Primary hepatocellular carcinoma (HCC) is one of the most common solid tumors in many countries of the world, especially in Asia and Africa, representing the third cause of mortality among deaths from cancer (1). Chronic infections with hepatitis B virus or hepatitis C virus and alcoholic cirrhosis are responsible for the majority of HCC cases. Other risk factors include prolonged dietary exposure to aflatoxin (2), primary hemochromatosis, and cirrhosis associated with genetic liver diseases (3); however, the principal risk factor varies among countries. Although much is known about both the cellular and molecular basis of hepatocarcinogenesis, a precise understanding with respect to prognosis does not exist; ref. 4).

Homeobox proteins are known to play essential roles in the determination of cell fate and the development of the body plan. The roles of homeobox proteins have been documented individually, although these total physiologic roles remain unclear. Several homeobox genes are the targets of chromosomal translocations in malignancies and are thought to be potential oncogenes. Deregulation of such a homeobox gene may give rise to tumorigenesis in target organ. The homeobox gene Prox1 is related to the Drosophila prospero gene, which mediates cell fate decisions of neuroblasts (5). Prox1 is the master gene of lymphangiogenesis but it is also expressed in potential oncogenes. Deregulation of such a homeobox gene may give rise to tumorigenesis in target organ. The homeobox gene Prox1 is related to the Drosophila prospero gene, which mediates cell fate decisions of neuroblasts (5). Prox1 is the master gene of lymphangiogenesis but it is also expressed in potential oncogenes. Deregulation of such a homeobox gene may give rise to tumorigenesis in target organ. The homeobox gene Prox1 is related to the Drosophila prospero gene, which mediates cell fate decisions of neuroblasts (5). Prox1 is the master gene of lymphangiogenesis but it is also expressed in potential oncogenes. Deregulation of such a homeobox gene may give rise to tumorigenesis in target organ.

Prox1 may be involved in the tumorigenesis of hepatocellular carcinoma (HCC), human clinical samples were analyzed. Experimental Design: To screen prox1 as a potential tumor suppressor gene, its expression was analyzed in HCC cell lines and in human HCC tissues. Its growth-conferring abilities were assessed by transiently overexpressing Prox1 in HCC cell lines and by knocking down its expression by RNA interference. Results: We found that there was a significant correlation between Prox1 expression and the differentiation scores of the tumors. Subsequently, we also showed that low expression of Prox1 in tumors was closely associated with a poor prognosis. The specific knockdown of Prox1 by RNA interference strongly accelerated in vitro cell growth, whereas the overexpression of Prox1 greatly suppressed the growth. Conclusions: Our results suggest that Prox1 is involved in the differentiation and progression of HCC, and thus it may be a candidate for the development of novel diagnostic and therapeutic strategies for HCC.

A Homeobox Protein, Prox1, Is Involved in the Differentiation, Proliferation, and Prognosis in Hepatocellular Carcinoma

Masayuki Shimoda,1,2 Meiko Takahashi,1 Takanobu Yoshimoto,1 Tomoya Kono,1 Iwao Ikai,2 and Hajime Kubo1

Abstract Purpose: It has been shown that a lymphatic differentiation master gene, prox1, also plays an essential role in fetal hepatocyte migration. Its expression is detected in embryonic hepatoblasts and in adult hepatocytes. Hepatoma cells are similar to embryonic hepatoblasts to a certain extent because they both proliferate and invade the surrounding tissue. To address the possibility that Prox1 may be involved in the tumorigenesis of hepatocellular carcinoma (HCC), human clinical samples were analyzed.

Experimental Design: To screen prox1 as a potential tumor suppressor gene, its expression was analyzed in HCC cell lines and in human HCC tissues. Its growth-conferring abilities were assessed by transiently overexpressing Prox1 in HCC cell lines and by knocking down its expression by RNA interference.

Results: We found that there was a significant correlation between Prox1 expression and the differentiation scores of the tumors. Subsequently, we also showed that low expression of Prox1 in tumors was closely associated with a poor prognosis. The specific knockdown of Prox1 by RNA interference strongly accelerated in vitro cell growth, whereas the overexpression of Prox1 greatly suppressed the growth.

Conclusions: Our results suggest that Prox1 is involved in the differentiation and progression of HCC, and thus it may be a candidate for the development of novel diagnostic and therapeutic strategies for HCC.

Authors’ Affiliations: 1Molecular and Cancer Research Unit, HMRO, and 2Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Received 3/22/06; revised 5/19/06; accepted 6/30/06.

Grant support: Ministry of Education, Culture, Sports, Science, and Technology, Special Coordination Funds for promoting Science and Technology, Ichiro Kanehara Foundation, Takeda Science Foundation, and the Princess Takamatsu Cancer Research Fund.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

M. Shimoda and M. Takahashi contributed equally to this work.

Requests for reprints: Hajime Kubo, Molecular and Cancer Research Unit, HMRO, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. Phone: 81-75-753-9307; Fax: 81-75-753-9371; E-mail: kuboflt@kuhp.kyoto-u.ac.jp.

©2006 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-06-0712
that inactivation of Prox1 caused abnormal cellular proliferation and down-regulated expression of the cell cycle inhibitors (10). Interestingly, a report showed that Prox1 controlled progenitor cell proliferation and horizontal cell genesis in mammalian retina from the analysis of the gene-targeting mice (13). In some situations, Prox1 seems to suppress cell proliferation, probably by regulating the cell cycle, and thus proxl may have a role in tumorigenesis. In fact, mutations and aberrant DNA methylation of prox1 have been observed in hematologic malignancies (14).

These information prompted us to screen prox1 as a potential tumor suppressor gene. Hepatoma cells are similar to embryonic hepatoblasts to a certain extent because they both proliferate and invade the surrounding tissue, and Prox1 could be the driving force behind these cellular activities. Is there a relationship between HCC and Proxl? In this study, we report the down-regulation of Prox1 in HCC, and investigate its involvement in human hepatocellular carcinogenesis.

Materials and Methods

Cell lines and human tissue specimens. Hep3B, Huh7, Alexander, Hepg2, and HeLa cell lines were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics at 37°C in humidified air containing 5% CO2.

Cancerous and corresponding noncancerous frozen tissues and optimum cutting temperature–embedded tissues obtained by surgical resection of 52 cases of HCC were retrieved from the 1998-2003 surgical pathology files of Kyoto University Hospital (see Table 1). All samples were obtained with informed consent and their use was approved by the ethics committee of the institution.

Semiquantitative reverse transcription-PCR. Total RNAs were extracted from clinical samples of HCC and cultured cells employing TRIzol reagent (Invitrogen, San Diego, CA) according to the protocol of the manufacturer. Extracted RNA was treated with DNase I (Boehringer Mannheim, Mannheim, Germany). First strand cDNAs were synthesized with oligo dT primer and SuperScipt II RNase H Reverse Transcriptase (Invitrogen). Each single-stranded cDNA was diluted for subsequent PCR amplification. Standard PCR procedures were carried out in 25-μl volumes of PCR buffer (10X ExTaq Buffer, TaKaRa Bio, Otsu, Japan). For detecting prox1, the following primers were used: 5’-CAGATGGAGAAGTACGCAC-3’, reverse 5’-CTTCTCC-GACGTTAACTCAAGCGCCCTG-3’. The primer set for GAPDH was 5’-CTTCTGC-CAGCTAATGAGAGGCCTGTG-3’, reverse 5’-CTTCTCGGGATGCTTGTTATCTG-3’. The sequencing analysis was done with ABI PRISM 3100 Avant Genetic Analyzer and ABI PRISM DNA Sequencing Analysis software (Applied Biosystems, Foster City, CA) according to the instructions of the manufacturer.

Real-time PCR. The primer set for Prox1 was as follows: forward 5’-AACGCAAAGCTCTGGTACCATG-3’ and reverse 5’-GAAACTCAGGAATTGTCATGACGTG-3’. The probe for Prox1 was 5’-CAGCGCATATTTACCAGAGAC-3’, reverse 5’-CTTCTCC-GACGTTAACTCAAGCGCCCTG-3’. GAPDH was used as an endogenous control using commercially available primers (Applied Biosystems). Real-time PCR was done in triplicate using a thermal cycler ABI PRISM 7700 (Perkin-Elmer Applied Biosystems, Foster City, CA) in accordance to the instructions of the manufacturer. Standard curves for templates of prox1 and GAPDH were generated by serial dilution of the PCR products. The Ct values obtained for Prox1 were normalized by the corresponding Ct values of GAPDH.

Immunohistochemistry. Cryosections of 5-μm thickness were prepared and fixed in 4% parafomaldehyde. Endogenous peroxidase was quenched with 3% H2O2 in methanol for 10 minutes, and they were blocked with TBN buffer (TSA Biotin system kit/NE1700, Perkin-Elmer) for 30 minutes. Anti-human prox1 antibody (Relia Tech GmbH, Braunschweig, Germany) was diluted 1:100 and incubated with the sections at 4°C for 16 hours. The slides were washed thrice with TNT buffer (Perkin-Elmer) and incubated with biotin-conjugated antirabbit antibody (1:1,000; DakoCytomation, Glostrup, Denmark) at room temperature for 30 minutes. TSA Biotin System (Perkin-Elmer) was used to enhance staining, and peroxidase activity was envisioned with diaminobenzidine kit (DakoCytomation). A subset of the sections was counterstained with hematoxylin. All the sections were mounted in Entellan (Merck, Darmstadt, Germany).

| Table 1. Clinicopathologic variables in 52 HCC patients |
|-----------------|------------|
| Variable        | n (%)      |
| Sex             |            |
| Female          | 40 (76.9)  |
| Male            | 12 (23.1)  |
| Metastasis status |        |
| T0              | 3 (5.8)    |
| T1              | 19 (28.8)  |
| T2              | 22 (42.3)  |
| T3              | 12 (23.1)  |
| Nodal status    |            |
| N0              | 49 (94.2)  |
| N1              | 3 (5.8)    |
| Pathological status |       |
| I               | 3 (5.8)    |
| II              | 13 (25.0)  |
| III             | 18 (34.6)  |
| IVa             | 11 (21.2)  |
| IVb             | 7 (13.5)   |
Survival rate. The differential expression of Prox1 was divided into two groups (high versus low) according to the value of real-time PCR. The cutoff value was set up at \( C_l \text{t value} = 5.5 \). The survival rate was analyzed by the Kaplan-Meier method. SPSS software was used for the statistics.

RNA interference. Two target sequences were assigned at different locations of human prox1 mRNA, designed to match nonconserved sequences. siRNAs were 21-nucleotide-long double-stranded RNA oligos with a two-nucleotide overhang at the 3’ end: siRNA-1 (sense 5’-GGAGGACGUUGUAUGUUATT-3’, antisense 5’-UAACAUAUC-CAGCClUCOCCAT-3’) and siRNA-2 (sense 5’-CGUUUAUUCGGGAAG-GLUGCAATT-3’, antisense 5’-UIUCACUUCCGAAIAAGGTG-3’). Negative control siRNA was purchased from Qiagen (Hilden, Germany).

Western blotting. Whole-cell extracts were prepared in radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40] and protein concentration was measured with Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL). Extracts (10-20 \( \mu \)g) were resolved on 12.5% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against Prox1 (1:200) or \( \text{h-actin} \) (1:10,000) for 1 hour. The blot was washed with TBS containing 0.05% Tween 20 and exposed to horseradish peroxidase–conjugated secondary antibodies for 1 hour, and finally detected by the enhanced chemiluminescence method (Western Lighting Chemiluminescence Reagent Plus, Perkin-Elmer).

Tet-off system. pTet-off regulator plasmid, which contains the tTA transactivator gene under the control of the cytomegalovirus promoter/ enhancer, pTRE2hyg response plasmid, which contains a multicloning site immediately downstream of the Tet response element (TRE), and pTRE2hyg-Luc plasmid, which contains the gene encoding firefly luciferase cloned into the pTRE2hyg, were purchased from BD Biosciences. Which had no known homology with any mammalian genes (sense 5’-UUUCCGGAACUUGUGUGUTT-3’, antisense 5’-ACGUCAACAG-GUUCCGGAATT-3’). Cell cultures of 50% to 80% confluence were prepared in a six-well plate and siRNA was introduced using Lipofectamine 2000 (Invitrogen) according to the recommended protocol. In brief, 1.67-\( \mu \)L siRNA (1 \( \mu \)g/\( \mu \)L solution) was incubated with 3.33-\( \mu \)L Lipofectamine in 133-\( \mu \)L Optimem (Invitrogen) for 30 minutes; the mixture was then added to the cells in a final volume of 0.8 mL.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell numbers were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye uptake method (Cell Titer 96 Aqueous One Solution Reagent, Promega, Madison, WI). In brief, the cells were incubated in a six-well plate in a final volume of 1 mL medium including 1/5 MTT solution at 37°C. After 1-hour incubation, the absorbance value was measured using a plate reader (ARVO 1420, Bio-Rad, Hercules, CA) at 490 nm for 0.1 second. Triplicate wells were assayed and SDs were determined.

Fig. 1. Semiquantitative RT-PCR of prox1 expression normalized to GAPDH. A, Prox1 expression in four general HCC cell lines. B, Prox1 expression in nine human HCCs as compared with their corresponding normal liver. T, HCC; N, corresponding normal liver. Well, well differentiated; Moderate, moderately differentiated; Poor, poorly differentiated. Ratio of gray values, confer Materials and Methods.

Fig. 2. Immunohistochemical detection of Prox1 in human HCC and normal human liver with anti-Prox1 antibody. Representative images (magnification, \( \times 400 \) (A); \( \times 100 \) (B)). A, human HCC. B, human normal liver. Left, Prox1; right, Prox1 + hematoxylin. Note that Prox1 was abundantly expressed in the nuclei of both cancer cells and normal hepatocytes.
Biosciences Clontech (Palo Alto, CA). Human prox1 was subcloned and inserted into the Mul1-EcoRV site of pTRE2hyg plasmid to create the pTRE2hyg-prox1 mammalian expression plasmid. The tetracycline transactivator system was employed to generate Hep3B and Huh7 cell lines that express Prox1 and the luciferase protein in a regulated manner. Hep3B and Huh7 were transfected with pTet-off regulator plasmid using Lipofectamine 2000 (Invitrogen) as previously described. Clones were then selected with 400 µg/mL (Hep3B) or 800 µg/mL (Huh7) of G418 for 4 weeks, and the effect of the transfection was analyzed by luciferase assay system (Promega). Briefly, the clones were harvested 48 hours after transfection of pTRE2hyg-Luc plasmid and cells were lysed in Passive Lysis Buffer (Promega) as indicated by the manufacturer. Insoluble material was pelleted by centrifugation for 1 minute at 13,000 × g. Twenty microliters of the supernatant were mixed with 200 µL of luciferin reagent. The light emitted was measured in a luminometer (ARVO 1420, Bio-Rad) comparing the light in the absence or presence of 1 µg/mL doxycycline to select the most effective clone (Hep3B-pTet-off, Huh7-pTet-off).

Loss of heterozygosity assessment. Genomic DNA was extracted from a total of 30 primary human HCCs and corresponding noncancerous liver tissues with a QiAamp Tissue Kit (Qiagen) after proteinase K digestion. Eight microsatellite markers (Supplemental data 1), which were mapped on human chromosome 1 (1996 Genethon Microsatellite Map, GenLink), were used for loss of heterozygosity (LOH) analysis. Each primer pair was fluorescent dye labeled. The PCR mixture contained ≥0 ng of genomic DNA, 200 µmol/L of each deoxynucleotide triphosphate, 0.25 units of Ex Taq polymerase, 0.4 µmol/L of each primer, and 10× ExTaq Buffer (TakaRa Bio) in a final volume of 10 µL. After denaturation at 94°C for 5 minutes, DNA amplification was done for 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. Samples were loaded on a 6% polyacrylamide 8 mol/L urea gel and run for 2.5 hours in a 377XL Automated Sequencer (Applied Biosystems, Chuo-ku, Tokyo, Japan). The data were collected automatically and analyzed with the GeneScan software and Genotyper software (Applied Biosystems). LOH was quantitatively assessed by calculating the LOH index, which was defined as the allele ratio in the normal tissue divided by the allele ratio in the tumor tissue. The allele ratio was calculated as the peak height of the smaller allele divided by the peak height of the larger allele. If the LOH index was <0.5 or >2.0, we defined that the case was LOH.

Results

Expression of prox1 in human HCC. We carried out RT-PCR on some hepatoma cell lines to assess the level of prox1 mRNA expression and detected the highest prox1 expression in Hep3B and lower levels in Huh7, Alexander, and HepG2 (Fig. 1A). We next examined prox1 expression in human HCC and corresponding noncancerous liver tissues by semiquantitative RT-PCR. Prox1 expression was found in cancer tissues as well as normal tissues, and the density value ratio of tumor to non-tumor was evaluated. Well-differentiated tumors tended to exhibit the highest prox1 expression levels, and its expression decreased in proportion to the tumor stage, although there was no significant difference statistically (Fig. 1B).

To confirm the expression of prox1 in cancer cells, we carried out immunohistochemical staining in cancerous human liver samples (Fig. 2A and B). Immunostaining revealed high levels of Prox1 staining in the nuclei of cancerous cells in 3 of 6 HCC tissues examined, and stronger staining could be detected in hepatocytes in 4 of 12 noncancerous tissues. We could not detect Prox1 staining in a number of HCC, which were all proven to have little or no prox1 expression by real-time PCR. Notably, lymphatic vessels were hardly observed in cancerous tissues.

Prox1 expression is positively associated to the differentiation scores of HCC. We analyzed the expression of prox1 in 60 tumor samples by real-time PCR. After excluding the eight samples that were determined to be inadequate because total RNA could not be extracted, we evaluated the association between the pathologic stage of the tumors and the expression of prox1 in 52 samples. We found the highest prox1 expression in well-differentiated tumors, and its expression decreased in
proportion to the tumor stage (Fig. 3A). The mean relative expression of prox1 in 9 well-differentiated tumors was 0.043, that in 22 moderately differentiated tumors was 0.021, and that in 21 poorly differentiated tumors was 0.008 ($P = 0.045$, between well and moderate; $P = 0.010$, between moderate and poor).

**Correlation between Prox1 expression and survival rate.** The overall cancer-specific survival was defined as the period from the date of operation to the date of cancer death. We compared the overall survival rate of 52 HCC patients with the expression of prox1 using the Kaplan-Meier method. The differential expression of prox1 was divided into two groups (high and low)

---

**Fig. 4.** siRNA directed against prox1 specifically inhibits its expression in cell lines. A, ratio of mRNA expression of prox1 assessed by real-time PCR 48 hours after the transfection of siRNA (control, 1.0). The level of prox1 mRNA was reduced to 2.4% to 41.0% in the cells treated with both siRNAs as compared with control siRNA–treated cells. Representative of three independent experiments. B and C, Prox1 protein expression detected by Western blot 72 hours (B) and 96 hours (C) after siRNA transfection. siRNA-transfected cells were harvested and cell lysates were subjected to Western blot analysis with antibodies against prox1 and actin (loading control). Hep3B and Huh7 cells in which there are high levels of endogenous Prox1 expression and HeLa cells in which there are no detectable endogenous Prox1 expression were analyzed. D to F, siRNA directed against prox1 promotes in vitro growth of HCC cell lines. D and E, MTT assay showing the effect of Prox1-siRNA on the in vitro proliferation of Hep3B (D) and HeLa (E) cells. Hep3B cells transfected with siRNA1 or siRNA2 increased by ~30% compared with the control siRNA–treated cells (*: $P < 0.05$), whereas there was no significant difference between siRNA and control siRNA–treated HeLa cells, in which there is hardly detectable endogenous Prox1 expression. F, MTT assay at 72 hours after siRNA transfection in three cell lines. The suppression of Prox1 significantly promoted the proliferation of cells. All experiments were done in triplicate; bars, SD. Control, negative control siRNA.
human prox1 gene in HCC. Molecular characterization of tumors caused by tumor suppressor genes revealed that the second mutational "hit" usually involved large portions of the particular chromosome, which could be detected by LOH analyses. Genetic events that result in LOH play a major role in the development of tumors, as they convert a heterozygous configuration of a tumor suppressor gene into homozygosity. To investigate whether any loss could be observed in the vicinity of prox1, we carried out LOH analyses in 30 HCC cases using eight polymorphic markers on chromosome 1 (Supplemental data 1). The mean heterozygosity of the microsatellite markers was 75%. The frequency of LOH for each marker in the tumors ranged from 0% to 22.2%, with an average of 8.0 ± 6.8% (mean ± SD). Although 12 tumors exhibited LOH at one or more locus, none showed loss at the two microsatellite markers F (D1S213) and G (D1S2811) spanning the prox1 region (1q32.2-32.3). Therefore, LOH may have no important effect on the suppression of Prox1 in HCC. To then investigate whether mutations exist in the coding region of prox1, cDNAs derived from 30 human HCCs were amplified by PCR and sequenced as a population. We compared the sequences with the published human prox1 sequence but could not find any mutations in all 30 samples. Therefore, the mutation of prox1 gene is not essential for liver carcinogenesis.

Knockdown of prox1 expression by RNA interference promotes cell proliferation in Hep3B and Huh7. Previous data suggested that Prox1 played important roles in control of proliferation and differentiation (13). To more rigorously explore this possibility, we employed two sets of siRNA to knock down endogenous Prox1 expression in two HCC cell lines, Hep3B and Huh7, which had shown the highest expression of prox1. To confirm gene-specific knockdown, we used a negative control siRNA, which had no known homology with mammalian genes. By real-time PCR, we found that the level of prox1 mRNA was reduced to 2.4% to 41.0% in the cells treated with both siRNAs as compared with control siRNA–treated cells (Fig. 4A). The maximal knockdown effect was observed at 48 hours after transfection for both siRNAs. To corroborate the RT-PCR analysis, we carried out a Western blot analysis to detect Prox1 protein at 48, 72, and 96 hours after siRNA transfection. We showed a significant decrease of Prox1 protein as compared with control siRNA–treated cells at all time points taken, and the most marked effect was found at 72 hours after siRNA (Fig. 4B). Taken together, these data showed that Prox1 expression was efficiently knocked down in the cells by RNAi technique.

To assess the effects of Prox1 knockdown in cell proliferation, siRNAs and control siRNA were transfected into Huh7, Hep3B, and HeLa cells. After 24 hours, 5.0 × 10⁴ cells treated with and without siRNAs were seeded and cultured in medium containing serum. Cells were collected at 0, 24, 48, and 72 hours after seeding (24, 48, 72, and 96 hours after transfection, respectively), and MTT assay and Western blot were done. Western blot results showed that both siRNA1 and siRNA2 were effective for 72 hours (Fig. 4C). After 72 hours, the MTT assay value of Hep3B treated with siRNA1 or siRNA2 exhibited a statistically significant increase by ~30% compared with the control siRNA–treated cells (P = 0.019, between siRNA1 and control siRNA; P = 0.025, between siRNA2 and control siRNA; P = 0.019, between siRNA1 and control siRNA; P = 0.025, between siRNA2 and control siRNA; Fig. 4D). Furthermore, the two siRNAs affected the cell proliferation to the same extent. On the contrary, there was no significant difference between siRNA- and control siRNA–treated HeLa cells (Fig. 4E), in which there is hardly detectable endogenous Prox1 expression (Fig. 4C). In addition, a similar result was obtained using Huh7. After monitoring cell growth...
for 72 hours at 24-hour intervals, we showed that knockdown of Prox1 protein significantly promoted cell proliferation in the cells (Fig. 4F).

The effect of prox1 overexpression on the proliferation of HCC cell lines. To explore the biological function of Prox1, we next investigated the effects of overexpression of Prox1 on cell proliferation. pTRE2hyg-prox1 and pTRE2hyg-Luc plasmids were transfected into Hep3B-pTet-off cells and incubated with doxycycline. After 24 hours, the transfected cells were counted and plated onto six-well plates at 1 × 10^5 per well with and without doxycycline. To test whether the Tet-off system was effective, Western blot analysis was carried out on days 3 and 5. The presence of doxycycline inhibited the expression of Prox1 protein at both time points tested (Fig. 5A). Cell growth was monitored by MTT assay at days 1, 3, and 5 after the passage (Fig. 5B). The assay showed that overexpression of Prox1 suppressed cell proliferation by >30%, whereas that of the control gene luciferase had no effect.

Discussion

In this study, we have shown that the level of Prox1 expression is associated with both clinical and biological aspects of HCC. Various levels of proxi mRNAs were expressed in HCC cell lines and tumor samples, indicating that they have a diverse influence on the differentiation and prognosis of HCC. In our clinical study, a lower expression level of proxi considerably corresponded to a poorer differentiation of HCC. Furthermore, we showed that proxi gene expression was associated with a poor prognosis for patients with HCC. The results of these experiments reveal new insights into the specific expression patterns of Proxi within HCC tumors and suggest novel functions for Proxi (i.e., as a tumor suppressor gene or a factor to regulate the progression of malignant character of HCC cells).

In that case, how is proxi gene regulated? A previous study reported that the expression of the proxi gene was silenced in various hematologic cell lines, and bisulfate sequencing analysis revealed that DNA methylation of intron 1 might cause this silencing and other cell lines had DNA mutations in the proxi gene (14). To address the mechanism of down-regulation of proxi gene expression in HCC, hepatoma cell lines (Hep3B and Huh7) and HeLa cells were treated with the demethylating agent 5-azacytidine (0, 0.02, 0.5, and 1 μmol/L). However, this had no effect on restoring proxi gene expression in the three cell lines (data not shown). Subsequently, we carried out mutation screening of proxi mRNA and LOH analyses of proxi in 30 HCC tumors, but we could not find any mutations or LOH in these samples. Previously, it was reported that a high frequency of LOH was not observed at the chromosome arm 1q, which is the location of proxi gene (15). Our results are in good agreement with theirs. However, the mechanism of down-regulation of proxi gene expression remains undisclosed. We surmise that there is an unknown mechanism that regulates the proxi gene.

In attempting to determine the possibility of proxi as a tumor suppressor gene, we employed the RNAi technique for knockdown of its expression and analyzed its phenotype. We found that a transient knockdown of proxi significantly accelerated the growth of HCC cell lines in vitro, and we also showed that overexpression of proxi resulted in suppression of cell proliferation. These results suggest that overexpression of Prox1 can strongly inhibit tumorigenesis, and our findings indicate that Proxi is an attractive candidate for a diagnostic and therapeutic target. We have recently found that Proxi overexpression conferred a slower growth phenotype to some cancer cell lines and enabled them to form much smaller tumors in nude mice.3

It remains to be determined how the reduction of Prox1 expression contributes to the differentiation of HCC and influences the survival of patients with HCC. Because of the proliferation and/or differentiation activity in HCC cells, decreased Prox1 expression may enhance the unregulated growth or recurrence of HCC cells.

In summary, we have presented evidence of Proxi expression in malignant hepatoma cell lines and in HCC specimens. Furthermore, we have shown that decreased Proxi levels are correlated with the progression of differentiation of HCC and predict poor prognosis for patients with HCC after surgery. Our findings indicate that proxi may be a novel candidate gene for the development of diagnosis and therapeutic strategies for HCC.

References


3 Unpublished data.
A Homeobox Protein, Prox1, Is Involved in the Differentiation, Proliferation, and Prognosis in Hepatocellular Carcinoma

Masayuki Shimoda, Meiko Takahashi, Takanobu Yoshimoto, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/20/6005

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/12/20/6005.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.