Androgen receptor reverts dexamethasone-induced inhibition of prostate cancer cell proliferation and migration

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Received October 9, 2017; Accepted December 21, 2017

DOI: 10.3892/mmr.2018.8566

Abstract. The aim of the present study was to determine the role of androgen receptor in the effect of dexamethasone on cell proliferation and migration of multiple prostate cancer cells. The prostate cancer cell lines LNCaP, 22Rv1, C4-2 and PC3 were cultured *in vitro*. For glucocorticoid-induced experiments, the cells were transferred and cultured in RPMI-1640 medium with 10% charcoal-stripped serum from RPMI-1640 medium with 10% fetal bovine serum for at least 24 h. The effects of dexamethasone on the proliferation and migration of various cell lines were analyzed by MTT and migration assays. Dexamethasone exhibited no effect on LNCaP, C4-2 and 22Rv1 cell lines, but suppressed proliferation of glucocorticoid receptor $(GR)^+$ androgen receptor $(AR)^-$ PC3 cell line. Dexamethasone suppressed PC3 cell migration, and did not affect migration of PC3-AR9 cells. Dexamethasone positively or negatively regulated proliferation of various prostate cancer cells based on AR and GR expression profiles. The data presented in the present study indicates that androgen receptor reverts the dexamethasone-induced inhibition of prostate cancer cell proliferation and migration.

Introduction

Prostate cancer has been the most prevalent disease among men for decades and estimated 180,890 new prostate cancer cases have been diagnosed in 2016 which accounts for 21% of all cancer diagnoses that year (1). Androgen expression or androgen receptor (AR) activation serve a role in the prostate cancer (PCa) and androgen deprivation therapy (ADT) has been extensively applied as a treatment. The progress of battling PCa was made substantially based on ADT, such as combining radical radiotherapy with ADT may improve overall survival outcome in localized PCa patients (2). Following a median treatment of 24 months, almost all prostate cancer cases invariably progress to castration resistant prostate cancer (CRPC), maintaining AR activity and continuation of ADT is recommended for treatment (3,4).

In the process of ADT, adverse effects on bone, metabolic, cardiovascular, sexual and cognitive health as well as body composition are known by clinicians (5). To alleviate pain and improve the quality of life, glucocorticoids (GCs) combined with antitumor or antiandrogen agents (including Docetaxel and Abiraterone acetate) are a common class of adjuvant drugs for treatment of CRPC (6,7). Apart from ameliorating side effects caused by antitumor or antiandrogen agents, a direct effect has been identified on prostate cancer cell proliferation (8,9). GC inhibits prostate cancer cell proliferation *in vivo* and *vitro* (8,9). By contrast, an increasing amount of evidence demonstrates that glucocorticoid receptor confers resistance to antiandrogens (10,11). Therefore, GC serves a complex role in treatment, management and progression of CRPC and understanding the biological role of glucocorticoids in patients with prostate cancer is of major importance (12).

The effects of GC on prostate cancer cells, especially the castration resistance prostate cancer cells, remain to be elucidated. An association between AR and glucocorticoid receptor (GR) has been previously demonstrated and GR was negatively regulated by active androgen receptor signaling (13). Dexamethasone is a common GC agent used in the clinic (14,15). A recent study demonstrated that dexamethasone may be more effective compared with prednisolone for prostate cancer treatment (14). Therefore, the present study aimed to determine the effects of dexamethasone on prostate cancer cells. Recently, it has been demonstrated that dihydrotestosterone (DHT) can promote prostate cancer cell proliferation via glucocorticoid receptor (GR) (16). In the present study, prostate cancer cells were cultured in RPMI-1640 with 10% charcoal-stripped serum to investigate the effects of dexamethasone on prostate cancer cell proliferation and migration ability, and the role of AR in the effects.

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Key words: prostate cancer, androgen receptor, glucocorticoid receptor, proliferation, migration

Materials and methods

Cell culture. Human PCa cell lines, PC3-AR9 was provided as a gift from professor Niu (Tianjin Institute of Urology, the Second Hospital of Tianjin Medical University, Tianjin, China) (17), LNCaP, CWR22Rv1, C4-2, Du145 and PC3 (American Type Culture Collection; Manassas, VA, USA) were routinely maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml of penicillin and 100 mg/ml of streptomycin in a 5% CO_2 atmosphere at 37˚C. For glucocorticoid-induced experiments, the cells were transferred and cultured in RPMI-1640 with 10% charcoal-stripped serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) for 24 h in a 5% CO_2 atmosphere at 37˚C. The charcoal-stripped serum contains reduced hormone levels and is suitable for AR or GR signaling studies. DHT (MedChemExpress Co., Ltd., Princeton, New Jersey, USA) was dissolved in ethanol in 10^{-5} M concentration, DHT was diluted 1,000-fold in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% charcoal-stripped serum (HyClone; GE Healthcare Life Sciences) to reach a final concentration of 10 nM. Dexamethasone was dissolved in DMSO at 2×10^{-2} M final concentration, which was further diluted 100,000-fold in the RPMI-1640 medium with 10% charcoal-stripped serum to reach a final concentration of 100 nM.

Measurement of cell viability. Cell viability was assessed by MTT assay. MTT assay was modified (150 µl DMSO were used and a microplate reader at a wavelength of 490 nm instead of 50 ul DMSO at 570 nm) and performed to quantify cell proliferation (18). Briefly, LNCaP (3×10^4), C4-2 (10^4), 22Rv1 (10^4), PC-3 (10^3), Du145 (10^3) and PC3-AR9 (3×10^4). Cells were maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and were incubated in 96-well microplates with RPMI-1640 medium supplemented with 10% charcoal-stripped serum. Following 24 h, the medium was removed and replaced by either a medium containing different concentration of drug (RPMI-1640 medium supplemented with 10% charcoal-stripped serum with 10-8 M DHT, 10 nM dexamethasone, or 10 uM MDV3100; Selleck Chemicals, Shanghai, China) or a drug-free medium (control condition: DMSO or ethanol diluted in RPMI-1640 medium supplemented with 10% charcoal-stripped serum). Following 24, 48, 72 and 96 h or on day 2, 4 and 6 the media were removed and repla ced with 100 µl of 1 mg/ml MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in RPMI-1640. Following a 4-h incubation in a 5% CO_2 atmosphere at 37˚C, the MTT solution was removed and replaced with 150 µl DMSO, and the plates were shaken for 3 min on an oscillator. The optical density of each sample was determined using a microplate reader at a wavelength of 490 nm. Each experiment was performed in triplicate.

Western blot analysis. Harvested cells following the aforementioned treatment, were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 150 mM NaCl; 1 mM EDTA; 1 mM proteinase inhibitor; 1 mM Na_3VO_4; 1 mM NaF; 1 mM okadaic acid; and 1 mg/ml aprotinin, leupeptin and pepstatin). Cytoplasmic and nuclear extracts were prepared as previously described (19). Prior to the procedure, the following compounds were prepared: Buffer A: 10 mM HEPES (Sigma-Aldrich; Merck KGaA), pH 7.9, 1.5 mM $MgCl_2$ (Sigma-Aldrich; Merck KGaA), 10 mM KCl (Sigma-Aldrich; Merck KGaA), 300 mM sucrose (Sigma-Aldrich; Merck KGaA), 0.5% NP-40 (Sigma-Aldrich; Merck KGaA), stored at 4˚C; Buffer B: 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl (Sigma-Aldrich; Merck KGaA), 0.2 mM EDTA (Invitrogen; Thermo Fisher Scientific, Inc.), 2.5% glycerol (Sigma-Aldrich; Merck KGaA), stored at 4˚C; and Buffer D: 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 8% glycerol, stored at 4˚C. Medium was removed from the cultures and cells were washed in cold PBS, and harvested with a rubber scraper. Subsequently, cells were centrifuged at 550 x g for 5 min at 4˚C and supernatant was discarded. The following inhibitors were added to buffers A, B and D: 0.5 mM PMSF (Sigma-Aldrich; Merck KGaA), 1 mM Na_3VO_4 (Sigma-Aldrich; Merck KGaA), 0.5 mM DTT (Invitrogen; Thermo Fisher Scientific, Inc.), 1 µg/ml leupeptin (Sigma-Aldrich; Merck KGaA), 25 mM β-glycerophosphate (Sigma-Aldrich; Merck KGaA), 10 mM NaF (Sigma-Aldrich; Merck KGaA). The pellet was resuspended in 2X cell volume of buffer A with inhibitors and the solution was kept on ice for 10 min. Samples were vortexed briefly and centrifuged at 2,600 x g for 30 sec at 4˚C. The supernatant was collected which corresponds to cytoplasm proteins. The pellet was resuspended in buffer B with inhibitors. The mixture was sonicated for 5 sec at 4˚C and centrifuge at 10,400 x g for 5 min at 4˚C. The supernatant was diluted with equal volume of buffer D with inhibitors, and nuclear protein was extracted. Protein concertation was measured by coomassie brilliant blue. Samples (30 µg protein/lane) were separated on 8% SDS-PAGE gel and transferred to polyvinylidene fluoride membranes at 4˚C (250 mA, 2 h). Membranes were blocked in 5% fat-free milk in TBS with 1% Tween-20 for 1 h at room temperature and incubated with primary antibodies: Anti-GAPDH (Sanjian, Tianjin, China; cat. no. KM9002; 1:5,000; www.sungenebiotech.com), anti-GR (BIOSS, Beijing, China; cat. no. bs-0252R; 1:500), anti-Histone3 (used as nuclear control; Abcam, Cambridge, UK; cat. no. ab8580; 1:1,000), AR (Abcam; cat. no. ab9474; 1:1,000), Akt (Abcam; cat. no. ab8805; 1:1,000), p-Akt (Abcam; cat. no. ab81283; 1:1,000), vimentin (Abcam; cat. no. ab92547; 1:1,000) overnight at 4˚C. Subsequently, the membranes were washed in TBS with 1% Tween (TBST) for 10 min/wash 3 times and incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA; cat. no.) for 1 h at room temperature and washed for 10 min/wash 3 times. The blots were developed in Enhanced Chemiluminescence mixture (Amersham Biosciences; GE Healthcare, Chicago, IL, USA) and visualized by Imager (Image J, National Institutes of Health, version 1.48).

Migration assay. Cells (10^5 of PC3-AR9 and 5×10^4 of PC3) following different treatments (DMSO or dexamethasone) were re-suspended with RPMI-1640 with 10% charcoal-stripped serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and seeded in the upper chambers of the transwells (Corning Inc., Corning, NY, USA). A 10% solution

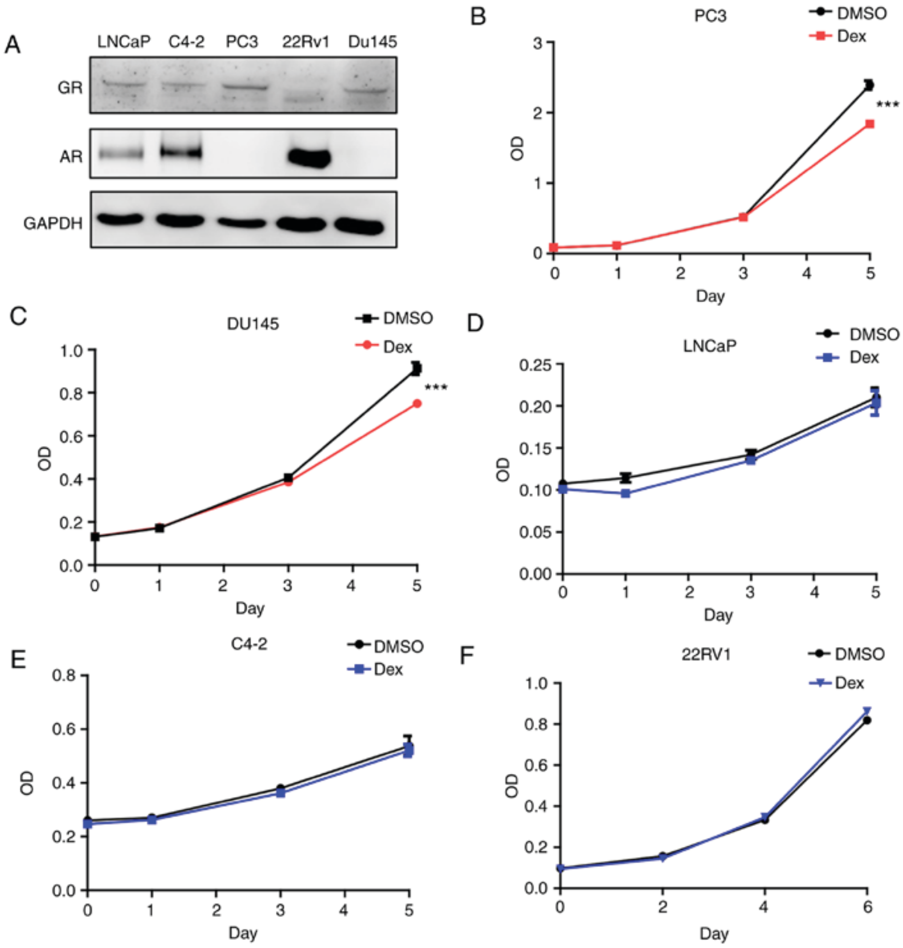

Figure 1. Effects of dexamethasone on the growth of various prostate cancer cells. (A) Western blot analysis indicated that LNCaP, C4-2 and 22Rv1 cells expressed AR and that GR expression was hardly detectable in these cells. PC3 and Du145 expressed GR but not AR. Dexamethasone demonstrated significant inhibitory effects on (B) PC3 and (C) Du145. Dexamethasone exhibited no effect on proliferation of (D) LNCaP, (E) C4-2 and (F) 22Rv1. Dex, dexamethasone; AR, androgen receptor; GR, glucocorticoid receptor. Data are presented as the mean ± standard deviation. $^{***}P<0.001$.

of FBS (Gibco; Thermo Fisher Scientific, Inc.) with or without 100 nM dexamethasone was applied in the lower chamber. As described previously (17), following a 24- or 48-h incubation (with PC3 or PC3-AR9 cells, respectively), the cells that invaded to the lower part of the membrane were harvested, fixed with 75% ethanol and stained with 0.1% crystal violet at room temperature for 25 min in PBS. Invaded cells were counted under a light microscope (magnification, x100). The standard deviation was calculated from three independent wells.

Statistical analysis. All values are presented as the mean ± standard error of the mean. Statistical evaluation of the results was performed by one-way analysis of variance followed by the Newman-Keuls method (SPSS software; version 18.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Dexamethasone affects prostate cancer cell proliferation. Glucocorticoids exert effects through non-genomic action or genomic action mediated by GR (15,20). To investigate the effects of glucocorticoids on prostate cancer cells, GR expression was initially assessed in various cell lines. PC3 and Du145 demonstrated elevated GR expression compared with other cells, whereas no AR expression was detected in these cells (Fig. 1A). AR was expressed in LNCaP, C4-2 and 22Rv1 cells, whereas GR expression was low or hardly detectable in these cells. Dexamethasone was used to treat the cell lines in the present study. Dexamethasone treatment inhibited the PC3 and Du145 cell proliferation (Fig. 1B and C), but exerted no effect on LNCaP, C4-2 and 22Rv1 cells (Fig. 1D-F). Therefore, dexamethasone exhibits distinct effects on different prostate cancer cells. Based on the above results, it can be hypothesized that the presence of AR reduced the effect of dexamethasone on cell proliferation.

AR reverts the inhibition of dexamethasone on prostate cancer cell proliferation. To determine the role of AR in the effect of dexamethasone on prostate cancer proliferation, PC3 cells were transfected with AR in to establish a PC3-AR9 cell line, as previously described (21). The transfected cell line expressed both AR and GR (Fig. 2A). By contrast to the PC3 cells, dexamethasone exerted positive effect on PC3-AR9 cell proliferation (Fig. 2B). This effect was not affected by AR agonist DHT (Fig. 2C) or antagonist MDV3100 (Fig. 2D). Therefore, AR reverted the inhibition of dexamethasone on

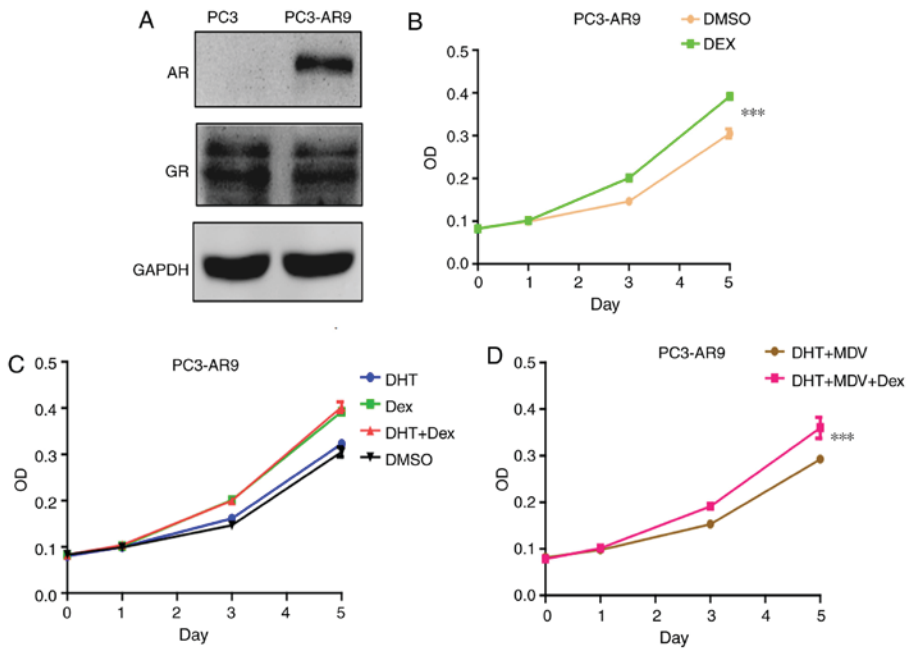

Figure 2. Effects of dexamethasone of PC3-AR9 cells. In PC3 cells (A) transfected with AR as demonstrated by western blotting, (B) dexamethasone promoted PC3-AR9 cell proliferation. This effect was not affected by (C) AR agonist DHT (D) or antagonist MDV3100. Dex, dexamethasone; AR, androgen receptor; GR, glucocorticoid receptor; DHT, dihydrotestosterone; dex, dexamethasone. Data are presented as the mean ± standard deviation. ***P<0.001.

prostate cancer cell proliferation. The treatment with an agonist and antagonist indicated that this alteration was depended on AR protein but not AR signal.

Phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)-RAC-alpha serine/threonine-protein kinase (Akt) pathway is involved in the distinct effect of dexamethasone on various prostate cancer cells. It has been previously demonstrated that AR expression and translocation influence prostate cancer cell proliferation (22). AR expression and distribution were investigated upon dexamethasone treatment; however, AR expression was not significantly affected by dexamethasone treatment (Fig. 3A) and no AR protein was detected in the nuclei (Fig. 3B). A direct interaction between Akt and AR was previously demonstrated by co-immunoprecipitation, and increased phosphorylation of Akt (Ser-473 and Thr-308) was associated with phosphorylation at Ser213 and Ser791, and AR degradation (22). The present study demonstrated that p-Akt^{473} increased following dexamethasone treatment in PC3 cells, but decreased in PC3-AR9 cells (Fig. 3C). p-Akt^{473} level was unaltered despite dexamethasone treatment in 22Rv1 and C4-2 cells (Fig. 3D). The present data are consistent with a previous study where Akt-AR interaction in androgen dependent prostate cancer (ADPC) and androgen independent prostate cancer (AIPC) was different (23).

AR rescues the inhibition of dexamethasone on prostate cancer migration. To assess the effect of dexamethasone on prostate cancer migration, a migration assay was performed using PC3 cells. Dexamethasone inhibited cell migration in PC3 cells (Fig. 4A and B). However, dexamethasone exhibited no influence on PC3-AR9 cell migration ability (Fig. 4C and D). Therefore, AR rescued the inhibition of dexamethasone on prostate cancer migration. The expression of mesenchymal marker vimentin was decreased following dexamethasone treatment in PC3 cells and unaltered in PC3-AR9 cells (Fig. 4E), which may indicate that dexamethasone may be linked with epithelial mesenchymal transition (EMT) and AR may alter dexamethasone's effect.

Discussion

It has been previously hypothesized that dexamethasone may inhibit prostate cancer proliferation and that the underlining mechanisms may involve interleukin (IL)-6, nuclear factor-κB inhibition (8,24). A previous study suggested that glucocorticoids exhibit a distinct effect on prostate cancer cells, suppress PC3 and Du145 cell proliferation and induce no effect on LNCaP cells (8). Glucocorticoids can also promote prostate cancer cell proliferation and previous data demonstrated that dexamethasone promotes proliferation of 22Rv1 cells (11,25). Recently published data indicated that DHT can affect AR$^-$ prostate cancer cell proliferation via GR (16). Therefore, the authors of the present study evaluated dexamethasone action in the absence of androgen.

In the present study, it was demonstrated that dexamethasone exhibits different action on LNCaP, PC3, 22Rv1 and C4-2 cells. Dexamethasone inhibited PC3 proliferation but did not affect the proliferation of LNCaP, 22Rv1, C4-2 cells. For the purpose of glucocorticoid-induced experiments, the cells were transferred and cultured in RPMI-1640 with 10% charcoal-stripped serum for at least 24 h to abolish the action exerted by androgen. Previous data suggested that glucocorticoids exert a negative effect on proliferation of androgen-independent prostate cancer and LNCaP-GR (LNCaP transfected with GR) cells (8,9). Increasing amount of evidence suggests that glucocorticoid receptor confers resistance to antiandrogens (10,11). The authors of the present

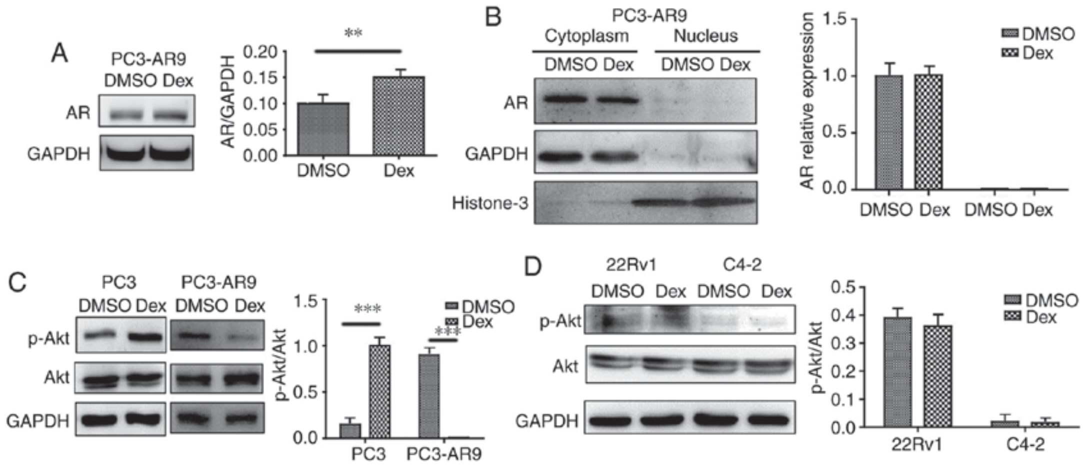

Figure 3. Akt pathway is involved in the distinct effect of dexamethasone on prostate cancer cells. (A) Western blotting indicated that dexamethasone exhibited no influence on AR expression in PC3-AR9 cells. (B) AR nucleus-cytoplasm distribution was not affected by dexamethasone. Dexamethasone (C) promoted Akt phosphorylation in PC3 cells and inhibited Akt phosphorylation in PC3-AR9, and (D) exhibited no effect on 22Rv1 or C4-2 cells. Akt, RAC-alpha serine/threonine-protein kinase; p, phosphorylated; AR, androgen receptor; dex, dexamethasone. Data are presented as the mean ± standard deviation. **P<0.01, ***P<0.001.

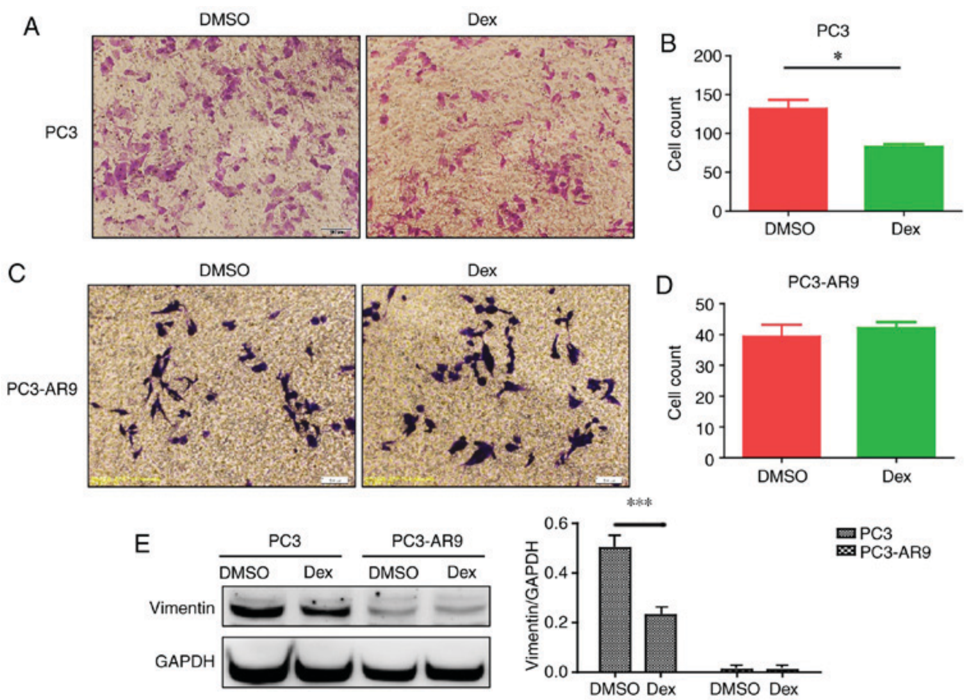

Figure 4. AR rescues the inhibition induced by dexamethasone on prostate cancer migration. Migration assay indicated that dexamethasone (A) inhibited PC3 cell migration, (B) as demonstrated by quantitative analysis, but (C) exhibited no effect on PC3-AR9 cell migration, (D) as demonstrated by quantitative analysis. (E) Western blotting indicated that dexamethasone inhibited vimentin expression in PC3 cells but not in PC3-AR9 cells. Data are presented as the mean ± standard deviation. *P<0.05, ***P<0.001.

study hypothesized that the action of dexamethasone on prostate cancer cells may depends on the presence of AR. Dexamethasone promoted cancer cell proliferation in PC3 cells transfected with AR (Fig. 2B).

The present study investigated the effect of dexamethasone on various prostate cancer cells. The association between AR and GR is complex and their interaction is influenced by disease progression (12). The expression of GR is negatively regulated by active androgen receptor signaling (13). By contrast, androgen receptor activity was inhibited by glucocorticoid action in human adipocytes (26). Whether androgen receptor activity was inhibited by glucocorticoid action has not been fully demonstrated in prostate cancer cells (12). Nevertheless, the immunophilin FKBP51 which is the downstream of AR regulates the function of GR (26,27). Increased expression of FKBP51 by AR inhibits GR nuclear translocation and therefore suppresses the function of GR (27,28). Therefore, prostate cancer cells should be divided into four types according to AR and GR expression (double AR/GR^{+} or $^{-}$ and AR/GR single positive).

Clinically, multiple studies investigated the utility of dexamethasone in CRPC (14,29-34). Prostate-specific antigen (PSA) response rate of dexamethasone is ~41-62%, median time to PSA progression of dexamethasone is ~5.4-9.7 months. Dexamethasone induces distinct effect in various clinical studies (reviewed in 15). In order to confirm the present findings, future *in vivo* experiments and experiments using more cell lines are needed.

When PCa is a localized disease, five year survival rates can be 100% but once it has spread, the survival rates decrease to 28% (35). Cancer metastases markedly decrease patients' survival time. The present study performed a migration assay to determine the effects of dexamethasone on prostate cancer migration. Dexamethasone inhibited PC3 cell migration but did not affect PC3-AR9 cell migration. In bladder cancer, dexamethasone increased glucocorticoid receptor-mediated reporter activity and cell proliferation; however, dexamethasone induced mesenchymal-to-epithelial transition by suppressing the expression of MMP-2/MMP-9, IL-6, VEGF, and the activity of MMP-2/MMP-9, thus inhibited bladder cell invasion (36). Although the mechanism of dexamethasone activity on PC3 migration remains to be elucidated, it appears that dexamethasone inhibited cell migration of AR^- but not AR^+ cancer cells. As demonstrated in previous studies, dexamethasone was sufficient to confer enzalutamide resistance, and substituted for AR to activate genes involved in proliferation and metastasis and were necessary for maintenance of the resistant phenotype (10).

There are certain limitations of the present study. A serial concentration of dex may be included in the future studies to support the initial results. Additionally, the potential mechanism was proposed based on experimental results from one pair of prostate cell lines and other cell lines should be added in the future. The experiments preformed in the present study also need to be repeated to confirm the results. There is also no loss of function studies to support the hypothesis that the AR protein itself is critical. The aforementioned issues should be addressed in future studies.

In conclusion, the present study suggested that dexamethasone positively or negatively regulated proliferation of various prostate cancer cells according to AR and GR expression.

Acknowledgements

The present study was supported by the Project of the Education Department of Jiangxi Province (grant no. GJJ160036).

Competing interests

The authors declare thay they have no competing interests.

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