

Tumor Suppressor PAX6 Functions as Androgen Receptor Co-Repressor to Inhibit Prostate Cancer Growth

Chih-Rong Shyr,^{1,2} Meng-Yin Tsai,^{2,3} Shuyuan Yeh,⁴ Hong-Yo Kang,^{2,3}
Yun-Chao Chang,² Pei-Ling Wong,² Chao-Cheng Huang,³
Ko-En Huang,^{2**} and Chawnshang Chang^{4*}

¹Department of Laboratory Medicine & Biotechnology, Tzu-Chi University, Hualien, Taiwan

²The Center for Menopause and Reproductive Medicine Research,
Chang Gung University/Memorial Hospital, Kaohsiung, Taiwan

³Graduate Institute of Clinical Medicine, Kaohsiung Division, Chang Gung University, Kaohsiung, Taiwan

⁴George Whipple Laboratory for Cancer Research, Department of Urology, Pathology, Radiation Oncology,
the Cancer Center, University of Rochester Medical Center, Rochester, New York

BACKGROUND. PAX6, a transcription factor, has currently been suggested to function as a tumor suppressor in glioblastoma and to act as an early differentiation marker for neuroendocrine cells. The androgen receptor (AR) plays a pivotal role in prostate cancer development and progression due to its transcriptional activity in regulating genes involved in cell growth, differentiation, and apoptosis. To determine the role of PAX6 in prostate cancer, we investigated whether PAX6 interacts with AR to affect prostate cancer development.

METHODS. We used immunostaining, RT-PCR, and Western blotting assays to show the expression status of PAX6 in prostate tissue and human prostate cancer cell lines. The role of PAX6 in cell growth and colony regeneration potential of LNCaP cells were evaluated by MTT assay and soft agar assay with PAX6-overexpressed LNCaP cells. Mammalian two-hybrid and co-immunoprecipitation (Co-IP) assays were used to demonstrate the interaction between PAX6 and AR. Reporter gene and Q-RT-PCR assays were performed to determine the effects of PAX6 on the function of AR.

RESULTS. In prostate cancer tissues, PAX6 expression was stronger in normal epithelial cells than cancer cells, and decreased in LNCaP cells compared to that of DU145 and PC3 cells. Enforced expression of PAX6 suppressed the cell growth of LNCaP cells and also inhibited the colony formation of LNCaP cells. PAX 6 interacted with AR and repressed its transcriptional activity. PAX6 overexpression decreased the expression of androgen target gene PSA in LNCaP cells.

CONCLUSIONS. In this study, we found that PAX6 may act as a prostate cancer repressor by interacting with AR and repressing the transcriptional activity and target gene expression of AR to regulate cell growth and regeneration. *Prostate* © 2009 Wiley-Liss, Inc.

KEY WORDS: androgen receptor; PAX6; prostate cancer; co-repressor

Chih-Rong Shyr and Meng-Yin Tsai contributed equally to this work.

Grant sponsor: National Institute of Health; Grant numbers: DK073414, CA122295.

*Correspondence to: Chawnshang Chang, PhD, George Whipple Laboratory for Cancer Research, Department of Urology, Pathology, Radiation Oncology, the Cancer Center, University of Rochester Medical Center, Rochester, NY. E-mail: chang@urmc.rochester.edu

**Correspondence to: Ko-En Huang, MD, The Center for Menopause and Reproductive Medicine Research, Chang Gung University/Memorial Hospital, Kaohsiung, Taiwan.

E-mail: khuang@adm.cgmh.org.tw

Received 5 May 2009; Accepted 5 August 2009

DOI 10.1002/pros.21052

Published online in Wiley InterScience
(www.interscience.wiley.com).

INTRODUCTION

PAX6, a member of the paired box gene family, functions as a transcription factor and is involved in various developmental processes [1]. It was first cloned in 1991 with a predicted 422-amino acid polypeptide product possessing a N-terminal paired domain (PD), a homeodomain (HD) in the middle, and a serine/threonine-rich C-terminal domain, all structural motifs characteristic of certain transcription factors [2]. The loss of function studies linked the gene to the development of the eye, pituitary gland, neuroendocrine cells in the pineal body, and pancreatic Langerhans islet cells [3–6]. As an early differentiation marker, PAX6 is also used to identify the precursors of neuron and neuroendocrine cells [7–10] in current stem cells studies.

In addition to its role on development and cell differentiation, PAX6 was also found to influence the fate of some cancer cells, possessing a tumor suppression function [11]. In animal studies, the PAX6 mutant small eye mice were easily susceptible to leukemia [12]. In human, Salem et al. [13] identified that the PAX6 is hypermethylated in colon and bladder cancer cells. The increased methylation of the PAX6 promoter was observed in bladder cancer as well [14]. Furthermore, PAX6 was also found to be hypermethylated in its promoter region and silenced in breast cancer lines and primary tumors, and furthermore, the transfection of PAX6 restored the expression of PAX6 back in MDA-MB-231 cells and consequently suppressed cancer cell growth [15]. These results suggest PAX6 could act as a cancer suppressor since the low expression of PAX6 could be linked to cancer development. The similar cancer repressor's role of PAX6 was also reported in the neuronal cancer, glioblastoma, in which PAX6 was found to suppress the growth and invasiveness of the cancer in vitro and in animal models [16,17]. In summary, these studies suggest that the PAX6 acts as a tumor repressor and its silencing promotes the cancer growth. However, the role of PAX6 in prostate cancer is still unknown.

Prostate cancer remains a great threat to men's health in the United States. The American Cancer Society estimated that 186,320 men would be diagnosed with prostate cancer in 2008, and ~28,660 men would die of the disease in the United States [18]. Therefore, it is of great interests to more clearly understand this cancer. Androgens and androgen receptor (AR) are involved in the normal development and maintenance of the prostate and thus the aberrant androgen/AR signaling plays a critical role in the growth and progression of prostate cancer [19]. This recognition has greatly influenced therapeutic concepts for human prostate cancer during the last several decades [20]. However, androgen deprivation therapy

is usually associated with a gradual transition of the cancer cells from androgen-dependence (sensitive) to androgen depletion-independent (ADI) [21] (refractory) [22]. A number of theories and mechanisms leading to the transition have been studied and one possibility is the ligand-independent activation of the AR [23]. Neuroendocrine differentiation in prostate cancer is another possible mechanism proposed to lead to androgen insensitivity. The stem cell model was proposed to explain the derivation of neuroendocrine cells that usually are associated with androgen independence and androgen resistance from a small stem cell population [24]. Thus, androgen deprivation therapy may force these cells to differentiate to androgen-independent neuroendocrine cells [24].

Although PAX6 may act as an early differentiation regulator for neuroendocrine cells as well as a tumor repressor [11], its dual roles in prostate has not been studied. As prostate cancer is an endocrine-related cancer and the neuroendocrine differentiation is closely correlated to the prognosis, we hypothesized that PAX6 may play a role in the prostate cancer development.

To test our hypothesis, we used the androgen-dependent prostate cancer cell line, LNCaP, to assess the cellular and molecular effects of PAX6. Our results showed that PAX6 repressed cell growth and colony formation of LNCaP cells and inhibited the transcriptional activity of AR. Using the mammalian two-hybrid system and co-immunoprecipitation (Co-IP) method, we demonstrated interaction between PAX6 and AR. Furthermore, the exogenous expression of PAX6 in LNCaP cells inhibited the expression of prostate-specific antigen (PSA) stimulated by androgen at the RNA and protein level. Taken together, these results suggest that PAX6 acts as a negative coregulator for AR by interacting with AR and repressing its function. This phenomenon could establish the tumor suppressor's role of PAX6 in prostate cancer and help us understand the development and progression of prostate cancer.

MATERIALS AND METHODS

Plasmids and Antibodies

The pCMX-PAX6 was a gift from Dr. Richard L. Maas. pSG5AR and VP-16AR were constructed as previously described [25]. The insertion fragments of PAX6 were generated by PCR methods. The primers were designed based on the sequence of human PAX6 mRNA available on the Genome Data Base. The *Bam*H1 restriction sequence was inserted at the 5' end of each primer for cloning purposes and constructed into the pCMX-Gal4, pCMX-VP16, and pcDNA3-flag vector for different experiments. The anti-AR (N-20), anti-

PSA, and anti-PAX6 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-flag antibody was from Sigma (St. Louis, MO).

Cell Culture and Transfection

The human prostate cancer cell line, LNCaP, was maintained in RPMI 1640 medium containing penicillin (25 U/ml), streptomycin (25 µg/ml), and 10% fetal bovine serum. The COS-1 cells were maintained in Dulbecco's minimum essential medium containing penicillin (25 U/ml), streptomycin (25 µg/ml), and 10% fetal bovine serum. For the Western blot, 8×10^5 LNCaP cells were plated on 100-mm dishes and transfected with different plasmids using *Superfect* (Qiagen, Valencia, CA). After transfection, the cells were maintained in the RPMI1640 supplemented with charcoal-dextran-treated fetal bovine serum (CD-FBS) for 16 hr, then subjected to different treatments for another 20–24 hr. For the transactivation assay and the mammalian two-hybrid assay, the COS-1 cells were transfected using calcium phosphate precipitation method as described previously [26] and the LNCaP cells were transfected using *Superfect*. For the stable clone selection, the LNCaP cells transfected with either pcDNA3-flag or pcDNA3-flag-PAX6 were selected using neomycin.

Immunohistochemistry

For immunohistochemistry, paraffin-embedded sections were heated at 55°C for at least 2 hr, deparaffinized in xylene, rehydrated, and washed in Tris-buffered saline (TBS), pH 8.0. For antigen retrieval, slides were microwaved in 0.01 M sodium citrate/pH 6.0, immersed with 1% hydrogen peroxide in methanol for 30 min, and blocked with 20% normal goat serum in TBS for 60 min. After washing with PBS, sections were incubated for 90 min in PAX6 antibodies diluted 1:100 in TBS containing 1% BSA, followed by goat anti-rabbit biotinylated secondary antibody diluted 1:300 in TBS containing 1% BSA. Sections were incubated with avidin-biotin-peroxidase complex solution for 30 min, followed by development with diaminobenzidine peroxidase substrate kit (Vector Laboratories) for 5 min. Slides were counterstained with hematoxylin for 30 sec, dehydrated, cleaned in xylene, and mounted. Primary antibody was replaced with normal rabbit IgG or 1% BSA in TBS for negative controls.

Co-Immunoprecipitations

COS-1 cells were transfected with 5 µg of pSG5 AR plus 5 µg of pcDNA3-flag-PAX6 and harvested after 48 hr. Nuclear extracts were prepared for Co-IP assays.

Four hundred microliters of each extract were first incubated with anti-Flag antibodies or anti-AR antibodies (N20, Santa Cruz Biotechnology) overnight at 4°C, and then with Protein G Sepharose for an additional 16 hr at 4°C. Protein G Sepharose containing the immune complex was then washed three times with the washing buffer (50 mM Tris-HCl/pH 7.4, 100 mM NaCl, 5 mM CaCl₂, 5 mM MgCl₂, 0.1% Nonidet P-40) and resuspended in SDS-containing sample buffer. The flag and AR associated complexes were immunoprecipitated by anti-flag and anti-AR antibody, respectively. The isolated protein complexes were fractionated, transferred, and immunodetected with anti-AR or anti-flag antibodies. The proteins were resolved through a 6% SDS-PAGE and immunoblotted with anti-flag antibody or anti-AR antibody.

Soft Agar Assay

We suspended 5×10^3 pcDNA3-Flag-PAX6 or pcDNA3-Flag transfected LNCaP cells in 0.3% Bacto-agar (BD, Lot. 5089021) and after transfection, layered the cells on top of 1 ml of 0.6% agarose in 6-well culture plates, incubated with 1 ml RPMI 1640 supplemented with 10% FBS for 4 weeks. After 4 weeks, the colonies were visualized by staining with 0.5% crystal violet. The experiment was analyzed in triplicate, and colonies larger than 100 µm in diameter were counted.

MTT Assay

LNCaP cells stably transfected with and without pcDNA3-PAX6 were incubated in 96-well dishes (1.5×10^3 cells/well) for 5 days, MTT reagent (Promega, Madison, WI) was added at days 1, 3, and 5 per the manufacturer's instruction. After 16 hr of reaction, absorbance of the converted dye was measured at a wavelength of 595 nm with background subtraction at 650 nm.

Western Blotting Analysis

Western blotting was performed as described previously [27]. In short, cell extracts from LNCaP cells transfected with various vectors were prepared for electrophoresis run on SDS/PAGE gel and then transferred onto nitrocellulose (Minipore). The antibodies for anti-AR (Santa Cruz Biotechnology SC-816), anti-flag (Sigma F-3165), anti-PSA (Santa Cruz Biotechnology SC-7638), anti-Nkx 3.1 (Santa Cruz Biotechnology T 19, sc-15022), or anti-tubulin (Santa Cruz Biotechnology SC-5274) were used for the detection. The images were shown using alkaline phosphatase substrate color development method (Bio-Rad, Hercules, CA).

Mammalian Two-Hybrid Assays and Reporter Gene Assays

For COS-1 cells, 2×10^5 cells were plated on 6-well plates 12 hr before being transfected with 0.5 μ g of luciferase reporter and other expression vectors as indicated in the figures and legends. After 24 hr transfection, the cells were treated with 10^{-8} M 5α -dihydrotestosterone (DHT) or ethanol for another 24 hr. For each transfection, SV40 promoter driven *Renilla luciferase* (SV40RL) was used as an internal control. For LNCaP cells, the same volume of cells were seeded in 6-well plates 36 hr before transfection, and the cells were transfected by *Superfect* (Qiagen) as described by the manufacturer. The treatment after transfection was the same as with the experiments performed in COS-1 cells.

RNA Extraction and Quantitative Real-Time PCR Analysis

RNA was extracted by using the Trizol reagent (Invitrogen), and first-strand cDNA was synthesized from 2 μ g of total RNA in 20 μ l reactions containing RT buffer with dNTPs, Oligo-dT, RNase inhibitor, and Moloney murine leukemia virus reverse transcriptase according to the manufacturer's protocol (Invitrogen). Reverse transcription reactions were incubated at 72°C for 5 min and then 25°C for 10 min, followed by 42°C for 60 min, and aliquots of the reaction products were used in later quantitative real-time PCR analysis. The quantitative PCR analysis was done using an ABI PRISM 7700 sequence detector system and the SYBR green PCR master mix kit (Applied Biosystems), according to the manufacturer's suggestions. The housekeeping gene, β -actin was used for normalization. Relative mRNA expression was calculated by $2^{-\Delta\Delta C_t}$ method as described [28]. The following primer pairs were used: PSA forward 5'-CCGCATCTAATCGCTGGAGAG-3', reverse 5'-CGATGTAGTTGGCGAAGCG-3'; β -actin forward 5'-CATATTCACCACCTCGGACAA-3', reverse 5'-TGACGCCACAGACCACAC-3'; kallikrein 2 (KLK2) forward 5'-CCTGGCAGGTGGCTGTGTAC-3', reverse 5'-TGTGCCGACCCAGCCA-3' [29]; and Nkx3.1 forward 5'-CGCAGCGGCAAGGC-3', reverse 5'-GGTGCTCAGCTGGTCTGTTCT-3' [30].

RESULTS

PAX6 Is Expressed in Prostate Tissue

Since higher PAX6 expression level indicates improved prognosis in malignant astrocytic gliomas, whereas lower levels correlate with unfavorable outcomes [11], PAX6 was proposed to play an essential

role in tumor progression. To determine whether PAX6 affects prostate cancer development, we examined the expression of PAX6 in normal prostate tissues by immunostaining. Expression of PAX6 in the normal prostate epithelial cells (Fig. 1A, upper panel) was higher compared to PAX expression in adjacent cancer cells in the same tissue sections (Fig. 1A, middle panel). We also examined the PAX6 expression in three different types of prostate cancer cells (PC3, DU145, and LNCaP cells). The RNA level of PAX6 was determined in prostate cells using reverse transcription-PCR analysis. The protein level of PAX6 was determined in prostate cells using Western blotting analysis. As shown in Figure 1B,C, PAX6 was expressed in all prostate cancer cells, but PAX6 was expressed higher in PC3 and DU145, which are AR-negative cells compared to that of LNCaP cells, which are AR-positive cells. Therefore, these results showed

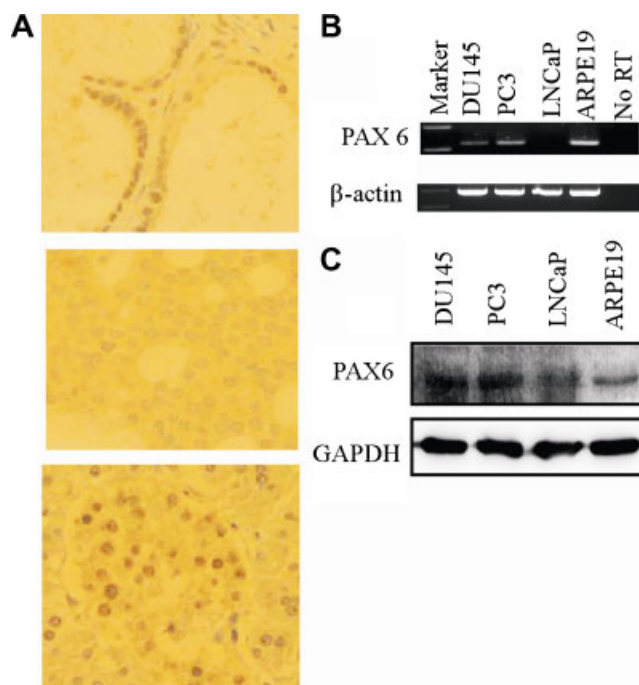


Fig. 1. The expression of PAX6 in prostate cancer cells. **A:** The prostate cancer specimens were stained with a rabbit polyclonal antibody against PAX6. The normal epithelial cells adjacent to the cancer cells were strongly stained positive for PAX6 (upper panel). The cancer cells in the same specimen had weaker PAX6 signals (middle panel). The pancreas specimen served as a positive control with PAX6 stained positive in the islet cells (lower panel). The staining results were examined by a pathologist to determine the cancer cells and normal epithelia cells in tissue sections. **B:** Regular reverse transcription-PCR was performed with RNAs from DU145, PC3, and LNCaP prostate cancer cells. **C:** Western blotting was assayed with lysates from DU145, PC3, and LNCaP prostate cancer cells. The human retinal pigment epithelium (ARPE19) cells were used as a positive control for PAX6.

that PAX6 is expressed in prostate tissues and the expression level is lower in AR-positive prostate cancer cells, as compared to AR-negative cells.

PAX6 Suppresses Cell Growth and Colony Formation of LNCaP Cells

Overexpression of PAX6 in glioblastoma cells suppresses cell growth [16], suggesting that PAX6 may act as a tumor repressor for certain cancers. Therefore, we evaluated the effects of PAX6 on prostate cancer growth. Because of the lower PAX6 expression in LNCaP cells, we stably transfected pcDNA3-flag-PAX6 into the LNCaP cells to establish PAX6-overexpressed LNCaP cells. The cell growth of PAX6-overexpressed LNCaP cells was assayed by the MTT assay and compared to that of parental LNCaP cells. As shown in Figure 2A, through days 1–4, PAX6-overexpressed LNCaP cells grew slower than parental LNCaP cells and on day 5, the growth of PAX6-overexpressed LNCaP cells showed about 57% lower than that of parental LNCaP cells. To determine whether the modulation of PAX6 expression affected the tumorigenic properties of the prostate cancer cells, we then tested whether PAX6 overexpression could affect the ability of LNCaP cells to form colonies in soft agar assay. LNCaP cells were transfected with either pcDNA3-Flag-PAX6 or pcDNA3-Flag. At 48 hr after transfection, the cells were placed into medium with soft agar, and colonies were counted after 4 weeks. The colony numbers in the PAX6 transfected cells significantly decreased (about 48% reduction) compared with those of LNCaP cells transfected with control vectors (Fig. 2B,C). This result showed that the exogenous expression of PAX6 decreased the ability of LNCaP cells to regenerate in soft agar. Taken together, these results suggest that PAX6 may suppress the LNCaP cell growth and reproduction.

PAX6 Represses AR Transcriptional Activity in LNCaP Cells

Androgens can activate the transcriptional activity of AR to affect the expression of genes that influence the prostate cancer cell growth. Therefore, the development and progression of prostate cancer are affected by the androgen/AR-signaling pathway. Because PAX6 was demonstrated to repress LNCaP cells growth in previous results, the interactions between PAX6 and AR were assessed for the functional association between PAX6 and AR. To determine whether PAX6 affects AR transcriptional activity, three sets of different androgen-response elements (AREs) constructed in luciferase (Luc) reporter plasmids were used for reporter gene assays. AR expression vectors with various amounts of PAX6 expression vectors were

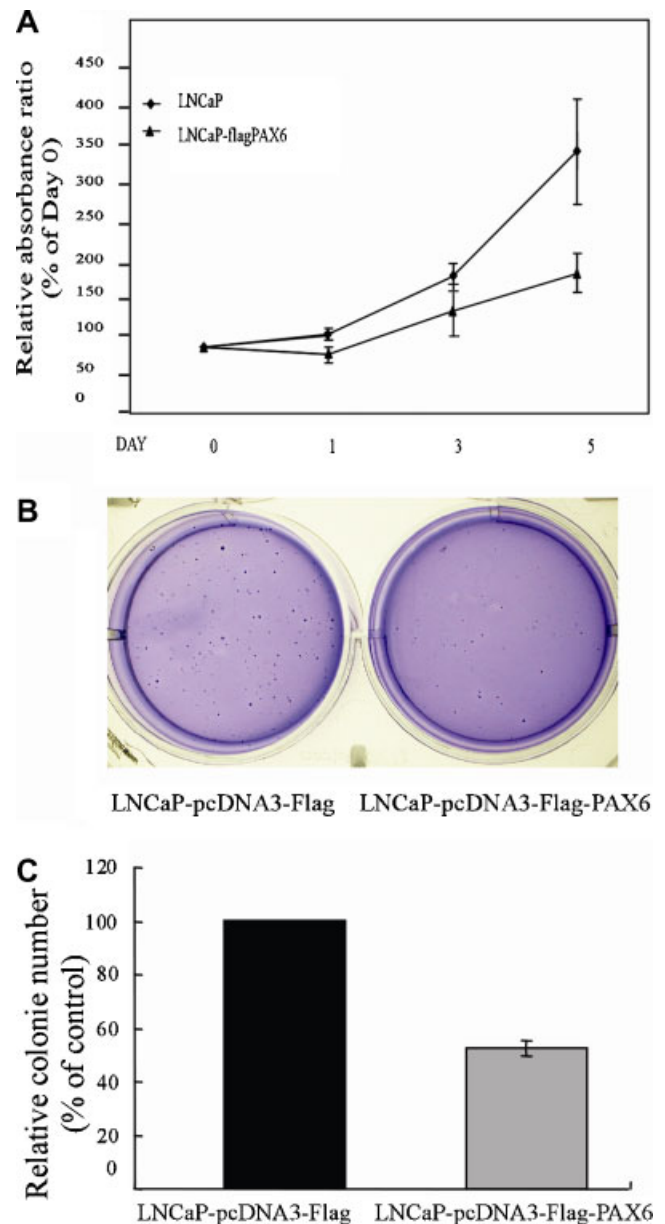


Fig. 2. The effect of PAX6 on the LNCaP cell growth and reproduction. **A:** The growth of LNCaP cells stably transfected with pcDNA3-flag-PAX6 or parent LNCaP cells was determined by MTT assays on the indicated days. **B:** The representative wells demonstrate the total number of colonies formed by LNCaP cells transfected with the indicated plasmids. For soft agar colony formation assay, the LNCaP cells were transfected with pcDNA3-Flag-PAX6 or pcDNA3-Flag as controls, and seeded in 0.3% Bacto-agar containing RPMI 1640 medium with 10% fetal bovine serum. After culture for 4 weeks, the colonies were counted under an inverted microscope. **C:** The numbers of colonies of LNCaP-pcDNA3-Flag-PAX6 cells standardized against the control cells (set at 100%). The data shown are means and SD from three sets experiments. The difference between treatments is statistically significant ($P = 0.001$). The colonies were counted under 40 \times light microscope.

transfected into COS-1 cells together with either mouse mammary tumor virus (MMTV), four copies of ARE4, or PSA Luc reporter plasmids. *Renilla* SV40 Luc reporter was used as an internal control. The results shown in Figure 3A–C revealed that the transfection of PAX6 suppressed the AR transactivation in all three sets of reporter gene assays in the presence of DHT. The dose-dependent effect was shown in MMTV-Luc. However, there was no significant dose-dependent effect in PSA-Luc and ARE4-Luc since the suppression is too profound to display the difference of the dose-dependent effect. These findings indicate that PAX6 represses AR transactivation.

PAX6 and AR Directly Bind Each Other

The physical interaction between two transcriptional factors is one proposed mechanism to inhibit each other's transcriptional activity. To clarify whether PAX6 directly interacts with AR, Co-IP and mammalian two-hybrid assays were performed. The interaction was first confirmed in the mammalian two-hybrid system. In the mammalian two-hybrid assay, pGal4-PAX6 and pVP16-AR plasmids were transfected into the COS-1 cells to generate Gal4-PAX6 fusion protein with the Gal4 DNA binding domain as well as VP16 activation domain. As shown in Figure 4A, the luciferase activity was increased fourfold over the DHT-treated cells with co-transfection of pGal4-PAX6 and pVP16-AR plasmids comparing with the cells transfected with pGal4 and pVP16, suggesting that AR and PAX6 interact in the system. The directly physical interaction was demonstrated in the Co-IP, pcDNA3-flag-PAX6, and pSG5-AR were transfected to COS-1 cells. After 2 days of transfection, the protein in the cells was harvested. The anti-AR antibody or anti-flag antibody was used to pull down the precipitate. Then the Western blot was performed with the AR or flag antibody (Fig. 4B). The results showed that using anti-flag antibody was able to immunoprecipitate PAX6 and pulled down AR and using anti-AR antibody pulled down AR and PAX6. These experiments indicate that PAX6 can interact with AR and form a protein complex in intact cells.

Pax6 Suppresses the PSA Expression Stimulated by Androgen

To determine the significance of PAX6 inhibitory effect on AR signaling, we examined the expression of AR target genes: PSA, KLK2 and the homeobox gene Nkx 3.1 in LNCaP cells [31,32]. We compared the AR-regulated gene level induced by DHT in LNCaP cells with or without the exogenous expression of PAX6 by transfecting PAX6 expression vectors or parent vectors. Using q-RT-PCR to determine the expression of PSA,

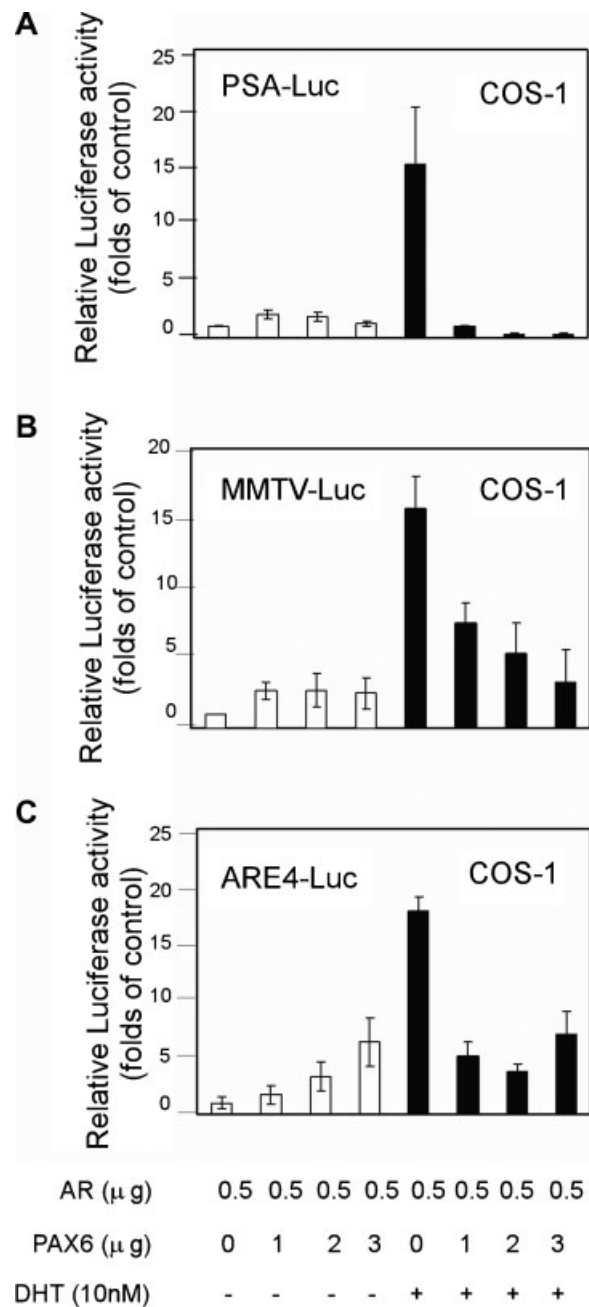


Fig. 3. The repression of AR transcriptional activity by PAX6. **A:** COS-1 cells were transfected with 0.5 μg of reporter plasmids containing different AREs: PSA promoter/enhancer, **(B)** MMTV and **(C)** four copies of synthetic androgen-response element (ARE), 0.5 μg of pSG5AR, and increasing amounts of pCMX-PAX6 plasmids (0, 1, 2, and 3 μg). The pSV40-PRL plasmids were also co-transfected as internal control. After 24 hr the cells were treated with either ethanol or 10⁻⁸ M DHT. The relative luciferase activity of the control cells without pCMX-PAX6 plasmids transfected and treated with ethanol was set as onefold. All values represent the mean ± SD of three independent experiments.

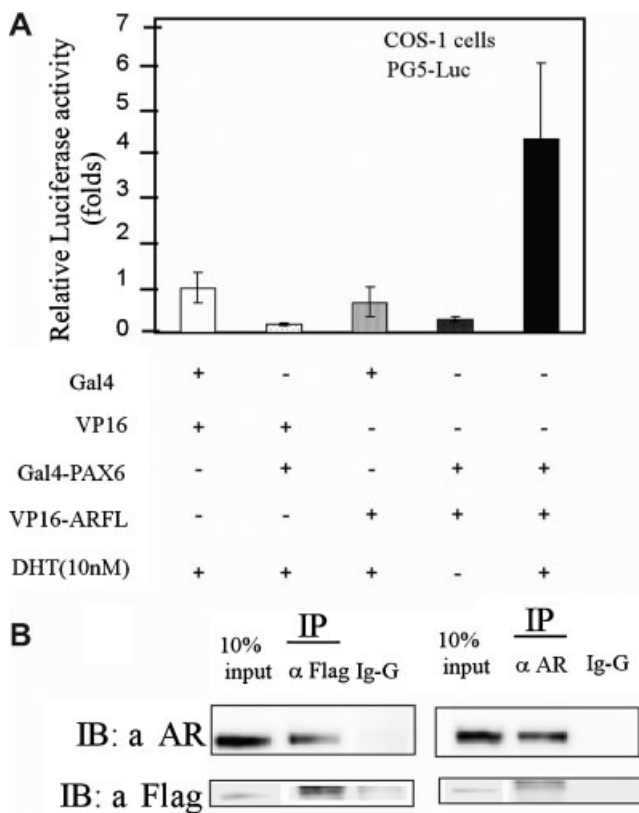


Fig. 4. The direct interaction between PAX6 and AR. **A:** To perform the mammalian two-hybrid assay, the pG5-LUC reporter plasmids were transiently co-transfected with different sets of plasmids (pCMX-Gal4 plus pCMX-VP16, pCMX-Gal4-Pax6 plus pCMX-VP16, pCMX-Gal4 plus pCMX-VP16-AR, or pCMX-Gal4-PAX6 plus pCMX-VP16-AR) as indicated into COS-1 cells. **B:** COS-1 cells were seeded in 100-mm dishes and were transfected with 5 μ g of pSG5 AR plus 5 μ g of pcDNA3-flag-PAX6. After 48 hr, cells were harvested and nuclear extracts were prepared for Co-IP assay. The flag and AR associated complexes were immunoprecipitated by either anti-FLAG or anti-AR antibodies, respectively. The isolated protein complexes were fractionated, transferred, and immunodetected with anti-AR or anti-flag antibodies. We loaded 10% nuclear extract inputs as reference. IgG precipitated complexes were used as control. A representative blot of three independent experiments is shown.

we showed that PAX6 decreased the PSA (Fig. 5A), KLK2 (Fig. 5B), and Nkx 3.1 (Fig. 5C) mRNA expression stimulated by DHT. In order to check whether the protein expression was also affected by PAX6, we performed Western blot analysis in the LNCaP cells transfected with vector or pcDNA3-flag-PAX6 and treated with either ethanol or 10 nM DHT. As shown in Figure 5B, cells transfected with pcDNA3-flag-PAX6 expressed a flag-tagged PAX6 fusion protein of 47 kDa detected by anti-flag Abs, but cells transfected with pcDNA3-flag-PAX6 only expressed flag protein of 1,012 Da, which was not detected in the position of

flag-tagged PAX6 fusion protein on Western blot. Similar to the down-regulation of mRNA levels, we found that the LNCaP cells transfected with PAX6 and treated with DHT expressed less PSA and Nkx 3.1 proteins than the cells transfected with parental vectors (Fig. 5D). These results suggest that PAX6 is capable of repressing AR action on its positively regulated genes.

DISCUSSION

In this study, we found that PAX6 acts as a prostate cancer repressor by inhibiting AR action. Beside the tissue sites such as neuron, eye, pancreas, and embryo, the expression of PAX6 is found in small intestine, testis, and thymus [33]. The diverse expression pattern of PAX6 suggests that it has a versatile role in tissue development and maintenance. Here, the results of immunohistochemistry performed on tissues sections (Fig. 1A) demonstrated that PAX6 expression is higher in normal prostate tissue adjacent to the cancer cells than cancer cells. After examining the expression of PAX6 in prostate cancer cells, we also found that the expression of PAX6 varied among three different prostate cancer cell lines with lowest expression in androgen-sensitive LNCaP cells (Fig. 2B,C). The differential expression profile of PAX6 in prostate cancer tissues and prostate cancer cell lines may be linked to prostate cancer development and progression related to androgen sensitivity.

Besides being an important protein for regulating differentiation, it is also possible that PAX6 plays a critical role in control of cell growth and differentiation in prostate. Since PAX6 is a key player in lens developmental processes and is involved in almost every step as the development proceeds, PAX6 frequently shifts its role between the repressor and the activator in different stages [34,35]. PAX6 also interacts with numerous transcriptional factors during the processes of eye, neuron, and pancreas development, forming a possible network of transcriptional factors, which cooperate with PAX6 to fine-tune the expression of key genes related to development [5,34,36,37]. However, in adult tissues, PAX6 may have another role. In pancreatic islet cells, the direct interaction between PAX6 and PPAR γ resulted in decreasing PAX6 transactivation activity and reducing the glucagons secretion [19]. This study and our results suggest the regulatory role of PAX6 by interacting with transcriptional factors such as PARR γ and AR to modulate gene expression, which could be the function of PAX6 after finishing tissue development in adult tissues.

In addition, the results of our growth assay and soft agar assay showed that PAX6 suppressed LNCaP cell growth and reproduction (Fig. 2), possibly by

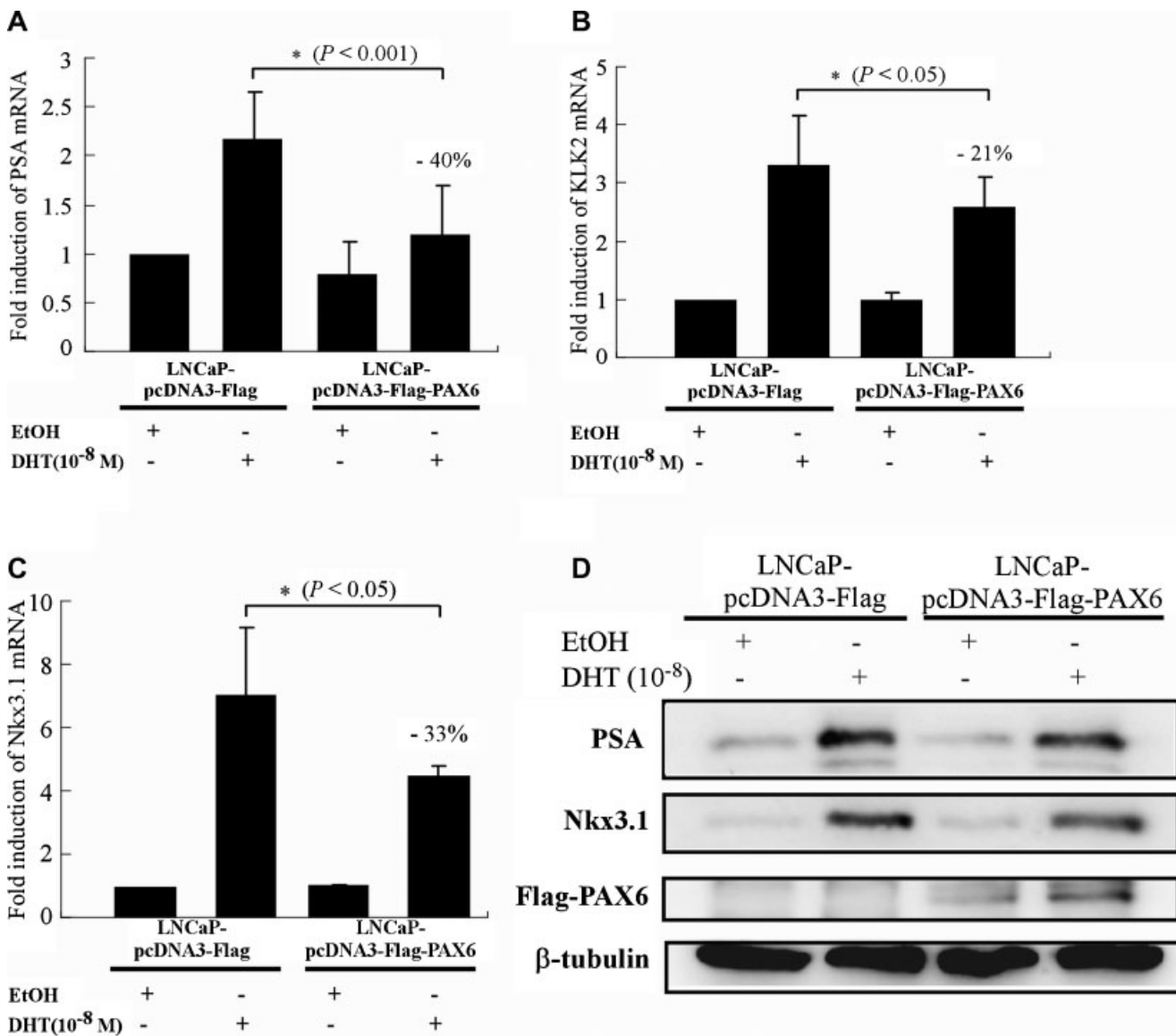


Fig. 5. PAX6 decreases androgen-induced PSA expression in LNCaP cells. LNCaP cells were transfected with pcDNA3-Flag-PAX6 or pcDNA3-Flag and then treated with EtOH or DHT in RPMI 1640 medium with 10% CD-FBS. The RNAs from treated cells were used for qRT-PCR experiments to determine their (A) PSA, (B) KLK2, and (C) Nkx3.1 RNA levels. D: LNCaP cells were transfected with pcDNA3-Flag-PAX6 or pcDNA3-Flag treated with EtOH or 10^{-8} M DHT. The cell lysates were used to determine their PSA and Nkx3.1 protein levels expression by Western blot analysis. β Tubulin was used as an internal control. Data are presented as mean values \pm SD from three independent experiments.

repressing the transcriptional activity of AR (Fig. 3). This is consistent with its tumor suppression role in glioblastoma [16]. However, little is known about the mechanism of PAX6 suppression in cancer growth. One proposed mechanism regarding the influence of PAX6 on cancer physiology is that it may suppress the matrix metalloproteinase-2 (MMP-2) to reduce the invasiveness of glioblastoma cells [38]. In contrast, androgen was reported to increase MMP-2 expression in prostate cancer [39]. As PAX6 suppresses androgen action, it may also reduce MMP-2 in prostate cancer and suppress the progression of prostate cancer.

The AR mediates the action of androgens in the normal development and maintenance of prostate [40]. Proteins that interact with AR and affect its transcriptional activity could contribute to prostate cancer progression due to the aberrant regulation of AR activity. Here, we demonstrated that PAX6 suppresses the AR transcriptional activity through the direct interaction with AR. As shown in Figure 5, PAX6 was also able to decrease the expression of PSA, one of AR's target genes in prostate and its expression is linked to the progression of prostate cancer. These findings establish a role of PAX6 in regulating prostate cancer

development and progression. AR can interact with the other transcriptional factors as both belong to the family of DNA binding regulatory proteins. For examples, AR has been reported to interact with AP-1 and consequently disrupt the formation of the AR-ARE complex, resulting in the repression of PSA gene induced by androgen [41]. Nuclear factor κ B was shown to form a complex with AR, causing the repression of AR transactivation [42,43]. And sex-determining region Y was demonstrated to interact with and negatively regulate AR transcriptional activity [44]. The transcriptional factor, AP-1/c-Jun, was also shown to interact with AR and suppress AR transcriptional activity [41]. In summary, these findings provide the mutually interactive links among transcriptional factors, which may be essential for normal cell functions and once deregulated, may contribute to the development and progression of cancer.

CONCLUSIONS

In conclusion, we have found that PAX6 was expressed in the prostate tissue. It may have tumor cell suppression roles in the androgen-sensitive LNCaP cells. The direct interaction between PAX6 and AR, which leads to the decreasing AR transactivation activity and its target gene expression, may contribute to the tumor cell suppression effect of PAX6.

REFERENCES

1. Strachan T, Read AP. PAX genes. *Curr Opin Genet Dev* 1994; 4(3):427–438.
2. Ton C, Miwa H, Saunders G. Small eye (Sey): Cloning and characterization of the murine homolog of the human aniridia gene. *Genomics* 1992;13:251–256.
3. St-Onge L. PAX6 glucagon. *Nature* 1997;387:406–409.
4. Guillemot F. Cellular and molecular control of neurogenesis in the mammalian telencephalon. *Curr Opin Cell Biol* 2005;17(6): 639–647.
5. Dohrmann C, Gruss P, Lemaire L. Pax genes and the differentiation of hormone-producing endocrine cells in the pancreas. *Mech Dev* 2000;92(1):47–54.
6. Bamiou DE, Musick FE, Sisodiya SM, Free SL, Mitchell TN, Davies RA. Defective auditory interhemispheric transfer in a patient with a PAX6 mutation. *Neurology* 2004;62:489–490.
7. Goolsby J, Marty M, Heletz D, Chiappelli J, Tashko G, Yarnell D, Fishman P, Dhib-Jalbut S, Bever CJ, Pessac B, Trisler D. Hematopoietic progenitors express neural genes. *Proc Natl Acad Sci USA* 2003;100(25):14926–14931.
8. Culig Z, Steiner H, Bartsch G, Hobisch A. Mechanisms of endocrine therapy-responsive and -unresponsive prostate tumours. *Endocr Relat Cancer* 2005;12(2):229–244.
9. Kioussi C, O'Connell S, St-Onge L, Treier M, Gleiberman A, Gruss P, Rosenfeld M. Pax6 is essential for establishing ventral-dorsal cell boundaries in pituitary gland development. *Proc Natl Acad Sci USA* 1999;96(25):14378–14382.
10. Bentley C, Zidehsarai M, Grindley J, Parlow A, Barth-Hall S, Roberts V. Pax6 is implicated in murine pituitary endocrine function. *Endocrine* 1999;10(2):171–177.
11. Robson EJ, He SJ, Eccles MR. A PANorama of PAX genes in cancer and development. *Nat Rev Cancer* 2006;6(1):52–62.
12. Nitta Y, Yoshita K, Satoh K, Senba K, Natagata N, Peters J, BM C. Spontaneous and radiation induced leukemogenesis of the mouse small eye mutant, Pax6 (Sey3H). *J Radiat Res (Tokyo)* 2004;45(2):245–251.
13. Salem C, Markl I, Bender C, Gonzales F, Jones P, Liang G. PAX6 methylation and ectopic expression in human tumor cells. *Int J Cancer* 2000;87:179–185.
14. Hellwinkel OJ, Kedia M, Isbarn H, Budaus L, Friedrich MG. Methylation of the TPEF- and PAX6-promoters is increased in early bladder cancer and in normal mucosa adjacent to pTa tumours. *BJU Int* 2008;101(6):753–757.
15. Ballestar E, Paz M, Valle L, Wei S, Fraga M, Espada J, Cigudosa J, Huang T, Esteller M. Methyl-CpG binding proteins identify novel sites of epigenetic inactivation in human cancer. *EMBO J* 2003;22(23):6335–6345.
16. Zhou YH, Wu X, Tan F, Shi YX, Glass T, Liu TJ, Wathen K, Hess KR, Gumin J, Lang F, Yung WK. PAX6 suppresses growth of human glioblastoma cells. *J Neurooncol* 2005;71:223–229.
17. Myers D, Hu Y, Teng Y, Siegel E, Wu X, Panda K, Tan F, Yung W, Zhou Y. PAX6 suppresses the invasiveness of glioblastoma cells and the expression of the matrix metalloproteinase-2 gene. *Cancer Res* 2006;66(20):9809–9817.
18. Jemal A, Siegel R, Ward E, Hao YP, Xu JQ, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008;58(2):71–96.
19. Schinner S, Dellas C, Schroder M, Heinlein C, Chang C, Fischer J, Knepel W. Repression of glucagon gene transcription by peroxisome proliferator-activated receptor gamma through inhibition of Pax6 transcriptional activity. *J Biol Chem* 2002; 277(3):1941–1948.
20. Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA Cancer J Clin* 1972;22(4):232–240.
21. Roy-Burman P, Tindall DJ, Robins DM, Greenberg NM, Hendrix MJ, Mohla S, Getzenberg RH, Isaacs JT, Pienta KJ. Androgens and prostate cancer: Are the descriptors valid? *Cancer Biol Ther* 2005;4(1):4–5.
22. Denis L, Murphy G. Overview of phase III trials on combined androgen treatment in patients with metastatic prostate cancer. *Cancer* 1993;72(12 Suppl):3888–3895.
23. Sadar M, Hussain M, Bruchofsky N. Prostate cancer: Molecular biology of early progression to androgen independence. *Endocr Relat Cancer* 1999;6(4):487–502.
24. Bonkhoff H, Remberger K. Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: A stem cell model. *Prostate* 1996;28(2):98–106.
25. Schoenmakers E, Verrijdt G, Peeters B, Verhoeven G, Rombauts W, Claessens F. Differences in DNA binding characteristics of the androgen and glucocorticoid receptors can determine hormone-specific responses. *J Biol Chem* 2000;275(16):12290–12297.
26. Mizokami A, Yeh S, Chang C. Identification of 3',5'-cyclic adenosine monophosphate response element and other cis-acting elements in the human androgen receptor gene promoter. *Mol Endocrinol* 1994;8(1):77–88.
27. Altuwaijri S, Lin H, Chuang K, Lin W, Yeh S, Hanchett L, Rahman M, Kang H, Tsai M, Zhang Y, Yang L, Chang C.

- Interruption of nuclear factor kappaB signaling by the androgen receptor facilitates 12-O-tetradecanoylphorbolacetate-induced apoptosis in androgen-sensitive prostate cancer LNCaP cells. *Cancer Res* 2003;63(21):7106–7112.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25(4):402–408.
 29. Dai Y, Ngo D, Forman LW, Qin DC, Jacob J, Faller DV. Sirtuin 1 is required for antagonist-induced transcriptional repression of androgen-responsive genes by the androgen receptor. *Mol Endocrinol* 2007;21(8):1807–1821.
 30. Asatiani E, Huang WX, Wang A, Rodriguez Ortner E, Cavalli LR, Haddad BR, Gelmann EP. Deletion, methylation, and expression of the NKX3.1 suppressor gene in primary human prostate cancer. *Cancer Res* 2005;65(4):1164–1173.
 31. Gregory CW, Hamil KG, Kim D, Hall SH, Pretlow TG, Mohler JL, French FS. Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res* 1998;58(24):5718–5724.
 32. Sun Z, Pan J, Balk SP. Androgen receptor-associated protein complex binds upstream of the androgen-responsive elements in the promoters of human prostate-specific antigen and kallikrein 2 genes. *Nucleic Acids Res* 1997;25(16):3318–3325.
 33. Salem C, Liang G, Tsai Y, Coulter J, Knowles M, Feng A, Groshen S, Nichols P, Jones P. Progressive increases in de novo methylation of CpG islands in bladder cancer. *Cancer Res* 2000;60(9):2473–2476.
 34. Goudreau G, Petrou P, Reneker LW, Graw J, Loster J, Gruss P. Mutually regulated expression of Pax6 and Six3 and its implications for the Pax6 haploinsufficient lens phenotype. *Proc Natl Acad Sci USA* 2002;99(13):8719–8724.
 35. Simpson T, Price D. Pax6: A pleiotropic player in development. *Bioessays* 2002;24(11):1041–1051.
 36. Kozmik Z. Pax genes in eye development and evolution. *Curr Opin Genet Dev* 2005;15(4):430–438.
 37. Hevner R, Hodge R, Daza R, Englund C. Transcription factors in glutamatergic neurogenesis: Conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci Res* 2006;55(3):223–233.
 38. Mayes D, Hu Y, Teng Y, Siegel E, Wu X, Panda K, Tan F, Yung W, Zhou Y. PAX6 suppresses the invasiveness of glioblastoma cells and the expression of the matrix metalloproteinase-2 gene. *Cancer Res* 2006;66(20):9809–9817.
 39. Liao X, Thrasher J, Pelling J, Holzbeierlein J, Sang Q, Li B. Androgen stimulates matrix metalloproteinase-2 expression in human prostate cancer. *Endocrinology* 2003;144(5):1656–1663.
 40. Cooke PS, Young P, Cunha GR. Androgen receptor expression in developing male reproductive organs. *Endocrinology* 1991;128(6):2867–2873.
 41. Sato N, Sadar M, Bruchofsky N, Saatcioglu F, Rennie P, Sato S, Lange P, Gleave M. Androgenic induction of prostate-specific antigen gene is repressed by protein-protein interaction between the androgen receptor and AP-1/c-Jun in the human prostate cancer cell line LNCaP. *J Biol Chem* 1997;272(28):17485–17494.
 42. Palvimo J, Reinikainen P, Ikonen T, Kallio P, Moilanen A, Janne O. Mutual transcriptional interference between RelA and androgen receptor. *J Biol Chem* 1996;271(39):24151–24156.
 43. Aarnisalo P, Palvimo J, Janne O. CREB-binding protein in androgen receptor-mediated signaling. *Proc Natl Acad Sci USA* 1998;95(5):2122–2127.
 44. Yuan X, Lu M, Li T, Balk S. SRY interacts with and negatively regulates androgen receptor transcriptional activity. *J Biol Chem* 2001;276(49):46647–46654.