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Genistein Induces Phenotypic Reversion of Endoglin Deficiency in Human Prostate Cancer Cells

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ABSTRACT

Genistein has been shown to inhibit human prostate cancer (PCa) cell motility. Endoglin has been identified as an important suppressor of PCa cell motility, and its expression is lost during PCa progression. It is therefore important to determine whether endoglin loss affects genistein's efficacy and, if so, by what mechanism. In the current study, genistein was shown to induce reversion of endoglin-deficient cells to a low motility, endoglin-replete phenotype. Because endoglin suppresses PCa cell motility in an activin-like kinase receptor-2 (ALK2)- and Smad1-dependent manner, we sought to determine whether genistein was activating the ALK2-Smad1 pathway. Although treatment with genistein or overexpression of Smad1 or ALK2 all increased Smad1-responsive promoter activity and decreased cell motility, genistein's efficacy was abrogated by either Smad1 or ALK2 knockdown. Furthermore, transfection of cells with a kinase dead mutant of ALK2 abrogated genistein's efficacy. Together, these findings demonstrate that genistein therapeutically induces reversion to a low-motility phenotype in aggressive endoglin-deficient PCa cells. It does so by activating ALK2-Smad1 endoglin-associated signaling. These findings support the notion that individuals with low endoglin-expressing PCa will benefit from genistein treatment.

Prostate cancer (PCa) is a leading cause of cancer-associated death in the United States and worldwide (Jemal et al., 2006). Death from PCa is almost invariably caused by the development of metastatic disease (Carroll et al., 2001). Cancer metastasis, including PCa, follows a multistep pathway appropriately named the "metastatic cascade" (Woodhouse et al., 1997). The ultimate development of metastasis requires that cells successfully transition through initial steps in the cascade. Inhibition of initial steps precludes the ultimate development of metastasis. As such, the metastatic cascade and, in particular, early steps in the metastatic cascade represents a rational pathway to target therapeutically. Increased cell invasion (i.e., increased cell motility) is an early step in the metastatic cascade and is thus a rational therapeutic target.

We have demonstrated previously that genistein (4′,5,7-trihydroxyflavone) inhibits PCa cell invasion (Huang et al., 2005; Xu et al., 2006). Genistein is a constituent of soy, and epidemiological studies have associated dietary consumption of genistein with a reduced risk of death from PCa (Severson et al., 1989). Genistein has undergone phase I testing in humans (Takimoto et al., 2003) and has been well tolerated. Phase II efficacy studies are underway.

Dysregulated cell motility is a basic characteristic of cancer, including PCa, and is seen during PCa progression. Molecular changes that relate to the regulation of cell motility underlie this abnormal cellular phenotype. To be effective, anticancer therapeutics must retain efficacy in the face of molecular aberrations associated with cancer progression. Alternatively, their use must be tailored to specific molecular profiles. In either situation, optimal therapeutic implementation requires an understanding of the relationship between therapeutic intervention and the underlying molecular profile.

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ABBREVIATIONS: PCa, prostate cancer; ALK, activin-like kinase receptor; β-gal, β-galactosidase; ENG, endoglin; HA, hemaglutinin; KD, kinase dead; PC3, parental prostate cancer cell line; PC3-M, metastatic prostate cancer cell line; siRNA, small interfering RNA; siNeg, small interfering RNA negative control; TGFβ, transforming growth factor β; RI, type I transforming growth factor β superfamily receptor; qRT/PCR, quantitative reverse transcription/polymerase chain reaction; VC, empty vector; siENG, small interfering RNA-targeting endoglin; FACS, fluorescence-activated cell sorting; BRE2-Luc, BRE2-Luciferase; Sd1, Smad1; siSd1, small interfering RNA-targeting Smad1; siA2, small interfering RNA-targeting ALK2; WT, wild type; MAP, mitogen-activated protein.

A series of prior studies by us have identified endoglin as a key regulator of PCa cell motility and have shown that its expression is lost during PCa progression (Jovanovic et al., 2001; Liu et al., 2002). Specifically, altered endoglin expression was uniquely identified by gene array technology during changes in human prostate cell motility (Jovanovic et al., 2001). Endoglin expression was then found to be lost during PCa cell progression, and this was shown to increase cell invasion (Liu et al., 2002). Endoglin is a 180-kDa homodimeric type I transmembrane auxiliary receptor in the TGFβ superfamily (Gougos and Letarte, 1990).

A consideration of a series of studies by us and others supports the notion that genistein may exert effects on the endoglin signaling pathway. Both endoglin and genistein act to suppress PCa cell invasion. Furthermore, we have demonstrated recently that endoglin suppresses PCa cell motility by activating Smad1 in an ALK2-dependent manner (Craft et al., 2007). Smads act as transcription factors after they are activated by a type I TGFβ superfamily receptor (RI) (Shi and Massague, 2003). ALK2 is an RI subtype. In that same study, we also showed that endoglin-mediated activation of Smad1 and endoglin-mediated suppression of PCa cell motility did not require exogenous TGFβ ligand. Yu et al. (2005) have reported that genistein can induce Smad activation in colon cancer cells, again in the absence of exogenous TGFβ. Finally, anecdotal reports suggest that genistein may have therapeutic efficacy in people with hereditary hemorrhagic telangiectasia type 1 (Korzenik et al., 1998). Endoglin is expressed at high levels in blood vessel endothelial cells. In hereditary hemorrhagic telangiectasia type 1, mutations in endoglin lead to aberrant A-V malformations, uncontrollable bleeding, and early death (Fernández-L et al., 2006). Taken together, these considerations support the hypothesis that genistein has the potential to therapeutically compensate for endoglin deficiency and that effects on endoglin-associated signaling pathways are likely.

The current study was undertaken to determine whether genistein retained its anti-invasion efficacy in human PCa in the face of endoglin loss and to determine whether there was any mechanistic overlap between the endoglin pathway and genistein. Here we demonstrate for the first time that genistein can cooperate with endoglin-associated signaling molecules ALK2 and Smad1 to inhibit cell invasion in endoglin-deficient PCa cells.

Materials and Methods

Materials. Genistein (4′,5,7-trihydroxyflavone) (Sigma Chemical Co., St. Louis, MO), was prepared and stored as described previously (Liu et al., 2000). Unless otherwise stated, genistein was used at a final concentration of 50 μM for 24 h in serum-free media, and control cells were treated with dimethyl sulfoxide. Antibodies were as follows: anti-endoglin-phycoerythrin (R&D Systems, Minneapolis, MN), anti-endoglin (clone 35; BD Biosciences, San Jose, CA), anti-Smad1 (Upstate Biotechnology, Lake Placid, NY), anti-glyceraldehyde-3-phosphate dehydrogenase (clone, CSA-335E; Stressgen, Victoria, AB, Canada), and anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-mouse and anti-rabbit IgG-horseradish peroxidase were from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Vectors included β-galactosidase (pCMV-β-gal; Stratagene, La Jolla, CA), BRE2-Luciferase pGL3 (described and provided by Peter ten Dijke, Netherlands Cancer Institute; Monteiro et al., 2004), Smad1 pCDNA3.1 (described and provided by Mark de Caestecker, Vanderbilt-Ingram Cancer Center; de Caestecker et al., 1997), endoglin-long isoform pcDNA3 (Liu et al., 2002), and HA-ALK2 pCMV5 (provided by Andreas Lux, University of Applied Sciences Mannheim, and described

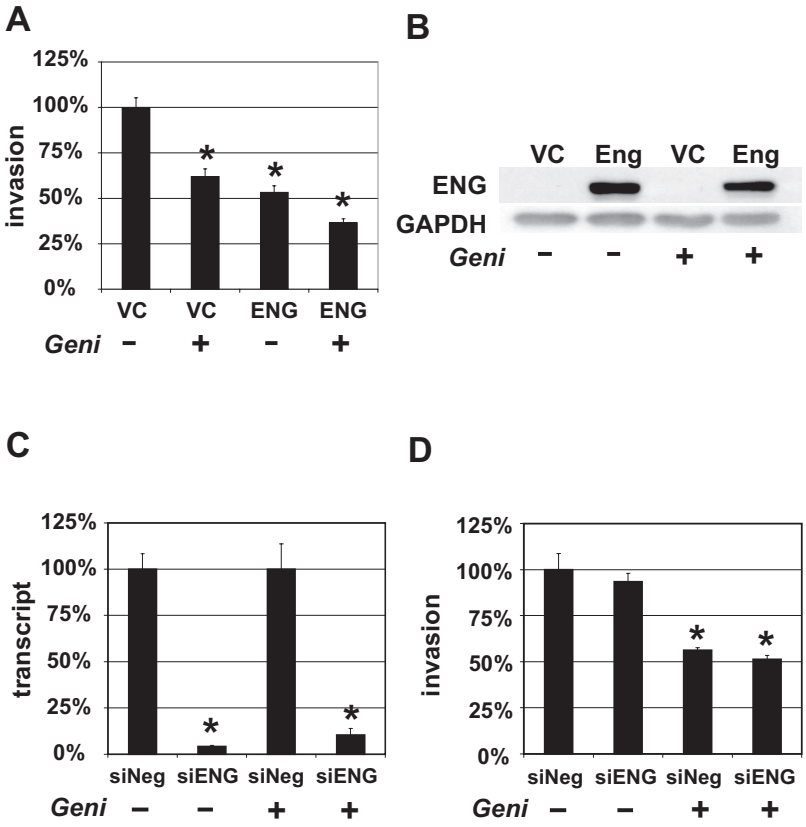

Fig. 1. Genistein-mediated decreases in cell invasion are not affected by endoglin expression. PC3-M cells were transfected with ENG, VC, siENG, or siNeg and then treated with genistein (or not), as indicated. A, endoglin and genistein both inhibit cell invasion. The invasion of ENG and VC cells was measured and expressed as a percentage compared with untreated VC cells. Data for all invasion assays are the mean ± S.E.M. (n = 4) of a single experiment, with similar results seen in a separate experiment performed at a separate time (also n = 4). B, the expression of endoglin protein in equal amounts of protein lysate from transfected cells was confirmed by Western blot. C, siENG knocks down endoglin. The expression of endoglin was measured by qRT/PCR in RNA isolated 24 h after cells were transfected with siENG or siNeg. Values are the percentage of endoglin transcript compared with siNeg control cells. Values represent the mean ± S.D. of a single experiment (n = 2) with similar results seen in a separate experiment (also n = 2). D, genistein-mediated decreases in cell invasion do not require endoglin. The invasion of siENG and siNeg cells was measured and expressed as a percentage compared with untreated siNeg cells. Cell invasion, Western blot, and qRT/PCR assays were all performed as described under *Materials and Methods*. *, values that differ from control as defined by a two-sided t test p value of $\leq$ 0.05; controls are VC/genistein− (A) and siNeg/genistein− (C and D).

by Jeff Wrana; Attisano et al., 1993). Kinase-dead (K233R) ALK2 pCMV5 (kd-ALK2) was engineered as described previously (Wieser et al., 1995). Constructs were confirmed by sequencing.

Cell Culture and Transfection. The origin, culture conditions, and phenotypic characterization of metastatic PC3 and PC3-M cells, early-stage (i.e., localized) PCa 1532CPTX and 1542CPTX cells, and transformed normal prostate 1532NPTX and 1542NPTX epithelial cells, all of human origin, have been described previously (Liu et al., 2001). Cell viability, as determined by trypan blue exclusion, was monitored under all experimental conditions. It was not adversely altered in any of the experimental conditions, compared with control. Transient transfection of plasmids was performed with Mirus LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's instructions. Transfection of Smad1, ALK2, endoglin, and negative control SMARTpool siRNA used DharmaFECT (all from Dharmacon, Lafayette, CO) and was performed 5 h after plasmid transfection according to the manufacturer's instructions.

Cell Invasion Assays. Cell invasion assays were performed as described previously (Huang et al., 2005; Craft et al., 2007). Cells were cotransfected with β-gal and expression vector. Cells invaded through a gelatin-coated Nuclepore Track-Etch Membrane with 8-μm pores (Whatman, Clifton, NJ) toward serum-free NIH-3T3-conditioned medium. The cell invasion time ranged from 18 to 24 h. It was adjusted for each cell type such that for $\geq$200 cells counted, 5 to 10% of cells were invading. Furthermore, similar results upon repeat were required. Transfected cells were visualized with a β-gal staining kit (Stratagene), and the percentage of invaded-transfected cells was counted.

Flow Cytometry. Flow cytometric analysis was performed as described previously (Craft et al., 2007). Cell surface endoglin was detected using an anti-endoglin-phycoerythrin-conjugated IgG (R&D Systems) according to the manufacturer's instructions. Median fluorescent intensity was determined on a Beckman Coulter (Fullerton, CA) Epics-XL-MCL flow cytometry machine.

Western Blot. Western blotting of equal amounts of resultant protein was performed as described previously (Craft et al., 2007).

Smad1 Promoter Luciferase Reporter Assays. Cells were cotransfected with BRE2-Luciferase (BRE2-Luc) and β-gal, and luciferase and β-gal activity were measured as described previously (Hayes et al., 2003; Craft et al., 2007) using Luciferase and β-Galactosidase Assay Systems (Promega, San Luis Obispo, CA) according to the manufacturer's instructions. Luciferase activity was then normalized to total protein and to β-gal.

Quantitative Reverse Transcription/Polymerase Chain Reaction. RNA isolation and real time quantitative reverse transcription/polymerase chain reaction (qRT/PCR) were performed as described previously (Ding et al., 2006). Reactions were run in duplicate on a single Applied Biosystems 7500 Real Time PCR workstation using a TaqMan universal PCR kit and validated gene-specific exon spanning primers and probe sets (all from Applied Biosystems, Foster City, CA). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase.

Results

Genistein Induced a Low-Motility Phenotype in Endoglin-Deficient Cells. Human PC3-M PCa cells express low levels of endoglin (Liu et al., 2002). PC3-M cells were transfected with endoglin (ENG) or empty vector (VC) treated with genistein or not, and cell invasion was measured, Fig. 1, A and B. Genistein significantly (two-sided t test, p value $\leq$ 0.05) decreased the invasion of VC cells to nearly 50% of that of untreated VC cells, and endoglin had similar effects. There was no significant difference between untreated ENG cells and genistein-treated VC cells. However, genistein further decreased the invasion of ENG cells by 40% relative to untreated ENG cells.

Because PC3-M cells express endoglin, albeit at low levels, we used siRNA technology to further suppress endoglin and to further evaluate genistein's efficacy (Fig. 1, C and D). PC3-M cells were treated with siRNA-targeting endoglin (siENG) or with nontargeting siRNA (siNeg) for control. Endogenous endoglin was effectively and specifically knocked down by siENG (Fig. 1C). However, siENG had no significant effect on PC3-M cell invasion (Fig. 1D), thus providing another measure of the low levels of endoglin in those cells. It is noteworthy that genistein was equally effective in siENG and

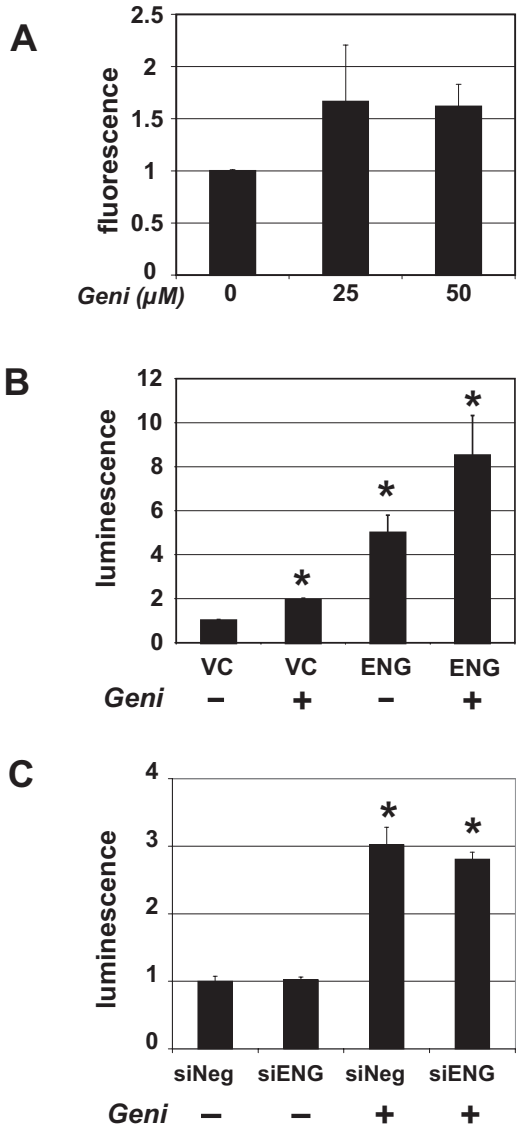

Fig. 2. Genistein activates Smad1. A, genistein has little effect on endoglin expression. PC3-M cells were treated with the indicated concentrations of genistein for 24 h, and cell surface endoglin expression was measured by FACS. The resultant level of endoglin expression, normalized to untreated cells, was then determined. Values are the mean $\pm$ S.D. fluorescent intensity of two separate experiments performed at different times (n = 2). B and C, genistein increases Smad1 transcriptional activity. Cells were cotransfected with BRE2-Luc and β-gal along with ENG, VC, siENG, or siNeg and treated with genistein (or not), as indicated. Normalized luciferase activity was then expressed as the mean $\pm$ S.E.M. (n = 6 for B, n = 2 for C), performed at separate times relative to that observed with untreated VC (B) or untreated siNeg (C). FACS and reporter assays were all performed as described under *Materials and Methods*. *, values that differ from control by a p value of $\leq$ 0.05; controls are genistein 0 μM (A), VC/genistein$-$ (B), and siNeg/genistein$-$ (C).

siNeg cells and decreased invasion by approximately 50%. Together, these findings demonstrate that both genistein and endoglin exert similar anti-invasion efficacy on low-endoglin-expressing cells. Furthermore, they demonstrate that endoglin is not necessary for genistein efficacy. Finally, they suggest that endoglin and genistein have additive effects.

Genistein Increased Smad1 Promoter Activity. The above findings raised the possibility that genistein could be activating the endoglin pathway. Others have reported that genistein could increase endoglin expression in human PCa cells (Rokhlin and Cohen, 1995). We investigated this possibility by treating PC3-M cells with 0, 25, or 50 μM genistein for 24 h and measuring endoglin expression by FACS. Endoglin is a cell surface protein, and we have used FACS previously to measure cell surface endoglin expression (Craft et al., 2007). As can be seen in Fig. 2A, genistein did increase endoglin expression by approximately 1.5-fold; however, this was not statistically significant. Given the small magnitude of the increase and the lack of statistical significance, this increase was believed not to be responsible for genistein's effects. This notion is supported by other findings. First, genistein retained efficacy in the face of endoglin knockdown. Second, although endoglin expression did not increase with increases in genistein concentration, we have shown previously enhanced anti-invasion efficacy by genistein across this concentration range (Huang et al., 2005). Given these considerations, we sought to determine whether genistein could be affecting proteins other than endoglin in the endoglin signaling pathway.

Because endoglin has been shown to suppress PCa cell invasion by activating Smad1, we hypothesized that genistein was activating Smad1 (Craft et al., 2007). Smad1 is a transcription factor whose activation by cell surface TGFβ superfamily receptors can be detected by use of the Smad1-responsive promoter BRE2-Luc construct (Monteiro et al., 2004). We have shown recently that measurement of Smad1-responsive promoter activity provides a more accurate measure of Smad1 activation in human prostate cells than does measurement of Smad1 phosphorylation status (Craft et al., 2007). Human prostate cells contain high levels of acid phosphatase that serves to disrupt the accurate measurement of protein phosphorylation status (Hayes et al., 2003). To evaluate whether genistein was activating Smad1, cells were first transfected with BRE2-Luc, β-gal (for normalization), and either ENG or VC. Cells were then treated with genistein (or not), and luciferase activity was measured (Fig. 2B). Both genistein and endoglin significantly increased BRE2 promoter activity. In ENG cells, genistein further increased BRE2 activity compared with untreated ENG

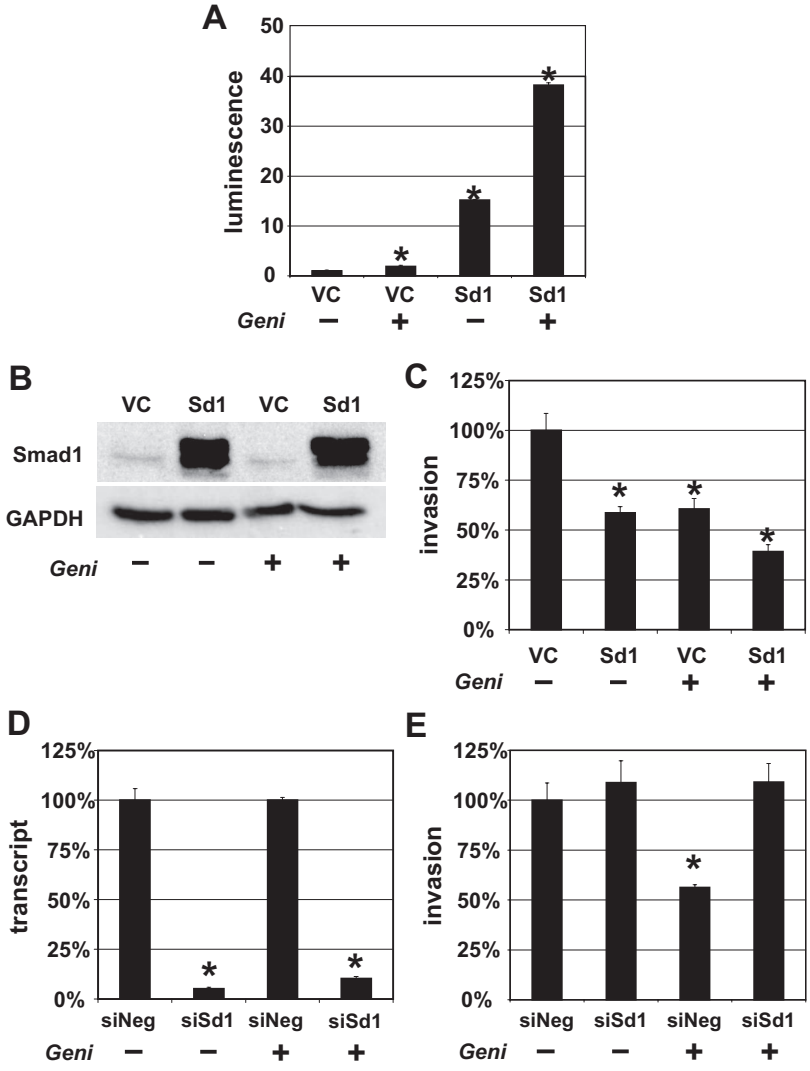

Fig. 3. Genistein cooperates with Smad1 to inhibit PCa cell invasion. A and B, genistein and Smad1 have additive effects on Smad1-responsive promoter activity. PC3-M cells were transfected with VC or Sd1, BRE2-Luc, and β-gal treated with genistein (or not), and luciferase activity was measured (A) as described in Fig. 2. Normalized luciferase activity was then expressed as the mean ± S.D. relative to that observed with untreated VC cells. Smad1 protein expression in equal amounts of total protein was evaluated by Western blot, as described in Fig. 1 (B). C, genistein and Smad1 have additive anti-invasion effects. PC3-M cells were transfected with Smad1 or VC and treated with genistein (or not), and the percentage of invasion relative to untreated VC cells was determined as in Fig. 1. Data are the mean ± S.E.M. (n = 4) of a single experiment, with similar results seen in a separate experiment (also n = 4). D and E, Smad1 is necessary for genistein's action. PC3-M cells were transfected with siSd1 or with siNeg and treated with genistein (or not). The resultant effects on Smad1 transcript levels, as measured by qRT/PCR (D), and on cell invasion (E) were then measured. Values are from a single experiment, run in replicates of n = 2 for promoter and qRT/PCR assays and n = 4 for invasion assays. For all assays, separate experiments run at separate times (with identical replicates) gave similar results. *, values that differ from control by a p value of ≤0.05; controls are VC/genistein− (A and C) and siNeg/genistein− (D and E).

cells. These findings demonstrate that genistein activates Smad1 and that its effects in this regard seem additive with that of endoglin.

To determine whether genistein-mediated activation of Smad1 was dependent on endoglin, cells were transfected with siENG or siNeg, treated with genistein or not, and BRE2 promoter activity was measured (Fig. 2C). Knockdown of endoglin had no effect on BRE2 promoter activity. It is noteworthy that genistein's ability to increase BRE2 promoter activity was not altered by endoglin knockdown.

Genistein Cooperated with Smad1 to Inhibit PCa Cell Invasion. Because genistein increases Smad1 activation, additional studies were performed that focused on Smad1. First, cells were transfected with either Smad1 (Sd1) or VC and then treated with genistein or not, and BRE2 promoter activity was measured (Fig. 3, A and B). In VC cells, genistein significantly increased BRE2 promoter activity 2-fold. Compared with VC cells, BRE2 promoter activity in Sd1 cells increased by 15-fold. It is noteworthy that for Sd1 cells, genistein increased BRE2 promoter activity 2.5-fold compared with untreated Sd1 cells.

Taking a similar approach, we went on to evaluate the functional relevance of these findings by measuring the effect on cell invasion (Fig. 3C). In VC cells, genistein significantly decreased cell invasion to 60%. Compared with untreated VC cells, the invasion of untreated Sd1 cells was significantly decreased to 58%. Consistent with BRE2 findings, genistein retained anti-invasion efficacy in Sd1 cells. Specifically, the invasion of genistein-treated Sd1 cells was significantly decreased to 39% of that of untreated Sd1 cells.

The above studies suggested that genistein and Smad1 have additive effects. This is consistent with our endoglin findings and with the fact that endoglin's effects are mediated through Smad1. Investigations were next conducted to evaluate whether genistein's effects were dependent on Smad1. Cells were therefore transfected with siRNA-targeting Smad1 (siSd1) or siNeg. After confirming siSd1 efficacy and specificity (Fig. 3D), effects on invasion were evaluated (Fig. 3E). Compared with siNeg, siSd1 had little effect on invasion. It is noteworthy that although genistein significantly decreased the invasion of siNeg cells, knockdown of Smad1 completely abrogated genistein's anti-invasion effects. These findings demonstrate that Smad1 is necessary for genistein-mediated inhibition of cell invasion.

ALK2 Was Necessary for Genistein-Mediated Inhibition of Cell Invasion. Type I (RI) TGFβ superfamily receptors have kinase domains that function as activators of Smad proteins (Shi and Massague, 2003). We demonstrated previously that ALK2, an RI receptor, cooperates with endoglin to inhibit cell motility and to promote Smad1 transcriptional

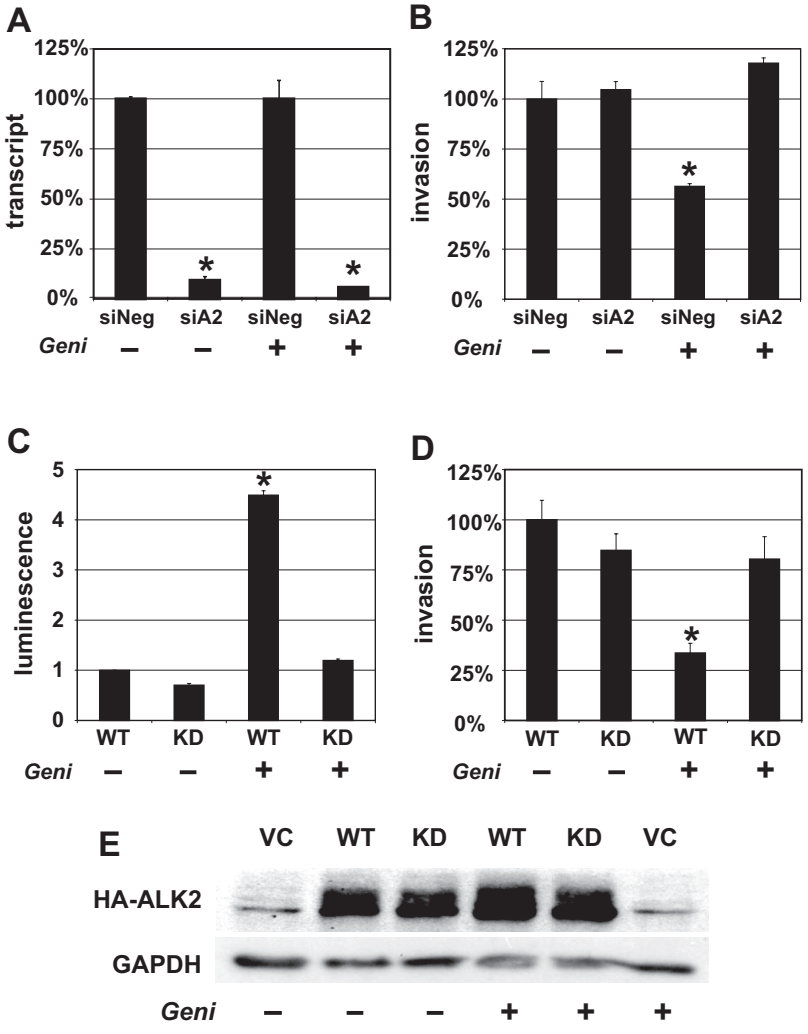

Fig. 4. ALK2 is necessary for genistein-mediated inhibition of cell invasion. PC3-M cells were transfected with siA2, siNeg, WT-ALK2, or KD and treated with genistein (or not), as indicated. Resultant effects on ALK2 expression by qRT/PCR (A), on cell invasion (B and D), Smad1 activation (C), and on protein expression by probing for HA-tagged ALK2 by Western blot (E) are shown. All values are from a single experiment, run in replicates of $n = 2$ for promoter and qRT/PCR assays and $n = 4$ for invasion assays. For all assays, separate experiments run at separate times (with identical replicates) gave similar results. $*$, values that differ from control by a p value of ≤ 0.05; controls are siNeg/genistein$-$ (A and B) and WT-ALK2/genistein$-$ (C and D).

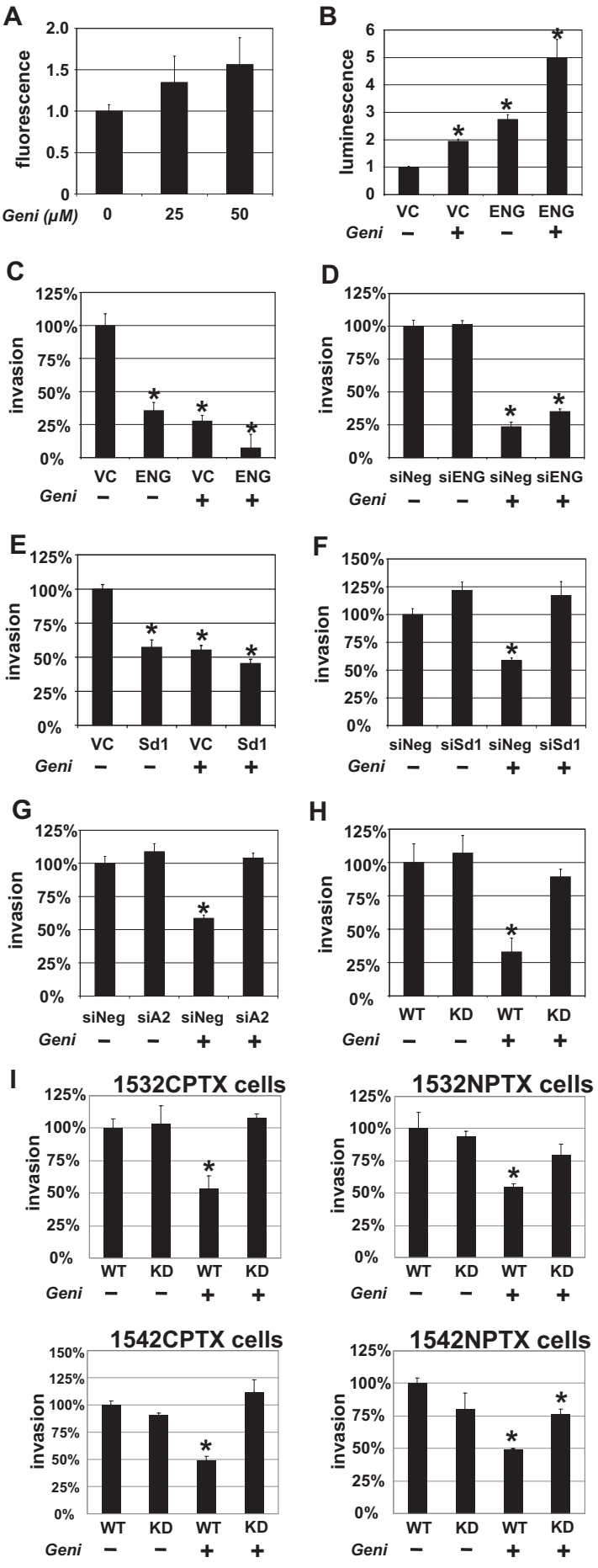

Fig. 5. Effects in other human prostate cells. A, genistein treatment has a modest effect on endoglin protein cell surface expression. As described in Fig. 2, nontransfected PC3 cells were treated with the indicated concentrations of genistein, and surface expression of endoglin was measured by FACS analysis. B to H, PC3 cells were then transfected with the indicated expression construct or siRNA and treated with genistein (or not), and effects on BRE2 promoter activity (B) and cell invasion (C–H) were measured as in Fig. 4. I, ALK2 is necessary for genistein-mediated inhibition of invasion in early-stage human prostate cell lines. 1532CPTX, 1532NPTX, 1542CPTX, and 1542NPTX were transfected with either WT-ALK2 or with KD-ALK2 and treated with genistein (or not), and effects on cell invasion were measured. All values are from a single experiment run in replicates of $n = 2$ for FACS assay, $n = 6$ for promoter assay (three experiments), and $n = 4$ for invasion assays. For all assays, separate experiments run at separate times (with identical replicates) gave similar results. $*$, values that differ from control by a p value of ≤ 0.05; controls are genistein 0 μM (A), VC/genistein$-$ (B, C, and E); siNeg/genistein$-$ (D, F, and G), and WT-ALK2/genistein$-$ (H and I).

activity (Craft et al., 2007). As the studies above demonstrated that genistein's effects are additive with that of Smad1, but require Smad1, we evaluated the role ALK2 in modulating genistein-mediated inhibition of invasion. The efficacy and specificity of siRNA-targeting ALK2 (siA2) was first confirmed (Fig. 4A). Next, cells were transfected with siA2 or siNeg and treated with genistein or not, and cell invasion was measured (Fig. 4B). Cell invasion was not affected by siA2 compared with siNeg. However, siA2 abrogated genistein's anti-invasion activity.

Because the above findings implicated ALK2 in mediating genistein function, additional studies were performed. Human prostate cells contain high levels of acid phosphatase, complicating the accurate measurement of protein phosphorylation. Experience has taught us that measurement of in vivo function represents the optimal approach (Hayes et al., 2003). Cells were therefore transfected with either wild-type (WT) or K233R kinase dead (KD) ALK2 (Fig. 4, C–E). The K233R mutation results in an nonphosphorylated and kinase inactive receptor (Wieser et al., 1995). As can be seen in Fig. 4C, there was a small decrease in BRE2 promoter activation in KD cells compared with WT cells. With genistein treatment, BRE2 promoter activity increased significantly by 4.5-fold, compared with untreated WT cells. It is noteworthy that KD-ALK2 completely abrogated genistein-mediated increases. When cell invasion was evaluated (Fig. 4D), KD-ALK2 had no significant effect compared with WT-ALK2. It is noteworthy that KD-ALK2 completely abrogated genistein's anti-invasive activity. These studies demonstrate that ALK2 is necessary for genistein activity.

Effects in Other Prostate Cells. To ensure that the above findings were not limited to a single cell line, a series of additional investigations was performed. Initial studies used PC3 cells. PC3 cells are the parental line for PC3-M cells, also express low levels endoglin, and also represent an aggressive metastatic phenotype (Liu et al., 2002). First, PC3 cells were treated with 0, 25, or 50 μM genistein for 24 h, and the level of cell surface endoglin was measured by FACS (Fig. 5A). With PC3 cells, there was a dose-dependent increase in endoglin expression, but again, it was not significant. Cells were then transfected with ENG or VC and treated with genistein or not, and effects on BRE2 activation (Fig. 5B) and cell invasion (Fig. 5C) were measured. In both assays, genistein's effects closely approximated those of endoglin. Furthermore, in both assays, genistein displayed additional activity in the face of endoglin expression. Next, cells were transfected with siENG or siNeg and treated with genistein or not, and invasion was measured (Fig. 5D). Although siENG did not decrease invasion, genistein significantly decreased invasion to a similar degree in both siENG and siNeg cells. Likewise, genistein's anti-invasion activity was maintained in siENG cells, confirming that genistein can exert anti-invasion efficacy similar to endoglin in low-endoglin-expressing cells. These studies also demonstrate that genistein induces a low-motility phenotype in endoglin-deficient PC3 cells.

Studies next evaluated Smad1. As can be seen in Fig. 5E, cell invasion was decreased to a similar extent in genistein-treated and in Sd1 cells. However, for Sd1 cells, genistein's additional effects were only modest. That is, the invasion of genistein-treated Sd1 cells was only 20% lower than that of untreated Sd1 cells. Knockdown of Smad1 by siSd1 abrogated genistein's anti-invasion effect (Fig. 5F). For ALK2, knockdown by siA2 (Fig. 5G) or transfection with KD (Fig. 5H) both abrogated genistein's anti-invasion effect. In total, findings in PC3 cells corroborate those found in PC3-M cells.

The final series of studies demonstrates that ALK2 is also necessary for genistein's effect in early-stage human prostate cells. These studies used 1532CPTX, 1532NPTX, 1542CPTX, and 1542NPTX cells, which are endoglin-replete (Liu et al., 2002). Cells were transfected with either WT-ALK2 or with KD-ALK2 and treated with genistein or not, and the resultant effects on cell invasion were measured. As can be seen in Fig. 5I, genistein decreased invasion by ~50% in WT-ALK2 cells in all cell lines evaluated. It is noteworthy that in all cell lines evaluated, KD-ALK2 abrogated genistein's effect compared with KD-ALK2 cells not treated with genistein.

Discussion

We demonstrate for the first time that treatment with genistein can compensate for endoglin deficiency. This was demonstrated by showing that genistein causes low-endoglin-expressing PCa cells to revert to a low-motility, endoglin-replete phenotype. This has important implications for the therapeutic use of genistein in humans. This is because endoglin expression seems to be lost relatively early during the transition to a metastatic phenotype (Liu et al., 2002), and genistein is being used relatively early in the clinical course of PCa as a chemopreventative agent (Takimoto et al., 2003). These findings suggest that it may be possible to therapeutically compensate for molecular aberrations that enhance motility, by directly activating antimotility pathways. Furthermore, these findings suggest that individuals with endoglin-deficient PCa may in fact experience a greater therapeutic benefit from therapy than those with normal endoglin expression.

We also show for the first time that genistein compensates for endoglin deficiency by activating endoglin-associated signaling pathways. In particular, endoglin activated Smad1 transcriptional activity. This in turn was shown to require ALK2 and, in particular, a kinase-competent ALK2. We have shown recently that endoglin inhibits PCa cell motility through a mechanism involving the type I TGFβ superfamily receptor ALK2 and Smad1 (Craft et al., 2007). Therefore, it was not surprising that the current study identified ALK2-Smad1-dependent activation of Smad1 transcriptional activity as the endoglin-linked mechanism by which genistein can compensate for endoglin deficiency. In addition to genistein's effect on this pathway, other findings support the notion of specificity. In particular, we have shown previously that under the current treatment conditions, genistein decreases PCa cell invasion but not cell viability

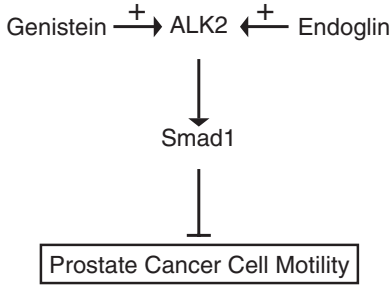

Fig. 6. Proposed model of genistein's effect on the endoglin signaling pathway in human prostate cancer.

(Huang et al., 2005). Furthermore, we have demonstrated previously that although engineered changes in endoglin expression affected cell motility, they did not affect viability (Liu et al., 2002). Finally, in the current study, cell viability was closely monitored, and was not adversely altered under experimental conditions compared with relevant controls. We thus propose the schema depicted in Fig. 6.

We have shown previously that genistein inhibits TGFβ-mediated increases in cell invasion by blocking TGFβ-mediated activation of p38 MAP kinase and its downstream effector heat shock protein 27 (Huang et al., 2005; Xu and Bergan, 2006; Xu et al., 2006). Furthermore, we have shown that p38 MAP kinase can activate Smad3 through signaling pathway cross-talk and that Smad3 is proinvasive (Hayes et al., 2003). The effect of TGFβ ligand was not evaluated in the current study for a number of reasons. Recently, we demonstrated that endoglin inhibits cell motility and activates Smad1 regardless of the activation state of the TGFβ-Smad3 pathway (Craft et al., 2007). In the same study, it was shown that endoglin-ALK2-Smad1 signaling does not interfere with TGFβ-Smad3 signaling. Thus, the presence of exogenous TGFβ ligand is irrelevant to endoglin signaling and function in our system. In addition, given that genistein is known to inhibit TGFβ-mediated activation of the p38 MAP kinase proinvasive pathway, the use of TGFβ in the current study would only serve to confound our ability to evaluate genistein's mimicry of endoglin. Taken together, these considerations support the notion that genistein seems to function through at least two distinct mechanisms. One involves activation of Smad1 signaling, thereby augmenting anti-invasion pathways. The other involves inhibition of Smad3 signaling, thereby inhibiting proinvasive pathways.

The current study identifies ALK2 and, in particular, kinase-competent ALK2 as necessary for genistein-mediated reversion to an endoglin replete phenotype. However, additional studies will be required to further elucidate the underlying mechanism. One possibility is that genistein may alter the molecular makeup of heteromeric cell surface receptor complexes. Canonical signaling through TGFβ superfamily receptors requires the formation of a multiprotein cell surface complex, which contains two or more RI subtypes, two or more RII subtypes, and with and without one or more endoglin subunits (Shi and Massague, 2003). It should also be noted that there are also many ligands associated with the TGFβ superfamily. At this time, the involvement of a ligand or a combination of ligands cannot be ruled out. For example, expression of endoglin or treatment with genistein could initiate ligand production and/or secretion in PCa cells, which would potentially result in autocrine-like signaling. We are currently pursing these possibilities.

In summary, genistein was shown to induce reversion of low-endoglin PCa cells to a low-motility, high-endoglin phenotype. This was due to genistein-mediated activation of Smad1, which in turn was dependent on kinase-competent ALK2. Because endoglin is lost during PCa progression and contributes to its metastatic phenotype, the current study supports the notion that individuals with low endoglin expressing PCa may derive relatively high therapeutic benefit from genistein. These findings may help interpret ongoing phase II molecular efficacy studies of genistein in prostate and other cancers.

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References

Attisano L, Carcamo J, Ventura F, Weis FM, Massague J, and Wrana JL (1993) Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75:**671–680.

Carroll PR, Lee KL, Fuks ZY, and Kantoff PW (2001) Cancers of the genitourinary system, in *CANCER: Principals and Practice of Oncology*, 6th ed (DeVita VT, Hellman S, and Rosenberg SA eds) pp 1343–1490, Lippincott Williams & Wilkins, New York.

Craft CS, Romero D, Vary CPH, and Bergan RC (2007) Endoglin inhibits prostate cancer motility via activation of the ALK2-Smad1 pathway. *Oncogene* **26:**7240–7250.

de Caestecker MP, Hemmati P, Larisch-Bloch S, Ajmera R, Roberts AB, and Lechleider RJ (1997) Characterization of functional domains within Smad4/DPC4. *J Biol Chem* **272:**13690–13696.

Ding Y, Xu L, Chen S, Jovanovic BD, Helenowski IB, Kelly DL, Catalona WJ, Yang XJ, Pins M, Ananthanarayanan V, et al. (2006) Characterization of a method for profiling gene expression in cells recovered from intact human prostate tissue using RNA linear amplification. *Prostate Cancer Prostatic Dis* **9:**379–391.

Fernández-L A, Sanz-Rodriguez F, Blanco FJ, Bernabeu C, and Botella LM (2006) Hereditary hemorrhagic telangiectasia, a vascular dysplasia affecting the TGF-beta signaling pathway. *Clin Med Res* **4:**66–78.

Gougos A and Letarte M (1990) Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem* **265:**8361–8364.

Hayes SA, Huang X, Kambhampati S, Platanias LC, and Bergan RC (2003) p38 MAP kinase modulates Smad-dependent changes in human prostate cell adhesion. *Oncogene* **22:**4841–4850.

Huang X, Chen S, Xu L, Liu YQ, Deb DK, Platanias LC, and Bergan RC (2005) Genistein inhibits p38 MAP kinase activation, MMP-2, and cell invasion in human prostate epithelial cells. *Cancer Res* **65:**3470–3478.

Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, and Thun MJ (2006) Cancer statistics, 2006. *CA Cancer J Clin* **56:**106–130.

Jovanovic BD, Huang S, Liu Y, Naguib KN, and Bergan RC (2001) A simple analysis of gene expression and variability in gene arrays based on repeated observations. *Am J Pharmacogenomics* **1:**145–152.

Korzenik J, Barnes S, and White RJ (1998) Possible efficacy of isolated soy protein in treatment of hereditary hemorrhagic telangiectasia-associated epistaxis, gastrointestinal hemorrhage, and migraine: a pilot study. *Am J Clin Nutr* **68:**1530S.

Liu Y, Jovanovic B, Pins M, Lee C, and Bergan RC (2002) Over expression of endoglin in human prostate cancer suppresses cell detachment, migration and invasion. *Oncogene* **21:**8272–8281.

Liu YQ, Kyle E, Patel S, Housseau F, Hakim F, Lieberman R, Pins M, Blagosklonny MV, and Bergan RC (2001) Prostate cancer chemoprevention agents exhibit selective activity against early stage prostate cancer cells. *Prostate Cancer Prostatic Dis* **4:**81–91.

Liu YU, Kyle E, Lieberman R, Crowell J, Kelloff G, and Bergan RC (2000) Focal adhesion kinase (FAK) phosphorylation is not required for genistein-induced FAK-b-1-integrin complex formation. *Clin Exp Metastasis* **18:**203–212.

Monteiro RM, de Sousa Lopes SM, Korchynskyi O, ten Dijke P, and Mummery CL (2004) Spatio-temporal activation of Smad1 and Smad5 in vivo: monitoring transcriptional activity of Smad proteins. *J Cell Sci* **117:**4653–4663.

Rokhlin OW and Cohen MB (1995) Differential sensitivity of human prostatic cancer cell lines to the effects of protein kinase and phosphatase inhibitors. *Cancer Lett* **98:**103–110.

Severson RK, Nomura AM, Grove JS, and Stemmermann GN (1989) A prospective study of demographics, diet, and prostate cancer among men of Japanese ancestry in Hawaii. *Cancer Res* **49:**1857–1860.

Shi Y and Massague J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113:**685–700.

Takimoto CH, Glover K, Huang X, Hayes SA, Gallot L, Quinn M, Jovanovic BD, Shapiro A, Hernandez L, Goetz A, et al. (2003) Phase I pharmacokinetic and pharmacodynamic analysis of unconjugated soy isoflavones administered to individuals with cancer. *Cancer Epidemiol Biomarkers Prev* **12:**1213–1221.

Wieser R, Wrana JL, and Massague J (1995) GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J* **14:**2199–2208.

Woodhouse EC, Chuaqui RF, and Liotta LA (1997) General mechanisms of metastasis. *Cancer* **80 (Suppl):**1529–1537.

Xu L and Bergan RC (2006) Genistein inhibits matrix metalloproteinase type 2 activation and prostate cancer cell invasion by blocking the transforming growth factor β-mediated activation of mitogen-activated protein kinase-activated protein kinase 2–27-kDa heat shock protein pathway. *Mol Pharmacol* **70:**869–877.

Xu L, Chen S, and Bergan RC (2006) MAPKAPK2 and HSP27 are downstream effectors of p38 MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer. *Oncogene* **25:**2987–2998.

Yu Z, Tang Y, Hu D, and Li J (2005) Inhibitory effect of genistein on mouse colon cancer MC-26 cells involved TGF-beta1/Smad pathway. *Biochem Biophys Res Commun* **333:**827–832.

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