Original Research

Resistin stimulates PC-3 prostate cancer cell growth through stimulation of SOCS3 and SOCS5 genes

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Impact statement

The development of prostate cancer (PCa) is characterized by an increased number of abnormal prostate cells due to mitogenesis and is associated with obesity. Adipocyte resistin has been found to associate with obesity and stimulate PCa cell proliferation. However, the exact mechanism of its action on PCa growth in association with SOCS is still not clear. This study investigated the pathway of SOCS3 and SOCS5 involvement in resistin modulation of PCa cell growth. In PC-3 PCa cells, we found that resistin upregulates the expression of *SOCS3* and *SOCS5*, but not *SOCS7*, genes and induces cell growth via the TLR4, ERK, p38 MAPK, JNK, PI3K, and JAK2 proteins. In addition, silencing *SOCS3* or *SOCS5* antagonizes resistin-increased cell proliferation. Therefore, we found that the SOCS3- and SOCS5-regulated pathway mediates the mitogenic effect of resistin on PC-3 cells.

Abstract
Resistin and suppressors of cytokine signaling (SOCSs) have been reported to regulate prostate cancer (PCa) cell proliferation and survival, respectively. Whether any of the SOCS molecules mediate the mitogenic effect of resistin on PCa cells is unknown. Using PC-3 human PCa cells, we found that resistin upregulates the expression of *SOCS3* and *SOCS5* mRNA, but not *SOCS7* mRNA, in a dose- and time-dependent manner. The resistin-induced increases in *SOCS3* and *SOCS5* expression and cell proliferation were prevented by pretreatment with specific inhibitors of the TLR4, ERK, p38 MAPK, JNK, PI3K, and JAK2 proteins. However, pretreatment with a TLR2 inhibitor had no effect on resistin-mediated *SOCS3* and *SOCS5* expression. In addition, the effects of resistin on *SOCS3*, *SOCS5*, and *SOCS7* mRNA levels were cell type-specific. Overexpression of either *SOCS3* or *SOCS5* enhanced further resistin-stimulated growth of PC-3 cells, whereas silencing *SOCS3* or *SOCS5* antagonized resistin-increased cell growth. Further PCa tissue analysis demonstrated higher levels of *RETN*, *TLR4*, *SOCS3*, and *SOCS5* mRNAs in cancer tissues than benign prostate hyperplasia and indicated positive correlations among *RETN*, *TLR4*, and *SOCS5*. These data suggest that SOCS5, TLR4, and, to a lesser extent, SOCS3 can mediate the mitogenic effect of resistin on PC-3 PCa cells.

Introduction

Prostate cancer (PCa) is the most common reproductive cancer in elderly men.[1] The American Cancer Society estimates that 164,690 new cases of PCa and 29,430 deaths due to PCa occurred in the United States of America in 2018.[1] The development of PCa is characterized by an increased number of abnormal prostate cells due to mitogenesis and is associated with age, race, and obesity.[2–9] The mitogenic process is regulated by endocrine, genetic, environmental, and nutritional factors.[4,6–16] Resistin, a cysteine-rich peptide hormone discovered in the adipose tissues of rodents that has a functional link between obesity and type II diabetes,[17,18] has been found to stimulate the proliferation of androgen-independent PCa cells via the PI3K/AKT pathway.[19] Although suppressor of cytokine signaling (SOCS) 3, the major resistin signaling protein, has been found to control the liver X receptor-mediated and cAMP-mediated growth of PCa cells,[20–22] whether SOCS3 mediates the stimulatory effect of resistin on the growth of PCa cells has not been demonstrated. Notably, a recent study reported that SOCS5 may transduce an oncogenic signal of microRNA (miR)-9 in the human prostate.[23] Unfortunately, the results did not demonstrate whether SOCS5 acts as a resistin signaling molecule to control PCa cell growth. No

reports have investigated whether resistin selectively regulates SOCS gene expression in PCa cells or whether any of the SOCSs can mediate the mitogenic effect of resistin on androgen-independent PCa cells. Careful examination of the signaling pathway through which resistin affects the growth of PCa cells, particularly androgen-independent PCa cells, would help in our understanding obesity-related PCa.

The signaling actions of resistin have been described extensively in non-PCa cells, but not much in PCa cells.[19,20,24,25] In non-PCa cells, resistin stimulates smooth muscle cell proliferation through the activation of ERK1/2 and PI3K,[24] and SOCS3 acts as a signaling molecule, mediating the insulin-resistant effect of resistin.[20] A recent study reported that Toll-like receptor (TLR) 4, but not TLR2, could serve as a receptor for the actions of resistin in human leukocytes.[25] In PCa cells, the overexpression of resistin increases intracellular phosphorylation of Akt and proliferation of PC-3 PCa cells.[19] *SOCS3* and *TLR4* have been associated with the development and progression of PCa, respectively.[26–34] For example, SOCS3 methylation occurs more frequently in PCa subjects compared to BPH and normal control subjects and inhibits its mRNA and protein expression,[26] and downregulation of SOCS3 causes apoptosis of PC-3, DU145, LNCaP-IL-6 + PCa cells but not parental LNCaP cells.[33] In contrast, overexpression of SOCS3 reduces cell growth and induces cell apoptosis in DU145 and TRAMP-C2 cells, but not in PC-3 cells.[27] These disparate findings may be explained by the presence of various signaling molecules or isoforms of SOCSs in different PCa cell types. This notion may be supported by the downregulation of SOCS5 during miR-9-mediated migration and invasion of M12 human PCa cells.[23] These results, taken together with the fact that SOCS3 is the major resistin signaling protein,[20] have created much controversy surrounding the possible role of the various resistin signaling molecules in regulating SOCS3 and SOCS5 expression in PCa cells and the possible involvement of SOCS3, SOCS5, and TLR4 in the resistin regulation of androgen-independent PCa cell growth. Thus, we hypothesized that resistin may regulate the expression of SOCS3 and SOCS5 genes in PCa cells in a TLR4- and PCa cell type-dependent manner in association with PCa cell growth.

This study was designed to understand the influence of resistin on the expression of *SOCS3*, *SOCS5*, and *SOCS7* in PC-3 human PCa cells. We show that resistin stimulates the expression of *SOCS3* and *SOCS5*, but not *SOCS7*, and confirm that this stimulation is mediated through the TLR4, ERK, p38 MAPK, JNK, PI3K, and JAK2 pathways, but not the TLR2 pathway. In addition, we demonstrate the specific effect of resistin on *SOCS3*, *SOCS5*, and *SOCS7* mRNA expression in different cell types. We also show that SOCS3 and SOCS5 are required for resistin-stimulated PC-3 cell growth. Our results indicate positive correlations among the levels of *SOCS5*, *RETN*, and *TLR4* mRNAs in human prostate carcinoma tissue.

Materials and methods

Chemical reagents

RPMI1640 medium, fetal bovine serum (FBS), trypsin, the protein marker, and Trizol reagent were purchased from Thermo Fisher Scientific (Waltham, MA). TissueScan Prostate Cancer cDNA Arrays I, II, and III were purchased from OriGene Technologies, Inc. (Rockville, MD). All other materials were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) unless otherwise stated.

Cell culture and treatment

PC-3 cells are the most commonly used cell line and derived from bone metastases.[35] They do not express androgen receptor (AR) and are androgen-independent.[35] To the best of our knowledge, androgen can regulate SOCS3 gene expression[36] and PCa proliferation.[29] To avoid interference by androgen, we used PC-3 cells to evaluate the effect of resistin on SOCS gene expression and PCa cell growth. In some experiments, LNCaP-FGC, DU145, and U937 cells were used for cell type-specific comparisons. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere. All human cell lines were purchased from the American Type Culture Collection (Manassas, VA). PC-3, LNCaP-FGC, DU145, and U937 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin).[9] To study the effect of resistin on SOCS gene expression in PC-3 cells, we plated 8×10^5 cells in a 6-cm dish and allowed them to attach. After 48 h, the medium was exchanged for medium lacking FBS. After 12–16 h, serum-starved cells were treated with 50 ng/mL human recombinant resistin (PeproTech, Inc., Rocky Hill, NJ) for 0, 1, 2, 4, 6, 12, or 24 h, followed by 0, 5, 12.5, 25, 50, or 100 ng/mL human recombinant resistin for 2 h. The *SOCS* mRNA levels were determined relative to *GAPDH* at each time point as described below for the real-time PCR analysis.

Real-time PCR

We used real-time PCR to determine the mRNA levels of *SOCS3*, *SOCS5*, *SOCS7*, *RETN*, *TLR4*, and *GAPDH* according to the methods of Chang *et al.*[37] Total RNA was isolated using the Trizol kit and cDNA synthesized from equal amounts (5 μg) of RNA using 100 units of M-MLV reverse transcriptase (Thermo Fisher Scientific) in the presence of 40 units of RNase inhibitor (Thermo Fisher Scientific). The adapter primer was 5′-GGCCACGCGTCGACTAGTAC(T)$_{19}$-3′. Real-time PCR was performed twice in duplicate using the power SYBR green PCR master mix (Kapa Biosystems, Boston, MA) and ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturing cycle at 95°C for 5 min, followed by 40 amplification cycles comprising denaturation at 95°C for 3 s and annealing at 60°C for 30 s. The forward and reverse primers are given in Table 1. *GAPDH* mRNA levels were determined in parallel reactions for normalization. The relative expression of *SOCS* transcripts to *GAPDH* transcript was calculated as described elsewhere[37,38] and then expressed as a fold or percentage of the control. To validate the amplification specificity of the primers for qRT-PCR, a dissociation curve analysis performed from 60°C to 95°C at a rate of 0.1°C/s after the PCR indicated a single peak for *RETN*, *TLR4*, *SOCS3*, *SOCS5*, *SOCS7*, or *GAPDH* (Supplemental Figure S1). The amplification efficiency for each amplicon was close to or greater than 83% (Supplemental Figure S2).

Table 1. Primers for the detection of *SOCS3*, *SOCS5*, *SOCS7*, *RETN*, *TLR4*, and *GAPDH* genes expression by real-time PCR.

Genes	Sequence of primers	Accession number (sizes of PCR product)
SOCS3	FP: 5′-GGGGAGTACCACCTGAGTCT-3′	NM_003955.4
	RP: 5′-CGAAGTGTCCCCTGTTTGGA-3′	(128 bp)
SOCS5	FP: 5′-ATCTGGAGACAGCCATACCCA-3′	NM_014011.4
	RP: 5′-CAAATCAGGCACGAGGCAGT-3′	(91 bp)
SOCS7	FP: 5′-CCTTCAGCCTGTGGTGTCAT-3′	NM_014598.4
	RP: 5′-TCGGGACACTGGATAGAGCA-3′	(170 bp)
RETN	FP: 5′-AGCCATCAATGAGAGGATCCAG-3′	NM_001193374.2
	RP:5′-TCCAGGCCAATGCTGCTTA-3′	(72 bp)
TLR4	FP: 5′-AAGCCGAAAGGTGATTGTTG-3′	NM_003266.4
	RP:5′-CTGAGCAGGGTCTTCTCCAC-3′	(153 bp)
GAPDH	FP: 5′-GGAGCCAAAAGGGTCATCATCTC-3′	NM_001256799.3
	RP: 5′-GAGGGGCCATCCACAGTCTTCT-3′	(233 bp)

Western blot analysis

Immunoblot analysis was performed essentially as described by Ku *et al.*[39] Briefly, cells were lysed using radioimmuno-precipitation assay (RIPA) buffer (150 mM NaCl, 20 mM Na_2HPO_4 (pH 7.4), 100 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 1% Triton X-100) containing aprotinin and leupeptin, and then centrifuged at 14,500 rpm for 10 min at 4°C. Protein samples (100 μg) were separated by 12% SDS-PAGE in 2× gel-loading buffer (100 mM Tris-HCl (pH 6.8), 0.2% bromophenol blue, 4% SDS, 10% β-mercaptoethanol, and 20% glycerol). Antibodies specific for SOCS3, β-actin, GAPDH, and donkey anti-rabbit IgG conjugated with horseradish peroxidase (HRP) were obtained from Cell Signaling Technology (Danvers, MA). All other antibodies (i.e. anti-SOCS5 and anti-SOCS7) were purchased from Abcam (Cambridge, MA). The primary antibodies were used at a dilution of 1:1000 (0.2 μg/mL) and secondary antibody at a dilution of 1:2000 (~0.2 μg/mL). After washing, the immunoblots were visualized using the TOPBIO ECL Substrate Kit (TOP BIO, New Taipei City, Taiwan) for 3 min, and chemiluminescence was detected by the chemiluminescence imaging system MultiGel-21 (TOP BIO, New Taipei City, Taiwan). Using a Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD), each scanned band was quantified, the integrated optical density (IOD) calculated, and the data normalized to β-actin according to the IOD. Protein levels were expressed as a percentage of the control.

Cell proliferation assay

PC-3 cells (5000 cells/well) were plated in triplicate wells of a 96-well plate.[40] To evaluate whether resistin dose-dependently stimulated the growth of PC-3 PCa cells, we treated the cells with different dosages of resistin (0, 5, 12.5, 25, 50, 100, and 200 ng/mL) for 24, 48, and 72 h. In addition, after treatment with different inhibitors for 0.5 h, serum-starved cells were treated with 50 or 100 ng/mL human recombinant resistin for 24 and 48 h. We followed the MTT assay method from our previous report[41] and Mosmann[42] to detect cell proliferation. After incubating cells for the indicated times, we added tetrazolium dye, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazlium bromide), and incubated the cells in the dark at 37°C for 2 h. Next, the medium was removed, and we added 100 μL of 100% dimethyl sulfoxide (DMSO) to each well to stop the reaction. The cells were then incubated for 10 min on a shaker at 300 rpm. The insoluble product (formazan) dissolved in the DMSO, and the absorbance was read at 570 nm. We also used a commercial bromodeoxyuridine (BrdU) ELISA kit (Roche Applied Science, Mannheim, Germany) to measure cellular proliferation. Briefly, 36 hour serum-starved PC-3 cells (5000 cells/well) seeded in RPMI 1640 medium in four wells of a 96-well microplate were incubated with or without resistin for 18 h at 37°C, and then BrdU (10 μM) was added for an additional 16 h. After incubation, cell pellets were washed with 10 mM phosphate buffered saline (PBS) and dried at 55°C for 0.5 h. Cell pellets were fixed with FixDenat solution (200 μL/well) for 30 min at room temperature, probed with mouse anti-BrdU-POD (10 μL) for 1.5 h, and visualized by the addition of 3,3,5,5-tetramethylbenzidine substrate (100 μL/well) for 5 min for color development. To stop the reaction, we added 1 N H_2SO_4 (25 μL/well) to the culture medium for an additional 5 min on a shaker at 300 rpm. The absorbance was read at a wavelength of 450 nm using the BMG LABTECH's SPECTROstar® Nano. Blank controls for non-specific binding were culture medium alone and cells incubated with anti-BrdU-POD in the absence of BrdU.

Overexpression of wild-type and short hairpin SOCS

Following our previous method using Lipofectamine 2000 (Thermo Fisher Scientific),[41–43] 5×10^5 cells/well in a 6-well plate were transiently transfected with empty vector (pCDNA3.1, TRC1, or EG5 vectors), the recombinant wild-type SOCSs plasmid (2 μg), scramble RNA, or shSOCSs for 48 h and incubated with or without 100 ng/mL resistin for 72 h. After transfection, real-time PCR and Western blot analysis were performed to detect differences in the levels of *SOCS3* and *SOCS5* expression in PC-3 cells. After 72 h incubation with resistin, MTT was added for 1 h, and then 100 μL of DMSO was added to dissolve the remaining MTT formazan crystals. The absorption was read at 570 nm. The cells were co-transfected with pSV-β-galactosidase cDNA to determine the transfection efficiency. Expression of SOCS3 and SOCS5 genes and proteins were validated by qPCR and Western blot analysis, respectively.

Statistical analysis

Data were expressed as mean ± standard error (SE). Unpaired Student's *t*-test was used to examine differences between the control and resistin-treated groups. One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple-range test was used to examine differences among multiple groups. The Kruskal–Wallis test followed by the Mann–Whitney U test was used to examine differences between prostate gland, benign prostatic hyperplasia (BPH), and prostate carcinoma tissue samples. Differences were considered significant if $P < 0.05$. Pearson correlation analysis was used to examine correlations between different genes. Statistical analyses were performed using SigmaPlot (Jandel Scientific, Palo Alto, CA) and SPSS software (SPSS Inc., Chicago, IL).

Results

Resistin stimulated expression of *SOCS3* and *SOCS5* in PC-3 cells

Resistin increased the steady-state levels of *SOCS3* and *SOCS5* mRNA, but not *SOCS7* mRNA, in a time-dependent (Figure 1(A)) and dose-dependent (Figure 1(B)) manner. Resistin (50 ng/mL) significantly altered the levels of *SOCS3* and *SOCS5* mRNA at 0.5 to 2 h, increasing them 21–31% and 80–133%, respectively (Figure 1(A)). After 2 h of treatment, we observed no significant alterations in the levels of *SOCS3* and *SOCS5* mRNAs. The dose-dependent effect was observed with resistin doses of 5–100 ng/mL (Figure 1(B)).

To determine whether the stimulatory effects of resistin on the expression of *SOCS3* and *SOCS5* require new mRNA synthesis or are attributable to an alteration in mRNA stability, we pretreated PC-3 cells with the transcriptional inhibitor Acti-D for 30 min and analyzed changes in the levels of the *SOCS3* and *SOCS5* mRNAs (Figure 1(C)). Acti-D alone reduced the *SOCS3* and *SOCS5* mRNA levels, but not *SOCS7* mRNA levels. In the presence of resistin, Acti-D prevented resistin stimulation of *SOCS3* and *SOCS5* expression.

Next, we examined the possibility that resistin affects SOCS3, SOCS5, and SOCS7 protein levels (Figure 2). At 2 h, resistin was able to increase the protein levels of SOCS3 and SOCS5, but not SOCS7, in a concentration-dependent manner, with significant induction at 100 ng/mL resistin.

Resistin increased SOCS3 and SOCS5 gene expression via the TLR4 pathway

To examine whether the resistin-induced increase in the expression of *SOCS3* and *SOCS5* mRNA was mediated by the TLR4 pathway, cells were pretreated with either the TLR4 antagonist TAK-242 (1 μM),[44] or the TLR1/2 antagonist CU-CPT22 (1 μM)[45] for 0.5 h, and then incubated with or without 50 ng/mL resistin for 2 h (Figure 3). Neither TAK-242 nor CU-CPT22 alone altered the steady-state levels of *SOCS3*, *SOCS5*, or *SOCS7* mRNAs. However, TAK-242 prevented the resistin-induced increases in *SOCS3* and *SOCS5* mRNAs.

Functional ERK MEK, JNK MAPK, p38 MAPK, PI3K, and JAK2 proteins were necessary for resistin to stimulate SOCS3 and SOCS5 gene expression

We attempted to find the downstream signaling transducers involved in activating the resistin-induced expression of *SOCS3* and *SOCS5*, such as the MAPK pathway (e.g. ERK MEK, JNK MAPK, p38 MAPK), PI3K/AKT, and JAK2 proteins (Figures 4 to 6). PC-3 PCa cells were pretreated with ERK MEK inhibitors U-0126 and PD-98059, JNK inhibitor SP-600125, p38 MAPK inhibitor SB-203580, PI3K inhibitors wortmannin and LY-294002, or JAK2 inhibitor AG-490, followed by 50 ng/mL resistin for 2 h. U-0126, PD-98059, SB-203580, SP-600125, wortmannin, LY-294002, and AG-490 all blocked the resistin-stimulated expression of *SOCS3* and *SOCS5* mRNAs. In the absence of resistin, SB-203580, SP-600125, wortmannin, LY-294002, and AG-490 alone did not change the *SOCS3*, *SOCS5*, and *SOCS7* mRNA levels. However, U-0126 and PD-98059 alone decreased the *SOCS3* or *SOCS5* mRNA level approximately 21%.

### The cell type–specific effect of resistin on SOCS gene expression

To evaluate whether the effect of resistin on the expression of *SOCS3* and *SOCS5* mRNAs was cell type-specific, we compared the results in PC-3 cells to the same assays performed in LNCaP-FGC, DU145, and U937 (lymphoma) cells (Figure 7). Resistin at 50 and 100 ng/mL for 2 h induced a significant 53–77% increase in *SOCS5* mRNA levels in LNCaP-FGC cells, another PCa cell line (Figure 7(A)). Similar to PC-3 cells, resistin did not alter the expression of *SOCS7* mRNA levels in LNCaP-FGC cells. Interestingly, in LNCaP-FGC cells, 25, 50, or 100 ng/mL resistin for 2 h did not affect *SOCS3* mRNA levels. In DU145 cells, 25, 50, or 100 ng/mL resistin for 2 h did not affect *SOCS3*, *SOCS5*, or *SOCS7* mRNA levels. In U937 lymphoma cells, 50 and 100 ng/mL resistin alone for 2 h significantly increased the levels of *SOCS3* and *SOCS7* mRNA by approximately 19–70% (Figure 7(B)), but 25, 50, or 100 ng/mL resistin for 2 h did not affect the *SOCS5* mRNA levels. However, resistin induced higher *SOCS7* mRNA levels in U937 cells compared to the control.

Resistin signaling molecules were required for the stimulatory effect of resistin on PC-3 cell growth

MTT analysis showed that resistin dose-dependently (12.5–200 ng/mL resistin) induced the growth of PC-3 cells at 24, 48, and 72 h (Figure 8(A)) by 12–35%, 30–57%, and 33–45%, respectively. BrdU analysis also showed the dose-dependent effect of resistin on PC-3 cells growth (Figure 8(B)). To evaluate which, if any, of the resistin signaling pathways were responsible for modulating PCa cell growth, we pretreated PC-3 cells with different specific inhibitors of the individual protein kinases for 0.5 h, and then treated them with 50 or 100 ng/mL resistin (Figure 8(C) and (D)). CU-CPT22, TAK-242, U-0126, SB-203580, SP-600125, LY-294002, and AG-490 prevented the resistin-stimulated growth of PC-3 cells.
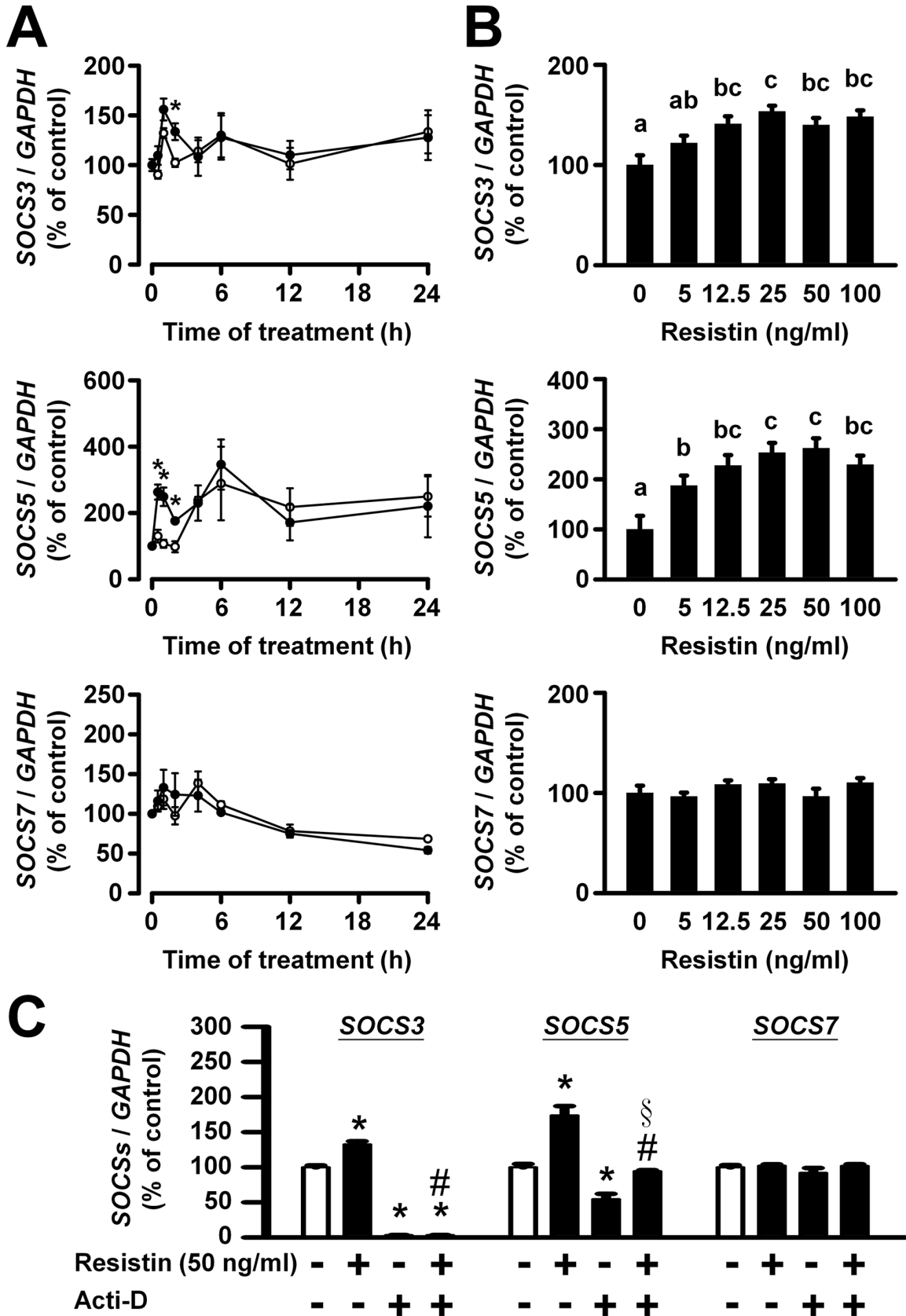
Figure 1. Resistin time-dependently and dose-dependently stimulated *SOCS3* and *SOCS5* mRNA expression in PC-3 cells. (A) Serum-starved PC-3 cells were incubated in the presence (experimental group; •) or absence (control group; ○) of resistin hormone (50 ng/mL) for 0, 1, 2, 4, 8, 12, and 24 h. *$P < 0.05$ versus the control at same time. (B) Serum-starved PC-3 prostate cancer cells were incubated in the presence or absence (control group) of different doses of resistin hormone (0, 5, 12.5, 25, 50, and 100 ng/mL) for 2 h. Groups with different letters are significantly different ($P < 0.05$) from each other. (C) The effect of resistin was blocked by actinomycin D (Acti-D, 5 μg/mL) pretreatment for 0.5 h. After 2 h of resistin treatment, *SOCS* mRNAs were analyzed by real-time PCR. Controls were not treated with resistin or Acti-D. *$P < 0.05$ versus control; #$P < 0.05$, resistin versus resistin + Acti-D; §$P < 0.05$, Acti-D versus resistin + Acti-D. The levels of *SOCS* mRNAs were determined by real-time PCR. Data are expressed as mean ± SE from replicates of three experiments.
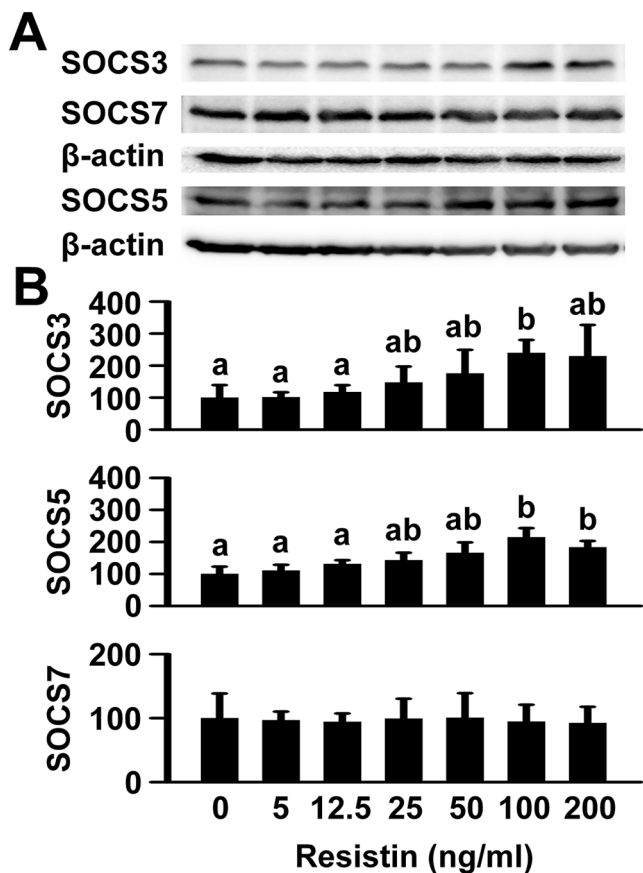
Figure 2. Resistin dose-dependently stimulated SOCS3 and SOCS5 protein expression in PC-3 cells. Serum-starved PC-3 prostate cancer cells were incubated in the presence or absence (control group) of different doses of resistin hormone (0, 5, 12.5, 25, 50, 100, and 200 ng/mL) for 2 h. (A) After 2 h of resistin treatment, SOCS proteins were determined by Western blot. Data are expressed as mean ± SE from replicates of three experiments. (B) Groups with different letters shown in (B) are significantly different ($P < 0.05$) from each other.
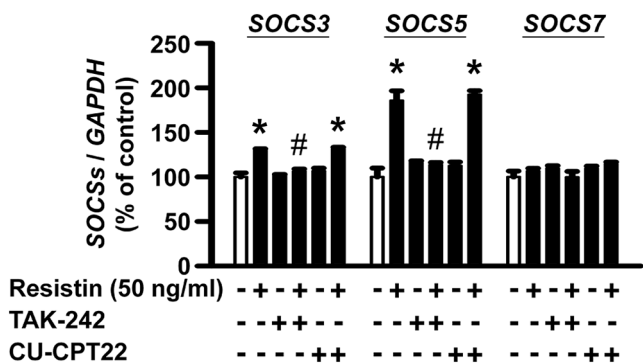
Figure 3. The toll-like receptor 4 (TLR4) antagonist TAK-242, but not the toll-like receptor 1/2 (TLR1/2) antagonist CU-CPT22, blocked resistin-altered levels of *SOCS3* and *SOCS5* mRNAs. Serum-starved PC-3 cells were pretreated with or without TAK-242 (1 μM) or CU-CPT22 (1 μM) for 0.5 h and then stimulated with 50 ng/mL resistin. After 2 h of resistin treatment, levels of *SOCS3*, SOCS5, and *SCOS7* mRNAs were analyzed by quantitative real-time PCR. Data are expressed as mean ± SE from replicates of three experiments. *$P < 0.05$ versus the control; #$P < 0.05$, resistin versus inhibitor + resistin.

Overexpression or knockdown of *SOCS3* or *SOCS5* altered the effect of resistin-induced PC-3 cell growth

Overexpression or knockdown of *SOCS3* or *SOCS5* was achieved to demonstrate whether SOCS3 and SOCS5 are
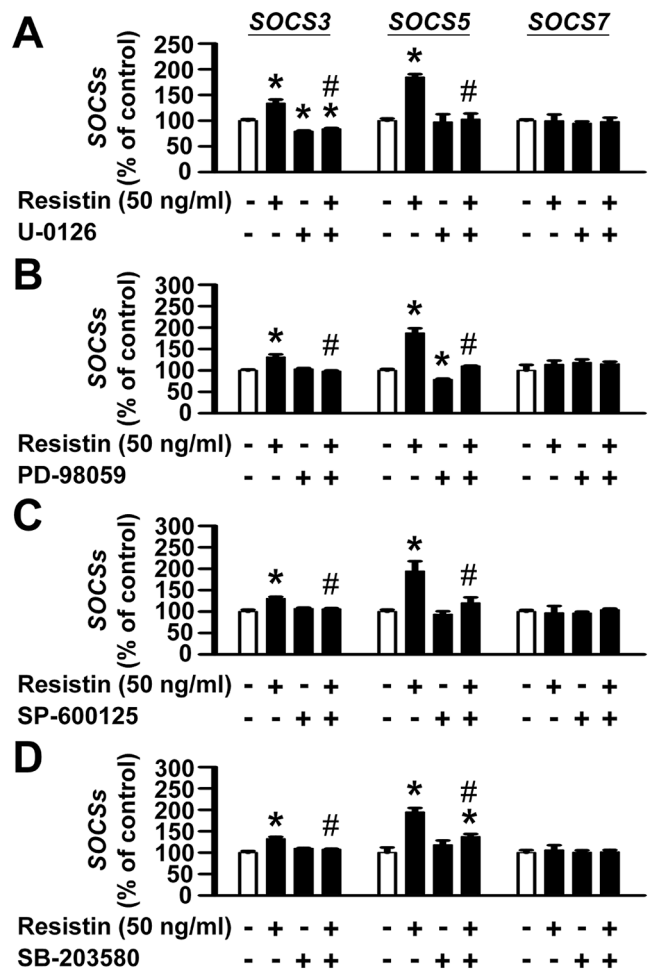
Figure 4. Specific inhibitors of the mitogen-activated protein kinases, such as U-0126, PD-98059, SP-600125, and SB-203580, reduced resistin-induced increases in the levels of *SOCS3* and *SOCS5* mRNA. (A) Serum-starved PC-3 cells were pretreated with U-0126 (25 μM; in A), (B) PD-98059 (50 μM), (C) SP-600125 (20 μM), or (D) SB-203580 (20 μM) for 0.5 h and then stimulated with resistin (50 ng/mL) for 2 h. Levels of *SOCS3*, *SOCS5*, and *SOCS7* mRNAs were analyzed by real-time PCR. Data were expressed as mean ± SE from replicates of three experiments. *$P < 0.05$ versus the control; #$P < 0.05$, resistin versus resistin + kinase inhibitor.

both required for resistin to stimulate the growth of PC-3 cells (Figure 9). After transfection, we validated significant changes in the levels of *SOCS3* and *SOCS5* expression at both the mRNA and protein level (Figure 9(A) to (D)). Our results indicate that transient overexpression of *SOCS3* or *SOCS5* enhanced the resistin-stimulated growth of PC-3 cells (Figure 9(E) and (F)) and that knockdown of *SOCS3* or *SOCS5* suppressed the resistin-increased growth of PC-3 cells (Figure 9(G) and (H)).

Expression of *SOCS3*, *SOCS5*, *SOCS7*, *RETN*, and *TLR4* mRNAs in prostate tissues

For clinical validation, TissueScan Prostate Cancer cDNA Arrays I, II, and III were used. The mRNA expression profiles of *SOCS3*, *SOCS5*, *SOCS7*, *RETN*, and *TLR4* in the prostate gland ($n = 24$), BPH ($n = 11$), and prostate carcinoma ($n = 105$) are presented in Figure 10. Patients with BPH had lower levels of *SOCS5* and *TLR4* mRNAs, but no significant differences were found in the levels of *SOCS3*, *SOCS7*, and *RETN* mRNAs compared to the normal prostate gland. Although
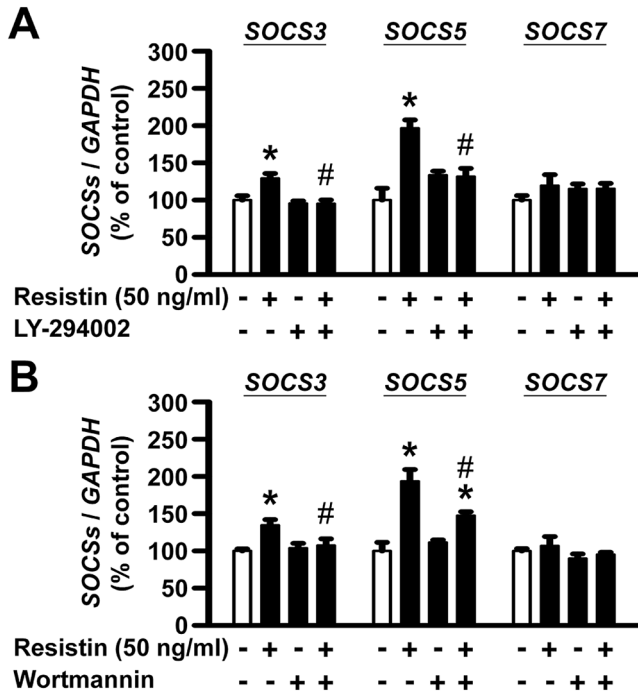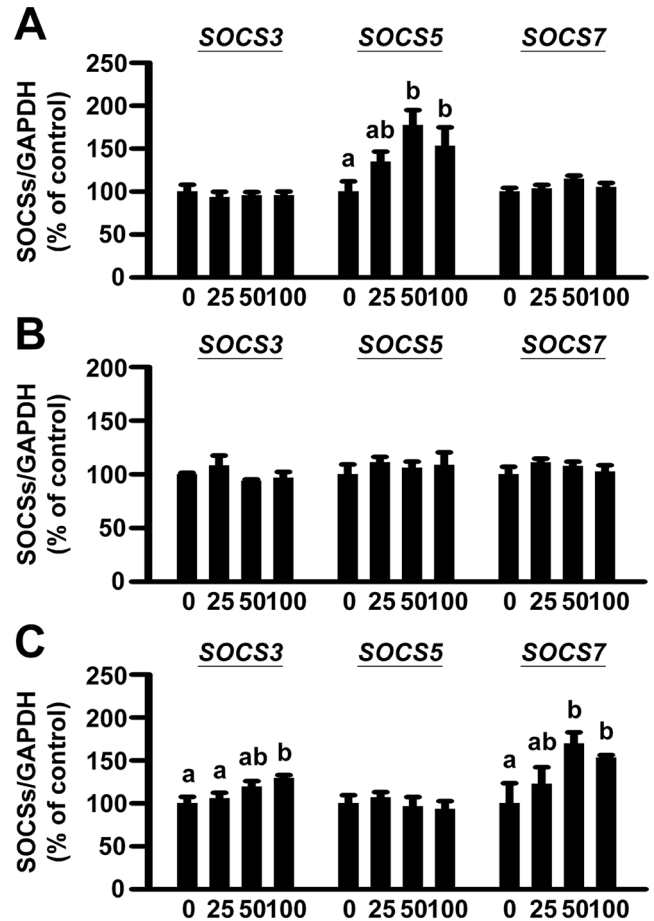
Figure 5. The PI3K inhibitors, such as LY-294002 and wortmannin, reduced resistin-induced increases in levels of *SOCS3* and *SOCS5* mRNAs. (A) Serum-starved PC-3 cells were pretreated with LY-294002 (50 μM; in A) or (B) wortmannin (0.2 μM) for 0.5 h and then stimulated with resistin (50 ng/mL) for 2 h. Levels of *SOCS3, SOCS5,* and *SOCS7* mRNAs were analyzed by real-time PCR. Data were expressed as mean ± SE from replicates of three experiments. *$P < 0.05$ versus the control; #$P < 0.05$, resistin versus resistin + kinase inhibitor.
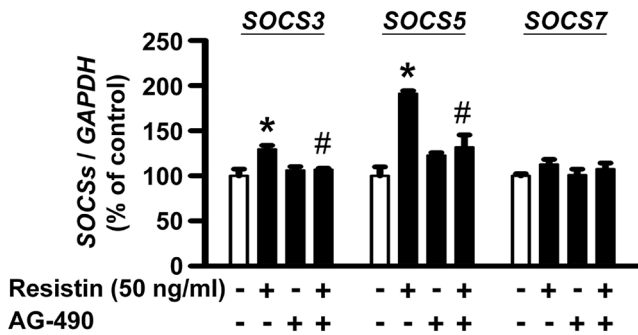
Figure 6. The JAK2/STAT3 inhibitor AG-490 reduced resistin-induced increases in levels of *SOCS3* and *SOCS5* mRNAs. Serum-starved PC-3 cells were pretreated with AG-490 (10 μM) for 0.5 h and then stimulated with resistin (50 ng/mL) for 2 h. Levels of *SOCS3, SOCS5,* and *SOCS7* mRNAs were analyzed by real-time PCR. Data were expressed as mean ± SE from replicates of three experiments. *$P < 0.05$ versus the control; #$P < 0.05$, resistin versus resistin + kinase inhibitor.

Figure 7. Resistin altered *SOCS3*, *SOCS5*, and *SOCS7* mRNA expression in cancer cells. (A) LNCaP-FGC prostate cancer cells, (B) DU145 prostate cancer cells, and (C) U937 lymphoma cells. Serum-starved cells were incubated with 0 (control group), 25, 50, or 100 ng/mL of resistin for 2 h. Levels of *SOCS3*, *SOCS5*, and *SOCS7* mRNAs were analyzed by quantitative real-time PCR. Data are expressed as mean ± SE from replicates of three experiments. Groups with different letters for expression of in a given *SOCS* mRNA significantly differed ($P < 0.05$) from each other.

significant differences in the levels of *SOCS3*, *SOCS5*, *SOCS7*, *RETN*, and *TLR4* mRNAs were not observed between normal prostate gland and prostate carcinoma, significant differences were found in the levels of *SOCS5* ($P < 0.01$) and *TLR4* ($P < 0.01$) mRNA, but not *SOCS3*, *SOCS7*, or *RETN* mRNA, in prostate carcinoma compared to BPH. Interestingly, when we compared changes in *RETN* mRNA levels between different stages of PCa with normal prostate gland, we observed greater *RETN* mRNA expression in stages II, III, and IV PCa relative to normal prostate gland (Supplemental Figure S3).

The correlations among *SOCS3*, *SOCS5*, *SOCS7*, *RETN*, and *TLR4* in a given group of tissues are summarized in Table 2. In the prostate gland, we found positive correlations between *SOCS5* and *RETN* ($P < 0.001$) or *TLR4* ($P < 0.01$), and between *SOCS7* and *TLR4* ($P < 0.05$). In prostate carcinoma, positive correlations occurred between *SOCS5* and *SOCS7* ($P < 0.001$), *RETN* ($P < 0.001$), or *TLR4* ($P < 0.001$), and between *RETN* and *TLR4* ($P < 0.001$). However, we found no significant correlations between *SOCS3*, *SOCS5*, *SOCS7*, *RETN*, and *TLR4* in BPH.

Discussion

This study demonstrated that resistin selectively affects particular types of SOCS family members in PC-3 PCa cells. Resistin likely upregulates *SOCS3* and *SOCS5* mRNA levels by stimulating gene transcription. This conclusion is supported by Acti-D treatment preventing the resistin-induced increases in *SOCS3* and *SOCS5* mRNA levels. However, resistin antagonized the Acti-D suppression of *SOCS5*, but not *SOCS3*, mRNA levels compared to Acti-D treatment
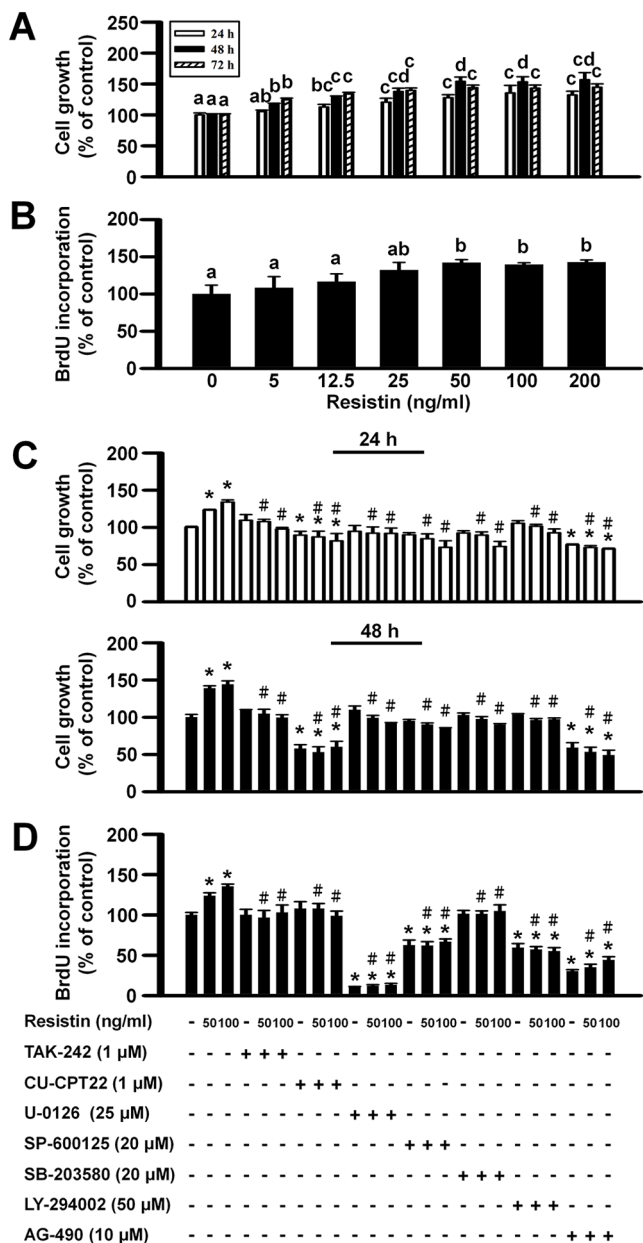
Figure 8. TAK-242, CU-CPT22, U-0126, SP-600125, SB-203580, LY-294002, and AG-490 reduced resistin-stimulated growth of PC-3 cells. (A and B) Serum-starved PC-3 cells were incubated in the presence or absence (control group) of different doses of resistin hormone for MTT assay and BrdU assay. Group with different letters are significantly different ($P < 0.05$) from each other. (C and D) Serum-starved PC-3 cells were pretreated with CU-CPT22 (1 µM), TAK-242 (1 µM), U-0126 (25 µM), SP-600125 (20 µM), SB-203580 (20 µM), LY-294002 (50 µM), or AG-490 (10 µM) for 0.5 h and then stimulated with resistin (50 or 100 ng/mL) for MTT assay and BrdU assay. Data are expressed as mean ± SE from replicates of three experiments. *$P < 0.05$ versus the control; #$P < 0.05$, resistin versus resistin + kinase inhibitor.

alone (Figure 1(C)). This suggests that mRNA stability is also required for the effect of resistin on *SOCS5* but not *SOCS3*.

We attempted to determine the signaling proteins required for resistin induction of *SOCS3* and *SOCS5* gene expression. A recent study demonstrated that TLR4 can act as a resistin receptor in human leukocytes.[25] In support of this notion, we found that pretreatment of PC-3 cells with the TLR4 inhibitor, but not the TLR2 inhibitor, significantly blocked the resistin-stimulated expression of *SOCS3* and

SOCS5 mRNAs. This suggests that functional TLR4 is necessary for the effect of resistin. This is also consistent with the reported effects of resistin on interleukin-6 release from leukocytes,[25] fractalkine expression in smooth muscle cells,[46] and *SOCS3* expression in colon cancer cells.[47] Because the TLR2 inhibitor did not block the resistin-induced increases in *SOCS3* and *SOCS5* mRNA levels in PC-3 cells, the results suggest a TLR2-independent effect of resistin. Therefore, it is likely that resistin regulates *SOCS3* and *SOCS5* mRNA expression in PC-3 cells through particular types of TLR family molecules.

In this study, when kinase signaling proteins downstream of resistin were examined, we observed that increases in the level of *SOCS3* and *SOCS5* mRNAs induced by resistin could be attributed to its activation of the respective MAPK, PI3K/AKT, and JAK2/STAT3 proteins. Any specific inhibitors of these protein kinases significantly prevented resistin-induced increases in the levels of *SOCS3* and *SOCS5* mRNAs in PC-3 cells. Our findings are consistent with those reported for the ERK-dependent effect of resistin on *SOCS3* expression in colon cancer cells,[47] as well as those reported for the p38 and JNK MAPK-dependent effect of resistin on fractalkine expression in human endothelial cells.[48]

The functions of SOCS3 and SOCS5 are necessary for resistin to stimulate the growth of PC-3 PCa cells. In particular, knockdown of *SOCS3* or *SOCS5* suppresses resistin-stimulated growth of PC-3 cells. Furthermore, overexpression of *SOCS3* or *SOCS5* enhanced the resistin-stimulated growth of PC-3 cells. In support of the SOCS3- and SOCS5-dependent effect of resistin on PC-3 cell growth, treatment with specific inhibitors of the TLR4, ERK, JNK, p38 MAPK, PI3K, and JAK2 proteins decreased the resistin-stimulated growth of PC-3 cells, which accompanied the decrease in *SOCS3* and *SOCS5* mRNA levels. Taken together, these observations suggest that the effect of resistin on PC-3 cell growth is mediated through the SOCS3 and SOCS5 proteins in a TLR4, ERK, JNK, p38 MAPK, PI3K, and JAK2-dependent pathway.

Resistin also dose- and time-dependently stimulated the growth of DU145 cells (Supplemental Figure S4). In addition, resistin dose-dependently stimulated *SOCS5* mRNA expression in LNCaP cells and increased *SOCS3* mRNA levels in U937 cells. However, resistin did not alter *SOCS7* mRNA levels in DU145 cells or LNCaP cells. Interestingly, the effects of resistin on *SOCS3* and *SOCS5* mRNA expression in DU145 cells were somewhat different from the effects in PC-3 (Figure 1(A) and (B)) and LNCaP-FGC cells (Figure 7). These observations indicate a PCa cell type-dependent effect of resistin on the regulation of *SOCS3* and *SOCS5* gene expression. The notion is consistent with the reported cell type-specific regulation of SOCS3 by ET-1[37] and IL-6.[49] Possible explanations for the cell type-dependent effect of resistin on *SOCS3* and *SOCS5* mRNA expression are that the distinct types of SOCS family members respond to resistin at varying levels, and that signaling pathways required for the actions of resistin vary with the nature of PCa cell types, SOCS members, or kinase signaling cascades.[19] The notion is supported by our findings that high levels of TLR4, MYD88, SOCS3, and SOCS7 proteins were found in LNCaP-FGC cells, and high levels of SOCS5 protein were found in DU145 cells (Supplemental Figure S5). Notably, other reports have
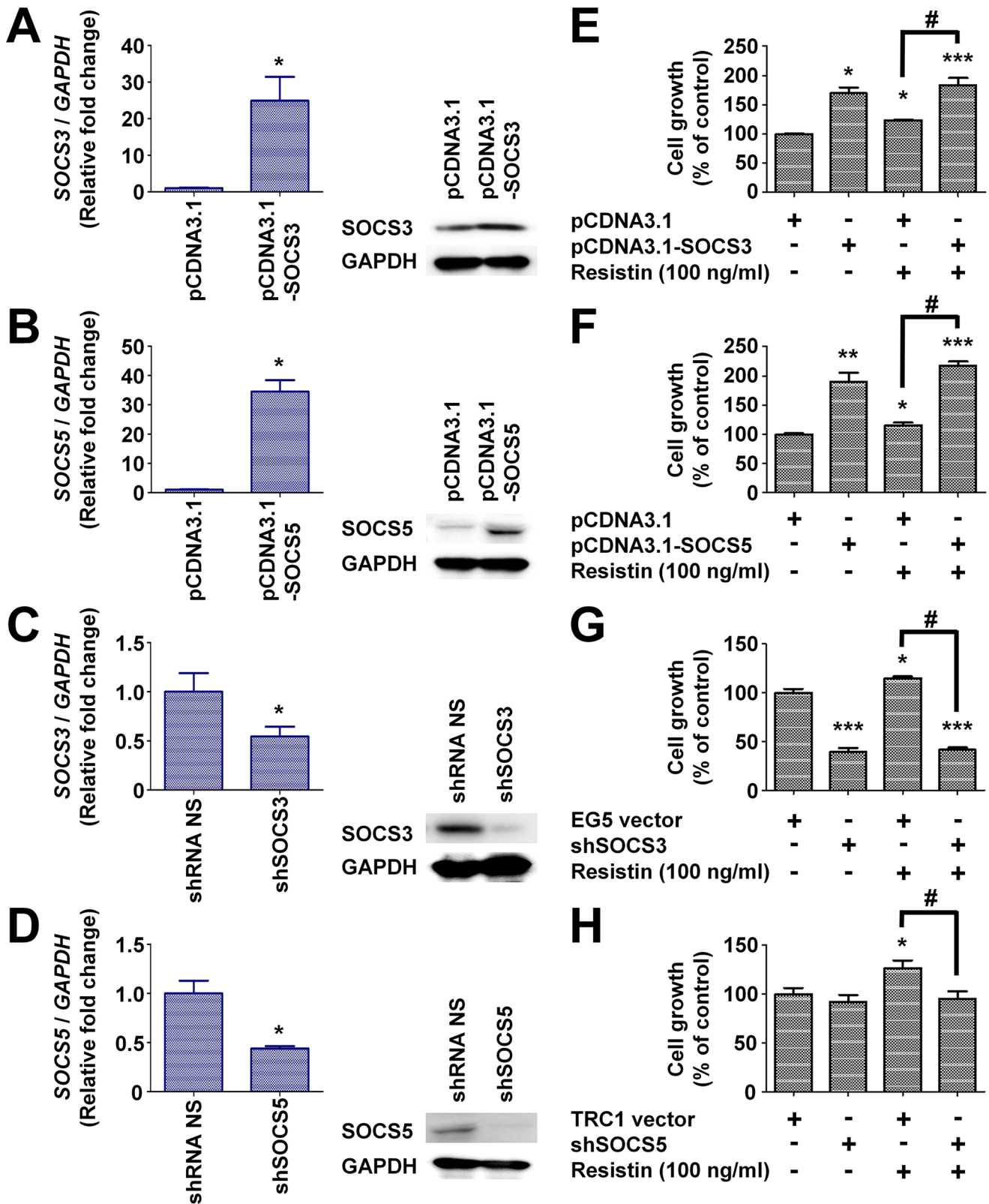
Figure 9. (A, B, E, F) Overexpression of *SOCS3* or *SOCS5* enhanced the stimulatory effect of resistin on the growth of PC-3 cells. (C, D, G, H) Knockdown of *SOCS3* or *SOCS5* reduced resistin-stimulated growth of PC-3 cells. PC-3 cells transfected with empty vector, SOCSs or shSOCSs were treated with 100 ng/mL resistin. After 72 h incubation, cell growth was measured by the MTT assay (E-H). To verify the mRNA (bar graphs in A–D) and protein levels (gel graphs in A–D) of SOCS3 and SOCS5, we used real-time PCR and Western blot, respectively. Data are expressed as mean ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the control. #$P < 0.05$, resistin versus resistin + vector.
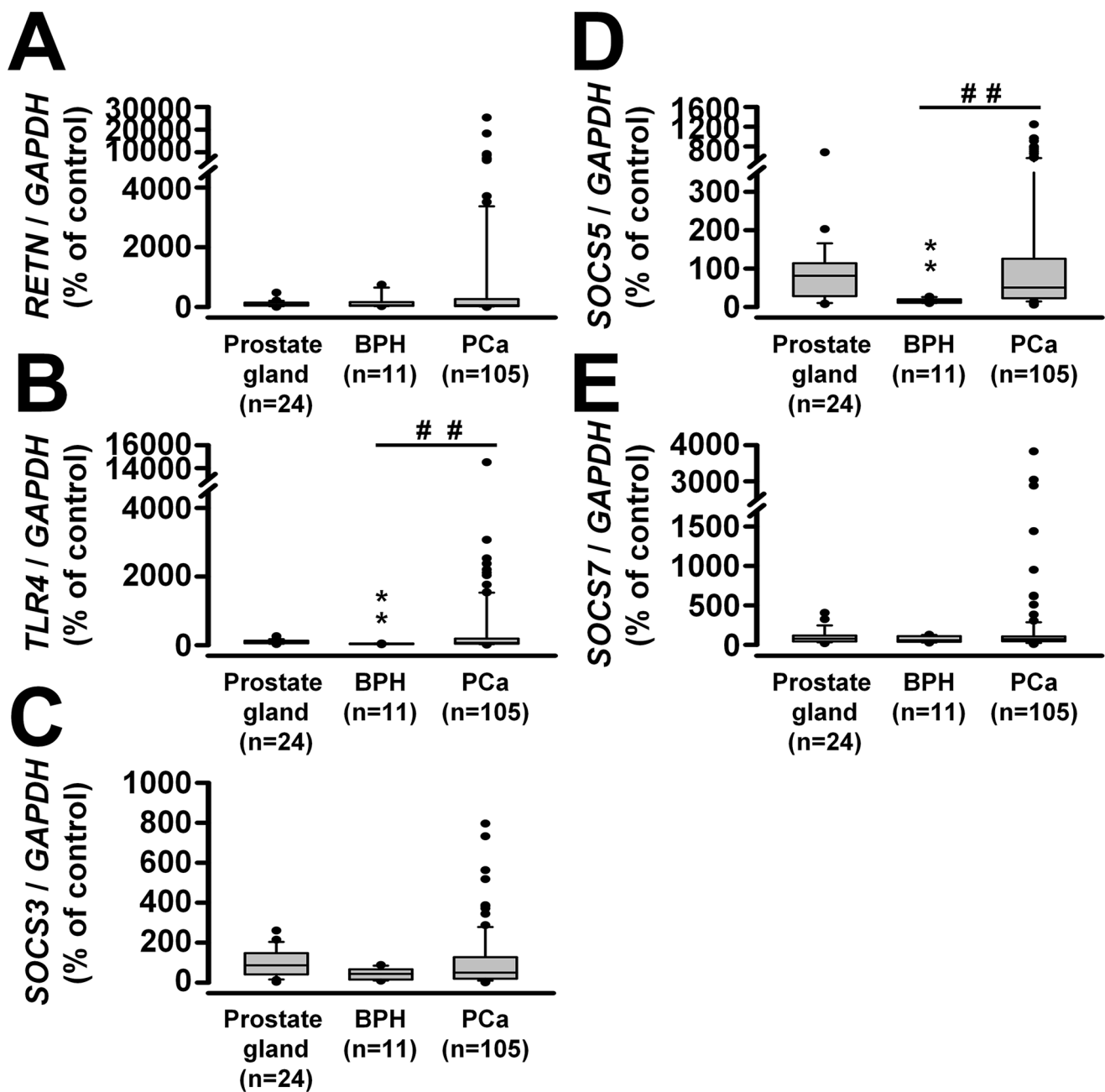
Figure 10. Differences in mRNA levels of (A) *RETN*, (B) *TLR4*, (C) *SOCS3*, (D) *SOCS5*, and (E) *SOCS7* in prostate gland ($n = 24$), benign prostate hyperplasia (BPH, $n = 11$), and prostate carcinoma ($n = 105$) samples. **$P < 0.01$ versus prostate gland; ##$P < 0.01$, BPH versus prostate carcinoma.

indicated that overexpression of SOCS3 decreases phosphorylation of pSTAT3 and influences cell growth and apoptosis in DU145 and TRAMP-C2 cells, but not in PC-3 cells, which do not express pSTAT3.[27] Furthermore, downregulation of SOCS3 causes apoptosis of PC-3 cells, DU145 cells, and LNCaP-IL-6-positive cells, but not parental LNCaP cells.[33] In addition, *SOCS5* mRNA levels are not significantly different between P69 and M12 PCa cells after transfection of the miR-9 inhibitor. However, the miR-9 inhibitor reduces SOCS5 protein levels in M12 PCa cells. Moreover, different signaling pathways control cell growth in PC-3, LNCaP, and DU145 cells, which express different levels of the SOCS family members.[27–33] We used real-time PCR to detect *RETN*

mRNA in normal (RWPE-1) and cancerous (PC-3, DU145, and LNCaP-FGC) human prostate cells relative to U937 lymphoma cells and found that all RWPE-1, DU145, PC-3, and LNCaP-FGC cells expressed less *RETN* mRNA than U937 cells (Supplemental Figure S6(A)). However, changes in the *RETN* mRNA levels were not altered in PC-3, LNCaP-FGC, and U937 cells after 2 h of resistin (25, 50, and 100 ng/mL) treatment (Supplemental Figure S6(B)). The presence of *RETN* mRNA expression in RWPE-1, DU145, and PC-3 cells is consistent with the report by Kim *et al*.[19] Interestingly, our observations indicate that stages II, III, and IV of PCa from tissue array analysis exhibited higher levels of *RETN* mRNA than normal prostate gland. These findings are also consistent

Committee of Landseed Hospital, Taiwan (LSHIRB IRB No./Protocol No. 17-018-C0) and the Ethics Committee of Taoyuan General Hospital, Taiwan (TYGHIRB IRB No./Protocol No. 110-08).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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