Sprouty2 regulates growth and differentiation of human neuroblastoma cells through RET tyrosine kinase

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The Sprouty (SPRY) family of proteins includes important regulators of downstream signaling initiated by receptor tyrosine kinases. In the present study, we investigated the role of SPRY proteins in intracellular signaling via the RET receptor tyrosine kinase activated by glial cell line-derived neurotrophic factor (GDNF). Expression of SPRY1, SPRY2, SPRY3 and SPRY4 in HEK293T cells transfected with RET and GDNF receptor family α1 (GFRα1) genes significantly reduced sustained ERK activation as well as ELK-1 activation. Because expression of SPRY2 was efficiently induced by GDNF in TGW human neuroblastoma cells expressing RET and GFRα1, we further investigated the role of SPRY2 in the growth and differentiation of TGW cells. Expression of wild-type SPRY2 (WT-SPRY2) decreased the growth of TGW cells. In contrast, expression of a dominant negative form of SPRY2 (MT-SPRY2, with a mutated tyrosine residue) enhanced cell proliferation. In addition, expression of WT-SPRY2 reduced GDNF-dependent neurite outgrowth of TGW cells, whereas expression of MT-SPRY2 enhanced it. Taken together, our results suggest that SPRY2 regulates GDNF-dependent proliferation and differentiation of TGW neuroblastoma cells mediated by RET tyrosine kinase. (*Cancer Sci* 2007; 98: 815–821)

The RET receptor tyrosine kinase (RTK) regulates a variety of cellular processes, including proliferation, survival, differentiation, migration, chemotaxis, branching morphogenesis and synaptic plasticity.[1–5] RET signaling is activated by the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GDNF, neurtuin, artemin and persephin). Unlike other RTK, however, the ligands do not bind to RET directly, but require glycosylphosphatidylinositol-anchored cell surface proteins called GDNF family receptor α 1–4 (GFRα1–4) as ligand-binding components.[1] GDNF, neurtuin, artemin and persephin use GFRα1, GFRα2, GFRα3 and GFRα4, respectively, as their preferred receptors. Complex formation results in the activation of several signaling pathways, including the RAS/ERK, phosphatidylinositol-3 kinase/Akt, p38 MAPK, phospholipase Cγ, and RAC/c-Jun amino terminal kinase (JNK) pathways.[6–12] Gene knockout studies have demonstrated that the GDNF/RET signaling pathway is crucial for development of the enteric nervous system and the kidneys, as well as spermatogenesis.[13–17]

RET mutations are responsible for the development of several human diseases, including papillary thyroid carcinoma, multiple endocrine neoplasia (MEN) types 2A and 2B, familial medullary thyroid carcinoma and Hirschsprung's disease.[5,18] MEN 2A and 2B share the clinical features of medullary thyroid carcinoma and pheochromocytoma, whereas familial medullary thyroid carcinoma is characterized by the development of medullary thyroid carcinoma alone. In addition, 10–30% of MEN 2A patients develop parathyroid hyperplasia, whereas MEN 2B patients show a more complex phenotype that includes ganglioneuromatosis of the gastrointestinal tract, mucosal neuroma and marfanoid habitus. Papillary thyroid carcinoma is caused by somatic rearrangement of *RET*, and MEN 2A, MEN 2B and familial medullary thyroid carcinoma are caused by its germ-line point mutations. These mutations lead to *RET* gain-of-function. However, loss-of-function mutations of *RET* lead to the development of Hirschsprung's disease, which is a congenital malformation associated with the absence of enteric neurons.

Sprouty (SPRY) proteins have been found to antagonize fibroblast growth factor (FGF) signaling during tracheal branching in *Drosophila*.[19] *Drosophila* SPRY proteins are conserved as inhibitors of RTK signaling.[20] They specifically suppress RAS/ERK signaling activated by RTK, leaving the phosphatidylinositol-3 kinase/Akt and other MAPK pathways unaffected. The mechanism by which SPRY blocks ERK activation remains unclear, and may depend on the cellular context or the specific RTK.[21] The mouse and human genomes each contain four SPRY genes (*SPRY1–4*) encoding proteins that have molecular masses of 32–34 kDa. It has been reported that SPRY1, SPRY2 and SPRY4 inhibit FGF- and vascular endothelial growth factor (VEGF)-induced ERK activation, but do not affect epidermal growth factor (EGF)-induced ERK activation.[22,23] These findings suggest the possibility that SPRY proteins are rather selective inhibitors of RTK.

A recent report revealed that *Spry2*-deficient mice develop hyperganglionosis in the enteric nervous system, probably resulting from enhancement of ERK activation in the GDNF/RET signaling pathway.[24] In the present study, we investigated the role of SPRY in GDNF/RET signaling in cell growth and differentiation using TGW human neuroblastoma cells.

Materials and Methods

Plasmids. The 6 × Myc epitope-tagged wild-type human *SPRY2* and mouse *SPRY4*, and mutant (Y55A) *SPRY2* cDNAs, which were subcloned into pcDNA3 (Invitrogen, San Diego, CA, USA), were generously provided by A. Yoshimura (Kyushu University, Fukuoka, Japan).[25] Human *SPRY1* and *SPRY3* cDNAs were isolated from a TGW human neuroblastoma cell cDNA library, and subcloned into pcDNA3 with the 6 × Myc epitope tag. Wild-type and mutant *SPRY2* cDNAs were subcloned into the pEGFP-C1 vector (Takara Bio, Shiga, Japan). Human *RET* and *GFRα1* cDNAs were inserted into the pcDNA3.1 vector (Invitrogen). The FLAG-tag was inserted after the signal sequence of *GFRα1* cDNA.

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Cell lines. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The SK-N-MC primitive neuroectodermal tumor cell line was transfected with an expression plasmid carrying the wild-type *RET* gene (SK-N-MC [RET] cells),[11] and maintained in DMEM supplemented with 8% FBS and geneticin (100 μg/mL). TGW cells were maintained in RPMI medium supplemented with 10% FBS. HEK293T and TGW cells were transfected with plasmids using FuGENE 6 Transfection Reagent (Roche Applied Science, Basel, Switzerland) and Lipofectamine 2000 (Invitrogen), respectively. Stable transfectants were selected in medium supplemented with geneticin (200 μg/mL).

Antibodies. Rabbit anti-SPRY2 and anti-SPRY4 polyclonal antibodies were purchased from Upstate (New York, NY, USA) and Zymed Laboratories (San Francisco, CA, USA), respectively. Rabbit anti-RET polyclonal antibody was developed against the C-terminal 19 amino acids of the RET long isoform, as described previously.[26] Anti-phosphotyrosine mouse monoclonal antibody (4G10) was purchased from Upstate. Anti-p42/44 ERK1/2, antiphospho-p42/44 ERK1/2, anti-AKT, antiphospho-AKT, antiphospho-p38 MAPK antibodies, anti-p38 MAPK, antiphospho-SAPK/JNK and anti-SAPK/JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-FLAG and anti-β-tubulin antibodies were purchased from Sigma (St Louis, MO, USA). Anti-c-myc and anti-green fluorescent protein (GFP) antibodies were purchased from Roche Applied Science and MBL (Nagoya, Japan), respectively.

Western blotting. Cells were stimulated with GDNF (100 ng/mL) after 24 h starvation and subsequently lysed in sodium dodecyl sulfate (SDS) sample buffer (20 mM Tris-HCl, pH 6.8, 2 mM ethylenediaminetetracetic acid [EDTA], 2% SDS, 10% sucrose, 20 μg/mL bromophenol blue and 80 mM dithiothreitol). The resulting lysates were ultrasonicated, boiled for 4 min, and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% albumin in phosphate-buffered saline containing 0.05% Tween 20 and probed with the primary antibody. After washing, they were incubated with the secondary antibody for 1 h and specific binding was detected by the enhanced chemiluminescence system (ECL or ECL plus; Amersham Biosciences, Piscataway, NJ, USA).

Immunoprecipitation. Cells were lysed in immunoprecipitation assay buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 100 μM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride), supplemented with protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablet; Roche Applied Science). After centrifugation at 15 000*g* at 4°C for 20 min, cell lysates were added to 50 μL of EZview red antic-myc affinity gel (Sigma), incubated at 4°C for 1 h and centrifuged at 8200*g* for 30 s. After washing three times with lysis buffer, the gel beads were mixed with 100 mM glycine-HCl (pH 2.9) and removed by centrifugation. Samples were neutralized with 1 M Tris-HCl (pH 9.0), suspended in SDS sample buffer and boiled for 3 min. Samples were subjected to western blotting.

Luciferase assay. ELK-1 activation was measured using the luciferase reporter gene assay (PathDetect *in vivo* signal transduction pathway *trans*-reporting system; Stratagene, La Jolla, CA, USA). HEK293T cells were transfected with *RET* and *GFRα1* expression plasmids (300 ng each), pFA-ELK1-specific fusion *trans*-activator plasmid (30 ng), pFR-Luc reporter plasmid (300 ng), tk-Renilla (30 ng), and Myc-SPRY or pcDNA (400 ng). SK-N-MC(RET) cells were also transfected with pFA-ELK1-specific fusion *trans*-activator plasmid (30 ng), pFR-Luc reporter plasmid (300 ng), tk-Renilla (30 ng) and Myc-SPRY or pcDNA (600 ng). Cells were subsequently serum-starved for 24 h, then incubated in the absence or presence of GDNF (100 ng/mL) for 6 h, and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). Data are presented as means ± SE.

Reverse transcription–polymerase chain reaction. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was transcribed using Transcriptor First-Strand cDNA Synthesis (Roche Applied Science). Reverse transcription–polymerase chain reaction (RT-PCR) was performed with primers specific to human *SPRY1–4*. The following PCR primer sets were used: *SPRY1* forward primer, 5′-ACATGGCAGTGG-CAGTTCGT-3′; *SPRY1* reverse primer, 5′-GTCTTGGTGCTG-TCCGAGGAG-3′; *SPRY2* forward primer, 5′-AGATCAGAG-CCATCCGAAACA-3′; *SPRY2* reverse primer, 5′-AGAATGG-ACCTGCGAGTGC-3′; *SPRY3* forward primer, 5′-ACTTCTCT-CCCCCGCAGTCT-3′; *SPRY3* reverse primer, 5′-CAGGTTGGG-TTCGGATGATG-3′; *SPRY4* forward primer, 5′-TTTGGTGC-AGGGCATCTTCT-3′; and *SPRY4* reverse primer, 5′-GTGGC-AGGCAGGTAGCAGA-3′.

Northern blotting. Total RNA from GDNF-untreated or -treated TGW cells was separated on 1% agarose-formamide gels with formaldehyde and transferred to Hybond-XL nylon membranes (Amersham Biosciences). *SPRY2* and *SPRY4* cDNA fragments were labeled with [α-^{32}P]dCTP using the High Prime DNA-labeling system (Roche Applied Science) and used as probes for northern hybridization at 68°C for 3 h in QuickHyb Solution (Stratagene). Signals were detected on Fuji X-ray films (Fuji, Tokyo, Japan) following appropriate exposure.

Cell proliferation assay. TGW cells (expressing myc-SPRY2) were cultured overnight in triplicate (1500 cells/well in 96-well trays) in RPMI containing 10% FBS. On the following day, the medium was replaced with RPMI containing 2% FBS in the absence or presence of GDNF (25 ng/mL). After a further 48 h culture, cell proliferation was measured using the WST-1 assay (Cell Proliferation Reagent WST-1; Roche Applied Science). WST-1 reagent (10 μL) was added to 100 μL of cell suspension and incubated for 4 h. The enzyme-linked immunosorbent assay reader was set at a wavelength of 450 nm with a reference wavelength of 620 nm. Data are presented as percentage increase of cell proliferation at 48 h after cells were incubated in RPMI containing 2% FBS in the absence or presence of GDNF. Three independent experiments were carried out and data are presented as the mean ± SD for each triplicate sample. Statistical significance was evaluated using Student's *t*-test.

Quantification of neurite outgrowth. TGW cells were transfected with EGFP-wild-type or mutant *SPRY2* or EGFP-empty vector. After serum starvation for 4 h, cells were incubated in the absence or presence of GDNF (5 ng/mL) for 12 h, and neurite length measured. Neurite length was determined by tracing the entire length of the process, and total length calculated using the WinROOF software program (Mitani Corp., Fukui, Japan). At least 100 TGW cells were evaluated in each culture. Data are presented as mean ± SE. Statistical significance was evaluated using Student's *t*-test.

Results

SPRY family proteins inhibit GDNF-induced ERK activation. To investigate the effects of the SPRY family of proteins on RET receptor signaling, we transfected myc-*SPRY1–4* with the *RET*- and FLAG-*GFRα1*-expressing plasmids into HEK293T cells. ERK1/2 activation was induced by GDNF stimulation in HEK293T cells when transfected with the *RET* and *GFRα1* genes (Fig. 1a). Expression of SPRY1–4 significantly inhibited sustained activation of ERK1/2 after GDNF stimulation, although there was little impact on transient ERK activation 5 min after stimulation (Fig. 1b,c). In contrast, AKT activation by GDNF was not impaired by SPRY expression (Fig. 1b).

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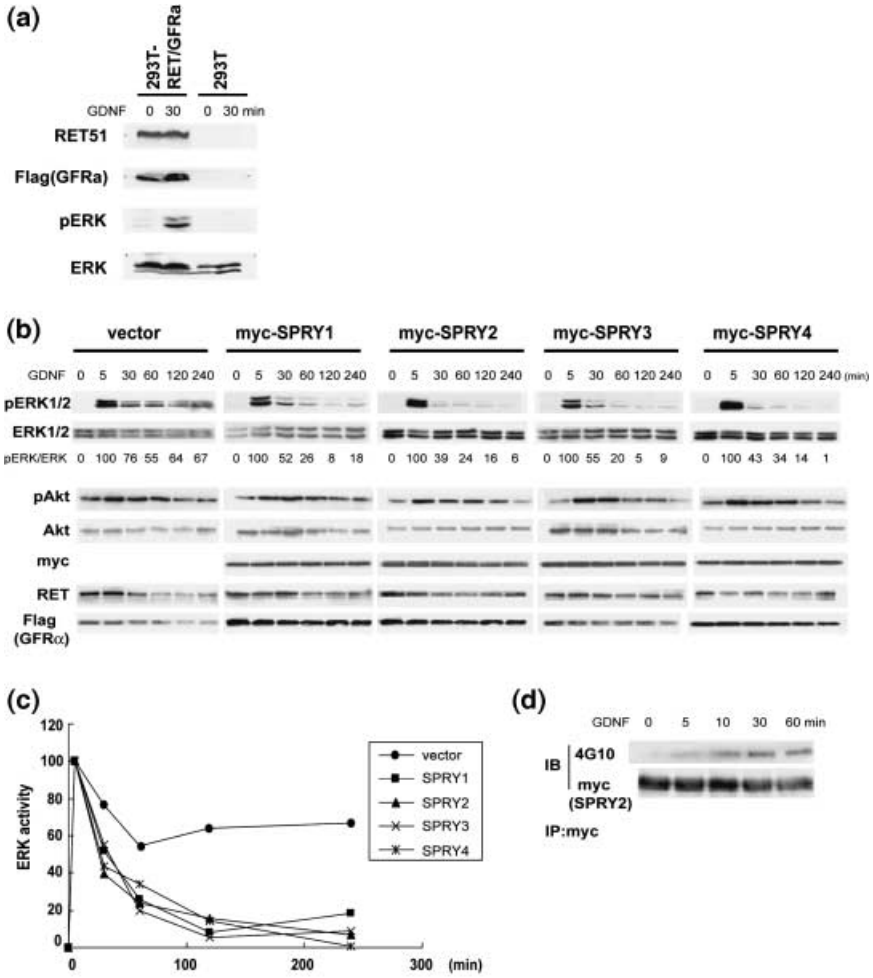

Fig. 1. Sprouty (SPRY) family proteins inhibit glial cell line-derived neurotrophic factor (GDNF)-induced ERK activation. (a) HEK293T cells were transfected with *RET* and Flag-*GFRα1* expression plasmids and cultured for 24 h. After 24 h serum starvation, cells were treated with GDNF (100 ng/mL) for 30 min. Lysates from HEK293T cells (right) and HEK293T cells transfected with *RET* and Flag-*GFRα1* genes (left) were analyzed by immunoblotting with the antibodies indicated. (b) Myc-*SPRY1–4* or empty vector were transfected with *RET* and Flag-*GFRα1* expression plasmids into HEK293T cells and cultured for 24 h. After 24 h serum starvation, cells were treated with GDNF (100 ng/mL) for the indicated times. Cell lysates were analyzed by immunoblotting with indicated antibodies. (c) Quantitative analysis of ERK activity in each transfectant. Activity at 5 min after GDNF stimulation was set as 100%. (d) Tyrosine phosphorylation of SPRY2 protein by GDNF stimulation. HEK293T cells transfected with *RET*, Flag-*GFRα1* and Myc-*SPRY2* expression plasmids were treated with GDNF. Cell lysates were immunoprecipitated with antimyc antibody, followed by immunoblotting with antiphosphotyrosine antibody (4G10).

When GDNF-mediated phosphorylation of exogenous SPRY2 was examined in the transfectant, it was low 5 min after GDNF stimulation and thereafter increased (Fig. 1d). This finding may explain why exogenous SPRY expression does not significantly inhibit transient ERK1/2 activation.

ELK-1 is a nuclear target of ERK. Thus, GDNF-induced activation of ERK can be monitored by measuring the activation of ELK-1 activity with a luciferase gene reporter assay (Fig. 2a). The assay was conducted with HEK293T cells cotransfected with human *RET* and *GFRα1* cDNA. In agreement with the data shown in Fig. 1b, expression of SPRY 1–4 impaired GDNF-dependent ELK-1 activation (Fig. 2b). When SK-N-MC human primitive neuroectodermal cells transfected with human *RET* cDNA were used, inhibition of ELK-1 activation by expression of SPRY 1–4 was more significant (Fig. 2c). These results indicate that SPRY proteins inhibit the downstream ERK/ELK-1 signaling pathway activated by RET.

Induction of SPRY in TGW neuroblastoma cells by GDNF. Regulation of SPRY proteins in the GDNF/RET signaling pathway was examined by determining whether GDNF stimulation induced SPRY expression. TGW neuroblastoma cells were used as they express both RET and GFRα1 endogenously. First, induction of *SPRY1–4* gene expression was investigated by RT-PCR. *SPRY2* was induced rapidly and strongly by GDNF, although its induction appeared to be biphasic (Fig. 3a). *SPRY4* was also induced by GDNF stimulation. In contrast, induction of *SPRY1* and *SPRY3* was below the limits of detection (Fig. 3a). Induction of *SPRY2* and *SPRY4* was confirmed by northern blot analysis (Fig. 3b). To extend those results, we characterized the expression of SPRY2 and SPRY4 proteins by western blotting. As shown in Fig. 3c, SPRY2 protein expression was clearly induced by GDNF, whereas induction of the SPRY4 protein was weak.

Effect of SPRY2 expression on cell proliferation. Based on the finding that SPRY2 is induced by GDNF in TGW cells, we investigated the effect of SPRY2 expression on the proliferation of TGW cells. Stable transfectants expressing myc-wild type-SPRY2 (TGW-WT-SPRY2) were established. In addition, we established a cell line expressing a mutant SPRY2 in which tyrosine 55 was replaced with alanine (designated myc-MT-SPRY2). This tyrosine is conserved among the four proteins of the SPRY family (Fig. 4a) and its mutation was shown to enhance growth-factor-dependent ERK activation.[25,27] As shown in Fig. 4b, sustained activation of ERK by GDNF was lower in TGW-WT-SPRY2 cells and higher in TGW-MT-SPRY2 cells compared to control TGW cells. In addition, expression of MT-SPRY2 increased transient ERK1/2 activation.

We next used the WST-1 assay to compare the proliferation of TGW-WT-SPRY2 or TGW-MT-SPRY2 cells with that of control TGW cells. Each cell line was maintained in RPMI containing 2% FBS in the presence or absence of GDNF and cell growth was measured 48 h after GDNF stimulation. The proliferation of TGW cells in the presence of GDNF was significantly repressed by expression of WT-SPRY2 and accelerated by expression of MT-SPRY2 (Fig. 4c), indicating that the level of ERK activation affects GDNF-dependent cell growth. Interestingly,

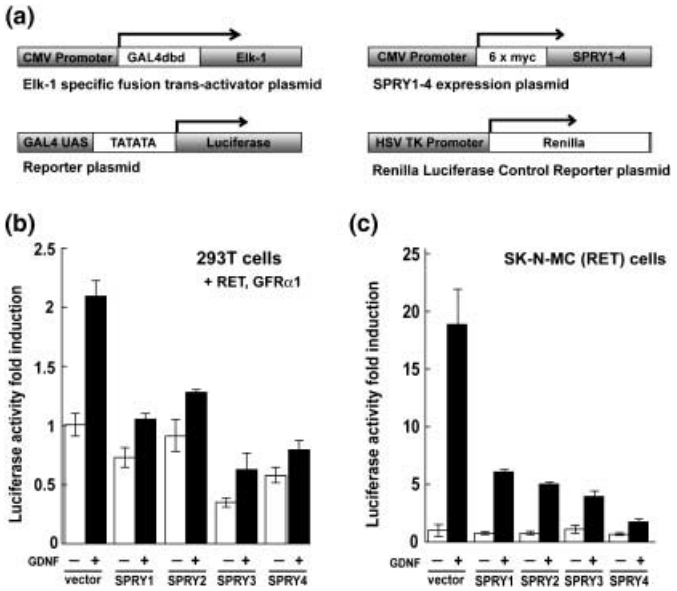

Fig. 2. Inhibition of ELK-1 activity by Sprouty (SPRY) expression. (a) Constructs for the ELK-1 luciferase assay. (b) *RET*, Flag-*GFRα1* and *Myc-SPRY* expression plasmids were cotransfected into HEK293T cells with ELK-1-specific fusion *trans*-activator and reporter plasmids. Cells were treated with (+; closed bar) or without (–; open bar) glial cell line-derived neurotrophic factor (GDNF) (100 ng/mL) for 6 h and then analyzed. Data are presented as mean ± SE. (c) Myc-*SPRY* plasmids were cotransfected into SK-N-MC (RET) cells with ELK-1-specific fusion *trans*-activator and reporter plasmids. Cells were treated with (+; closed bar) or without (–; open bar) GDNF (100 ng/mL) for 6 h and then analyzed. Data are presented as mean ± SE.

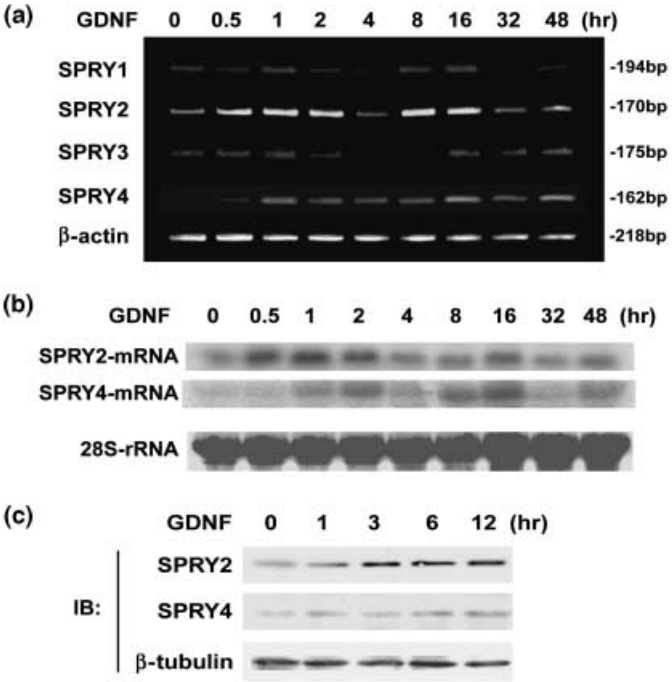

Fig. 3. Upregulation of Sprouty (SPRY) 2 by glial cell line-derived neurotrophic factor (GDNF) in TGW neuroblastoma cells. (a) Expression of SPRY mRNA in TGW human neuroblastoma cells. Total RNA was isolated from TGW cells treated with GDNF (100 ng/mL) for the indicated time, and reverse transcription–polymerase chain reaction was carried out. β-Actin was amplified as a control. (b) TGW human neuroblastoma cells were stimulated with GDNF (100 ng/mL) for the indicated times, and total RNA (10 μg) was subjected to northern blotting with a *SPRY2* or *SPRY4* cDNA fragment as a probe. *SPRY* transcript and 28S ribosomal RNA from each sample are shown. (c) After serum starvation for 24 h, TGW cells were treated with GDNF (100 ng/mL) for the indicated times. Then, cell lysates were analyzed by immunoblotting with anti-SPRY2 antibody, anti-SPRY4 antibody, or anti-β-tubulin antibody.

the growth of MT-SPRY2-expressing TGW cells increased compared with control TGW cells in the absence of GDNF (Fig. 4c).

Moreover, to investigate the effect of knockdown of endogenous SPRY2 in TGW cells, we designed several small interfering RNAs. However, none of them could effectively repress its expression (date not shown).

Expression of SPRY2 inhibits GDNF-dependent neurite outgrowth of TGW cells. We previously demonstrated that GDNF induces neurite outgrowth of TGW cells.[28] To investigate the effect of SPRY2 expression on neurite outgrowth, we generated EGFP-WT-SPRY2 and EGFP-MT-SPRY2 constructs. Again, expression of EGFP-WT-SPRY2 in HEK293T cells inhibited sustained ERK activation whereas expression of EGFP-MT-SPRY2 enhanced it (Fig. 5a).

These constructs were transiently transfected into TGW cells, and neurite outgrowth of TGW cells expressing EGFP-SPRY2 proteins was monitored. Transfected TGW cells were incubated with GDNF for 12 h, and evaluated for the degree of neurite extension. Consistent with the levels of sustained ERK activation, TGW cells expressing WT-SPRY2 showed short neurites in response to GDNF stimulation. In contrast, MT-SPRY2 expression significantly enhanced neurite outgrowth (Fig. 5b). The effects of WT-SPRY2 and MT-SPRY2 on neurite outgrowth were also confirmed using TGW transfectants that stably express WT-SPRY2 or MT-SPRY2 (data not shown).

Discussion

RET receptor tyrosine kinase controls a variety of cellular processes including proliferation, differentiation and migration.[1–3] Its activity is tightly controlled through the coordinated action of both positive and negative regulators that function at multiple levels of the signal transduction cascade, and at different time points within the GDNF-induced response. When this process goes awry, developmental defects and malignancy may result. It has been demonstrated that *SPRY* expression is positively regulated by the ERK pathway (that it antagonizes), yielding a negative feedback loop.[29] In agreement with this model, GDNF treatment induced apparent upregulation of *SPRY2* expression in TGW neuroblastoma cells, although upregulation of *SPRY4* expression in TGW cells by GDNF was weak, and expression of *SPRY1* and *SPRY3* was not apparent. Thus, individual *SPRY* genes may be regulated by specific combinations of factors to allow optimal control of signaling and function in a tissue-specific manner.[30]

Overexpression of SPRY1–4 proteins inhibited sustained ERK activation by GDNF, whereas overexpression did not appear to affect transient ERK activation. This is in agreement with the finding that activation of exogenous SPRY2 was low 5 min after GDNF stimulation (Fig. 1d). In contrast, expression of dominant-negative MT-SPRY2 enhanced transient ERK activation as well as sustained ERK activation. This result suggests that MT-SPRY2 expression may significantly inhibit the activity of endogenous SPRY2 even 5 min after GDNF stimulation.

It is well established that GDNF/RET signaling plays a crucial role in regulating the proliferation, differentiation and migration of enteric neural crest cells.[31,32] Thus, defects in signaling result

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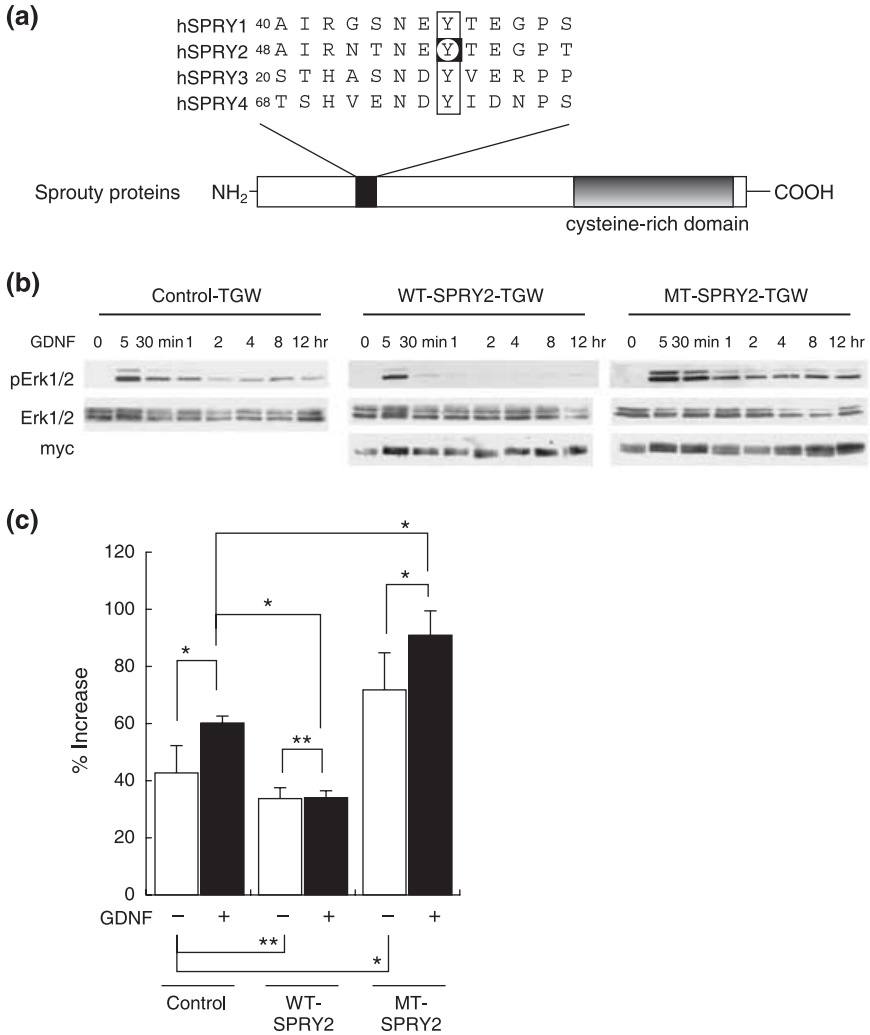

Fig. 4. Effect of Sprouty (SPRY) 2 on cell proliferation. (a) The domain structures of mammalian SPRY family proteins. A conserved cysteine-rich domain is present at the C-terminus of all mammalian SPRY proteins. In the N-terminal half, an invariant tyrosine residue (Y) is located in a short, conserved motif. (b) Control TGW cells and TGW cells stably expressing myc-wild-type-SPRY2 (WT-SPRY2-TGW) or myc-mutant-SPRY2 (MT-SPRY2-TGW) were stimulated with glial cell line-derived neurotrophic factor (GDNF) (25 ng/mL) for the indicated time, and cell extracts subjected to immunoblotting analysis with the indicated antibodies. (c) Control-TGW, WT-SPRY-TGW and MT-SPRY2-TGW cells were plated in triplicate in RPMI containing 10% fetal bovine serum (FBS) and incubated overnight. On the following day, the medium was replaced with RPMI containing 2% FBS with (+; closed bar) or without (–; open bar) GDNF (25 ng/mL). After 48 h, cell proliferation was measured using the WST-1 assay. Three independent experiments were carried out, and data are presented as mean ± SD. Two clones of each transfectant were used, and similar results were obtained. $*P < 0.05$, $**P > 0.05$.

in the development of Hirschsprung's disease, a congenital malformation of the enteric nervous system.[1–5,18] In addition, it was reported that GDNF/RET signaling is involved in proliferation and differentiation of neuroblastoma cells.[33] In the present study, we investigated the effect of *SPRY2* expression on proliferation or differentiation of TGW neuroblastoma cells. Consistent with the finding that SPRY2 expression inhibits ERK activation in TGW cells, GDNF-dependent proliferation of TGW cells was significantly impaired by expression of WT-SPRY2. In contrast, expression of the dominant-negative MT-SPRY2 (in which tyrosine 55 was replaced by alanine) enhanced cell proliferation. This finding suggests that SPRY2 regulates the growth of neuroblastoma cells by modulating ERK activity downstream of RET tyrosine kinase.

GDNF treatment can induce neurite outgrowth of TGW cells.[28] Neurite outgrowth is an established marker of neuronal differentiation that requires concerted intracellular signaling cascades.[34] Our recent study revealed that GDNF-dependent neurite outgrowth in TGW neuroblastoma cells was accelerated by sustained ERK activation.[28] We observed that expression of WT-SPRY2 significantly reduced sustained ERK activation by GDNF whereas expression of MT-SPRY2 enhanced it. Consistent with this finding, TGW cells expressing WT-SPRY2 produced shorter neurites in the presence of GDNF than did control TGW cells. In contrast, GDNF treatment of TGW cells expressing MT-SPRY2 developed longer neurites, indicating that SPRY2 also regulates neuronal differentiation.

In mice, *Spry2* deficiency leads to ganglionic megacolon.[24] That report suggests that the hyperresponsiveness of enteric neurons to GDNF leads to enteric nervous system (ENS) hyperganglionosis in these mice. This finding suggests that Spry negatively regulates proliferation of enteric neural crest cells during embryogenesis. In addition, *Spry1*-deficient mice show kidney abnormalities resulting from hyperresponsiveness of the Wolffian duct to GDNF.[35,36] Thus, the SPRY family of proteins plays critical roles in normal development of both the enteric nervous system and the kidney by regulating ERK signaling downstream of RET tyrosine kinase.

SPRY1 and/or *SPRY2* are downregulated in several types of cancers, including breast, prostate and liver cancers,[37] suggesting that the change in *SPRY* expression may be associated with the malignant phenotypes of these cancers. Conversely, *SPRY2* expression was found to be upregulated in melanoma with *B-RAF* mutations. For example, SPRY2 protein binds to wild-type B-RAF but not to mutant B-RAF (V599E mutation), resulting in decreased inhibition of ERK signaling by mutant B-RAF.[38] Complete understanding of the role of these proteins in carcinogenesis will require analysis of the mechanisms by which each protein in the SPRY family inhibits RTK signaling.

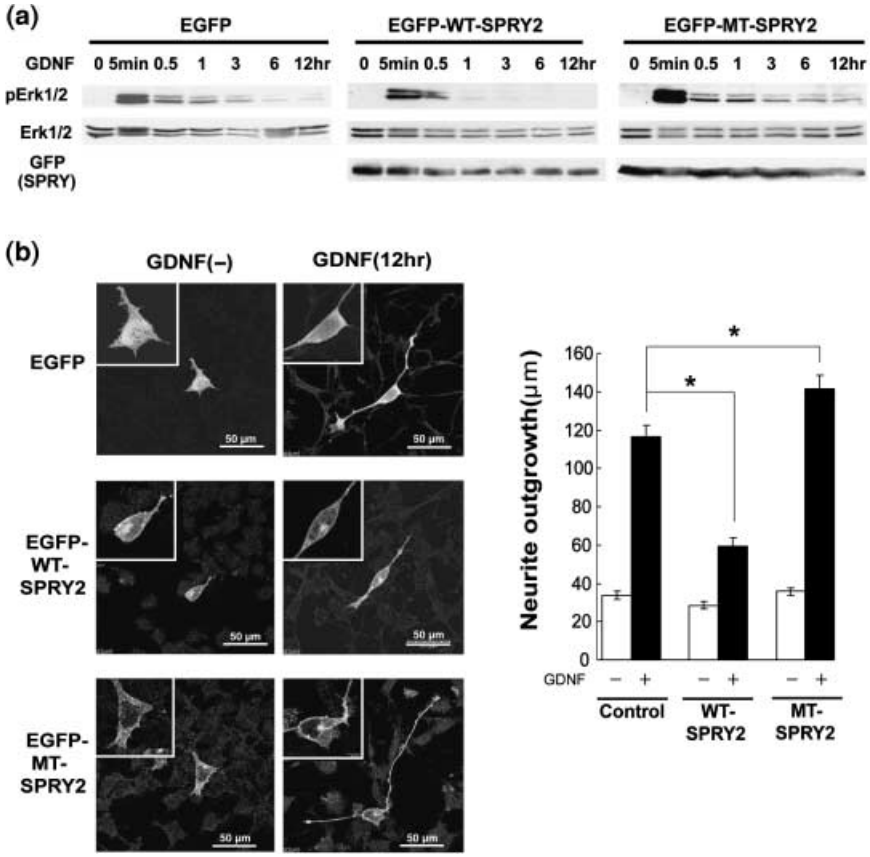

Fig. 5. Effect of Sprouty (SPRY) 2 on neurite outgrowth of TGW neuroblastoma cells. (a) EGFP-WT-*SPRY2*, EGFP-MT-*SPRY2* or EGFP-empty-vectors were transfected with the *RET* and Flag-*GFRα1* expression plasmids into 293T cells. Cells were serum-starved for 24 h, and treated with glial cell line-derived neurotrophic factor (GDNF) (25 ng/mL) for the indicated times. Then, cell extracts were immunoblotted with the indicated antibodies. (b) TGW cells were transfected with EGFP-WT/MT-*SPRY2* or EGFP vector, serum-starved and incubated for 12 h in the absence or presence of GDNF (5 ng/mL) (left panel). The mean of the longest neurite was determined for each culture from measurements of 100 neurons in three different experiments (right panel). Each data point represents the mean ± SE. **P* < 0.01.

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