Sprouty 2, an Inhibitor of Mitogen-Activated Protein Kinase Signaling, Is Down-Regulated in Hepatocellular Carcinoma

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Abstract

The Sprouty proteins are increasingly being recognized to be deregulated in various types of cancers. This deregulation is often associated with aberrant signaling of receptor tyrosine kinases and its downstream effectors, leading to the mitogen-activated protein kinase (MAPK) signaling pathway. In human hepatocellular carcinoma, where the MAPK activity is enhanced via multiple hepatocarcinogenic factors, we observed a consistent reduced expression of the sprouty 2 (Spry2) transcript and protein in malignant hepatocytes compared with normal or cirrhotic hepatocytes. The expression pattern of Spry2 in hepatocellular carcinoma resembles that of several potential tumor markers of hepatocellular carcinoma and also that of several angiogenic factors and growth factor receptors. In contrast to previous studies of Spry2 down-regulation in other cancers, we have ruled out loss of heterozygosity or the methylation of promoter sites, two common mechanisms responsible for the silencing of genes with tumor suppressor properties. Functionally, we show that Spry2 inhibits both extracellular signal-regulated kinase signaling as well as proliferation in hepatocellular carcinoma cell lines, whereas knocking down Spry2 levels in NIH3T3 cells causes mild transformation. Our study clearly indicates a role for Spry2 in hepatocellular carcinoma, and an understanding of the regulatory controls of its expression could provide new means of regulating the angiogenic switch in this hypervascular tumor, thereby potentially controlling tumor growth.

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Introduction

Hepatocellular carcinoma, the primary adult liver malignancy, is the fifth most common cancer and the third leading cause of cancer deaths worldwide (1). The major risk factors (accounting for 70-85% of all cases) of hepatocellular carcinoma are chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV), although other nonviral causes, such as environmental carcinogens (e.g., aflatoxin B1, and alcohol abuse) and inherited disorders (e.g., hemochromatosis, Wilson disease, α1-antitrypsin deficiency, and tyrosinemia), also play a role (2, 3). Once predominant in Asian and African countries due to endemic HBV infections, the incidence of hepatocellular carcinoma is steadily on the rise in Western countries due to HCV infections and nonviral causes (1, 4).

Although the etiology of hepatocellular carcinoma is more well defined than other types of cancers, the underlying molecular mechanisms leading to hepatocarcinogenesis remain incompletely understood despite current research efforts into the molecular genetics and pathogenesis of hepatocellular carcinoma.

Extensive studies have shown that the development of hepatocellular carcinoma from preneoplastic lesions are associated with complex and heterogeneous genetic or chromosomal abnormalities, such as loss of heterozygosity (LOH), microsatellite instability, gene alterations, and aberrant global gene expression profiles (2). Additionally, there is convincing evidence that HBV and HCV, the prominent etiologic factors of hepatocellular carcinoma, are involved directly in the carcinogenic process (5). There are fundamental differences between HBV and HCV: HBV is a Hepadnavirus with a double-stranded DNA genome, whereas HCV is a Flaviviridae virus with a single-stranded RNA genome; HBV DNA is integrated into the host cell genome, whereas the HCV genome is not. Despite these major differences, similarities exist in the pathways of HBV and HCV induced hepatocarcinogenesis (3). Most notably, the product of the HBV X gene (HBx protein) and the core protein of HCV have been shown to have oncogenic potential (5) and to activate the Ras/extracellular signal-regulated kinase (ERK) signaling cascade (6-10). Indeed, ERK expression and activity both have been found to be activated in hepatocellular carcinoma, suggesting an important role of this signaling pathway in hepatocarcinogenesis (11-14). ERK is a key molecule that transduces signals from convergent pathways into the nuclei, resulting in multiple cellular responses, such as proliferation and differentiation (11). Additionally, it is a protein serine/threonine kinase that can be activated by a number of growth factors and receptors, among which and pertinent to the liver is the hepatocyte growth factor (HGF) and its receptor the e-met proto-oncogene (15, 16).

Given the significance of the mitogen-activated protein kinase pathway in hepatocellular carcinoma, which can be activated by important etiologic factors (HBV and HCV) and mitogenic growth factors, modulation of this pathway in hepatocellular carcinoma could have profound effects on the development or progression of hepatocellular carcinoma. From an earlier gene expression study of hepatocellular carcinoma, we observed that Sprouty 2 (Spry2), an inhibitor of the Ras/ERK pathway (17), was among the top 600 genes found to be significantly differentially expressed between hepatocellular carcinoma and nontumor liver (18). Spry2 is one of four members of the mammalian sprouty family of signal transduction proteins. There is emerging evidence that these proteins may be important modulators of vital pathways central to the development or progression of cancer, such as angiogenesis (19, 20), cell growth, invasion, migration, and cytokinesis (21).
addition to the current observation of Spry2 down-regulation in hepatocellular carcinoma, we had also previously reported that Spry2 was down-regulated in breast cancer (22). Other Spry members also seem to have important roles in cancer: Spry1 was reported to be down-regulated in breast cancer (22) and prostate cancer (23), whereas Spry4 was identified as a potential response marker of Gleevec in gastrointestinal stromal tumors (24).

Because of the implied significance of Spry2 in human hepatocellular carcinoma, we were compelled to study the functional roles, if any, of Spry2 in hepatocellular carcinoma. In this article, we confirm that Spry2 is indeed down-regulated, at both the mRNA and protein levels, in hepatocellular carcinoma compared with nontumor liver. The transcript expression profile of several growth factors and receptors across hepatocellular carcinoma samples mimic that of Spry2, offering insights into the pathways potentially modulated by Spry2. We further investigated the functional contributions of Spry2 to see whether it has tumor suppressive properties in hepatocellular carcinoma.

Materials and Methods

Gene expression data analysis. Gene expression data based on an earlier study (18) were retrieved from the Stanford Microarray Database according to the following selection criteria: all nonflagged spots with >1.5-fold intensities over local background in either channel, 75% good data, and genes whose log 2 of red/green normalized ratio (mean) was >3-fold for at least four arrays. We retrieved data for 4,841 cDNA clones in 75 liver tumor and 72 nontumor liver tissues and uploaded these data onto the web-based microarray data analysis program Genetic Analysis By Rules Incorporating Expert Logic (GABRIEL; http://gabriel.stanford.edu) for further analysis.

GABRIEL is a rule-based computer program designed to apply domain-specific and procedural knowledge systematically for the analysis and interpretation of data from DNA microarrays (25). In the Chen et al. study (18), both the tumor samples (T) and nontumor samples (N) were compared with a pooled universal cell line reference (U) such that expression ratios were represented as log 2 (T/U) and log 2 (N/U), respectively. To derive more biologically meaningful data, we rescaled the data set such that the relative T versus N expression ratios [log 2 (T/N)] is calculated by log 2 (T/U) − log 2 (N/U). If an hepatocellular carcinoma sample did not have a corresponding nontumor sample, the global mean of the nontumor gene expression ratios were used. We used the t score pattern based rule to identify genes that are significantly differentially expressed between tumor and nontumor liver tissues: the t score (average/SD) allows variability among samples to be taken into consideration during the calculation. To search for genes that have expression profiles similar to that of Spry2, we used the Proband-based analysis function using Spry2 as the proband.

Quantitative real-time PCR. To validate expression data from microarray, we did quantitative real-time PCR on 11 patients (included in the microarray study) with matched nontumor and tumor liver tissues. Total RNA was extracted from surgically resected, snap-frozen samples as previously described (18), with the appropriate approval from the Institutional Review Board at Stanford University and in compliance with the regulations for using human subjects for medical research. Before surgery, all patients gave informed consent to have their resected tissues stored for research purposes only. Total RNA was further purified with an RNAqueous kit (Ambion, Austin, TX), including DNase I digestion to remove any genomic DNA contamination. Human ribosomal 18S (h18S) RNA was used as the normalization standard in the quantitative analysis. PCR was done using the ABI Prism 7900HT sequence detection system (Applied Bio-systems, Foster City, CA) via a two-step nonmultiplexed Taqman 5′−3′ exonuclease assay using Taqman reverse transcription reagents kit and Taqman PCR core reagents kit (Applied Biosystems) according to the relative standard method. Calibration curves were generated for each transcript and validated using linear regression analysis ($r^2 \geq 0.99$).

Transcript quantification was done in triplicates for each sample and reported relative to h18S. The primers and probes used for Spry1 and Spry2 were purchased from the Applied Biosystem Gene Expression Assay-on-Demand inventory.

Nonradioactive in situ hybridization of paraffin sections. We used two tissue microarrays for this study, both consisting of archived tissues retrieved from surgical pathology files. In total, there were 17 normal livers, 14 cirrhotic livers, and 67 malignant hepatocellular carcinomas, among other control tissues. Tissue arrays were constructed as previously described (26, 27), with each core being 1.6 mm on one array and 2 mm on the other array.

The hybridization probes for Spry2 were prepared as previously described (22) and used in nonradioactive in situ hybridization as detailed in Chen et al. (28). Arrays were scored using a four-tier scale: 0, negative; 1, insufficient or equivocal; 2, weak staining; 3, strong staining. Tissues with scores of 1 were not included in statistical analysis.

Immunohistochemistry. The same tissue microarrays used for in situ hybridization were used for immunohistochemistry, which was done as previously described using microwave heat induced epitope retrieval in citrate buffer (29). To avoid interference from endogenous biotin, a biotinfree method, EnVision, was used for amplification of the signal (DAKO, Carpinteria, CA). The Spry2 antibody was produced under contract by Biogenes (Berlin, Germany) and used at 7.7 ng/μL for immunohistochemistry. The same scoring system as for in situ hybridization was used, and tissues scoring 1 were again not further analyzed.

Tissue culture, stable expression of Spry2 and small interfering RNA knockdown. SNU449 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI supplemented with 10% fetal bovine serum (Hyyclone, Logan, UT), 2 mmol/L l-glutamine (Sigma, St. Louis, MO), 100 units/mL penicillin (Sigma), and 100 μg/mL streptomycin (Sigma). NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum (Life Technologies, Gaithersburg, MD) and other supplements as above. The Spry2 and Spry2(555F) expression constructs used for stable expression were prepared as previously described (22). Cells stably expressing Spry2 and Spry2(555F) were selected by using up to 1 mg/mL of geneticin (Life Technologies) in culture media. For small interfering RNA (siRNA) knockdown assay, Spry2 siRNA duplexes (D1-D4) and a negative control siRNA (C) were obtained from Dharmacon (Lafayette, CO) and transfected into cells using siPORT Amine (Ambion) according to the manufacturer’s instructions.

LOH. Genomic DNA was extracted from surgically resected, snap-frozen samples obtained with patient consent and with the appropriate approval from the Institutional Review Board at Stanford University. Paired tumor and nontumor liver tissues of 12 hepatocellular carcinoma patients were used in this study.

For LOH assay, we used the microsatellite markers D13S1263, D13S170, D13S1277, and D13S266. Genomic DNA from the patient samples was extracted upon overnight digestion with Proteinase K (Roche, Indianapolis, IN) and purified using Qiagen Genomic tip 20G (Hilden, Germany) according to the manufacturer’s instructions. A “reduced nucleotide” PCR reaction was set up with the following components: 25 μmol/L of all nucleotides except dCTP (4.8 μmol/L), 0.3 mmol/L of each primer, 1 unit Taq Polymerase (Roche), 40 μCi [γ-32P]dCTP (Perkin-Elmer Life and Analytical Sciences, Boston, MA), and 0.2 μg genomic DNA in a total volume of 20 μL. The PCR protocol used was as follows: denaturation at 95°C for 30 seconds, annealing at 57°C for 45 seconds, and amplification at 72°C for 1 minute, repeating the cycle for 30 times. The radiolabeled PCR products were separated on a 5% TBE-Urea Ready Gel (Bio-Rad, Hercules, CA), air-dried, and autoradiographed.

Methylation-specific PCR. The methylation-specific PCR (MSP) assay was carried out according to McKie et al. (30). The sequencing primers for CpG1A (5′ putative promoter amplicon) used in the current study are Fseq primer, 5′-GAAATTAGATAAAATTGTGTTAG-3′ and Rseq primer, 5′-CTACATGCTACACGTACCATC-3′.

Proliferation assay. The proliferation assay was done as described previously (22). SNU449 cells stably expressing Spry2 or Spry2(555F) were seeded into 96-well plates at a density of 3 × 103 per well. The following day,
the culture medium was replaced with serum-free medium and incubated for 48 hours before obtaining a day zero absorbance reading (at 485 nm) using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer’s instructions. HGF (50 ng/mL) was added to the wells and left to incubate for another 2 days, after which the cell proliferation assay was repeated.

Adenovirus system. The AdEasy Adenoviral Vector System was purchased from Stratagene (La Jolla, USA). Spry2 was cloned into the NotI and XhoI restriction sites of pShuttle-IRES-hrGFP1 and subsequently recombined with the adenovirus vector pAdEasy-1 to form viable adenoviruses. Production of Spry2 adenoviruses was done in AD293 cells (Stratagene) following Bio-safety level 2 guidelines.

Results

Spry2 transcript is down-regulated in hepatocellular carcinoma. As our laboratory had previously discovered that both Spry1 and Spry2 are down-regulated in breast cancer (22), we therefore extended our study to other cancers including liver cancer. In an earlier gene expression study of hepatocellular carcinoma, Spry2 was one of the top 600 genes observed to be differentially expressed in hepatocellular carcinoma compared with nontumor liver tissue ($P < 0.01$ by Student’s $t$ test with Bonferroni correction; ref. 18). We used a more stringent and biologically relevant analytic approach to verify this observation: first, we rescaled the data set such that the

Figure 1. A, relative expression of Spry1 and Spry2 in hepatocellular carcinoma tumor compared with nontumor liver. Of the total of 75 patients, 57 are matched hepatocellular carcinoma and nontumor liver samples. In the study by Chen et al. (18), both the tumor samples (T) and normal tissue samples (N) were compared with a pooled universal cell line reference (U) such that expression ratios were represented as log $2 (T/U)$ and log $2 (N/U)$, respectively. The relative $T$ versus $N$ expression ratios [log $2 (T/N)$] depicted here is calculated by log $2 (T/U) / C0$ log $2 (N/U)$. If a hepatocellular carcinoma sample did not have a corresponding nontumor sample, the global mean of the nontumor gene expression ratios was used. The relative abundance of transcripts of each gene is represented by a color scale (bottom right). Real-time PCR measurement of (B) Spry2 and (C) Spry1 in 11 paired tumor/nontumor samples confirmed our observations that only Spry2 is significantly down-regulated in hepatocellular carcinoma.
relative tumor (T) versus nontumor (N) expression ratios \( [\log 2 (T/N)] \) is calculated by \( \log 2 (T/U) - \log 2 (N/U) \), where \( U \) is the pooled cell line reference used in the microarray study. If an hepatocellular carcinoma sample did not have a corresponding nontumor sample, the global mean of the nontumor gene expression ratios was used. We then used a \( t \) score pattern based algorithm to find genes satisfying the pattern of being down-regulated in tumor compared with nontumor, using a \( t \) score of less than -2. The \( t \) score is a measure of variability (average/SD) and thus provides an indication of the consistency of the expression values across all samples. We found 1,557 genes satisfying this defined pattern of \( t \) score less than -2, with false-positive rate of 0.008 and false-negative rate of 0.002. Spry2 (\( t \) score = -8.023) was among the top 7% of the genes with an absolute \( t \) score of >8, suggesting that it is consistently (69 of 75, 92% of matched samples) and significantly differentially down-regulated in tumor versus nontumor liver (Fig. 1A).

We verified the observed gene expression data using semiquantitative real-time PCR to measure the transcript level of Spry2 in 11 matched tumor and nontumor liver tissues and consistently observed that Spry2 is down-regulated in hepatocellular carcinoma compared with nontumor liver (8 of 11, 73%; Fig. 1B). Unlike in breast cancer, Spry1 was not significantly down-regulated in hepatocellular carcinoma (Fig. 1A and C).

**Expression profile of Spry2 in hepatocellular carcinoma correlates with those of genes with diagnostic and functional implications.** To assess the potential diagnostic value and to gain functional insights of Spry2 down-regulation in hepatocellular carcinoma, we used the Proband-based analysis to compare the expression profile of Spry2 with those of all differentially expressed genes in hepatocellular carcinoma to identify genes that have similar expression patterns as Spry2. We found a total of 303 known genes correlating with Spry2 at correlation coefficients above 0.6 (Supplementary Table S1); genes of particular interests are displayed in Fig. 2A.

IGFBP3, a potential hepatocellular carcinoma marker that has been shown to be consistently down-regulated in hepatocellular carcinoma (31), correlated strongly with Spry2 (correlation coefficient of 0.8), suggesting that Spry2 may have diagnostic potential as well. IGFBP3 additionally controls cell proliferation in hepatocellular carcinoma (32). Three genes (EPHA2, THBS1, and ADAMTS1) involved in the angiogenic process showed strong correlation with Spry2 expression. EPHA2 is a proangiogenic gene that is expressed during tumor angiogenesis (33); THBS1 has been shown to be associated with tumor invasiveness and progression in hepatocellular carcinoma, and a proangiogenic function has been implied based on its positive correlation with vascular endothelial growth factor (VEGF) expression in hepatocellular carcinoma (34). Conversely, ADAMTS1 is a matrix metalloprotease with angiinhibitory properties (35) that has been shown to be consistently down-regulated in breast cancer (36). These observations suggest potentially important functional roles of Spry2 in hepatocellular carcinoma. Other genes showing good correlation with Spry2 expression in hepatocellular carcinoma include several growth factor receptors (PDGFRα, NGER, and FGFR1); downstream signaling effectors, such as ARHGEF (the ras homologue gene family, member E); a protein tyrosine kinase proto-oncogene FYN; the oncogene FOSB; and the zinc finger transcription factor early growth response gene 1 (EGR1).

We also looked at genes that correlated negatively with Spry2 (i.e., genes that are consistently overexpressed in hepatocellular carcinoma compared with nontumor liver). This may elucidate genes that are negatively regulating Spry2 expression or genes that are negatively controlled by Spry2. Using a correlation coefficient cutoff of -0.5, 621 known genes were found to be negatively correlated with Spry2 (Supplementary Table S2), of which those of interest are displayed in Fig. 2B. Notably, several genes that have recently been reported to be good potential markers of hepatocellular carcinoma show opposite expression profiles than Spry2: CD34, RGS5, and PDXL2 are endothelial cell markers of hepatocellular carcinoma (28); GPC3 is a serum and histologic marker of hepatocellular carcinoma (37, 38). SHC1 (the signaling adaptor that couples activated growth factor receptors to signaling pathways) and MET (the hepatocyte growth factor receptor) both show weak negative correlations to Spry2.

**Spry2 is differentially expressed in normal, cirrhotic, and hepatocellular carcinoma liver.** We used in situ hybridization on...
We next studied whether methylation Spry2 down-regulation.

et al. (40), in which no homozygous deletion was found for Spry2 results. Our result correlates with a recent study by Pineau prostate cancer); other microsatellite markers gave similar down-regulation of in hepatocellular carcinoma (39), we investigated whether the is located on 13q, a chromosomal region that is frequently deleted not due to loss of heterozygosity.

The LOH result for microsatellite marker D13S266 is shown in 47% of prostate cancer samples exhibit LOH at these loci (30). Microsatellite markers D13S1277 and D13S266 were chosen because up to tocellular carcinoma tumor/nontumor tissues. The microsatellite A, right deoxycytidine treatment was effective (Fig. 5B). Treatment with the deacetylase inhibitor trichostatin gave similar findings upon 5-aza-deoxycytidine treatment (Fig. 5A, left). RASSF1A is one of the most frequently inactivated genes described thus far in human solid tumors (43). Inactivation of RASSF1A most commonly involves methylation of the promoter and CpG islands and can be up-regulated by 5-aza-deoxycytidine treatment. We used it as a positive control to show that the 5-aza-deoxycytidine treatment was effective (Fig. 5A, right). Treatment with the deacetylase inhibitor trichostatin gave similar findings for both Spry2 and RASSF1A (data not shown).

We further investigated the extent of methylation in the hepatocellular carcinoma paired samples by bisulfite treatment of the extracted genomic DNA followed by MSP and direct sequencing of Spry2 CpG islands. MSP of the 5′ putative promoter (CpG1A) indicates a heterogeneous pool of methylated and nonmethylated sequences in a small number of samples (Fig. 5B). The MSP of the exon 1 CpG1B region, however, suggests clear methyl-specific bands for hepatocellular carcinoma samples T4, T5, and T9 (Fig. 5C). There is also a basal level of methylation observed in sample N7. The methylated sequences were cloned into TA-cloning vector for subsequent sequencing analysis. Figure 5D is a schematic representation of the methylation status for both the putative promoter and exon1 CpG regions. Hepatocellular carcinoma patient sample T9 is the only one showing a substantial level of methylation within the two CpG sites. In essence, methylation of CpG sites

Of the Spry2 promoter region (or of genes upstream of Spry2) is a possible mechanism for the silencing of Spry2 in hepatocellular carcinoma. Hypermethylation of promoter regions is a common mechanism of gene silencing. This process works in concert with histone deacetylation to repress gene transcription (41, 42). We studied the expression of Spry2 in two hepatocellular carcinoma cell lines that expressed Spry2 at low levels (SNU449 and HepG2) and one with moderate levels of expression (Hep3B; about 4-fold higher) following treatment with the DNA methyl-transferase inhibitor 5-aza-deoxycytidine according to the protocol by McKie et al. (30). Real-time PCR analysis of Spry2 mRNA instead show a slight reduction in the levels of Spry2 in all the hepatocellular carcinoma cell lines upon 5-aza-deoxycytidine treatment (Fig. 5A, left). RASSF1A is one of the most frequently inactivated genes described thus far in human solid tumors (43). Inactivation of RASSF1A most commonly involves methylation of the promoter and CpG islands and can be up-regulated by 5-aza-deoxycytidine treatment. We used it as a positive control to show that the 5-aza-deoxycytidine treatment was effective (Fig. 5A, right). Treatment with the deacetylase inhibitor trichostatin gave similar findings for both Spry2 and RASSF1A (data not shown).

To assess the correlation of methylation with Spry2 expression, we performed MSP analysis of the Spry2 promoter CpG island in two hepatocellular carcinoma cell lines that expressed Spry2 at low levels (SNU449 and HepG2) and one with moderate levels of expression (Hep3B; about 4-fold higher) following treatment with the DNA methyl-transferase inhibitor 5-aza-deoxycytidine according to the protocol by McKie et al. (30). Real-time PCR analysis of Spry2 mRNA instead show a slight reduction in the levels of Spry2 in all the hepatocellular carcinoma cell lines upon 5-aza-deoxycytidine treatment (Fig. 5A, left). RASSF1A is one of the most frequently inactivated genes described thus far in human solid tumors (43). Inactivation of RASSF1A most commonly involves methylation of the promoter and CpG islands and can be up-regulated by 5-aza-deoxycytidine treatment. We used it as a positive control to show that the 5-aza-deoxycytidine treatment was effective (Fig. 5A, right). Treatment with the deacetylase inhibitor trichostatin gave similar findings for both Spry2 and RASSF1A (data not shown).

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<th>Table 1.</th>
<th>A. In situ hybridization of Spry2 in liver tissues</th>
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<td></td>
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<td>Malignant hepatocellular carcinoma</td>
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<th>B. Immunohistochemistry of Spry2 in liver tissues</th>
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<th>Strong staining (score 3)</th>
<th>P (Fisher’s exact t test)</th>
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upstream of Spry2 does not seem to be a common occurrence in hepatocellular carcinoma and hence does not account for the down-regulation of Spry2.

Spry2 inhibits HGF-stimulated ERK and exerts an anti-proliferative effect in the hepatoma cell line SNU449. Functional inhibition of Spry2 has been reported to enhance proliferation of the breast cancer cell line MCF-7 (22), suggesting that Spry2 has a growth inhibitory role in breast cancer. To test whether this functional implication of Spry2 is conserved in liver cancer, we used a similar approach by first generating stably expressing clones of Spry2 and its reported dominant-negative mutant Spry2(Y55F) using the human hepatocellular carcinoma cell line SNU449. In the absence of any growth stimulatory factors, inhibition of Spry2 function in the SNU449 cells stably expressing Spry2(Y55F) mutant resulted in an enhanced proliferation rate compared with the control parental cell line (Fig. 6A). The growth inhibitory effect of overexpressing Spry2 was evident only in the presence of HGF (50 ng/mL); this effect is significant at $P = 0.03$ (Student’s $t$ test from three independent experiments).

To further determine the molecular effect that Spry2 may exert on hepatocellular carcinoma cells upon HGF stimulation, we generated recombinant adenoviruses carrying the Spry2 gene using the AdEasy Adenoviral Vector System (Stratagene). This enabled us to infect SNU449 cells at close to 100% efficiency. The activation of ERK was found to be inhibited at earlier time points of HGF stimulation when compared with uninfected SNU449 control cells (Fig. 6B). This is consistent with a previous study (21), in which Spry2 was shown to inhibit ERK activation, although a leiomyosarcoma and not a hepatocellular carcinoma cell line was used in that study.

Knocking down Spry2 levels transforms NIH3T3 cells in the presence of fibroblast growth factor. As the current and that of the breast and prostate cancer studies (22, 30) suggest that a reduced level of Spry2 may play a central role in enabling certain

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**Figure 3.** Spry2 expression is relatively higher in normal and cirrhotic liver compared with hepatocellular carcinoma (HCC) liver, as detected by both (A) *in situ* hybridization and (B) immunohistochemistry. All images are taken at ×20 magnification.
stages of tumor progression, we therefore attempted to knock down the levels of Spry2 in the NIH3T3 cell line and look for foci formation. NIH3T3 cells are chosen because these cells are predisposed to cellular transformation by activation of the Ras signal transduction pathway, which Spry proteins regulate (17). The siRNA duplexes (Dharmacon) were first transfected into mouse Neuro2A cells (which exhibits higher transfection efficiency), and real-time PCR analysis showed ~50% knockdown for each of the four duplexes (D1-D4; data not shown). The siRNAs were subsequently transfected into NIH3T3 cells, which were either left unstimulated or stimulated with basic fibroblast growth factor (bFGF; 20 ng/mL) for 2 weeks with regular replacement of the media and ligand every 3 days. FGF was used as the NIH3T3 cells do not respond to HGF stimulation (data not shown) probably due to low levels of endogenous Met receptor expression. Crystal violet stained dishes indicated no difference in cell morphology among the unstimulated dishes (Fig. 6). A mild but noticeable transforming phenotype was observed for cells transfected with Spry2 siRNA duplexes when the FGF ligand was added. This suggests that a lower Spry2 level coupled with a high concentration of growth factor can indeed facilitate the tumorigenesis process and hence clearly indicate a role for Spry2 in cancer.

Discussion

There is emerging evidence indicating the possible roles of the sprouty family of proteins in cancer, particularly because some of them are specific inhibitors of the Ras/ERK signaling pathway that is commonly dysregulated in various types of cancers (22, 23, 30). In hepatocellular carcinoma, the Ras/ERK pathway can be activated by major etiologic factors (such as HBV and HCV) and mitogenic growth factors (6–10). We observed consistent underexpression of Spry2 in the majority of hepatocellular carcinoma livers compared with their matched normal livers, implying a potential diagnostic value of Spry2 in hepatocellular carcinoma. On the other hand, Spry1 did not show any consistent down-regulation in tumor versus nontumor liver samples, based on either gene expression or real-time PCR data, which is in contrast to our previous study of its level in breast cancer (22). The down-regulation of Spry2 in hepatocellular carcinoma may confer a proliferative advantage for tumor cells by allowing unchecked activation of the Ras/ERK pathway, which would normally be controlled by relatively higher expression levels of Spry2 in normal liver. An interesting observation is that the gene encoding the HGF receptor (Met) is overexpressed whenever Spry2 is expressed at low levels (Fig. 2B), suggesting that enhanced signaling through the HGF receptor may play an important role in hepatocellular carcinoma. Other Spry genes (Spry3 and Spry4) were not included in the cDNA arrays used to generate the gene expression profiles; thus, we have no insights into their potential roles in hepatocellular carcinoma.

Validation using in situ hybridization and immunohistochemistry on an independent set of archived liver tissues confirmed the presence of Spry2 transcript and protein in normal liver tissues and their absence in hepatocellular carcinoma liver tissues. Interestingly, the level of Spry2 transcript and protein were the highest in cirrhotic liver. Cirrhosis is characterized anatomically by widespread nodules in the liver combined with fibrosis, which together cause distortion of the normal liver architecture, interference with blood flow through the liver, and disruption of the normal biochemical functions of the liver. Cirrhosis can result from almost any chronic liver disease that causes damage to the liver cells. In patients with chronic hepatitis, the development of cirrhosis increases their risk of developing hepatocellular carcinoma. In general, hepatocellular carcinoma in patients with cirrhosis tend to be less well differentiated than those in patients without cirrhosis, and the presence of cirrhosis in turn portends a poorer prognosis (44). The abundance of Spry2 in cirrhosis raises the interesting possibility that it could serve as an early marker heralding later onset of hepatocellular carcinoma. Potentially, high levels of Spry2 may be expressed due to the increased tissue remodeling during cirrhosis and is necessary to prevent uncontrolled cellular proliferation. It will be interesting to monitor the levels of Spry2 in cirrhotic samples undergoing the transition to hepatocellular carcinoma.

The mechanisms regulating Spry2 expression in various types of cancer are incompletely understood. As in breast cancer, we ruled out the possibility of DNA methylation of promoter sites as an underlying mechanism (22) contributing to the observed silencing of Spry2.
Figure 5. A, epigenetic silencing via methylation of Spry promoter (or the promoter of genes upstream of Spry) is not responsible for the down-regulation of Spry expression in liver tumors. Treatment of Hep3B, HepG2, and SNU449 liver cancer cells with 5-aza-deoxycytidine (25 μmol/L) for 6 days (with alternate rest days) did not up-regulate the expression of Spry2. RASSF1A is a gene silenced by hypermethylation and was used as a positive control to show that the 5-aza-deoxycytidine treatment was effective. The prostate cancer cell line PC-3M was used as a positive control in the experiment. All real-time PCR analysis was normalized using the gene β-macroglobulin and expressed as folds versus control (untreated) samples. B, MSP for CpG1A (5' putative promoter). C, CpG1B (exon 1) were done on genomic DNA extracted from eight paired normal (N) and hepatocellular carcinoma (T) samples. T73-PCa (a prostate cancer sample) was used as a positive control for the methyl-specific primers, whereas DU145 (a prostate cancer cell line) was used as a positive control for the unmethyl-specific primers. D, methylation status of 16 and 13 CpG dinucleotide residues from CpG1A and CpG1B amplicons, respectively, generated from sequencing cases T4, T5, and T9 (CpG1A) and T4, N7, and T9 (CpG1B). The degree of methylation was depicted by the intensity of shading of each circle representing a single CpG (see Key).
Spry2 in hepatocellular carcinoma. This is in contrast to the study of Spry2 in prostate cancer where McKie et al. (30) found a high proportion of samples methylated. Similarly, no LOH was observed in the microsatellite markers flanking the Spry2 gene locus in hepatocellular carcinoma unlike that in prostate cancer (30). We believe that genetic variability between the different cancers may account for the differences. In the case of hepatocellular carcinoma, the Spry2 exon 2 locus was previously checked for homozygous deletion in hepatocellular carcinoma cell lines by Pineau et al. (40), but none was found. In addition, Jou et al. (45) had scanned for minimal deleted regions (MDR) in hepatocellular carcinoma and found the cytogenetic loci 13q13.1 to 13q22.1 (MDR regions D13S171 to D13S156) and 13q31.3 to 13q32.2 (MDR regions D13S265 to D13S159) to be deleted. As the Spry2 locus is located at chromosome 13q31.1 and is therefore between these two MDR regions, our finding is in agreement with these previous studies.

Recent functional studies of the promoter region of human Spry2 uncovered the presence of binding sites for numerous transcription factors that could modulate the basal and tissue-specific expressions of Spry2 (46). It is likely that the complex interactions of multiple transcription factors together control the expression of Spry2 in a cell-specific and context-specific manner, eventually accounting for dysregulation of Spry2 in human cancers. We are currently looking into whether the loss of activators or increased activity or levels of repressors acting on the Spry2 promoter is involved in the down-regulation of Spry2 in hepatocellular carcinoma.

Despite unknown mechanisms regulating Spry2 expression in hepatocellular carcinoma, we observed that Spry2 plays functionally important roles in hepatocellular carcinoma, including the inhibition of HGF-stimulated proliferation and HGF-activated ERK pathway in hepatocellular carcinoma cell lines. The inhibition of overactive ERK pathway in hepatocellular carcinoma is a promising mode of therapeutic intervention that is currently being exploited: a recent clinical trial is investigating the use of a Raf kinase inhibitor (Sorafenib) to treat hepatocellular carcinoma by arresting...
proliferation and angiogenesis resulting from inappropriate signal-
ing in the RAF/mitogen-activated protein kinase/ERK and VEGF-2/PGDFR-β cascades, respectively.

Hepatocellular carcinoma is a hypervascular lesion with a rich supply of blood vessels; the angiogenic switch is regulated by various angiogenic growth factors and inhibitors, most commonly VEGF, bFGF, and insulin-like growth factor II (47). Recently, new angiogenic pathways and regulators of these pathways have been discovered, among which includes Sprouty (19). Specifically, mouse Spry4 was shown to inhibit angiogenesis (20), apparently via inhibition of receptor tyrosine kinase signaling upstream of or at the level Raf (48). A reduction in both basic and bFGF-induced or VEGF-induced ERK phosphorylation by Spry4 was postulated, resulting in the reduction of proliferation, migration, and differentiation during angiogenesis. Whether other Sprouty proteins have similar angiogenic properties is still unknown; this topic warrants further investigation as it can offer valuable insights into the molecular pathogenesis of hepatocellular carcinoma, as well as point towards novel approaches of therapeutic intervention.

Finally, our current study using Spry2 siRNA to knock down the expression levels in NIH3T3 cells suggests that it is possible to lower the threshold for cell transformation. By mimicking the high levels of growth factor environment where the liver tumor may be exposed to, the reduction of Spry2 protein in the siRNA duplex transfected dishes induces a mild transforming phenotype in the presence of basic FGF. This suggests that Spry2 may play a role in preventing cell transformation, and a down-regulation of this gene may reduce the threshold for cells to become malignant.

In conclusion, our study clearly indicates a role for Spry2 in hepatocellular carcinoma. Further studies into the functional roles of Spry2 in the progression to liver cancer will not only enhance our understanding of the intricate molecular pathophysiology of this malignancy but also help in the design of novel therapeutic strategies against it.

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Sprouty 2, an Inhibitor of Mitogen-Activated Protein Kinase Signaling, Is Down-Regulated in Hepatocellular Carcinoma

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