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PAX2 promoted prostate cancer cell invasion through transcriptional regulation of HGF in an *in vitro* model

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ABSTRACT

Elucidating the mechanism of prostate cancer cell invasion may lead to the identification of novel therapeutic strategies for its treatment. Paired box 2 (PAX2) and hepatocyte growth factor (HGF) proteins are promoters of prostate cancer cell invasion. We found that PAX2 protein activated the *HGF* gene promoter through histone H3 acetylation and upregulated *HGF* gene expression. Deletion analysis revealed that the region from −637 to −314 of the *HGF* gene was indispensable for *HGF* promoter activation by PAX2. This region contains consensus PAX2 binding sequences and mutations of the sequences attenuated *HGF* promoter activation. Using an *in vitro* invasion model, we found that PAX2 and HGF promoted prostate cancer cell invasion in the same pathway. Knockdown of *HGF* expression attenuated the cells' invasive capacity. Moreover, in tissue samples of human prostate cancers, HGF and PAX2 expression levels were positively correlated. These results suggested that upregulation of *HGF* gene expression by PAX2 enhanced the invasive properties of prostate cancer cells. The PAX2/HGF pathway in prostate cancer cells may be a novel therapeutic target in prostate cancer patients.

1. Introduction

Cancer cell invasion is an important step in metastasis, a process that requires multiple steps [1,2]. Uncovering the mechanism of cancer cell invasion is crucial for identifying new therapeutic targets. Previously, we reported that paired box 2 (PAX2) protein functioned as a promoter of prostate cancer cell invasion [3]. PAX2 is a member of the *PAX* gene family. There are nine *PAX* genes in humans, and they are divided into four groups according to their structure [4]. *PAX* genes code for transcription factors and they regulate the development of various tissues through their modulation of target gene expression. Functional dysregulation of *PAX* genes is also known to be associated with the progression of cancer. Hyper-expression of *PAX2* was observed in metastatic prostate cancer cells and knockdown of *PAX2* reduced the invasiveness of prostate cancer cells in *in vitro* assays [3]. The expression levels of several cadherins were downregulated by *PAX2* knockdown. Cadherin, which is expressed in the plasma membrane of cells, is a meditator of cell–cell adhesion. Dysregulation of cadherin expression occurs during cancer cell invasion [5,6]. In kidney cancer cell lines, several direct target genes of PAX2 protein, such as *ADAM10* [7] and the Wilms tumor suppressor gene 1 (*WT1*) [8] have been reported. In prostate cancer cells, genes directly targeted by PAX2 protein remain to be identified. Analysis of the target genes of PAX2 protein in prostate cancers cells may lead to better understanding of the mechanism of prostate cancer cell invasion.

Some intracellular signal pathways promoting cancer cell invasion are thought to be the same as those promoting migration and cell growth during embryogenesis and tissue repair [4,9]. Hepatocyte growth factor (HGF) is a ligand of the Met receptor, a transmembrane receptor tyrosine kinase and a growth factor for epithelial cells. HGF activates Shp2 Ras and ERK/MAPK pathways by association with the Met receptor. Receptor activation induces invasion, migration and proliferation of cells during development and regeneration, especially in the liver [9]. Dysregulation of HGF and Met signaling is observed in metastasis of cancer cells [10]. HGF is known to promote invasion of cells such as Madin–Darby canine kidney (MDCK) cell lines in *in vitro* assays [11]. Recombinant HGF treatment of MDCK cells increased the E-cadherin protein level [12]. It was reported that HGF produced by prostate stroma increased the invasiveness of epithelial prostate cancer

Abbreviations: ADAM10, ADAM metallopeptidase 10; AIPC, androgen-independent prostate cancer; AR, androgen receptor; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; MAPK, mitogen-activated protein kinase; MDCK, Madin–Darby canine kidney; *MET*, MET proto-oncogene, receptor tyrosine kinase; PAX2, paired box 2; ras, resistance to audiogenic seizures; RT-PCR, reverse transcription polymerase chain reaction; STAT3, signal transducer and activator of transcription 3; UGM, urogenital sinus mesenchyme; UGM, urogenital sinus mesenchyme; UGS, urogenital sinus; *WT1*, Wilms tumor suppressor gene 1.

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cells through its paracrine activation of the Met receptor [13]. 22Rv1 is a human androgen-independent prostate cancer (AIPC) cell line. In an autocrine manner, 22Rv1 cells produce their own HGF that induces cell growth through activation of the Met receptor [14]. In pancreatic β-cells, interleukin-6 and glucocorticoids activate the HGF promoter through enhanced binding of STAT3 to the *HGF* promoter [15]. Here, we investigated the mechanism underlying HGF expression in prostate cancer cells with the intent of better understanding the process of prostate cancer progression. We focused on the *in vitro* relationship between PAX2 and HGF using prostate cancer cell lines.

2. Material and methods

2.1. siRNA

siRNAs targeting *PAX2* (HSS107605 and HSS10706) and those targeting *HGF* (HSS179212, HSS179213 and HSS179214) were purchased from Invitrogen. Nonspecific control siRNA was also purchased from Ambion (catalogue number 12935-200) and used as siControl. Each experiment was performed using all the above siRNAs to confirm the results. The results obtained using *siPAX2* (HS107605) and *siHGF* (HSS179212) are shown in the figures.

2.2. Antibodies

Antibodies used to target specific proteins were as follows: IgG (I5006, Sigma), PAX2 (9666, Cell Signaling Technology, Danvers, MA, USA), HGF (24865, Abcam, Cambridge, MA, USA), acetyl-histone H3 (06-599, Millipore, Temecula, CA, USA) and β-actin (A5441, Sigma, St. Louis, MO, USA). Peroxidase-conjugated secondary antibodies for immunoblot were purchased from Jackson Immuno Research (West Grove, PA, USA) or GE Healthcare (Pittsburgh, PA, USA).

2.3. Plasmids

FLAG-tagged fused human *PAX2* isoform c (NCBI Reference Sequence: NM_003988.3) was inserted into the pcDNA3 vector (Invitrogen). A reporter plasmid, *HGF-luc*, was constructed by inserting the upstream region of the *HGF* gene into the pGL3-basic vector (Promega, Madison, WI, USA).

2.4. Cell culture and transfection

The 22Rv1 cell line was purchased from ATCC and cultured in RPMI 1640 with 10% fetal bovine serum (FBS). PC3 and LNCaP cell lines were provided by the RIKEN BRC (the National Bio-resource Project of the MEXT, Japan) and were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). For knockdown of specific genes in 22Rv1 cells, transfection of siRNA was performed using Lipofectamine RNAiMAX (Invitrogen) with antibiotic-free medium for more than 3 days. The efficacy of silencing was assessed by both by qPCR and western blotting. Transfection of cDNAs into PC3 cell was performed using Lipofectamine 2000 (Invitrogen) for 20–72 h.

2.5. Invasion assays

Matrigel invasion assays were performed using uncoated or Matrigel-coated Transwell inserts (Becton Dickinson, East Rutherford, NJ, USA) according to the manufacturer's instructions as previously described [3,16]. Two days after 22Rv1 cells had been transfected with each siRNA, 2.5×10^5 live cells were added to the inserts. Cells were incubated with or without 100 mM recombinant HGF (TP315593, OriGene, Rockville, MD, USA) at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. Cells that had invaded through the inserts were stained using a Diff-Quik staining kit (Sysmex, Hyogo, Japan), and the total number of stained cells was counted.

2.6. Quantitative RT-PCR

One microgram of total RNA was extracted from each sample using TRIzol (Invitrogen). RNA was transcribed into first-strand cDNA using PrimeScript RT Master Mix (Takara). Quantitative RT-PCR was performed using a Thermal Cycler Dice TP900. Primer sets for PCR are as follows:

Gene	Sequence
PAX2 (*3′-UTR*) used in Figs. 1A, C and Fig. 6	Forward 5′-CCCAGCGTCTCTTCCATCA-3′ Reverse 5′-GGCGTTGGGTGGAAAGG-3′
PAX2 (*ORF*) used in Fig. 1D	Forward 5′-CCCAGCGTCTCTTCCATCA-3′ Reverse 5′-GGCGTTGGGTGGAAAGG-3′
HGF	Forward 5′-TGGTGTGAATCCAGTAGTCCCATTT-3′ Reverse 5′-CGCCGCCCTATATTCTGTGGA-3′
GAPDH	Forward 5′-ACCACAGTCCATGCCATCAC-3′ Reverse 5′-TCCACCACCCTGTTGCTGTA-3′

2.7. Western blotting

Whole cell lysates were extracted with lysis buffer (10 mM Tris–HCl [pH 7.8], 1% NP40, 0.15 M NaCl, 1 mM EDTA). Western blotting was then performed using standard methods [17].

2.8. Chromatin immunoprecipitation (ChIP) assay

Cells were fixed with 1% formalin for 10 min at 37 °C. Cell pellets were diluted with 1% sodium dodecyl sulfate (SDS), 10 mM EDTA (pH 7.9), and 50 mM Tris–HCl (pH 8.1) and reacted with 0.3 U/μL MNase (Takara) for 10 min at 4 °C. ChIP assays were performed using previously reported protocols [17]. Immunoprecipitated DNA fragments were amplified with several primer sets and quantified with Thermal Cycler Dice TP900 (Takara). Primer sets for PCR are described as follows:

Primers	Sequence
Region-*a*	Forward 5′-TATGCTGCTTCCCCTTCCTC-3′ Reverse 5′-GATCCCAGTGGCTCCTATCC-3′
Region-*b*	Forward 5′-CGAGTGAGGAAAGGAGGGG-3′ Reverse 5′-GTGCCTAAAAGAGCCAGTCG-3′
Region-*c*	Forward 5′-TTTTGTGAAGTGGCCCGTTT-3′ Reverse 5′-CGACCCTGTGAAGCGATTTT-3′

2.9. Luciferase assay

PC3 cells (70% confluent) were transfected with *PAX2* expression plasmids and reporter plasmids using Lipofectamine for 20 h. As a reference plasmid to normalize transfection efficiency, pRL-CMV plasmid (Promega) was cotransfected in all experiments. After 24 h of transfection, total RNA was isolated, subjected to RT, and quantified by real-time PCR [18]. All values are the means ± standard deviations of at least 3 independent experiments. The primers are as follows:

Primers	Sequence
Firefly luciferase	Forward 5′-AAGGTTGTGGATCTGGATAC-3′ Reverse 5′-GATTGTTTACATAACCGGAC-3′
Renilla luciferase	Forward 5′-CTTCGTGGAAACCATGTTGCC-3′ Reverse 5′-CTTCGTGGAAACCATGTTGCC-3′,

2.10. DNA pull-down assay

DNA pull-down assays were performed as previously described [19]. Dynabeads M-280 Streptavidin (250 μg; Invitrogen) were washed in buffer A (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 2 M NaCl). They were then incubated with annealed 5′-biotinylated oligonucleotides (125 pmol) for 15 min at room temperature in buffer A. Beads were then washed twice with buffer A and three times with buffer C

(20 mM Tris–HCl pH 8.0, 1 mM EDTA, 10% glycerol, 1 mM DTT, 50 mM NaCl). Beads (250 μg) were incubated in buffer C with 0.05 mg/mL poly(dI-dC) and 10 μL of recombinant PAX2 for 15 min at room temperature and washed with buffer C. Proteins were then analyzed by SDS-PAGE and immunoblots using anti-PAX2 antibody. 5′-Biotinylated oligonucleotides are as follows:

Oligos	Sequence
wt	Sense 5′-Bio-TGTATTTTCCTGTTTAAATTGAGCG-3′ Antisense 5′-Bio-CGCTCAATTTAAACAGGAAAATACA-3′
Mutant	Sense 5′-Bio-TGTATTCCGCTCTTGATATTGAGCG-3′ Antisense 5′-Bio-CGCTCAATATCAAGAGCGGAATACA-3′.

Recombinant PAX2 was expressed using an *in vitro* translation system according to the manufacturer's instructions (Promega).

2.11. Patient tumor samples

Human prostate tumor samples were obtained from University Hospital, Kyoto Prefectural University of Medicine, in accordance with the protocols approved by the hospital's Institutional Ethical Committee. Written informed consent was obtained from each patient. Tumor samples were taken from freshly isolated surgical resections or needle biopsy.

2.12. Statistical analysis

Statistical analyses were carried out by a *t*-test as appropriate. All data are reported as means ± SD. A *P*-value of <0.05 was considered significant.

3. Results

3.1. PAX2 increased the HGF expression level in prostate cancer cells

Previously, we performed genomic screening to identify *PAX2* target genes by DNA microarray analysis using *PAX2*-knockdown 22Rv1 cells which expressed PAX2 at higher level than other prostate cancer cell lines [3]. A total of 645 genes among the tested 28,768 genes exhibited a more than 2-fold decrease in mRNA expression, including *HGF*. To verify the results of the gene array experiments, we used quantitative PCR (qPCR) to measure the expression of *HGF*. We found that the mRNA expression level of *HGF* was indeed reduced in *PAX2* knockdown cells (Fig. 1A). Protein levels of HGF were also reduced by knockdown of *PAX2* (Fig. 1B). Notably, the *PAX2* expression level was not altered by knockdown of HGF (Fig. 1A, B). Next, we compared expression level of PAX2 and HGF between different prostate cancer cell lines (Fig. 1C). The 22Rv1 cell line is derived from a human primary prostate cancer [20]. PC3 and LNCaP cell lines are derived from prostate cancer cells metastasized to bone and lymph node, respectively [21,22]. qPCR results showed that 22Rv1 cells expressed both PAX2 and HGF at higher level than PC3 and LNCaP cells. To confirm that overexpression of *PAX2* caused upregulation of *HGF* expression, *PAX2* overexpression was induced in PC3 cells. Overexpression of *PAX2* increased *HGF* mRNA expression level in PC3 cells (Fig. 1D). These findings confirmed that PAX2 increased HGF expression levels in prostate cancer cells.

3.2. HGF promoter was transcriptionally activated by PAX2

To assess whether PAX2 activated transcription on the *HGF* promoter, we performed luciferase assays using PC3 cells that were transfected with reporter plasmids containing the *HGF* promoter region (−904 to +81) fused with the luciferase gene (Fig. 2A). Cotransfection with PAX2 expression plasmids did indeed increase relative luciferase

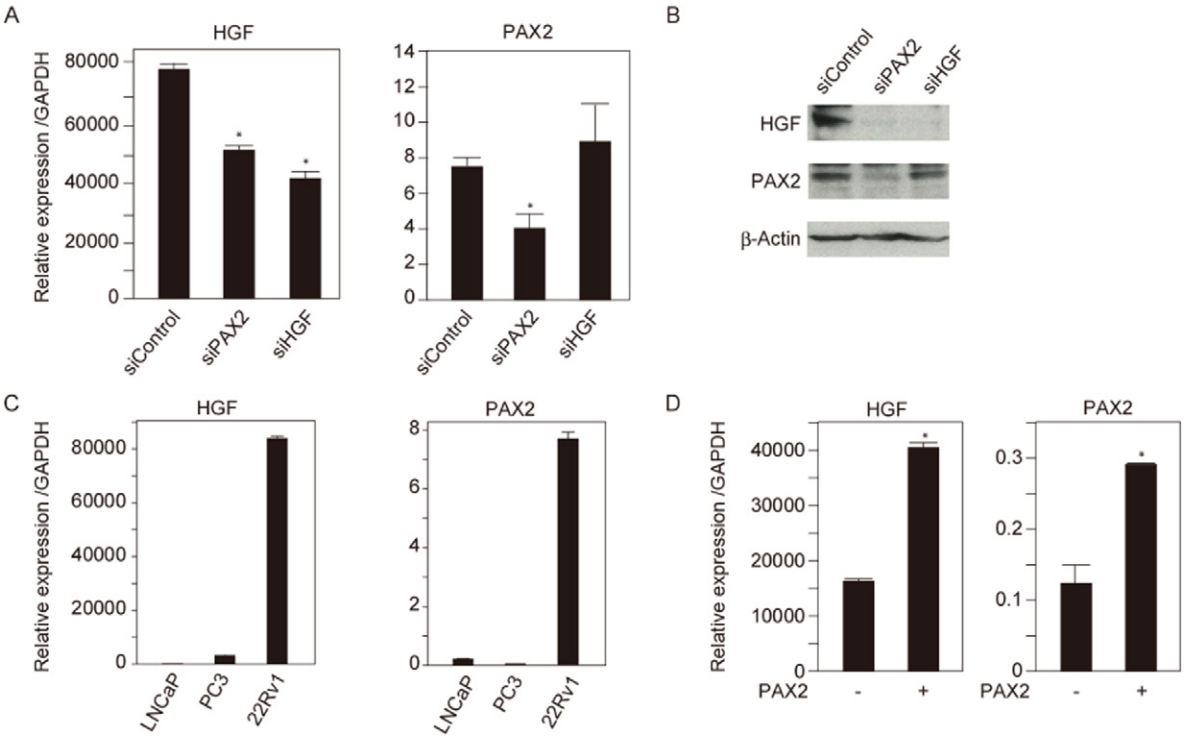

Fig. 1. PAX2 increased HGF expression levels in prostate cancer cells. (A) Regulation of *HGF* gene expression by PAX2 in 22Rv1 cells. siRNA for PAX2 or HGF was transfected into 22Rv1 cells for 3 days. qPCR was used to measure the expression levels of the *HGF* and the *PAX2* genes. Each measurement shows the average values of 3 independent measurements that were normalized to the expression levels of *GAPDH* mRNA. *$P < 0.05$. (B) Immunoblots using *PAX2*- or *HGF*-knockdown 22Rv1 cell lysates with anti-HGF or anti-PAX2 antibodies. (C) Comparative analysis of PAX2 and HGF expression levels among various cell lines. The expression of PAX2 relative to GAPDH as a control is shown for each cell line. (D) *HGF* gene expression in PAX2-expressing PC3 cells. PC3 cells were transfected with PAX2 expression plasmid or an insert-less plasmid for 3 days. mRNA expression levels in the cells were quantified by qPCR. *$P < 0.05$. Each experiment was reproduced three times.

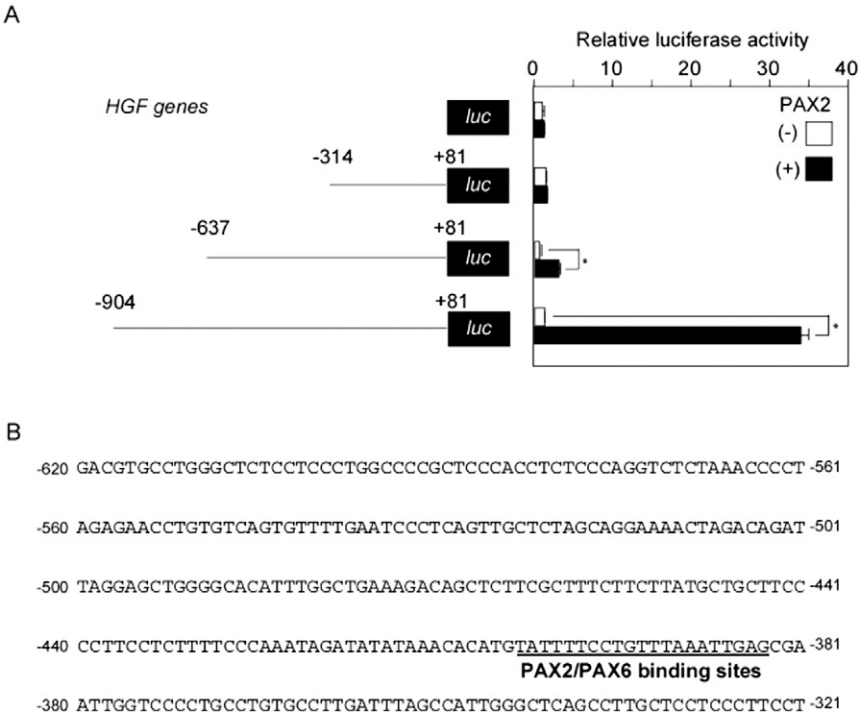

Fig. 2. PAX2 upregulated *HGF* gene expression. (A) Luciferase assay using PC3 cells transfected with a series of constructs encoding a luciferase reporter gene driven by an upstream regulatory region of the *HGF* gene plasmids. FLAG-*PAX2* expression plasmid or an insert-less plasmid was cotransfected with the reporter plasmid for 20 h. Transcripts of firefly luciferase and *Renilla* luciferase were quantified by qPCR. Each measurement shows the average value of 3 independent measurements that were normalized to the level of *Renilla* luciferase mRNA. $^{*}P < 0.05$. The same results were reproduced in independent three experiments. (B) DNA sequence of the upstream region of the human *HGF* gene. Underlined sequences are PAX2 and/or PAX6 binding consensus sites.

expression through the *HGF* promoter region. This result indicated that PAX2 protein upregulated *HGF* gene expression at the transcriptional level. Next, to determine the region responsible for the induction of *HGF* transcription by PAX2 protein, portions of the *HGF* gene promoter were deleted. When the luciferase gene was fused with the −637 to +81 region, expression was activated by PAX2, whereas the −314 to +81 region was not activated by PAX2 (Fig. 2A). These observations suggested that the region from −637 to −314 was indispensable for

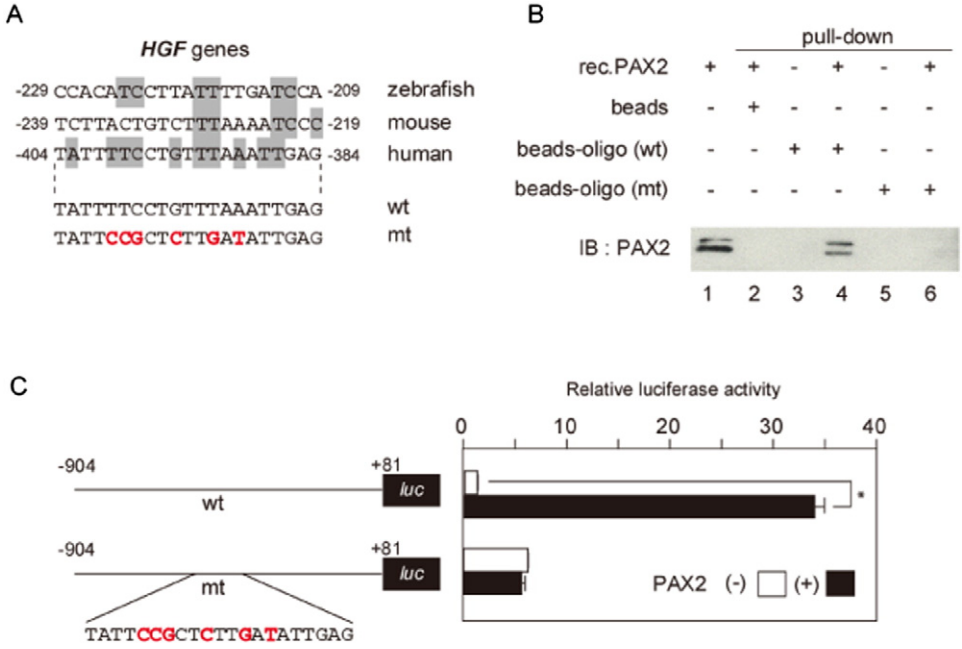

Fig. 3. PAX2 interacted upstream of the *HGF* gene. (A) DNA sequence alignment of the upstream region of the *HGF* genes (upper sequences). Gray boxes are consensus sequences of PAX2 and/or PAX6 binding sites. A schematic alignment of the oligonucleotides used in DNA pull-down assay (bottom sequences). Six nucleotides in the PAX2 and/or PAX6 binding site were replaced with other nucleotides (red characters in mt sequences). (B) DNA pull-down assay using recombinant PAX2 protein and oligonucleotides derived from upstream of the *HGF* gene (shown in Fig. 4A). (C) Luciferase assay using PC3 cells cotransfected with *HGF-luc* reporter plasmids (wt or mt, −904–+81 of *HGF* gene) and FLAG-*PAX2* expression plasmids. The same results were reproduced for each experiments three times.

activation of the *HGF* promoter by PAX2. PAX2 and/or PAX6 consensus binding sites are found in this region (Fig. 2B) [23], therefore we hypothesized that PAX2 bound to DNA around the *HGF* promoter.

3.3. PAX2 bound to DNA in the HGF promoter

The region described above contained a *PAX2* and/or *PAX6* consensus binding sequence that is conserved in several species (Fig. 3A). We used synthetic oligonucleotides to analyze the consensus binding sites' ability to bind PAX2 and confirmed that the wild-type (wt) oligo was able to bind PAX2 (Fig. 3B). Replacement of six nucleotides with other residues in the consensus binding sites sharply diminished PAX2 binding (mt in Fig. 3B). Luciferase activity of the *HGF* promoter mediated by PAX2 was decreased by mutation in the consensus binding sites (Fig. 3C). The results suggested that PAX2 protein activated the *HGF* gene through binding to the DNA in the *HGF* promoter region.

3.4. PAX2 associated with the HGF promoter and induced HGF gene activation through histone H3 acetylation

To determine whether PAX2 associated with the promoter region of *HGF* in prostate cancer cells, we conducted ChIP assays using anti-PAX2 antibody. The results revealed that PAX2 associated with region-*b* containing the *PAX2* consensus binding site (Fig. 4A). Transcriptional activation is well known to be marked by acetylation of histones or methylation of specific lysines (H3K4 and H3K36) [24]. Thus, we assessed whether the acetylation level of the *HGF* promoter was altered by PAX2. The results of the ChIP assay using anti-histone acetylated-H3 antibody showed that the histone H3 acetylation level of region-*b* was reduced by knockdown of PAX2 (Fig. 4C). These results suggested that PAX2 interacted with the *HGF* promoter and increased histone H3 acetylation level in the region.

3.5. HGF mediated the invasive properties of prostate cancer cells downstream of PAX2

To investigate the relationship between PAX2 and HGF in prostate cancer cell invasion, matrigel invasion assays were performed using 22Rv1 cells. 22Rv1 cells were used as a model of prostate cancer cell behavior. 22Rv1 cells are the most invasive of the prostate cancer cell lines that we investigate previously [3]. Though cancer cell invasion was reduced by single knockdown of PAX2 or HGF, reduced rates of invasion were not altered by double knockdown of both *PAX2* and *HGF* (Fig. 5A, B). These data raised the possibility that PAX2 and HGF functioned in the same pathway regulating cancer cell invasion. To confirm that HGF substantially mediated the invasive properties of prostate cancer cells downstream of PAX2, we added recombinant HGF to the medium after knockdown of PAX2 in the invasion assay. The effect of the PAX2 knockdown on invasion was rescued by addition of recombinant HGF.

3.6. PAX2 expression positively correlated with HGF expression in prostate cancer patients

To investigate the correlation between PAX2 and HGF expression, mRNA expression levels of the two genes were quantified by qPCR in tissues obtained from prostate cancer patients (Fig. 6, Table S1). In these prostate cancer samples, there was a positive correlation between PAX2 and HGF expression ($r = 0.616$). These results suggested PAX2 protein promoted prostate cancer cell invasion through induction of HGF expression in prostate cancer patients.

4. Discussion

We previously suggested that overexpression of the *PAX2* gene promoted cancer cell invasion in AIPC. In this report, we showed that HGF, a well-known regulator of cancer cell invasion, was a direct target gene of

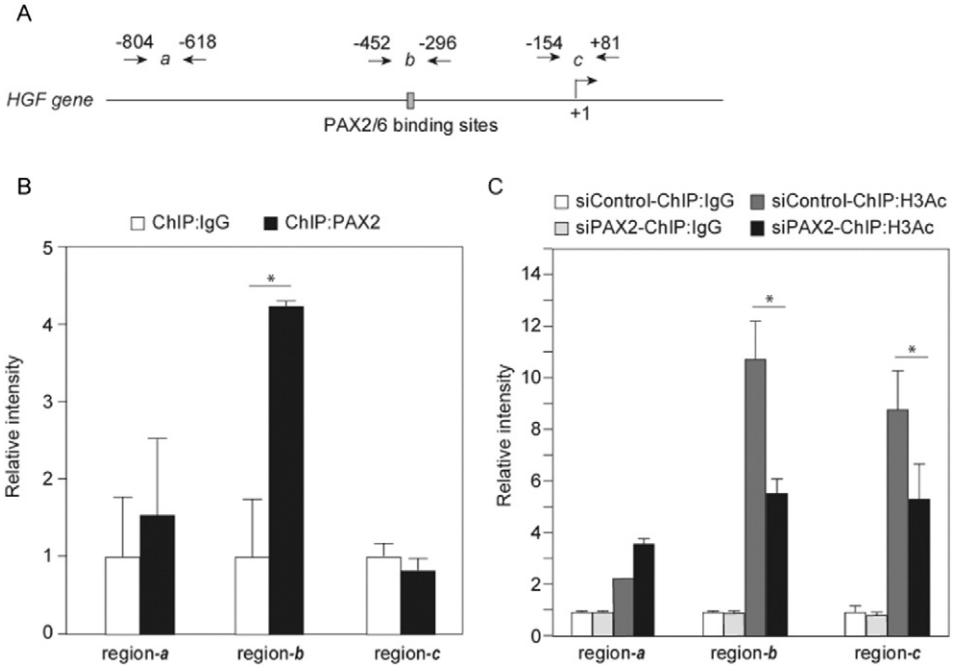

Fig. 4. PAX2 promoted histone acetylation in the upstream region of the *HGF* gene. (A) Schematic diagram of the human *HGF* gene. Black arrows indicate amplified regions for ChIP assays. A gray box shows PAX2 and/or PAX6 consensus binding site. (B) ChIP assay with anti-PAX2 antibody in 22Rv1 cells. Immunoprecipitated DNA was amplified and quantified by qPCR. Each bar represents an average of 3 independent experiments as the relative intensity compared to the input (mean + S.D.). $^*P < 0.05$. (C) ChIP assay with anti-acetylated histone H3 antibodies in 22Rv1 cells transfected for siPAX2 for 3 days. Each experiment was reproduced three times.

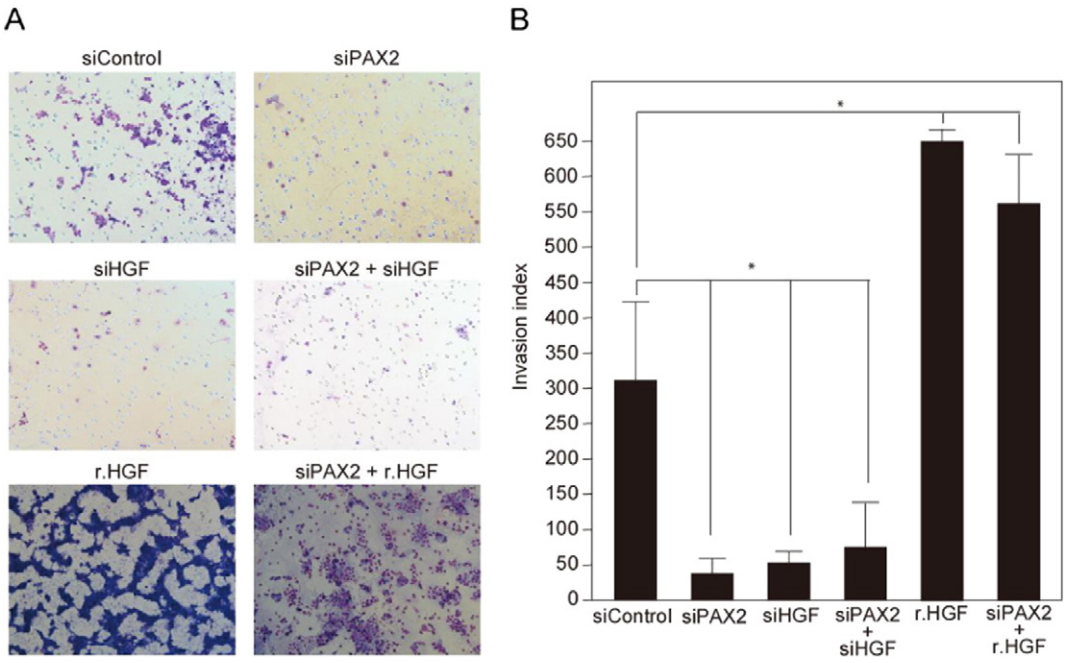

Fig. 5. PAX2 enhanced invasive activity of androgen-independent prostate cancer cells upstream of the HGF pathway. (A) Invasion assay using 22Rv1 cells. 22Rv1 cells were transfected with siRNAs for HGF or PAX2 for 2 days. Then, these cells were cultured in Matrigel invasion chambers with or without 100 pM recombinant HGF for 24 h. (B) The number of invading cells was counted. Invasion index shows the average values of 3 independent measurements, which was normalized to cell number of control inserts. $^{*}P < 0.05$. The same results were reproduced in independent three experiments.

PAX2. PAX2 associates upstream of the *HGF* gene and upregulates *HGF* gene expression through alteration of histone modification.

The primary therapy for metastatic prostate cancer is blockade of the androgen signaling pathway through the androgen receptor (AR) [25]. Though the patient responds to the therapy initially, most patients become resistant to the therapy in a few years [25]. There are several proposed mechanisms by which prostate cancer cells become resistant to androgen blockade therapy. One such is activation of a signaling pathway other than that of androgen [26]. Dysregulation of the HGF/MET signaling pathway has been associated with emergence of prostate cancer cells resistant to androgen blockade therapy [27]. Reflecting the importance of HGF/MET signaling in prostate cancer progression, several clinical studies have tested new drugs targeting the HGF/MET signal pathway [28]. Rilotumumab, a monoclonal anti-HGF antibody, was administered to metastatic castration-resistant prostate cancer patients in a randomized phase II study [27]. Although the toxicities of rilotumumab were manageable, the effectiveness was inadequate. A previous report using an *in vitro* system showed that administration of anti-HGF antibody had no effect on the growth of 22Rv1 cells, whereas knockdown of MET suppressed the growth of 22Rv1 cells [14]. These results suggested that the HGF/MET signaling pathway was activated through different mechanisms in androgen-dependent and AIPC cells. Although anti-HGF antibody could inhibit the growth of androgen-dependent prostate cancer cells in which paracrine activation of HGF/MET signaling took place and the antibody failed to block autocrine activation of the pathway in AIPC. Our study suggested that the PAX2/HGF pathway may be a new therapeutic target. From our observation that PAX2 overexpression induced HGF expression (Fig. 1C), high production of PAX2 protein may be one of the mechanisms underlying HGF expression in AIPC cells. Inhibition of such an autocrine loop by *PAX2* knockdown may be a more effective therapy than administration of HGF-antibody when AIPC patients are treated.

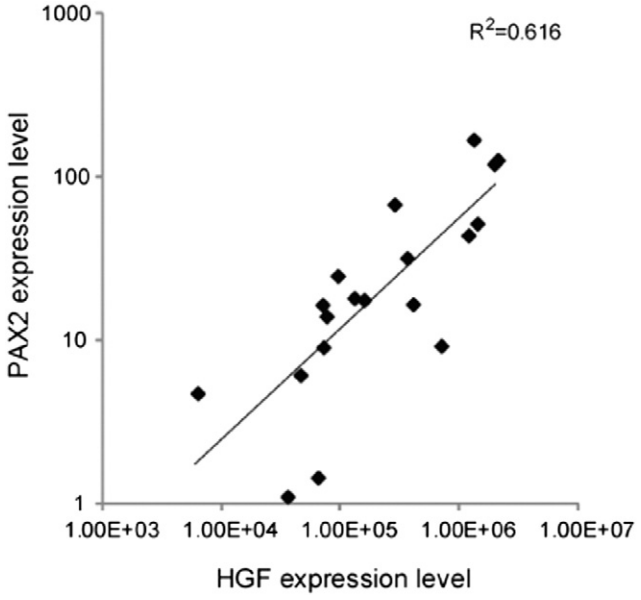

Fig. 6. PAX2 expression correlated with HGF expression in prostate cancer patients. *HGF* and *PAX2* mRNA expression levels in prostate cancer tissues (n = 19). mRNA expression levels were quantified by qPCR. Each measurement was normalized to *GAPDH* mRNA levels.

Understanding the mechanism of normal prostate development may lead to the identification of new therapies for AIPC because dysregulated function of the factors regulating normal organogenesis is associated with carcinogenesis. Several factors reportedly regulate prostate development. Among them, AR is expressed in the urogenital sinus mesenchyme (UGM) of the prostate at an early stage and initiates prostatic bud formation [29]. AR is a ligand-dependent transcription factor that exerts its function through the regulation of target gene expression. *NKX3-1* is one of the target genes of AR and it functions in prostate development and carcinogenesis [30]. PAX2, a ligand-independent transcription factor, is expressed in the urogenital sinus (UGS) and regulates prostatic ductal growth and branching. *HGF*, a target gene of PAX2, may function in prostate development. Though HGF is known to regulate the development of the liver, the role of HGF in prostate development is unknown. HGF-knockout mice are embryonic

lethal and the phenotype in the prostate at the embryonic stage cannot be easily examined. Examination of HGF expression in the prostate at various developmental stages may be useful for understanding the mechanism of prostate development and carcinogenesis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.08.008.

COI disclosure information

The authors declare that they have no financial support for this study to disclose.

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References

[1] S. Valastyan, R.A. Weinberg, Tumor metastasis: molecular insights and evolving paradigms, Cell 147 (2011) 275–292.
[2] P. Friedl, K. Wolf, Tumour-cell invasion and migration: diversity and escape mechanisms, Nat. Rev. Cancer 3 (2003) 362–374.
[3] T. Ueda, S. Ito, T. Shiraishi, P. Kulkarni, A. Ueno, H. Nakagawa, Y. Kimura, F. Hongo, K. Kamoi, A. Kawauchi, T. Miki, Hyper-expression of PAX2 in human metastatic prostate tumors and its role as a cancer promoter in an in vitro invasion model, Prostate 73 (2013) 1403–1412.
[4] E.J. Robson, S.J. He, M.R. Eccles, A PANorama of PAX genes in cancer and development, Nat. Rev. Cancer 6 (2006) 52–62.
[5] H. Tanaka, E. Kono, C.P. Tran, H. Miyazaki, J. Yamashiro, T. Shimomura, L. Fazli, R. Wada, J. Huang, R.L. Vessella, J. An, S. Horvath, M. Gleave, M.B. Rettig, Z.A. Wainberg, R.E. Reiter, Monoclonal antibody targeting of N-cadherin inhibits prostate cancer growth, metastasis and castration resistance, Nat. Med. 16 (2010) 1414–1420.
[6] U. Cavallaro, G. Christofori, Cell adhesion and signalling by cadherins and Ig-CAMs in cancer, Nat. Rev. Cancer 4 (2004) 118–132.
[7] K. Doberstein, J. Pfeilschifter, P. Gutwein, The transcription factor PAX2 regulates ADAM10 expression in renal cell carcinoma, Carcinogenesis 32 (2011) 1713–1723.
[8] M.J. McConnell, H.E. Cunliffe, L.J. Chua, T.A. Ward, M.R. Eccles, Differential regulation of the human Wilms tumour suppressor gene (WT1) promoter by two isoforms of PAX2, Oncogene 14 (1997) 2689–2700.
[9] C. Birchmeier, W. Birchmeier, E. Gherardi, G.F. Vande Woude, Met, metastasis, motility and more, Nat. Rev. Mol. Cell Biol. 4 (2003) 915–925.
[10] L. Trusolino, P.M. Comoglio, Scatter-factor and semaphorin receptors: cell signalling for invasive growth, Nat. Rev. Cancer 2 (2002) 289–300.
[11] J.P. Thiery, Epithelial–mesenchymal transitions in tumour progression, Nat. Rev. Cancer 2 (2002) 442–454.
[12] D.F. Balkovetz, V. Sambandam, Dynamics of E-cadherin and gamma-catenin complexes during dedifferentiation of polarized MDCK cells, Kidney Int. 56 (1999) 910–921.
[13] B.S. Knudsen, M. Edlund, Prostate cancer and the MET hepatocyte growth factor receptor, Adv. Cancer Res. 91 (2004) 31–67.
[14] K. Nakashiro, S. Hara, Y. Shinohara, M. Oyasu, H. Kawamata, S. Shintani, H. Hamakawa, R. Oyasu, Phenotypic switch from paracrine to autocrine role of hepatocyte growth factor in an androgen-independent human prostatic carcinoma cell line, CWR22R, Am. J. Pathol. 165 (2004) 533–540.
[15] K. Nakagawa, S. Takasawa, K. Nata, A. Yamauchi, A. Itaya-Hironaka, H. Ota, K. Yoshimoto, S. Sakuramoto-Tsuchida, T. Miyaoka, M. Takeda, M. Unno, H. Okamoto, Prevention of Reg I-induced beta-cell apoptosis by IL-6/dexamethasone through activation of HGF gene regulation, Biochim. Biophys. Acta 1833 (2013) 2988–2995.
[16] S. Ito, T. Ueda, A. Ueno, H. Nakagawa, H. Taniguchi, N. Kayukawa, T. Miki, A genetic screen in *Drosophila* for regulators of human prostate cancer progression, Biochem. Biophys. Res. Commun. 451 (2014) 548–555.
[17] S. Ito, S. Fujiyama-Nakamura, S. Kimura, J. Lim, Y. Kamoshida, Y. Shiozaki-Sato, S. Sawatsubashi, E. Suzuki, M. Tanabe, T. Ueda, T. Murata, H. Kato, F. Ohtake, R. Fujiki, T. Miki, A. Kouzmenko, K. Takeyama, S. Kato, Epigenetic silencing of core histone genes by HERS in *Drosophila*, Mol. Cell 45 (2012) 494–504.
[18] S. Ito, T. Ueda, A. Ueno, H. Nakagawa, H. Taniguchi, F. Hongo, K. Kamoi, K. Okihara, A. Kawauchi, T. Miki, Paired box 2 upregulates androgen receptor gene expression in androgen-independent prostate cancer, FEBS J. 281 (2014) 4506–4518.
[19] V. Drewett, H. Molina, A. Millar, S. Muller, F. von Hesler, P.E. Shaw, DNA-bound transcription factor complexes analysed by mass-spectrometry: binding of novel proteins to the human c-fos SRE and related sequences, Nucleic Acids Res. 29 (2001) 479–487.
[20] R.M. Sramkoski, T.G. Pretlow II, J.M. Giaconia, T.P. Pretlow, S. Schwartz, M.S. Sy, S.R. Marengo, J.S. Rhim, D. Zhang, J.W. Jacobberger, A new human prostate carcinoma cell line, 22Rv1, In Vitro Cell. Dev. Biol. Anim. 35 (1999) 403–409.
[21] M.E. Kaighn, K.S. Narayan, Y. Ohnuki, J.F. Lechner, L.W. Jones, Establishment and characterization of a human prostatic carcinoma cell line (PC-3), Investig. Urol. 17 (1979) 16–23.
[22] J.S. Horoszewicz, S.S. Leong, E. Kawinski, J.P. Karr, H. Rosenthal, T.M. Chu, E.A. Mirand, G.P. Murphy, LNCaP model of human prostatic carcinoma, Cancer Res. 43 (1983) 1809–1818.
[23] J. Epstein, J. Cai, T. Glaser, L. Jepeal, R. Maas, Identification of a Pax paired domain recognition sequence and evidence for DNA-dependent conformational changes, J. Biol. Chem. 269 (1994) 8355–8361.
[24] M.L. Suva, N. Riggi, B.E. Bernstein, Epigenetic reprogramming in cancer, Science 339 (2013) 1567–1570.
[25] S.M. Green, E.A. Mostaghel, P.S. Nelson, Androgen action and metabolism in prostate cancer, Mol. Cell. Endocrinol. 360 (2012) 3–13.
[26] B.J. Feldman, D. Feldman, The development of androgen-independent prostate cancer, Nat. Rev. Cancer 1 (2001) 34–45.
[27] C.J. Ryan, M. Rosenthal, S. Ng, J. Alumkal, J. Picus, G. Gravis, K. Fizazi, F. Forget, J.P. Machiels, S. Srinivas, M. Zhu, R. Tang, K.S. Oliner, Y. Jiang, E. Loh, S. Dubey, W.R. Gerritsen, Targeted MET inhibition in castration-resistant prostate cancer: a randomized phase II study and biomarker analysis with rilotumumab plus mitoxantrone and prednisone, Clin. Cancer Res. 19 (2013) 215–224.
[28] J.L. Wozney, E.S. Antonarakis, Growth factor and signaling pathways and their relevance to prostate cancer therapeutics, Cancer Metastasis Rev. 33 (2014) 581–594.
[29] K.P. Keil, L.L. Abler, J. Laporta, H.M. Altmann, B. Yang, D.F. Jarrard, L.L. Hernandez, C.M. Vezina, Androgen receptor DNA methylation regulates the timing and androgen sensitivity of mouse prostate ductal development, Dev. Biol. 396 (2014) 237–245.
[30] R. Bhatia-Gaur, A.A. Donjacour, P.J. Sciavolino, M. Kim, N. Desai, P. Young, C.R. Norton, T. Gridley, R.D. Cardiff, G.R. Cunha, C. Abate-Shen, M.M. Shen, Roles for Nkx3.1 in prostate development and cancer, Genes Dev. 13 (1999) 966–977.