Table 1. Demographic and Baseline Characteristics of Patients in the Original Study Population*

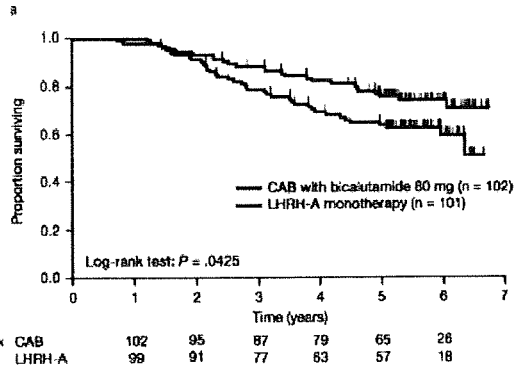
Characteristic	No. of Patients (%)	
	CAB With Bicalutamide 80 mg	LHRH-A Monotherapy
All patients	102 (100)	101 (100)
Age, y		
<75	53 (52)	50 (49.5)
≥75	49 (48)	51 (50.5)
PSA level, ng/mL		
<60	40 (39.2)	37 (36.6)
≥60	62 (60.8)	64 (63.4)
Histological grade		
Well differentiated	3 (2.9)	6 (5.9)
Moderately differentiated	52 (51)	55 (54.5)
Poorly differentiated	47 (46.1)	40 (39.6)
Clinical stage		
C,D1	59 (57.8)	57 (56.4)
D2	43 (42.2)	44 (43.6)
Disease stage		
T2	3 (2.9)	1 (1)
T3	83 (81.4)	77 (76.2)
T4	16 (15.7)	23 (22.8)
Nodal stage		
N0	74 (72.5)	63 (62.4)
N1	28 (27.5)	38 (37.6)
Metastatic stage		
M0	59 (57.8)	58 (57.4)
M1	43 (42.2)	43 (42.6)
Location of metastases†		
Bone	40 (39.2)	40 (39.6)
Lymph node	28 (27.5)	38 (37.6)
Other	2 (2)	3 (3)
LHRH-A		
Goserelin acetate	77 (75.5)	79 (78.2)
Leuprorelin acetate	25 (24.5)	22 (21.8)
Performance status		
0, 1	99 (97.1)	99 (98)
2	3 (2.9)	2 (2)

CAB indicates combined androgen blockade; LHRH-A, luteinizing hormone-releasing hormone agonist; PSA, prostate-specific antigen.
*See Usami 2007.[9]
†Some patients had metastases at more than 1 site.

63.4% for LHRH-A monotherapy. The results from the subgroup analysis according to disease stage (stage C/D1 and stage D2) are shown in Figure 3.

Cause-specific Survival

CAB also was associated with fewer cause-specific deaths compared with LHRH-A monotherapy (14 deaths vs 22 deaths, respectively). The difference in cause-specific survival between the 2 groups was not significant (Cox regression analysis: HR, 0.79; 95% CI, 0.55-1.11; $P = .1703$; log-rank test: $P = .0918$) (Fig. 4).



	CAB	LHRH-A
Cause-specific death	14	22
Other death	12	16

b

Overall survival		
	Hazard ratio (95%CI)	P-value
Treatment		
LHRH-A	1	
CAB	0.78 (0.60-0.99)	.0498
Clinical stage		
C, D1	1	
D2	2.68 (1.55-4.76)	0004
Age		
< 75 years	1	
≥ 75 years	0.83 (0.49-1.37)	4671
PS		
0 or 1	1	
2	2.46 (0.39-8.19)	2779
PSA at diagnosis		
< 60 ng/mL	1	
≥ 60 ng/mL	1.02 (0.56-1.85)	9428
Histological grade		
Well or moderate	1	
Poor	1.27 (0.76-2.11)	3628
LHRH-A		
Goserelin acetate	1	
Leuprorelin acetate	1.04 (0.76-1.36)	.7720

FIGURE 2. Overall survival was analyzed at a median follow-up of 5.2 years: (a) Kaplan-Meier curve by randomized treatment; (b) results of multivariate analysis. CAB indicates combined androgen blockade; LHRH-A, luteinizing hormone-releasing hormone agonist; CI, confidence interval; PS, performance status; PSA, prostate-specific antigen.

Overall Survival and Prostate-Specific Antigen Nadir Level

During the original study, PSA levels decreased to ≤1 ng/mL in 137 of 203 patients (67%). Overall survival was prolonged significantly in patients who attained a PSA nadir ≤1 ng/mL compared with those who did not (death rate: 19.7% [27 of 137 patients] vs 56.1% [37 of 66 patients], respectively; HR, 0.34; 95% CI, 0.20-0.59; $P = .0001$; log-rank test: $P < .0001$) (Fig. 5). In total, 75%

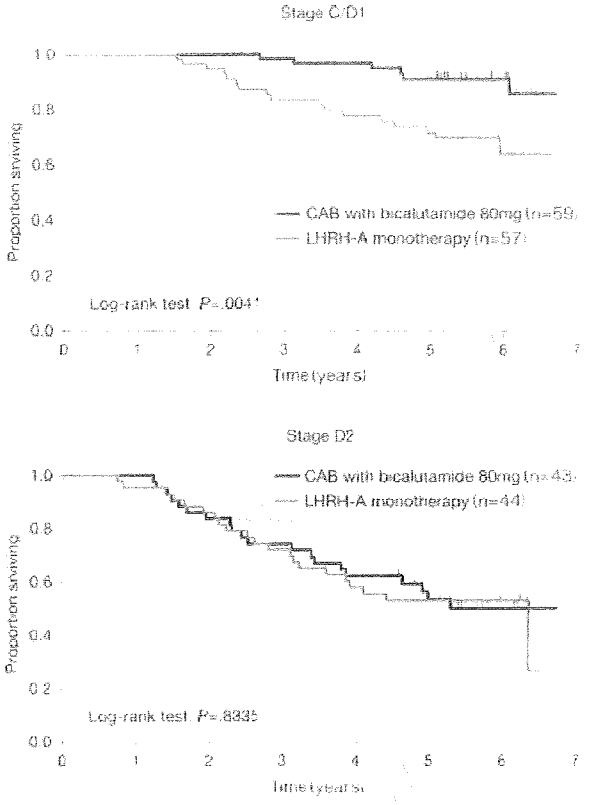
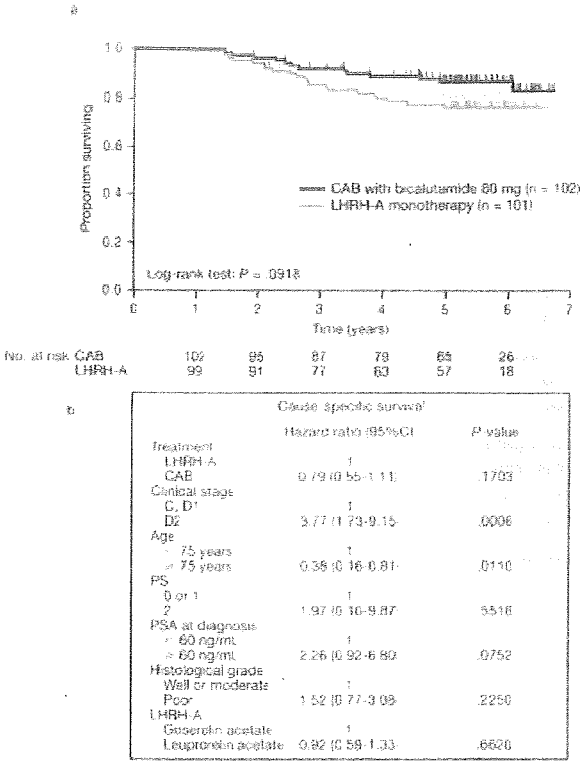


FIGURE 3. Overall survival was analyzed according to disease stage in patients with (*Top*) stage C/D1 disease and (*Bottom*) stage D2 disease at a median follow-up of 5.2 years. CAB indicates combined androgen blockade; LHRH-A, luteinizing hormone-releasing hormone agonist.



b

Cause-specific survival	Hazard ratio (95% CI)	P value
Treatment		
LHRH-A	1	
CAB	0.79 (0.55-1.11)	.1703
Clinical stage		
C, D1	1	
D2	3.77 (1.73-9.15)	.0006
Age		
< 75 years	1	
≥ 75 years	0.58 (0.16-0.81)	.0110
PS		
0 or 1	1	
2	1.97 (0.10-9.87)	.5516
PSA at diagnosis		
< 60 ng/mL	1	
≥ 60 ng/mL	2.26 (0.92-6.80)	.0752
Histological grade		
Well or moderate	1	
Poor	1.52 (0.77-3.09)	.2250
LHRH-A		
Goserelin acetate	1	
Leuprorelin acetate	0.82 (0.59-1.33)	.6626

FIGURE 4. Cause-specific survival was analyzed at a median follow-up of 5.2 years: (a) Kaplan-Meier curve by randomized treatment; (b) results of multivariate analysis. CAB indicates combined androgen blockade; LHRH-A, luteinizing hormone-releasing hormone agonist; CI, confidence interval; PS, performance status; PSA, prostate-specific antigen.

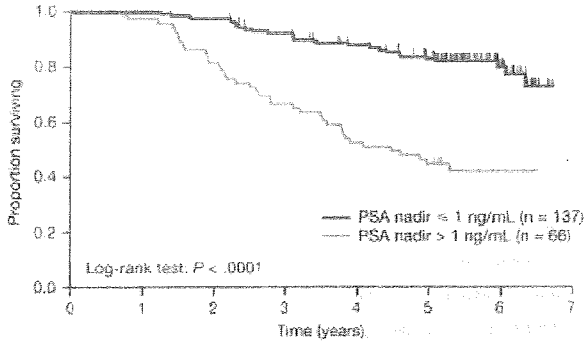
of patients who achieved a PSA nadir $\leq$1 ng/mL had reached that level within the first 192 days of the study (Fig. 6). During randomized treatment, PSA nadir concentrations $\leq$1 ng/mL were achieved by 83 of 102 patients (81.4%) who received CAB and by 34 of 101 patients (33.7%) who received LHRH-A monotherapy (Fisher exact test: $P < .001$) (Table 2).

DISCUSSION

In this report, long-term follow-up data from a phase 3 study of CAB with bicalutamide 80 mg versus LHRH-A monotherapy alone have demonstrated a significant overall survival advantage in favor of CAB. The overall survival advantage for CAB is consistent with previous observations from this study of prolonged time to treatment failure and time to progression.[9] In particular, the magnitude of the reduction in risk of death reported for CAB with bicalutamide 80 mg (22%) concurs with that estimated by Klotz and colleagues[6] for CAB with bicalutamide 50 mg (20%). In most countries, bicalutamide is licensed at a dose of 50 mg daily for use in CAB. However, based on pharmacokinetic and pharmacodynamic data,[13] the only approved dose of bicalutamide in Japanese men is 80 mg per day for monotherapy. A previous pilot study of LHRH-A in combination with bicalutamide 80 mg identified no safety concerns[14]; therefore, the 80 mg dose of bicalutamide is used both for monotherapy and for CAB in Japan. A comparison between our study results and Western CAB data with bicalutamide 50 mg should be considered as the next step.

In total, 30 patients in the CAB group experienced disease progression during the original phase 3 study, including at least 18 patients who were observed for antiandrogen withdrawal syndrome, and 7 patients (39%) responded (median response duration, 58 weeks).[9] Of 57

a



	CAB	LHRH-A
PSA nadir ≤ 1 ng/mL	85*	52*
PSA nadir > 1 ng/mL	17	49

*These numbers include the patients who achieved PSA nadir ≤ 1 ng/mL after randomized treatment

b

Overall survival (using PSA nadir)		
	Hazard ratio (95%CI)	*P* value
PSA nadir		
> 1 ng/mL	1	
≤ 1 ng/mL	0.34 (0.20-0.59)	.0001
Treatment		
LHRH-A	1	
CAB	0.82 (0.48-1.40)	.4710
Clinical stage		
C, D1	1	
D2	2.22 (1.31-3.78)	.0032
Age		
< 75 years	1	
≥ 75 years	0.80 (0.48-1.33)	.3875
PS		
0 or 1	1	
2	2.76 (0.66-11.73)	.1640
Histological grade		
Well or moderate	1	
Poor	1.01 (0.99-1.04)	.3673
LHRH-A		
Goserelin acetate	1	
Leuprorelin acetate	0.81 (0.45-1.48)	.4965

FIGURE 5. The relation between prostate-specific antigen (PSA) nadir and overall survival was analyzed at a median follow-up of 5.2 years: (a) Kaplan-Meier curve by PSA nadir; (b) results of multivariate analysis using PSA nadir level as a covariate. CAB indicates combined androgen blockade; LHRH-A, luteinizing hormone-releasing hormone agonist; CI, confidence interval; PS, performance status.

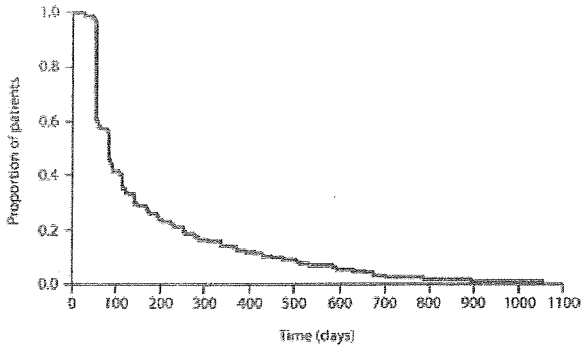


FIGURE 6. This graph illustrates the time to achieve a prostate-specific antigen level ≤1 ng/mL (n = 137).

Table 2. Patients Who Achieved Defined Prostate-Specific Antigen Nadir Levels During Randomized Treatment in the Original Phase 3 Study

PSA Nadir, ng/mL	No. of Patients	
	CAB With Bicalutamide, 80 mg (n = 102)	LHRH-A Monotherapy (n = 101)
>4	6	42
From ≤4 to >1	13	25
From ≤1 to >0.2	9	20
≤0.2	74	14

PSA indicates prostate-specific antigen; CAB, combined androgen blockade; LHRH-A, luteinizing hormone-releasing hormone agonist.

patients in the LHRH-A monotherapy group who had disease progression during the phase 3 study, at least 40 patients subsequently received second-line CAB with bicalutamide 80 mg, and 31 patients (78%) responded to that treatment (median response duration, 40 weeks).[9] Currently, CAB is used widely in Japan and accounts for approximately 70% of primary hormone therapy for prostate cancer.[15] For patients who receive LHRH-A monotherapy as initial treatment and subsequently experience disease progression, second-line therapy is usually the addition of an antiandrogen to their regimen (deferred CAB therapy). Because the majority of patients who progressed in the LHRH-A monotherapy group received second-line CAB therapy, our study can be considered a comparison of immediate versus deferred CAB. Consequently, results from the current follow-up study suggest that immediate CAB may be superior to deferred CAB in terms of prolonging overall survival.

Although it was not predefined in the protocol, a subgroup analysis of overall survival by clinical stage was performed for reference. Consequently, the difference in overall survival between CAB and LHRH-A monotherapy was greater in the patients who had stage C/D1 disease. In the original phase 3 study, the same tendency was observed in the time to progression for CAB in the patients who had stage C disease, suggesting that the long-term prognosis for patients who have stage C disease and

are treated with CAB can be expected to be markedly better than that of the patients who are treated with LHRH-A monotherapy.[9] Sylvester et al. reported that, among patients with stage D2 prostate cancer who either underwent orchiectomy or received CAB (goserelin + flutamide), the survival benefit of CAB was greater for patients who had mild bone metastasis than for those who had more advanced disease.[16] On the basis of these results, the survival benefit of CAB versus LHRH-A monotherapy is expected to be much greater for patients who have early stage disease.

Our follow-up study revealed no significant difference in cause-specific survival between CAB and LHRH-A monotherapy ($P = .0918$). This is unsurprising, because the analysis lacked statistical power to detect a significant difference in cause-specific mortality in light of the low number of prostate cancer-related deaths (14 patients on CAB and 22 on LHRH-A monotherapy). To observe a treatment difference in cause-specific survival, longer follow-up or a larger patient population may be necessary.

Previous studies have suggested that the normalization of PSA by hormone therapy may be associated with prolonged time to progression and survival.[17,18] Because of an exploratory multivariate analysis with PSA cutoff levels of 4 ng/mL, 2 ng/mL, 1 ng/mL, 0.5 ng/mL, and 0.2 ng/mL, the use of 1 ng/mL produced a stable and better fitting model with a small P value and variance of estimated values. Therefore, we used a cutoff level of 1 ng/mL for our analysis of overall survival. Data from our study indicated that patients who attained a PSA nadir $\leq$1 ng/mL survived significantly longer than patients who had PSA levels that remained >1 ng/mL. It also was apparent that patients who received CAB achieved lower PSA nadir levels than patients who received LHRH-A monotherapy. It is noteworthy that PSA levels fell below 0.2 ng/mL (the detection limit) in 89% of patients who had a PSA nadir $\leq$1 ng/mL in the CAB group, compared with only 41% of patients who had a PSA nadir $\leq$1 ng/mL in the LHRH-A monotherapy group. Therefore, the PSA reduction associated with CAB appears to be important clinically in terms of prolonging overall survival. Among the patients who achieved a PSA nadir $\leq$1 ng/mL in the original study, 75% had attained this nadir within approximately 6 months (192 days). This suggests that, if no therapeutic effect is observed within the first 6 months of treatment, then a change of therapy should be considered.

A primary obstacle to the wider use of CAB is the potential for increased side effects and costs compared with castration alone. Indeed, compared with castration alone, CAB with flutamide is associated with an increased incidence of gastrointestinal disorders and hepatotoxicity, whereas CAB using nilutamide is associated with an increased incidence of visual disorders.[19] However, in the phase 3 study of CAB with bicalutamide 80 mg versus LHRH-A monotherapy, there was no difference between the 2 treatment arms regarding the percentage of withdrawals because of adverse drug reactions (primary safety endpoint) or adverse drug reaction profiles.[8,9] QoL was assessed as a secondary endpoint in this study using the Japanese version of the Functional Assessment of Cancer Therapy-Prostate questionnaire.[20] These data demonstrated that, compared with LHRH-A monotherapy, CAB with bicalutamide did not reduce overall QoL and provided an early improvement in QoL related to micturition disorder and pain.[21]

Nishimura and colleagues[22] conducted a cost-effectiveness analysis of CAB with bicalutamide 80 mg based on efficacy data from the phase 3 study and medical costs in Japan. Those authors concluded that CAB was a cost-efficient therapy with an incremental cost effectiveness ratio of approximately ¥1,560,000 (approximately $14,000 in US dollars). This is consistent with results from similar analyses conducted in the United States. For example, Ramsey and colleagues[23] demonstrated that the incremental cost per quality-adjusted life-year (QALY) gained for CAB with bicalutamide 50 mg versus CAB with flutamide was $22,000 at 5 years and $16,000 at 10 years. Likewise, Penson and colleagues[24] estimated that the cost per QALY of CAB with bicalutamide 50 mg was $33,677 and $20,053 at 5 years and 10 years, respectively, compared with castration alone. These studies support CAB with bicalutamide as a cost-effective treatment strategy for patients with advanced prostate cancer.

In conclusion, the long-term follow-up of the first double-blind controlled study to directly compare CAB with bicalutamide 80 mg versus LHRH-A monotherapy has demonstrated a statistically significant overall survival benefit in favor of CAB. The advantage in overall survival, together with the previously reported significant improvements in time to treatment failure and time to

progression, which were achieved without reducing tolerability, indicate that CAB with bicalutamide is a recommendable first-line therapy option for patients with locally advanced or metastatic prostate cancer.

Conflict of Interest Disclosures

Financial sponsorship for this study and publication was provided by the Advanced Clinical Research Organization (a nonprofit organization).

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ORIGINAL ARTICLE

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

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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.
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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.

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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

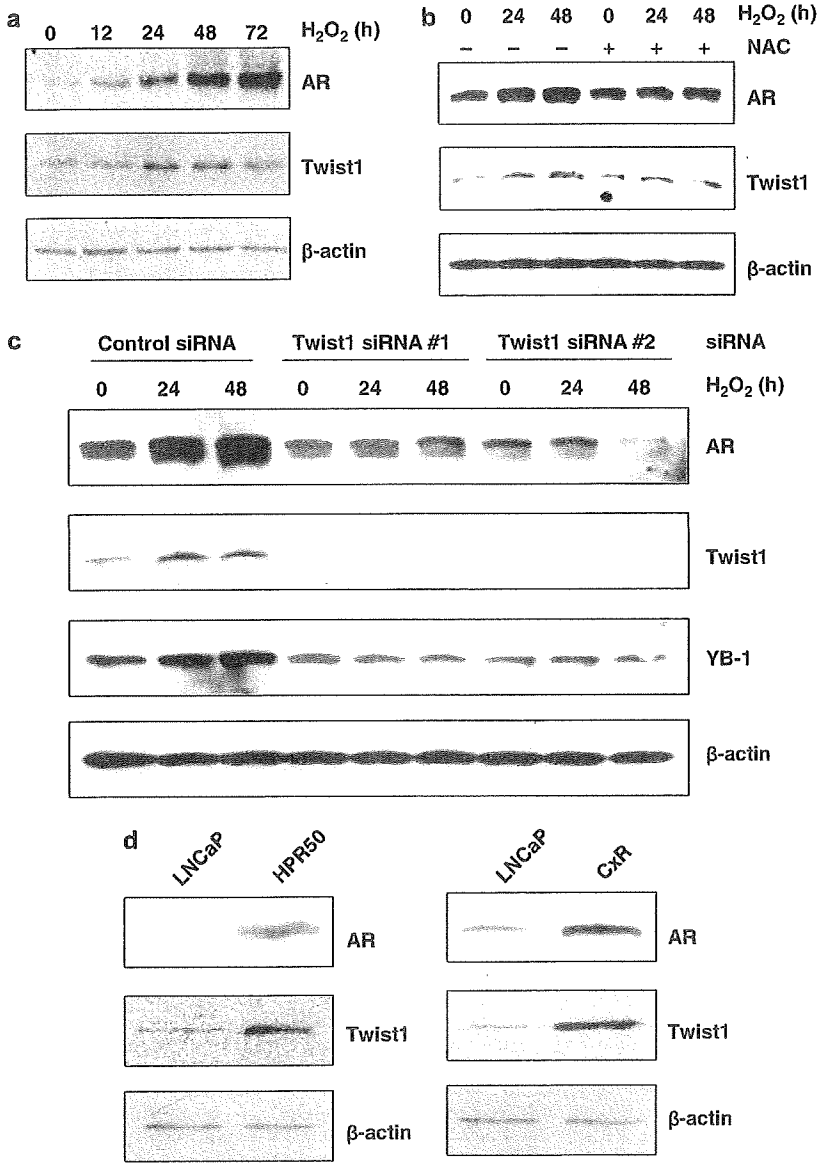


Figure 1 Twist1 and androgen receptor (AR) expressions are both upregulated in response to hydrogen peroxide and in both HPR50 and CxR cells. (**a**) LNCaP cells were cultured with 10 μM of hydrogen peroxide for 2 h, and the medium was refreshed. At the indicated times after the addition of hydrogen 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

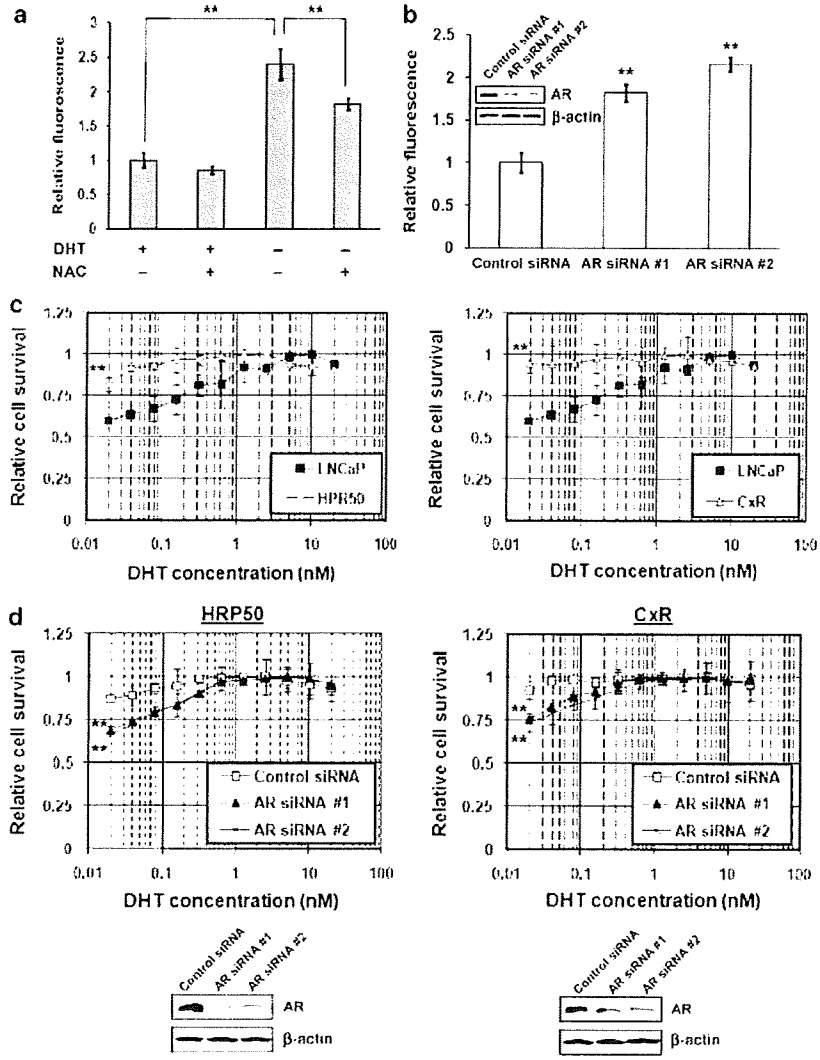


Figure 2 Blockade of androgen/androgen receptor (AR) signaling increases intracellular reactive oxygen species (ROS) level and both HPR50 and CxR cells are resistant to androgen depletion through AR overexpression. (**a**) LNCaP and CxR cells (2×10^3) were seeded into 96-well plates and incubated. On 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 (2×10^3) 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 (2×10^3) 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 (2×10^3) 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

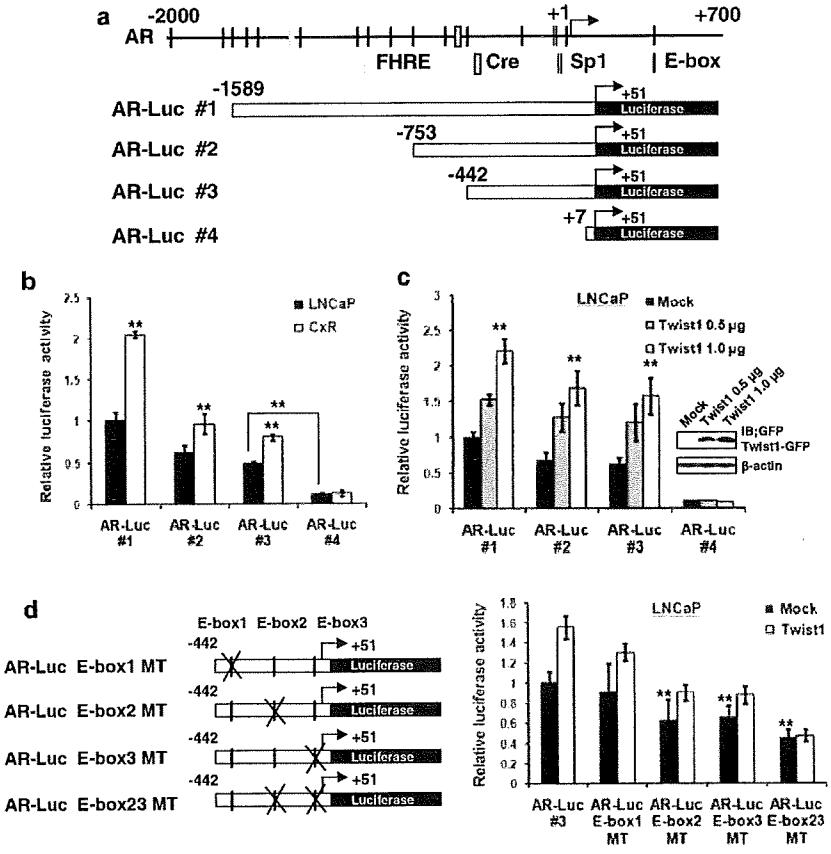


Figure 3 Twist1 upregulates androgen receptor (AR) transcription E-box-dependently. (**a**) Schematic representations of the promoter region of the AR gene and AR-Luc #1–#4 used in (**b**) and (**c**) are shown. (**b**) LNCaP and CxR cells were cotransfected with 1.0 μg of the various AR-Luc plasmids 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).

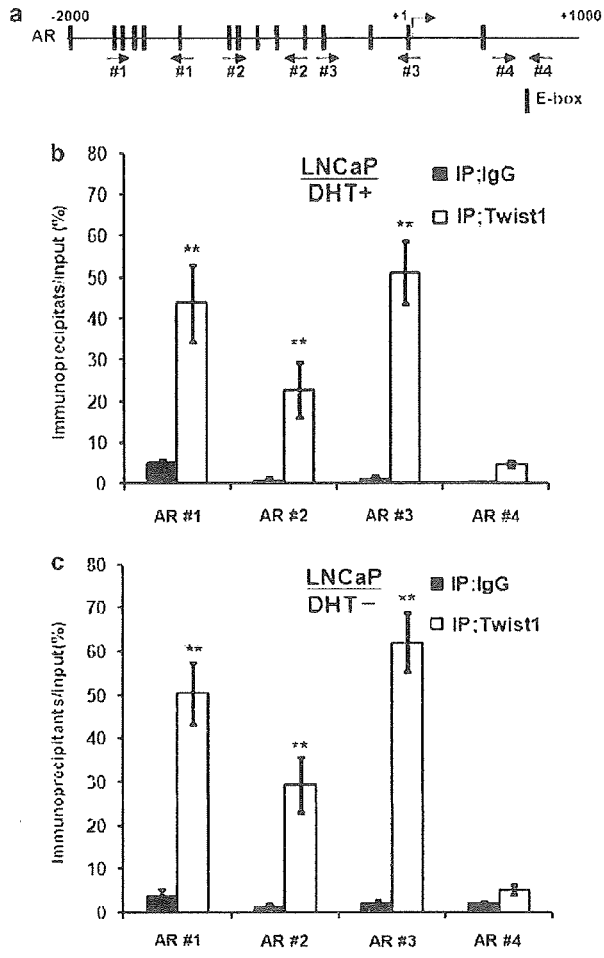


Figure 4 Twist1 binds to E-boxes in the promoter region of androgen receptor (AR). (**a**) Schematic representation of the promoter region and 5′ end of the AR gene. Black boxes, E-boxes (5′-CANNTG-3′); arrows, primer pairs used in chromatin immunoprecipitation (ChIP) assays. (**b**) and (**c**) ChIP 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

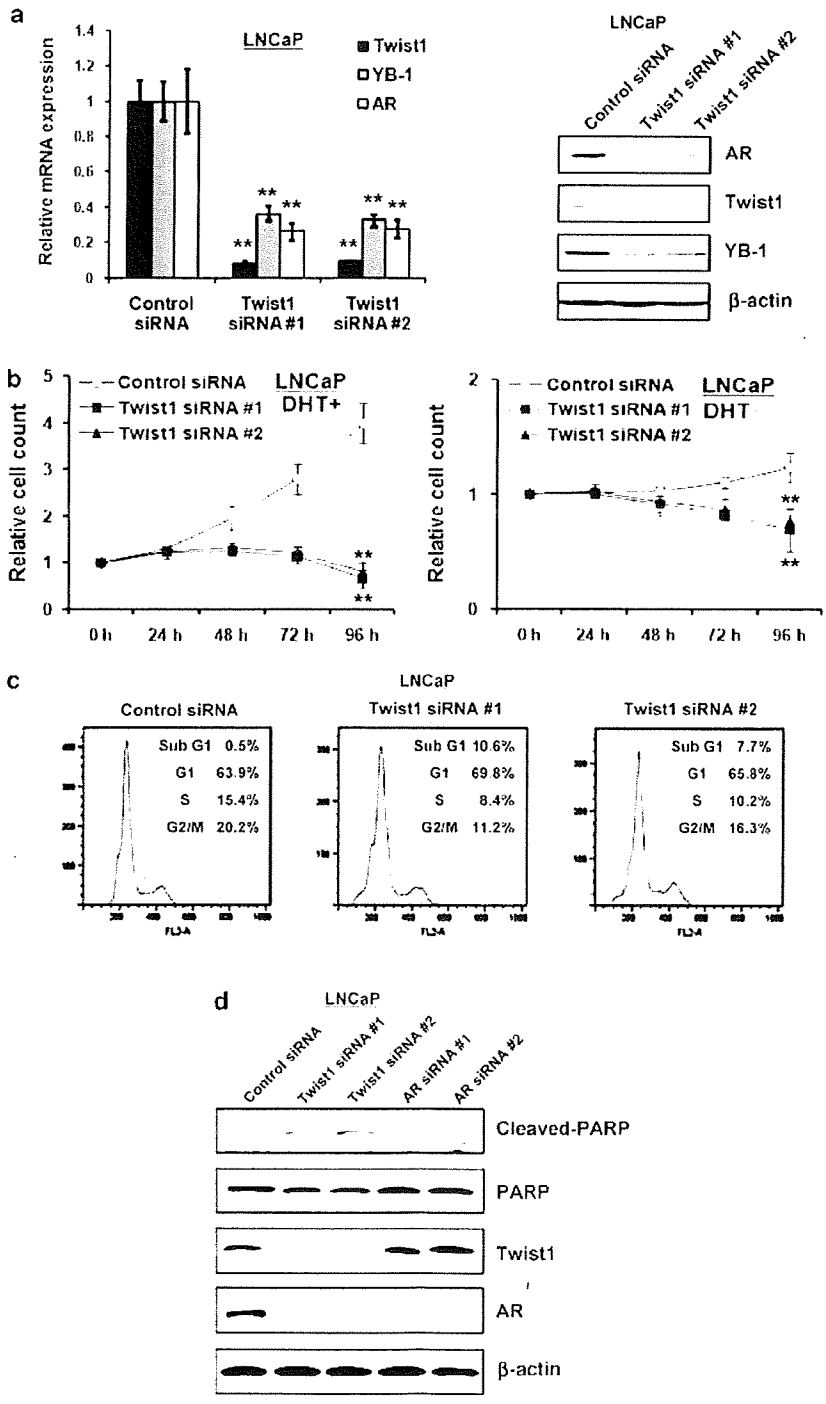
As Twist1 was found to regulate the expression of AR as well as YB-1 in PCa cells, we investigated whether Twist1 knockdown-induced cell growth suppression resulted from regulation of YB-1 or AR expression. Although Twist1 knockdown significantly reduced the colony-formation ability of LNCaP cells, AR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

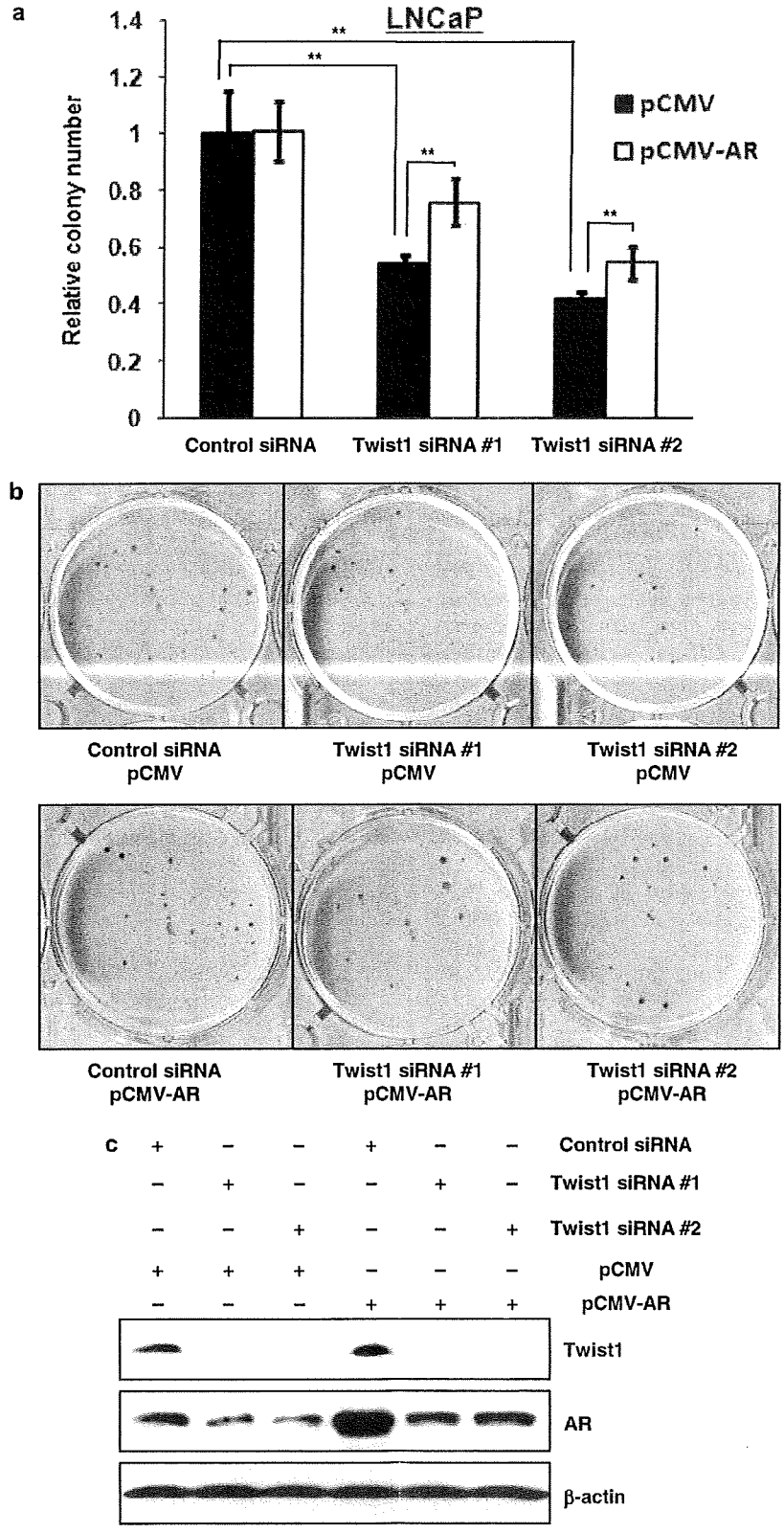
recovered the colony-formation ability up to about 50%, indicating that the effect of Twist1 knockdown on cell growth of AR-expressing cells was partially due to downregulation of AR expression (Figures 6a and b). Western blotting analysis confirmed that siRNAs and expression plasmids functioned properly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

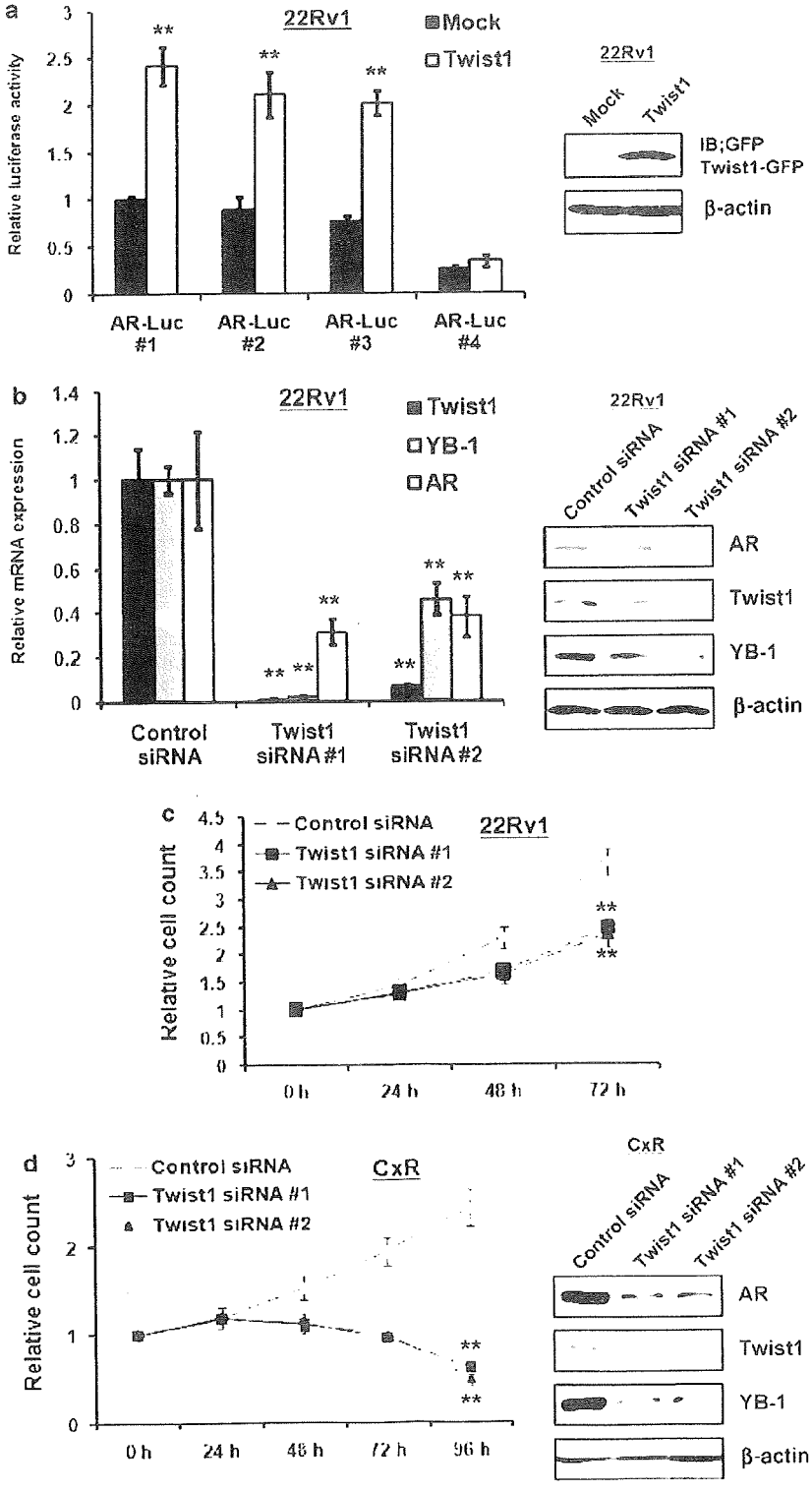
To the best of our knowledge, AR expression is regulated by various transcription factors, such as Sp1, Foxo3a, CREB (cAMP-response element-binding protein), TCF (T-cell factor) and NFκB (Lee and Chang, 2003; Reddy *et al.*, 2006). However, the regulation of AR expression in CRPC remains unclear. Twist1 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 (2×10^3) 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 (3×10^3) 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 (2.5×10^2) 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.

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

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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

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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.