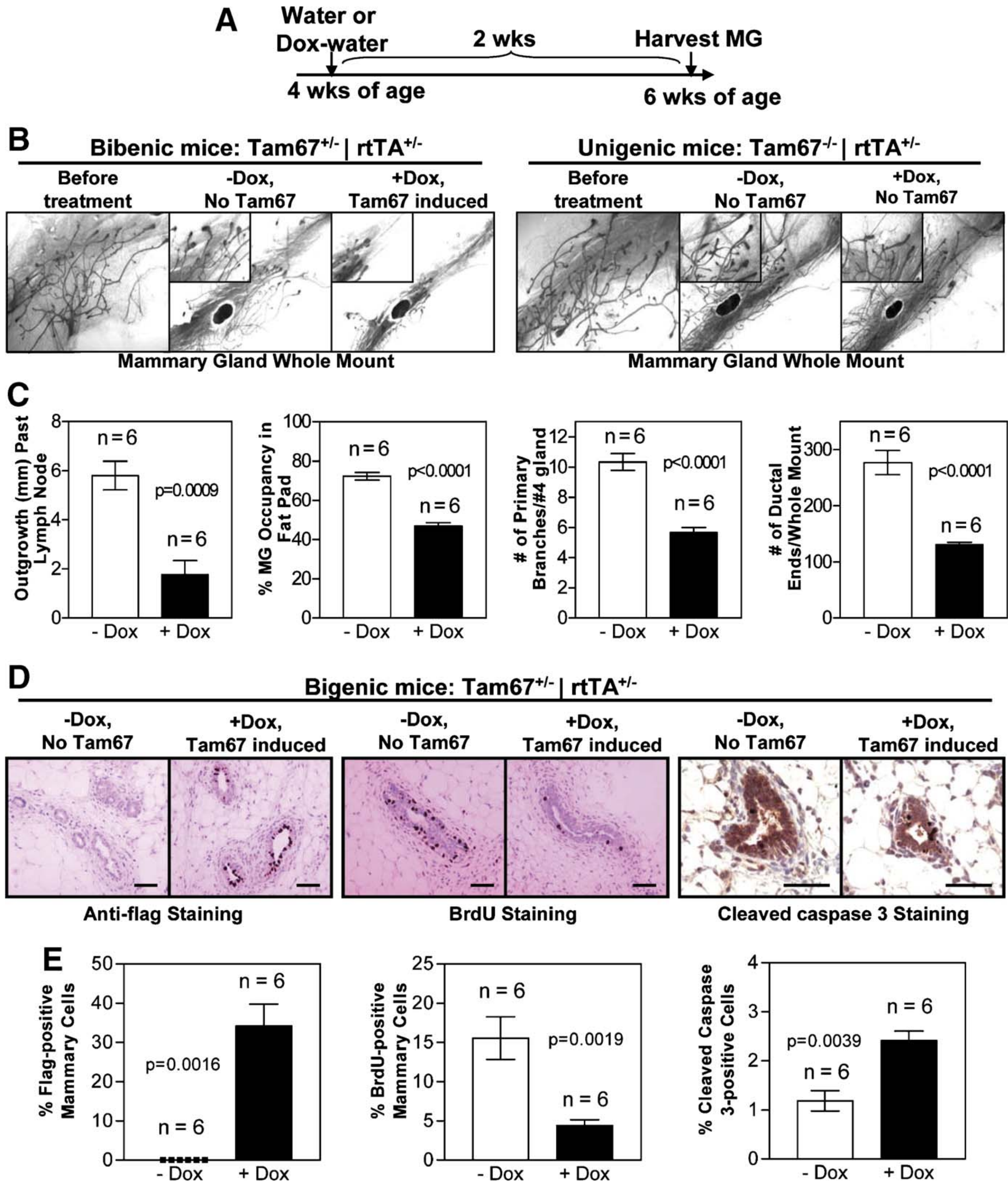
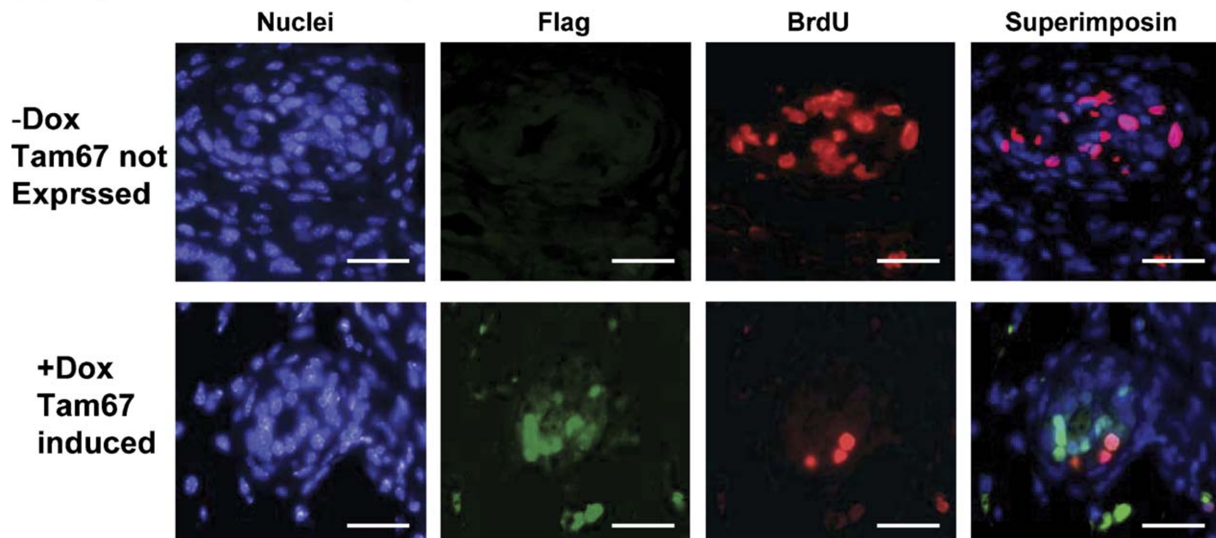
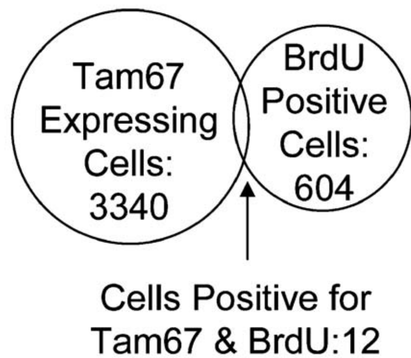
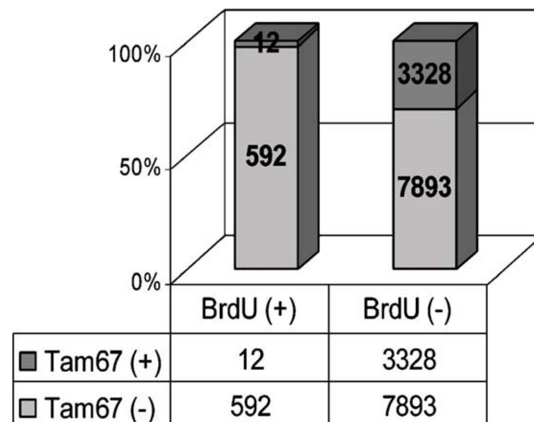


Fig. 3. AP-1 blockade suppresses mammary gland development in pubertal bigenic mice by decreasing proliferation and increasing apoptosis. (A) Bigenic Tam67/rtTA mice were fed with water or Dox-water at 4 weeks old for 2 weeks, then mammary glands were collected. (B) Representative mammary gland whole mount (#4 inguinal gland) analysis of pubertal bigenic Tam67/rtTA mice fed with water or Dox-water (4× magnification of whole mounts before treatment, 2× magnification of whole mounts after treatment). Left panel: the ductal tree was less developed globally and locally (see inserts) in Tam67-expressed mammary glands. Right panel: control group of mice with only rtTA transgene. (C) Data from 6 bigenic mice treated with water or Dox-water. Total number of primary branches, outgrowth past the lymph node, fat pad occupancy, and total number of ductal ends are plotted. Error bars represent SEM. (D) Representative IHC for the induction of Tam67 (left panel, original magnification 200×, scale bar: 50 μm), proliferation (middle panel, original magnification 200×, scale bar: 50 μm), and apoptosis (right panel, original magnification 400×, scale bar: 50 μm), analyzed by IHC using antibodies against Flag, BrdU, and cleaved caspase 3, respectively. (E) Data from 6 bigenic mice treated with water or Dox-water. Error bars represent SEM.







	BrdU (+)	BrdU (-)
Tam67 (+)	12	3328
Tam67 (-)	592	7893

Fig. 4. Proliferating mammary epithelial cells rarely express Tam67. (A) Bigenic Tam67/rtTA mice were fed with water or Dox-water at 4 weeks old for 2 weeks, then mammary glands were collected. Mammary gland sections from paraffin block were stained with anti-Flag and BrdU antibodies. Tam67 expression was labeled with FITC and BrdU was labeled with Texas Red. Representative images are shown for comparing control and Tam67-expressed mammary gland. Original magnification 400×, scale bar: 10 μm. (B and C) A total of 65 fields (from 6 mice) that had Tam67-expressing cells and BrdU-positive cells were counted. Data from 6 bigenic mice treated with Dox-water are presented as bar graph.

the neonatal–prepubertal stage, which is typically considered "hormone-independent" (Hennighausen and Robinson, 1998, 2001).

AP-1 blockade inhibits estrogen + progesterone (E + P) stimulated mammary gland development

Since the proliferation and growth of the mammary gland in early and mid-pregnancy can be mimicked by estrogen and progesterone stimulation, we examined the effect of AP-1 blockade on E + P-stimulated mammary gland growth. Silastic tubing containing E + P powder was implanted into the back of adult 13-week-old female mice for 9 days. Dox was then given for 1 week (starting 2 days after E + P implantation). The mammary glands were then harvested at day 9 after E + P treatment, and examined by whole mount processing and IHC staining (Figs. 6A–B). AP-1 blockade caused decreased branching and fewer terminal ends (Fig. 6B, whole mounts and inserts). Induced Tam67 expression was verified with anti-Flag staining by IHC (Fig. 6B). Approximate 65% of mammary epithelial cells expressed Tam67 upon Dox treatment (Fig. 6C). Proliferation was increased after E + P stimulation in both AP-1-blocked and normal AP-1 (AP-1 not blocked) mammary glands, as compared with the mammary glands without E + P stimulation (Fig. 2D). However, there were fewer BrdU-positive cells in Tam67-expressing mammary glands (9%) than in E + P stimulated glands (18%, $P = 0.007$, Figs. 6B, D). This is the first in vivo evidence that AP-1 blockade suppresses estrogen and progesterone-induced proliferation of mammary epithelial cells. AP-1 blockade also resulted in a significantly increased apoptosis, as measured by cleaved caspase 3 staining ($P = 0.004$, Figs. 6B, E).

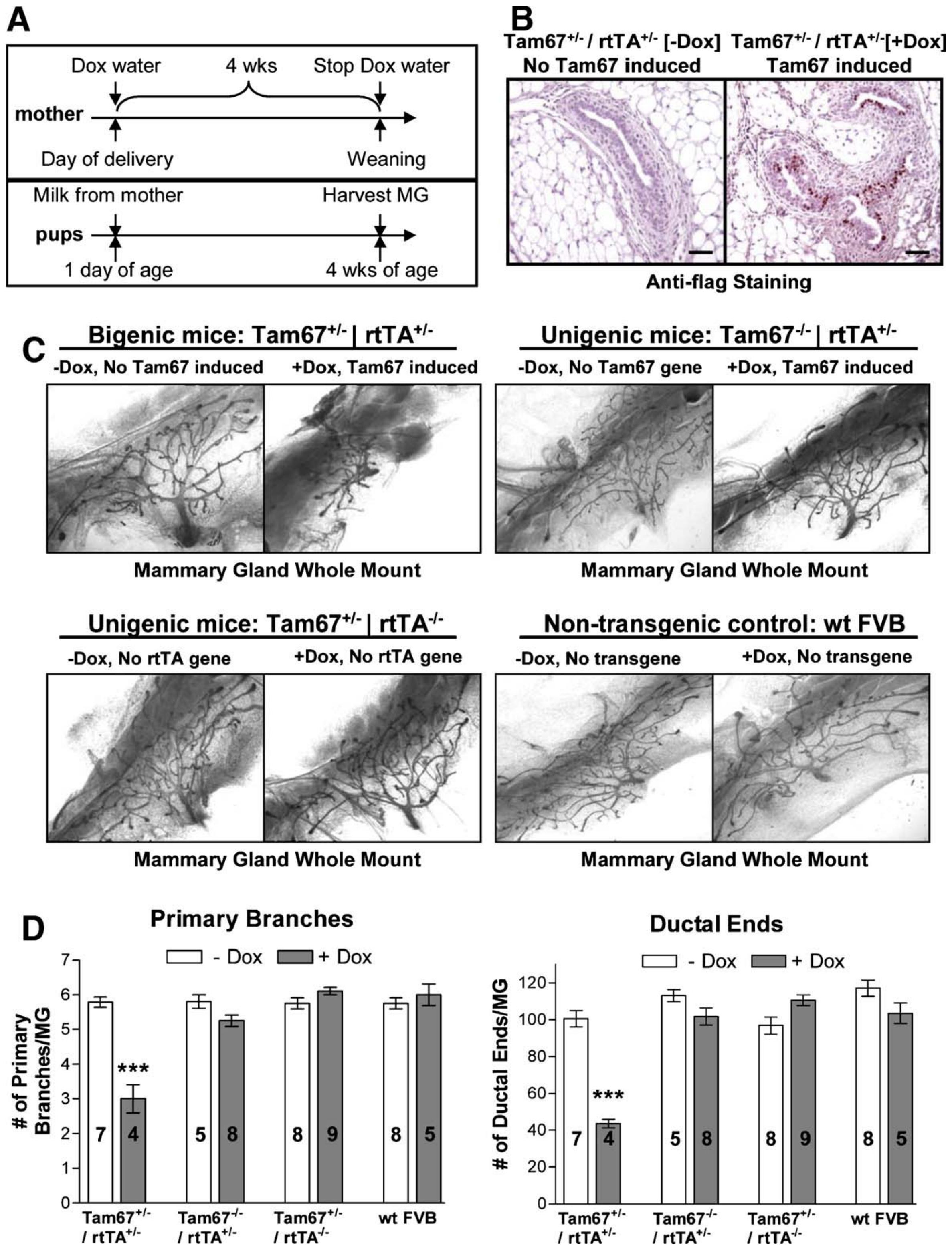


Fig. 5. AP-1 blockade suppresses mammary gland development in prepubertal bigenic mice. (A) After birth, the bigenic Tam67/rtTA mice were fed with milk from their mother that drank Dox-water for 4 weeks. The mammary glands were collected from the prepubertal mice at 4 weeks of age. (B) Representative IHC staining for the induction of Tam67 confirmed in the mammary glands of prepubertal female mice. Original magnification 200×, scale bar: 50 μm. (C) Representative mammary gland whole mount (#4 inguinal gland) of prepubertal bigenic Tam67/rtTA mice (4× magnification). Left upper panel: the ductal tree was less developed in Tam67-expressed mammary gland. Right upper panel: control group of mice with only rtTA transgene. Left lower panel: control group of mice with only Tam67 transgene. Right lower panel: control wild-type FVB mice with no transgene. (D) Data from quantifying mammary glands of the transgenic mice in panel C. Total numbers of primary branches and ductal ends are plotted. The number within each bar represents the total mice in each group. Error bars represent SEM. *** Indicates $P < 0.0001$.

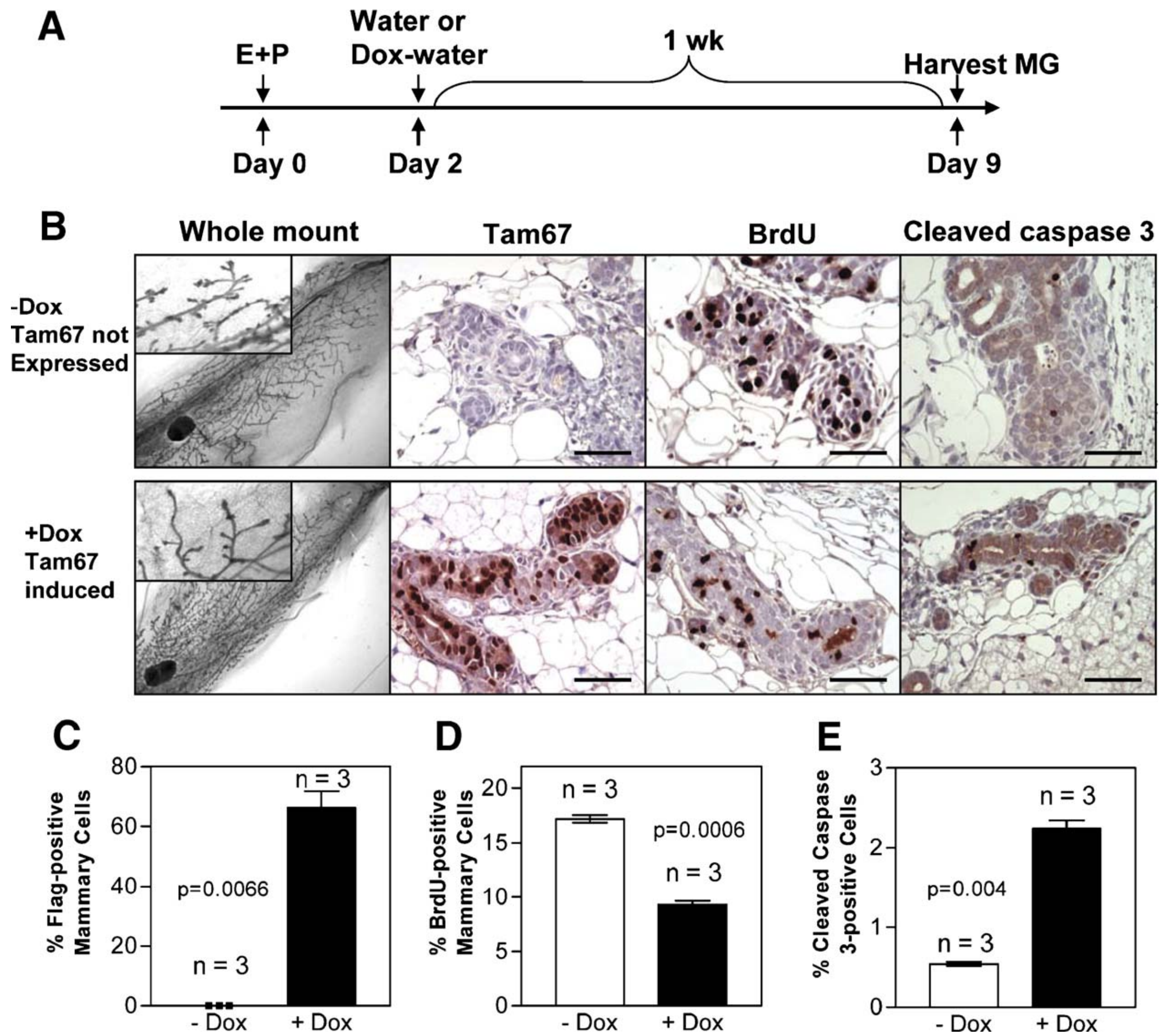


Fig. 6. AP-1 blockade inhibits E + P-stimulated mammary gland development in adult bigenic mice. (A) Adult female bigenic Tam67/rtTA mice were given E + P stimulation at day 0, and then fed with water or Dox-water from day 2 for 1 week. The mammary glands were collected at day 9 from these mice. (B) Representative mammary gland whole mount (#4 inguinal gland, 2× magnification) and paraffin block sections are shown. IHC staining for Tam67, BrdU, and cleaved caspase 3 was aligned for comparing the control and Tam67-expressed mammary glands. Original magnification 400×, scale bar: 50 μm. (C–E) Data from 3 transgenic mice in panel B. Percent of cells positive for Flag, BrdU, and cleaved caspase 3 are plotted. Error bars represent SEM.

AP-1 blockade suppresses AP-1-regulated genes and cell cycle regulators

We continued to explore the underlying molecular mechanisms by which in vivo AP-1 blockade suppresses mammary gland development and epithelial cell proliferation. We first examined the expression of AP-1-regulated genes and then cell cycle regulators in mammary glands from mice expressing Tam67. Each RNA sample was prepared from mammary epithelial cells isolated from inguinal mammary glands from five to seven mice fed with or without Dox. Three independent sets of RNA samples (each from 5–7 mice) were used to measure gene expression. This method had the advantage that the mRNA will reliably reflect the expression of AP-1-regulated genes in mammary epithelial cells. Quantitative RT-PCR was performed to measure the expression of several AP-1-regulated genes: *TIMP1*, *vimentin*, *Fra-1*, and *fibronectin*. We found that the mRNA levels of these genes were significantly reduced in AP-1-blocked mice that were stimulated with E + P (Fig. 7A) and a similar downregulation was observed in pubertal mammary epithelial cells from mice treated with Dox (data not shown). These data demonstrated that Tam67 did cause in vivo AP-1 blockade.

We have previously shown that the expression of estrogen-regulated genes is critically dependent on crosstalk between the AP-1 transcription factor and ER (DeNardo et al., 2005). ER/AP-1-regulated genes that are important regulators of growth include *cyclin D1* and *c-myc*. We therefore measured the expression of these genes in the mammary epithelial cells from mice treated with or without Dox. Using quantitative RT-PCR, we found that both *cyclin D1* and *c-myc* transcript levels were reduced in mammary epithelial cells that were collected from mice treated with E + P and Dox (Fig. 7A). We also found that this suppression of *cyclin D1* and *c-myc* occurred in pubertal

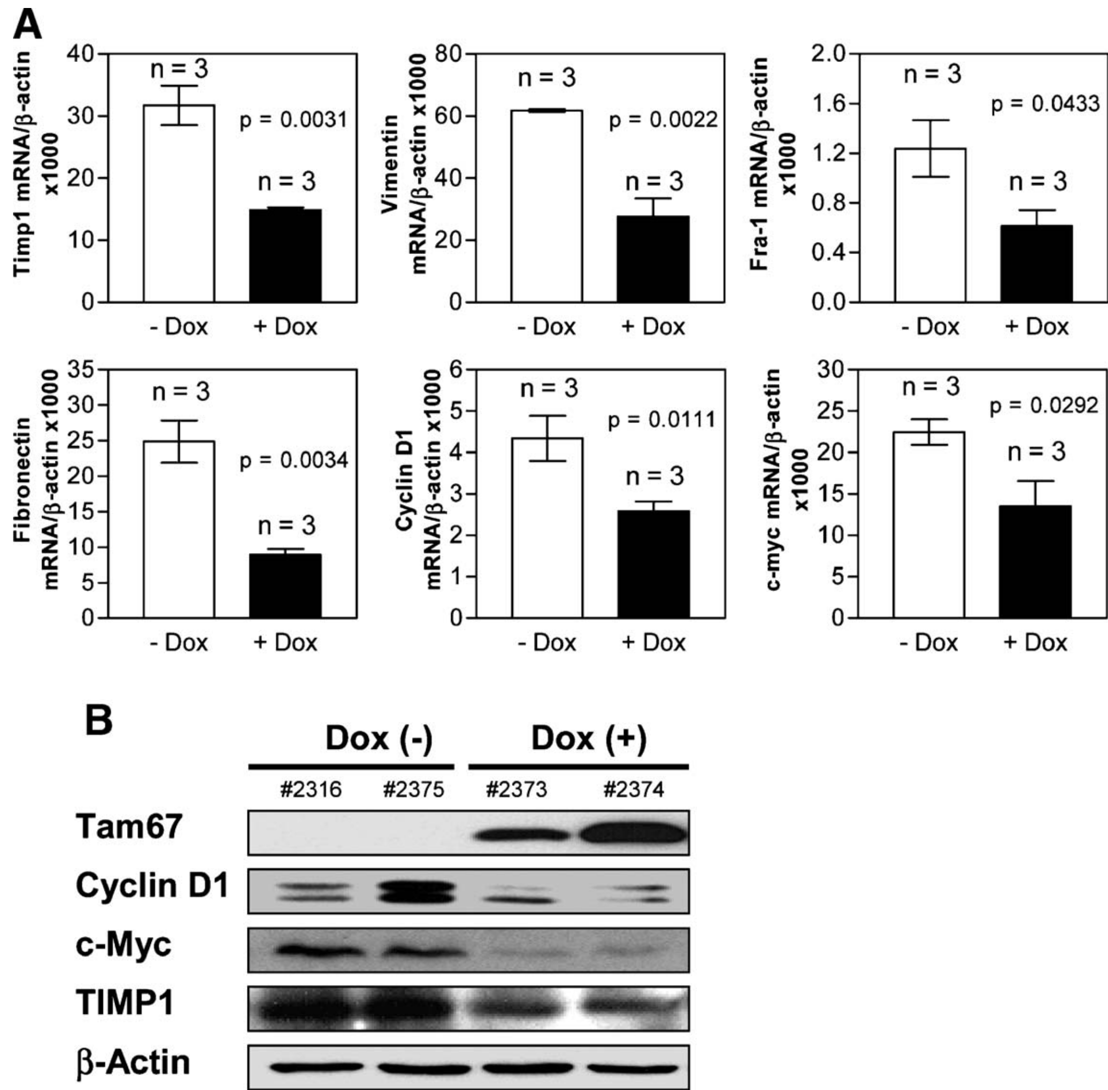


Fig. 7. AP-1 blockade downregulates AP-1-regulated genes and cell cycle regulators. (A) Total RNA samples from control and Tam67-expressing mice that were treated with E + P were used as templates for quantitative real-time PCR analysis. The mRNA levels of *Timp1*, *vimentin*, *Fra-1*, *fibronectin*, *cyclin D1*, and *c-myc* (all AP-1-regulated genes) were plotted. Error bars represent SEM. (B) Protein levels of cyclin D1, c-Myc, and TIMP1 were detected by Western blotting. β-Actin was shown as an internal loading control. Anti-flag antibody was used for confirmation of Tam67 induction upon Dox treatment.

mammary cells as well. Protein levels of cyclin D1, c-Myc, and TIMP1 were also studied using Western blotting (Fig. 7B). These results demonstrate that the AP-1 inhibitor Tam67 suppresses AP-1-regulated genes and cell cycle regulators *cyclin D1* and *c-myc* in vivo, which, in turn, suppress the growth of the mammary gland at different postnatal developmental stages.

Discussion

The AP-1 factor is an important transcription factor that regulates multiple cellular functions such as proliferation, apoptosis, and development. In order to investigate the in vivo role of the AP-1 transcription factor in normal mammary gland development, we generated a unique bigenic transgenic mouse that expresses an inducible AP-1 inhibitor, Tam67, in the mammary gland. Our data demonstrate that AP-1 blockade suppresses mammary gland development in prepubertal, pubertal, adult, and hormone-stimulated mice. Furthermore, AP-1 blockade suppresses mammary cell proliferation and inhibits AP-1-regulated gene expression in mammary epithelial cells. We speculate that this suppression of proliferation is caused by suppression of critical growth regulatory genes such as *cyclin D1* and *c-myc*. These studies demonstrate that the AP-1 transcription factor is critical for mammary gland development. Our results demonstrate that in vivo AP-1 blockade causes suppression of mammary gland development at multiple stages. This is the first evidence that the AP-1 factor has an important role in early and pubertal mammary gland development, and in hormone-stimulated adult mammary gland development.

Mammary gland development is divided into distinct stages including embryo, prepuberty, puberty, pregnancy, lactation, and involution stages. Known molecules that regulate

mammary gland development include prolactin (PRL), PRL receptor (PRLR), Stat5a, ER, PR, C/EBP, cyclin D1, and others (Hennighausen and Robinson, 1998). After birth and before puberty (up to weaning, 3–4 weeks of age), the mammary glands continue to grow from embryonic stage without the influence of endocrine hormones, though stromal EGFR and ERα are required (Bocchinfuso et al., 2000; Wiesen et al., 1999). During pubertal development of mammary glands, the ovarian hormones, estrogen and progesterone, stimulate ductal growth. Terminal end buds lead to the development of secondary and tertiary duct structures by elongation and lumen formation (Humphreys et al., 1996; Imagawa et al., 2002). These hormones, along with ERα and its downstream effector IGF-Is and IGF-IR, PR-B, glucocortocoid receptor, and other factors, modulate the proliferation, apoptosis, and organogenesis of the developing mammary gland (Bonnette and Hadsell, 2001; Kleinberg et al., 2000; Mueller et al., 2002; Mulac-Jericevic et al., 2000). During pregnancy, PRL and its receptor, and placental lactogens are the key hormones modulating mammary alveolar and lobular development (Brisken et al., 1999; Ormandy et al., 1997). In addition, the Stat5 signaling pathway, transcription factor C/EBP, cyclin D1,

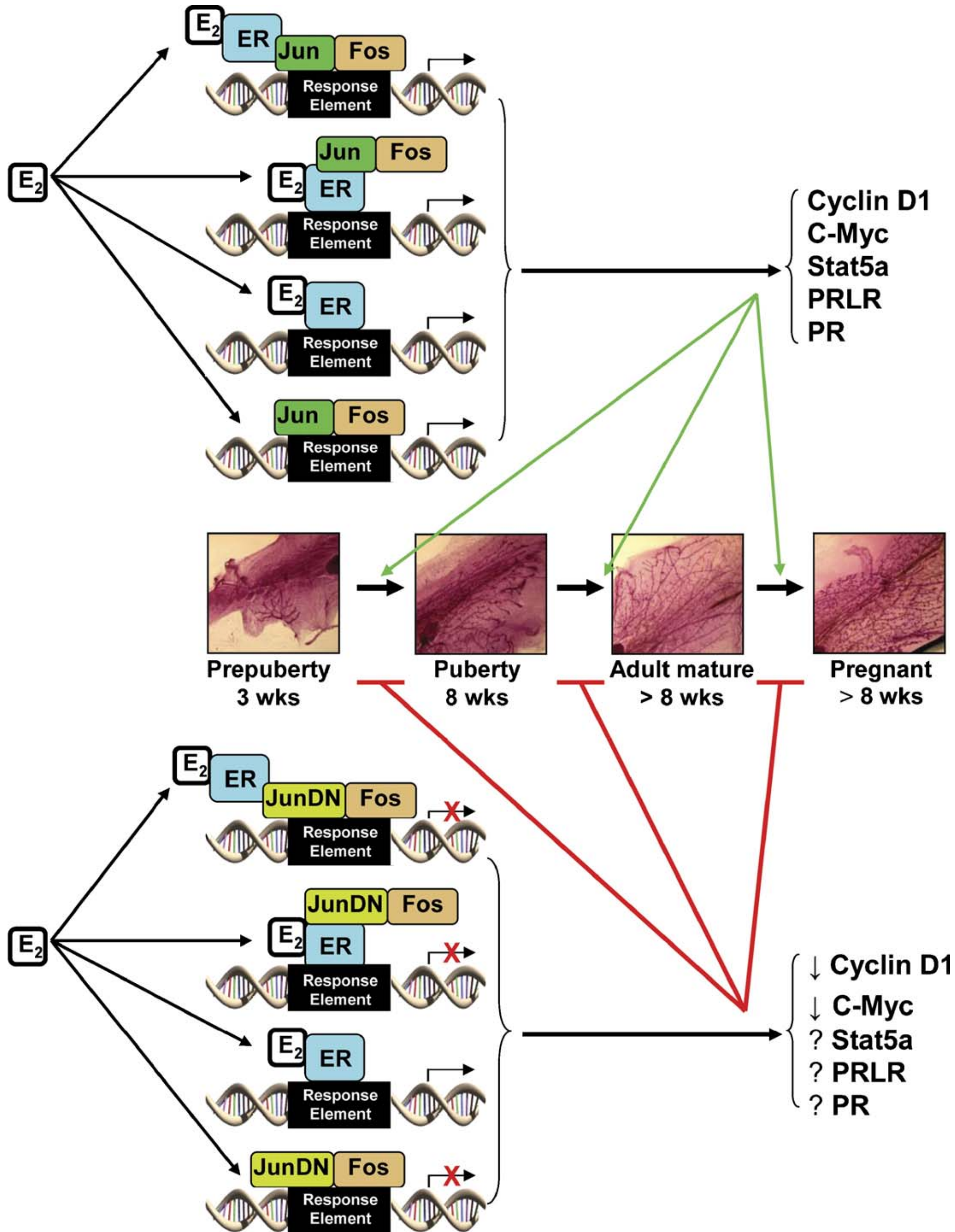


Fig. 8. Schematic model of the mechanism by which AP-1 regulates mammary cell proliferation and gland development.

Akt, and NF-κB are involved in the alveolar formation and accompanying proliferation and apoptosis (Hennighausen and Robinson, 1998; 2001; Seagroves et al., 2000; Sicinski et al., 1995). ER is critical for the development of adult mammary glands as well as embryonic mammary glands as shown by Korach's group using ERα knockout mice (Bocchinfuso et al., 2000; Korach et al., 1996). Peptide hormones (such as FGF and PTHR/PTHrP), hormone receptors (such as FGFR, EGFR, and ER in the stroma), and other oncogenes (such as Wnt) are also involved in the early development of mammary gland (Bocchinfuso et al., 2000; Dunbar et al., 1999; Foley et al., 2001; Mailleux et al., 2002; Veltmaat et al., 2004; Wiesen et al., 1999; Wysolmerski et al., 1998, 2001). These growth factors and hormones activate signal transduction pathways that ultimately activate transcription factors such as AP-1 to regulate gene expression.

A role of AP-1 in postnatal mammary gland development has not previously been found. The AP-1 factors may regulate AP-1-dependent gene expression by several mechanisms (as shown in Fig. 8). Estrogen stimulates gene expression by activating transcription factors such as ER or AP-1 directly or indirectly (shown in the top half of Fig. 8). Activation of these transcription factors induces gene expression which then regulates mammary gland development. The AP-1 complex (shown as dimers of Jun and Fos proteins) and ER may interact at AP-1 binding sites or estrogen response elements (EREs) to regulate specific AP-1-dependent and ER-dependent genes that regulate genes with either AP-1 binding sites or EREs within their promoters (as shown in the top half of Fig. 8). It is well documented that *cyclin D1*, *Stat5a*, *PR*, *PRLR*, and others are estrogen-induced genes that are required for postnatal mammary gland development. We recently found that the AP-1 factor directly and indirectly regulates cyclin D1 expression through AP-1 and E2F response elements within this promoter (unpublished observation). AP-1 binding sites have also been identified in the Stat5a promoter (Crispi et al., 2004) and in the prolactin gene promoter (Caccavelli et al., 1998; Farrow et al., 2004; Manfroid et al., 2001, 2005; Van De Weerdt et al., 2000). Jun and Fos proteins also regulate PR expression via an AP-1 binding site (Petz et al., 2004). In addition, AP-1 may regulate cyclin D1, PR, and PRL expression through AP-1/ER crosstalk (DeNardo et al., 2005; Petz et al., 2002). Our results demonstrate that AP-1 blockade by Tam67 inhibits the expression of cell cycle regulators such as cyclin D1 and cMyc to suppress postnatal mammary gland development. Taking these together, we speculate that AP-1 may also regulate Stat5a, PRL, PRLR and possibly other growth regulatory proteins. The role of AP-1 in regulating the expression of these proteins in postnatal mammary glands is now under investigation.

The results presented here show that AP-1 blockade leads to reduced mammary epithelial cell proliferation with a slight increase in apoptosis. Using the MMTV-driven inducible system, Tam67 expression and the resulting AP-1 blockade occurred only in normal mammary epithelial cells. However, in our previous studies, AP-1 blockade induced by Tam67 can inhibit proliferation of normal mammary epithelial cells, and some, but not all breast cancer cell lines grown in vitro (Liu et al., 2002; Ludes-Meyers et al., 2001). Other studies have shown that Tam67 expression in mouse keratinocytes did not affect proliferation of these skin cells (Young et al., 1999, 2003). The differential effect of Tam67 in the mammary gland as compared to keratinocytes suggests that mammary epithelial cells are more dependent on AP-1 for their growth than are keratinocytes.

Tam67 has previously been shown to form an inactive complex with its dimerizing partners that will occupy the AP-1 binding site and suppress target gene expression (Brown et al., 1994). Here we show that a set of AP-1-regulated genes such as *TIMP1*, *vimentin*, *fibronectin*, *Fra-1*, *cyclin D1* and *c-myc* were downregulated in mouse mammary glands after AP-1 blockade. The growth suppressive effects of AP-1 blockade are likely due to downregulation of cyclin D1 and c-Myc, and perhaps other regulatory proteins. Our finding that in vivo AP-1 blockade suppresses cyclin D1 and c-Myc expression in the mouse mammary glands strongly supports our conclusion that AP-1 factor regulates postnatal mammary epithelial proliferation and mammary gland development. These data raise the intriguing possibility that AP-1 transcription factor may also be required for mammary gland transformation, making this transcription factor a promising target for future drugs for the treatment or prevention of human breast cancer.

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