Simvastatin Up-Regulates Annexin A10 That Can Inhibit the Proliferation, Migration, and Invasion in Androgen-Independent Human Prostate Cancer Cells

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BACKGROUND. Statins have recently been studied for their proapoptotic and antimetastatic effects. However, the exact mechanisms of their anticancer actions remain unclear. Using microarrays, we discovered up-regulation of annexin A10 (ANXA10) in PC-3 cells after simvastatin treatment. ANXA10 reportedly has antitumor effects. In this study, we evaluated the effects of simvastatin on ANXA10 signaling in androgen-independent prostate cancer cells.
METHODS. PC-3, LNCaP-LA (which were derived from LNCaP cells and cultured in 10% charcoal-stripped fetal bovine serum for 3 months), and DU145 human prostate cancer cell lines were used. Prostate tissues were collected from 60 patients (benign prostatic hyperplasia [BPH], n = 20; prostate cancer with a Gleason score of 7, n = 20; prostate cancer with a Gleason score of 8–10, n = 20) at the time of prostate biopsies performed. We used a nude mouse tumor xenograft model with administration of simvastatin or phosphate-buffered saline via intraperitoneal injection.
RESULTS. Simvastatin inhibited the proliferation, migration, and invasion of PC-3, LNCaP-LA, and DU145 cells. The expression level of ANXA10 was up-regulated by simvastatin in PC-3, LNCaP-LA, and DU145 cells. Transfection with ANXA10 inhibited PC-3 and LNCaP-LA cells proliferation, migration, and invasion. Knockdown of ANXA10 by siRNA increased the proliferation of PC-3 and LNCaP-LA cells. In a nude mouse xenograft model of PC-3 cells, simvastatin induced both reduction in the tumor size and up-regulation of ANXA10 expression. In human prostate biopsy samples, ANXA10 mRNA expression was significantly lower in the prostate cancer group than in the BPH group. Next, we found that up-regulation of ANXA10 in PC-3 resulted in down-regulation of S100 calcium binding protein A4 (S100A4), which is reportedly correlated with aggressiveness and a worse prognosis for patients with different types of carcinomas. Expression of S100A4 was down-regulated by simvastatin. In PC-3 cells, knockdown of S100A4 by siRNA inhibited the proliferation, migration, and invasion of PC-3 cells.
CONCLUSION. Our results suggest that statins inhibit the proliferation, migration, and invasion of androgen-independent prostate cancer cells by up-regulation of ANXA10. Additionally, it is possible that S100A4 plays a role in these effects. Statins may be beneficial in the prevention and/or treatment of prostate cancer. *Prostate* © 2016 Wiley Periodicals, Inc.

KEY WORDS: **prostate cancer; statin; annexin A10; S100 calcium binding protein A4**

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy and one of the leading causes of cancer death in the United States. Prostate cancer accounts for approximately 27% (233,000 new cases) of incident cases in the United States. Additionally, prostate cancer accounts for 10% (29,480 cases) of the total cancer deaths among men in the United States [1]. Prostate cancer has also been on the rise in Japan in

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recent years [2]. When treating localized prostate cancer, a radical cure can be expected from surgical treatment, radiotherapy alone, or a combination of radiotherapy and androgen deprivation therapy (ADT) [3]. ADT is widely used and has become a first-line treatment since Huggins et al. [4] described the occurrence of recurrent and metastatic prostate cancer after localized treatment. ADT is effective for the treatment of many cases of recurrent or metastatic prostate cancer. Although treatment outcomes are dependent on the degree of malignancy, many patients with prostate cancer receiving this therapy can expect a good long-term prognosis.

However, tumor progression may be observed in patients in a castrated state after successful ADT. This is known as castration-resistant prostate cancer and has a negative effect on the prognosis and quality of life in patients [3]. Various drugs that focus on androgen receptors (ARs) have been developed and used clinically. In addition to "conventional" AR inhibitors such as flutamide [5] and bicalutamide [6], enzalutamide has been reported to prolong survival time before and after chemotherapy [7,8]. Abiraterone, an androgen biosynthesis enzyme inhibitor, also contributes to improved patient prognosis [9]. In addition, docetaxel and cabazitaxel have been used as anticancer drugs [10,11]. These novel drugs have widened the range of therapeutic measures that do not target ARs or androgen synthesis. Although the effects of these novel drugs have been confirmed, there have also been cases in which exacerbation of castration-resistant prostate cancer has been observed [12]. We believe that further examination of the mechanisms of action of these novel drugs is urgently required.

Cholesterol is a major material in androgen synthesis. In the present study, we focused on the relationship between prostate cancer and cholesterol metabolism. In particular, we investigated the effect of statins, which influence cholesterol metabolism. Various reports have examined the effects of statins on prostate cancer; however, these effects remain unclear [13]. Because some reports have indicated that statins improve prognosis, we focused on molecules that are strongly expressed in prostate cancer cell lines following statin administration to search for new therapeutic targets.

We administered statins to PC-3 cells (a human prostate cancer cell line) and investigated gene expression using microarrays. We found that the annexin-A10 (ANXA10) gene was more strongly expressed in cells receiving statins than those not receiving statins. ANXA10 is the most recently identified member of the annexin family of calcium- and phospholipid-binding proteins [14]. In previous studies, down-regulation of ANXA10 was correlated with dedifferentiation, invasion, and tumor progression, pointing to a possible tumor suppressor role [14]. However, whether ANXA10 has a specific function in prostate cancer remains unclear. In studies of liver cancer, patients with low expression of ANXA10 were found to have a poor prognosis, early recurrence, and significantly more vascular infiltration by the tumor [15]. Moreover, in studies of bladder cancer, patients with low expression of ANXA10 had a significantly poorer prognosis and a higher degree of malignancy [16]. Accordingly, we investigated whether ANXA10 could be a new therapeutic target gene in prostate cancer.

MATERIALS AND METHODS

Cells and Chemicals

The human prostate cancer cell lines PC-3, LNCaP, and DU145 were purchased from Dainippon Pharmaceutical (Tokyo, Japan) and cultured in RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Moregate, Bulimba, Australia). LNCaP-LA was used as an in vitro model of castration-resistant prostate cancer; these cells were derived from LNCaP cells cultured with 10% charcoal-stripped FBS for 3 months.

Rabbit anti-human β-actin monoclonal antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-ANXA10 polyclonal antibody was purchased from Abnova (Taipei, Taiwan). Anti-S100A4 rabbit anti-S100A4 antibody was purchased from Novus Biologicals (Littleton, CO). Simvastatin was purchased from Calbiochem (San Diego, CA).

cDNA Microarray

Cells were seeded into a 12-well microtiter plate with 10% FBS for 48 h. The medium was then aspirated and the cells were incubated with culture medium containing simvastatin (0 or 5 μM). After incubation at 37°C in 5% CO_2 for 48 hr, total RNA was extracted using an miRNeasy Mini Kit (Qiagen, Valencia, CA). RNA quality and quantity were measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Microarray experiments were carried out using a Human Gene Expression ver. 2 4×44 K Microarray Kit (Agilent Technologies, Santa Clara, CA) with 50 ng of total RNA as starting material according to the manufacturer's protocol (Hokkaido Systems Science Co., Ltd., Sapporo, Japan). Probes showing significantly different expression were extracted with the filtering criteria of a 8.0-fold change and $P < 0.001$

using GeneSpring GX (ver. 7.3) after per-chip and per-gene normalization. Differences between the values were evaluated by unpaired *t*-test.

Cell Proliferation Assay of Human Prostate Cancer Cells

Cells were seeded into a 96-well microtiter plate in 100 μl of medium with 10% FBS for 48 hr. The medium was then aspirated and the cells were incubated with medium containing various concentrations of simvastatin and various reagents (siRNA for ANXA10 or S100A4). After incubation at 37°C in 5% CO_2 for 48 hr, the number of living cells was measured using an MTS assay (Celltiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI). The absorbance of the cell lysate was expressed as the fold change over the control.

Migration Assay

Cells were plated on a 12-well plate and grown to confluence. The medium was then aspirated, and the cells were incubated in medium containing 10% FBS for 24 hr before each experiment. One-thousand-microliter tips were used to make a denuded area. Cells were washed twice with phosphate-buffered saline (PBS) and incubated with or without simvastatin for 24 hr. Mitomycin C (0.5 mM) was added to block cell proliferation during the entire 48-hr period of the study. Photographs of PC-3 were taken at 0 and 48 hr, LNCaP-LA were taken at 0 and 96 hr, and the cell migration distance was determined by subtracting the values. Quantitation was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Migration distances are expressed as the fold change over the control.

Invasion Assay

Matrigel invasion chamber plates (Becton Dickinson/Biocoat, Bedford, MA) were used for this assay. Cells were incubated in RPMI-1640 containing 10% FBS for 24 hr before each experiment. Cells were plated in the upper chamber with RPMI-1640 containing simvastatin; the lower chamber contained RPMI-1640 plus 10% FBS and the same test ligands as the upper chamber. After 48 hr, non-invading cells were removed using a cotton swab. The number of cells that adhered to the bottom surface of the membrane was counted with a microscope in several fields of the membranes. The invasion index was expressed as the fold change over the control.

Quantification of mRNA Levels

Transcript levels were quantified using a CFX96 Real-Time System (Bio-Rad, Hercules, CA). Total RNA extraction and cDNA synthesis were performed as described previously. Amplification was performed in 10 μl Premix Ex Taq (TaKaRa, Tokyo, Japan) using 2 μl cDNA and ANXA10 (No. Hs01105012_m1) and S100A4 (No. Hs00243202_m1) primer (Applied Biosystems, Foster City, CA). Next, PCR was performed for one cycle of 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. The transcription of β-actin (Applied Biosystems) was also examined as an internal control. Relative quantitation values were calculated using the comparative C_T method, also known as the $2^{-\Delta\Delta CT}$ method.

Western Blotting Assays

Cell lysates were prepared in RIPA buffer (Pierce, Rockford, IL) and protease inhibitors (Complete, without EDTA; Roche Diagnostics, Penzberg, Germany). Equal amounts of proteins (30–40 μg/lane) were electrophoresed on 4–12% SDS–PAGE gels and then transferred to nitrocellulose membranes. Each membrane was incubated with the primary antibodies described above. Blots were developed using a 1:1,000 dilution of a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Inc.). Proteins were visualized using Immobilon Western HRP Reagents (Millipore, Billerica, MA).

siRNA

Cells were seeded into a 12-well microtiter plate with 10% FBS. The cells were then transfected with ANXA10 siRNA (On-Target plus Human ANXA10 siRNA; GE Healthcare, England) or S100A4 siRNA (On-Target plus Human S100A4 siRNA, GE Healthcare) using DharmaFect 2 (GE healthcare). After transfection, the cells were incubated for 48 hr at 37°C in a 5% CO_2 atmosphere. We used another ANXA10 siRNA (siGENOME Human ANXA10 siRNA; Dharmacon, GE Healthcare, England) and S100A4 siRNA (siGENOME Human S100A4 siRNA; Dharmacon, GE Healthcare, England) to rule out potential off-target effects of siRNAs.

Overexpression of ANXA10

We used plasmid DNA that encoded ANXA10 and Myc-DDK-tagged ORF clone ANXA10 as transfection-ready DNA (OriGene Technologies Inc., Rockville, MD) for overexpressing ANXA10 in PC-3 cells. We prepared an empty vector (OriGene Technologies Inc.)

as a control. We transfected plasmid DNA to PC-3 cells with jetPRIME reagent (Polyplus Transfection, Illkirch, France) (2 μg DNA per 2×10^6 cells in 5 μl jetPRIME reagent).

Animal Studies

Four-week-old male BALB/c/nu mice (Charles River Laboratories Japan, Yokohama, Japan) were injected subcutaneously into the right flank with approximately 3×10^6 PC-3 cells in BD Matrigel basement membrane matrix (BD Biosciences, Bedford, MA). Simvastatin treatment was initiated 1 day earlier. Five mice were used per group. Mice also received three intraperitoneal injections per week with PBS alone (control) or 5 or 50 mg/kg simvastatin. Tumor size was measured weekly during the following 4 weeks, and tumor volume was calculated using the formula $A \times B \times C \times \pi/6$, where A, B, and C are the length, width, and height of the tumor, respectively. Mice were killed 29 days after the xenograft, and the tumors were collected.

All animal experiments were approved by the Animal Care and Experimentation Committee, Gunma University, Showa Campus, Japan and conducted according to the guidelines of the committee.

Prostate Biopsy Sample Analysis

Prostate biopsy samples from 60 patients were collected at Gunma University Hospital from 2002 to 2007. Of them, 20 patients had benign prostatic hyperplasia (BPH), 20 had prostate cancer with a Gleason score of 7, and 20 had prostate cancer with a Gleason score of 8–10. None had received any therapy before the prostate biopsy. ANXA10 mRNA levels were measured in prostate biopsy samples using quantitative real-time PCR as described above, and β-actin was used as a control. The Ethical Committee of Gunma University approved this study.

Immunohistochemistry Assay

Prostate biopsy samples from 15 patients were collected at Gunma University Hospital from 2002 to 2007. Of them, five patients had benign prostatic hyperplasia (BPH), five had prostate cancer with a Gleason score of 7, and five had prostate cancer with a Gleason score of 8–10. None had received any therapy before the prostate biopsy. For Immunohistochemistry (IHC) assay, tissue sections were de-paraffined with xylene, and rehydrate through an ethanol series and PBS. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min, followed by incubation with Protein Block (Genostaff, Japan). The sections were incubated with anti-ANXA10 rabbit polyclonal antibody (Abnova, Taiwan) at 4°C overnight. They were incubated with histofine simple stain MAX-PO (Multi) (Nichirei, Japan) for 30 min. Peroxidase activity was visualized by diaminobenzidine. The sections were counterstained with Mayer's Hematoxylin (Muto, Tokyo, Japan), dehydrated, and then mounted with Malinol (Muto). To quantify the state of ANXA10 protein expression in human samples, we calculated IHC score. The ANXA10 IHC scores are calculated as follows: IHC score = Proportion score(0: 0%, 1: <1%, 2: 1–10%, 3: 11–33%, 4: 34–66%, 5: 67–100%) + Intensity score(0: none, 1: weak, 2: intermediate, 3: strong). Two urologists, neither of whom had knowledge of the patients' clinical status, made these judgments.

Statistical Analysis

All data, unless otherwise indicated, are expressed as mean ± SD. Differences between values were evaluated by one-way ANOVA, with Tukey's post hoc analysis for more than three groups. ANOVA was also used to compare tumor size in mice after different treatments. In all analyses, P values of <0.05 were considered to indicate statistical significance.

RESULTS

Microarray

Microarray analysis was performed on PC-3 cells after simvastatin treatment. Many genes changed in response to simvastatin. To identify candidate genes affected by simvastatin, the criterion of an >8-fold change was adopted for gene selection. More than 100 genes were up-regulated (Supplemental Table SI). Of these genes, we focused on ANXA10, which has been correlated with dedifferentiation, invasion, and tumor progression, pointing to a possible tumor suppressor role [14].

Simvastatin Inhibited Proliferation, Migration, Invasion, and ANXA10 Expression of Prostate Cancer Cells

First, we examined the effects of simvastatin on prostate cancer cell proliferation using the MTS assay and cell counting. Numbers of viable PC-3, DU145, and LNCaP-LA cells in 10% FBS medium decreased

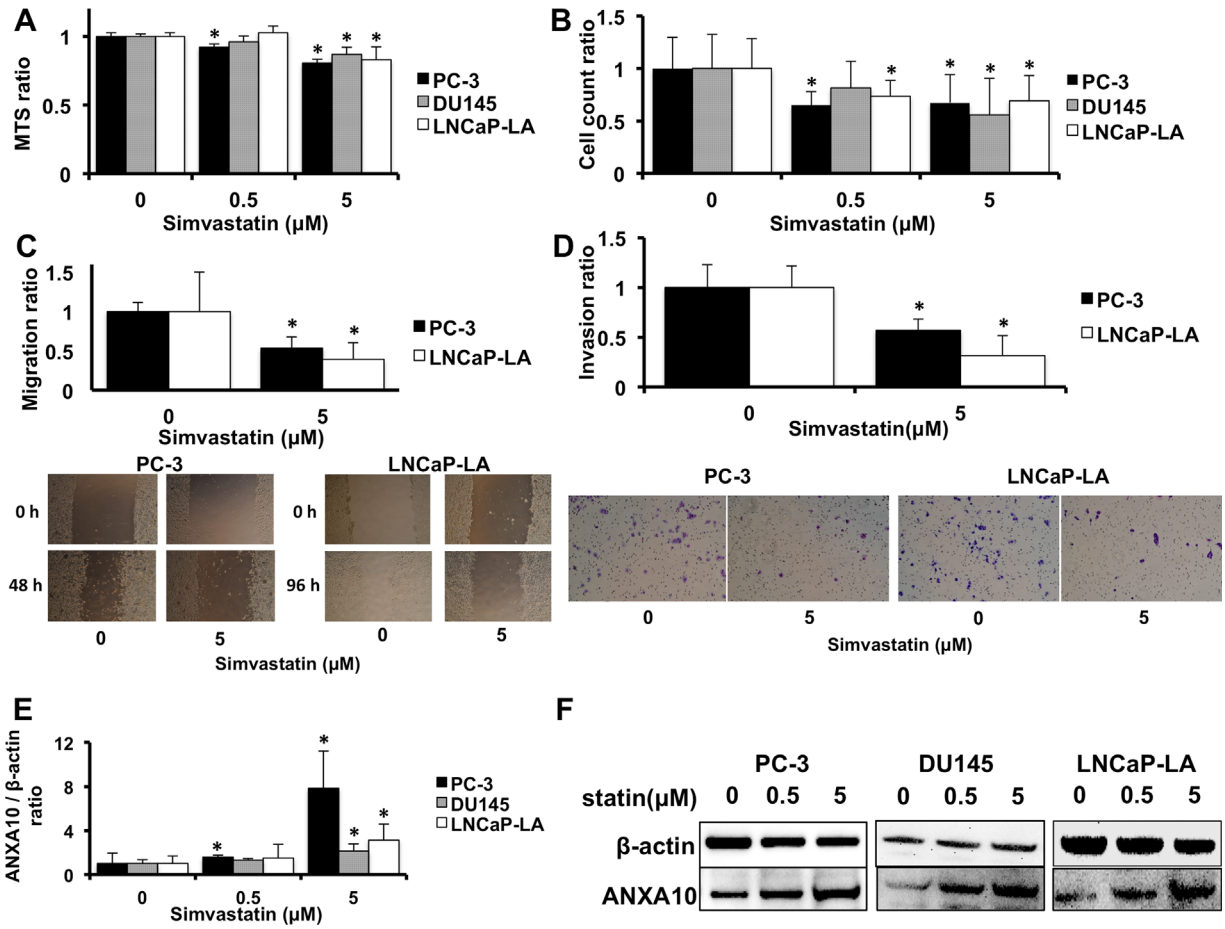

Fig. 1. Effects of simvastatin on androgen-independent prostate cancer cells. (**A**) Cells were incubated in culture medium containing 10% FBS with or without simvastatin (0, 0.5, and 5 μM). After 48 hr, the number of viable cells was determined using the MTS assay. Columns, mean (n = 8); bars, SD; $^{*}P < 0.01$ versus simvastatin 0 μM. (**B**) Cells were incubated in culture medium containing 10% FBS with or without simvastatin(0, 0.5, and 5 μM). After 48 hr, the number of viable cells was determined by cell counting. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus simvastatin 0 μM. (**C**) Cells were wounded and then cultured in medium containing 10% FBS with or without simvastatin (0 or 5 μM) for 48 hr (PC-3) and 96 hr (LNCaP-LA). Cell migration into the wound was examined via phase-contrast microscopy. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus simvastatin 0 μM. (**D**) After culturing cells with simvastatin, the numbers of cells that invaded through the Matrigel were counted under the microscope. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus simvastatin 0 μM. (**E**) Cells were incubated in culture medium containing 10% FBS with or without simvastatin (0, 0.5, and 5 μM). After 48 hr, mRNA was isolated and ANXA10 mRNA levels were measured via quantitative real-time PCR. Columns, mean (n = 4); bars, SD; $^{*}P < 0.01$ versus 0 μM. (**F**) ANXA10 protein levels were measured by western blotting. β-actin protein levels were used as the internal control. Cells were incubated in medium containing 10% FBS with or without simvastatin (0, 0.5, and 5 μM). After 48 hr, total cell proteins were extracted.

significantly after 48 hr of incubation with simvastatin (Fig. 1A and B).

We also examined whether simvastatin inhibited the migration of PC-3 and LNCaP-LA cells in a wound healing assay. We found that simvastatin significantly inhibited the migration of PC-3 and LNCaP-LA cells (Fig. 1C). It also significantly inhibited the invasion of PC-3 and LNCaP-LA cells in a Matrigel invasion chamber (Fig. 1D).

Next, we examined whether simvastatin could increase the expression of ANXA10 in PC-3, DU145, and LNCaP-LA cells. ANXA10 mRNA expression levels increased significantly after 48 hr of incubation in the presence of simvastatin in PC-3, DU145, and LNCaP-LA cells (Fig. 1E). ANXA10 protein expression increased in all cell lines (Fig. 1F).

Overexpression of ANXA10 Inhibited Proliferation, Migration, and Invasion of Prostate Cancer Cells

We examined whether overexpression of ANXA10 could inhibit the proliferation of PC-3 and LNCaP-LA cells. After transfection of ANXA10 to PC-3 and LNCaP-LA cells, we confirmed that ANXA10 protein expression increased in PC-3 and LNCaP-LA cells

(Fig. 2A). The numbers of viable PC-3 and LNCaP-LA cells in 10% FBS medium decreased significantly after 48 hr of incubation with overexpression of ANXA10 (Fig. 2B). We found that the migration of PC-3 and LNCaP-LA cells with overexpression of ANXA10 was significantly inhibited (Fig. 2C). The invasion of PC-3 and LNCaP-LA cells in a Matrigel invasion chamber was also significantly inhibited (Fig. 2D).

ANXA10 Knockdown-Induced Proliferation, Migration and Invasion of Prostate Cancer Cells

Next, we used siRNA to inhibit ANXA10 gene expression in PC-3 and LNCaP-LA cells. ANXA10 protein expression decreased significantly following siRNA treatment in PC-3 and LNCaP-LA cells (Fig. 3A). We then evaluated the effect of ANXA10 knockdown by siRNA on PC-3 and LNCaP-LA cell proliferation, migration, and invasion. ANXA10 knockdown significantly induced PC-3 and LNCaP-LA cell proliferation, migration, and invasion (Fig. 3B–D). Considering the significance of ruling out the potential off-target effects of siRNA, we perfomed the same experiments using another ANXA10 siRNA. We also observed ANXA10 knockdown by another siRNA significantly induced PC-3 and LNCaP-LA cell proliferation, migration, and invasion (Supplemental Fig. S1). Next, we evaluated whether there is no effect of statins following ANXA10 siRNA treatment in PC-3 and LNCaP-LA. We found that simvastatin has effects on proliferation, migration, and invasion similarly when ANXA10 is knocked down (Fig. 3E–G). These results indicated that the pathway via ANXA10 is one of the antitumor mechanisms by statins.

Effect of Simvastatin on PC-3 Cell Growth and ANXA10 Expression In Vivo

To determine whether simvastatin affected tumor growth in vivo, we evaluated a xenograft model with PC-3 cells. Simvastatin did not affect body weight (data not shown). However, treatment with

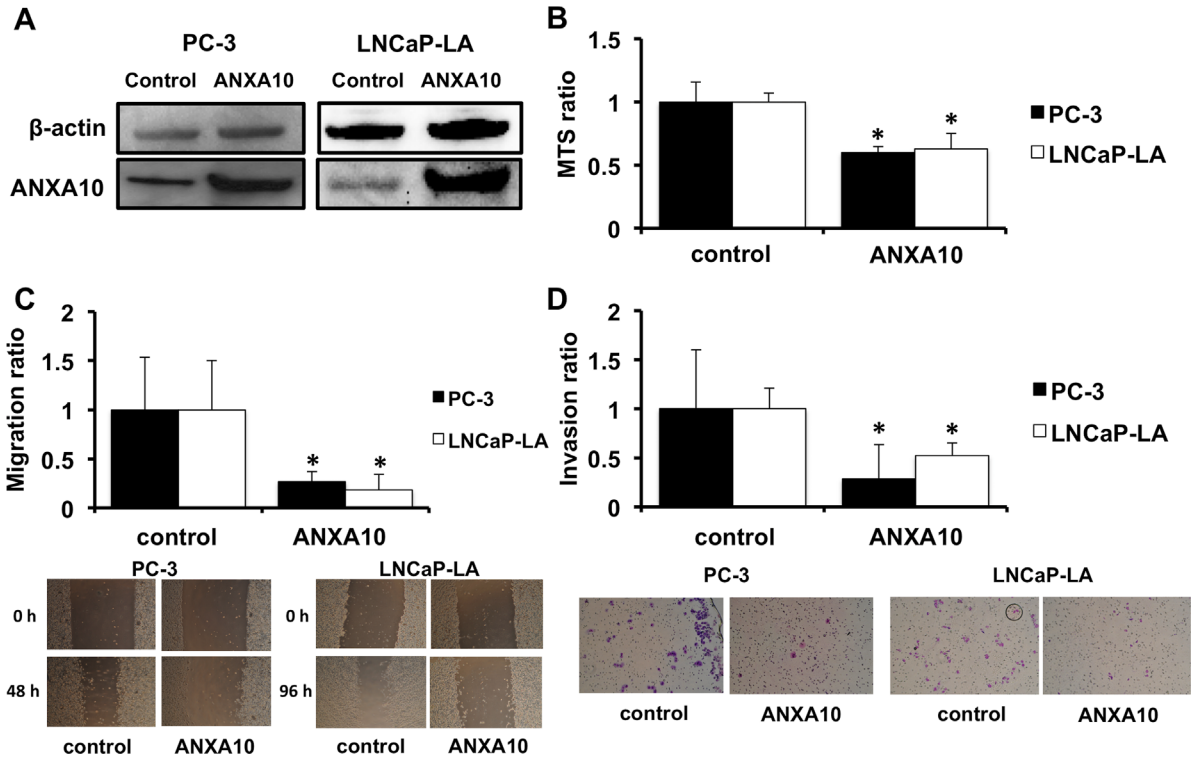

Fig. 2. Effects of ANXA10 overexpression on PC-3 and LNCaP-LA cells. (**A**) The effects of the ANXA10 vector on ANXA10 expression in PC-3 and LNCaP-LA cells were evaluated. Cells transfected with ANXA10 or control vector were incubated for 48 hr before western blotting. (**B**) Cells were incubated in culture medium containing 10% FBS with or without ANXA10 vector transfection. After 48 hr, the number of viable cells was determined using the MTS assay. Columns, mean (n = 8); bars, SD; $^{*}P < 0.01$ versus control. (**C**) Cells were wounded and then cultured in medium containing 10% FBS with or without ANXA10 vector transfection. Cell migration into the wound was examined via phase-contrast microscopy. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus control. (**D**) After culturing cells with ANXA10 vector transfection, the numbers of cells that invaded through the Matrigel were counted under the microscope. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus control. Control, empty vector-transfected; ANXA10, ANXA10 vector-transfected.

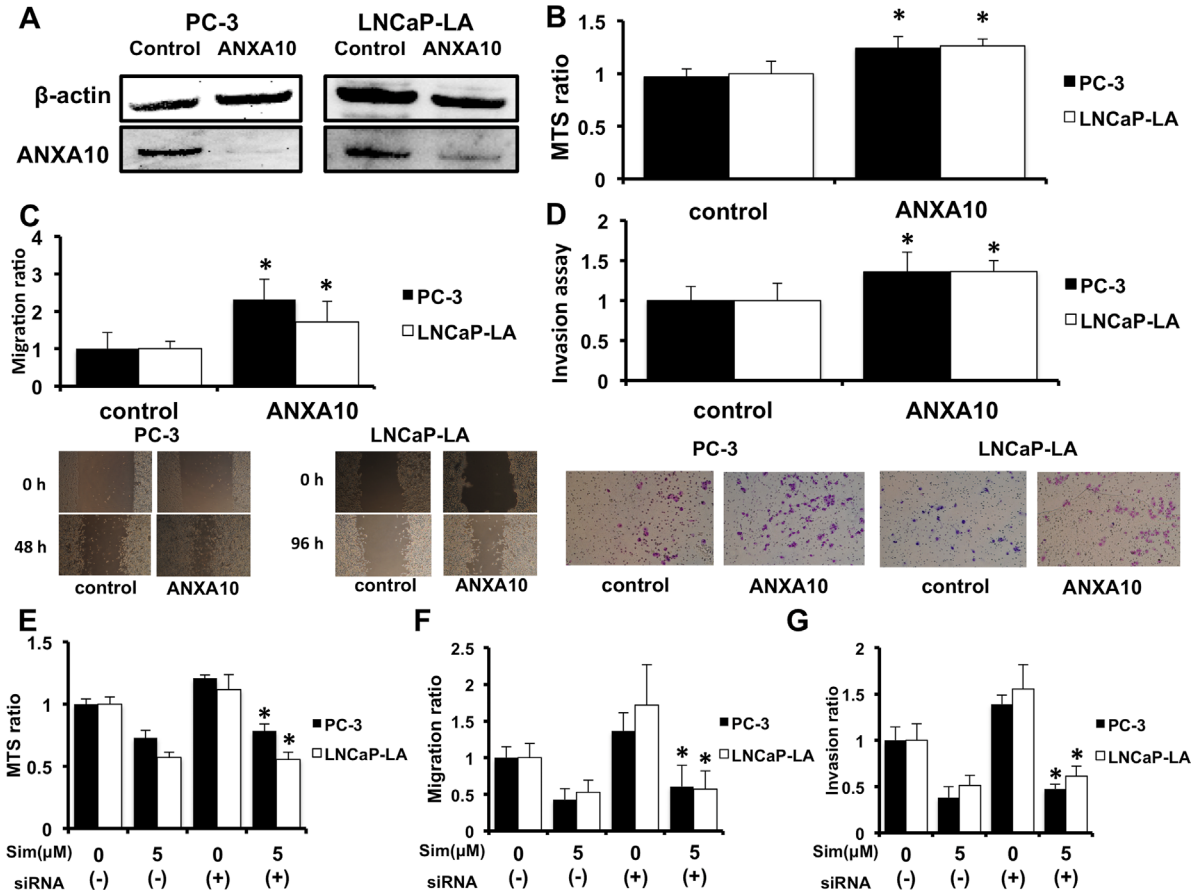

Fig. 3. Effects of ANXA10 knockdown via siRNA in PC-3 and LNCaP-LA cells. (**A**) ANXA10 expression in PC-3 and LNCaP-LA cells transfected with ANXA10 siRNA was evaluated. Cells transfected with ANXA10 siRNA or control were incubated for 48 hr before western blotting. (**B**) Cells were incubated in culture medium containing 10% FBS with or without ANXA10 siRNA transfection. After 48 hr, the number of viable cells was determined using the MTS assay. Columns, mean (n = 8); bars, SD; $^{*}P < 0.01$ versus control. (**C**) Cells were wounded and then cultured in medium containing 10% FBS with or without ANXA10 siRNA transfection. Cell migration into the wound was examined via phase-contrast microscopy. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus control. (**D**) After culturing cells with ANXA10 siRNA transfection, the numbers of cells that invaded through the Matrigel were counted under the microscope. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus control. Control, transfected with negative siRNA; ANXA10, transfected with ANXA10 siRNA. (**E**) Cells were incubated in culture medium containing 10% FBS with or without ANXA10 siRNA transfection and with or without 5 μM simvastatin. After 48 hr, the number of viable cells was determined using the MTS assay. Columns, mean (n = 8); bars, SD; $^{*}P < 0.01$ versus Sim 0 μM/siRNA(+). (**F**) Cells were wounded and then cultured in medium containing 10% FBS with or without ANXA10 siRNA transfection and with or without simvastatin. Cell migration into the wound was examined via phase-contrast microscopy. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus Sim 0 μM/siRNA(+). (**G**) After culturing cells with or without ANXA10 siRNA transfection and with or without simvastatin, the numbers of cells that invaded through the Matrigel were counted under the microscope. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus Sim 0 μM/siRNA(+).

simvastatin (5 and 50 mg/kg) significantly reduced tumor growth by 36–41% relative to the PBS-injected control (Fig. 4A). To determine whether simvastatin affected ANXA10 transcription in vivo, we examined ANXA10 mRNA expression in xenografts after treatment with simvastatin. Consistent with the tumor growth data, simvastatin significantly increased AXNA10 mRNA expression in tumor cells at doses of 5 and 50 mg/kg/day (Fig. 4B). These results suggest that similar to our in vitro observations, simvastatin decreased tumor growth and increased ANXA10 expression in PC-3 cells in vivo.

ANXA10 Gene Expression in Human Prostate Tissue

To evaluate the relationship between ANXA10 and prostate cancer, ANXA10 mRNA levels were examined by RT-PCR in 60 prostate biopsy samples. Compared with biopsy samples from patients with

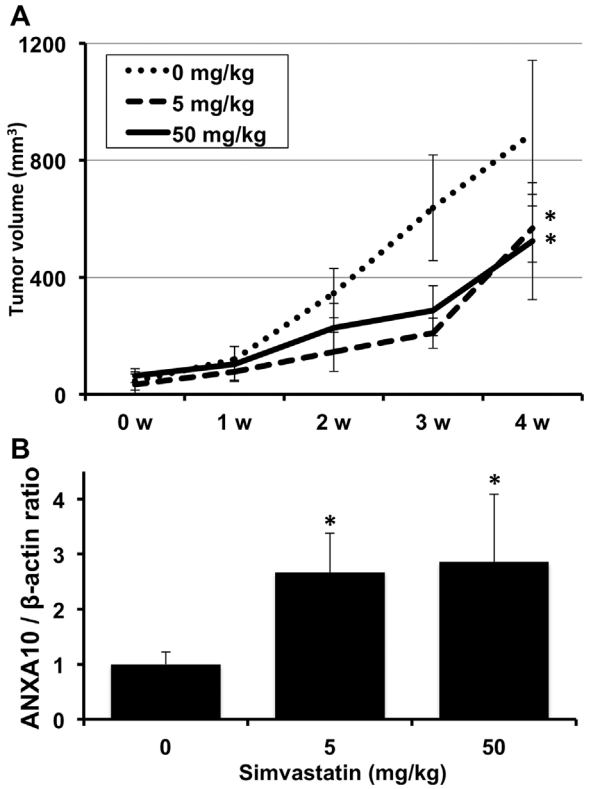

Fig. 4. Effects of simvastatin on tumor growth and ANXA10 expression in vivo using a PC-3 xenograft model. (**A**) Graphic view of mean tumor volumes in PC-3 xenografts. Mice were injected with PC-3 cells (3×10^6 per site) and then subsequently injected intraperitoneally with PBS (control) or 5 or 50 mg/kg of simvastatin thrice per week. Line graphs, mean ($n = 5$); bars, SD; $^*P < 0.01$ versus control at 5 weeks. (**B**) ANXA10 mRNA expression was analyzed via quantitative real-time PCR and compared with that in PBS-treated controls. Columns, mean ($n = 5$); bars, SD; $^*P < 0.01$ versus control.

BPH, samples from patients with prostate cancer with a Gleason score of 7 had, on average, 20% of the level of ANXA10 mRNA observed in BPH samples (Fig. 5A). Biopsy samples from patients with more advanced cancer (Gleason score of 8–10) also had decreased ANXA10 mRNA and contained 33% of the levels observed in BPH samples (Fig. 5A). We found that ANXA10 mRNA levels were significantly lower in human prostate cancer samples than in BPH samples. There was no significant difference in ANXA10 mRNA levels between the group with a Gleason score of 7 and the group with a Gleason score of 8–10. To determine whether ANXA10 was mainly expressed in luminal epithelial cells of human prostate tissue, we performed IHC assay in human biopsy samples. We found that ANXA10 was mainly expressed in cytoplasm of luminal epithelial cells and ANXA10 was stained strongly and widely in BPH samples as compered to GS 7 and GS 8–10 samples. The ANXA10 IHC scores of BPH samples were significantly higher than GS 7 and GS 8–10 samples (Fig.5B). We showed representative images of IHC assay (Fig.5C).

Up-Regulation of ANXA10 Inhibited the Proliferation, Migration, and Invasion of PC-3 Cells via Down-Regulation of S100A4

We found that up-regulation of ANXA10 may decrease the proliferation, migration, and invasion in PC-3 cells. Next, we focused on the relationship between ANXA10 and S100A4. S100A4 is a member of the S100 family of calcium-binding proteins and is directly involved in tumor metastasis [17]. S100A4 expression is higher in prostate cancer than in normal tissue, suggesting that enhanced S100A4 expression contributes to the manifestation of a metastatic phenotype [17]. Munksgaard et al. [16] reported that down-regulation of ANXA10 induced up-regulation of S100A4 in bladder cancer.

We examined whether overexpression of ANXA10 could inhibit the expression of S100A4. We found that the expression of S100A4 mRNA in PC-3 cells was significantly inhibited by overexpression of ANXA10 (Fig. 6A). We found that simvastatin induced down-regulation of S100A4 mRNA (Fig. 6B). We also examined whether simvastatin could inhibit the expression of S100A4 in PC-3 cells. S100A4 protein expression also decreased in PC-3 cells administered simvastatin (Fig. 6C). Next, we used siRNA to inhibit S100A4 gene expression. S100A4 protein expression decreased significantly following siRNA treatment in PC-3 cells (Fig. 6D). S100A4 knockdown significantly decreased cell proliferation, migration, and invasion (Fig. 6E–G, respectively). We perfomed the same experiment using another S100A4 siRNA to rule out potential off-target effects of siRNAs. We also observed S100A4 knockdown by another siRNA significantly decreased PC-3 and LNCaP-LA cell proliferation, migration, and invasion (Supplemental Fig. S2). Next, we evaluated S100A4 mRNA and protein expressions in PC-3 cells with or without knockdown of ANXA10. We found that S100A4 mRNA and protein expressions were up-regulated in PC-3 cells with ANXA10 knockdown versus PC-3 cells without ANXA10 siRNA transfection (Fig. 6H and I). We observed down-regulation of S100A4 mRNA and protein with simvastatin administration, but down-regulation of S100A4 mRNA and protein expressions were not observed in PC-3 cells that were transfected with ANXA10 siRNA with administration of simvastatin (Fig. 6H and I). These results suggest that ANXA10 plays an important role

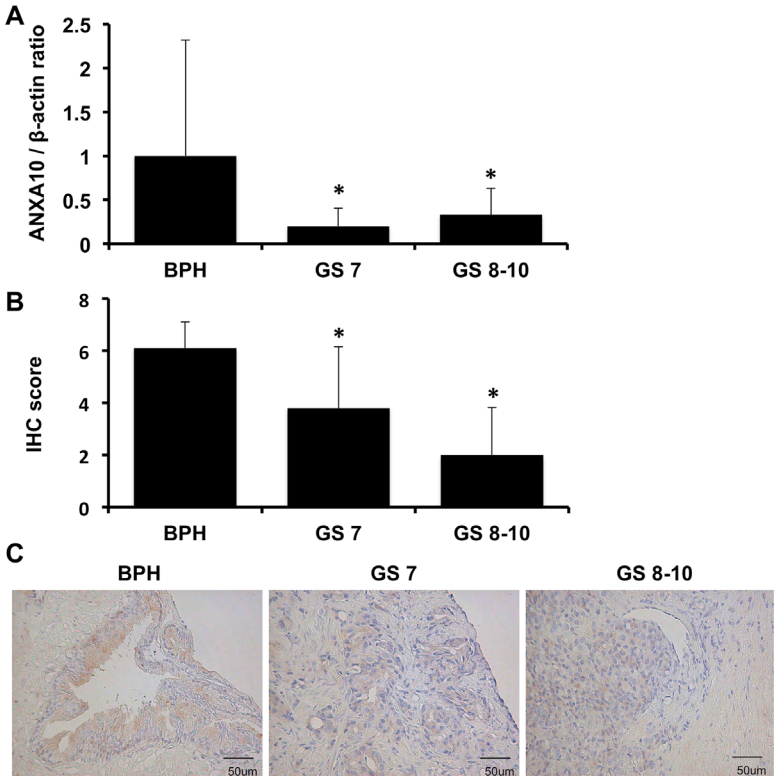

Fig. 5. ANXA10 gene and protein expressions in prostate biopsy samples. (**A**) mRNA expressions of ANXA10 in benign prostatic hyperplasia (BPH; n = 20), prostate cancer with a Gleason score of 7 (GS 7; n = 20), and prostate cancer with a Gleason score of 8–10 (GS 8–10; n = 20) were evaluated by real-time PCR. Expression values are expressed as the fold change compared with BPH. Values are expressed as the mean + SD. $^*P < 0.05$ versus BPH. (**B**) Protein expressions of ANXA10 were evaluated by IHC assay in human biopsy samples. Values are expressed as the mean + SD. $^*P < 0.05$ versus BPH. (**C**) We showed representative images of IHC assay in BPH, GS 7, and GS 8–10 samples. Length of bar indicated 50 μm in image.

in the down-regulation of S100A4 caused by administration of simvastatin in PC-3 cells.

DISCUSSION

The main finding from this study is that the pathway via ANXA10 is one of the antitumor mechanisms by statins in prostate cancer cells. To our knowledge, this is the first report to show that statins increase the expression of ANXA10 and that up-regulation of ANXA10 induces decreased expression of S100A4, a well-known inducer of invasion and metastasis [17].

There has been some discussion regarding the association of statin use and prostate cancer. Bansal et al. [18] examined the association of statin use and the risk of prostate cancer. They evaluated 27 studies (15 cohort and 12 case-control) from the PubMed database. They reported that statin use significantly reduced the risk of total prostate cancer by 7% (relative risk [RR] = 0.93, 95% confidence interval [CI] = 0.87–0.99, $P = 0.03$) and clinically important advanced prostate cancer by 20% (RR = 0.80, 95%CI = 0.70–0.90, $P = 0.001$) [18]. Katz et al. [19] found that statin use was associated with a reduced risk of all-cause mortality after radical prostatectomy (hazard ratio [HR] = 0.35, 95%CI = 0.21–0.58) and radiation therapy (HR = 0.59, 95% CI = 0.37–0.94). Geybels et al. [20] investigated 1,001 patients with prostate cancer and found a large reduction in prostate cancer-specific mortality in those who were administered statins (HR = 0.19, 95%CI = 0.06–0.56). However, Platz et al. [21] performed a cohort study of 9,457 men aged ≥55 years at randomization and estimated the multivariable-adjusted HR of prostate cancer (574 cases in 62,192 person-years) for statin drug use. They reported that statin use was not associated with the risk of total prostate cancer (HR = 1.03, 95%CI = 0.82–1.30) or lower-grade (HR = 0.96, 95%CI = 0.71–1.29) or higher-grade (HR = 1.27, 95%CI = 0.85–1.90) prostate cancer. They concluded that statin drug use did not protect against prostate cancer [21]. Park et al. [22] evaluated the association between statin use and the recurrence of prostate cancer after radical prostatectomy and radiation therapy.

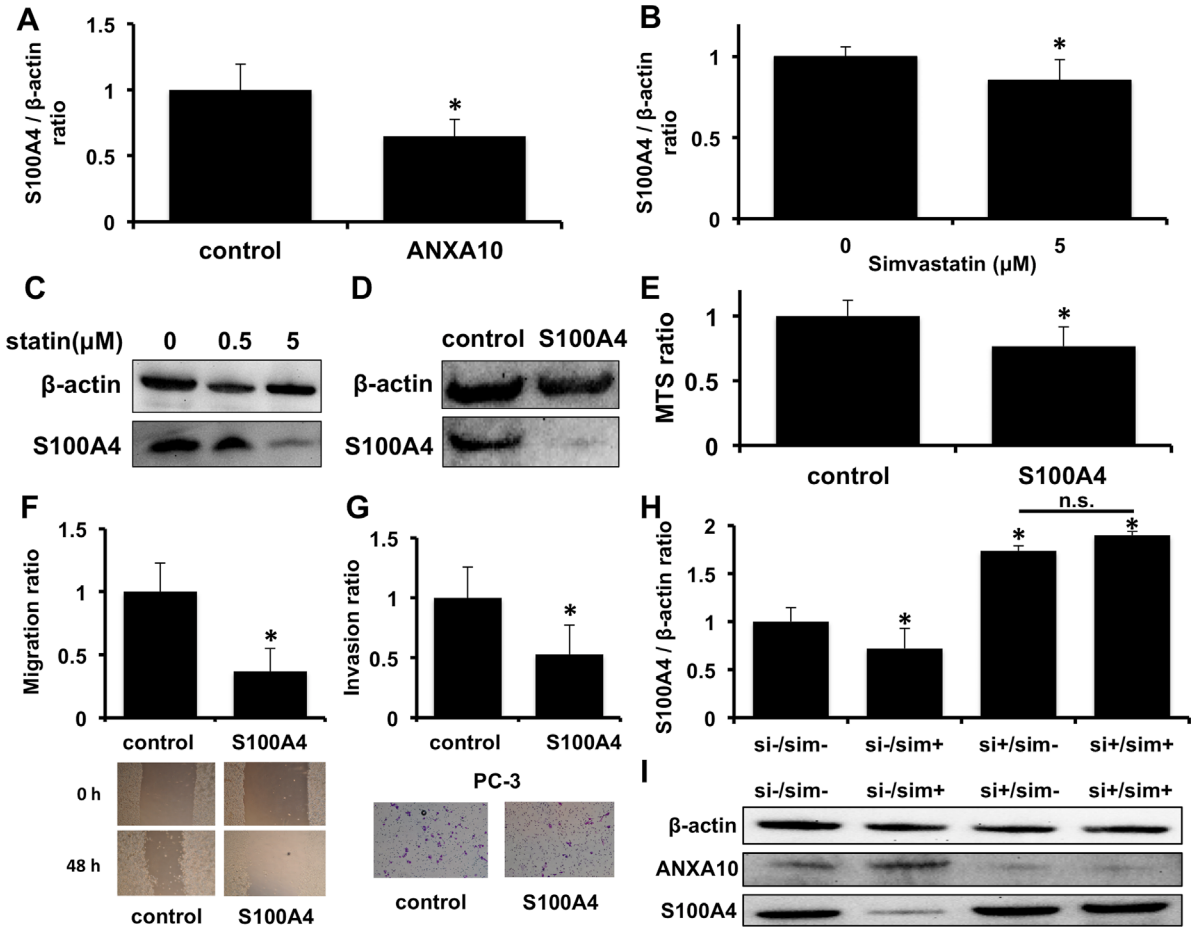

Fig. 6. Effects of S100A4 on PC-3 cells. (**A**) Cells were incubated in culture medium containing 10% FBS with or without ANXA10 vector transfection. After 48 hr, mRNA was isolated and S100A4 mRNA levels were measured via quantitative real-time PCR. Control; empty vector-transfected, ANXA10; ANXA10 vector-transfected. Columns, mean (n = 4); bars, SD; $^{*}P < 0.01$ versus control. (**B**) Cells were incubated in culture medium containing 10% FBS with or without simvastatin (0 or 5 μM). After 48 hr, mRNA was isolated and S100A4 mRNA levels were measured via quantitative real-time PCR. Columns, mean (n = 4); bars, SD; $^{*}P < 0.01$ versus 0 μM. (**C**) S100A4 protein levels were measured by western blotting. β-actin protein levels were used as the internal control. Cells were incubated in medium containing 10% FBS with or without simvastatin (0, 0.5 and 5 μM). After 48 hr, total cell proteins were extracted. (**D**) S100A4 expression in PC-3 cells transfected S100A4 siRNA was evaluated. Cells transfected with S100A4 siRNA or control were incubated for 48 hr before western blotting. Control, negative siRNA transfected; S100A4, S100A4 siRNA transfected. (**E**) Cells were incubated in culture medium containing 10% FBS with or without S100A4 siRNA transfection. After 48 hr, the number of viable cells was determined using the MTS assay. Columns, mean (n = 8); bars, SD; $^{*}P < 0.01$ versus control. Control, negative siRNA-transfected; S100A4, S100A4 siRNA-transfected. (**F**) Cells were wounded and then cultured in medium containing 10% FBS with or without S100A4 siRNA transfection. Cell migration into the wound was examined via phase-contrast microscopy. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus control. Control, negative siRNA-transfected; S100A4, S100A4 siRNA-transfected. (**G**) After culturing cells with S100A4 siRNA transfection, the numbers of cells that invaded through the Matrigel were counted under the microscope. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus control. Control, negative siRNA-transfected; S100A4, S100A4 siRNA-transfected. (**H**) After culturing PC-3 cells with or without ANXA10 siRNA transfection, cells were incubated in culture medium containing 10% FBS with or without 5 μM simvastatin. S100A4 mRNA levels measured via quantitative real-time PCR. Columns, mean (n = 6); bars, SD; $^{*}P < 0.01$ versus si−/sim−. si−/sim−; negative siRNA transfected without simvastatin (control). si−/sim+, negative siRNA transfected with simvastatin; si+/sim−, ANXA10 siRNA transfected without simvastatin; si+/sim+, ANXA10 siRNA transfected with simvastatin; n.s., not significant. (**I**) ANXA10 and S100A4 protein levels were measured by western blotting. The results shown are representative of three independently performed experiments. si−/sim−; negative siRNA transfected without simvastatin (control). si−/sim+, negative siRNA transfected with simvastatin; si+/sim−, ANXA10 siRNA transfected without simvastatin; si+/sim+, ANXA10 siRNA transfected with simvastatin.

They suggested a potentially beneficial effect of statin use on prostate cancer in patients treated with radiation therapy (HR = 0.68, 95%CI = 0.49–0.93) but not among patients treated with radical prostatectomy (HR = 1.05, 95%CI = 0.90–1.24) [22]. Thus, whether statin use reduces the incidence or mortality of prostate cancer or recurrence after radical prostatectomy or radiation therapy remains controversial.

Recent studies have suggested that statins affect prostate cancer progression through cholesterol-mediated and pleiotropic effects [13]. Much attention has been paid to mechanisms of statins' effects on prostate cancer. Papadopoulos et al. [23] suggested that statins affect prostate carcinogenesis in two ways: first, by lowering cholesterol levels and second, by pleiotropic cholesterol-independent effects. It has been reported that membrane cholesterol seems to activate the pro-survival PI3K/Akt and EGFR signaling pathways in prostate cancer cells. Thus, lowering cholesterol levels may suppress tumor cell growth via inactivation of the PI3K/Akt and EGFR signaling pathways. Most effects of pleiotropic statins are reportedly mediated via inhibition of the synthesis of isoprenoids. Isoprenylated Rho and Ras proteins exist in the cellular membrane and control the signal transduction of various membrane receptors with tyrosine kinase activity. This activation induces a downstream cascade that leads to the recruitment of several proteins that promote cell survival. As a result, statins may inhibit prostate tumor cell growth by blocking isoprenylation [23]. Other pathways of cell signaling were reported in several studies. We examined the effects of simvastatin on IGF-1R signaling in prostate cancer PC-3 cells, and statin use was a potent inhibitor of IGF-1/IGF-1R signaling [24]. IGF-1/IGF-1R signaling is well known to occur in association with the malignant behavior of tumor cells [25].

ANXA10 is one of a large group of calcium-binding proteins participating in diverse and important biological processes. ANXA10 is the most recently identified member of the annexin family, and its biological functions are not yet well defined [14]. Several recent reports have shown that down-regulation of ANXA10 has malignant effects in tumor cells. ANXA10 down-regulation is associated with a malignant phenotype in hepatocytes, vascular invasion, and progression of hepatocellular carcinoma [15]. Additionally, ANXA10 and p53 expression were inversely correlated with each other and with clinical outcomes in patients of hepatocellular carcinoma [15]. In bladder cancer, low expression of ANXA10 was correlated with shorter progression-free survival in patients and an unfavorable prognosis [16]. Kim et al. [26] also suggested that down-regulation of ANXA10 might be involved in gastric carcinogenesis. In conclusion, it seems that ANXA10 may have an antitumor effect.

We showed that a statin induced up-regulation ANXA10 mRNA and protein in the prostate cancer cell lines PC-3, DU145, and LNCaP-LA (Fig. 1E and F) and up-regulation of ANXA10 mRNA in a xenograft mouse model that was administered statins (Fig. 4). Up-regulation of ANXA10 decreased cell proliferation, migration, and invasion in PC-3 cells. Correspondingly, down-regulation of ANXA10 increased cell proliferation, migration, and invasion in PC-3 cells, and cell proliferation and invasion in LNCaP-LA cells (Figs. 2 and 3). Our results suggest that ANXA10 may have a role in regulating cell growth, migration, and invasion in androgen independent prostate cancer cells, similar to several other reports. Interestingly, our investigation of prostate biopsy samples showed that ANXA10 mRNA expression was higher in patients with BPH than in those with prostate cancer (Fig. 5). Our results indicate that ANXA10 might be a useful clinical marker for predicting the presence of prostate cancer.

Next, we investigated the association between ANXA10 and S100A4, which is known for its role in the metastatic spread of tumor cells [17]. Munksgaard et al. [16] reported that down-regulation of ANXA10 induced up-regulation of S100A4 in a bladder cancer cell line (SW780). Matsubara et al. [27] also reported that S100A4 suppressed the expression of ANXA10. These data show that there are relationships between ANXA10 and S100A4. S100A4 is a member of the S100 family of calcium-binding proteins and is directly involved in tumor metastasis. Siddique et al. [28] suggested that S100A4 is not merely a metastatic protein but also an oncoprotein that promotes prostate tumor genesis via regulation of NF-κB through the RAGE receptor. Agerbaek et al. [29] investigated the expression of S100A4 protein in 108 patients with bladder cancer and found that S100A4 protein expression was an independent predictor of distant metastatic relapse and metastasis-free survival in a multivariate analysis. In another study, S100A4 levels were increased in prostatic tissues of patients with prostate cancer [30], and S100A4-positive tumors grew at a faster rate than S100A4-negative tumors in vitro and in a xenograft mouse model [31]. Expression of S100A4 mRNA decreased in PC-3 cells transfected with ANXA10 in the present study (Fig. 6A).

We showed statin-induced down-regulation of S100A4 mRNA and protein in PC-3 cells (Fig. 6B and C) and found that down-regulation of S100A4 decreased cell proliferation, migration, and invasion in PC-3 cells (Fig. 6E–G). The biological

role of ANXA10 remains unclear, but it seems that ANXA10 is associated with S100A4 (Fig. 6H). Down-regulation of S100A4 may be a signal induced by up-regulation of ANXA10 in the antitumor effects of statins. This study is important because it indicates that ANXA10, as a target, could be used to develop new therapeutic agents that would help in inhibiting the progression of prostate cancer as well as its metastasis.

There is a limitation in our study. Simvastatin inhibited proliferation, migration, and invasion of prostate cancer cells similarly when ANXA10 was knocked down (Fig. 3E–G). It seems that, even if we inhibited expression of ANXA10 by siRNA transfection, statin plays the role of tumor suppressor via other pathways. Many antitumor pathways by statin were reported up to date. Therefore, we think that ANXA10 knockdown did not affect simvastatin inhibition of prostate cancer cells, which cannot negate that the pathway via ANXA10 is one of the antitumor mechanisms by statins.

CONCLUSIONS

In conclusion, we found that simvastatin inhibited the proliferation, migration, and invasion of androgen-independent human prostate cancer cells via up-regulation of ANXA10. The biological role of ANXA10 remains unclear, but it seems that ANXA10 is associated with S100A4, which is a known oncoprotein and metastatic protein. It seems that statins may be beneficial in the prevention and/or treatment of prostate cancer.

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