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ORIGINAL ARTICLE

EWS-FLI1-mediated suppression of the RAS-antagonist Sprouty 1 (SPRY1) confers aggressiveness to Ewing sarcoma

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Ewing sarcoma is characterized by chromosomal translocations fusing the *EWS* gene with various members of the *ETS* family of transcription factors, most commonly *FLI1*. EWS-FLI1 is an aberrant transcription factor driving Ewing sarcoma tumorigenesis by either transcriptionally inducing or repressing specific target genes. Herein, we showed that *Sprouty 1* (*SPRY1*), which is a physiological negative feedback inhibitor downstream of fibroblast growth factor (FGF) receptors (FGFRs) and other RAS-activating receptors, is an EWS-FLI1 repressed gene. EWS-FLI1 knockdown specifically increased the expression of SPRY1, while other *Sprouty* family members remained unaffected. Analysis of SPRY1 expression in a panel of Ewing sarcoma cells showed that SPRY1 was not expressed in Ewing sarcoma cell lines, suggesting that it could act as a tumor suppressor gene in these cells. In agreement, induction of SPRY1 in three different Ewing sarcoma cell lines functionally impaired proliferation, clonogenic growth and migration. In addition, SPRY1 expression inhibited extracellular signal-related kinase/mitogen-activated protein kinase (MAPK) signaling induced by serum and basic FGF (bFGF). Moreover, treatment of Ewing sarcoma cells with the potent FGFR inhibitor PD-173074 reduced bFGF-induced proliferation, colony formation and *in vivo* tumor growth in a dose-dependent manner, thus mimicking SPRY1 activity in Ewing sarcoma cells. Although the expression of SPRY1 was low when compared with other tumors, SPRY1 was variably expressed in primary Ewing sarcoma tumors and higher expression levels were significantly associated with improved outcome in a large patient cohort. Taken together, our data indicate that EWS-FLI1-mediated repression of SPRY1 leads to unrestrained bFGF-induced cell proliferation, suggesting that targeting the FGFR/MAPK pathway can constitute a promising therapeutic approach for this devastating disease.

INTRODUCTION

Ewing sarcomas are aggressive bone and soft-tissue sarcomas mostly affecting children and young adults.[1] Although the 5-year survival in patients with localized disease increased significantly on the addition of systemic chemotherapy to protocol treatments in the 70–80 s,[2] the prognosis and survival of patients with metastatic or recurrent disease remained generally very poor.[3] Indeed, Ewing sarcoma features high rates of early metastasis with ~ 20% of patients having detectable metastasis at diagnosis.[4]

The molecular hallmarks of Ewing sarcoma are nonrandom chromosomal translocations generating in-frame fusion of the *EWS* gene on chromosome 22 and the C-terminus of a member of the *ETS* family of transcription factors (that is, *FLI1*, *ERG*, *ETV1*, *FEV*, *ETV4* and *POU5F1*) including the DNA-binding domain[5] (reviewed in Mackintosh *et al.*[1]). This fusion gives rise to aberrant EWS-ETS transcription factors, EWS-FLI1 being present in 85% of cases.

EWS-FLI1 induces massive deregulation of protein expression by either transcriptionally inducing or repressing specific target genes, many of which are involved in the oncogenic process.[6] For instance, EWS-FLI1 induces the expression of *NR0B1* (*DAX1*), *EGR2*, *NKX2.2*, *CCK*, *PRKCB* or *STEAP1*,[7–11] while suppressing *IGFBP3*, *LOX*, *DKK1* or *TGFBIIR*.[12–15] All these genes have been shown to be important in Ewing sarcoma pathogenesis.

Here we report on the tumor suppressive role of another repressed EWS-FLI1-targeted gene, namely *Sprouty 1* (*SPRY1*), which is a negative feedback inhibitor of the RAS/mitogen-activated protein kinase/extracellular signal-related kinase (RAS/MAPK/ERK) pathway downstream of the fibroblast growth factor receptor (FGFR).

SPRY1 is part of the mammalian *Sprouty* gene family consisting of four members (SPRY1–4), which share important sequence similarities[16] such as a highly conserved cysteine-rich domain in the C-terminal region (which is also found in the SPRED family of proteins) and a short amino acid sequence in the N-terminus.[17] SPRY proteins differ largely in their tissue distribution, activity and interaction partners,[18] thus suggesting non-redundant functions. SPRY1 is an upstream antagonist of RAS that is activated by ERK, providing a negative feedback loop for RAS signaling. Of note, about one-third of all human cancers are thought to carry a mutated *RAS* gene that activates downstream signaling.[19] It has been suggested that SPRY1 may have a tumor suppressor function in specific tumors, as its expression is decreased in several human cancers such as breast and prostate cancer.[20–22] Indeed, several studies showed that SPRY1 overexpression in tumor cell lines inhibits cell proliferation, migration and anchorage-independent growth *in vitro*.[21,23,24]

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In this study, we show that SPRY1 acts as a tumor suppressor in Ewing sarcoma cells, and that SPRY1 repression is necessary for cell proliferation and migration. Interestingly, SPRY1 repression was important to ERK pathway activation. Moreover, FGFR inhibition mimicked SPRY1 effect on proliferation and growth, indicating that SPRY1 has an important role in Ewing sarcoma. Finally, elevated SPRY1 expression correlated with improved overall survival of Ewing sarcoma patients and inversely correlated with metastasis at diagnosis. Collectively, our data indicate that EWS-FLI1-mediated repression of SPRY1 confers a growth advantage to Ewing sarcoma cells, and that SPRY1 levels constitute a novel biomarker for outcome prediction of Ewing sarcoma patients. Taken together, these results suggest a rationale for targeting FGFR/SPRY1/RAS/MAPK/ERK pathway as a new therapeutic approach in this devastating disease.

RESULTS

SPRY1 expression is strongly inhibited by EWS-FLI1 in Ewing sarcoma cell lines

Analysis of a gene expression profile of A673 Ewing sarcoma cell line genetically modified to express a specific small hairpin RNA directed against EWS-FLI1 mRNA on doxycycline stimulation (A673/TR/shEF) (Gene Expression Omnibus accession code: GSE36007) indicated that SPRY1 is strongly downregulated by EWS-FLI1. These microarray results were confirmed by reverse transcription–quantitative PCR experiments. As depicted in Figure 1a, EWS-FLI1 knockdown led to a dramatic re-expression of SPRY1 mRNA (up to 1000-fold compared with controls), whereas the mRNA levels of the other members of the SPRY family (SPRY2, 3 and 4) were only minimally affected. Analysis of SPRY1 protein levels in the A673/TR/shEF cell model confirmed these results. As shown in Figure 1b, SPRY1 protein was undetectable by western blotting in

A673/TR/shEF grown in the absence of doxycycline. However, a strong induction of SPRY1 protein was observed on doxycycline-mediated EWS-FLI1 knockdown.

We next studied whether the inhibition of SPRY1 expression could be a common feature of Ewing sarcoma cells. We first analyzed the levels of SPRY1 mRNA and protein in a panel of eight Ewing sarcoma cell lines harboring different EWS-FLI1 or EWS-ERG fusion proteins (Supplementary Table 1). As shown in Figures 1c and d, SPRY1 mRNA and protein were undetectable in all Ewing sarcoma cell lines analyzed. Interestingly, the mRNA levels of the other members of the SPRY family were variably expressed in this panel of Ewing sarcoma cells. These data could also be confirmed assessing larger public data sets. For instance, analysis of Cancer Cell Line Encyclopedia data set[25] (http://www.broad institute.org/ccle/home) showed that Ewing sarcoma cell lines exhibited the lowest SPRY1 levels among all tumor cell lines analyzed (Supplementary Figure 1).

SPRY1 induction impairs cell proliferation of Ewing sarcoma cells

The strong downregulation of SPRY1 by EWS-FLI1, its absence of expression in Ewing sarcoma cell lines and the finding that it acts as a negative feedback inhibitor of the RAS/MAPK/ERK cascade suggest a potential function of SPRY1 inhibition in Ewing sarcoma. To test this hypothesis, we generated three doxycycline-inducible SPRY1 Ewing sarcoma cell lines (A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1) and subjected them to several functional assays. As shown in Figure 2a, these genetically modified Ewing cell lines express high levels of SPRY1 protein on doxycycline stimulation, whereas the levels of the EWS-FLI1 oncoprotein remain unaffected. Thus, they constitute a suitable model to test the consequences of exclusive SPRY1 re-expression in Ewing sarcoma without affecting the levels of EWS-FLI1 oncoprotein.
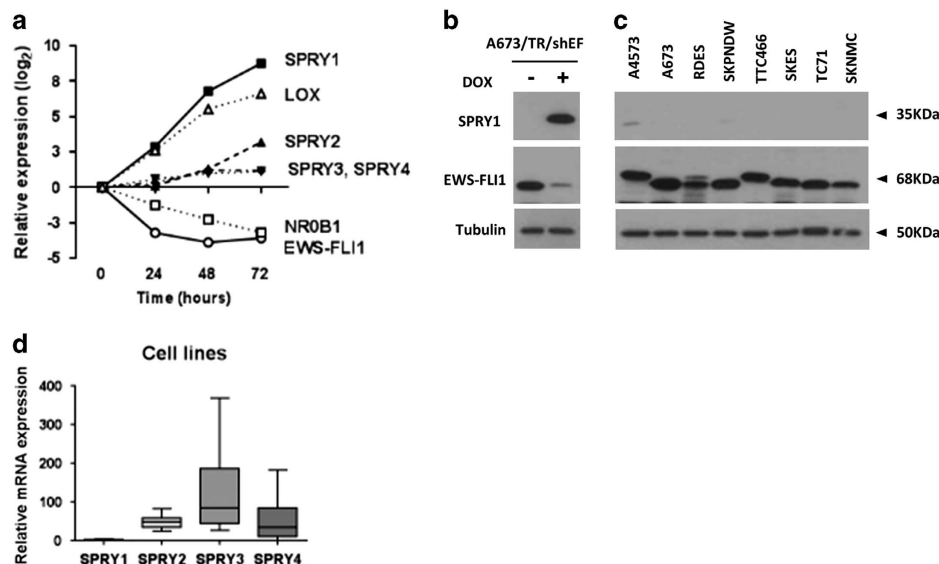
Figure 1. SPRY1 is negatively regulated by EWS-FLI1 oncoprotein. (**a**) Time course of SPRY1, 2, 3 and 4 on EWS-FLI1 doxycycline-inducible knockdown in A673/TR/shEF. EWS-FLI1 expression and two known target genes such as LOX and NR0B1 were included as controls. mRNA levels were quantified by real-time reverse transcription–quantitative PCR (RT–qPCR), normalized to that of TBP (reference gene) and referred to unstimulated cells. Figure shows data of one out of three independent experiments done in triplicate with equivalent results. EWS-FLI1 inhibition in A673/TR/shEF cells selectively upregulates SPRY1 more than 1000 times over the rest of the members of the SPRY family of genes. As expected, LOX appears upregulated[13] and NR0B1 downregulated[51] on EWS-FLI1 knockdown. (**b**) SPRY1 protein is re-expressed on EWS-FLI1 knockdown in A673/TR/shEF cells. SPRY1 protein is undetectable by western blotting in A673 cells grown in the absence of doxycycline and thus expressing EWS-FLI1. Incubation of A673/TR/shEWSFLI1 cells with doxycycline (1 μg/ml, 72 h) inhibits EWS-FLI1 expression and dramatically induces re-expression of SPRY1 protein. Tubulin was used as a control for loading and transferring. (**c**) SPRY1 is undetectable at protein level by western blotting in eight Ewing sarcoma cell lines. Expression of the different EWS-ETS proteins is also shown. Tubulin was used as a control for loading and transferring. (**d**) SPRY1, 2, 3 and 4 mRNA levels in Ewing sarcoma cell lines. Box plot shows the absence of SPRY1 expression in all Ewing sarcoma cell lines tested relative to other members of the SPRY family. The figure shows the expression levels normalized to that of TBP (reference gene).
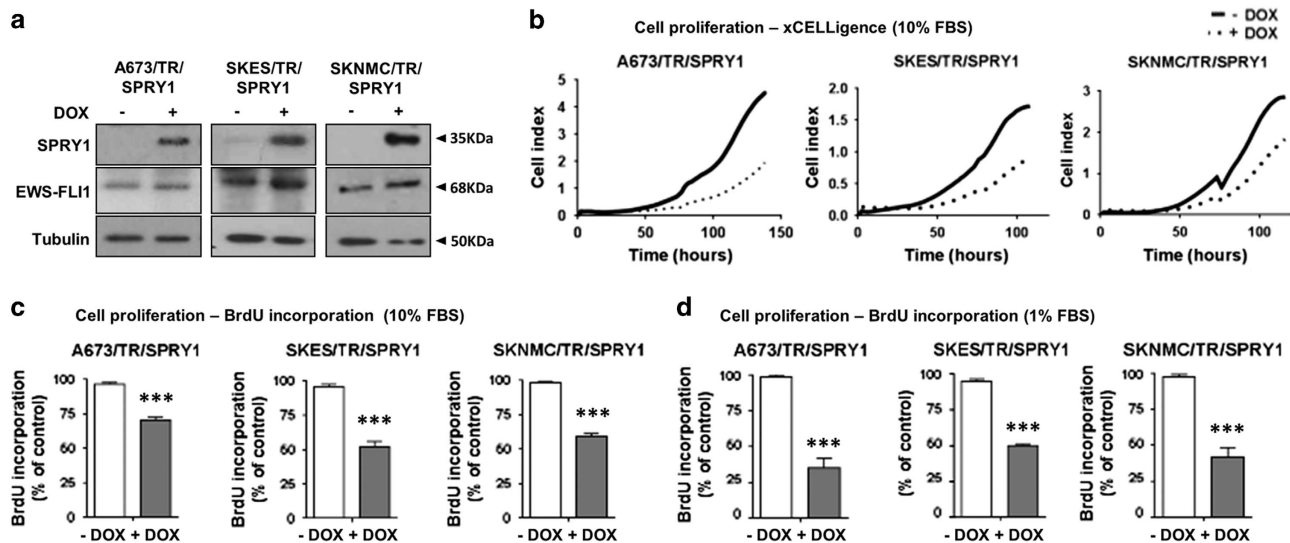
Figure 2. SPRY1 re-expression impairs proliferation in Ewing sarcoma cell lines. (**a**) A673/TR, SKES/TR and SKNMC/TR Ewing cell lines expressing constitutively the tetracycline repressor (TR) were infected with a doxycycline-inducible lentiviral vector encoding the SPRY1 cDNA. The figure shows the expression of SPRY1 protein in whole protein extracts isolated from A673/TR/SPRY1 (clone 1), SKES/TR/SPRY1 (clone 7) and SKNMC/TR/SPRY1 (clone 2) cells stimulated with doxycycline (DOX, 1 μg/ml, 72 h). High SPRY1 levels were detected in all three cell lines after doxycycline stimulation. EWS-FLI1 expression was not affected by SPRY1 ectopic expression. The same blot was stripped and incubated with anti-tubulin as a control for loading and transferring. (**b**) Cell proliferation was assayed in A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells using an xCELLigence assay with or without re-expression of SPRY1 (DOX, 1 μg/ml). Graphs depict the growth curves of the cells cultured in the absence or presence of doxycycline during 120 h and they show one representative experiment out of three independent experiments performed. Re-expression of SPRY1 produces a significant inhibition of cell proliferation. Slight artifacts in the graphs at 72 h are a consequence of media change and subsequent readjustment of the conditions in the xCELLigence device and do not affect the final result. (**c**) A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells were plated in octuplicates and cultured in the presence or absence of doxycycline (DOX, 1 μg/ml) for 72 h in 10% tetracycline-free FBS-supplemented media (standard culture conditions). Cell proliferation was assayed by bromodeoxyuridine (BrdU) incorporation into DNA. Graphs depict the percentage of cell proliferation of doxycycline-treated cells (expressing SPRY1) versus control. Figure depicts one representative experiment (mean ± s.d.) out of three independent experiments performed (***$P < 0.005$). (**d**) Cells were plated and cultured as described in **c**, but kept in 1% FBS-supplemented media (low-serum conditions). Cell proliferation is significantly inhibited in doxycycline-treated cells (expressing SPRY1) versus control. Figure depicts one representative experiment (mean ± s.d.) out of three independent experiments performed (***$P < 0.005$).

First, we studied the effect of SPRY1 induction on cell proliferation (Figures 2b–d). Induction of SPRY1 in these Ewing sarcoma cell lines on doxycycline stimulation significantly reduced their proliferation. This was observed using real-time monitoring of cell number (xCELLigence instrument, ACEA Biosciences, San Diego, CA, USA) (Figure 2b) and by bromodeoxyuridine incorporation assays (Figures 2c and d). Notably, no effect on cell proliferation was observed in cells carrying the empty vector, both in the absence and in the presence of doxycycline (data not shown). Cell proliferation inhibition was observed in standard culture media supplemented with 10% fetal bovine serum (FBS) (Figures 2b and c) and in low-serum (1% FBS) conditions as well (Figure 2d). Induction of SPRY1 in cells grown in low-serum conditions exhibited an even stronger reduction of cell proliferation (Figure 2d), probably suggesting that in conditions where there is a diminished availability of growth factors, such as in the tumor microenvironment, SPRY1 is able to markedly impair cell proliferation.

Similarly, induction of SPRY1 expression reduced clonogenic growth of the three Ewing sarcoma cell lines plated at very low density in medium supplemented with 5% FBS, whereas cells carrying the empty vector remained unaffected on doxycycline treatment (Figure 3a and Supplementary Figure 2A). When cells were tested for anchorage-independent growth in soft agar, no significant differences were observed in the number of colonies formed, whereas there was a significant difference in the size of the individual colonies (Figure 3b). No significant differences in anchorage-independent growth were observed in cells transfected with the empty vector when treated accordingly (Supplementary Figure 2B).

Finally, the three Ewing cell lines harboring the SPRY1 construct were cultured in the presence or absence of doxycycline and assayed for cell cycle in non-synchronized cells by flow cytometry. As shown in Supplementary Figure S3, the impairment in cell proliferation in SPRY1-re-expressing cells seems partially due to a cell cycle arrest in the G1 phase, although these results were not statistically significant.

Taken together, these results provide evidence that SPRY1 induction impairs cell proliferation as well as clonogenic and anchorage-independent growth of Ewing sarcoma cell lines.
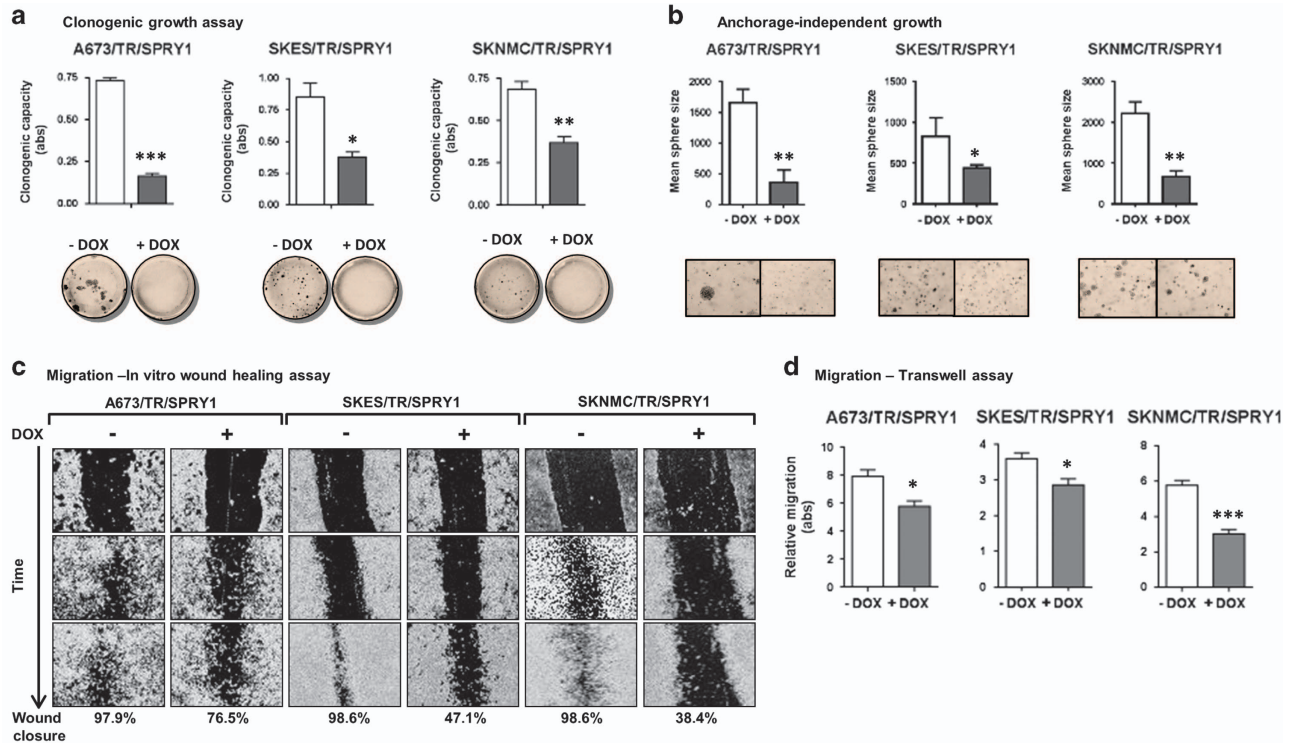
SPRY1 induction impairs migration of Ewing sarcoma cells
We next analyzed the effect of SPRY1 induction in Ewing sarcoma cells on cell migration. As shown in Figure 3c, SPRY1 induction reduced the ability of A673, SKES and SKNMC Ewing sarcoma cells to close an artificial wound produced in a confluent cell monolayer (in vitro wound-healing assay). In addition, SPRY1 re-expression significantly impaired migration of Ewing sarcoma cells through a porous membrane (transwell assay) (Figure 3d). No differences in the migratory properties were observed in cells carrying the empty vector (data not shown).

SPRY1 repression is necessary for ERK activation and proliferation in Ewing sarcoma cells
SPRY1 has been described to inhibit MAPK/ERK pathway, which is one of the most relevant proliferative pathways in cancer. For that reason we investigated the effect of SPRY1 induction on ERK activation mediated by serum. As shown in Figure 4, SPRY1 induction reduced the levels of phospho-ERK both in low (1%) and

Figure 3. SPRY1 re-expression impairs Ewing sarcoma cell clonogenicity, anchorage-independent growth, migration and invasion of Ewing sarcoma cells. (**a**) A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells were platted in triplicates at low densities and treated with or without doxycycline (DOX, 1 μg/ml) for 9 days. Colony formation was measured by crystal violet staining. Pictures show representative wells of one out of three independent experiments. Graphs depict a quantification of absorbance measured after cell de-staining (one representative experiment out of three performed) (mean ± s.d.). Clonogenic growth is significantly impaired in all three cell lines on SPRY1 re-expression (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$). (**b**) A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells were platted in triplicate in soft agar and cultured in the presence or absence of doxycycline (DOX, 1 μg/ml) during 25 days and subsequently stained with crystal violet. Pictures show representative images of sphere formation taken at the end of the experiment. Graphs depict the mean area per particle after 25 days (mean ± s.d.). SPRY1 re-expression inhibits sphere formation in all three cell lines (3 independent experiments) (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$). (**c**) A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells were platted in triplicates and treated with or without doxycycline (DOX, 1 μg/ml) for 72 h. A 'wound gap' was created by scratching the cell monolayer using a micropipette tip. Pictures depict the healing of the gap as a consequence of cell migration at the beginning, middle and end of the experiments. Relative wound closure for each cell line at the end of the experiment is stated in percentages. Images show a representative experiment out of three performed. (**d**) A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells were incubated in the absence or presence of doxycycline (DOX, 1 μg/ml) during 48 h, to induce the expression of SPRY1 protein. Afterwards, cells were starved for another 24 h. Next, they were placed in the upper compartment of a transwell and allowed to migrate through the membrane in response to serum. Migrating cells were quantified by crystal violet staining. Figure shows mean ± s.d. of two experiments performed in triplicate. Data are shown as arbitrary units of absorbance (abs) (*$P < 0.05$ and ***$P < 0.005$).

standard (10%) serum conditions. Next, we explored the effect of SPRY1 induction on the ERK activation mediated by basic FGF (bFGF), an established and potent RAS-activating growth factor. Using bFGF stimulation we observed a similar effect on ERK activation (Figure 4) in the three Ewing cell lines.

Collectively, these results indicate that EWS-FLI1-mediated SPRY1 repression in Ewing sarcoma cells contributes to the activation of MAPK/ERK pathway and thus to the malignant features observed.

FGFR inhibitors mimic the effects of SPRY1 re-expression.

As SPRY1 proved to be capable of inhibiting ERK phosphorylation, especially when the FGF pathway was activated, we assessed the effect of four FGFR inhibitors (PD-173074 [PD-74], PD-166866 [PD-66], SU5402 [SU54] and NVP-BGJ398 [BG-98]) on Ewing sarcoma cells (A673, SKNMC, SKES, RDES and POE), in order to test whether FGFR inhibition can mimic SPRY1 effect on Ewing cell lines. Complementary to our previous finding that bFGF can induce proliferation in Ewing sarcoma cell lines (that is, A673, SKNMC and POE cells),[26] we observed that FGFR inhibition reduces proliferation of these Ewing sarcoma cells (Figure 5a), whereas it did not affect normal cells such as fibroblasts (IMR90).

Consistently, FGFR inhibition through any of the four FGF inhibitors severely impairs clonogenic growth of A673, SKNMC and POE Ewing sarcoma cell lines (Figure 5b).

As POE cells exhibited high sensitivity toward this FGFR inhibiton compared with the other cells tested (Supplementary Table 2), we chose this cell line to perform in vivo experiments to test whether PD-74 has an antitumoral effect in a xenograft model in mice. As shown in Figure 5c, PD-74 treatment significantly inhibited tumor growth ($P = 0.004$) of Ewing sarcoma xenografts. These tumors showed an ~ 50% decrease in the number of mitoses ($P = 0.001$) along with a 40% increase in the number of apoptotic cells per high-power field ($P = 0.001$) when comparing vehicle versus PD-74 treatment. Moreover, Ki-67 staining for proliferation showed a significant reduction in the number of Ki67-positive cells in the tumor samples treated with PD-74 ($P < 0.01$) (Figure 5d).

To confirm whether PD-74 had an antitumoral effect in other Ewing sarcoma cell lines we performed an in vivo experiment using SKES cells, which presented less sensitivity to it in vitro (Figure 5a). As shown in Supplementary Figure S4A, PD-74 had a dose-dependent effect on SKES xenograft growth in mice, with 20 mg/kg being the most effective dose ($P = 0.005$). Again,
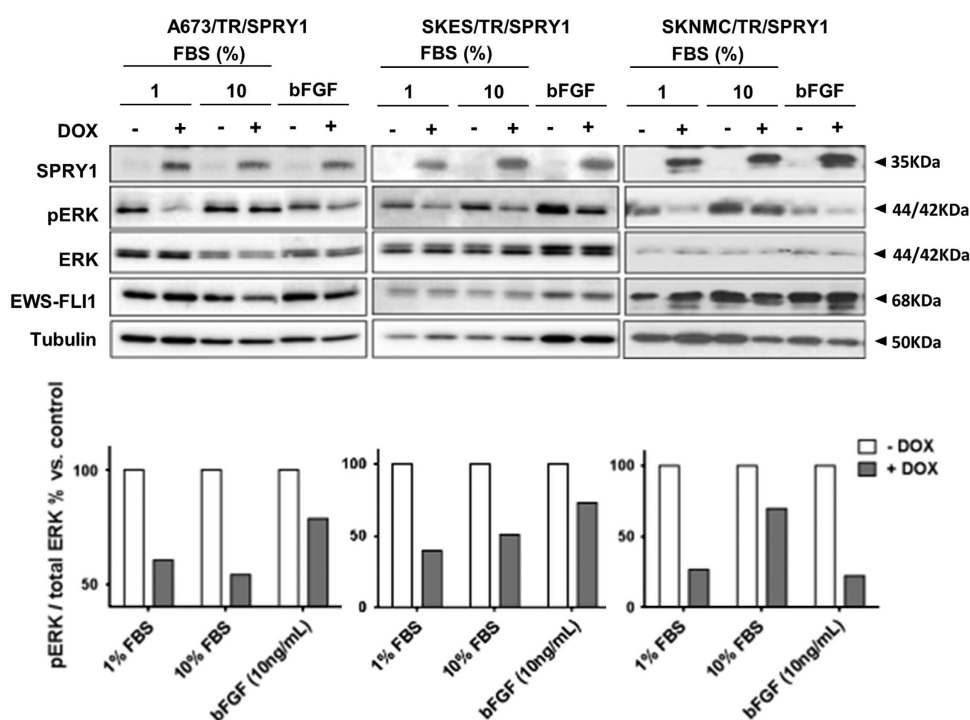
Figure 4. SPRY1 inhibits MAPK pathway in Ewing sarcoma cells by inhibiting ERK phosphorylation induced by bFGF or serum. A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells were incubated in the absence or in the presence of doxycycline (DOX, 1 μg/ml) during 48 h, to induce the expression of SPRY1 protein. Afterwards, cells were starved for an extra 24 h (1% FBS) and finally stimulated for 15 min with 10% FBS or bFGF (bFGF, basic fibroblast growth factor) (10 ng/ml) where indicated. SPRY1, phospho-ERK (pERK), ERK and EWS-FLI1 proteins were detected by specific antibodies. Anti-tubulin was used as a control for loading and transferring. SPRY1 re-expression is capable of inhibiting ERK phosphorylation induced by bFGF or serum in all three cell lines. Graphs depict densitometries corresponding to the western blotting bands showing pERK/total ERK ratios in percentage versus cells cultured in the absence of doxycycline (control). The figure shows one representative experiment out of three performed.

we observed a significant reduction of the number of mitoses ($P = 0.01$) and a significant increase in the number of apoptotic cells per high-power field ($P < 0.001$) on treatment with PD-74 (20 mg/kg) (Supplementary Figure S4B). Similarly, we detected significantly less Ki-67 positive cells on treatment of with PD-74 ($P < 0.01$) (Supplementary Figure S4B).

We next explored the combined effect of FGFR inhibition and SPRY1 re-expression. SPRY1 was re-expressed in the three Ewing sarcoma cell lines and they were concomitantly treated with either bFGF or PD-74 alone or a combination of both (Figure 6). In analogy to the results presented in Figure 2, SPRY1 significantly inhibited cell proliferation induced by bFGF in the three cell lines studied. Moreover, the effect of SPRY1 re-expression and PD-74 on cell proliferation was similar in A673 and SKNMC cells (Figure 6). Furthermore, when the three cell lines were treated with other FGF inhibitors (BG98, PD-66 and SU54), two of them (BG98 and PD-66) were able to significantly further reduce the proliferation beyond the effect of SPRY1 alone (Supplementary Figure S5). However, when SPRY1 was re-expressed concomitantly with any of the three new inhibitors tested, none of them produced a further impairment in proliferation on any of the cells tested, which is in agreement with what was previously observed with PD-74 (Supplementary Figure S5).

SPRY1 expression positively correlates with improved overall survival of Ewing sarcoma patients
Our results indicate that SPRY1 repression leads to a constitutive activation of MAPK/ERK pathway in response to external stimuli such as bFGF. Thus, we wondered whether the expression levels

of SPRY1 in situ could be associated with clinical outcome in Ewing sarcoma patients.

First, SPRY1 mRNA levels were examined in a cohort of 117 Ewing sarcoma samples studied with gene expression microarrays and compared them with published microarray data sets comprising 24 different solid tumor types.[27] This analysis revealed that Ewing sarcoma range among the ones with the lowest SPRY1 expression (Figure 7a), although in in situ tumors there was more heterogeneity in the SPRY1 mRNA levels as compared with Ewing sarcoma cells in culture (Figure 7b). Moreover, there was statistically less SPRY1 expression in Ewing sarcoma cell lines as compared with primary tumors. In fact, all cell lines except for one exhibited less SPRY1 expression than the median sample of primary tumors. In contrast, there was no statistical difference in LOX, NR0B1 and CD99 expression in cell lines when compared with primary tumors (Figure 7b).

Next, we analyzed the correlation between SPRY1 levels in primary tumors and clinical outcome in a cohort of 162 Ewing sarcoma patients.[28] The median expression value of SPRY1 was used as a cutoff to define moderate and low SPRY1 expression levels. Using this cutoff, moderate SPRY1 expression levels were significantly associated with a better overall survival (5-year overall survival 0.70 vs 0.38, $P = 0.002$; log-rank test) and event-free survival (5-year event-free survival 0.72 vs 0.45, $P = 0.0015$; log-rank test) (Figures 7c and d). Interestingly, low SPRY1 levels were associated with a higher risk for the presence of metastasis at diagnosis ($P = 0.002$, Fisher's exact test) (Figure 6e). Collectively, these results strongly support a relationship between the levels of SPRY1 and Ewing sarcoma aggressiveness.
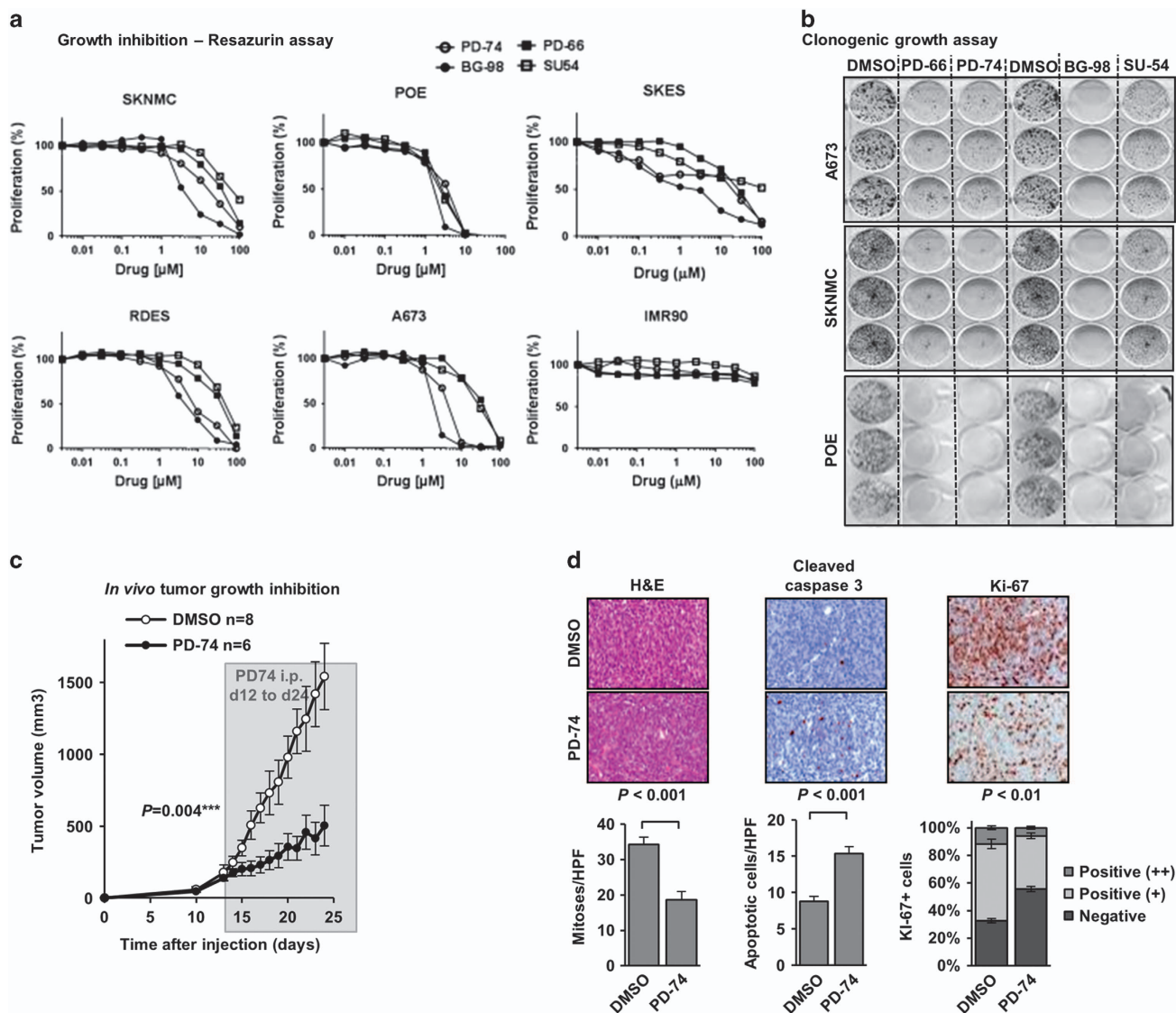
Figure 5. FGFR inhibitors block Ewing sarcoma cell line proliferation. (**a**) Four FGFR inhibitors, namely PD-173074 (PD-74), PD-166866 (PD-66), SU5402 (SU54) and NVP-BGJ398 (BG-98), inhibit A673, SKNMC, POE, RDES and SKES Ewing cell growth *in vitro* in a dose-dependent manner, whereas normal cells (IMR90 fibroblasts) remained unaffected. PD-74 proved to be most effective in four out of five Ewing sarcoma cell lines tested. Cells were grown in 10% FBS conditions and cell proliferation was measured after 72 h using a Resazurin assay. (**b**) PD-173074 (PD-74), PD-166866 (PD-66), SU5402 (SU54) and NVP-BGJ398 (BG-98) impair A673, SKNMC and POE Ewing sarcoma cells clonogenic growth *in vitro* when cells are grown at 5% FBS for 10–12 days. (**c**) C.B17/SCID mice were injected with POE cells and randomly split in groups. Each group was treated intraperitoneally once a day with PD-74 or placebo. The figure shows the evolution of tumor volume (mean ± s.e.m. of six to eight animals per group) versus time. PD-74 treatment significantly inhibits tumor growth ($P = 0.004$) of Ewing sarcoma xenografts. (**d**) Immunohistochemistry images of tumors obtained in the *in vivo* experiments. Tissue sections were stained with Ki-67 to detect proliferation and cleaved caspase 3 to detect apoptosis. The graphs show how PD-74 treatment reduces the number of mitoses ($P = 0.001$) and increases the number of apoptotic cells per field ($P = 0.001$). Ki-67 staining and graph show a reduction in the number of Ki67-positive (++ or +) cells when treated with PD-74 ($P < 0.01$).

DISCUSSION

EWS-ETS fusion proteins have a central role in the pathogenesis of Ewing sarcoma by regulating the expression of other key factors. In this sense, the identification of these regulated genes may help characterize the pathways involved in Ewing sarcoma pathogenesis and aggressiveness, and to therefore open new opportunities for targeted therapies.[29]

In this study, we showed that SPRY1, a member of the Sprouty family of proteins, is repressed by EWS-FLI1 and is not expressed in established Ewing sarcoma cell lines. The exact mechanism through which EWS-FLI1 regulates SPRY1 is still unknown. However, analysis of two independent chromatin immunoprecipitation sequencing studies[30,31] indicates that EWS-FLI1 does not bind to SPRY1 promoter directly (Supplementary Figure S6). Interestingly, on EWS-FLI1 knockdown there is an increase of H3K27ac marks located at the putative SPRY1 promoter comprising SPRY1 exon 1 and intron 1 (Supplementary Figure S6). This suggests an epigenetic mechanism of *SPRY1* regulation involving histone modifications, instead of a direct binding of EWS-FLI1 to the *SPRY1* promoter. Moreover, there were no significant differences in the percentage of SPRY1 CpG islands' methylation on modulation of EWS-FLI1 expression levels.[32] Accordingly, we propose that the actual mechanism underlying *SPRY1* regulation in Ewing sarcoma may be different from the one operating in other tumors where SPRY1 downregulation is associated with promoter methylation.[22,33,34]
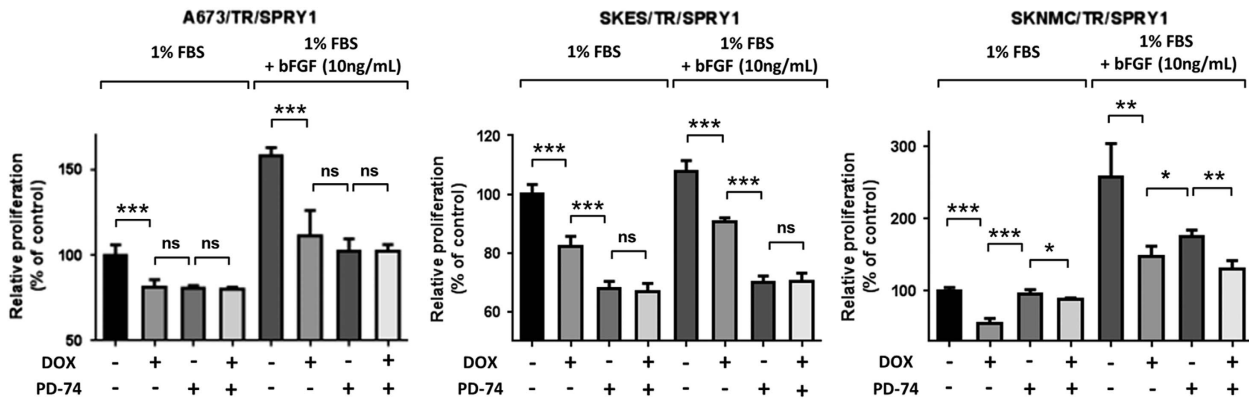
Figure 6. bFGF induces proliferation of Ewing sarcoma cells, which can be antagonized by FGFR inhibition. A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells were incubated in the absence or in the presence of doxycycline (DOX, 1 μg/ml), to induce the expression of SPRY1 protein, and were concomitantly cultured with 1% FBS, bFGF (10 ng/ml), PD-173074 (PD-74, 5 μM) or a combination of bFGF and PD-74 where indicated. Cell proliferation was measured after 72 h using the Resazurin assay. Graphs depict one independent experiment (mean ± s.d.) out of three performed. SPRY1 re-expression and PD-74 inhibit cell proliferation-induced serum or bFGF treatment (*P < 0.05 and **P < 0.005, ns, nonsignificant).
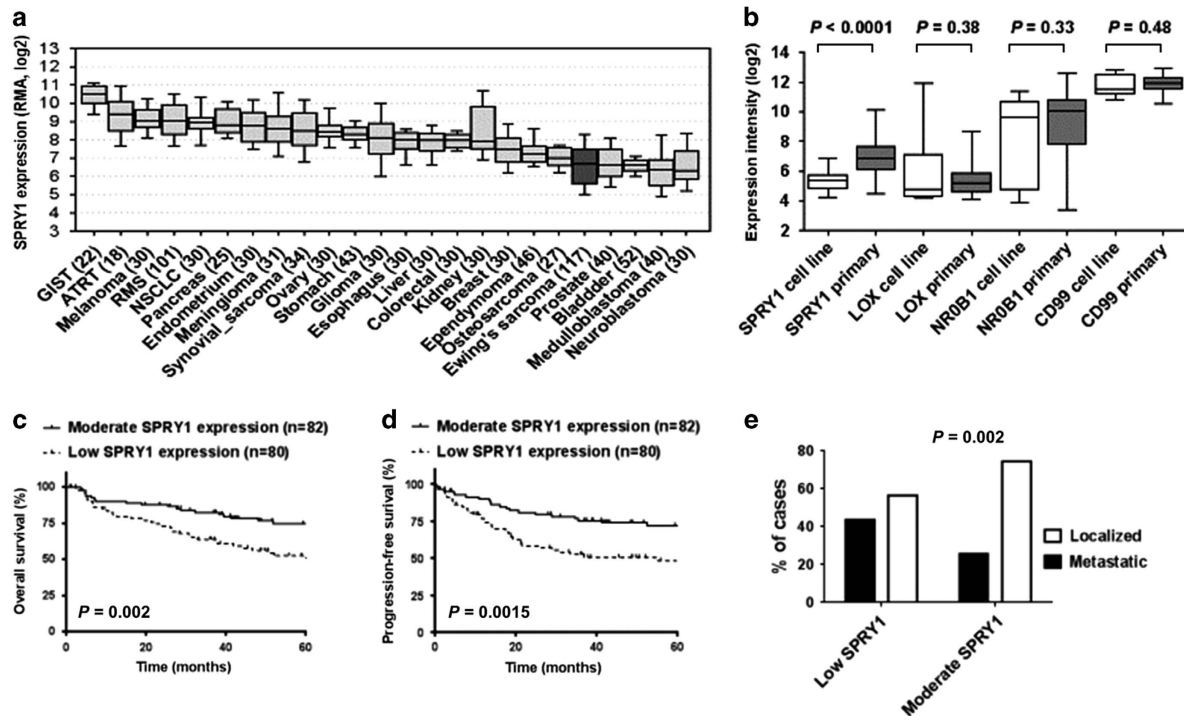
Figure 7. SPRY1 expression is positively correlated with improved overall survival of Ewing sarcoma patients. (**a**) SPRY1 mRNA expression levels in 24 different solid tumor entities as determined by Affymetrix HG-U133plus2.0 DNA microarrays. Data were retrieved from the Gene Expression Omnibus (GEO) or the European bioinformatics Institute (EBI) and simultaneously normalized by RMA using brain array CDF files (v17, ENTREZG) as previously described.[27] Data are represented as medians with boxes representing the interquartile range. Whiskers indicate the 10th and 90th percentile of the data. The number of analyzed samples is given in parentheses. Ewing sarcoma tumors are shown in gray. ATRT, atypical teratoid rhabdoid tumor; Ca-, carcinoma; GIST, gastrointestinal stromal tumor; NSCLC, non-small cell lung carcinoma; RMS, rhabdomyosarcoma. (**b**) Relative expression of *SPRY1* as compared with other EWS-FLI1 target genes (*LOX* and *NR0B1*) and CD99 in 15 individual Ewing sarcoma cell lines versus 117 primary Ewing sarcoma samples (all Affymetrix HG-U133Plus2.0 microarrays). Data were retrieved from the GEO (accession codes: GSE8596, GSE36133, GSE70826 and GSE34620) and simultaneously normalized by RMA using brainarray CDF files (v17, ENTREZG) as previously described.[27] Unpaired two-tailed Student's *T*-test. (**c**) Kaplan–Meier survival estimates (overall survival) in the Ewing sarcoma patient cohort. Patients were classified as being either SPRY1 low or moderate (cutoff: median SPRY1 expression); P = 0.002, log-rank test). (**d**) Graph depicts the relapse-free survival probability versus SPRY1 level of expression (low or moderate, cutoff: median SPRY1 expression). SPRY1 expression positively correlates with improved relapse-free probability (P = 0.0015, log rank test). (**e**) Graph shows the percentage of cases with metastasis at diagnosis versus SPRY1 level of expression (low or moderate, cutoff: median SPRY1 expression). Moderate SPRY1 expression correlates with lower risk of metastasis at diagnosis (P = 0.002, Fisher's exact test).

As SPRY1 has been shown to be a potent negative regulator of the RAS/MAPK/ERK signaling pathway,[35] we hypothesized that SPRY1 may act as a tumor suppressor gene in Ewing sarcoma. In support of this notion, induction of SPRY1 in three independent Ewing sarcoma cell lines significantly impaired cell proliferation and migration. This is consistent with a tumor supressor function of SPRY1 in Ewing sarcoma and in agreement with previous reports showing that SPRY1 overexpression impairs cell growth, proliferation, migration and invasion of a variety of cancer cell lines including ovarian carcinoma, breast cancer, lung adenocarcinoma, colon carcinoma or osteosarcoma.[24,36–38]

Our results also demonstrate that SPRY1 downregulation is necessary for bFGF-mediated proliferation and activation of RAS/MAPK/ERK pathways in Ewing sarcoma cells. Thus, SPRY1 re-expression in the three Ewing sarcoma cell lines used in this study impaired cell proliferation and ERK phosphorylation induced by bFGF. bFGF is known to mediate proliferation, migration and differentiation in various cellular contexts[39–42] and FGF-regulated pathways have a preponderant role in cancer (reviewed in Touat et al.[43]). Notably, an important role for FGF-dependent pathways in Ewing sarcoma pathogenesis is emerging. We have recently reported that bFGF increases proliferation of Ewing sarcoma cells in vitro, and that EGR2, which is a downstream component of the FGF pathway, is an EWS-FLI1-induced target gene.[26] Other studies have demonstrated that bFGF regulates motility and invasion of Ewing sarcoma cells in the bone microenvironment.[44] In agreement, Agelopoulos et al.[45] recently showed that constitutive knockdown of FGFR1 abolishes engraftment of Ewing sarcoma xenografts in mice. Interestingly, over 75% of Ewing sarcoma biopsy samples present moderate-to-high levels of FGFR1 phosphorylation,[44] although activating FGFR1 mutations are extremely rare in this disease.[45]

In light of these facts and our new results, we propose that constitutive activation of FGFRs and downstream pathways are key contributors to the pathogenesis of Ewing sarcoma, and that EWS-FLI1-mediated suppression of the negative-feedback regulator SPRY1 constitutes a major mechanism for sustained FGFR phosphorylation and thus unrestrained FGF-induced signal transduction and tumor progression. In synopsis, our results support that SPRY1 downregulation is pre-requisite for enhanced proliferation and migration of Ewing sarcoma cells induced by either EWS-FLI1 itself, external growth factor stimulation or a combination of both as part of an autocrine loop.

The importance of this pathway in Ewing sarcoma pathogenesis is additionally illustrated by FGFR-inhibition-mediated impairment of cell proliferation and clonogenic growth of Ewing sarcoma cells in vitro. Interestingly, the search for more efficient and specific FGFR inhibitors is an active field in the pharmaceutical industry, as FGF signaling pathways is one of the most commonly mutated systems in cancer.[43] In this regard, the Ewing sarcoma research community can take advantage of the development of these new drugs, some of which are being tested in clinical trials with promising results, particularly in tumors harboring aberrant FGFR signaling (reviewed in Touat et al.[43]).

FGFR signaling can be aberrantly activated in Ewing sarcoma either through downregulation of SPRY1 (as observed in most cases), through overexpression of FGFRs (as observed in subset of patients[45]), or very rarely through somatic mutations.[45] For that reason, we anticipate that Ewing sarcoma patients may benefit from targeted drugs directed against FGFRs or its downstream targets. In support of this notion, Agelopoulus et al.[45] reported on a single patient affected by relapsed Ewing sarcoma, who was treated with an FGFR-tyrosine kinase inhibitor (ponatinib), which led to a reduction in 18-FDG-PET activity and thus glucose uptake by the tumor.

Interestingly, although SPRY1 was undetectable in established Ewing sarcoma cell lines, its levels in primary tumors were variable. Currently, the reasons for the differences between SPRY1 levels in established cell lines and tumors in situ are still unknown;

however, it can be hypothesized that SPRY1 levels remain variable in tumors, and that the harsh conditions of in vitro cell culture favor the growth of cells with lower SPRY1 levels during establishment of Ewing sarcoma cell lines. In fact, established Ewing sarcoma cell lines harbor a much higher rate of STAG2, TP53 and CDKN1A mutations than that observed in primary tumors specimens,[46] suggesting that cells derived from more aggressive tumors are favored in culture.[47,48]

Ewing sarcoma is a very aggressive pediatric malignancy in which primary metastasis is the most unfavorable risk factor, very often leading to fatal outcome despite highly intense and toxic treatment.[49] Here we show that low SPRY1 expression levels correlate with a significantly worse overall and event-free survival in a large cohort of Ewing sarcoma patients. More interestingly, primary tumors displaying low levels of SPRY1 were more frequently observed in patients harboring metastasis at diagnosis. This is compatible with a more aggressive behavior of SPRY1-low tumors and in agreement with the results observed in the in vitro experiments. We speculate that tumors expressing low levels of SPRY1 would present a higher response to external growth factor stimulation and thus exhibit higher rates of proliferation and migration, making them more aggressive. This may have a potential clinical application, as SPRY1 has been recently proposed as a possible tissue biomarker to differentiate aggressive from indolent prostate carcinomas.[50]

In summary, our data provide evidence that EWS-FLI1-mediated SPRY1 downregulation is an important mechanism in Ewing sarcoma pathogenesis. Moreover, our results strongly suggest that bFGF-mediated stimulation of cell proliferation could be more important than initially acknowledged in Ewing sarcoma, and that FGFR inhibitors may constitute promising drugs for treatment of Ewing sarcoma patients.

MATERIALS AND METHODS

Cell culture
A673/TR/shEF cells, which have been described elsewhere,[7] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% tetracycline-free FBS (Clontech, Mountain View, CA, USA), 2 mM L-glutamine, 100 μg/ml zeocin and 3 μg/ml blasticidin. Induction of a small hairpin RNA against EWS-FLI1 was started by the addition of 1 μg/ml doxycycline (Sigma, St Louis, MO, USA). Ewing sarcoma cell lines A4573, TC-71, RD-ES, POE and TTC-466, and the normal fibroblast cell line IMR90 were maintained in RPMI 1640 medium, SK-PN-DW and SKNMC wild-type cells were maintained in Iscove's modified Dulbecco's medium, and wild-type A673 and SKES cells were maintained in Dulbecco's modified Eagle's medium. All media were supplemented, if not otherwise stated, with 10% FBS, 2 mM L-glutamine (Invitrogen, Paisley, UK) and 1% penicillin and streptomycin. All cells were routinely tested for mycoplasma contamination (Mycoalert mycoplasma detection kit, Lonza #LT07-318, Basel, Switzerland) and were authenticated by short tandem repeats profiling at the Genomic Facility at Biomedical Research Institute, CSIC, Madrid, Spain).

Establishment of Ewing sarcoma cell lines stably expressing doxycycline-inducible SPRY1 cDNA
The complete coding region of SPRY1 was reverse transcription–PCR amplified from A673/TR/shEF cells stimulated with doxycycline using primers 5′-GCGGTCGACGAGATCACTACACATGGATCC-3′ (forward) and 5′-CGGCGGCCGCTCATCATCATGATGGTTTACCCTGACC-3′ (reverse). The amplified fragments were digested with SalI and NotI, cloned into the pENTR2B plasmid (Invitrogen) and transferred by recombination to the lentiviral doxycycline-inducible plasmid pLenti4-TO-V5-DEST (Invitrogen). Next, A673/TR, SKES/TR and SKNMC/TR Ewing sarcoma cells expressing the tetracycline repressor constitutively were infected with lentiviruses containing the SPRY1 cDNA. Control cells were infected with empty lentiviral vector. Stable clones were selected with zeocin (100 μg/ml). Induction of SPRY1 was assayed by western blottings on doxycycline (1 μg/ml) stimulation. Clones displaying the highest levels of protein expression on doxycycline stimulation were chosen for additional studies.

Reverse transcription–quantitative PCR

Reverse transcription–quantitative PCR conditions, primer and TaqMan probe sequences specific for EWS-FLI1, LOX, NR0B1(DAX1) and TBP were described elsewhere.[7,13,51] TaqMan probes for SPRY1, 2, 3 and 4 were purchased to Life Technologies (San Diego, CA, USA). Reactions were run on a RotorGene 6000 (Corbett Research, Sydney, NSW, Australia) and relative expression was calculated as previously described.[51]

Western blot analysis and antibodies

Procedure was described elsewhere.[13] Primary antibodies were purchased to the following companies: anti-FLI1 polyclonal antibody from NeoMarkers (#RB-9295-P) (Fremont, CA, USA), anti-SPRY1 monoclonal antibody from Santa Cruz Biotechnology (#100861) (Dallas, TX, USA), Tubulin monoclonal antibody from Sigma Aldrich (#T9026) (St Louis, MO, USA), and anti-Phospho-p44/42 (pERK, #9106) and anti-p44/42 ($_{total}$ERK, #9102) were from Cell Signaling (Danvers, MA, USA). Anti-mouse (#2055) and anti-rabbit IgG (#2054) horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology.

Bromodeoxyuridine proliferation assay

Cells were plated in octaplicates (1×10^3 cells per well in 96 multi-well plates) and cultured in the presence or absence of doxycycline (1 µg/ml) for 72 h in 10% or 1% tetracycline-free FBS (Clontech). Thereafter, bromodeoxyuridine chemiluminescent assay (Roche, Basel, Switzerland) was performed according to manufacturer's instructions. Chemiluminescence was measured using an Infinite M200 (Tecan, Mannerdorf, Switzerland) microplate reader.

Resazurin proliferation assay

Cells were plated in octaplicates (2.5×10^3 cells per well in 96 multi-well plates) and concomitantly cultured in the presence or absence of doxycycline (1 µg/ml) and stimuli (1% or 10% tetracycline-free FBS or 10 ng/ml bFGF) for 72 h. For bFGF-inhibitor testing, cells were grown at 10% FBS for 72 h in the presence of PD-173074 (PD-74) (Selleckchem, Houston, TX, USA), PD-166304 (PD-66) (#PZ0114, Sigma Aldrich), SU5402 (SU54) (#S7667, Selleckchem) or NVP-BGJ398 (BG-98) (#S2183, Selleckchem). Thereafter, Resazurin (#R7017, Sigma Aldrich) was added to the media at 0.15 µg/ml and incubated for 2 h at 37 °C. Fluorescence was recorded using a 560-nm excitation/590-nm emission filter set in an Infinite M200 microplate reader (Tecan).

xCELLigence proliferation assay

Cell proliferation was assayed in real time with a bioelectric xCELLigence instrument (Roche/ACEA Biosciences). In each well, $3–4 \times 10^3$ Ewing sarcoma cells were seeded in 200 µl media containing 10% tetracycline-free FBS and treated with doxycycline (1 µg/ml) or vehicle (triplicate wells/group). Cellular impedance was measured periodically and media with or without doxycycline were changed once after 72 h.

Flow cytometry analysis of cell cycle

Cells were treated with doxycycline (1 µg/ml) for 72 h to induce the expression of SPRY1 and fixed with 70% ethanol for 24 h at 4 °C. Next, they were stained with a solution of 0.005% (w/v) of propidium iodide and RNAase A as recommended by the manufacturer (BD Biosciences, San José, CA, USA) and were incubated at 37 °C for 30 min. They were then analyzed in a MACS Quant Analyzer flow cytometer (Miltenyi Biotec, Cologne, Germany).

Wound-healing assay

Cells were plated in triplicates ($2–4 \times 10^4$ cells per well in 24 multi-well plates) and were incubated with or without doxycycline (1 µg/ml) for 72 h before the assay. At the end of this period, a 'wound gap' in the cell monolayer was created using a micropipette tip. The healing of the gap by cell migrating was monitored by photographing the progress every 6–12 h until wound closure. Quantification of relative cell migration is described elsewhere.[52]

Transwell assay

Cells were pre-treated with doxycycline (1 µg/ml) for 24 h to induce the expression of SPRY1 protein. Next, they were starved (0.5% FBS) for another additional 24 h in the same doxycycline conditions. Then, 3×10^5 pretreated cells were re-suspended in 2 ml of medium containing 0.5% tetracycline-free FBS and placed in the upper chamber of transwells (8.0 µm pore size) (Corning Costar, Cambridge, MA, USA) following the procedure described elsewhere.[13]

Soft agar assays

Cells were plated by triplicate (5×10^5 cells per 60 mm dishes) in soft agar and cultured in the presence or absence of doxycycline during 25 days. Fresh culture medium was added to plates every 2–3 days. At the end of the experiment, three random fields for each plate were photographed. The number of colonies per field and its respective area were calculated using NIH ImageJ software (National Institute of Health, Bethesda, MD, USA).

Clonogenic assay

A673/TR/SPRY1, SKES/TR/SPRY1 and SKNMC/TR/SPRY1 cells were plated in triplicates at 0.5×10^3, 1×10^3 and 2×10^3 cells per well, respectively, in a 24-well plate. They were subsequently treated with or without doxycycline (1 µg/ml) and maintained for 9 days in culture media supplemented with 5% tetracycline-free FBS. Media was changed every 3–4 days and doxycycline treatment was continued. Finally, colonies were fixed, stained with crystal violet and photographed. Cells were de-stained using 50% ethanol 0.1 M sodium citrate pH 4.2. Absorbance was quantified at 560 nm using an Infinite M200 (Tecan) microplate reader.

Tumor xenografts in mice

POE and SKES cells were resuspended in PBS/matrigel (BD Biosciences, Le Pont de Claix Cedex, France) (1:1) and injected (8×10^6/200 µl) subcutaneously in the flanks of 6-week old C.B17/SCID male and female mice (Charles River Laboratories, Lyon, France). When tumor volume reached 150 mm^3 (calculated with the formula length × width × depth × 0.5432), mice were injected intraperitoneally once a day with the indicated dose of PD-173074 (5, 10 or 20 mg/kg) dissolved in 10% dimethyl sulfoxide–90% Corn Oil (Sigma) or placebo in the control group. Tumor growth was monitored with a caliper and mice were killed when tumors reached a volume of 1500 mm^3. Experiments were carried out in accordance with recommendations of the European Community (86/609/EEC), the French Competent Authority, the UKCCCR guidelines (guidelines for the welfare and use of animals in cancer research), the Ethics Committee at ISCIII (CBA #64_2015-v2) and the Spanish Competent Authority (PROEX 009/16).

Histology and immunohistochemistry

Immunohistochemistry analyses were done on formalin-fixed, paraffin-embedded xenograft tumors. All tissue samples were collected at the Institute of Pathology of the LMU Munich for immediate immunohistochemistry staining, for which 4-µm sections were cut. Antigen retrieval was carried out by microwave treatment in Dako target retrieval solution (S2369). The following primary antibodies were used: polyclonal rabbit anti-cleaved-caspase-3 (1:100 at room temperature for 60 min; #9661, Cell Signaling) or monoclonal rabbit anti-Ki67 (1:200 at room temperature for 60 min; #275R-15 clone SP6, Cell Marque, Rocklin, CA, USA). The ImmPRESS Reagent Kit anti-rabbit IgG (MP-7401, Vector Laboratories, Burlingame, CA, USA) was used for antigen detection. Sections were counterstained with hematoxylin Gill's Formula (H-3401, Vector Laboratories). The average number of positive cells was determined by analysis of 10 high-power fields (×40 magnification) for each xenograft tumor. Statistical differences between groups were calculated with an unpaired tow-tailed Student's T-test.

Patients

A total of 162 Ewing sarcoma patients with available clinical data and tumor samples were used in this study. This cohort consists of 117 Ewing patients for which gene expression profiles in primary tumors were analyzed with HG-U133 plus2.0 microarrays (Affymetrix, Santa Clara, CA, USA) (Gene Expression Omnibus accession number: GSE34620) and 45 patients whose gene expression profiles were studied with Uniset Human 20 K I microarrays (Codelink Amersham Bioscience, Piscataway, NJ, USA). All patients received a similar protocol treatment.

Statistical analysis

For a single comparison of two groups, two-tailed Student's t-test was used and a normal distribution was assumed. Variances between the groups that were compared were similar. For animal studies, the sample size was estimated to be six to eight mice considering a signal/noise ratio of 1.6–1.8, 80% power, assuming a 5% significance level and a two-sided test. No investigator blinding was done during the experiment. For *in situ* studies including overall survival and relapse-free survival probabilities, log-rank test was used. For proportions, Fisher's exact test was used. For all analyses, the level of significance was set at $P = 0.05$ and the variance was similar between groups. All statistical calculations were performed using the GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)