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Research Article

Identification of a Biphasic Role for Genistein in the Regulation of Prostate Cancer Growth and Metastasis

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Abstract

Considered a chemopreventive agent, the ability of genistein to modulate the progression of existing prostate cancer (CaP) is not clear. We show here that the consumption of genistein (250 mg/kg diet) by 12-week-old transgenic adenocarcinoma mouse prostate (TRAMP-FVB) mice harboring prostatic intraepithelial neoplasia lesions until 20 weeks of age induces an aggressive progression of CaP, as evidenced by a 16% increase in the number of well-differentiated and poorly differentiated prostates, coinciding with a 70% incidence of pelvic lymph node (LN) metastases as opposed to 0% and 10% in 0 and 1,000 mg/kg groups, concomitant with elevated osteopontin (OPN) expression in prostates and LNs. Equivalent nanomolar (500 nmol/L) concentrations of genistein recapitulated these effects in human PC3 CaP cells as evidenced by increased proliferation, invasion, and matrix metalloproteinase-9 (MMP-9) activity (~2-fold), accompanied by an up-regulation of OPN expression and secretion, compared with vehicle-treated cells. A pharmacologic dose (50 μmol/L) decreased proliferation, invasion, and MMP-9 activity (>2.0-fold) concomitant with OPN reduction. Upon OPN knockdown by short hairpin RNA, genistein was no longer effective in up-regulating PC3 cell proliferation, invasion, and MMP-9 activation, which were significantly reduced in the absence of OPN, highlighting the requirement for OPN in mediating the effects of genistein. Proliferation, invasion, and OPN levels were also nonsignificantly induced by genistein in the presence of ICI 182,780 or wortmannin, indicating a dependence on phosphatidylinositol 3-kinase and estrogen signaling. Our results suggest the presence of a biphasic regulation of CaP growth and metastasis by genistein, warranting careful examination of the effects of genistein on hormone-dependent cancers in a chemotherapeutic setting. [Cancer Res 2009;69(8):3695–703]

Introduction

An estimated ~186,320 cases of prostate cancer (CaP) will be diagnosed in the United States in 2008. About 1 man in 6 will be diagnosed with CaP during his lifetime, but only 1 man in 35 will succumb to it, making CaP accountable for ~9% of cancer-related deaths in men (1). Initially treatable when localized, CaP is known to progress to a clinically hormone-refractory cancer and metastatic disease to bone, lungs, and regional lymph nodes (LN) when no effective treatment is available (2).

Observations have suggested that osteopontin (OPN) expression may be significantly altered in cancer and playing a role in bone metastasis (3). OPN expression is increased upon neoplastic transformation (4) and induced by tumor promoters (5) as well as estrogens (6). Specifically to CaP, OPN is elevated in androgen-independent and tumorigenic prostatic cell lines compared with normal prostate epithelial and benign prostatic hyperplasia cells. Increased expression is associated with elevated Gleason scores (7), increased tumor burden (8), and reduced patient survival (9). Serum OPN levels are substantially elevated in the blood of patients with metastatic cancer (10), rendering OPN a useful diagnostic marker in metastasis detection and an attractive potential target for CaP therapy.

Epidemiologic observations linking increased soy consumption and decreased incidence of clinically relevant CaP have generated a lot of attention about the chemopreventive/chemotherapeutic effects of genistein, a major component in soy, on the initiation and progression of CaP (11). We and others have previously shown that dietary genistein (250 and 1,000 mg/kg diet) inhibits the incidence of poorly differentiated cancer when consumed by tumor-free transgenic adenocarcinoma mouse prostate (TRAMP) mice dose dependently (12, 13). More recently, we have examined the effect of consumption of previously established chemopreventive genistein doses on CaP progression in mice with prostatic intraepithelial neoplasia (PIN) and observed a surprising increase in CaP growth at the lower dose used (14). This study represents a closer examination of dietary effects of genistein on TRAMP CaP progression when consumed after tumor initiation. We show that the 250 mg/kg dose, which resulted in nanomolar serum genistein concentrations, induced a 70% incidence of pelvic LN metastases, concomitant with activation of Akt and up-regulation of OPN. *In vitro* studies using PC3 cells recapitulated the induction of proliferation and invasion by nanomolar but not micromolar doses of genistein, concomitant with Akt activation, increased OPN expression, and MMP-9 activation, suggesting a biphasic modulation of proliferation and invasion by genistein in CaP cells. These effects were no longer observed upon knockdown of OPN by short hairpin RNA (shRNA) and treatment of PC3 cells with the estrogen receptor (ER) antagonist, ICI 182,780, or phosphatidylinositol 3-kinase (PI3K) inhibition by wortmannin. This is the first report showing that consumption of genistein, resulting in nanomolar concentrations, can enhance the proliferative and metastatic potential of undiagnosed early-stage CaP and thereby exacerbate it, via an estrogen- and PI3K-dependent mechanism, involving the up-regulation of the metastasis promoter OPN.

Materials and Methods

Animal handling and treatment. TRAMP (The Jackson Laboratory) and FVB mice (Charles River Laboratories) colonies were maintained at

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

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Georgetown University animal facilities in accordance with approved protocol guidelines. Heterozygous male offspring, from male and female TRAMP mated with FVB, were confirmed by genotyping as described (15). Four- or 12-wk-old transgenic males were fed purified AIN-76A pellets (Harlan Teklad) supplemented with 0, 250, and 1,000 mg/kg genistein diet (n = 15/diet group; Sigma) until 20 wk of age. A third group was fed a regular diet and mice were sacrificed at 5, 9, 18, and 24 wk of age (n = 10/age group).

Tissue processing and histopathologic evaluation. Mice were euthanized; blood was collected; and major organs and LNs were dissected out, fixed, and paraffin embedded. Histopathologic evaluation/scoring was done as previously described (12) and was reflective of prostatic lobes minus the anterior prostate.

Serum genistein levels. Serum was extracted and total genistein was unconjugated as previously described (12) and measured by Time-Resolved FluoroImmunoAssay according to the manufacturer's protocol (Labmaster TRF-genistein).

Immunohistochemistry. LN sections were stained with mouse anti-OPN (Santa Cruz Biotechnology) and mouse anti-SV40-Tag (NeoMarkers) overnight at 4°C followed by incubation with biotin-labeled (SV40-Tag; Invitrogen) or Alexa Fluor–tagged antibody (OPN) for 1 h. Slides were incubated in Vectastain avidin-biotin complex peroxidase (Vector Laboratories) for 30 min, developed with 3,3′-diaminobenzidine, and counterstained with hematoxylin or propidium iodide. Slides were photographed using a camera-equipped microscope (Zeiss AxioPlan2 Imaging System).

Cell lines. PC3, MCF-7 (American Type Culture Collection), or $PC3^{(OPN-)}$ cells were maintained at 37°C with 5% CO_2 in phenol red–free IMEM with 2 mmol/L glutamine, penicillin-streptomycin, and 10% fetal bovine serum (FBS) unless otherwise specified. For OPN knockdown stable cell line [$PC3^{(OPN-)}$], four different OPN shRNA and two scrambled shRNA constructs (OriGene Technologies, Inc.) were transfected into retroviral packaging Phoenix Ampho cells (gift from Dr. Dean Rosenthal, Georgetown University Medical Center, Washington, DC) with GeneJammer. Forty-eight hours after transfection, medium was filtered through 0.45 μm and added to PC3 cells with polybrene (5 μg/mL). Forty-eight hours after infection, cells were selected with puromycin, individually cloned, and screened for OPN expression.

Proliferation assay. PC3 or $PC3^{(OPN-)}$ cells, in triplicates, were treated with (a) genistein (0, 500, 1,000, and 50,000 nmol/L) for 72 h or (b) ± genistein (500 nmol/L) ± ICI 182,780 (50 nmol/L) for 72 h or (c) ± genistein (500 nmol/L) ± wortmannin (50 nmol/L) for 72 h and living cells were counted.

Invasion assay. A PC3 suspension (60,000 cells) in serum-free medium treated (a) ± genistein (500, 1,000, and 50,000 nmol/L) or (b) ± genistein (500 nmol/L) ± ICI 182,780 (50 nmol/L) or (c) ± genistein (500 nmol/L) ± wortmannin (50 nmol/L) or (d) $PC3^{(OPN-)}$ ± genistein (500 nmol/L) for 72 h was subjected to the Boyden chamber assay (BD Biosciences) as described (16).

Estrogen response element reporter assay. PC3 or MCF-7 cells, in triplicates, were transfected with ERE-TATA-luciferase (200 ng) and *Renilla* luciferase (20 ng) reporter constructs. Forty-eight hours after transfection, cells were treated with 5 pmol/L estradiol or 500 nmol/L genistein for 5 h. Luciferase activity was measured in cell lysates using the Dual Luciferase Assay kit (Promega) following the manufacturer's protocol and normalized to *Renilla* luciferase activity.

Reverse transcription-PCR. RNA extracted from prostatic tissue or LNs as described (12) was subjected to reverse transcription-PCR (RT-PCR) using the following primers: mouse OPN, 5′-TGGCAGCTCAGAGGAGAAGCTTTA-3′ (forward) and 5′-TCCTGGCTCTCTTTGGAATGCTCA-3′ (reverse); mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GGCCATGTCT-GAGAGATTGGTCTT-3′ (forward) and 5′-AATAGAAAGGCACAGAGCCA-GAGG-3′ (reverse). PCRs were as follows: 1 min at 94°C, 28 cycles of 94°C for 30 s, annealing temperature (58°C) for 30 s, 72°C for 45 s, extension at 72°C for 5 min. Yielded PCR products were 627 and 259 bp, respectively.

Western blot analysis. Protein isolation from prostates (minus anterior prostate lobe) and cell lines was performed as described (12). Membranes were probed with anti-OPN antibody, Akt, and phosphorylated Akt (pAkt; Ser^{473}; Cell Signaling) and reprobed for GAPDH (Abcam) to ensure equal loading.

Human OPN immunoassay. Secreted OPN was measured with Quantikine Human OPN ELISA (R&D Systems, Inc.) according to the manufacturer's protocol. PC3 cells were treated with 0, 500, 1,000, and 50,000 nmol/L of genistein for 72 h. Medium was collected every 24 h, pooled together, and concentrated (1:10) using an Amicon concentrator with a 50-kDa cutoff (Millipore). Samples and standards were run in triplicates.

Zymography. Conditioned media collected and pooled from PC3 cells treated with (a) genistein (0, 500, 1,000, and 50,000 nmol/L), (b) scrambled shRNA or OPN shRNA stable PC3 cells ± genistein (500 nmol/L), and (c) vehicle- and genistein-treated (500 nmol/L) PC3 cells ± ICI 182,780 or ± wortmannin for 72 h were concentrated (~10-fold) and loaded onto SDS-PAGE containing 0.1% gelatin for electrophoresis as described by Desai and colleagues (17).

Statistical analyses. Histologic data were evaluated using χ^2 analysis and Fisher's exact test. Western blots, agarose gels, and gelatin zymography band intensities were quantified with ImageJ software (NIH, Bethesda, MD) and presented as mean ± SE from three independent experiments. One-way ANOVA in Prism 3 (GraphPad Software, Inc.) was used to compare OPN levels across age groups and diet groups *in vivo*, genistein treatments/inhibitors in WB analyses, and ELISA and active MMP-9 across genistein doses, ± inhibitors, and in $PC3^{(OPN-)}$. Cells were counted in triplicates and invaded cells were counted from all filters from three independent experiments, and analysis was carried out between different treatments using one-way ANOVA followed by Dunnett's test with a confidence interval of 95%.

Results

A chemopreventive dose of genistein induces pelvic LN metastasis in TRAMP-FVB mice harboring PIN lesions. We have previously shown that genistein (250 mg/kg diet), when consumed by TRAMP-FVB mice with PIN, from 12 to 20 weeks of age, results in growth stimulation as evidenced by increased prostate weights, compared with control, independently of SV40-Tag modulation while resulting in serum genistein concentrations of 429.732 ± 83.709 nmol/L (Fig. 1*A, top*; Supplementary Fig. S1*A* and *B*; ref. 14). This aggressive phenotype is supported by a 16% increase in prostates with well-differentiated and poorly differentiated cancer in the 250 mg/kg diet group compared with 0 and 1,000 mg/kg groups and a 2-fold reduction in prostate numbers at PIN stage (P < 0.01) with a higher incidence of expression of the neuroendocrine marker synaptophysin (Fig. 1*A, bottom*; data not shown), suggesting an accelerated cancer progression in the 250 mg/kg group. Carcass examination revealed that the 250 mg/kg dose resulted in a 70% incidence of pelvis LN metastasis in TRAMP-FVB mice with poorly differentiated cancer as opposed to 0% (no enlarged LNs) and 10% of TRAMP-FVB mice in the 0 and 1,000 mg/kg diet groups (Fig. 1*B*); representative photographs of three LNs from three TRAMP-FVB mice consuming 250 mg/kg diet are shown alongside three LNs from control TRAMP-FVB mice for comparison (Fig. 1*C*). LN sections from the 250 mg/kg diet group revealed consistent SV40-Tag expression showing that metastasizing prostatic cells underlie LN enlargement (Supplementary Fig. S1*C*).

Dietary genistein differentially regulates OPN expression depending on exposure time in TRAMP-FVB mice. With this work in progress, Mentor-Marcel and colleagues (18) reported OPN reduction as a possible mechanism by which genistein reduces CaP metastasis and increases TRAMP survival. To determine its possible involvement in genistein-induced metastasis, we examined OPN expression in CaP progression of TRAMP-FVB mice on a regular diet at 5, 9, 18, and 24 weeks of age. OPN levels were barely

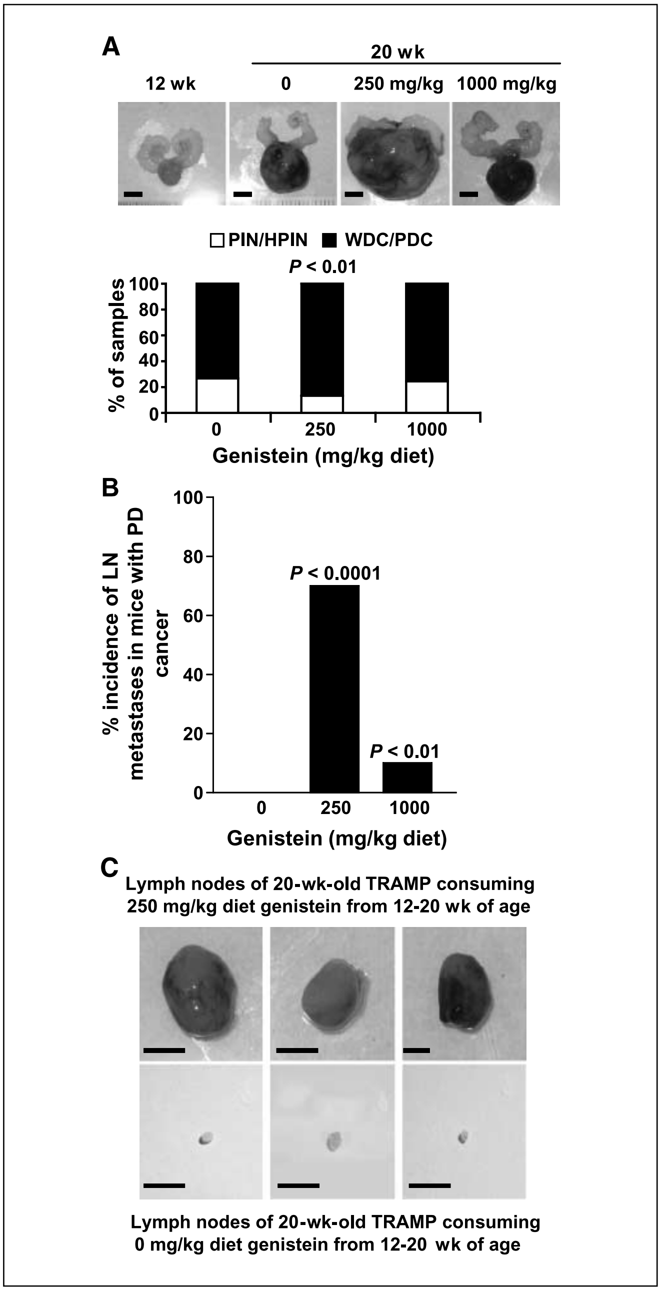

Figure 1. Genistein induces the metastasis of CaP cells to pelvic LNs. *A,* photographs (*top*) and histology (*bottom*) of prostates from TRAMP-FVB mice (n = 15/group) fed a diet containing 0, 250, and 1,000 mg/kg genistein AIN-76A from 12 to 20 wk of age, with a photograph representing beginning of treatment (12 wk). *HPIN,* high PIN; *WDC,* well-differentiated cancer; *PDC,* poorly differentiated cancer. *B,* pelvic LN incidence in TRAMP-FVB mice consuming 0, 250, and 1,000 mg/kg genistein diet from 12 to 20 wk of age, with a poorly differentiated cancer histopathology. *C,* photographs of pelvic LNs removed from 20-wk-old TRAMP-FVB mice fed control (*bottom*) and 250 mg/kg genistein diet (*top*) from 12 wk of age. *Scale bar,* 1 cm.

detectable at 5 weeks at mRNA and protein levels. By 9 weeks, levels increased by 2-fold and continued to increase up to 10-fold by 24 weeks (Fig. 2*A*). We then examined OPN levels in the prostates of TRAMP-FVB mice consuming genistein at 4 or 12 weeks of age. Mice consuming 250 and 1,000 mg/kg diets from 4 to 20 weeks of age (prevention regimen) displayed a dose-dependent reduction in OPN mRNA and protein expression (∼2-fold in the 1,000 mg/kg group; Fig. 2*B*), corroborating above-mentioned study (18). Alternatively, prostates of mice consuming 250 and 1,000 mg/kg from 12 to 20 weeks of age (intervention regimen) displayed an increase (∼2.5-fold) in OPN (mRNA and protein levels) in the 250 mg/kg group, whereas in 1,000 mg/kg group, OPN levels were not significantly altered, statistically, with only a single mouse showing elevated OPN, comparable with 250 mg/kg group levels and coinciding with LN metastasis (Fig. 2*B*; data not shown). LNs derived from TRAMP-FVB mice consuming 250 mg/kg genistein from 12 to 20 weeks of age expressed OPN (Fig. 2*C*), as opposed to lymphatic vessels from control TRAMP-FVB mice that did not (Supplementary Fig. S2).

Genistein biphasically regulates PC3 cell proliferation and invasion *in vitro*. To delineate whether the genistein-induced metastasis in TRAMP-FVB is an inherent characteristic of the model or a consistent biphasic effect of genistein at low versus high concentrations, human PC3 cells were treated with various concentrations of genistein [0, 500, and 1,000 nmol/L representing physiologically achievable concentrations *in vivo* (12) and 50,000 nmol/L (pharmacologic dose)] for 72 hours and counted. We observed a biphasic effect of genistein on PC3 proliferation; 500 to 1,000 nmol/L induced a significant 1.5-fold increase in cell number, as opposed to >3.0-fold decrease with 50,000 nmol/L genistein, compared with vehicle-treated cells ($P < 0.01$; Fig. 3*A*).

Similarly treated cells were subjected to the Boyden chamber with Matrigel assay with 10% FBS medium as a chemoattractant for 24 hours to assess invasion. A 2.0-fold increase in invaded cell number was observed with 500 nmol/L genistein compared with vehicle ($P < 0.01$), whereas 50,000 nmol/L genistein reduced invasion by 2.0-fold ($P < 0.05$; Fig. 3*B*). The 1,000 nmol/L dose failed to significantly increase invasion (Fig. 3*B*). Representative photographs of filters with toluidine blue–stained invading cells are shown (Fig. 3*B, bottom*). Zymography analysis of conditioned medium from these samples revealed that genistein (500 and 1,000 nmol/L) induced a ∼2.0-fold increase in active MMP-9 compared with vehicle, whereas 50,000 nmol/L decreased MMP-9 activity >2-fold ($P < 0.01$; Fig. 3*C*).

Physiologically achievable doses of genistein increase OPN levels that are necessary for up-regulation of proliferation and invasion of PC3 cells. To examine whether PC3 cells recapitulate the effects of genistein on OPN, expression and secreted levels of OPN in PC3 cells treated with 0, 500, 1,000, and 50,000 nmol/L of genistein for 72 hours were examined. Genistein (500 nmol/L) resulted in a 2.0-fold increase in OPN protein expression (Fig. 4*A*) as well as secretion as detected by ELISA (Supplementary Fig. S3). To determine the requirement for OPN in the genistein-induced increase in proliferation and invasion, scrambled and OPN shRNA stable cell lines were established via retroviral infection. The OPN shRNA stable cell line had undetectable OPN levels, which were not altered by genistein (Fig. 4*A, bottom*; Supplementary Fig. S4).

An equal number of OPN shRNA–expressing and scrambled shRNA–expressing cells were seeded and treated ± genistein (500 nmol/L) for 72 hours, living cell number was recorded, and 60,000 cells/cell type or treatment were transferred to the Boyden chamber with Matrigel and allowed to invade for 24 hours. Genistein treatment (500 nmol/L) resulted in a 1.5-fold increase in the number of scrambled shRNA–expressing cells (Fig. 4*B*), recapitulating the effect of genistein on paternal PC3 cells. Basal proliferation of OPN shRNA–expressing cells was reduced compared with scrambled shRNA cells by ∼35%, and genistein induced a 1.16-fold induction of proliferation compared with vehicle

(Fig. 4*B*), suggesting that OPN induction by genistein contributes to the proliferation increase. Knockdown of OPN also resulted in a 75% reduction of invasion compared with scrambled shRNA cells, and genistein (500 nmol/L) resulted in a ∼1.4-fold increase in invasion compared with vehicle-treated OPN shRNA cells, which is significantly lower than the 2-fold increase observed in the genistein-treated scrambled shRNA cells (Fig. 4*C*). OPN knockdown also resulted in a 70% reduction in MMP-9 activity ($P < 0.001$), which was no longer inducible by genistein (500 nmol/L; Fig. 4*D*). These results suggest that OPN not only contributes to basal PC3 invasion but that its induction is one of the mechanisms mediating the genistein-induced invasion.

Induction of OPN, proliferation, and invasion by genistein in PC3 cells are estrogen and PI3K dependent. OPN expression and activity are regulated by estrogen and PI3K signaling (19). The ability of genistein to modulate these pathways was examined. PC3 cells were transfected with ERE-TATA-luciferase and treated ± 500 nmol/L genistein. Genistein induced >1.6-fold increase in luciferase activity compared with vehicle-treated transfected cells (Fig. 5*A*). Induction was abolished by addition of ICI 182,780, an

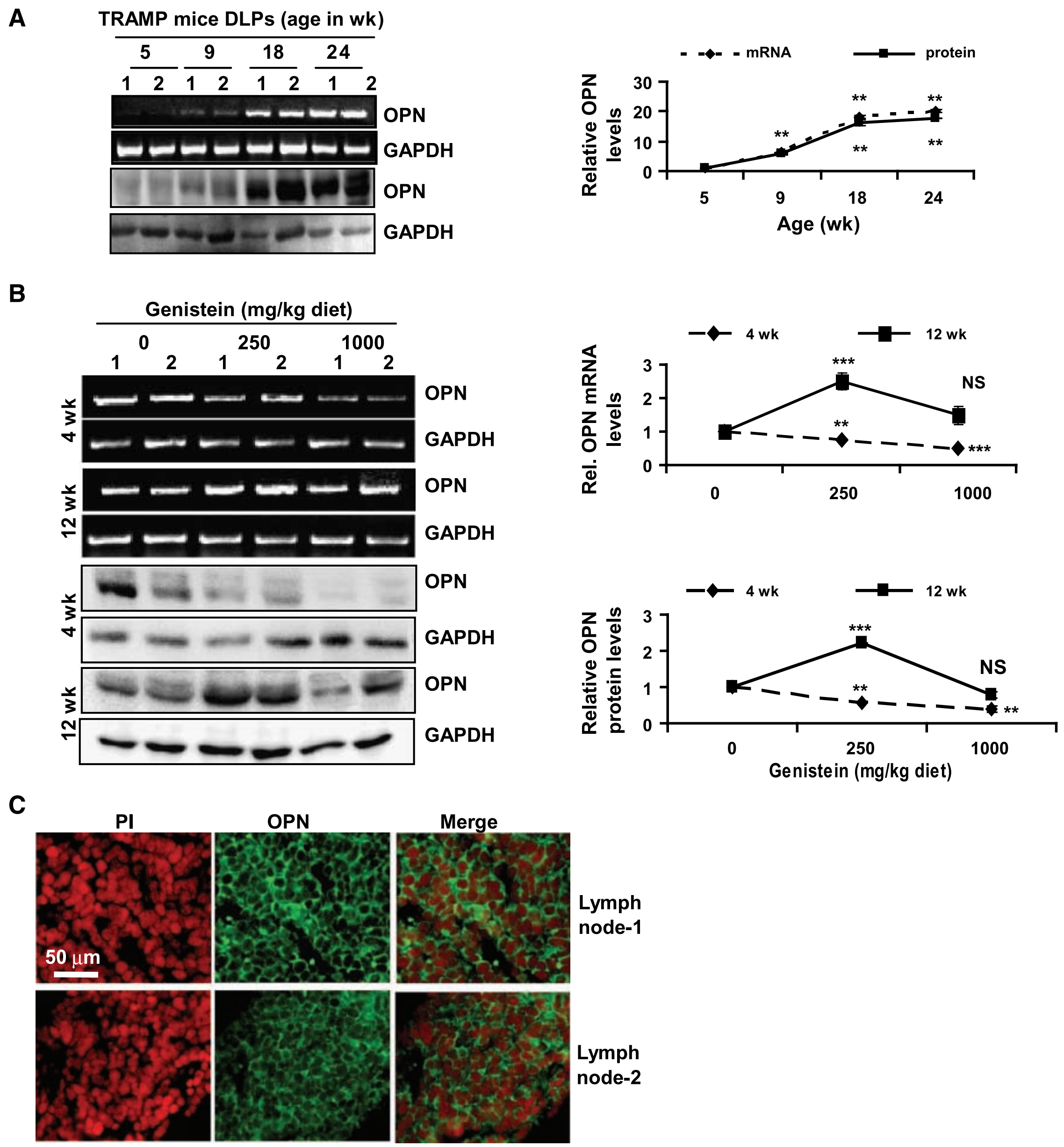

Figure 2. Age-dependent expression of OPN and its modulation by genistein (prevention versus intervention regimens). *A,* RT-PCR and Western blot analysis of OPN levels in two representative samples/age group (*1* and *2*) of TRAMP-FVB mice on a regular diet. *B,* RT-PCR (*top*) and Western blot analysis (*bottom*) showing OPN expression in prostates of TRAMP-FVB mice consuming dietary genistein from 4 or 12 wk until 20 wk of age. Quantifications from three independent experiments are shown in respective right panels. NS, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *C,* OPN expression in LN (*1* and *2*) sections derived from TRAMP-FVB mice fed genistein (250 mg/kg) from 12 to 20 wk of age by immunofluorescence with Alexa Fluor–tagged antibodies with propidium iodide counterstaining.

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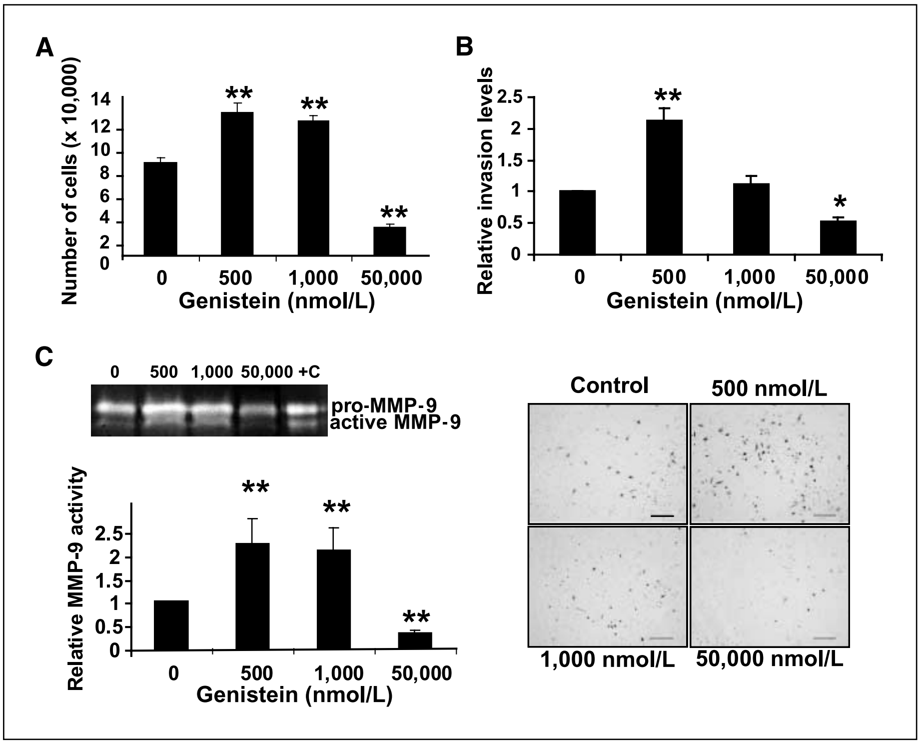

Figure 3. Low doses of genistein increase the proliferation and invasion of PC3 cells. *A,* quantification of genistein-treated PC3 cells for 72 h. Viable cells (as assessed by trypan blue) were counted and plotted as mean cell number ± SE based on three independent experiments. **, $P < 0.01$, compared with control. *B,* quantification of invaded genistein-treated PC3 cells for 72 h. Forty thousand cells were placed in invasion chambers with 10% FBS as chemoattractant for 24 h. All invaded cells stained with toluidine blue, from all filters, were viewed and counted at a ×2.5 magnification. Graphs are representative of three independent experiments, with values normalized to vehicle-treated cells and represented as mean fold ± SE. *, $P < 0.05$; **, $P < 0.01$. *Bottom,* representative filters. *Scale bar,* 200 μm. *C,* zymogram of pro-MMP-9 and active MMP-9 levels in conditioned medium from genistein-treated PC3 cells for 72 h. +*C,* MMP-9 standards (positive control). Quantification of active MMP-9 levels in above-mentioned samples from three independent experiments (*bottom*), presented as mean activity ± SE. **, $P < 0.01$, compared with vehicle-treated cells.

ER antagonist. Due to low induction levels of ERE-TATA-luciferase by genistein, we examined its modulation by estradiol in PC3 cells. Estradiol (5 pmol/L) induced a 1.5-fold increase in luciferase activity relative to control in PC3 cells (Supplementary Fig. S5*A*) compared with >8-fold induction in the estrogen-responsive breast cancer cell line MCF-7 (Supplementary Fig. S5*B*), suggesting that genistein exerts estrogenic activity in PC3 cells similar to estradiol. Genistein (500 nmol/L) induced a significant 2.0-fold increase in pAkt (S473) as opposed to a 2.0-fold reduction by 50,000 nmol/L (Fig. 5*B, top left*), with no effect on total Akt. Interestingly, prostatic lysates from TRAMP-FVB mice consuming genistein-supplemented diets also showed a biphasic pAkt regulation with a significant 2-fold up-regulation in the 250 mg/kg diet group and a slight nonsignificant decrease in the 1,000 mg/kg diet compared with control (Fig. 5*B, top right*). Inhibition of estrogen and PI3K signaling *in vitro* (Fig. 5*A*; Supplementary Fig. S6) abolished OPN induction by genistein in the presence of ICI 182,780 and wortmannin, respectively (Fig. 5*C, top* and *middle*). Although PC3 proliferation was not significantly affected by ICI 182,780 alone, the increase in proliferation by genistein (500 nmol/L) was abolished in its presence, showing that estrogen signaling is necessary for the proliferation increase by genistein (Fig. 5*D, top*). The significant ability of genistein to increase PC3 invasion was also mitigated in the presence of ICI 182,780 and was decreased from 2.1- to 1.18-fold (Fig. 5*D, top*). About PI3K signaling, a significant 2.0-fold reduction in PC3 cell number was observed in the presence of wortmannin alone (Fig. 5*D, top*). Moreover, wortmannin eliminated the 1.5-fold induction in cell number by genistein, suggesting that PI3K and/or Akt activation is needed for both basal and genistein-induced increase in PC3 proliferation. Wortmannin alone induced a 2.0-fold decrease in invasion compared with vehicle (Fig. 5*D, top*). Addition of genistein (500 nmol/L) in the presence of wortmannin not only failed to increase invasion but further reduced invading cell numbers, suggesting that PI3K activity is necessary for the genistein-induced invasion increase but also that low genistein doses might inhibit cell invasion in the absence of PI3K activity. Zymography revealed that genistein (500 nmol/L) failed to induce MMP-9 activity in the presence of ICI 182,780 or wortmannin in PC3 cells (Fig. 5*D, bottom*), suggesting MMP-9 as a possible mediator of the estrogen- and PI3K-dependent effects of genistein on PC3 cells.

Discussion

In this study, we showed that consumption of low genistein doses (250 mg/kg diet) accelerates CaP progression in TRAMP-FVB mice when consumed after PIN initiation. This phenotype was characterized by Akt activation, OPN up-regulation, and occurrence of pelvic LN metastases. Furthermore, the previously established chemopreventive dose (1,000 mg/kg diet; ref. 12) lost its efficacy when consumed at 12 weeks of age. To delineate the mechanisms underlying these observations, *in vitro* studies using PC3 cells treated with physiologic versus pharmacologic genistein doses recapitulated the proliferation and invasion increases observed *in vivo*. These increases were dependent on OPN up-regulation and required active estrogen and PI3K signaling, involving MMP-9 activation. To the best of our knowledge, this is the first report documenting an increase in metastasis by genistein in the TRAMP-FVB model while pinpointing an estrogen- and PI3K-dependent induction of OPN as a necessary mechanism.

Considered potentially chemopreventive, increased usage of soy products prompted the examination of effects of physiologically achievable concentrations of genistein on breast and uterine cancer cell lines and animal models (20). At low doses (<10 μmol/L), genistein stimulates the growth of estrogen-sensitive cell lines (21–23) while decreasing proliferation at higher doses (>10–20 μmol/L; refs. 22, 23). This suggests that genistein exerts a biphasic effect on growth and proliferation of cancer cells,

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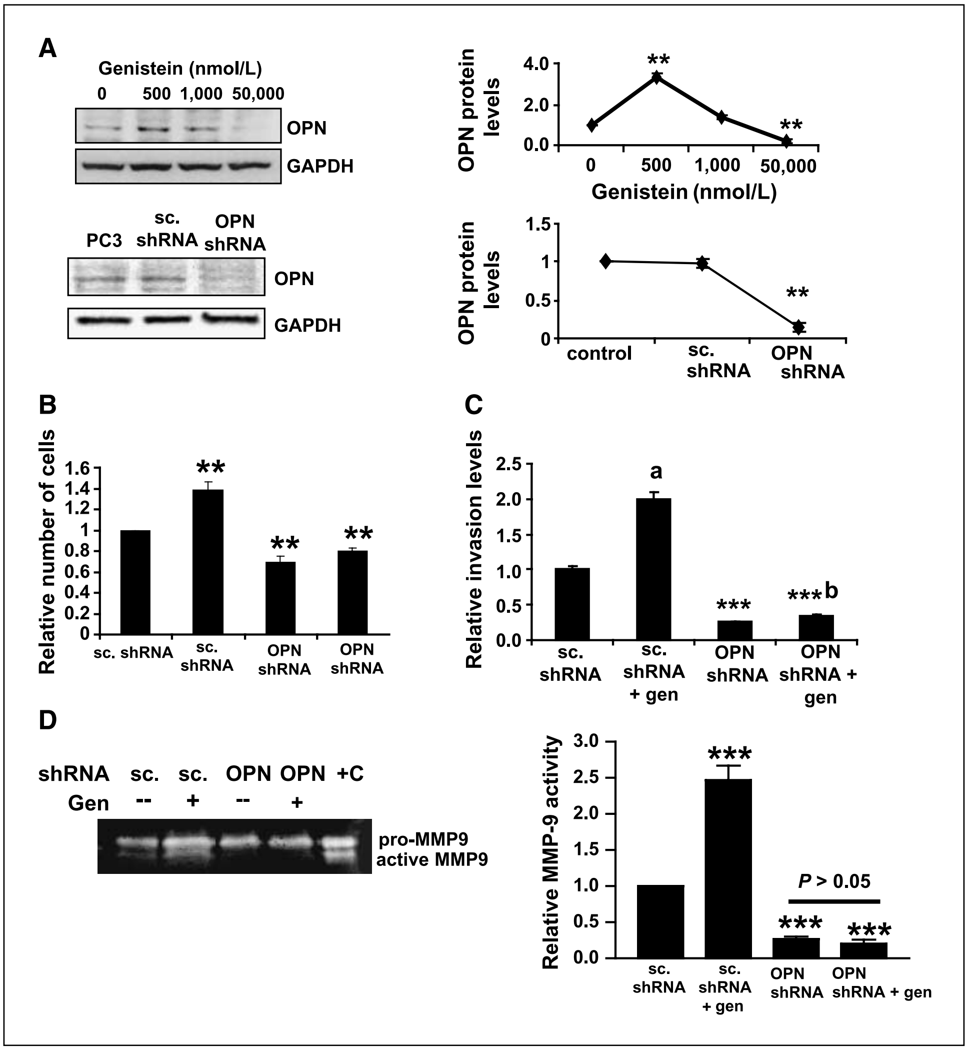

Figure 4. Induction of OPN by genistein is necessary for the increase in proliferation and invasion of PC3 cells. *A,* Western blot of OPN levels in genistein-treated PC3 cells for 72 h (*top*) and in PC3, scrambled shRNA, and OPN shRNA stably transfected PC3 cells (*bottom*) with respective quantification (*right*). *B,* quantification of cell count from three independent experiments of scrambled plasmid and OPN shRNA stably transfected PC3 cells treated ± genistein (500 nmol/L) for 72 h in triplicates. Values were normalized to vehicle-treated scrambled shRNA PC3 cells. *Columns,* mean fold; *bars,* SE. **, $P < 0.01$. *C,* quantification of invaded scrambled shRNA and OPN shRNA stably transfected PC3 cells treated ± genistein (500 nmol/L) for 72 h. After treatment, 60,000 cells were placed in Boyden chambers with Matrigel, with 10% FBS as chemoattractant for 24 h, and counted as earlier. Values were normalized to scramble-transfected, vehicle-treated PC3 cells and represented as mean fold ± SE from three independent experiments. [a], significantly different from scramble-transfected, vehicle-treated PC3 cells with $P < 0.01$; [b], $P < 0.05$, compared with vehicle-treated OPN shRNA cells. *D,* zymogram of pro-MMP-9 and active MMP-9 levels in conditioned medium from scrambled and OPN shRNA stably transfected PC3 cells treated ± 500 nmol/L genistein for 72 h. *+C,* MMP-9 standards (positive control) with quantification of active MMP-9 levels in above-mentioned samples from three independent experiments, presented as mean activity ± SE. ***, $P < 0.001$, compared with vehicle-treated cells (*right*).

similarly to what we have observed in prostate tumors of TRAMP-FVB mice and human PC3 cells, with low nanomolar concentrations inducing growth (14) and metastasis of prostate tumor cells when treatment started after PIN *in vivo* as well as proliferation and invasion *in vitro*, and a higher dose (50,000 nmol/L) reducing proliferation and invasion.

Increased tumor growth, metastasis, and OPN up-regulation were not seen when genistein is consumed (same dose) by 4-week-old, tumor-free TRAMP-FVB mice or 12-week-old nontransgenic C57BL/6×FVB mice (12),[1] suggesting a dependence on exposure time or tumor presence and is not an inherent issue in the model used.

OPN expression concomitant with pelvic LN metastases in TRAMP-FVB mice and proliferation/invasion induction in PC3 cells by low genistein doses is significant in the context of CaP metastasis. CaP cells preferentially metastasize to bone, a process facilitated by OPN in various ways (24, 25). Here, we present data with reference to LN metastasis as opposed to bone because the former occurs more frequently in the TRAMP model with 100% incidence in mice over 28 weeks as opposed to 25% incidence of bone metastases in 32-week-old TRAMP mice (26, 27), and LN metastases were the most striking observation upon mice dissection. Interestingly, OPN expression has been correlated with LN metastases in a variety of cancers (28–31). However, the possibility that bone metastasis occurs on OPN induction by genistein in other models of CaP or humans cannot be eliminated.

We have observed OPN increases as early as 9 weeks of age, coinciding with PIN initiation and a highly proliferative stage (12) in TRAMP-FVB mice, suggesting a role in proliferation. In fact, OPN induction promoted tumor growth, and its knockdown reduced Ras-transformed 3T3 cell growth in soft agar and animal implants (32). This is also confirmed by the decreased proliferation of $PC3^{(OPN-)}$ cells. Importantly, PC3 proliferation and invasion were no longer enhanced by genistein (500 nmol/L) to the same extent in $PC3^{(OPN-)}$ cells, indicating that OPN and/or its downstream effectors mediate at least the stimulatory part of the proposed biphasic genistein effect.

MMP-9 activity was also increased by low genistein doses (500 and 1,000 nmol/L). Interestingly, elevated OPN expression correlates with MMP-9 levels (33) and MMP-9 mediates OPN-induced cell migration and chemoinvasion in B16F10 cells (34). In PC3 cells, OPN overexpression increases MMP-9 activity (17), presenting strong evidence that MMP-9 is a downstream effector of OPN in

[1] Unpublished data.

invasion. Our results agree with these reports in that PC3$^{(OPN-)}$ cells exhibit reduced MMP-9 activity and inhibition of genistein-induced OPN induction by ICI 182,780 or wortmannin abrogated MMP-9 activation by genistein, suggesting that genistein (500 nmol/L) may stimulate invasion via up-regulation of OPN and subsequent activation of MMP-9.

The mechanism underlying OPN induction by genistein is unknown. However, OPN increase was paralleled by an increase in Akt phosphorylation *in vivo* and *in vitro* and was consistently abrogated *in vitro* by wortmannin or ICI 182,780. OPN is a transcriptional target of the ER-related receptor α (35) and is induced by estradiol *in vivo* (36). Therefore, OPN induction in prostates of TRAMP-FVB mice consuming 250 mg/kg genistein might be of estrogenic nature. OPN is also under the control of the PI3K pathway and is induced by PTEN deletion in colon cancer, whereas Ras-induced expression of OPN is PI3K dependent (37), which agrees with our wortmannin studies.

Estrogen sensitivity of cell lines/models used seems to determine the growth and metastasis-promoting effects of genistein. Studies reporting proliferation increase by genistein in estrogen-responsive cell lines have failed to observe similar effects in ER-negative cells (38). Consumption of 250 mg/kg diet before orthotopic implantation of PC3-M, a metastatic subline of PC3 lacking ERs (39), decreased lung metastases (40), whereas LN weights increased in

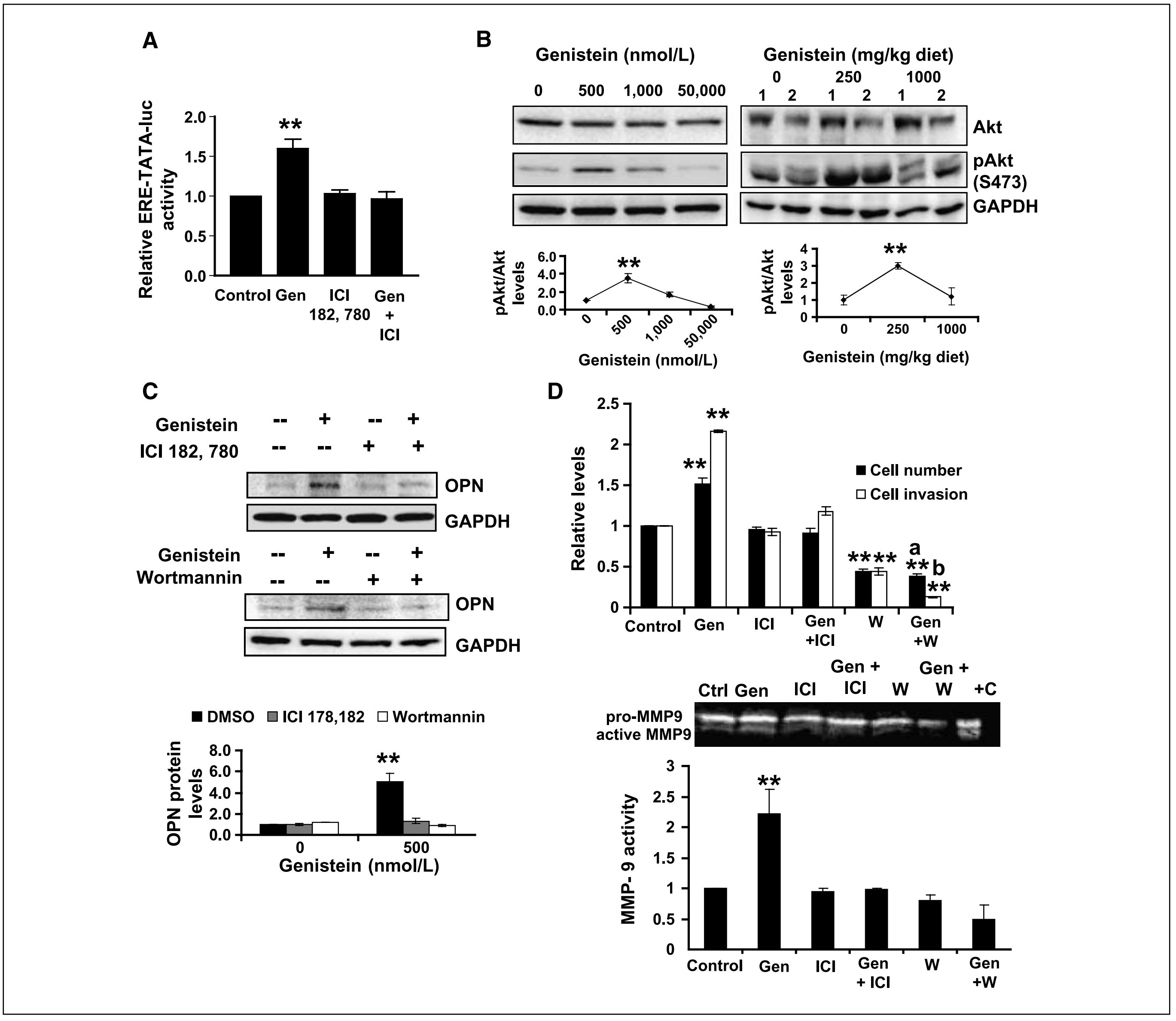

Figure 5. Induction of OPN by genistein is dependent on estrogen and PI3K signaling. *A,* 48 h after transfection with ERE-TATA-luciferase plasmid, PC3 cells were treated with 500 nmol/L genistein ± 50 nmol/L ICI 182,780 for 5 h and assayed using the Dual Luciferase Assay kit, with values normalized to *Renilla* and to values obtained in vehicle-treated transfected cells. Results are representative of three independent experiments. **, $P < 0.01$. *B,* Western blot of Akt and pAkt (S473) in genistein-treated PC3 cells for 72 h (*left*) and two prostate samples of TRAMP-FVB mice consuming genistein from 12 to 20 wk of age (*right*) with quantification from all samples below. **, $P < 0.01$, compared with control. *C,* Western blot of OPN levels in PC3 cells treated ± genistein (500 nmol/L) for 72 h ± 50 nmol/L ICI 182,780 (*top*) or ± 50 nmol/L wortmannin (*middle*) with quantification from three independent experiments (*bottom*). Immunoblots were reprobed with GAPDH to ensure equal loading. *D,* cell numbers and invasion levels of PC3 cells treated ± genistein (500 nmol/L) for 72 h ± 50 nmol/L ICI 182,780 or 50 nmol/L wortmannin. **, $P < 0.01$, compared with untreated PC3 cells; [a], nonsignificant compared with own control; [b], $P < 0.01$, compared with own control. Representative zymogram and quantification (*bottom*) of active MMP-9 levels in medium from above-mentioned treatments from three independent experiments. **, $P < 0.01$.

genistein-treated mice harboring PC3 xenografts (41). Our study agrees with these observations in that TRAMP-FVB prostates[1] and PC3 cells express ERα and ERβ (39). The need for estrogen signaling was also highlighted by the administration of ICI 182,780, which reduced the induction of proliferation and invasion by genistein. However, the importance of estrogen signaling on the *in vivo* effects of genistein remains to be determined.

We and others have pinpointed Akt inhibition by genistein as one mechanism by which genistein exerts its chemopreventive actions (12, 42). Recently, an increase in Akt phosphorylation by 10 μmol/L genistein was reported in porcine aortic endothelial cells *in vivo* (43). Although not made in a tumor cell setting, this observation agrees with our findings about Akt activation by genistein *in vitro* and *in vivo*.

We have also observed that PI3K inhibition by wortmannin abrogated the proliferation and invasion increase by genistein, suggesting that genistein acts in a PI3K-dependent manner in PC3 cells. Furthermore, the inhibitory effects on invasion by nanomolar doses of genistein in the absence of PI3K activity suggest that there might be a balance between inhibitory and activating effects of genistein, with the balance shifted toward inhibition upon PI3K inactivation. Experiments are under way to determine whether PI3K/Akt and estrogen signaling activation by genistein are independent events or an interaction between both pathways.

The inhibition of metastasis by genistein in a chemopreventive setting has been reported extensively (44). However, genistein increased the size of LN metastases but not tumor size when administered to PC3/nude mouse xenograft model (41), postulating that LN metastasis increase is due to the antiangiogenic effects of genistein and subsequent hypoxia. However, in our study, genistein resulted in increased tumor size and metastasis, suggesting a direct effect on tumor cell proliferation when administered to TRAMP-FVB mice with PIN lesions. Furthermore, proliferation and invasion were potentiated in the same cells used for the xenografts *in vitro*, eliminating the hypoxia theory in our model at least.

Recent findings showed that poorly differentiated carcinomas in the TRAMP-FVB strain are derived from neuroendocrine cells (45). In this study, 250 mg/kg diet genistein increased the number of synaptophysin-expressing poorly differentiated carcinomas, which was also expressed in pelvic LNs (data not shown). One hypothesis is that a low genistein environment (provided by 250 mg/kg diet) targets the synaptophysin-expressing neuroendocrine population in the prostate, resulting in the up-regulation of OPN, the positive selection of this population (considered highly proliferative and a candidate for the transit-amplifying population in the prostate; ref. 46), and emergence of a more aggressive phenotype in this group. More experiments characterizing the neuroendocrine population in our model, its possible differences at 4 and 12 weeks of age, and its ER and Akt status would prove/disprove this hypothesis and further highlight the potential detrimental effects of low genistein doses.

In this work, we have shown that timing of genistein exposure as well as the dose used had a major effect on CaP outcome and progression in TRAMP-FVB mice. This highlights the importance of examining the effects of physiologically achievable levels of genistein and its deleterious effects on undiagnosed CaP.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Identification of a Biphasic Role for Genistein in the Regulation of Prostate Cancer Growth and Metastasis

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