SPRY1 IS A NEW TARGET OF THE EWS/FLI1 ONCOPROTEIN IN EWING SARCOMA CELLS

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Introduction

Ewing sarcoma (ES) is an aggressive solid bone and soft tissue malignancy of children and young adults. ES is originated by nonrandom chromosomal translocations that yield in-frame fusion of the amino terminus of the EWS gene on chromosome 22 and the carboxyl terminus of a member of the ETS family of transcription factors. There are several EWS-ETS aberrant transcription factors of which EWS-FLI1 fusions are the most frequent[1]. The fusion protein EWS-FLI1 plays a role in different aspects of tumor development, maintenance and progression through the regulation of EWS-FLI1 target genes. Some of most relevant EWS-FLI1 target genes identified up to date are NKX2.2, cholecystokinin[2], NR0B1[3], TGFBR2, IGFBP3 and *LOX*[4] among others. Identification of **EWS-ETS target genes** and their role in tumor development will facilitate the translation into new treatment modalities for these neoplasms.

In this work we identified a new target of EWS-FLI1 oncoprotein: ***Sprouty 1*** **(SPRY1).** SPRY1 is an upstream antagonist of RAS and it has been suggested that it may have a tumour suppressor function since its expression is decreased in human cancer, including tumors from breast, prostate, and liver tissue[5].

Objectives

1. Identify new EWS/FLI1 target genes.
2. Study the mechanism involved in the regulation of SPRY1 expression.
3. Analyse the functional relevance of SPRY1 in Ewing Sarcoma.

Materials and Methods

Establishment of Ewing Sarcoma cell lines stably expressing doxycycline-inducible EWS/FLI1 shRNA or SPRY1 cDNA: Ewing A673, SKES and SKNMC cells were infected with a plasmid containing the tetracycline repressor and afterwards with a plasmid containing EWS-FLI1 shRNA or SPRY cDNA regulated by doxycycline. When these cells are treated with doxycycline at a concentration of 1ug/ul, they express the EWS/FLI1-specific shRNA, which subsequently knockdown EWS-FLI1 levels, or the SPRY1 mRNA.

Quantitative reverse transcription–polymerase chain reaction (RT-PCRq): Reactions were run on a RotorGene 6000 (Corbett Research) using specific Taqman probes for the genes analyzed. Relative expression was calculated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{cDNA\ of\ interest} - Ct_{TBP}$

Western blot analysis and antibodies: Following electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore), then blocked with 5% milk and incubated with primary antibodies. Finally they were incubated with horseradish peroxidase-conjugated secondary antibodies and subjected to chemiluminescence (GE Healthcare) detection analysis.

Proliferation assay: $5 \cdot 10^5$ cells were platted in presence or absence of doxycyclin for 4-5 days. Then they were trypsinized, counted and replated for a minimum of 15 days.

Chemical Agents: Trichostatin A (TSA) (Sigma), Vorionostat (SAHA) (Sigma), 5-aza-2'-deoxycytidine (Sigma) and Valproic Acid (VPA) (Sigma) were dissolved in DMSO first and then were further dissolved in PBS until desired concentration. bFGF (Peprotech) was used at 10ng/uL; IGFI (Peprotech) at 5ng/uL; VEGF (Peprotech) at 10ng/uL in PBS 1X.

Results

SPRY1 expression is downregulated by EWS/FLI1 in Ewing sarcoma cells

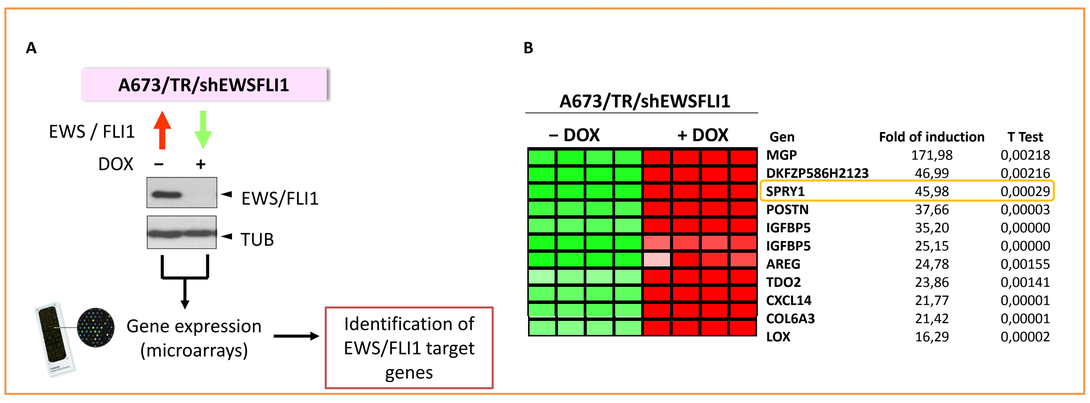


Figure 1. Identification of EWS/FLI1 target genes. A) A673/TR/shEWSFLI1 cells were cultured in presence or absence of doxycyclyne (1ug/ul) for 72 hours to induce the expression of the specific EWS/FLI1 shRNA in order to knockdown EWS/FLI1 levels. B) Gene expression profiles were analysed in A673/TR/shEWSFLI1 cells (four different clones) cultured in absence or presence of doxycycline. (*green: minimal expression; red: maximal expression).* Figure shows the main genes repressed by EWS/FLI1 where SPRY is in third place.

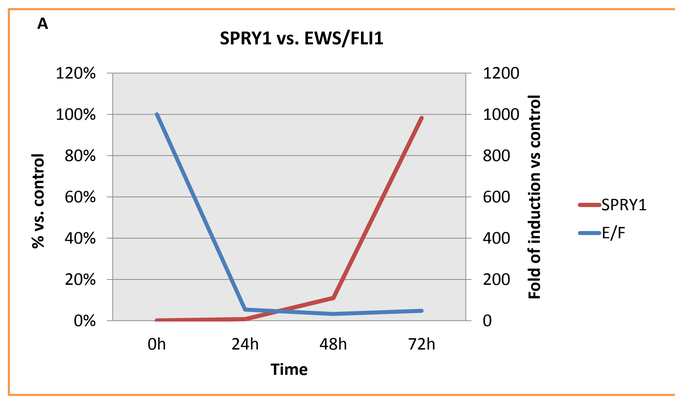


Figure 2. A) A673/TR/shEWSFLI1 cells were stimulated with doxycycline at different times (0, 24, 48 and 72 hours). SPRY1 and EWS/FLI1 mRNA were quantified by RT-PCRq using specific Taqman probes. The figure shows how EWS/FLI1 silencing produces a dramatic increment in SPRY1 mRNA. **B)** A673/TR/shEWSFLI1 cells were tested by Western Blot with specific antibodies against SPRY1, EWS/FLI1 and tubulin. The figure shows how doxycycline treatment generates a time-dependent increment in SPRY1 expression that correlates with the decrease in EWS/FLI1 protein levels. α-tubulin was used as a loading control. Graph depicts the relative SPRY1/tubulin protein level.

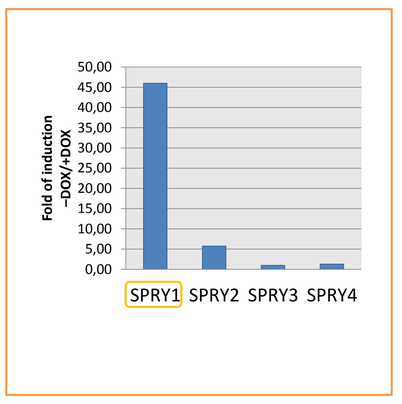


Figure 3. SPRY1 is selectively repressed by EWS/FLI1. Expression of the different members of the SPRY gene family (SPRY1, SPRY2, SPRY3 and SPRY4) was analyzed by RT-PCRq in A673/TR/shEWSFLI1 upon doxicycline stimulation (72 h). SPRY1 appears to be a selective target of EWS/FLI1 repression over the rest of members of the SPRY family of genes.

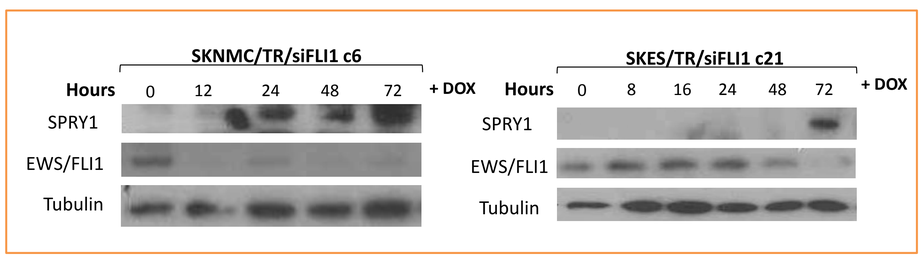


Figure 4. EWS/FLI1 knockdown correlates with SPRY1 induction in other Ewing sarcoma cell lines. The figure shows how SPRY1 protein is induced after 24-72 hours of EWS/FLI1 inhibition in SKNMC and SKES Ewing sarcoma cell lines.

SPRY1 is not expressed in Ewing Sarcoma cells

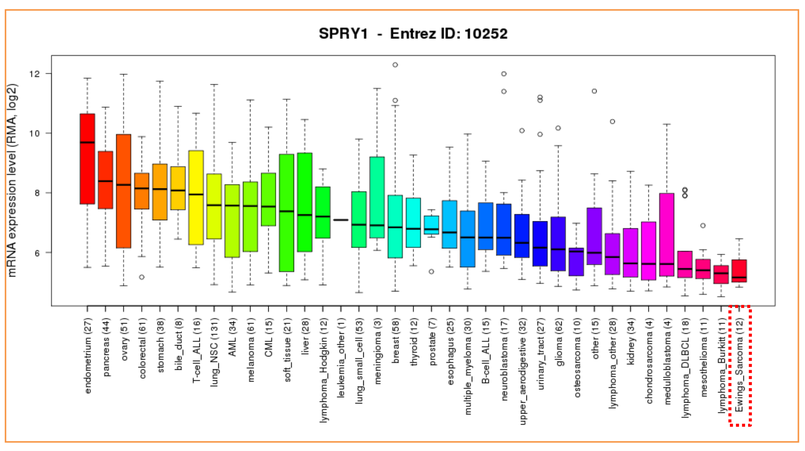


Figure 4. SPRY1 is not expressed in Ewing Sarcoma cells. Gene Expression data for SPRY1 extracted from Cancer Cell Line Expression (CCLE) dataset (http://www.broadinstitute.org/ccle/home). Gene-centric RMA-normalized mRNA expression data.

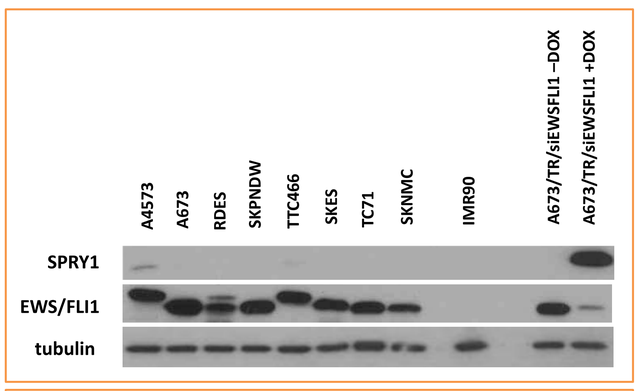


Figure 5. SPRY1 protein is not expressed in Ewing sarcoma cell lines. Total protein extracts from Ewing sarcoma cell lines (A4573, A673, RDES, SKPNDW, TTC-466, SK-ES, TC71 and SKNMC) were assayed by Western Blot with specific antibodies against SPRY1 and EWS/FLI1 proteins. IMR90 cells were used as a SPRY1 negative control. A673/TR/shEWSFLI1 incubated 72 hours with doxicycline were used as a positive control for SPRY1 expression. α-tubulin was used as a loading control.

HDACs are partially involved in the repression of SPRY1

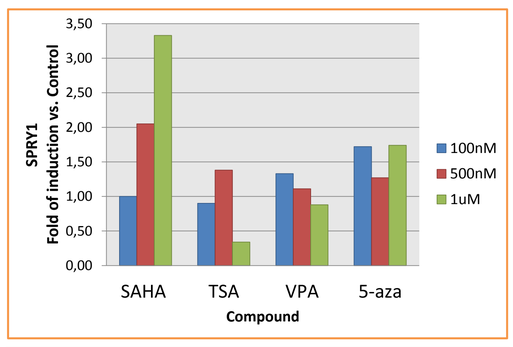


Figure 6. SAHA partially reverts SPRY1 downregulation. A673 Ewing sarcoma cells were treated with SAHA, TSA, 5-aza and VPA at different concentrations (100,nM 500nM and 1uM) for 24 hours. mRNA was extracted and SPRY1 mRNA was quantified by RT-PCRq using specific Taqman probes. The figure shows how SAHA treatment produces a moderate increment in SPRY1 mRNA expression.

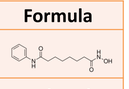
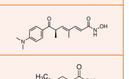
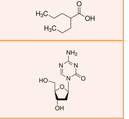
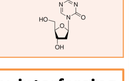
Compound	Activity	Formula
SAHA (Vorinostat)	Inhibits both class I and class II HDACs	
TSA (Trichostatin A)	Inhibits both class I and class II HDACs	
VPA (Valproic acid)	Inhibits class II histone deacetylase	
5-Aza-2'deoxycytidine	Produces DNA demethylation o hemi-demethylation	

Table I. Compounds that alter gene expression by interfereing with the epigenetic machinery. Both histone deacetylases (HDACs) such as SAHA, TSA and VPA, and demethylases such as 5-aza-2'deoxycytidine interfere with gene expression by inhibiting the function of histone deacetylases and methylases respectively.

Establishment of Ewing sarcoma cells expressing inducible SPRY1

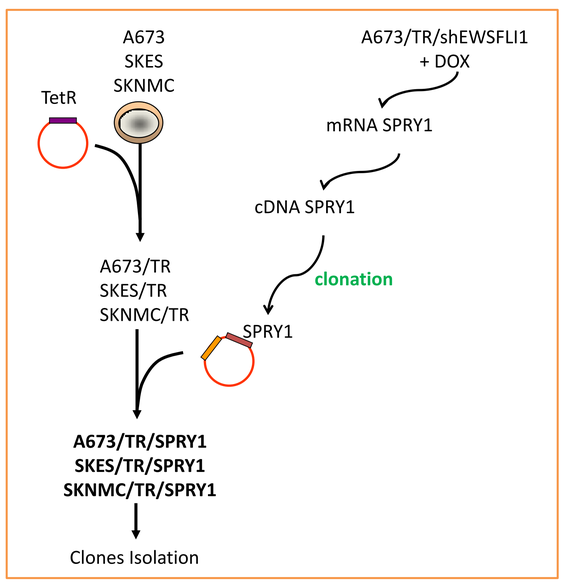


Figure 7. **SPRY1 inducible system.** A673, SKES and SKNMC Ewing Sarcoma cell lines were first transfected with the TetR gene. On the other hand, SPRY1 mRNA was transcribed to cDNA and cloned on a lentiviral vector under the control of a doxicycline regulated promoter. A673/TR, SKES/TR and SKNMC/TR cells were then infected with this plasmid and selected to obtain Ewing sarcoma cells in which SPRY1 expression can be regulated by incubation with doxicycline.

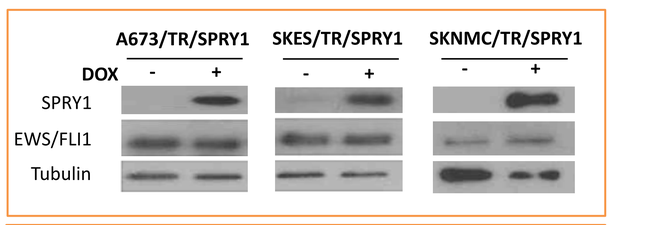


Figure 8. Inducible expression of SPRY1 in three Ewing Sarcoma cell lines. The figure shows SPRY1 protein induction after treatment with doxicycline 1ug/uL in A673, SKES and SKNMC cells transfected with a inducible form of the SPRY1 gene. Note that EWS/FLI1 levels remain constant.

SPRY1 induction in Ewing sarcoma cells affects cell proliferation

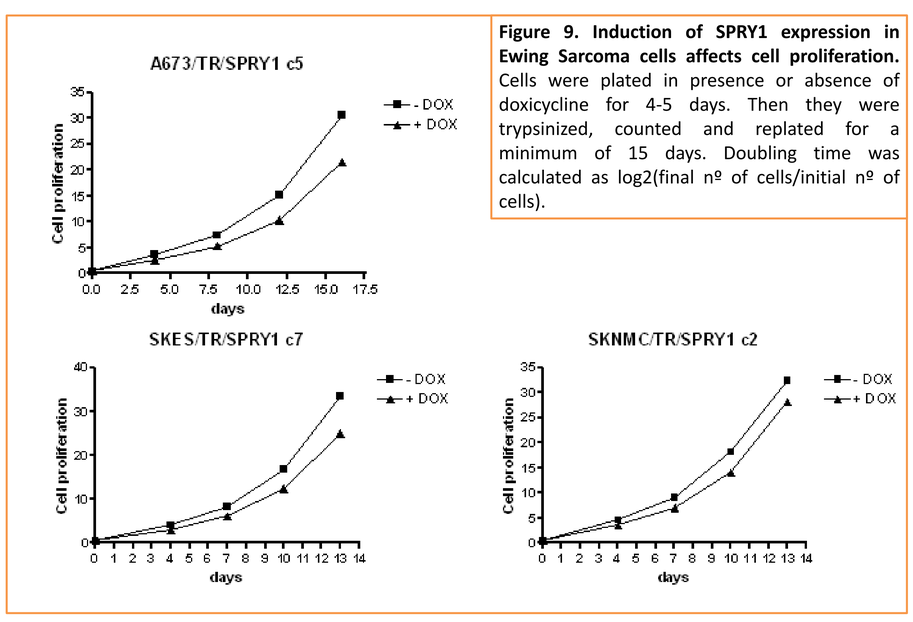


Figure 9. Induction of SPRY1 expression in Ewing Sarcoma cells affects cell proliferation. Cells were plated in presence or absence of doxicycline for 4-5 days. Then they were trypsinized, counted and replated for a minimum of 15 days. Doubling time was calculated as log2(final nº of cells/initial nº of cells).

SPRY1 regulates MAPK pathway by inhibiting ERK phosphorilation

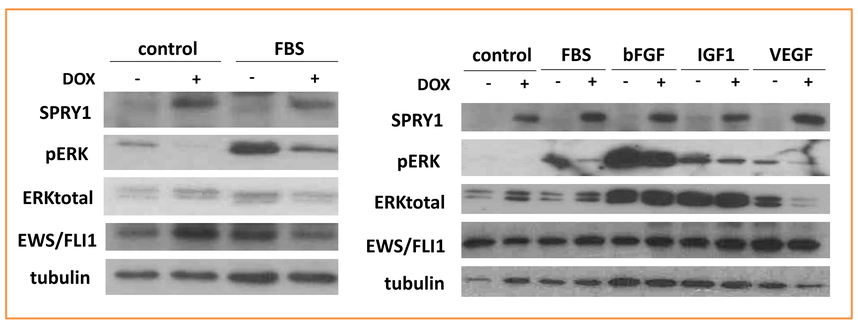


Figure 10 SPRY1 induction regulates MAPK pathway. A) Induction of SPRY1 in SKES/TR/SPRY1 cells produces an important decrease in the levels of ERK phosphorilation after FBS treatment for 15 minutes. **B)** Induction of ERK phosphorilation by bFGF, IGF1 and VEGF is inhibited upon SPRY1 expression in SKES/TR/SPRY1 cells.

Conclusions

1. **SPRY1 expression is strongly downregualted by EWS/FLI1 in Ewing sarcoma cells**
2. **These effect is partially mediated by epigenetic mechanism.**
3. **Induction of SPRY1 expression in Ewing sarcoma cell lines moderately inhibits cell proliferation**
4. **SPRY1 regulates MAPK pathway in Ewing sarcoma by inhibiting ERK activation.**

Acknowledgments

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