EVIDENCE THAT SPROUTY 2 IS NECESSARY FOR SARCOMA FORMATION BY HRAS ONCOGENE TRANSFORMED HUMAN FIBROBLASTS

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Sprouty-2 (Spry2) acts as an inhibitor of receptor tyrosine kinase signaling in various cellular contexts. Interestingly, Spry2 also prevents the c-Cbl-induced degradation of epidermal growth factor receptor (EGFR). We compared human fibroblasts malignantly transformed by overexpression of HRasV12 oncogene to their non-transformed parental cells and found that the malignant cells express a high level of Spry2. These cells also exhibited an increase in the level of EGFR compared to their precursor cells. We found that intact EGFR was required if HRas-transformed cells were to grow in the absence of exogenous growth factors or form large colonies in agarose. When we decreased expression of Spry2, using a Spry2-specific shRNA, the HRasV12-transformed fibroblasts could no longer form large colonies in agarose, grow in reduced levels of serum, or form tumors in athymic mice. The level of active HRas in these cells remained unaltered. A similar, but less pronounced, effect in tumor formation was observed when Spry2 was down-regulated in human patient-derived fibrosarcoma cell lines. In HRas-transformed cells Spry2 sustained the level and the downstream signaling activity of EGFR. In the parental, non HRas-transformed, fibroblasts expression of Spry2 resulted in the inhibition of HRas and ERK activation, suggesting that the positive effect of Spry2 in tumor formation is specific to HRas-transformation. Co-immunoprecipitation studies with HRas-transformed cells revealed that Spry2 and HRas interact, and that HRas interacts with Spry2-binding partners, c-Cbl and CIN85, in a Spry2-dependent manner. These data show that Spry2 plays a critical role in the ability of HRas-transformed cells to form tumors in athymic mice.

Carcinogenesis is a multistep process by which normal cells acquire characteristics of malignant cells through a series of genetic and/or epigenetic changes. To study this process, McCormick and colleagues developed a model system that mimics the pattern by which normal human fibroblasts become malignant (1). In those experiments, foreskin-derived human skin fibroblasts from a normal neonate, designated LG1, were transfected with a v-Myc oncogene, giving rise to the MSU1.0 cell strain, which has a normal diploid karyotype (2). As the MSU1.0 cells were being propagated, one of the cells spontaneously underwent two chromosomal translocations, giving rise to a cell strain, designated MSU1.1, that is chromosomally stable, near-diploid, and partially growth factor independent (2). Further studies showed that in contrast to MSU1.0, its parental strain, MSU1.1 cell strain, can be transformed into malignant cells by various means, e.g., by transfection of a Ras oncogene engineered to be expressed at a high level, followed by assaying for cells with the ability to form distinct foci (3-5) or by exposing the cells to a carcinogen, followed by similarly assaying the surviving population for focus-forming cells (6,7). When MSU1.1 transfectants expressing high levels of HRasV12 oncoprotein (4), (or other Ras oncoproteins (3,5)), are injected subcutaneously into athymic mice, they form fibrosarcomas at the site of injection with a short latency. For the present study, we used PH3MT cells, i.e., a cell strain...
derived from a malignant tumor that formed in an athymic mouse following injection of MSU1.1 cells that had been transformed by HRasV12 oncogene overexpression (4). The PH3MT cells are growth factor independent, form large-sized colonies in agarose, (i.e., exhibit anchorage independent growth), and form fibrosarcomas in athymic mice at the site of injection with a very short latency (4).

To determine whether the cell strains derived from the MSU1 lineage contain genes that are significantly overexpressed or underexpressed, compared to their precursor cell strains, we carried out a genome-wide comparison of mRNA expression profiles of MSU1.0, MSU1.1, and PH3MT cells. The array data indicated that expression of Spry2 was increased in MSU1.1 cells compared to their parental MSU1.0 cells, and that its level of expression in the tumor-derived PH3MT cells was increased at an even higher level (unpublished results). This discovery of very high levels of Spry2 mRNA in the tumor-derived HRasV12 oncogene-transformed PH3MT fibroblasts led us to examine whether Spry2 plays a role in malignant transformation of human fibroblasts by HRasV12.

Sprouty protein was first identified in Drosophila as an inhibitor of fibroblast growth factor (FGF)-induced tracheal branching (8) and epidermal growth factor (EGF)-induced eye development (9). Mammals have been shown to express four isoforms of Sprouty (8,10,11) that act as inhibitors of growth factor-induced cellular differentiation, migration, and proliferation. The inhibitory function of Spry2 is directed at the Grb2-SOS interaction or at the activation of Raf (12-17). In addition to its inhibitory function, Spry2 sustains EGFR signaling (12,18-23). This results from the interaction of Spry2 with c-Cbl, an E3 ubiquitin ligase that catalyzes ubiquitination of EGFR, targeting this receptor for lysosomal degradation (24,25). By binding to c-Cbl, Spry2 prevents interaction between c-Cbl and EGFR, blocking the degradation of that receptor, and this leads to sustained EGFR-induced ERK activity (12,20-22).

Recent studies show that Spry2 is involved in tumor formation (26). Spry2 is down regulated and has tumor suppressive properties in breast, prostate and liver cancer (27-29). Furthermore, Spry2 participates in a negative feedback mechanism in oncogene-induced senescence (30). Spry2 was found to be elevated in KrasG12V-expressing transgenic mice, were it acts in a negative feedback fashion to suppress lung tumorigenesis (31). Elevated expression of Spry2 has been reported in human melanomas harboring NRas and Braf mutations, but Spry2 was found to have an inhibitory function only in melanomas with wild type Ras and Raf (25, 26, 32).

The present study was designed to investigate the role of Spry2 in tumor formation by malignant human fibroblasts of the MSU1 lineage that express the HRasV12 oncoprotein. To do so, we stably down-regulated expression of Spry2 in PH3MT cells using shRNA and found that, expression of Spry2 was necessary for tumor formation in this context. A similar effect was observed when Spry2 was down-regulated in patient-derived fibrosarcoma cell lines. HRas-transformation was associated with an elevation of EGFR levels, a property that was also dependent on Spry2 expression. Enhanced expression of Spry2 in the parental cell strain, MSU1.1, resulted in an inhibition of the Ras/mitogen activated protein kinase (MAPK) cascade, suggesting that the ability of Spry2 to sustain EGFR signaling and tumor formation in this system requires expression of the HRas oncogene. We further determined that in PH3MT cells, Spry2 interacted with HRas and enabled HRas to interact with Cbl and CIN85. Together, these data indicate that Spry2 is necessary for tumor formation by HRas-transformed human fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Cells and cell culture** - The derivation of the cells of the MSU1 lineage has been described (2,4). Cell lines SL68 and SL89 are normal neonatal foreskin-derived fibroblasts. Cell lines SHAC, HT1080, VIP:FT and NCI are derived from patients’ fibrosarcomas (33). The microarray data were obtained from studies by Nielsen et al. (34) and Baird et al. (35). Cells were routinely cultured in Eagle’s minimal essentials medium, supplemented with L-aspartic acid (0.2 mM), L-serine (0.2 mM), pyruvate (1 mM) and 10% supplemented calf...
serum (Hy clone, Logan, UT). Penicillin (100 U/ml), and streptomycin (100 μg/ml) were included. The cells were grown in a humidified incubator with 5% CO2 at 37°C.

Western blot analysis- Whole cell lysates were prepared as described (36). The protein content was quantified with Coomasie protein reagent from Pierce (Rockford, IL). Whole cell lysate (50 μg) was separated by SDS-PAGE. The protein was transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA), and immunoblotting was carried out using standard techniques. Ku80 protein was used as a loading control. The signal was detected with the Super Signal reagent (Pierce). Antibodies for EGFR, ERK, pERK, c-Cbl and HRas were purchased from Santa Cruz (Santa Cruz, CA); Spry2 from Calbiochem (San Diego, CA); Pan-Ras from Cytoskeleton (Denver, CO) and Ku80 from Serotec (Raleigh, NC).

Preparation of Spry2-shRNA constructs- The down-regulation of Spry2 in PH3MT cells was carried out with the pSuper.retro system by Oligoengine (Seattle, WA). The shRNA constructs were designed according to the manufacturer’s protocol. Briefly, the coding region of Spry2 was analyzed with Oligoengine’s RNAi design tool, and two 19-nucleotide long regions, centered on positions 399 and 492 of the Spry2 coding region, were selected as target sites. For each of these target sites, a 65-base pair, double-stranded oligonucleotide was synthesized, which encodes the sense and antisense orientations of the Spry2 target site, separated by a hairpin sequence of nine base pairs. These double stranded oligonucleotides were synthesized to contain BglII and HindIII overhangs. The sequences of the oligonucleotides targeting positions 399 and 492 on the Spry2 coding region are:

3'GATCCCCGAGACTGCTAGGATCATCCTTCAAGAGGAGGTGATCTACGACTCTTTTGGA3' and 5'GATCCCCGCGCACTGAGATTTCAGAGATCTTCTGTTGGCTAGTGTGGCTTTTGGA5', respectively. In addition to the two Spry2-specific oligonucleotides, we synthesized a nonspecific oligonucleotide in which the sense and antisense sequences were scrambled. Following synthesis, the double stranded oligonucleotides were ligated into the pSuper.retro vector.

Stable infection- The pSuper.retro vectors encoding the Spry2-specific or the nonspecific shRNA constructs and the gene coding for neomycin resistance were transiently transfected into the Phoenix packaging cell line, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions. In each case, we used 5 μg of DNA and a 1:4 DNA to Lipofectamine ratio. After 48 h, the medium on the cells was collected, centrifuged at 1,500 rpm for 5 min, and the supernatant containing the retrovirus was collected. One ml of medium containing retrovirus was added to PH3MT cells that had been plated at a density of 2 x 10^5 cells per 60 mm dish, 24 h previously. The retrovirus-containing medium was removed from the cells after 18 h, and the cells were allowed to recover for 30 h. Then the cells were passaged into 100 mm-diameter dishes at a 1:10 dilution, and 24 h later, the infected cells were selected for resistance to puromycin (20 μg/mL).

Immunoprecipitation reactions- Whole cell lysates (250 μg) were precleared with an appropriate IgG antibody for 30 min, and then incubated for 2 h with an antibody specific for HRas, followed by incubation at 4°C with protein-G for 1-8 h. When an HRas agarose conjugate was used (Ras-cCbl/CIN85), the lysates were incubated with the agarose conjugate for 3 or more hours. The immunoprecipitated fraction was washed several times with lysis buffer and assayed by Western blotting. The Immunoprecipitation experiments were repeated three times for the Ras-Spry2 and Ras-c-Cbl interactions, and twice for the Ras-CIN85 interaction.

Anchorage independence assay- Cells were assayed for their ability to form colonies in agarose as described (36). The colony number and size was determined by using the NIH Image software. Only colonies greater than 120 μm in size are shown in the graphs.

Tumorigenicity assay- The derivatives of PH3MT cells were assayed for their ability to form tumors in athymic mice as described (36). Cell lines immediately derived from MSU1.1 cells were injected into an absorbable gelatin sponge (1 cm³) (Pharmacia, Kalamazoo, MI) which had been implanted subcutaneously into athymic mice 1 wk earlier.
Ras activation assay- Whole cell lysates (2 mg) were pulled down with Raf-Ras binding domain (RBD)-conjugated beads from Cytoskeleton (Denver, CO) according to the manufacturer’s instructions. The pulled down fractions were immunoblotted with an HRas-specific antibody.

Statistics- The anchorage independence assays for each of the two control cell strains and the three cell strains with down-regulated Spry2 were repeated at least twice. Growth curves were repeated at least twice. Statistical significance was determined by using a two tailed non paired Student’s t-test.

RESULTS

Determination of the level of expression of Spry2 in HRasV12-transformed human cell line PH3MT- To determine whether HRasV12-transformation altered the level of expression of Spry2, we compared the cells of the MSU1 lineage using Northern and Western blotting (Fig. 1A and 1B). Compared to the parental MSU1.0 cell stain, the non-transformed, cell strain derived from it, MSU1.1, exhibited only a modest increase in Spry2 expression. In contrast, the malignant cell line PH3MT, exhibited a significant increase in Spry2 expression. An independent HRasV12-transformed cell strain (PH2MT), also had high expression of Spry2 (Fig. 1B). These results are expected if HRas oncogene is responsible for the high level of Spry2 protein found in PH3MT cells. To determine whether expression of oncogenic NRasV12 causes the same effect, we compared the levels of expression of Spry2 protein in two independent, tumor-derived, MSU1.1-derivative cell lines malignantly transformed by transfection of the NRasV12 oncogene, i.e., cell strains NRas-2T and NRas-3T (5). As shown in Fig. 1C, these NRasV12-transformed malignant cell lines also expressed Spry2 at a high level, similar to the level seen in HRasV12-transformed cell lines PH2MT and PH3MT. To determine whether cells from patient-derived fibrosarcomas also express high levels of Spry2, we examined cell lines established from four patient-derived fibrosarcomas. Compared to the levels of Spry2 seen in normal, healthy, fibroblasts derived from the foreskin of two normal human newborns, i.e., SL68 and SL89, all four fibrosarcoma-derived cell lines were found to express high levels of Spry2 (Fig. 1D). In addition, we examined the expression of Spry2 mRNA by mining existing microarray data from a panel of soft tissue sarcomas (34). This panel included cell lines derived from synovial sarcoma (SS), gastrointestinal stromal tumors (GIST), leiomyosarcoma (LMS), malignant fibrous histiocytoma (MFH), and liposarcoma (LS). The relative change in the expression of Spry2 and Spry1 in the sarcomas as compared to a mixture of eleven cell lines is shown in Fig. 1E. In another study, Baird et al. (35) profiled a wider group of sarcomas, including a number of fibrosarcomas and dermatofibrosarcomas. In this study, Spry2 was elevated in 2/7 fibrosarcomas and in 4/5 dermatofibrosarcomas (35). The reference cell line used in this study was comprised of a mixture of five other sarcoma cell lines, including HT1080.

Effect of HRas-transformation on the level of epidermal growth factor receptor protein- Several studies demonstrate that Ras regulates the signaling activity and endocytosis effect of EGFR through Ras-effector proteins (37-41). Consistent with these findings we found that transformation of the parental MSU1.1 cells with HRasV12 was associated with an increase in the levels of EGFR, upon EGF stimulation (Fig 2A). The stimulation of MSU1.1 cells with EGF resulted in a decrease in the level of EGFR protein. Although a similar effect was observed in PH3MT cells (HRas-transformed), these cells exhibited an increased and sustained levels of EGFR, upon EGF stimulation, compared to their parental cells (Fig. 2A). In addition to exhibiting a change in the level of Spry2, PH3MT cells also exhibited a decrease in the level of c-Cbl (Fig. 2A), an E3 ubiquitin ligase shown to target EGFR for degradation.

To determine whether EGFR is necessary for the ability of HRas-transformed cells to replicate in the absence of exogenous growth factors, we measured the ability of PH3MT cells to grow in the presence of AG1478, a selective inhibitor of EGFR tyrosine kinase activity (42). As shown in Fig. 2B, inhibition of EGFR activity by AG1478 resulted in a significant decrease in the ability of the PH3MT cells to grow in medium with 0.1%
serum. What is more, this decrease in PH3MT cells was significantly greater than that observed in its parental MSU1.1 cell strain (Fig. 2B), which is partially growth factor independent (2). Furthermore, when we examined the ability of HRas-transformed cells to form colonies in agarose, we found that addition of AG1478 decreased the number of colonies greater than 120 μm (Fig. 2C). Interestingly, this effect was diminished at higher doses of inhibitor, but, even at its highest dose (4 μM), AG1478 caused a statistically significant decrease in the number of colonies. Inhibition of MAPK signaling by PD98059 reduced the number of large colonies to near zero (Fig. 2C), perhaps because signals downstream of oncogenic HRas are more important compared to upstream signaling, at least with respect to growth in agarose. These results support the notion that EGFR activity contributes to the phenotype of HRasV12-transformed cells consistent with similar findings by Hamilton et al. (43).

Effect of Spry2 on tumor formation by HRas-transformed cells- To study the role of Spry2 in HRasV12-transformation, we down-regulated its expression in the malignant PH3MT cells using short hairpin RNA (shRNA) molecules, designed to target either position 399 or 492 of the Spry2 coding region. Cell strains expressing a vector without a shRNA molecule (PH3MT-VC), or a vector encoding the scrambled shRNA molecule (PH3MT-SC), were used as controls. The two cell strains expressing the Spry2-specific shRNA molecule that targets position 399 (PH3MT-2A3 and -2B9), as well as a cell strain expressing the Spry2-specific shRNA molecule that targets position 492 (PH3MT-5A3), expressed lower levels of the Spry2 protein, compared to the level in the two control cell strains (Fig.3A). The level of Spry1 was not affected by the Spry2-shRNAs (Fig. 3A). We then tested the effect of Spry2 depletion on the ability of these PH3MT-derived cell strains to form large-sized colonies in agarose. The three cell strains with depleted expression of Spry2 produced significantly fewer colonies with a diameter greater than 120 μm than did the parental cells, regardless of serum concentration (Fig.3B,C).

To further examine the role of Spry2 in HRas-malignant transformation, we compared the three cell strains with decreased levels of Spry2 and the appropriate controls for their ability to form tumors in athymic mice. As shown in Table 1, the tumor-derived parental PH3MT cell strains, as well as its vector and scrambled control derivative cell strains formed tumors with a volume of 0.5 cm³ in 32 days. In contrast, the three cell strains with down-regulated expression of Spry2, (PH3MT-2A3, -5A3 and -2B9, Table I), as well as the non-transformed parental cell strain MSU1.1, failed to form any tumors over a six month period. A representative photo of the tumors that formed in athymic mice, compared to mice without tumors, is shown in Fig. 3D. These data indicate that expression of Spry2 is necessary for tumor formation by the HRas oncogene-transformed PH3MT cells.

In view of the significant effect that the down-regulation of Spry2 had on the tumorigenicity of HRasV12-transformed cells, we used a Ras-activation assay to investigate whether this down-regulation of Spry2 had reduced the level of activated HRas protein. Down-regulation of Spry2 had no effect on the levels of active HRas expression levels (Fig. 3C). These results strongly suggest that in PH3MT cells Spry2 acts downstream of HRas oncogene to regulate HRas-induced malignant transformation.

To determine if this mode of Spry2 function was present in additional cellular contexts, we down-regulated the expression of Spry2 in two human patient-derived fibrosarcoma cell lines, i.e. VIP:FT and HT1080, which express high levels of Spry2 (Fig.4A and B). Control cells and cells with down-regulated Spry2 were injected in athymic mice and the frequency of tumor formation is shown in Table II. Loss of Spry2 expression in VIP:FT cells resulted in a decrease in the number of tumors formed. When tumor-derived cells from the injections of control (-SC) and Spry2-down-regulated (-B1) VIP:FT cells were examined for the level of Spry2 expression, we found that those VIP:FT-B1 cells which were able to form tumors did not have down-regulated expression of Spry2 (Fig 4C). The effect of Spry2 down-regulation in HT1080 cells was less pronounced than that in VIP:FT cells. Note that HT1080-C12 cells, which express a similar level of Spry2 as that of control cells (Fig 4B), did not exhibit a significant
change in tumor formation when compared to the control cells. In both fibrosarcoma cell lines, the effect of Spry2 was less pronounced compared to the effect of Spry2 in PH3MT cells.

Effect of depletion of Spry2 protein on the level of EGFR- Because Spry2 prevents EGFR ubiquitination and degradation, we examined if Spry2 was responsible in part for the elevated EGFR levels noted in HRas-transformed cells. To this end, we examined the time course of EGFR decrease in one control cell strain (PH3MT-SC) and in one cell strain with down-regulated Spry2 (PH3MT-2A3, Fig. 5A). The cell strain with down-regulated Spry2 showed a marked reduction in the level of EGFR, upon EGF stimulation, compared to that seen in the scrambled control cell strain, indicating that Spry2 expression is necessary if the HRas oncoprotein-transformed cells are to sustain the level of EGFR. The levels of c-Cbl were unaffected by the down-regulation of Spry2, or EGF stimulation (Fig. 5A).

To determine whether the expression of Spry2 in HRas-transformed cells was associated with an increase in activation of the MAPK cascade, we examined the EGF-induced activation of ERK. Compared to the level found in the scrambled control cell strain (PH3MT-SC), the cell strain with down-regulated Spry2 (PH3MT-2A3) had decreased levels of activated ERK (Fig. 5A).

Similar results were obtained when we examined the levels of EGFR expression, after 10 min stimulation with EGF, in the other cell strains with down-regulated Spry2 (i.e., PH3MT-5A3 and -2B9) and in the control cell strains (Fig. 5B). We did not include an evaluation of MAPK in this experiment because we did not find a significant change in the level of active ERK after 10 min of EGF stimulation, when the time course experiment was performed (Figure 5A).

We also determined whether depletion of Spry2 affected the growth factor independence of PH3MT cells. As shown in Fig. 5C, HRas-transformed cells with decreased levels of Spry2 (PH3MT-2A3) grew slower than did control cells (PH3MT-SC) in media with 0.1% serum. These findings indicate that in the HRas transformed PH3MT cell line, Spry2 sustains EGFR levels and its downstream signaling activity.

Effect of Spry2 expression in MSU1.1 parental fibroblasts with wild type Ras signaling- To determine the role of Spry2 in a background of wild type Ras, we stably overexpressed Spry2 in human fibroblast cell strain MSU1.1 (Fig. 6A). Compared to the vector control cells (MSU1.1-VC), MSU1.1 cells expressing a low level of Spry2-V5 (MSU1.1-S41) exhibited a decrease in EGF-induced phosphorylation of ERK (Fig. 6B), consistent with previous findings in NIH3T3 fibroblasts (44-46). In contrast, in MSU1.1 cells expressing a higher level of Spry2-V5 (MSU1.1-S62) ERK activation was similar to that of control cells, with a decrease in pERK observed only after 60 min (Fig 6C). MSU1.1-S62 cells exhibited similar level of EGFR as did the control cells (Fig 6C). The level of Spry2-V5 expression in MSU1.1S62 cells was similar to the level of endogenous Spry2 expression in MSU1.1-S62 cells, an effect that was less apparent in MSU1.1-S62 cells (Fig. 6D).

Consistent with these findings, low levels of exogenous Spry2 expression resulted in a decrease in the ability of MSU1.1 cells to grow in reduced serum (Fig. 6E, MSU1.1-S41) compared to vector control cells or MSU1.1-S62 cells. Finally, neither of the two cell lines expressing Spry2 (MSU1.1-S41 and -S62) were able to form tumors in athymic mice after six months. These data show that in MSU1.1 cells Spry2 can have an inhibitory function on EGFR/Ras/MAPK signaling. This suggests that the ability of Spry2 to sustain EGFR signaling and to support tumor formation in this system is associated with oncogenic HRas signaling.

Interaction of Spry2 with HRas- Because the role of Spry2 tumor formation appears associated with of oncogenic HRas signaling, we sought to determine if a complex is formed between HRas and Spry2 in HRas-transformed cells (PH3MT). To this end, we carried out co-immunoprecipitation experiments and found that endogenous Spry2 co-immunoprecipitated with exogenous HRas (Fig. 7A), indicating that Spry2 interacts with HRas in these cells. We have been unable to detect an interaction between exogenous Spry2 and wild type Ras, when Spry2 was overexpressed in MSU1.1 cells (data...
Interaction of HRas with c-Cbl and CIN85 in a Spry2-dependent fashion - Spry2 regulates EGFR turnover through its interaction with c-Cbl (25). Furthermore, Haglund et al. (47) show that Spry2, c-Cbl, and CIN85 form a tertiary complex, which results in the inhibition of the lysosomal degradation of EGFR. As shown in Fig. 7B, we found that c-Cbl co-immunoprecipitated with HRas in cells expressing Spry2 suggesting an interaction between c-Cbl and HRas. This interaction was not observed in HRas-transformed cells with down-regulated Spry2 (Fig. 7B). In a similar fashion, CIN85 co-immunoprecipitates with HRas in cells expressing Spry2, but not in cells with down-regulated Spry2 (Fig. 7C). These data argue for a Spry2-mediated interaction between HRas with c-Cbl and CIN85, and suggest that HRas regulates the turnover of EGFR at the level of the Spry2/c-Cbl/CIN85 complex.

DISCUSSION

In this study we examined the role of Spry2 in tumor formation and the regulation of EGFR/Ras/MAPK signaling pathway in pre-malignant (MSU1.1) and in malignant (PH3MT) cells derived from HRasV12 overexpression in MSU1.1 cells. In pre-malignant cells Spry2 acted upstream of HRas to inhibit the Ras/MAPK signaling cascade and cellular growth. This finding strongly suggests that the inhibitory function of Spry 2 in MSU1.1 cells is mediated by the ability of Spry2 to inhibit RTK signaling at the level of Grb2:SOS, reported previously (13). In malignant cells Spry2 acted downstream of oncogenic HRas to sustain EGFR levels and signaling activity, indicating that Spry2 is capable of regulating the EGFR/Ras/MAPK pathway at two different levels, resulting in different effects. It is very likely that, in PH3MT cells, constitutively active Ras excludes an inhibitory effect that Spry2 would have upstream of HRas. This is similar to the finding that Spry1 can activate or inhibit T cell receptor signaling depending on the status of T cell activation (48).

We also found that HRas-transformed cells (PH3MT) have elevated levels of EGFR compared to their parental cells. Furthermore, intact EGFR was required for the growth factor and anchorage independence of these cells. These findings are consistent with previous studies showing that HRasV12 recruits EGFR, through the secretion of EGFR ligands, to promote transformation (27-30). The elevated level of EGFR in PH3MT cells was dependent on Spry2 expression, which supports the conclusion that Spry2 is responsible for the increase in EGFR levels associated with HRas-transformation of MSU1.1 cells. Consistent with this, Spry2 was found to interact with HRas and appeared to facilitate the interaction between HRas and EGFR-regulatory proteins, such as c-Cbl and CIN85, in PH3MT cells. With these in mind, it is possible that Spry2 is also involved in the recruitment of EGFR by HRas during cellular transformation. By relying on these initial findings, we suggest that the contribution of Spry2 to tumor formation by PH3MT cells is dependent, at least in part, on its ability to sustain EGFR levels, a critical factor for the transformed phenotype of these cells. Our future efforts are focused in elucidating the exact mechanism by which Spry2 contributes to tumor formation, in the context of oncogenic HRas signaling.

Our data support the conclusion that Spry2 can have an inhibitory function when expressed in specific immortalized human fibroblasts with wild type HRas, but in their HRas-transformed derivatives, Spry2 sustains EGFR signaling and is required for sarcoma formation. One concern is that PH3MT overexpress HRas oncogene, which could influence our conclusions. It is important to note, however, that expression of a lower level of HRas oncogene in MSU1.1 cells did not result in malignant transformation, and was not associated with an increase in the level of Spry2 (data not shown). We have attempted several times to express Spry2 in these cells, in order to assess whether this change would, function in a co-operative fashion with HRas, and, result in malignant transformation, but we have been unsuccessful in obtaining cells that express both HRas and Spry2. Nevertheless, we found that Spry2 was, at least in part, necessary for tumor formation by some patient-derived fibrosarcoma cell lines, which suggest that the
ability of Spry2 to contribute to tumor formation extends beyond our model system.

Interestingly, the role of Spry2 in supporting tumor formation in VIP:FT and partially in HT1080 cell lines appears to be independent of the activation status of Ras, given that VIP:FT cells express wild-type Ras, whereas HT1080 express NRasQ59. Although consistent with our observations in PH3MT cells, the partial effect of Spry2 down-regulation in tumor formation by HT1080 cells may reflect isoform-specific differences. Our findings in VIP:FT cells suggest that the association between the role of Spry2 and the status of Ras is an observation limited to our model system. It is not clear, however, if VIP:FT cells express activated Ras-effector, or other, pathways, which may influence the mode of Spry2 activity.

Others have shown that Spry2 negatively regulates RTK signaling (34, 37, 38), and that it is down-regulated in breast, lung and prostate cancer, were it may have tumor suppressive properties (23, 24, 39, 49). Recently, Shaw et al. (31) found that transgenic mice harboring KRasG12D express elevated Spry2 levels and Spry2 was found to inhibit tumor formation in a negative feedback manner. In light of these findings, our results may reflect differences between Ras isoforms, i.e. isoform-specific regulation and modes of activity (50), as well as differences inherent to the cellular context of these studies (25,48).

To our knowledge, this is the first report to show that Spry2 has the potential to positively contribute to the malignant phenotype. If this ability is unique to human fibroblasts, or if it has a broader context, remains to be seen.

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FOOTNOTE

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FIGURES

Fig. 1. Level of expression of Spry2 in human fibroblast cell lines. (A) Northern blot of MSU1.0, MSU1.1, and PH3MT cells. (B) Western blot of the same cells. (C) Western blot of the expression of Spry2 and of Ras in MSU1.1 cells, and MSU1.1 cells malignantly transformed by HRas or NRas oncogenes. (D) The expression of Spry2 protein in patient-derived fibrosarcoma cell lines and in normal, foreskin-derived, fibroblast cell lines (SL68 and SL89). Ku80 was used for a loading control in Western blots. (E) The expression of Spry2 mRNA in a panel of soft tissue sarcomas. These results were obtained from mining existing array data (34). Shown is a non-clustered representation of the change in expression of Spry2 (bars) and Spry1 (line), as compared to a mixture of eleven cell lines. Abbreviations- wt: wild type Ras, SS: synovial sarcoma, GIST: gastrointestinal stromal tumors, LMS: leiomyosarcoma, MFH: malignant fibrous histiocytoma, and LS: liposarcoma.

Fig. 2. Effect of HRas transformation on the level of EGFR protein. (A) Parental human fibroblasts (MSU1.1 cells) and their HRasV12-transformed tumor derived derivative (PH3MT) were deprived of serum for 12 h and then stimulated with EGF (100 ng/mL) for the indicated times. Whole cell lysates (WCL) were assayed by Western blotting to determine the levels of the indicated proteins. (B) MSU1.1 cells and PH3MT cells were grown in medium with 0.1% serum in the presence or absence of the selective EGFR inhibitor AG1478 (6 μM). The effect of AG1478 on the growth of PH3MT cells was greater than the effect of the same inhibitor in MSU1.1 cells (p<0.001, N=4). (C) MSU1.1 and PH3MT cells were grown in agarose in the presence or absence the of either AG1478 (1, 2 or 4 μM) or PD98059 (5 μM). Only the number of colonies greater than 120 μm in diameter is shown. The p-value reflecting the statistical significance of the effect of AG1478 in PH3MT cells is 0.0004, 0.002 and 0.02 for the indicated doses respectively (N=4).

Fig. 3. Effect of Spry2 on the anchorage independent growth of HRas-transformed fibroblasts. (A) PH3MT cell strains stably expressing the indicated constructs were analyzed by Western blotting to determine the expression of Spry2. The PH3MT-2A3 and PH3MT-2B9 cell strains were infected with a shRNA targeting position 399 of the Spry2 coding region, whereas the PH3MT-5A3 cell strain was infected with a shRNA targeting position 492 of the Spry2 coding region. PH3MT-VC cells were infected with an empty vector, and PH3MT-SC cells with a scrambled shRNA. The levels of Spry1 are also shown. (B) The indicated cell lines were grown in agarose with a culture medium containing 10% serum or 2.5% serum. This graph represents the number of colonies with a diameter greater than 120 μm (N=4). (C) Representative pictures of the colonies that formed in agarose. (D) Representative photographs of
tumors formed in athymic mice by transformed MSU1.1 cells, compared to mice without tumors. (E) Whole cell lysates from the indicated cell lines were pulled down with Raf-RBD conjugated beads. The total amount of Ras in the whole cell lysate was determined by using a Pan-Ras-specific antibody.

**Fig. 4.** Down-regulation of Spry2 in human patient-derived fibrosarcoma cell lines. VIP:FT (A) and HT1080 (B) cells were stably infected with a vector encoding a scrambled shRNA or a Spry2-specific shRNA. Whole cell lysates from the indicated cell lines were assayed by Western blotting to determine the level of Spry2 expression. (C) The cells derived from tumors formed in athymic mice by the indicated VIP:FT cells were assayed to determine the level of Spry2 protein.

**Fig. 5.** Effect of Spry2 down-regulation in HRas-transformed cells. (A) The indicated cell strains were analyzed as were those in Fig. 2A. (B) Control cell strains and cell strains with down-regulated Spry2 were serum starved as indicated above and then stimulated with EGF (100 ng/mL) for 10 min. Whole cell lysates were assayed to determine the level of EGFR. (C) The indicated cell strains were grown in medium with 0.1% serum. One of two experimental repeats is shown (N=4).

**Fig. 6.** Effect of Spry2 expression in immortalized, non-transformed, MSU1.1 cells. (A) MSU1.1 cells were stably transfected with an empty vector (MSU1.1-VC), or a vector encoding V5-tagged Spry2 (MSU1.1-S41 and -S62). Whole cell lysates from the indicated cell strains were analyzed by Western blotting to determine the expression of Spry2. Ku80 served as a loading control. (B,C) The indicated cell lines were analyzed as in Fig. 4A. (D) Whole cell lysates from the indicated cell lines were analyzed as in Fig. 3C. (E) The indicated cell strains were grown in medium containing 0.1% serum and cell counts were performed at the indicated time points. One of two experimental repeats is shown (N=4).

**Fig. 7.** Interaction of HRas with Spry2 and Spry2 binding-partners c-Cbl and CIN85. (A) Whole cell lysates from HRas-transformed cells (PH3MT) were immunoprecipitated with a HRas-specific antibody or a nonspecific IgG antibody, and immunoblotted with the indicated antibodies. (B,C) Whole cell lysates from control PH3MT cells (PH3MT-SC) and cells with down-regulated Spry2 (PH3MT-2A3) were immunoprecipitated with anti-HRas and immunoblotted with the indicated antibodies. Whole cell lysates were analyzed by Western blotting to determine the level of the proteins in the cells. IP: immunoprecipitation

### Table I

Table I. Down-regulation of Spry2 eliminates the ability of HRas-transformed cells to form tumors in athymic mice

| Cell strain | HRas<sup>v12</sup> | Spry2-shRNA | Tumor incidence<sup>†</sup> | Tumor latency<sup>‡</sup> |
|-------------|-----------------|-------------|--------------------------|--------------------------|
| MSU1.1      | -               | -           | 0/6                      | -                        |
| PH3MT       | ++              | -           | 6/6                      | 32 ± 0                   |
| PH3MT-VC    | ++              | -           | 12/12                    | 36 ± 2                   |
| PH3MT-SC    | ++              | -           | 12/12                    | 33 ± 0                   |
| PH3MT-2A3   | ++              | +           | 0/12                     | -                        |
| PH3MT-5A3   | ++              | +           | 0/12                     | -                        |
| PH3MT-2B9   | ++              | +           | 0/12                     | -                        |

<sup>†</sup>Number of tumors that arose per injection, i.e., 2 sites per mouse

<sup>‡</sup>Number of days required for tumor to reach 0.5 cm³
Table II. Effect of Spry2 down regulation in the ability of fibrosarcoma cells to form tumors in athymic mice

| Cell strain | Spry2-shRNA | Tumor incidence† | Tumor latency' |
|-------------|-------------|------------------|----------------|
| VIP:FT      | na          | 6/6              | 50 ± 0         |
| VIP:FT-SC   | -           | 4/6*             | 50 ± 12        |
| VIP:FT-B1   | +           | 6/16             | 82 ± 16        |
| HT1080      | na          | 6/6              | 30 ± 0         |
| HT1080-SC   | -           | 7/8              | 44 ± 0         |
| HT1080-C10  | +           | 6/10             | 43 ± 5         |
| HT1080-C12  | +           | 9/10^            | 44 ± 3         |

* Tumors formed in two more sites, but they regressed to normal.
† Included in this measurement are two tumors that did not reach 0.5 cm³.
‡ Number of tumors that arose per injection, i.e., 2 sites per mouse.
§ Number of days required for tumor to reach 0.5 cm³.
Figure 1
Figure 2
Figure 3
Figure 4
### Figure 5

#### Figure 5A

|          | PH3MT-SC | PH3MT-2A3 |
|----------|----------|-----------|
| 0        | 0        | 0         |
| 10       | 10       | 10        |
| 30       | 30       | 30        |
| 60       | 60       | 60        |
| min. (EGF)|          |           |
| EGFR     |          |           |
| c-Cbl    |          |           |
| Spry2    |          |           |
| p-ERK    |          |           |
| ERK1/2   |          |           |
| Ku80     |          |           |

#### Figure 5B

|          | PH3MT-SC | PH3MT-2A3 |
|----------|----------|-----------|
| VC       | -        | -         |
| SC       | -        | -         |
| 2A3      | +        | +         |
| 5A3      | +        | +         |
| 2B9      | -        | -         |
| EGF      | -        | -         |
| EGFR     | -        | -         |
| Spry2    | -        | -         |
| Ku80     | -        | -         |

#### Figure 5C

**Cell number (x10^6)**

| Days | PH3MT-SC | PH3MT-2A3 |
|------|----------|-----------|
| 1    | 10       | 10        |
| 2    | 30       | 30        |
| 3    | 50       | 50        |
| 4    | 70       | 70        |
Figure 6
Figure 7
Evidence that sprouty 2 is necessary for sarcoma formation by HRAS oncogene transformed human fibroblasts
Piro Lito, Bryan D. Mets, Susanne Kleff, Sandra O'Reilly, Veronica M. Maher and J. Justin McCormick

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