Overexpression of *Sprouty 2* in Mouse Lung Epithelium Inhibits Urethane-Induced Tumorigenesis

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Members of the *Sprouty* family encode novel proteins that are thought to function primarily as intracellular antagonists of the Ras-signaling pathway. Increased Ras signaling is a critical characteristic of human lung adenocarcinoma, the most common type of non–small cell lung cancer. *Sprouty 2* is expressed in the lung epithelium, the tissue layer from which lung cancers arise. We hypothesized that overexpression of *Sprouty 2* in the distal lung epithelium would inhibit lung tumorigenesis. To test the hypothesis, the consequences of overexpressing *Sprouty 2* in the distal lung epithelium on urethane-induced mouse lung tumorigenesis were determined. Urethane is a chemical carcinogen found in tobacco smoke that causes activating mutations in *Kras* and induces lung tumors in mice. *Sprouty 2*–overexpressor mice developed significantly fewer lung tumors compared with their littermate controls (13.2 ± 1.1 versus 18.1 ± 1.3, $P = 0.006$). Tumor diameter was also significantly smaller in *Sprouty 2* overexpressors (0.85 mm ± 0.03 versus 0.95 mm ± 0.02, $P = 0.005$). *Sprouty 2* overexpression did not alter *Kras* mutational frequencies in urethane-induced tumors, suggesting that the tumor-suppressing effect of *Sprouty 2* overexpression acts at a stage after *Kras* mutation, perhaps by interfering with receptor tyrosine kinase–induced signaling. These results demonstrate that *Sprouty 2* overexpression inhibited both tumor initiation and subsequent tumor growth.

Keywords: Sprouty; lung cancer; mouse; chemoprevention; Ras

Lung cancer is the leading cause of cancer death in men and women in the United States. Smoking accounts for roughly 90% of cases in men and 70% in women. Non–small cell lung cancer (NSCLC) accounts for approximately 80% of lung cancers. NSCLC is subdivided into squamous cell carcinoma, large cell carcinoma, and adenocarcinoma, with the latter being most common (~ 40% of all lung cancers) (1, 2).

Fifteen to twenty percent of human NSCLCs, particularly adenocarcinomas, have point mutations in the *KRAS* proto-oncogene, in most cases at codon 12. These mutations prevent hydrolysis of KRAS-GTP (3). As a result, KRAS signaling becomes constitutive, leading to a strong proliferative stimulus (1). While a minority of human NSCLCs contain gain-of-function mutations in *KRAS*, increased Ras signaling, via several different mechanisms, appears to occur in most NSCLCs. For example, the receptor tyrosine kinase (RTK) class of growth factor receptors classically signal through Ras. Growth factors and their receptors appear to affect human lung cancer development. Members of the *epidermal growth factor* (EGF) and *EGF receptor* (EGFR) families appear to function as autocrine loops in many NSCLCs. In the vast majority of NSCLC, *EGFR* and/or one of its ligands is over expressed. Another member of the *EGFR* family, *HER2/neu*, is expressed in approximately 35% of NSCLCs. Increased expression of these ligands and/or their receptors is thought to result in increased intracellular signaling through the Ras/Raf/MAP kinase pathway, leading to proliferation. Blockade of these receptors with various reagents in human lung cancer cell lines inhibits proliferation (1). Basic fibroblast growth factor (bFGF; aka FGF 2) binds to members of the FGFR family. Forty-nine to seventy percent of adenocarcinomas of the lung express *bFGF*. FGFRs, like members of the EGFR family, are RTKs, and they classically signal via Ras (1). It remains unclear whether this enhanced Ras signaling plays early, late, multiple, or continuous roles in the many stages leading to overt human lung cancer.

CLINICAL RELEVANCE

Sprouty 2 is an inhibitor of selected receptor tyrosine kinases and Ras signaling. This research suggests that Sprouty mimetics may have utility in the prevention and treatment of lung cancer.

The mouse as a model system for the study of lung cancer has many strengths (4). Inbred strains of mice have different genetic susceptibilities for developing spontaneous lung tumors, most of which resemble adenomas. In mice, susceptibility to spontaneous lung tumor development generally correlates with susceptibility to chemically induced lung tumors. Like spontaneous lung tumors, the induced tumors usually are adenomas.

Similar to some human adenocarcinomas, many spontaneous and induced mouse lung tumors have an activating mutation in murine *Kras*, usually codon 12 or 61. For example, urethane induces mutations at codon 61, and induction of a *Kras* mutation appears to be an early (if not the earliest) event in urethane-induced tumorigenesis (4). Recent studies have shown that an activating mutation in *Kras* is sufficient to induce mouse lung tumors (5, 6). In addition, persistent Kras signaling appears to function as a survival signal in this model, since tumors disappeared if such signaling was abolished (6).

Thus, much has been learned about gain-of-function mutations that lead to increased Kras signaling in human and mouse lung tumors. In contrast, little is known about the potential role antagonists of the Kras signaling pathway may play in lung carcinogenesis. Loss-of-function mutations in these genes would also be predicted to increase Kras signaling, and therefore may play a role in lung tumorigenesis.

One example of a negative regulator is the *Sprouty* (*Spry*) family, whose members encode proteins that function as in-

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tracellular antagonists of the Ras signaling pathway (7, 8). There are four vertebrate members of the family. Sproutys have been shown to antagonize EGFR (8), FGFR (7, 9), VEGF-R (10), and c-MET (aka hepatocyte growth factor receptor [HGF]) mediated Ras signaling (11). However, Sproutys do not appear to antagonize signaling by all RTKs. The mechanism(s) that generates this selective antagonism of specific RTK-mediated Ras signaling is not known. In addition, it remains unclear if specific Sproutys antagonize specific RTKs. However, *Spry* has been overexpressed in Drosophila, frogs, chicks, zebrafish, and mice. In each instance, Sprouty appeared to antagonize RTK-induced Ras signaling (12–14). When *Spry2* was specifically overexpressed in embryonic mouse lung epithelium, defects in lung development were observed (15). These defects were consistent with inhibition of EGFR and/or FGFR-mediated Ras signaling. However, recent *in vitro* studies have suggested that overexpression of *Spry2* can enhance EGFR signaling (12–14).

Spry2 is expressed in adult lung epithelium. Recently, *Spry2* expression has been reported in a number of human NSCLC tumors and cell lines (16). While considerable variation occurs between tumors and cell lines, *Spry2* protein and mRNA expression is generally repressed. Ectopic expression of *Spry2* decreased cell proliferation and migration and blocked tumor formation in immunodeficient mice. In a latent Kras murine model of adenocarcinoma, conditional null *Spry2* mice developed increased numbers of lung tumors, supporting a tumor-suppressive effect (17). Whether *Spry2* overexpression would suppress tumorigenesis has not been tested.

We hypothesized that overexpression of this putative Ras signaling antagonist in the lung epithelium would inhibit lung tumor development. To test this hypothesis, lung tumor formation was characterized in adult mice that overexpressed *Spry2* in lung epithelium and their littermate controls after they were exposed to the chemical carcinogen urethane, which causes lung tumors in mice and induces gain-of-function mutations in *Kras* (4).

MATERIALS AND METHODS

Generation of Mice with Lung Epithelium–Specific Overexpression of *Spry2*

The generation of lung epithelial–specific *Spry2* overexpressor mice was based on that used by Lobe and coworkers to construct the Z/AP Cre recombinase reporter mouse (18). A modified version of this vector was obtained (kind gift of C. Lobe, Sunnybrook and Women's Health Sciences Center, Toronto, ON, Canada). Several unique cloning sites replaced the human placental *alkaline phosphatase* (*AP*) cDNA. An internal ribosomal entry site (IRES) *AP* cDNA was inserted downstream of the cloning sites. The coding region of *Spry2* was PCR amplified, sequenced, and cloned downstream of the 3′ loxP site and upstream of the IRES *AP* (*see* Figure 1). Importantly, the promoter in this construct is ubiquitous and constitutively active. Thus, in the unrecombined configuration, the β-galactosidase–neomycin fusion cDNA, which is flanked on either side by loxP sequences, is constitutively expressed in all cells, but the downstream *Spry2* IRES *AP* cDNAs are not expressed. Cre recombinase recognizes loxP sequences, and irreversibly deletes intervening DNA. Consequently, in the presence of tetracycline-inducible Cre, the β-galactosidase–neomycin fusion cDNA is removed, and *Spry2* and IRES *AP* are now constitutively expressed even when doxycycline is no longer present. Thus, AP is a reporter for Cre-mediated recombination and marks cells that overexpress *Spry2*.

Spc-rtTA and *Tet-o-Cre* transgenic mice were kindly provided by Dr. Jeffrey Whitsett (Cincinatti Children's Hospital Medical Center). The development and characterization of these transgenic mouse lines have been described previously (19). The *Spry2*^{GOF} line was backcrossed to FVBN mice for three to four generations. The *Spc-rtTA* and *Tet-o-Cre* transgenic mice were backcrossed to FVBN for five to six generations. These mice were then bred to generate triple transgenic mice and littermate controls.

A PCR-based strategy was used to identify presence or absence of each transgene from genomic DNA extracted from tails. Previously published SPC and Cre primer sequences and conditions were used (19, 20). The following primers and conditions were used to detect the unrecombined *Spry2*^{GOF} transgene: forward, 5′-gcttgggtggagaggctattc-3′; reverse, 5′-caaggtgagatgacaggagatc-3′; annealing temperature was 54°C for 30 seconds; 72°C extension time was 45 seconds; 35 cycles.

To induce constitutive overexpression of *Spry2,* all 30- to 35-day-old mice received doxycycline (0.5 mg/ml final concentration; Sigma, St. Louis, MO) in their drinking water for 1 week (19). Fresh doxycycline drinking water was provided every 48 hours. After 1 week, mice drank regular water for the duration of the protocol.

Western Blot

Five- to seven-week-old triple transgenic mice and littermate controls received doxycline for 1 week followed by 1 week of regular water. After mice were killed, the lungs were dissected free of the body and homogenized in 1.5 ml of ice-cold lysis buffer (50 mM Tris-HCL, pH 7.4; 150 mM NaCl; 0.25% sodium deoxycholate; 1% IGEPAL; 0.1% SDS) containing 50 μl of protease inhibitor cocktail (Sigma). The lung homogenate was transferred to a microfuge tube and spun in a microfuge at 16,000 × *g* for 10 minutes at 4C. The supernatant was collected, aliquotted, and stored at −80°C until further analysis.

Total protein concentration in the lung homogenates was determined using the BCA kit (Pierce, Rockford, IL) following the manufacturer's directions. Ten micrograms of protein were loaded in to each well of a 15% SDS-PAGE Criterion gel (Bio-Rad, Hercules, CA). Proteins were separated and then transferred to an Immobilon membrane (Millipore, Bedford, MA) using the Criterion system (Bio-Rad).

The membrane was blocked with 5% nonfat dried milk powder in PBS + 0.1% Tween (PBST). Rabbit polyclonal anti-Spry2 antibody (Upstate, Charlottesville, VA) was used at 1:2,500 dilution, and the blot incubated overnight at 4°C. The blot was washed with PBST, and then incubated with a horseradish peroxidase–tagged goat anti-rabbit secondary antibody at a 1:5,000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). After washing with PBST, bands were visualized using the ECL plus Western Blotting kit (Amersham, Piscataway, NJ).

Detection of Human Placental Alkaline Phosphatase Activity

Immunologic reagents for the detection of *Spry2* protein by immunohistochemistry are not readily available; therefore, we used expression of the co-expressed *IRES AP* as a marker of *Spry2* expression. Triple

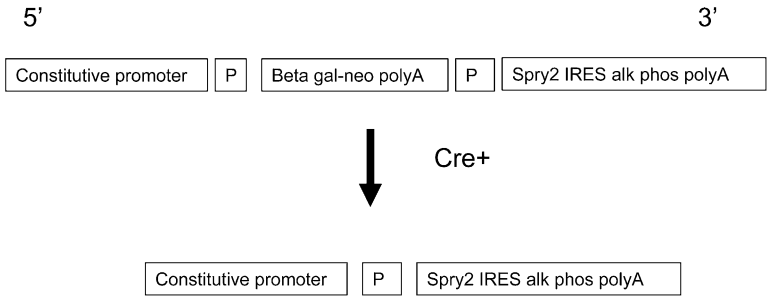

Figure 1. Schematic representation of *Spry2* conditional gain-of-function transgene. The unrecombined allele constitutively expresses the β-galactosidase–neomycin fusion cDNA. In the presence of the Cre recombinase, the β-galactosidase–neomycin fusion cDNA is deleted, and the *Spry2* IRES *HPLAP* cDNA is constitutively expressed. "Constitutive promoter" = CMV enhancer element fused to the Chick β actin promoter; *P* = loxP; IRES = internal ribosomal entry site.

transgenic mice and littermate controls were killed after receiving doxycyline for 1 week. The trachea was cannulated with an 18-gauge angiocatheter (BD, Franklin Lakes, NJ) and the lungs were inflation-fixed with 10% buffered formalin (Fisher, Pittsburgh, PA) under 25 cm of water pressure for 15 minutes. The lungs and trachea were then removed and immersed in 10% formalin overnight. Lungs were washed extensively in phosphate-buffered saline (PBS). Fixed lungs in PBS were then immersed in a 70°C water bath for 30 minutes to inactivate endogenous alkaline phosphatases. After a brief rinse in distilled water, lungs were incubated in BM purple (Roche, Indianapolis, IN) for 24 hours with gentle agitation, and then washed in PBS with 5 mM EDTA to stop the reaction. Lungs were placed in 30% sucrose overnight at 4°C, and then embedded in Tissue Tek OCT (Miles, Elkhart, IN). Ten-micrometer frozen sections were cut and collected on Superfrost Plus slides (Fisher, Pittsburgh, PA). Slides were washed in PBS, coverslipped, and images captured on a Leica upright microscope (Leica, Wetzlar, Germany) fitted with a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Carcinogenesis Protocol

Five- to seven-week-old *Spry2^{GOF}* mice and their littermate controls were given doxycycline in their drinking water for 1 week. The mice then drank regular water for the duration of the protocol. Six- to eight-week-old mice were weighed the day before injection. Mice were given a single intraperitoneal injection of urethane (1 mg urethane dissolved in normal saline/gram of body weight; Sigma); total volume injected was 300 microliters. Sixteen weeks after injection, mice were anesthetized by an intraperitoneal injection of 70 mg/kg of ketamine and 14 mg/kg xylazine, and then killed by transection of the abdominal aorta and the vena cava. The lungs were removed and placed in ice-cold PBS. The experimental protocol was approved by the Case Western Reserve University IACUC.

Enumeration and Measurement of Lung Tumors

Lungs were examined, and tumors were identified and dissected out of the lung parenchyma under a dissecting microscope. Tumor diameter was measured using a digital micrometer. Tumors were fixed in 10% buffered formalin (Fisher, Pittsburgh, PA) and then submitted to a core histology lab for paraffin embedding and sectioning. For RNA extraction, freshly dissected tumors were stored in RNALater (Ambion, Austin, TX) at −80°C.

Immunohistochemistry and TUNEL

Five-micrometer-thick sections of paraffin-embedded tumors were collected. TUNEL assay was performed according to the manufacturer's directions (Roche, Indianapolis, IN). Anti–Ki-67 (1:100 dilution; Abcam, Cambridge, MA), anti-phosphohistone (1:50; Upstate), and anti-activated caspase 3 (1:50; R&D, Minneapolis, MN) antibodies were purchased. Biotinylated secondary antibodies were purchased (Vector, Burlingame, CA), and the ABC detection kit was used according to the manufacturer's directions. Sections were counterstained with hematoxylin (Vector). For each antibody, immunohistochemistry was performed with or without antigen retrieval. For antigen retrieval, slides were immersed in 10 mM sodium citrate pH 6.0 (Sigma), heated under pressure in a pressure cooker for 3 to 5 minutes, pressure relieved, and the slides allowed to cool for 20 minutes. For Ki-67, pressure cooker time was increased to 15 minutes.

TUNEL-positive cells and cells positive for Ki-67, phosphohistone, and activated caspase 3 expression from representative fields were manually counted. At least six fields were counted for each assay. Tumors from three control and three *Spry2^{GOF}* mice were examined.

RNA Extraction, RT-PCR, and Sequencing

Tumors were homogenized and RNA extracted using the RNA Easy kit (Qiagen, Valencia, CA) following the manufacturer's directions. RT-PCR was performed using the 1-step RT-PCR kit (Qiagen). *Kras*-specific primer pair (forward primer, 5′-gcctgctgaaaatgactgagta-3′; reverse primer, 5′-tgtcttgtctttgctgaggtct-3′) was used to amplify a product, including codons 12 and 61. The PCR product was sequenced in a core sequencing facility.

Statistics

Statistical analysis was performed using Prism4 software (GraphPad, San Diego, CA); *t* test was used to compare groups. A P value less than 0.05 was considered significant. Bar graphs show mean values ± SEM.

RESULTS

Lung Epithelium–Specific Overexpression of *Spry2*

To determine if the *Spry2^{GOF}* transgene could be recombined specifically in the lung epithelium, which would result in expression of *Spry2* and the IRES *AP*, previously characterized *SP-C rtTA* and *Tet-O-Cre* transgenic mouse lines were obtained. Mice that inherit the *SP-C rtTA* and *Tet-O-Cre* transgenes have been shown to express *Cre* specifically in the lung epithelium in the presence of doxycycline (19). These mice were bred to the *Spry2^{GOF}* transgenic line to generate triple transgenic offspring that could constitutively overexpress *Spry2* specifically in the lung epithelium. Administration of doxycycline to the drinking water of pregnant females beginning at about Embryonic Day (E)6.5 resulted in transgenic *Spry2* being overexpressed throughout the developing lung epithelium of triple transgenic embryos. Littermate controls only expressed endogenous *Spry2* in normal domains (data not shown). In the absence of doxycycline, lungs of triple transgenic embryos only expressed endogenous *Spry2* in normal domains through about E16.5 to E17. As described previously, the *SP-C rtTA* transgene becomes leaky during late gestation and the perinatal period, presumably due to the same factors that led to the pronounced up-regulation of endogenous *SP-C* during this same time period (19). In our triple transgenic mice, this leakiness results in patchy but widespread expression of *Cre* in distal lung epithelium. Where expressed, Cre deletes the β-galactosidase–neomycin fusion cDNA, and *Spry2* and IRES *AP* become constitutively expressed even if doxycycline is subsequently removed because Cre-mediated deletion is irreversible and the promoter is constitutive (data not shown). Subsequent postnatal administration of doxycycline results in more uniform and widespread expression of *Cre*, leading to more uniform and widespread constitutive overexpression of *Spry2* and the *IRES AP* in the distal lung epithelium as shown by AP histochemical staining in Figures 2A and 2B. However, low-power views of the lung reveal considerable variation in the level of expression of *IRES AP*, a surrogate for *Spry2* (Figure 3), with some areas showing intense AP histochemical activity and others below the threshold of detection.

The lungs of these mice are histologically indistinguishable from littermate controls 1 year after constitutive overexpression was induced in the lung. In addition, lung overexpressors are fertile, and are behaviorally indistinguishable from littermate controls at 16 months of age.

Western blot analysis of littermate control and *Spry2* overexpressor lung homogenates showed significantly more Spry2 protein in overexpressor lung homogenates compared with littermate controls (Figure 2C). The two bands detected likely represent a baseline and a post-translationally modified form of the protein, as Sproutys can undergo modifications such as phosphorylation (13). The Western blot also demonstrates the constitutive nature of the promoter driving *Spry2^{GOF}* transgene expression, since the *Spry2* overexpressor mice were killed 1 week after their last exposure to doxycyline-supplemented water, but their lung homogenates continued to express significantly more Spry2 protein than did those of littermate controls. In addition, 1 month after exposure to doxycycline, levels of Sprouty 2

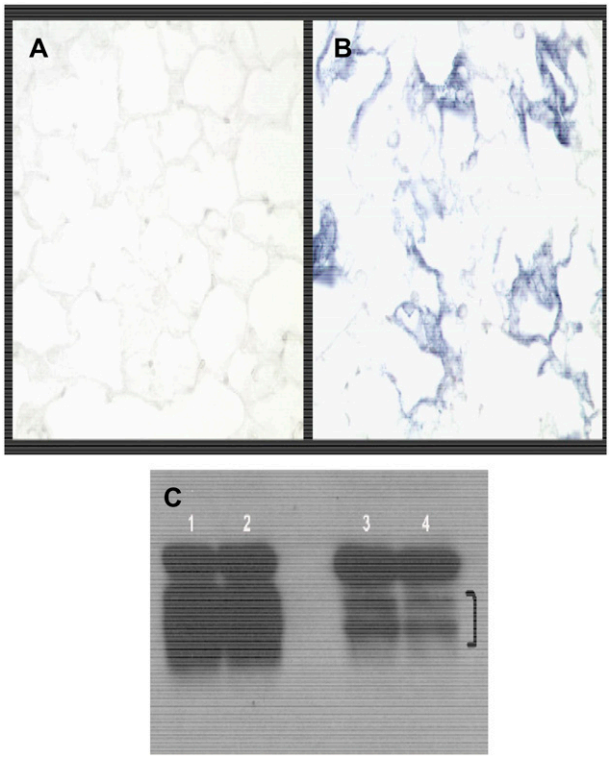

Figure 2. Overexpression of the recombined *Spry2* IRES *AP* transgene in the lung. *SPC-rtTA, Tet-o-Cre, Spry*^{GOF} triple transgenic adult mouse and a littermate control drank water supplemented with doxycycline for 1 week. Mice were then killed, and lungs were processed for alkaline phosphatase histochemical staining and then cryosectioned. (*A*) Littermate control lungs show no alkaline phosphatase staining. (*B*) In contrast, triple transgenic lungs show alkaline phosphatase staining in alveoli due to Cre-mediated recombination of the *Spry2*^{GOF} transgene, which results in expression of both *Spry2* and the AP reporter cDNAs. *Magnification* = ×200. (*C*) Western blot demonstrating doublet of Spry2 protein in lung homogenates from two *Spry2* overexpressors (*lanes 1* and *2*) and littermate controls (*lanes 3* and *4*). *Bracket* marks doublet.

protein in lung homogenates are not different from those seen from mice killed at the end of 1 week of doxycycline administration (data not shown).

Fewer Tumors in Mice Overexpressing *Spry2* in the Lung Epithelium

The *Spry2*^{GOF} lines were established on a mixed genetic background. Since genetic background can have a profound effect on susceptibility to urethane-induced lung tumors (4), the *Spry2*^{GOF} line was backcrossed three to four generations to the FVBN inbred strain. The *SPC-rtTA* and *tet-O-Cre* transgenic lines were backcrossed to FVBN at least six generations each before they were crossed to the *Spry2*^{GOF} line to produce the triple transgenic and littermate control mice. Thus, the experimental mice should be in a greater than 93% FVBN genetic background.

Five- to six-week-old experimental mice were given doxycycline in their drinking water beginning 1 week before injection of urethane. In triple transgenic mice, widespread lung epithelium overepression of *Spry2* should be induced after approximately 72 hours of doxycycline (19). After 1 week of doxycycline water, all mice received a single intraperitoneal injection of urethane. Sixteen weeks later, the mice were killed and examined.

All mice developed tumors. Thus, tumor incidence was 100% in each group (n = 21 for *Spry2*^{GOF} group; n = 26 for littermate control group).

A standard measure of tumor burden is to count the number of tumors visible from inspection of the pleural surface with the aid of a dissecting scope (4). As shown in Figure 4, mean number of lung tumors per mouse was significantly lower than for mice that overexpressed *Spry2* in the lung epithelium compared with their littermate controls (13.2 ± 1.1 versus 18.1 ± 1.3, mean ± SEM; P = 0.006). This 27% reduction in lung tumors suggests that overexpression of *Spry2* in the lung epithelium inhibited tumor initiation.

In our system, Cre-mediated recombination results in overexpression of both *Spry2* and human placental *AP*. To address the possibility that the anti-tumor effects were due to overexpression of human placental *AP*, the *SP-C rtTA* and *Tet-O-Cre* transgenic mice were bred to the *Z/AP* transgenic mouse line, which contains the same promoter and human placental *AP* as found in the *Spry2*^{GOF} transgene. Triple transgenic offspring and littermate controls underwent the same doxycycline and urethane protocols. Urethane-induced lung tumor number was not significantly different between mice that only overexpressed human placental *AP* in the lung epithelium compared with their littermate controls (data not shown). This result indicates that it is overexpression of

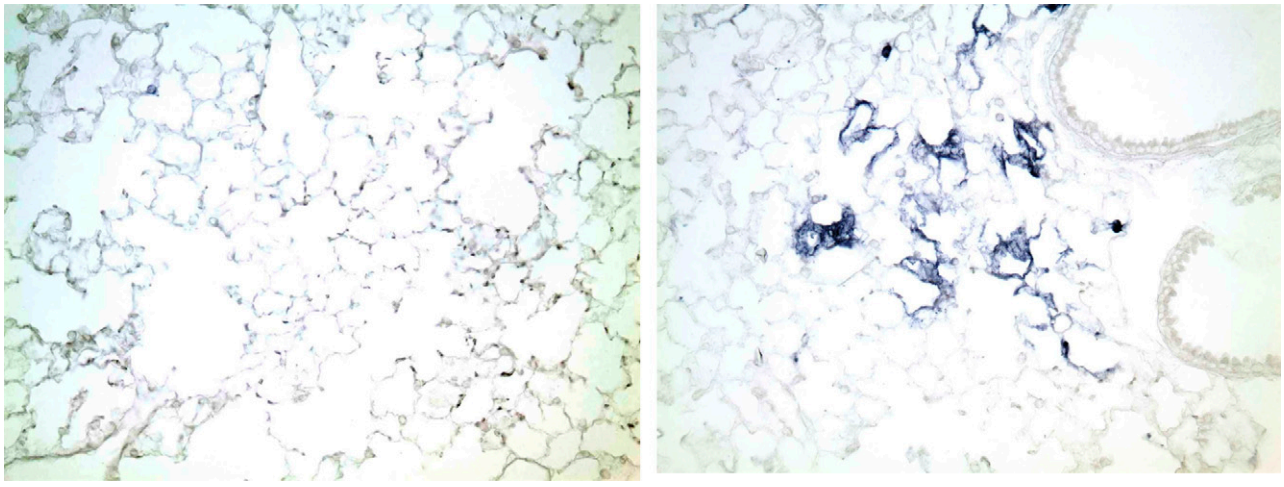

Figure 3. Low-power view of alkaline phosphatase histochemical stained lung from littermate control (*left panel*) and triple transgenic (*right panel*) mice from Figure 2, demonstrating significant patchiness in staining for alkaline phosphatase, a surrogate for *Spry2* expression.

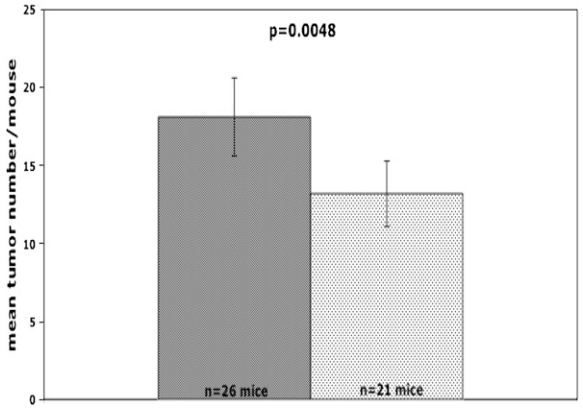

Figure 4. Mice that overexpress *Spry2* (*lightly shaded bars*) in the lung epithelium developed significantly fewer urethane-induced lung tumors than littermate controls (*darkly shaded bars*) 16 weeks after injection of urethane (13.2 ± 1.1 versus 18.1 ± 1.3, mean ± SEM; $P = 0.006$).

Spry2 and not *AP* that is responsible for the effects on urethane-induced tumorigenesis.

Effect of Overexpressing *Spry2* in the Lung Epithelium on Urethane-Induced *Kras* Mutations

Urethane-induced tumors typically have a gain-of-function point mutation at codon 61 of *Kras*. A codon 61 mutation that results in glutamine becoming leucine is associated with adenoma formation, while a glutamine becoming arginine is associated with a more malignant phenotype (21). Since adenomas are benign, the associated *Kras* mutation is thought to signal less efficiently than the mutation associated with the more malignant phenotype (4). We hypothesized that fewer tumors were found in mice overexpressing *Spry2* because this Ras-signaling antagonist was able to antagonize the less efficient mutant Kras signal and therefore able to inhibit adenoma development. This hypothesis predicted that significantly fewer tumors in the $Spry2^{GOF}$ group would express the glutamine to leucine mutation.

To begin to test this hypothesis, RNA was extracted from 30 randomly selected tumors from the $Spry2^{GOF}$ mice and 33 from littermate controls. RNA was reverse transcribed and PCR was performed using *Kras*-specific primers. The PCR product was sequenced. At codon 61, 24/30 (80%) $Spry2^{GOF}$ tumors and 28/33 (85%) control tumors had a CAA to CGA mutation. This mutation results in glutamine becoming arginine, and is associated with adenocarcinomas. The other tumors had a CAA to CTA mutation at codon 61, which leads to a leucine, and is associated with adenomas. The difference in mutation rates was not statistically significant and is not likely to represent a biologically important phenomenon.

Smaller Tumors in Mice Overexpressing *Spry2* in the Lung Epithelium

Transgenic expression of *Kras* with a gain-of-function mutation was sufficient to induce lung tumors (5, 6). This model also demonstrated that continued expression of *Kras* with a gain-of-function mutation was required to sustain tumor growth. In its absence, tumor cells underwent apoptosis (6). Since Sprouty 2 appears to function as an antagonist of Ras signaling, we hypothesized that urethane-induced lung tumors would be smaller in $Spry2^{GOF}$ mice because of inhibition of this survival signal. All tumors were dissected out of the lung and measured with a digital micrometer. Four hundred seventy-two tumors were found in littermate control mice, and 276 in $Spry2^{GOF}$ mice. As shown in Figure 5, mean tumor diameter in control mice was significantly larger than in $Spry2^{GOF}$ mice (0.95 mm ± 0.02, and 0.85 mm ± 0.03, mean ± SEM; $P = 0.005$). Assuming approximately spherical shape of the tumors, this difference translates to an approximately 40% smaller average tumor volume in the *Spry2* overexpressor mice.

Apoptosis Rates in Lung Tumors

Having found that mean urethane-induced tumor size was smaller in $Spry^{GOF}$ mice, we investigated if tumors in these mice were smaller because they had a higher rate of apoptosis. To determine cell death rates, TUNEL assays were performed on representative sections from tumors 16 weeks after urethane injection. The apoptosis rate within tumors from control mice (7.77/10x field ± 2.0) was not significantly different from $Spry2^{GOF}$ mice (7.4/10x field ± 2, $P = 0.800$). Similar results were obtained using an anti-activated Caspase 3 antibody (data not shown).

Proliferation Rates in Lung Tumors

Increased Ras signaling induces proliferation of many cell types. Transgenic expression of *Kras* with a gain-of-function mutation was sufficient to induce proliferation of lung epithelial cells, which went on to form lung tumors (5, 6). Overexpression of *Spry2*, a Ras signaling antagonist, would be predicted to inhibit proliferation. The difference in mean urethane-induced tumor size could be due to a slower proliferation rate in the $Spry2^{GOF}$ tumors.

To address this possibility, proliferation rate was determined by anti–Ki-67 immunohistochemistry on representative paraffin sections of 16-week-old tumors. The proliferation rate within tumors from control mice (5.77% ± 1.16) was not significantly different from the proliferation rate found in the $Spry2^{GOF}$ tumors (5.48% ± 1.07, $P = 0.870$). Similar results were obtained using an anti-phosphohistone antibody (data not shown).

DISCUSSION

This study demonstrates that lung epithelium specific constitutive overexpression of *Spry2*, a putative intracellular antagonist of Ras signaling, resulted in a 27% reduction in multiplicity of urethane-induced lung tumors. Moreover, the tumors that developed were on average smaller than those found in littermate

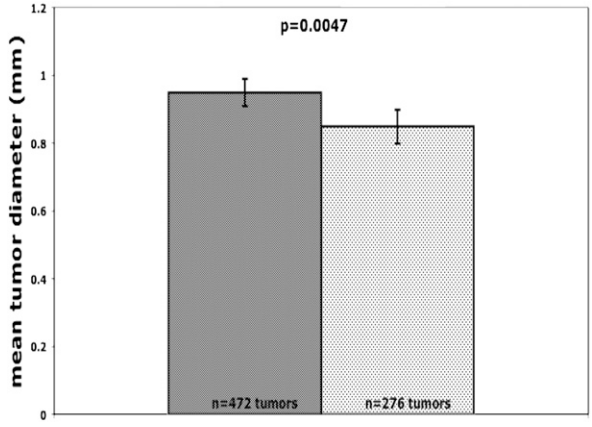

Figure 5. Mice that overexpress *Spry2* (*lightly shaded bars*) in the lung epithelium developed significantly smaller urethane-induced lung tumors than littermate controls (*darkly shaded bars*) 16 weeks after injection of urethane (0.85 mm ± 0.03 versus 0.95 mm ± 0.02, mean ± SEM; $P = 0.005$).

controls. Both the reduction in tumor multiplicity and size were statistically significant.

The finding of fewer tumors suggests that overexpression of *Spry2* inhibited urethane-induced tumor initiation. We hypothesized that the reduction in tumor development was due to suppression of tumors arising with a mild Kras mutation at codon 61 (i.e., associated with adenoma formation) (21). Sequencing the *Kras* mRNA extracted from tumors did not reveal a significant difference in mutation frequencies at codon 61 between $Spry2^{GOF}$ mice and their littermate controls. Thus a skewed mutational spectrum does not explain the 27% reduction in tumor number observed in the $Spry2^{GOF}$ mice. The mechanism by which overexpression of *Spry2* in the lung epithelium leads to fewer urethane-induced lung tumors remains unclear, but may occur at stages of carcinogenesis subsequent to *Kras* mutation. Further investigation will be necessary to understand the mechanism by which *Spry2* antagonizes tumorigenesis.

The significantly smaller mean diameter in the *Spry2*-overexpressing mice suggested that overexpression of *Spry2* affected not only tumor initiation, but also subsequent tumor growth. Smaller tumors could arise because of decreased proliferation and/or increased apoptosis. We determined proliferation rates in tumors from *Spry2*-overexpressing mice and their littermate controls with two different markers. We found no significant difference in proliferation rates using anti–Ki-67 or anti-phosphohistone antibody staining. Our results are in accord with previous studies which demonstrated that urethane-induced lung tumors grow continuously, but at a continuously decreasing rate. Doubling time of tumor volume was approximately 3.5 times faster 4 to 6 weeks after urethane injection compared with 7 to 12 weeks after injection (22, 23). As our analysis was performed 16 weeks after urethane injection, it remains possible that *Spry2* overexpression exerted a significant antiproliferative effect at an earlier time point.

Similarly, cell death rates as determined by TUNEL and anti-activated caspase 3 antibody staining were very low and not significantly different between the two groups. Earlier studies showed that cell death is almost undetectable in established urethane-induced lung tumors (22). However, it is clear that low rates of apoptosis can have profound effects on tissue size over a relatively small number of cell cycles (23, 24). In addition, as with tumor cell proliferation, it remains possible that significantly different detectable differences in cell death rates occur earlier. Thus, we cannot exclude the possibility that smaller tumor size in *Spry2*-overexpresssing mice is due to effects on cell death rates.

One limitation of this study is that we did not formally assess the level of *Spry2* expression in the tumors arising in the triple transgenic and control mice. It is possible that a fraction of alveolar type II cells not expressing the transgene gave rise to most of the tumors. The variability of AP histochemical activity, a surrogate for *Spry2*, on low-power views (Figure 3) of the lungs of triple transgenics given doxycycline supports this idea. We do not know the quantitative relationship between AP histochemical staining and *Spry2* expression, so it is possible that the AP-negative regions actually do express biologically active levels of *Spry2*. The likely variability in *Spry2* expression does not affect the basic conclusion that *Spry2* overexpression inhibits lung tumorigenesis and may suggest that our results are actually an underestimation of this effect. The inhibitory effect of *Spry2* overexpression on urethane-induced lung tumorigenesis was modest, but comparable to many other potential lung cancer–chemopreventive agents, including green tea (25), lipoxygenase and leukotriene pathway inhibitors (26), and nonsteroidal anti-inflammatory drugs such as indomethacin (27), ibuprofen, and piroxicam (28). Sprouty 2 mimetics alone may not be effective chemopreventive agents, but may be one component of a multifaceted chemopreventive regimen. In addition, it remains possible that even higher levels of *Spry2* overexpression may have greater inhibitory activity against lung tumorigenesis. Unfortunately, inheriting two copies of the $Spry2^{GOF}$ transgene was not compatible with postnatal life. Thus, we were not able to determine if there may be a dose response effect.

Our findings also provide insight into the normal function of Sprouty 2. Most of the published data indicate that Sprouty 2 functions as an antagonist of selected growth factor signaling. Both biochemical and genetic data indicate that Sprouty interacts with components of the Ras signaling pathway that are both upstream and downstream of Ras. Moreover, these studies have shown interactions with components known to promote or inhibit Ras signaling (13, 14). A model reconciling these findings is one in which Sprouty functions as part of a macromolecular complex whose net effect is inhibition of Ras signaling. However, recent *in vitro* data has shown that overexpression of vertebrate Sprouty 2 can promote EGFR-initiated Ras signaling by interacting with Cbl, and thereby preventing Cbl from interacting with EGFR and targeting the latter for degradation (12). Many pathologic insults to the lung induce EGFR expression, and increased EGFR signaling is found in most human lung cancers. Thus, if Sprouty 2 promotes EGFR-mediated signaling, urethane would be predicted to induce more and/or larger tumors in the $Spry2^{GOF}$ mice. We observed the opposite. Thus, our *in vivo* findings are consistent with Sprouty 2 functioning as an antagonist of Ras signaling. However, our findings do not exclude the possibility that Sprouty 2 may promote RTK signaling in other contexts. For example, Sprouty 2 may have different functions in different cell types. The *in vitro* studies were not performed with lung epithelial cell lines.

In the past, Sproutys have been suggested to play a role in breast and prostate cancer (29, 30). Interestingly, loss of rather than enhanced *Spry* expression was associated with human cancer. It has been suggested that Sproutys may function as tumor suppressors (11, 30). More recently, low *Spry2* expression has been reported in human NSCLC; ectopic expression antagonized cell migration (16). *Spry2*-null mice have increased numbers of lung tumors in a conditional *KRAS* tumorigenesis model, further supporting a tumor suppressor effect in lung cancer. We now demonstrate that overexpression of *Spry2* decreases lung tumor numbers and size in the urethane model, in which *KRAS* mutation is an early and critical event. This suggests that *Spry2* mimetic strategies might be of utility in lung cancer chemoprevention.

In summary, we have shown that overexpression of the Ras signaling antagonist *Spry2* in the lung epithelium inhibits, but does not prevent, urethane-induced lung tumor initiation. In addition, such overexpression also inhibits subsequent growth of these tumors. The mechanisms by which overexpression of *Spry2* inhibits tumor initiation and tumor growth remain unclear. Moreover, our findings raise the possibility that Sprouty 2 mimetics may have a role in preventing and/or treating lung cancer.

Conflict of Interest Statement: G.M does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.E.M. is a co-inventor on a pending patent for the use of prostacyclin and analogs to prevent cancer.

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