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.

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

Materials and Methods

Animal handling and treatment. TRAMP (The Jackson Laboratory) and FVB mice (Charles River Laboratories) colonies were maintained at...
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 Tekdak) 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, 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 genitin levels.** Serum was extracted and total genitin was unconjugated as previously described (12) and measured by Time-Resolved FluorolImmuNoAssay according to the manufacturer's protocol (Lambmaster TRF-genitin).

**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 Tag; Invitrogen) or Alexa Fluor–tagged antibody (OPN) for 1 h. Slides were photographed using a camera-equipped microscope (Zeiss AxiosPlan2 Imaging System).

**Cell lines.** PC3, MCF-7 (American Type Culture Collection), or PC3(OPN−/−) cells were maintained at 37°C with 5% CO2 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 Amphi 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) genitin (0, 500, 1,000, and 50,000 nmol/L) for 72 h or (b) ± genitin (500 nmol/L) ± ICI 182,780 (50 nmol/L) for 72 h or (c) ± genitin (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 with (a) genitin (500, 1,000, and 50,000 nmol/L) or (b) ± genitin (500 nmol/L) ± ICI 182,780 (50 nmol/L) or (c) ± genitin (500 nmol/L) ± wortmannin (50 nmol/L) or (d) PC3(OPN−/−) ± genitin (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 pmol/L genitin 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′-TGGCAGCTCAGAGGAAGTCTTTA-3′ (forward) and 5′-TCTCGGCTCTCTTGGAGATCTCA-3′ (reverse); mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GGCCATGGCTCTGAGAGATTGTCC-3′ (forward) and 5′-AATAGAAAGCGACAGGACGAGG-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 prostate (minus anterior prostate lobe) and cell lines was performed as described (12). Membranes were probed with anti-OPN antibody, Akt, and phosphorylated Akt (pAkt; Ser473; 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 genitin 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) genitin (0, 500, 1,000, and 50,000 nmol/L), (b) scrambled shRNA or OPN shRNA stable PC3 cells ± genitin (500 nmol/L), and (c) vehicle- and genitin-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 χ² 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, genetin treatments/inhibitors in WB analyses, and ELISA and active MMP-9 across genitin 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 genitin induces pelvic LN metastasis in TRAMP-FVB mice harboring PIN lesions. We have previously shown that genitin (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 genitin concentrations of 429.73±83.709 nmol/mL (Fig. 1A, top; Supplementary Fig. S1A 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. 1A, 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 pelvic 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. 1B); 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. 1C). LN sections from the 250 mg/kg diet group revealed consistent SV40-Tag expression showing that metastasizing prostatic cells underlie LN enlargement (Supplementary Fig. S1C).

**Dietary genitin 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 genitin reduces CaP metastasis and increases TRAMP survival. To determine its possible involvement in genitin-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
of 250 mg/kg genistein diet (Fig. 2B), 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. 2B; data not shown). LNs derived from TRAMP-FVB mice consuming 250 mg/kg genistein from 12 to 20 weeks of age expressed OPN (Fig. 2C), 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. 3A).

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. 3B). The 1,000 nmol/L dose failed to significantly increase invasion (Fig. 3B). Representative photographs of filters with toluidine blue–stained invading cells are shown (Fig. 3B, 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. 3C).

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. 4A) 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. 4A, 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. 4B), recapitulating the effect of genistein on parental 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

![Figure 1. Genistein induces the metastasis of CaP cells to pelvic LNs.](image-url)

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 250 mg/kg diet genistein from 12-20 wk of age. Mice consuming 250 and 1,000 mg/kg diets from 12 weeks of age. Mice consuming 250 and 1,000 mg/kg diets from 12 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. 2B), 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. 2B; data not shown). LNs derived from TRAMP-FVB mice consuming 250 mg/kg genistein from 12 to 20 weeks of age expressed OPN (Fig. 2C), as opposed to lymphatic vessels from control TRAMP-FVB mice that did not (Supplementary Fig. S2).

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(Fig. 4B), 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. 4C). 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. 4D). 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 with 500 nmol/L genistein. Genistein induced >1.6-fold increase in luciferase activity compared with vehicle-treated transfected cells (Fig. 5A). Induction was abolished by addition of ICI 182,780, an

![Figure 2](image-url). 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.
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. S5A) compared with a 8-fold induction in the estrogen-responsive breast cancer cell line MCF-7 (Supplementary Fig. S5B), 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 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 x 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), represented as mean activity ± SE. **, P < 0.01, compared with vehicle-treated cells.

**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 x 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), represented as mean activity ± SE. **, P < 0.01, compared with vehicle-treated 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 effects of genistein on PC3 cells.
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/C2FVB mice (12), 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 mouse 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/C0) cells. Importantly, PC3 proliferation and invasion were no longer enhanced by genistein (500 nmol/L) to the same extent in PC3(OPN/C0) 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...
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](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-08-2958)

**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 transected cells. Results are representative of three independent experiments. **,** \( P < 0.01 \), compared with control. **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). Immunblots were reprobed with GAPDH to ensure equal loading. **,** \( P < 0.01 \), 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 xenographs (41). Our study agrees with these observations in that TRAMP-FVB prostates 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 vitro 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 increase induced 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/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 estrogen 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.

References

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