Original Article
Androgen deprivation therapy induces androgen receptor-dependent upregulation of Egr1 in prostate cancers

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Abstract: Early growth response gene-1 (Egr1) has a crucial function in the development and progression of prostate cancer. However, whether Egr1 contributes to the transition of advanced androgen-independent prostate cancer (AIPC) from androgen-dependent prostate cancer (ADPC) remains largely unknown. To the best of our knowledge, through immunohistochemical staining methods, we were the first to identify that Egr1 is more highly expressed in AIPC clinical specimens than in androgen-dependent prostate cancer (ADPC). An in vitro study with quantitative RT-PCR and Western blot demonstrated that Egr1 also has a higher expression in androgen-independent PC3 cells than in the androgen-dependent LNCaP cells. Egr1 expression in LNCaP cells was significantly upregulated during the androgen deprivation treatment (ADT) and was re-downregulated through the addition of dihydrotestosterone. Although no variation in PC3 cells was identified, Egr1 responded to dihydrotestosterone and flutamide in the androgen receptor (AR)-transfected PC3 cells. Further investigation with Egr1 agonist and specific siRNA-targeting Egr1 revealed that Egr1 upregulation or downregulation was accompanied by a change in inhibitors of differentiation and DNA binding-1 (Id1) in the same direction in both LNCaP and PC3 cells. The variation is shown to be negatively regulated by androgen through AR during ADT. Our data suggested that upregulated Egr1 might partially contribute to the emergence of AIPC after prolonged ADT. This study also elucidated the potential mechanism underlying Id1 participation in the progression of prostate cancer. Understanding the key molecular events in the transition from ADPC to AIPC may provide new therapeutic intervention strategies for patients with AIPC.

Keywords: Prostate cancer, Egr1, Id1, androgen deprivation treatmen, androgen receptor

Introduction

Prostate cancer is the most common malignancy in American men and the second leading cause of male cancer mortality [1]. In 2012, prostate cancer was expected to account for 29% (241,740) of all newly diagnosed cancers and 9% (28,170) of all male cancer deaths [2]. The development and progression of prostate cancer are initially androgen-dependent. Hence, androgen deprivation therapy (ADT) is generally employed to treat advanced or metastatic prostate cancer. However, prolonged androgen deprivation generally results in relapse and androgen-independent tumor growth [3]. No effective treatment strategy for advanced androgen-independent prostate cancer [AIPC, also known as castration-resistant prostate cancer (CRPC)] is available. The molecular events that enable prostate cancer cells to proliferate in reduced androgen conditions are poorly understood. Therefore, at present, the most challenging issue in the clinical treatment of prostate cancer is the emerging AIPC [4]. A more in-depth understanding of the variations in gene expression during the transition from androgen-dependent prostate cancer (ADPC) to AIPC may lead to new paradigms and possible improvements in the treatment of AIPC.

The family of early growth response (Egr) transcription factors has a highly conserved DNA-binding domain consisting of three zinc finger motifs, which bind to a GC-rich region in the promoters of their target genes [5]. The induction of Egr1 by external stimuli is generally transient

Table 1. Clinicopathologic details of the 15 case studies

Stage	ADPC	AIPC
Number of cases	10	5
Age (years)	53 to 81 (median, 68)	61 to 74 (median, 69)
Time of ADT before operation (month)	0	11 to 17 (median, 15)
PSA (Pre-ADT, ng/ml)	6.13 to 86.14 (median, 18.72)	> 100

Footnotes: ADPC: androgen-dependent prostate cancer; AIPC: androgen-independent prostate cancer; ADT: androgen deprivation therapy; PSA: prostate-specific antigen.

but sustained in a large fraction of prostate tumors, suggesting that Egr1 has a critical function in the initiation and/or progression of prostate cancer [6, 7]. These previous findings suggest that Egr1 protein may be an important candidate molecular marker for aggressive human prostate cancer. However, to date, whether Egr1 participates in AIPC remains unknown.

Previous studies have suggested the critical role of Egr1 in cancer metastasis and tumor invasion [8]. Our recent evidence shows that inhibitors of differentiation and DNA binding-1 (Id1) are negatively regulated by androgen through androgen receptors (AR) which contribute to the development of AIPC [9]. In addition, Id1 transcription was at least partially regulated by a protein complex containing the Egr1 [10]. Combining the present results with those from previous studies, we hypothesize that Egr1 might contribute to the negative regulation of AR on Id1 and participate in the formation of AIPC or CRPC.

Materials and methods

Tissue specimens

Study protocols involving human materials were approved by the institutional ethics committee of Changhai Hospital (Shanghai, China). Formalin-fixed and paraffin-embedded tissue specimens were obtained from the archives of the hospital's Department of Pathology. ADPC specimens were obtained from 10 ADPC patients by puncture biopsy, and AIPC specimens were obtained from 5 AIPC patients during transurethral resection of the prostate (TURP), who received ADT for 11 to 17 months before TURP. Clinicopathologic details of the 15 cases are described in **Table 1**.
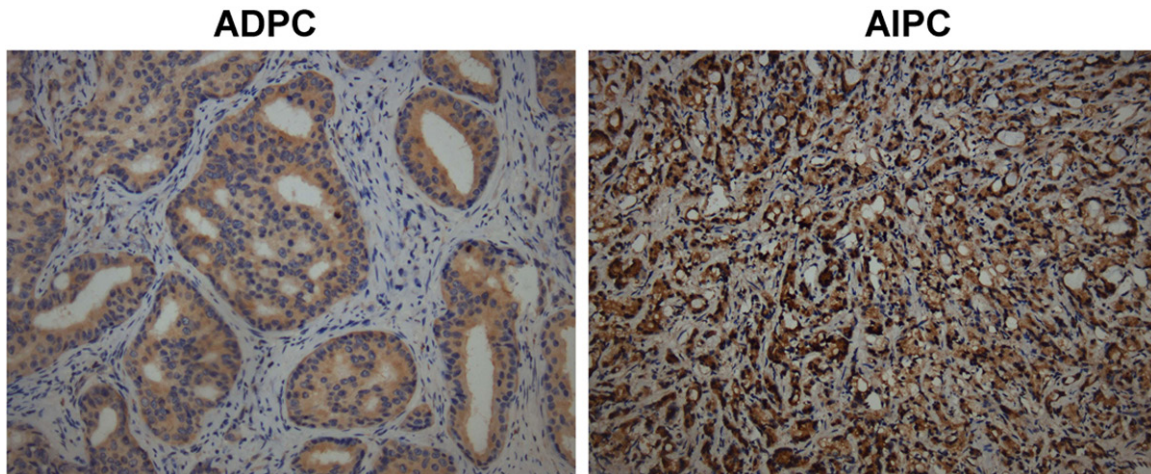
Immunohistochemistry

Paraffin-embedded prostate tissue specimens were sliced to 5 μm sections, deparaffinized with xylene, rehydrated with decreasing concentrations of ethanol, blocked with methanol/hydrogen peroxide, and incubated overnight with 0.66 Ag/ml rabbit polyclonal Egr1 antibody (588; Santa Cruz Biotechnology, Santa Cruz, CA). Egr1 immunocomplexes were detected using a biotinylated-labeled anti-rabbit secondary antibody and streptavidin-peroxidase with the Vector Nova Red substrate kit for peroxidase (Vector Laboratories, Burlingame, CA). Tissue sections were counterstained with hematoxylin. Semi-quantitative determination of Egr1 was performed. The proportion of positively stained cells was judged as follows: 0 = no cells were stained, 1 = 0% to 1% cells, 2 = 1% to 10% cells, 3 = 10% to 33% cells, 4 = 33% to 66% cells, and 5 = 66% to 100% cells. In addition to the proportion score, an intensity score was obtained based on the mean intensity of staining: 0 = negative, 1 = weakly positive, 2 = intermediately positive, and 3 = strongly positive. The intensity and the proportion scores were added to obtain a total score. The scoring values were analyzed and grouped as follows: 7 to 8, high Egr1 expression; 3 to 6, low Egr1 expression; and 0 to 2, negative Egr1 expression. Slides were blindly evaluated by two investigators independently, who were unaware of the pathologic characteristics.

Cell culture

Human prostate cancer cell line PC-3 was obtained from the Institution of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Human prostate cancer LNCaP cells were provided by Prof. Klaus Jung (Department of Urology, University Hospital Charité, Humboldt University, Germany). All cell lines were maintained in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37°C in 5% CO_2. Phenol-red free RPMI-1640 medium (Gibco) with 10%

ADPC   **AIPC**

Figure 1. Egr1 was significantly upregulated in clinical AIPC samples. Egr1 expression was determined by immunohistochemistry in the representative paired slides of the prostate cancer in ADPC or AIPC groups, respectively (×200).

charcoal-stripped FBS (CS-FBS, Biological Industries, M.P. Ashrat, Israel) was used as the androgen deprivation condition.

Quantitative RT-PCR

Total RNA was extracted from the prostate cancer cells using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA), and reversely transcribed to cDNA using PrimeScript™ Reagent Kit (Takara, Tokyo, Japan). One milligram cDNA was used as the template for quantitative PCR (SYBR Green, Takara), which was performed using the Lightcycler Detection System (Roche, Basel, Switzerland) with $^{\Delta\Delta}$Ct method, according to the manufacturer's instructions. The expression level of housekeeping gene GAPDH was used to normalize the Egr1 and Id1 mRNA expression. The PCR was set at 95°C (15 s) and 60°C (30 s) in a total of 40 cycles with a final extension step at 60°C for 5 min. The primers used in this study were as follows: 5'-TGA CCG CAG AGT CTT TTC CT-3' and 5'-TGG GTT GGT CAT GCT CAC TA-3' for Egr1; 5'-GTA AAC GTG CTG CTC TAC GAC ATG-3' and 5'-AGC TCC AAC TGA AGG TCC CTG-3' for Id1; and 5'-GAC AAC TTT GGC ATC GTG GA-3' and 5'-ATG CAG GGA TGA TGT TCT GG-3' for GAPDH. Values represent the mean ± SD from at least three independent experiments. Each experiment was performed in triplicates.

Western blot

Changes in the Egr1 expression in the LNCap and PC3 cells, as well as the AR expression in AR-transfected PC-3 cells, were detected by Western blot analysis. Cells were collected and lyzed in 100 μl M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA) containing 3% Halt Phosphatase inhibitor Cocktail (Pierce). All samples were normalized according to the protein concentration, separated in 10% SDS-PAGE gel, and then transferred to polyvinylidene difluoride membranes (Gelman Pall Life. Sciences, Ann Arbor, MI USA) using the wet transfer blotting system (Bio-Rad, Hercules, CA, USA). After blocking in Tris-buffered saline (TBS: 120 mmol/l NaCl, 50 mmol/l Tris-HCl, pH 7.4) containing 0.05% Tween 20 and 1% BSA, the blot was probed with Egr1 polyclone antibodies (1:500, Santa Cruz, 588) or AR monoclonal antibodies (1:1000, Santa Cruz, sc-7305) and incubated overnight at 4°C. Subsequently, the membranes were washed extensively, incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, Cell Signaling Technology, Danvers, MA, USA) for 1.5 h at room temperature, and washed thrice with TBS containing 0.05% Tween-20 for enhanced chemiluminescence detection.

Plasmid transfection

PC-3 cells were seeded at a density of 2.5×10^5 cells/well into a six-well culture plate (Nalge Nunc, Rochester, NY, USA) with RPMI-1640 medium containing 10% FBS or phenol-red free RPMI-1640 medium containing 10% CS-FBS.

Table 2. Egr1 expression in AIPC and ADPC patients

Egr1 level	Total score	Distribution of included cases	
		ADPC (10)	AIPC (5)
Negative	≤2	0	0
	3	0	0
	4	2	0
Low	5	6	0
	6	1	0
	7	1	0
High	8	0	5

Footnotes: ADPC: androgen-dependent prostate cancer; AIPC: androgen-independent prostate cancer.

After adherence, PC-3 cells were transfected with full-length human AR expression plasmid pSGAR2 packaged by Lipofectin 2000 as described [9]. Then, 48 h post-transfection, cells were collected and prepared for further Western blot and/or qPCR analysis.

Short interfering RNA (siRNA) preparation and transfection

The sequence of siRNA oligonucleotide-target Egr1 and the control oligonucleotides were as previously described [11] and synthesized by Shanghai Shenggong Co. LNCaP and PC3 cells were cultured 24 h before transfection, resulting in an 80% confluence of the cell monolayer. Specific Egr1 and control siRNA were transfected to the cells mixed with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendation. After 6 h at 37°C, the medium was changed, and the cells were cultivated in RPMI-1640 supplemented with 10% CS-FBS. The expression inhibition rates of Egr1 in both cells were approximately 70% at mRNA levels (data not shown).

Statistical analysis

Variation in the results obtained between two groups was calculated by Student's t-test. Variation in the results obtained from two different group tissue specimens were analyzed with Wilcoxon's Signed Rank Sum test. SAS 9.1.3 (SAS Institute, Cary, NC, USA) was used to complete data analyses. All of the above hypothesis tests were two-sided and two-tailed. A P value of 0.05 or less was considered to indicate statistical significance.

Results

High Egr-1 expression was confirmed in AIPC patients

To understand the potential biological and clinical role of Egr1 protein in prostate cancer of ADPC and AIPC stages, the Egr1 protein expression level was detected in 10 ADPC patients and 5 AIPC patients. Immunohistochemistry showed that Egr1 protein in prostate cancer was located mainly in the cytoplasm, which was consistent with previous studies (**Figure 1**) [12, 13]. High Egr1 protein levels was found in all AIPC specimens with the highest total scores of 8, whereas 9 of 10 cases in ADPC specimens exhibited low expression with total scores ranging from 4 to 6. Only one ADPC specimen was classified as a high expression group, having the total score of 7 (**Table 2**, *P* < 0.001). Thus, Egr1 was identified to be significantly more highly expressed in AIPC patients than in ADPC patients.

Androgen downregulates the Egr1 expression in prostate cancers in vitro

To further investigate the possible correlation of Egr1 expression with the formation of AIPC, we first determined Egr-1 expression *in vitro* in ADPC (LNCaP) and AIPC (PC-3) cells. Quantitative real-time-PCR analysis showed that Egr1 mRNA expression was higher in PC-3 cells than in LNCaP cells, which was consistent with the result of the Western blot (**Figure 2A** and **2B**). Considering that these two cancer cell lines represent the difference in androgen dependence, the different Egr1 expression patterns might correlate with the biological function of androgen during the transition from ADPC to AIPC. To further elucidate how Egr1 expression was regulated after androgen deprivation in ADPC cells *in vitro*, LNCaP and PC-3 cells were cultured in phenol-red free RPMI-1640 medium containing 10% CS-FBS for 4 d, respectively, and Egr1 mRNA and protein level expression was measured. Egr1 mRNA and protein expression was gradually upregulated in LNCaP cells cultured in androgen deprivation medium (**Figure 2C** and **2D**), whereas no significant difference was identified in the PC-3 cells (**Figure 2E** and **2F**). These data suggest that Egr1 was downregulated by androgen, and the gradual upregulation of Egr1 in prostate cancer
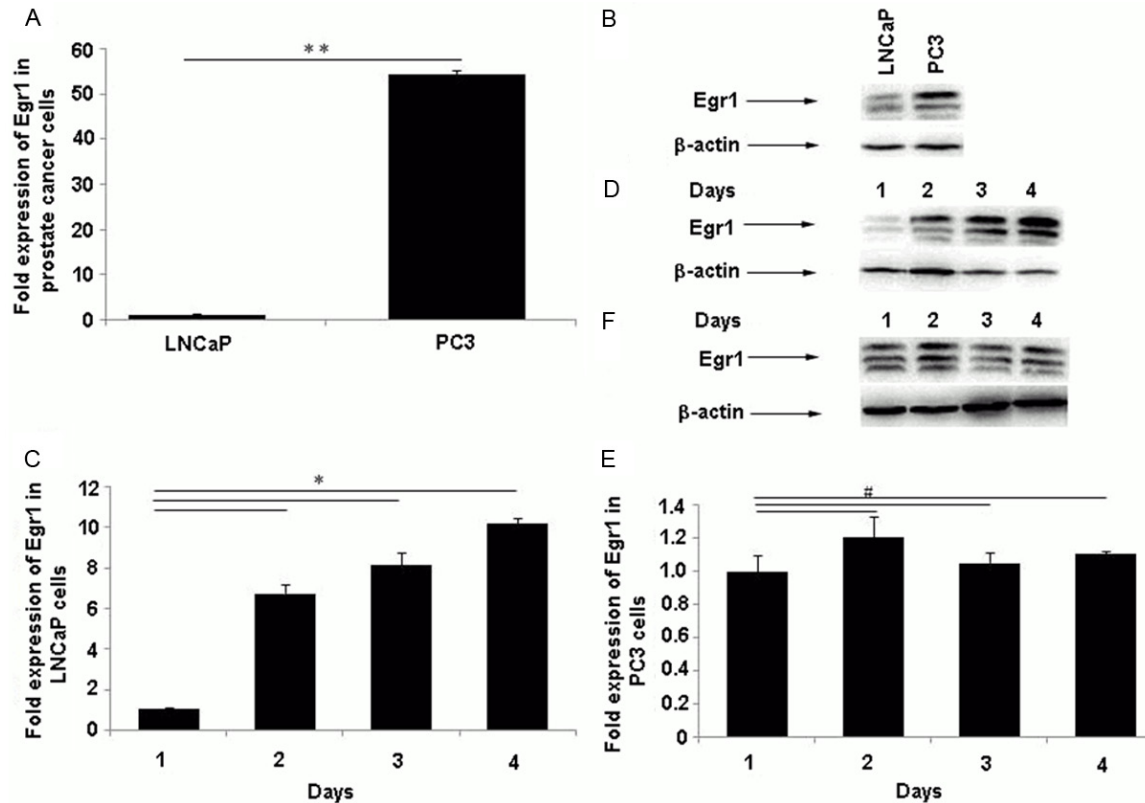
Figure 2. Egr1 was more highly expressed in PC3 cells and was evidently upregulated in LNCaP cells after ADT *in vitro*. LNCaP and PC3 cells were seeded and cultured *in vitro* in RPMI-1640 containing 10% FBS for 48 h, and were subsequently collected and detected for Egr1 mRNA (A) and protein (B) levels using qRT-PCR and Western blot. Egr1 was more highly expressed in PC3 than in LNCaP cells (**$P < 0.001$); (C to F) After incubation in phenol-red free RPMI-1640 containing 10% CS-FBS for 1 d to 4 d, LNCaP and PC3 cells were collected and these cells underwent qRT-PCR and Western blot analysis. Expression of Egr1 mRNA (C) and protein (D) were both gradually upregulated in LNCaP cells, whereas no significant difference was identified in PC-3 cells (E and F) (*$P < 0.01$, #$P > 0.05$).

during ADT might contribute to the transition of prostate cancers from ADPC to AIPC.

AR mediated the downregulation of Egr1 by androgen

As shown above, no significant difference in Egr1 expression was found when AR-negative PC-3 cells were cultured in androgen-free conditions. This phenomenon indicates the possibility that the variation in Egr1 expression might be mediated by androgen through AR. DHT and AR antagonist flutamide (Sigma-Aldrich) were then used to probe the potential correlation between the Egr1 expression and androgen-AR pathway. One group of LNCaP cells were cultured for 2 d in phenol-red free RPMI-1640 medium containing 10% CS-FBS, followed by administration of different concentrations of DHT for 2 d. The other group of LNCaP cells was cultured for 4 d in RPMI-1640 medium contain-

ing 10% FBS medium after administration of different concentrations of flutamide. As shown in **Figure 3A** and **3B**, DHT administration significantly downregulated Egr1 mRNA expression, whereas flutamide significantly upregulated its expression; both regulations were in a dose-dependent manner. As expected, these phenomena could not be repeated in AR-negative PC-3 cells (**Figure 3C** and **3D**).

To further confirm whether AR mediates the regulation of Egr1 expression, AR-negative PC-3 cells were transfected with AR expression plasmid pSGAR2 containing EGFP expression sequence to investigate the effect of DHT and flutamide on Egr1 mRNA expression during the course of androgen deprivation treatment. AR expression in PC-3 cells after transfection was confirmed by highly coexpressed EGFP (**Figure 4A**) and by specific AR protein bands using Western blot analysis, compared with control
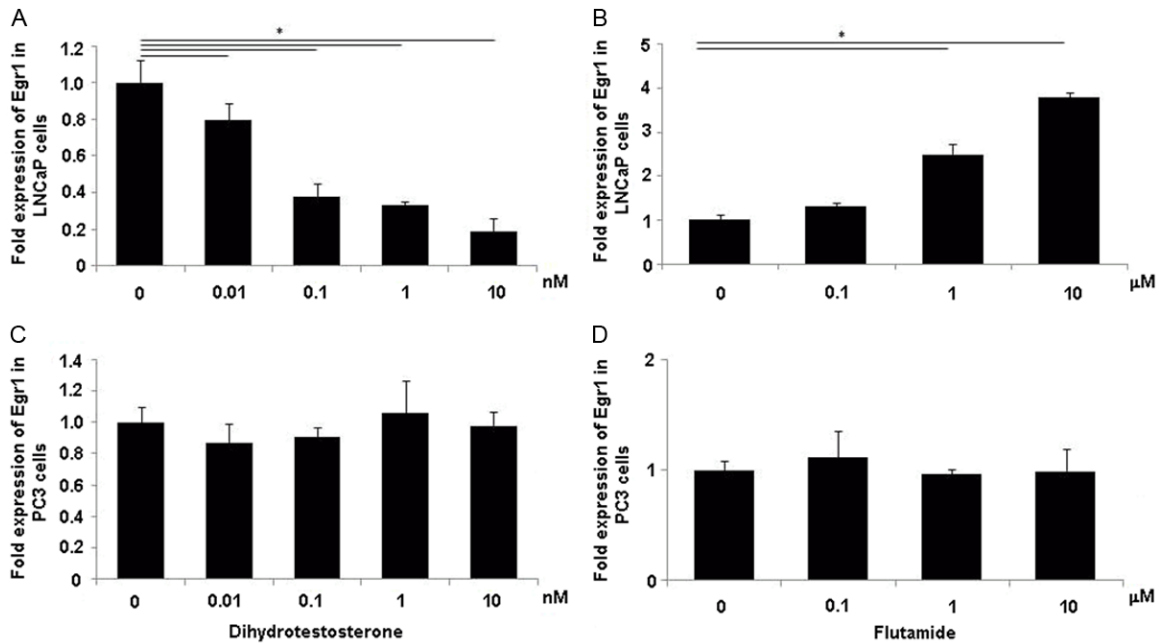
Figure 3. Androgenic regulation of Egr1 in LNCaP or PC-3 cells. LNCaP (A) and PC-3 (C) cells were seeded in six-well culture dishes and incubated in phenol-red free RPMI-1640 medium containing 10% CS-FBS for 48 h. The cells were then treated with indicated concentrations of dihydrotestosterone for another 48 h. LNCaP (B) and PC-3 (D) cells were seeded in six-well culture dishes, incubated in RPMI-1640 medium containing 10% FBS and the indicated concentrations of flutamide for 4 d. After incubation, total RNA of all these cells was isolated and subjected to qRT-PCR analysis. All data have been normalized to GAPDH RNA and plotted relative to the Egr1 expression levels in LNCaP cells incubated in medium without drugs (*P < 0.01). Egr1 was down-regulated by DHT and up-regulated by flutamide in LNCaP cells, but was not affected by the two drugs in PC3 cells.

cells transfected with empty plasmid (**Figure 4B**). The group of PC-3 cells transfected in phenol-red free RPMI-1640 medium containing 10% CS-FBS was administered with different DHT concentrations for 2 d. The other group of cells transfected in RPMI-1640 medium containing 10% FBS were administered with different flutamide concentrations for 4 d. Similar to the LNCaP cells, Egr1 mRNA expression was downregulated by DHT (**Figure 4C**) and upregulated by flutamide (**Figure 4D**). These data confirmed that the upregulation of Egr1 expression in prostate cancer cells after androgen deprivation treatment was mediated by AR.

Id1 is regulated by Egr1 in prostate cancer cells

Id1 was also found to be negatively regulated by androgen through AR [9], and the transcription of Id1 was at least partially regulated by a protein complex containing the Egr1 [10]. To investigate whether the negative regulation of Id1 by androgen was mediated by Egr1 in prostate cancer cells, we used Egr1 agonist bombe-

sin to upregulate Egr1 and investigate the Id1 mRNA expression in prostate cancer cells. LNCaP and PC3 cells were both cultured in phenol-red free RPMI-1640 medium containing 10% CS-FBS for 2 d, and were administered with bombesin for 3 d. As shown in **Figure 5**, bombesin administration significantly upregulated Egr1 expression in both LNCaP and PC3 cells (**Figure 5A** and **5B**), and simultaneously upregulated Id1 mRNA expression in a dose-dependent manner (**Figure 5C** and **5D**).

To further confirm the direct regulation of Egr1 on Id1, we used siRNA to silence Egr1 and investigated the Id1 changes in LNCaP and PC3 cells. LNCaP and PC3 cells were both transfected with specific or with control siRNA cultured in phenol-red free RPMI-1640 medium containing 10% CS-FBS for 2 d and were administered with 1 nM bombesin for 3 d. Cells were then collected, and Egr1 and Id1 mRNA expression was quantified using qRT-PCR. As shown in **Figure 5E** and **5F**, Egr1 expression was downregulated significantly after transfection with specific Egr1 siRNA in both LNCaP and PC3
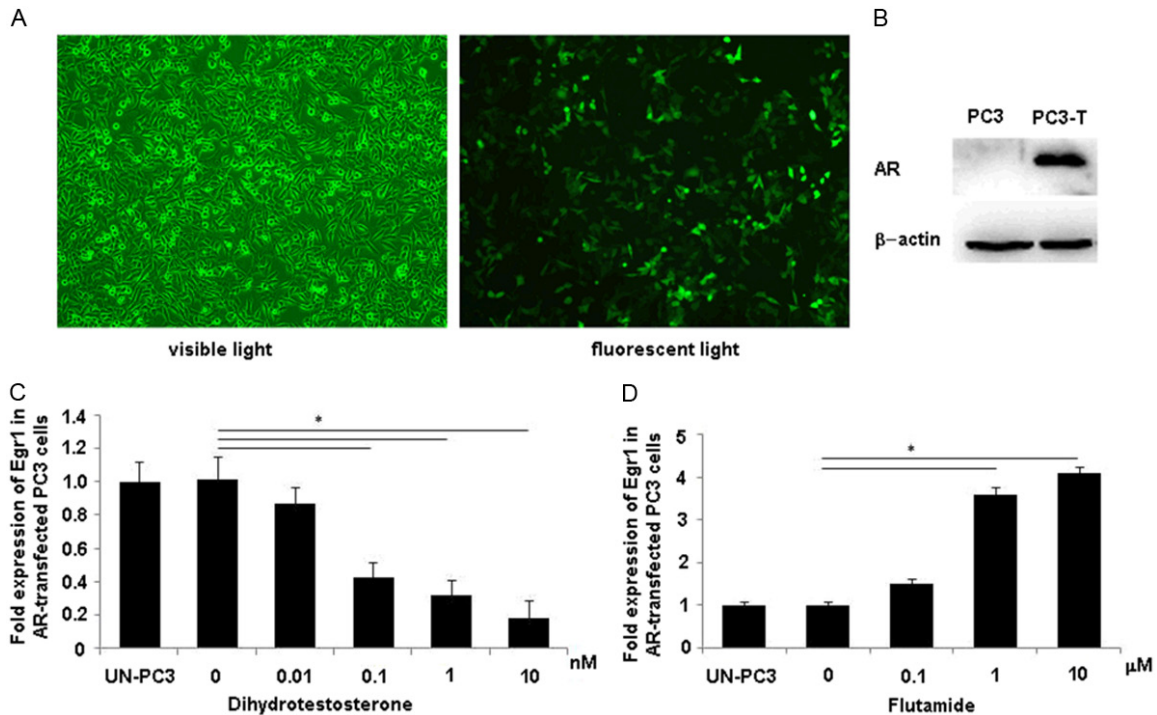
Figure 4. Egr1 was negatively regulated by AR. PC-3 cells were transiently transfected with AR expression plasmid pSGAR2 containing EGFP expression sequence, and 48 h later the cells were detected for EGFP expression using fluorescence microscopy. Most of the transfected cells expressed EGFP, suggesting high AR expression (A); (B) The transfected PC3 cells were collected and analyzed for the AR protein expression using Western blot assay, and a specific AR protein band was detected as compared with the empty plasmid control. PC-3-T: plasmid pSGAR2 transfected PC-3 cells. (C) PC-3 cells were transiently transfected with plasmid pSGAR2 and cultured in phenol-red free RPMI-1640 medium containing 10% CS-FBS for 48 h. Then, the cells were treated with different DHT concentrations for another 48 h before the cell lysates were prepared and measured using qRT-PCR. (D) PC3 cells were transiently transfected in RPMI-1640 medium containing 10% FBS for 48 h. Then, the cells were treated with different flutamide concentrations for another 4 d before mRNA levels were measured using qRT-PCR. All data were normalized to GAPDH RNA and plotted relative to the Egr1 expression levels in PC-3 cells transfected with empty plasmid (*P < 0.01).

cells and not very surprisingly, Id1 expression was also downregulated in the experiment groups. Therefore, Egr1 might have participated in the AIPC formation through the Id1 regulation during ADT in patients with prostate cancers.

Discussion

ADT has been used to shrink androgen-dependent tumors for many years. However, prolonged androgen deprivation often results in relapse and AIPC, which progresses and undergoes metastasis. The evolution of prostate cancer from an androgen-dependent state to AIPC is always a lethal progression. The AR is essential in both, although its function in androgen-independent cancers is poorly understood [14]. Developing diagnostic and therapeutic approaches that target AIPC, therefore, has significant potential for improving survival and quality of life of prostate cancer patients [15]. Earlier research performed to understand hormone-refractory prostate cancer focused on molecular alterations, such as AR overexpression or mutations, which could allow AR to respond to low levels of androgens or be directly activated by other ligands. More recently, AR has been suggested to be indirectly activated by cell-surface receptors, such as HER2, interleukin-6 receptor, and G-protein-coupled receptors [16]. Despite awareness on the important function of AR signaling in prostate cancer, the exact effect of ADT on AR signaling has not been well characterized [17]. In this study, we expanded our understanding of Egr1 expression regulation in prostate cancer after androgen deprivation and have provided new insights into the importance of the Egr1 action in the Id1 regulation by androgen through AR.
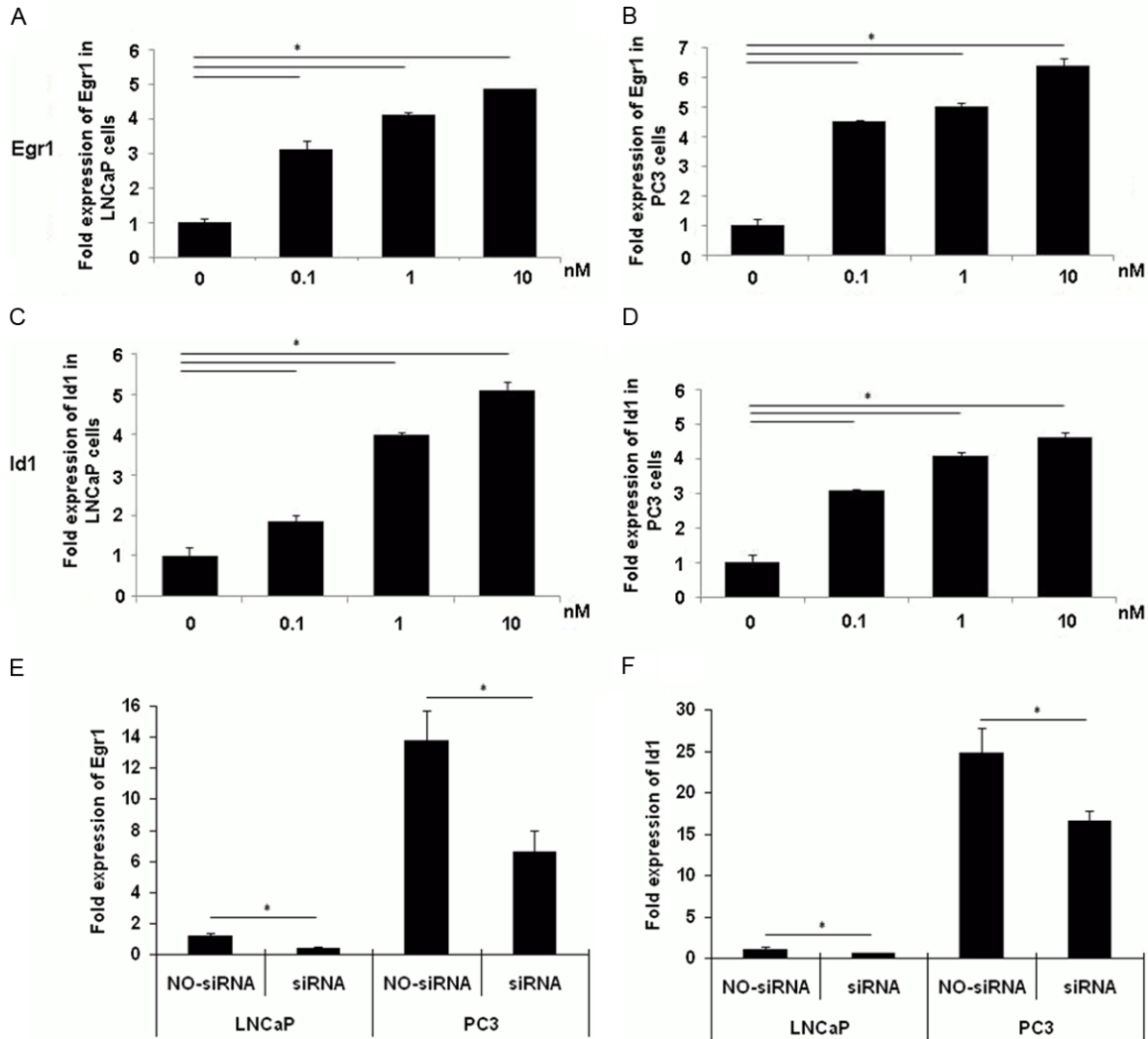
Figure 5. Egr1 directly regulates the Id1 expression in prostate cancer. LNCaP and PC3 cells were first incubated in phenol-red free RPMI-1640 medium containing 10% FBS for 48 h. The cells were then treated with indicated concentrations of bombesin for another 72 h before mRNA levels were measured using qRT-PCR. Bombesin induced a dose-dependent upregulation of Egr1 both in LNCaP (A) and PC3 (B) cells. Id1 was also upregulated in the same pattern in LNCaP (C) and PC3 (D) cells. When specific siRNA targeting at Egr1, or the control siRNA were used before 1 nM of bombesin was supplemented, the upregulation of Id1 became withdrawn following the silencing of Egr1 in both cells (E, F, *$P < 0.01$).

Egr1 is a prototypic member of the zinc finger transcription factor family that also includes Egr2, Egr3, and Egr4. These four proteins have a high degree of homology at their DNA-binding zinc finger domains, but they have divergent sequences outside the DNA-binding domains. In normal tissues, Egr1 expression is generally low or undetectable. In response to growth factors and cytokine signaling, Egr1 regulates cell growth, differentiation, and apoptosis. Aberrant Egr1 expression is linked to human diseases, such as ischemic injury, cancers, inflammation, atherosclerosis, and cardiovascular pathogenesis [17]. The function of Egr1 in cancer pathogenesis is complex, because it functions both in cell proliferation and apoptosis [18]. In several types of human tumor cells, Egr1 exhibits prominent suppressor gene activity by binding to and direct transactivation of major tumor suppressor factors, including transforming growth factor-β1, p53, p73, fibronectin, and PTEN. The downstream pathways of these factors display multiple nodes of interaction with each other. This condition suggests the existence of a functional network of suppressor factors that serve to maintain normal growth

regulation and resist the emergence of trans-formed variants. Suppression of Egr1 expres-sion is common in non-small cell lung cancers, breast cancer, glioblastomas, and acute myelogenous leukemia [18-20]. Paradoxically, increased Egr1 expression is consistently observed in epithelial ovarian and prostate cancers, where growing evidence indicates that Egr1 is oncogenic [13, 21, 22]. The basis of the oncogenic function of Egr1 in prostate cancer is not well known. Egr1 has been suggested to directly regulate genes that function in the development of prostate cancer, including IGF-II, TGF-β1, and PDGF-A, which were previously implicated in enhancing tumor progression [7]. In mouse prostate cancer cells, Egr1 was found to promote cell growth, cell cycle progression, and increased cell resistance to apoptotic sig-nals by upregulating cyclin D2, inhibiting P19^{ink4d}, and FAS expression [23]. Insufficient Egr1 significantly impaired tumor progression but did not affect the tumor initiation and tumor growth rate in prostate cancers [24]. Egr1 is also upregulated in structurally intact adjacent prostate tissues and defines the field cancer-ization. Field cancerization at prostatectomy specimen surgical margins may be a predictor of cancer recurrence [25]. More recently, Yang et al. showed that Egr1 expression enhanced the androgen-independent growth of prostate carcinoma cells both *in vitro* and *in vivo*. Egr1 partially mediated these effects through the AR signaling pathway [26]. However, how Egr1 was regulated after androgen deprivation in pros-tate cancer and whether the elevated Egr1 expression contributes to the transition of ADPC to AIPC (or CRPC) remains unknown. Whether Id1 is involved and regulated by Egr1 during the transformation is also undete-rmined.

To the best of our knowledge, we were the first to identify that Egr1 was significantly more highly expressed in well-characterized AIPC patients than in ADPC patients. This result, together with the aforementioned previous research, identified the possibility that Egr1 is involved in the transition from ADPC to AIPC during ADT in prostate cancers. Further *in vitro* investigation of Egr1 expression in ADPC (LNCaP) and AIPC (PC-3) cells confirmed the results obtained from clinical samples. The gradual upregulation of Egr1 in androgen-dependent LNCaP cells during ADT, and down-

regulation after retreatment with DHT, suggest-ed that Egr1 was negatively regulated by androgen in prostate cancers. AR antagonist and agonist experiments, especially the andro-gen-dependent variation of Egr1 in PC-3 cells before and after the knock in of AR, further con-firmed that Egr1 was negatively mediated by the androgen-AR pathway. Upregulation of Egr1 in ADPC cells after prolonged androgen depri-vation *in vitro* and the relatively high expression of Egr1 in both AIPC model system and in clini-cal cases suggest that Egr1 might have an essential function during the transition of pros-tate cancers from ADPC to AIPC.

Helix-loop-helix (HLH) proteins are transcrip-tional factors that control a variety of develop-mental pathways, including hematopoiesis, myogenesis, neurogenesis, and mammary gland development [5]. The Id family proteins, including Id1, Id2, Id3, and Id4, belong to the family of HLH proteins and function as domi-nant negative regulators of basic HLH tran-scriptional factors. Of all the members of the Id protein family, Id1 is the one mostly linked to tumorigenesis, moreover regulating cellular senescence as well as cell proliferation and survival. Id1 is overexpressed in prostate, lung, breast, gastric, esophageal, and cervical can-cers; especially in the poorly- differentiated cancers [27]. The intensity of Id1 staining in carcinomas was significantly stronger than that in the normal prostate and benign prostatic hyperplasia, and it was significantly increased with increasing malignancy of carcinomas, as measured by the Gleason score [28]. Over-expression of Id1 was observed in multidrug resistant cells in prostate cancer [29] and could result in an androgen-independent phenotype of prostate cancer cells (LNCaP) [30]. We have examined the potential role of Id1 in the malig-nant progression of prostate cancer and have shown that Id1 is significantly overexpressed in all prostate cancer cell lines and clinical speci-mens examined. The observed upregulation during ADT both *in vivo* and *in vitro* indicated that Id1 might have an important function in the transition from ADPC to AIPC. Meanwhile, Id1 expression in ADPC was negatively regulat-ed by androgen in a receptor-dependent way [9]. However, the potential molecular mecha-nism of Id1 negative regulation by AR is still poorly understood. Previous studies have found a 210-bp enhancer element containing a con-

sensus Egr1 binding site in the upstream region of the Id1 gene. The regulation of the Id1 response to serum has been suggested to be mediated in part by the Egr1 early response gene in muscle cells and fibroblasts [9]. In fibroblast growth factor-2 stimulated human neuroblastoma SK-N-MC cell line, Egr1 was found to directly bind to the Id1 promoter [31]. To further confirm whether Egr1 is a key element in the regulation of Id1 by AR signal during ADT in our experiment system, we investigated the Id1 variation after the Egr1 pathway was activated or inactivated *in vitro*. Here, we demonstrated that upregulation of Egr1 with bombesin, an agonist of Egr1, was accompanied by a significant upregulation of Id1 in both LNCaP and PC3 cells. Moreover, silencing Egr1 with specific siRNA also down-regulated the Id1 expression. Therefore, this study is the first to confirm that Egr1 was involved in the negative regulation of androgen on Id1 through AR in prostate cancers.

In summary, our data suggest that Egr1 might at least partially contribute to the negative regulation of AR on Id1 and participate in the formation of AIPC (or CRPC). The present study further expands our understanding of the regulation of Id-1 expression after androgen deprivation in prostate cancer. Additionally, new insights into the action of AR in the regulation of Id1 and Egr1 by androgen are provided. Further studies are needed to clarify the underlying mechanism of the AR signaling pathway in AIPC and to identify the cross-talk between Egr1, Id1, and AR, which may finally lead to new strategies to neutralize aggressive androgen-independent progression of prostate cancer.

Acknowledgements

Disclosure of conflict of interest

None.

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