Regression of progestin-accelerated 7,12-dimethylbenz[a]anthracene-induced mammary tumors in Sprague–Dawley rats by p53 reactivation and induction of massive apoptosis: a pilot study

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Abstract

p53 Reactivation and induction of massive apoptosis (PRIMA-1) is a small-molecule compound that reactivates mutant p53, restoring its normal tumor suppressor function. PRIMA-1 effectively suppresses the growth of homogeneous p53-deficient tumor xenografts in immunosuppressed mice; however, the ability of PRIMA-1 to suppress the growth of mammary tumors in rodents and other species is not well characterized. Here, we examined the ability of PRIMA-1 to suppress the growth of 7,12-dimethylbenz[a]anthracene (DMBA)-induced and progestin-accelerated DMBA-induced mammary tumors in Sprague–Dawley rats. Mammary tumors were induced in female rats with DMBA or DMBA plus progestin treatment. After tumors had reached 5–25 mm^2 in size, PRIMA-1 was administered twice a day for 3 days via tail vein injection (20 or 50 mg/kg). Tumor size was monitored every day following PRIMA-1 for at least 15 days prior to killing. PRIMA-1 caused regression of $\sim$40% of progestin-accelerated DMBA-induced mammary tumors, but did not induce regression of native non-progestin-accelerated DMBA-induced tumors. Importantly, PRIMA-1 also suppressed the emergence of any new progestin-accelerated tumors in this model. Immunological studies with an antibody that selectively reacts with mutant p53 suggested that none of the native DMBA-induced tumors expressed mutant p53. By contrast, six out of eight progestin-accelerated DMBA-induced tumors stained for mutant p53 protein. In PRIMA-1-treated tumor-bearing rats, tumor regression correlated with conversion of mutant to wild-type p53 conformation, reduced expression of vascular endothelial growth factor and estrogen receptor, lack of blood vessel perfusion, increased expression of p21, and massively increased expression of anti-angiogenic protein, secreted protein acidic and rich in cysteine. These pre-clinical results suggest that PRIMA-1, as a single agent or in combination with other anti-cancer compounds, has potential as a novel chemotherapeutic treatment for progestin-accelerated human breast cancer.

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Introduction

p53 is the most commonly mutated gene in human cancer cells (Lacroix *et al*. 2006). Over 50% of breast tumor cells express a high level of mutant p53 protein, which is not effectively degraded by the proteasome system in human cells (Blaszyk *et al*. 2000, Lai *et al*. 2002).

Intensive research efforts are presently being directed towards developing new methods to suppress the growth and progression of cancer cells in humans using small-molecule therapeutics (Wang *et al*. 2003, Seemann *et al*. 2004). p53 Reactivation and induction of massive apoptosis (PRIMA-1), a small-molecule compound that

reactivates resident mutant p53 and restores its normal tumor suppressor functions in tumor cells (Bykov *et al.* 2002, 2003), is considered to be such a promising novel therapeutic agent for human tumors. Although the mechanism of action of PRIMA-1 is not yet known, it has been proposed that the effects of PRIMA-1 may be mediated by cellular chaperones (i.e., *HSP90*), which can facilitate functional reactivation of mutant proteins via protein refolding (Rehman *et al.* 2005).

Recent studies suggest that human breast cancer risk is influenced by exposure to exogenous progestins (i.e., hormone replacement therapy (HRT)). For example, several recent clinical trials demonstrated higher breast cancer risk in women treated with estrogen and synthetic progestin than in women treated with estrogen alone or placebo (Ross *et al.* 2000, Chlebowski *et al.* 2003). It has been proposed that synthetic progestins cause rapid progression of latent and/or quiescent breast tumor cells (Chen *et al.* 2004, Benakanakere *et al.* 2006). Consistent with this idea, progestins stimulate expression of vascular endothelial growth factor (VEGF) and accelerate the growth of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced tumors in rats (Benakanakere *et al.* 2006) and human breast cancer xenograft tumors in nude mice (Liang *et al.* 2007). Our recent studies demonstrate that progestin-stimulated expression of VEGF only occurs in progesterone receptor (PR)-positive cells that express mutant p53 (Liang & Hyder 2005, Liang *et al.* 2005, 2007). In model rodent systems (e.g., DMBA-induced mammary tumors in mice), p53 mutations in the pre-neoplastic lesions of the mammary gland are frequent (Jerry *et al.* 1993), though frank tumors seem to develop from the cells that retain their wild-type p53 status (Kito *et al.* 1996). However, it is not yet known whether medroxyprogesterone acetate (MPA)-accelerated DMBA-induced tumors in rodents express mutant p53 as a result of potential proliferative effects of MPA on both wild-type and mutant p53 expressing cells in the pre-neoplastic lesions. The presence of mutant p53 protein may make these tumors a good candidate for PRIMA-1 treatment.

This study examines the ability of PRIMA-1 to inhibit the progression of DMBA-induced and MPA-accelerated DMBA-induced mammary tumors in rats (Benakanakere *et al.* 2006). The effect of PRIMA-1 on VEGF expression was also examined in this rat model system. The results show that PRIMA-1 causes complete or partial regression of ∼40–50% of progestin-accelerated mammary tumors, and inhibits the growth and progression of majority of MPA-accelerated DMBA-induced mammary tumors. By contrast, PRIMA-1 does not inhibit the growth of native non-MPA-accelerated DMBA-induced

mammary tumors. The implications of these data for the treatment of progestin-exposed women with breast cancer are discussed.

Materials and methods

Animals

Forty- to forty-five-day-old virgin female Sprague–Dawley (SD) rats (Harlan, Indianapolis, IN, USA) were maintained in cages under a 12h light:12h darkness cycle with *ad libitum* food and water. All of the animal surgical procedures and experimentation were approved by the Institutional Animal Care and Use Committee of the University of Missouri, Columbia, USA, and were in accordance with the NIH 'Guide for Care and Use of Laboratory Animals' (NIH publication 85–23). Five animals were used per experimental group (exceptions noted), and each experiment was repeated at least twice.

Hormones

Where indicated, animals were dosed with 60-day release hormone pellets prepared by Innovative Research (Sarasota, FL, USA). The pellets were implanted s.c. in the dorsal area as described previously (Benakanakere *et al.* 2006). Animals were anesthetized prior to implantation.

Experimental design

Female rats, 45–50 days old, were given a single 20 mg dose of DMBA (Sigma Chemical Co.) in peanut oil by gavage. Where indicated, 25 mg/60-day release MPA pellets (Innovative research) were implanted 6 weeks after dosing with DMBA. Animals were examined for tumor growth every 2 days, and the size of all palpable tumors was determined and recorded. Two or three weeks after pellet implantation, animals were dosed with PRIMA-1 by tail vein injection (Tocris Biosciences, Ellisville, MO, USA). PRIMA-1 was administered twice daily for 3 days at 20 or 50 mg/kg at week 8 after DMBA administration. Animals in the 20 mg/kg dosing group were killed 15 days after the first injection. Animals in the 50 mg/kg dose group were maintained until week 11 after DMBA (15 days after the initial PRIMA-1 injection), after which they were injected with 20 mg/kg PRIMA-1 twice weekly for 4 weeks. Animals were killed at day 101 (15 weeks after DMBA). Control animals received placebo pellets or PBS. Experimental protocols are summarized in Figs 1A, 2A, and 3A.

Palpable tumors were measured during this period and tumor size recorded. Tumor diameter was

measured using a micrometer caliper; tumor size was calculated using the formula $L/2 \times W/2 \times$ (Chen *et al.* 2003). Tumor number, size, and location were recorded for each animal.

Tumors were classified as 'regressing' if their size decreased by at least 50% at the end of the experiment, following treatment with PRIMA-1. Other tumors were considered to be PRIMA-1 non-responsive or 'progressing' if their size increased or remained unchanged (stable). Tissues and tumors were fixed in 10% buffered formalin or 4% paraformaldehyde for histopathology and flash frozen in liquid nitrogen and stored at $-80\,^{\circ}\text{C}$ for subsequent RNA extraction and analysis or immunohistochemistry.

Histology and immunohistochemical analysis

Staining for mutant p53 in cryosections

Frozen biopsies were placed in cryomolds filled with the embedding medium (FSC22; Surgipath Medical industries Inc., Richmond, IL, USA) and frozen for cryosectioning. Eight micrometer cryosections were mounted on charged glass slides (X-tra Slides; Surgipath Medical Industries Inc.), thawed at room temperature, rehydrated in Tris-buffered saline with Tween 20 (wash buffer; DAKO, Carpenteria, CA, USA) at room temperature, and rinsed twice with the buffer to remove the embedding medium. Tissues were quenched with 3% hydrogen peroxide, rinsed, and incubated with 5% BSA to minimize non-specific antibody binding. The sections were then incubated with the antibody that recognized mutant p53 (PAb240 at 1:10; Calbiochem, San Diego, CA, USA) for 60 min at room temperature (Delfino *et al.* 2002). Control frozen sections of BT-474 and MCF human breast cancer cells (ATCC) were similarly probed with PAb240. The sections were rinsed and then incubated for 30 min with either biotinylated rabbit anti-mouse antibody for PAb240-labeled tissues (E0464 at 1:200; DAKO) or with biotinylated swine anti-rabbit antibody for wild-type p53-labeled tissues (E0353 at 1:200; DAKO). After rinsing, the sections were incubated with peroxidase-labeled streptavidin (DAKO) for 30 min prior to treatment with diaminobenzidine peroxidase substrate solution (DAKO) for 5 min. Slides were rinsed and counterstained with Mayer's hematoxylin, dehydrated in graded alcohols and xylene, and mounted with a coverslip.

For the studies involving fluorescently labeled secondary antibodies, the sections were incubated with antibody-recognizing mutant p53 (PAb240 at 1:10; Calbiochem) or wild-type p53 (PAb246 at 1:20; Calbiochem) for 60 min at room temperature. Co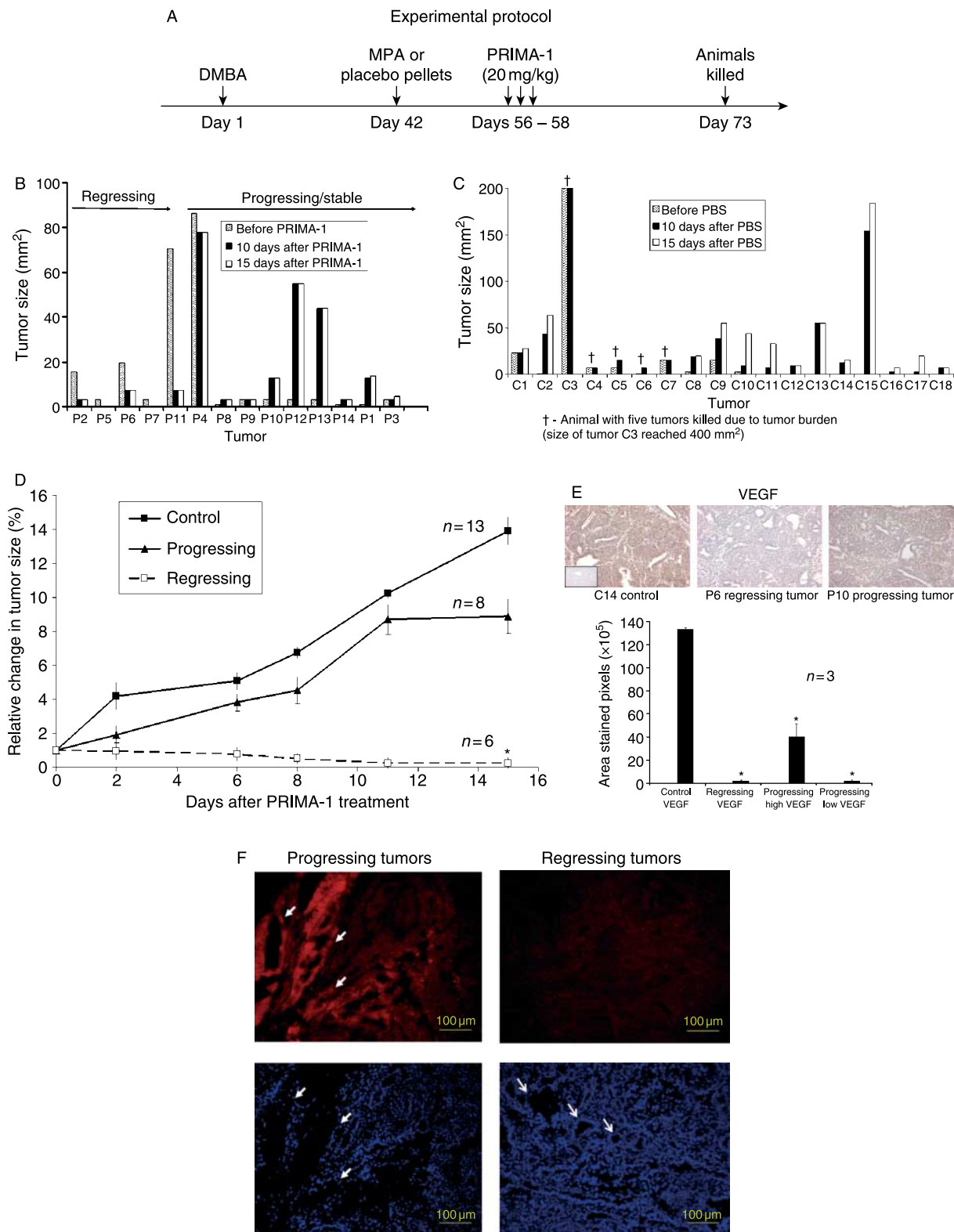ntrol frozen sections of xenografts in nude mice obtained from BT-474 (for mutant p53) and MCF (for wild-type p53) human breast cancer cells (ATCC) were similarly probed with PAb240 or PAb246. The sections were rinsed and then incubated for 30 min with either Texas Red-conjugated rat anti-mouse antibody for PAb240-labeled samples (415-075-166 at 1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or with a FITC-conjugated goat anti-mouse antibody for wild-type p53-labeled tissues (02-18-06 at 1:200; Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA). After rinsing, the sections were incubated with 4% paraformaldehyde, rinsed again, and mounted with DAPI (VECTASHIELD Hardset with DAPI; Vector Laboratories, Burlingame, CA, USA) and coverslipped.

Immunohistochemical analysis of paraffin sections

Expression of ERα, ERβ, PR, VEGF, and factor VIII was determined immunochemically, as described previously (Benakanakere *et al.* 2006). Anti-p53 antibody that recognizes total (mutant + wild type) p53 (sc6243, dilution 1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and secreted protein acidic and rich in cysteine (SPARC) antibody (anti-human osteonectin, AON-5031, 1:10 000 dilution; Haematologic Technologies Inc., Essex Junction, VT, USA) were detected by sequential 30-min incubations at room temperature with biotinylated swine anti-rabbit serum (1:200 dilution; DAKO), streptavidin-linked HRP (Pharmingen, San Diego, CA, USA) followed by $3,3'$-diaminobenzidine peroxidase (0.016% with 0.05% H_2O_2 in PBS) solution (DAKO) for 3–5 min. The sections were counterstained with Meyer's hematoxylin, dehydrated, cleared, and coverslipped for microscopic examination. Distribution of immunolabeled cells or nuclei in histological sections of tumors was determined by the use of a morphometric software (Fovea Pro 3.0, 2005 Reindeer Graphics) on images photographed at $40\times$ magnification. Sections from at least three tumors per treatment group were analyzed. Hormone receptor prevalence was measured by counting immunoreactive cells in three distinct fields. SPARC and VEGF distribution was determined on all cells in each of three tumor sections photographed at $20\times$ magnification. Results are expressed as area stained in pixels for various treatments.

Blood vessel perfusion assay

At 40–45 days of age, rats were given a single dose of 20 mg DMBA in peanut oil by gavage. Six weeks post-DMBA animals were implanted with 25 mg/60-day release MPA pellets (Innovative Research). Animals

A

Experimental protocol

DMBA — Day 1

MPA or placebo pellets — Day 42

PRIMA-1 (20 mg/kg) — Days 56 – 58

Animals killed — Day 73

B

Regressing → Progressing/stable →

Tumor size (mm²)

Before PRIMA-1
10 days after PRIMA-1
15 days after PRIMA-1

Tumor: P2 P5 P6 P7 P11 P4 P8 P9 P10 P12 P13 P14 P1 P3

C

Tumor size (mm²)

Before PBS
10 days after PBS
15 days after PBS

Tumor: C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15 C16 C17 C18

† - Animal with five tumors killed due to tumor burden
(size of tumor C3 reached 400 mm²)

D

Relative change in tumor size (%)

Control
Progressing
Regressing

$n=13$
$n=8$
$n=6$
*

Days after PRIMA-1 treatment

E

VEGF

C14 control — P6 regressing tumor — P10 progressing tumor

Area stained pixels (×10⁵)

$n=3$
*

Control VEGF — Regressing VEGF — Progressing high VEGF — Progressing low VEGF

F

Progressing tumors — Regressing tumors

100 µm

100 µm

100 µm

100 µm

were given PRIMA-1 at a dose of 20 mg/kg body weight twice a day for 3 days. Animals were palpated every 2 days, tumors measured, and recorded. Seven days after PRIMA-1 injections, as tumors began to regress, animals were given 0.5 ml of 1 mg/ml of Texas Red-conjugated tomato lectin (Vector Laboratories). Tomato lectin remains sequestered within blood vessels and therefore highlights functional perfused vessels (Kurozumi *et al.* 2007). Rats were killed after 10 min and tissues fixed in a tissue freezing medium (cat # H-TFM; Triangle Biomedical Sciences, Durham, NC, USA) over dry ice. Frozen tissues were sectioned at 8–10 μm, mounted, and fixed for 15 min with 4% paraformaldehyde. Samples were cover-slipped with aqueous mounting medium containing 1.5 μg/ml DAPI (VECTASHIELD Hardset mounting medium with DAPI; Vector Laboratories). All sections were viewed with u.v.-filtered light appropriate for the fluorophore. In order to visualize Texas Red-labeled tomato lectin, the samples were imaged with a 590 nm bandpass filter at 1.5 s exposure times. DAPI-labeled nuclei were viewed with a 390 nm bandpass filter at 0.2 s exposure times.

Statistical analysis

Statistical significance of tumor response was evaluated by χ^2 test for each group. The difference in response to 20 or 50 mg PRIMA-1 was not statistically significant; the response to PRIMA-1 versus controls was statistically significant in each group separately ($P < 0.01$). Slides from at least three tumors were analyzed for hormone receptor expression and statistical significance of immunochemical labeling prevalence was evaluated using a Mann–Whitney rank-sum test. Expression levels of VEGF and SPARC, measured using the Fovea Pro 3.0 imaging program, were compared for significance employing ANOVA and, as *post hoc* test, Fisher's probable least-squares difference test (Sigma Stat software version 3.1, SPSS Inc). All measurements are expressed as mean $\pm$ s.e.m.. Differences were considered to be significant when $P < 0.05$.

Results

Dosing regimens used in this study are summarized in Figs 1A, 2A, and 3A. Because the rate of tumor growth was altered by treatment with MPA (Benakanakere *et al.* 2006), PRIMA-1 injections (20 or 50 mg/kg, twice daily for 3 days) were initiated on day 56 or 63, in MPA-treated and non-MPA-treated animals respectively. When these protocols were followed, tumor size was 5–25 mm^2 in both treatment groups at the start of PRIMA-1 treatment (exceptions noted below). None of the treatment protocols were toxic, since no animals lost weight during and after PRIMA-1 treatment.

PRIMA-1 causes regression of MPA-accelerated DMBA-induced tumors

SD rats were treated with DMBA followed by MPA, via slow-release pellet, or with DMBA alone according to the protocols shown in Figs 1A and 2A (also see Materials and Methods). Control animals were dosed with placebo or vehicle. Animals bearing MPA-accelerated DMBA-induced tumors had a higher tumor multiplicity, as reported previously by us (Benakanakere *et al.* 2006) and tumors grew more rapidly in MPA-treated than in non-MPA-treated rats. Starting at day 56, MPA-treated rats were given injections of PRIMA-1 (20 mg/kg) twice daily for 3 days. The size of 14 tumors in five animals was monitored after PRIMA-1 injections. Two of these

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Figure 1 (A) Experimental protocol for PRIMA-1 treatment of MPA-implanted rats. Animals (45- to 50-day-old Sprague–Dawley rats) were treated with DMBA by oral gavage (20 mg/rat). This is designated as day 1. MPA pellets (25 mg/60-day release) were introduced at 42 days (6 weeks) post-DMBA treatment. Rats were treated with PRIMA-1, 20 mg/kg bodyweight in PBS by tail vein injection twice a day for 3 days at days 56–58 (week 8 post-DMBA). All animals were killed 15 days after the last PRIMA-1 injection at day 73. (B) PRIMA-1 treatment caused regression of MPA-accelerated tumors. Rats were treated as described in (A) above. All tumors were monitored and measured every day post-PRIMA-1 treatment. Tumors P7 and P5 showed complete regression 10 days after PRIMA-1 treatment. Out of 14 total tumors treated, 6 showed regression, P9 was stable, and no new tumors appeared during this time period. (C) PBS treatment did not affect MPA-induced tumor progression. (D) Relative growth rate of DMBA-induced MPA-accelerated tumors treated with PBS or PRIMA-1 (20 mg/kg body weight) twice daily for 3 days as described in (A). MPA pellets were introduced 28 days after animals were given a single dose of 20 mg DMBA/rat at 50–55 days of age. *Significantly different from control and PRIMA-1-treated progressing tumors ($P < 0.001$, ANOVA). (E) VEGF expression in control, progressing, and regressing tumors following PRIMA-1 treatment. (magnification, 64×; bar, 50 μm). The inset in figure represents non-immune antibody control. Bar graph represents quantitation of staining as described in the Methods section. *Significantly different from control tumors ($P < 0.001$, ANOVA). (F) Blood perfusion assay of DMBA-induced MPA-accelerated tumors with fluorescently labeled tomato lectin as described in the Methods section. (magnification, 20×; bar, 100 μm). In sections viewed with 590 nm filter, Texas Red-positive regions correspond to perfused vessels. The same section viewed with 390 nm filter demonstrates tumor cellularity (bottom panels). White arrows point to identifiable blood vessels in tumor sections. Quantification of images using Fovea Pro imaging system to measure stained pixels showed a significant difference in intensity between progressing and regressing tumors (285 ± 59 ($n = 4$) vs 27 ± 13 ($n = 3$), mean $\pm$ s.e.m.; $P < 0.05$, Student's *t*-test).
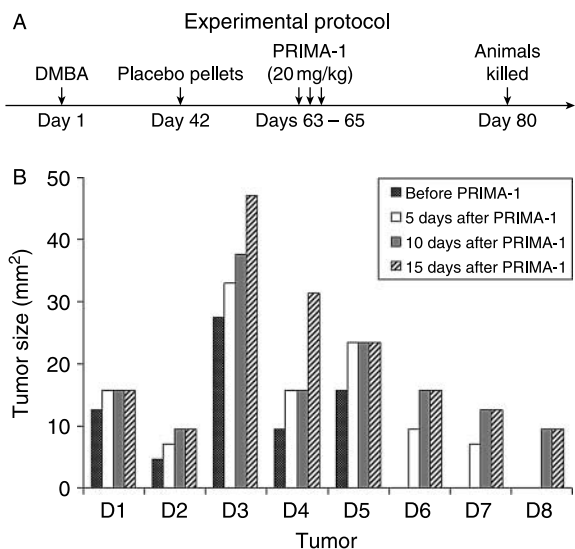
Figure 2 (A) Experimental protocol. Animals (Sprague–Dawley rats) were treated with DMBA by oral gavage (20 mg/rat) on day 1. Placebo pellets (25 mg/60-day release) were introduced at 42 days (6 weeks) post-DMBA treatment. Rats were treated with PRIMA-1 (20 mg/kg bodyweight) in PBS by tail vein injection twice a day for 3 days at days 63–65 (week 9 post-DMBA). All animals were killed 15 days after the last PRIMA-1 injection at day 80. (B) DMBA-induced tumors do not respond to PRIMA-1 treatment. Animals were treated with DMBA by oral gavage as described in the protocol above. DMBA-induced tumors did not regress with PRIMA-1 treatment; three new tumors appeared in this time frame.

tumors exhibited complete regression (P5 and P7) and three tumors exhibited partial regression in response to PRIMA-1. Nine tumors did not regress (Fig. 1B, right-hand columns). In some animals carrying multiple tumors, some tumors regressed, while the size of other tumors became stable. Overall, ~40% of MPA-accelerated tumors regressed more than 50% in volume in response to PRIMA-1 in the time frame tested. Importantly, new tumors did not emerge in PRIMA-1-treated rats during the 15 days after injection with PRIMA-1. By contrast, tumors continued to grow, and eight new tumors emerged in a comparable time frame in control animals (Fig. 1C, C11–C18). One rat in the control group was killed due to the presence of five tumors (Fig. 1C, C3–C7), one of which became extremely large (~400 mm^2) during the course of the experiment.

PRIMA-1 does not increase the growth rate of MPA-accelerated tumors

In keeping with our expectations, PRIMA-1 treatment of DMBA-induced MPA-accelerated tumors did not bring about an increase in tumor growth rate (Fig. 1D). In fact, PRIMA-1 treatment either caused tumor
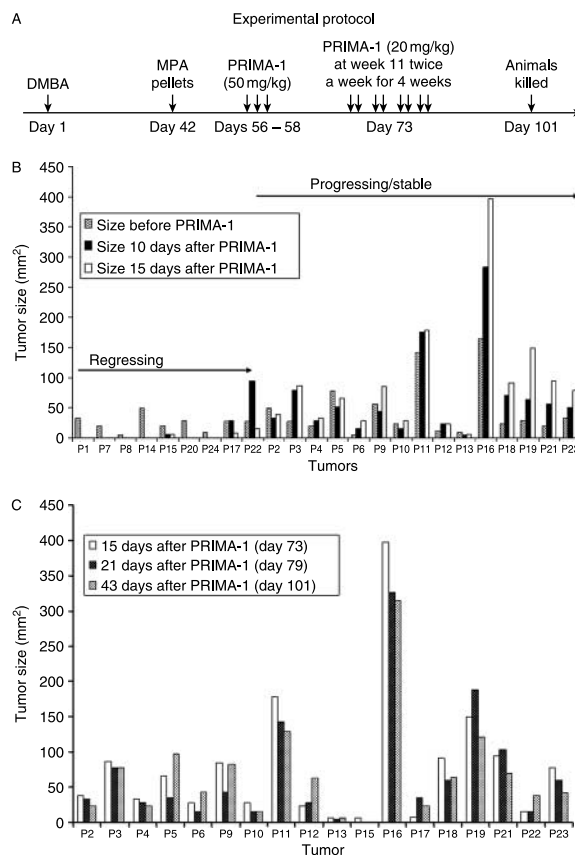

Figure 3 (A) Experimental protocol for extended treatment of MPA-accelerated tumors. Animals (Sprague–Dawley rats) were treated with DMBA by oral gavage (20 mg/rat) at ages 45–50 days. MPA pellets (25 mg/60-day release) were introduced at 42 days (6 weeks) post-DMBA treatment. Rats were treated with PRIMA-1 (50 mg/kg bodyweight) in PBS by tail vein injection twice a day for 3 days at days 56–58 (week 8 post-DMBA). All animals were further treated with PRIMA-1 at 20 mg/kg starting on day 73 (week 11 post-DMBA) single injection by tail vein twice a week for 4 weeks. All animals were killed on day 101. (B) PRIMA-1 treatment at a higher dose does not improve the response of the MPA-accelerated tumors. Rats were treated with DMBA by oral gavage (20 mg/rat) at ages 45–50 days. MPA pellets (25 mg/60-day release) were introduced at 42 days (6 weeks) post-DMBA treatment. Rats were treated with PRIMA-1, 50 mg/kg bodyweight in PBS by tail vein injection twice a day for 3 days at days 56–58 (week 8 post-DMBA). All tumors were monitored and measured every day post-PRIMA-1 treatment. Tumors P1, P7, P8, P14, P20, and P14 showed complete regression within 10 days following PRIMA-1 treatment. Out of 24 total tumors treated, 14 regressed and no new tumors appeared during this time period. (C) Treatment for extended periods does not improve outcome. After day 73 (15 days after the initial dose of PRIMA-1), animals continued to receive PRIMA-1 at the dose of 20 mg/kg body weight twice a week for 4 weeks. Only P21 fully regressed within 21 days after the initial PRIMA-1 treatment.

regression, as shown, or slightly reduced the growth rate of even those tumors that were progressing (up to day 10 post-PRIMA-1 treatment). Between days 11 and 15, tumors in PRIMA-1-treated animals became

static with respect to tumor growth. However, the difference in growth rate between the control and the PRIMA-1-treated progressing tumors did not reach a statistical significance using ANOVA to compare the three groups shown in Fig. 1D.

PRIMA-1 inhibits expression of VEGF in mammary gland tumors

Expression of VEGF was quantified by immunohistological methods in PRIMA-1-treated mammary tumors, some of which regressed in response to PRIMA-1 and some of which did not. VEGF expression was also examined in non-PRIMA-1-treated tumors, which were actively proliferating (Fig. 1E). Tumors in control animals (Fig. 1E, left panel) expressed high levels of extracellular and cytoplasmic VEGF in epithelial and stromal tissues. In general, VEGF was expressed at a high level in untreated tumors, at a moderate level in PRIMA-1-resistant tumors that did not progress and remain stable (Fig. 1E, right panel), and at a moderate level or relatively low level in regressing tumors (Fig. 1E, middle panel). A bar graph of quantitative results of VEGF distribution is demonstrated in Fig. 1E (bottom panel). Overall, there was a 60% reduction in VEGF levels in PRIMA-1-treated tumors compared with controls. Quantitation of immunohistochemical staining for factor VIII revealed a similar level of expression in all tumors (not shown), indicating that variations in VEGF staining were not likely to be an artifact of the experimental system and that loss of blood vessels was not the cause of tumor regression.

Blood vessel perfusion in PRIMA-1-treated progressing and regressing tumors
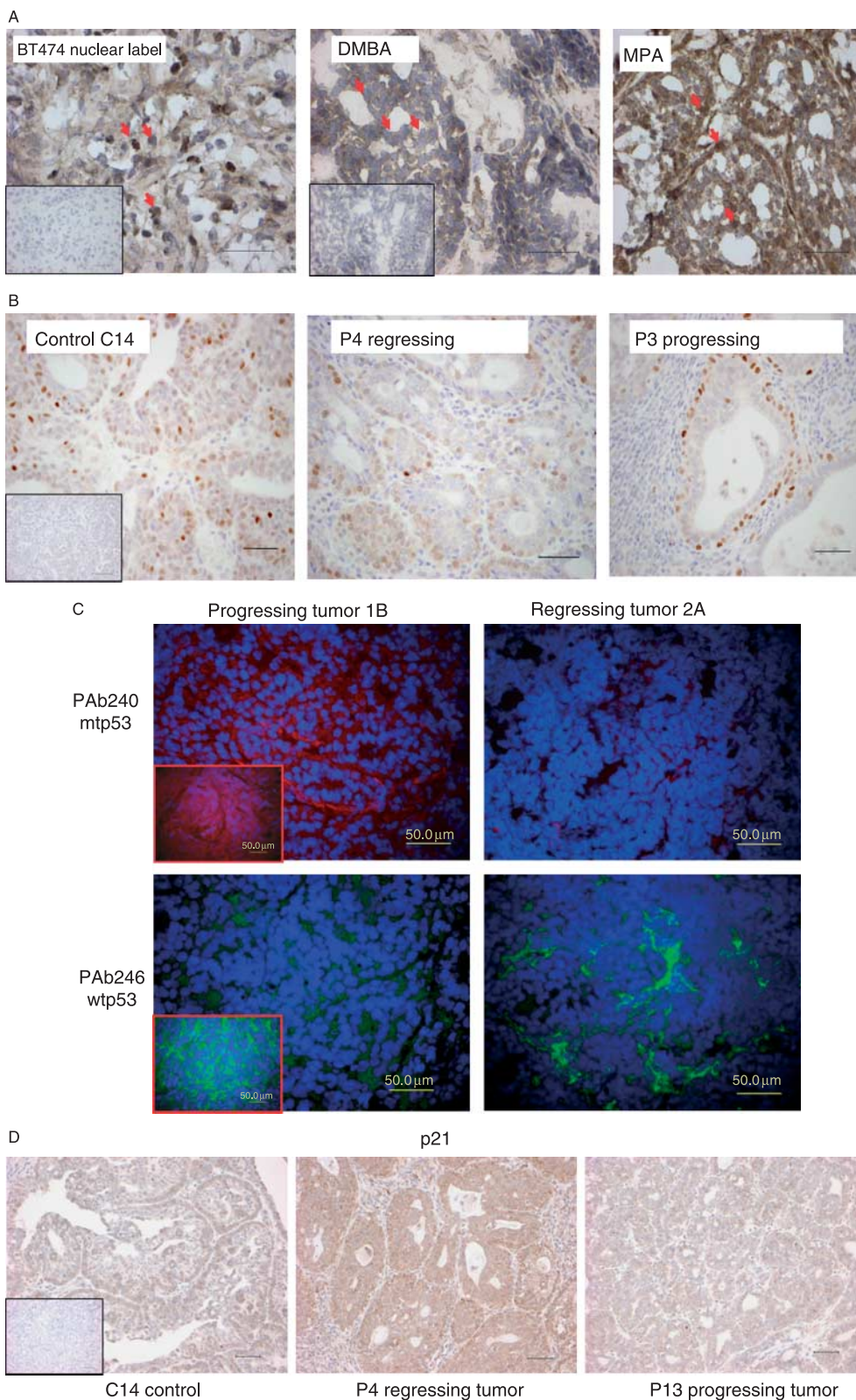
Factor VIII staining indicated no difference in the blood vessel density in PRIMA-1-treated progressing and regressing tumors (data not shown). Consequently, we studied blood vessel perfusion in these tumors to determine whether PRIMA-1 reduced blood flow and thereby brought about tumor shrinkage in the responsive tumors. Seven days following PRIMA-1 treatment both progressing and regressing tumors were identified. Fluorescently labeled tomato lectin was injected via tail vein of tumor-bearing rats and animals were killed 10 min later. Tumors were removed, fixed, and analyzed as described in the Methods section. As shown in Fig. 1F, PRIMA-1-treated progressing tumors showed extensive staining within clearly defined blood vessels, indicating extensive perfusion, while within regressing tumors staining was absent, demonstrating that these tumors either lacked blood flow or that blood flow was severely reduced and therefore inadequate for tumor nourishment.

PRIMA-1 does not cause regression of non-MPA-accelerated DMBA-induced mammary tumors

Placebo pellets were implanted into DMBA-treated animals (Fig. 2A). Three weeks post-implantation, animals were dosed with PRIMA-1 as described above (i.e., 20 mg/kg via tail vein injection twice daily for 3 days) and tumor size was monitored for 15 days. The results showed that PRIMA-1 did not cause regression in all of five tumors (D1–D5), and tumor size increased significantly in three of these tumors in the 15 days post-PRIMA-1 treatment (Fig. 2B). The remaining tumors became static over time, but this was also observed in animals treated with vehicle alone (not shown). As expected, none of the tumors regressed spontaneously (Fig. 2B). Furthermore, three new tumors emerged and continued to increase in size in control animals after injection with PRIMA-1. These results suggest that PRIMA-1 does not cause regression of non-MPA-accelerated DMBA-induced tumors.

Increasing PRIMA-1 dose does not increase overall tumor response

In the initial experiments described above, MPA/DMBA-treated rats were dosed with 20 mg/kg PRIMA-1. Similar experiments were also performed using a dose of 50 mg/kg PRIMA-1 2 weeks post-MPA administration. Animals bearing PRIMA-1-resistant tumors were also given additional injections of PRIMA-1 (20 mg/kg, twice a week for 4 weeks starting at week 11 after DMBA). The effects of these treatments on 24 tumors in five animals are summarized in Fig. 3. Out of 24 tumors ($\sim$40%), 9 responded to PRIMA-1; six tumors regressed completely in response to PRIMA-1 (Fig. 3B). However, the remaining 15 tumors continued to grow or became stable, despite exposure to a higher dose of PRIMA-1 (Fig. 3B). Nevertheless, because two tumors, p17 and p22, began to regress 15 days after injection with PRIMA-1, we examined the effect of additional injections of PRIMA-1 (20 mg/kg, twice a week for 4 weeks starting at week 11) on tumor growth (Fig. 3A and C). The effect of these injections on the tumors that regressed initially but then relapsed and resumed aggressive growth (p2, p5, p9, p10, and p13) was also examined. Unfortunately, extended administration of PRIMA-1 caused complete regression of only one tumor (p15) from the 18 tumors in this series, while 17 tumors showed a partial response, remained stable or were larger than at the beginning of treatment (p5, p6, p12, p17, and p22; Fig. 3C). These data suggest that chronic and/or higher dosing with PRIMA-1 may be no more effective on tumor inhibition than lower doses or

A

BT474 nuclear label

DMBA

MPA

B

Control C14

P4 regressing

P3 progressing

C

Progressing tumor 1B

Regressing tumor 2A

PAb240 mtp53

PAb246 wtp53

D

p21

C14 control

P4 regressing tumor

P13 progressing tumor

acute treatment with PRIMA-1. Significantly, PRIMA-1 suppressed the emergence of new tumors until approximately the 100 day point during the course of the experiment, at which point new tumors emerged. These results suggest that PRIMA-1 suppresses an early step in tumor development in this model system.

Expression of mutant p53 in MPA-accelerated mammary gland tumors

Previous studies showed that progestin stimulates expression of VEGF, and that PRIMA-1 can suppress expression of VEGF in human breast cancer cells that contain mutant p53 (Hyder *et al.* 2001, Liang *et al.* 2005, 2007). These earlier results suggest that the effect of PRIMA-1 on the expression of VEGF is dependent on the expression of mutant p53 in the target cell. Here, the expression of mutant p53 was examined in MPA-accelerated and non-MPA-accelerated DMBA-induced tumors. This experiment utilized a conformation-specific antibody, PAb240, which recognizes mutant p53 but not wild-type p53 (Bykov *et al.* 2002, Delfino *et al.* 2002). The results show that six out of eight (75%) MPA-accelerated tumors express mutant p53 (predominantly in the nucleus), while non-MPA-accelerated DMBA-induced tumors do not express mutant p53 (Fig. 4A). Additional immuno-chemical studies with an antibody that recognizes wild-type and mutant p53 revealed intense local p53 protein expression in the nuclei of actively proliferating mammary tumor cells. Interestingly, p53 expression was more diffuse and less frequent in tumors that were regressing in response to PRIMA-1 (Fig. 4B).

In order to ensure that PRIMA-1 was promoting the conversion of mutant p53 to wild-type protein, we utilized a fluorescence-based immunohistochemical assay with conformation-specific p53 antibodies and adjacent sections of either progressing or regressing tumors. As shown in Fig. 4C (top and bottom panels on left), PRIMA-1-treated tumors that continued to progress exhibited mainly the mutant form of p53 with minor levels of the wild-type form, indicating that

the mutant protein was resistant to change, while PRIMA-1-treated regressing tumors displayed diminished intensity of mutant p53 protein and intense labeling with FITC, demonstrating the presence of the wild-type conformation of p53 that could be responsible for promoting tumor cell apoptosis (Fig. 4C, panels on right). This result was observed in three regressing tumors analyzed. Consistent with this result, p21 was strongly upregulated in PRIMA-1-treated mammary gland tumors that were regressing (Fig. 4D), indicating the presence of functional p53.

Expression of ER and PR in PRIMA-1-treated MPA-accelerated tumors

Previous studies showed that DMBA-induced tumors express ER. Here, the expression of ER was compared in PRIMA-1-resistant and PRIMA-1-sensitive MPA-accelerated DMBA-induced mammary gland tumors. ER expression was evaluated immunohistochemically, by counting ER-positive cells (three fields/slide, $40\times$ magnification). The results indicated a distinct difference in the distribution of ER-α in progressing and regressing tumors compared with placebo-treated tumors. Placebo-treated progressing tumors (not exposed to PRIMA-1; Fig. 5A, left panel) expressed ERα in the nucleus of numerous ductal epithelial cells (218 ± 14 positively labeled cells) when compared with PRIMA-1-treated tumors. By contrast, approximately only 32% (71 ± 8 positively labeled cells) of epithelial cells in regressing PRIMA-1-treated tumors (Fig. 5B, middle panel) expressed ERα This represents a significantly lower level of ER in regressing tumors compared with placebo-treated tumors ($P<0.001$, Mann–Whitney rank-sum test; Fig. 5A and B). Additionally, ERα was also downregulated in PRIMA-1-treated progressing tumors (P1, P3, P10, P13, P12; 110 ± 12 positively labeled cells) and was significantly lower compared with placebo-treated tumors ($P<0.001$, Mann–Whitney rank-sum test; Fig. 5B). By contrast, expression of ERβ was similar in progressing and regressing tumor cells (not shown).

Figure 4 (A) Mutant p53 distribution in DMBA-induced and DMBA-induced progestin-accelerated tumors. DMBA-treated rats were implanted with MPA or placebo pellets as described in protocol in Fig. 3. Tumors were excised at week 10 post-DMBA treatment and stained for mutant p53 with a conformation-specific antibody PAb240 (upper panel). Xenograft obtained from BT-474 human breast cancer cells were used as positive control (top left panel). (B) Total p53 distribution in PRIMA-1-treated MPA-accelerated tumors. Tumors were excised 15 days following PRIMA-1 treatment and regressing and progressing tumors tested for the distribution of total p53 as described in the Methods section. (C) Conversion of mtp53 to wtp53 conformation following PRIMA-1 treatment. The staining procedure with fluorescently labeled secondary antibodies is described in the Methods section (magnification, $64\times$; bar, 50 μm). The insets in figures represent staining of mtp53 in the sections of xenografts from BT-474 breast cancer cells that express mutant p53 protein (top left panel) and in the sections of xenografts from MCF-7 cells that express the wild-type p53 protein (bottom left panel). (D) Expression of p21 in PRIMA-1-treated regressing and progressing DMBA-induced MPA-accelerated tumors (Magnification, $64\times$; bar, 50 μm). The insets in figures represent controls minus antibody.
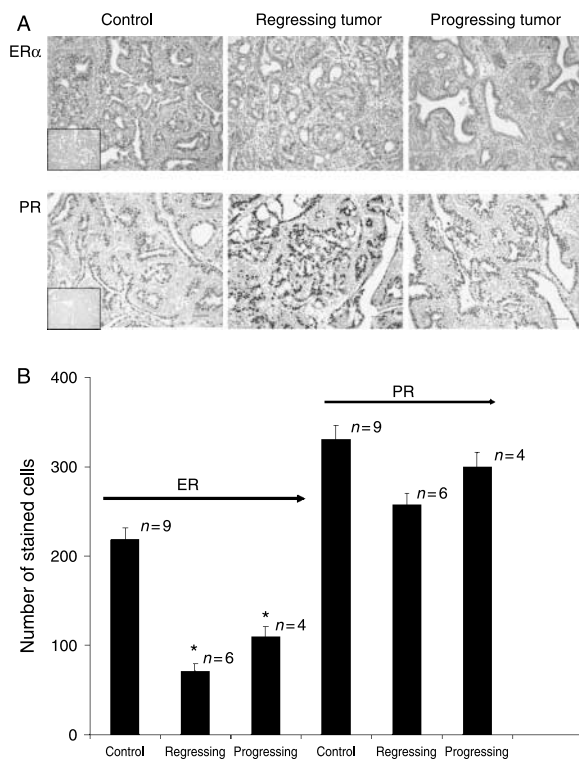
Figure 5 ERα and PR expression in PRIMA-1-treated progressing and regressing tumors. DMBA-treated rats were implanted with MPA pellets 6 weeks post-DMBA. Animals were treated with PRIMA-1 (20 mg/kg bodyweight) in PBS by tail vein injection twice a day for 3 days at days 56–58 (week 8 post-DMBA). All progressing and regressing tumors were excised 15 days after the last PRIMA-1 treatment. (A) Stained with specific ER and PR antibody (magnification, $10\times$; bar, 50 μm). (B) The number of cells staining positive with antibody were counted. $*P < 0.001$ when compared with controls with Mann–Whitney rank-sum test. The insets in control figures represent controls minus antibody.

All MPA-accelerated (PBS-treated) tumors, including those tumors that progressed following PRIMA-1 treatment, expressed nuclear PR in ductal epithelial cells (Fig. 5A and B). Surprisingly, regressing tumors expressed PR at a higher intensity level, even though there was no significant change in the number of PR-positive cells. A representative image is shown in Fig. 5A, and analysis of the prevalence of PR-positive cells is shown in Fig. 5B.

Histopathology of PRIMA-1-sensitive and -resistant MPA-accelerated tumors

Histopathological analysis of mammary lobules and ducts in PRIMA-1-sensitive tumors revealed degenerative changes in mammary epithelium with vacuolation, increased apoptosis, and infiltrating inflammatory cells (e.g., eosinophils, lymphocytes,

macrophages; data not shown). By contrast, PRIMA-1-resistant tumors had no degenerative lesions and actively mitotic epithelium was noted in neoplastic glands. Surprisingly, PRIMA-1-resistant tumors also contained a large number of infiltrating inflammatory cells (i.e., lymphocytes and eosinophils) as was also observed in the regressing tumors (data not shown).

Expression of SPARC in regressing PRIMA-1-treated mammary gland tumors

Histological analysis indicated disrupted nests of tumor cells of PRIMA-1-sensitive regressing mammary gland tumors (data not shown). Preliminary microarray analysis of gene expression in regressing (P2 and P6) and two progressing tumors (P10 and P12) with a focus on the expression of matrix metalloproteins indicated a fourfold upregulation of SPARC, an inhibitor of angiogenesis, in PRIMA-1-sensitive tumors (data not shown). We confirmed SPARC protein expression by immunohistochemical methods in PRIMA-1-treated progressing and regressing tumors. Indeed, the results showed that SPARC was expressed extracellularly at a high level in PRIMA-1-sensitive regressing tumors. By contrast, SPARC was expressed at a much lower level in control and PRIMA-1-resistant tumors, and was equally abundant in the nucleus, cytoplasm, and extracellular space (Fig. 6). The relative levels of SPARC between different tumors is shown in Fig. 6
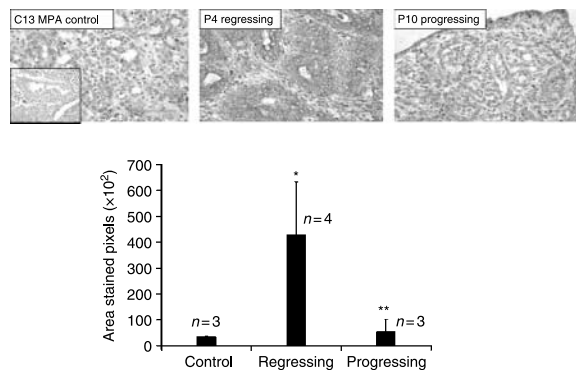

Figure 6 SPARC expression in MPA-accelerated PRIMA-1-treated progressing and regressing tumors. DMBA-treated rats were implanted with MPA pellets 6 weeks post-DMBA. Animals were treated with PRIMA-1 (20 mg/kg bodyweight) in PBS by tail vein injection twice a day for 3 days at days 56–58 (week 8 post-DMBA). All progressing and regressing tumors were excised 15 days after the last PRIMA-1 treatment and assessed for SPARC expression using immunohistochemistry (magnification, $64\times$; bar, 50 μm). The inset shows negative control. Bar graph represents quantitation of staining as described in the Methods section. $*$Significantly different from control and progressing tumors ($P < 0.001$, ANOVA). $**$Significantly different from regressing tumors ($P < 0.001$, ANOVA).

(bottom panel); quantitation was performed as described in the Methods section and the level of expression was found to be significantly different in the regressing tumors compared with progressing or control-treated tumors ($P < 0.01$, Fisher's least significant difference (LSD)).

Discussion

This study examines the ability of PRIMA-1 to inhibit the growth of MPA- and non-MPA-accelerated DMBA-induced mammary tumors in rats. We demonstrated that PRIMA-1 was an effective drug to treat and prevent emergence of progestin-accelerated DMBA-induced mammary tumors in rats. The results of this study may have significant implications for the treatment and prevention of human breast cancer, at least in part because such a large fraction of human breast cancers are dependent on estrogens and progestins for growth. In addition, postmenopausal women worldwide are exposed to exogenous progestins in the form of HRT and clinical studies have associated combined exposure to progestin and estrogen with an increased incidence of human breast cancer in this population (Ross *et al.* 2000, Chlebowski *et al.* 2003), thus confirming the public health relevance of this issue.

The results presented here demonstrate that PRIMA-1 causes regression of about 40% of MPA-accelerated DMBA-induced tumors in rats (Figs 1B and 3B). Furthermore, PRIMA-1 also slightly reduces the growth rate of non-regressing (PRIMA-1 resistant) MPA-accelerated DMBA-induced tumors in this model system. This suggests strongly that MPA-accelerated DMBA-induced tumors are heterogeneous at the molecular level, containing a mixed population of tumor cells. It is likely that the fraction of cells expressing mutant p53, the target of PRIMA-1, is highly variable from one tumor to another. This provides a molecular explanation for the fact that different tumors in a single animal are differentially sensitive or resistant to PRIMA-1. It is also consistent with the observation that PRIMA-1 blocks the formation of new tumors.

By contrast, PRIMA-1 does not inhibit the growth of non-MPA-accelerated DMBA-induced tumors, and does not block the formation of new tumors in DMBA-treated rats not treated subsequently with MPA (Fig. 2), indicating that PRIMA-1 does not suppress the progression of neoplastic or pre-neoplastic lesions in non-MPA-accelerated DMBA-induced tumors. This is not surprising, because a large fraction of DMBA-induced tumors express wild-type p53 (Kito *et al.* 1996), while PRIMA-1 only inhibits proliferation

of tumor cells expressing mutant p53 (Bykov *et al.* 2002, Liang *et al.* 2005). One possible explanation for our observations is that MPA preferentially stimulates pre-neoplastic lesions that contain mutant p53 (Jerry *et al.* 1993), thus enriching the population of mutant p53 cells in MPA-accelerated DMBA-induced mammary tumors. These cells are sensitive to growth inhibition by PRIMA-1, while the cells that contain wild-type p53 protein are not.

It remains to be determined why some MPA-accelerated tumors continue to grow in PRIMA-1-treated animals. This may indicate that some forms of mutant p53 are resistant to PRIMA-1 (Bykov *et al.* 2002), or that some MPA-accelerated tumors contain a relatively small fraction of cells that express mutant p53. Alternatively, the growth of these tumors might be inhibited by higher dose or more frequent exposure to PRIMA-1 at the onset of the study. These possibilities will be addressed in future studies, some of which will require molecular analysis of biopsies from PRIMA-1-sensitive and -resistant tumors to determine their p53 status. These studies will be important to perform since the data related to the presence of mutant p53 protein in pre-neoplastic lesions, and the presence of wild-type protein in frank tumors, are available only for DMBA-treated mouse mammary gland. However, our immunochemical data in the present report suggest that mutant p53 exists in the rat mammary gland.

Our previous studies showed that MPA-accelerated tumors express a high level of VEGF (Benakanakere *et al.* 2006, Liang *et al.* 2007). This study shows that PRIMA-1 strongly inhibits expression of VEGF in regressing tumors and surprisingly also suppresses the expression of VEGF in progressing tumors. Thus, PRIMA-1 may prevent tumor progression via multiple pathways, including reactivation of mutant p53, which ultimately leads to apoptosis. It is important to note that blood vessel density was similar in regressing and progressing tumors, despite the fact that VEGF was expressed at a lower level in the former. This phenomenon, which has been reported previously (Mizukami *et al.* 2005), may indicate that the vasculature in PRIMA-1-treated regressing tumors is functionally deficient (possibly due to reduced permeability). We conducted an experiment to determine the functionality of blood vessels by measuring their ability to perfuse using fluorescently labeled tomato lectin that binds to the endothelial cells in a functional vessel. Our results demonstrated extensive fluorescence in progressing tumors, with almost no label in PRIMA-1-treated regressing tumors. It is our belief that regressing tumors do not receive a sufficient supply of nutrients to support aggressive tumor growth

and therefore shrink. Blood perfusion analysis also identified regions that lacked perfusion in growing tumors since the vessels remained intact but lectin staining could not be detected. The lack of blood perfusion in some vessels could be due to the fact that these are new vessels (angiogenesis) that are not yet fully functional; however, this is speculative.

In this study, MPA-accelerated DMBA-induced tumors were exposed to two different doses of PRIMA-1 (25 or 50 mg/kg). Because similar results were observed at both PRIMA-1 doses, we conclude that dose was not a limiting factor. Nevertheless, long-term studies indicate that PRIMA-1 inhibits the progression of most MPA-accelerated DMBA-induced tumors, even though regression is only observed in a subset of these tumors. When PRIMA-1 was injected directly into pre-existing PRIMA-1-resistant tumors (data not shown), such tumors failed to respond, supporting the idea that a subset of DMBA-induced tumors is intrinsically insensitive to PRIMA-1 and perhaps the rate of apoptosis in these tumors is counterbalanced with MPA-induced cellular prolifer-ation in these tumors. Because PRIMA-1 blocked the formation of new tumors following progestin stimu-lation in this experimental model system, it is tempting to speculate that this agent could be used to prevent progestin-accelerated tumors in women on HRT. However, this idea remains to be tested thoroughly in a clinical setting.

Immunohistochemical analysis of tissue from PRIMA-1-sensitive and -resistant tumors confirmed the fact that MPA-accelerated PRIMA-1-sensitive tumors express mutant p53, while non-MPA-acceler-ated tumors do not. This observation reinforces the idea that PRIMA-1 mainly targets cells expressing mutant p53, and tumors that become stable in size following PRIMA-1 treatment contain a mixture of mutant and wild-type p53. This also reinforces the idea that some tumor cells that express mutant p53 may be responsive to PRIMA-1, while others may be resistant. The notion that PRIMA-1 reactivates mutant p53 was confirmed using conformation-specific antibodies and adjacent sections from progressing or regressing tumors. Our analyses showed that the wild-type p53 conformation occurs chiefly within regressing tumors and that mutant p53 conformation is reduced following PRIMA-1 treatment. The wild-type p53 conformation was detected both in the cell nucleus and in the extra-nuclear space of tumor cells, though it was predomi-nantly detected in the extra-nuclear space; this could be attributable to the fact that wild-type p53 in the nucleus is tagged by ubiquitination for degradation. It is also possible that due to the time factor involved (7 days

post-PRIMA-1), many cells with nuclear wild-type p53 protein have either been destroyed due to apoptosis or are in the process of undergoing cell death. Regressing tumors show increased levels of p21 expression, indicating that PRIMA-1-induced p53 activation is responsible for cell cycle arrest and apoptosis.

Interestingly, expression of ER-α was drastically reduced in PRIMA-1-sensitive MPA-accelerated tumors (Fig. 5A and B). However, somewhat unexpectedly, PR is expressed at a higher level in these tumors, suggesting either reduced turnover or increased synthesis of PR. The precise mechanism involved in increasing the steady-state level of PR in these tumors remains to be determined. Loss of ER could explain why they fail to grow, because DMBA-induced tumors are estrogen dependent and fail to thrive in the absence of functional ER. However, the tumors that progress in the presence of PRIMA-1 also express a lower level of ER compared with controls, so loss of ER alone cannot explain loss of tumor growth in PRIMA-1-treated tumors. It remains to be determined whether ER is a direct target for PRIMA-1 (possibly via activation of mutant p53). Recent studies have demonstrated that p53 suppresses ER-mediated effects (Yu *et al.* 1997, Sayeed *et al.* 2007).

Histological analysis studies presented here indicate that control and MPA-accelerated PRIMA-1-resistant tumors are histologically similar, as observed pre-viously (Benakanakere *et al.* 2006), with mitotically active neoplastic nests and glands. By contrast, regressing PRIMA-1-sensitive tumors are structurally defective in general, containing disrupted glands, apoptotic cells, and vacuolar degeneration. Both progressing and regressing PRIMA-1-treated tumors contained inflammatory cells (lymphocytes and mac-rophages), indicating that PRIMA-1 may directly or indirectly stimulate an immune response through an unknown mechanism. The basement membrane appeared disrupted in PRIMA-1-responsive tumors, suggesting that proteases may be targeted to the basement membrane in regressing tumors. This idea was supported by preliminary gene expression micro-array studies, which demonstrate that SPARC was strongly upregulated in regressing tumors. SPARC is a matricellular protein that is overexpressed in several human cancers and is associated with anti-angiogen-esis. SPARC expression has also been associated with reduced tumor growth, reduced angiogenesis, and with structural changes in the tumor microenvironment (Chlenski *et al.* 2006). The association between PRIMA-1 and SPARC warrants further investigation. For example, it is not yet clear what mechanism leads to the upregulation of SPARC in PRIMA-1-sensitive

MPA-accelerated tumors. However, it is possible that SPARC is a target for PRIMA-1-reactivated p53.

In summary, this study strongly supports the idea that PRIMA-1 reactivates mutant p53 and inhibits the growth of progestin-dependent DMBA-induced mammary gland tumors in rats. Further studies are needed to understand the specific mechanism by which PRIMA-1 exerts its anti-tumor effects in this and other experimental systems. It is essential that we understand why some progestin-accelerated tumors do not regress following PRIMA-1 treatment, even though they grow more slowly and express low levels of VEGF. Perhaps, one of the most important observations from this study is that PRIMA-1 prevents the emergence of new progestin-accelerated DMBA-induced tumors in this model. Future studies will explore the efficacy of PRIMA-1 in blocking initiation of progestin-dependent tumors, as well as the efficacy of combination therapy with PRIMA-1 and anti-progestins or other chemotherapeutic anti-angiogenic or vascular disrupting agents. Such approaches may prevent both progestin-dependent and -independent cancers in the rodent mammary gland, leading to eventual applications to prevent and/or treat human breast cancer.

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