to chemotherapeutic drug resistance, such as DNA repair, apoptosis etc.[25] Bcl2 is a key contributor to apoptosis. Bachmann et al found that the *bcl2* –938C/A polymorphism is associated with prognosis in patients with breast cancer.[18] Our results agree with that study. Our current findings may shed new light on the mechanism behind this observation.

CONCLUSIONS

To our knowledge this is the first report of a significant effect of the *bcl2* –938C/A polymorphism on overall survival in patients with RCC. Furthermore, we noted that carriers with a *bcl2* promoter region –938C/A+A/A genotype have significantly higher Bcl2 expression and lower cell proliferative activity in RCC tissues than C/C genotype carriers. These data suggest that the *bcl2* –938C/A polymorphism is associated with survival in patients with RCC via altered Bcl2 expression.

ACKNOWLEDGMENTS

Dr. Roger Erickson assisted with the manuscript.

REFERENCES

1. Greenlee RT, Murray T, Bolden S et al: Cancer statistics, 2000. CA Cancer J Clin 2004; **50:** 7.
2. Eggener SE, Yossepowitch O, Pettus JA et al: Renal cell carcinoma recurrence after nephrectomy for localized disease: predicting survival from time of recurrence. J Clin Oncol 2006; **24:** 3101.
3. Kudo-Saito C, Wansley EK, Gruys ME et al: Combination therapy of an orthotopic renal cell carcinoma model using intratumoral vector-mediated costimulation and systemic interleukin-2. Clin Cancer Res 2007; **13:** 1936.
4. Chowdhury S, Larkin JM and Gore ME: Recent advances in the treatment of renal cell carcinoma and the role of targeted therapies. Eur J Cancer 2008; **44:** 2152.
5. Zinkel S, Gross A and Yang E: BCL2 family in DNA damage and cell cycle control. Cell Death Differ 2006; **13:** 1351.
6. Faderl S, Keating MJ, Do KA et al: Expression profile of 11 proteins and their prognostic significance in patients with chronic lymphocytic leukemia (CLL). Leukemia 2002; **16:** 1045.
7. Stackhouse GB, Sesterhenn IA, Bauer JJ et al: p53 and bcl-2 immunohistochemistry in pretreatment prostate needle biopsies to predict recurrence of prostate cancer after radical prostatectomy. J Urol 1999; **162:** 2040.
8. Bilim V, Tomita Y, Kawasaki T et al: Prognostic value of Bcl-2 and p53 expression in urinary tract transitional cell cancer. J Natl Cancer Inst 1996; **88:** 686.
9. Ofner D, Riehemann K, Maier H et al: Immunohistochemically detectable bcl-2 expression in colorectal carcinoma: correlation with tumour stage and patient survival. Br J Cancer 1995; **72:** 981.
10. Zhang GJ, Kimijima I, Tsuchiya A et al: The role of bcl-2 expression in breast carcinoma (review). Oncol Rep 1998; **5:** 1211.
11. Silvestrini R, Benini E, Veneroni S et al: p53 and bcl-2 expression correlates with clinical outcome in a series of node-positive breast cancer patients. J Clin Oncol 1996; **14:** 1604.
12. Fontanini G, Vignati S, Bigini D et al: Bcl-2 protein: a prognostic factor inversely correlated to p53 in non-small-cell lung cancer. Br J Cancer 1995; **71:** 1003.
13. Kallio JP, Hirvikoski P, Helin H et al: Renal cell carcinoma MIB-1, Bax and Bcl-2 expression and prognosis. J Urol 2004; **172:** 2158.
14. Itoi T, Yamana K, Bilim V et al: Impact of frequent Bcl-2 expression on better prognosis in renal cell carcinoma patients. Br J Cancer 2004; **90:** 200.
15. Seto M, Jaeger U, Hockett RD et al: Alternative promoters and exons, somatic mutation and deregulation of the Bcl-2-Ig fusion gene in lymphoma. EMBO J 1988; **7:** 123.
16. Nückel H, Frey UH, Bau M et al: Association of a novel regulatory polymorphism (−938C>A) in the BCL2 gene promoter with disease progression and survival in chronic lymphocytic leukemia. Blood 2007; **109:** 290.
17. Tanaka Y, Hirata H, Chen Z et al: Polymorphisms of catechol-O-methyltransferase in men with renal cell cancer. Cancer Epidemiol Biomarkers Prev 2007; **16:** 92.
18. Bachmann HS, Otterbach F, Callies R et al: The AA genotype of the regulatory BCL2 promoter polymorphism (−938C>A) is associated with a favorable outcome in lymph node negative invasive breast cancer patients. Clin Cancer Res 2007; **13:** 5790.
19. Sinicrope FA, Hart J, Michelassi F et al: Prognostic value of bcl-2 oncoprotein expression in stage II colon carcinoma. Clin Cancer Res 1995; **1:** 1103.
20. Winter JN, Andersen J, Reed JC et al: BCL-2 expression correlates with lower proliferative activity in the intermediate- and high-grade non-Hodgkin's lymphomas: an Eastern Cooperative Oncology Group and Southwest Oncology Group cooperative laboratory study. Blood 1998; **91:** 1391.
21. Zhang X and Takenaka I: Cell proliferation and apoptosis with BCL-2 expression in renal cell carcinoma. Urology 2000; **56:** 510.
22. Huang DC, O'Reilly LA, Strasser A et al: The anti-apoptosis function of Bcl-2 can be genetically separated from its inhibitory effect on cell cycle entry. EMBO J 1997; **16:** 4628.
23. Huang RS and Ratain MJ: Pharmacogenetics and pharmacogenomics of anticancer agents. CA Cancer J Clin 2009; **59:** 42.
24. Dai Z, Papp AC, Wang D et al: Genotyping panel for assessing response to cancer chemotherapy. BMC Med Genomics 2008; **1:** 24.
25. Bandrés E, Zárate R, Ramirez N et al: Pharmacogenomics in colorectal cancer: the first step for individualized-therapy. World J Gastroenterol 2007; **13:** 5888.

Oncogene (2010) 29, 237–250

www.nature.com/onc

ORIGINAL ARTICLE

Castration resistance of prostate cancer cells caused by castration-induced oxidative stress through Twist1 and androgen receptor overexpression

M Shiota[1], A Yokomizo[1], Y Tada[1], J Inokuchi[1], E Kashiwagi[1], D Masubuchi[1], M Eto[3], T Uchiumi[2] and S Naito[1]

[1]Department of Urology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [2]Department of Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and [3]Department of Urology, Graduate School of Medicine, Kumamoto University, Kumamoto, Japan

There are few successful therapies for castration-resistant prostate cancer (CRPC). Recently, CRPC has been thought to result from augmented androgen/androgen receptor (AR) signaling pathway, for most of which AR overexpression has been observed. In this study, Twist1, a member of basic helix-loop-helix transcription factors as well as AR was upregulated in response to hydrogen peroxide, and the response to which was abolished by an addition of *N*-acetyl-L-cysteine and Twist1 knockdown. In addition, castration-resistant LNCaP derivatives and hydrogen peroxide-resistant LNCaP derivatives exhibited a similar phenotype to each other. Then, both castration and AR knockdown increased intracellular reactive oxygen species level. Moreover, Twist1 was shown to regulate AR expression through binding to E-boxes in AR promoter region. Silencing of Twist1 suppressed cell growth of AR-expressing LNCaP cells as well as castration-resistant LNCaP derivatives by inducing cell-cycle arrest at G1 phase and cellular apoptosis. These findings indicated that castration-induced oxidative stress may promote AR overexpression through Twist1 overexpression, which could result in a gain of castration resistance. Modulation of castration-induced oxidative stress or Twist1/AR signaling might be a useful strategy for developing a novel therapeutics in prostate cancer, even in CRPC, which remains dependent on AR signaling by overexpressing AR.
Oncogene (2010) **29,** 237–250; doi:10.1038/onc.2009.322; published online 5 October 2009

Keywords: AR; CRPC; oxidative stress; prostate cancer; ROS; Twist1

Introduction

Prostate cancer (PCa) is the most common noncutaneous cancer and the second leading cause of cancer-related death in men in developed countries. The incidence of PCa has been increasing significantly as a result of rapid increase of aging populations and the prevalence of high-fat diets (Hsing and Devesa, 2001; Gronberg, 2003). The risk of PCa increases dramatically after the age of 50 years and by 80 years of age about 80% of men have prostate carcinoma (Billis, 1996; Sakr *et al.*, 1996; Landies *et al.*, 1999). Although PCa is known to involve androgen, which is thought to promote prostate epithelial carcinogenesis, the incidence of PCa increases according to aging, whereas testosterone declines (Mitsiades *et al.*, 2008). Aging is closely related to a shift in the prooxidant–antioxidant balance of many tissues toward an oxidative status with reactive oxygen species (ROS) damage, which leads to an increased risk of carcinogenesis (Benz and Yau, 2008; Maynard *et al.*, 2009). Suppressed androgen levels in elderly men may be correlated with the increased incidence of PCa and castration-resistant PCa (CRPC), in which repression of the androgen might augment androgen signaling by an increase of androgen receptor (AR) expression. As the prostate gland is recognized to be an AR-expressing organ, androgen suppression may act on the prostate gland through androgen/AR signaling. These well-known findings indicate that androgen suppression may lead to a prooxidant status in elderly males, resulting in increased risks of prostate carcinogenesis and CRPC occurrence through AR overexpression.

The majority of PCa are androgen-dependent at diagnosis, and most of them respond to androgen-deprivation therapy (ADT). However, most tumors relapse in a castration-resistant manner during ADT, which is designated as CRPC (Debes and Tindall, 2002). As there are few successful therapies for CRPC, overcoming CRPC is a serious problem. Several studies have shown that progression to CRPC could be associated with an increased level of AR expression, indicating that AR downregulation should suppress tumor growth, even in CRPC (Gregory *et al.*, 1998; Zegarra-Moro *et al.*, 2002; Chen *et al.*, 2004; Scher and Sawyers, 2006). Less than 10% of CRPCs were found to possess somatic AR gene mutations (Taplin *et al.*, 2003). In addition, AR is overexpressed in most CRPCs, among which 10–20% exhibit amplification of the AR gene (Linja *et al.*, 2001). This discrepancy indicates that AR overexpression in CRPC may result from transcriptional upregulation of AR.

Correspondence: Dr A Yokomizo, Department of Urology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8252, Japan.
E-mail: yokoa@uro.med.kyushu-u.ac.jp
Received 11 June 2009; revised 31 August 2009; accepted 3 September 2009; published online 5 October 2009

Twist1, which belongs to the family of basic helix-loop-helix transcription factors, has been proposed as an oncogene (Olson and Klein, 1994; Maestro *et al.*, 1999). Recently, gene profiling analyses revealed that upregulation of Twist1 is associated with malignant transformation (Hoek *et al.*, 2004; van Doorn *et al.*, 2004). In addition, increased Twist1 expression is detected in rhabdomyosarcomas and gastric carcinomas compared with that in nonmalignant tissues (Maestro *et al.*, 1999; Rosivatz *et al.*, 2002) and correlated with a poor outcome and shorter survival (Hoek *et al.*, 2004). Recent evidence has also indicated that Twist1 is a key factor responsible for metastasis (Yang *et al.*, 2004). In PCa, Twist1 was shown to be upregulated and

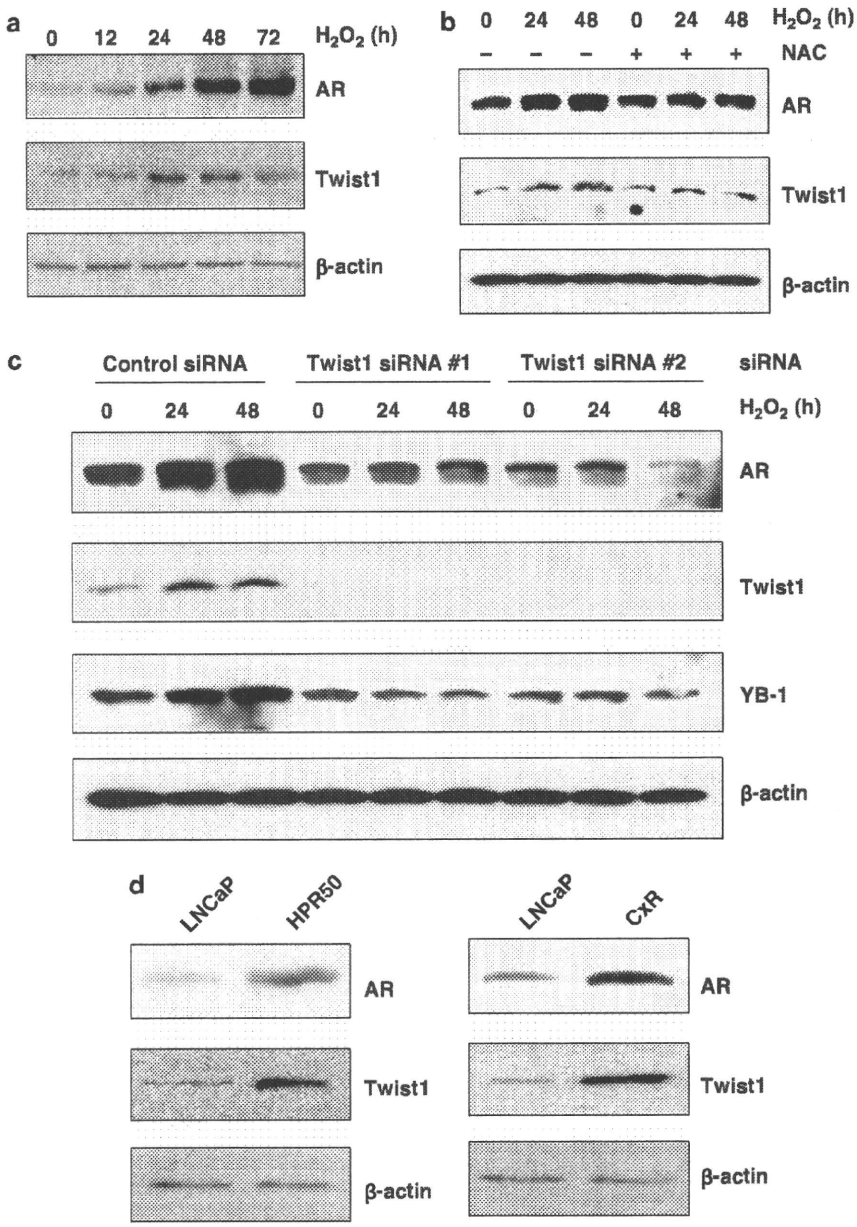


Figure 1 Twist1 and androgen receptor (AR) expressions are both upregulated in response to hydrogen peroxide and in both HPR50 and CxR cells. (**a**) LNCaP cells were cultured with 10 μM of hydrogen peroxide for 2 h, and the medium was refreshed. At the indicated times after the addition of hydrogen peroxide, the cells were harvested. Whole-cell extracts were analyzed for AR, Twist1 and β-actin (loading control) by SDS–PAGE and western blotting with specific antibodies. (**b**) LNCaP cells were cultured with 10 μM of hydrogen peroxide and/or 5 mM of *N*-acetyl-L-cysteine (NAC) for 2 h, and the medium was refreshed. At the indicated times after the addition of hydrogen peroxide, the cells were harvested. Western blot analysis was performed as described in (**a**). (**c**) LNCaP cells were transfected with 40 nM of control siRNA, Twist1 siRNA #1 or Twist1 siRNA #2. On the following day, cells were cultured with 10 μM of hydrogen peroxide for 2 h, and the medium was refreshed. At the indicated times after the addition of hydrogen peroxide, cells were harvested. Western blot analysis was performed as described in (**a**). (**d**) Whole-cell extracts of LNCaP, HPR50 and CxR cells were analyzed for AR, Twist1 and β-actin (loading control) by SDS–PAGE and western blotting with specific antibodies. PAGE, polyacrylamide gel electrophoresis; YB-1, Y-box-binding protein-1.

involved in the colony-formation and invasive abilities (Kwok *et al.*, 2005). We previously showed that Twist1 is involved in both cisplatin resistance and tumor growth through Y-box-binding protein-1 (YB-1) expression (Shiota *et al.*, 2008a). Moreover, p53 and programmed cell death protein 4 downregulate the transcriptional activity of Twist1 and YB-1 expression (Shiota *et al.*, 2008b, 2009). Twist1 is associated with

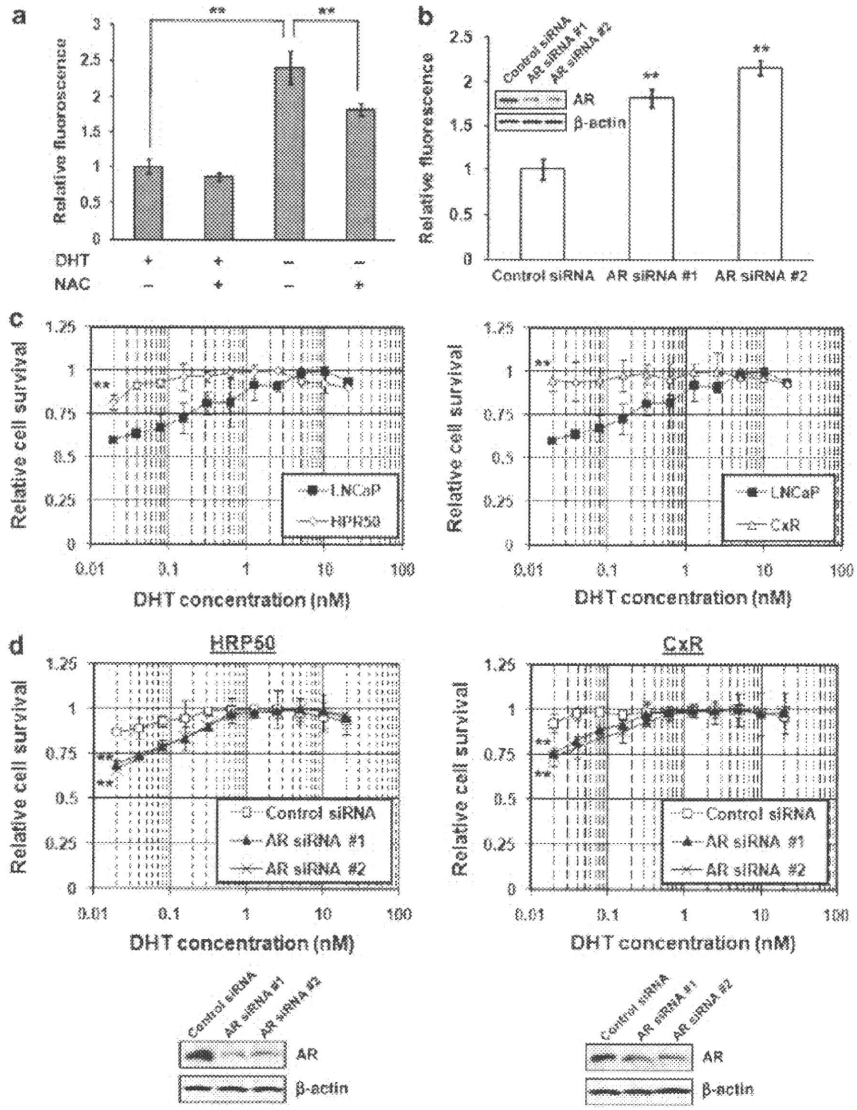


Figure 2 Blockade of androgen/androgen receptor (AR) signaling increases intracellular reactive oxygen species (ROS) level and both HPR50 and CxR cells are resistant to androgen depletion through AR overexpression. (**a**) LNCaP and CxR cells (2×10^5) were seeded into 96-well plates and incubated. On the following day, the media were replaced with charcoal-stripped medium with or without 10 nM of dihydrotestosterone (DHT) and/or 5 mM of *N*-acetyl-L-cysteine (NAC). After 48 h, the cells were stained with CM-H_2DCFDA and measured for their florescence intensities. All values are representative of at least three independent experiments. The fluorescence intensity of LNCaP cells with DHT and without NAC was set as 1. Boxes, mean; bars, ±s.d. $^{**}P<0.05$. (**b**) LNCaP cells (2×10^5) transfected with 40 nM of control siRNA, AR siRNA #1 or AR siRNA #2 were seeded into 96-well plates and incubated. After 48 h, the intracellular ROS levels were measured as described in (**a**). The fluorescence intensity of LNCaP cells transfected with control siRNA was set as 1. Boxes, mean; bars, ±s.d. $^{**}P<0.05$ (compared with that of LNCaP cells transfected with control siRNA). Whole-cell extracts of LNCaP cells transfected with the indicated siRNAs were analyzed for AR and β-actin (loading control) by SDS–PAGE and western blotting with specific antibodies. (**c**) LNCaP, HPR50 and CxR cells (2×10^5) were seeded into 96-well plates. On the following day, the indicated concentrations of DHT were applied in charcoal-stripped medium. After 48 h, the cell survival rates were analyzed by cytotoxicity assays. Cell survival at the DHT concentration with the best cell proliferation was set as 1. All values are the representative of at least three independent experiments. Boxes, mean; bars, ±s.d. $^{**}P<0.05$ (compared with that of LNCaP cells). (**d**) HPR50 and CxR cells (2×10^5) transfected with 40 nM of control siRNA, AR siRNA #1 or AR siRNA #2 were seeded into 96-well plates. On the following day, the indicated concentrations of DHT were applied in charcoal-stripped medium. Cytotoxicity assays were performed as described in (**c**). Boxes, mean; bars, ±s.d. $^{**}P<0.05$ (compared with that of cells transfected with control siRNA). Whole-cell extracts of HPR50 and CxR cells transfected with the indicated siRNAs were analyzed for AR and β-actin (loading control) by SDS–PAGE and western blotting with specific antibodies. PAGE, polyacrylamide gel electrophoresis.

resistance to doxorubicin and paclitaxel (Kwok *et al.*, 2005). These findings that Twist1 is involved in resistance to anticancer agents indicate that Twist1 could respond to various stresses caused by anticancer agents. As cisplatin, paclitaxel and doxorubicin are known to exert oxidative stress, Twist1 may be implicated in oxidative stress.

In this study, we attempted to elucidate a mechanism for AR overexpression in CRPC. As several Twist1-binding sites (5′-CANNTG-3′) exist in the promoter region of the AR gene, we focused on Twist1 (Castanon *et al.*, 2001). Twist1 is implicated in resistance to anticancer agents exerting oxidative stress. Therefore, we investigated the relationship between oxidative stress and Twist1/AR expressions in PCa cells, and the implications of oxidative stress signaling in CRPC.

Results

Twist1 and AR expressions are both upregulated in response to hydrogen peroxide and in both hydrogen peroxide-resistant LNCaP derivatives (HPR50 cells) and castration-resistant LNCaP derivatives (CxR cells)

Twist1 is upregulated in response to cisplatin (unpublished data) as well as hypoxia through upregulation of HIF-1α (Yang *et al.*, 2008), suggesting that Twist1 is a stress-inducible transcription factor. As Twist1 is involved in drug resistance to cisplatin, paclitaxel and doxorubicin exerting oxidative stress, we investigated whether Twist1 was induced by oxidative stress. When LNCaP cells were exposed to 10 μM of hydrogen peroxide for 2 h, Twist1 expression was induced and reached a peak between 24 and 48 h. When we

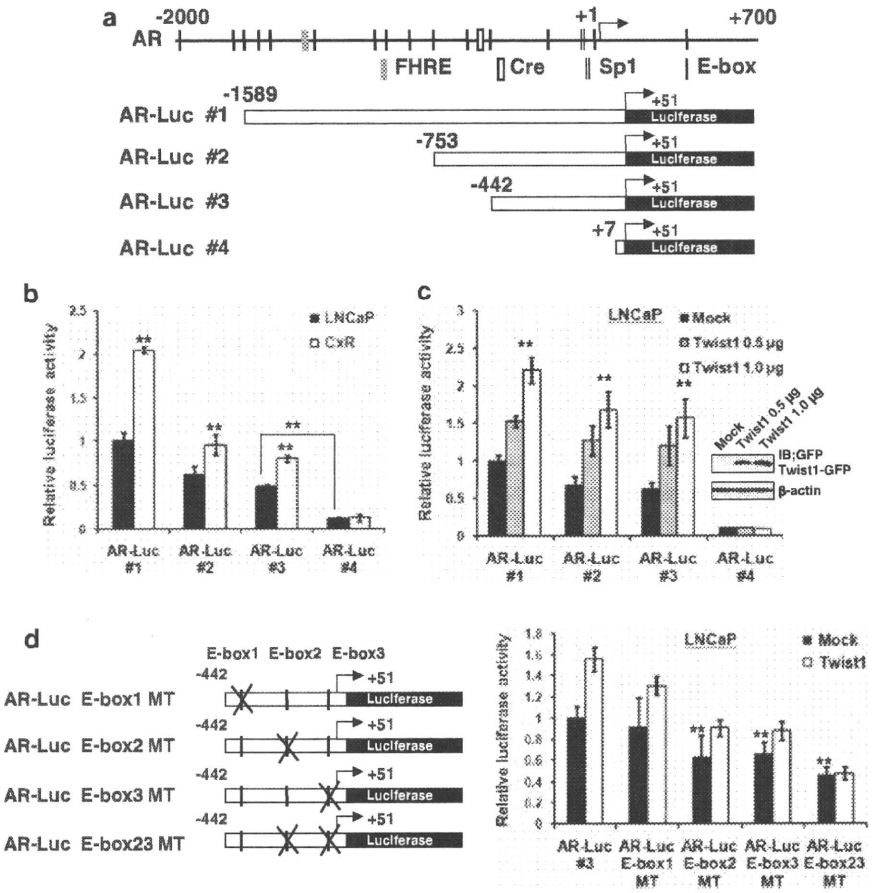


Figure 3 Twist1 upregulates androgen receptor (AR) transcription E-box-dependently. (**a**) Schematic representations of the promoter region of the AR gene and AR-Luc #1–#4 used in (**b**) and (**c**) are shown. (**b**) LNCaP and CxR cells were cotransfected with 1.0 μg of the various AR-Luc plasmids shown in (**a**) and 0.05 μg of pRL-TK. All values are representative of at least three independent experiments. The luciferase activity of AR-Luc #1 in LNCaP cells was set as 1. Boxes, mean; bars, ± s.d. $^{**}P<0.05$ (compared with that of LNCaP cells). (**c**) LNCaP cells were cotransfected with 0.5 μg of the various AR-Luc plasmids shown in (**a**), the indicated amounts of GFP or Twist1-GFP and 0.05 μg of pRL-TK. All values are representative of at least three independent experiments. The luciferase activity of AR-Luc #1 alone was set as 1. Boxes, mean; bars, ± s.d. $^{**}P<0.05$ (compared with that of LNCaP cells transfected with mock). Whole-cell extracts of LNCaP cells transfected with the indicated amount of Twist1-GFP expression plasmid were analyzed for Twist1-GFP and β-actin (loading control) by SDS–PAGE and western blotting with anti-GFP and anti-β-actin antibodies, respectively. (**d**) Schematic representations of AR-Luc E-box1 MT, AR-Luc E-box2 MT, AR-Luc E-box3 MT and AR-Luc E-box23 MT are shown. LNCaP cells were cotransfected with 0.5 μg of the various AR-Luc plasmids, 0.5 μg of GFP or Twist1-GFP and 0.05 μg of pRL-TK. All values are representative of at least three independent experiments. The luciferase activity of AR-Luc #3 alone was set as 1. Boxes, mean; bars, ± s.d. $^{**}P<0.05$ (compared with that of LNCaP cells transfected with AR-Luc #3). FHRE, Forkhead-responsive element; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis.

performed immunoblotting using an anti-AR antibody, expression of AR was surprisingly upregulated gradually for up to 72 h after hydrogen peroxide exposure (Figure 1a). To confirm that this effect resulted from oxidative stress caused by hydrogen peroxide, LNCaP cells were exposed to hydrogen peroxide and the free-radical scavenger *N*-acetyl-L-cysteine (NAC). Addition of NAC almost completely abolished the increase of Twist1 as well as AR expression by hydrogen peroxide (Figure 1b). Next, we investigated whether the AR response to hydrogen peroxide was regulated by Twist1. As shown in Figure 1c, upregulated AR expression by hydrogen peroxide was completely abolished by Twist1 silencing using Twist1-specific siRNAs. We further examined both Twist1 and AR expressions in HPR50 cells. As expected, both Twist1 and AR were overexpressed in HPR50 cells compared with that in parental cells (Figure 1d), suggesting that Twist1 expression is possibly linked to AR expression. It is known that AR is upregulated in CRPC and that AR overexpression contributes to castration-resistant progression of PCa. Therefore, we investigated the AR and Twist1 expression levels in CRPC cells. As shown in Figure 1d, AR as well as Twist1 expression was upregulated in CxR cells compared with that in parental cells.

Blockade of androgen/AR signaling increases intracellular ROS level, and both HPR50 and CxR cells are resistant to androgen depletion through AR overexpression

The facts that both Twist1 and AR were overexpressed in HPR50 and CxR cells led us to speculate a possible association between androgen deprivation and oxidative stress. Therefore, we investigated whether blockade of AR signaling could exert oxidative stress. First, we compared the intracellular ROS levels in LNCaP cells cultured in medium with or without androgen. When LNCaP cells were cultured in androgen-deprivation medium for 48 h, their intracellular ROS levels were elevated by about 2.5-fold, which increase was blunted by NAC addition (Figure 2a). Then, we investigated whether AR suppression affected intracellular ROS levels in a similar manner to androgen deprivation. AR suppression using AR-specific siRNAs induced approximately twofold increases of the ROS levels in LNCaP cells (Figure 2b). As these findings indicated that androgen/AR signaling blockade was associated with oxidative stress, we examined the androgen-sensitivities of HPR50 and CxR cells. HPR50 and CxR cells were resistant to low concentrations of dihydrotestosterone, suggesting that common factor in both cells, probably AR overexpression, may be responsible for the castration-resistant phenotype (Figure 2c). To investigate whether castration-resistant phenotype in HPR50 and CxR cells resulted from AR overexpression, we performed cytotoxicity assay after AR knockdown. The results showed that both HPR50 and CxR cells became sensitive to androgen deprivation by AR silencing (Figure 2d).

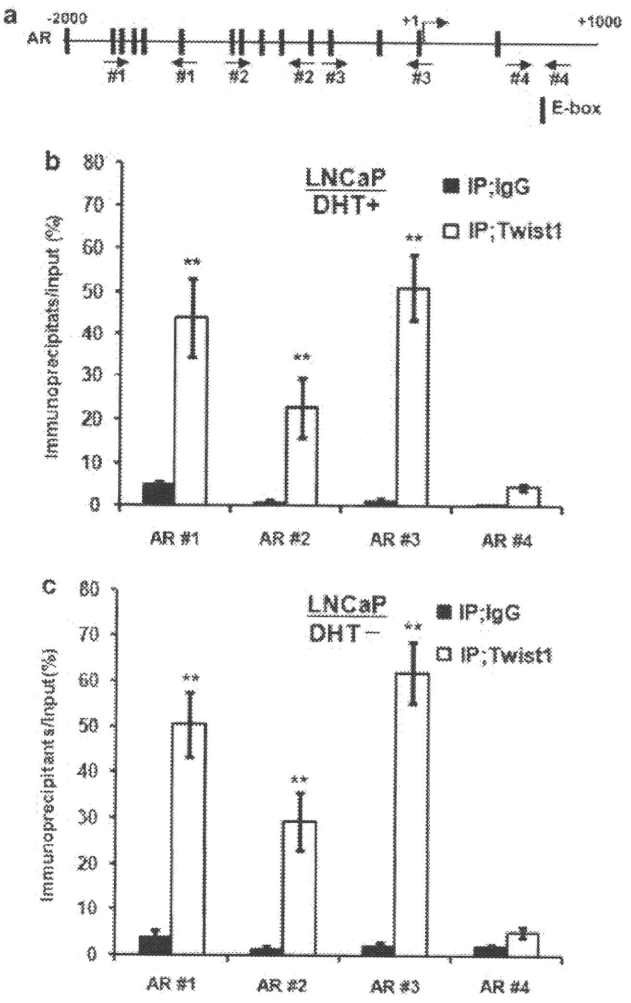


Figure 4 Twist1 binds to E-boxes in the promoter region of androgen receptor (AR). (**a**) Schematic representation of the promoter region and 5′ end of the AR gene. Black boxes, E-boxes (5′-CANNTG-3′); arrows, primer pairs used in chromatin immunoprecipitation (ChIP) assays. (**b**) and (**c**) ChIP assays were performed on nuclear extracts from LNCaP cells cultured with (**b**) or without (**c**) 10 nM of dihydrotestosterone (DHT) for 48 h using 2.0 μg of mouse IgG or anti-Twist1 antibody and 20 μl of Protein A/G PLUS-agarose. The quantitative real-time PCR was performed using immunoprecipitated DNAs, soluble chromatin and specific primer pairs for the AR promoter (AR #1, −1589 to −1187 bp; AR #2, −974 to −539 bp; AR #3, −442 to +51 bp; AR #4, +620 to +812 bp). The results of immunoprecipitated samples were corrected for the results of the corresponding soluble chromatin samples. All values represent at least three independent experiments. Boxes, mean; bars, ±s.d. $^{**}P<0.05$ (compared with that of primer pairs for AR #4).

AR transcription is upregulated in CxR cells and Twist1 upregulates AR transcription E-box-dependently

The above findings prompted us to investigate the mechanism of AR transcription by Twist1, which is known to bind to E-box motifs (5′-CANNTG-3′) and affect the transcription of its target genes. As shown in Figure 3a, an investigation of the AR gene revealed the presence of multiple E-boxes in the AR promoter region. Therefore, we cloned the AR promoter region and constructed various lengths of AR reporter

plasmids (AR-Luc #1–#4). Then, luciferase reporter assays were conducted using these reporter plasmids. AR-Luc #3 exhibited higher luciferase activities in LNCaP cells compared with AR-Luc #4, suggesting that the AR promoter region from −442 to +6 bp was most responsible for AR transcription. Similar results were obtained when CxR cells were used for luciferase reporter assays. Moreover, luciferase activities of AR-Luc #1, AR-Luc #2 and AR-Luc #3 were higher than those in LNCaP cells (Figure 3b).

To examine whether Twist1 is involved in AR transcription, LNCaP cells were transfected with AR reporter plasmids and a Twist1 expression plasmid. Twist1 overexpression upregulated the luciferase activities of the AR reporter plasmids in dose-dependent manners (Figure 3c). To confirm the upregulation of AR transcription by Twist1, we introduced mutations into the E-boxes of the AR promoter region, as shown in Figure 3d. Introduction of mutations into E-box2 (AR-Luc E-box2 MT) and E-box3 (AR-Luc E-box3 MT) located in the AR proximal promoter region decreased the luciferase activity in LNCaP cells, whereas introduction of a mutation into E-box1 (AR-Luc E-box1 MT) had little influence compared with the wild-type reporter plasmid. Furthermore, introduction of double mutations into E-box2 and E-box3 (AR-Luc E-box23 MT) almost completely abolished the increase of luciferase activity in response to Twist1 overexpression (Figure 3d).

Finally, we carried out chromatin immunoprecipitation assays using primer pairs for several AR gene regions (Figure 4a). When LNCaP cells cultured in androgen-containing medium were examined, Twist1 bound to the AR promoter region (#1, #2 and #3), but not to the AR 5′-UTR region (#4) (Figure 4b). Similar results were obtained when LNCaP cells were cultured under androgen deprivation (Figure 4c).

Twist1 knockdown decreases AR transcript and protein expressions, and induces growth retardation and cellular apoptosis in LNCaP cells

We examined whether Twist1 knockdown influenced the AR transcript and protein expression levels. As expected, the AR transcript and protein expression levels in LNCaP cells were reduced (Figure 5a). Similarly, Twist1 knockdown decreased YB-1 expression in LNCaP cells as we previously reported (Shiota *et al.*, 2008a). To investigate the influence of Twist1/AR signaling on Pca-cell growth, we performed Twist1 knockdown and counted the cell numbers after culture for the indicated times. LNCaP cell growth was retarded in androgen-containing medium and the number of LNCaP cells decreased after Twist1 knockdown. Next, LNCaP cells transfected with Twist1-specific siRNAs were cultured in androgen-deprivation medium and subjected to cell proliferation assays. LNCaP cells cultured in androgen-deprivation medium grew to a lower extent than cells cultured in androgen-containing medium. Number of LNCaP cells in androgen-deprivation medium decreased after 72 h of culture similar to that in androgen-containing medium (Figure 5b). This finding may be due to YB-1 suppression in addition to blockade of AR signaling. As Twist1 knockdown in LNCaP cells retarded cell growth and decreased the cell number, we carried out flow cytometry analyses. Silencing of Twist1 expression led to slight increases in the G1 fraction and significant increases in the sub-G1 fraction indicating cell death (Figure 5c). These data are consistent with previous reports (Valsesia-Wittmann *et al.*, 2004; Stasinopoulos *et al.*, 2005; Shiota *et al.*, 2008a). The cellular apoptosis in LNCaP cells by silencing of Twist1 was shown in other experimental way. After Twist1 or AR knockdown, LNCaP cells were subjected to immunoblotting using an antibody against cleaved PARP (Poly (ADP-ribose) polymerase), which indicates degradation products by caspase cascade. Twist1 knockdown induced cleavage of PARP, whereas AR knockdown did not (Figure 5d).

AR expression partially rescues the Pca-cell growth retardation induced by Twist1 knockdown

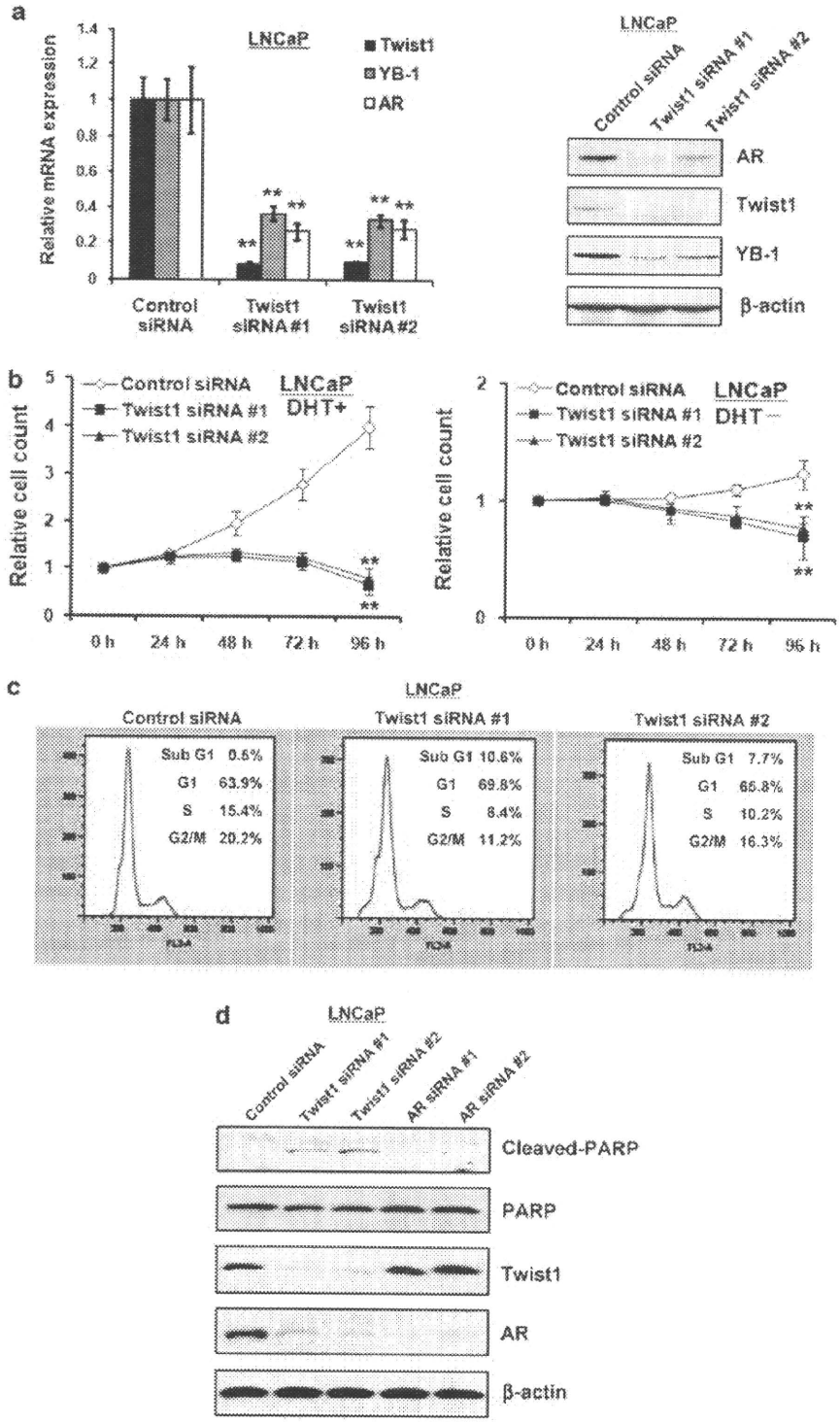
As Twist1 was found to regulate the expression of AR as well as YB-1 in PCa cells, we investigated whether Twist1 knockdown-induced cell growth suppression resulted from regulation of YB-1 or AR expression. Although Twist1 knockdown significantly reduced the colony-formation ability of LNCaP cells, AR overexpression using an AR expression plasmid partially

Figure 5 Silencing of Twist1 reduces androgen receptor (AR) transcript and protein expression levels, and induces growth arrest and cellular apoptosis in LNCaP cells. (**a**) LNCaP cells were transfected with 40 nM of control siRNA, Twist1 siRNA #1 or Twist1 siRNA #2. At 72 h after transfection, quantitative real-time PCR was performed using the primers and probes for Twist1, Y-box-binding protein-1 (YB-1), AR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The target transcript levels were corrected by the corresponding GAPDH transcript levels. All values are representative of at least three independent experiments. Each transcript level from cells transfected with control siRNA was set as 1. Boxes, mean; bars, ± s.d. $^{**}P<0.05$ (compared with that of LNCaP cells transfected with control siRNA). Whole-cell extracts were analyzed for AR, Twist1, YB-1 and β-actin (loading control) by SDS–PAGE and western blotting with specific antibodies. (**b**) LNCaP cells were transfected with 40 nM of control siRNA, Twist1 siRNA #1 or Twist1 siRNA #2, and cultured in charcoal-stripped medium with or without 10 nM of dihydrotestosterone (DHT). At the indicated time points, the cell numbers were counted. The results were normalized by the cell numbers at 0 h. All values are representative of at least three independent experiments. Boxes, mean; bars, ± s.d. $^{**}P<0.05$ (compared with that of LNCaP cells transfected with control siRNA). (**c**) LNCaP cells were transfected with 40 nM of control siRNA, Twist1 siRNA #1 or Twist1 siRNA #2. At 72 h after transfection, the cells were stained with propidium iodide and analyzed by flow cytometry. The cell-cycle fractions are shown at the top right of each graph. (**d**) LNCaP cells were transfected with 40 nM of control siRNA, Twist1 siRNA #1, Twist1 siRNA #2, AR siRNA #1 or AR siRNA #2. At 72 h after transfection, the cells were harvested and whole-cell extracts were analyzed for cleaved PARP, PARP, Twist1, AR and β-actin (loading control) by SDS–PAGE and western blotting with specific antibodies. PAGE, polyacrylamide gel electrophoresis; PARP, Poly (ADP-ribose) polymerase.

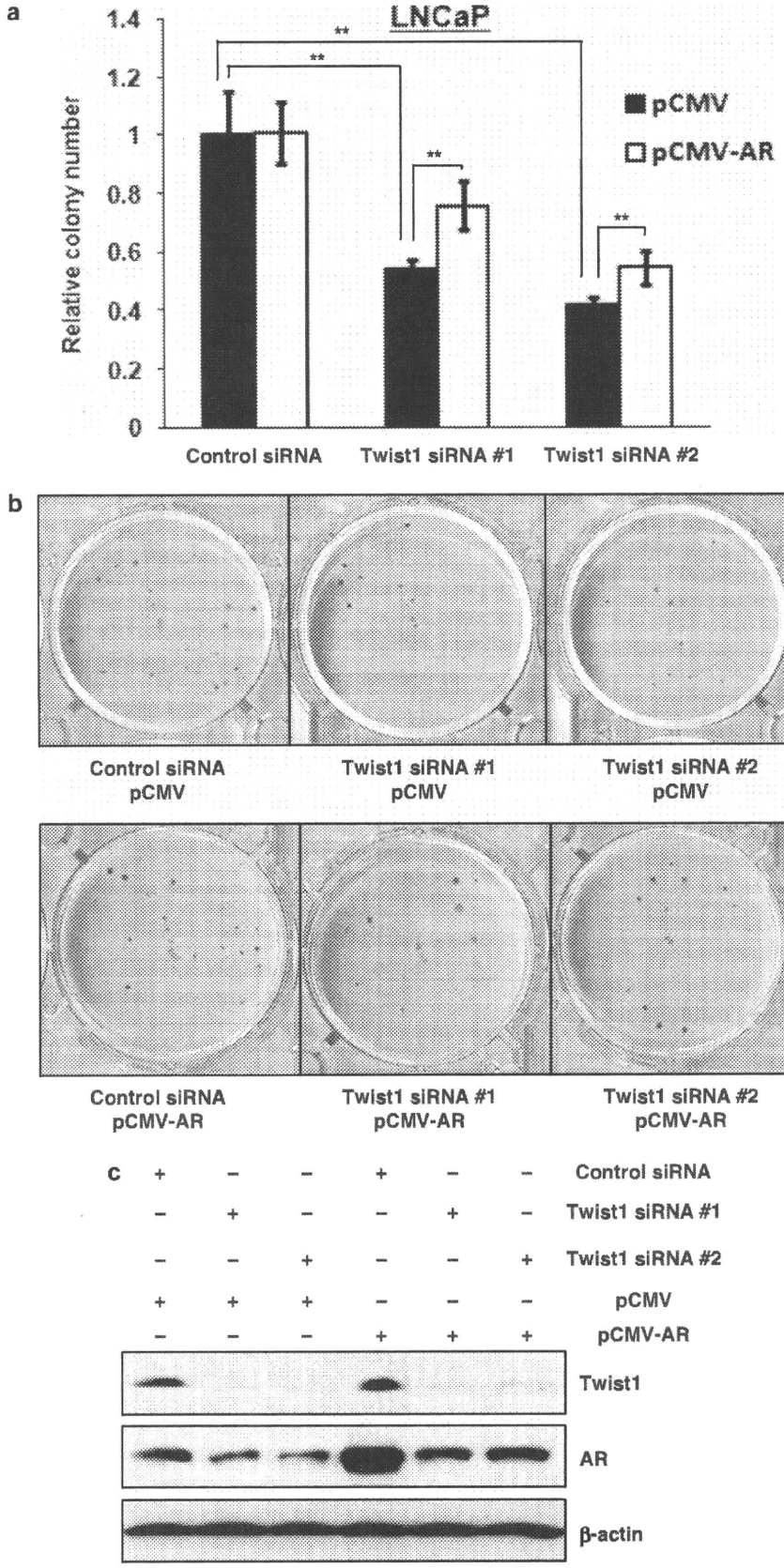
recovered the colony-formation ability up to about 50%, indicating that the effect of Twist1 knockdown on cell growth of AR-expressing cells was partially due to downregulation of AR expression (Figures 6a and b). Western blotting analysis confirmed that siRNAs and expression plasmids functioned properly in these experiments (Figure 6c).

Twist1 regulates AR expression and cell growth in other AR-expressing PCa cells

To confirm that AR expression regulation by Twist1 is applicable to other PCa cells, human PCa 22Rv1 cells were employed. When 22Rv1 cells were used for luciferase reporter assay, similar result was obtained as LNCaP cells (Figure 7a). Then, YB-1 and AR expres-



sions were investigated after Twist1 knockdown in 22Rv1 cells. As shown in Figure 7b, YB-1 as well as AR expressions were reduced by Twist1 knockdown both in mRNA and protein levels. Moreover, cell proliferation decreased after Twist1 knockdown also in 22Rv1 cells (Figure 7c).



Finally, to investigate whether silencing of Twist1 in CRPC cells could affect cell growth, we knocked down Twist1 expression in CxR cells and subjected them to cell proliferation assays. As shown in Figure 7d, YB-1 and AR expressions were also reduced by Twist1 knockdown in CxR cells. Moreover, similar to LNCaP cells, CxR cell growth and cell number significantly decreased, indicating that Twist1 knockdown also induced cell-cycle arrest and cell death in CxR cells.

Discussion

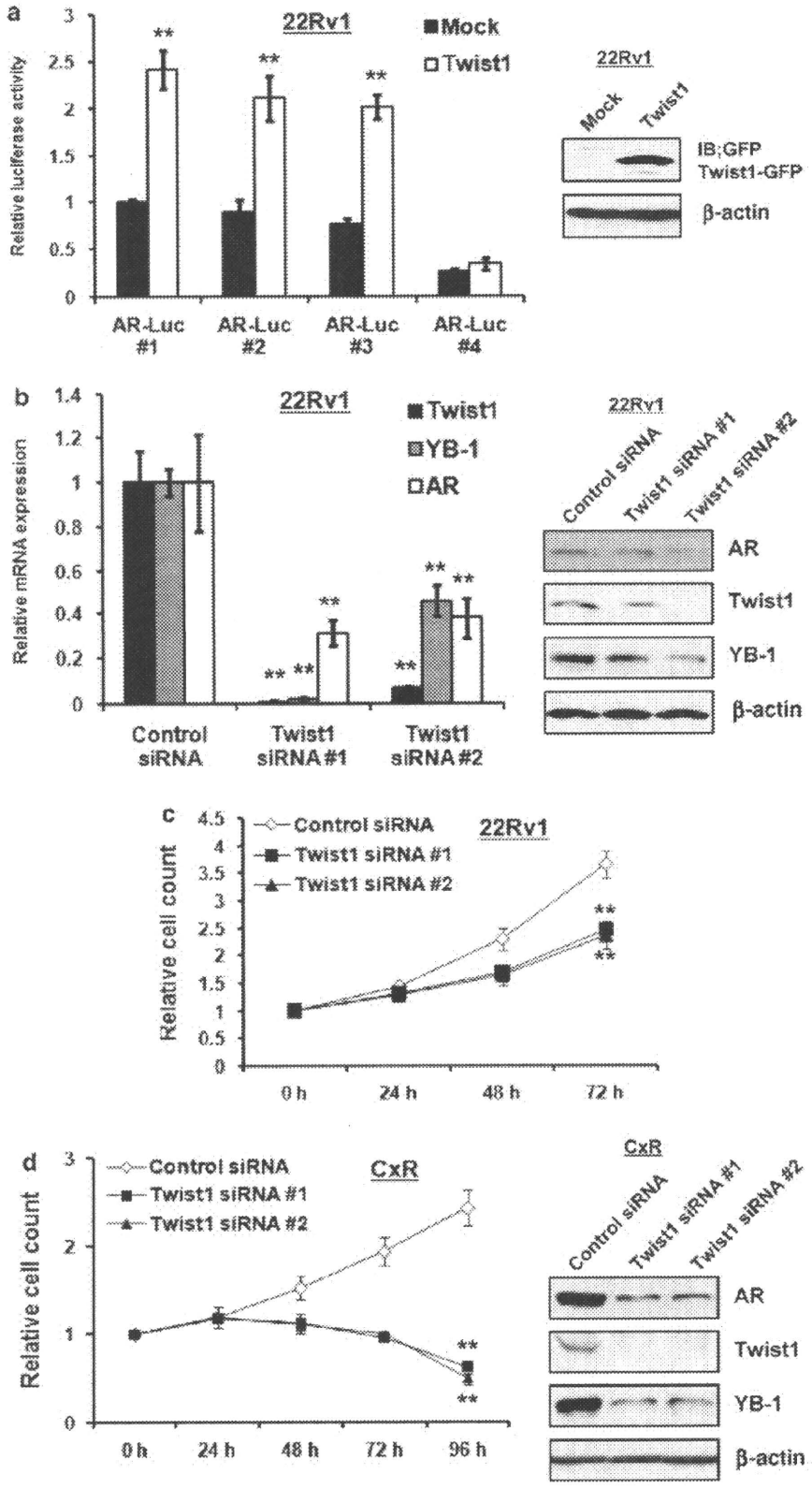
To the best of our knowledge, AR expression is regulated by various transcription factors, such as Sp1, Foxo3a, CREB (cAMP-response element-binding protein), TCF (T-cell factor) and NFκB (Lee and Chang, 2003; Reddy *et al.*, 2006). However, the regulation of AR expression in CRPC remains unclear. Twist1 was found to regulate AR expression using various experimental methods in this study and thought to be involved in resistance to various anticancer agents (Kwok *et al.*, 2005; Shiota *et al.*, 2008a, 2009). We found that Twist1 expression as well as AR protein expression was induced in response to oxidative stress by hydrogen peroxide and that these expressions were upregulated in hydrogen peroxide-resistant LNCaP cells as well as castration-resistant LNCaP cells, suggesting that Twist1 and AR are involved in resistance to both oxidative stress and castration. In addition, HPR50 cells exhibited a similar phenotype to CRPC cells through AR overexpression. On the basis of these findings of a connection between oxidative stress and androgen starvation, we hypothesized that androgen deprivation may produce oxidative stress in PCa cells. As expected, our results clearly showed that androgen deprivation increased the ROS levels in PCa cells, and this effect was partially abolished by the free-radical scavenger NAC. These findings seem to be inconsistent with previous reports that androgen signaling increases oxidative stress (Ripple *et al.*, 1997; Pinthus *et al.*, 2007; Pathak *et al.*, 2008). However, these studies were conducted using overdoses of androgen beyond physiological levels. Our results are supported by previous observations of increased oxidative damage to cellular molecules with the development of malignancies (Bostwick *et al.*, 2000; Oberley *et al.*, 2000) and aging (Ghatak and Ho, 1996; Lu and Finkel, 2008; Maynard *et al.*, 2009), accompanied by declining testosterone levels. Furthermore, our results are supported by the finding that ADT for PCa may increase the risk of death from cardiovascular disease, which is closely implicated in oxidative stress (Hakimian *et al.*, 2008). It has also been shown that increased oxidative stress in rats after castration results from dramatic increases in ROS-generating NAD(P)H oxidases and significant reductions in ROS-detoxifying enzymes (Tamm *et al.*, 2003). In addition, the major ROS scavenger MnSOD (mitochondrial superoxide dismutase-2) shows decreased mRNA levels in PCa after ADT (Best *et al.*, 2005), and oxidative stress-related genes, including thioredoxin, peroxiredoxin 5 and MnSOD, are reduced in the rat prostate after castration (Pang *et al.*, 2002). Taken together, these findings may indicate that both androgen deprivation and overload can increase oxidative stress. Further, MnSOD locates in mitochondrion and is implicated in the protection of mitochondrial DNA from oxidative stress, which can evoke mitochondrial DNA damage. Recently, mitochondrial gene mutation was shown to lead to the upregulation of intracellular ROS level and more malignant phenotype (Ishikawa *et al.*, 2008), suggesting that gene expression changes by castration-induced oxidative stress are responsible for prostate carcinogenesis as well as progression to CRPC.

As we previously showed that Twist1 is involved in cancer cell proliferation (Shiota *et al.*, 2008a), we investigated the effect of Twist1 silencing on AR-expressing PCa-cell proliferation. Under androgen-containing conditions, LNCaP cell growth was significantly reduced by Twist1 knockdown, whereas it was slightly reduced under androgen deprivation. These results indicate that Twist1 suppressed LNCaP cell growth partially through AR suppression, but probably partially through YB-1 suppression, as we showed that cell growth was even retarded in PC-3 cells, which express no AR protein (Shiota *et al.*, 2008a). However, Twist1 knockdown seemed to be more effective in LNCaP cells than in PC-3 cells, as indicated by the flow cytometry analysis data that the sub-G1 fraction increases after Twist1 knockdown were more notable in LNCaP cells than in PC-3 cells (7.7 and 10.6% in LNCaP cells vs 4.1% in PC-3 cells). These differences may result from additive effects of AR knockdown, suggesting that Twist1 knockdown may be a more effective therapeutic strategy for AR-expressing PCa. Moreover, under androgen-deprivation conditions, CxR cell growth was significantly reduced by Twist1 knockdown, indicating that Twist1 suppression may even be a novel therapeutic strategy for CRPC, which is suggested to be dependent on androgen/AR signaling by AR overexpression and AR cofactors.

In summary, we have revealed that castration induces oxidative stress, which upregulates Twist1 expression in

Figure 6 Androgen receptor (AR) expression partially rescues PCa cell growth retardation induced by Twist1 knockdown in LNCaP cells. (**a**) LNCaP cells (2.5×10^2) were cotransfected with 20 nM of control siRNA, Twist1 siRNA #1 or Twist1 siRNA #2 and 0.5 μg of pCMV or pCMV-AR. At 14 day after transfection, colonies were stained and colony number was counted. All values are representative of at least three independent experiments. The number of colonies transfected with the control siRNA and pCMV was set as 1. Boxes, mean; bars, ± s.d. $^{**}P<0.05$. (**b**) Representative plates treated with the indicated siRNAs and expression plasmids. (**c**) Whole-cell extracts of LNCaP cells transfected with the indicated siRNA and expression plasmid were analyzed for Twist1, AR and β-action (loading control) by SDS–PAGE and western blotting with specific antibodies. PAGE, polyacrylamide gel electrophoresis.

PCa cells. In turn, Twist1 overexpression leads to AR overexpression, which is closely implicated in CRPC. This functional link between Twist1 and AR suggests that Twist1 represents a promising molecular target for the treatment of various human cancers as well as PCa, particularly CRPC. Furthermore, reduction of the oxidative stress induced by castration may lead to successful ADT through the prevention of Twist1 and



AR overexpressions. Therefore, we are currently searching for effective strategies to reduce castration-induced oxidative stress, which may lead to a new stage of ADT.

Materials and methods

Cell culture

Human PCa LNCaP and 22Rv1 cells were purchased from American Type Cell Collection (Manassas, VA, USA), and cultured in RPMI1640 purchased from Invitrogen (San Diego, CA, USA) and supplemented with 10% fetal bovine serum. LNCaP cells propagated between 10 and 30 times were used. Castration-resistant derivatives of LNCaP cells (LNCaP-CxR cells, referred to as CxR cells) were established by culture under androgen starvation using charcoal-stripped medium for more than 4 months as described previously (Patel *et al.*, 2000; Tso *et al.*, 2000) and maintained in 87.5% androgen-reduced medium, which was prepared by mixture of 87.5% charcoal-stripped medium and 12.5% non-charcoal-stripped medium. CxR cells grew in the 87.5% androgen-reduced medium similarly to their parental cell growth in non-charcoal-stripped medium. Hydrogen peroxide-resistant derivatives of LNCaP cells (LNCaP-HPR50 cells, referred to as HPR50 cells) were established by long-term culture in medium containing gradually increasing concentrations of hydrogen peroxide and maintained in medium containing 50 μM of hydrogen peroxide. HPR50 cells were about 12-fold more resistant to hydrogen peroxide than the parental cells (data not shown). All cell lines were maintained in a 5% CO_2 atmosphere at 37 °C.

Antibodies

Antibodies against AR (sc-815), PARP (sc-1561), GFP (green fluorescent protein) (sc-8334) and Twist1 (sc-81417) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti β-actin, anti-cleaved PARP and anti-YB-1 antibodies were purchased from Sigma (St Louis, MO, USA), Promega (Madison, WI, USA) and Epitomics Inc. (Burlingame, CA, USA), respectively.

Plasmid construction

The Twist1-GFP plasmid expressing C-terminally GFP-tagged Twist1 protein was purchased from OriGene (Rockville, MD, USA). The pCMV-AR plasmid expressing wild-type AR was kindly provided by Dr Chawnshang Chang (University of Rochester, Rochester, NY, USA).

Various lengths of the promoter and partial first exon of the wild-type AR gene were amplified by PCR using genomic DNA and the following primer pairs: 5′-AGATCTCAATGA GTATTCAAATGAG-3′ and 5′-AAGCTTCTTGCTCCGGA CCGTCCC-3′ for AR-Luc #1; 5′-AGATCTCACTCTCCCAT CTGCGCGC-3′ and 5′-AAGCTTCTTGCTCCGGACCGTC CC-3′ for AR-Luc #3. The obtained PCR products were cloned and ligated into the pGL3-basic vector (Promega). AR-Luc #2 and AR-Luc #4 were constructed from AR-Luc #1 by deletion of the *Sac*I and *Sma*I fragments, respectively. Mutations were introduced into the E-boxes of AR-Luc #3 using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA) with the following primer pairs: 5′-GACTCTCCCACT CTCCACTCTGCGCGCTCTTATC-3′ and 5′-GATAAGAG CGCGCAGAGTGGAGAGTGGGAGAGTC-3′ for AR-Luc E-box1 MT; 5′-GCCCACGCTGCGCCAGACCTTGTTTCT CCAAAGC-3′ and 5′-GCTTTGGAGAAACAAGGTCTGG CGCAGCGTGGGC-3′ for AR-Luc E-box2 MT; 5′-CGA CTCGCAAACTGTTGACTTTGCTCTCCACCTCCC-3′ and 5′-GGGAGGTGGAGAGCAAAGTCAACAGTTTGCGAGT CG-3′ for AR-Luc E-box3 MT. AR-Luc E-box23 MT was constructed by introducing a mutation into E-box3 of AR-Luc E-box2 MT. The underlined nucleotides indicate the mutated sequences.

Western blot analysis

Whole-cell extracts were prepared as described previously (Shiota *et al.*, 2008a, b, 2009). The protein concentrations were quantified using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Whole-cell extracts (30 μg) were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ, USA) using a semi-dry blotter. The blotted membranes were sequentially incubated with appropriate primary antibodies for 1 h, and peroxidase-conjugated secondary antibodies for 40 min at room temperature. The bound antibodies were visualized using an ECL Kit (GE Healthcare Bio-Science) and the membranes were exposed to X-OMAT film (Kodak, Tokyo, Japan).

Knockdown analysis using siRNAs

Knockdown analysis using siRNAs was performed as described previously (Shiota *et al.*, 2008a, 2009). Briefly, the following double-stranded 25-bp siRNA oligonucleotides were commercially generated (Invitrogen): 5′-UAGAGAGCAAGG CUGCAAAGGAGUC-3′ (sense) and 5′-GACUCCUUUGC

Figure 7 Twist1 regulates androgen receptor (AR) expression and cell growth in other AR-expressing PCa cells. (**a**) 22Rv1 cells were cotransfected with 0.5 μg of the various AR-Luc plasmids shown in Figure 3a, 0.5 μg of GFP or Twist1-GFP and 0.05 μg of pRL-TK. All values are representative of at least three independent experiments. The luciferase activity of AR-Luc #1 alone was set as 1. Boxes, mean; bars, ±s.d. $^{**}P<0.05$ (compared with that of 22Rv1 cells transfected with control siRNA). Whole-cell extracts of 22Rv1 cells transfected with the indicated plasmids were analyzed for Twist1-GFP and β-actin (loading control) by SDS–PAGE and western blotting with anti-GFP and anti-β-actin antibodies, respectively. (**b**) 22Rv1 cells were transfected with 40 nM of control siRNA, Twist1 siRNA #1 or Twist1 siRNA #2. At 72 h after transfection, quantitative real-time PCR was performed using the primers and probes for AR, Twist1, Y-box-binding protein-1 (YB-1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The target transcript levels were corrected by the corresponding GAPDH transcript levels. All values are representative of at least three independent experiments. Each transcript level from cells transfected with the control siRNA was set as 1. Boxes, mean; bars, ±s.d. $^{**}P<0.05$ (compared with that of 22Rv1 cells transfected with control siRNA). Whole-cell extracts were analyzed for Twist1, YB-1, AR and β-actin (loading control) by SDS–PAGE and western blotting with specific antibodies. (**c**) 22Rv1 cells were transfected with 40 nM of control siRNA, Twist1 siRNA #1 or Twist1 siRNA #2. At the indicated time points, the cell numbers were counted. The results were normalized by the cell numbers at 0 h. All values are representative of at least three independent experiments. Boxes, mean; bars, ±s.d. $^{**}P<0.05$ (compared with that of 22Rv1 cells transfected with control siRNA). (**d**) CxR cells were transfected with 40 nM of control siRNA, Twist1 siRNA #1 or Twist1 siRNA #2. At the indicated time points, the cells were subjected to cell proliferation assays as described in (**c**). Boxes, mean; bars, ±s.d. $^{**}P<0.05$ (compared with that of CxR cells transfected with control siRNA). Whole-cell extracts of CxR cells were analyzed for AR, Twist1, YB-1 and β-actin (loading control) by SDS–PAGE and western blotting with specific antibodies. GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis.

AGCCUUGCUCUCUA-3′ (antisense) for AR siRNA #1; 5′-CAUAGUGACACCCAGAAGCUUCAUC-3′ (sense) and 5′-GAUGAAGCUUCUGGGUGUCACUAUG-3′ (antisense) for AR siRNA #2; 5′-CUUCCUCGCUGUUGCUCAGGCUGUC-3′ (sense) and 5′-GACAGCCUGAGCAACAGCGAGGAAG-3′ (antisense) for Twist1 siRNA #1; 5′-UUGAGGGUCUGAAUCUUGCUCAGCU-3′ (sense) and 5′-AGCUGAGCAAGAUUCAGACCCUCAA-3′ (antisense) for Twist1 siRNA #2. PCa cells were transfected with various amounts of the siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cytotoxicity analysis
Cytotoxicity analysis was performed as described previously (Shiota *et al*., 2008a). LNCaP, CxR and HPR50 cells (2×10^5) were seeded into 96-well plates. On the following day, various concentrations of dihydrotestosterone were applied in charcoal-stripped medium. After 48 h, the surviving cells were stained with the alamarBlue assay (TREK Diagnostic Systems, Cleveland, OH, USA) for 180 min at 37 °C. The absorbances of the wells were measured using a plate reader (ARVO MX; Perkin Elmer Inc., Waltham, MA, USA).

Measurement of intracellular ROS
LNCaP cells (3×10^5) seeded into 96-well plates were incubated with charcoal-stripped medium with or without 10 nM of dihydrotestosterone and/or 5 mM of NAC for 48 h. Intracellular ROS levels were measured using CM-H_2DCFDA (Invitrogen) according to the manufacturer's protocol. Briefly, cells were incubated with 5 μM of CM-H_2DCFDA in phosphate-buffered saline (PBS) for 30 min. Then, cells were washed and returned to fresh medium for a 30-min recovery period. The fluorescence intensities of the wells were measured using the ARVO MX plate reader. At the same time, surviving cells were measured using the alamarBlue assay. The intracellular ROS levels were corrected by the corresponding results of the alamarBlue assay. The results are representative of at least three independent experiments.

Luciferase reporter assay
LNCaP cells (2×10^5) were cotransfected with various amounts of AR reporter plasmids, various amounts of expression plasmids or siRNAs and 0.05 μg of pRL-TK as an internal control using Lipofectamine 2000. After 48 h, the luciferase activities were detected using a Dual-Luciferase Reporter Assay System (Promega). The light intensities were measured using the ARVO MX plate reader. The firefly luciferase activities were corrected by the corresponding *Renilla* luciferase activities. The results are representative of at least three independent experiments.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation assay was performed as described previously (Shiota *et al*., 2008a). Briefly, soluble chromatin from LNCaP cells (1×10^6) was incubated with 2.0 μg of anti-mouse immunoglobulin G or anti-Twist1 antibody and 20 μl of Protein A/G PLUS-agarose (Santa Cruz Biotechnology). The purified DNA was dissolved in 20 μl of distilled H_2O and 2.0 μl of DNA was used for PCR analysis with the following primer pairs: 5′-AGATCTCAATGAGTATTCAAATGAG-3′ (forward) and 5′-CTTTATCTTGTGCACAGCCAAAC-3′ (reverse) for AR #1; 5′-AGATCTCCATTCCCACTTGCATCTC-3′ (forward) and 5′-GGCATTGTGCCATTTGCTCTAGG-3′ (reverse) for AR #2; 5′-AGATCTCACTCTCCCATCTGCGCGC-3′ (forward) and 5′-AAGCTTCTTGCTCCGGACCGTCCC-3′ (reverse) for AR #3; 5′-TCTCTCTCCACCTCCTCCTG-3′ (forward) and 5′-CCTCCACCTTCCAAATTCAG-3′ (reverse) for AR #4. The quantitative real-time PCR assay with the diluted DNA, the above primer pairs and SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan) was performed using ABI 7900HT System (Applied Biosystems, Foster City, CA, USA). The results are representative of at least three independent experiments.

RNA isolation and reverse transcriptase–PCR
Total RNA was prepared from cultured cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1.0 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol.

Quantitative real-time PCR
The synthesized cDNA was diluted 1:2, and 2.0 μl of the diluted sample was used. Quantitative real-time PCR with TaqMan Gene Expression Assay (Applied Biosystems) and TaqMan Gene Expression Master Mix (Applied Biosystems) was performed using an ABI 7900HT System. The expression level of each target gene was corrected by the corresponding GAPDH (glyceraldehyde 3-phosphate dehydrogenase) expression level. The results are representative of at least three independent experiments.

Cell proliferation assay
Cell proliferation assay was performed as described previously (Shiota *et al*., 2008a, 2009). Briefly, PCa cells (2.5×10^4) were seeded into 12-well plates and transfected with the indicated siRNA. The time point of 12 h after transfection was set as 0 h. The cells were harvested with trypsin and counted daily using a cell counter (Beckman Coulter, Fullerton, CA, USA). The results were normalized by the cell counts at 0 h, and are representative of at least three independent experiments.

Flow cytometry analysis
Flow cytometry analysis was performed as described previously (Shiota *et al*., 2008a). Briefly, LNCaP cells (2.5×10^5) were seeded into 6-well plates, transfected with the indicated siRNA and cultured for 72 h. The cells were then harvested, washed twice with ice-cold PBS containing 0.1% bovine serum albumin and resuspended in 70% ethanol. After two washes with ice-cold PBS, the cells were resuspended in PBS containing 0.1% bovine serum albumin, incubated with RNase (Roche Applied Science) and stained with propidium iodide (Sigma). The stained cells were analyzed using a FACS Calibur (BD Biosciences, San Jose, CA, USA).

Colony-formation assay
Colony-formation assay was performed as described previously (Shiota *et al*., 2008a). Briefly, LNCaP cells (2.5×10^5) transfected with 20 nM of the indicated siRNA and 0.5 μg of pCMV or pCMV-AR were seeded into 6-well plates. At 14 days after transfection, cells were washed with PBS and fixed in 100% methanol for 30 min followed by staining with 2% Giemsa solution for 1 h. The wells were washed with H_2O and dried. Colonies with >50 cells were counted on an inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis
The Mann–Whitney's U-test was used for statistical analysis, and significance was set at the 5% level.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported in part by Health Sciences Research Grants for Clinical Research for evidence-based medicine and grants-in-aid for Cancer Research (016), from the Ministry of Health, Labor and Welfare, Japan, Young Researcher Promotion Grant from the Japanese Urological Association, Japan. We are grateful to Dr Chawnshang Chang for providing the pCMV-AR plasmid. We would like to thank Dr Dongchon Kang (Kyushu University, Fukuoka, Japan) for helping with quantitative real-time PCR and flow cytometry, and Noriko Hakoda, Hitomi Matoba and Seiko Kamori for their technical assistances.

References

Benz CC, Yau C. (2008). Ageing, oxidative stress and cancer: paradigms in parallax. *Nat Rev Cancer* **8**: 875–879.

Best CJ, Gillespie JW, Yi Y, Chandramouli GV, Perlmutter MA, Gathright Y *et al.* (2005). Molecular alterations in primary prostate cancer after androgen ablation therapy. *Clin Cancer Res* **11**: 6823–6834.

Billis A. (1996). Age and race distribution of high-grade prostatic intraepithelial neoplasia: an autopsy study in Brazil (South America). *J Urol Pathol* **5**: 1–7.

Bostwick DG, Alexander EE, Singh R, Shan A, Qian J, Santella RM *et al.* (2000). Antioxidant enzyme expression and reactive oxygen species damage in prostatic intraepithelial neoplasia and cancer. *Cancer* **89**: 123–134.

Castanon I, Setina SV, Kass J, Baylies MK. (2001). Dimerization partners determine the activity of the Twist bHLH protein during Drosophila mesoderm development. *Development* **128**: 3145–3159.

Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R *et al.* (2004). Molecular determinants of resistance to antiandrogen therapy. *Nat Med* **10**: 33–39.

Debes JD, Tindall DJ. (2002). The role of androgens and the androgen receptor in prostate cancer. *Cancer Lett* **187**: 1–7.

Ghatak S, Ho SM. (1996). Age-related changes in the activities of antioxidant enzymes and lipid peroxidation status in ventral and dorsolateral prostate lobes of Noble rats. *Biochem Biophys Res Commun* **222**: 362–367.

Gregory CW, Hamil KG, Kim D, Hall SH, Pretlow TG, Mohler JL *et al.* (1998). Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res* **58**: 5718–5724.

Gronberg H. (2003). Prostate cancer epidemiology. *Lancet* **361**: 859–864.

Hakimian P, Blute Jr M, Kashanian J, Chan S, Silver D, Shabsigh R. (2008). Metabolic and cardiovascular effects of androgen deprivation therapy. *BJU Int* **102**: 1509–1514.

Hoek K, Rimm DL, Williams KR, Zhao H, Ariyan S, Lin A *et al.* (2004). Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. *Cancer Res* **64**: 5270–5282.

Hsing AW, Devesa SS. (2001). Trends and patterns of prostate cancer: what do they suggest? *Epidemiol Rev* **23**: 3–13.

Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H *et al.* (2008). ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science* **320**: 661–664.

Kwok WK, Ling MT, Lee TW, Lau TC, Zhou C, Zhang X *et al.* (2005). Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target. *Cancer Res* **65**: 5153–5162.

Landies SH, Murray T, Bolden S, Wingo PA. (1999). Cancer statistics. *CA Cancer J Clin* **49**: 8–31.

Lee DK, Chang C. (2003). Expression and degradation of androgen receptor: mechanism and clinical implication. *J Clin Endocrinol Metab* **88**: 4043–4054.

Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T. (2001). Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer Res* **61**: 3550–3555.

Lu T, Finkel T. (2008). Free radicals and senescence. *Exp Cell Res* **314**: 1918–1922.

Maestro R, Dei Tos AP, Hamamori Y, Krasnokutsky S, Sartorelli V, Kedes L *et al.* (1999). Twist is a potential oncogene that inhibits apoptosis. *Genes Dev* **13**: 2207–2217.

Maynard S, Schurman SH, Harboe C, de Souza-Pinto NC, Bohr VA. (2009). Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis* **30**: 2–10.

Mitsiades N, Correa D, Gross CP, Hurria A, Slovin SF. (2008). Cognitive effects of hormonal therapy in older adults. *Semin Oncol* **35**: 569–581.

Oberley TD, Zhong W, Szweda LI, Oberley LW. (2000). Localization of antioxidant enzymes and oxidative damage products in normal and malignant prostate epithelium. *Prostate* **44**: 144–155.

Olson EN, Klein WH. (1994). bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev* **8**: 1–8.

Pang ST, Dillner K, Wu X, Pousette A, Norstedt G, Flores-Morales A. (2002). Gene expression profiling of androgen deficiency predicts a pathway of prostate apoptosis that involves genes related to oxidative stress. *Endocrinology* **143**: 4897–4906.

Patel BJ, Pantuck AJ, Zisman A, Tsui KH, Paik SH, Caliliw R *et al.* (2000). CL1-GFP: an androgen independent metastatic tumor model for prostate cancer. *J Urol* **164**: 1420–1425.

Pathak S, Singh R, Verschoyle RD, Greaves P, Farmer PB, Steward WP *et al.* (2008). Androgen manipulation alters oxidative DNA adduct levels in androgen-sensitive prostate cancer cells grown *in vitro* and *in vivo*. *Cancer Lett* **261**: 74–83.

Pinthus JH, Bryskin I, Trachtenberg J, Lu JP, Singh G, Fridman E *et al.* (2007). Androgen induces adaptation to oxidative stress in prostate cancer: implications for treatment with radiation therapy. *Neoplasia* **9**: 68–80.

Reddy GP, Barrack ER, Dou QP, Menon M, Pelley R, Sarkar FH *et al.* (2006). Regulatory processes affecting androgen receptor expression, stability, and function: potential targets to treat hormone-refractory prostate cancer. *J Cell Biochem* **98**: 1408–1423.

Ripple MO, Henry WF, Rago RP, Wilding G. (1997). Prooxidant-antioxidant shift induced by androgen treatment of human prostate carcinoma cells. *J Natl Cancer Inst* **89**: 40–48.

Rosivatz E, Becker I, Specht K, Fricke E, Luber B, Busch R *et al.* (2002). Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am J Pathol* **161**: 1881–1891.

Sakr WA, Grignon DJ, Haas GP, Heilbrun LK, Pontes JE, Crissman JD. (1996). Age and racial distribution of prostatic intraepithelial neoplasia. *Eur Urol* **30**: 138–144.

Scher HI, Sawyers CL. (2006). Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *J Clin Oncol* **23**: 8253–8261.

Shiota M, Izumi H, Onitsuka T, Miyamoto N, Kashiwagi E, Kidani A *et al.* (2008a). Twist promotes tumor cell growth through YB-1 expression. *Cancer Res* **68**: 98–105.

Shiota M, Izumi H, Onitsuka T, Miyamoto N, Kashiwagi E, Kidani A *et al.* (2008b). Twist and p53 reciprocally regulate target genes via direct interaction. *Oncogene* **27**: 5543–5553.

Shiota M, Izumi H, Tanimoto A, Takahashi M, Miyamoto N, Kashiwagi E *et al.* (2009). Programmed cell death protein 4

down-regulates Y-box binding protein-1 expression via a direct interaction with Twist1 to suppress cancer cell growth. *Cancer Res* **69**: 3148–3156.

Stasinopoulos IA, Mironchik Y, Raman A, Wildes F, Winnard P, Raman V. (2005). HOXA5-Twist interaction alters p53 homeostasis in breast cancer cells. *J Biol Chem* **280**: 2294–2299.

Tamm NN, Gao Y, Leung YK, Ho SM. (2003). Androgenic regulation of oxidative stress in the rat prostate: involvement of NAD(P)H oxidases and antioxidant defense machinery during prostatic involution and regrowth. *Am J Pathol* **163**: 2513–2522.

Taplin ME, Rajeshkumar B, Halabi S, Werner CP, Woda BA, Picus J *et al.* (2003). Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663. *J Clin Oncol* **21**: 2673–2678.

Tso CL, McBride WH, Sun J, Patel B, Tsui KH, Paik SH *et al.* (2000). Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. *Cancer J* **6**: 220–233.

Valsesia-Wittmann S, Magdeleine M, Dupasquier S, Garin E, Jallas AC, Combaret V *et al.* (2004). Oncogenic cooperation between H-Twist and N-Myc overrides failsafe programs in cancer cells. *Cancer Cell* **6**: 625–630.

van Doorn R, Dijkman R, Vermeer MH, Out-Luiting JJ, van der Raaij-Helmer EM, Willemze R *et al.* (2004). Aberrant expression of the tyrosine kinase receptor EphA4 and the transcription factor twist in Sezary syndrome identified by gene expression analysis. *Cancer Res* **64**: 5578–5586.

Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C *et al.* (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**: 927–939.

Yang MH, Wu MZ, Chiou SH, Chen PM, Chang SY, Liu CJ *et al.* (2008). Direct regulation of TWIST by HIF-1α promotes metastasis. *Nat Cell Biol* **10**: 295–305.

Zegarra-Moro OL, Schmidt LJ, Huang H, Tindall DJ. (2002). Disruption of androgen function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res* **62**: 1008–1013.

MOLECULAR ENDOCRINOLOGY

Peroxisome Proliferator-Activated Receptor {gamma} Coactivator-1{alpha} Interacts with the Androgen Receptor (AR) and Promotes Prostate Cancer Cell Growth by Activating the AR

Masaki Shiota, Akira Yokomizo, Yasuhiro Tada, Junichi Inokuchi, Katsunori Tatsugami, Kentaro Kuroiwa, Takeshi Uchiumi, Naohiro Fujimoto, Narihito Seki and Seiji Naito

Mol. Endocrinol. 2010 24:114-127 originally published online Nov 2, 2009; , doi: 10.1210/me.2009-0302

Peroxisome Proliferator-Activated Receptor γ Coactivator-1α Interacts with the Androgen Receptor (AR) and Promotes Prostate Cancer Cell Growth by Activating the AR

Masaki Shiota, Akira Yokomizo, Yasuhiro Tada, Junichi Inokuchi, Katsunori Tatsugami, Kentaro Kuroiwa, Takeshi Uchiumi, Naohiro Fujimoto, Narihito Seki, and Seiji Naito

Departments of Urology (M.S., A.Y., Y.T., J.I., K.T., K.K., N.S., S.N.) and Clinical Chemistry and Laboratory Medicine (T.U.), Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8252, Japan; and Department of Urology (N.F.), School of Medicine, University of Occupational and Environmental Health 807-8555, Kitakyushu, Japan

There are currently few successful therapies for castration-resistant prostate cancer (CRPC). CRPC is thought to result from augmented activation of the androgen/androgen receptor (AR) signaling pathway, which could be enhanced by AR cofactors. In this study, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) was found to be an AR cofactor. PGC-1α interacted with the N-terminal domain of AR, was involved in the N- and C-terminal interaction of AR, and enhanced the DNA-binding ability of AR to androgen-responsive elements in the prostate-specific antigen enhancer and promoter regions to increase the transcription of AR target genes. Silencing of PGC-1α suppressed cell growth of AR-expressing prostate cancer (PCa) cells by inducing cell-cycle arrest at the G_1 phase, similar to inhibition of androgen/AR signaling. Furthermore, PGC-1α knock-down also suppressed cell growth in the castration-resistant LNCaP-derivatives. These findings indicate that PGC-1α is involved in the proliferation of AR-expressing PCa cells by acting as an AR coactivator. Modulation of PGC-1α expression or function may offer a useful strategy for developing novel therapeutics for PCa, including CRPC, which depends on AR signaling by overexpressing AR and its coactivators. (***Molecular Endocrinology*** **24: 114–127, 2010)**

Prostate cancer (PCa) is the most common noncutaneous cancer and the second leading cause of cancer-related death in men in developed countries. The incidence of PCa has increased significantly because of the prevalence of high-fat diets and massive increase in the aging population (1, 2). Also, screening using prostate-specific antigen (PSA) has dramatically improved the early detection of PCa. However, 20–30% of patients with localized PCa who received surgical or radiation therapy still suffer from the relapse of the disease (3–5). Also, many patients with PCa are still only diagnosed at an advanced stage of disease. Most PCas are androgen dependent at diagnosis and, in most patients, androgen-deprivation therapy (ADT) is effective and prevents further growth and often leads to tumor regression. However, most tumors will relapse in a castration-resistant manner after a median of 13 months after ADT, and are, thus, designated as castration-resistant prostate cancer (CRPC) (6). There are currently few successful therapies for CRPC. Therefore, CRPC remains a serious obstacle to overcome.

The androgen/androgen receptor (AR) signaling pathway is thought to have a key role in prostate carcinogenesis and PCa progression. Several studies using PCa cell

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

doi: 10.1210/me.2009-0302 Received July 31, 2009. Accepted September 22, 2009.
First Published Online November 2, 2009

Abbreviations: ADT, Androgen-deprivation therapy; AR, androgen receptor; ARE, androgen-responsive elements; CRPC, castration-resistant prostate cancer; DHT, dihydrotestosterone; GAPDH, glyceraldehyde 3-phosphatase; LBD, ligand-binding domain; MMTV, mouse mammary tumor virus; NTD, N-terminal domain; PCa, prostate cancer; PGC-1α, PPARγ coactivator-1α; PPARγ, peroxisome proliferator-activated receptor γ; PSA, prostate-specific antigen; siRNA, small interfering RNA; TAD, transactivation domain.

lines and mouse models have shown that the progression to CRPC could be associated with enhanced AR expression, as indicated by findings from AR down-regulation using dominant-negative AR mutants, small interfering RNA (siRNA), or small molecules, whereas increased AR expression converts androgen-dependent PCa cells to CRPC (7–10). The *AR* gene is overexpressed in most CRPCs, 10–20% of which show amplification of the *AR* gene (11). Also, less than 10% of CRPCs were found to have somatic mutations in the *AR* gene, which could confer promiscuous activity to the receptor, allowing its activation by nonandrogen steroids and antiandrogens (12). Furthermore, the AR pathway in CRPC was considered to rely on changes in expression of growth factors, such as IGF (13), HER-2 (14), and IL-6 (15), which could modify AR activity. Also, AR signaling could be modulated by AR cofactors such as heat-shock protein 27 (Hsp27) (16), peroxiredoxin1 (17), Tip60, histone deacetylase 1 (HDAC1) (18), ARA 54 (19), ARA55 (20), ARA70 (21), GRIP1 (22), HMGB1, HMGB2 (23), PIAS1, PIAS3 (24), and SRC1 (25), some of which have been reported to be implicated in CRPC. Modification of these growth factors and cofactors in CRPC may cause androgen-dependent PCa to gain castration-resistant status.

Peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) was isolated based on its abilities to interact with PPARγ in a two-hybrid screening system and to enhance glucocorticoid responses in a functional genetic screening system (26, 27). It has previously been shown that PGC-1α has a novel role in adaptive thermogenesis, where it enhances the ability of PPARγ and nuclear respiratory factors (NRF1 and NRF2) to induce the synthesis of the enzymes required for oxidative metabolism (28). PGC-1α has been shown to be expressed and highly regulated in brown adipose tissue and skeletal muscle (26). Also, PGC-1α is expressed in the heart, kidney, and brain, suggesting that it is involved in processes other than thermogenesis (26). Because AR is also expressed in these tissues, we hypothesized that PGC-1α might have a role in various tissues together with AR. PGC-1α is known to interact with and enhance the transactivation of other nuclear receptors such as estrogen receptor α (ERα) (29, 30). Also, PGC-1α interacts with and activates ERα and ERβ in a ligand-independent manner with a particularly high-binding affinity to ERβ (31). In contrast, PGC-1β, also known as PERC, selectively binds to ERα in a ligand-dependent manner and activates its transcriptional ability (29, 32). Similar to a relationship between androgen and AR in carcinogenesis and development of PCa, a relationship between estrogen and ER has also been established, and it was reported that reduced levels of ovarian steroids and ERα significantly decrease the breast cancer risk (33, 34). Furthermore, antiestrogen therapies that inhibit estrogen synthesis or block ER activity are used to treat breast cancer similar to antiandrogen agents to treat PCa. Wirtenberger *et al.* (35) recently showed that a polymorphism of PGC-1α was associated with familial breast cancer, high-risk familial breast cancer, and bilateral familial breast cancer.

However, the function of PGC-1α in association with AR and in the progression of CRPC currently remains unknown. In this study, we intended to resolve the function of PGC-1α in association with AR and PCa. Our data showed that PGC-1α interacted with AR and was involved in the proliferation of androgen-dependent and CRPC cells. Together, PGC-1α appears to be a key factor involved in the progression to PCa, and is a promising molecular target for treating PCa, even CRPC.

Results

PGC-1α interacts with AR *in vitro* and *in vivo*

We intended to research the mechanisms responsible for carcinogenesis and progression of PCa in terms of AR function. Accordingly, we found that PGC-1α might interact with AR. First, the interaction between AR and PGC-1α was investigated by a glutathion S-transferase (GST) pull-down assay using GST-fused AR and Myc-Flag-tagged PGC-1α proteins. As shown in Fig. 1A, Myc-Flag-tagged PGC-1α was found to interact with GST-AR. To confirm this finding, a coimmunoprecipitation assay using the overexpression method was performed. PC-3 cells, which expressed no AR mRNA and protein, were transfected with GFP-tagged AR and Myc-Flag-tagged PGC-1α expression plasmids, and a coimmunoprecipitation assay was performed. Myc-Flag-tagged PGC-1α reproducibly interacted with the GFP-tagged AR protein. Simultaneously, we assayed whether the interaction between PGC-1α and AR could be influenced by dihydrotestosterone (DHT). PC-3 cells were transfected with the GFP-tagged AR and Myc-Flag-tagged PGC-1α expression plasmids, and then cultured under charcoal-stripped medium with or without DHT. The results of the coimmunoprecipitation assay showed that DHT did not influence this interaction (Fig. 1B). Last, we investigated whether endogenous proteins interacted with each other. Using cellular extracts of LNCaP cells expressing AR protein that were cultured under charcoal-stripped medium with or without DHT, the endogenous AR was immunoprecipitated using anti-AR antibody, and the immunoprecipitated samples were blotted with anti-PGC-1α antibody. As expected, endogenous AR interacted with PGC-1α, and reproducibly, this interaction was not affected by DHT (Fig. 1C).

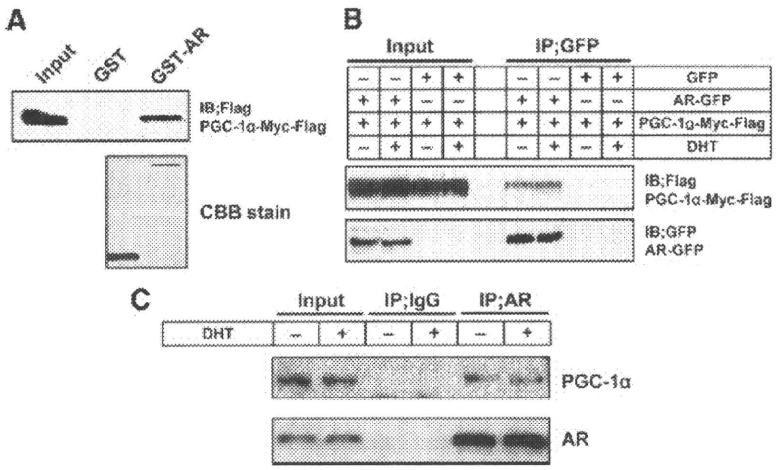


FIG. 1. PGC-1α interacts with AR *in vitro* and *in vivo*. A, Equal amounts of GST and GST-AR fusion proteins were immobilized on glutathione-sepharose 4B and were incubated with nuclear extracts from PC-3 cells transfected with PGC-1α-Myc-Flag plasmid. The bound protein and 10% of the input were subjected to SDS-PAGE, and Western blot analysis was performed using the anti-Flag antibody. Purified GST and GST-AR fusion proteins stained with Coomassie Brilliant Blue (CBB; Wako, Osaka, Japan) are also shown. B, PC-3 cells were cotransfected with 1.0 μg of each of the indicated expression plasmids and incubated in charcoal-stripped medium with or without 10 nM of DHT. Whole-cell extracts (300 μg) were immunoprecipitated with agarose-conjugated anti-GFP antibody. The resulting immunocomplexes and whole-cell extracts (30 μg) were subjected to SDS-PAGE, and Western blot analysis was performed using anti-Flag and anti-GFP antibodies. C, Whole-cell extracts (500 μg) were prepared from LNCaP cells incubated in charcoal-stripped medium with or without 10 nM of DHT and were immunoprecipitated (IP) with 2.0 μg of rabbit IgG or anti-AR antibody (C-19) and 20 μl of protein A/G agarose. The resulting immunocomplexes and whole-cell extracts (50 μg) were subjected to SDS-PAGE, and Western blot analysis was performed using anti-PGC-1α and anti-AR (C-19) antibodies.

PGC-1α is overexpressed in PCa cells and PGC-1α knock-down reduces PSA expression

To investigate a role of PGC-1α in PCa, we examined PGC-1α expressions in human normal prostate epithelial cells (RWPE-1 cells) and a panel of PCa cells (DU145, PC-3, VCaP, 22Rv1, LNCaP, and castration-resistant LNCaP derivatives CxR cells). PGC-1α was overexpressed in PCa cells compared with that in normal prostate epithelial cells. Also, PGC-1α expression level was similar between LNCaP and CxR cells (Fig. 2A). Furthermore, PGC-1α expression was not affected by DHT in LNCaP and CxR cells (Fig. 2B). To confirm the function of PGC-1α on AR, we next examined the expression of a well-known AR target gene, PSA, after knock-down of PGC-1α. After LNCaP and CxR cells were transfected with PGC-1α-specific siRNAs, quantitative real-time PCR and Western blot analysis for PSA were performed. The results showed that the expression of PSA mRNA was decreased by PGC-1α knock-down in the presence of DHT, although basal PSA expression was decreased by androgen starvation, but not in the absence of DHT. In addition, PSA mRNA expression level both in the presence or absence of DHT was decreased after PGC-1α knock-down also in CxR cells (Fig. 2C), in which AR could locate in nucleus and have a potential to transactivate its target genes even without ligand (Shiota, M., A. Yokomizo A., D. Masubichi, Y. Tuda, J. Inokuchi, M.Eto, T. Uchiumi, N. Fujimoto, S. Naito, manuscript submitted). Similar findings in terms of the protein PSA level were obtained when androgen-dependent LNCaP cells and CxR cells were transfected with PGC-1α-specific siRNAs. Also, transfection efficiencies of PGC-1α-specific siRNAs seemed to be equivalent between LNCaP and CxR cells as indicated by decrease of PGC-1α protein expression. Furthermore, as previously reported, the expression of AR was increased in the CxR cells compared with that in the parental LNCaP cells (Fig. 2D) (9–11).

PGC-1α activates AR transcriptional activity

Because PGC-1α was thought to interact with AR and regulate PSA expression, we determined the effect of PGC-1α on AR transcriptional activity using a luciferase assay. First, the PC-3 cells were transfected with a PSA reporter plasmid, pGLPSAp5.8, possessing PSA enhancer and promoter regions (~5.8 kb) with three putative androgen-responsive elements (AREs) and a PGC-1α expression plasmid in addition to pCMV-AR expressing wild-type AR protein. Without DHT, luciferase activity was hardly detected even with PGC-1α overexpression. However, luciferase activity was significantly increased with DHT. Also, PGC-1α overexpression increased the transcriptional activity of PSA. In PCa, several AR mutations such as T887A in LNCaP cells have been found. Simultaneously, we investigated whether the AR mutation influences the PGC-1α function as a coactivator of AR in PC-3 cells. PGC-1α overexpression enhanced luciferase activity of PSA reporter plasmid even when mutated AR (T887A) was expressed in the PC-3 cells. Next, mouse mammary tumor virus (MMTV)-Luc possessing an MMTV promoter region with a putative ARE was used, and similar results were obtained. PGC-1α overexpression increased the transcriptional activities of MMTV when DHT was applied in addition to AR expression (Fig. 3A). Last, to confirm the above results, a knock-down assay using PGC-1α-specific siRNAs was performed. As expected, PGC-1α knock-down decreased the luciferase activity of the PSA and MMTV reporter plasmids to approximately 10–40% in LNCaP cells with DHT, whereas basal luciferase activity without DHT was not affected by PGC-1α

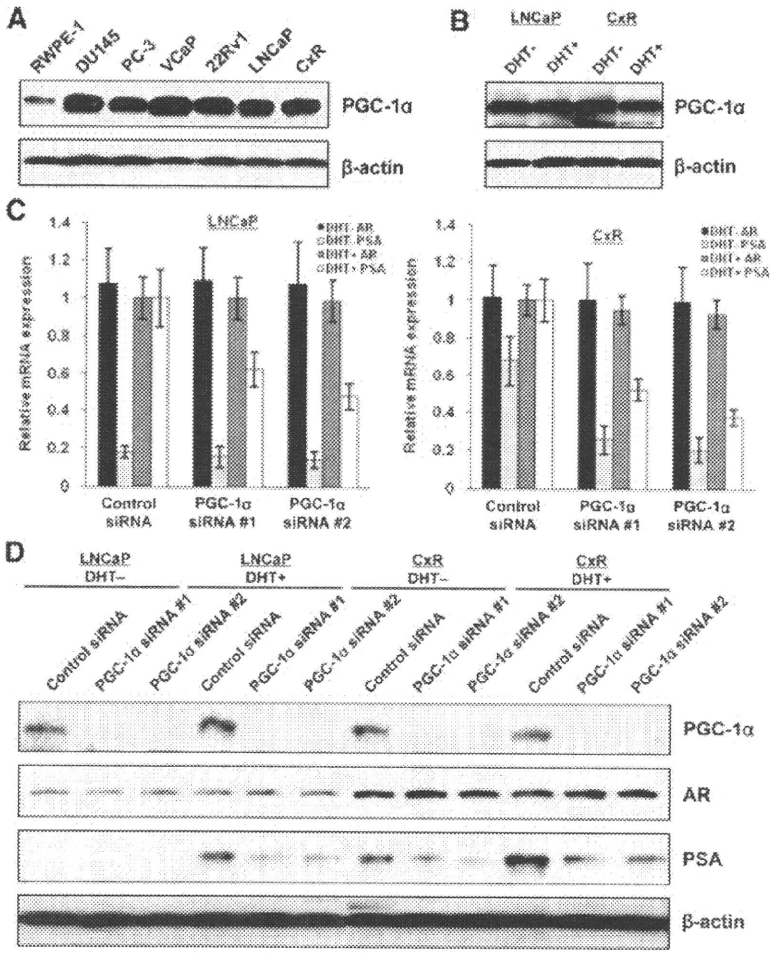


FIG. 2. PGC-1α is overexpressed in PCa cells and PGC-1α knock-down reduces PSA expression. A, Whole-cell extracts from the indicated cells were subjected to SDS-PAGE, and Western blot analysis was performed using anti-PGC-1α and anti-β-actin antibodies. B, Whole-cell extracts from LNCaP, CxR cells incubated in charcoal-stripped medium with or without 10 nM of DHT for 72 h were subjected to SDS-PAGE, and Western blot analysis was performed using anti-PGC-1α and anti-β-actin antibodies. C, LNCaP and CxR cells were transfected with 50 nM of control siRNA, PGC-1α siRNA no. 1 or PGC-1α siRNA no. 2, and incubated in charcoal-stripped medium with or without 10 nM of DHT for 72 h. After extraction of total RNA and synthesis of cDNA, quantitative real-time PCR was performed using the primers and probes for AR, PSA, and GAPDH. The transcription levels of AR and PSA were corrected for the corresponding GAPDH transcript level. All values represent at least three independent experiments. The level of each transcript from cells transfected with control siRNA and incubated with DHT corresponds to 1. *Boxes*, Mean; *bars*, ± SD. D, Whole-cell extracts from cells prepared in C were subjected to SDS-PAGE, and Western blot analysis was performed using anti-PGC-1α, anti-AR (C-19), anti-PSA, and anti-β-actin antibodies.

knock-down (Fig. 3B). These results indicate that PGC-1α can affect PSA transcription androgen/AR signaling-dependently.

N-terminal domain (NTD) of PGC-1α interacts with the N-terminal transactivation domain (TAD) of AR

The finding that PGC-1α interacted with AR and had a functional role with AR prompted us to examine which domains are involved. First, a GST pull-down assay was performed using GST-AR and its series of deletion mutants with Myc-Flag-tagged PGC-1α (Fig. 4A). As shown in Fig. 4B, PGC-1α interacted with the TAD of AR. Next, GST-PGC-1α and its series of deletion mutants were used for the GST pull-down assay with nuclear extracts of LNCaP (Fig. 4C). As shown in Fig. 4D, AR interacted with the NTD of PGC-1α.

TAD of AR is indispensable for augmentation of AR-transcriptional activity by PGC-1α

Because PGC-1α was found to interact with the TAD of AR, we investigated whether the transcriptional ability of TAD-deleted AR was affected by PGC-1α manipulation. First, we constructed an AR-GFP 508-920 plasmid expressing GFP-tagged TAD-deleted AR protein. It is known that the TAD of AR interacts with a C-terminal ligand-binding domain (LBD) and can form a homodimer in a head-to-tail fashion binding to the ARE drive the expression of its target genes (36). Therefore, a coimmunoprecipitation assay was performed using AR-GFP 508-920 and PGC-1α-Myc-Flag expression plasmids in LNCaP cells. An interaction between AR-GFP 508-920 and endogenous AR protein was found to be augmented by PGC-1α expression, probably through an interaction between the LBD of AR-GFP 508-920 and the TAD of endogenous AR (Fig. 5A). To confirm augmentation of an interaction between TAD and LBD of AR, we constructed pCMV-AR 1-567 plasmid expressing LBD− and most part of DBD-deleted AR protein, and performed coimmunoprecipitation assay using PC-3 cells transfected with AR-GFP 508-920, pCMV-AR 1-567, and PGC-1α-Myc-Flag expression plasmids. The result clearly showed that PGC-1α expression increased an interaction between TAD and LBD of AR (Fig. 5B). Then, a luciferase reporter assay was performed to confirm the effects of PGC-1α against AR-GFP 508-920. Although the transcription of PSA was increased in PC-3 cells transfected with the PGC-1α-Myc-Flag expression plasmid, full-length AR-GFP expression plasmid, and PSA reporter plasmid, the transcriptional ability of AR-GFP 508-920 was not affected by PGC-1α expression (Fig. 5C). Similar results were obtained when the MMTV-reporter plasmid was used (Fig. 5D).

Knock-down of PGC-1α decreases the DNA-binding ability of AR

Disruption of the interaction between the TAD and LBD of AR has a potential to inhibit the DNA binding ability of AR (37, 38). Because PGC-1α interacted with