·*Original Article*·

Expression of human AR cDNA driven by its own promoter results in mild promotion, but not suppression, of growth in human prostate cancer PC-3 cells

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Abstract

Aim: To examine the physiological role of the androgen receptor (AR) in the PC-3 cell line by transfecting full-length functional AR cDNA driven by its natural human AR promoter. **Methods:** We generated an AR-expressing PC-3(AR)9 stable clone that expresses AR under the control of the natural human AR promoter and compared its proliferation to that of the PC-3(AR)2 (stable clone that expresses AR under the control of the cytomegalovirus (CMV) promoter, established by Heisler *et al.*) after androgen treatment. **Results:** We found that dihydrotestosterone (DHT) from 0.001 nmol/L to 10 nmol/L induces cell cycle arrest or inhibits proliferation of PC-3(AR)2 compared with its vector control, PC-3(pIRES). In contrast, PC-3(AR)9 cell growth slightly increased or did not change when treated with physiological concentrations of 1 nmol/L DHT. **Conclusion:** These data suggest that intracellular control of AR expression levels through the natural AR promoter might be needed for determining AR function in androgen-independent prostate cancer (AIPC) PC-3 cells. Unlike previous publications that showed DHT mediated suppression of PC-3 growth after transfection of viral promoter-driven AR overexpression, we report here that DHT-mediated PC-3 proliferation is slightly induced or does not change compared with its baseline after reintroducing AR expression driven by its own natural promoter, as shown in PC-3(AR)9 prostate cancer cells. *(Asian J Androl 2007 Mar; 9: 181–188)*

Keywords: androgen receptor; proliferation; apoptosis; prostate cancer; androgen ablation therapy

1 Introduction

Prostate cancer is the commonest cancer in men and is the second leading cause of cancer related deaths in men in the USA [1]. Like normal prostate tissues, prostate cancer proliferation and maintenance of tissue differentiation depends on androgen signaling; this property has been targeted therapeutically by androgen deprivation treatment in prostate cancer [2]. Although androgen ablation is effective at slowing initial progression of tumor growth, it is commonly known that long-term treatment eventually results in loss of efficacy as a result of the development of androgen-independent prostate can-

cer (AIPC) [3]. Although AIPC cells lose their dependence on androgen as a proliferative signal, immunohistochemical data suggest that cells from many androgen-independent tumors continue to express the androgen receptor (AR) [4, 5]. Thus, the role of AR in AIPC remains unclear.

PC-3 is an androgen insensitive human prostate cancer cell line derived from a bone metastasis that has been used to study AIPC for several years [6]. These cells lack expression of AR, despite having normal AR gene. Commonly, the PC-3 cell line has been used to study AIPC *in vitro* or *in vivo*, and furthermore the use of PC-3 has increased in studying the role of the AR in AIPC through transfection of functional AR cDNA. However, results remain controversial. Marcelli *et al.* [6] reported that the transfection of a constitutively active AR driven by the cytomegalovirus (CMV) promoter resulted in growth inhibition in PC-3 cells. In support of this, Heisler *et al.* [5] also created a series of AR expressing PC-3 clones stably transfected with the CMV promoter-driven AR expressed at various levels; from one-quarter to twice the AR expression concentration of AR-positive LNCaP prostate cancer cell line. Even with the wide range of AR expression levels, dihydrotestosterone (DHT) treatment resulted in an inhibition of cellular growth in PC-3 clones with low-level (PC-3[AR]13), moderate-level (PC-3[AR]2) and high-level (PC-3[AR]10) AR expression [5]. In addition to the use of the CMV promoter to drive the AR expression, AR expressing PC-3 clones were also created with the pSG5-AR vector in which expression of the AR was controlled by the SV40 promoter. Similarly, the expression of the AR in this PC-3 clone caused androgen-sensitivity resulting in a suppression of the cell growth [7]. With these results, however, the AR expression within different PC-3 cell lines is artificially controlled by viral promoters and might not represent the control of the AR function under physiological and cellular conditions. Observations of the AR function in AIPC PC-3 cell lines with continual AR expression might be insufficient for showing the physiological contributions of androgen stimulated AR signaling for cell growth and proliferation.

In the present study, we generated PC-3(AR)9, a PC-3 cell line that expresses the AR under the control of the intrinsic AR promoter, and compared the proliferation of this cell line to that of PC-3(AR)2, which consistently expresses high levels of the AR under androgen treatment. Because the intrinsic AR promoter is regu-

lated by various intracellular responses [8, 9], we hope to examine the AR function in AIPC PC-3 cell expressed under physiological control. We now report that DHT induces cell cycle arrest and inhibits the proliferation of PC-3(AR)2. In contrast, growth of PC-3(AR)9 cells is increased mildly or does not change when exposed to physiological conditions (1 nmol/L) of DHT [10].

2   Materials and methods

2.1 Cell culture, plasmids and reagents

PC-3(AR)2 was a generous gift from Dr T. J. Brown [5]. Human prostate cancer cell lines PC-3(pIRES) (a mammalian expression vector, internal ribosome entry site), PC-3(AR)2 (AR expression driven by human CMV), and PC-3(AR)9 (AR expression driven by its own natural promoter) were maintained in RPMI 1640 media containing 10% fetal calf serum (FCS), 25 U/mL penicillin and 25 μg/mL streptomycin. DHT and hydroxyflu-tamide (HF) were purchased from Sigma (St. Louis, MO, USA). Anti-AR polyclonal antibody, NH27, was produced as previously described [11, 12]. An anti-actin monoclonal antibody was purchased from Amersham Biosciences (Piscataway, NJ, USA). The natural promoter driven AR plasmid was constructed by inserting a 3.6-kDa hAR promoter, the entire hAR 5'-UTR, and the full-length AR cDNA into the pIRES plasmid. Expression of the AR was placed under the control of the 3.6 kDa proximal AR promoter region cloned into pIRES. Neomycin resistant cells were selected by incubation with 500 mg G418/mL.

2.2 Transient transfections and reporter gene assays

Transfections were carried out using the calcium phosphate precipitation method [13]. Luciferase (Luc) assays were carried out as described previously [17]. Briefly, 1–4×10^5 cells were plated on 35- or 60-mm dishes 24 h before adding the DNA precipitation mix containing either mouse mammay tumor virus (MMTV)-Luc or androgen response element 4-Luc (ARE[4]-Luc) reporter plasmid DNA. The medium was changed to phenol-red-free RPMI 1640 with 10% charcoal-dextran treated FCS (CD-FCS) 1 h before transfection. In each experiment, the total amount of transfected DNA/dish was equalized by the addition of empty expression vector (pCMV) to make a total of 10 μg/60-mm dish. After 24-h transfection, the medium was changed, the cells were treated with various concentrations of DHT, harvested,

and whole cell extracts were used for Luc assay. Luc activity was determined using a Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) and a luminometer.

2.3 Western blot analysis

PC-3(pIRES), PC-3(AR)2 and PC-3(AR)9 cells were treated with ethanol or 1 nmol/L DHT CD-FCS for 24 h. The medium was removed and the attached cells were washed with phosphate buffered saline (PBS). Protein extraction was accomplished by cell lysis with sodium dodecyl sulfate (SDS) [14]. Protein concentrations were measured using bicinchoninic acid protein (BCA) protein reagent (Pierce Chemical, Rock-ford, IL, USA). Equal amounts of total protein (50 μg) were loaded onto an SDS-polyacrylamide gel with a Tris/glycine running buffer system and subjected to electrophoresis. Proteins were then transferred to a 0.2-μm polyvinylidene difluoride membrane (PVDF) in a mini-electrotransfer unit (Bio-Rad, Hercules, CA, USA). Membranes were probed with anti-AR and anti-actin antibodies. Immunoblot analysis was carried out with horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG using enhanced chemiluminescence Western blotting detection reagents purchased from Amersham Biosciences (Piscataway, NJ, USA).

2.4 Thiazolyl blue assay

Thiazolyl blue (MTT) assay is a quantitative colorimetric assay for mammalian cell survival and proliferation. Briefly, 5×10^4 PC3-3(pIRES), PC-3(AR)2 and PC-3(AR)9 cells were seeded in 24-well plates in RPMI 1640 with 10% CD-FCS. After 24 h, cells were treated with either vehicle or selected concentrations of DHT for 9 days. Daily cell growth was determined by adding 50 μL of thiazolyl blue (5 mg/mL MTT; Sigma, St. Louis, MO, USA) into each well of a plate with 500 μL of medium for 2 h. After incubation, 400 μL of 0.04 mol/L HCL in isopropanol was added to each well. Absorbency was read at a test wavelength of 590 nm.

2.5 Cell cycle analysis (flow cytometry assay)

PC-3(pIRES), PC-3(AR)2 and PC-3(AR)9 cells grown in 100-mm dishes were treated with either ethanol or 1 nmol/L DHT in CD-FCS for the indicated times. At the end of each time period, the cells were digested by trypsin–EDTA. As many as 1×10^6 cells were harvested and fixed in 70% ethanol at 4°C. After 12 h, cells were centrifuged (1 000 × *g*, 7 min, 4°C), resuspended in PBS containing 0.05 mg/mL RNase A (Sigma, St. Louis, MO, USA), and then incubated at room temperature for 30 min. After washing, the cells were stained with 10 mg/mL propidium iodide, filtered through a 60-mm mesh, and 10 000 cells per treatment were analyzed by flow cytometry (FACSCalibur; BD Company, San Jose, CA, USA) [15] with MODFIT software (Verity Software House, Topsham, MA, USA).

3 Results

3.1 Expression of AR in PC-3 cells stably transfected with a full length AR cDNA under the control of the natural promoter (p-3.6 hAR)

Cell lines expressing AR under the control of the natural AR promoter were generated by transfecting PC-3 cells with p-IRES-3.6hAR. The plasmid was constructed using a 3.6-kDa proximal human AR promoter region to drive expression of a full-length human AR cDNA (Figure 1A). Several G418-resistant colonies were selected from the original transfected pool using cloning cylinders, followed by expansion and an assay for the AR expression. A rabbit anti-human AR polyclonal antibody was used to probe Western blots of the protein extracts from each cell line. A protein band with a molecular weight of 110 kDa was present in several transfected lines designated as PC-3(AR)7, PC-3(AR)8 (data not shown) and PC-3(AR)9. No AR protein band was detected in PC-3(pIRES) cells. The 110 kDa proteins detected in the natural promoter transfected PC-3(AR)9 cell were the same size as those from the CMV promoter derived cell line PC-3(AR)2 (Figure 1B, C). Furthermore, the level of AR protein detected in PC-3(AR)9 cells using the natural AR promoter was consistently less then that detected in the CMV promoter-driven cell line in the presence or absence of 1 nmol/L DHT and the levels of AR expression in PC-3(AR)9 varied between vehicle or 1 nmol/L DHT treatment (Figure 1B, C).

3.2 The functional assay of AR in PC-3(AR)9 compared with AR in PC-3(AR)2

Androgen responsiveness was tested using transient transfection assays. Cell lines PC-3(AR)2 and PC-3(AR)9 were transfected with a Luc reporter plasmid driven by the MMTV promoter, treated with various concentrations of DHT, and assayed for Luc activity (Figure 2A). Both PC-3(AR)2 and PC-3(AR)9 showed an increase in
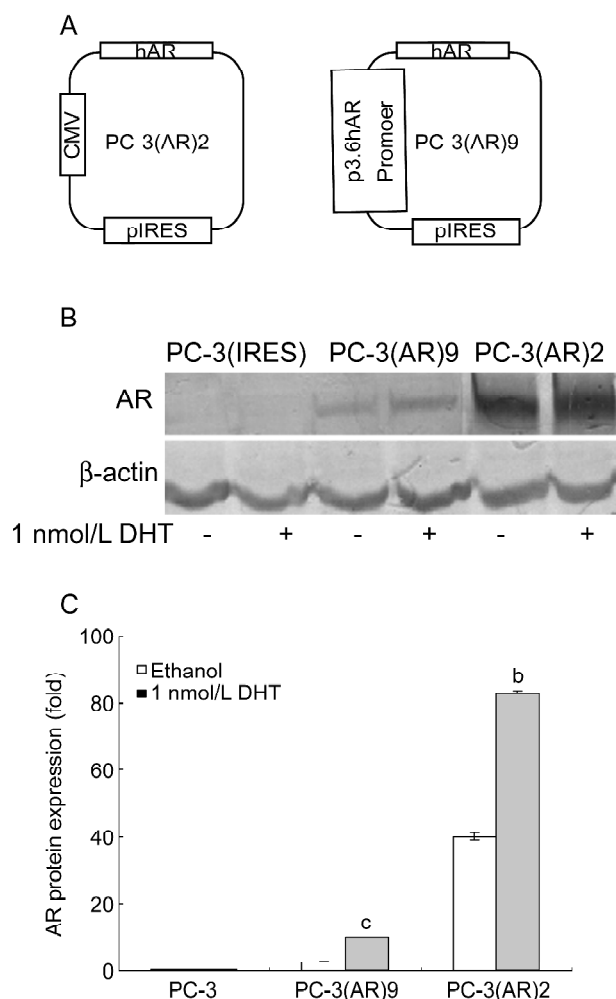
Figure 1. The androgen receptor (AR) protein expression and activity in PC-3 cell line transfected with AR cDNA under the control of the natural AR promoter region. (A): Diagram of plasmid pIRES-3.6AR constructed with the human AR cDNA under the control of the cytomegalovirus (CMV) promoter or a 3.6-kDa proximal human AR promoter region. (B): Western blot analysis using a polyclonal antibody against human AR carried out on protein lysate derived from PC-3(pIRES); PC-3 cells transfected with pIRESneo vector alone; PC-3(AR)9, cell line established by transfection with pIRES-3.6AR; and PC-3(AR)2, cell line expressing human AR under the control of a CMV promoter, in the presence or absence of 1 nmol/L DHT. (C): Levels of AR expression in the PC-3, PC-3(AR)9 and PC-3(AR)2 cell lines in the presence or absence of 1 nmol/L DHT. Data are mean ± SD. [b]$P < 0.05$, [c]$P < 0.01$, compared with corresponding controls.

relative Luc activity with the addition of DHT from 0.0001 nmol/L to 10 nmol/L. The addition of 1 nmol/L DHT resulted in a maximal 20-fold induction of Luc re-

porter gene in PC-3(AR)2, whereas PC-3(AR)9 showed a 5-fold induction. This is consistent with the higher level of the AR protein expressed in the CMV promoter-driven cell line PC-3(AR)2 (Figure 1B). The level of the AR protein expressed correlates with the degree of AR transactivation observed using both MMTV promoter driven reporter plasmid and ARE reporter plasmid. Finally, the transactivation activity was inhibited by HF in both the PC-3(AR)2 and PC-3(AR)9 cell lines, indicating that expression of luciferase activity from the reporter plasmid is androgen/AR-mediated (Figure 2B, C).

3.3 Differential modulation of cell culture growth in PC-3(AR)2 and PC-3(AR)9 cells

A MTT assay in PC-3(pIRES), PC-3(AR)2 and PC-3(AR)9 cell lines were used to determine the effect of androgen treatment on cell viability. Cells were plated in 24-well tissue culture plates (5×10^4 cells/well) and the number of viable cells was determined after 9 days. In PC-3(AR)2, DHT inhibits cell proliferation at all tested concentrations of DHT (Figure 3B, E). This is consistent with a previous report in which DHT was shown to induce cell cycle arrest and inhibit cell proliferation in PC-3(AR)2 cells [5]. In contrast, PC-3(AR)9 cells showed a slight increase or no change in cell proliferation when treated with 1 nmol/L DHT (Figure 3C, F).

3.4 The high level of AR expression inducing cell cycle arrest

It has been proposed that androgen inhibits cell proliferation in PC-3(AR)2 cells by inducing cell cycle arrest [7]. Using flow cytometry, we examined the effect of DHT on cell cycle phase distribution. After incubating cells for 9 days in medium containing 1 nmol/L DHT, flow cytometry analysis with propidium iodide staining showed that 1 nmol/L DHT treatment increased the proportion of PC-3(AR)2 cells in the G_0–G_1 phase from 39% to 62% ($P < 0.05$; Figure 4C). This was completely prevented by the addition of HF (data not shown). However, in PC-3(pIRES) and PC-3(AR)9 cells, DHT had little effect on the cell cycle (Figure 4A, B), suggesting that DHT-induced G_1 cell cycle arrest is dependent on the expressed level of the AR protein.

4 Discussion

In contrast to experimental results in which the commonly used AIPC cell lines DU145 and PC-3 were AR-
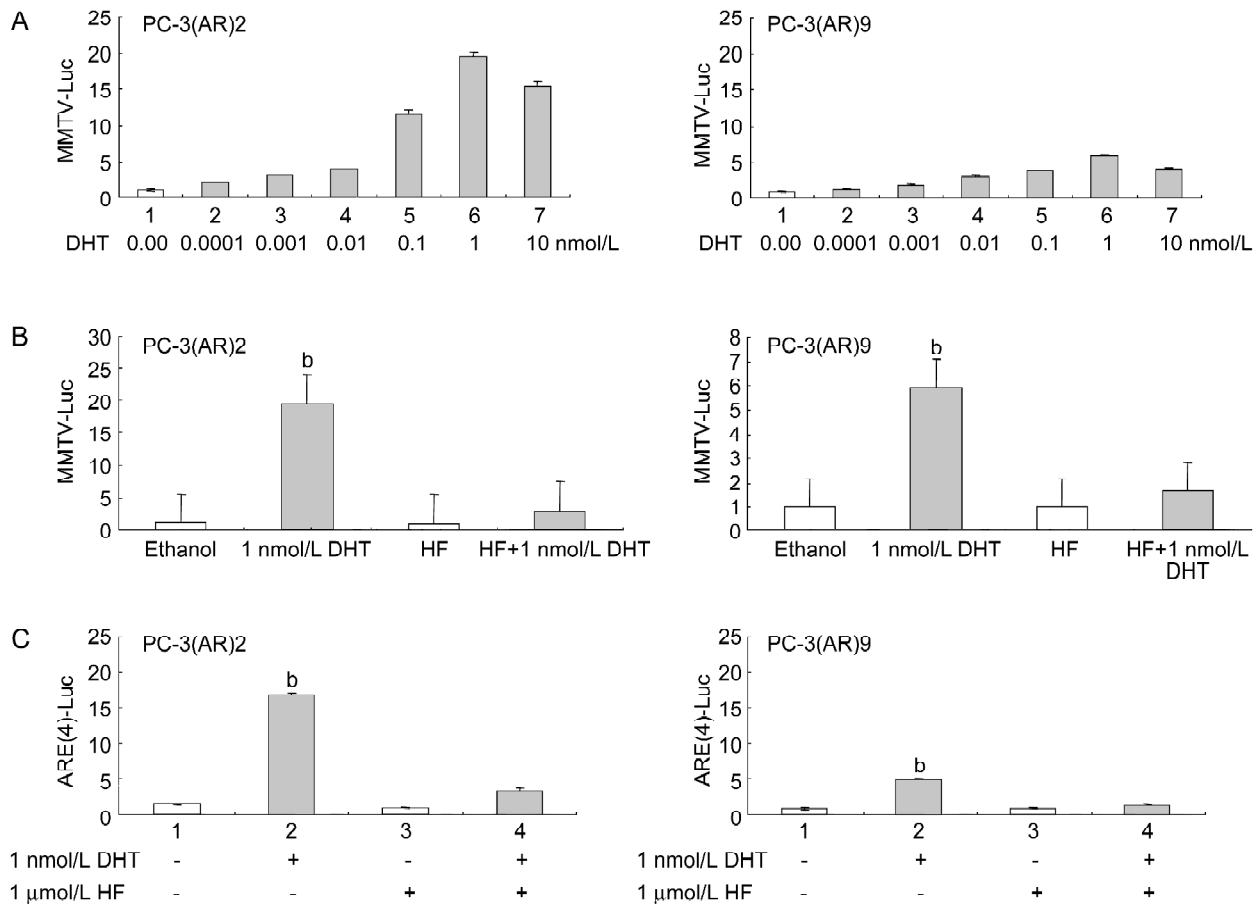
Figure 2. (A): The androgen receptor (AR) transcriptional activity was measured using the MMTV-Luc reporter gene transfected into PC-3(AR)2 or PC-3(AR)9. Both cell lines were treated with ethanol or 10^{-12}–10^{-8} mol/L DHT as indicated. (B)–(C): AR transcriptional activity in PC-3(AR)2 and PC-3(AR)9 using the MMTV-Luc and the ARE(4)-Luc reporter gene. Cells were treated with ethanol, 1 nmol/L DHT, 1 μmol/L HF or a combination of DHT and HF as indicated. Data are mean ± SD. [b]$P < 0.05$, compared to ethanol control.

negative, more than 90% of prostatic cancers obtained directly from patients failing androgen ablation actually overexpress the AR; 30% of this overexpression is to the result of genetic amplification [16]. Despite this high percentage of AR expression in AIPC, the role of the AR in AIPC is unknown. In the present study, using the AIPC cell line PC-3, we created PC-3(AR)9 stable cell line transfected with the normal human AR natural promoter driven AR vector in order to examine the AR role in AIPC under physiological conditions.

Numerous reports have shown that androgen/AR can modulate AIPC cell growth [13, 16]. However, the precise role of androgen/AR remains unclear. By disrupting AR in two different androgen-refractory sublines of LNCaP (LNCaP-Rf and LNCaP-C4), Zegarra-Moro *et al.* [3] and Xu *et al.* [17] showed the dependence of AIPC

on the AR for proliferation. In contrast, using AR restoration strategies in the PC-3 AR negative AIPC cell line, multiple groups have shown that an overexpression of AR resulted in AIPC growth inhibition [5–7]. Therefore, whether the AR stimulates or inhibits cell growth is a question that remains controversial. In related studies, it has been reported that estrogen insensitive breast cancer cells transfected with the estrogen receptor (ER) cDNA showed a similar controversial cell growth inhibition. Like the AR, the ER is a DNA-binding protein that can interact with general transcription factors. Thus, the growth inhibitory effects seen in the transfected breast cancer cells might be the result of non-specific effects caused by overexpression of the ER [5, 18]. This might also be the case in the AR transfected PC-3 related studies in which the strong viral promoter-driven AR vectors (CMV
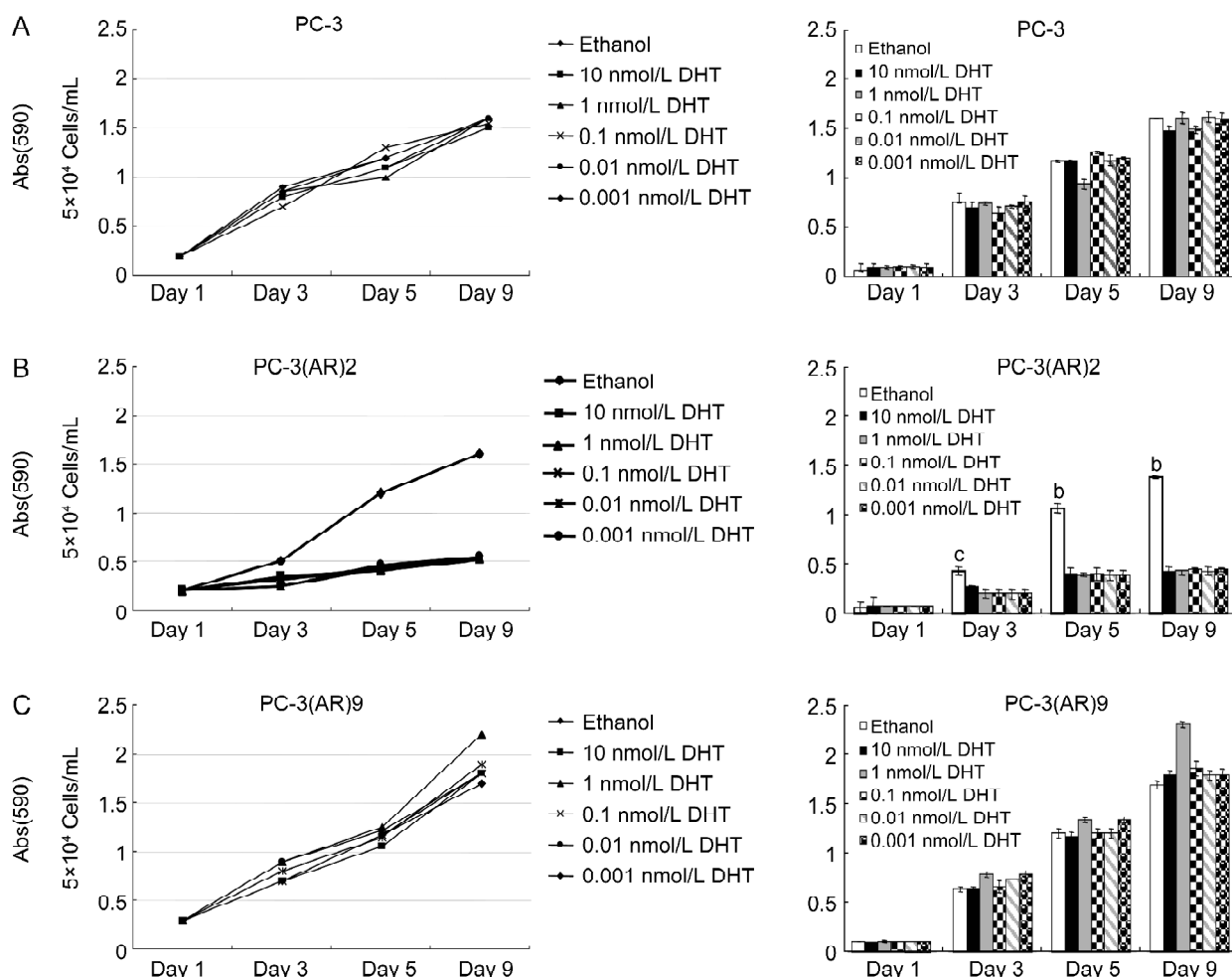
Figure 3. Thiazolyl blue (MTT) assay showing cell growth usingPC-3(pIRES) (A), PC-3(AR)2 (B) and PC-3(AR)9 (C) cell lines in response to dihydrotestosterone (DHT). Equal numbers of cells (50 000 cells/mL) were seeded into 24-well tissue culture plates. Cells were grown in DMEM 10% CD-FBS serum treated with either vehicle or DHT. Data are mean ± SD. $^bP < 0.05$, $^cP < 0.01$, compared to ethanol control.

or SV40 promoters) were used [5]. Therefore, in order to mimic the physiological levels of androgen and allow for intracellular modifications of the AR expression, we constructed a natural promoter driven AR plasmid for transfection into PC-3 cells. This was carried out in order to establish a stable PC-3(AR)9 clone in which androgen/AR mediates cell proliferation and other functions including cell cycle and cell invasion.

Having established the PC-3(AR)9 clone, we first compared the AR protein level as well as AR transactivation to that of the previously established PC-3(AR)2 cell line [5]. Levels of AR in PC-3(AR)9 were consistently less than that of PC-3(AR)2. In addition, the AR protein levels of PC-3(AR)9 with 1 nmol/L DHT treatment showed

an increase compared to that of vehicle treatment. Lin *et al.* [19] reported that testosterone and DHT under normal conditions act as moderate up-regulators of AR mRNA expression in smooth muscle cells *in vitro*. It has been reported that various stimuli might also modify the CMV promoter driven expression levels [20]; thus, this change in the AR protein level might be an artificial cause of the CMV promoter-modification in PC-3(AR)2. In order to characterize the function of AR in PC-3(AR)9, we used the MMTV promoter-driven Luc reporter gene to show the AR transactivation after DHT treatment. Treatment of PC-3(AR)2 with 1 nmol/L DHT resulted in a maximal 20-fold increase in Luc activity as compared with PC-3(AR)9, in which DHT treatment induced a 5-
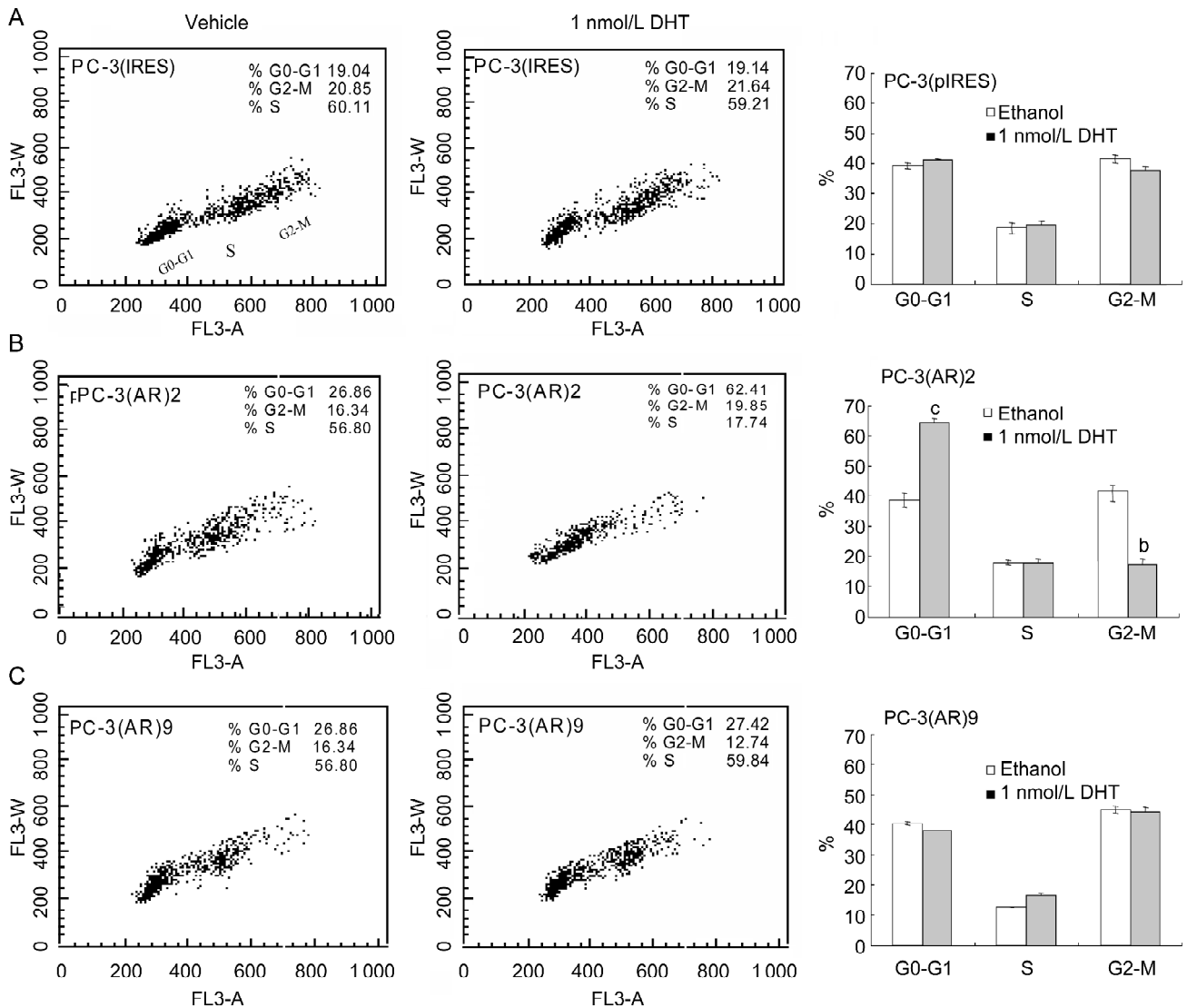
Figure 4. (A)–(C): Summary of cell density plates of PC-3(pIRES), PC-3(AR)2, and PC-3(AR)9 treated with 1 nmol/L dihydrotestosterone (DHT) or ethanol for 9 days in DMEM 10% CD-FCS. Cells were harvested, stained with propidium iodide, and analyzed by flow cytometry, which displayed three populations of cells; G1, S and G2-M. Data are mean ± SD. $^b P < 0.05$, $^c P < 0.01$, compared with corresponding controls.

fold increase. This correlates with the difference in the AR protein levels detected in the two cell lines. In order to characterize the AR role in PC-3 AIPC cell proliferation, an MTT assay was carried out. In agreement with previous publications, PC-3(AR)2 showed a decrease in cell growth when treated with various concentrations of DHT [5]. In contrast, after treating PC-3(AR)9 with physiological levels (1 nmol/L) of DHT, we observed a slight increase in cell growth in comparison with vehicle treatment. Furthermore, in comparing cell cycle analy-

sis using flow cytometry, treating PC-3(AR)2 cells with 1 nmol/L of DHT resulted in an increase in G0/G1 cell cycle arrest. This phenomenon was also observed previously by Heisler *et al.* [5]. As for PC-3(AR)9, little change in cell cycle was observed.

The rationale for the use of a natural AR promoter in creating a PC-3 AR positive cell line is to allow cellular modification of AR expression according to its physiological needs. We observed that under the control of the normal AR promoter, PC-3(AR)9 AR expression was

significantly less than that in the PC-3(AR)2 cell line. Furthermore, DHT treatment of PC-3(AR)9 showed that androgen/AR increased cell proliferation or did not change in PC-3, in contrast to the inhibition reported previously by groups that used viral promoter driven AR expression. Whether this difference in the role of androgen/AR in AIPC cell proliferation is a result of the AR protein level is still debatable. Heisler *et al*. [5] created a series of PC-3(AR) stable clones that expressed the AR at levels one-quarter (PC-3[AR]13) to twice that of LNCaP endogenous AR. At various concentrations of DHT treatment, the investigators observed a consistent inhibition of PC-3(AR) growth through the androgen/AR. The AR expression level of the PC-3(AR)13 cell line exceeded the necessary threshold for inducing androgen/AR-mediated increase in cell proliferation. This is possible because the CMV promoter drives its AR expression, however, further investigation is needed. We believe that PC-3(AR)9 will be an excellent model that most closely resembles the physiological function of the AR in AIPC. This will give us further insight into the AR function in the PC-3 AIPC cell line, and has introduced greater considerations and criteria for the use of AR overexpression strategies.

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