ARTICLE

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Down-Regulation of Notch-1 Is Associated With Akt and FoxM1 in Inducing Cell Growth Inhibition and Apoptosis in Prostate Cancer Cells

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ABSTRACT

Although many studies have been done to uncover the mechanisms by which down-regulation of Notch-1 exerts its anti-tumor activity against a variety of human malignancies, the precise molecular mechanisms remain unclear. In the present study, we investigated the cellular consequence of Notch-1 down-regulation and also assessed the molecular consequence of Notch-1-mediated alterations of its downstream targets on cell viability and apoptosis in prostate cancer (PCa) cells. We found that the down-regulation of Notch-1 led to the inhibition of cell growth and induction of apoptosis, which was mechanistically linked with down-regulation of Akt and FoxM1, suggesting for the first time that Akt and FoxM1 are downstream targets of Notch-1 signaling. Moreover, we found that a ''natural agent'' (genistein) originally discovered from soybean could cause significant reduction in cell viability and induced apoptosis of PCa cells, which was consistent with down-regulation of Notch-1, Akt, and FoxM1. These results suggest that down-regulation of Notch-1 by novel agents could become a newer approach for the prevention of tumor progression and/or treatment, which is likely to be mediated via inactivation of Akt and FoxM1 signaling pathways in PCa. J. Cell. Biochem. 112: 78–88, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: NOTCH-1; PROSTATE CANCER; CELL GROWTH; APOPTOSIS; Akt; FoxM1

Although prostate cancer (PCa) mortality has been decreased in recent years, it is still the second leading cause of cancer-related deaths in men in the United States [Jemal et al., 2009]. Therefore, there is a tremendous need for the development of mechanism-based strategies by which PCa could be treated with a better outcome. Notch signaling has been very attractive due to its functions in a variety of cellular processes, including differentiation, proliferation, and survival [Rizzo et al., 2008]. Four Notch receptors (Notch 1–4) and five ligands (Jagged-1, 2, Delta-1, 3, 4) have been described in mammals [Miele et al., 2006]. Binding of ligand to its receptor induces metalloproteinase-mediated and gamma secretase-mediated cleavage of the Notch receptor. The Notch intracellular domain (ICN) is released from the plasma membrane and translocates into the nucleus and activates its target genes [Miele, 2006; Wang et al., 2008]. Notch signaling pathway was found to be over-expressed in PCa cell lines [Shou et al., 2001; Wang et al., 2010b]. Moreover, Notch signaling pathways play important roles in prostate development and progression [Leong and Gao, 2008; Bin et al., 2009].

Recently, another signaling pathway, namely FoxM1, has been shown to be over-expressed in PCa and studies have shown that alterations in FoxM1 signaling were associated with carcinogenesis [Kalin et al., 2006; Chandran et al., 2007; Pandit and Gartel, 2010]. Specifically, FoxM1 signaling network is frequently up-regulated in most human malignancies including lung cancer, glioblastomas, PCa, basal cell carcinomas, hepatocellular carcinoma, breast cancer, and pancreatic cancer [Gartel, 2008, 2010; Wang et al., 2010a], suggesting that FoxM1 is a major player in human cancers. Moreover, it has been shown that higher expression of FoxM1 was associated with poor prognosis in breast cancer and gastric cancer patients [Bektas et al., 2008; Li et al., 2009]. These results suggest that FoxM1 may have a critical role in the development and progression of human cancers especially PCa. Therefore, it is believed that inactivation of FoxM1 could represent a promising

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strategy for the development of novel and selective anti-cancer therapies. It has been shown that Akt is Notch downstream gene [Wang et al., 2010b] and Akt can control FoxM1 expression in osteosarcoma [Major et al., 2004], and thus we sought to determine whether FoxM1 expression could be controlled by Notch and Akt in PCa cells in the present study. Although several chemical agents such as gamma secretase inhibitors, siomycin A, and thiostrepton have been shown to inhibit Notch and FoxM1 activity, respectively, they also demonstrated unwanted toxicity in mice and human. Therefore, we also investigated whether a non-toxic "natural agent" could be useful for the inhibition of Notch signaling which consequently may also inactivate Akt and FoxM1 signaling, and thus it could be beneficial for the prevention of tumor progression and/or therapy for PCa.

Taxotere (Docetaxel) has shown clinical activity in a wide spectrum of solid tumors including PCa [Chiuri et al., 2009]. Taxotere has been reported to inhibit cell growth and induce apoptosis in PCa [Li et al., 2005a,b,c]. Clinical trials have shown that the combination chemotherapy using taxotere with other agents improves survival in PCa patients [Falci et al., 2009]. However, the combination treatment contributes to a certain degree of dose-related toxicity. Therefore, there is a dire need for the development of therapeutic strategies to improve efficacy and reduce side effects of taxotere-based treatment. Naturally occurring agents such as genistein is a prominent isoflavone found in soybeans, has been found to inhibit cell growth and induce apoptosis in vitro and in vivo without toxicity [Banerjee et al., 2008]. Studies from our laboratory have also found that genistein can inhibit NF-κB and Akt activation in PCa cells, suggesting its anti-tumor activity against PCa [Banerjee et al., 2005, 2007b; Li et al., 2005a]. It has been reported that NF-κB is regulated by Notch signaling in human cancer [Wang et al., 2006a, 2008; Osipo et al., 2008], which became the basis for conducting the current study to test whether genistein could inhibit the Notch signaling in PCa cells and how it is related to other signaling pathways.

In the present study, we sought to gain molecular evidence in support of the mechanistic consequence of Notch-1 down-regulation in cell growth and apoptosis using human PCa cells. Our results show that down-regulation of Notch-1 could be an effective approach for inhibiting cell growth and inducing apoptotic cell death, which was mechanistically associated with inactivation of Akt and FoxM1. Moreover, we found that genistein could inhibit cell growth and also could induce apoptotic cell death in PCa, which appears to be in part mediated via inactivation of Notch-1/Akt/FoxM1 signaling pathways. Together, we believe that inactivation of Notch-1 by novel non-toxic agents could be a potential targeted approach for the prevention of tumor progression and/or treatment of human PCa, which we believe would be due to inactivation of Notch-1 downstream genes such as Akt and FoxM1.

MATERIALS AND METHODS

CELL CULTURE AND EXPERIMENTAL REAGENTS

Human PCa cell lines including PC-3, DU145, LNCaP, and C4-2B were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. The cell lines have been tested and authenticated through our Genomic Core Facility, Applied Genomics Technology Center at Wayne State University. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). Experimental reagents were described in Supplementary Methods.

PLASMIDS AND TRANSFECTION STUDIES

The Notch-1 ICN cDNA plasmid encoding the Notch-1 intracellular domain was described as before [Weijzen et al., 2002]. PCa cells were transfected with Notch-1 siRNA and siRNA control, respectively, using Lipofectamine 2000.

REAL-TIME REVERSE TRANSCRIPTION-PCR ANALYSIS FOR GENE EXPRESSION STUDIES

The total RNA from treated and untreated cells was isolated by Trizol (Invitrogen) and purified by RNeasy Mini Kit and RNase-free DNase Set (Qiagen, Valencia, CA) according to the manufacturer's protocols. The primers used in the PCR reaction and real-time PCR amplifications were performed as described earlier [Wang et al., 2006a, 2007].

WESTERN BLOT ANALYSIS

Cells were lysed in lysis buffer by incubating for 20 min at 4°C. The protein concentration was determined using the Bio-Rad protein assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated using SDS–PAGE and transferred onto a nitrocellulose membrane for Western blotting as described before [Wang et al., 2006a].

CLONOGENIC ASSAY

To test the survival of cells treated with genistein, PCa cells were plated (50,000–100,000 per well) in a six-well plate and incubated overnight at 37°C. After 72 h exposure to various concentrations of genistein, the cells were subjected to clonogenic assay as described earlier [Wang et al., 2009]. The surviving fraction was normalized to untreated control cells with respect to clonogenic efficiency.

HISTONE/DNA ELISA FOR DETECTION OF APOPTOSIS

The Cell Death Detection ELISA Kit was used for assessing apoptosis according to the manufacturer's protocol. Briefly, treated cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with anti-histone antibody for detection of apoptosis as described earlier [Wang et al., 2006b].

FLOW CYTOMETRY AND CELL-CYCLE ANALYSIS

The cells were synchronized in G_0 by serum starvation for 24 h in phenol red-free RPMI with 0.1% serum. Subsequently, cells were released into complete media containing 10% FBS. The cell cycle was analyzed by flow cytometry as described earlier [Wang et al., 2006b].

CELL GROWTH INHIBITION STUDIES BY MTT ASSAY

The PCa cells (5×10^3) were seeded in a 96-well culture plate and subsequently treated with taxotere and incubated with MTT reagent

(0.5 mg/ml) at 37°C for 2 h and MTT assay was performed as described earlier [Wang et al., 2006b].

IMMUNOFLUORESCENCE MICROSCOPY

Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, and incubated in PBS and 10% goat serum blocking solution for 1 h. The cells were incubated for 2 h with anti-FoxM1 in 5% goat serum and were stained, and viewed as described earlier [Wang et al., 2010b].

MMP-9 AND VEGF ACTIVITY ASSAY

The cells were seeded in six-well plate and incubated at 37°C. After 24 h, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium for 72 h. MMP-9 and VEGF activity in the medium was detected as described before [Wang et al., 2010b].

CELL INVASION ASSAY

The invasive activity of the cells was tested using the BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) as described earlier [Wang et al., 2006a].

ANIMAL EXPERIMENTS

The severe combined immunodeficiency (SCID)-human model of experimental PCa was used for our study as described earlier [Banerjee et al., 2007a]. Briefly, suspensions of PC-3 and C4-2B cells were injected, respectively, by insertion of a 27-gauge needle through the mouse (Taconic Farms) skin directly into the marrow surface of the previously implanted bone. The mice were divided into two groups of seven animals in each group. In the genistein treatment groups, the mice were fed a genistein containing diet (1 g/kg diet) beginning on the 30th day after intraosseous PC-3 cell injection as described before [Li et al., 2006]. The C4-2B xenograft mice were treated with 7.5 mg genistein/100 μl/mice by gavage every day for 4 weeks beginning on the 30th day after intraosseous C4-2B cell injection. All mice were sacrificed on the 28th day after genistein treatment because big tumors were formed in control mice. H&E staining confirmed the presence of tumor.

DENSITOMETRIC AND STATISTICAL ANALYSIS

The statistical significance of differential findings between experimental groups and control was statistically evaluated using GraphPad StatMate software (GraphPad Software, Inc., San Diego, CA). *P*-values lower than 0.05 were considered statistically significant.

RESULTS

NOTCH SIGNALING PATHWAY IN PROSTATE CANCER CELLS

First, the baseline expression of Notch signaling molecules was determined using real-time RT-PCR and Western blotting analysis, respectively, in a panel of human PCa cell lines that included PC-3, DU145, LNCaP, and C4-2B. The results showed that the Notch signaling pathway was frequently but differentially dysregulated in different human PCa cell lines (Fig. 1). It is important to note that we focused our studies on the cleaved Notch because it is the active functional form of Notch. Therefore, Notch in our all figure legends means active cleaved Notch. We found that Notch-1 was highly expressed in PC-3, LNCaP, and C4-2B. Previous studies have shown that down-regulation of Notch-1 inhibited cell growth, induced apoptosis, and decreased cell invasion of PCa cells [Zhang et al., 2006; Wang et al., 2010b]. In an effort to confirm our results, and to further investigate the precise molecular roles of Notch-1 in PCa cells, we have used stable clones (name PC-3 ICN) of PC-3 cells transfected with ICN plasmid. We found that the over-expression of Notch-1 promoted cell growth, inhibited apoptosis, increased S-phase fractions, and increased cell invasion, all of which were associated with up-regulation of pAkt, NF-κB, and its target gene VEGF and MMP-9 (Supplementary Figs. 1 and 2), which provided direct molecular evidence in support of the role of Notch-1 in tumor aggressiveness.

DOWN-REGULATION OF Akt INHIBITED FoxM1 EXPRESSION

Recently, high expression of FoxM1 was reported in PCa. Down-regulation of FoxM1 by siRNA inhibited cell growth in PCa cell lines [Kalin et al., 2006]. Moreover, FoxM1 has been shown to cross-talk with the PI3K/Akt pathway [Major et al., 2004; Wang et al., 2010a,b]. Therefore, we assessed whether FoxM1 is downstream of Akt pathway or not, and for which we designed the experiments as shown below. First, we detected the basal level of FoxM1 in PC-3, DU145, LNCaP, and C4-2B cell lines using real-time RT-PCR and Western blotting, respectively. We found that FoxM1 is highly expressed in PC-3 and C4-2B, which is consistent with pAkt expression (Fig. 2A). It has been shown that Akt can control FoxM1 expression in osteosarcoma [Major et al., 2004], and thus we sought to determine whether FoxM1 expression could be controlled by Akt in PCa cells. As expected, over-expression of pAkt by Akt cDNA plasmid increased FoxM1 expression in PC-3 cells (Fig. 2B). However, down-regulation of pAkt by siRNA inhibited FoxM1 expression in PC-3 cells (Fig. 2B). Moreover, we found that LY294002 and Wortmanin, the PI3K inhibitors, eliminated the expression of FoxM1 (Fig. 2C), suggesting that FoxM1 is regulated by Akt pathway in PCa cells, and further suggesting that inactivation of Notch-1 could inactivate Akt which, in turn, leads to the inactivation of FoxM1. To further confirm our results in order to document whether FoxM1 is a downstream target of Akt or not, we examined cell growth, which was reduced in Akt knock-out MEFs (Supplementary Fig. 3A). We also examined the expression of FoxM1 in Akt WT, Akt-1KO, and Akt DKO MEF cell lines, and found that FoxM1 was significantly decreased in Akt DKO cell lines, which was consistent with decreased pAkt pathway (Supplementary Fig. 3B). Further experiments were done in PTEN KO MEF, which showed high expression of pAkt and FoxM1 (Supplementary Fig. 3C), suggesting that Akt regulates the expression of FoxM1.

DOWN-REGULATION OF NOTCH-1 EXPRESSION INHIBITED FoxM1

Next, we assessed whether Notch-1 could regulate the FoxM1 expression because Akt is one of the Notch-1 downstream target genes, which appears to regulate FoxM1. Down-regulation of Notch-1 by siRNA transfection and gamma secretase inhibitor (GSI, L-685,458, DAPT) treatments showed lower expression of Notch-1 protein as confirmed by Western blotting (Fig. 3A,B). We also found

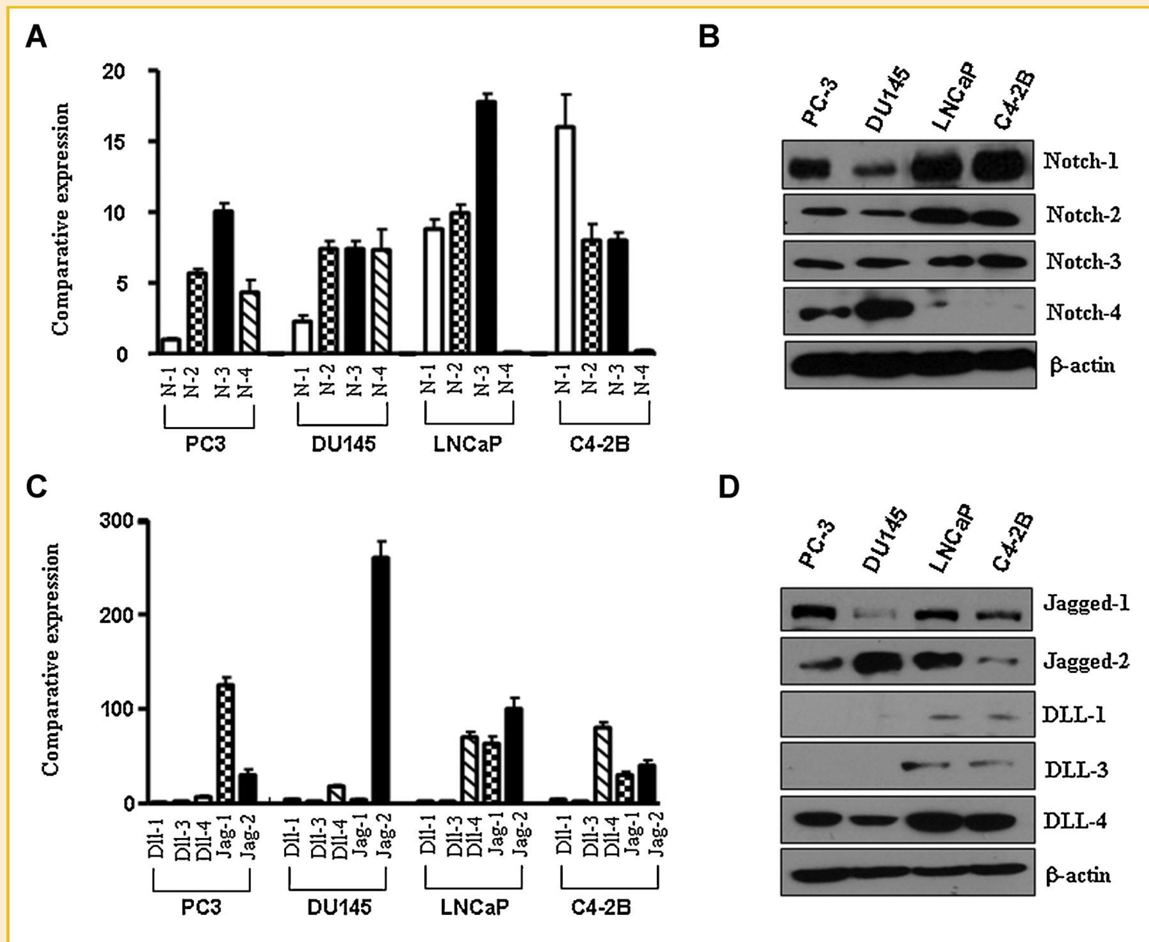

Fig. 1. Notch signaling pathway in PCa cell lines. The baseline expression of Notch signaling pathway was determined in a panel of PCa cell lines using real-time RT-PCR (A,C) and Western blotting analysis (B,D), respectively.

that the inhibition of Notch-1 significantly decreased pAkt and FoxM1 expression in PC-3 and C4-2B cell lines (Fig. 3A,B). Conversely, over-expression of Notch-1 by ICN transfection showed increased expression of FoxM1 at the mRNA and protein levels (Fig. 3B). Moreover, Notch-1 siRNA decreased pAkt and FoxM1 in Akt WT cell lines (Supplementary Fig. 3C). Furthermore, immunofluorescent staining showed higher levels of FoxM1 protein in PC-3 ICN cells (Fig. 3C), suggesting that FoxM1 is regulated by Notch-1, which could be due to inactivation of Akt. Although these molecular studies clearly suggest that Notch-1 inactivation could be an important strategy for the prevention of tumor progression and/or therapy, we sought to investigate whether we could find any ''natural'' non-toxic agent that could down-regulate Notch-1 and consequently could inactivate Akt and FoxM1.

DOWN-REGULATION OF NOTCH-1 EXPRESSION BY GENISTEIN

Our previous studies have shown that genistein inhibited cell growth and induced cell apoptotic death in PCa cells. However, genistein did not inhibit the normal prostate cell growth [Banerjee et al., 2008]. In order to further understand the molecular mechanism involved in genistein-induced apoptosis of PCa cells, alterations in the cell survival pathway were investigated. Notch signaling is over-expressed in PCa and is involved in the inhibition of apoptosis and potentiation of cell growth and thus considered as a putative target for drug development. Therefore, we investigated whether genistein could regulate Notch signaling pathway. Notch-1 mRNA and protein expression in PCa cell lines treated with genistein for 72 h were assessed. We found that Notch-1 was down-regulated by genistein in all three cell lines (Fig. 4A). To confirm the downstream effect on Notch down-regulation by genistein, we also assessed the expression of Notch-1 target gene pAkt in PCa cells after genistein treatment. We found that genistein inhibited the pAkt expression (Fig. 4A). We also assessed the expression of FoxM1 in PCa cells treated with genistein, and as expected, we found down-regulation of FoxM1 in genistein-treated cells (Fig. 4A). To further confirm our results, we also did immunofluorescent staining. Indeed, we observed lower level of FoxM1 protein in genistein-treated cells (Fig. 4B).

DOWN-REGULATION OF NOTCH-1 EXPRESSION BY siRNA POTENTIATES GENISTEIN-INDUCED CELL GROWTH INHIBITION AND APOPTOSIS

In order to gain further molecular insight, we assessed whether inactivation of Notch-1 by Notch-1-specific siRNA could lead to potentiate the effects of genistein. We found that the down-

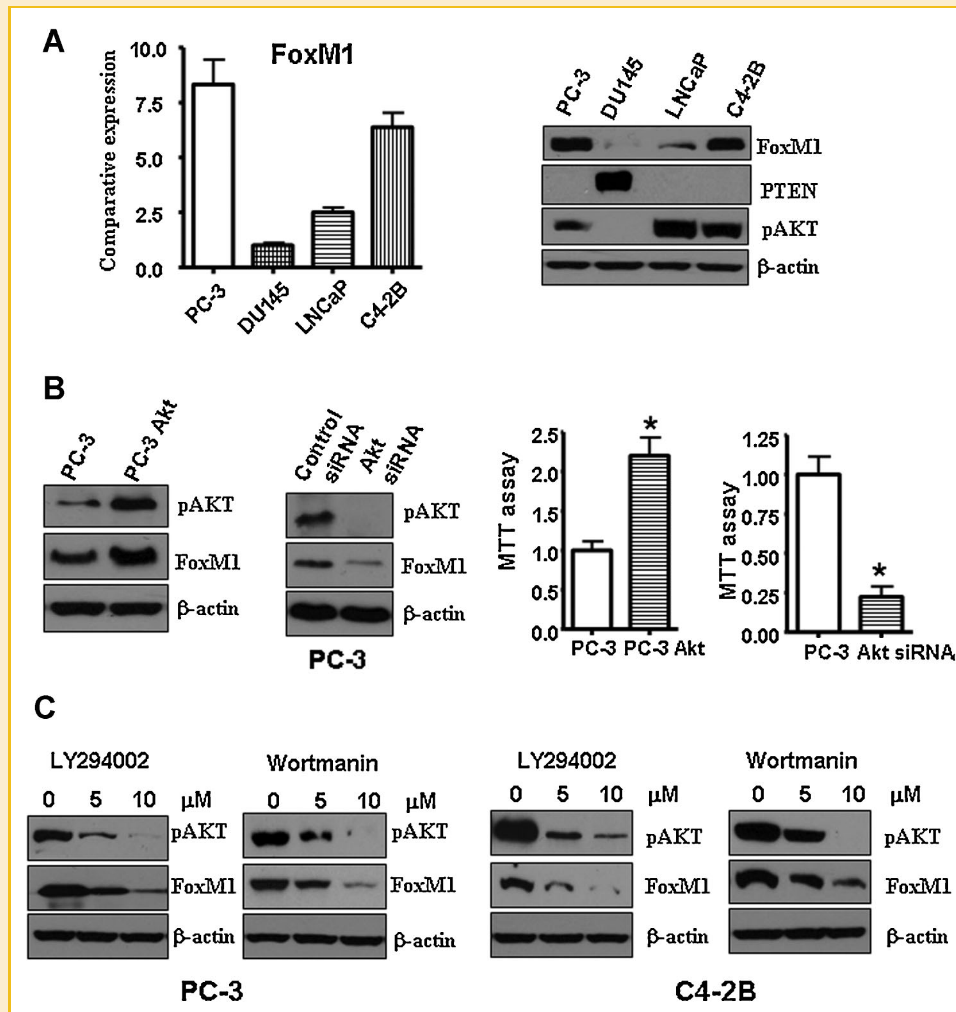

Fig. 2. Down-regulation of Akt inhibited FoxM1. A: The baseline expression of FoxM1 was determined between a panel of PCa cell lines using real-time RT-PCR and Western blotting analysis, respectively. B: Down-regulation of Akt by siRNA inhibited FoxM1 expression, whereas up-regulation of Akt by cDNA plasmid transfection resulted in increased expression of FoxM1. Akt siRNA inhibited cell growth, while Akt cDNA transfection promoted cell growth. C: Inactivation of Akt by PI3K inhibitors (LY294002, Wortmanin) inhibited the expression of pAkt, which was consistent with decreased expression of FoxM1 as assessed by Western blot analysis.

regulation of Notch-1 expression significantly inhibited cell growth induced by genistein (Fig. 5). Genistein plus Notch-1 siRNA inhibited cell growth to a greater degree compared to genistein alone. Moreover, Notch-1 siRNA-transfected PC-3 cells were significantly more sensitive to spontaneous and genistein-induced apoptosis (Fig. 5A). However, over-expression of Notch-1 rescued genistein-induced cell growth inhibition and abrogated genistein-induced apoptosis to a certain degree (Fig. 5B). These results provide molecular evidence suggesting that genistein induced cell growth inhibition and apoptosis is in part mediated through Notch-1 signaling pathway in PCa cells.

GENISTEIN-MEDIATED EFFECTS ON PCa CELLS WERE ENHANCED BY TAXOTERE IN INHIBITING CELL GROWTH AND CAUSING INDUCTION OF APOPTOSIS

First, we found that taxotere inhibited cell growth and induced apoptosis in PC-3, LNCaP, and C4-2B cells (Supplementary Fig. 4). Second, we found that taxotere did not significantly inhibit the Notch-1 and pAkt expression (data not shown). However, taxotere inhibited FoxM1 expression in all three PCa cells (Fig. 5C). Next, we tested whether genistein could synergize with taxotere leading to enhanced suppression of cell growth as assessed by MTT assay. As can be seen from the results presented in Figure 5D, 30 μM genistein alone or 1 nM taxotere alone caused 40–50% cell growth inhibition, whereas the combination of genistein and taxotere showed drastic inhibition (about 80%) in cell growth, which was also found to be synergistic in inducing apoptotic cell death (Fig. 5D).

GENISTEIN INHIBITED TUMOR GROWTH IN VIVO

To test whether genistein has similar effects in vivo, we conducted an animal experiment using SCID-human model of experimental bone metastasis of PCa. We found that genistein significantly inhibited PC-3 and C4-2B tumor growth, demonstrating an inhibitory effect of genistein in the in vivo model of PCa (Fig. 6A). The body weight of mice in each group did not show any significant difference, suggesting non-toxic nature of genistein.

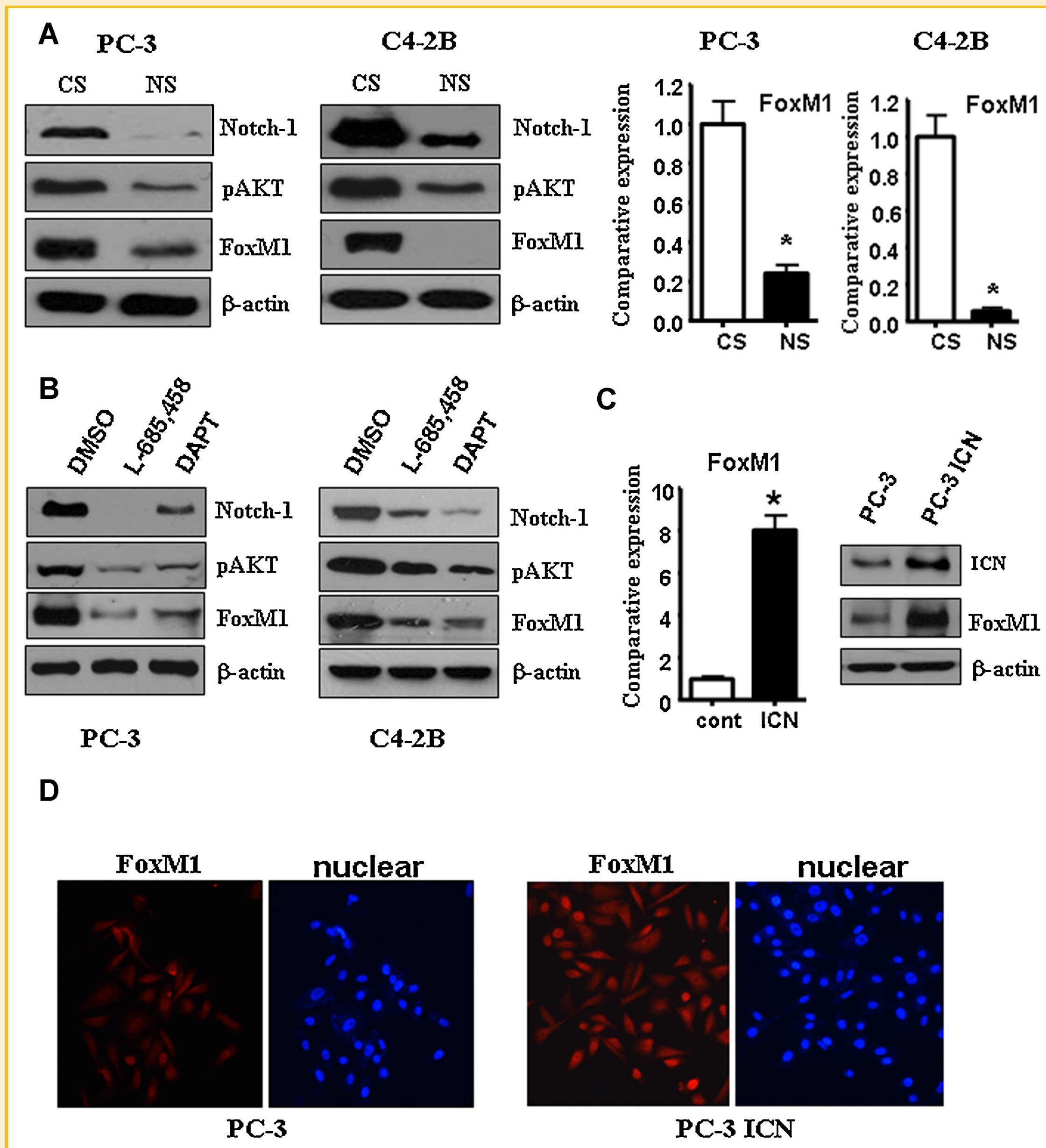

Fig. 3. Inhibition of FoxM1 expression by Notch-1 siRNA and GSI. A: The expression of FoxM1 was detected by Western blotting analysis (left panel) and real-time RT-PCR (right panel) in PCa cells transfected with Notch-1 siRNA. B: The expression of FoxM1 was detected by Western blotting analysis in PCa cells treated with GSI for 72 h. C: The expression of FoxM1 was detected by real-time RT-PCR (left panel) and Western blotting analysis (right panel) in PC-3 ICN cells. D: The PC-3 and PC-3 ICN cells were subjected to immunofluorescent staining using anti-FoxM1 antibody. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In order to further explore the molecular mechanism by which genistein caused anti-tumor activity, we analyzed the gene expression altered by genistein treatment, and we found that genistein inhibited the expression of Notch-1, pAkt, and FoxM1 in tumor remnants (Fig. 6B).

DISCUSSION

Notch signaling plays important roles in maintaining the balance between cell proliferation, differentiation, and apoptosis [Kopan and Ilagan, 2009]. The Notch gene is abnormally activated in many human malignancies [Miele, 2006; Rizzo et al., 2008]. It has been reported that the Notch signaling is involved in PCa cell survival and that Notch signaling pathway components and Notch target genes are up-regulated in PCa [Villaronga et al., 2008; Bin et al., 2009]. Moreover, Notch-1 expression in human PCa tissues increased with increasing tumor grade [Bin et al., 2009]. In our previous studies, we have shown that down-regulation of Notch-1 inhibits cell growth and induced apoptosis in PCa cells [Wang et al., 2010b]. Therefore, the inhibition of Notch signaling is likely to have beneficial effects toward designing strategies for the prevention of tumor progression and/or therapy for PCa.

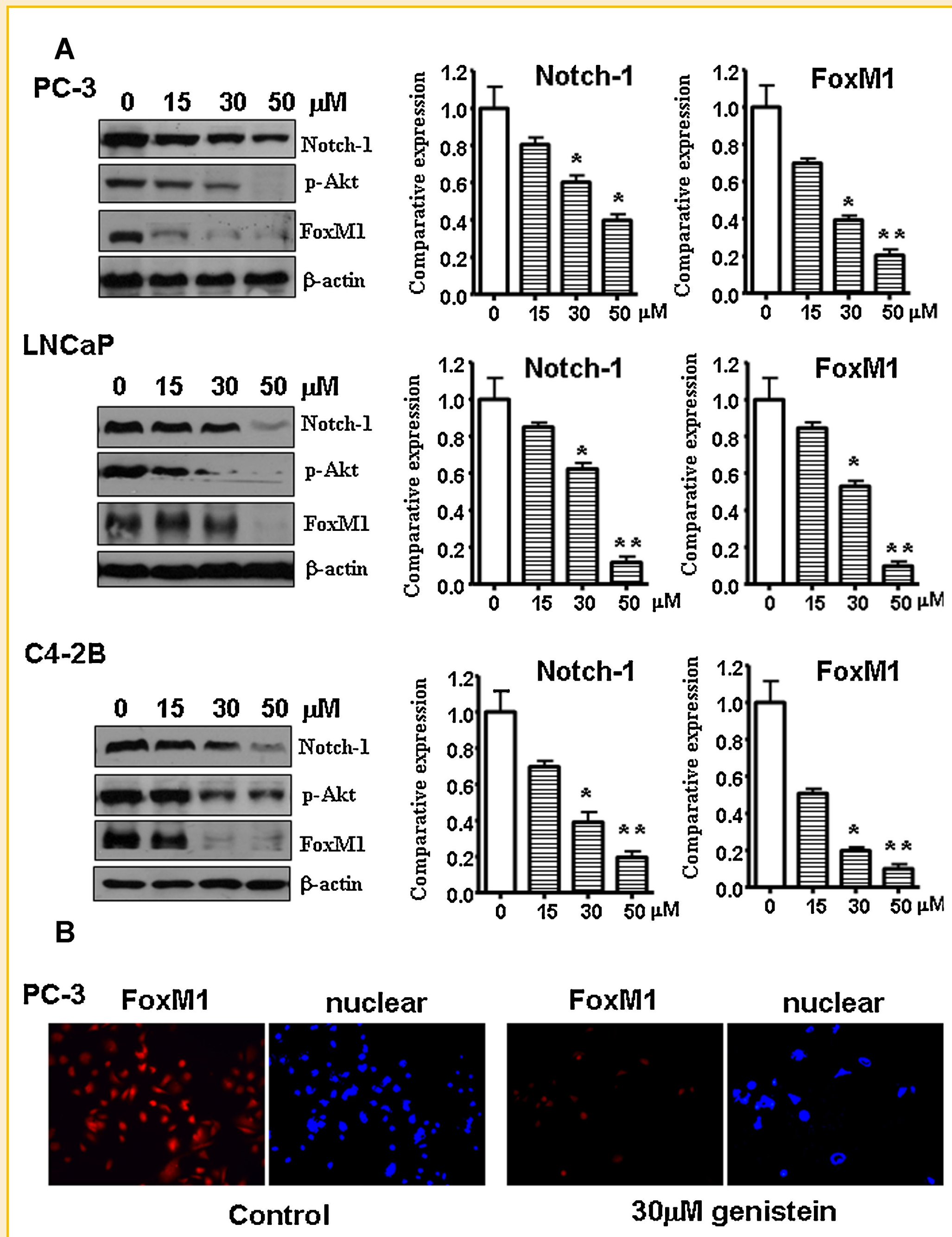

Fig. 4. Inhibition of Notch, Akt, and FoxM1 expression by genistein. A: PCa cells were treated with varied concentrations of genistein for 72 h. Left panel: The expression of Notch, pAkt, and FoxM1 protein was detected by Western blotting analysis. Middle and right panel: Notch-1 mRNA and FoxM1 mRNA were detected by real-time RT-PCR. B: Immunofluorescent staining showing lower levels of FoxM1 protein in the cytoplasm and nucleus in the genistein-treated PC-3 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Although several studies have shown the functional significance of Notch signaling, the role of Notch pathway in prostate carcinogenesis remains poorly understood. Therefore, in the present study, we investigated the mechanisms of Notch-1 in cell proliferation in PCa cells. Recently, Notch has been shown to regulate the Akt pathway. It has been reported that Notch-1 activation enhanced melanoma cell survival via activation of the Akt pathway [Liu et al., 2006]. Palomero et al. [2008] found that Notch-1 up-regulated the PI3K–Akt pathway, which negatively controls the expression of phosphatase and tensin homolog on chromosome 10 (PTEN) in T-ALL. In the current study, we found that down-regulation of Notch-1 by siRNA or GSI decreased Akt phosphorylation in PCa cells. Recently, Akt pathway has been shown to cross-talk with the FoxM1 pathway [Major et al., 2004; Park et al., 2009] and FoxM1 has been shown to be over-expressed in many human cancers including PCa [Wang et al., 2010a]. Given

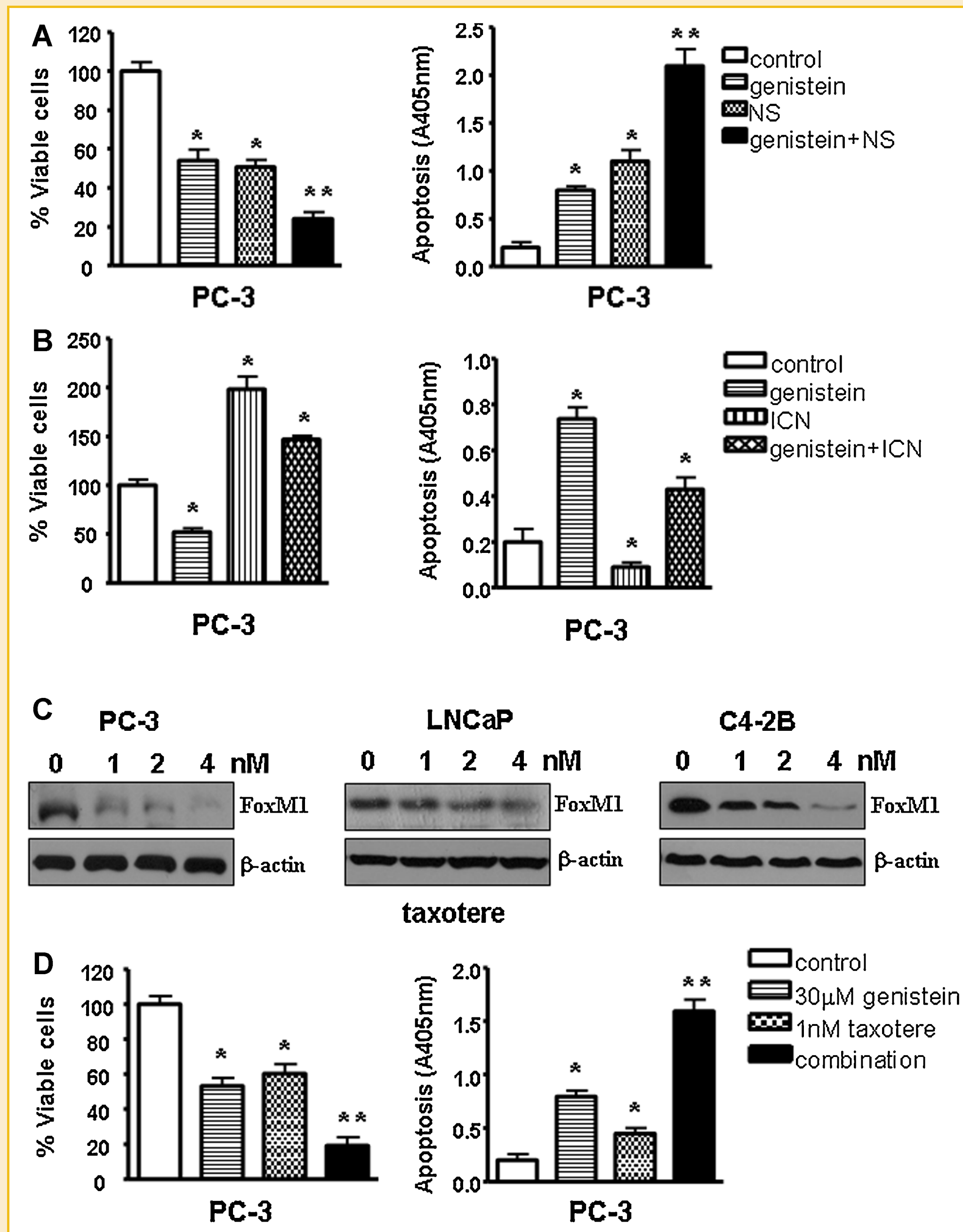

Fig. 5. Down-regulation of Notch-1 by siRNA promotes genistein-induced cell growth inhibition and apoptosis in PC-3 cells. Genistein: 30 μM genistein; NS: Notch-1 siRNA; ICN: ICN cDNA; genistein + siRNA: 30 μM genistein + Notch-1 siRNA; genistein + ICN: 30 μM genistein + ICN cDNA. A: Left panel: Down-regulation of Notch-1 by siRNA significantly inhibited PC-3 cell growth. Genistein plus Notch-1 siRNA inhibited cell growth to a greater degree compared to genistein alone. Right panel: Down-regulation of Notch-1 expression significantly increased apoptosis induced by genistein. Notch-1 siRNA-transfected cells were significantly more sensitive to spontaneous and genistein-induced apoptosis. B: Over-expression of Notch-1 expression significantly promoted cell growth. Over-expression of Notch-1 rescued cells from genistein-induced cell growth inhibition. Over-expression of Notch-1 by Notch-1 cDNA transfection abrogated genistein-induced apoptosis to a certain degree. C: The expression of FoxM1 was detected by Western blotting analysis in PCa cells treated with different concentrations of taxotere for 72 h. D: Left panel: Genistein synergize with taxotere leading to enhanced suppression of cell growth as assessed by MTT assay. Right panel: Genistein combined with taxotere led to synergistic induction of apoptotic cell death.

the emerging data describing the important role of FoxM1 in the progression of human cancers, Radhakrishnan et al. [2006] have rightly pointed out that it should be possible to target multiple facets of tumorigenesis by inhibiting only this single transcription factor. In our study, we investigated whether Akt could regulate FoxM1 expression in PCa cells. Indeed, we found that down-regulation of Akt by siRNA inhibited FoxM1 expression, whereas over-expression of Akt increased FoxM1 expression. Moreover, inactivation of Akt by LY294002 and Wortmanin decreased FoxM1 expression. Furthermore, we found that down-regulation of Notch-1 by siRNA

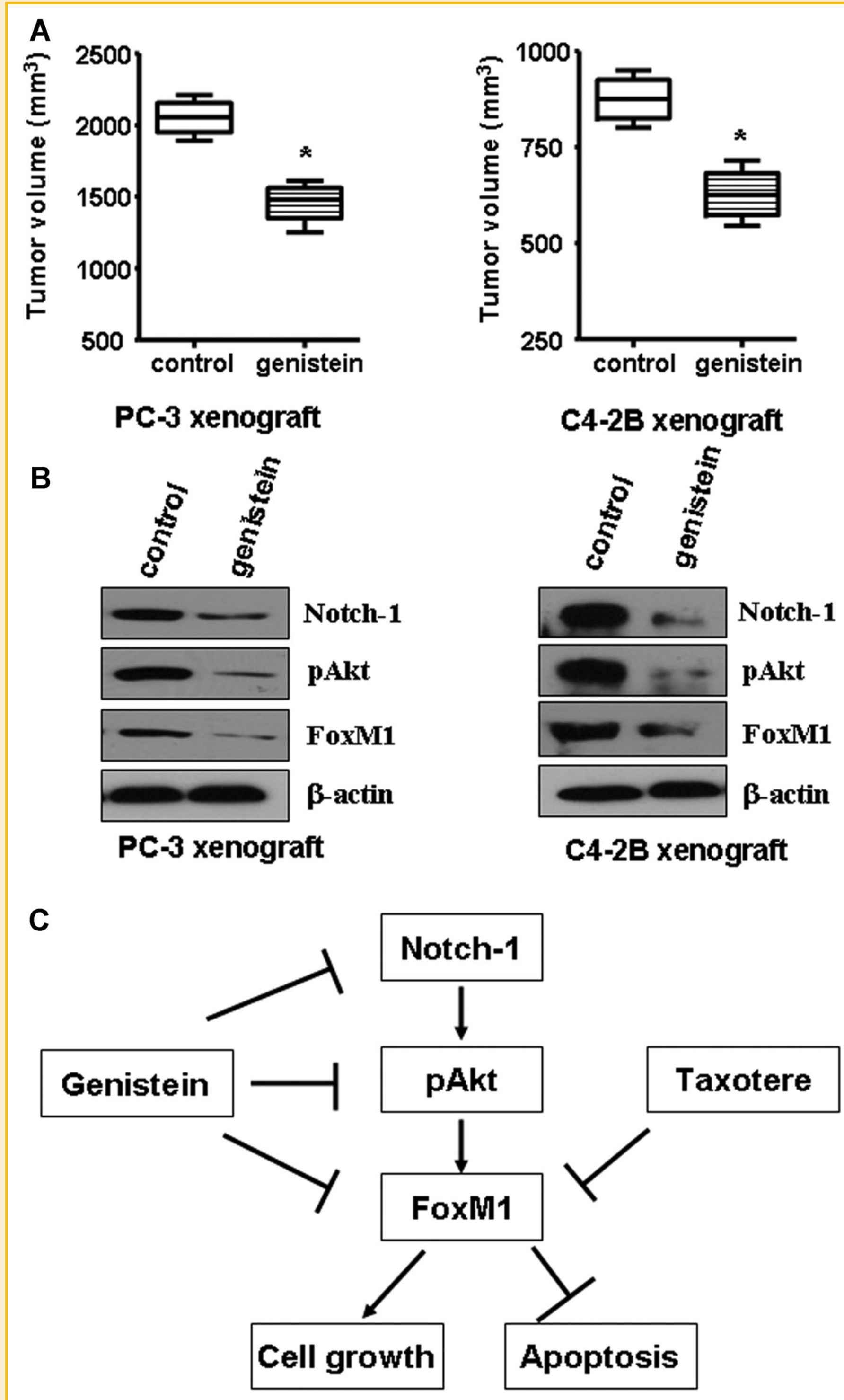

Fig. 6. Genistein inhibited tumor growth in animal model. A: Inhibitory effects of genistein on the growth of tumors formed by PC-3 or C4-2B cells in SCID-human mice (control, n = 7; genistein, n = 7). Comparison of the tumor volumes in each group on the day when all mice were sacrificed ($^{*}P < 0.05$, genistein vs. control). B: Genistein inhibited the expression of Notch-1, pAkt, and FoxM1 in tumor remnants as assessed by Western blot analysis. C: The schematic presentation of our proposed mechanism of how genistein inhibits cell growth and induces apoptosis.

or GSI decreased FoxM1 expression. We also found that FoxM1 was decreased significantly in Akt DKO cell lines, which was consistent with decreased pAkt pathway. These results suggest that Notch-1 could mediate FoxM1 signaling through Akt.

In the present study, we also found that a ''natural agent'' discovered from soybean such as genistein could inhibit Notch-1 activity in PCa cells, suggesting that this could be one mechanism by which genistein inhibits cell growth and induces apoptosis. Moreover, down-regulation of Notch-1 by siRNA together with genistein treatment inhibited cell growth and induced apoptosis to a greater degree in PC-3 cells compared to genistein treatment alone, which suggest that inactivation of Notch-1 could be mechanistically linked with the biological effects of genistein. Moreover, we have shown, for the first time, that genistein could inhibit the expression of FoxM1 in vitro and in vivo in PCa. The similar results were also found in pancreatic cancer cells and its orthotopic animal model in vivo (data not shown), and the anti-tumor activity of genistein was correlated with decreased expression of Notch-1, pAkt, and FoxM1 in tumor remnants (data not shown), which was consistent with in vivo findings in PCa. Therefore, genistein-mediated cell growth inhibition could be partly mediated via inactivation of FoxM1 activity. In view of these findings, we strongly believe that inactivation of FoxM1 by genistein appears to be mechanistically linked with genistein-induced cell growth inhibition and apoptosis in PCa cells. It has been found that taxotere down-regulated some genes for cell proliferation, transcription factors, and oncogenesis, and up-regulated some genes related to induction of apoptosis and cell-cycle arrest in PCa cells. Here, we found that taxotere alone could down-regulate FoxM1 and the combination of taxotere with genistein showed greater down-regulation in the expression of FoxM1, which appears to be consistent with the inhibition of cell growth and induction of apoptosis in PCa, further suggesting that the combination of taxotere with genistein could be an useful therapeutic strategy for the treatment of metastatic PCa.

In summary, we presented experimental evidence which strongly suggest that the role of Notch-1 down-regulation could be a potential anti-tumor and anti-metastatic approach toward the treatment of PCa. Moreover, our current data provided mechanistic information showing that genistein exerts its pro-apoptotic effects on PCa cells, which is in part due to inactivation of Notch-1, Akt, and FoxM1 signaling (Fig. 6C). On the basis of our results, we propose a hypothetical pathway by which genistein inhibits cell growth of PCa cells although further in-depth studies are needed to ascertain how genistein regulates these pathways. However, we believe that Notch-1, Akt, and FoxM1 are intimate partners of the same crime of tumor aggressiveness, and thus targeted inactivation of these pathways by genistein together with taxotere may prove to be a novel therapeutic approach for the treatment of PCa in the future.

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