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Original Article

Clostridium scindens metabolites trigger prostate cancer progression through androgen receptor signaling

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KEYWORDS
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Bacterial metabolite;
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Abstract Prostate cancer (PCa) is one of the most common malignancies in men; recently, PCa-related mortality has increased worldwide. Although androgen deprivation therapy (ADT) is the standard treatment for PCa, patients often develop aggressive castration-resistant PCa (CRPC), indicating the presence of an alternative source of androgen. *Clostridium scindens* is a member of the gut microbiota and can convert cortisol to 11β-hydroxyandrostenedione (11β-OHA), which is a potent androgen precursor. However, the effect of *C. scindens* on PCa progression has not been determined. In this study, androgen-dependent PCa cells (LNCaP) were employed to investigate whether *C. scindens*-derived metabolites activate androgen receptor (AR), which is a pivotal step in the development of PCa. Results showed that cortisol metabolites derived from *C. scindens*-conditioned medium promoted

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proliferation and enhanced migration of PCa cells. Furthermore, cells treated with these metabolites presented activated AR and stimulated AR-regulated genes. These findings reveal that *C. scindens* has the potential to promote PCa progression *via* the activation of AR signaling. Further studies on the gut—prostate axis may help unravel an alternative source of androgen that triggers CRPC exacerbation.

Introduction

Prostate cancer (PCa) is the second most commonly diagnosed life-threatening cancer in men, as reported by the Global Cancer Observatory.[1] Many risk factors, including age, family history, diet, and ethnicity, are considered challenging in preventing PCa and determining management strategies.[2] Androgen is an essential steroid hormone that contributes to the growth and survival of PCa cells through androgen receptor (AR) signaling.[3] In the presence of male hormones (androgen and testosterone), AR can be activated and translocated to the nucleus. The binding of ARs to androgen response elements (ARE) can initiate the transcription of a subset of genes responsible for PCa growth and survival.[4,5] Owing to the proliferation-promoting effects of AR signaling, androgen deprivation therapy (ADT) in the treatment of advanced-stage PCa often involves the suppression of androgen synthesis and/or AR signaling.[6] Despite continuous androgen deprivation, a large proportion of patients on ADT develop castration-resistant PCa (CRPC), which is inevitable.[7] CRPC is a lethal form of PCa that is refractory to hormone therapy and often increases tumor cell proliferation in spite of reduction in serum testosterone and androgen levels therapeutically.[8,9]

Despite subjecting patients to ADT therapy to treat the castrate levels of circulating testosterone, there are chances of PCa relapse, followed by the increase in serum prostate-specific antigen (PSA).[10] CRPC is androgen-dependent, as a pool of potent androgens that can activate AR signaling can be detected in CRPC tumors.[11] 11β-hydroxyandrostenedione (11β-OHA) is produced by human adrenal gland and is a precursor for the production of potent androgens 11-ketotestosterone (11KT) and 11keto-dihydrotestosterone (11KDHT).[12] 11KT was found to activate AR in the same manner as testosterone.[13] Although it is known that in the absence of testosterone, precursor androgens serve as substrates for the production of active androgens, which are prerequisites in PCa progression, the source of these precursor androgens has not been clearly determined.

The gut microbiota can produce hormones, including endogenous ones.[14,15] *Clostridium scindens* has recently been demonstrated as a particularly interesting member of the gut microbiota that cleaves the side chains of corticoids by steroid-17,20-desmolases (SDase).[16] SDase, encoded by *desABCD* operon in *C. scindens*, converts cortisol or other glucocorticoids into 11β-OHA.[17,18] The complex gut microbiota plays a vital role in maintaining human health; however, certain changes may have clinical implications in several diseases.[19—21] In this study, we have demonstrated that 11β-OHA in *C. scindens* metabolites plays a crucial role as a potent precursor androgen that enhances PCa progression through AR signaling. Our results reveal an alternative source of precursor androgen biosynthesis derived from *C. scindens* and emphasize the underlying mechanism of its impact on PCa development.

Materials and methods

Bacterial strain, growth conditions, and 11β-OHA induction

C. scindens (ATCC35704) obtaining from Bioresource Collection and Research Center, Hsinchu, Taiwan, was cultured on Chocolate Agar II plate (BD Biosciences, USA) at 37 °C under anaerobic condition. After 48 h of incubation, the bacteria were expanded in 10 ml anaerobic Brain Heart Infusion (BHI) broth. To induce the sterol metabolites, the overnight inoculum was then transferred to 10 ml BHI broth containing 50 μM cortisol. 11β-OHA contained in bacterial metabolites was verified by high-performance liquid chromatography (HPLC) (Merck, Rahway, NJ, USA).

Cell culture

LNCaP cells (ATCC CRL-1740) were maintained in the RPMI-1640 (HyClone, Logan, UT, USA) containing 10% charcoal stripped fetal bovine serum (HyClone) and supplemented with 100 U/mL penicillin and 100 μg/ml streptomycin (Sigma—Aldrich). The cells were routinely cultured at 37 °C, 5% CO_2 in humidified incubator for the following studies.

Cell proliferation assay

PCa cell proliferation was measured by MTT assay. LNCaP cells (1×10^4) were seeded in a 96-well plate for 12 h prior to the treatment with various dilution (1:5, 1:10, 1:20, 1:50, and 1:100) of *C. scindens*-conditioned medium, dihydrotestosterone (DHT, 10 nM), or cortisol (50 μM). After conducting the treatments for 6 days, the culture supernatant was discarded and incubated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] reagent for 2 h at 37 °C. Cell viability was measured with the absorbance at 562 nm by using a spectrophotometer (Molecular Devices, San Jose, CA, USA). Data were normalized to day 0 and cell growth was represented in fold over starting.

Wound healing assay

Wound healing assay was performed using SPLcar™ blocks (SPL Life science, Korea). LNCaP cells (4×10^4 cells/ml) were seeded into each block of 12 well culture plates (Thermo Fisher). After incubation for 24 h, SPLCar™ blocks were carefully removed. RPMI-1640 supplemented with either DHT or *C. scindens* metabolites were added to each well. The snapshot picture of wound closure was taken at 0, 24, 48 h after removing the blocks. The distance of two sides of the wound was measured and quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell migration assay

In vitro cell migration assay was performed using 24-well transwell plates (8 μm insert) (Corning, Lowell, MA USA). LNCaP cells (1×10^4/ml) in serum-free medium were plated onto the upper chamber of transwell plates and incubated with *C. scindens* metabolites for 48 h. Cells were fixed with 4% paraformaldehyde and stained with 0.01% crystal violet at room temperature for 5 min. Photographs were taken by Olympus IX51 (Olympus, Japan) and the number of cells in different fields of view were counted.

Luciferase reporter assay

LNCaP cells were co-transfected with PSA-Luc reporter (10 μg) and pRL-SV40 Renilla luciferase (10 μg) by jetPEI reagent (Polyplus transfection, France). The cells were lysed and the luciferase activity was measured by Dual-luciferase Reporter Assay System (Promega, Madison, MA, USA) according to the manufacturer's protocol.[22]

Western blot assay

Cells were washed with PBS (pH 7.4), and lysed in RIPA lysis buffer (Roche, Indianapolis, IN, USA) on ice for 30 min. The proteins were separated by 8% SDS-PAGE and resolved proteins were transferred to a polyvinylidene difluoride membrane (Milipore, Temecula, CA, USA). The membranes were incubated with primary antibodies and then probed with anti-rabbit secondary antibodies. The protein expression levels were detected using ECL western blotting detection reagents (GE Healthcare system, USA) and analyzed by Azure 400 (Azure Biosystems, Dublin, CA, USA).

Immunofluorescence staining

LNCaP cells were seeded on 6-well plates and incubated with either 2% culture supernatant of *C. scindens* or 10 nM DHT for 24 h. The cells were fixed in 4% paraformaldehyde for 1 h, followed by incubation with rabbit anti-AR antibody (Cell signaling technology, USA) and probed with 555-conjugated anti-rabbit IgG. Cells were mounted using Hoechst 33342. The samples were analyzed using LSM780 Confocal Laser Scanning Microscope (ZEISS, Germany). The fluorescence intensity was measured by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Corrected total cell fluorescence (CTCF) was taken as a reference source. Measurement was started with selecting the cell border and then determined the fluorescence intensity of each cell. CTCF = Integrated Density − (Area of selected cells × Mean fluorescence of background readings). AR intensity of cytosol and nucleus was measured by using CellProfiler software (Broad Institute of MIT and Harvard, Cambridge, MA, USA).[23,24]

Quantitative reverse transcription PCR (qRT-PCR)

Cells were harvested in 1 ml Trizol. Total RNA was extracted using PureLink™ RNA Mini Kit (Thermo Fisher), and DNase treated on-column with PureLink DNase (Thermo Fisher). For the synthesis of cDNA, 5 μg total RNA were reversed transcript by the Deoxy$^+$ HiSpec Reverse Trascriptase kit (Yeastern Biotech). The primers used for performing qRT-PCR were shown in Supplemented Table 1. The mRNA levels were analyzed by qRT-PCR using SYBR Green I Master Mix and a model 7900 Sequence Detector System. The program was pre-incubated at 50 °C for 2 min and 95 °C for 10 min; PCR was performed with 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Each gene quantity was determined by relative calculation using the $2^{-\Delta\Delta Ct}$ method. The method was used to calculate fold changes in each treatment group.

Statistical analysis

Standard unpaired two-tailed *t*-test were conducted to analyze the statistical differences between two groups. *P*-value less than 0.05 was considered statistically significant. Statistical analysis was performed using Prism 8 (Graph Pad, San Diego, CA, USA).

Results

C. scindens converts cortisol to 11β-hydroxyandrostenedione

The key feature of *Clostridium sindens* steroid-17,20-desmolase is that it is capable of side-chain cleavage of cortisol under anaerobic conditions (Fig. 1A), as previously described.[25] Two genes, *desA* and *desB*, which encode SDase, were validated to catalyze cortisol to 11β-OHA.[17,26] However, the biochemical reaction of enzyme catalysis may differ from the actual bacterial activity. Therefore, an alternative approach was applied to directly identify cortisol side-chain cleavage in *C. scindens*-cultured conditional medium. Standard 11β-OHA has a retention time of 5.15 min in the HPLC chromatogram (Fig. 1B). 11-OHA in *C. scindens*-conditioned media supplemented with cortisol was visible at a retention time of 5.15 min (Fig. 1C).

C. scindens metabolites promote proliferation of androgen-dependent prostate cancer cells

11β-OHA produced by the adrenal gland is a potent androgen precursor that drives PCa progression.[17,27] The

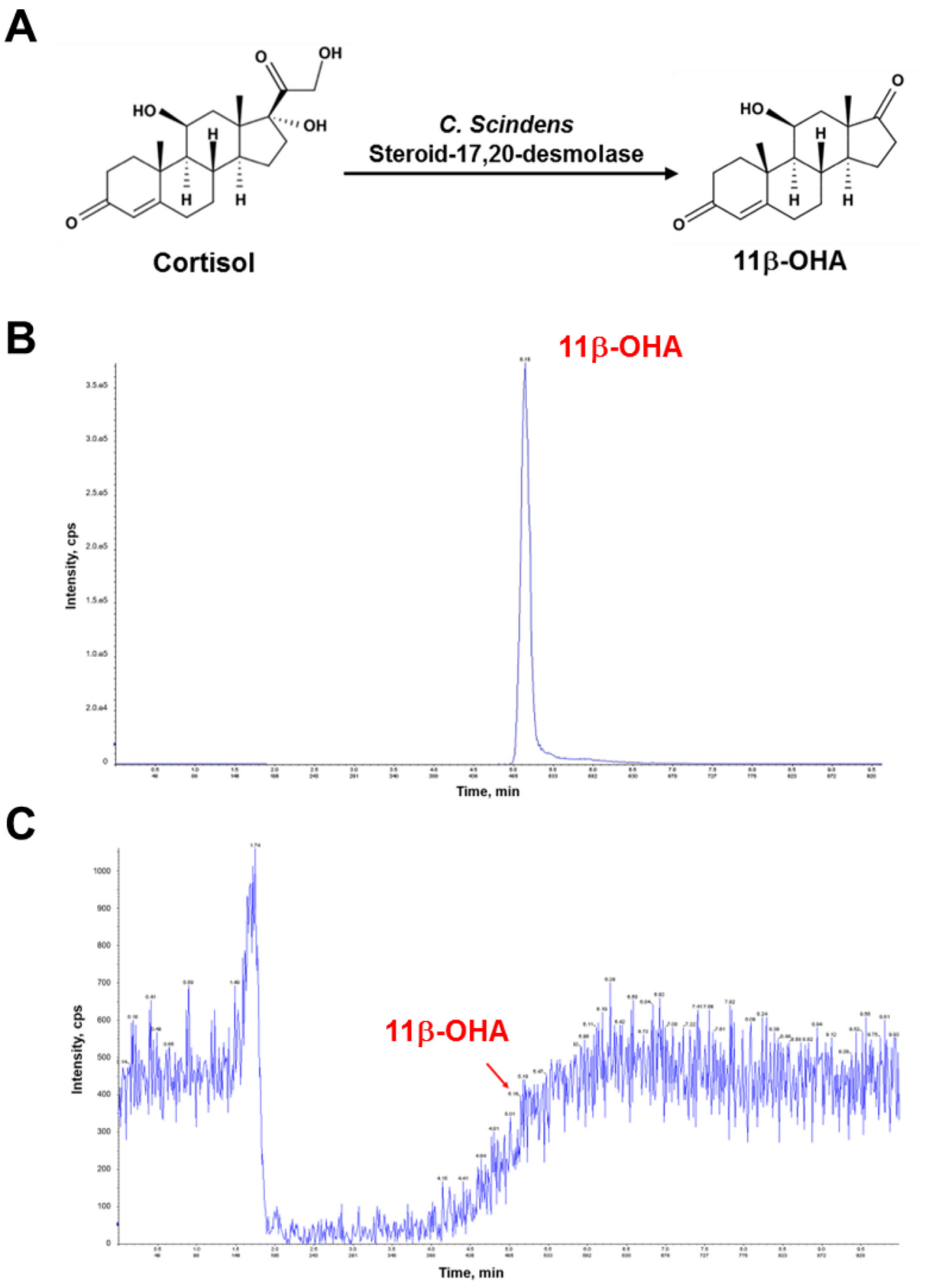

Figure 1. Analysis of 11β-OHA in *C. scindens* culture conditional medium. (A) *C. scindens* steroid-17,20-desmolase converts cortisol to 11β-OHA. HPLC chromatograms (intensity measured in counts per second, cps) of (B) pure 11β-OHA and (C) *C. scindens*-cultured medium supplemented with 50 μM cortisol.

derivation of 11β-OHA led to the production of predominantly 11KT and 11KDHT, which activated androgen receptors in androgen-dependent PCa cells (i.e., LNCaP).[8,9] The capability of *C. scindens* SDase to convert cortisol to 11β-OHA, which promotes PCa progression was further examined. As shown in Fig. 2A, the addition of either DHT or 11β-OHA significantly induced LNCaP cell proliferation. To optimize the *C. scindens*-conditioned medium with the highest activity for subsequent studies, LNCaP cells were incubated with various dilutions of *C. scindens*-cultured medium for 6 days, and cell proliferation was then analyzed. As shown in Fig. 2B, *C. scindens*-conditioned medium with a dilution of 1:50 displayed highest proliferation, and this optimal dilution was chosen for subsequent experiments. Similar to DHT and 11β-OHA, the LNCaP cells proliferated much higher in the present of metabolite from *C. scindens* cultured supplemented with cortisol than that without cortisol ($P < 0.05$) (Fig. 2C).

C. scindens metabolites enhance migration of androgen-dependent prostate cancer cells

To further explore the role of *C. scindens* in PCa cell motility, transwell cell migration and wound healing assays using the SPLScar block were performed. Transwell assays showed that both DHT and 11β-OHA significantly enhanced cell migration compared to that in control group (Fig. S1). Our results further showed that *C. scindens*-conditioned medium supplemented with cortisol remarkably increased PCa cell migration activity compared with that without cortisol (Fig. 3A and B). In parallel, the wound healing assay also showed that treatment with DHT, 11β-OHA, and *C. scindens* cultured with cortisol for 48 h markedly promoted LNCaP cell migration compared to that in control group (Figs. 3C, D, and S2). These results demonstrate that cortisol metabolites derived from *C. scindens*-conditioned medium promote proliferation and increase the migration of androgen-dependent PCa cells.

C. scindens metabolites activate androgen receptor-regulated signaling in prostate cancer cells

AR is an essential transcription factor that contributes to PCa progression and is a crucial target for therapeutic intervention.[8] In the presence of its ligand, the AR is activated and translocated to the nucleus, where it binds to androgen response elements (AREs) and initiates transcription of downstream genes.[28,29] The ability of *C. scindens* SDase to convert cortisol to 11β-OHA, which manipulates AR activity to promote PCa progression, was examined. As shown in Fig. 4, the expression level of phosphorylated AR increased in cells treated with DHT, 11β-OHA, and cortisol. Notably, markedly elevated levels of phosphorylated AR were observed in cells treated with *C. scindens* cultured with cortisol compared to those without cortisol. To verify the role of *C. scindens* in facilitating AR translocation from cytosol to the nucleus, an immunofluorescence assay was performed and the fluorescence intensity ratio of nucleus and cytosol was calculated (Figs. 5 and S3). Our results showed that nuclear AR expression in cells treated with *C. scindens* converted cortisol metabolites was much more prominent than that in cells without metabolite treatment (Fig. 5B). These findings indicate that *C. scindens*-derived metabolites promote AR translocation into the nucleus, which activates AR signaling, thereby facilitating PCa cell proliferation and migration.

C. scindens metabolites induce androgen receptor-regulated gene expression in prostate cancer cells

The AR is a crucial transcription factor that regulates a subset of genes involved in PCa cell survival, proliferation, and migration.[28] To determine whether *C. scindens* SDase converts cortisol to metabolites that activate AR downstream signaling in PCa cells, qRT-PCR was employed to analyze AR-regulated genes. Our results showed that both

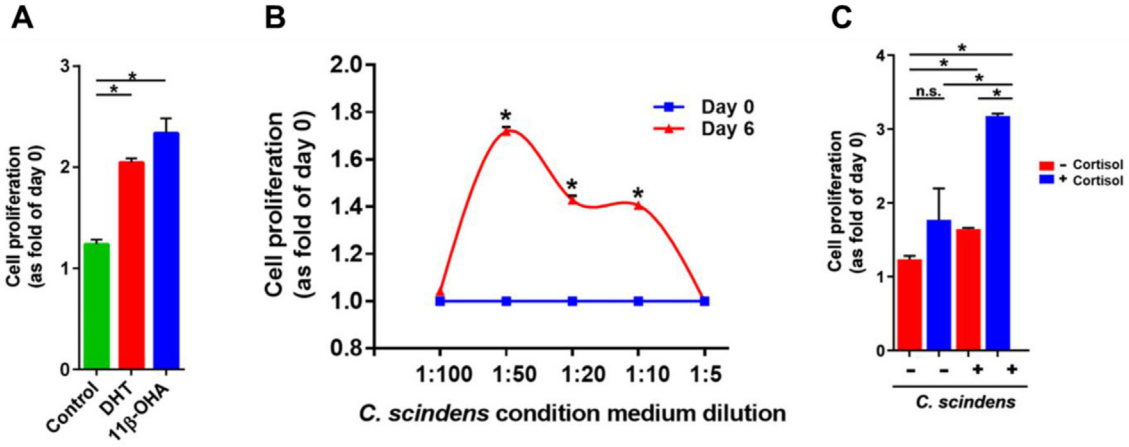

Figure 2. *C. scindens* promotes androgen-dependent PCa cell proliferation. (A) LNCaP cells were treated with DHT (red) or 11β-OHA (blue) for 6 days, and cell proliferation was analyzed using MTT assay. (B) Cells were exposed to *C. scindens*-cultured medium containing cortisol (50 μM) with various dilution (1:5, 1:10, 1:20, 1:50, and 1:100) for 6 days and then subjected to cell proliferation analysis. (C) LNCaP cells were treated with 2% *C. scindens* cultured-medium supplemented with or without cortisol (50 μM) for 6 days, and cell proliferation was performed. *, $P < 0.05$; n.s., non-significance.

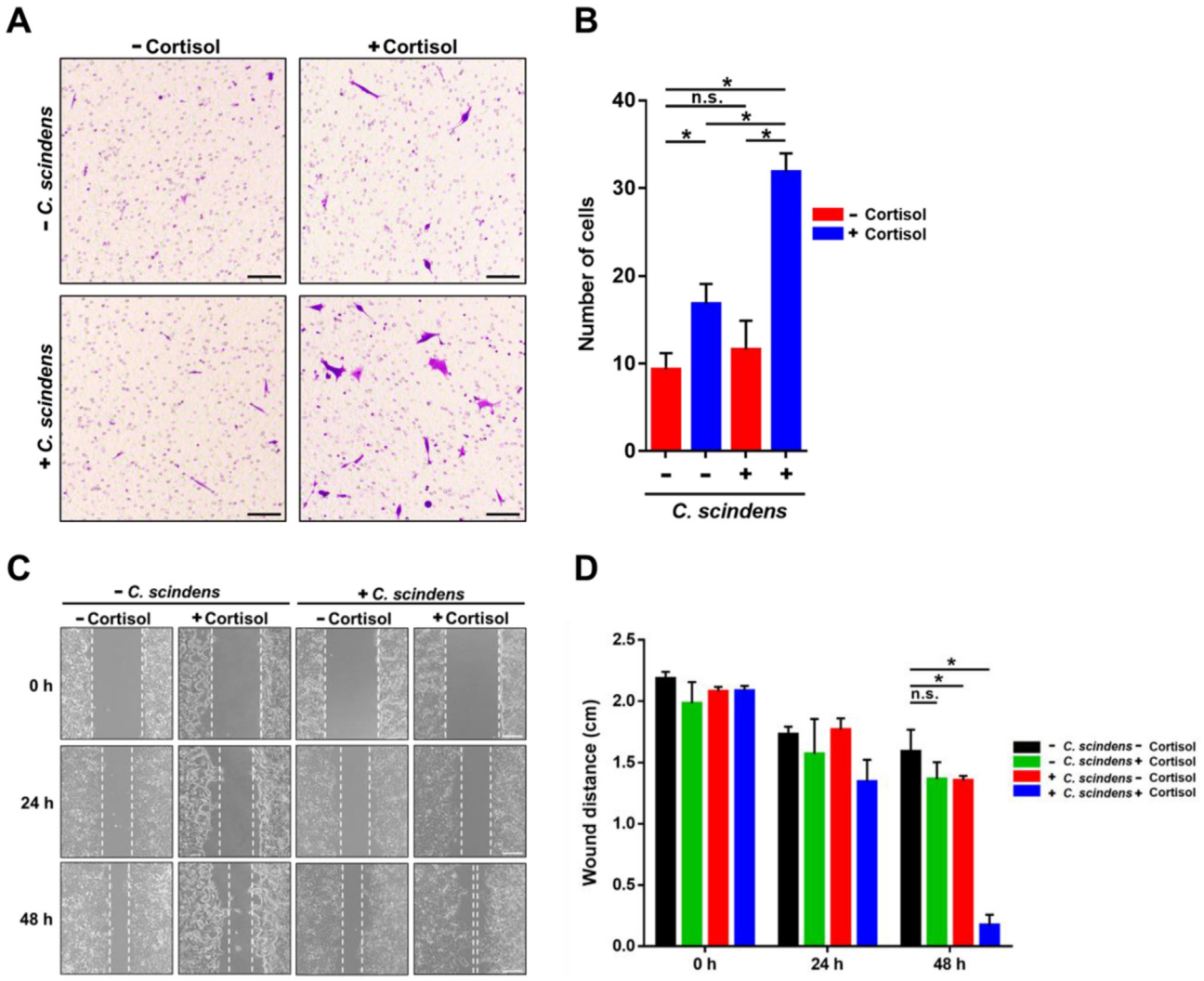

Figure 3. *C. scindens* facilitates PCa cell migration. (A) LNCaP cells were exposed to 2% *C. scindens*-conditioned medium with or without 50 μM cortisol for 48 h in an 8 μm transwell plate. Cells were stained by 0.05% crystal violate. Bar, 500 μm. (B) The number of cell migration in each field was counted. (C) LNCaP cells were seeded on SPLScar blocks and incubated with 2% *C. scindens*-conditioned medium with or without 50 μM cortisol for 48 h to analyze cell migration activity. Bar, 250 μm. (D) Cell migration activity was analyzed by determining the relative wound closure. *, $P < 0.05$; n.s., non-significance.

DHT and 11β-OHA increased PSA reporter activity and elevated the mRNA levels of AR-regulated genes (*PSA*, *Nkx3.1*, and *TMPRSS2*) (Fig. S4). Similarly, the metabolites from cortisol converted by *C. scindens* not only increased PSA reporter activity, but also activated AR-regulated genes (Fig. 6). Collectively, these results demonstrate that *C. scindens* converts cortisol metabolites to promote AR phosphorylation and translocation into the nucleus, leading to the manipulation of a subset of genes responsible for PCa progression.

Discussion

Mounting evidence has demonstrated that microbiome can influence every stage of cancer, from initiation to progression and treatment outcomes.[30] *C. scindens* is a low-abundance member of the gut microbiota (10^3—10^5 CFU/g wet weight stool).[31,32] Although *C. scindens* was first isolated in 1984, it gathered interest in 2015 owing to the formation of hydrophobic bile acids that prohibit *Clostridium difficile*-caused life-threatening diarrhea.[33] Our study demonstrated the adverse effects of *C. scindens* in PCa; its metabolites can promote PCa cell proliferation and migration. A high level of 11β-OHA in *C. scindens*-conditioned medium was detected by HPLC, which is similar to the results of previous studies using recombinant steroid-17,20-desmolase.[26,27] To the best of our knowledge, this study provides the evidence that *C. scindens* plays a certain role in promoting PCa progression.

Biosynthesis of 11β-OHA from human adrenal glands leads to the production of predominantly 11KDHT *via* 11 KT in LNCaP cells, which is sufficient for AR activation.[34] Our results showed that *C. scindens*-conditioned medium containing 11β-OHA can enhance LNCaP cell proliferation and migration. Androgen binding to AR induces AR

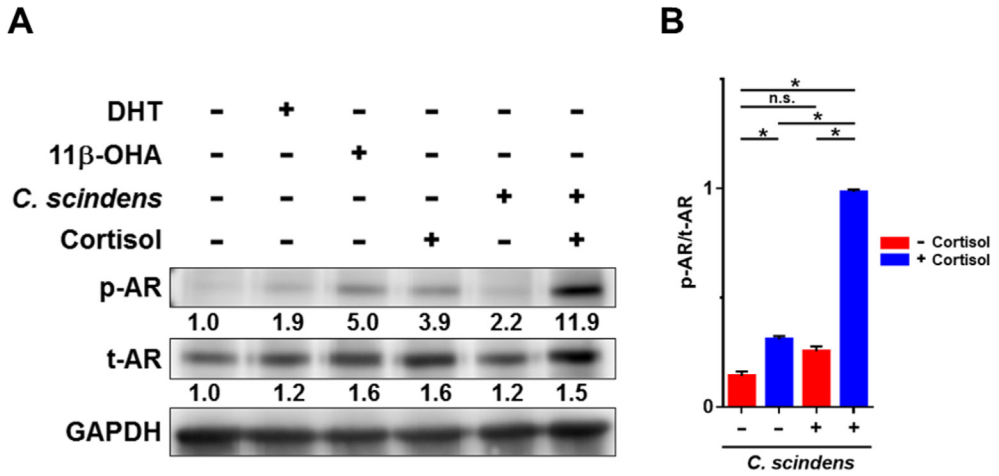

Figure 4. *C. scindens* induces AR phosphorylation in PCa cells. (A) LNCaP cells were treated with 2% *C. scindens*-conditioned medium with or without 50 μM cortisol for 24 h. The expression levels of phosphorylation of AR (p-AR) and total AR (t-AR) were analyzed by Western blot assay. GAPDH was used as a loading control. Relative expression levels were normalized to the control group and indicated under each band. (B) The relative expression of p-AR and t-AR was determined. *, $P < 0.05$; n.s., non-significance.

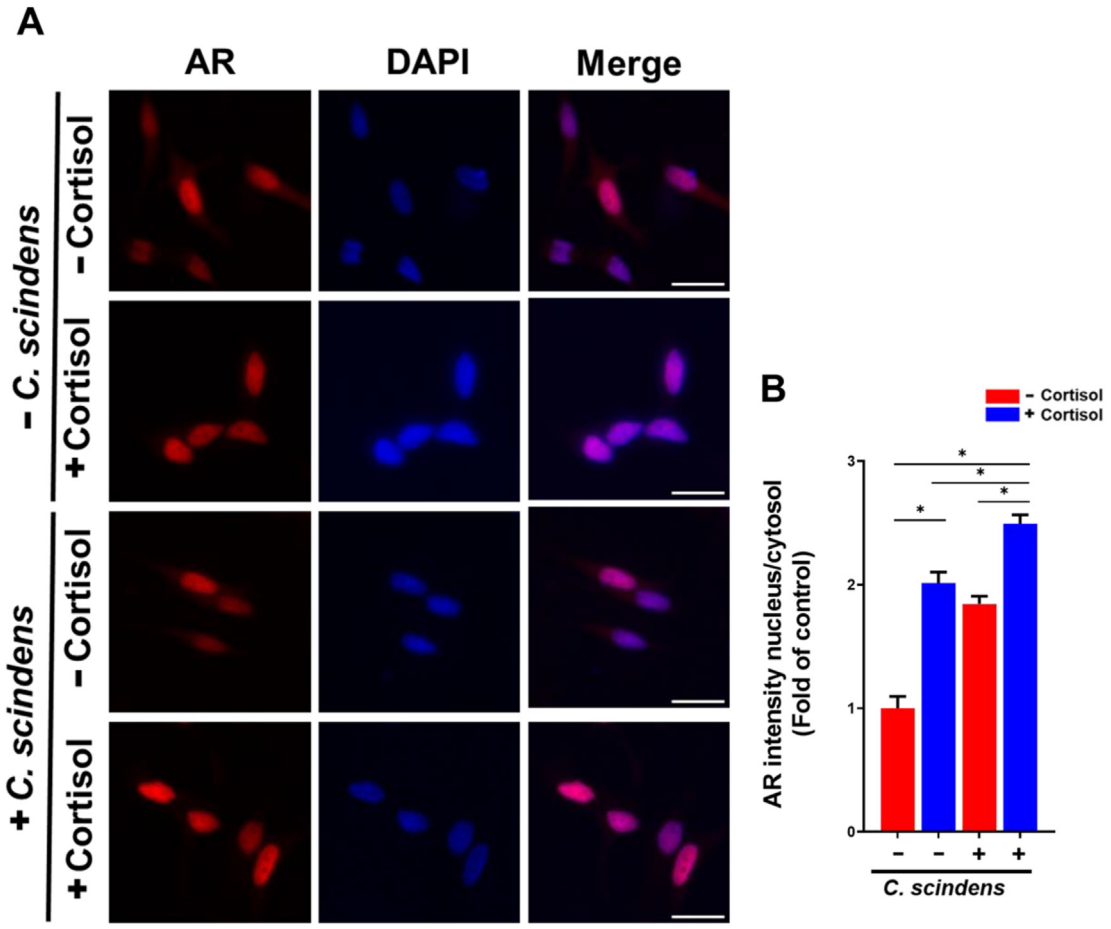

Figure 5. *C. scindens* promotes AR translocation into the nucleus. (A) LNCaP cells were treated with 2% *C. scindens*-conditioned medium with or without 50 μM cortisol for 24 h. Cells were probed with anti-AR (red), and the nucleus was stained with Hoechst 33342 (blue). The images were analyzed by a confocal laser scanning microscope with 40×objective. Scale bars, 50 μm. (B) Fluorescence intensity of AR in the nucleus and cytosol was measured in each field by total 20 fields using CellProfiller software. The ratio between nucleus and cytosol AR signal was calculated, as described in Methods section. *, $P < 0.05$; n.s., non-significance.

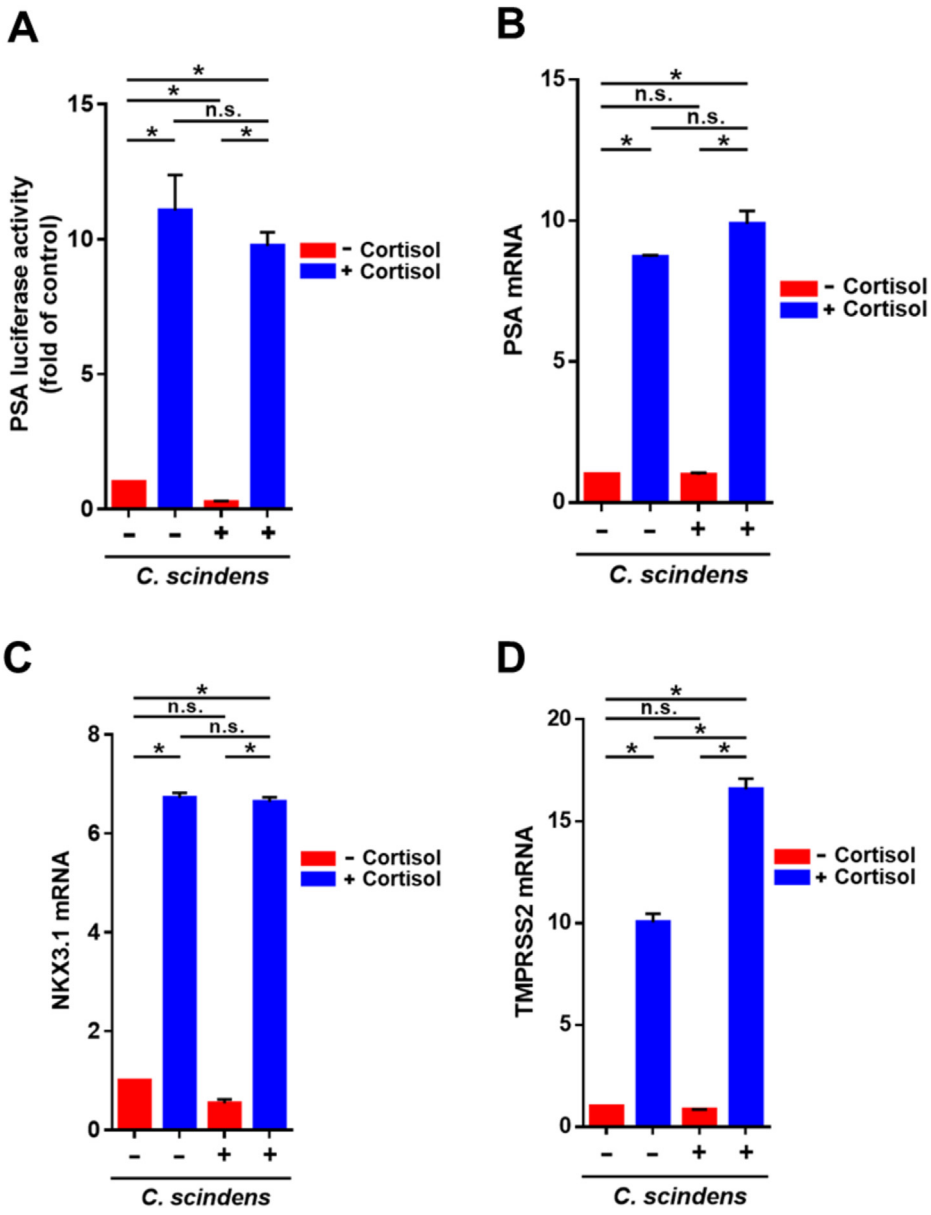

Figure 6. *C. scindens* elevates AR-regulated genes. (A) LNCaP cells were simultaneously transfected with PSA-Luc reporter and Renilla plasmid followed by exposure to 2% *C. scindens*-conditioned medium with or without 50 μM cortisol for 48 h. The level of PSA reporter activity was determined by dual-luciferase assay. (B–D) mRNA level of AR-regulated genes, including *PSA*, *Nkx3.1*, and *TMPRSS2* was analyzed using qRT-PCR. *, $P < 0.05$; n.s., non-significance.

phosphorylation at multiple sites to stabilize AR following by activating AR-regulated genes.[35] AR, on the other hand, is quickly degraded without interacting with androgen.[36] Our results showed that AR was highly expressed in the nucleus after treatment with *C. scindens* metabolites compared to that in the control group. These findings indicate that *C. scindens* converts cortisol to 11β-OHA, an androgen precursor that activates AR signaling to elicit PCa progression (Fig. 7).

Various mechanistic pathways have been suggested to explain the development of CRPC, including mutation of AR, overexpression of AR enzyme synthesis, drug efflux, and localized synthesis of androgens within PCa.[37,38] Notably, glucocorticoids are often included in chemotherapy regimens for treating patients with PCa.[39,40] The long-term administration of synthetic glucocorticoids leads to adrenocorticotropic hormone suppression and eventual atrophy of the adrenal glands to synthesize cortisol.[11,41,42] Cortisol is the main human glucocorticoid that can bind to the glucocorticoid receptor (GR) and mediate a wide range of metabolic processes, immunity, and growth.[28,41] GR signaling is essential for PCa cell proliferation through activation of AR expression.[43] In addition, GR substituted for the AR to induce a distinguishable set of target genes that necessary for maintenance of CRPC.[44] Consistently, using cortisol promoted the proliferation and migration of PCa cells. The presence of *C. scindens* in combination with cortisol treatment induced a

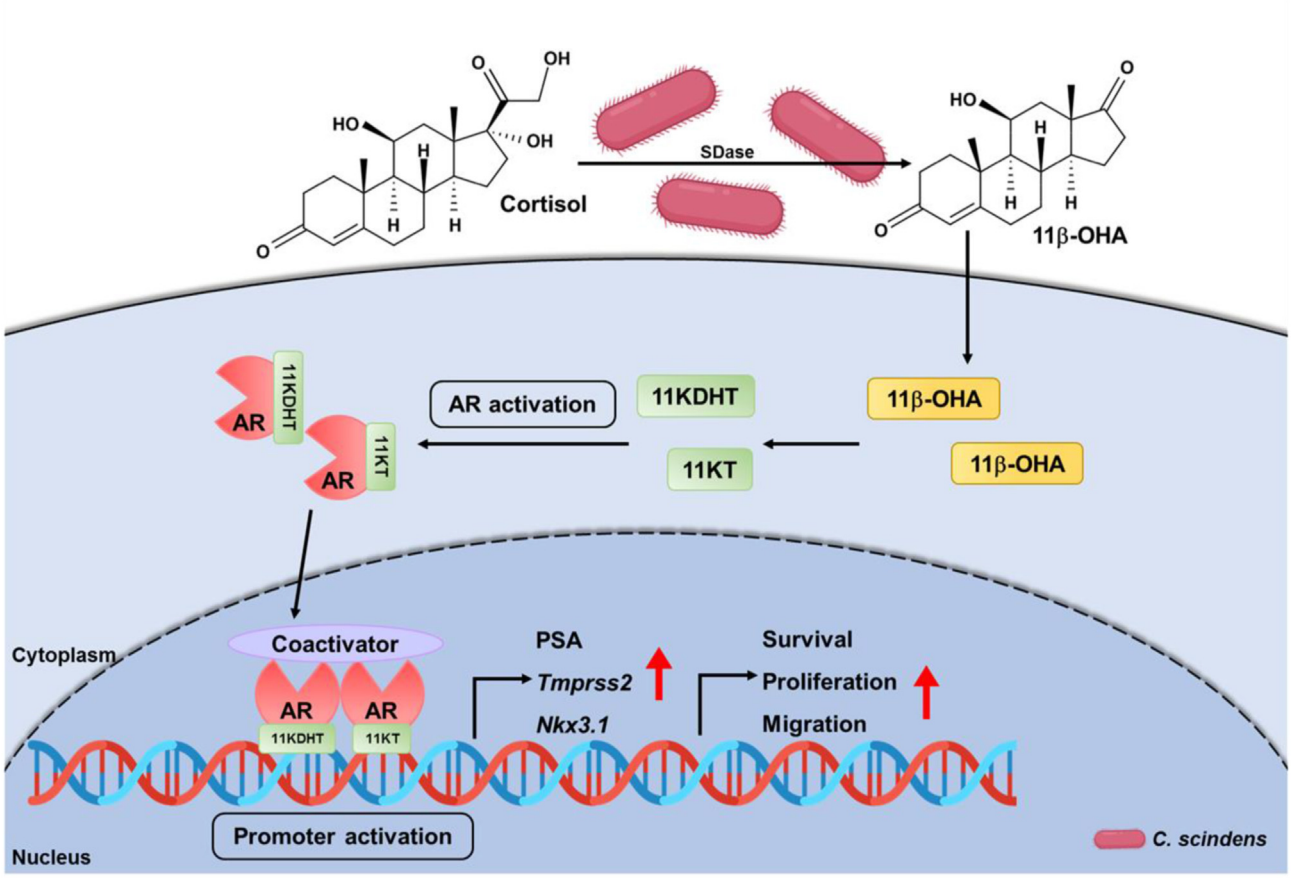

Figure 7. Molecular mechanism of *C. scindens* promotes androgen-dependent PCa cell progression. *C. scindens* converts cortisol to 11β-OHA to enhance AR phosphorylation and translocation into the nucleus, and then induces the activation of AR-regulated genes, resulting in PCa cell proliferation and migration.

significantly higher proliferation and migration of LNCaP cells upon compared to cortisol treatment alone. These lines of evidence suggest that *C. scindens* in the gut microbiota should be emphasized in the management of patients with PCa receiving glucocorticoids.

Although our current studies using cell-based experiments showed effective functions for *C. scindens* metabolites triggering PCa cell development, several limitations of the present study should be considered. First, AR remains the key target in PCa therapy, whereas ADT is highly effective in causing initial PCa regression. Increasing serum PSA and AR expression levels can be observed at low concentrations of circulating testosterone, indicating CRPC to be driven by this signaling. However, the induction of *PSA* mRNA level increased only required low levels of androgen treatment but not at high levels.[45] In this study, a high concentration of cortisol (50 μM) was used to induce a high level of 11β-OHA, which likely resulted in the plateau mRNA expression of *PSA* and *NKX3.1*. Consequently, even the presence of *C. scindens* metabolites did not significantly increase *PSA* and *NKX3.*1 mRNA levels. Second, *Clostridium* spp. exhibit high resistance to many antibiotics[46,47] that are commonly used for patient prophylaxis before radical prostatectomy.[48,49] The increased abundance of *C. scindens* after antibiotic treatment, or in the presence of glucocorticoids, may be responsible for PCa recurrence. Therefore, clinical studies on glucocorticoid therapy and/or additional diagnosis of *C. scindens* in patients with PCa relapse are warranted.

Conclusions

One of the mechanisms of CRPC development relies on AR activation by intra-tumoral androgens.[50] The circulating androgen precursors of adrenal origin are converted to the potent androgens DHT and 11KT.[8,51] However, the source of precursor androgen from the gut microbiota remains elusive. In the present study, we demonstrated that *C. scindens*-conditioned medium enhances PCa cell progression. The underlying mechanism by which *C. scindens* metabolites promote PCa development is mediated through AR-related signaling. The results of this study provide evidence that *C. scindens* plays an important role in driving PCa progression. Further research on the role of microbiota in the gut—prostate axis may develop new strategies for the prevention and treatment of PCa.

Author contributions

Conception or design of this work: HL, JTH, and CHL. Experimental study: NNB, CYL, YAC, WHK, and YTL.

Data analysis and interpretation: NNB, CYL, CWL.
Writing the manuscript: NNB, CYL, and CHL.
Final approval: all authors.
[†]NNB and CYL were equally contributed to this work.

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Conflicts of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2022.12.009.