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·*Original Article*·

Prostate androgen-regulated gene: a novel potential target for androgen-independent prostate cancer therapy

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Abstract

Aim: To investigate the involvement of the prostate androgen-regulated (*PAR*) gene in the androgen receptor (AR) signaling pathway and the malignant phenotype of androgen-independent prostate cancer (PCa) cells. **Methods:** The difference in *PAR* expression between LNCaP and PC3 cells was detected by reverse transcription-polymerase chain reaction (RT-PCR). Androgen and anti-androgen effects on *PAR* expression were evaluated by RT-PCR in LNCaP, PC3 cells and PC3 cells stably transfected with vector containing wild-type AR. To determine the importance of *PAR* in the malignant proliferation of androgen-independent PCa cells, we used small interfering RNA (siRNA) transfection to knock down the expression of the gene in PC3 cells. The changes in the malignant phenotype of PCa cells after transfection were analyzed by cell count, colony formation in soft agar and flow cytometry. **Results:** *PAR* expression was 3-fold higher in PC3 cells than that in LNCaP cells. Dihydrotestosterone (DHT) regulated *PAR* mRNA expression in LNCaP cells and the effect was inhibited by the AR antagonist, flutamide. By contrast, DHT did not affect *PAR* expression in PC3 cells. The reintroduction of AR into PC3 cells by stable transfection restored the androgen effect on *PAR* upregulation. After the knockdown of the *PAR* gene by siRNA, PC3 cells exhibited a reversal of the malignant phenotype. **Conclusion:** Because of the possibility that *PAR* is downstream from the AR, and because of its contribution to malignant proliferation in androgen-independent PCa cells, the gene could be a potential therapeutic target for androgen-independent PCa with AR signaling pathway alteration. *(Asian J Androl 2006 Jul; 8: 455–462)*

Keywords: prostate androgen-regulated gene; prostate cancer; androgen receptor; dihydrotestosterone; small interfering RNA

1 Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer in American men [1] and its incidence rate is steadily rising in China. At the time of diagnosis approximately half of the patients have metastatic disease. The androgen ablation therapy administered through interfering with the androgen receptor (AR) signaling pathway is the major treatment for patients in this stage [2]. However, the majority of PCa patients usually relapse, with tumors becoming androgen-independent [3, 4] for which there is no effective therapy. The underlying molecular mechanism involved in androgen-independent PCa and the therapies aimed at it are the active areas of current research. The alterations in the AR or the AR signaling pathway

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are the potential explantations for the transition to androgen-independent tumors [5, 6]. New treatments that inhibit the function of an androgen-regulated, growth-related gene downstream from the AR would be an ideal second line of therapy for androgen-independent PCa. Numerous investigators are searching for such a therapeutic target.

The novel prostate androgen-regulated (*PAR*) gene is overexpressed in all prostatic carcinoma cell lines compared to the normal prostatic tissue [7]. The expression of the *PAR* gene was higher in androgen-resistant PCa cell lines DU145, PC3 and LNCaP-OM compared to androgen-sensitive LNCaP cells [7]. Furthermore, *PAR* was involved in cellular malignant transformation and related to proliferation of PCa [8, 9]. More meaningfully, the expression of this gene could be regulated by androgen R1881 in androgen-dependent PCa cells, but not in androgen-independent PCa cells [7]. It is suggested that *PAR* is probably related to AR or is downstream from AR signaling pathway. If this hypothesis proves to be the case, targeting *PAR* might lead to treatments for androgen-independent PCa with AR signaling pathway alteration.

In the present study, we investigated whether *PAR* is downstream from the AR signaling pathway and confirmed the possibility that androgen-independent PCa could be inhibited by knockdown of *PAR* instead of interfering with the AR itself.

2 Materials and methods

2.1 Cell culture and stable transfections

LNCaP cells were purchased from the Basic Medical Cell Center of Peking Union Medical College (Beijing, China). PC3 cells were purchased from China Center for Type Culture Collection (Wuhan, China). The cell lines were maintained in RPMI–1640 medium supplemented with 10% fetal bovine serum (FBS) at 37ºC with 5% CO_2. For stable transfectants, PC3 cells were transfected with wild-type AR cDNA cloned in the plasmid vector pcDNA3 (kindly provided by Dr G. Wang, Florence, Italy) then incubated for 2 weeks in the medium containing 400 μg/mL G418. The G418-resistant cells were cultured in the medium supplemented with G418 to a final concentration of 200 μg/mL and were examined for the expression of AR by Western blotting.

2.2 Androgen stimulation procedures

Dihydrotestosterone (DHT; Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol at a concentration of 1 mmol/L and reconstituted in the culture media at concentrations ranging from 0.001 nmol/L to 100 nmol/L. Culture media containing flutamide (Sigma, St. Louis, MO, USA) at a concentration of 10 μmol/L was prepared in the same manner. The control medium contained 0.01% ethanol. The cells were aliquoted into 24-well culture plates and grown for 24 or 48 h before the addition of DHT or DHT plus 10 μmol/L flutamide. Flutamide was added to the cells 15 min before DHT. The cells were grown for 48 h (or the time indicated), after which time they were harvested for PAR expression analyses.

2.3 Small interfering RNA (siRNA) synthesis and transient transfection

Chemically synthesized sense and antisense RNAs corresponding to the *PAR* cDNA sequence (CGT-CCT-GAT-AGA-TCC-TCT-GCT, nucleotides 257–277 from AF115850, National Center for Biotechnology Information) were purchased from United Gene Holdings (Shanghai, China) and annealed at 95ºC for 1 min in annealing buffer (pH 7.4) containing 2 mmol/L $Mg(AC)_2$, followed by incubation at 37ºC for 1 h.

One day before transfection, the cells were seeded in a 12-well plate at 2×10^5 cells per well. Cells grown at 50–70% confluence were transfected with synthetic siRNA at a final concentration of 200 nmol/L.

2.4 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Promega, Madison, WI, USA) at 37ºC for 30 min. RT was performed with an RT kit (Promega, Madison, WI, USA). The primers 5'-GTCAGCAAGCACCTCAAAT-3', and 5'-GAAGAAGATGGGGAAAAGG-3' were used to amplify 451 bp transcripts of *PAR*, and the primers 5'-GTGCCACCAGACAGCACTGTGTTG-3' and 5'-TGGAGAAGAGCTATGAGCTGCCTG-3' were used to amplify 202 bp transcripts of *β-actin*. Using 1 μg cDNA as the template, the PCR program for *PAR* and *β-actin* was one cycle at 94ºC for 5 min, 31 cycles at 94ºC for 30 s, 55ºC for 30 s, 72ºC for 1 min, and one cycle at 72ºC for 10 min. The PCR products were subjected to electrophoresis in 1% agarose gel and visualized by ethidium bromide staining.

2.5 Western blotting

For detection of AR expression, equal numbers of

cells (2×10^6) were harvested then lysed with 150 μL of lysis buffer containing 1 × phosphate-buffered saline (PBS), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate (SDS) supplemented with protease inhibitors (10 mg/mL phenylmethylsulfonyl fluoride, 30 μg/mL aprotinin, and 10 μL/mL sodium orthovanadate [100 mmol/L]). Cell lysate was prepared and transferred to cold microcentrifuge tubes and cooled on ice for 20 min. Cellular debris was pelleted by centrifugation at 14 000 × *g* for 1 min, and the lysate was transferred to new tubes. Equivalent amounts of lysate were added to sample loading buffer, boiled for 5 min, and then fractionated by 8% SDS–polyacrylamide gel electrophoresis (PAGE). The PAGE-fractionated protein extracts were then transferred to nitrocellulose membranes and probed with anti-AR polyclonal antibodies at 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit antihuman β-actin antibodies followed by horseradish peroxidase-conjugated goat antirabbit IgG (Promega, Madison, WI, USA). Immunoblot signals were detected by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Gaithersburg, MD, USA) according to the manufac-turer's instructions.

2.6 Cell proliferation assays

For determining the effects of knockdown of the *PAR* gene on the proliferation of the PC3 cell line, the cells (1×10^4/well) were plated in 24-well plates in RPMI-1640 medium containing 10% FBS. Cells were transfected with siRNA targeting *PAR*. The cells were harvested and counted daily for six days.

2.7 Anchorage-independent proliferation assay

Three milliliters of 0.6% agar prepared in RPMI-1640 medium supplemented with 10% FBS was poured into 60 mm culture dishes and left to solidify. Then 200 cells mixed with 2 mL 0.3% agar in complete medium were layered over the bottom agar. The cultures were incubated at 37ºC in a 5% CO_2 atmosphere for 15 days. The colonies were scored under an inverted microscope. Each soft agar assay was performed in triplicate. The experiment was repeated twice.

2.8 Flow cytometric analysis

PC3 cells were transfected with or without siRNA for 24 h and 48 h, then harvested with trypsin and washed three times with PBS. After the final wash, the cells were resuspended in 0.5 mL PBS, then 5 mL of ice-cold 85% ethanol was added, and the samples were stored at –20ºC until staining. In preparation for staining, the cells were washed twice with PBS and incubated for 20 min at room temperature in 1 mL of phosphate-citric acid buffer (0.2 mol/L Na_2HPO_4 and 0.1 mol/L citric acid, pH 7.8). Cells were centrifuged and resuspended in 1 mL of propidium iodide solution (1% Triton X-100, 10 μg/mL propidium iodide and 0.1 mg/mL DNase-free RNase A). The cells were incubated at room temperature for 30 min in the dark, and the DNA content was analyzed in a FACS flow cytometer (Becton Dickinson, Mountain View, CA, USA). The cell distribution pattern was assessed using CellQuest software version 3.1 (Becton Dickinson, Mountain View, CA, USA).

2.9 Statistical analysis

All numerical data were expressed as the mean ± SEM. Differences in means among different treatment groups were compared by one-way ANOVA, followed by Student–Newman–Keuls' test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3 Results

3.1 PAR differentially expressed in LNCaP and PC3 cells

The expression of *PAR* was studied in PCa cells in LNCaP and PC3 cell lines (Figure 1). The *PAR* mRNA level in the androgen-independent cell line PC3 was 3-fold higher than that in the androgen-dependent LNCaP cell line.

3.2 Androgen regulates PAR expression in AR-positive PCa cell line LNCaP but not in AR-negative PCa cell line PC3

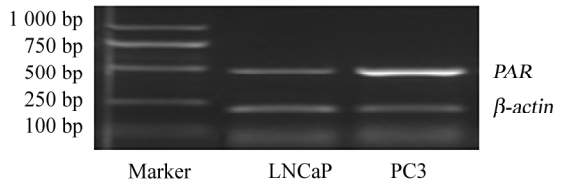

Figure 1. Differential expression of the prostate androgen-regulated gene between LNCaP and PC3 cells was detected by reverse transcription-polymerase chain reaction.

To investigate the relationship between androgen and the expression levels of the *PAR* gene in AR-positive and AR-negative PCa cells, we analyzed gene expression levels after DHT treatment in LNCaP and PC3 cells using RT-PCR analysis (Figure 2). *PAR* mRNA showed an increase of (172.42 ± 8.24)% in the LNCaP cells exposed to 0.1 nmol/L DHT for 16 h ($P < 0.05$, compared with the control). After 4-hour exposure to the hormone, no significant change in the *PAR* mRNA level was detected. Exposure for 48 h resulted in an increase of *PAR* by (118.03 ± 3.05)% ($P < 0.05$, compared with control). However, exposure of the PC3 cells to DHT did not cause any increase of PAR expression ($P > 0.05$, compared with control; Figure 3).

3.3 Androgen regulates PAR expression in PCa cells through a mechanism dependent on the AR

To confirm that the regulation of *PAR* expression by androgen was indeed downstream of the AR signaling pathway, we carried out studies in LNCaP cells and PC3 cells transfected with AR expression vector. PAR expression in LNCaP cells were examined at various doses of DHT with or without 10 μmol/L flutamide, an AR antagonist (Figure 4A, C). The highest expression level of *PAR* was observed at 0.1 nmol/L DHT. The increase of *PAR* mRNA expression compared to untreated cells was (172.42 ± 8.24)% ($P < 0.05$). Flutamide prevented the promotion of PAR expression by DHT (Figure 4B, C). These results indicated that androgen regulates PAR expression through the AR in LNCaP cells.

To determine whether androgen regulates *PAR* expression through the AR signaling pathway in androgen-independent PCa cells as well, PC3AR cells were used. As shown in Figure 5, PC3AR cells had an AR expression level only slightly lower than LNCaP cells; there was no AR expression in untransfected PC3 cells. The cells were exposed to 0.1 nmol/L DHT for 16 h. The treatment resulted in an increase of (43.27 ± 5.02)% of *PAR* in PC3AR cells ($P < 0.05$, compared with control), whereas exposure of untransfected PC3 cells to DHT did not cause any increase in *PAR* expression.

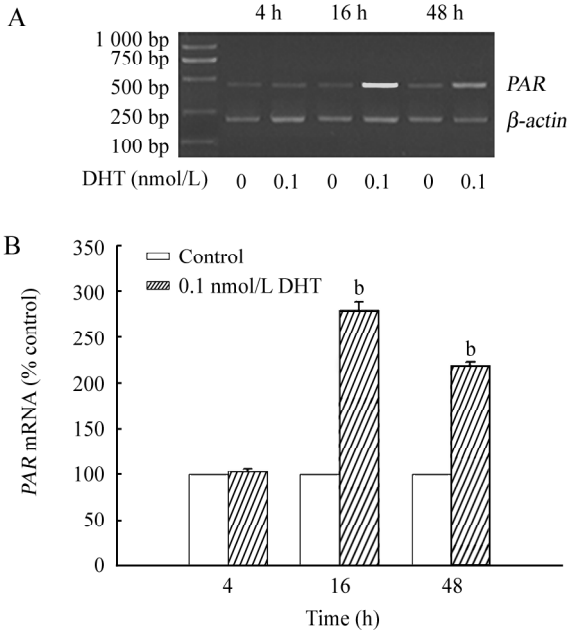

Figure 2. Confirmation of regulation of the prostate androgen-regulated (*PAR*) gene by dihydrotestosterone (DHT) in LNCaP cells. (A): Reverse transcription-polymerase chain reaction (RT-PCR) of *PAR* in LNCaP cells treated with 0.1 nmol/L DHT for 4, 16 and 48 h; (B): Histogram representing quantitative results from RT-PCR. The data shown are mean ± SEM of three independent experiments. $^bP < 0.05$, compared with the corresponding control.

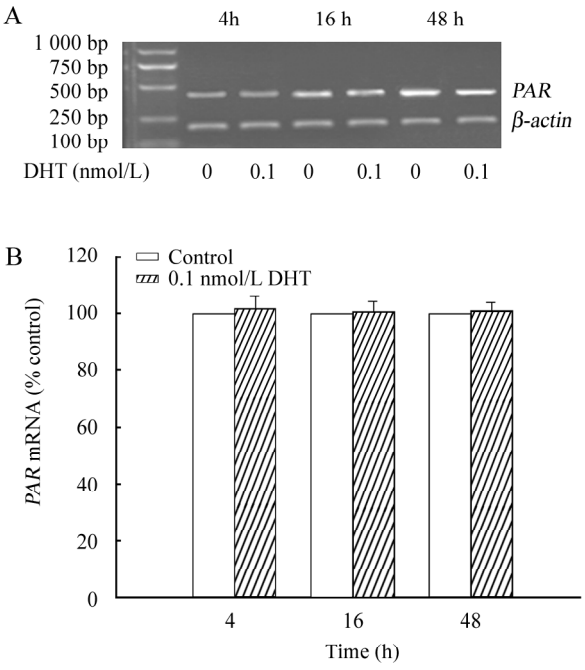

Figure 3. Confirmation of regulation of the prostate androgen-regulated (*PAR*) gene by dihydrotestosterone (DHT) in PC3 cells. (A): Reverse transcription-polymerase chain reaction (RT-PCR) of *PAR* in PC3 cells treated with 0.1 nmol/L DHT for 4, 16 and 48 h; (B): Histogram representing quantitative results from RT-PCR. The data shown are mean ± SEM of three independent experiments. $P > 0.05$, compared with the corresponding control.

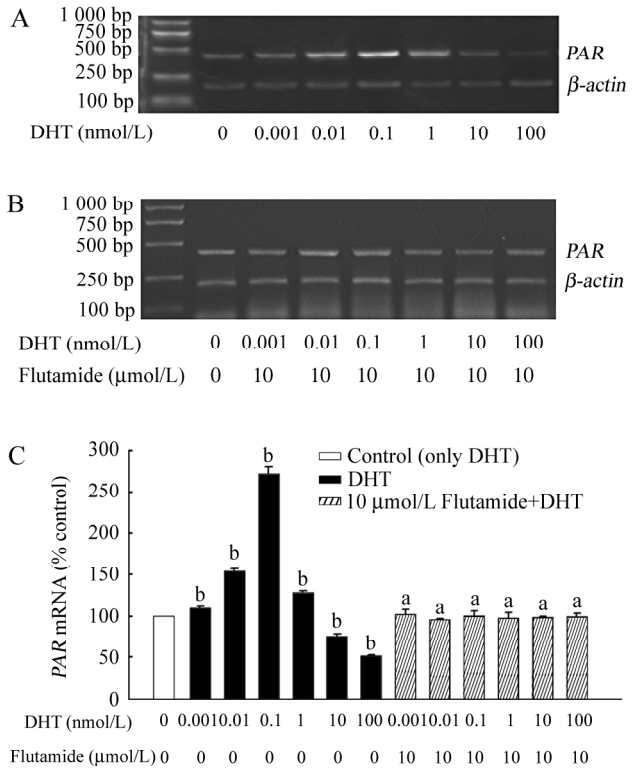

Figure 4. Effects of various concentrations of dihydrotestosterone (DHT) in the presence and absence of 10 μmol/L flutamide on the mRNA expression for the prostate androgen-regulated (*PAR*) gene of LNCaP cells. (A): reverse transcription-polymerase chain reaction (RT-PCR) of *PAR* in LNCaP cells treated with various concentrations of DHT for 16 h; (B): RT-PCR of *PAR* in LNCaP cells treated with various concentrations of DHT in the presence of 10 umol/L flutamide for 16 h; (C): histogram representing quantitative results from RT-PCR. The data shown are mean ± SEM of three independent experiments. $^{a}P > 0.05$, $^{b}P < 0.05$, compared with the corresponding control.

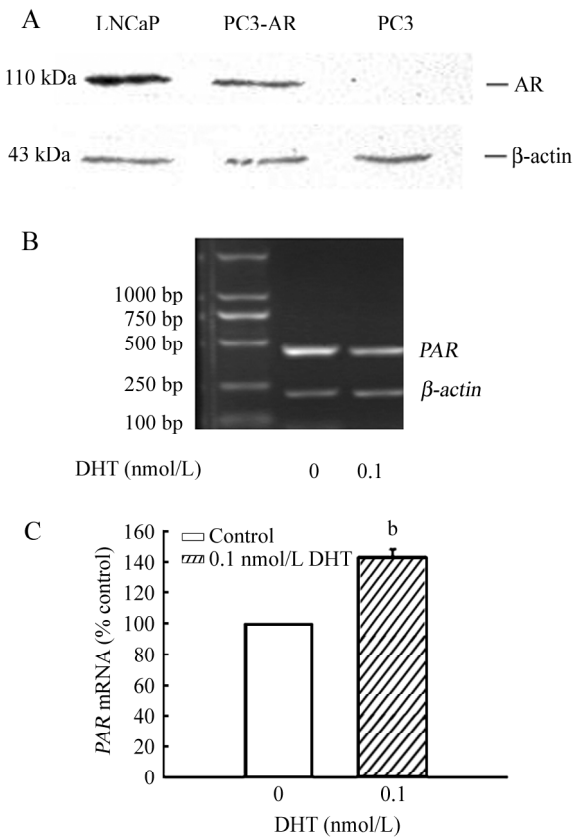

Figure 5. Confirmation of regulation of the prostate androgen-regulated (*PAR*) gene by dihydrotestosterone (DHT) in AR-transfected PC3 cells. (A): Western blotting showing androgen receptor (AR) expression in different PC3 cell clones stably transfected with AR expression vector (PC3–AR) and untransfected PC3 cells; (B): Reverse transcription–polymerase chain reaction showing the expressions of *PAR* in PC3–AR cells exposed to 0.1 nmol/L DHT for 16 h. (C): Histogram representing quantitative results from RT-PCR. The data shown are mean ± SEM of three independent experiments. $^{b}P < 0.05$, compared with control.

3.4 PAR is required for the proliferation of androgen-independent PCa cells in vitro

Next we investigated the functional importance of *PAR* to the proliferation of PCa cells. We used siRNA to decrease the expression of *PAR*. We chose the PC3 cell line because it is androgen-independent and has a higher level of *PAR* expression than androgen-dependent PCa cells. A 21-nucleotide double-stranded RNA corresponding to human *PAR* was transfected into PC3 cells. Cells transfected with siRNA showed significant decreases in *PAR* mRNA expression measured by RT-PCR (Figure 6A, B). The expressions of the PAR mRNA were (71.04 ± 3.95)%, (18.82 ± 1.68)% and (31.33 ± 1.00)% of the control at 24, 48 and 72 h after transfection, respectively ($P < 0.05$, compared with control).

The consequences of decreased *PAR* expression on cell growth were evaluated. SiRNA targeting *PAR* suppressed the proliferation of PC3 cells (Figure 6C).

After the transfection of siRNA, the cells were subjected to flow cytometric analysis at 48 h (Figure 6D, E). In transfected clones, the fraction of cells in G_0/G_1 phase decreased to (29.38 ± 2.17)% compared to (55.68 ± 3.20)% of the control ($P < 0.05$), whereas the fraction in G_2/M phase increased to (29.95 ± 3.25)% compared to (23.79 ± 3.16)% of the control ($P < 0.05$). In addition, at 48 h there was an increase fraction of (20.61

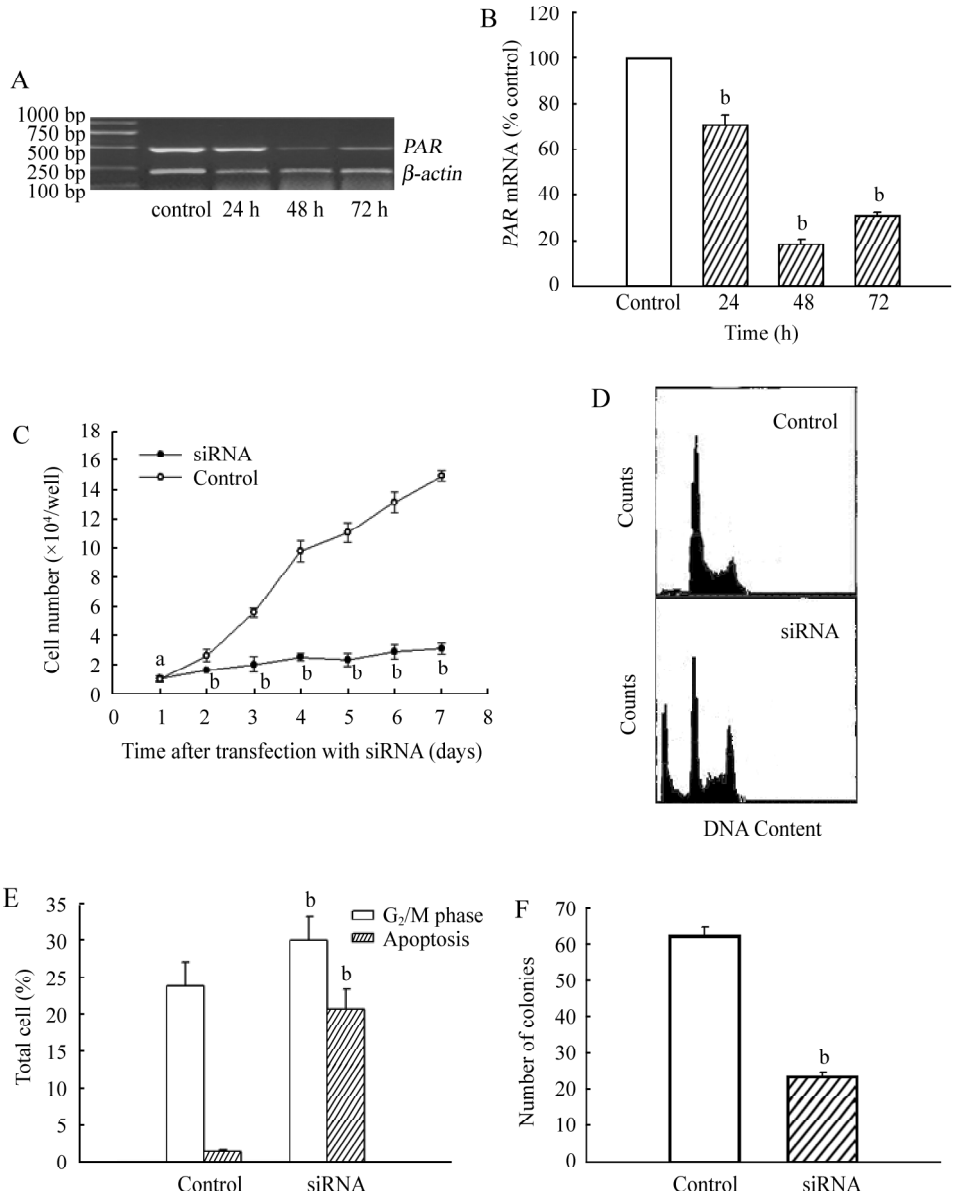

Figure 6. Growth analyses of PC3 cells after small interfering RNA (siRNA) treatment targeting the prostate androgen-regulated (PAR) gene. (A): Reverse transcription-polymerase chain reaction (RT-PCR) of *PAR* in PC3 cells treated with siRNA for 24, 48 and 72 h; (B) Histogram representing quantitative results from RT-PCR. The data shown are mean ± SEM of three independent experiments. $^{b}P < 0.05$, compared with control; (C): Proliferation of PC3 cells was analyzed by cell count. The results shown are representative of triplicate experiments. $^{a}P > 0.05$, $^{b}P < 0.05$, compared with the corresponding control; (D): DNA content-based cell cycle and apoptosis analysis of the same siRNA-treated PC3 cells 48 h after the transfection; (E): Bar graph summarizing the percentage of cells in apoptosis and the G_2/M phase of the cell cycle under different treatment conditions. $^{b}P < 0.05$, compared with the corresponding control; (F): Colony-forming assay at 15 days after transfection of siRNA. The results are representative of triplicate experiments. $^{b}P < 0.05$, compared with the control.

± 2.73)% in apoptosis in the clones with lower *PAR* expression compared to (1.49 ± 0.13)% in the control ($P < 0.05$). These results revealed G_2/M-phase cell cycle arrest with apoptosis induced by the knockdown of *PAR* expression.

To test the prolonged effects of temporary inhibition of *PAR* expression, the PC3 cells were inoculated on soft agar plates and cultured for 15 days. The number of colonies formed in siRNA-treated cells was significantly reduced (Figure 6F). This result suggests that

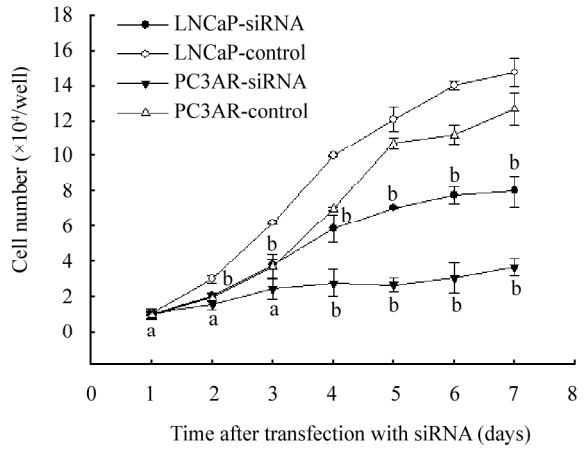

Figure 7. Growth curves of PC3–androgen receptor (AR) and LNCaP cells after short interfering RNA (siRNA) treatment targeting the prostate androgen-regulated (*PAR*) gene. The results shown are representative of triplicate experiments. $^{a}P > 0.05$, $^{b}P < 0.05$, compared with the corresponding control.

decreased *PAR* expression imparted a possible long-term growth disadvantage on PC3 cells.

3.5 PAR mediates the proliferation of PC3AR and LNCaP cell lines

We also studied the role of *PAR* in the proliferation and survival of PC3AR and LNCaP cells. After the transfection of siRNA targeting *PAR*, the proliferation of the cells was inhibited (Figure 7).

4 Discussion

Prostatic neoplasms are initially dependent on growth stimulation by androgens [10]. The major circulating androgen is testosterone, which is converted to DHT in prostate cells by the enzyme 5α-reductase. Androgen response is mediated by binding of DHT or other androgens to the AR, a ligand-activated transcription factor. This then translocates to the nucleus, binds response elements in the promoters of a diverse range of target genes involved in prostate cell homeostasis, angiogenesis, differentiation and apoptosis, and activates transcription from these promoters, resulting in the production of proteins involved in mitosis and differentiation of the prostate [11]. Treatment for advanced PCa primarily involves compounds that interfere with this signaling process, by blocking the circulating androgen and/or by inhibiting the AR itself [12]. This androgen ablation therapy can produce significant inhibition of androgen-dependent Pca. However, the treatment ultimately fails, and PCa progresses to an androgen-independent state [13]. The mechanism underlying the transition to androgen-independence is the focus of the present study. Evidence suggests that PCa progression occurs through the alteration of the AR or the AR signaling pathway by dysregulation of AR activity through signal transduction cascades [14–16], alteration in the expression of AR co-regulators [17], AR amplification [18] and mutations of AR that enable it to become transcriptionally active in response to ligands in addition to testosterone and DHT [19].

New genes downstream from the AR and related to the growth of androgen-independent PCa would be ideal therapeutic targets. Recently, during research on the mechanism of hormone resistance in prostate carcinomas, a novel gene designated PAR was isolated from LNCap-OM, an androgen-independent PCa subline. The gene was implicated in malignant transformation and its expression could be regulated by androgens in androgen-dependent PCa cells [7].

We corroborated that *PAR* expression was higher in the androgen-independent cell line PC3 than that in the androgen-independent cell line LNCaP. Platica *et al.* [7] reported that treatment of LNCaP cells with 0.1 nmol/L and 1.0 nmol/L R1881 (methyltrienolone, a synthetic androgen) decreased *PAR* expression. In the present study, the androgen DHT was used in a wider range of concentration (0.001–100.00 nmol/L). DHT in higher concentrations (10–100 nmol/L) downregulated *PAR* expression in LNCaP cells, which was similar to the result obtained by Platica *et al.*, whereas DHT in lower concentrations (0.001–1.00 nmol/L) upregulated *PAR* expression. However, the androgen-dependent regulation of *PAR* was not seen in PC3, a PCa cell line that is AR-negative. The upregulation of *PAR* expression in LNCaP cells was in a dose-dependent manner and could be blocked by AR antagonist flutamide. In addition, PC3 cells stably transfected with AR expression vector also demonstrated an androgen-dependent increase of (43.27 ± 5.02)% in *PAR* expression after exposure to 0.1 nmol/L DHT for 16 h. These effects were not seen in the parental AR-negative cells. It is reasonable to hypothesize that the expression of *PAR* might be downstream of the AR signalling pathway and directly related to AR action following stimulation by DHT.

To determine whether the knockdown of *PAR* could inhibit the proliferation of androgen-independent PCa without interfering with AR itself, PC3 cells will be used

for further study because of their androgen-independent and AR-negative status. Decreased *PAR* levels were obtained by transfecting PC3 cells with the siRNA targeting *PAR*. Knockdown of the *PAR* gene in PC3 cells inhibited cell proliferation, as well as the efficiency of colony formation in soft agar. Flow cytometric analysis revealed that downregulated *PAR* expression induced cell cycle arrest in G_2/M phase and an increase in apoptosis. Inhibition of *PAR* could induce not only growth arrest but also apoptosis. Simply inducing growth arrest is not sufficient because, if still alive, the cell will find ways of getting around that block. The inhibition of *PAR* led to the killing off of tumor cells. These results suggest that *PAR* is essential for the optimal proliferation of PC3 cells and might be a novel target for androgen-independent PCa after the androgen ablation therapy directed against the AR loses efficiency.

To determine the true nature of *PAR*, its role in the regulation of proliferation and survival was investigated in PC3AR and LNCaP cell lines. After transfection with siRNA targeting *PAR*, cell growth was suppressed. The result showed that *PAR* mediated the cell proliferation and survival regulation action of the AR in androgen-dependent and androgen-independent cells with AR expression.

In conclusion, *PAR* is a novel gene expressed higher in androgen-independent PCa cells than in androgen-dependent PCa cells. It might contribute to malignant proliferation in androgen-independent PCa cells. Because of the possibility that *PAR* is downstream from the AR, this gene could be a potential therapeutic target for androgen-independent PCa with AR signaling pathway alteration.

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