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Androgens regulate SMAD ubiquitination regulatory factor-1 expression and prostate cancer cell invasion

Xiaokun Gang^{1,2}, Guixia Wang^{1,*}, and Haojie Huang^{2,3,4,*}

¹Department of Endocrinology and Metabolism, The First Hospital of Jilin University, Changchun, Jilin 130021, China

²Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

³Department of Urology, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

⁴Mayo Clinic Cancer Center, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

Abstract

BACKGROUND—Prostate cancer (PCa) is the most commonly diagnosed male cancer in the United States and is a hormone-driven disease. Androgens have been recognized as a major promoter of PCa development and progression. However, the mechanism of androgen action in PCa, especially in PCa cell invasion and metastasis remains largely unclear. SMAD ubiquitination regulatory factor-1 (SMURF1) is a C2-WW-HECT-domain E3 ubiquitin ligase that plays important roles in cancer cell metastasis. Whether there is a relationship between androgens and SMURF1 expression is not known.

METHODS—The effect of androgens on the expression of SMURF1 in PCa cell lines was examined by Western blot analyses and reverse transcription-polymerase chain reaction (RT-PCR). Gene transfection was performed by electroporation to manipulate the expression levels of proteins studied. The binding of AR to the SMURF1 gene enhancer was determined by chromatin immunoprecipitation (ChIP) assay. Cell migration and invasion was measured by wound healing and Matrigel invasion assays, respectively.

RESULTS—We found that expression of SMURF1 is upregulated by androgens in PCa cell lines and that this effect of androgens is mediated through the androgen receptor (AR). We further showed that androgens regulate SMURF1 expression at transcriptional level and provided evidence that AR transcriptionally activates SMURF1 by binding to its enhancer that contains a canonical half androgen responsive element (ARE). Finally, we demonstrated that SMURF1 is important for androgen-induced invasion of PCa cells.

CONCLUSIONS—We demonstrate for the first time that SMURF1 is a *bona fide* target gene of the AR. Our findings also suggest a potential role of SMURF1 in PCa metastasis.

*Corresponding authors: Guixia Wang, M.D., Ph. D., Department of Endocrinology and Metabolism, The First Hospital of Jilin University, Changchun, Jilin, China, 130021. Phone: +8615843081103. gxwang6688@yahoo.com, Haojie Huang, Ph. D., Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905. Phone: +15072931311. huang.haojie@mayo.edu.

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Keywords

Androgen; AR; SMURF1; prostate cancer; invasion; and metastasis

Introduction

In the United States, prostate cancer (PCa) is the most commonly diagnosed and one of the most predominant causes of cancer death in men (1). Since Huggins and Hodges found that castration of patients results in regression of advanced PCa, the strong connection between androgens and PCa has been a major focus of PCa research. PCa is a hormonally driven disease. Recently, a number of studies support a role for re-activated AR in castration-resistant prostate cancer (CRPC). Several clinical studies with metastatic CRPC suggest that PCa growth and metastasis remain dependent on androgen supply (2,3). Androgens have always been the top driving factor for PCa. Unfortunately, the mechanism of androgen action in PCa, especially the metastatic disease remains poorly understood.

SMURF1, a member of the HECT family of E3 ubiquitin ligases, was identified due to its abilities to modulate transforming growth factor- β (TGF- β)/bone morphogenetic protein (BMP) signaling by inducing ubiquitin modification on SMADs. However, accumulating evidence suggests that SMURF1 is a pleiotropic factor, with its substrates extended beyond the members of the TGF- β /BMP superfamily to those involved in cell migration, proliferation, differentiation and senescence. Notably, the substrates of SMURF1 play important roles in development of cancers, including breast, esophageal, pancreatic and renal cell carcinomas (4). The discovery of SMURF1-mediated RHOA (Ras homolog family member A) ubiquitination represents the direct evidence for the role of SMURF1 in cell migration (5,6). In addition, SMURF1 can also trigger degradation of TALIN head and hPEM-2 (Posterior End Mark-2) to regulate cell adhesion and abnormal cell migration (7). These findings suggest a potential role of SMURF1 in cancer metastasis.

In this study, we found that androgens regulate SMURF1 expression in PCa cells. We identified SMURF1 as a novel AR downstream target gene. We provided evidence that AR bound to the SMURF1 enhancer, transcriptionally activated SMURF1 expression. We also showed that androgens promoted PCa cell migration and invasion and these effects were largely diminished by knocking down SMURF1. These findings suggest that SMURF1 is a potential target for the treatment of metastatic PCa.

Materials and Methods

Cell lines, cell culture and chemicals

LNCaP cells were purchased from American Type Culture Collection (Manassas, VA), and C4-2 cells were purchased from Uro Corporation (Oklahoma City, OK). LAPC-4 cells were kindly provided by C. L. Sawyers. LNCaP and C4-2 cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 μ g/ml streptomycin, 100 U/ml penicillin, and 0.25 μ g/ml amphotericin B. LAPC4 cells were maintained in Iscove's Modified Dulbecco's Media with 10% FBS. Cells were incubated at 37°C with 5% CO₂. For androgen dose and

time-course studies, cells were plated in medium with 10% charcoal-stripped serum (CSS). After 48 h, mibolerone (mib), a potent synthetic androgen or ethanol (EtOH) was added. For protein and RNA synthesis inhibition, LNCaP cells were treated with cycloheximide (CHX, 25 µg/ml) and actinomycin D (5 µM), respectively. Cycloheximide and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO). Mibolerone was purchased from Steraloids Inc (Newport, RI). MDV3100 (enzalutamide) was acquired from Medivation (San Francisco, CA). To measure the proliferation of C4-2 cells following androgen treatment, cells were treated with vehicle (ethanol) or 10 nM of mibolerone for 24 h and cell number were measured by trypan blue assays (Mediatech, Inc, Manassas, VA).

Cell transfections

Transfections were performed by electroporation using an Electro Square Porator ECM 830 (BTX) (8) or by using Lipofectamine 2000 (Invitrogen). Small interfering RNA (siRNA) specific for AR, SMURF1 and nonspecific (NS) control siRNAs were purchased from GE Health Dharmacon (Lafayette, CO). Cells were harvested 48–72 h after transfection. Approximately 75–90% transfection efficiencies were routinely achieved.

Western blot analysis

Protein samples were prepared by lysing cells in modified RIPA buffer [1× PBS, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)]. Lysates (50–100 µg) were separated on a 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with the specific primary antibody and HRP-conjugated secondary antibody and then visualized by chemiluminescence. Antibodies against AR (N-20), SMURF1 (H-60), ERK2 (D-2) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Semi-quantitative and real-time RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was done with cDNA samples using the iQ SYBR Green Supermix and an iCycler iQTM detection system (BioRad) according to manufacturer's instructions. The 2⁻Ct method was used to calculate the relative expression level by normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. The following primer sequences were used: SMURF1, forward 5'-TGAATCACCAGTGCCAACTCAAGG-3' and reverse 5'-TCGTGTCTGAGGACTTTCAGCTTC-3'; PSA, forward 5'-TGCCCACTGCATCAGGAACAAA-3' and reverse 5'-GGAAGCTGTGGCTGACCTGAAATA-3'; GAPDH, forward 5'-ACCCACTCCTCCACCTTTGAC-3' and reverse 5'-TGTTGCTGTAGCCAAATTCGTT-3'.

Chromatin immunoprecipitation (ChIP) assay

LNCaP cells were treated with 1 nM mib or vehicle for 24 h after 48 h of plating and the ChIP assay was performed as described previously (9). The soluble chromatin was incubated with 2 µg of rabbit IgG or AR (N-20) antibodies. PCR was performed using primers specific

for the AR binding region in the SMURF1 enhancer: forward 5'-ACTACCACGCATTGCTTTCA-3' and reverse 5'-AATGGTAGTGCCGGTAACCTC-3' or AR binding region in the PSA enhancer (positive control): forward 5'-TGGGACAACCTTGCAAACCTG-3' and reverse 5'-CCAGAGTAGGTCTGTTTTCAATCCA-3'. Quantitative real-time PCR was performed with ChIP samples using the iQ SYBR Green Supermix and an iCycler iQTM detection system (BioRad) according to manufacturer's instructions.

Wound healing assay

C4-2 cells were transfected with non-specific (NS) control or SMURF1-specific siRNAs. At 24 h after transfection, cells were plated in culture inserts (IBIDI, Martinsried, Germany). The culture inserts were removed 24 h later, which created a gap. The cells were treated with vehicle (EtOH) or 10 nM mib. Photographs of the same area of the wound were taken at 0, 48 and 72 h after wounding to measure the width of the wound. The areas of the gaps and percent wound areas filled were determined.

Invasion assay

The invasive properties of PCa cells were assessed using commercially available Matrigel invasion chambers (BD Biosciences). C4-2 cells were transfected with NS control or SMURF1-specific siRNAs and cultured in 10% CSS medium. At 48 h after transfection, cells were re-suspended in serum free RPMI. Add serum free medium into the upper and lower chambers, and incubated at 37°C for 2 h. Then the medium in lower chambers were changed to 10% CSS medium with or without 10 nM of mib. About 2×10^5 cells were added into the upper chamber in the serum-free RPMI 1640 with or without 10 nM of mib. The incubation was carried out for 24 h. The medium in the top and bottom chambers was carefully aspirated. The membrane was washed with 1×PBS and fixed in 100% methanol, and residual cells were removed off the upper surface of the membrane. The cell invasion was quantified by counting the cells stained by crystal violet (average cell counts in six random visual fields).

Statistical analysis

All values were expressed as means±SD. Comparison between two mean values was made by independent-sample t-test. $P < 0.05$ was considered statistically significant.

Results

Androgens regulate the expression of SMURF1 protein

To explore the effect of androgens on SMURF1 expression, we treated LNCaP cells with different doses of mibolerone (mib), a synthetic potent anabolic androgen which is both of high affinity and selective for AR. To effectively monitor the effect of mibolerone, cells were cultured in medium supplemented with 10% CSS for 48 h prior to androgen treatment. Treatment of LNCaP cells with different concentrations (0 ~ 10 nM) of mibolerone increased expression of SMURF1 proteins in a dose dependent manner (Fig. 1A). Quantitative analysis showed that mibolerone at 1 nM had the most dramatic effect on induction of SMURF1 expression comparing with other concentrations (Fig. 1B). Similar

results were obtained in LAPC-4 cells (Fig. 1C and D). Treatment of C4-2 CRPC cells resulted in a similar result, but the most effective dose is 10 nM (Fig. 1E and F). The effectiveness of mibolerone was evident by the increased mRNA expression of prostate-specific antigen (*PSA*), a well-studied AR downstream target gene (Fig. 1B, D and F). Thus, expression of SMURF1 can be induced by androgens in various human PCa cell lines.

Androgen-induced increase in SMURF1 expression is mediated through the AR

Given that under the stimulation of different concentrations of mibolerone, the increase in SMURF1 protein level was always associated with the elevated mRNA levels of *PSA* (Fig. 1B, D and F), we sought to determine whether AR plays a role in androgen-induced SMURF1 expression. LNCaP cells were transfected with non-specific (NS) or AR-specific siRNA. Right after transfection, cells were cultured in 10% CSS medium for 48 h and then treated with or without 1 nM of mibolerone for 24 h. Consistent with the finding shown in Fig. 1A, SMURF1 protein level increased following the treatment of mibolerone. However, knockdown of endogenous AR not only decreased basal levels of SMURF1 in mibolerone-unstimulated cells, but also almost completely abrogated androgen-induced increase in SMURF1 expression (Fig. 2A). AR knockdown in C4-2 cells was not effective as that in LNCaP cells because the residual AR level was detected and AR expression was slightly increased following mibolerone treatment (Fig. 2B). In agreement with these observations, SMURF1 was modestly induced by mibolerone in AR knockdown cells although the effect was largely reduced in these cells compared to control knockdown cells (Fig. 2B). Next, we examined the role of AR in androgen regulation of SMURF1 by treating cells with the second-generation AR antagonist MDV3100 (enzalutamide). As expected, MDV3100 treatment diminished mibolerone-mediated induction of AR proteins in both LNCaP and C4-2 cells (Fig. 2C and D). Importantly, mibolerone-induced upregulation of SMURF1 was almost completely abrogated by MDV3100 (Fig. 2C and D). Thus, using both genetic and pharmacological approaches we demonstrate that androgen-stimulated expression of SMURF1 is mediated through the AR.

Androgens regulate SMURF1 expression at the transcriptional level

Given that expression of SMURF1 is increased following stimulation by different concentrations of androgens (Fig. 1), we focused our efforts on understanding the molecular basis of androgenic regulation of SMURF1. After treatment with 1 nM of mibolerone for 48 h, LNCaP cells were treated with the *de novo* protein synthesis inhibitor cycloheximide (CHX) and protein levels of SMURF1 were measured by Western blot analyses. Consistent with the data shown in Fig. 1A, the overall levels of SMURF1 protein were higher in androgen-treated than untreated cells (Fig. 3A). Quantitative analysis indicated that androgen treatment had little or no effect on the stability of SMURF1 protein in LNCaP cells (Fig. 3B). These data suggest that androgen-increased SMURF1 expression was not mediated through decreased degradation of the protein. To further explore the molecular mechanism of androgen regulation of SMURF1, we focused on the mRNA level. As demonstrated by RT-PCR, treatment of LNCaP cells with different concentrations of mibolerone increased expression of *SMURF1* mRNA. Consistent with the protein changes, *SMURF1* mRNA was increased most dramatically by treatment with 1 nM of mibolerone in

LNCaP cells comparing with other concentrations (Fig. 3C). A similar result was obtained in C4-2 cells (Fig. 3D). Thus, androgens affect *SMURF1* expression at the transcriptional level.

We sought to determine whether androgen-induced up-regulation of *SMURF1* mRNA is due to increased synthesis or stability of *SMURF1* mRNA. LNCaP cells were pretreated with the RNA synthesis inhibitor actinomycin D (Act D) 30 min prior to androgen treatment. Little or no difference in the half-life of *SMURF1* mRNA was detected in cells treated with or without mibolerone (Fig. 4A). These data suggest that androgens do not affect the stability of *SMURF1* mRNA. To determine whether androgens increase the synthesis of *SMURF1* mRNA, we performed time-course studies. We demonstrated that expression of *SMURF1* mRNA levels began to increase at 2 h (Fig. 4B) and *SMURF1* protein started to increase at 4 h after androgen treatment (Fig. 4C and D). The early response suggests that androgenic regulation of *SMURF1* expression could be mediated by a direct mechanism.

***SMURF1* is a novel downstream target gene of AR**

Based on the results described above, we sought to determine whether *SMURF1* is a direct downstream target gene of AR. To this end, we analyzed the AR ChIP-seq data in LNCaP cells (Yu and Huang, unpublished data). We found that there is an obvious binding peak of AR at an area approximately 16 kb from the transcription start site of *SMURF1* gene (Fig. 5A). The abundance of AR binding at this site was markedly increased following mibolerone treatment (Fig. 5A). Histone h3 lysine 4 monomethylation (H3K4Me1) and trimethylation (H3K4Me3) are established marks for gene enhancer and promoter, respectively with a typical bilateral enrichment pattern (10,11). Further analyses indicate that the AR binding site belongs to the enhancer region of the *SMURF1* gene because the AR binding area is H3K4me1-positive but H3K4me3-negative (Fig. 5A), which represents a signature characteristics of potential enhancers (10,11). By examining *SMURF1* expression in the RNA-seq data obtained from LNCaP cells (Yu and Huang, unpublished data), we found that expression of *SMURF1* exons was readily detected in LNCaP cells (Fig. 5B). However, expression of *SMURF1* mRNA was largely inhibited by MDV3100 (Fig. 5B). These data suggest that *SMURF1* is a downstream target of AR in PCa cells.

Next, we performed motif search in the promoter/enhancer regions of the *SMURF1* gene. We found that in the AR-bound enhancer region, there exists a canonical half ARE AGAACA (Fig. 5C and D). To further verify whether *SMURF1* is an authentic AR target gene, we employed a ChIP assay to assess the binding of AR to the enhancer of *SMURF1* in LNCaP cells in the presence or absence of androgen stimulation. LNCaP cells were cultured in 10% CSS medium for 48 h and then treated with or without 1 nM of mibolerone for 24 h. Cells were harvested and subjected to ChIP assays. ChIP primers were designed based on the DNA sequences surrounding the ARE motif in the *SMURF1* enhancer. A basal level of AR binding to the *SMURF1* enhancer was detected in LNCaP cells without androgen treatment. Importantly, the binding was increased by approximately 10 folds after androgen treatment (Fig. 5E). As a positive control, AR binding to the *PSA* enhancer was readily detected in LNCaP cells without mibolerone treatment and markedly increased following androgen stimulation (Fig. 5E). A similar result was obtained in C4-2 cells (Fig. 5F). These

data indicate that *SMURF1* is a direct binding target of AR in PCa cells and that AR binds to the *SMURF1* enhancer and mediates androgen-induced expression of *SMURF1* in PCa cells.

SMURF1 plays an important role in androgen-induced invasion of PCa cells

As an E3 ubiquitin ligase, SMURF1 plays many roles during cancer progression. One of these functions is that SMURF1 regulates cancer cell adhesion and migration (12,13). Androgens have also been reported to regulate tumor metastasis (14). Therefore, we examined whether SMURF1 is involved in androgen-induced PCa cell migration and invasion. While we have shown that expression of SMURF1 can be regulated by androgens in both C4-2 and LNCaP cell lines, because previous studies have shown that C4-2 cells have more metastatic potential than LNCaP cells (15,16), the C4-2 cell line was chosen for migration and invasion studies. To this end, we knocked down SMURF1 in C4-2 cells, and then treated cells with mibolerone and performed wound healing assay. The effectiveness of SMURF1 knockdown by two independent siRNAs (siSM1 and siSM3) and androgen stimulation on SMURF1 expression was confirmed by Western blot analysis in C4-2 cells (Fig. 6A). When cells were treated with androgens, cells migration was substantially increased, but these effects were partially diminished by the silence of SMURF1. In agreement with the finding that siSM3 had a better effect on SMURF1 knockdown, its treatment resulted in better inhibition of androgen-induced cell migration (Fig. 6B). Quantitative analyses were performed and the quantified data are shown in Fig. 6C. In the same manner, endogenous SMURF1 was knocked down with siSM3 and treated with mibolerone, followed by invasion assay to detect the effect of SMURF1 on androgen-induced invasion of PCa cells. Knocking down SMURF1 completely blocked androgen-induced invasion of C4-2 cells (Fig. 6D and E). Notably, we found that 24 h-treatment of C4-2 cells with 10 nM of mibolerone had modest, but not significant inhibitory effect on cell proliferation (Fig. 6F), which is consistent with a slight reduction in expression of pro-proliferation genes CDK2 and CDK4 (Fig. 6G). Similarly, knockdown of SMURF1 had little or no effect on C4-2 cell proliferation in the absence or presence of androgen treatment (Fig. 6F). These results rule out the possibility that the effect of SMURF1 on androgenic regulation of cell invasion is mediated by its effect on cell proliferation. Together, our data indicate that SMURF1 plays an important role in androgen-induced invasion of PCa cells.

Discussion

SMURF1 belongs to the HECT family of E3 ubiquitin ligases that play important roles in regulating cell response to TGF- β /BMP signaling pathway. SMURF1 selectively regulates the steady-state level of regulatory SMADs (R-SMADs), such as SMAD1, SMAD5, SMAD8 and SMAD2 proteins, and had little or no effect on SMAD3 or SMAD4 (17). Besides, SMURF1 also interacts with inhibitory SMADs (I-SMADs) SMAD6 and SMAD7 (18). SMURF1 can also utilize SMAD1 and SMAD6 as adaptors to mediate degradation of RUNX2, a bone-specific transcription factor frequently overexpressed in invasive breast and prostate cancer, thereby regulating bone development and cancer bone metastasis (18). However, there is little report about SMURF1 in PCa, especially the functional interaction between SMURF1 and AR. In this report, we demonstrate that androgens induce SMURF1 expression in a dose-dependent manner in various PCa cell lines. This effect of androgens is

not caused by the changes in SMURF1 protein stability. Instead, it is mediated by androgenic regulation of *SMURF1* mRNA, as demonstrated by RT-PCR and RNA-seq data. Therefore, we demonstrate for the first time that the androgens induce upregulation of SMURF1 expression PCa cells.

We provide evidence that androgen regulation of SMURF1 is mediated through the AR. First, we demonstrate that depletion of endogenous AR by siRNAs abrogates androgen-induced expression of SMURF1 in LNCaP cells. Second, we show that treatment with the AR antagonist enzalutamide largely abolishes androgen regulation of SMURF1 expression in both LNCaP and C4-2 cells. Third, by analyzing the AR ChIP-seq data, we find that AR binds to the SMURF1 enhancer and this result is further confirmed by ChIP-qPCR. Thus, we identify SMURF1 as a *bono fide* target of AR and demonstrate that AR binds to the SMURF1 enhancer and mediates its expression in response to androgen stimulation in PCa cells.

As a novel downstream target gene, what is the role of SMURF1 in AR signaling in PCa cells? AR not only mediates invasiveness of androgen-dependent PCa cells, but also is necessary for the enhanced invasiveness of CRPC cells (14). Several clinical studies with metastatic CRPC have demonstrated that PCa growth and metastasis remain dependent on androgen supply and reactivation of the AR (2). To date, however, our knowledge regarding the mechanism by which these effects are exerted remains limited. SMURF1 has been implicated in cancer progression and enables to regulate cancer cell adhesion and migration (13,19). In agreement with previous reports (20,21), androgens induce PCa cell migration and invasion. Importantly, the androgenic effect on invasion is completely abolished by knockdown of endogenous SMURF1. Thus, SMURF1 is a very important factor that is required for androgen-induced invasion of PCa cells.

In summary, we have identified *SMURF1* as a novel downstream target gene of AR. We have also provided molecular insight into the mechanisms by which androgens regulate SMURF1 expression in PCa cells. We further demonstrate that upregulation of SMURF1 plays an essential role in androgen-induced invasion of PCa cells. Understanding the molecular mechanisms of androgen regulation of SMURF1 and the functional interaction between AR and SMURF1 may lead to the identification of novel therapeutic targets for treatment of advanced/metastatic PCa.

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Fig. 1.

Androgens regulate the expression of SMURF1 protein. **A, B, E and F:** LNCaP (**A and B**) and C4-2 (**E and F**) cells were grown in 10% CSS for 48 h and then incubated for an additional 24 h with the given concentrations of mibolerone (mib). Protein expression levels of SMURF1, and ERK2 (loading control) were analyzed by immunoblotting. The expression levels of *PSA*, and *GAPDH* (internal control) mRNA were analyzed by RT-PCR (**B and F**). Quantification of the SMURF1 and ERK2 protein signal intensities were obtained from exposures in which the signals were not saturated during the entire time course (**B and F**). Signal intensities were normalized to the signal intensities obtained at the point that the concentration of mib was zero. Columns, mean values among three replicates; error bars, SD. **C and D:** LAPC4 cells were grown in 10% CSS for 48 h and then incubated for an additional 72 h with the given concentrations of mibolerone (mib). Protein expression levels of SMURF1, and ERK2 (loading control) were analyzed by immunoblotting (**C**). The expression levels of *PSA*, and *GAPDH* (internal control) mRNA were analyzed by RT-PCR (**D**). Quantification of the SMURF1 and ERK2 protein signal intensities were obtained from exposures in which the signals were not saturated during the entire time course (**D**). Signal intensities were normalized to the signal intensities obtained at the point that the concentration of mib was zero. Columns, mean values among three replicates; error bars, SD.

Fig. 2.

Androgen-induced increase in SMURF1 expression is mediated through the AR. **A:** LNCaP cells were transfected with non-specific (NS) control or AR siRNA for 48 h and then treated with or without mib (1 nM) for an additional 24 h. SMURF1, AR, and ERK2 protein expression was analyzed by Western blotting. The levels of SMURF1 were quantified, normalized to ERK2 levels, and given relative to mock-treated cells. Similar results were obtained from three independent experiments. **B:** C4-2 cells were transfected with NS control or AR siRNA for 48 h and then treated with or without mib (10 nM) for an additional 24 h. SMURF1, AR, and ERK2 protein expression was analyzed by Western blotting. The level of SMURF1 were quantified, normalized to ERK2 levels, and given relative to mock-treated cells. Similar results were obtained from three independent experiments. **C:** LNCaP cells were grown in 10% CSS for 48 h and then treated with or without mib (1 nM) in the presence or absence of 10 μ M of MDV3100 (enzalutamide, an androgen receptor antagonist) for additional 24 h. Cells were harvested and lysed for Western blot analysis with indicated antibodies. The levels of SMURF1 were quantified, normalized to ERK2 levels, and given relative to mock-treated cells. Similar results were obtained from three independent experiments. **D:** C4-2 cells were grown in 10% CSS medium for 48 h and then treated with or without mib (10 nM) in the presence or absence of 10 μ M of MDV3100 for an additional 24 h. Cells were harvested and lysed for Western blot analysis with antibodies indicated. The level of SMURF1 were quantified, normalized to ERK2 levels, and given relative to mock-treated cells. Similar results were obtained from three independent experiments.

Fig. 3.

Androgens regulate SMURF1 expression at the transcriptional level. **A:** After grown in 10% CSS medium for 48 h, LNCaP cells were treated with 1 nM of mib for 24 h and then treated with cycloheximide (25 μ g/ml). At the time points indicated cells were harvested and lysed for Western blot analysis with antibodies of SMURF1 and ERK2. **B:** Quantification of the SMURF1 protein signal intensities were obtained from exposures in which the signal was not saturated during the entire time course. Signal intensities were normalized to the signal intensity obtained at the time zero. Mean values were obtained from three independent experiments. Error bars, SD. **C** and **D:** LNCaP (**C**) and C4-2 (**D**) cells were grown in 10% CSS medium for 48 h, and then treated with different doses of mib as indicated for 24 h. Cells were harvested, and mRNA levels of *SMURF1* was determined by real-time PCR and normalized relative to that of *GAPDH*. Columns, mean values among three replicates; error bars, SD. * $P < 0.05$ and ** $P < 0.01$.

Fig. 4. *SMURF1* is an early response gene of androgens. **A:** LNCaP cells were grown in 10% CSS medium for 48 h, pretreated with 5 μ M Act D for 30 min and then treated with or without 1 nM of mib. At the time points indicated, cells were harvested and RNAs were isolated and subjected to real-time PCR for *SMURF1* expression. *GAPDH* cDNA was used as a control for the normalization of RNA used. **B:** Time-course study of the androgenic effect on *SMURF1* mRNA expression. LNCaP Cells were grown in 10% CSS medium for 48 h, treated with 1 nM of mib for varying lengths of time, *SMURF1* mRNA expression was examined by real-time PCR. **C:** Time-course study of the androgenic effect on *SMURF1* protein expression. LNCaP Cells were grown in 10% CSS medium for 48 h, treated with 1 nM of mib for varying lengths of time, *SMURF1* protein expression was examined by Western blotting. **D:** The protein level of *SMURF1* was quantified, normalized to ERK2 levels. Similar results were obtained from three independent experiments. Columns, mean values among three replicates; error bars, SD. * $P < 0.05$ compared with mib untreated (–) group.

Fig. 5.

SMURF1 is a novel downstream target gene of AR. **A:** Screen shots from UCSC genome browser showing signal profiles of AR binding (ChIP-seq), and H3K4me1 and H3K4me3 modifications (ChIP-seq) (22). **B:** Screen shots from UCSC genome browser showing signal profiles of *SMURF1* mRNA expression (strand-specific RNA-seq) in LNCaP cells treated with or without MDV3100 (MDV). **C:** Canonical full- and half- androgen response element (ARE) sequences. **D:** Genomic DNA sequence surrounding a potential ARE (in red) in the *SMURF1* enhancer. **E:** For ChIP assay, LNCaP cells were grown in 10% CSS medium for 48 h, and then treated with or without 1 nM mib for 24 h and harvested. Chromatin proteins cross-linked to DNA were immunoprecipitated with AR antibody or control IgG. DNA pulled down was analyzed by real-time PCR using primers that amplify the AR binding region in the *SMURF1* enhancer. As a positive control, DNA pulled down in LNCaP cells was analyzed by real-time PCR using primers that amplify the AR binding region in the

PSA enhancer. **F:** C4-2 cells were grown in 10% CSS medium for 48 h, then treated with or without 1 nM mib for 24 h and harvested. Chromatin proteins cross-linked to DNA were immunoprecipitated with AR antibody or control IgG. DNA pulled down was analyzed by real-time PCR using primers that amplify the AR binding region in the SMURF1 enhancer. Columns, mean values among three replicates; error bars, SD. *P<0.05.

Fig. 6.

SMURF1 plays an important role in androgen-induced migration and invasion of PCa cells.

A: C4-2 cells were transfected with NS control or SMURF1 siRNAs (siSM1 and siSM3) using Lipofectamine 2000, and then cultured in 10% CSS medium. At 48 h after transfection, cells were treated with or without 10 nM of mib. SMURF1, AR, and ERK2 protein expression was analyzed by Western blotting. **B:** C4-2 cells were transfected with NS control or SMURF1 siRNAs using Lipofectamine 2000 as in (A), and then cultured in 10% CSS medium. At 48 h after transfection, cells were treated with or without 10 nM of mib, the wound healing assay was conducted. The area of the gaps and percent wound

closure were determined at 0 h, 48 h and 72 h after mib treatment. **C:** The qualification of wound healing assays in **(B)**. Similar results were obtained from three independent experiments. Columns, mean values among three replicates; error bars, SD. **D:** C4-2 cells were transfected with NS control or SMURF1 siRNAs (siSM3) using Lipofectamine 2000 as in **(A)**, and then cultured in 10% CSS medium. At 48 h after transfection, cells were re-suspended in serum free RPMI and treated with or without 10 nM of mib. The invasion assay was performed. **E:** The qualification of invasion assays in **(D)**. Similar results were obtained from three independent experiments. Columns, mean values among three replicates; error bars, SD. **F:** C4-2 cells were seeded into six-well plates with 4×10^5 /well, and transfected with NS control or SMURF1 siRNAs (siSM3) using Lipofectamine 2000 as in **(A)**, then cultured in 10% CSS medium. At 48 h after transfection, cells were treated with or without 10 nM of mib for another 24 h. Cells were harvested for trypan blue assay. Columns, mean values among three replicates; error bars, SD. * $p < 0.05$; siSM1, number 1 siRNA for SMURF1; siSM3, number 3 siRNA for SMURF1; +, mib-treated. **G:** C4-2 cells were grown in 10% CSS medium for 48 h, then treated with or without 10 nM of mib for 24 h and harvested for Western blot analysis.