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Sprouty2 but not Sprouty4 is a potent inhibitor of cell proliferation and migration of osteosarcoma cells

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ABSTRACT

As negative regulators of receptor tyrosine kinase-mediated signalling, Sprouty proteins fulfil important roles during carcinogenesis. In this report, we demonstrate that Sprouty2 protein expression inhibits cell proliferation and migration in osteosarcoma-derived cells. Although earlier reports describe a tumour-promoting function, these results indicate that Sprouty proteins also have the potential to function as tumour suppressors in sarcoma. In contrast to Sprouty2, Sprouty4 expression failed to interfere with proliferation and migration of the osteosarcoma-derived cells, possibly due to a less pronounced interference with mitogen-activated protein kinase activity. Sequences within the NH_2-terminus are responsible for the specific inhibitory function of Sprouty2 protein.

1. Introduction

Cell proliferation is largely controlled by extracellular cues interpreted by the action of pathways downstream of receptor tyrosine kinases (RTKs). These receptors are activated by ligand binding and activate different intracellular signalling pathways like the Ras/mitogen activated protein kinase (MAPK), the PI3K/Akt or the phospholipase C (PLC)-mediated pathways [1]. Intensity and duration of stimulation is restricted by many negative regulatory mechanisms [2].

Sprouty (Spry) proteins have been identified as inhibitors of RTK-mediated processes in *Drosophila* [3–5]. In *Drosophila* as well as in many other organisms Spry proteins play a pivotal role during branching morphogenesis, including lung tracheal network formation, nephrogenesis and angiogenesis (reviewed in [6]).

In mammalians there are four orthologues identified [7,8]. Whereas Spry3 mRNA can only be detected in the brain and testis of adults, the other three Spry family members are ubiquitously expressed in all embryonic and adult tissues [8] and show common but also distinct domains of expression [9]. Although all Spry proteins can be detected in both epithelial as well as mesenchymal tissue [9], it was found that in many organs such as the developing lung [7] or the teeth [10] Spry1 and Spry2 are restricted to cells of epithelial origin, whereas Spry4 localises to the mesenchymal part [9].

Functionally the Spry proteins are not able to fully complement each other, therefore loss of Spry1 [11,12], Spry2 [13,14] and Spry4 [15] causes specific phenotypes similar to those observed in case of growth factor overdoses. A double knockout of Spry2 and Spry4 is embryonic lethal [15].

In accordance with their cellular functions Spry proteins fulfil a bivalent role in cancers derived from different origin. Spry2 and Spry4 function as tumour suppressors in lung cancer, while Spry1 mRNA and protein level are rather increased in the malignant tissue of the lung cancer patients [16]. Additionally, published data document that in prostate cancer [17,18] and in breast [19] decreases in Spry1 and Spry2 levels contribute to malignant

Abbreviations: AA, aminoacids; FCS, fetal calf serum; GST, glutathione S-transferase; MAPK, mitogen activated protein kinase; PAGE, polyacrylamidgelelectrophoresis; PLC, phospholipase C; pERK, phosphorylated extracellular signal-regulated kinase; prPS6, phosporylated ribosomal protein S6; RTK, receptor tyrosine kinase; Spry, Sprouty

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transformations. In hepatocellular carcinomas Spry2 but not Spry1 expression levels are frequently repressed [20].

Contrary to these findings, Lito et al. published investigations showing that Spry2 is necessary for sarcoma formation and transformation of fibroblasts by the H-Ras oncogene [21] and that Spry1 is positively involved in cancerogenesis of rhabdomyosarcoma [22].

In this report, we investigated the role of Spry2 and Spry4 proteins as inhibitor of cell proliferation and migration in osteosarcoma-derived cells.

2. Materials and methods

2.1. Cell culture

The human osteosarcoma cell lines U2OS, MG63, SaOS-2, 143B and HOS as well as the chondrosarcoma-derived cell line SW-1353, the fibrosarcoma cell line HT-1080, the synovial-derived SW-982, the liposarcoma cell line SW-872 and the primary human lung fibroblasts WI-38 were purchased from the American Type Culture Collection. Additionally cell lines established at our institute from a osteosarcoma (HLNG), a leiomysarcoma (VLMS1), and from a lung metastasis of a Ewing sarcoma (VEs1) were used. The gliosarcoma cell line LN40 was kindly provided by Dr. Tribolet (Lausanne). All cells were cultured in the recommended medium containing 10% fetal calf serum (FCS) and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 7.5% CO_2.

2.2. Recombinant adenovirus generation

The coding sequence of Spry4 was amplified by PCR using the upstream primer 5′-TAGCGAATTCGGATCCATGCTCAGCCCC-3′ (orfSpry4s) and downstream 5′-TAGAATTCCTCGAGTCAGAAAGGCTTG-3′ (orfSpry4as). The 2/4 hybrid was generated by PCR amplification of the Spry2 N-terminus using the following primers: 5-TAGCGAATTCGGATCCATGGAGGCCAGAGCTCAGAG-3′ (orfSpry2s) and 5′-ACAGGCCTCGCACCTGTAGGCGTGCAGGCC-3′ (S2/4hyas) and fused by PCR to the C-terminus of Spry4 which was modified by a PCR with the following primers: 5′-CACGCCTACAGGTGCGAGGCCT GTGGGAAG-3′ (S2/4hys) and orfSpry4as. To construct the virus expressing a protein fusing the N-terminus of Spry4 to the C-terminus of Spry2, sequences amplified using the primers orfSpry4s and S4/2hyas (5′-CACAGTCCTCACACAGCAAGAAGTGCTTGTC-3′) were combined with the Spry2 sequences amplified by using the primers S4/2hys (5′-GCACTTCTTGCTGTGTGAGGACTGTGGCAAG-3′) and orfSpry2as (5′-TAGCGAATTCCTCGAGTCAGAAAGGCTTG-3′). All sequences were digested BamH1/EcoR1 and cloned into a pADlox backbone. The recombinant adenoviruses were generated and used as described [16].

2.3. Growth curve

Twenty-four hours post infection, 2–5 × 10^4 cells were reseeded in cell culture dishes in the appropriate medium containing 10% FCS, and counted every day using a Bürker–Türk cell-counting chamber. Growth curves were generated and doubling times were calculated in exponential growth equations using GraphPadPrism software. Each experiment was performed at least twice in duplicates.

2.4. Clonogenic assay

Twenty-four hours after adenoviral infection, 100 cells were seeded in triplicates and cultured one to four weeks. After this time cells were stained with GIEMSA solution (Merck). Visible colonies were counted and pictured.

2.5. Scratch assay

Scratch assay was performed as described [16]. Migration velocity was calculated as linear regression curve using GraphPadPrism software.

2.6. Immunoblot

Immunoblotting was carried out as described [23] using affinity purified antibodies against the NH_2-terminus of Spry2 (characterised in [16]) and Spry4 (described in [24]). As a loading control primary antibodies against β-actin (Novus Biologicals) were used. Antibodies recognizing phosphorylated extracellular signal-regulated kinase (pERK), phoshorylated ribosomal protein S6 (prPS6) and cRaf were purchased from Cell signalling (New England Biolabs).

2.7. GST pulldown

The construct expressing the glutathione S-transferase (GST)-tagged NH_2-terminal 150 amino acids of human Spry2 (pGEX-Spry2N150) was constructed earlier [16]. *Escherichia coli* BL21 strain was transformed with pGEX and pGEX-Sprouty2N150. A colony was inoculated in LB-medium containing ampicillin and incubated at 37 °C. The overnight cultures were diluted 1:10 with medium and incubated for 1 h before induction of the protein synthesis by addition of 100 mM isopropyl-β-D-thiogalactopyranosid. After 3 h bacteria were collected by centrifugation and resuspended in phosphate buffered saline. According to the manufactures (GE Healthcare) instructions lyses and protein purification was performed. Twenty microlitre of the beads with the purified GST containing proteins were loaded on a SDS polyacrylamid gel electrophoresis (PAGE) and stained with coomassie. 5 μl of the beads were mixed with protein extracts of U2OS cells transfected with a cRaf expressing plasmid and incubated on a head-over-head rotation at 4 °C. Beads were washed four times with TNN-buffer (50 mM Tris pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% NP-40) for 10 min and an immunoblot was performed. The cRaf expressing construct was kindly provided by Richard Marais.

3. Results

3.1. In sarcoma-derived cell lines Spry2 and Spry4 protein levels correlate

In an initial experiment we measured Spry2 and Spry4 protein levels in logarithmically growing sarcoma-derived cell lines.

In all 13 tested cell lines Spry2 was detected, although a wide variation in expression could be observed. When compared to primary WI-38 fibroblasts, about half of the cell lines ($n = 6$) had increased levels of Spry2 while Spry2 expression was decreased in the other seven lines (Fig. 1A). In case of Spry4 expression, only four of the tested cell lines showed slightly increased levels versus WI-38 expression. The cell lines with higher levels of Spry4 also expressed significantly more Spry2 than the cells exhibiting low level of Spry4 (Fig. 1B) and vice versa cell lines with reduced levels of Spry2 were also expressing significantly lower amounts of Spry4 protein (Fig. 1C).

These data suggest that in sarcoma-derived cells Spry4 and Spry2 protein levels are determined by similar mechanisms.

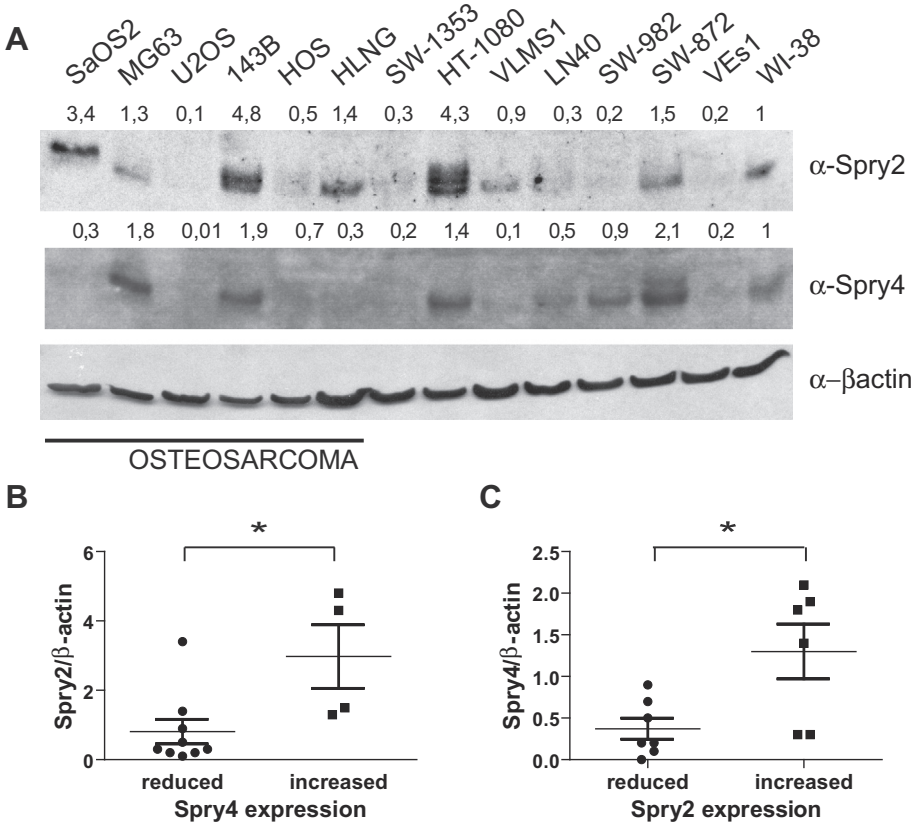

Fig. 1. Endogenous Spry2 and Spry4 expression in sarcoma-derived cell lines. (A) Endogenous Spry2 and Spry4 levels in 13 different sarcoma-derived cell lines were determined compared to normal primary fibroblasts WI-38. The immunoblots were sequentially probed with the indicated antibodies. (B) Spry2 and Spry4 expression was determined using Image Quant software 5.0 and calculated as ratio to β-Actin. Spry4 levels of cell lines with low Spry2 expression were compared with high expressing cell lines. Mean ± S.E.M. are shown. (C) Calculated Spry2 protein levels of high and low Spry4 expressing cell lines were compared by an unpaired *t*-test. Mean values ± S.E.M. are shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3.2. Ectopic expression of Spry2, but not Spry4 inhibits cell proliferation in osteosarcoma-derived cell lines

Next we wanted to test the influence of Spry proteins on cell proliferation of sarcoma-derived cells. We selected two osteosarcoma-derived cell lines with low and high endogenous Spry protein levels as representative to measure the influence of ectopic Spry2 and Spry4 expression on cell proliferation. U2OS cells have the lowest endogenous Spry2 as well as Spry4 protein levels. Since 143B cells were hardly to infect with adenoviruses, the MG63 cell line was selected to represent osteosarcoma with high endogenous levels of Spry proteins. Logarithmically growing cells were infected and clonogenic assays as well as growth curves were performed. In both cell lines, the number of clones measured after 10 days was not reduced when Spry4 was ectopically expressed. In contrast, Spry2 expression halved the amount of clones in U2OS cells when compared to control treated cells (Fig. 2A). In MG63 cells showing high endogenous levels of Spry2, the additionally expressed Spry2 reduced the number of clones to about 60% compared with the one measured in mock treated cells (Fig. 2A). In line with these data, Spry2 overexpressing U2OS cells doubled not even twice within the monitoring period (96 h), while the control cells have almost quadrupled. Although in MG63 cells the observed inhibition of proliferation by Spry2 expression (0.84 ± 0.09 compared to 1.15 ± 0.02 doublings) was less pronounced than in U2OS cells, the measured interference of Spry2 with cell doubling was considerable. In case of Spry4 expression, cell proliferation was not significantly changed when compared to mock treated control osteosarcoma cells (Fig. 2B). Both Spry4 and Spry2 were clearly overexpressed by the respective recombinant adenovirus as verified by immunoblot (Fig. 2C).

These data demonstrate that in osteosarcoma Spry2 interferes with cell proliferation.

3.3. Spry2 expression interferes with cell migration of osteosarcoma-derived cells

To evaluate if the interference with proliferation is also detected in other mitogen-dependent processes, we measured the influence of ectopic Spry2 and Spry4 expression on cell migration. To this end MG63 cells were infected and 48 h post infection a scratch assay was performed.

The time to close the gap was significantly delayed when cells expressed ectopic Spry2 (Fig. 3A). On average Spry2 overexpressing cells cover a distance of 12 μm in an hour, while control treated cells move about 1.3-fold faster. Spry4 overexpression rather increases the velocity of gap closure than inhibiting it (Fig. 3A and B).

These data demonstrate that like proliferation, cell migration of osteosarcoma-derived cells is inhibited specifically by Spry2.

3.4. Inhibition of MAPK pathway is more pronounced in case of Spry2 expression when compared to the influence of increased Spry4 levels

In response to mitogens Spry proteins are mainly described as inhibitors of MAPK activation, but also PI3K induced pathways can be negatively influenced via PTEN [6]. In order to investigate the influence of Spry expressions on signalling pathways, U2OS cells were serum starved for 24 h before infected with adenovirus

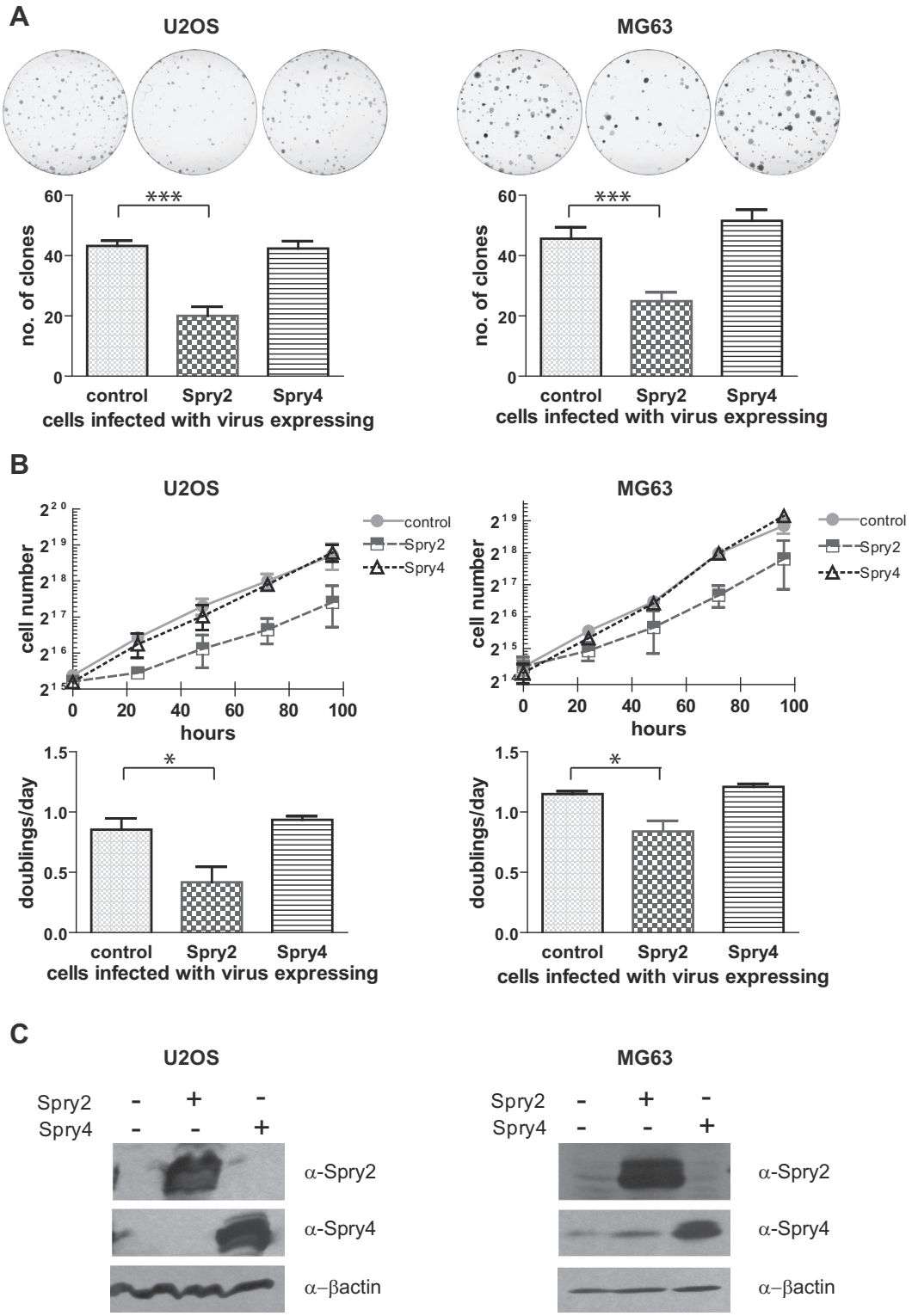

Fig. 2. Influence of Spry2 and Spry4 expression on cell proliferation of osteosarcoma-derived cells. U2OS (left panel) and MG-63 (right panel) were infected with adenoviruses expressing the indicated protein and clonogenic assays as well as growth curves were performed at least three times. (A) A representative plate of a clonogenic assay is depicted and the numbers of clones are presented as mean values ± S.D. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) A representative growth curve is depicted. The calculated doublings per day obtained in four experiments are shown as blots. (C) Ectopic expression was verified by immunoblotting with the indicated antibodies.

expressing either lacZ, Spry2 or Spry4 proteins. One day after infection serum was added for 5, 10, 15 or 30 min before protein extraction. MAPK activation was evaluated by measuring pERK levels in the cellular extracts. As depicted in Fig. 4A and B, serum addition caused an immediately induction of pERK levels in control cells as well as in Spry4 expressing cells. In U2OS cells with elevated Spry2 levels phosphorylation of ERK was slightly delayed and peaked after 10 min. Furthermore it was obvious that the levels of pERK in serum deprived cells were reduced to one half and a fourth in Spry4 and Spry2 expressing cells, respectively. Inhibition of ERK phosphorylation in serum deprived cells was significantly stronger in cells expressing Spry2 when compared to cells expressing Spry4

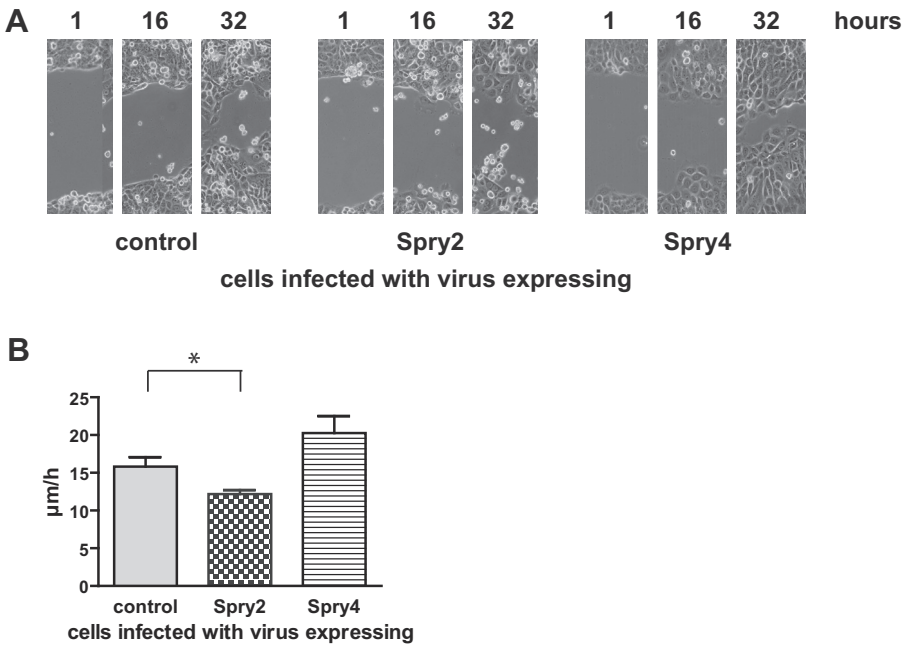

Fig. 3. Influence of Spry2 and Spry4 on migration. (A) A scratch assay of MG63 cells infected with adenoviruses expressing the indicated proteins was performed. Pictures were taken 1, 16 and 32 h after the scratch was set. (B) Migration velocities of cells with adenoviral overexpression of Spry2 or Spry4 and control-treated cells were calculated and analysed by using the *t*-test. A summary of the results from three independent experiments shows means and standard deviation of the different migration velocities. Migration velocities were calculated from linear regressions and analysed using GraphPadPrism. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

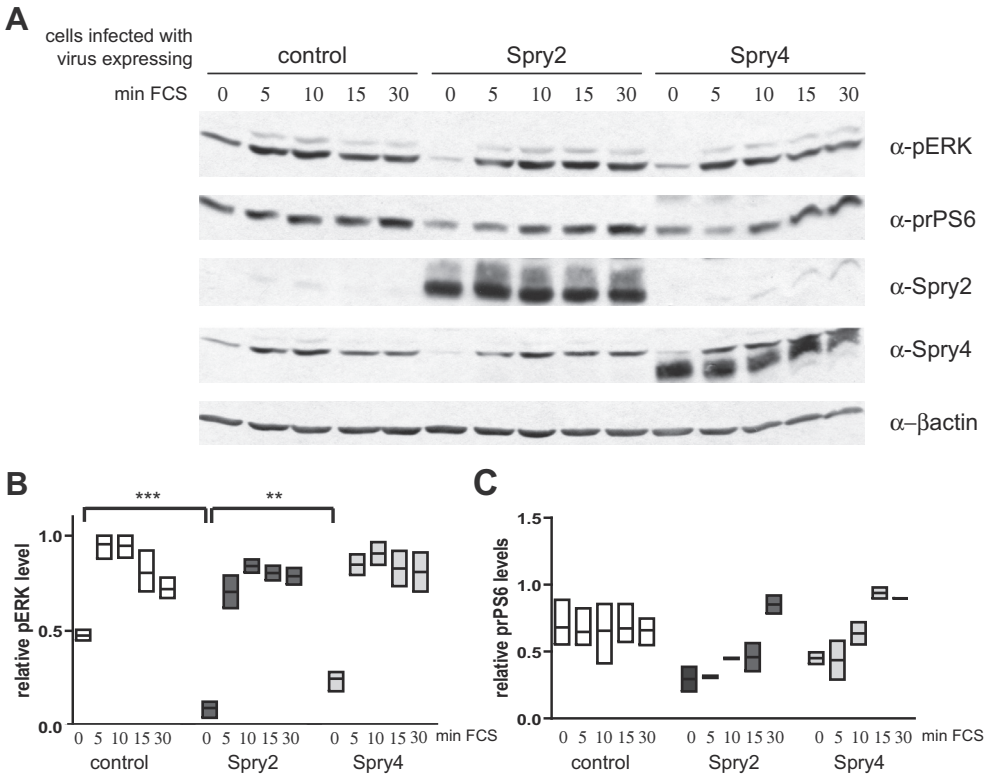

Fig. 4. Influence of Spry proteins on cell signalling. U2OS cells were serum-deprived for 24 h and then infected with adenoviruses expressing the indicated proteins. As control lacZ expressing viruses were used. A day after virus addition, serum was added to a final concentration of 20% and the cells were harvested for expression analysis at 0, 5, 10, 15 and 30 min. Immunoblots were sequentially probed with the indicated antibodies. (A) A representative immunoblot is shown. (B) Summary of calculated values for pERK from 2 to 4 experiments. The indicated expressions were calculated after densitometric analysis of the respective bands using Image Quant 5.0. All signals were normalised to the values obtained for β-actin. The highest values were set arbitrarily as one. (C) Summary of calculated values for prPS6 from two experiments. The indicated expressions were calculated after densitometric analysis of the respective bands using Image Quant 5.0. All signals were normalised to the values obtained for β-actin. The highest values were set arbitrarily as one. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(Fig. 4B). In addition to MAPK activation, cell signalling via PI3K was evaluated by measuring phosphorylation of ribosomal protein S6 (Fig. 4A and C). While in control treated U2OS cells serum induction had no influence on the levels of phosphorylated ribosomal protein S6 (prPS6), expression of both Spry2 and Spry4 reduced the levels of prPS6 and after serum addition the levels were clearly induced. In contrast to the influence of Spry proteins in case of MAPK activation, signalling via PI3K in response to serum addition was comparable in Spry2 and Spry4 expressing cells. These data indicate that the more potent inhibition of Spry2 on ERK activation could be necessary for the Spry2 specific interference with cell proliferation, although both Spry2 and Spry4 influence mitogen induced cell signalling in U2OS cells.

3.5. The NH_2-terminus of Spry2 exerts the specific inhibitory effect in osteosarcoma-derived cells

Spry proteins are characterised by a conserved cystein-rich domain at the COOH-terminus. Their NH_2-terminus is variable but contains two short homologous sequences [25]. In order to study which domains are important for the Spry2 specific inhibitory influence on RTK mediated processes in osteosarcoma, hybrids between Spry2 and Spry4 proteins were constructed. To this end the COOH-terminus harbouring the cystein rich Spry domain of Spry2 was fused to the NH_2-terminus of Spry4. The resulting protein was termed Spry4/2. In the so called Spry2/4 construct the NH_2-terminal 182 amino acids (aa) of Spry2 were combined with the COOH-terminal 136 aa of Spry4 (Fig. 5A). As depicted in Fig. 5B Spry2/4 clearly interfered with cell proliferation. Like Spry2-expressing cells, U2OS infected with this adenoviral construct almost stopped proliferation. In contrast, irrespective of the origin of the COOH-terminus, expression of Spry proteins containing the NH_2-terminus of Spry4 doubled comparably and significantly faster than Spry2 expressing U2OS cells (Fig. 5C). Both, Spry4 and Spry4/2 were prominent overexpressed when cells were incubated with the respective adenoviruses (Fig. 5E), but the affected cells showed doubling times comparable to the one calculated for control-treated cells (Fig. 5D).

These data demonstrate that the NH_2-terminus of Spry4 misses sequences necessary for Spry2 to interfere with cell proliferation of osteosarcoma-derived cells.

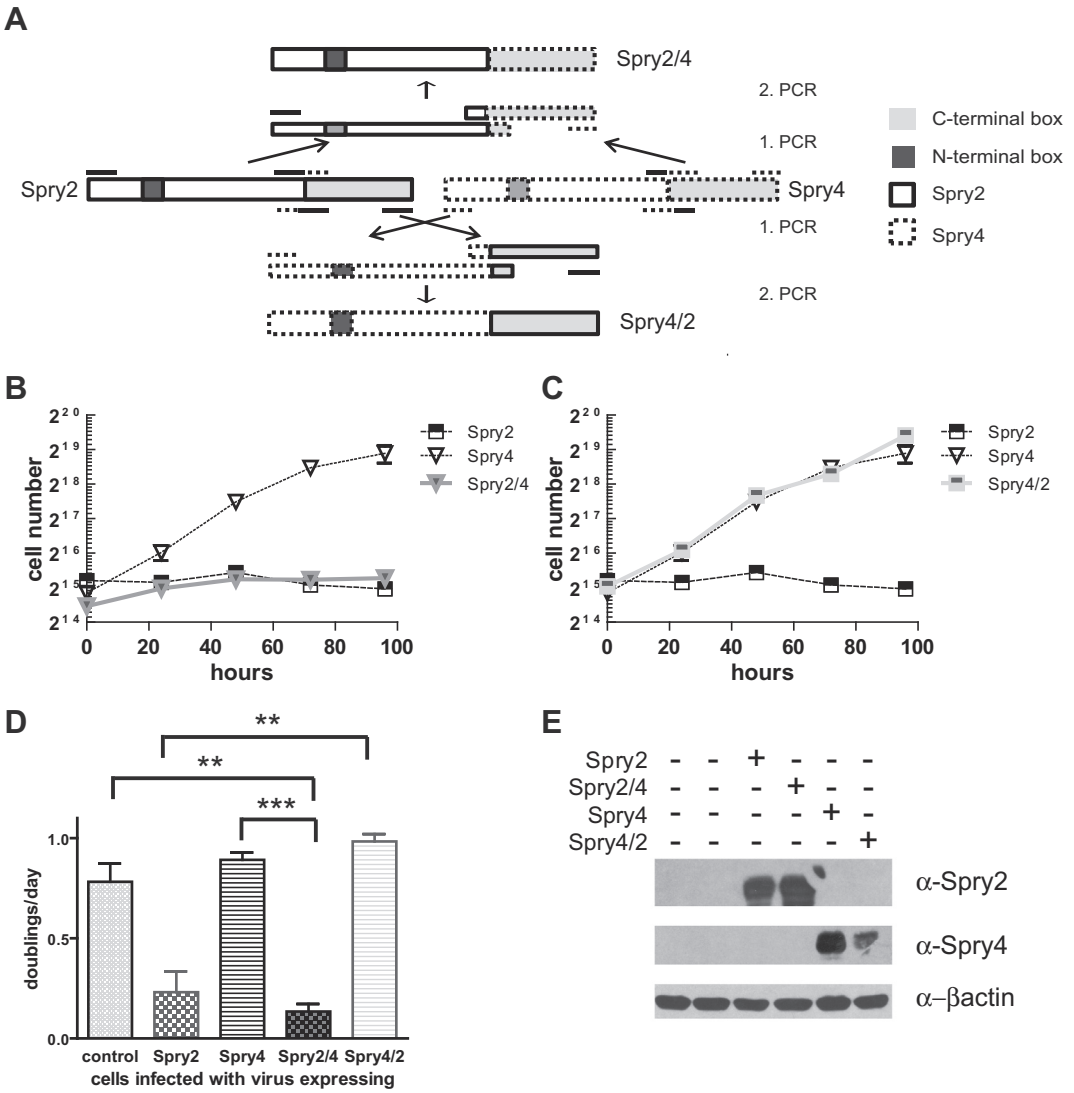

Fig. 5. Influence of Spry hybrids on cell proliferation. (A) A diagram showing the construction of the Spry2/4 and Spry4/2 hybrids. (B) A representative growth curve of U2OS cells comparing the proliferation of cells expressing a protein fusing the N-terminus of Spry2 to the C-terminus of Spry4 (Spry2/4) with the one of Spry2 and Spry4 expressing cells is shown. (C) Representative growth curves of cells infected with viruses expressing Spry2, Spry4 or a Spry 4/2 hybrid protein are depicted. (D) Doublings per day from three experiments were calculated using GraphPadPrism and significance was analysed using an unpaired *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (E) Verification of virus expression as detected by immunoblot.

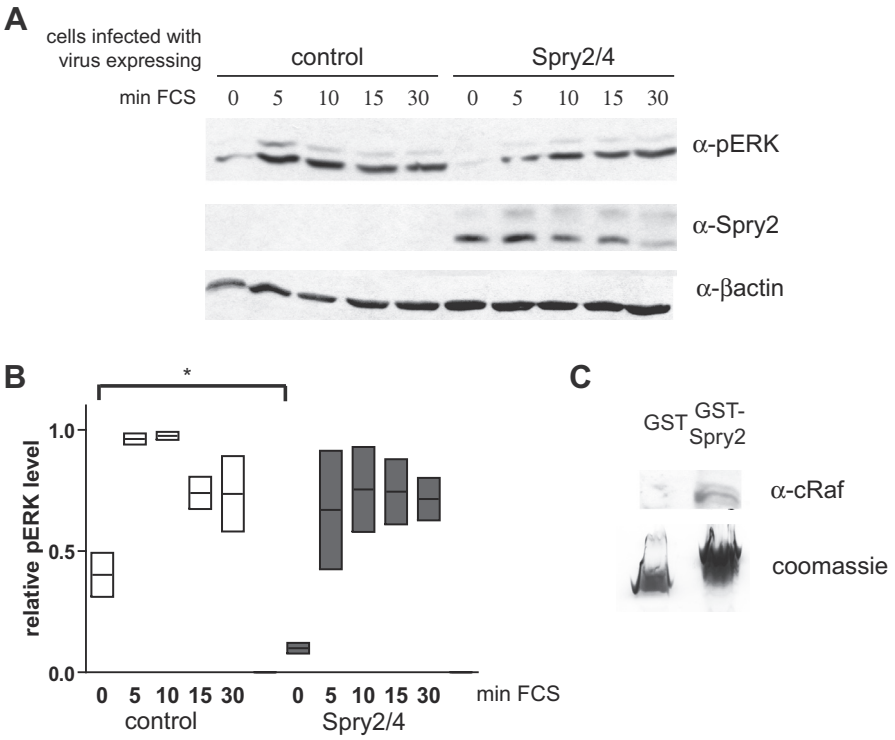

Fig. 6. Influence of Spry 2/4 on cell signalling. Serum deprived U2OS cells were infected with adenoviruses expressing lacZ (control) or Spry2/4 proteins. 24 h post infection cells were serum induced for 0, 5, 10, 15 and 30 min. Cells were harvested and analysed by immunoblotting using the indicated antibodies. (A) A representative immunoblot is shown. (B) Depiction of the calculated values for pERK from two independent experiments after densitometric analysis using Image Quant 5.0. The signals were normalised to β-actin. The highest values were set arbitrarily as one. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (C) A pull down assay with GST and GST-Spry2 was performed. Both bacterial expressed protein were purified and incubated with protein extracts from U2OS 48 h after transfection of cRaf expressing constructs. An immunoblot detecting cRaf bound to the GST-protein columns is shown. At the lower panel the Coomassie staining of the five-fold input is shown.

3.6. The NH_2-terminus of Spry2 is involved in inhibition of ERK phosphorylation

In order to investigate if the N-terminal sequences of Spry2 are required for the strong inhibition of Spry2 on MAPK activation, U2OS cells expressing Spry2/4 were serum deprived for 24 h and infected with adenovirus expressing the hybrid construct. After another 24 h serum was added to the cells. After 0, 5, 10, 15 and 30 min cells were lysed and immunoblots were performed. Like in the case of Spry2 expression (Fig. 4B), the levels of phosphorylated ERK in serum deprived U2OS cells were reduced to almost a fourth of the signal strength observed in control treated cells, when cells were expressing a Spry2/4 fusion protein (Fig. 6A and B). As consequence of serum addition ERK phosphorylation was induced, but the induction was delayed and slightly reduced when cells were expressing a Spry protein harbouring the NH_2 terminus of Spry2. To determine if the N-terminal Spry2 sequences are able to interfere with MAPK activation via the earlier described interaction with Raf, we performed a pull down experiment using a fusion protein of GST with the N-terminal 150 aa of Spry2. Bacterial expressed and GST purified N-terminus of Spry2 was able to bind detectable amounts of cRaf, when incubated with cellular extract of U2OS transfected with cRaf, while in parallel GST incubated with the second half of the cell extract failed to pull down Raf.

These data indicate that the NH_2-terminus of Spry2 is involved in inhibition of MAPK pathway.

4. Discussion

The presented data demonstrate that Spry2 expression in osteosarcoma-derived cells interferes potently with cell proliferation and migration. This indicates that Spry2 seems to function as an inhibitor of the malignant phenotype in osteosarcoma. Although most of the reports investigating the role of Spry proteins in malignant tissue show a tumour antagonistic function of the different Spry protein members [17,19,20,26–29], the few known studies investigating Spry proteins in sarcoma show that Spry proteins fulfil a tumour supporting function. In H-Ras^{V12}-transformed human fibroblasts as well as in fibrosarcoma Lito and colleagues observed that elevated Spry2 levels are required for colony formation in agar and tumour formation in mice [21]. In line with these observations a study performed in rhabdomyosarcoma demonstrated that in the embryonal rhabdomyosarcoma subtype Spry1 is beneficial for cancer cell proliferation and survival [22]. However, the tumour-supporting functions of Spry1 was only observed in cells harbouring oncogenic Ras, which can be detected in about 40% of the rhabdomyosarcom, but in osteosarcoma Ras is rarely mutated [30]. To our knowledge the present study is the first to provide data suggesting that a Spry protein member may have a protective role in sarcomas. Spry2 protein is a good candidate to act as tumour suppressor in osteosarcoma. Corroborating with our data it is known that the q arm of chromosome 13 containing the SPRY2 gene frequently shows gene copy number losses in osteosarcoma [31,32]. Additionally it is shown that Spry2 expression interferes with ERK activation after FGF stimulation of osteoblasts [33].

In contrast to Spry2, Spry4 fails to negatively influence cell proliferation and migration in osteosarcoma. Also in other tumour entities it was observed that specific Spry protein family members can antagonise malignant phenotypes, while others fail to do so. In prostate cancer for example Spry1 is able to interfere with cell proliferation and migration [17] while Spry4 only diminishes migration velocity [29]. Additionally Spry4 did not affect tumour outgrowth or malignancy in pancreatic cells [34]. Although Spry4

can interfere with cell proliferation and migration of cancer cells as it is demonstrated for NSCLC-derived cells [28], our data indicate that in osteosarcoma the cellular context is not appropriate for Spry4 to interfere with these biological processes. An experimentally validated explanation for the observed difference between Spry2 and Spry4 is still missing, but our data illustrate that differences in the N-terminus are responsible for the specific antiproliferative function of Spry2 in osteosarcoma. This part of the protein is the more variable part of the Spry family, therefore these data are not surprising [35]. Few proteins were shown to bind to the N-terminus of Spry2. Most of them, including Cbl [36], SIAH2 [37], Mnk-1 [38] or Nedd4 [39] are involved in regulating Spry2 stability in order to ensure its properly timed appearance and disappearance during cell cycle phases [40]. Additionally an intact N-terminus is necessary for the direct interaction of Spry1 and Spry2 with PLCγ and the inhibition of Ca^{2+} signalling via the PLCγ pathway [41]. The interaction between Spry4 and PLCγ was not explicitly explored, but it is shown that unlike Spry1 and Spry2, Spry4 expression is not associated with decreased PLCγ phosphorylation [42]. Additionally, in the case of Spry2 protein it is reported that Raf1 binding [33] as well as GRB2 binding [43] requires intact structures at the N-terminus. Spry4 can interact with Raf1 via the C-terminus, but this interaction is mainly involved in regulation of Ras-independent MAPK activation [44]. Concerning inhibition of ERK activation via Ras-dependent pathways Spry2 is shown to be much more efficient than Spry4 [45]. In accordance with these data we observed that although both Spry2 and Spry4 protein expression were able to modulate ERK activation in osteosarcoma-derived cells, Spry2 was more potent in inhibition of MAPK pathway. Phosphorylated ERK levels were significantly stronger reduced in serum deprived cells and activation of ERK was timely delayed. Therefore inhibition of MAPK activation via Spry2 unique elements in the NH_2-terminus can account for the observed Spry2 specific inhibition of cell proliferation. A hybrid expressing the NH_2-terminus of Spry2 fused to the COOH-terminus of Spry4 reduces the levels of phosphorylated ERK to comparable extents as observed in case of Spry2 expression. Accordingly the NH_2-terminus of Spry2 is able to bind Raf protein when incubated with osteosarcoma-derived protein extracts.

To elucidate if additionally other mechanisms are responsible for the Spry2-mediated inhibition of cell proliferation and migration in osteosarcoma further investigations are needed.

In summary our results propose Spry2 as tumour suppressor in osteosarcoma-derived cells.

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