PCOTH, a Novel Gene Overexpressed in Prostate Cancers, Promotes Prostate Cancer Cell Growth through Phosphorylation of Oncoprotein TAF-I\(^3\)/SET

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Abstract

Through genome-wide cDNA microarray analysis coupled with microdissection of prostate cancer cells, we identified a novel gene, prostate collagen triple helix (PCOTH), showing over-expression in prostate cancer cells and its precursor cells, prostatic intraepithelial neoplasia (PIN). Immunohistochemical analysis using polyclonal anti-PCOTH antibody confirmed elevated expression of PCOTH, a 100-amino-acid protein containing collagen triple-helix repeats, in prostate cancer cells and PINs. Knocking down PCOTH expression by small interfering RNA (siRNA) resulted in drastic attenuation of prostate cancer cell growth, and concordantly, LNCaP derivative cells that were designed to constitutively express exogenous PCOTH showed higher growth rate than LNCaP cells transfected with mock vector, suggesting the growth-promoting effect of PCOTH on prostate cancer cell. To investigate the biological mechanisms of this growth-promoting effect, we applied two-dimensional differential gel electrophoresis (2D-DIGE) to analyze the phospho-protein fractions in LNCaP cells transfected with PCOTH. We found that the phosphorylation level of oncoprotein TAF-I\(^3\)/SET was significantly elevated in LNCaP cells transfected with PCOTH than control LNCaP cells, and these findings were confirmed by Western blotting and in-gel kinase assay. Furthermore, knockdown of endogenous TAF-I\(^3\)/SET expression by siRNA also attenuated viability of prostate cancer cells as well. These findings suggest that PCOTH is involved in growth and survival of prostate cancer cells throughout, in parts, the TAF-I\(^3\)/SET pathway, and that this molecule should be a promising target for development of new therapeutic strategies for prostate cancers.

Introduction

Prostate cancer is the most common malignancy in males and the second leading cause of cancer-related death in the United States and Europe (1), and frequency of prostate cancer has been increasing significantly in most developed countries probably due to prevalent western-style diet and the explosion of the aging population (1, 2). Surgical and radiation therapies are effective to the localized disease, but nearly 30% of treated prostate cancer patients still suffer from the relapse of the disease (3–5). Most of the patients with relapsed or advanced disease respond well to androgen ablation therapy because prostate cancers are usually androgen-dependent at a relatively early stage. However, they often acquire androgen-independent phenotype and show no or very poor response to the androgen ablation therapy. No effective anticancer drug or therapy is presently available to the advanced or recurrent androgen-independent prostate cancer. Hence, development of new therapies based on the molecular mechanisms of prostate carcinogenesis or hormone refractory is urgently and eagerly required.

Because prostate cancer shows high degree of heterogeneity in histopathologic and molecular aspects, it is essential to characterize the detailed molecular features of prostate cancer cells. In this aspect, we did genome-wide cDNA microarray analysis of prostate cancer cells purified from clinical cancer tissues by means of laser microbeam microdissection (LMM) and identified dozens of genes whose expression levels were evidently increased in prostate cancer cells and/or its precursor prostatic intraepithelial neoplasias (PIN), comparing with normal prostatic epithelial cells (6). Among the trans-activated genes, we here report characterization of a novel gene, prostate collagen triple helix (PCOTH), which is very likely to play a significant role in proliferation or viability of prostate cancer cells. We also show that PCOTH may enhance the phosphorylation of oncoprotein TAF-I\(^3\)/SET and the cell proliferation. Our data should provide new insights in prostatic carcinogenesis and some clues to develop the new therapeutic strategy against prostate cancers.

Materials and Methods

Cell lines and tissues. Human prostate cancer cell lines LNCaP, DU-145, and PC-3 were obtained from American Type Culture Collection (Rockville, MD). All of the cells were cultured as monolayers in the following media: RPMI 1640 (Sigma-Aldrich, St. Louis, MO) for LNCaP, EMEM (Sigma-Aldrich) for DU-145, and F-12 (Invitrogen, Carlsbad, CA) for PC-3 with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma-Aldrich). Cells were maintained in incubators containing humidified air with 5% CO\(_2\) at 37°C. Frozen or paraffin-embedded prostate cancer tissues were obtained from prostate cancer patients who underwent radical prostatectomy at Department of Urology, Kochi Medical School and Department of Urology, Iwate Medical University, with appropriate informed consent as described previously (6).

Semi-quantitative reverse transcription-PCR. Total RNA was extracted from cell lines, microdissected prostate cancer cells, and bulk prostate cancer tissues using TRIzol reagent (Invitrogen) according to manufacturer’s instruction. Extracted total RNA was treated with DNase I (Roche Diagnostic, Mannheim, Germany) and reversely transcribed to single-stranded cDNA using oligo d(T)\(_{12-18}\) primer with Superscript reverse transcriptase II (Invitrogen). We prepared appropriate dilutions of each single-strand cDNA followed by normalizing cDNA content using β2-MG

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PCOTH, a Novel Gene Overexpressed in Prostate Cancers

Northern blotting analysis. Human multiple-tissue blots (BD Biosciences, Palo Alto, CA) were hybridized for 16 hours with 32P-labeled cDNA as PCR templates. Primer sequences were the following: β2-MG (forward, 5'-CACCACCCGCTGAAAAAGAAG-3' and reverse, 5'-TACCTGAGGCTCAGGAGCCCA-3'). The conditions for PCR are followed: initial denaturation at 94°C for 5 minutes, 20 cycles for β2-MG and 35 cycles for PCOTH of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA).

Antibodies and recombinant protein. The cDNA fragment encoding the second-99th amino acid of PCOTH (Genbank accession no. AB113650) was generated using KOD-Plus polymerase (TOYOBO) and cloned into pGEX-6P-1 vector (Amersham). The recombinant GST-TAF-I (forward, 5'-CCGACACTCTGGGTAGC-3' and reverse primer, 5'-TGCTGAGCT-3') was expressed exclusively in the testis and prostate and was deparaffinized, subjected to treatment with microwave at 700 W for 1-Min, and treated with peroxidase blocking reagent (DAKO) followed by protein glutathione sepharose 4B (Amersham) under native condition according to the supplier’s protocol. Further purification was done by use of High-performance Liquid Chromatography AKTA explorer (Amersham) equipped with Mono S HR 5/5 (Amersham). The protein was immunized into rabbits, and the immune sera were purified on affinity columns packed with Affi-Gel 10 activated affinity media (Bio-Rad, Hercules, CA) conjugating recombinant PCOTH protein with accordance of basic methodology. The affinity-purified anti-PCOTH polyclonal antibody was used for detection of PCOTH protein. The cDNA fragment encoding the first-276th amino acid of TAF-Iβ was generated using KOD-Plus polymerase (TOYOBO) and cloned into pGEX-6P-1 vector (Amersham). The recombinant GST-TAF-Iβ and glutathione S-transferase (GST) proteins were expressed in E. coli BL21 codon plus (Stratagene) and purified with glutathione sepharose 4B (Amersham) under native condition according to the supplier’s protocol. Anti-β-actin monoclonal antibody, anti-calreticulin polyclonal antibody, and anti-TAF-Iβ polyclonal antibody were purchased from Stressgen (Victoria, British Columbia, Canada) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Immunohistochemical analysis. Paraffin-embedded tissue sections were deparaffinized, subjected to treatment with microwave at 700 W for 4 minutes in antigen retrieval solution, high pH (DAKO, Carpinteria, CA), and treated with peroxidase blocking reagent (DAKO) followed by protein block reagent (DAKO). Tissue sections were incubated with a polyclonal antibody against PCOTH followed by horseradish peroxidase–conjugated

as a quantitative control, demonstrating PCR reaction using single-strand cDNA as PCR templates. Primer sequences were the following: β2-MG (forward, 5'-CACCCGCTGAAAAAGAAGA-3' and reverse, 5'-TACGAGGCTCAGGAGCCCA-3'). The conditions for PCR are followed: initial denaturation at 94°C for 5 minutes, 20 cycles for β2-MG and 35 cycles for PCOTH of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds on a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster, CA).

PCOTH mRNA expression level in prostate cancer cells and tissue distribution of PCOTH mRNA. A, semiquantitative RT-PCR analysis of PCOTH in cancer cells (T) and normal epithelial cells (N) microdissected from 12 prostate cancer tissues. Twelve horizontal lines are clinical N/T pair cases. β2-MG was used to quantify the each of cDNA contents. B, multiple tissue Northern blot analysis demonstrated that PCOTH was expressed exclusively in the testis and prostate and the length of major PCOTH transcript was about ~0.9 kb. β-Actin served as a loading control. P.B. leukocyte, peripheral blood leukocyte. C, genomic structure of PCOTH and PCOTH-b spanning at 3.22 kb on chromosome 13q12.12. Exon-intron organization and alternative transcripts of the PCOTH and PCOTH-b genes are presented. PCOTH encodes 100 amino acid residues (Genbank accession no. AB113650). The splicing-variant PCOTH-b that lacks cDNA of 3' region corresponding to the 69th to 147th base in exon 3 of PCOTH, resulting in open reading frame encodes 116-amino-acid residues. Arrows, positions of primers used in RT-PCR analysis. Black box, open reading frame. D, RT-PCR analysis with cDNA prepared from seven prostate cancer tissues purified with LMM. Primer sequences used in this study are the following: forward primer, 5'-AGACATTTTGCCACACGATG-3' and reverse primer, 5'-TCAGGGCGAGAAGGGAATAAGG-3'. Arrows, PCR products amplified from PCOTH or PCOTH-b as illustrated.

Immunochemical staining. Site specific PCR of cDNA from prostate cancer tissues demonstrated that the gene transcribed the splice variant, PCOTH-b, and activated full length PCOTH. As illustrated. Regional distribution of full length and splice variant were provided.
secondary antibody (DAKO). Antigen was visualized with diaminobenzidine (DAKO) staining and sections were counterstained with hematoxylin. To validate the specificity of this antibody in immunohistochemical analysis, 1 μg anti-PCOTH polyclonal antibody was first incubated with 50 μg of recombinant PCOTH protein for 30 minutes at 37°C and the reaction product was centrifuged at 12,000 × g for 15 minutes at 4°C to remove the immune complexes. The supernatant was used as a neutralized antibody for further analysis.

Establishment of LNCaP-PCOTH cells. Full-length PCOTH cDNA (Genbank accession no. AB113650) was PCR amplified using primers that were designed to contain HA-tag sequences at the NH2 terminus, and was cloned into the pCAGGS expression vector encompassing neomycin resistant gene (pCAGGSneo). LNCaP cells (5 × 104) were transfected with 6 μg empty pCAGGS neo-vector or gene-specific expression vectors. Cells were selected with appropriate medium containing 0.8 mg/mL of G418 (Sigma-Aldrich) for 2 weeks and used for protein expression analysis by Western blotting. Proliferation of LNCaP cells that stably expressed PCOTH (LNCaP-PCOTH) or those transfected with pCAGGSneo (LNCaP-mock) were examined by Cell-counting kit-8 (DOJINDO, Kumamoto, Japan). Each of LNCaP-PCOTH and LNCaP-mock cells was seeded at the concentration of 5 × 104 cells per well using a 12-well plate. The assay was done at every 24 hours for 7 days, according to the manufacturer’s instruction.

Construction of small interfering RNA expressing vector and cell viability assay. To investigate the biological function of PCOTH and TAF-I in prostate cancer cells, we used psiU6BX3.0 vector for expression of short hairpin RNA against a target gene as described previously (7). Plasmids designed to express small interfering RNA (siRNA) were prepared by cloning of double-stranded oligonucleotides into psiU6BX vector. The heterologous expression vectors psiU6-siEGFP using FuGene 6 reagent (Roche) according to supplier’s protocol and cultured in appropriate medium containing 800 μg/mL of G418 for 2 weeks. The cells were fixed with 100% methanol, stained with 0.1% of crystal violet-H2O for colony formation assay. In 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay, cell viability was measured using Cell-counting kit-8 (DOJINDO) at 10 days after transfection. Absorbance was measured at 490 nm and at 630 nm as reference with a Microplate Reader 550 (Bio-Rad). Preliminarily, the knockdown effects of these siRNA expression vectors on the target genes were validated by reverse transcription-PCR (RT-PCR) and Western blot analysis 48 hours after transfection. The primer sequences of RT-PCR for PCOTH were described above and those of TAF-I were as follows: forward, 5'-GGAGAGGATGAGAGGACACTGC-3' and reverse, 5'-GAATTGTTCAG-3. The antibody to PCOTH or TAF-I was used for Western blot analysis. For preparation of whole-cell lysates, adherent and detached cells were collected and resuspended in lysis buffer: 50 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, 10 mmol/L CHAPS, and 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA). The homogenate was centrifuged for 20 minutes in a microcentrifuge at 4°C.

Immunoblot analysis. For preparation of whole-cell lysates, adherent and detached cells were collected and resuspended in lysis buffer: 50 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, 10 mmol/L CHAPS, and 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA). The homogenate was centrifuged for 20 minutes in a microcentrifuge at 4°C.
and the supernatants were collected and boiled in SDS sample buffer. 30 μg each of SDS sample was loaded onto 10% and 12.5% SDS-PAGE gels and blotted onto nitrocellulose membranes or polyvinylidene difluoride (Amersham). Protein bands were visualized by chemiluminescent detection system (ECL, Amersham).

Two-dimensional differential gel electrophoresis and peptide fingerprinting analysis. Each of whole cell lysate from LNCaP-PCOTH clones 23, 33, and 80 and LNCaP-mock 6, 10, and 72 cells was prepared and subjected to phospho-protein enrichment kit (BD Biosciences) according to the manufacturer's instruction. The lysate from LNCaP-PCOTH, that from LNCaP-mock, and that of the mixture of LNCaP-PCOTH and LNCaP-mock (1:1) were labeled with CyDye differential gel electrophoresis (DIGE) Fluors Cy5, Cy3, and Cy2 (Amersham) under the supplier's recommendation. All of the labeled samples were mixed together before two-dimensional gel electrophoresis, passively rehydrated into 3 to 10 immobilized pH gradient (IPG) strips (Amersham) for 12 hours, and subjected to isoelectric focusing by means of the Ettan IPGphor Isoelectric Focusing system (Amersham) for a total of 61500 V hour (hold at 500 V for 500 V hours, hold at 1 kV for 1 kV hour, and hold at 8 kV for 60 kV hours). The Cy2, Cy3, and Cy5 signals were individually imaged with mutually exclusive excitation/emission wavelength of 480/530, 520/590, and 620/680 nm, respectively, using two-dimensional Master Gel Imager (Amersham). DeCyder software (Amersham) was used for pairwise comparisons of Cy2 and Cy3, Cy2 and Cy5, or Cy3 and Cy5 images and for quantitative determination of intensity changes of all spots among three combinations. Spots of interest were excised from the gel, subjected to in-gel trypsin digestion, and analyzed using AXIMA-CFR MALDI-TOF MS for peptide mass fingerprinting (PMF) analysis. Peptide masses were searched with 10 ppm mass accuracy and protein database searches were done using the database-fitting program IntelliMarque (Shimadzu Co., Tsukuba, Japan).

In-gel kinase assay. We examined the kinase activity in LNCaP-PCOTH and LNCaP-mock cells by means of kinase renaturation method as described previously (8). In brief, cells were lysed in lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10 mmol/L MgCl₂, 2 mmol/L CaCl₂, 1 mmol/L MnCl₂, 1 mmol/L DTT, 1% Triton X-100, and 0.1% protease inhibitor cocktail III (Calbiochem), incubated on ice for 30 minutes and centrifuged at 15,000 × g for 20 minutes at 4°C. The supernatant was used as a whole cell lysate; 30 μg each of cell lysates was run in 12.5% polyacrylamide/SDS gel containing myelin basic protein (MBP, Upstate Biotechnology, Waltham, MA), GST-TAFI or GST as a kinase substrate at 0.5 mg/mL in the separating gel. Gels were treated with 50 mmol/L Tris (pH 7.5) and 20% isopropanol to remove SDS for 30 minutes twice at room temperature followed by incubation in 50 mmol/L Tris (pH 7.5), 5 mM β-mercaptoethanol, and 6 mol/L guanidine for 1 hour at room temperature. Enzymes denatured in gels were allowed to be renatured in 50 mmol/L Tris (pH 7.5), 5 mM β-mercaptoethanol and 0.04% Tween 20 for 12 hours at 4°C. Gels were incubated in 40 mmol/L HEPES (pH 7.4), 2 mmol/L DTT, 15 mmol/L MgCl₂, 0.3 mmol/L Na₂VO₃.

Figure 3. Knockdown of PCOTH expression by siRNA caused attenuation of prostate cancer cell growth and cell viability. A and B, knockdown effect of siRNA on PCOTH in LNCaP prostate cell line. Semiquantitative RT-PCR (A) and Western blot analysis (B) were done using cells transfected with each of siRNA- expressing vectors to PCOTH (PCOTH-si1, PCOTH-2, PCOTH-3, and PCOTH-4) as well as a negative control vector (siEGFP). β-actin was used to quantify RNAs and β-actin to qualify proteins. C, colony formation assay of LNCaP cells transfected with each of indicated siRNA-expressing vectors to PCOTH (si1, si2, si3, and si4) and a negative control vector (siEGFP). Cells were visualized with 0.1% crystal violet staining after a 14-day incubation with G418. D, E, and F, MTT assay of each of the represented prostate cancer cell line (C, LNCaP; D, PC-3; E, DU-145) transfected with indicated siRNA-expressing vectors to PCOTH (PCOTH-si1, PCOTH-si2, PCOTH-si3, and PCOTH-si4) and a negative control vector (siEGFP); Columns, averages after a 14-day incubation with G418; bars, SD. ABS on Y-axis means absorbance at 490 nm and at 630 nm as reference measured with a microplate reader. Experiments were carried out in triplicate. **, P < 0.01 (Student’s t test).
Results

Identification of a novel gene, PCOTH, and its expression pattern. We previously reported the genome-wide expression profiles of prostate cancer cells and PINs purified from clinical prostate cancer tissues by means of cDNA microarray analysis representing 23,040 genes in combination with the LMM system (6). A number of genes were shown trans-activated in prostate cancer cells and/or PIN cells compared with normal prostate epithelial cells. Among them, we focused on one gene whose expression was highly elevated in both prostate cancer and PIN cells, which was later termed PCOTH according to the reason indicated below. Semiquantitative RT-PCR confirmed the elevated expression of PCOTH in prostate cancer cells in 10 of the 12 clinical prostate cancer samples as shown in Fig. 1A. Northern blot analysis for normal human adult tissues identified a ~0.9-kb PCOTH transcript exclusively in testis and prostate (Fig. 1B). To isolate a full-length cDNA sequence of this gene, we examined the expressed sequence tagged database and obtained a full-length cDNA sequence of this gene, we examined the expressed sequence tagged database and obtained a full-length cDNA sequence of this gene, represented by Genbank accession no. AB113650. A comparison of its genomic sequence with this cDNA sequence indicates that this gene consists of four exons. The peptide sequence revealed no significant homology to any other human proteins reported thus far, but the region encompassing the 37th to 95th amino acid residues was predicted to be a collagen triple-helix domain of Pro-X-Gly repeats by the SMART program (http://smart.embl-heidelberg.de/). Hence, we termed this novel protein as PCOTH. In the course of functional analysis shown later, we identified a splicing variant that was also considered one of the major transcripts of PCOTH. The splicing variant, termed PCOTH-b, which lacks the 3′ portion corresponding to exon 3 of PCOTH (Fig. 1C), encodes a peptide of 116 amino acids. To examine which variant (PCOTH and PCOTH-b) was dominantly expressed in prostate cancer cells, we did RT-PCR experiments and found that PCOTH but not PCOTH-b was highly up-regulated in prostate cancer cells (Fig. 1D).

Immunohistochemical analysis in prostate cancer tissues. To further investigate the expression of PCOTH protein in prostate cancer and PIN cells, we generated polyclonal antibody to PCOTH by immunizing recombinant PCOTH protein (rhPCOTH) and did immunohistochemical analysis. To clarify its specificity to PCOTH protein, we preincubated the antibody with rhPCOTH and did immunohistochemical analysis of prostate cancer tissues. As shown in Fig. 2A, the positive signal by anti-PCOTH antibody obtained in prostate cancer tissues was completely diminished by preincubation with rhPCOTH, indicating its high specificity to PCOTH protein. In the subsequent immunohistochemical analysis, strong immunohistochemical signal for PCOTH was detected predominantly in the cytoplasm of prostate cancer cells (Fig. 2B) and, more strongly, PIN (Fig. 2C) in all of 10 prostate cancer cases examined, although we also observed a weak signal in noncancerous prostate epithelial cells (Fig. 2B, arrowhead) as concordant to the results of multiple tissue Northern blot analysis.

Involvement of PCOTH expression in prostate cancer cell growth. To examine roles of PCOTH overexpression in prostate cancer cell growth, we constructed several expression vectors designed to express siRNA specifically to PCOTH and transfected them into three prostate cancer cell lines LNCaP, PC-3, and DU-145, all of which expressed PCOTH endogenously at some level. Among the four plasmids we tested in LNCaP cells, PCOTH-si3 showed the significant knockdown effect on endogenous PCOTH transcript and PCOTH protein (Fig. 3A), and this transfection resulted in reduction of the numbers of colonies (Fig. 3B) as well as those of the viable cells measured by MTT assay for all three prostate cell lines (Fig. 3C), whereas the transfection of other plasmids (PCOTH-si1, PCOTH-si2, PCOTH-si4 and a negative control of siEGFP) showed no or little knockdown effect on PCOTH expression. As shown in Fig. 3C, D, and E, the numbers of the viable cells transfected with the PCOTH-si3 construct were reduced to about 20% for LNCaP and PC-3 cells and to about 50% for DU-145 cells. These data indicated an important role of PCOTH in prostate cancer cell survival and/or growth. On the other hand, PCOTH-si3 did not affect the cell viability of other cell lines that did not express PCOTH, which excluded a possibility of the “off-targeting” effect of PCOTH-si3 (data not shown).

To further explore the potential oncogenic function of PCOTH, we established the LNCaP derivative cell line, LNCaP-PCOTH, in which exogenous PCOTH expressed constitutively at a much higher level than endogenous PCOTH in its parental cells. We

![Figure 4](image-url)
also prepared control LNCaP cells transfected with the mock vector (LNCaP-mock) and compared their proliferation. Western blot analysis shown in Fig. 4A validated high level of exogenous PCOTH expression in three derivative clones. MTT assay showed that the three derivative LNCaP-PCOTH clones 23, 33, and 80 grew significantly more rapidly than the three LNCaP-mock clones 6, 10, and 72 (Fig. 4B), indicating the higher levels of PCOTH expression enhanced prostate cancer cell growth.

Two-dimensional differential gel electrophoresis analysis of phospho-protein in PCOTH-overexpressing cells. To investigate the biological mechanisms of the growth-promoting effect of PCOTH on prostate cancer cells, we applied two-dimensional DIGE to analyze the phospho-protein fractions in LNCaP-PCOTH cells as indicated in Fig. 5A. After enrichment of phospho-protein fraction by the affinity columns, two-dimensional DIGE analysis detected 830 spots (average in triplicate) on a whole gel and indicated 30 protein spots were more phosphorylated in LNCaP-PCOTH cells comparing with LNCaP-mock cells (Fig. 5B). Among them, we concentrated on two spots whose signals were most significantly increased (32.07 and 9.58 times in the average of three experiments, respectively) on two-dimensional DIGE, and PMF analysis identified them to be TAF-I\(h\) [gi|4506891] and calreticulin [gi|4757900]. Western blot analysis after fractionation of the phosphorylated and non-phosphorylated proteins by the affinity column validated significantly enhanced phosphorylation of TAF-I\(h\) and calreticulin in LNCaP-PCOTH cells, comparing with those of LNCaP-mock cells (Fig. 5C). It was notable that almost all of the TAF-I\(h\) protein was phosphorylated in LNCaP-PCOTH cells, whereas a part of TAF-I\(h\) still remained to be non-phosphorylated in LNCaP-mock cells. Furthermore, our

Figure 5. Enhancement of phosphorylation of proteins in cells expressing high level of exogenous PCOTH. A, study flowchart of two-dimensional DIGE analysis with LNCaP-PCOTH and LNCaP-mock cells. Whole cell lysates from each LNCaP derivative cells (LNCaP-PCOTH and LNCaP-mock) was fractionated by the phospho-protein affinity column and labeled with indicated fluorescence. Mixture, 1:1 mix of LNCaP-PCOTH and LNCaP-mock sample. IEF, isoelectric focusing. B, fluorescence image of a representative two-dimensional DIGE gel. LNCaP-PCOTH and LNCaP-mock cells were labeled with Cy5 and Cy3, respectively, and an overlaid image is shown. Blue and red colored spots are proteins whose phosphorylation was relatively enhanced in terms of quantity in LNCaP-PCOTH, or LNCaP-mock cells, respectively. Two circles are protein spots that were subjected to PMF analysis because of showing the most significant enhancement of phosphorylation in LNCaP-PCOTH and turned out to correspond to TAF-I\(h\) [gi|4506891] and calreticulin [gi|4757900]. C, Western blot analysis on the eluent fraction (phosphorylated) and flow-through fraction (nonphosphorylated) separated by the phospho-protein affinity column validated enhanced phosphorylation of TAF-I\(h\) and calreticulin in LNCaP-PCOTH cells, comparing with those of LNCaP-mock cells. The eluent fraction and flow-through fraction from each of three clones of LNCaP-PCOTH and mock mixture organized with equal parts of individual three clones were blotted. \(\beta\)-Actin was known not phosphorylated under constitutive condition and served as a quality control for phospho-protein affinity column. Exogenous expression of PCOTH in LNCaP-PCOTH cells was confirmed using anti–HA-tag antibody. Immunoblots with anti–TAF-I\(h\) and anti-calreticulin antibody showed the elevated level of phospho-TAF-I\(h\) and phospho-calreticulin in LNCaP-PCOTH cells. SYPRO Ruby staining (Bio-Rad) served as a loading control. D, in-gel kinase assay revealed that high level of exogenous PCOTH expression modulated the kinase activity. Whole cell lysates from indicated cells expressing high level of exogenous PCOTH (three individual clones) or those transfected with mock vector (mixture of three individual clone) were separated in SDS-polyacrylamide gel containing the indicated substrate proteins (GST-TAF-I\(h\), GST, or MBP). Arrowheads, relative molecular weight of kinases involving phosphorylation of TAF-I\(h\). CBB stain served as loading control.
in-gel kinase assay showed that the kinase activity to TAF-Iβ was clearly elevated in LNCaP-PCOTH cells comparing to that in LNCaP-mock cells as seen on two intense bands of about 130 and 60 kDa (Fig. 5D). The kinase activity in LNCaP-PCOTH cells was also significantly elevated, comparing with that in LNCaP-mock cells (Fig. 5D), to MBP, which is a well-known in vitro substrate for numerous kinases, such as mitogen-activated protein kinase (9, 10), cyclic AMP–dependent protein kinase (11, 12), calcium-dependent protein kinase (13), and protein kinase C (14), and is often used for examining protein kinase activities in vitro. These observations suggested that PCOTH could serve as a functional modulator of various kinases, especially some kinases involved in TAF-Iβ phosphorylation.

**Knockdown of TAF-Iβ attenuated prostate cancer cell growth.** To further verify the biological role of TAF-Iβ on prostate cancer cell growth, we prepared five constructs designed to express siRNA specific to TAF-Iβ and examined the knockdown effect of each construct in LNCaP and PC-3 prostate cancer cell lines, which expressed TAF-Iβ as well as PCOTH at some level. Western blot analysis showed that TAF-Iβ-si1 and TAF-Iβ-si3 had strong knockdown effect on endogenous TAF-Iβ, whereas TAF-Iβ-si2, TAF-Iβ-si4, TAF-Iβ-si5, and negative control siEGFP had no or little knockdown effect on TAF-Iβ (Fig. 6A and D). Introduction of each of TAF-Iβ-si1 and TAF-Iβ-si3 constructs into LNCaP and PC-3 prostate cancer cell lines resulted in the reduced number of colonies (Fig. 6B and E) and the cell viability (Fig. 6C and F), whereas the transfections of TAF-Iβ-si2, TAF-Iβ-si4, TAF-Iβ-si5, and siEGFP revealed no or little effect on the colony number and the cell viability, suggesting that TAF-Iβ could have some important roles in prostate cancer cell growth and proliferation, presumably associated with PCOTH.

**Discussion**

In this study, we focused on a novel gene, PCOTH, as one of the trans-activated genes in prostate cancer cells. Northern blot analysis showed that PCOTH expression in normal adult tissues was highly specific to the prostate and testis. Our immunohistochemical study using polyclonal antibody to PCOTH clearly indicated up-regulation of PCOTH expression in PIN cells, the precursor of prostate cancer, as well as prostate cancer cells. The subsequent functional analysis implied that PCOTH overexpression was very likely to be involved in prostate tumorigenesis; knockdown of PCOTH expression by siRNA resulted in drastic reduction of the growth or viability of prostate cancer cells. Concordantly, exogenous addition of PCOTH expression into cells enhanced prostate cancer cell growth.

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**Figure 6.** Knockdown effect by siRNA on TAF-Iβ expression and cell growth of LNCaP and PC-3. A and D, Western blot analysis of cell lysate from indicated cell lines transfected with each of the indicated siRNA expressing plasmids (TAF-Iβ-si1, TAF-Iβ-si2, TAF-Iβ-si3, TAF-Iβ-si4, and TAF-Iβ-si5) or a negative control vector (siEGFP). TAF-Iβ-si1 and TAF-Iβ-si3 effectively knocked down the endogenous TAF-Iβ expression in LNCaP (A) and PC-3 cells (D), while TAF-Iβ-si2, TAF-Iβ-si4, and TAF-Iβ-si5 did not. β-Actin served as a loading control. B and E, colony formation assay for indicated cell lines to assess the cell number after transfection with each of the indicated siRNA expressing plasmids (TAF-Iβ-si1, TAF-Iβ-si2, TAF-Iβ-si3, TAF-Iβ-si4, and TAF-Iβ-si5) or a negative control vector (siEGFP). Cells were stained with 0.1% crystal violet after a 14-day incubation with G418. Experiments were done in triplicate. C and F, MTT assay in the indicated cell line to quantify the cell viability under knockdown of TAF-Iβ expression. Cells were transfected with each of the indicated siRNA expressing constructs to TAF-Iβ (TAF-Iβ-si1, TAF-Iβ-si2, TAF-Iβ-si3, TAF-Iβ-si4, and TAF-Iβ-si5), or the negative control (siEGFP). ABS on the Y-axis is the absorbance at 490 nm and at 630 nm as reference measured with a microplate reader. Columns, averages; bars, SD. Experiments were done in triplicate. **, P < 0.01 (Student’s t test).
To investigate the molecular mechanism of its growth-promoting effect on prostate cancer cells, we conducted two-dimensional DIGE to analyze phosphorylated protein fraction of LNCaP-PCOTH cells. Two-dimensional DIGE is a new emerging proteomics technology that makes it possible to comprehensively analyze the differences in protein levels including post-translational modification such as phosphorylation and glycosylation (15, 16). The reason we focused on phospho-proteins for the two-dimensional DIGE analysis was that phospho-proteins are known to play prominent roles in various biochemical processes and signal transduction pathways including cell proliferation and cell cycle control (17–19). We did two-dimensional DIGE in combination with phospho-protein enrichment by the affinity column and identified TAF-β- and calreticulin whose phosphorylation levels were most significantly increased in cells expressing a high level of PCOTH. Western blot analysis and the in-gel kinase assay validated that PCOTH expression in prostate cancer cells was associated with elevation of TAF-β phosphorylation. Calreticulin is known to be a major Ca²⁺ binding protein and is reported to act as an important modulator in gene transcription by nuclear hormone receptors (20), but there is no indications or reports about possibility of phosphorylated calreticulin or direct association with cell growth; we excluded calreticulin and focused on only TAF-β for the following reasons in addition to its notable change of phosphorylated status.

TAF-β was first identified as a partner of the fusion gene in acute undifferentiated leukemia as set-can gene (21) and was shown to be a multitasking protein such as a potent inhibitor of protein phosphatase 2A (22, 23), a target of granzyme A (24, 25), an inhibitor of histone acetyltransferase (26, 27), and also a regulator of cell cycle transition (28, 29), indicating that TAF-β should be a modulator of cell growth and proliferation. Indeed, our study also showed that knockdown of TAF-β expression by siRNA in prostate cancer cells lead to the decrease of the cell growth or viability. Furthermore, TAF-β was reported to be phosphorylated at its serine residues (30). Hence, we hypothesize that phosphorylated TAF-β might function as an active form and enhance cell growth of LNCaP derivative cells expressing a high level of PCOTH, although phosphorylation sites of TAF-β remains unknown. Our results supported this hypothesis that PCOTH would regulate the phosphorylated TAF-β level through active promotion of some kinases and/or through functional inhibition of phosphatases for TAF-β, because and other proteins with collagen triple helix motif are unlikely to have kinase activity, and that phosphorylated TAF-β would take a part in a wide range of cellular processes including cell growth, proliferation and cell cycle in a positive manner. Whereas TAF-β was ubiquitously expressed in various tissues and cell lines (31), PCOTH expression was exclusively observed in testis, prostate, and prostate tumors. Hence, PCOTH might serve as a prostate-, testis-, or prostate cancer-specific modulator in regulating, at least, phosphorylation of TAF-β, and then PCOTH overexpression in prostate cancer and PIN cells is likely to promote cell viability by modulating phosphorylation of TAF-β.

Taking these findings together, TAF-β phosphorylation modulated by PCOTH can be a specific event in prostate tumors, although the significance of elevated phosphorylated TAF-β level for cell growth still remains to be elucidated. The inhibition of PCOTH modulation of TAF-β phosphorylation may provide us a novel promising approach for molecular treatment of prostate cancer with high specificity.

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References


PCOTH, a Novel Gene Overexpressed in Prostate Cancers, Promotes Prostate Cancer Cell Growth through Phosphorylation of Oncoprotein TAF-Iβ/SET

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