SRC Signaling Is Crucial in the Growth of Synovial Sarcoma Cells

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Abstract

Synovial sarcoma is a soft-tissue malignancy characterized by a reciprocal t(X;18) translocation encoding a chimeric transcriptional modifier. Several receptor tyrosine kinases have been found activated in synovial sarcoma; however, no convincing therapeutic concept has emerged from these findings. On the basis of the results of phosphokinase screening arrays, we here investigate the functional and therapeutic relevance of the SRC kinase in synovial sarcoma. Immunohistochemistry of phosphorylated SRC and its regulators CSK and PTP1B (PTPN1) was conducted in 30 synovial sarcomas. Functional aspects of SRC, including dependence of SRC activation on the SS18/SSX fusion proteins, were analyzed in vitro. Eventually, synovial sarcoma xenografts were treated with the SRC inhibitor dasatinib in vivo. Activated phospho (p)-(Tyr416)-SRC was detected in the majority of tumors; dysregulation of CSK or PTP1B was excluded as the reason for the activation of the kinase. Expression of the SS18/SSX fusion proteins in T-REx-293 cells was associated with increased p-(Tyr416)-SRC levels, linked with an induction of the insulin-like growth factor pathway. Treatment of synovial sarcoma cells with dasatinib led to apoptosis and inhibition of cellular proliferation, associated with reduced phosphorylation of FAK (PTK2), STAT3, IGF-IR, and AKT. Concurrent exposure of cells to dasatinib and chemotherapeutic agents resulted in additive effects. Cellular migration and invasion were dependent on signals transmitted by SRC involving regulation of the Rho GTPases Rac and RhoA. Treatment of nude mice with SYO-1 xenografts with dasatinib significantly inhibited tumor growth in vivo. In summary, SRC is of crucial biologic importance and represents a promising therapeutic target in synovial sarcoma. Cancer Res; 73(8); 2518–28. ©2013 AACR.

Introduction

Synovial sarcomas account for 5% to 10% of all soft-tissue sarcomas. In the majority, synovial sarcoma arise mainly in adolescents and young adults with predominance in male gender. They are molecularly characterized by a reciprocal t(X;18) translocation, which juxtaposes the SS18 gene on chromosome 18 to either the SSX1, the SSX2, or rarely to the SSX4 gene on the X chromosome. The SS18/SSX chimeric proteins act as transcriptional coactivators, leading to deregulation of oncogenic pathways (1–3).

Current treatment protocols for synovial sarcoma are based on radical surgery and standardized chemo- and radiotherapy; however, prognosis is still poor in advanced disease (4). Targeted therapeutic approaches, which have significantly improved the clinical course of patients with gastrointestinal stromal tumors (GIST) or dermatofibrosarcoma protuberosa, are still lacking for synovial sarcomas (5, 6). Several receptor tyrosine kinases have been shown to be expressed in synovial sarcomas, including the EGF receptor (EGFR; ref. 7) and the insulin-like growth factor-I receptor (IGF-IR; ref. 8), leading to activation of the PI3K/AKT signaling pathway (9). Non-receptor tyrosine kinases, including members of the SRC family kinases (SFK), are important in tumor cell growth, survival, and motility in different tumor entities, including mesenchymal tumors as osteosarcoma and Ewing sarcoma (10).

The 60-kDa human c-SRC (SRC) tyrosine kinase contains 2 phosphorylation sites regulating its enzymatic activity. Phosphorylation at Tyr527 leads to a reduced activity, whereas
autophosphorylation at Tyr416 is associated with full kinase activity (11, 12). SRC phosphorylation status is modulated by the c-SRC tyrosine kinase (CSK) and the protein tyrosine phosphatase PTP1B, which modify SRC phosphorylation at Tyr527 (12, 13). CSK has been reported to be critical for SRC deregulation in colon cancer cells (14). Among the PTPs, PTP1B has been shown to be of particular importance, being overexpressed in breast cancer cell lines with elevated SRC activity (15–17). Different receptor tyrosine kinases including the IGF-IR and effectors of the PIK3/AKT, RAS/SRC activity (15 overexpressed in breast cancer cell lines with elevated PTP1B has been shown to be of particular importance, being deregulation in colon cancer cells (14). Among the PTPs, PTP1B, CSK, p-(Ser10)-histone H3 and cleaved caspase-3 (Asp175) antibodies from Cell Signaling Technologies. Tissue specimens (including xenografts) were cultured in medium supplemented with 1% FBS, 2 mmol/L L-glutamine, and 5 μg/mL blasticidin. SS18/SSX1, SS18/SSX2, SS18, SSX1, and SSX2 were amplified by PCR using templates expression vectors described before (27, 28). The cDNAs were then cloned into the tetracycline-regulated pTREx DEST30 Gateway expression vector (Invitrogen). Using Lipofectamine 2000 reagent, T-REx-293 cells were transfected with expression vectors for SS18/SSX1, SS18/SSX2, SS18, SSX1, and SSX2, respectively, and the pT-REX/GW-30/ LacZ vector (Invitrogen) expressing β-galactosidase was included as control. Forty-eight hours after transfection, T-REx-293 cells were selected in culture medium supplemented with 1 mg/mL geneticin (G418), and drug-resistant colonies were isolated after 6 weeks of selection. To induce gene expression, 1 μg/mL tetracycline (Sigma-Aldrich) was added to the T-REx-293 cell lines for 24 hours.

**Culture and treatment of human synovial sarcoma cells and MTT cell proliferation assays**

Cell lines were grown in monolayer cultures and maintained at 37°C in a humidified 5% CO₂ atmosphere as described (20). Because of their low proliferative rate, HS-SY-II synovial sarcoma cells were not suitable for further functional assays. For proliferation assays, cells were cultured in medium supplemented with 2% FBS in 96-well dishes at least in triplicate. Cell density was 5 × 10³ per well. Cells were exposed to increasing concentrations of dasatinib (0.01–10 μmol/L, Santa Cruz Biotech), and appropriate controls were included. For combination treatments with chemotherapeutic drugs, cell lines were incubated with increasing concentrations of doxorubicin, vincristine, and actinomycin D (0.1–100 ng/mL), alone or in combination with dasatinib in a concentration resulting in growth inhibition of 20% to 30%. Synergy was evaluated by the fractional product method (29). A difference of more than 10% between the observed and the predicted effect was considered to signify synergistic activity between dasatinib and the chemotherapeutic drug, a difference of less than 10% was defined as additive. All assays were conducted for 72 hours. MTT proliferation assays (Roche) were conducted according to the manufacturer’s instructions. The formazan dye was quantified using a scanning multwell spectrometer (BGM Labtech).
Knockdown of SRC and IGF-IR by RNA interference

1273/99 and CME-1 cells were cultured in 25-cm² flasks in medium supplemented with 10% FBS as described above. At a cell density of 50%, cells were transfected with 60 pmol Stealth RNAi (SRC: HSS186080, HSS186081, HSS186082; IGF-IR: HSS105253, HSS105254, HSS179797; Invitrogen) or nontargeting control siRNA (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. After 24 hours, cells were trypsinized, reseeded, and MTT assays were conducted as described above (2% FBS). To document SRC knockdown, 5 × 10⁵ siRNA-transfected cells were plated in 12-well dishes in medium supplemented with 2% FBS and cultured for 72 hours.

Western blot analysis

A total of 2 × 10⁴ to 4 × 10⁵ cells were seeded in 6-well dishes (Greiner) in medium supplemented with 2% FBS for 48 hours before treatment with increasing doses of dasatinib for 60 minutes. To document SRC activation via the IGF-IR pathway, 1273/99, FUJ1, and CME-1 cells were starved for 4 hours and treated with 200 ng/mL recombinant IGF-II (R&D Systems). Cell lysis and Western blots were conducted as described before (20). Following primary antibodies were used according to the manufacturer's instructions: β-actin (Sigma-Aldrich), c-SRC (Santa Cruz Biotech), p-(Tyr114)-SRC, p-(Tyr527)-SRC, STAT3, p-(Tyr705)-STAT3, FAK, p-(Tyr576/577)-FAK, p44/42 MAPK, p-(Thr202/Tyr204)-p44/42 MAPK, AKT, p-(Ser473)-AKT, IGF-IR, p-(Tyr1131/Tyr1146)-IGF-IR (all Cell Signaling Technologies), CSK, and PTP1B (Abcam). Secondary antibody labeling as well as filter development were conducted using the ECL kit (Amersham) as described before (26).

Flow cytometry

About 8 × 10⁵ cells were grown in 75-cm² cell culture flasks in medium supplemented with 2% FBS. After a preincubation of 18 hours, they were treated with 0.1 or 0.6 μmol/L dasatinib for 48 hours. For flow cytometric immunophenotyping, 1 × 10⁶ cells were fixed on ice in ice-cold 2% paraformaldehyde for 10 minutes. They were then washed in PBS, collected by centrifugation, resuspended and incubated in ice-cold PBS with 0.25% Triton X-100 for 5 minutes on ice. After another washing step, cells were resuspended in 100 μL PBS/0.5% bovine serum albumin (BSA) containing an Alexa Fluor 647–labeled phospho-(Ser10)-histone H3 antibody (Cell Signaling Technology: 1:20) and a phycoerythrin-labeled cleaved PARP (Asp214) antibody (BD Biosciences: 1:5) and incubated for 30 minutes at room temperature. After an additional washing step, 500 μL PBS containing 10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was added to stain DNA, and cells were incubated for an additional 30 minutes at room temperature. Analysis was conducted using a three-laser LSRII analytical flow cytometer (BD Biosciences). Each experiment was carried out at least in duplicate. At least 30,000 events were recorded per experiment. Only single cells were included in the analysis. Data were analyzed using FlowJo (Tree Star) analysis software. To document SRC specificity of the effects of dasatinib, the experiment was additionally carried out in CME-1 cells 48 hours after SRC siRNA transfection, which was done as described above.

Analysis of apoptosis by DAPI staining

Cells were cultured as described above and treated with 0.03 or 0.3 μmol/L dasatinib. They were harvested and washed in PBS, fixed in 3.7% paraformaldehyde for 10 minutes at room temperature, and washed again. After incubation with 1 μg/mL DAPI (Sigma-Aldrich) for 10 minutes and 2 further washing steps, cells were mounted on appropriate slides using Fluoromount-G medium (Southern Biotechnologies Associates). Nuclei were visualized and photographed using a Leica DMLB fluorescence microscope. Apoptotic cells were morphologically defined by chromatin condensation and fragmentation. For each assay, at least 300 cells were analyzed in triplicate.

Wound scratch, migration, and invasion assays

Cellular motility, migration, and invasion were analyzed by wound scratch, Boyden chamber, and invasion chamber assays. To exclude influences of growth effects on the results of migration and invasion assays, dasatinib was used in concentrations of 1 or 3 nmol/L, that is concentrations, which were shown to exert no significant effects on cell viability in MTT assays. CME-1 and SYO-1 cells were grown to confluence in 6-well dishes under serum-reduced conditions as described above. Cell monolayers were wounded by scratching with a sterile 100-μL pipette tip, medium was exchanged and the cells were treated with dasatinib or dimethyl sulfoxide (DMSO) as control. Photographs were taken at time points 0, 12, and 24 hours, using an AxioCam digital camera and the AxiosVision software (Zeiss). Boyden chamber motility assays were conducted with CME-1, SYO-1, and 1273/99 cells. A total of 2 × 10⁵ to 7 × 10⁵ tumor cells in 150 μL medium containing 2% FBS were added into the upper Transwell chambers (6.5-mm diameter, 8.0-μm pore size, Corning Costar Corporation) and treated with dasatinib or DMSO as control for 24 hours. FBS concentration in the lower chamber (500 μL) was 10%. Invasion chambers with Matrigel coating (6.4-mm diameter, pore size 8 μm; BD) were used to assess the effect of dasatinib on invasion in CME-1 and SYO-1 cells. Essentially, cells were seeded and treated as for migration assays. Dishes were incubated in a 37°C incubator (5% CO₂) for 24 hours. After removal of the nonmigrating cells from the upper chamber with a cotton swab, the membranes were fixed with 4% paraformaldehyde for 15 minutes, stained with Harris hematoxylin for 15 to 20 minutes, washed, removed from the plastic holders, and mounted on glass slides with Aquatex (Merck). Migrated cells were counted in five ×10 fields (× 100 magnification). All assays were conducted at least in triplicate. To document SRC specificity of the effects of dasatinib, Boyden chamber and invasion chamber assays were additionally conducted in CME-1 cells 48 hours after SRC siRNA transfection, which was done as described above.

RhoA and Rac activation assay

CME-1 and SYO-1 cells were cultured as described above to yield a density of 30% to 60% after 72 hours. Cells were

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then treated with 0.1 μmol/L of dasatinib for 20 minutes. DMSO was used as control. Lysates were prepared and immediately snap-frozen in liquid nitrogen. Protein lysates with equalized concentrations were analyzed. The assay was conducted in duplicate according to the manufacturer’s instructions (Cytoskeleton).

**In vivo experiments in SYO-1 xenografts**

All mouse procedures were conducted in accordance with the National and European Union guidelines and permission was obtained from the local authorities. A total of $5 \times 10^6$ SYO-1 synovial sarcoma cells were injected subcutaneously into the right flank of 5-week-old BALB/c nude mice (Charles River Laboratories). Tumor growth was assessed daily by measuring the tumor volume calculated as length $\times$ width $\times$ height $\pi/6$. Treatment was started when the tumor volume reached 100 mm$^3$. Dasatinib was injected intraperitoneally (i.p.) in a daily dosage of 10 mg/kg (days 1–7). As no side effects were observed during the first week of treatment, the dosage was escalated to 20 mg/kg (days 8–14). After 15 days of treatment, animals were sacrificed, tumors were explanted, and the tumor tissue was formalin-fixed and embedded in paraffin.

**Results**

**Phosphokinase arrays**

Phosphokinase array analysis of 1273/99 and HS-SY-II synovial sarcoma cells detected phospho-(Tyr416)-SRC as the most strongly phosphorylated protein kinase of 46 represented targets. Independent Western blotting confirmed this finding and detected p-(Tyr416)-SRC levels of different intensity in CME-1, SYO-1, FUJI, 1273/99, and HS-SY-II synovial sarcoma cells (Fig. 1; Supplementary Table S1). Because of their low proliferative rate, HS-SY-II synovial sarcoma cells were not suitable for further functional assays.

**Synovial sarcomas display elevated levels of Tyr416-phosphorylated SRC**

In a set of 30 synovial sarcomas, immunohistochemical stainings revealed strong expression levels of Tyr416-phosphorylated SRC in 13% of the samples, 60% showed moderate, and 27% weak expression levels. In contrast, strong Tyr527-phosphorylated SRC was detectable in only 7% of the samples, whereas moderate or weak expression was found in 10% and 30% of the tumors, respectively; in 53% of the samples, no
expression of Tyr527-phosphorylated SRC was detectable. Expression levels for CSK were strong in 30%, moderate in 17%, and weak in 50% of the samples, no CSK expression was found in one synovial sarcoma. Expression levels for PTP1B were strong in 13%, moderate in 37%, and weak in 40% of the samples, no expression of PTP1B was found in 10% of the samples. No significant correlation between SRC phosphorylation status and CSK or PTP1B protein expression levels was detected. Tyr416-phosphorylated SRC expression in the tumor samples did not correlate with the patients’ age, sex, tumor location, and tumor size or tumor grade. As in the immunohistochemical analysis, no correlation between SRC activity and PTP1B or CSK levels was observed in the cell lines analyzed in Western blots (Fig. 1 and data not shown).

**SRC activation in synovial sarcoma is induced by SS18/SSX translocation**

To functionally understand the mechanism of SRC activation in synovial sarcoma, T-REx-293 cells were stably transfected with vectors containing SS18/SSX1, SS18/SSX2, SSX1, SSX2, or SS18 cDNA to obtain an inducible cell culture model of the synovial sarcoma–specific chimeric translocation proteins. Western blot analysis showed elevated levels of activated p-(Tyr416)-SRC in T-REx-293 cells transfected with SS18/SSX1 and SS18/SSX2 (Fig. 2A); expression levels of PTP1B and CSK were not affected (data not shown). As it has been shown previously that receptor tyrosine kinase pathways including IGF-IR signaling are of particular importance in synovial sarcomas, we analyzed promoter-specific expression levels of IGF2 showing upregulation of promoter P2- and P4-dependent IGF2 transcripts in SS18/SSX1 and SS18/SSX2-expressing T-REx-293 cells (Fig. 2B). Stimulation of 1273/99, FUJI, and CME-1 synovial sarcoma cells with recombinant human IGF-II protein was associated with an increase of phosphorylation of IGF-IR at Tyr1131, AKT at Ser473, and SRC at Tyr416, which revealed IGF-IR signaling as a functionally relevant mechanism leading to SRC activation (Fig. 2C). A minor induction of phosphorylation of SRC at Tyr416 was observed upon SS18 overexpression alone as well; however, this activation was not associated with IGF2 transcriptional induction. Inversely, siRNA knockdown of the IGF-IR in CME-1 cells was associated with a significant decrease of p-(Tyr416)-SRC levels (Supplementary Fig. S2).

**SRC inhibition by dasatinib or RNA interference impairs growth of synovial sarcoma cells**

siRNA-mediated knockdown of SRC resulted in a significant decrease of growth of CME-1 and 1273/99 cells in MTT assays (t test: P < 0.001; Fig. 3A and data not shown). All analyzed synovial sarcoma cell lines displayed dose-dependent growth inhibition upon treatment with the SRC inhibitor dasatinib (Fig. 3B). This effect was particularly distinct in nanomolar concentrations of the inhibitor. Among the 4 synovial sarcoma cell lines investigated CME-1 (GI50 = 0.008 μmol/L), FUJI (GI50 = 0.01 μmol/L), and SYO-1 (GI50 = 0.013 μmol/L) were found to be slightly more sensitive to dasatinib than 1273/99 cells (GI50 = 0.077 μmol/L).

**Inhibition of SRC affects phosphorylation of its interaction partners**

To assess the effect of SRC inhibition on its interaction partners in synovial sarcomas, cells were treated with increasing concentrations of dasatinib (0.01–3 μmol/L) for 60 minutes (Fig. 3C). Dose-dependent dephosphorylation of p-(Tyr416)-SRC, p-(Ser473)-AKT, p-(Tyr576/577)-FAK, p-(Tyr705)-STAT3, and p-(Tyr1131)-IGF-IR was observed in all synovial sarcoma cell lines with nanomolar concentrations of dasatinib. Similarly, siRNA knockdown of SRC led to the dephosphorylation of
Dasatinib treatment increases apoptosis and decreases mitotic rate in synovial sarcoma cells

To determine the effect of dasatinib on the apoptotic and mitotic rate of synovial sarcoma cells, flow cytometric analyses were conducted. Cleaved PARP (Asp214) was used as a marker of apoptosis, and phospho-(Ser10)-histone H3 was used as a marker of mitosis. CME-1, 1273/99, and SYO-1 cell lines showed significantly increased rates of apoptosis and decreased mitotic fractions after treatment with dasatinib in concentrations of 0.1 and 0.6 μmol/L (Fig. 4A, Supplementary Table S2). These results were confirmed by microscopic analyses of DAPI-stained synovial sarcoma cells treated with dasatinib, showing increasing rates of chromatin condensation and fragmentation in a dose-dependent manner in CME-1 and SYO-1 cells (Fig. 4B). As an indicator of SRC specificity of the effects observed, dasatinib treatment of CME-1 cells after SRC knockdown did not show significant effects in terms of proliferation and apoptosis in flow cytometry (Supplementary Fig. S1).

Combination of SRC inhibition and conventional chemotherapy results in additive effects on cell growth

To determine the effects of combinations of conventional chemotherapy (vincristine, doxorubicin, actinomycin D) and dasatinib on the growth of synovial sarcoma cells, we investigated 1273/99 cells, which were the least responsive to monotherapies with dasatinib. Cells were exposed to increasing concentrations of conventional cytotoxic drugs and to a concentration of dasatinib that led to 20% to 30% growth inhibition after 72 hours. The combinations did not fulfill the...
criteria of synergy as defined above; the effects observed resulted from an additive, obviously independent action of the SRC inhibitor and conventional chemotherapeutic agents (Fig. 4C).

**Dasatinib inhibits motility and invasive potential of synovial sarcoma cells associated with an increased activity of RhoA and diminished Rac activity**

As SRC has been shown to modulate motility and invasiveness of tumor cells, we investigated the effect of SRC inhibition by dasatinib on cell migration and invasion. CME-1, SYO-1, and 1273/99 synovial sarcoma cells (treated with dasatinib in doses not affecting cell viability) showed a dose-dependent decrease of migratory and invasive potential in Boyden chamber and invasion chamber assays. Accordingly, wound healing was impaired in scratch assays in CME-1 and SYO-1 treated with dasatinib. In ELISA-based RhoA and Rac activation assays, treatment with dasatinib resulted in significantly increased levels of activated RhoA and decreased levels of activated Rac in SYO-1 and CME-1 cells (Fig. 5A–D, data not shown). As an indicator of SRC specificity of the effects observed, dasatinib treatment of CME-1 cells after SRC knockdown did not show significant effects in terms of migration and invasion in Boyden chamber and invasion chamber assays (Supplementary Fig. S1).

**Dasatinib displays antitumor activity in synovial sarcoma xenografts in vivo**

The antitumor activity of dasatinib was tested *in vivo* in a xenograft model of SYO-1 synovial sarcoma cells. The inhibitor significantly reduced tumor growth rate (Fig. 6A). No significant changes in the weight of the tumor-bearing mice were observed (data not shown). Consistent with the *in vitro* results, treatment was associated with diminished levels of Tyr416-phosphorylated SRC, a significant reduction of the mitotic fraction (*t* test: *P* < 0.001) and a significant increase of the...
apoptotic fraction (t test: $P < 0.001$) compared with control tumors (Fig. 6B and C).

**Discussion**

Considerable progress has been made in the understanding of soft-tissue tumors in the recent years. However, apart from few examples such as c-KIT or platelet-derived growth factor (PDGF) receptor inhibition in GIST and dermatofibrosarcoma protuberans (5, 6), the translation of molecular results into clinical care in terms of molecularly based therapies is still rare in this group of neoplasias. Despite elaborate treatment protocols involving radical surgery and standardized chemo- and radiotherapy, prognosis is poor in advanced cases of synovial sarcoma. Therefore, the identification of molecular targets, which are at the same time biologically essential and accessible to specific therapeutic drugs, represents an important issue for the development of innovative therapeutic approaches.

On the basis of a phosphokinase screen, we identified the SRC tyrosine kinase as one of the most strongly phosphorylated kinases in synovial sarcoma cells. Its particular relevance was confirmed immunohistochemically in biopsies of 30 synovial sarcomas, in which Tyr416-phosphorylated, that is activated,
SRC was found to be expressed in the majority of the cases. A consistent pattern of dysregulation of the SRC-regulating proteins CSK and PTP1B, analogous to what has been shown in some epithelial tumors could be excluded in synovial sarcomas (15–17, 30). Interestingly, SRC was found to be activated through the SS18/SSX translocation proteins. This activation was associated with an IGF-IR–dependent mechanism based on transcriptional induction of IGF2, which links SRC activation to the characteristic molecular aberration of synovial sarcomas. As shown, expression of the transcriptional cofactor SS18 alone is capable to (indirectly) induce SRC phosphorylation at lower levels as well; however, this appears to be independent from IGF2 induction. This finding underlines the oncogenic character of the SS18/SSX fusion proteins and distinguishes components of the IGF/SRC context from other therapeutic targets as molecularly based and tumor-specific. However, the finding of consistent expression of further growth factor receptors, such as PDGFR and EGFR, in synovial sarcomas makes it probable that other than IGF-IR–dependent pathways may mediate SRC activation in synovial sarcomas as well (31). Considering the IGF-IR and the SRC kinases as potential therapeutic targets, its central position within different oncogenic signaling pathways makes SRC an attractive candidate for specifically directed approaches. As shown here, synovial sarcomas display a fundamental dependence on SRC signals with regard to cellular proliferation and survival. This was observed in vitro in siRNA-mediated approaches and after pharmacologic intervention with the SRC inhibitor dasatinib as well as in vivo in murine synovial sarcoma xenografts. A role for dasatinib is clinically well-established in the treatment of chronic myelogenous leukemia and Philadelphia chromosome–positive acute lymphoblastic leukemia (ALL), in which the substance inhibits Abl kinases (32, 33). Effectivity of dasatinib has previously been shown for cells derived from solid tumors as well, including mesenchymal neoplasias, such as GIST and chondrosarcoma (34, 35). In chondrosarcoma, growth effects observed upon treatment with dasatinib were not consistently associated with diminished p-(Tyr416)-SRC levels, which makes SRC-independent modes of action probable (35). In contrast, in synovial sarcomas, dephosphorylation of SRC and its targets was a consistent feature detectable upon treatment with dasatinib. The low drug dosages resulting in dephosphorylation of the SRC targets argue in favor of SRC-dependent effects and against effects exerted through direct interaction of dasatinib with IGF-IR, FAK, and AKT (36). As an indirect proof of specific SRC-related

Figure 6. A, significantly reduced tumor growth in vivo in dasatinib-treated SYO-1 xenografts associated with diminished levels (B, C) of Tyr416-phosphorylated SRC, a reduction of the p-(Ser10)-histone H3–positive mitotic cell fraction, and an induction of the cleaved caspase-3 (Asp175)–positive apoptotic cell fraction. *** P < 0.001, Student t test.
action of dasatinib in synovial sarcoma cells. CME-1 did not display any significant effects upon dasatinib treatment after siRNA-mediated SRC knockdown. Interestingly, SRC inhibition was associated with a loss of phosphorylation of the IGF-IR at Tyr1131, which indicates activation of IGF-IR tyrosine kinase activity usually detectable upon ligand binding. This finding is particularly relevant for the option of IGF-IR directed therapeutic approaches, which have been proposed for synovial sarcomas recently (20, 37). Beyond that, because of the central position of SRC within intracellular signaling networks and its obvious capacity of cross-activating pathways as documented here, it is conceivable that targeting SRC as a central component integrating different signaling activities might be advantageous compared with individual receptor-directed approaches. As shown here, combined treatment of synovial sarcoma cells with chemotherapeutic drugs and dasatinib results in additive but not in synergistic effects. Therefore, SRC inhibitors might be useful in innovative therapeutic approaches, in which targeting of an activated pathway with specific inhibitory substances allows the reduction of the individual compounds’ dosages, thereby minimizing toxicity. In our in vivo experiments, dasatinib was found to be highly effective with regard to tumor growth and was well tolerated by the animals. As known for a variety of epithelial tumors (36, 38), on the basis of our data, the SRC signaling network appears to be of crucial relevance for cellular migration and invasion in synovial sarcomas. In all assays applied here, doses of dasatinib, which did not affect cellular proliferation, resulted in significantly impaired migratory and invasive capacities. These effects were associated with an SRC-dependent shift in activation levels of Rac and RhoA, small GTPases essentially involved in the regulation of cell mobility processes. This finding provides a functional background of the effects observed here and substantiates specificity, as increased levels of activated RhoA are associated with stress fiber formation, whereas diminished levels of activated Rac go along with the impairment of a "motile" phenotype (18). This finding is of particular importance with regard to therapeutic concepts, as prognostically unfavorable cases of synovial sarcomas frequently develop metastases. Using a dual-inhibition approach of SRC and Aurora kinases by SU6656, Ariai and colleagues recently observed high antitumor effectiveness in synovial sarcoma xenografts involving antiangiogenic mechanisms. This finding provides further evidence of the crucial role of SRC with regard to complex aspects of tumor biology and underlines its role in a oncogenic signaling network (39). In summary, our data in detail substantiate previous findings on the relevance of SRC in synovial sarcomas (40). For the first time, it is systematically shown that the SRC signaling network is commonly activated in synovial sarcomas and that targeting SRC results in substantial effects on tumor cell growth and motility. These findings argue in favor of SRC as a potential therapeutic target in synovial sarcomas.

Disclosure of Potential Conflicts of Interest
E. Wardelmann has honoraria from speakers’ bureau from Novartis Oncology, MSD, and Eisai and is a consultant/advisory board member for Novartis Oncology and MSD. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
The authors thank Alexandra Florin for excellent technical support.

Grant Support
This study was supported by the Deutsche Krebshilfe (KoStar-Sarcoma Net), The Wilhelm Sander-Stiftung, the BONFOR program of the Medical Faculty, University of Bonn, the Fortune program of the Medical Faculty, University of Cologne, and grant HBFG-119-517 to the Flow Cytometry Core Facility at the Institute of Molecular Medicine, University of Bonn.

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Received August 3, 2012; revised December 28, 2012; accepted January 16, 2013; published OnlineFirst April 10, 2013.

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