The Tumor Suppressor PTEN Is Necessary for Human Sprouty 2-mediated Inhibition of Cell Proliferation

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Sprouty family proteins are novel regulators of growth factor actions. Human Sprouty 2 (hSPRY2) inhibits the proliferation of a number of different cell types. However, the mechanisms involved in the anti-proliferative actions of hSPRY2 remain to be elucidated. Here we have demonstrated that hSPRY2 increases the amount of the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and decreases its phosphorylation. The resultant increase in PTEN activity is reflected in decreased activation of Akt by epidermal growth factor and serum. Consistent with increased PTEN activity, in hSPRY2-expressing cells, the progression of cells from the G1 to S phase is decreased. By using PTEN null primary mouse embryonic fibroblasts and their isogenic controls as well as small interfering RNA against PTEN, we demonstrated that PTEN is necessary for hSPRY2 to inhibit Akt activation by epidermal growth factor as well as cell proliferation. Overall, we concluded that hSPRY2 mediates its anti-proliferative actions by altering PTEN content and activity.

Over the past few years, the Sprouty (SPRY) family of proteins has emerged as an important modulator of growth factor actions. The ability of the Sprouty proteins to regulate the biological activity of growth factors has been conserved through evolution. Drosophila SPRY (dSPRY) was the first member of the family to be identified and has been shown to regulate tracheal branching in response to fibroblast growth factor (1). Later studies demonstrated that dSPRY also inhibited the actions of EGF (2). Like dSPRY, mammalian SPRY isoforms (SPRY1–4) have also been shown to modulate growth factor-mediated actions. For instance, mouse SPRY2 expression resulted in increased lung branching morphogenesis (3). These findings suggest that SPRY proteins have conserved function to modulate respiratory morphogenesis. The ability of mouse SPRY4 to inhibit angiogenesis (4) and cause pulmonary hypoplasia as well as the ability of SPRY2 and SPRY1 to decrease uteretic branching and kidney development (5, 6) also demonstrates that the SPRY proteins play a profound role in regulating tubular morphogenesis. Notably, however, SPRY proteins do not exclusively regulate tubular morphogenesis, but by opposing the actions of growth factors they may also play a role in the development of other organs, such as the brain and limbs (7–9).

At the cellular level, we and others have shown that overexpression of SPRY1 (10), SPRY2 (11, 12), and SPRY4 (5) inhibits migration and proliferation of cells in response to serum and growth factors. Treatment of cells with EGF results in translocation of the human SPRY2 (hSPRY2) protein from the vicinity of microtubules to membrane ruffles (11, 13). The abrogation of co-localization of hSPRY2 with microtubules or deletion of the region that is necessary for translocation to membrane ruffles obliterates the ability of the protein to inhibit cell migration and proliferation (11). We have shown previously that hSPRY2, in part, mediates its anti-migratory actions by increasing the amount of soluble protein-tyrosine phosphatase 1B (PTP1B) (14). Moreover, we have shown that hSPRY2 decreases growth factor-mediated activation of Rac1 and that constitutively active Rac1 protects against the anti-migratory, but not the anti-proliferative, actions of hSPRY2. Although some tenable mechanisms of hSPRY2-mediated inhibition of migration have been described, the mechanisms or signaling proteins that mediate the anti-proliferative actions of hSPRY2 in cells remain to be elucidated and form the subject of this study.

Here we demonstrate that hSPRY2 expression in HeLa cells increases the amount of the tumor suppressor PTEN. The phosphorylation of PTEN in hSPRY2-expressing cells is also decreased. Consistent with an increase in PTEN amount and activity in hSPRY2-expressing cells, the activation of Akt by EGF or serum is decreased. The ability of hSPRY2 to decrease proliferation was also obliterated in PTEN null, but not the isogenic control, PMEFs, and in HeLa cells where PTEN levels were knocked down by using siRNA approaches. These findings demonstrate that PTEN plays a pivotal role in mediating the anti-proliferative actions of hSPRY2.

MATERIALS AND METHODS

Cells and Culture Conditions—Control and HA-hSPRY2-expressing stable HeLa cell lines were used as reported previously (11). HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and maintained in 800 μg/ml G418. TAG-HA-tagged hSPRY2 and GFP were purified and used as described previously (11).

The PMEFs were derived from 14-day-old embryos of Pten+/−/Pten−/− mice. Following removal of the head and organs, embryos were rinsed with phosphate-buffered saline (PBS), minced, and digested with trypsin (0.05% trypsin containing 0.53 mM EDTA) for 20 min at 37 °C. Trypsin was inactivated by addition of PMEF medium (DMEM (4.5 g/liter glucose)) supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 0.2 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin. The PMEFs were maintained in medium supplemented with 10% fetal bovine serum.
0.1 mM minimum Eagle’s medium nonessential amino acids, 0.1 mM β-mercaptoethanol, and 100 units/ml penicillin/streptomycin. Cells from single embryos were plated onto one 100-mm diameter culture dish and incubated at 37 °C in a 5% CO₂ humidified incubator. The Pten/knockout MEFs (passage 1) were infected with retrovirus to express either GFP or Cre recombinase-GFP fusion protein, and PTEN expression in these cells was monitored by Western analyses to confirm the presence of either GFP or Cre recombinase-GFP fusion protein, and PTEN expression in these cells before they were used again. MEFs were used within passage 4.

Western Analyses—Cell lysates were prepared in the Laemmli sample buffer (15), and proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane for 1 h at 100 V in the cold. Membranes were blocked either in TBS containing 5% (w/v) nonfat dry milk with 0.1% Tween 20 or in PBS containing 3% (w/v) nonfat dry milk. The following primary antibodies were used: anti-HA (HA.11 monoclonal antibody, Covance Research Products, Berkeley, CA); anti-PTEN (MMAC1 Ab-5 polyclonal antibody, Labvision, Fremont, CA). The anti-phospho-PTEN (Ser-380/Thr-382/Thr-383 polyclonal antibody), anti-Akt (polyclonal antibody), and anti-phospho-Akt (Thr-380/ polyclonal; Ser-473, 4E2 monoclonal) antibodies were from Cell Signaling Technology, Beverly, MA. Anti-phospho-Ser-370 and Ser-385 PTEN antibodies were from BIOSOURCE and AnaSpec Inc. (San Jose, CA), respectively. Monoclonal anti-actin antibody was from MP Biomedicals (Aurora, OH). Anti-Erk1/2 antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY).

Semi-quantitative and Quantitative Real Time PCR—Total RNA was isolated from semi-confluent control and hSPRY2-expressing HeLa cells using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Quality and integrity of the RNA were checked by the A260/280 ratio and on formaldehyde/agarose gel, respectively. Equal amounts of RNA were reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) as per the manufacturer’s instructions. For semi-quantitative PCR, equal amounts of reverse-transcribed RNA were used with the following PTEN primers: forward primer, 5′-ACC AGG ACC AGA GGA AAC CT-3′, and reverse primer, 5′-GCT AGC CTC TGG ATT TGA CG-3′. Three sets of PTEN primers were designed using the primer design software Primer3 (16) and were analyzed for sequence homology for related proteins. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as endogenous control. Real time PCRs were performed on an iCycler iQ real time PCR detection system (Bio-Rad), and the data were analyzed using the provided iCycler software. The reaction mixture consisted of 5 μl of cDNA template, 25 μl of 2× SyberGreen PCR master mixture (Roche Applied Science), 5 μl of 4 μM forward primer, and 5 μl of 4 μM reverse primer in a 50-μl reaction volume. The PCR protocol consisted of one 10-min denaturation cycle at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Standard curves for both the PTEN and endogenous control GAPDH were used. The efficiency of PCR amplification was 100%, and the R² value was between 0.991 and 0.994, whereas the slope was between −3.302 and −3.31 in the standard curves. All real time PCR data are expressed as fold change in PTEN mRNA levels in hSPRY2-expressing cells with respect to control cells after normalizing to the levels of GAPDH. Data represented are from three independent experiments done in triplicate.

Stability of PTEN by Pulse-Chase Analyses—For pulse-chase analyses, semi-confluent control and hSPRY2-expressing HeLa cells were methionine-starved with methionine-free DMEM supplemented with 10% fetal bovine serum and antibiotics for 1 h at 37 °C in a humidified 5% CO₂ incubator. Cells were radiolabeled with [35S]methionine (100 μCi/ml; EasyTag, PerkinElmer Life Sciences) for 4 h, and the newly synthesized radiolabeled proteins were subsequently chased with normal media containing 1 mM cycloheximide for the indicated times or were lysed immediately. The lysis buffer contained 50 mM Hepes, pH 7.4, 0.1% SDS, 0.1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and protease inhibitors. PTEN was immunoprecipitated from 300 μg of cell lysate with PTEN antibody (MMAC1 Ab-5 polyclonal antibody, Labvision, Fremont, CA) or rabbit IgG alone. Samples were resolved on SDS-PAGE and analyzed by autoradiography.

The amount of radiolabeled PTEN at each time point was quantified by subtracting the background in each lane. Data are presented as the percent of PTEN at 0 h after cycloheximide addition.

Cell Proliferation Assays—For proliferation studies PTEn+/+ and PTEn−/− MEFs (30,000 cells/well) were grown in 24-well plates in triplicate for each condition. Cells were grown in MEF medium containing 10 μg/ml either TAT-HA-hSPRY2 or TAT-HA-GFP for 24 h. Media containing the TAT proteins were changed every 24 h. After 72 h of growth, cells were trypsinized and counted using a hemocytometer.

PTEN Knockdown Using siRNA—HeLa cells were plated at 50% confluence in 35-mm dishes. PTEN was knocked down as described in the transIT-TKO (Mirus, MI) protocol. Briefly, in 200 μl of Opti-MEM, 6 μl of transIT-TKO reagent was mixed thoroughly and incubated for 12 min at room temperature. 2.4 μl of 10 μM PTEN siRNA or mutant PTEN siRNA were added to this and incubated at room temperature for a further 12 min before dropwise addition to the dishes containing 1 ml of media. Cells were incubated for 48 h before experimentation. The PTEN siRNA duplex sequence, 5′-GUU AGC AGA AAC AAA AGG AGA UAU CAA-3′ (sense)/5′-UUG AUA UCU CCU UUU GUU UCU GCU AAC-3′ (antisense), and the mutant siRNA duplex sequence, 5′-GUC AGC AGA ACA AAA GUA GTT-3′ (sense)/5′-CUA CUU UUG UGU CUU CUG AC CAG ACT T-3′ (antisense), were custom-synthesized commercially. These sequences do not share homology with any known sequences in the data base.

[3H]Thymidine Incorporation Assay—HeLa cells (50,000 cells/well) previously treated with Sham, siRNA, or mutant siRNA were plated in triplicate in a 24-well tissue culture plate. Cells were serum-starved overnight with DMEM containing 0.5% bovine serum albumin and antibiotics before treating with or without serum for 20 h. One μCi of [3H]thymidine (PerkinElmer Life Sciences) was added to each well, and cells were incubated for 4 h at 37 °C. At the end of this period, plates were placed on ice, and cells were washed three times each with ice-cold PBS, then with ice-cold 10% trichloroacetic acid, and finally with ethanol/ether (2:1). The cells were dissolved with 0.1% SDS in 0.1 N NaOH by incubating overnight at room temperature. Aliquots were counted for [3H], and protein concentration was determined by using the bicinchoninic acid method as described previously (14).

Cell Cycle Analyses—Control and hSPRY2-expressing cells (300,000 cells) were grown in 60-mm dishes. The cells were serum-starved for 24 h with DMEM containing 0.5% bovine serum albumin and antibiotics before the addition of EGF (50 ng/ml) or vehicle. Twelve hours later, the cells were harvested, and single cell suspensions were prepared in PBS supplemented with 1% bovine serum albumin. Cells were washed twice, resuspended at a concentration of 1–2 × 10⁶ cells/ml, and fixed with 70% (v/v) ethanol for 1 h at −20 °C. They were then washed twice with PBS and then stained with propidium iodide solution (5 μg/ml) containing RNase A (100 μg/ml) at 37 °C for 20 min. Samples were analyzed on a flow cytometer.
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**FIGURE 1. hSPRY2 Increases Cellular PTEN Protein and mRNA Content but Decreases Its Stability.** Control or HA-hSPRY2-overexpressing HeLa cells were lysed in Laemmli buffer and analyzed for their PTEN content on immunoblots (A). Blots were probed for ERK1/2 to ensure equal loading. The anti-α-HA immunoblots show the expression of HA-hSPRY2. The right-hand panel shows quantification of PTEN levels in HeLa cells from at least three independent immunoblots similar to those in A. *p < 0.001; Student’s unpaired t test. B, total RNA was isolated from vector or HA-hSPRY2-expressing HeLa cells, and equal amounts were reverse-transcribed. By using specific primers, PTEN and GAPDH (internal standard) were amplified by PCR as described under “Materials and Methods.” Equal volumes of the PCR products were analyzed on 1.5% agarose gel. Real time PCR quantification is shown in the bar graph. For details see “Materials and Methods.” PTEN mRNA levels were normalized to GAPDH levels and represented as the fold change over control. The means ± S.E. (n = 3) are shown. C, control and HA-hSPRY2-expressing HeLa cells were methionine-starved for 1 h and labeled with [35S]methionine for 4 h before chasing with normal media for the indicated times. Cells were lysed, and PTEN was immunoprecipitated and resolved on 10% SDS-PAGE followed by autoradiography. Control immunoprecipitations were performed with rabbit IgG. The PTEN bands, designated by arrows, were quantified after correcting for background in each lane and plotted. The amount of PTEN at the 0-h time point in control and HA-hSPRY2 lanes was assigned as 100%.

**RESULTS AND DISCUSSION**

Previously, we have shown that the anti-migratory actions of hSPRY2 are mediated by an increase in the soluble, cytosolic PTPIB (14). In hSPRY2-expressing HeLa cells, an increase in cytosolic PTPIB can also explain the decrease in tyrosine phosphorylation of cellular proteins such as p130 Cas (14). Therefore, we reasoned that an increase in activity of other phosphatases might also contribute to the negative modulation of growth factor actions by hSPRY2. In our search for phosphatases whose activity or amount may be elevated by hSPRY2, we observed that the amount of PTEN was elevated by at least 2-fold in HeLa cells overexpressing hSPRY2 (Fig. 1A). Likewise, hSPRY2 also increased the amount of PTEN in primary mouse embryonic fibroblasts (PMEFs, Fig. 5C; discussed below). To determine the mechanisms that led to an increase in PTEN content, experiments were performed to determine whether the expression of hSPRY2 altered the turnover of the protein or increased PTEN transcript. As shown in Fig. 1B, by semi-quantitative RT-PCR, we determined that the amount of PTEN transcript is elevated in hSPRY2-expressing HeLa cells; by using real time PCR, we found that the PTEN mRNA content was increased by ~5-fold. Measurements of PTEN stability (Fig. 1C) showed that, most surprisingly, the t_1/2 of PTEN in hSPRY2-expressing cells was half (15 h) that of control cells. Thus, it would appear that despite a 2-fold increase in PTEN turnover in hSPRY2-expressing cells, the greater (5-fold) increase in PTEN transcript is responsible for the overall increase in PTEN content. The t_1/2 of ectopically overexpressed PTEN has been reported to be ~2–5 h (17, 18). However, the endogenous PTEN turns over with a significantly longer half-life (Fig. 1C), suggesting that the overexpressed protein is preferentially targeted to the degradation pathway in cells and does not really reflect the half-life of endogenous PTEN. PTEN has been shown to be phosphorylated on several residues in its C-terminal tail by casein kinase and other kinases (17, 19–21). The phosphorylation of PTEN by casein kinase 2 on Ser-370 and Ser-385 decreases its activity (21). Additionally, this post-translational modification interferes with the electrostatic membrane binding of PTEN and decreases its function (19, 20). Furthermore, the phosphorylation of PTEN on its C terminus also
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increases its stability (17, 19, 20) and may also have other as yet unidentified effects on the enzyme. Because we observed an increased turnover of PTEN protein and because PTEN function can be altered by its phosphorylation, using antibodies that recognize the phospho-forms of PTEN, we monitored the phosphorylation status of PTEN in control and hSPRY2-overexpressing cells. Because signaling cascades activated by EGF may alter the phosphorylation of PTEN, we investigated whether this agonist altered the phosphorylation status of PTEN. Fig. 2A shows that the amount of phospho-Ser-380, -Thr-382, and -Thr-383 PTEN in hSPRY2-expressing cells was decreased to ~50% that in control cells, and this phosphorylation status was not affected by EGF. According to the manufacturer (Cell Signaling, MA), this anti-phospho-PTEN Ser-380/Thr-382/Thr-383 antibody recognizes phospho-PTEN only when all three sites are phosphorylated. Total ERK1/2 shows equal loading of proteins. Because hSPRY2 decreased PTEN phosphorylation and increased PTEN content, we determined whether changes in phosphorylation status of PTEN altered the ability of the anti-PTEN antibody to recognize the protein. Essentially, we immunoblotted samples of cell lysates from control and hSPRY2-expressing cells before and after dephosphorylating the proteins with calf intestinal phosphatase. The data from these experiments (supplemental Fig. S1) demonstrated that the total PTEN content of hSPRY2-expressing cells was elevated even after PTEN was dephosphorylated; thus, the changes in PTEN content observed in Fig. 1A are not artifacts of changes in PTEN phosphorylation. By using antibodies against phospho-Ser-370 and phospho-Ser-385 PTEN, the two major casein kinase 2 phosphorylation sites (21), we also observed that in hSPRY2-expressing cells the phosphorylation of PTEN on Ser-370 and Ser-385 was also decreased by 50% (Fig. 2, B and C). EGF did not alter the phosphorylation of PTEN on Ser-370 and Ser-385 in either control or hSPRY2-expressing cells (not shown). A decrease in the C-terminal phosphorylation of PTEN would be predicted to decrease PTEN stability (17, 19, 20), consistent with the increased turnover of PTEN in the presence of hSPRY2. Phosphorylation may play a complex role in regulating PTEN activity, as decreased phosphorylation may also augment PTEN activity and function through enhanced association with membranes (19–21). Presently, how hSPRY2 causes decrease in PTEN phosphorylation is unknown and remains the subject of future investigations.

One of the principal substrates of PTEN is phosphatidylinositol 3,4,5-trisphosphate (PIP3) (22). An increase in PTEN function would be expected to decrease PIP3 levels and thereby decrease the activation of downstream kinases such as AKT that depend upon increases in PIP3 levels for their activation (23). Therefore, as a reporter of the functional significance of the increase in PTEN content and its decreased phosphorylation in hSPRY2-expressing cells, we monitored the ability of EGF to activate AKT. Although the amount of total AKT was not altered (Fig. 3A), the phosphorylation of AKT on Thr-308 (Fig. 3A) and Ser-473 (Fig. 3B) in response to EGF was markedly decreased in hSPRY2-expressing cells. Thus, consistent with increased PTEN content and decreased PTEN phosphorylation (Figs. 1 and 2), PTEN function was indeed elevated in hSPRY2-expressing cells.

PTEN is a well established inhibitor of cell proliferation. By opposing

FIGURE 3. AKT activation is decreased in hSPRY2-expressing cells. Control and hSPRY2-expressing HeLa cells were grown in serum-free medium for 24 h and then exposed to EGF (50 nm) for the times indicated, and the total cell lysates (50 μg) were applied to SDS-PAGE and immunoblotted for total AKT and phospho-Thr-308 AKT by using an anti-phospho-specific antibody (A). The bar graph on the right-hand panel depicts the ratio of phospho-AKT/total AKT and has been normalized by assigning the 2-min time point (where variability was minimal) in control cells a value of 1. The means ± S.D. of three experiments are shown. *, p < 0.05; **, p < 0.01; Student’s unpaired t test. B, same as A except that the blots were probed for phospho-Ser-473 AKT. The bar graph on the right is the quantification of three similar experiments in which the ratio of phospho-Ser-473AKT/total ERK1/2 is normalized by assigning the 2-min time point in control cells a value of 1. *, p < 0.02; **, p < 0.01; ***. p < 0.008.

FIGURE 4. hSPRY2 inhibits G1-S phase transition. Control and hSPRY2-expressing HeLa cells were serum-starved for 24 h and subsequently stimulated with or without EGF for 12 h. Cells were harvested, and cell cycle analysis was carried out on a flow cytometer as described under "Materials and Methods." The percent of cells in each phase of the cell cycle is presented as the means ± S.E. (n = 4). Statistical significance was assessed by Student’s unpaired t test.
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and inhibiting the actions of phosphatidylinositol 3-kinase and inhibiting activation of AKT, PTEN causes arrest of cells in the G1 phase of the cell cycle (24–26). In contrast, when PTEN is ablated, AKT activation is augmented, and the G1 to S phase transition of cells is enhanced (27). Because in hSPRY2-expressing cells we observed an increase in PTEN activity along with a decrease in phospho-AKT amounts and presumably AKT activity, we reasoned that the hSPRY2-expressing cells might be arrested in the G1 phase. Therefore, we monitored the distribution of EGF-stimulated control and hSPRY2-expressing HeLa cells in the different phases of the cell cycle. As shown in Fig. 4, hSPRY2 increased the fraction of cells in the G1 phase of the cell cycle. HeLa cells proliferate in a cell autonomous manner, and the stimulation of their cell cycle progression (decrease in G1) by growth factors such as EGF is modest but statistically significant (Fig. 4). Notably, however, the proportion of cells in the G1 phase is elevated by hSPRY2 even in the presence of EGF (Fig. 4). These data are consistent with what would be expected from increased PTEN amounts and decreased AKT activity. Thus, in hSPRY2-expressing cells, the inhibition of proliferation because of an increase in PTEN and a decrease in AKT activity provides a rational and tenable mechanism. SPRY4 expression has also been shown to arrest endothelial cells in G1 phase of the cell cycle (5).

To determine whether the hSPRY2-mediated attenuation of AKT activation and inhibition of cell proliferation requires PTEN, experiments were performed with PMEFs from PtenloxP/loxP animals (28). These fibroblasts were infected with retrovirus to express either Cre recombinase-GFP fusion protein or GFP alone to obtain Pten−/− and isogenic control cells, respectively. We have shown previously that the introduction of hSPRY2 in cells by protein transduction using TAT-HA-tagged SPRY2 is very efficient (>99% of cells are transduced), and the biological actions of the transduced proteins are the same as transfection-mediated hSPRY2 overexpression (11, 14, 29, 30). Therefore, for the experiments below, TAT-HA-hSPRY2 or TAT-HA-GFP (control) was transduced into the cells as described previously (11, 14, 29, 30). As observed with HeLa cells, transduction of hSPRY2 into control PMEFs decreased EGF-mediated phosphorylation of Thr-308 on Akt (Fig. 5A). Likewise, serum-induced activation of Akt in PMEFs was also decreased by hSPRY2 (Fig. 5B). However, in Pten−/− cells, transduction of hSPRY2 did not alter the activation of Akt by EGF or serum (Fig. 5, A and B). Our finding that in the Pten−/− cells, transduction of hSPRY2 did not alter the activation of Akt suggests that hSPRY2 is greater than that observed in PtenloxP/loxP PMEFs is similar to those of others and underscores the role of PTEN in regulation of Akt activation. The inability of hSPRY2 to alter Akt activation in the Pten−/− cells was not due to inadequate transduction of TAT-HA-hSPRY2 because the amount of TAT-hSPRY2 in these cells was the same as that in controls (see e.g. Fig. 5A). Overall, these data show that the hSPRY2-mediated decrease in Akt activation requires Pten and demonstrates that hSPRY2 inhibits serum- and growth factor-stimulated Akt activation by increasing Pten function. Notably, as observed with HeLa cells (Fig. 1A), in hSPRY2 transduced PMEFs, the amount of endogenous Pten was also increased (Fig. 5, A and C, and 6B). Thus, hSPRY2 increases Pten content in more than one cell type, and TAT-HA-hSPRY mediates the same biological actions as its transfected coun-

![Image](https://example.com/figure5.png)

**FIGURE 5.** hSPRY2 decreases Akt activation in Pten+/+ PMEFs but not in Pten−/− PMEFs. A, Pten−/− and Pten+/+ PMEFs were generated from PtenloxP/loxP PMEFs (passage 1) as described under “Materials and Methods.” The cells (250,000/35-mm plate) were serum-starved overnight in the presence of TAT-HA-GFP or TAT-HA-hSPRY2 and then treated with or without EGF (50 nM) for 5 min. Following lysis, 30 μg of proteins from each sample were analyzed by Western blotting with anti-phospho-Thr-308 Akt antibody. Total Akt was used as loading control. Complete quantification of phospho-Akt bands from three similar experiments is shown in the right panel. Data are presented as the mean ± S.E. (n = 3). *, p < 0.01 as compared with corresponding condition in cells treated with TAT-GFP. B, expression of Pten in Pten+/+ PMEFs after transduction with TAT-HA-GFP or TAT-HA-hSPRY2 was monitored. Equal loading is shown by actin. HA blot indicates the presence of transduced TAT proteins. The quantification of Pten bands from three different immunoblots after normalizing to actin is shown in the right panel. *, p < 0.02; Student’s unpaired t test. C, Pten−/− PMEFs were generated from PtenloxP/loxP PMEFs after transduction with TAT-HA-GFP or TAT-HA-hSPRY2. Total Akt was used as loading control. Quantification of phospho-Akt bands from three similar experiments is shown in the right panel. Data are presented as the mean ± S.E. (n = 4). Statistical significance was assessed by Student’s unpaired t test.
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FIGURE 6. PTEN is necessary for hSPRY2-mediated inhibition of cell proliferation. A. Pten−/− and Pten+/+ PMEFs (30,000 cells/well) were plated in triplicate in a 24-well plate. Cells were serum-starved overnight and subsequently stimulated with 10% serum in the presence of either 10 μg/ml TAT-HA-GFP or TAT-HA-hSPRY2 for 72 h. Media were changed every 24 h and supplemented with 10 μg/ml TAT proteins. The mean ± S.E. of three independent experiments are shown. Statistical significance was assessed by Student’s unpaired t test. B, the lack of expression of Pten in Pten−/− cells was confirmed in the presence and absence of hSPRY2. Actin was used as loading control, and the anti-HA antibody blot shows the presence of transduced TAT-HA-GFP and TAT-HA-hSPRY2 in these cells.

To determine the role of PTEN in the anti-proliferative actions of hSPRY2 in HeLa cells, we transfected the cells with nothing (sham) or siRNA targeted against PTEN or a mutant of this siRNA. Human SPRY2 inhibited the proliferation of sham or mutant siRNA-transfected HeLa cells to a similar extent (Fig. 7). As observed in PMEFs (Fig. 6), the knock down of PTEN in HeLa cells also increased mitogenesis (data not shown). Thus, the lack of an effect of hSPRY2 in Pten−/− PMEFs cannot be attributed to any artifact because of transfection of Cre-GFP.

The modulation of cell proliferation and migration by SPRY proteins has a number of roles ranging from embryonic development (3) to the incidence of tumor growth and progression (12, 31) as well as restenosis of injured arteries (29). Moreover, hSPRY2 levels have been shown to be decreased in human breast cancer tissues (31). Therefore, the elucidation of the signaling pathways involved in the anti-proliferative and anti-migratory actions of SPRY proteins is essential to understand their mechanism of action. Some studies have suggested that hSPRY2 may act at or above the level of Raf1 to inhibit downstream signaling from growth factors (32–34). Additionally, hSPRY2 by binding with c-Cbl has been shown to decrease the ubiquitination and degradation of the EGF receptor (35–38). This results in an up-regulation of the EGF receptor that then translates into a slight increase in Erk1/2 activation in response to EGF (35, 36). These latter findings demonstrate that the reported actions of hSPRY2 at or above the level of Raf1 (32–33) do not alter EGF-mediated activation of the Erk1/2 pathway. Up-regulation of the EGF receptor by hSPRY2 should result in an increase in Akt activation by EGF. However, we observe the opposite, i.e. hSPRY2 inhibits EGF-mediated activation of Akt (Figs. 3 and 5A). Moreover, in HeLa cells, we have not observed any significant effects of hSPRY2 on the activation of either the EGF receptor or ERK1/2 by EGF (supplemental data Fig. S2). Therefore, the hSPRY2-mediated decrease in AKT activation by EGF (Figs. 3 and 5) that we observe cannot be ascribed to alter-
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In EGF receptor activation. Rather, as shown by the data with Pten null PMEFs, Pten is necessary to observe hSPRY2-mediated inhibition of Akt activity. In this context, a recent study reported that Akt activation by glial derived neurotrophic factor in enteric nerve cells of Spry2 knock-out mice is enhanced as compared with wild type controls (39). Based on our data, in two different cell types it would not be surprising if Pten content in the enteric nerve cells of Spry2 knock-out mice is decreased. Likewise, because hSPRY2 (Fig. 4) and SPRY4 (5) both inhibit cell cycle progression from the G1 to the S phase, it would not be surprising if SPRY4 also increased Pten content and/or activity.

We have shown previously that an increase in cytosolic PTP1B content, at least in part, responsible for the anti-migratory actions of hSPRY2 (14) but not its anti-proliferative actions. An increase in PTP1B activity by hSPRY2 is associated with decreased phosphorylation of its substrate p130Cas (14). The complexes of phospho-p130Cas, CrkII, ELMO1/2, and DOCK180 have been shown to activate Rac1, a key regulator of cell migration (47–49). Thus an increase in PTP1B activity and the associated decrease in phospho-p130Cas could decrease Rac1 activation. Indeed, Rac1 activation is attenuated in hSPRY2-transduced cells, and the expression of constitutively active Rac1 obliterates the hSPRY2-mediated decrease of Rac1 activation is also manifested by an elevation of PTEN activity and decreased PIP3 content. This is perhaps why PTP1B ablation only partly rescues cells from the anti-migratory actions of hSPRY2, and Rac1 may present a locus where the PTP1B and PTEN pathways converge to regulate the anti-migratory actions of hSPRY2. These possibilities will be investigated in the future.

As evident from the data presented in this study, the anti-proliferative action of hSPRY2 requires PTEN, and SPRY2 expression results in both inhibition cell cycle progression from the G1 to the S phase, it would not be surprising if SPRY4 also increased Pten content and/or activity. hSPRY2. These possibilities will be investigated in the future.

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