Blockade of β-Catenin Signaling by Plant Flavonoid Apigenin Suppresses Prostate Carcinogenesis in TRAMP Mice

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Abstract

Deregulation of β-catenin signaling is an important event in the genesis of several human malignancies including prostate cancer. We investigated the effects of apigenin, a naturally occurring plant flavone, on prostate carcinogenesis in TRAMP mice and further elucidated its mechanism of action. Oral intake of apigenin by gavage at doses of 20 and 50 μg/mouse/d, 6 days per week for 20 weeks, significantly decreased tumor volumes of the prostate as well as completely abolished distant-site metastases to lymph nodes, lungs, and liver in TRAMP mice. Apigenin-treated mice had significantly diminished weights of their genitourinary apparatuses and dorsolateral and ventral prostate lobes, compared with the control group, and showed reduced proliferation and increased apoptosis in the dorsolateral prostates, which correlated with elevated plasma apigenin levels. Continuous intake of apigenin up to 50 weeks by TRAMP mice significantly improved their overall survival. P.o. administration of apigenin further resulted in increased levels of E-cadherin and decreased levels of nuclear β-catenin, c-Myc, and cyclin D1 in the dorsolateral prostates of TRAMP mice. Similar effects were noted in TRAMP mice with established tumors. Treatment of DU145 human prostate cancer cells with 10 and 20 μmol/L apigenin also increased protein levels of E-cadherin by 27% to 74%, inhibited nuclear translocation of β-catenin and its retention in the cytoplasm, and decreased c-Myc and cyclin D1 levels, an effect similar to the exposure of cells to β-catenin small interfering RNA. Our results indicate that apigenin effectively suppressed prostate carcinogenesis in TRAMP mice, at least in part, by blocking β-catenin signaling. [Cancer Res 2007;67(14):6925–35]

Introduction

Development and progression of prostate cancer has been shown to be associated with the loss of normal epithelial morphology, along with concomitant acquisition of invasive, metastatic, and ultimately fatal properties (1, 2). It is believed that cell adhesion molecules, especially those of the cadherin-catenin complex, play important roles in prostate carcinogenesis (3). In normal epithelial tissues, E-cadherin complexes with the actin cytoskeleton via cytoplasmic catenins to maintain the functional characteristics of the epithelia (4, 5). Disruption of this complex, primarily due to loss or reduced expression of E-cadherin and/or altered subcellular distribution of β-catenin, results in invasive behavior and poor clinical outcome in prostate cancer patients (3, 6). It has been shown that most of the β-catenin is located in the cell membrane where it is associated with the cytoplasmic region of E-cadherin, whereas a smaller pool of β-catenin is located in the nucleus and cytoplasm and mediates Wnt signaling. In the absence of a Wnt signal, β-catenin is constitutively down-regulated by a multicomponent destruction complex containing glycogen synthase kinase 3β, Axin, and a tumor suppressor gene product, adenomatous polyposis coli. These proteins promote the phosphorylation of serine and threonine residues in the NH_2-terminal region of β-catenin and thereby target it for degradation by the ubiquitin proteasome pathway. Wnt signaling inhibits this process, which leads to an accumulation of β-catenin in the nucleus and promotes the formation of transcriptionally active complexes with members of the T-cell factor/lymphoid enhancer factor (Tcf/LEF) family (3–6). Activation of Tcf/LEF and β-catenin targets has been shown to induce neoplastic transformation in epithelial cells, suggesting that the β-catenin signaling pathway may be a key molecular target for the prevention of prostate cancer and/or for therapeutic intervention in managing this malignancy.

We and others have shown that apigenin (4′,5,7,-trihydroxyflavone), a plant flavonoid, is a potent chemopreventive agent and inhibitor of various signal transduction pathways which are essential for the development of cancer (7). It is a nontoxic, nonmutagenic compound that is widely present in common fruits and vegetables and has proven anti-inflammatory and anticarcinogenic effects in cell culture and in various animal tumor model systems (ref. 7 and references therein). Apigenin has been shown to be capable of inhibiting growth in several different types of human cancer cell lines including leukemia and carcinomas of breast, colon, lungs, skin, thyroid, and prostate (8–14). Apigenin is a potent inhibitor of several protein tyrosine kinases including epidermal growth factor receptor and src tyrosine kinase (12, 15). Apigenin has been shown to modulate expression of phosphatidylinositol 3-kinase, protein kinase B/Akt, mitogen-activated protein kinases (extracellular signal-regulated kinase 1/2, c-Jun NH_2-terminal kinase, and p38), casein kinase 2, and other upstream kinases involved in the development and progression of cancer (8, 16–18). Apigenin has also been shown to suppress angiogenesis in melanoma and carcinomas of the breast, skin, and colon (19, 20). We have recently shown the role of apigenin in targeting the insulin-like growth factor growth axis in prostate tumor xenografts (21). Although several pathways have been proposed as targets of apigenin action in cell culture studies, it is unclear which mechanisms are instrumental *in vivo*.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Transgenic adenocarcinoma of the mouse prostate (TRAMP) has become well recognized as a relevant mouse model of prostate carcinogenesis (22, 23). TRAMP males spontaneously develop age-specific, multiple-stage prostatic adenocarcinoma that exhibits both histologic and molecular features similar to human prostate cancer. TRAMP was generated using a region of the androgen-regulated rat probasin promoter consisting of the minimal −426/+28 bp regulatory elements, targeting the expression of SV40 early-region tumor genes (T and t, Tag) to the prostate epithelium. The SV40 large tumor T antigen functions as an oncoprotein interacting with the Rb and p53 tumor-suppressor gene products, and the small t antigen interacts with the protein phosphatase involved in the regulation of the G_2-M transition of the cell cycle (23, 24). The temporally and spatially restricted expression of SV40 Tag antigens along with PB-Tag transgene results in the loss of cytoplasmic domains of E-cadherin, nuclear translocation of β-catenin, and transcriptional activation of specific target genes such as *c-Myc* and *cyclin D1*, events that correlate with disease progression (24–28). These unique features of the TRAMP model in an age-specific manner provide opportunities to conduct studies in cancer prevention and therapy at various stages of disease progression. In the present study, we evaluated the effects of apigenin on prostate cancer development and progression by targeting β-catenin signaling in TRAMP mice as well as in DU145 human prostate cancer cells.

Materials and Methods

Animals. Male and female heterozygous C57BL/TGN TRAMP mice, Line PB Tag 8247NG, were purchased as breeding pairs from The Jackson Laboratory. The animals were bred and maintained at the Association for Assessment and Accreditation of Laboratory Animal Care–accredited Animal Resource Facility of Case Western Reserve University. Transgenic males for these studies were routinely obtained as [TRAMP × C57BL/6] F1 or as [TRAMP X C57BL/6] F2 offspring. Identity of transgenic mice was established by PCR-based DNA screening as previously described (27–29).

Study design and apigenin feeding. Approximately 8-week-old male TRAMP mice and nontransgenic littermates were used in the first experimental studies. The animals received autoclaved Teklad 8760 high-protein diet and tap water *ad libitum* throughout the study. Apigenin (10 mg) was suspended in 1-mL vehicle material (0.5% methyl cellulose and 0.025% Tween 20) by sonication for 30 s at 4°C and further diluted for appropriate concentration. Apigenin, 20 and 50 μg/mouse/d (w/v), was administered by gavage in 0.2 mL of a vehicle consisting of 0.5% methyl cellulose and 0.025% Tween 20 to TRAMP and nontransgenic littermates beginning at 8 weeks of age and was continued until the animals were 28 weeks old, at which time the experiment was terminated. These doses are comparable to the daily consumption of flavonoid in humans as reported in previously published studies (21, 30, 31).

To determine the effect of apigenin on established tumors, in a second experiment, 16-week-old TRAMP mice with palpable tumors received 50 μg/d of apigenin for 12 weeks and were later sacrificed. At the termination of these experiments, blood was collected from the retro-orbital plexus under anesthesia from both experimental and control groups. The animals were then sacrificed by cervical dislocation and examined for the presence of prostate cancer and distant metastases. The genitourinary apparatus consisting of the bladder, urethra, seminal vesicles, ampullary gland, and the prostate was excised, removed, and weighed. The prostate gland was then separately excised using a dissecting microscope. The wet weight of the genitourinary apparatus was recorded to the nearest 0.01 g.

In the third experiment, to investigate the effect of apigenin intake on the growth of prostate tumors and overall survival, 30 male TRAMP mice, 12 weeks of age, were divided into three groups of equal size. The control group of animals was provided with only 0.2 mL of vehicle material by gavage for 6 days per week whereas the second and third groups of animals received 20 and 50 μg/mouse/d doses of apigenin in vehicle, respectively, until the animals died or reached 50 weeks of age. Animals in all the groups were observed weekly for body weight, tumor progression by abdominal palpation, and survival up to 50 weeks. The animals that were still alive at 50 weeks were sacrificed by CO_2 asphyxiation.

Magnetic resonance imaging. Six animals each from control and apigenin-treated groups, in the first experiment, were randomly selected and monitored for tumor growth and volume by magnetic resonance imaging (MRI) at 18 and 28 weeks of age as previously described (28, 29). Imaging of these animals was done by using a whole-body 1.5-T Siemens Sonata clinical MRI scanner with a custom-designed cylindrical (25-mm internal diameter) phased-array mouse coil. A T1-weighted spin echo acquisition (repetition time/echo time = 700 ms/14 ms) was used to acquire the high resolution (~200 μm) axial images. Images were transferred to a remote imaging workstation for volumetric analysis of prostate tumors. Tumor volume was measured by manually segmenting the region of interest in each slice and summing the tumor area from each slice.

Cells and treatment. DU145 human prostate cancer cells were cultured in standard condition and were treated with 10 and 20 μmol/L apigenin for 24 h or transfected with β-catenin small interfering RNA (siRNA) or control plasmid (SMARTpool, Dharmacon, Inc.) for 72 h. The cells were either imaged or harvested to obtain nuclear and cytoplasmic fractions as previously described (28).

Preparation and analysis of tissue. The dorsolateral prostates were excised and weighed, and a small portion was fixed overnight in 10% zinc–buffered formalin and then transferred to 70% ethanol. Sections (4 μm) were cut from paraffin-embedded tissue and mounted on slides. The sections were stained with H&E as previously described (27–29) and were evaluated for the presence or absence of the following lesions: prostatic intraepithelial neoplasia, well-differentiated adenocarcinoma, moderately differentiated adenocarcinoma, and poorly differentiated adenocarcinoma. The histologic characteristics of these lesions have been well established and described in a previous publication (23).

Metastases examination. Microscopic examinations of lymph nodes, liver, and lungs were done to evaluate for the presence of metastases. The India ink method was used to examine the lungs for metastasis as previously described (27).

Immunoblot analysis. The dorsolateral prostates were removed from both treated and control groups and then homogenized in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L Na_3VO_4, 0.5% NP40, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, pH 7.4) at 4°C to prepare cell lysates. The protein concentration was determined by DC Bio-Rad assay following the manufacturer's protocol (Bio-Rad Laboratories). Appropriate amount of protein (25–50 μg) was resolved on an 8% to 14% Tris-glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked with 5% nonfat dry milk and probed with appropriate primary antibody of E-cadherin, proliferation cell nuclear antigen proliferating cell nuclear antigen (PCNA), β-catenin, c-Myc, SV40 T antigen, and poly(ADP-ribose) polymerase cleavage (Santa Cruz Biotechnology, Inc.) and cyclin D1 (Lab Vision Corp.) in blocking buffer overnight at 4°C. The membrane was then incubated with antimouse or antirabbit secondary antibody horseradish peroxidase (HRP) conjugate (Amersham Life Sciences, Inc.) followed by detection with chemiluminescence ECL kit (Amersham Life Sciences, Inc.). Equal loading of protein was confirmed by stripping the membrane and reprobing it with appropriate housekeeping primary antibody and secondary HRP conjugate.

Immunohistochemistry and immunofluorescence. Immunohistochemistry for PCNA was done on formalin-fixed, paraffin-embedded prostate tissue sections using a standard protocol as previously described using 3,3′-diaminobenzidene and counterstaining with Mayer's hematoxylin (27, 29). Immunofluorescence staining for apoptosis was done using M30 CytoDEATH antibody (Boehringer Mannheim). DU145 cells were grown in Petri dish over the glass slide and treated with varying concentrations of apigenin or transfected with β-catenin siRNA and later incubated with β-catenin or E-cadherin antibodies using AlexaFluor-488

(Molecular Probes) and Texas red (Abcam) visualized under an inverted Olympus BX51 microscope equipped with a fluorescent light source as previously described (29).

High-performance liquid chromatography analysis for plasma apigenin levels. Plasma samples (0.2 mL) from the various experimental groups were deproteinized by adding 0.4 mL of methanol, vortex mixed for 60 s, and centrifuged at 1,100 rpm for 15 min at 4°C. The supernatant (0.6 mL) was collected into the tube and evaporated to dryness by vacuum freeze drying. The residue was dissolved in 200 μL of methanol and chromatographically analyzed by analytic reverse-phase high-performance liquid chromatography (HPLC) on Waters 600 System (Amphotech Ltd.) connected to a Waters UV detector as previously described (21).

Proliferation and apoptotic indices. The proliferation index was assessed by counting the distribution of PCNA-stained nuclei within the prostate tissue at ×40 magnification. Similarly, apoptotic index was determined by counting the number of M30 immunofluorescence positive cells in prostate tissue of TRAMP mice. The fields were randomly chosen and digitalized with the Microsoft suite software program.

Statistical analysis. Changes in prostate and body weight during the course of the experiments were analyzed by Kruskal-Wallis test, a nonparametric test based on Wilcoxon scores followed by pairwise comparison in which *P* values were not adjusted for multiple comparisons. The associations between apigenin exposure and tissue proliferation and between apigenin exposure and apoptosis were estimated by Pearson correlation coefficient. All tests were two sided and $P < 0.05$ was considered to be statistically significant. The Kaplan-Meier method was used to estimate survival and the differences were analyzed by the log-rank test.

Results

MRI analysis of TRAMP mouse prostates after intake of apigenin. To assess the effect of apigenin intake in TRAMP mice on prostate tumorigenesis, we measured the prostate growth by using MRI. Prostate volumes measured by MRI at 18 and 28 weeks of age in TRAMP mice were greater than prostate volumes of male nontransgenic littermates, consistent with the development of prostate cancer in the former group. Prostate volumes in apigenin-treated male TRAMP mice were substantially less than in those on vehicle treatment (Fig. 1*A* and *B*). Apigenin administration to TRAMP mice exhibited a significant reduction in proliferation of the prostate measured at 10 weeks on test (18-week-old animal) with 33% diminution of prostate volume in mice given 20 μg/d and 50% diminution in mice given 50 μg/d. Furthermore, 20 weeks of apigenin intake (28-week-old animal) resulted in 57% diminution in prostate volume in mice given 20 μg/d and 64% diminution in prostate volumes in mice given 50 μg/d, as observed by volumetric analyses of the prostate (Fig. 1*B*). This diminution in tumor volumes was also evident from abdominal pelvic palpation.

Effect of apigenin intake on prostate tumorigenesis in TRAMP mice. Mice given 20 and 50 μg of apigenin per day did not exhibit any symptoms of toxicity such as loss of appetite, decreased locomotion, or any other apparent signs of ill health throughout the study. No significant effects were observed in the body weight profiles in nontransgenic littermates receiving 50 μg/d of apigenin when compared with vehicle-fed nontransgenic controls (data not shown). TRAMP mice receiving 50 μg/d of apigenin exhibited a modest decrease in body weight (~5%) compared with control nontransgenics after 10 weeks of feeding, which persisted till the termination of the experiment. In comparison, TRAMP mice that received vehicle only showed an increase in body weight (~18%) compared with control nontransgenics, probably due to the greater degree of proliferation in the genitourinary region (Fig. 1*C*).

To investigate the effects of apigenin intake on prostate tumor growth and progression in TRAMP mice, two separate experiments were conducted using a control group and administering apigenin at doses of 20 and 50 μg/d to two other groups of TRAMP mice, beginning at 8 weeks of age and continuing for 20 weeks. In the first experiment, as expected, all six mice in the control group developed advanced prostate cancer that extensively infiltrated the abdominal region. In contrast, only 3 of the 6 (50%) animals receiving 20 μg/d of apigenin developed palpable tumors and only 1 of the 6 (17%) animals receiving 50 μg/d of apigenin developed a palpable tumor. Similarly, in the repeat experiment, 2 of the 6 (33%) animals receiving 20 μg/d of apigenin developed palpable tumors, and no animal receiving 50 μg/d of apigenin developed a palpable tumor. This inhibitory effect of apigenin administration on prostate tumorigenesis was also evident from assessment of the wet weight of the genitourinary apparatuses and prostates of these groups of mice. As summarized in Supplementary Table S1, apigenin intake to TRAMP mice resulted in a significant decrease in average dorsolateral prostate weight (28% reduction; $P < 0.033$) at 20 μg/d and 40% reduction ($P < 0.019$) at 50 μg/d doses, compared with the control group. Similarly, substantial reductions in the weights of ventral prostates were observed: 38% ($P < 0.016$) reduction at 20 μg/d and 40% ($P < 0.015$) reduction at 50 μg/d of apigenin administration. Apigenin intake also resulted in significant reductions in the genitourinary apparatus weights: 51% ($P < 0.0001$) reduction at 20 μg/d and 65% ($P < 0.0003$) reduction at 50 μg/d dose, compared with the control group (Fig. 2*A*).

We also studied the effects of apigenin administration on the development of systemic metastases. The cumulative data at the termination of the experiment (28 weeks of age) from 12 animals in the control group showed that all 12 animals (100%) in the control group had developed invasive cancers, with metastases to lymph nodes (75%), lungs (42%), and liver (33%). In sharp contrast, none of the 24 mice that received apigenin exhibited metastases to any of the distant organs studied (Supplementary Table S1).

Effect of apigenin intake on prostate histology in TRAMP mice. The histologic findings in TRAMP mice of various ages have previously been documented, and it is known that TRAMP mice by the age of 28 weeks have typically developed well-differentiated prostate adenocarcinoma, which subsequently progresses to poorly differentiated cancer with advancing age (23, 24). We evaluated the dorsolateral prostates of TRAMP mice in control and experimental groups of animals at 28 weeks of age (Fig. 2*B*). Prostates of vehicle-treated controls exhibited prostatic intraepithelial neoplasia (17%) and cancers of variable size, predominated by well-differentiated adenocarcinoma (>50%) followed by moderately differentiated cancer (>18%) and poorly differentiated cancer (~4%). About 5% of the prostate tissue was nonneoplastic. The histologic findings in the prostates of 20 μg/d apigenin–treated TRAMP mice at 28 weeks were notably different from findings in vehicle-treated TRAMP mice, showing a greater proportion of nonneoplastic prostate tissue (>25%) with concomitant decreases in prostatic intraepithelial neoplasia (~15%) and well-differentiated (~40%), moderately differentiated (~12%), and poorly differentiated (<2%) cancers. Histologic findings observed in the prostates of mice receiving higher dose of 50 μg/d apigenin; more than 50% of the prostate tissue was nonneoplastic, with significant reductions in the proportions of prostatic intraepithelial neoplasia (~14%) and well-differentiated (~30%), moderately differentiated (<5%), and poorly differentiated (<1%) cancers, respectively (Fig. 2*C*).

Effect of apigenin intake on β-catenin signaling and E-cadherin levels in the dorsolateral prostates of TRAMP mice. Loss of expression of cell adhesion molecules, particularly the

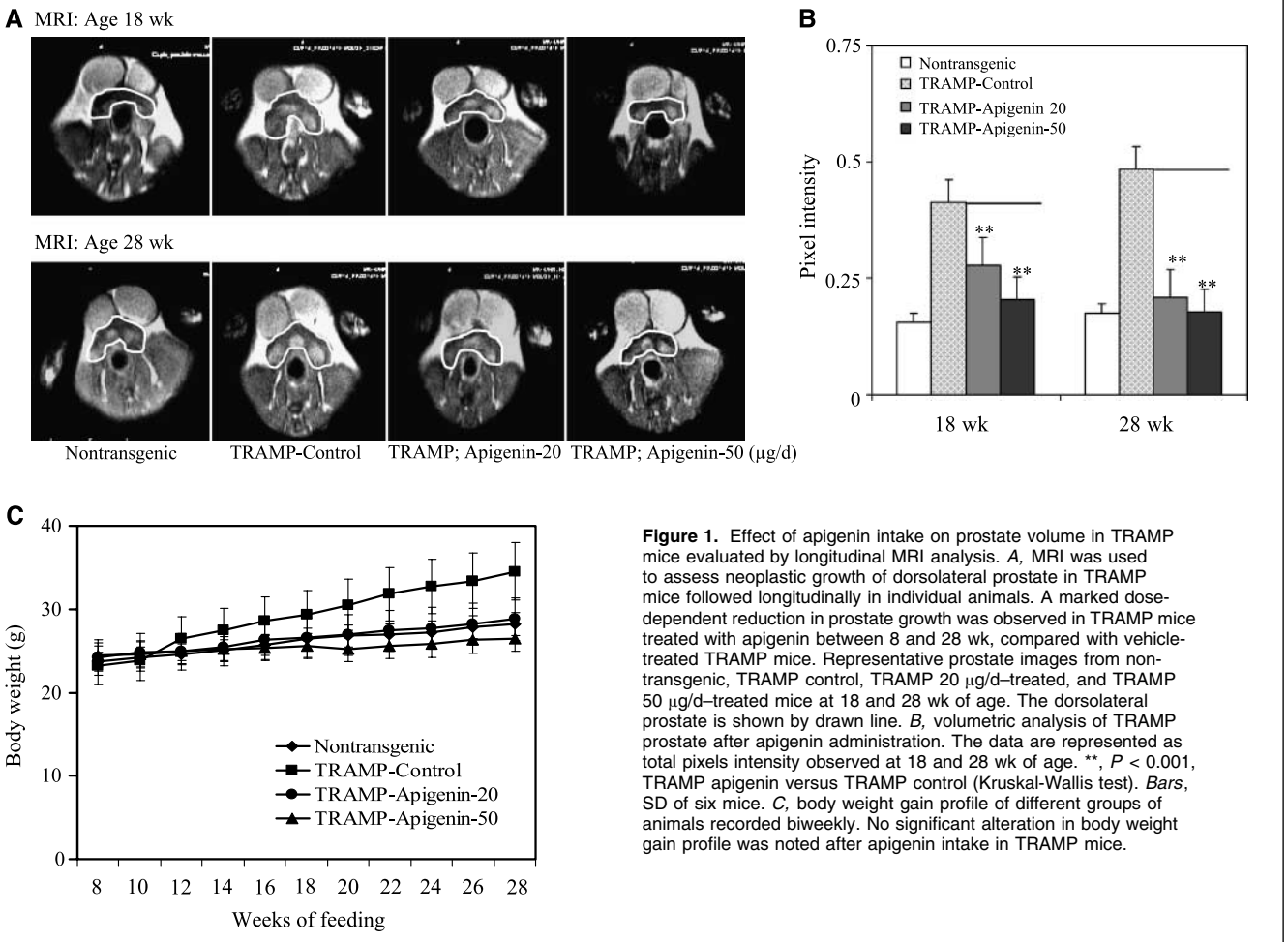

Figure 1. Effect of apigenin intake on prostate volume in TRAMP mice evaluated by longitudinal MRI analysis. *A,* MRI was used to assess neoplastic growth of dorsolateral prostate in TRAMP mice followed longitudinally in individual animals. A marked dose-dependent reduction in prostate growth was observed in TRAMP mice treated with apigenin between 8 and 28 wk, compared with vehicle-treated TRAMP mice. Representative prostate images from non-transgenic, TRAMP control, TRAMP 20 μg/d–treated, and TRAMP 50 μg/d–treated mice at 18 and 28 wk of age. The dorsolateral prostate is shown by drawn line. *B,* volumetric analysis of TRAMP prostate after apigenin administration. The data are represented as total pixels intensity observed at 18 and 28 wk of age. **, $P < 0.001$, TRAMP apigenin versus TRAMP control (Kruskal-Wallis test). *Bars*, SD of six mice. *C,* body weight gain profile of different groups of animals recorded biweekly. No significant alteration in body weight gain profile was noted after apigenin intake in TRAMP mice.

cadherin-catenin complex, in epithelial malignancies is associated with increased invasiveness and the development of metastasis (32, 33). Furthermore, β-catenin signaling has been shown to play a causative role in prostate cancer and is a critical event in the development of prostatic tumors in TRAMP mice; consequently, we made β-catenin signaling a focal point of our investigation (25, 32). By Western blot analysis, we measured nuclear levels of β-catenin in the dorsolateral prostates of TRAMP and nontransgenic mice. Because nuclear accumulation of β-catenin promotes transcription of proliferation genes including *c-Myc* and *cyclin D1*, we also measured the levels of these proteins. As shown in Fig. 3*A*, dorsolateral prostates from TRAMP mice exhibited significantly higher levels of nuclear β-catenin and c-Myc expression and higher levels of cyclin D1 in the cytoplasm compared with nontransgenic prostates. Apigenin intake for 20 weeks resulted in a marked reduction in the nuclear levels of β-catenin and c-Myc and reduced cytoplasmic levels of cyclin D1 in the dorsolateral prostates of TRAMP mice (Fig. 3*A* and *B*). These data suggest that the aberrant β-catenin signaling in the prostate tumors was suppressed by apigenin administration. One possible upstream event in the suppression of β-catenin signaling is the up-regulation of E-cadherin protein. As shown in Fig. 3*B*, p.o. administration of apigenin resulted in a significant increase of E-cadherin protein levels in dorsolateral prostates of TRAMP mice.

Effect of apigenin treatment on E-cadherin expression and localization of β-catenin in human prostate cancer DU145 cells. We examined whether similar effects of apigenin treatment on E-cadherin and nuclear β-catenin levels in prostate tumors of TRAMP mice could be recapitulated in apigenin-treated prostate cancer cells in culture. The DU145 cell line was chosen because these prostate cancer cells have low transcript levels of E-cadherin and high levels of nuclear β-catenin similar to prostate tumors in TRAMP mice (34). Using this cell line, we first investigated whether specifically reducing the levels of β-catenin results in decreased proliferation and invasiveness and increased apoptosis. For this analysis, RNA interference with siRNAs directed against β-catenin was used and comparisons were made between controls and cell lines exposed to apigenin. As shown in Fig. 4*A*, siRNA directly targeted against β-catenin resulted in a 40% to 50% decrease in cell proliferation and a 7- to 8-fold increase in apoptosis of DU145 cells seen at 72 h posttransfection in four separate experiments. The cell invasiveness could not be determined due to high rate of apoptosis in these cells. Treatment of DU145 cells with 20 μmol/L apigenin for 24 h resulted in a 30% to 35% decrease in cell proliferation and a 3- to 4-fold increase in apoptosis in these cells. Apigenin exposure significantly reduced the invasiveness of these cells: 38% to 47% inhibition compared with control (data not shown). As shown in Fig. 4*B* and *C*, targeting

β-catenin via siRNA transfection or apigenin decreased β-catenin levels by 90.7% and 21.2%. Increases in E-cadherin levels by 28% after siRNA transfection and 18% after apigenin treatment were observed in these cells. Inhibition of β-catenin signaling by siRNA decreased cyclin D1 by 11.6% and c-Myc by 67.6% whereas 32.8% and 54.6% decreases in cyclin D1 and c-Myc levels were noted after apigenin treatment (Fig. 4*B*). The marked reduction of cyclin D1 after apigenin exposure suggests the involvement of other pathways as well.

Next, we determined the effects of apigenin treatment in DU145 cells on the subcellular distribution of β-catenin and other associated proteins. As shown in Fig. 4*D*, treatment of DU145 cells with 10 or 20 μmol/L apigenin decreased β-catenin levels in both cellular compartments. Reductions of 25.8% and 36.0% in nuclear β-catenin levels and 11.8% and 19.1% in cytoplasmic levels were observed, which resulted in significant reductions in the nuclear to cytoplasmic ratio of β-catenin after apigenin treatment. This treatment also increased cytoplasmic E-cadherin protein levels by 27.1% and 73.7%, respectively, at the two different dosage levels. Apigenin treatment at 10 and 20 μmol/L doses also decreased the nuclear levels of cyclin D1 by 39.6% and 64.5% and cytoplasmic levels by 2.6% and 76.5%, respectively, as well as the nuclear levels of c-Myc by 14.6% and 26.3%. As shown in Fig. 4*E*, the altered distribution of β-catenin and E-cadherin after apigenin treatment by fluorescence microscopy is consistent with the view that apigenin treatment increased E-cadherin protein levels, increased E-cadherin-β-catenin complex formation in the cytoplasm, and prevented β-catenin from localizing in the nucleus (Fig. 4*D* and *E*). These results validate β-catenin as a key molecular target in preventive and therapeutic management strategies for prostate cancer.

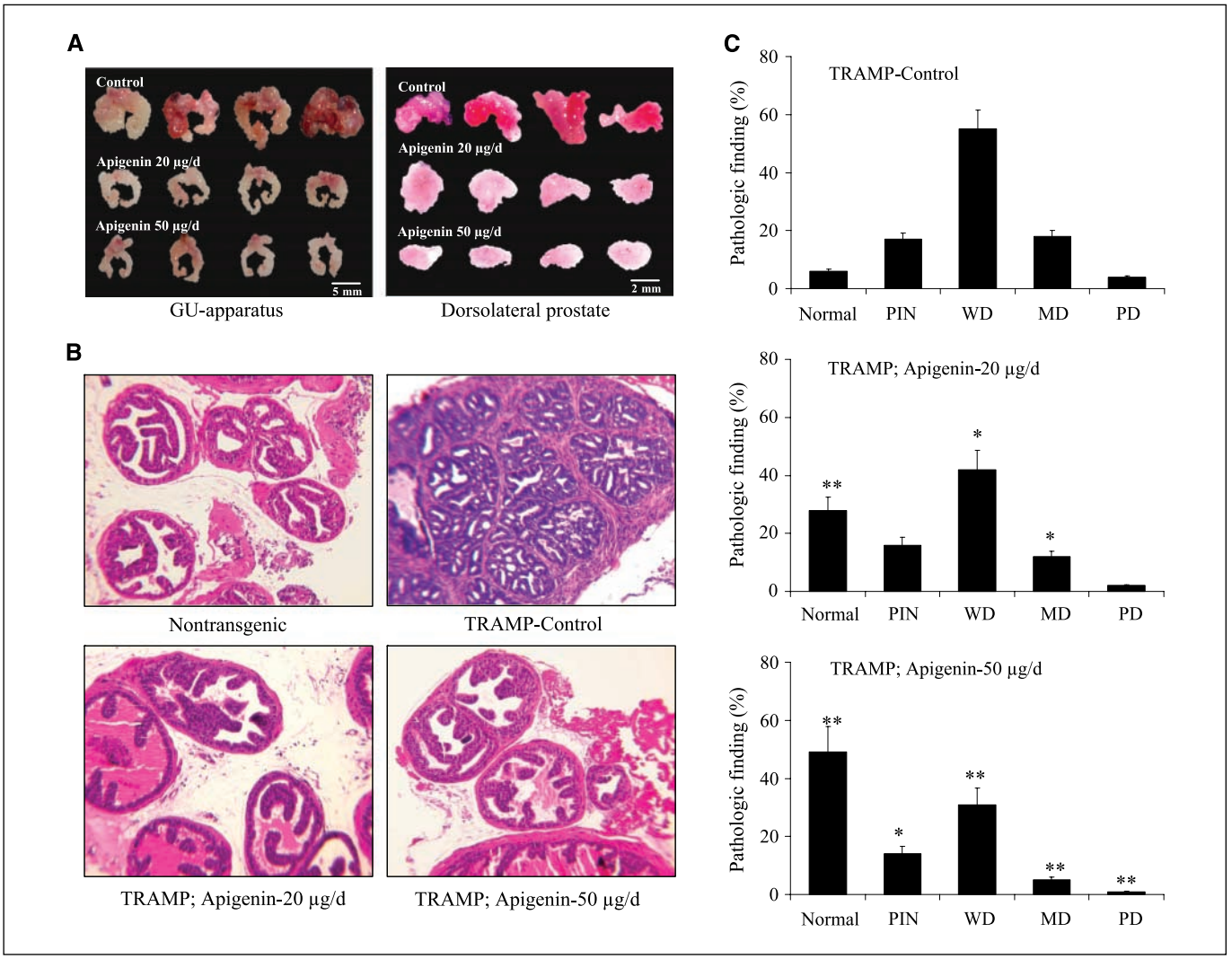

Figure 2. Effect of apigenin intake on prostate and genitourinary apparatus in TRAMP mice. *A,* photographs of genitourinary (*GU*) apparatus and dorsolateral prostate after apigenin intake. *B,* a typical dorsolateral prostate from a nontransgenic mouse exhibited acini with abundant eosinophilic intralumenal secretions. TRAMP mice (control) exhibited well-differentiated cancer with extensive epithelial stratification, crowded cribriform structures accompanied with marked thickening, remodeling, and hypercellularity of the fibromuscular stroma. Apigenin administration to TRAMP mice resulted in a marked reduction in epithelial stratification and cribriform structures. *C,* distribution of pathologic findings after apigenin feeding in the dorsolateral lobes of TRAMP mice. H&E-stained slides were evaluated by three independent scientists. Prostatic lobe was scored for percentage of each pathologic finding present in that lobe. The scores of the evaluators were averaged. *Columns,* average percentage of each pathologic finding in the dorsolateral prostate in TRAMP mice at 28 wk of age; *bars,* SE. Pathologic findings: *PIN,* prostatic intraepithelial neoplasia; *WD,* well-differentiated cancer; *MD,* moderately differentiated cancer; *PD,* poorly differentiated cancer. *, $P < 0.05$; **, $P < 0.001$, TRAMP apigenin versus TRAMP control (Kruskal-Wallis test). *Bars*, SE of eight mice.

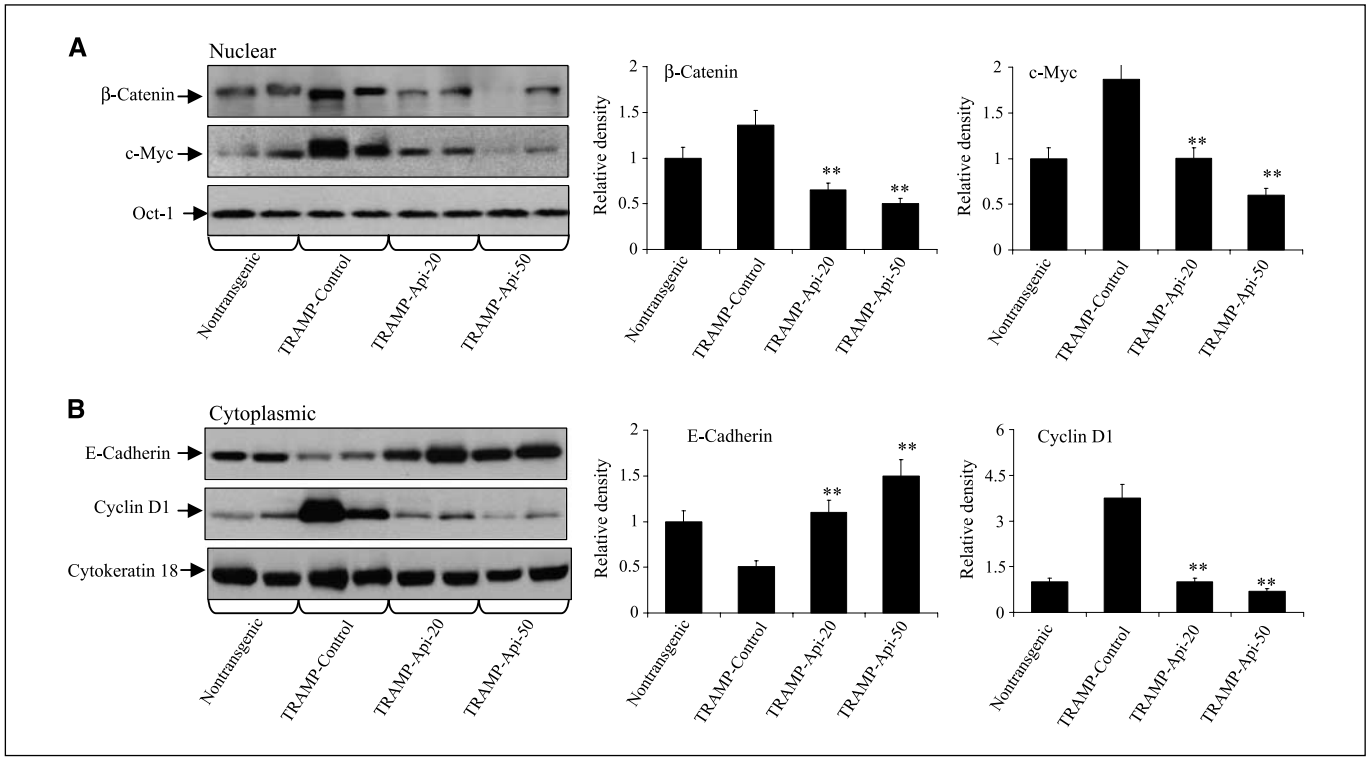

Figure 3. Effect of apigenin intake on β-catenin signaling and E-cadherin protein levels in the dorsolateral prostates of TRAMP mice. *A,* protein expression of β-catenin and c-Myc in the nuclear compartment and their corresponding densitometric analyses. *B,* E-cadherin and cyclin D1 in the cytoplasmic compartment and their corresponding densitometric analyses in the dorsolateral prostates of TRAMP mice. A significant retention of E-cadherin protein expression was observed whereas a significant decrease in the levels of cyclin D1, β-catenin, and c-Myc was observed after apigenin administration. Representative data from two mice per group. Equal loading of protein in the lanes was confirmed by stripping the membrane and reprobing it with appropriate housekeeping antibody. **, $P < 0.001$, TRAMP apigenin versus TRAMP control (Kruskal-Wallis test). *Bars,* SE of six mice.

Effect of apigenin intake on proliferation and apoptotic indices in the dorsolateral prostates of TRAMP mice. In previous experiments, we showed the functional consequences of inhibition of β-catenin signaling, which resulted in decreased proliferation and increased apoptosis in human prostate cancer cells. Therefore, we next determined the effects of apigenin feeding on cellular proliferation in mouse prostates by assessing the expression of a proliferation-related protein, PCNA. PCNA is a requisite auxiliary protein for DNA polymerase δ–driven DNA synthesis and is cell cycle regulated (ref. 29 and references therein). As shown in Fig. 5*A*, p.o. administration of apigenin markedly suppressed proliferation and PCNA protein expression in the dorsolateral prostates of TRAMP mice. Decreases of ~35% and ~62% in PCNA protein levels were observed after intake of 20 and 50 μg/d apigenin. We also determined the extent of apoptosis after apigenin intake in TRAMP mice. As shown in Fig. 5*B*, p.o. administration of apigenin significantly increased the extent of apoptosis in the dorsolateral prostates of TRAMP mice, shown by an immunofluorescent technique with the M30 CytoDEATH antibody that binds to a caspase-cleaved formalin-resistant epitope of the cytokeratin 18 cytoskeletal protein.

Plasma apigenin levels in TRAMP mice and its correlation with proliferation and apoptosis indices. Next, we determined the levels of apigenin in plasma and evaluated whether these levels correlate with tumor proliferation and apoptosis. To assess the level of apigenin in the plasma of apigenin-fed mice, a standard HPLC profile of apigenin was developed and its retention time was determined under a linear range of detection using area under curve of apigenin peak in HPLC profiles of these samples (data not shown). As shown in Fig. 5*C*, TRAMP mice receiving vehicle material only showed undetectable levels of apigenin in their plasma (*a*) whereas a sharp peak was observed from plasma treated with 2 μmol/L apigenin (*b*). Similar peaks were noted in the plasma of apigenin-treated mice (*c* and *d*). Apigenin administration to TRAMP mice resulted in 0.63 ± 0.16 μmol/L apigenin at 20 μg/d and 1.15 ± 0.12 μmol/L at 50 μg/d, which negatively correlated with proliferation indices ($R = -0.9$; $P < 0.0001$) and positively correlated with apoptotic indices ($R = 0.97$; $P < 0.0001$; Fig. 5*D*).

Effect of apigenin intake on β-catenin signaling and E-cadherin levels in the dorsolateral prostates of TRAMP mice with established tumors. To observe the effects of apigenin on established tumors, 16-week-old TRAMP mice with palpable tumors were provided with 50 μg/mouse/d of apigenin for 12 weeks and were sacrificed. As expected, apigenin treatment further arrested prostate tumor growth and proliferation. Dorsolateral prostates from apigenin-treated mice had significantly reduced nuclear levels of β-catenin and c-Myc and cyclin D1 in the cytoplasm, compared with control mice (Fig. 6*A* and *B*). Apigenin treatment significantly increased the protein levels of E-cadherin in the dorsolateral prostates of TRAMP mice.

Effect of apigenin intake on survival of TRAMP mice. Extended survival is one of the most desirable effects of any chemoprevention regimen. Therefore, we evaluated whether or not apigenin intake leads to increased survival of TRAMP mice. We studied 30 TRAMP males of ~12 weeks of age, which were equally divided into three groups. The first group received vehicle only and

served as a control group. The second and third groups received 20 and 50 μg/d of apigenin, respectively. Survival observations were continued until the animals died or reached 50 weeks of age. As shown in Fig. 6*C*, the survival for TRAMP mice in the control group was 90% at 30 weeks, 80% at 40 weeks, and 40% at 50 weeks. Only four animals in the vehicle-fed group were still alive after 50 weeks. In contrast, survival of TRAMP mice receiving 20 μg/d of apigenin was significantly increased (P = 0.087): 100% at 30 weeks, 90% at 40 weeks, and 80% at 50 weeks. Similarly, p.o. administration of apigenin to TRAMP mice at a higher dose of 50 μg/d further prolonged the life span of these mice (P = 0.02): 100% at 30 weeks, 100% at 40 weeks, and 90% at 50 weeks. Overall, a significantly improved survival was observed in TRAMP mice receiving apigenin compared with mice receiving vehicle treatment (P = 0.035).

Effect of apigenin intake on SV40 T and t antigens in the dorsolateral prostates of TRAMP mice. One major concern was that the observed preventive/therapeutic effects of apigenin might be a consequence of direct suppression of the probasin promoter by apigenin, resulting in reduced expression of the T and t, Tag transgene. As shown in Fig. 6*D*, the T and t Tag oncoprotein was expressed in the dorsolateral prostates of TRAMP treated with and without apigenin at 18 and 28 weeks of age, suggesting that the mechanism of apigenin action against prostate cancer is not related to Tag expression but rather to direct suppression of carcinogenesis.

Discussion

Our studies show that dietary intake of apigenin at doses of 20 and 50 μg/mouse/d for 20 weeks by TRAMP mice, starting at 8 weeks of age, effectively inhibits prostate carcinogenesis. Evidence for this inhibitory effect is provided by the observations that apigenin-treated mice have fewer palpable tumors, reduced tumor volumes, and complete absence of distant metastases, and their prostates contain smaller proportions of neoplastic tissue as compared with control animals. In addition, apigenin administration at a dose of 50 μg/d for 12 weeks arrested the growth of established tumors in TRAMP mice.

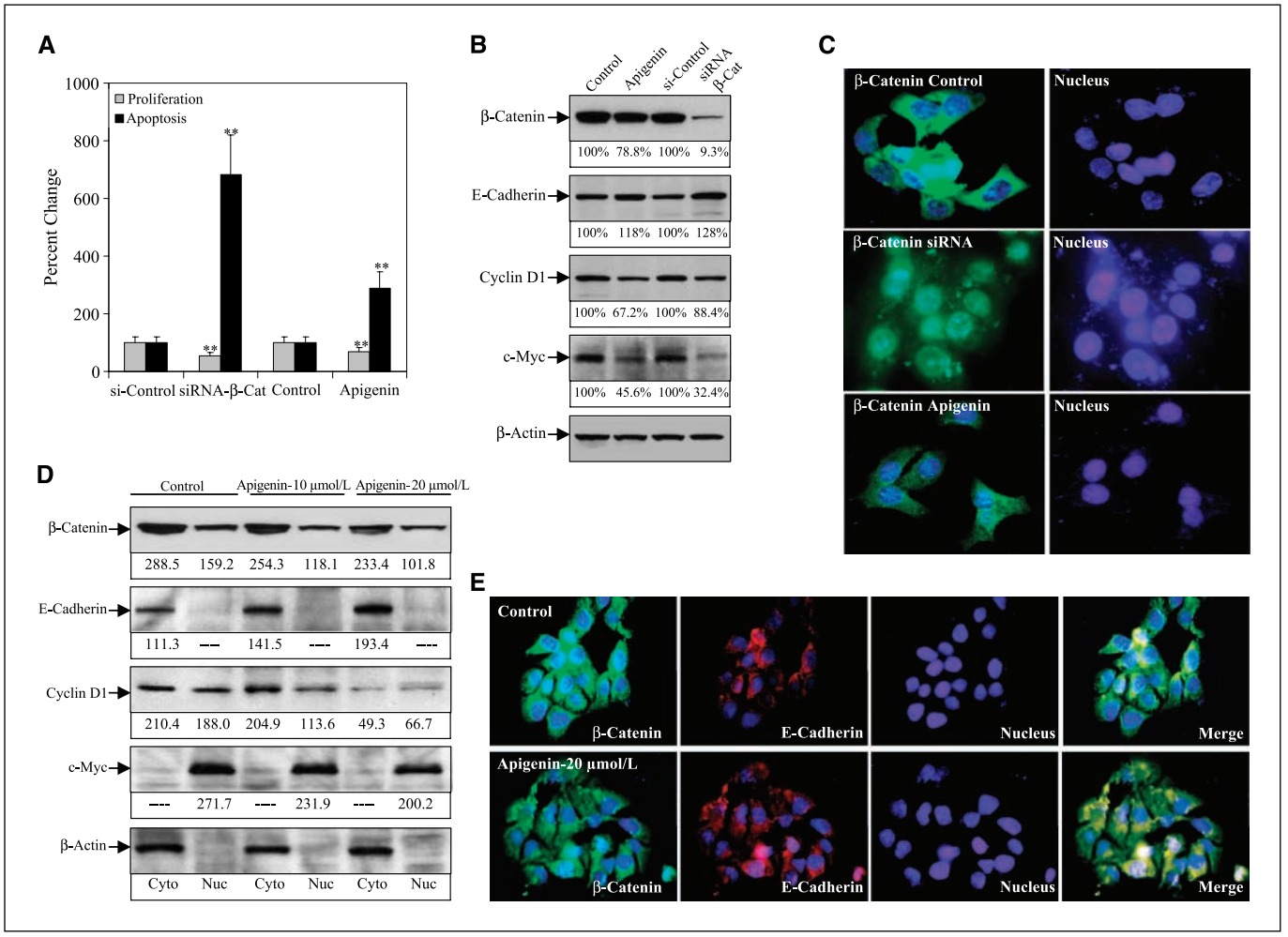

Figure 4. Effect of β-catenin gene silencing and its comparison with apigenin treatment on cellular functions and protein expression of β-catenin, c-Myc, cyclin D1, and E-cadherin in DU145 human prostate cancer cells. The cells were treated with 10 and 20 μmol/L apigenin for 24 h or transfected with β-catenin siRNA or control plasmid for 72 h. *A,* effect of β-catenin gene silencing and apigenin on cell proliferation and apoptosis. **, P < 0.001, compared with corresponding control (Kruskal-Wallis test). *B,* Western blot analysis of β-catenin, c-Myc, cyclin D1, and E-cadherin after β-catenin gene silencing and its comparison with apigenin treatment (after normalization by levels of β-actin). *C,* immunofluorescence detection of β-catenin after gene silencing and apigenin treatment. *D* and *E,* distribution of β-catenin, c-Myc, cyclin D1, and E-cadherin proteins in the nuclear and cytoplasmic fractions after apigenin exposure (*D*) and colocalization and distribution of β-catenin and E-cadherin after apigenin treatment by immunofluorescence microscopy (*E*). Apigenin treatment of DU145 cells caused a decrease in β-catenin signaling and its increased retention in the cytoplasm.

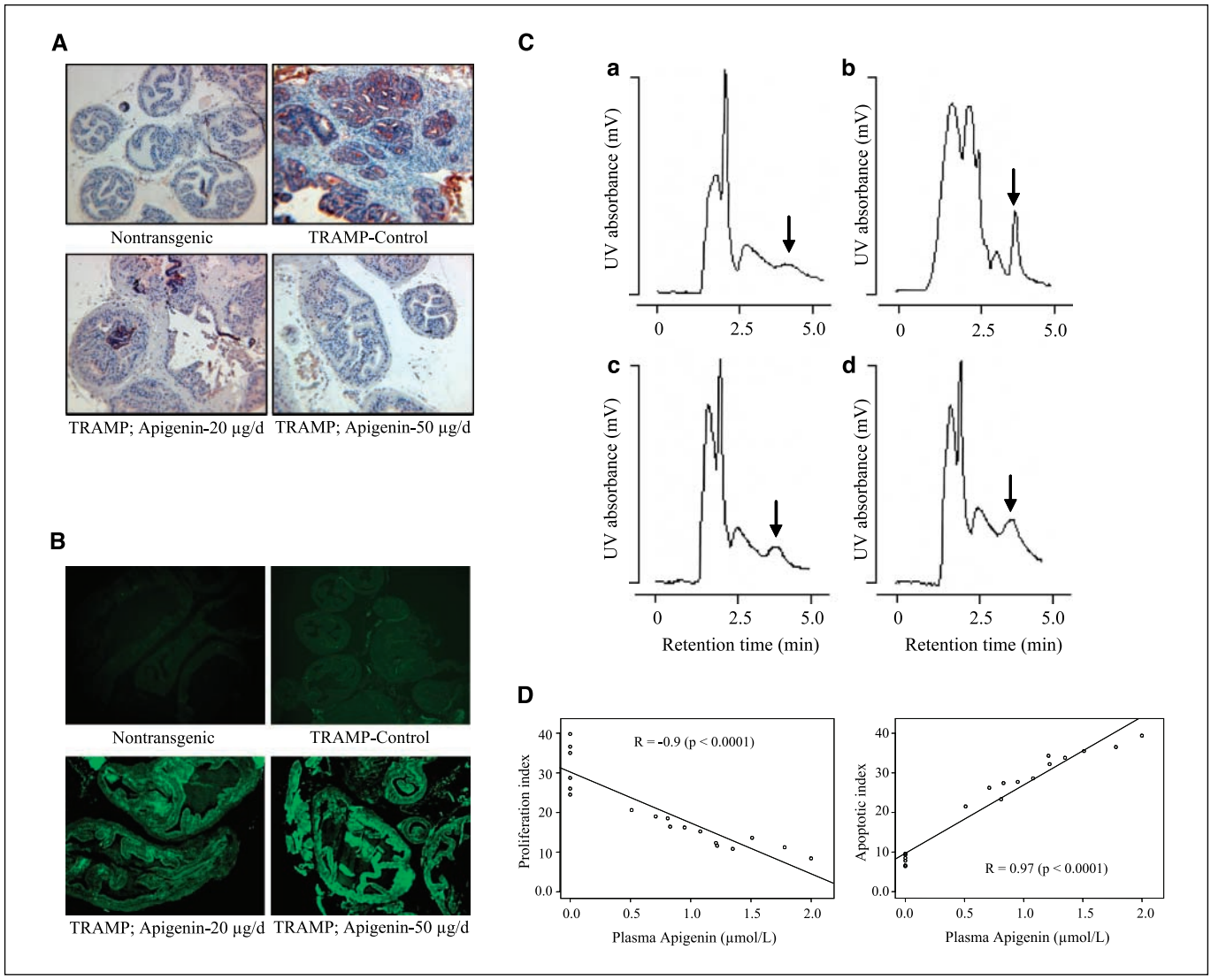

Figure 5. Effect of apigenin intake on the extent of proliferation and apoptosis and its correlation with plasma apigenin levels in TRAMP mice. *A,* immunohistochemical analyses of PCNA. In vehicle-treated TRAMP mice, extensive PCNA staining was observed in the nuclei of epithelial cells compared with nontransgenic mice. Apigenin administration resulted in marked reduction in the protein expression of PCNA in these mice in a dose-dependent manner. *B,* apoptosis detection by immunofluorescence staining of prostate tissue with M30 CytoDEATH antibody. *C,* plasma apigenin levels detected by HPLC. *a,* histogram from plasma of vehicle-treated control mice. *b,* histogram from plasma of 2 μmol/L apigenin–treated mice. *c,* histogram from plasma of 20 μg/d apigenin–fed mice. *d,* histogram from plasma of 50 μg/d apigenin–fed mice. A dose-dependent increase in apigenin peak was observed. *Arrow,* apigenin peak. *D,* correlation of plasma apigenin levels with proliferation and apoptotic indices. A strong negative association between plasma apigenin levels and proliferation index and a strong positive association between plasma apigenin levels and apoptotic index were observed in the dorsolateral prostates of TRAMP mice.

A number of molecular mechanisms for anticarcinogenic activity of apigenin have been proposed (7–21). However, most of the activities observed with apigenin *in vitro* may not be applicable to the *in vivo* situation due to variability of doses and variations in bioavailability of plant flavonoids. Therefore, it is important to investigate the mechanisms of the inhibitory action of apigenin *in vivo*. The present study is the first report showing that apigenin influences the subcellular distribution of β-catenin by suppressing its nuclear levels and signaling *in vivo* and restoring the E-cadherin-catenin complex in the cytoplasm by up-regulating the levels of E-cadherin. The adhesion protein E-cadherin plays a critical suppressive role in the transition from noninvasive to invasive malignancy in several types of carcinoma including prostate cancer (3–6, 32, 33). A similar increase in E-cadherin protein levels was observed *in vitro* following treatment of DU145 cells with apigenin, and this was accompanied by the retention of β-catenin in the cytoplasm. Suppression of β-catenin signaling with associated increase in E-cadherin expression has been reported and may be responsible for the chemopreventive activities of agents such as vitamin D, green tea polyphenols, indole-3-carbinol, and tangeretin (35–38).

Stimulation of the Wnt pathway results in increased levels of nuclear β-catenin, which binds to members of the Tcf/LEF family and activates several target genes including *c-Myc* and *cyclin D1* (25). Overexpression of c-Myc and cyclin D1 promotes G_1-S transition and cell cycling. Studies have shown that c-Myc–driven murine prostate cancer shares molecular features with human prostate carcinoma (26). Similarly, cyclin D1 has been found to be expressed in ~30% of prostate cancers, and an association between cyclin D1 expression and prostate cancer bone metastasis has been documented (39, 40).

Considering that the nuclear entry of β-catenin as a primary step is required for gene activation by the β-catenin/Tcf/LEF transcriptional complex, we determined the levels of these proteins after apigenin exposure in DU145 cells and in TRAMP mice. Apigenin treatment of DU145 cells decreased the nuclear levels of c-Myc and cyclin D1. Apigenin intake by TRAMP mice suppressed nuclear levels of β-catenin and aberrant β-catenin signaling, as evidenced by decreased protein expression levels of cyclin D1 and nuclear c-Myc. These results show the effectiveness of apigenin in targeting the β-catenin signaling pathway in prostate cancer.

Like most other cancers, prostate carcinogenesis in TRAMP mice involves a multistep progression from precancerous lesions to localized carcinoma followed by metastatic carcinoma. Loss of expression of cell adhesion molecules, especially E-cadherin, is of major significance in the development of metastatic lesions (24). Our results show retained expression of E-cadherin in prostate neoplasms after apigenin administration, an effect that may be responsible for the complete absence of metastases in TRAMP mice. A similar increase in E-cadherin levels has been observed in DU145 cells after apigenin treatment, which might be mediated by an attenuation of its transcriptional repression via the slug/snail zinc finger protein family, posttranscriptional modifications via reduction in protein internalization, and/or decrease in promoter hypermethylation (41, 42). Further research is required to

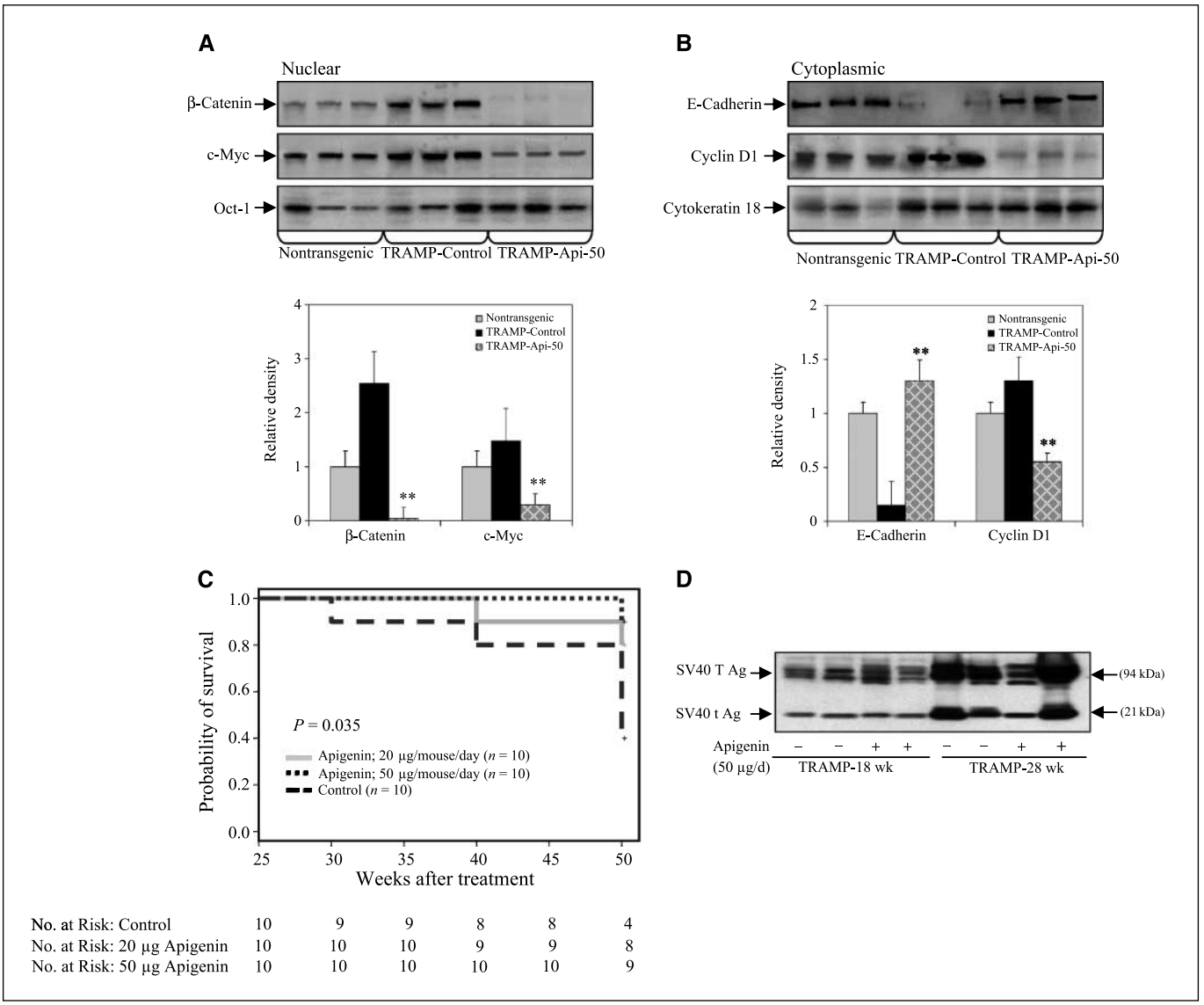

Figure 6. Effect of apigenin intake on β-catenin signaling and E-cadherin protein levels in established prostate tumors, overall survival, and SV40 T/t antigen in TRAMP mice. *A,* protein expression of β-catenin and c-Myc in the nuclear compartment and their corresponding densitometric analyses. *B,* E-cadherin and cyclin D1 in the cytoplasmic compartment and their corresponding densitometric analyses in the dorsolateral prostates of TRAMP mice. A significant retention of E-cadherin protein expression was observed whereas a significant decrease in the levels of cyclin D1, β-catenin, and c-Myc was observed after apigenin administration. Representative data from three mice per group. Equal loading of protein in the lanes was confirmed by stripping the membrane and reprobing it with appropriate housekeeping antibody. **, $P < 0.001$, TRAMP apigenin versus TRAMP control (Kruskal-Wallis test). *Bars,* SE of six mice. *C,* effect of apigenin intake on survival probability in TRAMP. Animals of 12 wk of age received 20 and 50 μg/mouse/d of apigenin for 6 d per week for 50 wk whereas the control group received vehicle only for similar time. Overall survival was measured from the start of apigenin feeding (12 wk of age) to date of death and censored at the date of last follow-up (50 wk) for survivors. *D,* effect of apigenin intake on SV40 T/t antigen. No significant alteration in the T and t Tag oncoprotein was observed in the dorsolateral prostates of TRAMP at 18 and 28 wk after apigenin intake.

determine the mechanisms involved in the increase of E-cadherin levels caused by apigenin.

Several studies have shown that increased nuclear localization of β-catenin and its transcriptional promoting activity induce apoptosis resistance in malignant cells (3–6). In prostate cancer, anomalous signaling through β-catenin has been shown to be associated with the acquisition of an apoptosis-resistant cell phenotype or therapeutic resistance (ref. 43 and references therein). In the present study, we have shown that transcriptional silencing of β-catenin by siRNA results in reduced proliferation and induction of apoptosis in DU145 cells. Similar results were noted *in vivo* in which apigenin intake by TRAMP mice resulted in reduced proliferation and invasiveness and induction of massive apoptosis of premalignant and malignant cells, which correlated with plasma apigenin levels. These results show that the doses of apigenin used in the study are physiologically attainable in suppressing prostate carcinogenesis.

Preneoplastic lesions such as high-grade prostatic intraepithelial neoplasia are frequently observed in asymptomatic men during the fourth and fifth decades of life, and it is believed that such precursors require two to three decades to develop into clinically relevant prostate cancer (44). Additionally, the fact that prostate cancer is typically a disease associated with advanced age suggests that agents that inhibit or delay the onset of clinical malignancy might significantly improve the quality of life in these patients (45). We found that apigenin administration to TRAMP mice significantly delayed the development of prostate cancer as well as delayed the occurrence of death from prostate cancer. These results suggest that regular consumption of plant flavones may prolong life expectancy and improve quality of life in human prostate cancer patients.

There is growing evidence from epidemiologic and case-control studies that higher intake of plant flavonoids reduces the risk of certain chronic diseases including cancer (46, 47). Reports have shown a strong inverse association between flavone intake and breast cancer risk (48). Our studies on the TRAMP mouse prostate cancer model have shown that apigenin, a plant flavone, is capable of suppressing prostate carcinogenesis at physiologically achievable concentrations. Our findings strongly support the development of clinical trials to determine whether apigenin can be useful as a chemopreventive or chemotherapeutic agent in the management of prostate cancer in humans.

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