Proliferation of Small Cell Lung Cancer Cell Line Reduced by Knocking-down PROX1 *via* shRNA in Lentivirus

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Abstract. *The present study aimed to find whether PROX1 is expressed in small cell lung cancer (SCLC) cell lines, and whether PROX1 knockdown with shRNA via lentivirus resulted in decreased cell proliferation. SCLC cell lines H69, H82, H187 and H889 were selected for the study. PROX1 mRNA and protein levels were determined with real-time reverse-transcription polymerase chain reaction(RT-PCR) and western blot, respectively. The localization and distribution of PROX1 was mapped by immunocytochemistry with a specific antibody. Three pairs of shRNA were selected from a pool of shRNA pairs, and packaged into lentivirus particles to infect the above cell lines. The non-target sequence (NT) and a house-keeping gene, glyceraldehyde 3 phosphate dehydrogenase (GAPDH), were employed as controls. SCLC cell proliferation rates were measured with bromine deoxyuridine (BrdU) incorporation method. The results indicated levels of that PROX1 mRNA were detected in SCLC cell lines in the following rank order H69>H889>H187>H82. A similar profile for PROX1 protein expression was captured. The majority of PROX1 was concentrated at the cell nucleus. H69 was selected to represent the above SCLC cell lines. The PROX1 level in H69 cells was successfully reduced with shRNA lentivirus, and the cell proliferation rate of infected H69 cells was dramatically reduced by 20-50%. Hence, it is concluded that PROX1 expression in SCLC cell line is high, and can be reduced with shRNA lentivirus, thereby reducing the cell proliferation rate.*

Prospero-related homeobox-1 (PROX1) is one of the diversified prospero family. PROX1 is composed of five exons and four introns, localized on chromosome 1q32.2-32.3. The PROX1 protein (MW: 82.3 kDa) consists of 737 amino acids and is encoded by its full-length cDNA (2,924 bp) (1), but numerous variant mRNA spliced forms are found across different species, different tissues, and even different developmental stages within the same tissue (2-4).

PROX 1 is characterized by an atypical C terminal and the nuclear docking signaling N-terminal. The homeodomain in the C-terminal is distinguished from other homeodomains consisting of the helix-loop-helix-turn-helix fold structure flanked with the prospero domain to form a single functional DNA-binding unit (3). The prospero-domain contains a functional proliferating cell nuclear antigen (PCNA) interacting motif. The prospero domain is self-adjusted to align for DNA binding. The N terminal of PROX1 harbors a nuclear docking signal and two nuclear receptor boxes (1-2).

It is well-known that PROX1 plays an important role in embryogenesis and development of many organs, because it interacts with NOTCH1, WNT/β-catenin-T-cell factor (TCF)/lymphoid enhancer factor (LEF) *etc*., particularly in the development of the central nervous system (2, 5-8).

However, a body of evidence has drawn a great deal of attention to the role of PROX1 in oncogenesis and metastasis. The expression levels of PROX1 in different tumors are quite different, and the different roles that PROX1 plays in different tumors, remain unclear. Briefly, higher levels of *PROX1* mRNA expression are found in neuroblastoma, glioma, colonic cancer, rhabdomyosarcoma, small cell lung carcinoma and lung carcinoid tumor, whereas lower levels found in hematological malignancies, breast cancer, hepatocellular carcinoma, biliary carcinoma, and variable protein expression occurs on, RNA mutations of *PROX1* in esophageal cancer (2, 9-13).

Recently, cancer stem cells (CSCs) have been deemed to play an important role in tumor therapy, because only CSCs are able to provide the driving force for tumor self-renewal and growth continuously (7).

PROX1 is a possible candidate involved in these processes. The purpose of the present study was to determine whether cell proliferation rates of SCLC cell lines with a high expression of PROX1 could be reduced by knocking-down PROX1 with shRNA lentivirus.

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Materials and Methods

Cell lines. Four SCLC cell lines (H889, ATCC number: CRL-5817; H187, ATCC number: CRL-5804; H82, ATCC number: HTB175; and H69, ATCC number: HTB119) were obtained from American Type Culture Collection (ATCC). Cell culture medium RPMI 1640 was purchased from the ATCC and 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin were added. Cell suspensions were cultured in plastic flasks at 37˚C, with 5% CO_2 and 95% in an air conditioned incubator. The media were changed every two days.

Real time RT-PCR. Cells were collected by centrifugation at 500 ×g and washed with ice-cold phosphate-buffered saline (PBS). Total RNA was extracted using QIAGEN RNease mini-kit (cat#74104) according to the manufacturer's instructions. The cell pellet was homogenized with 0.6 ml TLT buffer from the kit. The lysate was loaded onto a GIA shredder spin column (cat# 79654; QIAGEN) and centrifuged at 16,000 ×*g* for 2 min. The outflow from the column was mixed with an equal volume of 70% ethanol prepared using by diethylpyrocarbonate (DEPC)- treated distilled and deionized water (DDW). DNAse free RNA kit from Ambion Company (cat #1906) was used to remove residual DNA. The final RNA concentration was determined with a NanoDrop spectrophotometer (ND-1000) (NanoDrop Technologies, Inc., Wilmington, DE, USA).

The cDNA was synthesized by using the SuperScript III first-strand synthesis system for RT-PCR kit and the RNA residue was cleaned with RNAse H treatment. The following primers were adopted from a previous study (14): forward primer for human *PROX1* was 5'-AAAGCAAAG CTCATGTTTT TTTATA-3'; reverse primer of human *PROX1* was 5'-GTAAAACTCACGGAAA TTGCTAAA-3'; forward primer for human *actin* was 5'-TCACCCACACTGTGCCC ATCTACGA-3'; reverse primer of human *actin* was 5'-GGTAACCGTTACTCG CCAAGGCGAC-3'. An equal amount of cDNA (0.1 μg) template was assayed by using platinum SYBR green qPCR supermix-UDG kit (Cat#11733-038; Invitrogen). The final data were analyzed using $2^{-\delta\delta Ct}$ calculation by comparison between *PROX1* and *actin* (9).

Lentivirus construction. Lentiviral shRNA in pGIPZ vectors from Open Biosystems were constructed by the RNA Interference Core Facility at the Mayo Clinic. The pGIPZ-572 vector was designed to aim at the beginning sequence of human *PROX1* (5'-cgcgcccgggttgagaatat-3', 767-787, 20 nt), while pGIPZ-575 was aimed at the middle sequence of *PROX1* (5'-ggctctgaacatgcactacaat-3',2261-2282, 21nt) and pGIPZ-428 at the end of the *PROX1* gene sequence (5'-ccacttcatacatttaagtat-3', 2943-2963, 20nt). Turbo green fluorescent protein (GFP) marker in pGIPZ lentiviral vector was allowed to monitor shRNA expression in living cells. Besides this core lentiviral vector, trans-lentiviral packaging system (cat# TLP4614; Open Biosystems) was purchased and the final virus particles were packaged in our laboratory according to the product instructions. Every batch of viruses was titered using TLA-HEK293 cells, and viruses were then aliquoted and stored at –80˚C.

Cell transfection. The four cell lines were kept growing in 75 ml flasks. An equal number of cells were infected with each of five lentivirus sequences: non-targeting sequence (NT); targeting sequences for GAPDH, pGIPZ-428,pGIPZ-572,pGIPZ-575. The limit of multiplicity of infection was set at 0.1. After 48 h incubation, most cells expressed GFP under fluorescence microscopy; thus, the cell culture medium was refreshed. After one week, cells were harvested to determine the PROX1 protein level or to split for conducting cell proliferation assays.

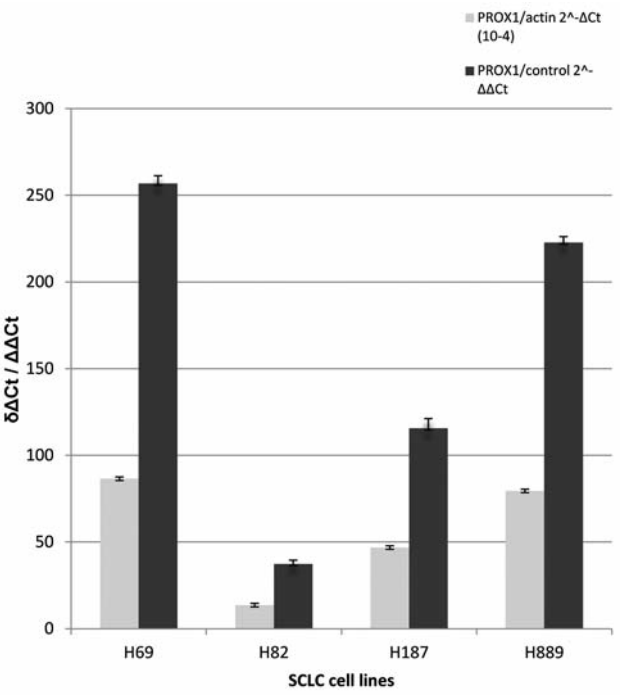

Figure 1. *Determination of mRNA levels of SCLC cell lines by real-time PCR. Equal amount of cDNA (0.1 μg) template was assayed by using platinum SYBR green qPCR supermix-UDG kit. The final data were analyzed using 2 ^- ΔΔCt Ct calculation by comparisons of normalized amounts of PROX1 relative to actin 2^-ΔCt (10^{-4}) and relative to control tissue 2^-ΔΔCt. (N=3, independent assay was replicated in three times).*

Western blot. The standard procedure was employed from our previous study (15). Briefly, harvested cells were washed with ice-cold PBS (pH7.4) once and digested with twice the volume of RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris (pH 8.0) plus a proteinase inhibitor cocktail (cat# 11836153001; Roche). Cell lysates were sonicated with a digital sonicator (Branson Ultrasonic Corporation, Danbury, CT, USA) at 30% intensity for 5 s, and centrifuged at 15,000 ×*g* for 10 min at 4˚C. Both sonication and centrifugation were repeated once. The milky suspension was collected and the cell debris was discarded. The protein concentration in the mixture was determined by the Lowry's method (16). An equal amount of each protein sample was loaded and ran on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with a primary antibody to PROX1 (1:800) (cat#51043-1-AP; Protein tech Group, Inc., Chicago, IL, USA) and β-actin for the control. After washing, the membrane

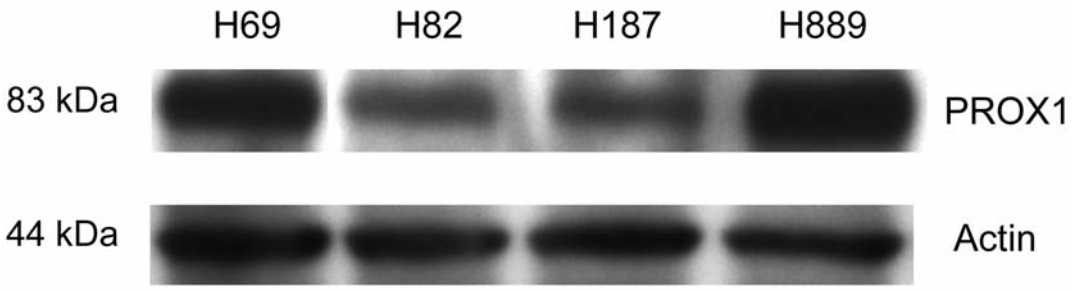

Figure 2. *PROX1 protein levels among small cell lung cancer cells were characterized by western blotting. 40 μg of protein from each sample were analyzed. PROX1 was recognized with a polyclonal antibody from Protein Tech. The same experiment was repeated twice.*

was incubated with a horseradish peroxidase (HRP)-linked secondary antibody. Finally, each membrane was developed with enhanced chemiluminescence (ECL) advance western blotting detection kit (cat# RPN2135; GE healthcare) and exposed to high-performance chemiluminescent film (GE Healthcare). At least two independent experiments were run.

Cell proliferation assay. The cell proliferation Biotrak ELISA kit was purchased from GE Healthcare (code: RPN250) and used according to the supplied instructions with slight modifications. Briefly, 10^5 cells from each cell line were seeded in 24 well plates with 1 ml medium. Cell proliferation was examined at 6, 12 and 24 h. Fifty micromoles of Br-deoxyuridine (BrdU) were added 2 h before each time point and cells were harvested by centrifugation at 300 ×*g* for 10 min. The supernatant was gently removed by tapping. The cell pellet was re-suspended with fixative at room temperature for 30 min. After centrifugation and discarding the supernatant, the cell pellet was re-suspended in blocking buffer for 30 min. This procedure was repeated twice and the re-suspended cells were incubated with peroxidase-labeled anti-BrdU for 90 min at 37˚C. The cell suspensions were equilibrated with 0.2 ml 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Color was allowed to develop for 1 min and reaction was stopped with 50 μl 1 M sulfuric acid. Aliquots (0.1 ml) were transferred to a 96-well plate and the optical density was read at 450 nm with a spectrophotometer. Blank control without cells was set up in parallel.The higher optical density (OD), the more BrdU incorporated, the more cell proliferated.

Immunocytochemistry. Suspensions of H187 and H889 cells were seeded on 1% poly-D-lysine pre-coated coverslips in a 6-well plate, and fixed with 4% paraformaldehyde at 37˚C for 15 min. After washing with PBS, the cells were blocked with 3% bovine serum albumin for 30 min at room temperature and permeated with 0.2% Triton X-100 in PBS for 10 min. After PBS washing, the cells were incubated with antibody to PROX1(1:500) for 1 h, washed again, and incubated with green fluorescein (FITC) conjugated with a second antibody (anti-rabbit, 1:100, Code: 111-096-144; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for another 1 h. After washing with PBS twice and with distilled deionized water (DDW) once, the coverslips were dried and mounted on the slides with gold anti-fade reagent with 4'-6-Diamidino-2-phenylindole (DAPI) (cat# P36931; Molecular Probe). The images were captured by a Zeiss 510 confocal microscope.

Statistical analysis. All the quantitative data were expressed as the mean±SD, and subjected to statistical analysis by using One-way ANOVA program (SigmaStat 3.0; Systat Software Inc., Chicago, IL, USA). *p*-Values of <0.05 were considered to be statistically significant when compared data in tested groups to data in the control. The control is parental H69 cells.

Results

High expression of PROX1 mRNA levels in SCLC cell lines. As shown in Figure 1, high levels of expressed *PROX1* mRNA were found in the following rank order of SCLC cell lines: H69>H889>H187>H82, as indicated by both 2^Δ Ct and 2^-ΔΔCt, respectively. Among these different SCLC cell lines, the expression levels of *PROX1* were markedly different in them: *PROX1* in H69 cells was 5-fold higher than that in H82 cells.

High expression of PROX1 protein levels in SCLC cell lines. As shown in Figure 2, protein levels of PROX1 were higher in cell lines H69 and H889 compared to H187 and H82, which is in accordance with the expression profile of *PROX1* mRNA shown in Figure 1.

Identification of PROX1 in the nucleus in SCLC cell lines. Regulation of PROX1 distribution occurs both at the RNA and protein level, and occurs through its subcellular location, *i.e*. PROX1 is inactive when in the cytoplasm but active for regulating gene transcription when in the nucleus. As shown in Figure 3, antibody staining for PROX1 indicated that expression of PROX1 was localized in the DAPI-stained nuclei. PROX1 staining in the cytoplasm was weak and scattered, whereas PROX1 was highly concentrated in the nucleus, which was consistent with our previous study (17).

Comparison of cell proliferation rates among SCLC cell lines. Cell proliferation was higher in the H187 than in the other cell lines. The proliferation rates for H82 and H889 were similar and that for H889 was statistically the lowest, as shown in Figure 4.

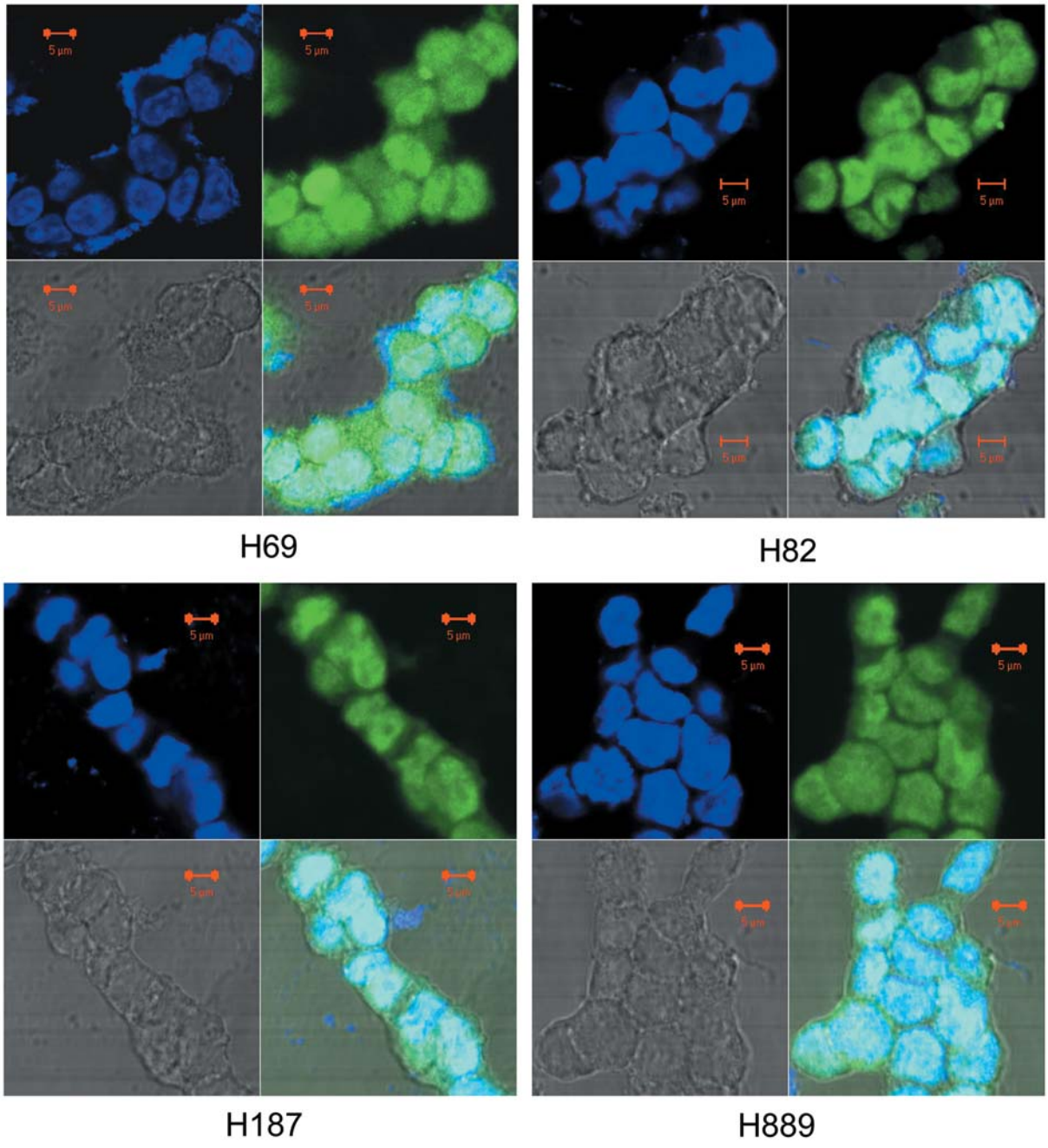

Figure 3. *PROX1 protein expression in H69 (A), H82 (B), H187 (C), H889 (D) cells was confirmed by immunocytochemistry. PROX1 was recognized by polyclonal antibody conjugated with FITC as green fluorescence (Ex 492 nm, Em 520 nm). Nuclei were stained as blue fluorescence with DAPI (Ex 345 nm, Em 455 nm). The images were captured by a Zeiss LSM510 laser confocal microscope (magnification ×250).*

H69 cell proliferation rate was reduced by knock-down of PROX1 with shRNA-lentivirus transfection. Five random constructed shRNA interfering with different regions of *PROX1* gene were packed into lentivirus particles, and screened with the SCLC cell lines. Three pairs of shRNA were found to be effective, and were eventually packaged into three lentivirus particles as pGIPZ-428, pGIPZ-572 and pGIPZ-575, and compared with non-targeting sequence (NT) and targeting of GAPDH. By virtue of *PROX1* gene sequence map, pGIPZ-572 was used to target the second exon of *PROX1*, where most mutations were found in glioblastoma, whereas pGIPZ-575 was used to target the

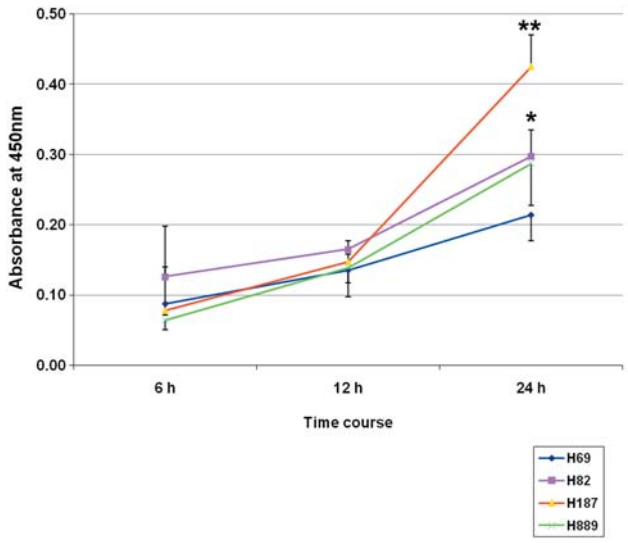

Figure 4. *Cell proliferation of four small cell lung cancer cell lines were characterized by BrdU incorporation: 50 μM BrdU was incorporated into 1×10^5 cells for 2 h before the cells were harvested at 6 h, 12 h and 24 h. Each sample was repeated four times. The same independent experiment was replicated. The data represented here are the mean±SD, and were statistically analyzed using SigmaStat software. One-way ANOVA followed by Dunnett's test was employed to evaluate statistical significance as *p<0.05 and **p<0.01 when other cells compared to H69 cells.*

fourth exon, where the prospero domain is self-aligned and interacts with DNA binding. The third shRNA-pGIPZ-428 was used to target the fifth exon of PROX1 gene, where the encoded protein region is the N-terminal that harbors a nuclear localization signal and two nuclear receptor boxes.

After parental SCLC H69 cells were transfected with the five different constructed shRNA-lentivirus, five different derived cell lines were developed and labeled as Control (parental H69), NT (shRNA non-targeted sequence), GAPDH (shRNA targeting GAPDH), PROX1-428 (shRNA-pGIPZ-428), PROX1-572 (shRNA-pGIPZ-572), PROX1-575 (shRNA-pGIPZ-575).

As shown in Figure 5A, high expression of PROX1 protein in H69 cells was successfully knocked-down by pGIPZ-428, 575 and 572 shRNA lentiviruses transfection, whereas NT shRNA and that targeting the housekeeping-gene GAPDH had no effect on PROX1 protein expression. The loading control used was β-actin. As shown in Figure 5B, cell proliferation rates after lentivirus transfection were determined by BrdU incorporation. The five different derived H69 cell lines were tested concurrently. The proliferation rates of PROX1-428, PROX1-572 and PROX1-575 were reduced at 21%-33%, and differences were statistically significant as compared to that of the Control ($p<0.01$). However, there was no statistical difference between the proliferation rates of PROX1-428, PROX1-572 and PROX1-575.

The SCLC cell lines H82, H187, H889 had similar expression profiles after the different shRNA-lentivirus transfections, consequently the data are not shown here.

Discussion

High amounts of PROX1 were found in the above SCLC cell lines at both mRNA and protein levels, and abundant PROX1 was localized in the nucleus. Thus, three pairs of shRNA were designed to aim at different regions of full-length gene *PROX1*, and packaged with lentivirus as pGIPZ428, 572 and 575. H69 cells were selected to represent the above four SCLC cell lines, and these results alone are reported here because other three cell lines displayed similar profiles. PROX1 protein expression in H69 cells was successfully knocked-down by the above lentiviruses, which finally led to a reduced cell proliferation rate.

PROX1 was found to be a master gene in inducing venous blood endothelial cells towards lymphatic endothelial phenotype (3). PROX1 was found to be abundantly expressed in lymphangiomas, venous hemangiomas and Kaposi sarcoma, whereas PROX1 was absent in capillary and cavernous hemangiomas (3). Nevertheless, one should be very cautious to use PROX1 as a bio-marker with predictive value in vascular tumors.

PROX1 has been detected to have undergone various alterations in different tumor types, such as overexpression, point-mutation, missense and non-sense mutation, decreased expression, hypermethylation, variable mRNA splicing, *etc*. (4, 10-11). It was found to be deleted, hypermethylated, or silenced in carcinomas of the biliary system (10), it was extensively hypermethylated (CpG island) or silenced in breast cancers (18), or showed reduction or point mutations (A to G) in pancreatic cancer (13). However, there are many studies demonstrating that PROX1 is closely-related to oncogenesis, and the level of PROX1 may even be proportional to the degree of tumor cell differentiation, and is a key factor in determining whether it is benign tumor or malignant tumor (14, 19). Petrova and colleagues recently showed that PROX1 promotes dysplasia in colonic adenomas and colorectal cancer progression; loss of PROX1 does not prevent tumor initiation but instead impairs tumor progression (4).

Our findings are in favor of the role of *PROX1* as an oncogene in SCLC. Delivering shRNA to knock-down PROX1, successfully reduced the cell proliferation rate, pointing out a direction to deal with SCLC.

Embryonic signaling pathways, including Wnt, Nocth, Hedgehog and TGFβ, are considered as targets for developing antitumor drugs. *e.g.*, WNT in colon, and then the blockade of WNT pathway is a means of treating colon cancer. As described above, PROX1 plays a crucial role in embryonic development of many organs, has direct or indirect interactions

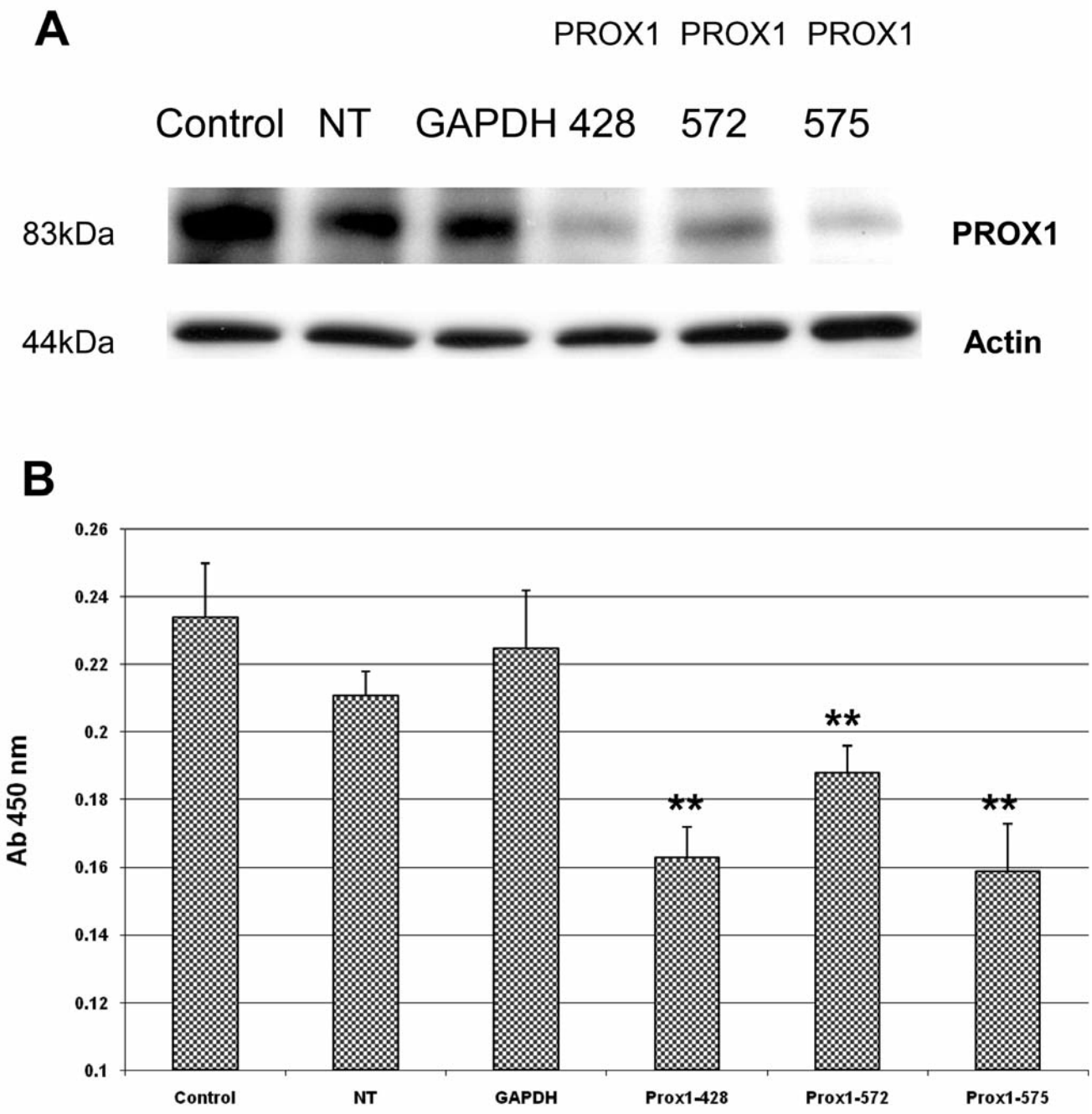

Figure 5. *A: PROX1 protein expression in H69 cells was knocked-down by shRNA lentivirus infection. Detailed method is described in Materials and Methods. Different viruses of NT (pGIPZ-NT), GAPDH (pGIPZ-GAPDH), 428(pGIPZ-428), 572(pGIPZ-572) and 575(pGIPZ-575) had different effects on PROX1 levels in H69 cells. B: PROX1 shRNA lentiviruses decrease H69 cell proliferation. The infection procedure was the same as in A. The absorbance at 450 nm represents the cell proliferation rate. The data were recorded as the mean±SD and the statistical significance was compared between treatment groups and control group using one-way ANOVA followed by Turkey's test with SigmaStat software and expressed as **p<0.01 against the control. (The control is parental H69 cells).*

with the embryonic signaling pathways, cross-linking with pathways, such as WNT and Notch (5-6, 9, 20-21). However, these interactions still require further investigation.

Our findings show that PROX1 is highly expressed in SCLC cell lines, and can be knocked-down with shRNA lentivirus, thereby providing the hope of treating SCLC.

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