Integration of Genomic Analysis and In Vivo Transfection to Identify Sprouty 2 as a Candidate Tumor Suppressor in Liver Cancer

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Hepatocellular carcinoma (HCC) is among the top 5 causes of cancer-related deaths worldwide.1 Epidemiological and molecular genetic studies have demonstrated that hepatitis B or hepatitis C are major risk factors for HCC development, especially when accompanied by liver cirrhosis. Treatment options of HCC are limited, and the 5-year survival rate for HCC patients remains at approximately 7% in the United States (statistic from http://www.cancer.org/).

Development of HCC is a multistep process. However, the molecular genetics and signaling pathways underlying hepatic carcinogenesis are still poorly understood.2 Molecular events frequently observed in HCC include mutations in p53 and β-catenin, and aberrant CpG island methylation of APC, E-cadherin, and p16.2 Among them, mutations of β-catenin occur in 15% to 30% of human HCCs.3,4 These β-catenin mutations tend to be point mutations or deletions at the N-terminus that lead to the stabilization of β-catenin. This stabilized β-catenin translocates into the nucleus and binds to the T-cell factor transcriptional factors to activate downstream genes. Another important pathway involved in HCC pathogenesis is the Ras/extracellular signal-regulated kinase (ERK) signaling pathway. Mouse models have demonstrated that activated Ras (RasV12) alone is
not sufficient to induce HCC in vivo. However, activated Ras and β-catenin together can promote hepatic carcinogenesis in mice. There is ubiquitous activation of the Ras/ERK pathway in human HCC, but Ras family members are rarely mutated. So how does Ras/ERK signaling become activated in human HCCs? One possible mechanism is the down-regulation of Spry2 in human HCCs.

Spry2 belongs to the Sprouty family of proteins, which are evolutionarily conserved inhibitors of receptor tyrosine kinases (RTKs). On activation of RTKs, Spry2 becomes phosphorylated at a conserved N-terminal tyrosine residue (Y55) and binds to a series of intracellular signaling molecules, including Raf, Grb2, and Cbl. This ultimately leads to the down-regulation of ERK phosphorylation and RTK signaling. Mutation of this conserved N-terminal tyrosine of Spry2 (Spry2Y55F) generates a dominant-negative Spry2 protein that enhances growth factor–dependent ERK signaling. In addition, Spry2 may interact with other signaling molecules, including caveolin, fibroblast growth factor receptor substrate 2 (FRS2), and protein tyrosine phosphatase receptor type 1 (PTP1B), to regulate other cellular processes, including cell growth and migration. Spry2 expression appears to be down-regulated in several tumor types, including HCC, breast, and prostate cancers.

This is thought to lead to abnormal activation of Ras/ERK signaling in tumor cells and to promote tumor development. However, most current studies are limited to in vitro or xenograft analysis of Spry functions. It is clearly imperative to investigate whether blocking Spry activity contributes to tumorigenesis, using in vivo mouse models.

DNA microarray technology provides a powerful tool to identify genes that are associated with tumor cells by surveying global gene expression in an unbiased way. Using this technology, we and several groups have reported the expression profiles of liver cancer cell lines and human samples. Such genomic studies provide us with a large number of genes that are differentially expressed by tumor and nontumor liver cells, as well as genes that may serve as prognostic markers. To characterize the molecular genetics of HCCs, we applied array-based comparative genomic hybridization (CGH) to study the DNA copy number variations among HCC samples. We identified recurrent DNA copy number gains at 1q, 8q, and 20q, as well as DNA copy number losses at 1p, 4q, 8p, 13q, 16q, and 17p. However, genes altered within these chromosomal regions remain largely unknown.

One of the major challenges during the postgenome era is how to effectively study the functions of large numbers of genes identified from genomic studies, especially in vivo. The traditional methods using transgenic or knockout mice are both time consuming and expensive. Recently, we and another group reported that the combination of hydrodynamic injection and sleeping beauty–mediated somatic gene integration is an efficient and flexible method to target long-term gene expression in mouse hepatocytes and induce liver cancer in vivo. In this manuscript, we showed that this approach can be readily integrated into oncogenetic studies to study the functional significance of candidate genes’ role in liver tumorigenesis in vivo.

Materials and Methods

Constructs and Reagents. Mouse Spry2 complementary DNA (cDNA) was provided by Dr. Gail Martin of UCSF; the hyperactive sleeping beauty construct (pCMV/SB) by Dr. Mark Kay of Stanford University; and pCAGgs-RasV12 by Dr. David Largaespada of University of Minnesota. The pT3-EF1α vector containing duplicated inverted repeats for sleeping beauty–mediated integration and elongation factor 1α (EF1α) promoter used for injection was described by Tward et al. Spry2Y55F was generated by site-directed mutagenesis using QuickChange (Stratagene, La Jolla, CA). Spry2Y55F (with a C-terminal V5 tag) and ΔN90-β-catenin were cloned into pT3-EF1α via the Gateway polymerase chain reaction (PCR) cloning strategy (Invitrogen, Carlsbad, CA). All plasmids were purified using the Endotoxin free Maxi prep kit (Sigma, St. Louis, MO) before injecting into mice.

Statistical Analysis of Microarray and Array CGH Data. The expression data of the 4,863 cDNA clones used in the previous gene expression analysis were retrieved. Mapping position for these cDNA clones was assigned using the National Center for Biotechnology Information genome assembly, accessed through the UCSC genome browser database. Of the 4,863 cDNA clones, 4,354 cDNA clones have chromosomal mapping information and were used for further analysis. The gene expression clones were mapped to the BAC clone within 1 megabase of the gene expression clone, which had the highest Pearson correlation between copy number and gene expression. Correlation was computed for each clone, and a correlation coefficient of 0.35 was used as the cutoff to identify clones having positive correlation between copy number and gene expression. P values were obtained based on permutation analysis and were corrected for multiple testing by controlling for the false discovery rate.

Hydrodynamic Injection and Mouse Monitoring. The procedures were described as previously. In brief, plasmids were diluted in 2 mL 0.9% NaCl, filtered, and injected into the lateral tail vein of 6-week-old to 8-week-old BALB/cByJ male mice. The mice were killed 48 hours after injection for analysis.
old FVB/N mice in 5 to 7 seconds. All mice were housed, fed, monitored, and treated in accordance with protocols approved by the committee for animal research at the University of California, San Francisco.

Immunohistochemistry, Western Blotting, and Real-Time RT-PCR. Immunohistochemical staining and Western blotting were performed using standard methods. Sybergreen-based real-time reverse transcription (RT)-PCR was carried out as described, and ribosomal RNA was used as an internal control. Transcript quantification was performed in triplicate for every sample and reported relative to ribosomal RNA. The primer pairs are listed in Supplementary Table 1.

See supplementary information for a detailed description of the materials and methods.

Results

Contribution of Genomic DNA Copy Number Variation to Global Gene Expression Changes in Human HCC Samples. To determine whether genomic DNA copy number variations contribute to global gene expression pattern changes, we examined the correlation between gene expression values and the corresponding DNA copy number changes from 44 human HCC samples with both expression array and array CGH data. Of the 4,354 cDNA clones analyzed, 747 cDNA clones (or 17.2% of total cDNA clones analyzed), representing approximately 542 unique genes, show statistical significant correlation between expression values and DNA copy number variations (correlation > 0.35 and adjusted P value is 0.016 with FDR less than 7.9%. See Supplementary Table 2 for the list of genes). To illustrate whether DNA copy numbers influence gene expression, we compared the pairwise correlation of gene expression data with CGH values of BAC clones close to the locus where each gene is located at (diagonal), or CGH values of BAC clones located at other regions of the genome. We found pairs of regions along the diagonal have higher positive correlation (median correlation, approximately 0.135) than the off-diagonal pairs (median correlation, approximately 0.005) (Supplementary Fig. 1A). A heatmap of the pairwise correlation between gene expression and copy number also demonstrates the positive correlation along the diagonal (Supplementary Fig. 1B).

Overall, our data confirm that genomic DNA copy number variations contribute to the regulation of regional gene expression profiles in human HCC samples.

Identification of Candidate Oncogenes or Tumor Suppressor Genes for Human HCCs. To pinpoint candidate oncogenes or tumor suppressor genes, we applied 2 criteria to the list of 747 cDNA clones. First, we searched for genes that showed consistent gain or loss in at least 8 (or approximately 20%) tumor samples. Second, we matched the gene list with the 1,946 cDNA clones that were identified to be differentially expressed among non-tumor liver and HCC samples in our previous studies. Thus, we narrowed our list to 134 cDNA clones, representing 113 unique genes. Among these genes, 76 genes are up-regulated in HCC samples and are frequently amplified at the genomic DNA level, whereas the remaining 37 genes are down-regulated in HCC samples and are frequently deleted at the genomic DNA level. These 2 sets of genes represent potential candidate oncogenes or tumor suppressor genes, respectively, for HCC pathogenesis. One of the genes identified from our screening is Jab1/CSN5, a gene that we previously demonstrated to be highly expressed in HCCs and that is frequently amplified. This confirms the reliability of our genome-wide correlation analysis.

Long-Term In Vivo Delivery of Target Genes in Mouse Hepatocytes and Induction of HCC by Hydrodynamic Transfection. One of the genes identified from our genomic analysis to be down-regulated and frequently deleted is Spry2, a well-characterized RTK/Ras pathway inhibitor. Our in vitro studies showed that overexpression of Spry2 inhibits HCC cell growth (Supplementary Fig. 2). The results are consistent with other in vitro studies that demonstrate Spry2 inhibits tumor cell proliferation. To determine whether Spry2 is a bona fide tumor suppressor for hepatic carcinogenesis, we investigated whether the loss of Spry2 activity contributes to liver cancer development using in vivo mouse models.

Because it has been shown that activated Ras and β-catenin cooperate to induce HCC development, we hypothesized that loss of Spry2 function together with activated β-catenin will also lead to hepatocarcinogenesis in mice. Spry2 knockout mice have been generated. However, these Spry2 null mice have a shortened life span and most die within 1 to 2 months after birth; therefore, they are not suitable for this study. To address our hypothesis, we took an alternative approach in which we used hydrodynamic transfection with a transposable vector to stably express exogenous genes in mouse liver. Most recently, we and another group showed that this method can be used to develop mouse models for liver cancer. We therefore generated a well-characterized dominant negative form of Spry2, Spry2Y55F, with a C-terminal V5 tag to block endogenous Spry2 function, as well as stimulated and activated β-catenin (ΔN90-β-catenin) for hydrodynamic transfection (Supplementary Fig. 3A). After injecting into mice, long-term expression of each of the transfected genes can be detected in 5%
| Gene Symbol | Chr | Percentage Gained/Lost* | Fold of Up/Down-Regulation† | Gene Symbol | Chr | Percentage Gained/Lost* | Fold of Up/Down-Regulation† |
|-------------|-----|--------------------------|-----------------------------|-------------|-----|--------------------------|-----------------------------|
| SF3B4       | 1q  | 68.2                     | 2.28                        | NEU1        | 6p  | 25.0                     | 2.22                        |
| APH1A       | 1q  | 68.2                     | 1.68                        | EHMT2       | 6p  | 25.0                     | 2.32                        |
| KIAA0460    | 1q  | 65.9                     | 2.01                        | RDBP        | 6p  | 25.0                     | 1.98                        |
| PRUNE       | 1q  | 65.9                     | 2.63                        | PDN6        | 6p  | 20.5                     | 1.66                        |
| SNX27       | 1q  | 65.9                     | 2.07                        | CUXA        | 6p  | 20.5                     | 1.62                        |
| ILF2        | 1q  | 65.9                     | 1.94                        | XPO5        | 6p  | 18.2                     | 1.80                        |
| NICE-3      | 1q  | 65.9                     | 1.99                        | SLC29A1     | 6p  | 18.2                     | 2.06                        |
| UBA2P2L     | 1q  | 65.9                     | 2.23                        | NUP205      | 7q  | 22.7                     | 1.48                        |
| UBE2Q1      | 1q  | 65.9                     | 1.96                        | TRIM24      | 7q  | 22.7                     | 2.01                        |
| PYG02       | 1q  | 65.9                     | 2.44                        | MULK        | 7q  | 22.7                     | 1.59                        |
| FLAD1       | 1q  | 65.9                     | 2.56                        | KCNH2       | 7q  | 22.7                     | 1.64                        |
| FDPS        | 1q  | 63.6                     | 2.70                        | ATP6V1H     | 8q  | 29.5                     | 1.53                        |
| DAP3        | 1q  | 63.6                     | 2.03                        | ARMCl       | 8q  | 34.1                     | 1.76                        |
| MF20        | 1q  | 63.6                     | 1.64                        | CNSN5/JAB1  | 8q  | 34.1                     | 1.56                        |
| PRCC        | 1q  | 63.6                     | 2.08                        | ARFGF1      | 8q  | 34.1                     | 1.52                        |
| NCSTN       | 1q  | 63.6                     | 1.97                        | NCOA2       | 8q  | 36.4                     | 1.87                        |
| BFGALT3     | 1q  | 63.6                     | 1.70                        | RDH10       | 8q  | 36.4                     | 1.64                        |
| TMCO1       | 1q  | 61.4                     | 1.86                        | PLEXH1F2    | 8q  | 45.5                     | 1.76                        |
| BATZD1      | 1q  | 61.4                     | 1.72                        | PGC1        | 8q  | 45.5                     | 1.63                        |
| SMG7        | 1q  | 56.8                     | 1.78                        | MTDH        | 8q  | 45.5                     | 1.64                        |
| UCH5        | 1q  | 54.5                     | 1.51                        | LAPTIM4B    | 8q  | 50.0                     | 2.58                        |
| JARID1B     | 1q  | 52.3                     | 1.65                        | RPL30       | 8q  | 45.5                     | 1.56                        |
| SNRPE       | 1q  | 50.0                     | 2.09                        | YWHAZ       | 8q  | 50.0                     | 1.85                        |
| NUCKS1      | 1q  | 47.7                     | 1.58                        | ZNF706      | 8q  | 50.0                     | 1.63                        |
| CD46        | 1q  | 47.7                     | 1.74                        | EST         | 8q  | 47.7                     | 2.06                        |
| LPGAT1      | 1q  | 50.0                     | 1.88                        | FAM83H      | 8q  | 45.5                     | 2.25                        |
| INTS7       | 1q  | 50.0                     | 1.75                        | SIAHBP1     | 8q  | 45.5                     | 1.64                        |
| PPP2R5A     | 1q  | 50.0                     | 1.70                        | GPAA1       | 8q  | 45.5                     | 1.77                        |
| CNHH4       | 1q  | 47.7                     | 2.07                        | CYC1        | 8q  | 45.5                     | 1.71                        |
| NUP133      | 1q  | 45.5                     | 2.06                        | MAF1        | 8q  | 45.5                     | 1.98                        |
| GNPAT       | 1q  | 45.5                     | 1.91                        | LOC441383   | 8q  | 45.5                     | 1.73                        |
| TOMM20      | 1q  | 45.5                     | 1.62                        | CBX1        | 17q | 18.2                     | 1.66                        |
| ARID4B      | 1q  | 45.5                     | 1.53                        | WDR68       | 17q | 20.5                     | 1.71                        |
| GGF51       | 1q  | 45.5                     | 2.09                        | NDRG3       | 20q | 22.7                     | 2.06                        |
| GMNN        | 6p  | 18.2                     | 3.64                        | PGGG        | 20q | 31.8                     | 1.91                        |
| HIST1H28K   | 6p  | 20.5                     | 1.89                        | NCOA3       | 20q | 29.5                     | 1.65                        |
| CSN2K8      | 6p  | 25.0                     | 1.49                        | ARFGF2      | 20q | 29.5                     | 1.74                        |
| MSH5        | 6p  | 20.5                     | 2.10                        | PRPF6       | 20q | 25.0                     | 1.71                        |

*Percentage of HCC samples with gain or loss at the specific gene loci. †Average fold of up-regulation or down-regulation of the gene comparing HCC versus nontumor liver samples.
10% of the hepatocytes by immunohistochemistry (Supplementary Fig. 3B,C). The transfection efficiency is similar to what has been described\textsuperscript{27} and our previous experience.\textsuperscript{24}

To further prove the reliability of our approach, we determined whether RasV12 and \( \Delta N90-\beta\)-catenin can cooperate to induce liver cancer in mice using hydrodynamic transfection. Consistent with previous reports,\textsuperscript{5,6} Co-expression of RasV12/\( \Delta N90-\beta\)-catenin or Spry2Y55F/\( \Delta N90-\beta\)-catenin induces liver cancer formation in mice. (A) Representative gross image of liver tumors from RasV12/\( \Delta N90-\beta\)-catenin or Spry2Y55F/\( \Delta N90-\beta\)-catenin co-injected mice. Note that there are numerous tumor nodules in RasV12/\( \Delta N90-\beta\)-catenin mice and 1 tumor nodule in Spry2Y55F/\( \Delta N90-\beta\)-catenin mice. (B) Tumor development incident curves in mice.

Table 2. Tumor Development in Mouse Coinjected with RasV12/\( \Delta N90-\beta\)-catenin or Spry2Y55F/\( \Delta N90-\beta\)-catenin.

| Injection                  | Code   | Sex | Age (Weeks Old) | Weeks Postinjection | Tumor* | Number of Tumors | Tumor Size (mm)† |
|----------------------------|--------|-----|-----------------|---------------------|--------|------------------|------------------|
| Y55FSpry2 + \( \Delta N90-\beta\)-catenin | YB-F1.1 | F   | 26              | 18                  | N      |                  |                  |
|                            | YB-F1.2 | F   | 26              | 18                  | N      |                  |                  |
|                            | YB-F1.3 | F   | 32              | 24                  | Y      | 1                | 5                |
|                            | YB-F1.4 | F   | 32              | 24                  | N      |                  |                  |
|                            | YB-M1.1 | M   | 29              | 22                  |        |                  |                  |
|                            | YB-M1.2 | M   | 36              | 27                  | Y      | 2                | 4, 18            |
|                            | YB-M1.3 | M   | 36              | 29                  | N      |                  |                  |
|                            | YB-M1.4 | M   | 36              | 29                  | Y      | 4                | 3, 2, 15, 16     |
|                            | YB-M1.5 | M   | 36              | 29                  | N      |                  | 1                |
|                            | YB-F2.1 | F   | 37              | 29                  | N      |                  |                  |
|                            | YB-F2.2 | F   | 37              | 29                  | N      |                  |                  |
|                            | YB-F2.3 | F   | 37              | 29                  | Y      | 1                | 1                |
|                            | YB-F2.4 | F   | 37              | 29                  | Y      | 1                | 14               |
|                            | YB-F2.5 | F   | 37              | 29                  | Y      | 1                | 10               |
| RasV12 + \( \Delta N90-\beta\)-catenin | RB-F1.1 | F   | 21              | 13                  | N      |                  |                  |
|                            | RB-F1.2 | F   | 21              | 13                  | Y      | TMC              | ND               |
|                            | RB-F1.3 | F   | 21              | 13                  | Y      | TMC              | ND               |
|                            | RB-F1.4 | F   | 21              | 13                  | Y      | TMC              | ND               |
|                            | RB-M1.1 | M   | 21              | 14                  | Y      | TMC              | ND               |
|                            | RB-M1.2 | M   | 26              | 18                  | N      |                  |                  |
|                            | RB-M1.3 | M   | 26              | 18                  | N      |                  |                  |
|                            | RB-M1.4 | M   | 26              | 18                  | N      |                  |                  |
|                            | RB-F2.1 | F   | 26              | 18                  | Y      | TMC              | ND               |
|                            | RB-F2.2 | F   | 23              | 16                  | Y      | TMC              | ND               |
|                            | RB-F2.3 | F   | 23              | 16                  | Y      | TMC              | ND               |
|                            | RB-F2.4 | F   | 26              | 18                  | Y      | TMC              | ND               |

Abbreviations: F, female; M, male; TMC, too many to count.
*Tumor: Y, liver tumor in liver; N, no tumor nodules observed in liver.
†ND: Not determined. Because there are too many tumor nodules in the mouse liver, it is difficult to distinguish the boundary of each individual tumor nodule. Therefore the tumor size was not determined.
we found that although none of the RasV12 or ΔN90-β-catenin–injected mice developed liver tumors, coexpression of RasV12 and ΔN90-β-catenin induced liver cancer formation in 67% of the mice (8/12) at 18 weeks after injection (Fig. 1, Table 2). In all cases, there were too many tumor nodules in the mouse liver to be counted.

**Spry2Y55F Cooperates with ΔN90-β-Catenin Signaling to Induce Liver Cancer Development in Mice.** To test our hypothesis that loss of Spry2 function cooperates with activated β-catenin to promote HCC development in vivo, we expressed Spry2Y55F, or ΔN90-β-catenin alone or Spry2Y55F plus ΔN90-β-catenin into mouse hepatocytes. All animals were sacrificed between 18 and 29 weeks after injection, and liver tissues were examined grossly and histologically for tumors. Analysis of these samples revealed that half of the mice (7/14) co-expressing Spry2Y55F and ΔN90-β-catenin developed liver tumors (Fig. 1, Table 2), whereas no tumors were observed in mice injected with Spry2Y55F or ΔN90-β-catenin alone (Fig. 1B). Interestingly, we noticed differences between tumors induced by RasV12/ΔN90-β-catenin and Spry2Y55F/ΔN90-β-catenin. In particular, mice co-expressing Spry2Y55F/ΔN90-β-catenin have lower tumor incidence (1-4 nodules per mouse) in comparison with the numerous tumors found in RasV12/ΔN90-β-catenin mice (Fig. 1A, Table 2). Furthermore, although RasV12/ΔN90-β-catenin can induce tumorigenesis as early as 13 weeks after injection, Spry2Y55F/ΔN90-β-catenin requires longer latency (approximately 24 weeks) for tumor development (Fig. 1B, Table 2).

Histological examination of liver tumor samples from Spry2Y55F/ΔN90-β-catenin mice revealed that tumor cells morphologically appear to be of hepatocellular origin and the neoplastic cells display cytological atypia and frequent trabecular disorganization, which are consistent with HCC (Fig. 2A). Real-time RT-PCR analysis revealed the high expression of liver tumor marker α-fetoprotein (Supplementary Fig. 4), further supporting that Spry2Y55F and ΔN90-β-catenin cooperate to induce HCC in vivo.

**Coexpression of Spry2Y55F and ΔN90-β-Catenin in Mouse HCC Samples.** We next examined whether all tumor nodules co-express Spry2Y55F and ΔN90-β-catenin. Immunohistochemistry with anti–β-catenin and anti-V5 antibodies demonstrated that all tumor cells exhibit nuclear/cytoplasmic staining of β-catenin as well as membrane expression of Spry2Y55F (Fig. 2B,C). These results
are consistent with the western blot analysis in which Spry2Y55-V5 and ΔN90-β-catenin are detected only in the HCC samples (Fig. 2D). Furthermore, the western blot depicts tumor tissues displaying a predominant lower band of β-catenin, which indicates that the activation of β-catenin in tumor cells is not due to random mutations of the endogenous β-catenin (which are typically point mutations) during tumorigenesis. Scattered Spry2Y55F or ΔN90-β-catenin–expressing cells are observed in the adjacent nontumor liver by immunostaining (Fig. 2B,C), but their expression levels may be too low to be detected by western blotting (Fig. 2D). Together our analysis supports that all tumor cells coexpress Spry2Y55F and ΔN90-β-catenin and the expression of both Spry2Y55F and ΔN90-β-catenin promotes liver cancer formation in vivo.

**Activation of ERK Pathway in Mouse Liver Tumors.** We next investigated the molecular mechanisms of Spry2Y55F in promoting HCC development by assaying the activity of key signaling components that may be downstream of Spry, including ERK and protein kinase (Akt). Using immunohistochemical staining, we found that although nontumor liver cells have no detectable levels of phospho-ERK, liver tumor cells display high levels of phospho-ERK (Fig. 3A). This observation is verified by western blotting (Fig. 3B). Total ERK expression remains unchanged in all samples (Fig. 3B). We observed no evidence of phospho-Akt expression in either nontumor liver tissues or liver tumor tissues (Fig. 3C). These results indicate that Spry2Y55F up-regulates ERK signaling, but has no effect on the Akt pathway.

To validate that phospho-ERK signaling observed in tumor samples is due to ectopic Spry2Y55F expression, but not due to the activation of the upstream RTKs, we measured the expression levels of total phosphorylated tyrosine by western blotting. We found the tumor showed no increase in the expression of total phosphorylated tyrosine (Fig. 3D), suggesting that there is no overall increased activation of RTKs in tumor cells.

**Molecular Features of HCCs Induced by Spry2Y55F and ΔN90-β-Catenin.** To determine whether liver tumors induced by Spry2Y55F/ΔN90-β-catenin resemble a subset of human HCCs, we characterized the molecular features of these mouse liver tumor samples. Ki67 staining revealed that tumor cells are highly proliferative (Fig. 4A). Furthermore, these tumor cells show high expression of genes involved in cell cycle and cell proliferation, including cyclins B1, D1, and E1 (Fig. 5A-C). Expression of a Cdk inhibitor, p21Cip1, is also increased in the HCC samples (Fig. 5D). P21Cip may be induced as a feedback inhibitor in response to the abnormal cell proliferation and has been reported to be frequently up-regulated in human HCCs. In addition, tumor cells express high levels of the anti-apoptotic protein survivin (Fig. 5E). Survivin has also been reported to be highly expressed in human HCC samples.
We next examined the expression of the cell-cell adhesion molecule E-cadherin. In the normal liver, hepatocytes show weak staining of E-cadherin around the periportal area. However, liver tumor samples induced by Spry2Y55F/ΔN90-β-catenin were found to have E-cadherin expression in virtually all neoplastic hepatocytes (Fig. 4B). The increased expression of E-cadherin in the tumor cell is also supported by RT-PCR analysis (Fig. 5F). The up-regulation of E-cadherin has been reported in multiple mouse liver cancer models, including HCCs induced by c-Myc/E2F1 or c-Myc/transforming growth factor alpha.30

One hallmark of HCC is the change in endothelial cells. In humans, although sinusoidal endothelial cells surrounding the normal hepatocytes do not express endothelial markers CD34 or podocalyxin kcl (PODXL1), the endothelial cells of HCC stain positively for these markers.31 This change in expression of these markers represents fundamental differences between endothelial cell structures in the normal liver and cancerous liver tissues. Using an antibody against PODXL1, we demonstrated that although the normal liver sinusoid endothelial cells do not express PODXL1, the endothelial cells in tumor tissues induced by Spry2Y55F/ΔN90-β-catenin express this marker at high levels (Fig. 4C).

The change in expression of endothelial cell markers in the mouse HCC samples implies that angiogenesis occurs during tumor development. We next investigated whether there are changes of expression of genes involved in angiogenesis. Although we did not detect any changes in the expression levels for angiogenic factors vascular endothelial growth factor (VEGF), VEGF120, and Ang1, as well as their receptors FLT1 and FLK1 in tumor versus non-tumor liver tissue (data not shown), we observed an increase in the expression of Ang2 in Spry2Y55F/ΔN90-β-catenin–induced HCCs (Fig. 5G). The data suggest that the up-regulation of Ang2 may be a key factor in promoting angiogenesis in our mouse model. Interestingly, Ang2 overexpression has been observed in human HCCs and is associated with tumor invasion and increased tumor microvessel densities.32

Although there are striking differences in tumor latency and incidence between the Spry2Y55F/ΔN90-β-catenin and RasV12/ΔN90-β-catenin–injected mice,
molecular features of both tumors are very similar, which include elevated expression of cyclins, survivin, E-cadherin, p21Cip1, and Ang2 in all HCC samples examined (Fig. 5). These observations support that the 2 sets of oncogenes share similar cellular responses and elicit similar molecular changes during hepatic carcinogenesis.

Altogether our studies support that HCCs induced by Spry2Y55F/H9004N90-/H9252-β-catenin mimic a subset of human HCCs characterized by the deregulation of genes associated with cell proliferation, apoptosis, and angiogenesis.

Discussion

In this study, we identified 76 candidate oncogenes and 37 candidate tumor suppressor genes by correlating expression arrays and array CGH data. Although most of the genes are not well characterized, some of these genes have been implicated in tumorigenesis. For example, MEF2D was identified as a candidate oncogene in 2 murine retroviral insertional mutagenesis studies.33,34 Strikingly, 3 transcriptional coactivators are identified as candidate oncogenes in our study: transcriptional intermediary factor (TIF1), nuclear receptor coactivator 2 (NCOA2) and nuclear receptor coactivator 3 (NCOA3). These transcriptional coactivators are involved in regulating estrogen or androgen signaling. In humans, liver cancer occurs more frequently in men than in women. The identification of co-activators involved in estrogen/androgen pathways as candidate oncogenes for human HCCs may provide clues to how these hormonal factors influence liver tumor development.

Although recent genomic studies have identified large numbers of genes whose expression levels are deregulated in human tumor samples, a major challenge remains as to how to effectively study the functions of these genes in tumor development, especially in vivo. The most common method is to study the individual gene in vitro using tumor cell lines. These studies have limited value because tumor cell lines cultured in vitro have distinct properties from normal or neoplastic cells in vivo. In addition, these studies do not account for the tumor microenvironment. Mouse models are critical tools in dissecting signaling pathways and genetic events that are important for tumor development. However, the development of mouse models for human cancers is hindered by the fact that generating transgenic or knockout mouse lines is both expensive and time consuming. In this manuscript, we present evidence that hydrodynamic transfection is an efficient and flexible method that can be integrated into genomic studies for initial characterization of target genes' function in vivo. As an in vivo screening technique, this will allow us to efficiently and rapidly identify the meaningful genetic interactions during hepatic tumorigenesis.

Almost all reports of Spry2 in tumor pathogenesis are in vitro or xenograft studies. However, whether Spry2 can...
function as a tumor suppressor and whether loss of Spry2 expression is directly involved in tumor development in vivo remains unclear. In a recent study, it was found that the knockdown of Spry2 expression using small interfering RNA accelerates Ras-induced lung cancer development in mice. In our study, we demonstrated that blocking Spry2 function using a dominant negative form of Spry2 cooperates with activated β-catenin signaling to induce liver cancer formation in mice. Our data provide compelling evidence that blocking Spry2 activity can directly participate in liver tumor development in vivo. Our results support a possible important mechanism of propagating Ras/ERK signaling in the absence of Ras mutations via loss of Spry2. Because Spry2 is down-regulated in other tumor types, this may also be a common approach for tumor cells to activate the ERK pathway.

Epidermal growth factor receptor (EGFR) is a well-characterized target of activated β-catenin in liver. Consistently, EGFR messenger RNA expression is elevated in HCC samples from Spry2Y55F/ΔN90-β-catenin mice (Supplementary Fig. 5). Because no tumors were observed in mice injected with activated β-catenin alone (Fig. 1B), it suggests that up-regulation of EGFR by β-catenin is not sufficient to promote tumor development. However, when Spry2Y55F is co-expressed in these cells, Spry2Y55F may further propagate the increased EGFR signaling and eventually lead to tumor development. If this hypothesis is correct, one might expect that the tumors induced by Spry2Y55F/ΔN90-β-catenin are dependent on EGFR expression. Further experiments treating Spry2Y55F/ΔN90-β-catenin mice with EGFR inhibitors will assist to test this hypothesis.

Whether activation of Ras/ERK signaling is the major pathway that promotes HCC pathogenesis remains to be further investigated. Experiments using RNA interference to silence MEK or ERK expression in vivo or treatment of tumor cells with MEK inhibitors will clarify the roles of Ras/ERK in transducing Spry2 signaling in our mouse models. In addition, Spry2 has been shown to interact with other signaling molecules, including caveolin, FRS2, and PTP1B. Further biochemical characterization is required to determine the proteins that interact with Spry2 in mouse hepatocytes. Together these studies will provide novel insight into the molecular mechanisms of Spry2 in promoting HCC development.

There are multiple Spry family proteins expressed in liver. Our dominant negative Spry2 construct may interfere with functions of other Spry family members. To definitively test whether loss of Spry2 is sufficient to cooperate with activated β-catenin to induce liver cancer, a conditional Spry2 knockout mice could be used in our system. This can be achieved by generating albumin-cre; Spry2<sup>flx/flx</sup> mice and then expressing ΔN90-β-catenin in these mice. These experiments will provide additional information as to whether Spry2 functions as a tumor suppressor or whether multiple Spry proteins have to be eliminated to cooperate with β-catenin to induce liver cancer in vivo.

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