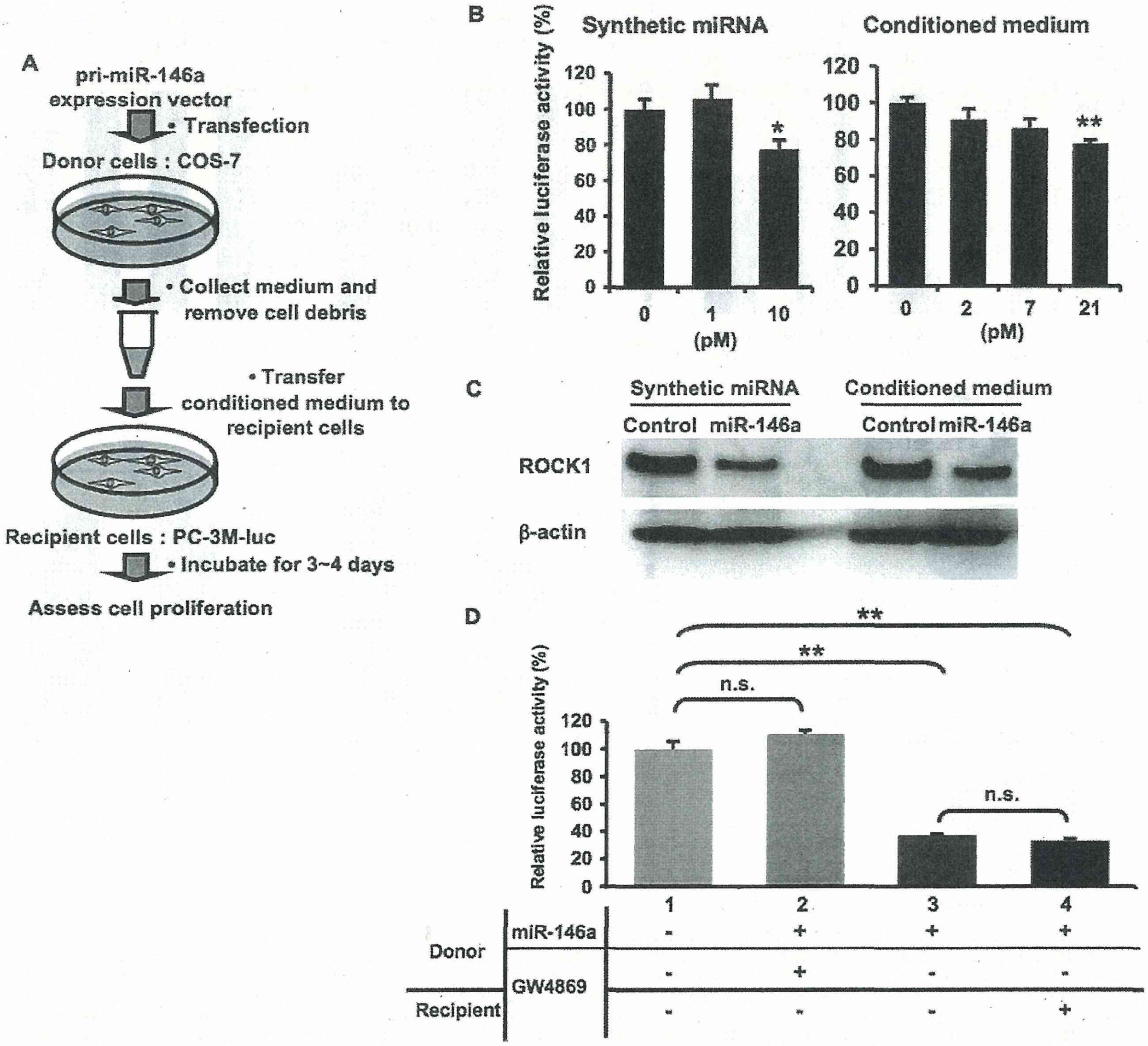

FIGURE 7. **Secretory tumor-suppressive miRNAs attenuated PC-3M cell proliferation.** *A*, schematic representation of a cell proliferation assay. *B*, cell growth inhibition by synthetic miR-146a and extracellular miR-146a. PC-3M-luc cells were transfected with synthetic miRNAs (*left graph*) or incubated in the conditioned medium containing extracellular miRNAs at the indicated concentrations (*right graph*) followed by cell growth assay as described under "Experimental Procedures." *C*, miR-146a-mediated ROCK1 suppression in PC-3M-luc cells. The final concentrations of synthetic and extracellular miR-146a are 10 and 21 pM, respectively. *D*, the treatment with GW4869 to donor cells restored the reduced cell growth by the exosomal miR-146a. Donor COS-7 cells transfected with pri-miR-146a expression vector were incubated in the presence (*lane 2*) or absence of 10 μM GW4869 for 3 days. The conditioned medium from COS-7 cells transfected with empty vector was used as a control (*lane 1*). GW4869-untreated conditioned medium enriched in miR-146a was divided into two aliquots, one of which was treated with 10 μM GW4869 (*lane 3*) and the other of which was not treated (*lane 4*) before the transfer to recipient cells. The following assay was conducted as described above. *B* and *D*, the values on the *y* axis are depicted relative to normalized *Renilla* luciferase activity of control cells, which is defined as 100%. Each *bar* is presented as mean S.E. ($n = 3$). (*, $p < 0.05$, **, $p < 0.005$, as compared with untreated PC-3M-luc cells; Student's *t* test); *n.s.* represents not significant.

Secretory miRNAs could be conducive to the maintenance and surveillance system against cancer progression.

Small RNAs including antisense, siRNA, and miRNA are emerging as promising therapeutic agents against a wide array of diseases (28). Effective delivery of these molecules is crucial to their successful clinical application. Now, exosomes have entered the limelight in delivery of therapeutic nucleic acids with their ability to transfer exogenous miRNAs into tumor cells. Exosomes are naturally produced membrane vesicles that circulate in the bloodstream and are taken up by various types of cells. These aspects might be beneficial for the improvement of durability, stability, and safety for *in vivo* delivery. In fact, tumor exosomes generated tumor regression *in vivo*, acting as a cancer vaccine through their capacity of antigen presentation (29). It is essential to examine whether exosomes can deliver their cargoes in an *in vivo* animal model.

In conclusion, we revealed the secretory machinery of miRNAs and their intercellular transfer. Our results raise the possibility that in the animal kingdom, circulating miRNAs could play a pivotal and general role as a signaling molecule in physiological and pathological events. We anticipate that our study could pave the way for explorations of signal network mediated by secretory miRNAs.

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Clinical Cancer
Research

Combined Functional Genome Survey of Therapeutic Targets for Hepatocellular Carcinoma

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Human Cancer Biology

Clinical Cancer Research

Combined Functional Genome Survey of Therapeutic Targets for Hepatocellular Carcinoma

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Abstract

Purpose: The outcome of patients with advanced hepatocellular carcinoma (HCC) has remained unsatisfactory. Patients with HCC suffer from chronic hepatitis or liver cirrhosis, and their reserve liver function is often limited.

Experimental Design: To develop new therapeutic agents that act specifically on HCC but interfere only minimally with residual liver function, we searched for genes that were upregulated in 20 cases of HCC [namely, discovery sets 1 (n = 10) and 2 (n = 10)] in comparison with corresponding nontumorous liver and a panel representing normal organs using high-density microarrays capable of detecting all exons in the human genome.

Results: Eleven transcripts whose expression was significantly increased in HCC were subjected to siRNA-based secondary screening of genes required for HCC cell proliferation as well as quantitative reverse transcription-PCR analysis [validation sets 1 (n = 20) and 2 (n = 44)] and immunohistochemistry (n = 19). We finally extracted four genes, *AKR1B10, HCAP-G, RRM2*, and *TPX2*, as candidate therapeutic targets for HCC. siRNA-mediated knockdown of these candidate genes inhibited the proliferation of HCC cells and the growth of HCC xenografts transplanted into immunodeficient mice.

Conclusions: The four genes we identified were highly expressed in HCC, and HCC cells are highly dependent on these genes for proliferation. Although many important genes must have been overlooked, the selected genes were biologically relevant. The combination of genome-wide expression and functional screening described here is a rapid and comprehensive approach that could be applied in the identification of therapeutic targets in any type of human malignancy. *Clin Cancer Res; 16(9); 2518–28. ©2010 AACR.*

Liver cancer is the fifth most common human cancer worldwide and the third most common cause of cancer mortality. Hepatocellular carcinoma (HCC) is the most common histologic subtype of liver cancer and is highly endemic in Southeast Asia and sub-Saharan Africa (1). HCC develops mainly in liver affected by chronic hepatitis or cirrhosis caused by persistent infection with hepatitis B or C virus; however, the precise molecular mechanisms that drive the transition from the background liver conditions to cancer are largely unknown. Liver resection, ethanol injection, radiofrequency ablation, and chemoembolization have been used successfully for the local management of HCC; however, no single cytotoxic chemotherapeutic agent has been proven effective for the systemic treatment of HCC; thus, the outcome for patients with locally advanced, multicentric, and/or metastatic HCC who are not eligible for these local treatments has remained unsatisfactory.

An increasing number of therapeutic agents targeting molecular components essential for cancer cell growth have begun to be incorporated into oncological practice: Imatinib, which blocks the Bcr-Abl fusion kinase of chronic myeloid leukemia (CML), is currently the first-line therapy for CML (2). The epidermal growth factor receptor inhibitors gefitinib and erlotinib have been used in the treatment of advanced non–small cell lung cancer (3). Recently, it was shown in a phase III study that sorafenib (BAY 43-9006), a multikinase inhibitor, significantly improved the overall survival of patients with advanced HCC (4, 5), and, consequently, sorafenib has since been approved for the treatment of patients with unresectable HCC by the American Food and Drug Administration. However, most patients enrolled in those studies retained relatively well-compensated

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

Microarray data from this study have been submitted to the Gene Expression Omnibus database (accession no. GSE12941).

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Translational Relevance

Liver cancer is the fifth most common human cancer worldwide and the third most common cause of cancer mortality. Recently, a multikinase inhibitor, sorafenib, has been approved as a systemic chemotherapeutic drug for advanced hepatocellular carcinoma (HCC); however, further improvement seems to be necessary. To identify an "Achilles heel" of HCC cells and develop new therapeutic agents that act specifically on HCC but interfere only minimally with residual liver function, we performed an unbiased survey of the whole genome. We finally identified four genes as candidates. siRNA-mediated knockdown of these candidate genes inhibited the proliferation of HCC cells and the growth of HCC xenografts transplanted into immunodeficient mice, confirming their feasibility as therapy targets.

liver function. In reality, the reserve liver function of HCC patients is often limited due to underlying liver conditions. Therefore, the safety and tolerability of sorafenib remain to be determined in HCC patients with compromised liver function. Therapeutic targeting molecules other than protein kinases have also been developed against various tumors of other organs (6–8). To identify a molecule essential for HCC cell growth and develop new therapeutic agents that would act specifically on HCC and only minimally interfere with residual liver function, a survey of the whole genome would be necessary.

In this study, we adopted a combined functional approach. We first searched for genes that were upregulated in HCC in comparison with the background nontumorous liver tissue. This was followed by siRNA-based screening of genes required for HCC cell proliferation. Recently, whole-genome RNA interference (RNAi)–based functional screening has been reported to successfully identify genes that sensitize lung cancer cells to a chemotherapeutic drug and genes required for proliferation and survival of several cancer cell lines; however, in those studies, the expressional specificity of the identified targets was not taken into consideration (9–12). Here, we report the identification of possible therapeutic target molecules of HCC through a combination of genome-wide expression and functional screening.

Materials and Methods

Patients and microarray analysis. Samples of HCC and surrounding nontumorous liver tissue were collected from 84 patients who underwent liver resection for HCC at the National Cancer Center Hospital (Tokyo, Japan) with informed consent. The clinical and histologic data for these patients are summarized in Supplementary Table S1. Total RNA of normal human organs was obtained from a commercial source (FirstChoice Human Total RNA Survey Panel, Ambion).

One microgram of total RNA was converted to end-labeled cRNA using a Whole Transcript Sense Target Labeling kit (Affymetrix). The fluorescent cRNA probes were hybridized to Human Exon 1.0 ST arrays (Affymetrix), as instructed by the supplier. Data analysis was carried out using the ArrayAssist software package (version 5.5.1, Stratagene). A GC content-based background correction followed by quantile normalization was done with an exonRMA algorithm available in the package. Multiple exonic expression data were also summarized into a single value using the same algorithm, as instructed by the supplier (http://www.stratagene.com/manuals/ArrayAssist.pdf).

The protocol of this study was reviewed and approved by the ethics committee of the National Cancer Center (Tokyo, Japan).

Cell lines. Three human cell lines derived from HCC were used in this study. KIM-1 was kindly provided by Dr. Masamichi Kojiro (Kurume University, Kurume, Japan). Hep3B was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). HLE was obtained from the Health Science Research Resources Bank (Osaka, Japan). KIM-1 and Hep3B were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. HLE was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum.

siRNA-based functional screening. The day before siRNA transfection, cells were seeded at 5×10^3 per well in 96-well plates to obtain 50% to 60% confluency. They were then transfected with siRNA using Lipofectamine 2000 (Invitrogen) at a concentration of 10, 20, or 50 nmol/L in KIM1, Hep3B, or HLE cells, respectively. Three days later, the relative proportion of living cells was assessed using a Premix WST-1 Cell Proliferation Assay System (Takara Bio) in accordance with the manufacturer's instructions. The siRNA was synthesized by Ambion, and the identification (ID) numbers of siRNAs used in this study are listed in Supplementary Table S4. Silencer Negative Control #1 siRNA (Ambion) was used as a nontargeting control. siRNA targeting *TOP2A* was described previously (13).

Real-time PCR. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript reverse transcriptase (Invitrogen). Real-time PCR was done as described previously (14). Primers and probes sets were obtained from Applied Biosystems, and their Assay IDs are provided in Supplementary Table S5. The amplification reaction was done according to the manufacturer's instructions (95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 50°C for 2 minutes, and 60°C for 1 minute).

Immunohistochemistry and immunoblot analysis. Anti-AKR1B10 (clone 1A6) and anti–HCAP-G (clone 4B1) monoclonal antibodies were purchased from Abnova. Anti-RRM2 antibody (E-16) was purchased from Santa

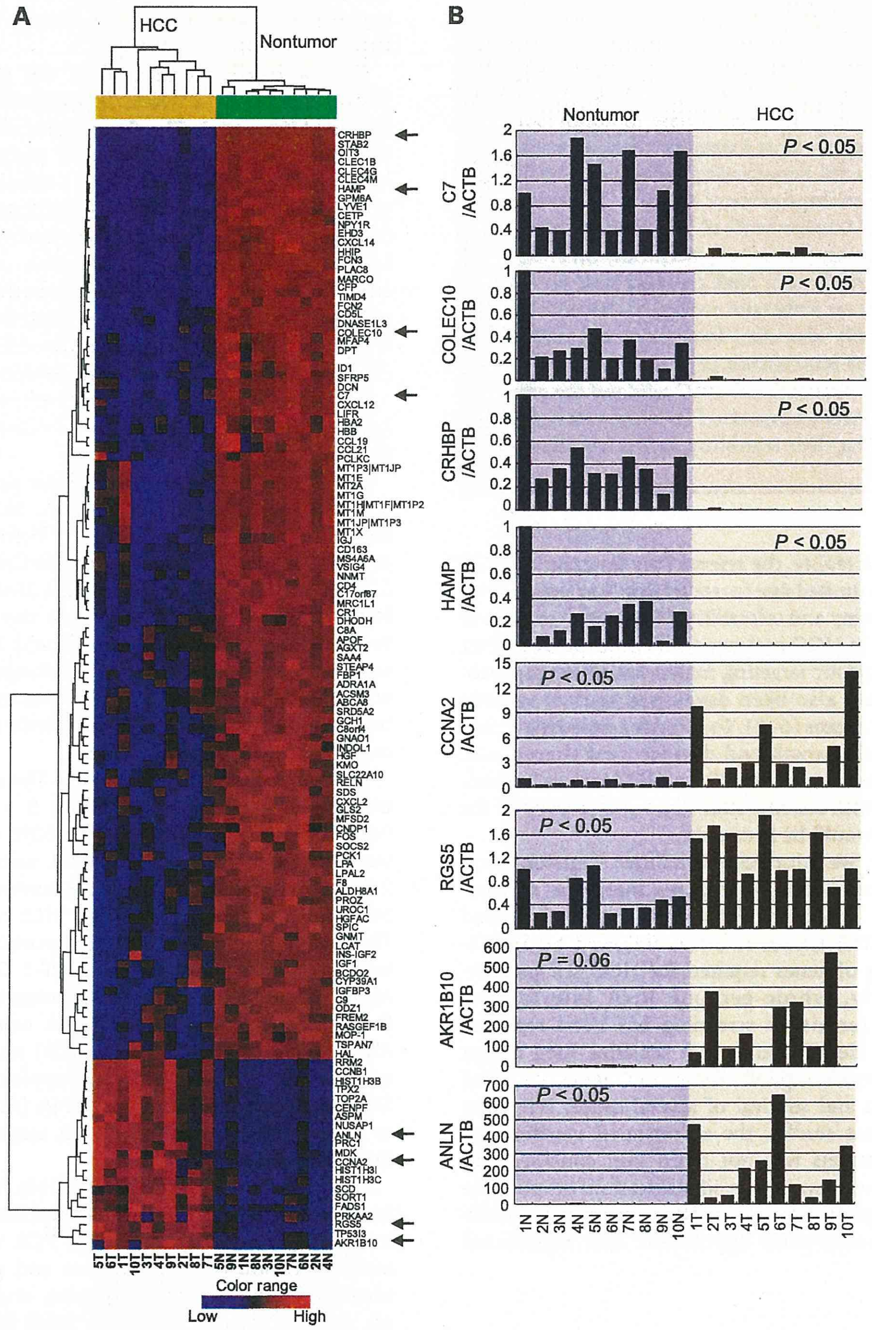

Fig. 1. Genes differentially expressed between HCC and nontumorous liver. A, hierarchical clustering of 124 genes whose expression differed significantly ($P < 0.001$ and >3-fold change) between HCC and adjacent nontumorous liver. Transcriptional signal intensity is shown as a heat map. Red indicates higher signals, whereas blue indicates lower signals. Arrows indicate eight genes selected for validation by real-time PCR (B). B, validation of the microarray data by real-time RT-PCR. The expression levels of eight representative genes whose expression differed significantly between adjacent nontumorous liver (left) and HCC (right) were validated by real-time RT-PCR (shown in arbitrary units). Significant correlation between array (discovery set 1) and real-time RT-PCR data was confirmed by calculating correlation coefficient values in eight randomly selected genes (indicated by arrows in A): *C7*, 0.96; *COLEC10*, 0.97; *CRHBP*, 0.98; *HAMP*, 0.98; *CCNA2*, 0.82; *RGS5*, 0.80; *AKR1B10*, 0.98; *ANLA*, 0.92. The significance of differential expression between HCC and adjacent nontumorous liver tissue was assessed using a permutation paired *t* test, and Bonferroni-corrected *P* values are provided.

Cruz Biotechnology. Anti-TPX2 antibody was purchased from Novus Biologicals.

Formalin-fixed and paraffin-embedded liver tissues containing HCC were obtained from the National Cancer Center Hospital, and stained as described previously (15, 16). Immunoblot analysis of the KIM-1 cell lysate was done as described previously (15).

Animal experiments. Eight million KIM-1 cells suspended in 0.1 mL of PBS were s.c. inoculated into the flanks of 5-week-old female BALB/c nu/nu nude mice (SLC). Eight days later, the tumor-bearing mice were treated with siRNA together with atelocollagen (Koken Co., Ltd.), as described previously (17, 18). The final concentration of siRNA and atelocollagen was 11 µmol/L and 0.5%, respectively, and 200 µL of the siRNA solution were injected directly into each tumor. Tumor volume was determined every 3 days using the formula $V = 1/2\ (A \times B^2)$, where A and B represent the largest and smallest dimensions of the tumor, respectively.

Animal experiments were reviewed by the institutional ethics committee and performed in compliance with the

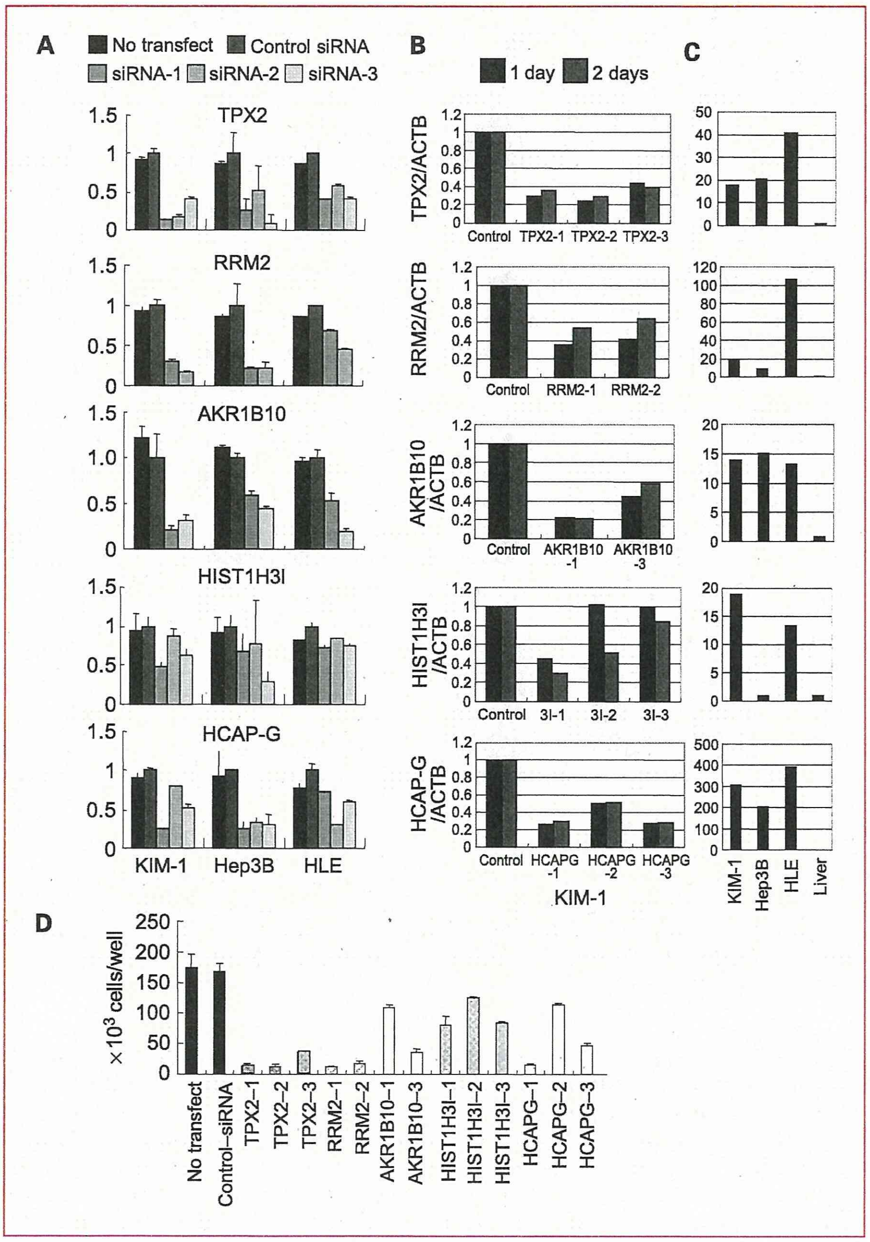

Fig. 2. siRNA-based functional screening. A, siRNA-mediated screening of genes required for proliferation of HCC cells. Three HCC cell lines (KIM-1, Hep3B, and HLE) were transfected with the indicated siRNAs, and the relative proportion of living cells was assessed 3 days later by measuring the mitochondrial succinate-tetrazolium reductase activity. Values for control siRNA were set at 1. B, reduction of the level of mRNA for each gene was determined by real-time PCR 1 and 2 days after transfection of KIM-1 cells with the indicated siRNAs. Values for control siRNA were set at 1. C, expression of each gene in HCC cell lines (KIM-1, Hep3B, and HLE) and normal liver tissue. D, confirmation of siRNA-mediated inhibition of HCC cell proliferation. KIM-1 cells were transfected with the indicated siRNAs, and the number of living cells was counted 3 days later by trypan blue dye exclusion using a hemocytometer.

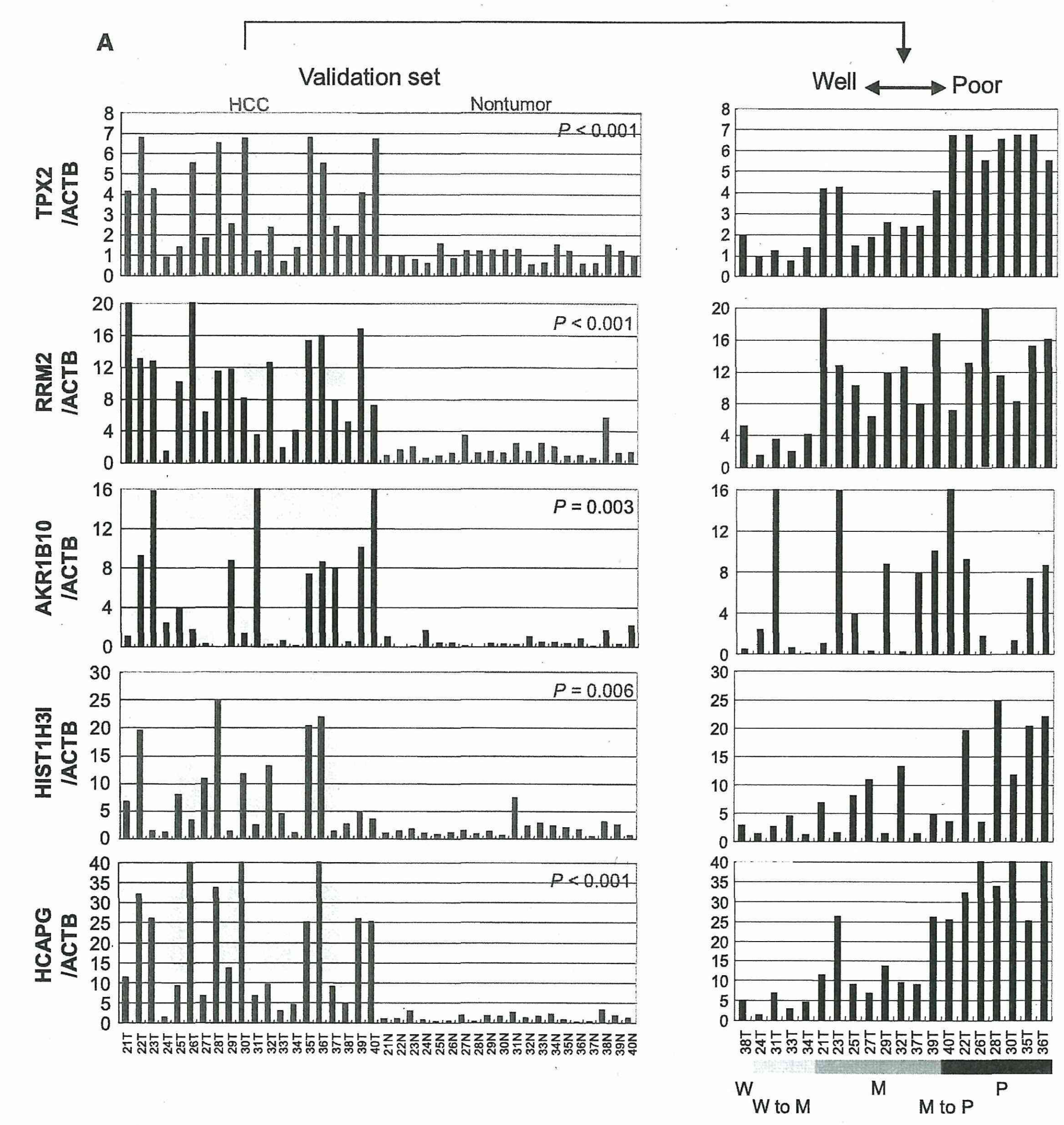

Fig. 3. Validation of differential expression. A, mRNA expression levels of selected genes in 20 independent pairs of HCC (21-40T) and adjacent nontumorous liver tissue (21-40N; validation set 1) determined by real-time PCR (left). The expression levels in HCC were realigned according to histologic differentiation (right). W, well differentiated; W to M, well to moderately differentiated; M, moderately differentiated; M to P, moderately to poorly differentiated, P, poorly differentiated.

guidelines for Laboratory Animal Research of the National Cancer Center Research Institute (Tokyo, Japan).

Statistical analysis. To extract differentially expressed genes from the array data, a paired *t* test with no correction was done (19) with asymptotic distribution to determine the *P* value. Correlations between array data and real-time PCR measurements were assessed using the Pearson correlation coefficient. The significance of differential gene expression between HCC and adjacent nontumorous liver tissue was assessed using the permutation paired *t* test followed by Bonferroni correction.

The weights and volumes of tumors are given as means (+SE). To evaluate the chronological effect of siRNAs on the growth of xenografts in comparison with control siRNA,

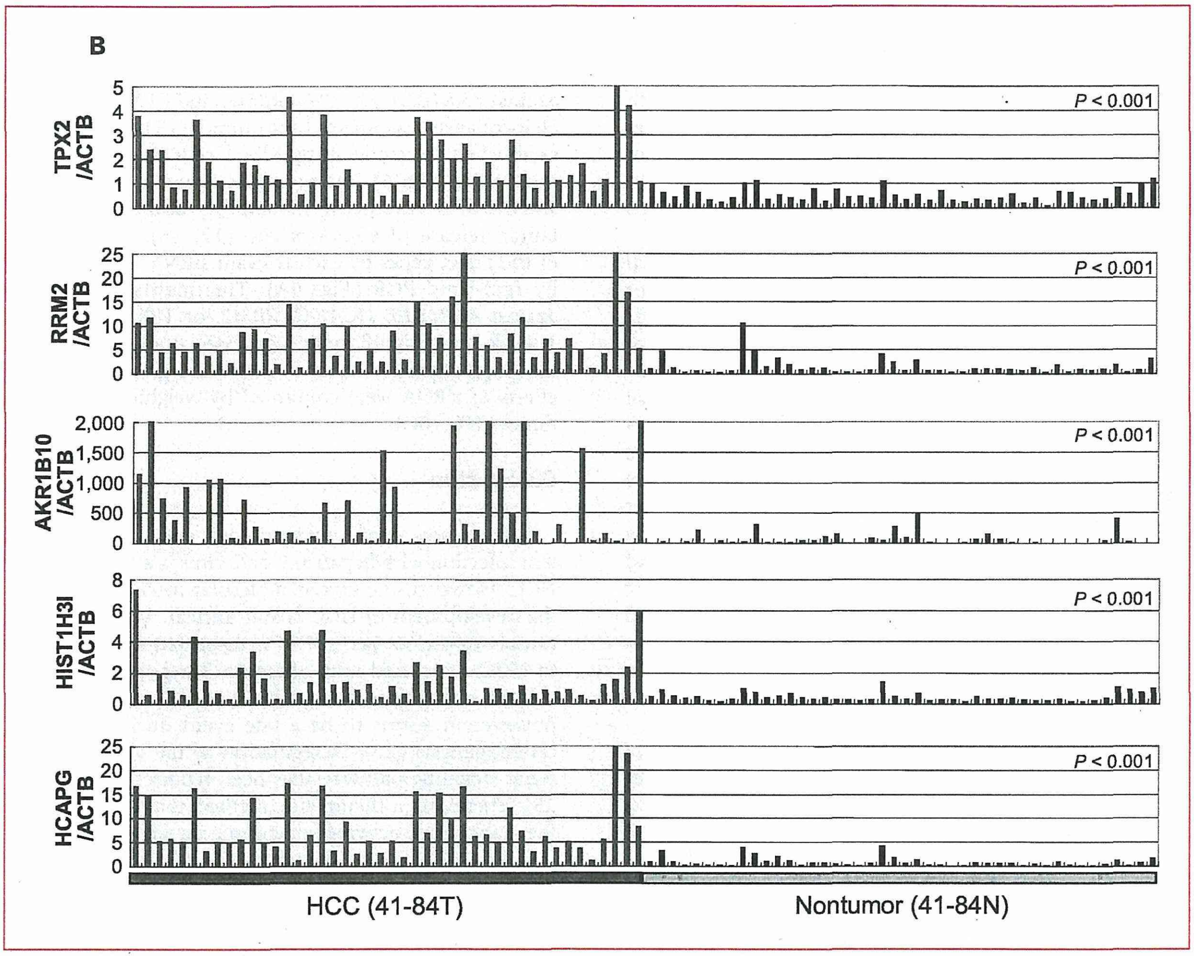

Fig. 3. *Continued.* B, expression levels of mRNAs for selected genes in 44 independent pairs of HCC (41-84T) and adjacent nontumorous liver tissue (41-84N; validation set 2) determined by real-time PCR.

a generalized linear mixed-effects model was used (20). The volume of the xenograft was modeled using γ-error distribution and log link function. This model considers each siRNA treatment as a fixed effect with control siRNA as an intercept and the number of days after implantation as a random effect. Estimates of variance components were obtained using the Laplacian approximation method, and the model fit was assessed using deviances. The significance of effects was estimated from the degree of freedom and t statistics followed by Bonferroni correction. Analysis was done using the lmer function for fitting generalized linear mixed-effects models, in the R statistical software package (version 2.6.0).

Results

Exon-based array analysis of HCC. Twenty paired samples of HCC and adjacent nontumorous liver tissue were subjected to genome-wide expression analysis using two different batches of the GeneChip Human Exon 1.0 ST arrays [discovery sets 1 (10 pairs) and 2 (10 pairs)]. Statistical analysis was done separately, and genes expressed differentially in the two sets were selected to eliminate any experimental bias caused by batch-to-batch variations. The exon array can detect mRNAs with low abundance as well as alternatively polyadenylated and spliced mRNA because the probes are designed to hybridize with the entire sequences of the transcripts (21). We identified 124 annotated genes that were differentially expressed between the background (nontumorous) liver tissue and HCC [at least a 3-fold change in transcription signal; $P < 0.001$ (paired t test with no correction)] in discovery set 1 (Supplementary Tables S2 and S3). The genes were clustered according to the similarity of their expression profiles (Fig. 1A), and the differential expression of representative genes was confirmed by real-time PCR (Fig. 1B). It was noteworthy that although 103 genes were found to be significantly downregulated, only 21 were apparently upregulated.

We selected 9 genes (*AKR1B10, ANLN, CCNB1, HIST1H3B, HIST1H3C, HIST1H3I, RRM2, TOP2A*, and *TPX2*) whose expression was upregulated in HCC (≥3-fold change in transcription signal; $P < 0.001$, *t* test) in both discovery sets 1 and 2. Furthermore, two additional genes (*HCAP-G* and *DEPDC1*) were selected using a different criterion (>2.5-fold change across all of the 20 cases in discovery sets 1 and 2, and a raw signal of <50 in all 20 of the nontumorous liver tissues; $P < 0.05$, *t* test).

RNAi-based screening of genes required for HCC cell proliferation. To identify genes that are essential for HCC cell proliferation, siRNA-based screening was done for the 11 genes that were upregulated in HCC. Two or three constructs of siRNA were designed for each gene. Relative cell viability was evaluated by the mitochondrial succinate-tetrazolium reductase activity-based assay 3 days after transfection (Fig. 2A). We selected five genes (*TPX2, RRM2, HCAP-G, HIST1H3I*, and *AKR1B10*) based on the criterion that at least two siRNAs per gene reproducibly suppressed cell proliferation by >20% in all of three cell lines (KIM-1, Hep3B, and HLE). Representative data are shown in Fig. 2A and B. The baseline expression of these genes was determined in the three cell lines by real-time reverse transcription-PCR (RT-PCR; Fig. 2C). We confirmed the cell proliferation–inhibitory activity of the siRNA by counting the numbers of cells (Fig. 2D).

Validation of differential gene expression in additional cases of HCC. The increased expression of the five genes selected using the siRNA-based screen was validated in 20 cases of HCC (validation set 1) by real-time PCR (Fig. 3A). The expression of all five genes was confirmed to be increased in HCC. The expression of *TPX2, RRM2, HCAP-G*, and *HIST1H3* was associated with loss of histologic differentiation (Fig. 3A, right). The expression of *AKR1B10* was upregulated in HCC regardless of differentiation. We further confirmed the differential expression of these genes between HCC and nontumorous liver tissues in 44 additional independent cases of HCC (validation set 2) by real-time PCR (Fig. 3B).

In the 18 normal organs examined, no significant expression of *TPX2, RRM2*, or *HCAP-G* was observed, except for the thymus (Fig. 4, left), which is largely involuted in nonjuvenile adults. No organs showed higher expression of *AKR1B10* than was the case in HCC. We did not select *HIST1H3I*, as this gene showed high expression in several vital organs (Fig. 4).

Protein expression analysis. Expression of the products of four candidate genes, *TPX2, HCAP-G, RRM2*, and *AKR1B10*, was examined immunohistochemically in 19 independent cases of HCC (Fig. 5). In 84% (16 of 19) of the cases, AKR1B10 protein was detected in the cancer but was hardly evident in the adjacent nontumorous liver tissue. The nuclear staining of HCAP-G and TPX2 was stronger in HCC than in the adjacent nontumorous liver in 42% (8 of 19) and 58% (11 of 19) of cases, respectively. Patchy staining of RRM2 was observed in 84% (16 of 19) of the HCCs.

***Inhibition of tumor growth* in vivo.** Finally, we performed an *in vivo* experiment to evaluate the feasibility of the four selected genes as therapeutic targets. siRNA against *AKR1B10, HCAP-G, RRM2*, and *TPX2* mixed with atelocollagen was injected into tumors (31.5 ± 1.9 mm^3) established by xenografting KIM-1 cells into the flank of nude mice (Fig. 6). Atelocollagen forms a complex with siRNA, thus enhancing its stability and allowing sustained release of siRNA *in vivo* (17, 18). The silencing of the target genes by each relevant siRNA was confirmed by real-time PCR (Fig. 6A). Treatments with siRNA against *AKR1B10, HCAP-G, RRM2*, or *TPX2* given twice, 1 week apart, significantly suppressed tumor growth (Fig. 6B; Supplementary Table S6), and the growth-inhibitory effects of siRNA were confirmed by weighing the excised tumors (Fig. 6C).

Discussion

There is now strong epidemiologic evidence that persistent infection with hepatitis B or C virus is a major cause of HCC. However, the precise molecular mechanism behind the development of HCC is still unclear. Mutation in the tumor suppressor gene *TP53* is most frequently observed in HCC associated with aflatoxin B exposure as well as chronic infection with hepatitis B and C viruses (22–24); however, it seems to be a late event during multistep carcinogenesis (22). Deregulation of the Wnt as well as other signaling pathways has been reported in HCC (22, 25). Therefore, a therapeutic method that can normalize these aberrantly activated oncogenic signals would be clinically valuable. In an attempt to discover therapeutic targets with high specificity for HCC, we searched for genes that are specifically upregulated in HCC in comparison with nontumorous liver tissue and normal vital organs using high-density microarrays designed to detect all the exons in the human genome (Figs. 1 and 4). This was followed by siRNA-based screening of genes required for HCC cell proliferation (Fig. 2) as well as quantitative RT-PCR analysis and immunohistochemistry of additional cases (Figs. 3 and 5). We finally identified four candidate genes and confirmed their functional involvement in the tumor growth of HCC xenografts (Fig. 6). These genes, *AKR1B10, HCAP-G, RRM2*, and *TPX2*, were expressed strongly and specifically in HCC, which is highly dependent on these genes for proliferation, and their feasibility as therapy targets also seems to be supported by the literature.

RRM2 is a subunit of ribonucleotide reductase that catalyzes the conversion of ribonucleoside 5′-diphosphates into their corresponding 2′-deoxyribonucleotides. Because this reaction is the rate-limiting step of DNA synthesis, and inhibition of ribonucleotide reductase stops DNA synthesis and cell proliferation, RRM2 has been considered a promising target for cancer therapy (26).

TPX2 (C20ORF1) is a microtubule-associated protein whose expression is restricted to the S, G_2, and M phases of the cell cycle. Suppression of TPX2 expression by RNAi causes defects in microtubule organization during mitosis,