

# The Ras/Mitogen-Activated Protein Kinase Pathway Inhibitor and Likely Tumor Suppressor Proteins, Sprouty 1 and Sprouty 2 Are Deregulated in Breast Cancer

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## ABSTRACT

Sprouty (Spry) proteins were found to be endogenous inhibitors of the Ras/mitogen-activated protein kinase pathway that play an important role in the remodeling of branching tissues. We investigated *Spry* expression levels in various cancers and found that *Spry1* and *Spry2* were down-regulated consistently in breast cancers. Such prevalent patterns of down-regulation may herald the later application of these isoforms as tumor markers that are breast cancer specific and more profound than currently characterized markers. *Spry1* and *2* were expressed specifically in the luminal epithelial cells of breast ducts, with higher expression during stages of tissue remodeling when the epithelial ducts are forming and branching. These findings suggest that Sprys might be involved as a modeling counterbalance and surveillance against inappropriate epithelial expansion. The abrogation of endogenous Spry activity in MCF-7 cells by the overexpression of a previously characterized dominant-negative mutant of Spry, hSpry2<sup>Y55F</sup> resulted in enhanced cell proliferation *in vitro*. The hSpry2<sup>Y55F</sup> stably expressing cells also formed larger and greater number of colonies in the soft-agar assay. An *in vivo* nude mice assay showed a dramatic increase in the tumorigenic potential of hSpry2<sup>Y55F</sup> stable cells. The consistent down-regulation of *Spry1* and *2* in breast cancer and the experimental evidence using a dominant-negative hSpry2<sup>Y55F</sup> indicate that Spry proteins may actively maintain tissue integrity that runs amok when their expression is decreased below normal threshold levels. This alludes to a previously unrecognized role for Sprys in cancer development.

## INTRODUCTION

Growth factor signaling by receptor tyrosine kinases regulates important processes in target cells, including activation of the Ras/mitogen-activated protein (Ras/MAP) kinase cascade, which has been shown to be central to both proliferation and differentiation of cells (1, 2). Since the discovery of the Ras/MAP kinase pathway over a decade ago, various genes or proteins that modulate the activity of the basic pathway components have been discovered (3, 4). However, given the putatively vital role of the Ras/MAP kinase pathway in such key processes, it would be expected that dysfunction of any of these key components would result in developmental disorders and inappropriate proliferation of cells, which are seen in various cancers (5, 6). Such deregulation has been documented with the inappropriate activation or overexpression of various receptor tyrosine kinases (*e.g.*, epidermal growth factor receptor and platelet-derived growth factor receptor) in cancers (7). Oncogenic forms of Ras proteins that have

been constitutively activated by a point mutation are also expressed frequently in tumors. This enables them to cause hyperactivation of the MAP kinase pathway, independent of any upstream ligand stimulation (7). Ras has been shown to be hyperactivated in 90% of pancreatic, 60% of thyroid, and 45% of colorectal cancers (8).

Ras/MAP kinase signaling can be down-regulated in a number of ways: (a) feed-back phosphorylation of the receptors on serine and threonine residues by MAP kinases; (b) dephosphorylation of key receptor tyrosine residues by tyrosine phosphatases; (c) competition with the binding of growth factors; and (d) by selective tagging and endosomal destruction of growth factor/receptor complexes (3, 4).

Recently, a specialist Ras/MAP kinase inhibitor protein was discovered in a *Drosophila* genetic screen designed to identify components in fibroblast growth factor (FGF)-induced tracheal branching (9). The *Drosophila* Sprouty (dSpry) protein is induced by activation of the Ras/MAP kinase pathway and acts back on the pathway to inhibit it by an unspecified mechanism (9–12). Later work indicated that dSpry is also an inhibitor of the Ras/MAP kinase pathway induced by other receptor tyrosine kinases, including the epidermal growth factor receptor during eye development and oogenesis (10–12).

Four mammalian *Sprouty* genes have since been identified, based on sequence similarities to *dSpry* (13, 14). Mammalian *Sprys* were found to be highly expressed within localized domains in the embryo, which overlapped with or are immediately adjacent to known expression domains of one or more *Fgf* genes (14–17). Conserved functions were found between dSpry and the mammalian *Sprys* in negative regulation of organogenesis. Mammalian *Sprys* have been shown to particularly function as negative regulators in FGF signaling during vertebrate embryonic development. The overexpression of mouse Spry2 or Spry4 results in the repression of FGF-mediated limb development in the chick (15) and inhibition of lung branching morphogenesis (14, 18, 19). In addition, the overexpression of mSpry4 in mouse embryos inhibited branching and sprouting of small blood vessels (20). The overexpression of Spry proteins *in vitro* was also found to inhibit FGF- and vascular endothelial growth factor-induced proliferation and migration by repressing pathways that lead to MAP kinase activation (21–23). However, Spry proteins did not affect the EGF- or phorbol ester-induced MAP kinase activation (21, 23). This data suggests that mammalian Spry proteins are not general inhibitors of the receptor tyrosine kinase-induced extracellular signal-regulated kinase signaling but rather selective inhibitors of particular receptor tyrosine kinase signaling. Currently, it is not known whether this mammalian specificity is because the target of the Sprys is exclusive to the FGF pathway, or because there is a tight spatial or temporal regulation of functionally related genes.

The four mammalian Spry proteins are truncated in comparison to dSpry. However, they have a highly conserved COOH-terminal cysteine-rich domain. Several short sequences in the NH<sub>2</sub>-termini of all of the Spry proteins distributed throughout the protein family are also conserved (24). There is good evidence that the cysteine-rich domain is a targeting domain that locates Spry proteins to membranes in

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activated cells (25, 26). Several groups have indicated that tyrosine phosphorylation is necessary for Spry proteins to function as MAP kinase inhibitors. The target tyrosine lies in one of the small conserved regions in the NH<sub>2</sub> terminus (Y<sup>55</sup> in hSpry2; ref. 23, 27, 28). Currently, the only protein that has been shown to bind to the phosphorylated Y<sup>55</sup> is the multifunctional E3 ubiquitin transferase and docker protein c-Cbl. The point-mutant hSpry2<sup>Y55F</sup> translocates from the cytosol to the membrane after FGF stimulation but does not bind c-Cbl and is not functional as a Ras/MAP kinase inhibitor (28). The exact mode of action of any of the Spry proteins remains to be elucidated.

Accumulated evidence indicates that Spry proteins provide a means to turn off the Ras/MAP kinase pathway and that this process, in coordination with pathway activation, may play an important role in modeling branching tissues, such as in development of the lung, kidney tubules, vascular system, and breast ducts. We reasoned that if Spry expression or function was deregulated, that this may be manifested in the form of abnormal development or in deregulated growth as seen in cancers. We decided to test the latter possibility and asked the question whether Spry may be deregulated in tumors to allow for hyperactivation of the Ras/MAP kinase pathway observed frequently in many cancers.

Our primary objective was to interrogate the expression level of *hSpry1* and *hSpry2* in a variety of human cancers and then to follow-up any consistent patterns of expression in a particular cancer with analysis of paired clinical samples. Ramaswamy *et al.* (29) created a public microarray database containing the gene expression profiles of 218 human tumors and 90 normal tissues samples, representing 14 common human cancer classes. We have queried this database, the Global Cancer Map ([www-genome.wi.mit.edu/MPR/GCM.html](http://www-genome.wi.mit.edu/MPR/GCM.html)) in our screen for deregulation of *Spry* genes in cancer. We show that *hSpry2* is down-regulated in breast cancers and provide evidence that Sprys may have tumor suppressing activity in the breast.

## MATERIALS AND METHODS

**Synthesis of [ $\alpha$ -<sup>32</sup>P]dCTP-Labeled cDNA.** NH<sub>2</sub>-terminal half of *hSpry1* (nucleotide 1–540) and *hSpry2* (nucleotide 1–531) cDNA fragment was purified from pBluescript KS(-) [Stratagene, San Diego, CA, and St. Louis, MO] by *Bam*HI/*Xho*I and *Eco*RI/*Xho*I restriction digestion, respectively. The cDNA was labeled by random oligonucleotide priming (High Prime DNA Labeling Kit, Roche, Mannheim, Germany) according to the manufacturer's instructions, in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP (6000Ci/mmol, Amersham Pharmacia, Freiburg, Germany). The labeled probes were purified by spin-column centrifugation (Probe QuantTM G-50 Micro Columns, Amersham Pharmacia) and met or exceeded the manufacturer's recommendation for specific activity.

**Hybridization of cDNA Probes to Cancer Profiling Array.** The [ $\alpha$ -<sup>32</sup>P]dCTP-labeled *hSpry* DNA probes were hybridized to commercial cDNA blot, the Cancer Profiling Array (Clontech, Palo Alto, CA). The probe was hybridized to each blot according to the manufacturer's instructions and exposed to X-ray film at -70°C for various lengths of times (24 hours to 3 days). To ensure equality of loading of the cDNA samples on the array, the array was stripped and reprobed with human ubiquitin.

**RNA Extraction and Reverse Transcription.** Primary breast tumors (including 18 ductal and 1 lobular carcinomas) and adjacent normal-appearing breast tissue were obtained from 19 patients who underwent surgery with malignant tumors. The samples were obtained with approval of the appropriate institutional ethics committee and in accordance with the National Health and Medical Research Council of Australia guidelines for the conduct of research involving humans and included one stage I carcinoma, 14 stage II carcinomas, two stage III breast carcinomas, and two unclassified samples. Total RNA was extracted from the primary breast tissues. RNA extraction was done using RNA kits, as recommended by the manufacturer (Qiagen, GmbH, Hilden, Germany). RNA was treated with RNase-free DNase I (Qiagen) for 30 minutes at room temperature. Reverse transcription reactions were done in 5  $\mu$ g of

DNase-treated RNA, 1  $\mu$ l of oligo(dT)<sub>12–18</sub>, and SUPERSCRIP<sup>TM</sup> II RNase H-, using the First-Strand Synthesis System for reverse transcription-PCR kit protocol (Invitrogen Life Technologies, Inc., Carlsbad, CA).

**Quantitative Real-Time PCR.** For each PCR reaction, 2  $\mu$ l of cDNA was mixed with 500 mM primers and 4 mM MgCl<sub>2</sub>, using the LightCycler-FastStart DNA Master SYBR Green Kit in a LightCycler (Roche) according to the manufacturer's instructions. PCR reactions were carried out in triplicates with the following PCR primers: human *Spry2*, forward 5' GCGATCACGGAGTTCAG 3' and reverse 5' GTGGAGTCTCTCGTGT 3'; human *Spry1*, forward 5' AGGGCTATCTTCTCTAGCA 3' and reverse 5' GTGAGAAGCATGGGGT 3'; and human *GAPDH*, forward 5' GGTGTGAACCATGAGAAG 3' and reverse 5' CCACAGTTTCCCGGAG 3'. The crossing points that are calculated by the LightCycler software take into account the difference in amplification efficiency of the target and the reference genes. Crossing points of *Spry* (target) amplification products were normalized to the crossing points of *GAPDH* (reference) amplification products. Fold changes in gene expression of *hSpry1* and *hSpry2* between normal and tumor tissue were computed.

**Histological Analysis.** Mammary glands were harvested and immediately fixed in 10% formalin (VWR Scientific, Brisbane, CA). Whole-mount preparations of no. 4 mammary glands or primary breast tissues were paraffin-embedded, sectioned at 4  $\mu$ m, and stained with Mayer's hematoxylin-and-eosin B-phloxine (Sigma, St. Louis, MO).

**In situ Hybridization Analysis.** For RNA *in situ* hybridization, the NH<sub>2</sub>-terminal of *hSpry1* and *hSpry2* cDNA sequence was subcloned into pBlue-script KS(-) [Stratagene], the plasmids were linearized, and the sense and antisense riboprobes were synthesized from the T3 or T7 transcription sites by using 5U of T3 or T7 RNA polymerase, respectively (Promega, Madison, CA). Cryosections were probed with sense or antisense digoxigenin-labeled riboprobes. Hybridization of the riboprobe was visualized immunohistochemically with an alkaline phosphatase-conjugated antidigoxigenin antibody (Roche).

**Immunohistochemistry.** Cryostat sections (10  $\mu$ m) were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. Sections were washed in PBS and incubated for 1 hour with rabbit polyclonal antibodies for *hSpry2* (Upstate Cell Signaling, Charlottesville, VA). After washing in PBS, the slides were incubated for 1 hour with biotinylated goat antirabbit from Dako LSAB 2 system peroxidase (Dako Corporation, Carpinteria, CA). After one wash in PBS, the slides were incubated for 20 minutes with streptavidin conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) before washing again with PBS for 10 minutes. Antibody staining was done by treatment with diaminobenzidine. Sections were washed in PBS and counterstained with Mayer's hematoxylin. All of the incubation and staining steps were done at room temperature. A negative control without primary antibody was done for all of the samples.

**Tissue Culture.** The MCF-7 and T47D cell lines were obtained from American Type Culture Collection (Manassas, VA). The cell lines were cultured in RPMI 1640 or DMEM high-glucose, supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine. Cells were cultured at 37°C and 5% CO<sub>2</sub>. Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT), and all of the other tissue culture materials were obtained from Sigma Chemical Co. (St. Louis, MO).

**Treatment of Cells with 5-Aza-Deoxycytidine and Trichostatin.** Cells were seeded at a density of 5  $\times$  10<sup>5</sup> in 100-mm dishes. The 5-aza-deoxycytidine was freshly prepared in deionized water and filter-sterilized. Trichostatin was freshly prepared in ethanol and filter-sterilized. Twenty-four hours later, cells were treated with 2.5  $\mu$ M 5-aza-deoxycytidine (Sigma Chemical Co.) or treated with 300 nM of trichostatin (Sigma Chemical Co.). The medium was changed every 24 hours. In instances where cells were doubly treated, cells were subjected to 96 hours of 5-aza-deoxycytidine treatment, and 300 nM of trichostatin was added in the last 24 hours. After treatment, cells were washed with PBS, and total RNA was isolated for reverse transcription-PCR.

**Reverse Transcription-PCR Analysis.** Reverse transcription was done as mentioned above. PCR reactions were carried out using 2  $\mu$ l of cDNA with the following PCR primers: The *hSpry2* and *hSpry1* primers for real-time quantitative PCR analysis were used, and primers for human *Maspin* were forward 5' GCTTTGCGGTGATCTGTTC 3' and reverse 5' GATCTGACCTTTCGTTTCTTCCA 3'. The PCR conditions are as follows: 1 cycle at 96°C for 2 minutes; and 35 cycles at 96°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Samples were kept at 72°C for 10 minutes after the last

cycle. The human  $\beta$ -microglobulin gene was coamplified as internal controls. Primers for  $\beta$ -microglobulin were as follows: forward, 5' CTCGCGC-TACTCTCTCTTTCTGG 3'; and reverse, 5' GCTTACATGTCCTGCATC-CCACTTAA 3'. The PCR products were resolved by electrophoresis on 1% agarose gels.

**Stable Transfection.** The hSpry2<sup>Y55F</sup> expression construct was made by subcloning the full-length hSpry2<sup>Y55F</sup> cDNA into the *Bam*HI-*Xho*I sites of the constitutive mammalian expression vector pCMVTag2B (Stratagene) to generate the pCMV-hSpry2<sup>Y55F</sup> (FLAG-tagged) construct. The MCF-7 cell line was transfected with pCMV-hSpry2<sup>Y55F</sup>, under the control of a cytomegalovirus promoter for constitutive expression. For control purposes, the MCF-7 cell line was transfected with a vector control. Cell lines are established as MCF-7-hSpry2<sup>Y55F</sup> and MCF-7 control cells.

**Proliferation Assay.** MCF-7 and its derived stable cell line were seeded ( $1 \times 10^4$ /well) in a 96-well plate in triplicates. After incubation at 37°C in 5% CO<sub>2</sub> for 12 hours, the number of living cells was measured using a CellTiter 96 AQueous One Solution Cell Proliferation Assay [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS); Promega, Madison, WI) according to the manufacturer's instructions. Assays were done with addition of a small amount of the CellTiter 96 AQueous One Solution Reagent directly to culture wells, incubated for 2 hours at 37°C in 5% CO<sub>2</sub>, and then recording absorbance at 485 nm with a 96-well plate reader. The quantity of formazan product as measured by the amount of 485-nm absorbance is directly proportional to the number of living cells in culture.

**Soft Agar Colony Formation.** MCF-7 and its derived cells were cultured in six-well plates first covered in soft agar layer (RPMI 1640 with 0.5% agar and 10% fetal bovine serum). The top layer contained  $5 \times 10^3$  cells in RPMI 1640 with 0.35% agar and 10% fetal bovine serum. Medium was added to the top layer to prevent drying of the agarose gel. After 14 days, the colonies were stained purple with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich).

**In vivo Tumor Formation.** A total of  $5 \times 10^5$  MCF-7-hSpry2<sup>Y55F</sup> or MCF-7 control cells were suspended in 200  $\mu$ l Matrigel (BD Biosciences, Palo Alto, CA) and injected into the mammary (axillary) fat pad of BALB/c nude mice, which simultaneously received a 60-day release pellet containing 0.72 mg of  $\beta$ -estradiol (Innovative Research of America, Toledo, OH). After 9 weeks, the tumors were excised, weighed, and tabulated.

**Statistics.** All of the data are presented as mean  $\pm$  SE. We assessed differences between groups by two-tailed nonpaired Student's *t* test using the Graphpad Prism statistical software.

## RESULTS

**The hSpry2 Expression Is Down-Regulated in Breast Cancer in a High Percentage of Analyzed Samples.** As an initial investigation, we interrogated the Global Cancer Map microarray database about the levels of expression of hSpry1 and 2. The hSpry1 and 2 were chosen because when all of the hSprys are compared, these two isoforms seem most similar to each other in function, protein sequence, and expression pattern. We also suspect that there might be some level of redundancy in the physiological function of the two proteins.

In the analysis of gene array data (Fig. 1), the gene expression levels in cancer tissues were compared with those levels in normal healthy tissue. Mammalian Spry2, in particular, has been shown to be associated particularly with FGF signaling molecules, Fgf8, and FGFR1 (15, 17, 30). Both of these genes, together with the various FGFRs, have been found to be overexpressed in various cancers (31, 32). We have tabulated them alongside the hSprys for comparison. We have shown previously that c-Cbl is functionally associated with hSpry2 (28); therefore, c-Cbl expression levels were also included in the analysis.

In terms of patterns of hSpry expression (Fig. 1), it was apparent that hSpry2 levels were diminished in breast cancer. There are other trends observable with other cancers, but the highlighted case was the most profound. Because these results are only indicative, we proceeded to investigate additionally the expression and tissue localization of the two Spry genes in breast development and cancer.

**The hSpry2 and hSpry1 Expression Is Down-Regulated in Breast Cancer as Assessed on a Matched-Tissue Cancer-Profiling Array.** We next investigated the expression levels of hSpry1 and 2 in a Cancer Profiling Array, where cDNA from matched normal and tumor tissue samples are spotted on a blot.

The hSpry2 probe was prepared and tested for specificity before subjecting it to hybridization to the Cancer Profiling Array. On the resultant blot, a high proportion of breast cancer samples were observed to have a repressed expression of hSpry2 (Fig. 2A). Using densitometry, the intensity of the radioactive probe signal from each cDNA sample was quantitated. Signals from breast tumor cDNA were compared with respect to that of normal breast tissue cDNA. The

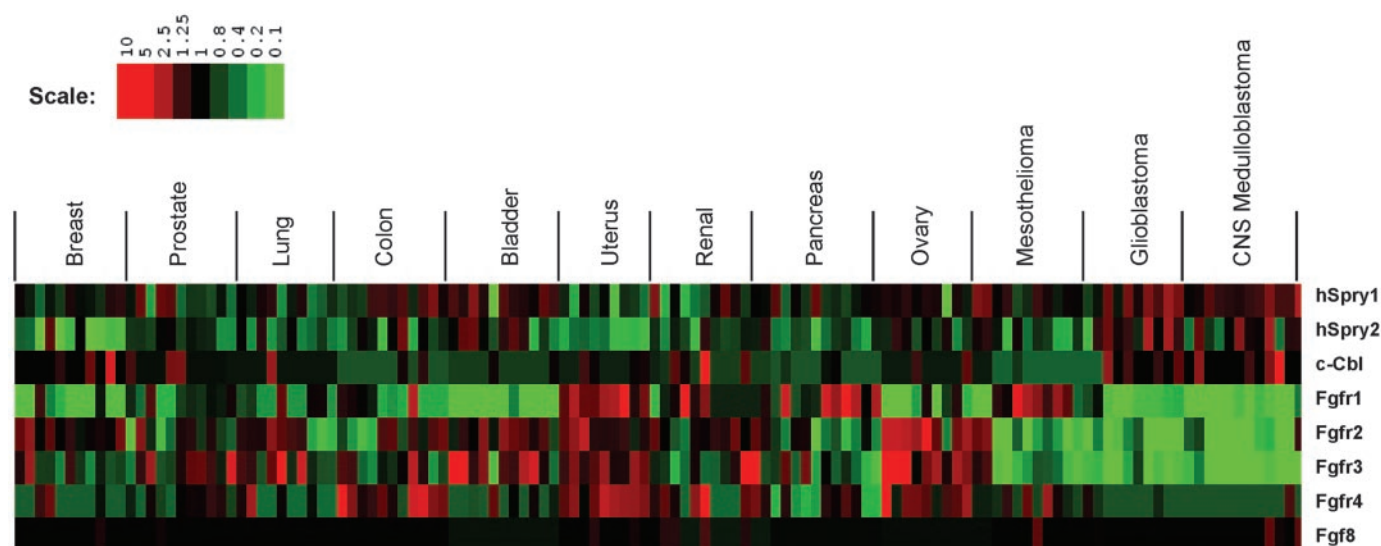


Fig. 1. Analysis of microarray database (Global Cancer Map) comparing the expression of Spry1 and 2 in human cancers with respect to normal tissues. Tumor tissues, spanning 12 common tumor types, and unmatched normal tissues were subjected to oligonucleotide microarray (Affymetrix GeneChips, Santa Clara, CA) gene expression analysis. The gene expression for the individual tumor tissues was compared with the mean values of that of the normal tissue. The panel shows the expression of hSpry1 and hSpry2 in human cancers, with respect to normal tissues. The hSpry2 is down-regulated in the breast cancer. (Explanation of scale: red, an up-regulation in gene expression in tumors with respect to normal tissue; and green, down-regulation in gene expression in tumors with respect to normal tissue. The scale indicates the fold change in gene expression.)



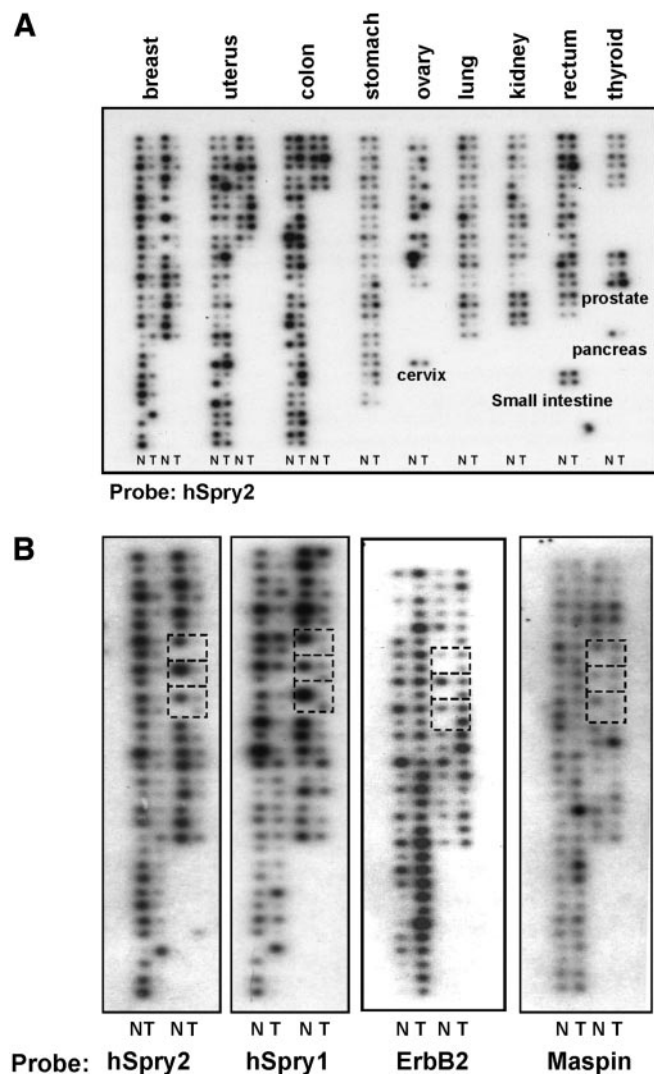


Fig. 2. The cDNA array studies show a down-regulation of *hSpry1* and 2 in breast tumors. **A**, The Cancer Profiling Array contains pairs of cDNAs generated from matched tumor and normal tissue samples from individual patients, spotted side by side on a nylon membrane. All of the samples on these arrays are normalized to two different housekeeping genes:  $\beta$ -actin and ubiquitin. There are 50 matched pairs of normal and tumor cDNA from 50 breast cancer patients. The [ $\alpha$ - $^{32}$ P]-labeled NH<sub>2</sub>-terminal half of *hSpry2* cDNA was hybridized to the Cancer Profiling Array. The *hSpry2* expression is down-regulated in breast cancer. A high (96%) proportion of breast cancer samples shows at least an arbitrary 1.2-fold down-regulation in the expression of *hSpry2*. Comparatively, the changes in expression of *hSpry2* in other tumors were not as significant as that for the breast tumors. **B**, The group of three spots of cDNAs outlined by a box represents a matched pair of normal (top left) and tumor (top right) cDNA from a patient as well as cDNA from the metastases in the same patient (bottom right). The [ $\alpha$ - $^{32}$ P]-labeled NH<sub>2</sub>-terminal half of *hSpry1* cDNA was hybridized to the Cancer Profiling Array. The *hSpry1* expression is down-regulated in breast cancer. A high (78%) proportion of breast cancer samples show at least an arbitrary 1.2-fold down-regulation in the expression of *hSpry1*. To show that the sample population of breast cancer tumors in the blot is a true representation of the population of breast cancers, as well as to show equal loading of cDNA samples on the blot, the blot was stripped and reprobbed for *ErbB2*. Fifty percent of the patients with breast cancer showed at least an arbitrary 2-fold up-regulation in *ErbB2*. The blot was stripped and reprobbed with a gene known to be down-regulated in breast cancer, *Maspin*. The extent of down-regulation of *Maspin* in breast cancer was not as profound and widespread as that observed for *hSpry2*. (T, tumor; N, normal)

breast cancers showing at least an arbitrary 1.2-fold down-regulation in signal were considered to exhibit a down-regulation in expression of *hSpry2*. Forty-eight of 50 (96%) of the paired samples showed a down-regulation of *hSpry2*. Similarly, when the *hSpry1* probe was hybridized to the Cancer Profiling Array, the same trend was observed: Thirty-nine of 50 (78%) of the paired samples showed a down-regulation of *hSpry1* (Fig. 2B).

To show that the sample population of breast cancer tumors in the blot is a true representation of the population of breast cancers, as well as to show equal loading of cDNA samples on the blot, the blot was stripped and reprobbed for *ErbB2*. *ErbB2* is a prognostic marker for breast cancer, and collated data indicates it is overexpressed in 25–30% of breast cancer (33). The *ErbB2* probe was prepared and hybridized to the Cancer Profiling Array. The breast cancers showing at least an arbitrary 2-fold up-regulation in signal were considered to exhibit an up-regulation in expression of *ErbB2*. *ErbB2* was found to be overexpressed in 25 of 50 (50%) breast cancer tumor samples in comparison with the normal samples (Fig. 2B). This is higher than the 30% typically observed in many breast cancer samples but deemed to be within acceptable limits. To additionally ensure equality in loading of the cDNA samples on the membrane, the membrane was reprobbed with a human *ubiquitin* control probe (provided by the manufacturer; data not shown).

Such prevalent patterns of down-regulation of *Sprys* may herald the later application of these isoforms as tumor markers that are breast cancer specific. We decided to examine other molecular markers that have been shown to be down-regulated in breast cancer. Down-regulation of the *Maspin* gene was reported recently in a significant percentage of breast tumors (34, 35), which prompted the authors to speculate that it may be useful as a diagnostic marker. For the sake of comparison, the *Maspin* probe was prepared and hybridized to the Cancer Profiling Array. *Maspin* was not found to be significantly down-regulated in breast tumor samples (Fig. 2B) as reported previously (34, 35). Comparatively, the extent of down-regulation observed with the *Sprys* was more profound and universal.

Although the cDNA array used does not encompass many cancers, and some of the paired samples are not present in statistically relevant numbers, the evidence that *hSpry1* and *hSpry2* are comprehensively down-regulated specifically in breast cancers was compelling, and we decided to further investigate this observation.

**Analysis of Breast Cancer Samples by Quantitative Real-Time PCR Shows a Profound Down-Regulation in Levels of *hSpry1* and 2 in Breast Cancer.** To confirm the data from the Global Cancer Map and Cancer Profiling Array, we needed to compare matched clinical samples and analyze them by an alternative method. To this end, we analyzed 19 matched normal and tumor breast samples (including 18 ductal and 1 lobular carcinomas) using real-time quantitative PCR. These included one stage I carcinoma, 14 stage II carcinomas, and two stage III breast carcinomas (two samples were not classified).

The expression levels of the *Spry* genes in each normal tissue sample (control) was deemed to be 100%, and the comparative level in the matching tumor sample was expressed as a percentage of the control. Each sample was analyzed three times, and a mean value was calculated. Eighteen of 19 (94.7%) of the tumor samples show a significant down-regulation of *hSpry2* (Fig. 3A) and *hSpry1* (Fig. 3B) expression, with respect to normal tissue. A 2–100-fold down-regulation of *hSpry1* and *hSpry2* was observed in the breast tumors.

As before, the expression levels of *ErbB2* were analyzed to show the 19 breast cancer samples used were from a representative patient population. Eleven of the 19 samples (57.8%) showed a significant overexpression of *ErbB2* (data not shown), which is comparable with the values obtained from the Cancer Profiling Array and previously documented reports.

In summary, a profound down-regulation in levels of *hSpry1* and *hSpry2* in breast tumors was observed. The results obtained from the three methods of analysis were essentially in accordance with each other, although a higher percentage of samples showing depressed *hSpry1* levels were observed using real-time quantitative PCR analysis.

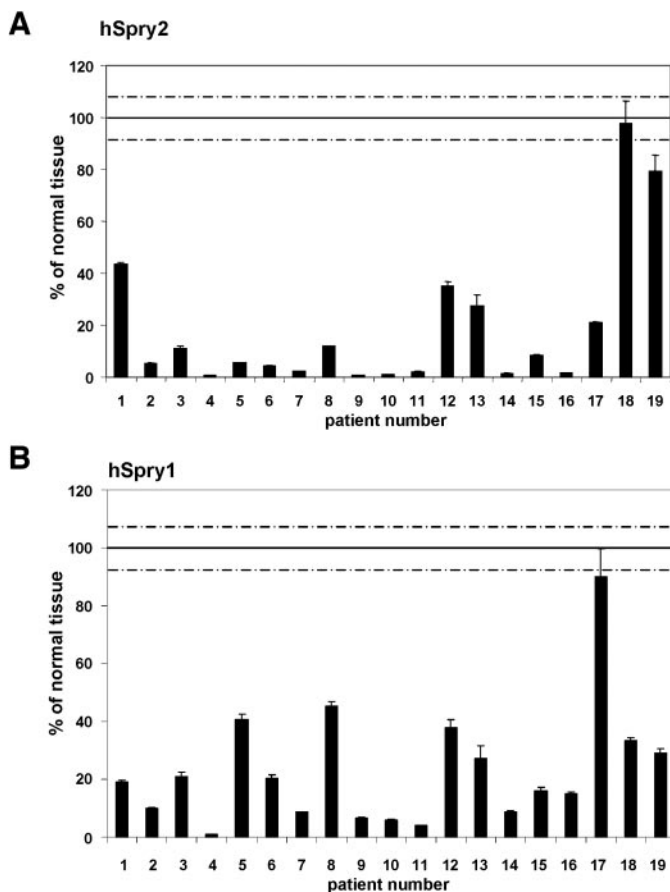


Fig. 3. Analysis of breast cancer samples by quantitative real-time PCR shows a profound down-regulation in levels of hSpry1 and hSpry2 in breast cancer. *A*, The expression of hSpry2 in 19 malignant breast tumors and paired normal tissue was analyzed. The expression of hSpry2 in the normal tissue was averaged and deemed to be 100% (solid horizontal line), and the SE is demarcated with the dotted lines. The tumor samples were expressed as a percentage of the mean expression of hSpry2 in normal tissues. Each sample was analyzed three times and a mean was calculated. Eighteen of 19 (94.7%) of the tumor samples show a significant down-regulation of hSpry2 expression, with respect to normal tissue. *B*, The expression of hSpry1 in 19 malignant breast tumors were analyzed and compared with the baseline expression (100%) of hSpry1 found in normal breast tissue. Eighteen of 19 malignant tumors showed a significant down-regulation of hSpry1. A 2–100-fold down-regulation of hSpry1 and hSpry2 was observed in the breast tumors. Bars,  $\pm$ SE.

**Expression of *mSpry1* and *mSpry2* in Developing Mouse Mammary Gland.** Thus far, it seems from the accumulated data that *Spry1* and *2* are expressed in normal human breast. Currently, there has been no data published on the disposition of *Spry1* and *2* during development of the breast. The breast is a unique organ in that it exhibits mostly postnatal development. At puberty, pubertal hormones stimulate the breast ducts to invade the mammary fat pad, branching by bifurcation until the ducts reach the limits of the fat pad. The final developmental fate of the mammary gland is fulfilled only when pregnancy and lactation occurs. There is an additional branching of the breast ducts as well as a proliferation of secretory alveoli during pregnancy that occurs to prepare for lactation. After weaning, the secretory epithelium involutes by apoptosis until the gland once again resembles that of a virgin (36, 37).

We wanted to investigate the expression levels and tissue localization of *Sprys* in the various stages of breast development. Although it is appreciated that there are differences between human and murine development, we next analyzed mouse tissue sections to identify the temporal and spatial localization of *mSpry1* and *2* over the described stages of development.

*In situ* hybridizations were carried out as described in Materials and

Methods, and representative results are shown in Fig. 4*A*. *Spry2* is highly expressed in developing mammary ducts in 2- and 6-week-old females and seems to be confined specifically to the epithelial lining of the mammary ducts and is absent in the stroma and adipose tissues. It is similarly expressed in the male mammary gland tissue at the same stages (data not shown). The level of expression starts decreasing as the mice reach sexual maturity, as evidenced by the lower levels apparent at 16 weeks. In pregnant mice, the level of *mSpry2* becomes elevated again when it is highly expressed in the actively developing alveoli (Fig. 4*B*). The *mSpry2* expression then diminishes in the lactating female and seems to be totally absent during the involution phase. Fig. 4*C* shows the sense control probe for *mSpry2* hybridized to a section of the mammary gland from a pregnant mouse, showing a lack of nonspecific staining.

In summary, it seems that *mSpry2* is expressed in development during the stages of tissue modeling when the epithelial ducts are forming and branching (puberty) and later when the alveoli are actively developing (pregnancy). Such a developmental scenario parallels the tissue modeling in the *Drosophila* trachea and mammalian lung formation, whereby tubular epithelium is remodeled into a branching system under the regulatory influence of the various *Sprys*.

We performed similar studies for *mSpry1* to examine the expression and localization in breast tissue. The *mSpry1* was found to be colocalized with *mSpry2*, albeit at lower expression levels in a pregnant mouse (Fig. 4*D*). This hints that the two isoforms of *Spry* may have some level of redundancy in their physiological functions during breast development.

***In situ* Analysis of Human Breast Tissue Shows the Down-Regulation of *Spry* Isoforms in Breast Cancer.** We next investigated the disposition of hSpry isoforms in human breast tissue, both normal and cancerous. It is desirable to locate and show the situation in an unaffected area within the same tissue selected for cancer state analysis. The disposition of hSpry2 and hFgf8 are shown within healthy tissue removed during cancer surgery (Fig. 5*A*). The staining shows clearly that both hSpry2 and hFgf8 are expressed in epithelial cells lining the breast ducts. When the tumor-encompassing tissue (invasive ductal carcinoma, grade 3) from the same sample was analyzed, it was apparent that there is no detectable hSpry2 or hSpry1 expression, although hFgf8 (shown previously to be up-regulated in breast cancer; ref. 38) is still apparent (Fig. 5*B*). The same observations were noted for another breast cancer specimen, which is an invasive ductal carcinoma (grade 2; data not shown).

Immunohistochemistry was then done to correlate the gene expression to protein levels. In a segment of healthy breast tissue, hSpry2 was shown to be present in the epithelial cells that line the lumen of mammary ducts (Fig. 5*C*). Correspondingly, when the epithelial tissue has become cancerous (in the same individual; invasive ductal carcinoma, grade 3), there is little or no Spry2 protein present (Fig. 5*D*). In the same cancerous tissue, both ErbB2 (stained with Neu-2 antibody) and Fgf8 are present in relatively high amounts (Fig. 5*D*). Both ErbB2 (33) and Fgf8 (38) have been shown previously to be up-regulated in breast cancer.

Taken together, the expression level and disposition of proteins indicate that both hSpry1 and, more profoundly, hSpry2 are present in normal luminal epithelial tissue. However, when this tissue becomes cancerous, the expression (and protein) levels decrease substantially in a high percentage of the breast tumors analyzed. The extent of down-regulation of *Sprys* was not found to be correlated to the staging of cancer in the patients we analyzed, although the sample size may not be large enough to be conclusive.

**Down-Regulation of hSpry2 in Breast Cancer Is Not Because of Epigenetic Silencing.** We have shown that a high percentage (94.7%) of breast cancers show a down-regulation of *Spry1* and *2*.



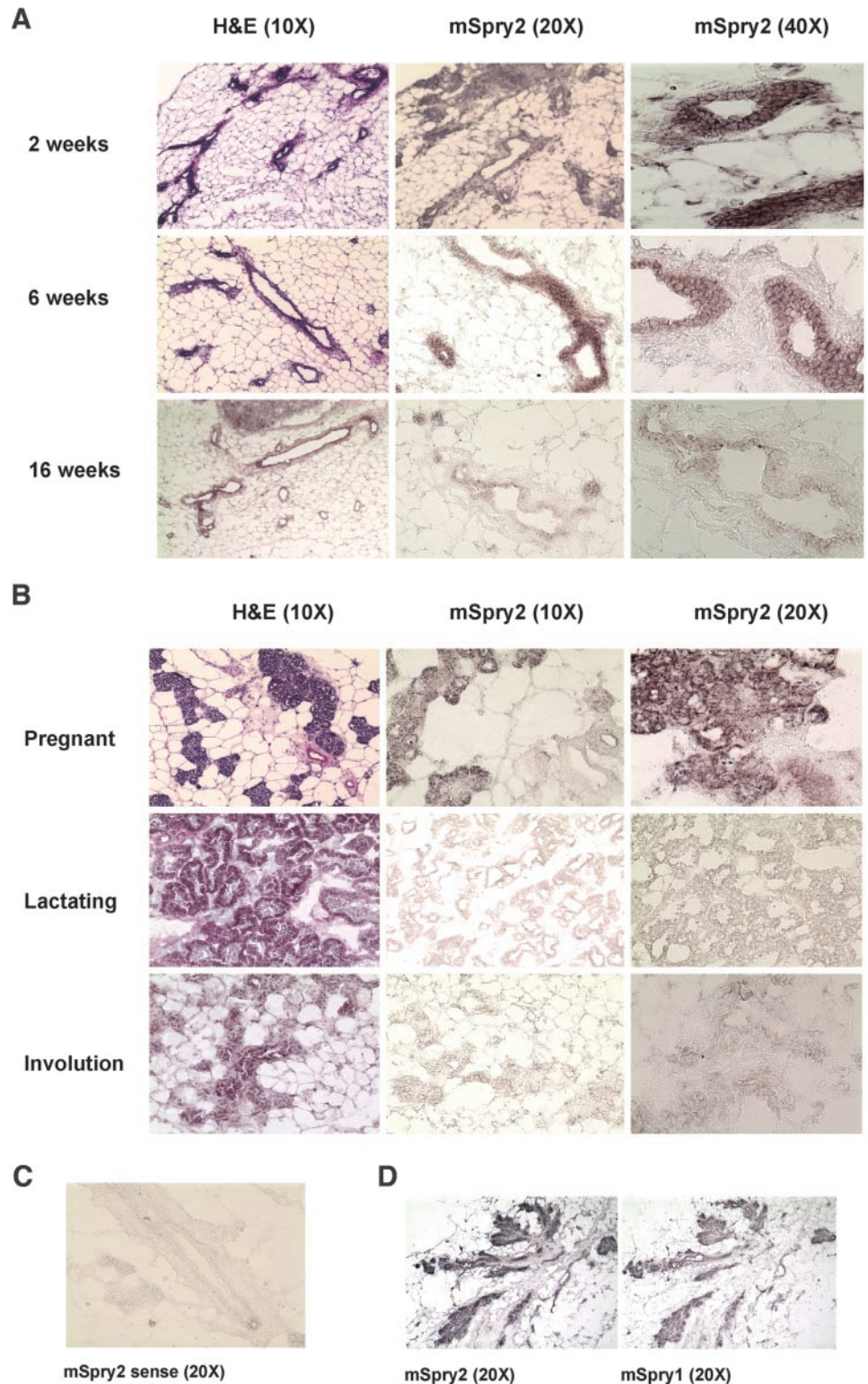


Fig. 4. Mouse mammary glands at different stages of development show that *mSpry1* and *2* are localized in the epithelium of the mammary ducts. **A**, H&E and *in situ* staining of mammary glands of 2 weeks, 6 weeks, and 16 weeks female mice. The *mSpry2* is highly expressed in developing mammary ducts in 2- and 6-week-old females and seems to be confined specifically to the epithelial lining of the mammary ducts and is absent in the stroma and adipose tissues. The level of expression starts decreasing as the mice reach sexual maturity, as can be evidenced by the lower levels apparent at 16 weeks. **B**, H&E and *in situ* staining of mammary glands of mice undergoing pregnancy, lactation, and involution. In pregnant mice, the level of *mSpry2* becomes elevated again when it is highly expressed in the actively developing alveoli. The *mSpry2* expression then diminishes in the lactating female and seems to be totally absent during the involution phase. **C**, the *hSpry2* sense control probe for *mSpry* hybridized to a section of the mammary gland from a pregnant mouse, demonstrating a lack of nonspecific staining. **D**, *in situ* staining for *mSpry2* (left) and *mSpry1* (right), respectively, showing colocalization of the two *Spry* isoforms, specifically in the luminal epithelial cells of the mammary ducts of a pregnant mouse.

This is unlikely to be caused by genetic changes such as chromosomal rearrangements or deletions. Besides, there is no evidence in the literature documenting alterations in the chromosomal regions where *hSpry1* and *hSpry2* are located. We next examined the possibility of epigenetic causes in contributing to the down-regulation of *Sprys*. Many genes are known to be silenced by methylation of gene promoter regions in cancer. Methyl-CpG-binding domain proteins are recruited to the methylated cytosines, and they work with histone deacetylases to repress the transcription of a gene (39, 40).

We chose breast cancer cell lines where previous analysis had shown low *hSpry2* levels. T47D cells were treated with the DNA methyl-transferase inhibitor, 5-aza-deoxycytidine, and the deacetylases inhibitor, trichostatin. As a positive control, we have monitored the expression of a *Maspin*, which is known to be silenced by methylation and/or histone acetylation in cancers (41). The results show that although *Maspin* expression increases when cells are treated with 5-aza-deoxycytidine, there was no increase in the expression of *hSpry2* (Supplementary Fig. 1). Likewise, *Maspin* expression in-

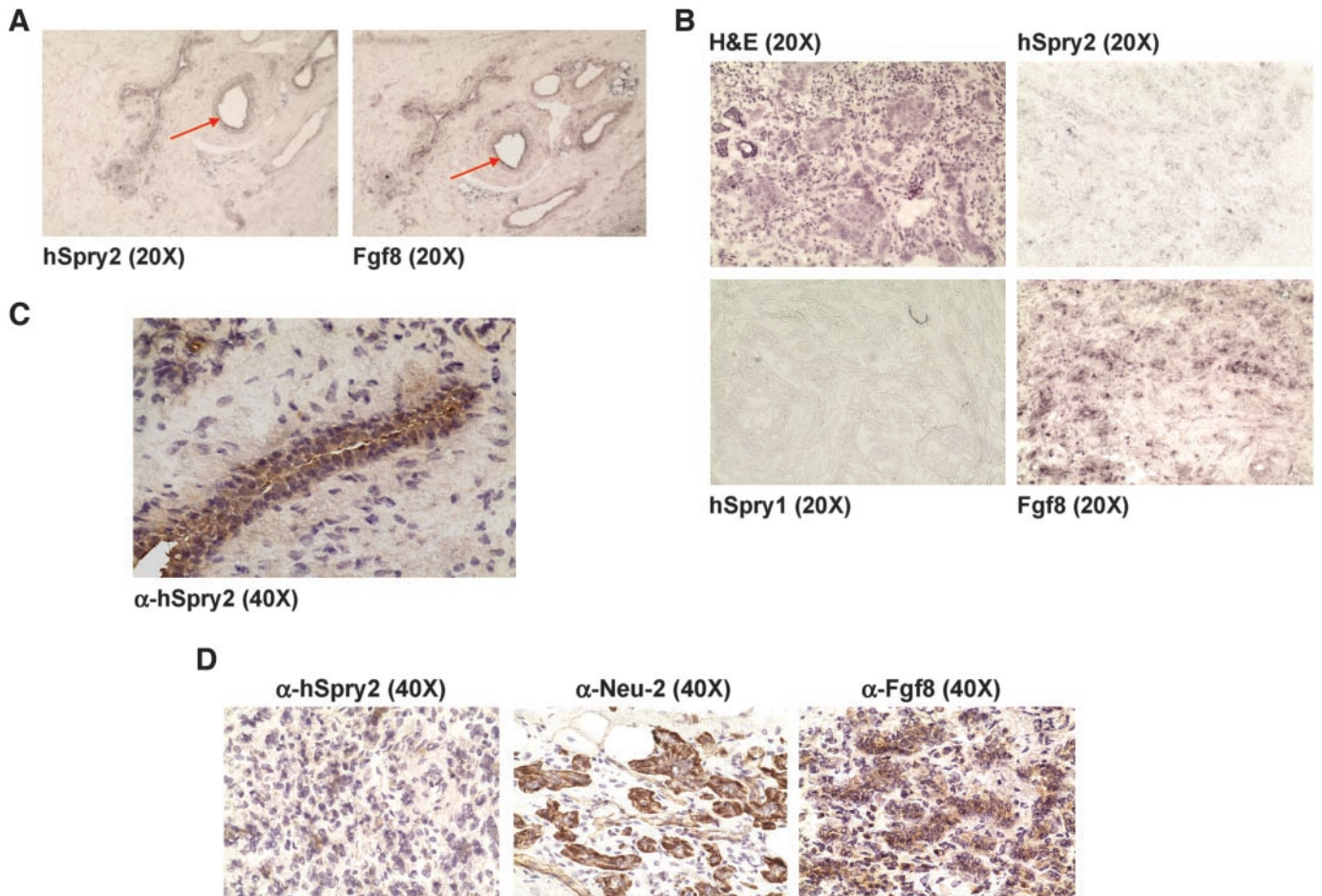


Fig. 5. *In situ* analysis of human breast tissue shows the down-regulation of *Spry* isoforms in breast cancer. A, *in situ* staining for *hSpry2* (left) and *hFgf8* (right) in a normal section of tissue found in a human ductal carcinoma. Both *hSpry2* and *hFgf8* were colocalized in the epithelial lining of breast ducts. B, H&E and *in situ* staining for *hSpry1*, *hSpry2*, and *hFgf8* in a human ductal carcinoma (grade 3). There is no detectable *hSpry2* or *hSpry1* staining, although *hFgf8* (previously shown to be up-regulated in breast cancer) staining is still apparent. C, immunohistochemical staining of *hSpry2* in normal human breast tissue. The *hSpry2* localizes specifically in the epithelial lining of the breast duct. D, immunohistochemical staining of *hSpry2*, ErbB2 (using Neu-2 antibody), and *Fgf8* in an invasive ductal carcinoma (grade 3). The *hSpry2* expression is down-regulated in breast cancer. In the same tissue, both ErbB2 (stained with Neu-2 antibody) and *Fgf8* are present in relatively high amounts. ErbB2 and *Fgf8*, which have been shown previously to be up-regulated in breast cancer, have been used as positive controls to show the integrity of the tissue.

creases when cells are treated with trichostatin, but there was no concurrent increase in the expression of *hSpry2* (Supplementary Fig. 1). To preclude the likelihood of an atypical, cell-specific result, the experiment was repeated on another breast cancer cell line, MCF-7, where the same observations were reiterated (data not shown).

To confirm additionally that methylation is not responsible for the silencing of *Spry2* expression in breast cancer, we used bisulphite PCR methods to sequence the CpG-rich islands found 5' upstream of the *hSpry2* gene in a human breast cancer specimen. We noted that the *hSpry2* gene has a CpG region extending from the 5' upstream sequence through the first exon and into the adjacent intron. We sequenced the region 600 bp upstream of the first exon, which was noted to be particularly CpG-rich, having a guanine cytosine content of 74.8% and an observed CpG/expected CpG ratio of 0.936 (using CpG Island Searcher).<sup>6</sup> We observed that the cytosine residues in these CpG-rich dinucleotides were not methylated (data not shown).

These results show clearly that the decreased expression levels of *hSpry2* seen in breast cancers are neither due to DNA methylation nor histone hypoacetylation. Taken together, it is likely that a transcriptional factor(s) upstream of *Spry* has been aberrated by a mechanism not involving epigenetic silencing. Gross *et al.* (42) highlighted *Spry1*

as the downstream transcriptional effector of WT1 in the developing kidney. The situation of the consistent down-regulation of *hSprys* in breast cancer is unlikely to be because of the status of WT1 expression, as it has been established that WT1 is up-regulated in breast cancer (43). When the WT1 probe was hybridized to the Cancer Profiling Array, no difference in WT1 expression was observed between normal and tumor breast tissue samples (data not shown). Additional characterization of the transcriptional mechanism of the *Spry* genes in normal *versus* cancerous breast tissue is required to shed light on the transcriptional factors involved.

**Inhibiting *Spry*'s Function in MCF-7 Cells Results in Cells Proliferating Faster and Exhibiting Anchorage-Independent Growth.** A central question arises when the gene that plays a pivotal role in a major signal transduction pathway shows consistently aberrant expression in a particular cancer—Is the change contributing to the tumorigenic process? We have seen that *Spry1* and 2 are highly expressed at times in the breast during extensive changes in ductal patterning, and that the expression in normal mature breasts may be sufficient to play a surveillance role against inappropriate cell growth or movement. It may be postulated that down-regulation of *Sprys* in cancer allows for uncontrolled proliferation because of unchecked upstream hyperactivation of the MAP kinase pathway and thereby permits the tumorigenic process to proceed.

<sup>6</sup> Web address: <http://cmt.hsc.usc.edu/cpgislands/>.



Therefore, we wanted to address whether down-regulation of hSpry2 in breast cancer has a role to play in tumorigenesis. To simulate the down-regulation in expression of *Sprys* in breast cancer, we decided to quench the function of endogenous wild-type Sprys by overexpressing the Y<sup>55F</sup> mutant of hSpry2 and compare the tumorigenic potential of these cells with parental cells in nude mice. There have been several reports that the tyrosine phosphorylation of Y<sup>55</sup> on hSpry2 is necessary for its Ras/MAP kinase inhibitory function (23, 27, 28). Some reports provide compelling evidence that the hSpry2<sup>Y55F</sup> mutant functions as a dominant-negative factor in inhibiting hSpry2 function (23, 27). Furthermore, there is a distinct possibility that hSpry1 and hSpry2 may play redundant roles fulfilling the general function of Spry; however, a cell line overexpressing hSpry2<sup>Y55</sup> will nullify the effect of both wild-type Spry1 and Spry2, because the tyrosine is highly conserved in all of the Spry isoforms, and the function that occurs because of phosphorylation on this residue will be blocked by an overexpression of such a point mutant, irrespective of its isoform.

The hSpry2<sup>Y55F</sup>-transfected stable MCF-7 cell lines were generated as described in Materials and Methods and as an initial analysis, the effect of overexpression of hSpry2<sup>Y55F</sup> was assessed based on the proliferation rate of the cells. The hSpry2<sup>Y55F</sup>-transfected clones showed a 22% significant increase in proliferation over control

MCF-7 cells ( $P = 0.0034$ ; Fig. 6A), indicating that nullifying the effect of wild-type Sprys impacts on the growth rate of the MCF-7 cells.

Next, we assessed the anchorage-independent growth of the stable hSpry2<sup>Y55</sup> cells in the formation of colonies, using the soft agar assay in comparison with the control MCF-7 cells. The hSpry2<sup>Y55</sup>-transfected clones formed larger and greater numbers of colonies compared with control MCF-7 cells (Fig. 6B). This indicates that the abrogation of the function of wild-type Sprys in MCF-7 cells causes cell transformation, resulting in the loss of contact inhibition and anchorage-independent growth.

**Inhibiting Spry's Function in MCF-7 Cells Results in the Formation of Larger Tumors.** The *in vivo* tumorigenic potential of the stable cells were assessed using an *in vivo* animal model of breast cancer that involves the growth of MCF-7 xenografts in nude mice. To avoid individual variation, hSpry2<sup>Y55</sup> MCF-7 and control MCF-7 cells were injected into each side of the nude mice. The progression of tumors was followed at weekly intervals over 9 weeks, after which the animals were sacrificed, and the tumors were extracted and weighed. It was immediately apparent that the hSpry2<sup>Y55F</sup> cells caused a significantly larger tumor mass when compared with the control cells ( $P = 0.0001$ ). An image of a typical mouse is shown in Fig. 6C. The respective tumors from 15 different animals were

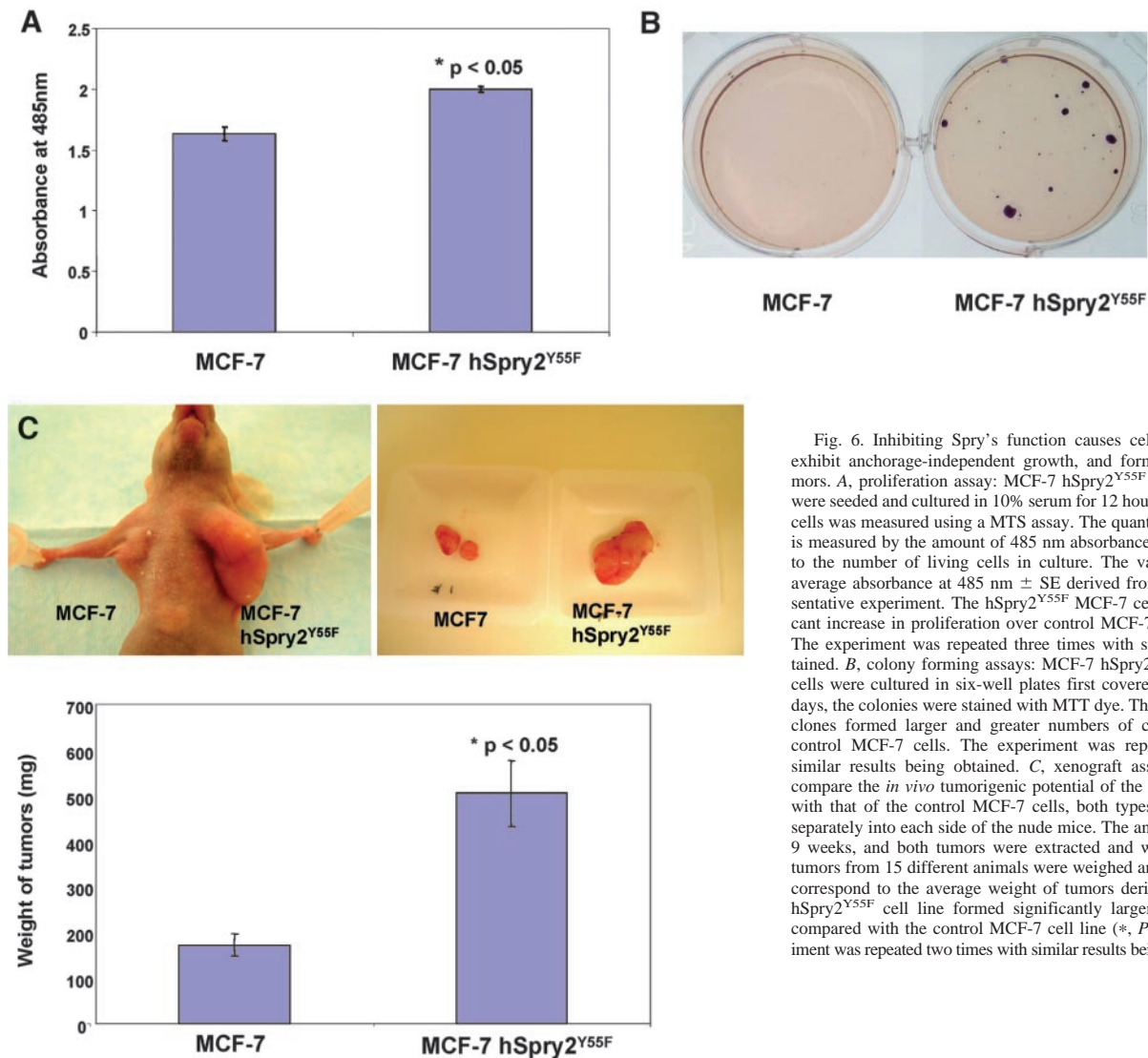


Fig. 6. Inhibiting Spry's function causes cells to proliferate faster, exhibit anchorage-independent growth, and form profoundly larger tumors. **A**, proliferation assay: MCF-7 hSpry2<sup>Y55F</sup> stable and control cells were seeded and cultured in 10% serum for 12 hours. The number of living cells was measured using a MTS assay. The quantity of formazan product is measured by the amount of 485 nm absorbance is directly proportional to the number of living cells in culture. The values correspond to the average absorbance at 485 nm  $\pm$  SE derived from triplicates of a representative experiment. The hSpry2<sup>Y55F</sup> MCF-7 cells show a 22% significant increase in proliferation over control MCF-7 cells (\*,  $P = 0.0034$ ). The experiment was repeated three times with similar results being obtained. **B**, colony forming assays: MCF-7 hSpry2<sup>Y55F</sup> stable and control cells were cultured in six-well plates first covered in soft agar. After 14 days, the colonies were stained with MTT dye. The hSpry2<sup>Y55</sup>-transfected clones formed larger and greater numbers of colonies compared with control MCF-7 cells. The experiment was repeated three times with similar results being obtained. **C**, xenograft assays in nude mice. To compare the *in vivo* tumorigenic potential of the MCF-7-hSpry2<sup>Y55</sup> cells with that of the control MCF-7 cells, both types of cells were injected separately into each side of the nude mice. The animals were sacrificed at 9 weeks, and both tumors were extracted and weighed. The respective tumors from 15 different animals were weighed and tabulated. The values correspond to the average weight of tumors derived from 15 mice. The hSpry2<sup>Y55F</sup> cell line formed significantly larger ( $\times 2.9$ ) tumor masses compared with the control MCF-7 cell line (\*,  $P = 0.0001$ ). The experiment was repeated two times with similar results being obtained. Bars,  $\pm$ SD.



weighed and the results displayed in the bar chart shown in Fig. 6C. The values correspond to the average weight of tumors  $\pm$  SE derived from 15 mice. The average weight of hSpry2<sup>Y55F</sup> tumors was 2.9 times greater than control tumors. To confirm that hSpry2<sup>Y55F</sup> is still expressed consistently in the xenograft tumors excised from the nude mice, proteins from both tumors were extracted, and hSpry2<sup>Y55F</sup> (FLAG-tagged) expression was confirmed using Western blotting techniques (data not shown).

These experiments indicate that the down-regulation or elimination of wild-type Spry function may contribute to transformation of cells, resulting in hyperproliferation and the larger tumor masses observed with hSpry2<sup>Y55F</sup> tumors. The results allude to a previously unrecognized role for Spry in cancer development.

## DISCUSSION

The preliminary analysis of *hSpry1* and 2 expressions in cancers via the Global Cancer Map revealed substantial *Spry2* down-regulation in breast cancer. We postulate that more cancer types may exhibit such trends of dysregulation in *Sprys*' expression. We are currently investigating the expression patterns of *Sprys* in other cancers which are not represented in the map array.

In the case of breast cancer, it was observed that in comparison to normal tissue, breast tumors showed an almost across-the-board down-regulation of *hSpry1* and 2. We showed additionally that *hSpry1* and 2 are expressed abundantly in normal breast tissues and that there is a 2–100-fold down-regulation in the expression of both genes in malignant breast cancer. Patient data accurately matched the prediction and preliminary data for the down-regulation of *hSpry2* in breast cancer; this also indicates that the down-regulation of *hSpry1* may be close to that of *Spry2*.

We have looked at the possible mechanisms by which the *Sprys* may be down-regulated and ruled out epigenetic causes, which is a relatively common reason for the observed down-regulation of genes in cancer. *WT1* is unlikely to be responsible for down-regulation of at least *hSpry1* in breast cancer, because it has been established that *WT1* is up-regulated in breast cancer (43), and we did not observe any difference in *WT1* expression between the normal and tumor breast tissue samples in the Cancer Profiling Array (data not shown). Only detailed analysis of the transcriptional mechanism of the *Spry* genes in normal *versus* cancer cells is likely to shed any light on the observed consistent down-regulation.

The colocalization of both isoforms in the normal breast tissue as well as the observed simultaneous down-regulation of both isoforms in breast cancer indicate that *Spry1* and 2 are likely to be similarly regulated and that there is possibly a redundancy in their physiological functions. To clarify additionally whether there is a functional redundancy between the expressed proteins, a comparison of the phenotype of single and double *Spry* knockout mice should provide some answers.

The expression of *Spry* was examined in the mammary glands of mice at different life stages. The *Sprys* were found to be expressed abundantly in actively developing mammary gland as well as during puberty and pregnancy. They were down-regulated once the mammary ducts have ceased to branch or proliferate. Given the evidence that *Spry* is an antagonist of tracheal branching morphogenesis, *Sprys* may have a role to play in negative regulation of the branching of breast ducts. It seems that the expression of *Spry* genes precludes random branching of ducts and most likely promotes branching at an active apical site. This leads us to suggest that *Spry* may have a tumor suppressor role in breast cancer. Although observed down-regulation of *Spry* in breast cancer is not the direct result of genetic or epigenetic causes (*i.e.*, mutation/deletion/methylation), the profound difference

in *Spry* expression between normal and malignant breast tissue at least mimics Knudson's two hit hypothesis modified to accommodate haploinsufficiency (44).

Although it is accepted that the causes of cancers are multifactorial, and because of certain genes (oncogenes), the deregulation of genes involved in growth and differentiation have a more significant impact. Conversely, the status of certain tumor suppressor genes such as p53 and Rb are important for their central surveillance and regulatory roles in cell cycle, such that the loss-of-function of these genes would relieve cells of the inhibitions that normally hold the cell cycle in check. It is possible that because of its putative role in the central Ras/MAP kinase pathway, *Spry* could be deemed a putative tumor suppressor gene, and loss of expression or function may allow the cell to be hypersensitive to growth signals.

We have examined this possibility and have shown that the over-expression of hSpry2<sup>Y55</sup> mutant quenches the function of endogenous wild-type *Spry* and causes cells to proliferate faster, exhibit anchorage-independent growth, and loss of contact inhibition. As a result, hSpry2<sup>Y55</sup> MCF-7 cells form larger tumors in the *in vivo* nude mice assay compared with control MCF-7 cells. The consistent down-regulation of *Spry1* and 2 in breast cancer and the experimental evidence using a dominant-negative hSpry2<sup>Y55F</sup> indicate that *Spry* proteins may actively maintain tissue integrity that runs amok when their expression is decreased below normal threshold levels. *Spry1* and *Spry2* may play tumor suppressive roles in the epithelium lining of breast ducts, a tubular system that undergoes additional development in the adult and undergoes cyclical morphological changes with respect to fluctuations in hormonal levels. This alludes to a previously unrecognized role for *Sprys* in cancer development. Future work will endeavor to provide additional evidence in this context by knocking out *Sprys* in the mammary glands *in vivo* or by "knocking down" *Sprys in vitro* in normal mammary epithelial cells and observing for changes in the phenotype of the tissue/cells.

We have taken a "candidate gene" approach to characterizing what may be a specific marker for a particular cancer situation with respect to the two *Spry* genes. By additionally analyzing clinical samples using different methods, we have strengthened the original array-based observation in terms of the percentage of samples that show irregular gene expression. There is normally a long road from discovery to clinic, which involves the analysis of many more samples and scenarios before a marker can be deemed to be medically useful in aiding the detection, prognostic evaluation, and treatment of cancer. Currently, we have highlighted *Sprys* as potential molecular markers. *Spry1* and *Spry2* show parallel down-regulation in breast cancer in over 90% of the observed cases. This would make *Sprys* ideal candidates as molecular markers in breast cancer.

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## The Ras/Mitogen-Activated Protein Kinase Pathway Inhibitor and Likely Tumor Suppressor Proteins, Sprouty 1 and Sprouty 2 Are Deregulated in Breast Cancer

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