HOXB13-mediated suppression of p21$^{WAF1/CIP1}$ regulates JNK/c-Jun signaling in prostate cancer cells

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Abstract. Many prostate cancer (PCa) patients die of recurrent disease due to the emergence of hormone-independent cancer cells of which the mechanism is not fully understood. Our previous studies demonstrated that most castration-resistant prostate cancers (CRPC) overexpress the HOXB13 transcription factor to confer positive growth signals. Since HOXB13 also suppresses p21$^{WAF1/CIP1}$ (p21) expression, we studied the correlation between HOXB13 and p21 in selected samples of PCa. While there was no statistically significant correlation between expression of HOXB13 and p21, HOXB13-deficient tumors had three times higher odds for expressing p21 than HOXB13-positive tumors. Moreover, CRPC showed more negative correlation than hormone-dependent PCa (HDPC). Further *in vitro* proliferation assay demonstrated that androgen did not affect the growth-suppressive function of p21 in androgen-dependent PCa cells, suggesting that p21 seems to override the growth-promoting function of androgen and suppression of p21 expression by HOXB13 is an important step in PCa cell survival under no androgen influence. HOXB13 also inhibited AP-1 signals via suppressed expression of JNK/c-Jun. While HOXB13 suppressed p21 expression via regulation of JNK signals, alteration of p21 expression also affected c-Jun and AP-1 activity. Taken together, overexpression of HOXB13 in CRPC is an important step in avoiding the growth-suppressive effect of p21 in a harsh condition such as an androgen-deprived environment.

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Introduction

HOXB13 is one of the 39 HOX homeodomain proteins in humans and manifests tissue-specific expression in prostate and rectum (1). Overexpression of HOXB13 has been reported in various cancers of the prostate, breast and ovary (2-4). However, the biological role of HOXB13 is not clearly understood, mainly due to the lack of phenotypes of genetically altered HOXB13 knockout mouse models (5,6). In our previous reports, HOXB13 was predominantly overexpressed in castration resistant prostate cancers (CRPC) to provide positive signals for cancer cell growth under harsh androgen-deprived conditions (7). The growth promoting function of HOXB13 seems to be mediated through the regulation of E2F1/RB pathways and intracellular zinc concentration (7,8). At the same time, we demonstrated that HOXB13 inhibits expression of p21$^{WAF1/CIP1}$ protein (p21) in prostate cancer cells grown under androgen-deprived conditions without gaining a clear mechanical understanding.

p21 is involved in the control of cell proliferation and differentiation. p21 is an effector of the p53 protein and it negatively regulates the cell cycle by inhibiting cyclin-dependent kinases (CDK) that directly inhibits the activity of cyclin-CDK2 and cyclin-CDK4 complexes. Thus, p21 is considered as a regulator of cell cycle progression at the G1/S phase checkpoint (9). The p21 gene has p53 transcriptional regulatory motifs, and the cells lacking functional p53 express very a low level of p21, suggesting that p53 directly regulates p21 expression. On the contrary, there are a number of agents that activate p21 transcription independent of the p53 pathway (10).

To explore HOXB13-mediated growth promotion of prostate cancer cells under androgen-deprived conditions, we studied the effect of p21 regulated by HOXB13. In the present study, HOXB13 inhibited both p21 expression and JNK signals. While it is known that c-Jun activates p21 expression, this study also demonstrated that HOXB13-mediated p21 inhibition affects JNK/c-Jun activity, suggesting that there seems to be reciprocal communication between p21 and JNK/c-Jun-mediated signals.

Table I. Prostate cancer patient cohort (N=28).

Characteristics	Data
Age median	68 (56-82)
Preoperative PSA (ng/ml)	
<10	12
10-20	6
>20	10
Pathological stage	
Stage 2	22
Stage 3	6
Invasion	
Extraprostatic invasion	8
Seminal vesicle invasion	2
Angiolymphatic invasion	8
Perineural invasion	13
Unknown	8
Castration resistant disease	8
Gleason score	
≤6	6
7	8
≥8	14

Materials and methods

Patient cohort and immunohistochemistry. Twenty-eight tumor specimens from patients were included in the present study, including 8 specimens of castration-resistant prostate cancers (CRPC). These paraffin-embedded human primary PCa specimens were acquired from patients who provided informed consent at Chonnam National University Hospital between 1997 and 2005. The tumors used in the present study were acquired by transurethral resection of the prostate. All cases had clinical follow-up of at least 10 years (Table I). Immunohistochemical studies were performed on both paraffin-embedded tissue sections of human hormone dependent prostate cancers (HDPC, 20 specimens) and CRPC (8 specimens) as previously described (7). The antibodies used were anti-p21 monoclonal antibody (DCS60; Cell Signaling Technology, Danvers, MA, USA) and anti-HOXB13 antibodies. The stained slides were evaluated by two different investigators, including a pathologist, who were blinded to the patient's clinical features. The intensity of the staining was classified into one of the two grades (+, positive; -, negative).

Plasmids and reagents. The synthetic testosterone R1881, was purchased from NEN Life Science (Boston, MA, USA) and used at a final concentration of 10 nM (nmol/l). Both fetal bovine serum (FBS) and charcoal dextran-treated (CDT) FBS were purchased from Invitrogen (Carlsbad, CA, USA). The pFLAG-p21 and pCDNA-FLAG-p21 were generated by PCR cloning. The pFLAG-HOXB13 was previously described (11). Antibodies to JNK, phosphorylated JNK (Thr183/185), ERK, AKT, STAT3 and p53 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG M5, c-Fos, c-Jun, phosphorylated c-Jun (Ser73) and β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against $p21^{WAF1/CIP1}$ were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The dominant negative c-Jun mutant was kindly provided by Dr Kyung Keun Kim (Chonnam National University, Gwangju, Korea). An inhibitor of c-Jun N-terminal kinase (SP600125) was obtained from Sigma-Aldrich. Reporter vectors, including AP1-luc and c-Jun-luc, were purchased from Stratagene (Santa Clara, CA, USA).

Cell culture. Human prostate cell lines, including LNCaP and PC3, were routinely cultured in RPMI media (Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS at 37˚C in an atmosphere containing 5% CO_2. HOXB13-suppressed LNCaP and HOXB13-overexpressed PC3 cells have been previously described (7). Wild-type HCT116-p21(+/+) and HCT116-p21(-/-) were kindly provided by Dr Bert Vogelstein. All cultures were fed with fresh medium every 3-4 days.

MTT in vitro cell proliferation assay. To determine the hormone effect, the cells were grown under 5% CDT-FBS for two days. The next day, cells were treated with either R1881 or ethanol and were grown for up to 7 days. After incubation for 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide *in vitro* proliferation assay was performed as previously described (11). Briefly, the cells were stained with 100 μl of 5 mg/ml MTT (Sigma-Aldrich) solution and incubated for 4 h at 37˚C. The reaction was stopped and then the absorbance at 570 nm was measured using a microplate reader with SoftMax PRO software (Molecular Devices, Sunnyvale, CA, USA). Densitometric values were analyzed with the Student's t-test, using GraphPad Prism software (San Diego, CA, USA).

Western blotting. The cells were grown to 80% confluence and then lysed in protein extraction RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) containing a cocktail of protease inhibitors and phosphatase inhibitors. Twenty micrograms of total cell lysates were loaded onto a 10% Bis-Tris gel and separated using the electroporation system (Bio-Rad Laboratories, Hercules, CA, USA). After the proteins were transferred to a PVDF membrane, primary antibodies were applied, followed by incubation with horse peroxidase-conjugated secondary antibodies. The blots were developed by the ECL detection system (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Luciferase assay. Approximately $1x10^5$ cells were plated in a 24-well plate 16 h before transfection. Each transfection was carried out using Lipofectamine 2000 (Invitrogen) with 0.1 μg of reporter and 2 ng of *Renilla* luciferase reporter as described in the manufacturer's protocol. Six hours later, the cells were washed and fed with medium containing 5% FBS. Addition of SP600125 was performed 18 h post-transfection. Subsequently, the cells were washed with PBS, harvested for 24 h, lysed with 100 μl of passive lysis buffer. Then luciferase activities were assayed as relative light units (RLU) using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) as per the instructions. The transfection experiments were performed in triplicate and the results are reported as the mean ± SD.

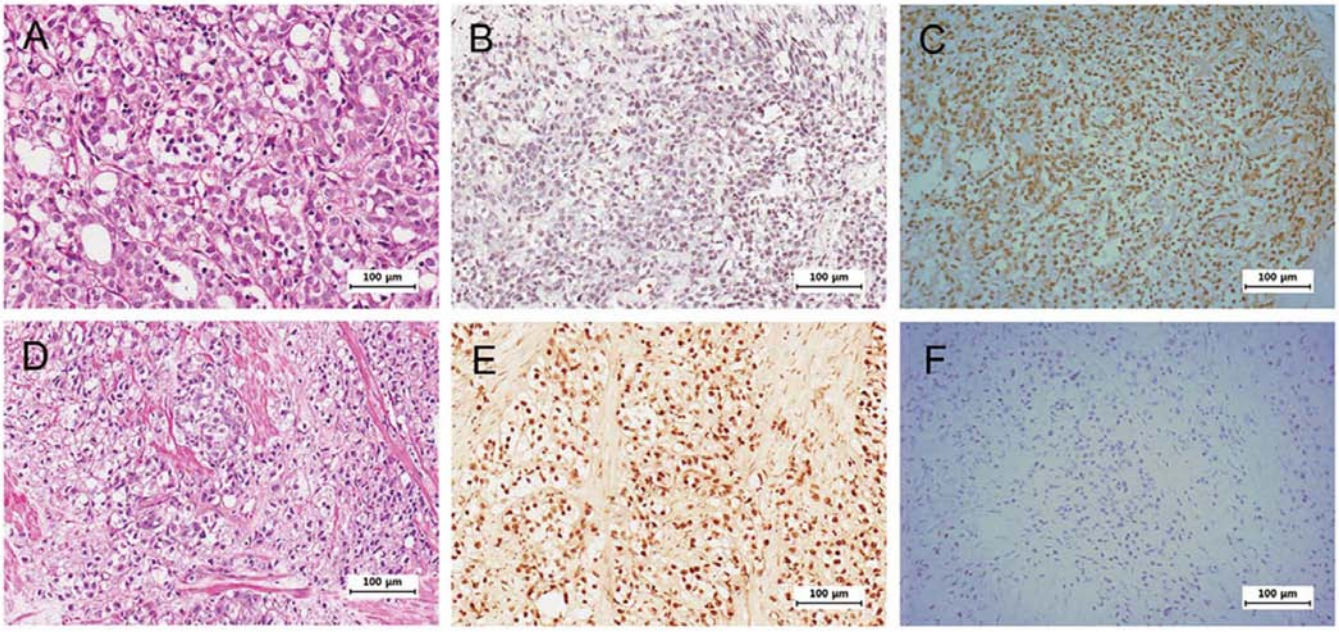

Figure 1. Photomicrographs of tissue sections of prostate cancer immunostained with either HOXB13 or p21. Selected tissue samples were stained for H&E (A and D), HOXB13 (B and E) or p21 (C and F) (original magnification, x200).

For each transfection, *Renilla* luciferase reporter was used to normalize for differences in transfection efficiency. The RLU from the experimental group were normalized to the control group and the values were represented as fold change.

Chromatin immunoprecipitation (ChIP). Chromatin immunoprecipitation was performed using antibodies against c-Jun, HOXB13 or IgG as previously described (12). The immunoprecipitated DNA was amplified by using specific primers as follows: p21 (forward, ctcacatcctccttcttcag and reverse, caca cacagaatctgactccc).

Statistical analysis. The data were expressed as mean ± standard deviation and processed using the SPSS software 17.0 (SPSS, Inc., Chicago, IL, USA). The Pearson's correlation coefficient test was used to estimate the correlation between tumors and HOXB13 and p21. Statistical significance was set at P-value <0.05.

Results

Expression of HOXB13 and p21 in castration-resistant prostate cancer. Our previous studies demonstrated that HOXB13 inhibits p21 expression without clearly indicating the underlying mechanism (7,8). Since HOXB13 is predominantly overexpressed in CRPC, we screened the expression of both HOXB13 and p21 in selected prostate cancers including CRPC in order to demonstrate correlated expression of these two proteins. Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tumor specimens. Fig. 1 shows negatively correlated expression of HOXB13 and p21 in some selected tumors. Intensity of HOXB13 staining was classified into one of the four grades (0, absent; 1, weak; 2, intermediate; and 3, strong) to statistically correlate the expression of HOXB13 and p21. We further classified the grades as follows: 0-1 as negative and 2-3 as positive. Due to the heterogeneous nature of prostate cancer and small sample size, there were no statistically significant correlations between HOXB13 and p21 expression (Table II). However, HOXB13-deficient tumors had higher odds for expressing p21 than HOXB13-positive tumors with negative p21 expression. Although the sample size was small resulting in lack of statistical significance, CRPC showed a more negative correlation between HOXB13 and p21 than HDPC.

Table II. Expression of HOXB13 and p21 in prostate cancer samples.

		p21		
Tumor type	HOXB13	Positive	Negative	Total
ADPC	Positive	2	4	6
	Negative	6	8	14
CRPC	Positive	2	3	5
	Negative	2	1	3

Expression of HOXB13 and p21 was determined by immunohistochemistry. ADPC, androgen dependent prostate cancer; CRPC, castration resistant prostate cancer.

The negative effect of p21 on cell growth is minimal in the absence of androgen. To demonstrate the growth-regulating role of p21 in the absence of androgen, androgen-responsive LNCaP PCa cells were transfected with either pCDNA or pCDNA-p21. Cells were grown up to 7 days in the presence of androgen (final concentration of 1 nM R1881) followed by MTT *in vitro* proliferation assays. As shown in Fig. 2, addition

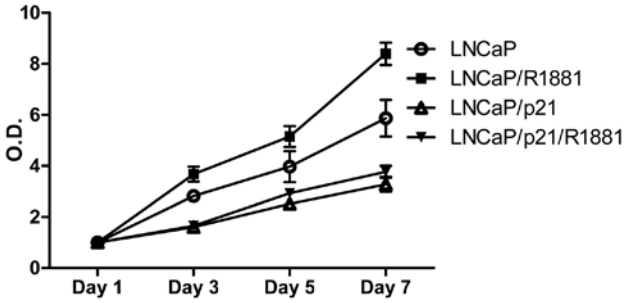

Figure 2. Role of p21 in LNCaP prostate cancer cell growth. LNCaP cells were grown under androgen-deprived conditions followed by the addition of R1881. After 1-7 days, cells were stained with MTT reagent and optical density was measured at 570 nm.

of R1881 promoted proliferation of LNCaP cells, while p21 generally suppressed cell proliferation. Noticeably, androgen did not stimulate cell proliferation in the presence of p21, suggesting that p21 suppression is an important step in survival of PCa cells under androgen-deprived conditions.

HOXB13 suppresses p21 expression and JNK-mediated signaling. To explore the detailed mechanism by which HOXB13 suppresses p21 expression, PC3 cells stably transfected with HOXB13 were used for western blot analysis. HOXB13 suppressed not only p21 expression but also c-Jun N-terminal kinase (JNK)-related proteins (Fig. 3A). There were no significant alterations in extracellular signal-regulated kinase (ERK), AKT, STAT3 and p53 proteins. While HOXB13 inhibited expression of total forms of JNK and c-Jun and phosphorylated forms of JNK and c-Jun, there was no significant difference in c-fos proteins. JNK is known to bind and phosphorylate the DNA binding protein c-Jun, which is a central component of the AP-1 family of transcription factors. AP-1 complex consists of homodimers or heterodimers of c-Jun and other transcription factors such as c-fos. Consequently, the reporter transcription assay demonstrated that HOXB13 suppressed c-Jun and AP-1 mediated transcription (Fig. 3B). Suppression of HOXB13 in LNCaP cells also indicated stimulation of AP-1 activity (Fig. 3C).

To confirm the effect of HOXB13 on c-Jun and AP-1 activity, TAM67 (dominant-negative c-Jun) and SP600125 (pharmacological inhibitor of JNK) were used in the reporter transcription assay. HOXB13 further downregulated AP-1 activity suppressed by TAM67 (Fig. 4A). Treatment with SP600125 blocked not only the activation of c-Jun but also the activation of p21 (Fig. 4B). Chromatin immunoprecipitation assay demonstrated that HOXB13 accommodated less amount of c-Jun on the p21 promoter region while HOXB13 association did not change (Fig. 4C). These results suggest that HOXB13 affects both AP-1 activity and p21 activity and there might be a mutual communication between these two proteins.

HOXB13-mediated suppression of p21 stimulates c-Jun activity. c-Jun negatively regulates transcription of p53 by binding to multiple AP-1 sites in the p53 promoter and represses its target p21 expression (13). As shown in Fig. 3A, however, HOXB13 suppressed the expression of both p21 and c-Jun, implying that there is no negative correlation between c-Jun and p21. To assess if there is c-Jun mediated regulation of p21 activity, p21-deficient HCT116 cells were tested for c-Jun

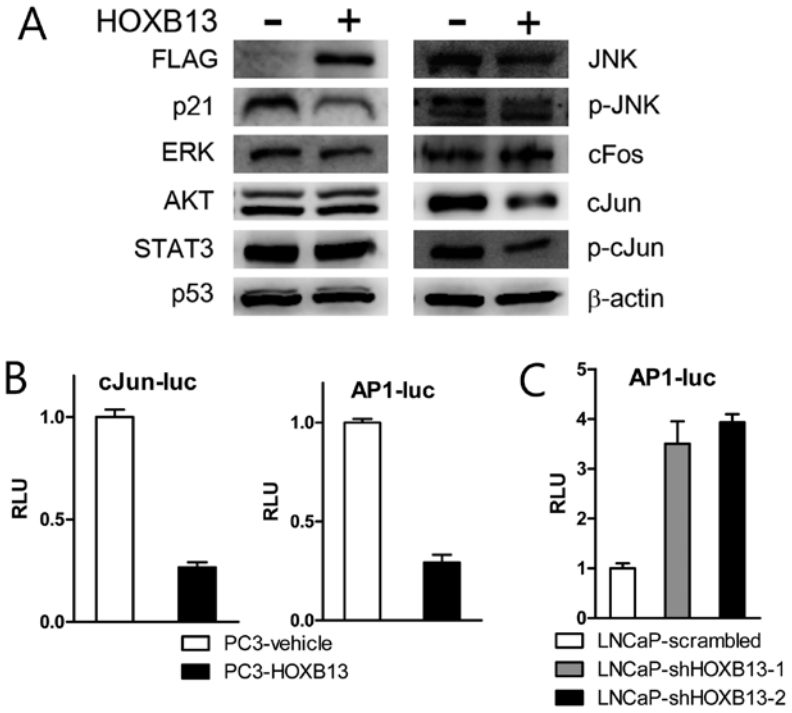

Figure 3. Effect of HOXB13 on the regulation of p21 and JNK/c-Jun. (A) Western blot analysis showed HOXB13-overexpressed LNCaP cells in addition to vehicle-transfected LNCaP. (B and C) By performing the reporter transcription assay, the effects on c-Jun and AP-1 activities were tested in the cells described. Cells were transiently transfected with 100 ng of either cJun-luc or AP1-luc and 2 ng of *Renilla*. Luciferase assays were performed 48 h post-transfection. Each bar represents the mean ± SD.

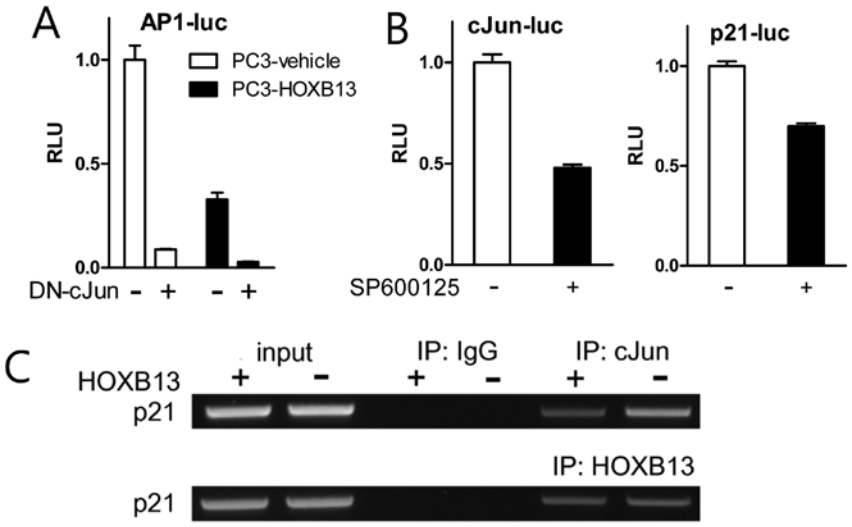

Figure 4. Association of HOXB13 with c-Jun in p21 regulation. By performing the reporter transcription assay, the effect on AP-1 was tested in HOXB13-overexpressed PC3 cells (A), and c-Jun and p21 activities were tested in PC3 cells (B). Cells were either transfected with DN-cJun or treated with SP600125. Luciferase assays were performed 48 h post-transfection. Each bar represents the mean ± SD. PC3 cells were transfected with pFLAG-HOXB13 and chromatin immunoprecipitation was performed using antibodies against c-Jun, HOXB13 or IgG. (C) The immunoprecipitated DNA was amplified by using p21 primers.

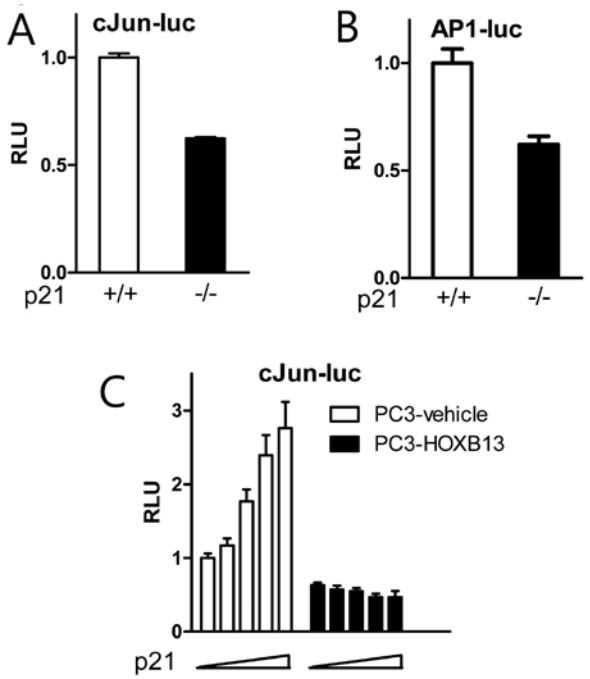

Figure 5. Mutual effects of p21 and JNK signaling. Both p21(-/-) and p21 intact HCT-116 cells were transfected with either (A) cJun-luc or (B) AP1-luc, followed by the luciferase assay. HOXB13-overexpressed cells were transfected with serial doses of pFLAG-p21 and cJun-luc and compared to vehicle-transfected PC3 cells (C).

activity. Compared to wild-type cells, p21(-/-) cells showed inhibited activity of c-Jun (Fig. 5A) and AP-1 (Fig. 5B). A further study demonstrated that exogenous p21 stimulated c-Jun activity in a dose-responsive manner in HOXB13-deficient PC3 cells, while the effect of p21 on c-Jun activity was minimal in HOXB13-overexpressed PC3 cells (Fig. 5C). These results suggest that the cell cycle inhibitor p21 may have a novel role in the regulation of c-Jun signaling in the presence of HOXB13.

Discussion

Androgens are the most dominant growth stimulatory factors for both normal and malignant prostate cells. Therefore, various androgen deprivation therapies are the most effective and final treatment for patients with recurrent PCa. The clinical response is, however, transient due to the presence of androgen-independent PCa cells. As a long-term goal with the involvement of HOXB13 homeodomain protein, we explored the mechanism by which PCa becomes androgen-independent and survives in an androgen-deprived environment. We have previously demonstrated that HOXB13 is overexpressed in most CRPC and it inhibits the expression of p21 as a result of which E2F1 signaling is activated to provide positive effects on prostate cancer cells under androgen-deprived conditions (7). More precisely, inhibition of p21 promotes the activity of CDK2 and consequently phosphorylates the RB protein to release E2F1 transcription factors. These oncogenic factors help prostate cancer cells to survive under harsh androgen-deprived conditions. This report demonstrates for the first time that HOXB13-mediated inhibition of p21 expression is also involved in JNK/c-Jun signaling. HOXB13 inhibited both c-Jun-mediated AP-1 activity and p21 promoter activity. While activation of c-Jun has been shown to promote p21 expression, we demonstrated that HOXB13-mediated p21 inhibition also reciprocally regulates c-Jun-mediated AP-1 signaling. We also showed that there is tendency for negative correlation between HOXB13 and p21 expression in PCa including CRPC and HDPC.

The p21 protein and JNK are two well-characterized cellular modulators that play important roles in regulating cell growth, differentiation and apoptosis. The p21 inhibits the cell cycle through its interaction with cyclin-CDK complexes (14). The expression of p21 is regulated via p53-dependent and p53-independent mechanisms (15,16). In response to stimuli, JNK binds and phosphorylates the c-Jun transcription factor and increases its transcriptional activity. c-Jun is a central

component of the AP-1 family of transcription factors, which consist of homodimers or heterodimers of either c-Fos or other transcription factors (17). Activation of JNK leads to both cell cycle arrest and cell cycle progression (18). Overexpression of c-Jun represses p53 and p21 expression and accelerates cell proliferation (13). c-Jun negatively regulates transcription of p53 by direct binding to a variant AP-1 site in the p53 promoter. On the other hand, JNK1 can also stabilize p21 protein by phosphorylation (19,20) and c-Jun can transactivate the p21 promoter by activation of Sp1 (21). Another study has also shown that androgen via p21 inhibits tumor necrosis factor α-induced JNK activation and apoptosis, suggesting that p21 may mediate crosstalk between androgen-androgen receptor signaling and JNK in prostate cancer cells (22). This study demonstrates that HOXB13 transcription factor inhibits the expression of p21 and JNK signaling. We have previously shown that HOXB13 suppresses the expression of p21 at the transcriptional level (7). Althogh HOXB13 inhibits AP-1 activity through the downregulation of JNK/c-Jun expression, there seems to be a sequential effect of JNK/c-Jun mediated suppression of p21 expression. In addition, our result also implies that p21 suppression by HOXB13 conversely affects JNK signaling. In conclusion, our findings demonstrate that HOXB13 inhibits both p21 expression and JNK signaling in prostate cancer cells and its regulation seems to be mainly accomplished through the mutual regulatory mechanism between p21 and JNK signaling. The present study provides insights suggesting that HOXB13 plays an important role in prostate tumorigenesis and malignant progression via the regulation of both p21 and JNK signaling and suggests that HOXB13 might be a new therapeutic target.

Acknowledgements

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