HOXB13 Homeodomain Protein Suppresses the Growth of Prostate Cancer Cells by the Negative Regulation of T-Cell Factor 4

Chaeyong Jung,1 Ran-Sook Kim,1 Sang-Jin Lee,1 Chihuei Wang,4 and Meei-Huey Jeng2,3

Departments of 1Urology, 2Microbiology and Immunology, and 3Medicine, Walther Oncology Center, Indiana University, Indianapolis, Indiana, and 4the Orthopedic Research Center and Department of Biology, Kaohsiung Medical University, Taiwan, Republic of China

ABSTRACT

In prostate gland, HOXB13 is highly expressed from the embryonic stages to adulthood. However, the function of HOXB13 in normal cell growth and tumorigenesis is not yet known. We investigated the role of HOXB13 and mechanism by which it functions in HOXB13-negative cells. Expression of HOXB13 was forced in HOXB13-negative PC3 prostate cancer cells using a liposome-mediated gene transfer approach. Compared with the control clones, HOXB13-expressing PC3 cells exhibited significant inhibition of in vitro and in vivo cell growth with G1 cell cycle arrest mediated by the suppression of cyclin D1 expression. Because cyclin D1 is mainly regulated by β-catenin-T-cell factor (TCF), TCF-4 response element was used in a reporter gene transcription assay, demonstrating that HOXB13 significantly inhibits TCF-4-mediated transcriptional activity in both prostate and nonprostate cells. This inhibition occurred in a dose-responsive manner and was specific to TCF-4 response element. Western blot analysis demonstrated that HOXB13 down-regulates the expression of TCF-4 and its responsive genes, c-myc and cyclin D1. HOXB13 also suppressed the activity of natural c-myc promoter. This study suggests that HOXB13, a transcription factor, functions as a cell growth suppressor by negatively regulating the expression of TCF-4, which eventually provides negative signals for cell proliferation. This observation will provide valuable insight into the molecular basis of prostate tumorigenesis.

INTRODUCTION

Prostate cancer (PCa) is the second leading cancer killer in North American males. The mechanisms controlling proliferation, differentiation, and tumorigenesis in the prostate are largely unknown, and therapeutic efforts to conquer this malignant disease have been limited. There have been attempts to identify genes that might be involved in prostatic development and carcinogenesis. Many studies have concentrated on the activation of oncogenes and inactivation of tumor suppressor genes. Several homeobox genes have been also described in the normal and malignant growth of prostate epithelial cells. Nkx3.1 is the best-studied homeobox gene in PCa. Expression of Nkx3.1 is very important for prostatic organogenesis and maintaining the normal growth of epithelial cells (1). Nkx3.1 knockout mice display reductions in prostate ductal tips and disrupted secretory function. At the same time, Nkx3.1 deletion mutants display a high incidence of prostatic intraepithelial neoplasia (2, 3). The loss of NKK3.1 is more common in tumors than in benign prostatic hyperplasia. Almost 80% of metastatic tumors lose NKK3.1 expression (3, 4).

HOX genes are known to regulate axial regional specification during embryonic development. They also have developmental roles in male accessory sexual organs, including the prostate (5–7). The Hox-13 paralogue is especially important to prostate development. All Hox-13 genes except Hoxc13 are expressed in the prostate during embryonic development, but Hoxa13 and Hoxd13 are no longer expressed after the embryonic stage (5, 8). Hoxa13 and/or Hoxd13-deficient mice showed morphological abnormalities in male accessory sex organs, including the seminal vesicle and prostate (5, 6, 9). Hoxb13, the last identified vertebrate Hox gene, has limited expression in the caudal extent of the spinal cord, tail bud, and urogenital sinus (10). In the prostate gland, embryologically derived from the urogenital sinus, expression of Hoxb13 is not regulated by androgen, in contrast to Nkx3.1 (11). Recently, Hoxb13 loss-of-function mutations showed overgrowth in all major structures derived from the tail bud (12) and malformation of the ventral prostate ducts, indicating complete loss of secretory proteins (13). The phenotype demonstrated in Hoxb13 mutant mice was similar to Nkx3.1 mutant mice, the development of swollen prostates in older mutant mice.

Although Hoxb13 expression is maintained throughout adulthood, only limited information is available on the biological function of HOXB13 in normal prostate development and human prostate tumorigenesis. We characterized the biological function of HOXB13 using the PC3 PCa cell line, which does not express this gene. Cell Lines and Tissues. Human PCA cell lines PC3 and CWR22Rv1 were cultured in RPMI media (Invitrogen) supplemented with 5% FCS at 37°C in an atmosphere containing 5% CO2, and described previously (14). MDA PCa 2b PCa cells were grown in Biological Research Faculty and Facility (BRFF)-HPC1 (AthenaES, Baltimore, MD) with 20% bovine serum (FBS). CV-1 monkey kidney and 293 human embryonic renal cells were maintained in DMEM containing 10% FBS. All cultures were fed with fresh medium every 3–4 days.

Constructions of HOXB13 Expression Vectors. HOXB13 full-length cDNA was obtained by reverse transcription-PCR (RT-PCR) (15) using human prostate total RNA purchased from Clontech. Amplified HOXB13 cDNA was first cloned into pFLAG-cytomegalovirus (CMV)12 vector (Sigma) that was linked to an NH2-terminal flag tag. The resulting pFLAG-HOXB13 vector was confirmed by sequencing analysis and transient transfection into 293 cells, followed by Western blot analysis. FLAG-HOXB13 (fHOXB13) was then excised from pFLAG-HOXB13 vector and subcloned into multiple cloning sites of CMV-driven pRES-neo expression vector (Clontech). An adenoviral HOXB13 expression construct was also developed by modifying adenoviral vector pAd1020SfisdA (OD260, Inc.). Briefly, the expression cassette CMV-multiple cloning sites-pA-CMV-GFP-pA was excised from pAdTrack-CMV (a generous gift from Dr. Bert Vogelstein) and ligated into pAd1020SfisdA vector. Then, fHOXB13 was cloned into the multiple cloning sites of modified pAd1020SfisdA (Invitrogen), generating pAd1020GFP-fHOXB13. The left arm of the adenovirus with the expression cassette was cut out by digestion with XbaI and PaeI and ligated into AdenoZapsfI2 (OD260, Inc.) in the presence of Pael. Purified DNA was transfected to 293 cells using LipofectAMINE 2000 (Invitrogen), and virus was produced and purified as described previously (15).

Transfection and Colony Formation. To determine the effects of HOXB13 on PCa cell growth, PC3 (1 × 106) cells were plated in 60 dishes and transfected with either pRES-neo or pRES-fHOXB13 vector using N-[1-(2,3-dioleoyloxy)propyl]N,N,N-trimethylammoniummethyl sulfate transfection reagent (Roche Molecular Biochemicals) as described by the manufacturer. Cells were selected in the presence of geneticin (200 µg/ml final concentration; Cellgro). When visible colonies (~200 cells) were formed, the number of colonies was counted. For the generation of stable transfectants, PC3

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Requests for reprints: Chaeyong Jung, Department of Urology, Indiana University, 1001 West 10th Street, OPW320, Indianapolis, IN 46202. Phone: (317) 278-7617; Fax: (317) 278-3432; E-mail: cjhung@iu.edu.

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cells were seeded at $1 \times 10^6$ cells onto a P100 tissue culture dish and transfected as described above. The resulting neomycin-resistant clones were ring cloned and expanded.

**Western Blot.** Cells were grown to 80% confluency in 60 culture dishes containing 5% FBS-T media. Cells were then lysed in protein extraction buffer (1× TBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors). Total cell lysates (20 μg) were loaded onto 10% Bis-Tris gel (Invitrogen) and separated using a Novex electroporation system (Invitrogen). After proteins were transferred to polyvinylidene difluoride membrane, the expression of FLAG-tagged HOXB13 was detected using FLAG M2 monoclonal antibody (Sigma) followed by the enhanced chemiluminescence detection system (Pierce).

**RT-PCR.** Total RNA from each cell line was extracted using the Ultraspec RNA isolation system (Biotecx Laboratories, Inc., Houston, TX) as described previously (16). cDNA was obtained from 1 μg of corresponding total RNA using Moloney murine leukemia virus reverse transcriptase. HOXB13 primers (5'-cccaacattgtcttcttta-3' and 5'-gctctgtcctgggtatgac-3') are designed to specifically amplify a coding region of 369–739. Cyclin D1 (5'-tctgcttgctcttctctggacag-3') and 5'-tagacaacctactatgaacct-3') T-cell factor 4 (TCF-4; 5'-cccacacacacacacactac-3' and 5'-tcgcttgctcttctctggacag-3') primers were also constructed. Primers for β-actin were used to act as equal loading (5'-gcaacaccatctttgagtccttc-3' and 5'-tagacaacctactatgaacct-3'). To be close to the linear range for PCR, equal amounts of RNA and cDNA products were applied with a minimum number of amplification cycles (≥25 cycles).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Cell Proliferation Assay. Stably transfected HOXB13-PC3 cells were plated at 1.5 × 10^6 cells/well into 96-well tissue culture plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂. At the indicated time points, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution were added to the cell cultures and incubated for 2 h at 37°C. The reaction was stopped by adding 100 μl of extraction buffer [50% formamide and 10% SDS (pH 4.7)]. After overnight incubation at 37°C, the absorbance at 570 nm was measured using a Spectra Microplate Reader ( Molecular Devices).

**Flow Cytometric Analysis.** Cells were grown in 5% FBS-T media containing 400 μg/ml gentamicin for 72 h, harvested, fixed, and treated with RNase. Cells were then stained with propidium iodide (50 μg/ml) and analyzed using a FACScan Flow Cytometer and the Cell Quest program.

**Tumor Growth in Vivo.** Male 8-week-old athymic nude mice (BALB/c-nu/nu) were obtained from Harlan. Mice were kept in groups of three according to the institutional guidelines for animal welfare. To characterize the tumorigenicity of HOXB13-transfected PC3 clones in vivo, HOXB13-PC3 clones 1 and 5 were injected into nude mice s.c. A vector-only transfected cells were selected by geneticin (Cellgro). After 3 weeks of selection, isolated colonies (~200–300 cells/colony) were counted. As shown in Fig. 1A, introduction of exogenous HOXB13 in HOXB13-negative PC3 cells resulted in statistically significant growth suppression, with a 20–40-fold decrease in the number of geneticin-resistant clones compared with neo control clones (Wald test; $P < 0.0001$). Growth suppression in PC3 cells was accompanied by dramatic changes in cell morphology to a gigantic cobblestone appearance (Fig. 1B), characteristic of terminal differentiated cells. By comparison, HOXB13 expression is down-regulated in developing dermis compared with adult skin, and the loss of HOXB13 causes adult skin to adopt a less differentiated and more proliferative mode (17, 18).

Thirteen neomycin-resistant clones were expanded and analyzed for HOXB13 expression using FLAG M2 antibody (Sigma). Only five clones expressed low levels of HOXB13 protein compared with the transiently transfected 293 cells, most likely because highly HOXB13-expressed clones were not expandable because of HOXB13-induced cell toxicity (Fig. 2A). HOXB13 mRNA levels were also verified by RT-PCR analysis, which also showed no ex-
expression of HOXB13 in parental PC3 cells using HOXB13-specific primers. HOXB13-PC3 clones were further analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay. Absorbance is shown as ±95% confidence interval (n = 8). Although clone 4 showed significant growth inhibition, this clone was ruled out, because it seemed to have an abnormal number of chromosomes (duplication of chromosomes is commonly seen in cancer cells). To reiterate the growth-inhibiting effect of HOXB13 on tumor growth in vivo, HOXB13-transfected and control cells were injected s.c. into the backs of nude mice. Compared with the control groups, including neo-transfected clone and wild-type PC3 cells, HOXB13-transfected clones showed a dramatic decrease in tumor volumes at 5 weeks after implantation (Fig. 2C; Wald test; P = 0.0014). At 5 weeks after implantation, the tumor volumes of HOXB13-transfected clones 1 and 5 were 92 ± 18 mm³ and 140 ± 45 mm³, respectively. On the other hand, wild-type PC3 and PC3-neo controls were 684 ± 117 mm³ and 522 ± 171 mm³, respectively.

We then investigated whether inhibition of cell growth was caused by apoptosis or cell cycle arrest. Flow cytometric analysis demonstrated that the growth-inhibiting effect was caused by significant abnormalities in cell cycle distribution. The majority of clones (three of four) showed a dramatic increase in the G₀-G₁ cell population (Fig. 2D), which may ultimately drive the cells into death. That there was no alteration of clone 3 cell distribution seems to be attributable to a low expression of HOXB13, as shown in Fig. 2A. Not all HOXB13-PC3 clones showed an alteration in apoptotic activity (data not shown). These results suggest that HOXB13 plays a role in the suppression of cell growth in highly malignant and metastatic PCa cells through cell cycle arrest in the G₁ phase. Because cyclin D1 is integral for accelerating the G₁ phase of the cell cycle (19), we used RT-PCR to test whether the expression of cyclin D1 was altered in HOXB13-expressing PC3 cells. Surprisingly, cyclin D1 expression was down-regulated in most clones (Fig. 2E). We failed to further characterize the function of HOXB13, because not all HOXB13-PC3 cells survived in culture, probably because of the cytotoxic effect of HOXB13.

We studied whether HOXB13 can inhibit cyclin D1 promoter activity. Because β-catenin/TCF-4 is an important transcription factor for cyclin D1 promoter, we used a luciferase reporter plasmid consisting of four copies of a TCF-4RE to test whether HOXB13 regulates this artificial promoter activity. Because both LNCaP and PC3 cells did not respond well to β-catenin-activated TCF signaling, as reported previously (data not shown; Ref. 20), CWR22RV and MDA PCa 2b PCa cells were used instead. CV-1 monkey kidney cells were also used for their high transfection efficiency and to simplify the reporter transcription assay. CV-1 cells did not require exogenous β-catenin to activate β-catenin-TCF activity in both PCa and CV-1 cells (Fig. 3A). HOXB13-mediated suppression was accomplished in a dose-responsive manner in CV-1 (Fig. 3B) but was not seen in the other promoters, including SV40, RSV, TATA, and CMV (Fig. 3C). Instead, HOXB13 moderately increased transcriptional activity in these promoters. Additionally, overexpression of another homeobox protein, NKX3.1, did not suppress the activity of β-catenin/TCF (Fig. 3D). These results suggest that HOXB13 specifically suppresses β-catenin/TCF-4-mediated transcription.
To test whether β-catenin acts as a modulator mediating HOXB13 suppressor function in this signaling pathway, a constitutively active form of β-catenin was cotransfected. The addition of this β-catenin did not restore the HOXB13-induced inhibition of TCF activity in CV-1 cells (Fig. 3E), suggesting that suppression of β-catenin/TCF signaling is not mediated through the alteration of β-catenin expression. Interaction of HOXB13 with β-catenin was not seen (data not shown).

To further investigate the mechanistic link between HOXB13 and β-catenin/TCF signaling, a recombinant adenovirus expressing HOXB13 (Ad-GFP-HOXB13) was constructed and infected into the aforementioned cells at 10 multiplicity of infection. As shown in Fig. 4A, Ad-GFP-HOXB13 virus showed a greater killing effect on CV-1 cells at day 10 after infection compared with Ad-GFP control virus. However, the same study could not be accomplished in PCa cells, including LNCaP, CWR22RV, PC3, and MDA PCa 2b, because of the viral cytotoxic effect on PCa cells, as reported previously (21). We then studied whether HOXB13 regulates the expression of TCF-4, the most abundant TCF in PCa, or TCF-4-regulated genes in PCa cells. As shown in Fig. 4B, HOXB13 suppression of TCF-4 in CV-1 cells could not be determined, because antihuman TCF-4 antibodies (Upstate Biotechnology) did not recognize the simian form of TCF-4. The expression of c-myc, a TCF-4 responsive protein, was markedly decreased in most cells. Suppression of cyclin D1 by HOXB13, however, was slight. Presumably this was because the regulation of cyclin D1 requires the long-term expression of HOXB13, rather than a transient expression from a recombinant virus.

As predicted in the reporter gene transcription assay shown in Fig. 3D, the level of β-catenin was not affected by HOXB13.

To see the transcription-suppressive effect of HOXB13 in natural promoter, we used c-myc promoter spanning a 2.5-kb region containing two copies of TCF-4RE (22). As shown in Fig. 4C, HOXB13 suppressed c-myc promoter activity in CWR22RV cells. Next, we investigated whether suppression of TCF-4 by HOXB13 is mediated at the RNA level. After viral infection into PC3 cells, total RNA was extracted, followed by RT-PCR analysis. As shown in Fig. 4D, HOXB13 virus significantly suppressed TCF-4 RNA levels. Attempts to define a mechanistic link between HOXB13 and the promoter region(s) of TCF-4 are in progress.

DISCUSSION

Alterations in the level of expression or mutant forms of HOX genes may be important in tumorigenesis, e.g., HOXA9 overexpression transforms myeloid cells, causing leukemia in animal models.
HOXB13 is constitutively expressed in melanomas, and transfection of antisense HOXB7 inhibits cellular proliferation and expression of basic fibroblast growth factor through which HOXB7 directly transactivates (25). Some HOX proteins, including HOXC6 and HOXC8, are involved in PCa progression and overexpressed in more advanced metastatic and recurrent PCa (26, 27).

HOXB13 is the most 5' gene in a cluster on chromosome 17q21, and the mouse and human homologues share 100% amino acid identity in the homeodomain and ~91% in the remainder of the gene (10). Expression of mouse Hoxb13 is restricted to the terminal spinal cord, hindgut, and urogenital sinus during embryogenesis (10), albeit very low expression of Hoxb13 is reported in skin (18, 28). In the urogenital sinus, Hoxb13 starts to express concomitantly with the formation of the prostatic bud in the later stage of embryogenesis. Its expression in adult mouse is confined to the epithelial cells of the prostate, distal colon, and rectum in an androgen-independent fashion (11). Hoxb13 knockout mice displayed overgrowth in all major structures derived from the tail bud (12) and ventral prostate-specific defects, including defects in epithelial cell morphology and complete loss of secretory proteins (13). These mice showed similar phenotypes to Nkx3.1 mutant mice and developed a swollen prostate in older mutant mice. However, Hoxb13 mutant mice did not reveal any phenotypes of prostatic intraepithelial neoplasia, which may require the loss of another regulatory protein, such as Nkx3.1. The miss-expression of CD44 in luminal epithelial cells observed in these mice is consistent with neoplastic lesions in many tissue types (29, 30).

The results shown here demonstrate that forced expression of HOXB13 inhibited the growth of PC3 PCa cells. HOXB13 is reported to be preferentially up-regulated in PCa cells (31, 32). Although we have seen that the level of HOXB13 expression is at least maintained in tumors, we believe that there are subpopulations of both HOXB13-positive and -negative PCa cells, which cannot be distinguished by routine RNA analyses. Although Johnson et al. (32) suggested, without showing data, that PC3 cells express HOXB13 and its antisense had an antiproliferative effect on PC3 cells, we have consistently observed that HOXB13 is not expressed in androgen receptor (AR)-deficient PCa cells (PC3 and DU145), although AR-expression PCa cells retain HOXB13 expression (LNCaP, MDA PCa 2b, and LAPC-4).

We have observed that the growth-suppressive effect of HOXB13 was accompanied by a remarkable change in cell morphology in PC3 cells, as shown in Fig. 1B. HOXB13 drove PC3 cells to adopt a gigantic cobblestone appearance, a characteristic phenotype of epithelial cells resulting from terminal differentiation to the mitotically inactive state. This phenomenon has been described to explain the antiproliferative activity of the Notch gene in PCa cells (33). HOX proteins are known to be transcription factors. A low level of nuclear HOXB13 was detected in epidermis but not developing dermis, implying that a decrease of HOXB13 is preferred in cells in a differentiated and proliferative mode (18). In addition, HOXB13 knockout mice exhibited a more fetal-like adult skin, along with enhanced wound healing and higher levels of hyaluronan, considered to inhibit differentiation and promote cell proliferation (17). Together, HOXB13 may inhibit cell growth by forcing PC3 cells into a terminal differentiated state. We demonstrated that HOXB13-mediated growth suppression of PC3 cells was accomplished through the down-regulation of TCF-4 expression and subsequently its responsive genes, including c-myc and cyclin D1. Suppression of TCF-4 expression was a specific effect by HOXB13; another homeodomain protein, NKX3.1, did not suppress TCF-4-mediated signaling. This result suggests that HOXB13-mediated growth suppression is not caused by nonspecific cell differentiation.

Involvement of HOXB13 in cell cycle arrest was somewhat inconsistent among the transfectants, although the growth-suppressing effect in three of four clones resulted from G1 arrest. Clone 3 seems to manifest no alteration of cell cycle distribution because of a low level of proliferation suppression.
of HOXB13, although clone 3 grew more slowly than a neo-clone in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in vitro proliferation assay. It is not unusual for random incorporation of DNA into chromosome to have a growth-regulating effect. A connection between HOX transcription factors and cell cycle progression has been reported, e.g., subgroups, such as HOXA13, HOXC10, and HOXC13, interacted with a sequence within the DNA region of replication associated with the human lamin B2 genes (34). HOX11 abrogated the G2 cell cycle checkpoint by interacting with protein phosphatase 2A (35).

TCF-4 belongs to a family of transcription factors possessing a high mobility group (HMG) DNA-binding box and facilitates the assembly of multiprotein enhancer complexes. Because TCF-4 is deeply involved in the adenomatous polyposis coli/β-catenin/TCF pathway, it is thought to be a key cell developmental and growth regulatory protein. In fact, TCF-4 knockout mice die shortly after birth and show an absence of proliferative crypt regions between the villi of the colon (36). TCF-4 has been known to be widely expressed in PCa cells (20). Because TCF-4 is negatively regulated by HOXB13, it is conceivable that tumors acquire a growth advantage from the loss of HOXB13. Although expression of HOXB13 did not seem to be altered in PCa, >70% of colorectal cancers lost HOXB13 expression, suggesting that expression of HOXB13 may be affected by the AR status. In fact, Shen et al. (37) have reported the possible involvement of HOXB13 in the regulation of hormone-mediated hormone receptor activation by its interaction with CBP/p300 coactivators. We also observed that HOXB13 is involved in the suppression of androgen-activated AR transcriptional activity. Therefore, the growth-suppressive function of HOXB13 seems to be accomplished by affecting multiple signaling pathways in prostate tumors. Additional studies are needed to elucidate the association between HOXB13 and AR as a key part of the process of prostate development and tumorigenesis.

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