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# Identification of Transcriptional Targets of HOXA5*[S]

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**The homeobox gene *HOXA5* encodes a transcription factor that has been shown to play important roles in embryogenesis, hematopoiesis, and tumorigenesis. In order to decipher downstream signaling pathways of HOXA5, we utilized oligonucleotide microarray analysis to identify genes that are differentially expressed in HOXA5-induced cells compared with uninduced cells. Comparative analysis of gene expression changes after 9 h of HOXA5 induction in Hs578T breast cancer cells identified 306 genes whose expression was modulated at least 2-fold. Ten of these 306 genes were also up-regulated by at least 2-fold at 6 h post-induction. The expression of all of these 10 genes was confirmed by semi-quantitative reverse transcription-PCR. Among these 10 genes, which are most likely to be direct targets of HOXA5, we initiated an investigation into the pleiotrophin gene by first cloning its promoter. Transient transfection assays indicated that HOXA5 can specifically activate the pleiotrophin promoter. Promoter deletion, chromatin immunoprecipitation assay, and gel-shift assays were performed to show that HOXA5 can directly bind to one binding site on the pleiotrophin promoter. These data strongly suggest that microarray analysis can successfully identify many potential direct downstream genes of HOXA5. Further functional analysis of these targets will allow us to better understand the diverse functions of HOXA5 in embryonic development and tumorigenesis.**

HOX genes, a subset of the homeobox genes that were originally identified in *Drosophila*, encode a family of transcriptional factors essential for axial and appendicular patterning and organogenesis (1). Recently, many *HOX* genes were found to be aberrantly expressed in a variety of cancers, including those of the breast, kidney, and skin, suggesting that they may also contribute to the progression of tumors (2–5). All 39 HOX transcriptional factors identified in vertebrates have in common a 60-amino acid DNA-binding homeodomain that binds DNA and that has been conserved with respect to sequence, structure, and mechanism of DNA binding (6). *In vitro* assays show that all HOX proteins can bind to similar DNA motifs with a core sequence of TAAT (7, 8). In sharp contrast to relatively simple binding motifs revealed in *in vitro* binding assays, each *HOX* gene has a variety of functions that cannot be completely compensated by other *HOX* genes. In order to understand the complexity of *HOX* gene functions extending from embryo development to hematopoietic maturation and tumorigenesis, it is important to identify the specific targets for each *HOX* gene.

The evidence for an important role of Hoxa5 in development comes mainly from the study of the *Hoxa5* knock-out mice (9, 10). The resultant heterozygous mutant mice were viable and indistinguishable from their wild type littermates. However, more than 60% of the homozygous pups died within 4 days of birth. In homozygous newborn mutants, improper tracheal and lung morphogenesis lead to tracheal occlusion and to respiratory distress associated with a marked decrease in the production of surfactant protein (10). Loss of *Hoxa5* also perturbed intestinal maturation and transiently affects thyroid development (11, 12). Analysis of the skeletal structures in the homozygote revealed consistent abnormalities affecting the region between sixth cervical vertebra (C6) and the first lumbar vertebra. In human, HOXA5 has been shown to play a role in blood cell differentiation (13, 14). Constitutive expression of HOXA5 expression in human hematopoietic progenitor cells causes a significant shift toward myeloid differentiation and away from erythroid differentiation (13). In addition, HOXA5 expression was lost in more than 60% breast cancer cell lines and primary breast carcinoma cells (15). Overexpression of HOXA5 in breast cancer cell line, MCF7, induced apoptosis by up-regulating the expression of p53 (15). However, in the p53-mutant breast cancer line Hs578T, we have presented evidence that HOXA5-mediated apoptosis by activating caspase-2 and caspase-8 (16). HOXA5 and TNF$\alpha$ acted synergistically to induce apoptosis (16). These studies indicated that HOXA5 may behave as a tumor suppressor gene in breast cells.

Despite knowledge of a variety of functions of the *HOXA5* gene that have been found to date in development and tumorigenesis, very few specific targets genes have been identified. The identity of the target genes could better define the multiple pathways through which HOXA5 may positively or negatively modulate growth. In this study, we utilized microarray analysis of an inducible HOXA5 breast cancer cell line, HS578T, to identify 306 genes whose expressions are significantly modified after HOXA5 induction. Furthermore, we demonstrated that HOXA5 can directly bind to and activate the promoter of one these genes, pleiotrophin (PTN).[1] Further sorting and function-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–4.

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[1] The abbreviations used are: PTN, pleiotrophin; RT, reverse transcription; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; nt, nucleotide; ChIP, chromatin immunoprecipitation; HBS, HOXA5-binding site.



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ally analyzing the candidate targets of HOXA5 will shed light on the underlying mechanisms of HOXA5 function.

## MATERIALS AND METHODS

*Cell Culture and Plasmids Construction*—The Hs578T breast cancer cell line was purchased from the ATCC and was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. SKBR3 breast cancer cells were cultured in McCoy 5A medium supplemented with 15% fetal bovine serum. The HOXA5-inducible Hs578T cell line was established using the two-step Tet-Off plasmid transfection system, where gene expression is induced by removing doxycycline from the medium, as described previously (16).

HOXB1-, HOXB3-, HOXB5-, and HOXD9-expressing plasmids were described previously (15, 17). pCB6-HOXA5M was constructed by restricting pCB6-HOXA5 with ApaI followed by treatment with Klenow enzyme to remove 4 bp followed by self-ligation. The 4-bp deletion in pCB6-HOXA5M resulted in a frameshift of the HOXA5-coding sequence. pCB6-HOXA5M encodes a truncated HOXA5 protein without its homeodomain and C terminus. We found unacceptably high levels of activation of the pGL2 promoterless constructs by HOXA5. To circumvent this problem, we modified the pLC-Gal plasmid (a kind gift from Dr. Linzhao Cheng, The Johns Hopkins University) that carries a $\beta$-galactosidase gene under the control of the cytomegalovirus promoter. The pLC-Luc plasmid was constructed by us by replacing the $\beta$-galactosidase gene sequence of pLC-Gal with the luciferase gene sequence from pGL-3 plasmid (Promega). The full-length and deleted PTN promoter sequences were PCR-amplified with primers (forward primers: P-1084, 5′-CTA CTT GCC ACA AGA CAA TG-3′; P-624, 5′-AAA TTC TTG GGT GTC AAA GG-3′; P-314, 5′-AAA TGC CCA TCA ATT GTC CA-3′; and reverse primer P+222, 5′-TTG CTA CCG CTG AGT CCA GG-3′) and cloned into the pGEM-T vector (Promega). The PTN promoter inserts, released from pGEM-T cloning vector by restriction enzymes SacII and NdeI, were cloned into pLC-Luc by replacing the cytomegalovirus promoter sequence between enzyme sites HindIII and NdeI. The resulting clones, which contained full-length and deletions of PTN promoter, were sequenced and referred to as PTN-Luc, PTN-D-604, and PTN-D-314. respectively. To construct the PTN promoter deletion plasmid PTN-D-880, PTN-Luc was digested with restriction enzymes NdeI and NheI to release a small fragment of 5′-end PTN promoter sequence and was followed by filling with Klenow enzyme and self-ligation with T4 ligase. Similarly, PTN-Luc was cut with NdeI and HindIII to construct PTN-D-101. PTN-D-604–106M was constructed by using a two-step PCR mutagenesis method (18).

*Transcriptional Profiling Microarray*—Total RNA was purified from HOXA5-inducible Hs578T cells with the RNeasy mini kit (Qiagen, Valencia, CA) after isolation with TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Two batches of RNA from HOXA5-inducible cells (0, 6, and 9 h) were purified for performing replicate microarray hybridization experiments. The U133A GeneChip® (Affymetrix, Santa Clara, CA) contains probes for detecting ~14,500 well characterized genes and expressed sequence tags. Hybridization was performed as described (19). Briefly, double-stranded cDNA was synthesized from 15 $\mu$g of total RNA with SuperScript Choice System (Invitrogen) by using oligo$(dT)_{24}$ primers, with a T7 RNA polymerase promoter site added to its 3′-end (Genset Corp, La Jolla, CA). The isolated cDNA was then labeled to generate biotinylated cRNA *in vitro* and amplified using the BioArray T7 RNA polymerase labeling kit (Enzo, Farmingdale, NY). After purification of the cRNA by RNeasy mini kit, 20 $\mu$g of cRNA was fragmented at 94 °C for 35 min. Approximately 12.5 $\mu$g of fragmented cRNA was used in a 250-$\mu$l hybridization mixture containing herring sperm DNA (0.1 mg/ml; Promega, Madison, WI), plus bacterial and phage cRNA controls (1.5 pM BioB, 5 pM BioC, 25 pM BioD, and 100 pM Cre) to serve as internal controls for hybridization efficiency as directed by the manufacturer (Affymetrix). Aliquots (200 ul) of the mixture were hybridized onto the array for 18 h at 45 °C in a GeneChip® Hybridization Oven 640 (Affymetrix). Each array was washed and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and amplified with biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) on the GeneChip® Fluidics Station 400 (Affymetrix). Each array was scanned with the GeneArray scanner (Agilent Technologies, Palo Alto, CA) to obtain image and signal intensities.

*Data Analysis Using Affymetrix Software*—Scanned output files were analyzed with the Affymetrix Microarray Suite 5.0, and global normalization was performed prior to comparison. To identify differentially expressed transcripts, pairwise comparison analysis was carried out with Data Mining Tool 3.0 (Affymetrix). The analysis compares the differences in values of perfect match to mismatch of each probe pair in the base-line array to its matching probe on the experimental array. $p$ values were determined by the Wilcoxon's signed rank test and denoted as increase, decrease, or no change. Analysis using Data Mining Tool also provides the signal log ratio, which estimates the magnitude and direction of change of a transcript when two arrays are compared (induced *versus* uninduced). We have converted the signal ration output into fold change for convenience using Equation 1 as recommended by Affymetrix,

$$\text{fold change} = \begin{cases} 2^{\text{signal log ratio}}, & \text{signal log ratio} > 0 \\ (-)2^{-(\text{signal log ratio})}, & \text{signal log ratio} < 0 \end{cases} \quad \text{(Eq. 1)}$$

In the present study, we performed 4 pairwise comparisons for each time point (induced, $n = 2$, and uninduced, $n = 2$). Only those altered genes that appeared in 4 of 4 comparisons were selected. This conservative analytical approach was used to limit the number of false-positives. The expressed sequence tags obtained in the data were searched for their recent annotation using the "Analysis Center" at the Affymetrix site (www.netaffx.com). The microarray data has been deposited to the GEO data base (series entry GSE2241; www.ncbi.nlm.nih.gov/geo).

*Quantitative Real Time RT-PCR Analysis*—Ten up-regulated genes as identified by microarray were selected for real time RT-PCR analysis to verify the array results. Reverse transcription reaction was performed as follows: 1 $\mu$g of DNase-treated total RNA, 0.5 $\mu$g of anchored oligo$(dT)_{15}$ primer, and 500 M dNTPs (New England Biolabs) were heated for 5 min at 65 °C; 1× first strand buffer (Invitrogen), 0.01 M dithiothreitol, and 200 units of Superscript II (Invitrogen) were added, and reverse transcription was carried out, in a 20-$\mu$l reaction, for 50 min at 42 °C and terminated by heating for 15 min at 70 °C. To assess for potential contamination of solutions, a control containing all reagents, but devoid of RNA, was included. In addition, a control containing all reagents, except the Superscript II, was included for each sample in order to monitor for possible residual genomic DNA in the RNA preparations.

The quantitative RT-PCR was performed using the fluorescent dye SYBR Green Master Mix following standard protocols on an ABI PRISM 7900 sequence detection system (Applied Biosystems, CA). The primers used for the PCR are shown in Table I. Native gel electrophoresis was used to characterize the final products. The data were first analyzed using the Sequence Detector Software SDS 2.0 (Applied Biosystems). Results were calculated and normalized relative to the GAPDH control by using the Microsoft Excel program. All of the PCRs were performed in triplicate, and mean values are shown in figures.

*Western Blot Analysis*—Twenty $\mu$g of protein was fractionated in a 4–12% NuPAGE gel and transferred to polyvinylidene difluoride mem-

TABLE I
*Primers used in the quantitative real time RT-PCR analysis*

| Gene name | Primer sequence |
|---|---|
| *EGR-1* | 5′-CGAGCAGCCCTACGAGCACCTGAC-3′ |
| | 5′-TGCGCAGCTCAGGGGTGGGCTCTG-3′ |
| *IL-8* | 5′-TCTTGGCAGCCTTCCTGATT-3′ |
| | 5′-AACTTCTCCACAACCCTCTG-3′ |
| *GADD45β* | 5′-CGGTGGAGGAGCTTTTGGTG-3′ |
| | 5′-CACCCGCACGATGTTGATGT-3′ |
| SAT | 5′-ATGGCTAAATTCGTGATCCG-3′ |
| | 5′-TTAGCAAGTACTCCTTGTCG-3′ |
| *TIEG* | 5′-CTCAACTTCGGTGCCTCTCT-3′ |
| | 5′-GGTTTGGCAGTATCTGAGAG-3′ |
| *ETR101* | 5′-TCGGTGTGGAAGATGTATCA-3′ |
| | 5′-TGTCCATTGGCTCCGGAAAC-3′ |
| *PTN* | 5′-ATGCAGGCTCAACAGTACCA-3′ |
| | 5′-TATGTTCCACAGGTGACATC-3′ |
| *EST1* | 5′-TTTGCACTTATTCCCATCTT-3′ |
| | 5′-GGTGCCCATTGTTGAACTAA-3′ |
| *EST2* | 5′-AGGAGTCACTGGTTTACAAG-3′ |
| | 5′-ACTTGGAAATTGTGCCAGTG-3′ |
| *EST3* | 5′-GAGTATTTCCAGCCATAACA-3′ |
| | 5′-TAAAGGACTCACACTGGAGA-3′ |
| *GAPDH* | 5′-GAAGGTGAAGGTCGGAGTC-3′ |
| | 5′-GAAGATGGTGATGGGATTTC-3′ |

branes. The membrane was blocked with 100 ml of TBS buffer (10 mM Tris base, pH 7.5, 0.9% NaCl) containing 5% dry milk and 0.1% Tween 20 for 1 h on the shaker at room temperature or overnight in cold room. The membrane was rinsed once with TBS buffer before incubating with an appropriate dilution of the 1° antibody in TBS buffer containing 5% milk and 0.02% Tween 20 on shaker for 1 h. The primary antibody-bound membrane was washed with TBS buffer containing 0.1% Tween 20 four times and then incubated with the 2° antibody (ECL kit anti-rabbit or anti-mouse from Amersham Biosciences) at about 1:1000 dilution for 1–1.5 h on a shaker. The filter was developed by using the ECL-Plus reagent (Amersham Biosciences). Rabbit anti-peptide antibodies to HOXA5 were provided by Zymed Laboratories Inc.

*Transient Transfection Assay*—$2 \times 10^5$ SKBR3 cells were seeded onto each well of 6-well plate 24 h prior to transfection. 1–2 $\mu$g of plasmids was transfected into cells using Gene Jammer (Stratagene Corp., La Jolla, CA) according to the manufacturer's instructions. 24 h post-transfection, cells were harvested for luciferase and $\beta$-galactosidase assay using the luciferase activity measuring kit (Promega, Madison WI) and the $\beta$-galactosidase assay kit (ICN Biomedicals Inc., Aurora, OH) according to the manufacturers' instructions. The luciferase activities were normalized to the $\beta$-galactosidase activities for each sample. The fold activation was calculated as the ratio of normalized luciferase activities in the cells transfected with the reporter plasmid in the presence of HOXA5-expressing plasmid to that in the absence of HOXA5-expressing plasmid. Each transfection was repeated at least three times, and the averaged data are shown in the figures.

*Chromatin Immunoprecipitation (ChIP) Assay*—ChIP assays were performed using the ChIP assay kit (Upstate Biotechnology, Inc., Lake Placid, NY) according to the manufacturer's instructions. In brief, $1 \times 10^6$ vector and HOXA5-induced cells were cross-linked by adding formaldehyde directly to the culture medium. Cells were harvested and sonicated to shear DNA to lengths between 200 and 1000 bp. After centrifuging samples for 10 min at 13,000 rpm at 4 °C, the supernatant was pre-cleared with 75 $\mu$l of salmon sperm DNA/protein A-agarose, 50% slurry for 30 min at 4 °C with agitation. 2 $\mu$g of HOXA5 antibody was then added to the supernatant fraction for incubation overnight at 4 °C with rotation. Then 60 $\mu$l of salmon sperm DNA/protein A-agarose was added to collect the antibody-histone complex. The protein A-agarose-antibody-histone complex was extensively washed for 5 min as suggested and heated at 65 °C for 4 h to reverse histone-DNA cross-links. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The PCR were performed using two pairs of primers (control: nt 1084, 5′-CTA CTT GCC ACA AGA CAA TG, and nt 725, 5′-ACG CTA AGG CAA TGC ATA GG-3′; HBS: nt 271, 5′-GAG ATC TGG CTT TGC ACT CAT CTG AA-3′, and nt 8, 5′-GCA TAT GGA GAA TGG GAG GGA ATG A-3′).

*Gel-shift Assay*—Nuclear extracts from HOXA5-inducible cells were prepared, and gel-shift assays were performed as described previously (15, 17). The reaction was carried out in a final volume of 20 $\mu$l. One microliter of nuclear extract (~2 $\mu$g of total protein) was added to binding buffer containing 2 $\mu$g of poly[d(I-C)] (Amersham Biosciences), 20 mM HEPES-HCl, pH 7.9, 50 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, 6% glycerol, and $2 \times 10^4$ cpm of $^{32}$P-end-labeled oligonucleotide. After 15 min on ice, loading buffer was added, and the protein-DNA complexes were resolved in nondenaturing 5% polyacrylamide gels by electrophoresis at 100 V for 3–4 h in 0.25× TBE (1× TBE: 0.089 M Tris base, 0.089 M boric acid, 2 mM EDTA). The gels were dried and exposed to Kodak-RP film (Eastman Kodak Co.). The 20-mer oligonucleotides, containing the canonical HOXA5-binding site within the PTN promoter and the mutated binding sites, were synthesized and purified by high performance liquid chromatography. Double-stranded oligonucleotides were end-labeled with [$\gamma$-$^{32}$P]ATP. To ascertain specificity of binding, unlabeled competitor wild type or mutated oligonucleotides were incubated with the protein extract prior to the addition of the labeled oligonucleotide. A supershift assay was also performed by incubating the DNA-protein complex with 2 $\mu$g of rabbit polyclonal HOXA5 antiserum (Zymed Laboratories Inc.) for 10 min on ice.

## RESULTS

*Identification of Genes That Are Differentially Expressed after Induction of HOXA5 Expression*—TheTet-Off HOXA5-inducible Hs578T cell line was established as described previously (16). In this system, HOXA5 expression is tightly controlled by the tetracycline-responsive promoter and can be rapidly induced by removal of doxycycline (a tetracycline analog) from the culture medium (Fig. 1). Similar to the parental

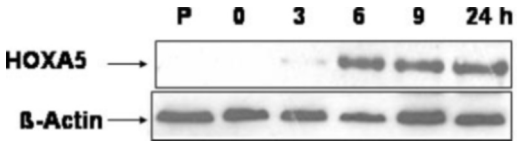


FIG. 1. **Time course of induction of HOXA5 expression in Hs578T cells.** $2 \times 10^6$ HOXA5-inducible Hs578T breast cancer cells were seeded onto a 10-cm plates 24 h prior to induction. The cells were harvested at time points indicated and subjected to Western blot analysis. *P*, parental Hs578T cells.

cells, under uninduced conditions (0-h time point), the expression of HOXA5 was undetectable by Western blot analysis. At 3 h post-induction, the expression of HOXA5 became detectable and continued to increase over the time course of induction until 6–9 h post-induction. At 12 h post-induction, cells began to undergo apoptosis (16).

These data suggested that HOXA5-mediated gene expression had occurred during the first few hours of induction. The earliest responding genes are more likely to be the direct targets of HOXA5. To this end, we harvested cells at 0, 6, and 9 h after induction. Total RNA was purified from these cells and subjected to oligonucleotide microarray analysis using Affymetrix chips.

The microarray experiments were repeated once with separately purified RNA samples for each of the time points. The gene expression profiles of cells induced for 6 and 9 h were compared with that under uninduced conditions (0 h) in two sets of experiments (_1 and _2). The differentially expressed genes in 4 of 4 comparisons (6 h_ 1 *versus* 0 h_ 1 and _ 2; 6 h_ 2 *versus* 0 h_ 1 and _ 2; 9 h_ 1 *versus* 0 h_ 1 and _ 2; 9 h_ 2 *versus* 0 h_ 1 and _ 2) are listed in the supplemental tables. At 9 h post-induction, when we observed the maximal number of genes that were differentially expressed, we identified 262 genes whose expression was up-regulated by at least 2-fold and 44 genes whose expression was down-regulated by at least 2-fold. Because HOXA5 is well established as a positive regulator of genes, we have focused only on the up-regulated genes in this study.

Among the 262 up-regulated genes, 10 genes or expressed sequence tags were also up-regulated at 6 h post-induction (Table II). In contrast, only one gene which is up-regulated at 6 h post-induction did not appear in the list of up-regulated genes at 9 h post-induction.

*Validation of Microarray Data by Real Time PCR Analysis*—The 10 genes whose expression was up-regulated at both 6 and 9 h post-induction are more likely to be the direct targets of HOXA5. Therefore, we examined the expression pattern of these genes over the time course of HOXA5 induction. Similar to the continued increase in the expression of HOXA5 during the time course of induction, we observed that compared with parent cells and uninduced cells, expression of most of these 10 genes increased in parallel to the expression of HOXA5 (Fig. 2). Hence, the data generated by the microarray was confirmed for each of these genes by real time PCR, thereby validating the robustness of the analysis.

*HOXA5 Specifically Activates the Promoter of the Pleiotrophin Gene*—Next, a search was performed of the promoter region of these 10 HOXA5-induced candidate genes. Among these, we observed the presence of multiple HOXA5 core-binding motifs (TAAT) in the promoter sequences of the PTN gene. Although the core binding sequences of HOX genes are TAAT, the specificity of binding for each HOX genes has been shown, in one study, to be attributable to the flanking sequences (8). But no consensus HOXA5-specific binding sites beside the TAAT core sequence were identified. PTN encodes a 136-amino acid cytokine that is an important contributor to growth, dif-

TABLE II
*Genes that are up-regulated in HOXA5-induced cells*

The genes that showed fold changes > 2.0 in four out of four comparisons at 6 h post-induction are listed in this table.

| GenBank™ accession no. | Gene | Folds (±S.E.) (at 6 h) | Folds (±S.E.) (at 9 h) |
|---|---|---|---|
| NM_001964 | Early growth response 1 (EGR1) | 4.4 (±1.7) | 6.4 (±1.8) |
| NM_004907 | Immediate early protein (*ETR101*) | 2.8 (±1.3) | 3.3 (±1.3) |
| NM_005655 | TGF$\beta$-inducible early growth response (*TIEG*) | 2.8 (±1.3) | 4.3 (±1.5) |
| NM_000584 | Interleukin-8 (*IL-8*) | 8.8 (±3.6) | 13.0 (±3.6) |
| NM_015675 | Growth arrest and DNA-damage-inducible, $\beta$ (*GADD45*$\beta$[$\rho$]) | 4.6 (±1.4) | 5.1 (±1.4) |
| NM_002825 | Pleiotrophin (heparin-binding growth factor 8) (PTN) | 8.1 (±1.3) | 7.7 (±1.2) |
| AF087853 | Growth arrest and DNA damage-inducible, $\beta$ (GADD45$\beta$[$\rho$]) | 4.5 (±1.5) | 4.5 (±1.4) |
| AF078077 | Growth arrest and DNA damage-inducible, $\beta$ (GADD45$\beta$[$\rho$]) | 3.5 (±2.0) | 3.0 (±1.4) |
| M55580 | Spermidine spermine $N^1$-acetyltransferase mRNA (*SAT*) | 2.5 (±1.1) | 5.0 (±1.1) |
| AI572079 | *EST1* | 2.0 (±1.1) | 2.9 (±1.0) |
| AL556438 | *EST2* | 2.3 (±1.0) | 3.2 (±1.2) |
| AU1544742 | *EST3* | 2.0 (±1.2) | 2.5 (±1.1) |
| AI809774 | *EST4* | 2.0 (±1.1) | NC[a] |

[a] No change.

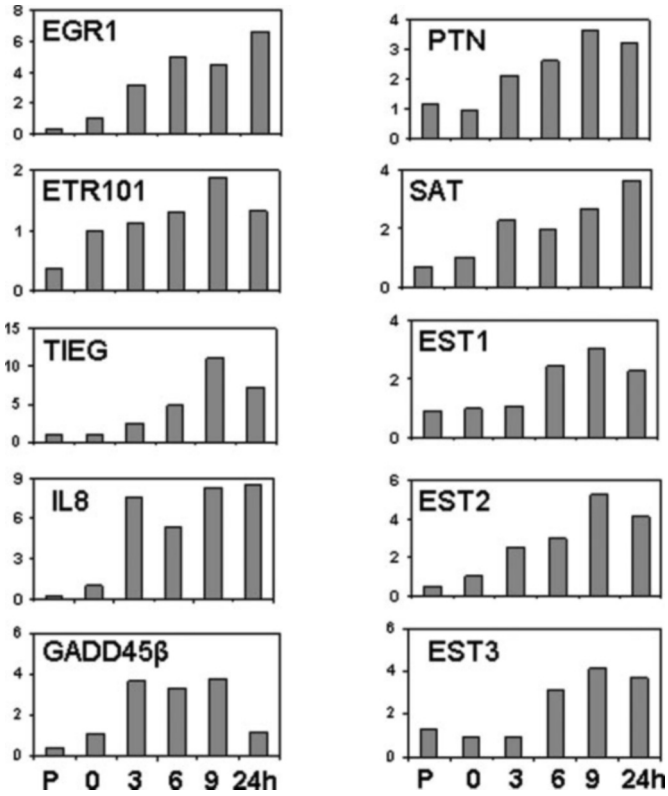


FIG. 2. **Quantitative real time RT-PCR analysis.** Total RNA was purified from parent and HOXA5-inducible Hs578T cells. 10 genes in which expressions are up-regulated at both 6 and 9 h post-induction by microarray analysis were tested by quantitative real time RT-PCR analysis. The PCR was performed as described under "Materials and Methods." The PCR primer sequences are listed in Table I. The relative expression level of each gene in the uninduced cells was referred to as one after normalization to GAPDH control for each sample. *P*, parent Hs578T cells.

ferentiation, and maintenance of viability within the mammalian nervous system during development (21, 22). Hoxa5 is also highly expressed in the nervous system during mouse embryogenesis. Moreover, in primary breast cancers, we analyzed multiple array data bases and determined that PTN expression is lost in breast cancer (www.oncomine.com). PTN expression level was significantly lower in breast tumor samples in three of three studies involving a total of 32 normal breast samples and 149 breast carcinoma samples. The adjusted $p$ values for these three studies were 0.002, 0.1, and $5.6 \times 10^{-6}$ respectively. Based upon the data that there is loss of HOXA5 (15) and PTN in a large proportion of breast cancers, we wanted to test whether HOXA5 directly activates the expression of PTN. To this end, we performed transient transfection assays. As shown in Fig. 3*B*, HOXA5 up-regulated the PTN promoter driven-luciferase gene expression in a dose-dependent manner. Luciferase activity increased by about 4-fold in the presence of 1 $\mu$g of HOXA5-expressing plasmid, when compared with activity in the absence of HOXA5-expressing plasmids (Fig. 3*B*). With the same amount of transfected mutant HOXA5-expressing plasmid, no increase in luciferase gene expression was observed (Fig. 3*B*).

Because many *HOX* genes are functionally redundant, we next tested the specificity of HOXA5 on activation of PTN promoter. PTN promoter was cotransfected with expression constructs of either HOXB1, HOXB3, HOXB5, or HOXD9. We found that among the *HOX* genes tested, only HOXD9 weakly activated the promoter of PTN (Fig. 3*E*). These results suggested that, among the HOX genes tested, it is likely that HOXA5 acts as a transcriptional regulator upstream of PTN.

*Homeodomain of HOXA5 Is Required for Its Ability to Transactivate the PTN Promoter*—The homeodomain of the HOX protein, which binds to DNA, is generally required for its transactivation abilities. However, HOX proteins can also regulate gene expression by interacting with other transcriptional factors independent of their binding domain (25). To investigate whether the DNA binding domain of HOXA5 is required for activation of PTN expression, we constructed a mutant HOXA5 plasmid that encoded a truncated HOXA5 protein, lacking most of its homeodomain (Fig. 3*A*). Transient transfection assays indicated that this truncated HOXA5 protein completely lost its ability to activate the promoter of PTN (Fig. 3*B*). The inability of truncated HOXA5 to activate gene expression was not because of failure of expression since Western analysis revealed that both wild type and truncated protein were expressed at similar levels (Fig. 3*C*). These results suggested that HOXA5 DNA binding domain is required for its ability to regulate the PTN promoter.

*In vitro* assays have shown that *HOX* gene family transcription factors bind to very similar binding sites with core sequences such as TAAT (8). The specificity for transcriptional regulation by *HOX* genes is often achieved either by interaction with cofactors or by the DNA context around the binding site (26–29). The N-terminal part of the HOX protein is known to play an important role in protein-protein interactions. For example, the pentapeptide (YPWMR), which is found N-terminal to the homeodomain of many HOX proteins, is the determinant for interaction with the cofactor PBX1A (30). Interaction with PBX1A has been shown to increase greatly the DNA binding affinity of HOX proteins, including HOXA5, and also altered DNA binding specificity (30–32). We hypothesized that the truncated HOXA5 protein (which had lost its binding capacity

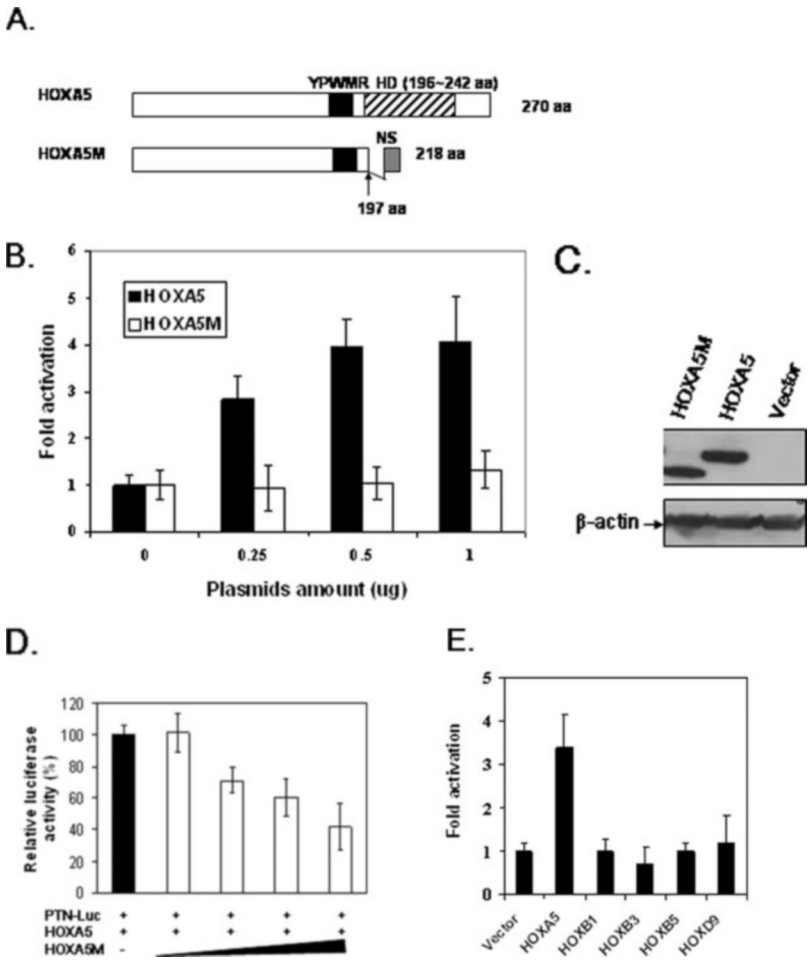


FIG. 3. **HOXA5 can specifically activate the PTN promoter.** *A*, wild type and mutant HOXA5-expressing constructs. A 4-bp deletion in the HOXA5M plasmid resulted in a frameshift from amino acid (*aa*) position 197. HOXA5M encodes a peptide that has the intact N-terminal part of HOXA5, and 22 amino acid nonspecific (*NS*) sequences due to the frameshift but lacks the entire homeodomain (*HD*) and the C-terminal part of HOXA5. *B*, transactivation of PTN promoter by wild type or mutant HOXA5. 100 ng of PTN-Luc was co-transfected into SKBR3 cells with different concentrations of either wild type or mutant HOXA5-expressing plasmids. 2.5 ng of β-galactosidase-expressing plasmids was co-transfected as internal control. At 24 h post-transfection, the cells were harvested for luciferase and β-galactosidase activities assay. The calculation for activation was done as described under "Materials and Methods." The transfection experiments were repeated at least three times. *C*, Western blot analysis of wild type and mutant HOXA5 protein. SKBR3 cells were transiently transfected with wild type and mutated HOXA5-expressing plasmids. At 48 h post-transfection, the cells were harvested for Western blot analysis. *D*, competition of HOXA5 transactivation abilities by HOXA5M. 100 ng of PTN-Luc was co-transfected with 100 ng of wild type HOXA5-expressing plasmid in the presence of different amounts of mutant HOXA5-expressing plasmids (0, 0.1, 0.5, and 1.0 μg). The normalized luciferase activity in the absence of mutant HOXA5-expressing plasmids was referred to as 100% activity. *E*, effects on PTN promoter activities by co-transfection of other HOX-expressing plasmids. 100 ng of PTN-Luc was co-transfected into SKBR3 cells with 1 μg of different HOX genes expressing plasmids. The experiments were done as described in legend *B*.

but retained its ability to interact with cofactors) may competitively inhibit the transactivation of the PTN promoter mediated by wild type HOXA5. As predicted, by cotransfecting cells with wild type HOXA5 and different concentrations of mutant HOXA5, we found that the truncated HOXA5 protein inhibited the transactivation ability of wild type HOXA5 in a dose-dependent manner. In the presence of 10-fold excess of mutant HOXA5-expressing plasmids, the transactivation of PTN promoter by wild type HOXA5 was inhibited by more than 50% (Fig. 3*D*). These results provide further evidence that HOXA5 can, with some specificity, increase the promoter activity of PTN.

*Mapping the Binding Sites of HOXA5 on the Promoter of PTN*—The DNA binding domain of HOXA5 is required for HOXA5-mediated activation of the PTN promoter. We next attempted to define the binding sites of HOXA5 in the promoter by serially deleting the PTN promoter. Within about 1 kb of PTN promoter sequences immediately upstream of the transcriptional start site, we found seven potential HOXA5-binding sites. We constructed a series of deletions aimed at removing these sites in a stepwise manner. As shown in Fig. 4*A*, deletion of the first six sites in the deletion constructs (PTN-D-880, PTN-D-604, and PTN-D-314) had no drastic effects on their abilities to be activated by HOXA5. All three of these deleted promoters were activated 3–4-fold by HOXA5. However, further deletion of the last site in the shortest construct, PTN-D-101, completely abolished its ability to be activated by HOXA5. Also, this deletion construct of the promoter still retains more than 50% basal transcriptional ability compared with the full-length promoter as reported by Li *et al.* (33). These results indicated that the last TAAT-containing sequences are likely to comprise the direct HOXA5-binding site (HBS). To test this further, a promoter construct (PTN-D-604–106M) with a point mutation in the HBS site (T**AA**T→T**GG**T) was made. Although it still carries two upstream TAAT sites, the mutated plasmid completely lost its ability to be activated by HOXA5 (Fig. 4*A*). These findings provided additional lines of evidence that HOXA5 binds to the PTN promoter and that the putative HBS is most likely *a bona fide* HOXA5-binding site in the PTN promoter.

To test whether HOXA5 can bind directly to the putative HBS site, we performed gel-shift assays with synthetic probes designed according to the sequence of HBS (Fig. 4*B*). In most

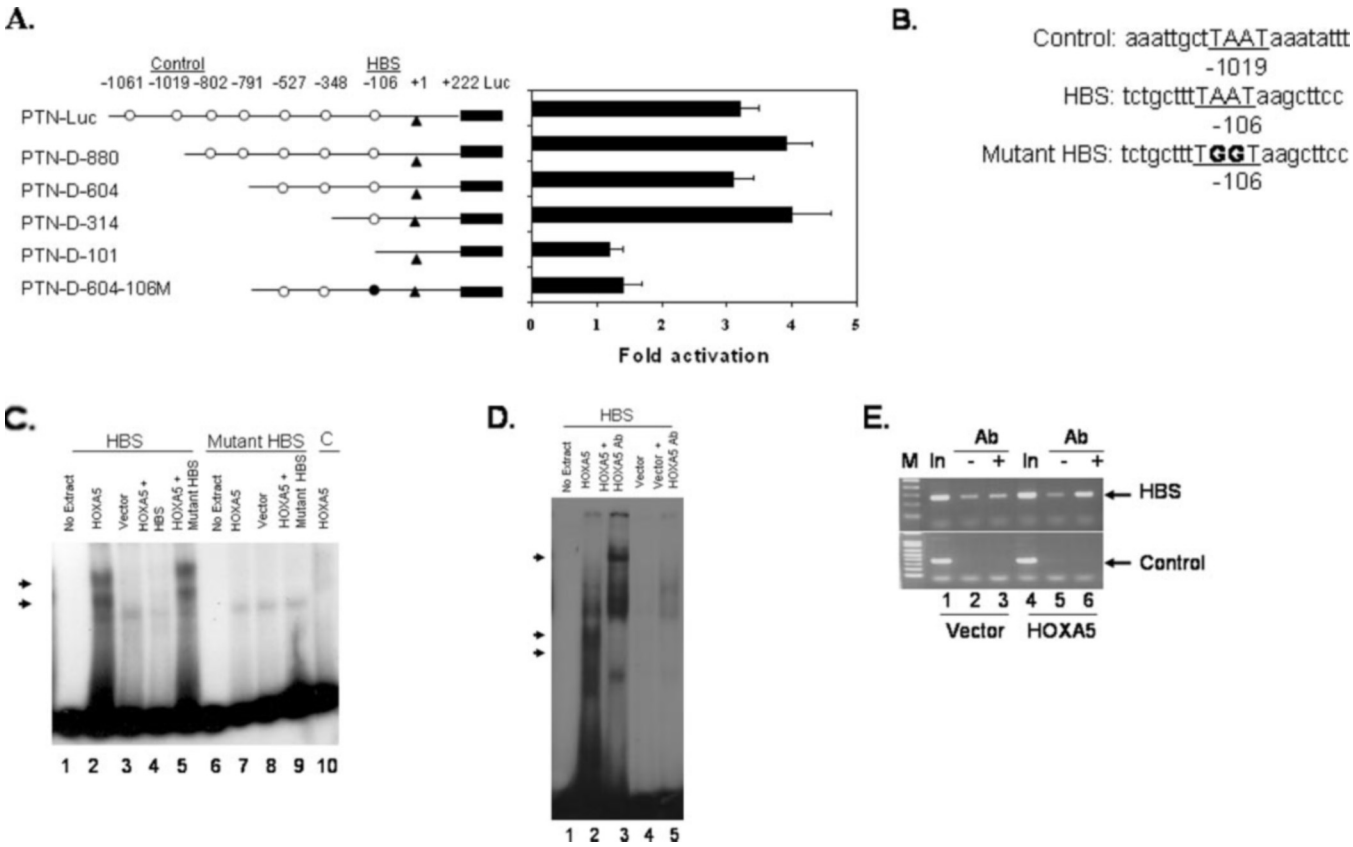


FIG. 4. **Mapping the HOXA5-binding sites on PTN promoter.** *A*, deletion analysis of the PTN promoter. 100 ng of each of these deletion constructs were co-transfected into cells with 0.5 $\mu$g of HOXA5-expressing plasmids. The luciferase activities were measured at 24 h post-transfection as described under "Materials and Methods." The results are an average of three independent experiments. PTN-Luc, which contains the PTN promoter sequence from −1084 to +222 bp, was referred to as the full-length promoter. PTN-D-604–106M carries a point mutation in the HBS site (nt 106, T**AA**T→T**GG**T). *HBS*, HOXA5-binding site. *B*, the wild type and mutant oligonucleotides containing the potential HBS and control oligonucleotide were synthesized for gel-shift assays. *C*, HOXA5 directly binds to HBS on PTN promoter. The wild type and mutant oligonucleotide probes were end-labeled and hybridized with nuclear extracts from HOXA5 and vector-inducible cells 16 h post-induction. For each probe, a negative control (no nuclear extract) was included, and the HOXA5-specific binding was competed with cold 50-fold excess of either wild type oligonucleotide or mutant HBS oligonucleotide. Two base mutations (AA→GG) in HBS completely abolished its ability to bind to HOXA5 (*lanes 6–9*). HOXA5 and vector represent nuclear extracts from HOXA5 and vector-inducible cells respectively. *Lane C*, control oligonucleotide. *D*, supershift assay using HOXA5-specific antibody. The end-labeled HBS oligonucleotide was incubated with HOXA5 or vector cell nuclear extract in the presence or absence of HOXA5 antibodies. The bands of HOXA5-oligonucleotide complex and the supershifted complex are indicated by *arrows*. *E*, binding of HOXA5 to HBS *in vivo* by ChIP assay. *M*, DNA marker; *In*, total input DNA; *Ab*, HOXA5 antibody; *HBS*, HOXA5-binding region; *Control*, upstream region of PTN promoter used as a negative control. Primers for PCR amplification of HBS and control regions were described under "Materials and Methods."

previous studies on HOX protein-DNA binding assays, purified HOX proteins were used for the gel-shift assay (8, 26). The strong binding sites found in these *in vitro* assays usually confer very weak transactivation ability to the consensus binding site-containing promoters in *in vivo* transfection assay (8, 26). Besides co-factors affecting the binding affinity *in vivo*, post-transcriptional modifications of HOX protein also alter their DNA binding abilities (34). We used nuclear extracts from HOXA5-induced cells to perform the gel-shift assays. The results showed that HOXA5 specifically bound to the putative HBS (Fig. 4*C, lane 2*). No specific shifted bands were observed when vector cell lysate was added (Fig. 4*C, lane 3*). The binding of HOXA5 to HBS was competitively inhibited by an excess of the corresponding unlabeled wild type HBS oligonucleotide (Fig. 4*C, lane 4*), but not by adding the mutant HBS oligonucleotide to the reaction (Fig. 4*C, lane 5*). Two base mutations (AA to GG) in the HBS completely abolished its binding ability to HOXA5 (Fig. 4*C*, *lane 7*), further indicating that the core binding sequence (TAAT) in HBS was important for HOXA5 binding. In addition, HOXA5 did not bind to another oligonucleotide probe corresponding to the DNA sequence (nt 1011–1030) containing a TAAT site at nt 1019 (Fig. 4*C, lane 10*). The binding specificity of HOXA5 to the HBS was further tested by performing supershift assays (Fig. 4*D*). Adding HOXA5-specific antibody to the reaction resulted in a shift of the HOXA5-specific bands (Fig. 4*D, lanes 2* and *3*) but had no effect on the migration of nonspecific bands (Fig. 4*D, lanes 4* and *5*).

To study if HOXA5 binds to HBS in intact cells, we performed a ChIP assay using a HOXA5-specific antibody. Consistent with the results of the gel-shift assay, the DNA fragment encompassing the HBS was specifically pulled down in the presence of the HOXA5 antibody (Fig. 4*E*, *lane 6*), but not in its absence (*lane 5*), in immunoprecipitates of the HOXA5-transfected cells but not in the vector control (*lane 3*). In contrast, a control DNA fragment cannot be pulled down by the HOXA5 antibody. These results, combined with evidence from deletion and mutation studies of the PTN promoter and DNA binding and supershift assays, provide strong experimental evidence that supports the conclusion that the TAAT-containing sequences located at −106 in the PTN promoter are the binding sites for HOXA5.

## DISCUSSION

The HOX family transcription factors are known to play pivotal roles in embryogenesis and tumorigenesis. A prerequisite for understanding the complex patterning functions of

*HOX* genes is to identify their downstream target genes. Although the number of Hox targets in *Drosophila* alone has been estimated to range in the thousands, to date only a few presumptive downstream genes have been isolated in both flies and vertebrates (35). Efforts to identify gene-specific targets are greatly hindered by the lack of knowledge of the sequence of binding sites specific to each *HOX* gene. As a master regulator, *HOX* genes may indeed regulate expression of many genes simultaneously *in vivo*. Because the recent development of subtractive hybridization and array technologies, a small number of studies have resulted in the identification of a number of other *HOX* gene targets (36–41). But similar studies have not been performed for HOXA5.

In this study, we utilized oligonucleotide microarray analysis to identify the downstream targets of HOXA5. We found that induction of HOXA5 expression can rapidly modulate the expression of a large group of genes representing a wide variety of functional categories. Semi-quantitative RT-PCR was able to confirm the gene expression changes identified by the microarray analysis in all 10/10 genes tested. Detailed transient transfection assays, promoter deletion assays, gel-shift assays, and ChIP assays demonstrated that HOXA5 can directly bind to and activate the promoter of the *PTN* gene. These results indicated that at least some of these genes may be direct targets of HOXA5. Many of the 262 up-regulated genes at 9 h post-induction are transcriptional factors, implying that a large number of these differentially expressed genes are indirect targets of HOXA5. However, all of these genes may get involved in HOXA5-signaling pathways.

HOXA5 plays an important role during embryogenesis. However, the signaling pathways downstream of *HOXA5* are largely unknown. Identification of PTN as a *bona fide* target of HOXA5 may provide insight into the functions of HOXA5 in development. PTN was initially isolated from early postnatal rat brain as a protein with neurite outgrowth-promoting activity on embryonic brain neuron cells in culture (42). During embryogenesis, PTN expression was expressed in a distinctive temporal and cell type-specific manner (43). Although *in vitro* studies showed that PTN expression can be regulated by platelet-derived growth factor and other stimuli (43, 44), it is difficult to reconcile the temporal-spatial expression pattern of *Ptn* during embryogenesis. The data presented in this paper show that like human HOXA5, mouse Hoxa5 may directly control the expression of *Ptn* during development. Most strikingly, the expression of *Hoxa5* coincides well with that of *Ptn* both in the time window and locations during development (24, 45–47). Because both of Hoxa5 and Ptn are expressed in the central nervous system, lung, and gut, it is possible that HOXA5 may directly dictate functions of PTN in the development of these tissues. For example, both Hoxa5 and Ptn are expressed in Purkinje cells (46, 48). Their expression appears to be activated perinatally and continues into adulthood. Most interestingly, Hoxa5 has been shown to activate a Purkinje cell-specific promoter, L7 (48). Another line of evidence for their interaction may be provided by the fact that the *Hoxa5* knock-out mice died of respiratory failure soon after birth. This phenotype, resulting from the absence of Hoxa5, corresponds to the high expression of both *Ptn* and *Hoxa5* in the lung of neonate animals (34, 42). Further studies are needed to confirm their colocalization in tissues and functional relevance during development.

In breast cancer cells, it was initially reported that PTN mRNA was detected in 25% of breast cancer cell lines and about 60% of breast carcinomas (23). However, the expression of PTN in normal breast tissue was not examined. The authors concluded that PTN was overexpressed in a large proportion of breast carcinomas. Because tumors that did not express PTN also contained similar proportions of normal tissue, they argue that the expression of PTN in normal breast tissue is too low to be detected by the RNase protection assay. In recent years, gene expression profiles of breast cancer using large numbers of clinical samples have been performed using microarray analysis (49–51). We searched through these gene expression data bases at www.oncomine.com and found that, in contrast to the published data (23), the PTN expression level was significantly lower in breast tumor samples in three of three studies involving a total of 32 normal breast samples and 149 breast carcinoma samples. Our data suggest that PTN expression was lost in breast tumors. Our previous study showed that HOXA5 expression was also lost in more than 60% of primary breast carcinomas. Identification of PTN as a direct target of HOXA5 implies that loss of HOXA5 expression may contribute to the loss of PTN expression in breast tumors. Both PTN and HOXA5 play diverse roles in development and tumorigenesis. PTN was reported to stimulate the proliferation of fibroblasts and endothelial and epithelial cells and act more like an oncogene (23). On the other hand, HOXA5 was found to induce apoptosis in breast cancer cells when it was overexpressed (15, 16). However, both of them have also been shown to promote cellular differentiation (13, 14, 42). Currently, we cannot integrate these diverse functions into a simple model. Further studies are needed to address the functional relationship between these two genes in breast tumorigenesis.

As mentioned above, overexpression of HOXA5 in breast cancer cells triggers apoptotic pathways (15, 16). Most interestingly, when we searched through genes that were up-regulated at 9 h post-induction, we identified many genes related to receptor-mediated apoptosis. Among them are two TNF receptor family members, TNFR9 and TNFR10b. TNF10b (also named as DR5) is a receptor for TRAIL, which is a well documented apoptotic inducer (52). Among the 12 gene probes that displayed unregulated genes at 6 h post-induction, 3 of them represent one single gene *GADD45β*. GADD45β is a putative target of NF-κB (53). Interleukin-8, which is another putative target of NF-κB (54), is also up-regulated at the 6 h post-induction. These results strongly suggest that the NF-κB pathway may be activated downstream of HOXA5. Our recent data showed that the p65 protein was translocated into the nucleus 30 min after induction of HOXA5 expression (data not shown), which has been shown to be one of the most important criteria for NF-κB activation (55, 56). Activation of NF-κB antagonizes apoptosis by numerous triggers, including the ligand engagement of the "death receptor" such as the TNF receptor (57). Consistent with these findings, we have recently shown that HOXA5 and TNFα can synergistically induce apoptosis, strongly suggesting that the receptor-mediated apoptotic pathway was activated (16). In conclusion, HOXA5 may trigger receptor-mediated apoptotic pathways that involve activation of the NF-κB signaling pathway.

In summary, we have successfully identified many direct or indirect targets of HOXA5. The HS578T-tet-inducible system allowed us to easily identify genes whose expression was up-regulated early, which are more likely to be the direct targets and may initiate HOXA5 downstream signaling pathways. Further comprehensive sorting and characterization of these downstream targets will help us to understand better how the HOXA5 signaling pathways contribute to the integration of proliferation, differentiation, and apoptosis in development and tumorigenesis.

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