Nkx3.1 Functions as Para-transcription Factor to Regulate Gene Expression and Cell Proliferation in Non-cell Autonomous Manner*

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Background: Transcription factor Nkx3.1 is classically considered as an intracellular tumor suppressor.

Results: Nkx3.1 is intercellularly translocated for regulating gene expression and inhibiting prostate epithelial cell growth.

Conclusion: Nkx3.1 is a paracrine transcription factor mediating gene expression and growth inhibition signals between cells.

Significance: Transcription factors can be secreted and taken up among mammalian cells as a direct pathway for intercellular gene regulation.

Nkx3.1 is a homeoprotein transcription factor (TF) that inhibits proliferation of prostate epithelial cells (PECs) and acts as a tumor suppressor for prostate cancer (PCa). Because TFs classically function within the cells that produce them, Nkx3.1-induced growth inhibition was considered to occur in a cell-autonomous manner. We, however, found that Nkx3.1 protein can be secreted from cultured PECs and is detectable in the prostatic fluid and urine. A PCa-related point mutation (T164A) abolished Nkx3.1 secretion. Amazingly, secreted Nkx3.1 protein can translocate into adjacent cells, bind to the regulatory sequence of Nkx3.1 target genes and impact the expression of these genes in these adjacent cells. Expression of Nkx3.1 in PECs can also affect gene expression in adjacent cells, and this effect is abolished by the T164A mutation. Nkx3.1 protein inhibits cell proliferation when added to the culture. Expression of Nkx3.1, not the T164A mutant, also inhibits the proliferation of co-cultured cells. These results indicate that Nkx3.1 functions as a “para-transcription factor (PTF),” with the ability to regulate genes and inhibit cell proliferation in a non-cell-autonomous manner. We also demonstrate that Nkx3.1 contains an evolutionarily conserved protein transduction domain essential for its PTF function, implicating potentially common PTF function among homeoproteins. In addition to the PEC-related T164A mutant, the secreted Nkx3.1 is reduced drastically in the prostatic fluid and urine of mice with PCa. These results indicate that Nkx3.1 can function as a PTF to suppress PCa and the urinary Nkx3.1 may be a potential biomarker for PCa diagnosis.

Regulatory pathways mediated by endocrine hormones, paracrine growth factors, cytokines, and neurotransmitters are responsible for cell-cell communication. In many typical cell signaling pathways, transcription factors (TFs)3 serve as the downstream terminal effectors to regulate gene expression in a cell autonomous manner. Studies investigating TFs that regulate transcription outside the cells that produce them have been rare.

Nkx3.1 is a TF critical for normal prostate development and function (1, 2). The Nkx3.1 gene maps to 8p21, a region deleted in 50–85% of human prostate cancer (PCa) specimens (3–7). A genome-wide association study has linked mutations at the Nkx3.1 locus to PCa susceptibility (8, 9). In mice, Nkx3.1 deletion causes prostatic intraepithelial neoplasia, a putative precursor of PCa (10–12). These findings indicate that Nkx3.1 is a prostate tumor suppressor. Intriguingly, the 8p21-deletion in human PCa is monoallelic (13, 14) and a subset of cells in PCa retains Nkx3.1 expression (15–20). Concomitant functional defects may therefore contribute to the loss of Nkx3.1 tumor suppressor activity. The nature for these putative defects on cell regulation is unknown.

The Nkx3.1 tumor suppressor activity is attributed to its ability to regulate genes responsible for cell proliferation. Forced Nkx3.1 expression in human PC3 and rodent AT6 PCa cells inhibits proliferation (10). Conversely, Nkx3.1 knock-out in mice increases prostate epithelial proliferation (1, 10, 11, 21, 22). Furthermore, Nkx3.1 overexpression suppresses the growth of Pten loss-induced prostate tumors (23). In prostate tumors, cells with lower Nkx3.1 have higher proliferation rates (19, 23). Because Nkx3.1 is a TF, it is assumed that its inhibitory effects on proliferation occurred in a cell autonomous manner. No studies have, however, investigated the potential that Nkx3.1 may also function in cells that do not produce it. To

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3 The abbreviations used are: TF, transcription factor; qPCR, quantitative PCR; PEC, prostate epithelial cell; PCa, prostate cancer; CPP, cell-permeable peptide; PTD, protein transduction domain; PHP, Penetratin homologous peptide; TRAMP, transgenic adenocarcinoma of the mouse prostate.
address this, we studied the non-cell autonomous effect of Nkx3.1 on the regulation of gene expression and cell growth.

**EXPERIMENTAL PROCEDURES**

**Cells and Expression Plasmids**—PZ-HPV-7 cells were cultured in PrEGM medium (Lonza). Nbe cells and C4-2 cells were cultured in T medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS). PNT1A cells were cultured in RPMI medium (Invitrogen) supplemented with 10% FBS. RWPE1 cells were maintained in keratinocyte-serum-free medium supplemented with EGF and bovine pituitary extract (Invitrogen). The human Nkx3.1 expression plasmid was provided by Dr. Charles J. Bieberich in University of Maryland and was used to construct other Nkx3.1 mutant plasmids. The double-expression plasmids pC/G and pC-Nkx3.1/G were constructed by inserting Cherry or Cherry-Nkx3.1 fusion sequence into vector pIRE2-EGFP (Clontech), respectively.

**Cell Growth Assay**—PZ-HPV-7 cells were transiently transfected with Nkx3.1 expression plasmids. Cell transfection efficiency was determined by co-expression of GFP using a GFP expression plasmid. Cell number was counted 3 days after transfection. For para-inhibition assay, PZ-HPV-7 cells were transfected overnight and replated in the upper chamber of the trans-well and co-cultured with untransfected PZ-HPV-7 cells in the bottom chamber for 3 days. To test the inhibitory effect of His-Nkx3.1 recombinant protein on cell growth, elution buffer (vehicle) or His-Nkx3.1 protein was added to the cell culture for 2 days. Relative cell numbers were determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent according to the manufacturer’s instructions (Promega). The relative inhibition index was calculated by using the following formula: (number of His-Nkx3.1-treated cells/number of vehicle-treated cells)/number of vehicle-treated cells.

**Nkx3.1 Translocation Assays**—For detecting translocation, cells were transfected with the pC/G and pC-Nkx3.1/G double-expression vectors using TransIT-2020 (Mirus) and incubated overnight. After the transfected cells were thoroughly washed, the same type of untransfected cells was added to the culture. The co-culture was maintained for 30–36 h and then, the translocation of the Cherry-red-labeled Nkx3.1 protein was determined by fluorescence microscopy. 100–200 cells in each randomly selected area were examined individually for red, green, or both red and green fluorescence. Four areas were examined for each group. Data were presented as percentages of red cells, green cells, or cells with both red and green colors.

**Luciferase and RT-qPCR Assays**—The luciferase assay was performed as described previously (24). Briefly, cells were transfected with Probasin-luciferase or PSA-luciferase plasmid. Eighteen hours later, the transfected cells were treated with 100 nm of His-Nkx3.1 protein or the elution buffer (vehicle) for 24 h. The luciferase activity was determined by normalizing to the total protein assayed in the cell lysates. The qPCR primers were designed according to human Pou2F2, GSTa4, and MRPL48 mRNA sequences. RT-qPCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems). For examining para-gene regulation, rat Nbe cells were transfected overnight with indicated expression plasmids and washed with PrEGM medium. The untransfected human PZ-HPV-7 cells were plated with the transfected Nbe cell culture with fresh PrEGM medium. Total RNA was isolated from the co-cultured cells after 24 h and subjected to RT-qPCR assays to determine mRNA levels in PZ-HPV-7 cells using human cDNA-specific primers.

**ChIP Assays for Association of Intercellularly Translocated Nkx3.1 with Its Target Genes in Adjacent Cells**—The rat Nbe prostate epithelial cells were transfected with HA-tagged Nkx3.1 (HA-Nkx3.1) expression plasmid for 24 h. Transfected Nbe cells were washed and harvested by trypsinization. When the human PZ-HPV-7 prostate epithelial cells were cultured to 40% confluence, the harvested Nbe cells were added to the PZ-HPV-7 cell culture to give a cell density of ~80% confluence. These mixed cells were co-cultured for 24 h before ChiP assay. The transfected Nbe cells and untransfected PZ-HPV-7 cells also were cultured separately and used as negative controls for ChiP assay. ChiP assay was performed using 2 μg/reaction of HA antibody or normal control rabbit IgG and protein-G/A beads as described previously (25). The human gene-specific PCR primers for ChIP assays were designed to amplify Nkx3.1-binding regions in known Nkx3.1 target genes (26), including the 5’ non-coding regions from bp −6519 to bp −6380 of the human GSTa4 gene and from bp −3228 to bp −2976 of the human MRPL48 gene. As a negative control, another pair of primers (5’-aagcttggagcctggagaag and 5’-cagtcgagctg-gcaaaa) was used to amplify an unrelated DNA region in the human VEGF gene that does not contain any Nkx3.1 binding site. All of these primer pairs do not amplify rat genes.

**Peptide Synthesis and Recombinant Protein Production**—All synthetic FITC-labeled Nkx3.1 polypeptides were gifts from Dr. Jung-Mo Ahn (University of Texas at Dallas). All peptides including Nkx3.1 peptide (TQVKIWFQNRRYKTKR) and Nkx3.1(3xP) peptide (TQPKIWFPNRRYPTKR) have a purity of 80−95% as determined by HPLC and mass spectrometry. The recombinant His-Nkx3.1 protein was expressed in bacteria by using a PET vector and the overnight expression autoinduction system (Novagen) and purified by a nickel column. The purified protein was dialyzed against PBS and kept at −20 °C before use. FITC was conjugated to the purified His-Nkx3.1 protein by using the N-hydroxy-succinimide-fluorescein reagent (Pierce).

**Peptide/Protein Uptake and Secretion Assay**—FITC-labeled peptide at indicated concentrations was added to the cell culture with RPMI medium supplemented with 0.5% FBS for 1 h at 37 °C. Cells were washed one time with 0.1% (v/v) trypsin blue in PBS and four times with PBS. Cells were collected in a lysis buffer consisting of PBS and 0.5% (v/v) Tween 20. The fluorescent intensity of the cell lysate was measured (excitation, 485 nm; emission, 528 nm). Cells with fluorescent peptide/protein were also imaged by confocal microscopy. For determining the uptake of recombinant His-Nkx3.1, the cell lysates were analyzed by Western blot using Nkx3.1 antibody (Novus). To examine Nkx3.1 secretion, the conditioned medium was collected after 48 h of cell culture, concentrated >10 times using Microcon (Millipore), and assayed by Western blot using Nkx3.1 antibody.
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FIGURE 1. Non-cell autonomous function of Nkx3.1. A, in the transfection (TXN)-based assay, PZ-HPV-7 cells were transfected with control (C) or Nkx3.1 expression plasmids and assessed for changes in cell number. In the PTF-based experiment, Transwell assay was employed. Nkx3.1-transfected PZ-HPV-7 cells were cultured in the upper chamber, whereas untransfected PZ-HPV-7 cells were cultured in the lower chamber and assessed for changes in cell number. Relative cell number was determined by MTT assay.

RESULTS

To study the effects on cell growth, Nkx3.1 was expressed in an immortalized human prostate epithelial cell (PEC) line (i.e. PZ-HPV-7). Transient Nkx3.1 expression strongly inhibited PZ-HPV-7 cell proliferation (Fig. 1A). This super-efficient inhibition of cell proliferation (~40%) by transient transfection suggests the growth inhibition was, at least partly, caused by a non-cell autonomous effect because the transfection efficiency was only ~5–10%. To test this hypothesis, we performed Transwell assays by plating Nkx3.1-transfected cells in the upper chamber and measuring the proliferation of untransfected cells in the lower chamber. Interestingly, the proliferation of these untransfected cells was also inhibited strongly (Fig. 1A), indicating that Nkx3.1 can function in a non-cell autonomous manner to inhibit cell growth. To test whether Nkx3.1 regulates cell proliferation in a paracrine manner, we produced recombinant His-tagged Nkx3.1 protein in bacteria and treated PZ-HPV-7 PECs and PC3 PCA cells in culture with purified His-Nkx3.1. Indeed, His-Nkx3.1 treatment effectively inhibited the growth of these cells in a dose-dependent manner (Fig. 1B).

Importantly, His-Nkx3.1 treatment suppressed endogenous expression of known Nkx3.1 target genes such as Pou2F2, Gsta4, and Mrpl48 (26) (Fig. 1C). In addition, either intracellular expression of Nkx3.1 or extracellular treatment with His-Nkx3.1 could suppress the transcriptional activities of PSA (prostate-specific antigen) and probasin gene promoters in cell-based transfection assays (Fig. 1, D and E). These results demonstrate that extracellular Nkx3.1 protein can regulate intracellular expression of its target genes.

The above findings unveil an unconventional para-cellular action for a TF. We name this type of TF as a “para-transcription factor (PTF).” We hypothesize that the Nkx3.1 PTF can be secreted from Nkx3.1-expressing cells and taken up by their adjacent cells where Nkx3.1 can regulate gene expression, inhibit cell proliferation, and promote cell differentiation.

To delineate the translocation of Nkx3.1 between cells, we developed a double-fluorescent labeling expression system consisting of a construct, pC-Nkx3.1/G, in which a Cherry (Cherry-red fluorescent protein)-Nkx3.1 fusion protein-coding sequence was separated from a GFP coding sequence by an internal ribosomal entry site sequence. A control plasmid (pC/G) consisted of the same double-expression system with-
out an Nkx3.1 fusion component. Cells transfected with pC/G vector showed yellow color due to co-expression of Cherry red and GFP (Fig. 2, A and B). Among cells transfected with pC-Nkx3.1/G vector, many had yellow nuclei and green cytosol, due to the nuclear localization of Cherry-Nkx3.1 and the nuclear/cytosolic localization of GFP. Importantly, many cells in the same culture dish had red nuclei and unlabeled cytosol (Fig. 2, A and B), indicating that these cells do not produce Cherry-Nkx3.1 but instead obtained it from other pC-Nkx3.1/G-transfected cells that express both Cherry-Nkx3.1 and GFP.

We further confirmed that endogenous Nkx3.1 protein is secreted from and taken up by PECs. By performing Western blot analysis with Nkx3.1 antibody, we faithfully detected endogenously produced Nkx3.1 protein in PZ-HPV-7 cell-conditioned medium, normal mouse prostatic fluids, and mouse urine samples (Fig. 3, A–C). These results clearly demonstrate that Nkx3.1 is secreted not only from cultured PECs but also from normal prostate epithelial cells in normal live mice where Nkx3.1 is produced in the prostate epithelial cells and released into prostatic fluids and urine. Importantly, we found that Nkx3.1 protein was drastically reduced in the prostatic fluids, urine samples, and prostatic tissues of mice with Pten deletion-induced or SV40 T/t expression-induced prostate tumors (Fig. 3, B–D). This suggests that the decreased Nkx3.1 in the urine of PCa patients may serve as a convenient biomarker for diagnosis of prostate tumorigenesis.

Nkx3.1(T164A) is a germ line mutant cosegregated with hereditary PCa and was suggested previously as a putative loss-of-function mutant (29). However, similar to wild type (WT) Nkx3.1, expression of this mutant in PZ-HPV-7 cells suppressed the activity of PSA promoter (Fig. 4A) and the expression of GSTa4 and MRPL48 target genes (Fig. 4B). To assess the association of this PCA-related mutation with the PTF function of Nkx3.1, we expressed WT and mutant Nkx3.1 in rat Nbe cells that had no detectable endogenous Nkx3.1 and co-cultured
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FIGURE 4. The PCa-associated Nkx3.1(T164A) mutant lacks of PTF function due to incapable secretion and the secreted, intercellularly translocated wild type Nkx3.1 protein is recruited to its target genes. A, both wild type Nkx3.1 and Nkx3.1(T164A) mutant suppressed the PSA promoter activity in transfected cells. In PZ-HPV-7 cells, PSA-luciferase reporter was co-transfected with control, wild type Nkx3.1, or Nkx3.1(T164A) mutant plasmid. The luciferase activity was normalized to total cellular protein assayed. The relative luciferase activity in control plasmid-transfected cells was set as 1. *, *p < 0.01 (one-tailed t test). B, PZ-HPV-7 cells were transfected with control, Nkx3.1, or Nkx3.1(T164A) plasmid. The levels of GSTa4 and MRPL48 mRNAs were measured by RT-qPCR and normalized to 18 S rRNA. *, p < 0.05 (one-tailed t test). C, the rat Nbe cells were transfected with control, Nkx3.1, or Nkx3.1(T164A) plasmid and co-cultured with human PZ-HPV-7 cells. The levels of human GSTa4 and MRPL48 mRNAs in PZ-HPV-7 cells were specifically measured by RT-qPCR using human mRNA-specific primers and normalized to 18 S rRNA. D, PZ-HPV-7 cells were transfected with control (C), WT Nkx3.1, or Nkx3.1(T164A) plasmids and cultured in the upper chambers. Untransfected PZ-HPV-7 cells were cultured in the lower chambers and assessed for cell proliferation by MTT assay. Data for cell growth inhibition were normalized to the control group and calculated by the formula: (value of testing group – value of control group)/value of control group. **, *p < 0.01 (one-tailed t test). E, PZ-HPV-7 cells were transfected with pC/G control (C), pC-Nkx3.1/G (WT), or pC-Nkx3.1(T164A) plasmid. Their conditioned media were concentrated and analyzed by Western blot. The levels of Nkx3.1 and Nkx3.1(T164A) mRNAs in the transfected cells were measured by RT-qPCR and normalized to 18 S rRNA. F, ChIP assays for association of the intercellularly translocated HA-Nkx3.1 with the regulatory DNA regions of the GSTa4 and MRPL48 genes in adjacent cells. Cross-linked DNA-protein complexes were prepared from a coculture with PZ-HPV-7 cells and transfected HA-Nkx3.1-expressing Nbe cells and individually cultured PZ-HPV-7, Nbe, and transfected HA-Nkx3.1-expressing Nbe cells. A small aliquot of the DNA-protein complex material prepared from the coculture was used as a positive input control for PCR (lane 1). The prepared DNA-protein complexes were immunoprecipitated with HA-tag antibody or normal IgG as indicated (lanes 2–6). The eluted DNA samples were assayed by PCR using a pair of primers for GSTa4 (upper panel), a pair of primers for MRPL48 (medium panel), and a pair of primers for a negative control (neg. ctrl) region in VEGF (lower panel). PCR reactions without any template DNA served as negative controls for PCR (lane 7). NS, not significant.

these cells with human PZ-HPV-7 cells. Analysis by human mRNA-specific qPCR revealed that WT Nkx3.1, when expressed in Nbe cells, effectively suppressed the expression of GSTa4 and MRPL48 in PZ-HPV-7 cells. On the contrary, expression of Nkx3.1(T164A) in Nbe cells did not affect the expression of GSTa4 and MRPL48 genes in PZ-HPV-7 cells (Fig. 4C). In a Transwell coculture system, WT Nkx3.1 expressed in cells in the upper chamber significantly inhibited the growth of untransfected cells in the lower chamber, whereas Nkx3.1(T164A) mutant failed to do so (Fig. 4D). To understand the defective PTF function of this PCa-associated mutant, we performed Western blot analysis for conditioned media from WT Nkx3.1 and Nkx3.1(T164A) transfected PZ-HPV-7 cells. Interestingly, although both mRNAs were detected inside the transfected cells, we only detected WT Nkx3.1 but not Nkx3.1(T164A) mutant in the media (Fig. 4E).

These results indicate that the PCa-associated Nkx3.1(T164A) mutant is unable to be secreted and thus has a diminished PTF function to inhibit gene expression and cell proliferation in adjacent cells.

To demonstrate the direct DNA binding capability of intercellularly translocated Nkx3.1 protein, we performed ChIP assays in the same co-culture. HA-Nkx3.1 was expressed epitopically in the rat Nbe cells and co-cultured with human PZ-HPV-7 cells. The ChIP assay was performed using HA tag antibody, followed by PCR analysis specific to the Nkx3.1-binding regions in its target genes GSTa4 and MRPL48 in human PZ-HPV-7 cells but not in the rat Nbe cells. The PCR reactions detected the expected products for both GSTa4 and MRPL48 genes from the input samples with whole genome DNA of the cocultured cells, suggesting that the PCR worked as designed. The PCR reactions also detected bands for both GSTa4 and
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MRPL48 genes from the ChIP samples from the cocultured cells by the HA tag antibody but not by normal IgG (Fig. 4F). Furthermore, no PCR product was detected from the ChIP samples from untransfected human PZ-HPV-7 and rat Nbe cells and transfected rat Nbe cells by the HA tag antibody (Fig. 4F). Moreover, as a negative control for ChIP assay background, another pair of primers that amplifies an unrelated DNA region was used to verify the ChIP background. The bands were not detected in the input samples or in the ChIP samples from untransfected human PZ-HPV-7 and rat Nbe cells (Fig. 4F). Taken together, these results prove that the rat Nbe cell-produced HA-Nkx3.1 protein is specifically translocated into the human PZ-HPV-7 cells where it becomes associated with the regulatory DNA regions of its target genes.

To understand the molecular basis for the PTF function of Nkx3.1, we analyzed the Nkx3.1 amino acid sequence and identified an Nkx3.1 region highly homologous to Penetratin (Fig. 5A), a cell-permeable peptide (CPP) derived from the protein transduction domain (PTD) of ANTP (Drosophila antennapedia protein) (30). Thr164 of Nkx3.1 is located in the region corresponding to the N-terminal putative secretion peptide (Sec peptide) within the SecPenetratin sequence of ANTP, which is the secretable version of the extended Penetratin peptide (30). To test whether this Penetratin homologous peptide (PHP) (Thr<sup>166</sup>–Arg<sup>181</sup>) in Nkx3.1 is a cell-permeable domain, we synthesized this peptide with FITC conjugation and assayed its cell permeability. To quench any fluorescence activity on the cell surface or in dead cells, we washed cells with trypan blue immediately before measurement (30). We observed a dose-dependent increase in the fluorescence intensity in both PZ-HPV-7 and Nbe PECs treated with FITC-labeled PHP (Fig. 5B). Fluorescent microscopy analysis also revealed that FITC-labeled PHP was specifically taken up by PZ-HPV-7, Nbe, PNT1A, and RWPE1 PECs (Fig. 5, C and D). Nevertheless, the kidney-derived HEK293 and COS cells and the bladder-derived T24 tumor cells did not take up the FITC-labeled PHP, suggesting a PEC-specific uptake of this peptide (Fig. 5D).

Mutation of three amino acids in Penetratin to prolines blocks the ability of the peptide to traverse cellular membranes (30). Similarly, making the same mutations to the corresponding amino acids (V168P; Q173P; K177P) in the FITC-labeled Nkx3.1 PHP abolished its cell-permeable ability to all PECs examined, including PZ-HPV-7, Nbe, PNT1A, and RWPE1 cells (Fig. 5, B and C). Cellular uptake of many CPPs can be blocked by dextran sulfate or protamine sulfate (31), presumably due to their interference with CPP-cell membrane interaction. The uptake of Nkx3.1 PHP by PZ-HPV-7, Nbe, PNT1A, and RWPE1 cells was blocked by dextran sulfate (Fig. 5, C and E), but its uptake was not blocked by protamine sulfate at least in PZ-HPV-7 cells (Fig. 5E), indicating that Nkx3.1 uptake requires a selective interaction with the membrane of PECs.
To prove Nkx3.1 is a cell-permeable protein, we incubated PZ-HPV-7 with His-Nkx3.1 protein. After washing cells to remove His-Nkx3.1 in the medium, we measured Nkx3.1 in the cell lysates by Western blot. Indeed, His-Nkx3.1 was detected in the cell lysates (Fig. 6A). Similar to the Nkx3.1 PHP, the selective uptake of His-Nkx3.1 by PZ-HPV-7 cells was blocked by dextran sulfate in a dose-dependent manner (Fig. 6B). Moreover, fluorescent microscopy analysis revealed that the FITC-conjugated full-length Nkx3.1 protein was localized in both the nucleus and cytoplasm after absorbed by either PZ-HPV-7 or Nbe cells (Fig. 6C). These results indicate that the full-length Nkx3.1 protein is a PHP domain-mediated cell-permeable transcription factor that can be translocated from extracellular environment to the cell nucleus.

**DISCUSSION**

We discovered that the Nkx3.1 TF is secreted from PECs. We found secreted Nkx3.1 not only in the PEC-conditioned medium but also in the prostatic fluids and urine of mice, indicating that it is a physiological event. Nkx3.1 secretion is a specific domain-dependent event and can be blocked by a PCa-associated point mutation (T164A) within this domain. We also provided multiple lines of compelling evidence showing that the secreted Nkx3.1 can be specifically taken up and used by co-cultured PECs as a PTF to regulate gene expression and cell proliferation. First, the Cherry-Nkx3.1 fusion protein was translocated from transfected PECs to non-transfected PECs; second, the added Nkx3.1 recombinant protein was efficiently taken up by PECs; third, the intercellularly translocated Nkx3.1 protein is located in both cytoplasm and nucleus and associated with the Nkx3.1-binding regions of its target genes; fourth, these known Nkx3.1 target genes were clearly regulated in PECs that either received recombinant Nkx3.1 treatment or were co-cultured with Nkx3.1-expressing cells; and fifth, the proliferation of these recipient cells also was inhibited. Finally, we demonstrated that the intercellular translocation property of Nkx3.1 is essential for its PTF function, as the secretion-deficient T164A mutation restricted Nkx3.1 at home cells as a cell autonomous TF. Taken together, we discovered that Nkx3.1 contains a cell-permeable PTD and is a PTF secreted from Nkx3.1-expressing PECs and selectively taken up by nearby PECs. The translocated Nkx3.1 regulates gene expression and inhibits PEC growth. To our knowledge, this is the first finding demonstrating a TF can function in a non-cell autonomous manner to regulate genes in adjacent cells and control their proliferation. Thus, this direct intercellular gene regulatory pathway mediated by a PTF such as Nkx3.1 represents a novel cell-cell communication pathway.

The Nkx3.1-mediated non-cell autonomous regulation of gene expression and cell growth implicates a novel mechanism for a PTF as a tumor suppressor. We have shown that the PTF function of Nkx3.1 is required for its tumor suppressor role in adjacent cells. Accordingly, the loss of Nkx3.1 PTF function caused by T164A mutation diminishes its tumor-suppressing activity to adjacent cells. Although this T164A germ line mutation of Nkx3.1 gene is rare in human PCs, these results illustrate the importance of PTF function for the tumor suppressor activity of Nkx3.1. In both TRAMP and Pten deletion-induced PCs mice, we found a drastic reduction of Nkx3.1 protein in the prostate gland, prostatic fluids, and urine, implicating a lost PTF function of Nkx3.1 during PCs carcinogenesis.

Cancer cells usually are believed to escape the control of tumor suppressors through genetic mutation or promoter repression of the tumor suppressor genes. Thus, transcriptional inhibition or loss-of-function mutations in cancer were a part of criteria for a gene to be considered as a tumor suppressor. However, the loss of tumor suppressor function by impaired PTF function of a TF like Nkx3.1 provides a new way to consider the functional loss of a tumor suppressor. Presumably, the intercellular translocation of a PTF could be blocked by many other factors without mutation or transcriptional inhibition of the PTF. For the Nkx3.1 gene, although multiple lines of evidence indicate that it has a tumor-suppressing function, including its linkage with PCa susceptibility (8, 9) and its growth inhibitory function in PECs (1, 10, 11, 21, 22), it is still unexplained why Nkx3.1 can always be detected and no Nkx3.1 somatic mutation is found in human PCs (13–20). These ambiguous correlations have raised a question whether Nkx3.1 is a tumor suppressor, and some investigators have considered Nkx3.1 as a non-classical tumor suppressor. The discovery of the PTF function of Nkx3.1 in this study may provide molecular basis for additional mechanisms by which cancer cells escape growth control of tumor suppressors.

Homeoprotein is defined by a homeodomain consisting of three α-helixes with 60 amino acids. We have identified the PTD domain of Nkx3.1 located at the C terminus of the three putative α-helix structures, the same location for the PTD domain of the antennapedia. The PTD sequences of human Nkx3.1 and *Drosophila* antennapedia are highly homologous.
and share 69% identity within their 16-amino acid sequence, indicating that this PTD domain is conserved evolutionarily. In the human genome, 300 homeobox loci have been identified (32). The homeobox genes play important roles in morphogenesis and tumorigenesis. Nkx3.1 belongs to the ANTP class, named after the evolutionarily related antennapedia (Antp) gene in Drosophila melanogaster (32). The ANTP class contains at least 90 gene members and is divided into the HOXL subclass that includes the Hox gene family and the NKL subclass that includes the Nkx3.1 gene. The presence of these highly conserved PTD domains in the homeobox supergene family suggests that Nkx3.1 could be just one of many homeoproteins that can function as a PTF. Considering the diverse physiological functions of homeobox genes, if these homeoproteins indeed function as PTFs, they would be well implicated in the regulation of many physiological processes.

Our findings may also have potential clinical applications. The cell-permeable, PEC-selective Nkx3.1 PHP may be used to deliver full-length Nkx3.1 or other cancer drugs into PCa cells for treating PCa. The discoveries of abundant Nkx3.1 protein in the prostatic fluids and urine of normal animals and its marked reduction in these specimens of PCA animals suggest that Nkx3.1 reduction may serve as a potential diagnostic marker for PCa.

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