ORIGINAL ARTICLE

SOCS1 gene therapy has antitumor effects in imatinib-resistant gastrointestinal stromal tumor cells through FAK/PI3 K signaling

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Abstract
Background Most of the gastrointestinal stromal tumors (GIST) have mutations in the *KIT* gene, encoding a receptor tyrosine kinase. Imatinib, a receptor tyrosine kinase inhibitor, is the first-line therapy for unresectable and metastatic GISTs. Despite the revolutionary effects of imatinib, some patients are primarily resistant to imatinib and many become resistant because of acquisition of secondary mutations in *KIT*. This study investigated the antitumor effects of SOCS1 gene therapy, which targets several signaling pathways.
Methods We used GIST-T1 (imatinib-sensitive) and GIST-R8 (imatinib-resistant) cells. We infected both cell lines with an adenovirus expressing SOCS1 (AdSOCS1) and examined antitumor effect and mechanisms of its agent.
Results The latter harboured with secondary *KIT* mutation and had imatinib resistance > 1000-fold higher than the former cells. We demonstrated that AdSOCS1 significantly decreased the proliferation and induced apoptosis in both cell lines. Moreover, SOCS1 overexpression inhibited the phosphorylation of signal transducer and activator of transcription 3 (STAT3), AKT, and focal adhesion kinase (FAK) in both of them. Inhibition of JAK signaling did not affect the proliferation enough. However, inhibition of the FAK signaling with an FAK inhibitor or RNA interference significantly showed inhibitory effect on cell growth and suppressed the phosphorylation of AKT, indicating a cross-talk between the AKT and FAK pathways in both the imatinib-sensitive and imatinib-resistant GIST cells.
Conclusions Our results indicate that the activation of FAK signaling is critical for proliferation of both imatinib-sensitive and -resistant GIST cells and the interference with FAK/AKT pathway might be beneficial for therapeutic target.

Keywords Gastrointestinal stromal tumor · SOCS1 · Imatinib resistance · Focal adhesion kinase · Secondary *KIT* mutation

Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor in the gastrointestinal tract [1, 2]. Most GISTs are characterized by mutations in

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the proto-oncogene *KIT,* encoding the receptor protein-tyrosine kinase KIT that is a key factor in sporadic GIST pathogenesis and proliferation [3, 4]. Activated KIT in turn activates various downstream pathways, including the phosphoinositide 3-kinase (PI3 K)/AKT, mitogen-activated protein kinase (MAPK), and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways, and induces cell proliferation. The PI3 K/AKT pathway is considered to be more important than the MAPK or JAK/STAT pathways in conducting the KIT oncogenic signal in GISTs [5]. This knowledge has facilitated the design of targeted therapies employing tyrosine kinase inhibitors (TKIs) and, specifically, the development of imatinib mesylate (Glivec®, Novartis Pharmaceuticals) for the treatment of GITS. Imatinib mesylate (commonly indicated as imatinib) is a selective inhibitor of KIT, platelet-derived growth factor receptor A (PDGFRA), and Abelson proto-oncogene (ABL) via competitive binding to their adenosine tri-phosphate (ATP)-binding domains: consequently, this drug exerts its antitumor activity by shutting down KIT-mediated signaling. The prognosis for patients with GIST has dramatically improved, since imatinib therapy was introduced to clinical practice [5, 6]. Despite the effectiveness of imatinib, however, several problems remain, such as susceptibility and resistance to imatinib. Although various attempts to treat patients with imatinib-resistant GIST have been made, the success of such treatments is still limited [7]. Most imatinib-resistant GISTs acquire, indeed, secondary *KIT* mutations and are characterized by re-activation of KIT downstream pathways. Therefore, new approaches that allow the suppression of KIT downstream pathways in imatinib-resistant GIST are needed.

The family of suppressor of cytokine signaling (SOCS), characterized by a central src homology 2 (SH2) domain and a conserved C-terminal SOCS box, is composed of eight structurally related proteins [8]. Of these, SOCS1 is the most potent negative regulator of pro-inflammatory cytokine signaling and interacts with phosphotyrosine residues on proteins [9]. We have previously reported that overexpression of SOCS1 in cells, using adenoviral vectors, has a potent anti-proliferative effect obtained through the targeting of several signaling pathways, including the JAK/STAT, MAPK, focal adhesion kinase (FAK), and p53 pathways [10–14]. Since SOCS1 targets multiple signaling pathways, it may be a promising therapeutic approach for the treatment of several tumors, by preventing and/or circumventing drug resistance in tumor cells [15].

In this study, we investigated the effect of SOCS1 overexpression on the progression of GIST using imatinib-sensitive and -resistant GIST cell lines. In addition, we examined the mechanisms through which SOCS1 induces antitumor effects in GIST.

Materials and methods

Cell lines

We previously established the human GIST cell line GIST-T1, which has a 57-nucleotide in-frame deletion in *KIT* exon 11, corresponding to the deletion valine (V)570- tyrosine (Y)578 in the KIT protein [16]. The cell line identity was confirmed by DNA fingerprinting through short tandem repeat profiling, as previously described [17]. The GIST-R8 cell line was established from GIST-T1 cells as an imatinib-resistant clone that arose from continuous culturing in 5 nM imatinib. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 100 U/mL penicillin and 100 μg/ml streptomycin (PS; Nacalai Tesque, Kyoto, Japan) at 37 °C, under a humidified atmosphere of 5% CO_2.

Cell proliferation assay

GIST cells were plated in 96-well plates at a density of 2×10^3 cells per well and incubated for 24 h. Cell proliferation was evaluated using the Cell Counting Reagent SF (Nacalai Tesque) at the indicated times, as previously described [14, 15]. The following inhibitors were used in this study: Imatinib (Novartis Pharmaceuticals, Basel, Switzerland), JAK inhibitor I (Calbiochem), FAK inhibitor (TAE226, Novartis Pharmaceuticals), and PI3 K inhibitor (LY294002, Cell Signaling Technology, Danvers, MA, USA). TAE226 is a small-molecule inhibitor that was recently synthesized to specifically target FAK: it exerts a potent anti-proliferative effect by suppressing FAK and its downstream effectors [18]. The half maximal inhibitory concentration (IC_{50}) values were measured 72 h after treatment with imatinib and calculated as: $IC_{50} = 10 \{\log (A/B) (50 - C)/(D - C) + \log (B)\}$ where A represents the higher concentration between the 50% of the inhibition rate, B represents the lower concentrations between the 50% of inhibition rate, C represents the inhibition rate at concentration B, and D represents the inhibition rate at concentration A.

A replication-defective recombinant adenoviral vector expressing the human *SOCS1* gene (AdSOCS1) was produced by Takara Bio (Kusatsu, Japan). An adenoviral vector expressing the *LacZ* gene (AdLacZ) was constructed. After incubation in culture medium containing 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin for 24 h, the GIST cells were infected with the indicated adenoviral vectors at a multiplicity of infection (MOI) of 10–160.

Western blotting analysis

GIST cell lines were harvested and lysed as previously described, [14, 15] and proteins were separated according to standard procedures. The following antibodies were used: anti-phospho (p)-KIT (Tyr703), anti-p-AKT (Thr308), anti-p-AKT (Ser473), anti-AKT, anti-p-p44/42 MAPK, anti-p44/42 MAPK, anti-p-STAT3, and anti-Cleaved-Caspase3 (1:1000 dilution) were from Cell Signaling Technology (Danvers, MA, USA); anti-KIT and anti-STAT3 (1:1000 dilution), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:2000 dilution) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-SOCS1 (1:2000 dilution) was from IBL (Fujioka, Japan); anti-p-FAK (Tyr397) and anti-FAK (1:1000 dilution) were from BD Transduction Laboratories (San Jose, CA, USA).

Caspase-3/7 activity assay

GIST cells were plated into 96-well white plates at a density of 2×10^3 cells per well and infected with AdLacZ or AdSOCS1 (MOI = 10) for 48 h. The activity of caspase-3 and -7 in cell culture was detected using the Caspase Glo® 3/7 Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luminometer readings were determined using a Spectra Max Gemini EM Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Small-interfering RNA (siRNA) transfection

The following ON-TARGETplus SMARTpool siRNAs were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA): non-targeting siRNA (D-001810-10-20) and human protein-tyrosine kinase (PTK) 2, a member of the FAK subfamily, siRNA (L-003164-00-0010). GIST cells were seeded in antibiotic-free DMEM medium with 10% FBS. The next day, the cells were transfected with the siRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For western blotting analysis, cells were seeded into 6-well plates at a density of 2.5×10^5 cells/well and incubated for 48 h.

Statistical analysis

Data are shown as the mean ± standard deviation (SD) for the indicated number of experiments. To test for statistically significant differences between two groups, the unpaired Student's t test was used. Two-sided P values less than 0.05 were considered significant. These analyses were

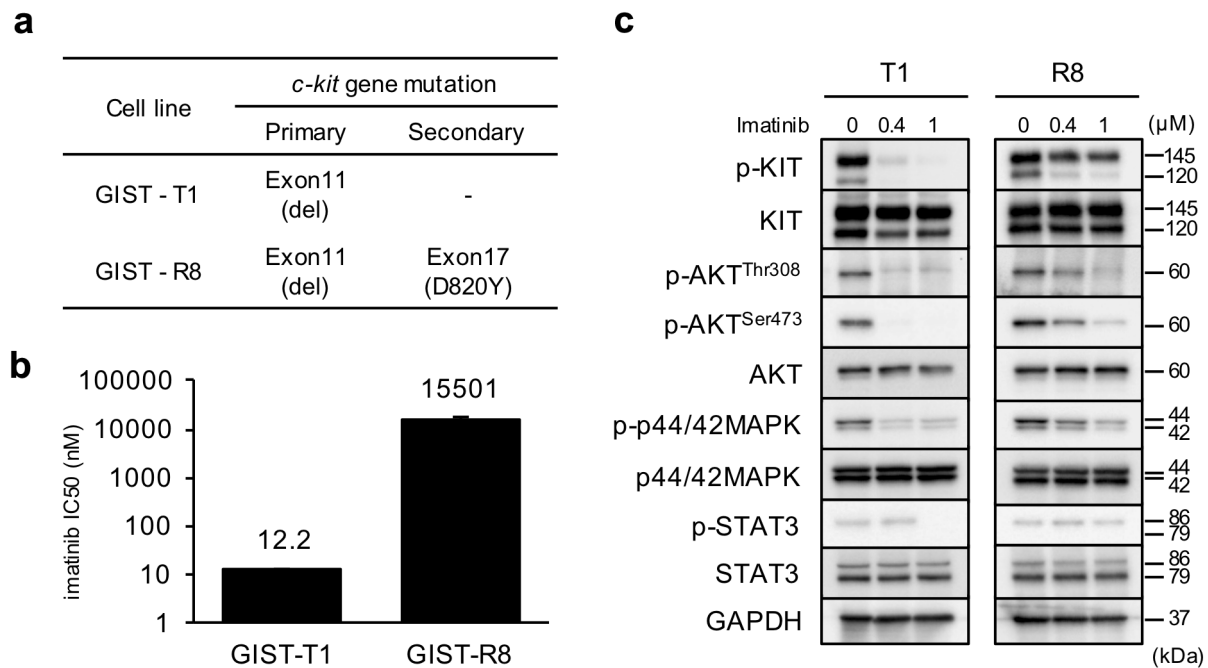carried out using the JMP software, version 13.0 (SAS Institute, Cary, NC, USA).

Results

Mutation analysis of GIST-T1 and R8 cells and effect of imatinib treatment

We have previously established an imatinib-resistant GIST cell line (GIST-R8) by exposing GIST-T1 cells to imatinib [19]. Sanger sequencing revealed that GIST-R8 cells harboured a secondary *KIT* mutation on exon 17 (D820Y) in addition to a deletion in exon 11 (Fig. 1a). This mutation has been reported as secondary mutation which resulted in imatinib resistance [20]. The imatinib IC_{50} values for GIST-T1 and R8 cells were 12.2 nM and 15501 nM, respectively: the imatinib resistance of GIST-R8 cells was more than 1000-fold higher than that of GIST-T1 cells (Fig. 1b). Next, we evaluated the effect of imatinib on GIST cells by western blotting analysis. The expression of p-KIT was suppressed in response to imatinib treatment in GIST-T1 cells only. KIT downstream pathways, such as the PI3 K, MAPK, and JAK/STAT pathways, were almost unchanged in GIST-R8 cells compared to those in GIST-T1 cells, upon imatinib treatment (Fig. 1c).

Inhibition of proliferation in GIST cells upon SOCS1 overexpression

We used a replication-defective recombinant adenoviral vector harbouring *SOCS1* (AdSOCS1) to overexpress SOCS1 in GIST cells and investigate its effect on cell proliferation; AdLacZ, expressing the *LacZ* gene, was used as a negative control. Both GIST-T1 and R8 cells showed a significant effect on growth inhibition in a concentration-dependent manner in the presence of AdSOCS1 (Fig. 2a). Western blotting analysis showed that the expression of p-STAT3 (Tyr705) was clearly inhibited upon overexpression of SOCS1 in both cell lines (Fig. 2b). Moreover, SOCS1 overexpression decreased the expression of p-AKT (Thr308 and Ser473) in both cell lines (Fig. 2c). In addition, the levels of total and phosphorylated FAK (Tyr397) were downregulated upon SOCS1 overexpression in GIST-T1 and R8 cells (Fig. 2d).

To evaluate if the overexpression of SOCS1 was associated with the induction of apoptosis, we measured the levels of caspase-3/7 activity using a luciferase luminescence assay after the infection of the cells with AdSOCS1 or AdLacZ. The levels of caspase-3/7 activity in AdSOCS1-infected cells were significantly higher than those in AdLacZ-infected cells (Fig. 3a). In addition, overexpression of SOCS1 was

a

Cell line	c-kit gene mutation	
	Primary	Secondary
GIST - T1	Exon11 (del)	-
GIST - R8	Exon11 (del)	Exon17 (D820Y)

b

c

Fig. 1 Mutation analysis in GIST cell lines and effects of imatinib treatment. **a** *KIT* gene mutation in two GIST cell lines, GIST-T1 and -R8. **b** Imatinib IC_{50} in GIST-T1 and -R8 cells, as determined by proliferation assays 72 h after imatinib treatment. **c** Western blotting analysis 24 h after imatinib treatment

associated with increased expression of cleaved-caspase3 (Fig. 3b).

JAK inhibition and FAK downregulation suppressed cell proliferation in GIST cells

To investigate whether the JAK/STAT, FAK, and PI3 K pathways regulated the proliferation of GIST-T1 and R8 cells, we used specific inhibitors of these signaling pathways. Inhibition of the JAK/STAT pathway slightly inhibited the growth of both cell lines, while inhibition of the FAK pathway had a considerable inhibitory effect (Fig. 4a, b). Inhibition of the PI3 K signaling also suppressed cell proliferation in both cell lines (Supplementary Fig. S1). The expression of p-STAT3 (Tyr705) decreased upon treatment of both cell lines with a JAK inhibitor I. High doses of this agent reduced the levels of p-AKT in GIST-T1 cells only and did not change the levels of p-FAK (Fig. 4c). As shown in Fig. 3d, p-FAK levels decreased in both cell lines upon treatment with an FAK inhibitor. In addition, inhibition of the FAK signaling was associated with decreased levels of p-AKT (Thr308 and Ser473) and p-STAT3 and increased levels of cleaved-caspase3 (Fig. 4d).

Next, we examined the association between the FAK signaling and GIST cell proliferation using FAK-directed RNA interference. Attenuation of FAK was associated with the inhibition of cell proliferation in both cell lines, and,

notably, with a decrease in p-AKT levels even in GIST-R8 cells (Fig. 5a, b).

Discussion

Malignant GISTs showed that the PI3 K/AKT pathway is the major player in conducting the KIT oncogenic signal in GISTs [5]. We also demonstrated that the inhibition of PI3 K signaling suppresses cell proliferation in imatinib-sensitive and -resistant GIST cells (Supplementary Fig. S1). In addition to these signaling pathways, Koon N. et al. reported that FAK pathway is up-regulated in GISTs and the extent of its upregulation correlates with malignancy and clinical outcome [21]. FAK is a ubiquitously expressed non-receptor protein-tyrosine kinase that has emerged as a crucial molecule involved in the integration of signals from integrins and receptor tyrosine kinases in processes such as cell survival, proliferation, and motility [22]. Recent reports indicated that GIST cells with imatinib resistance had increased FAK signaling and pointed to the inhibition of the FAK pathway as a potential therapeutic strategy for imatinib-resistant GISTs [23, 24].

Regarding the FAK pathway, both the Src homology 2 (SH2) and the kinase inhibitory region (KIR) domains of the SOCS proteins, which are SOCS1 and SOCS3, contribute to FAK binding, and interact with FAK in an FAK-Y397-dependent manner. Moreover, SOCS1 and SOCS3 recruit the
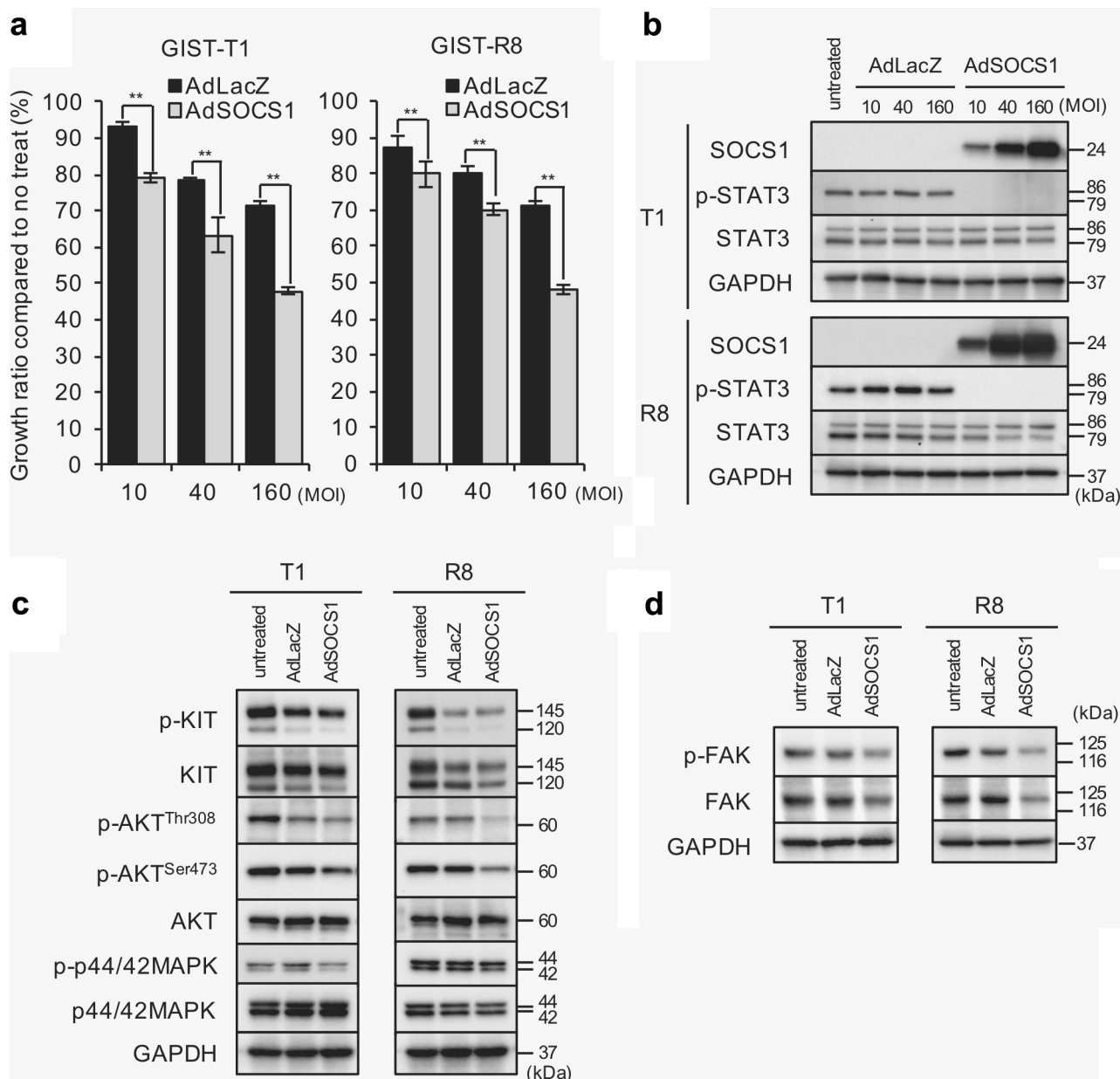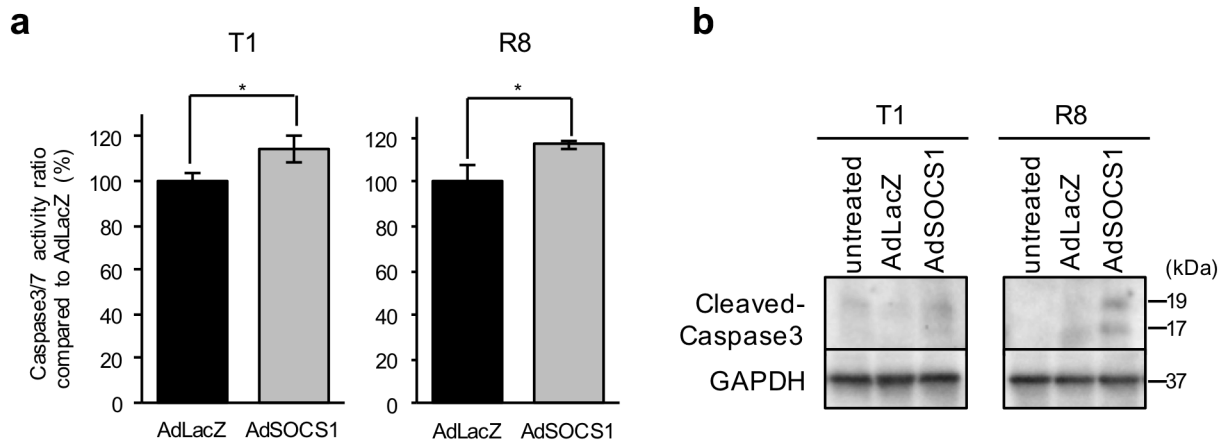
Fig. 2 SOCS1 overexpression inhibits proliferation in GIST cells. **a** Cell proliferation in GIST-T1 and -R8 cells 72 h after infection with AdSOCS1 or AdLacZ (negat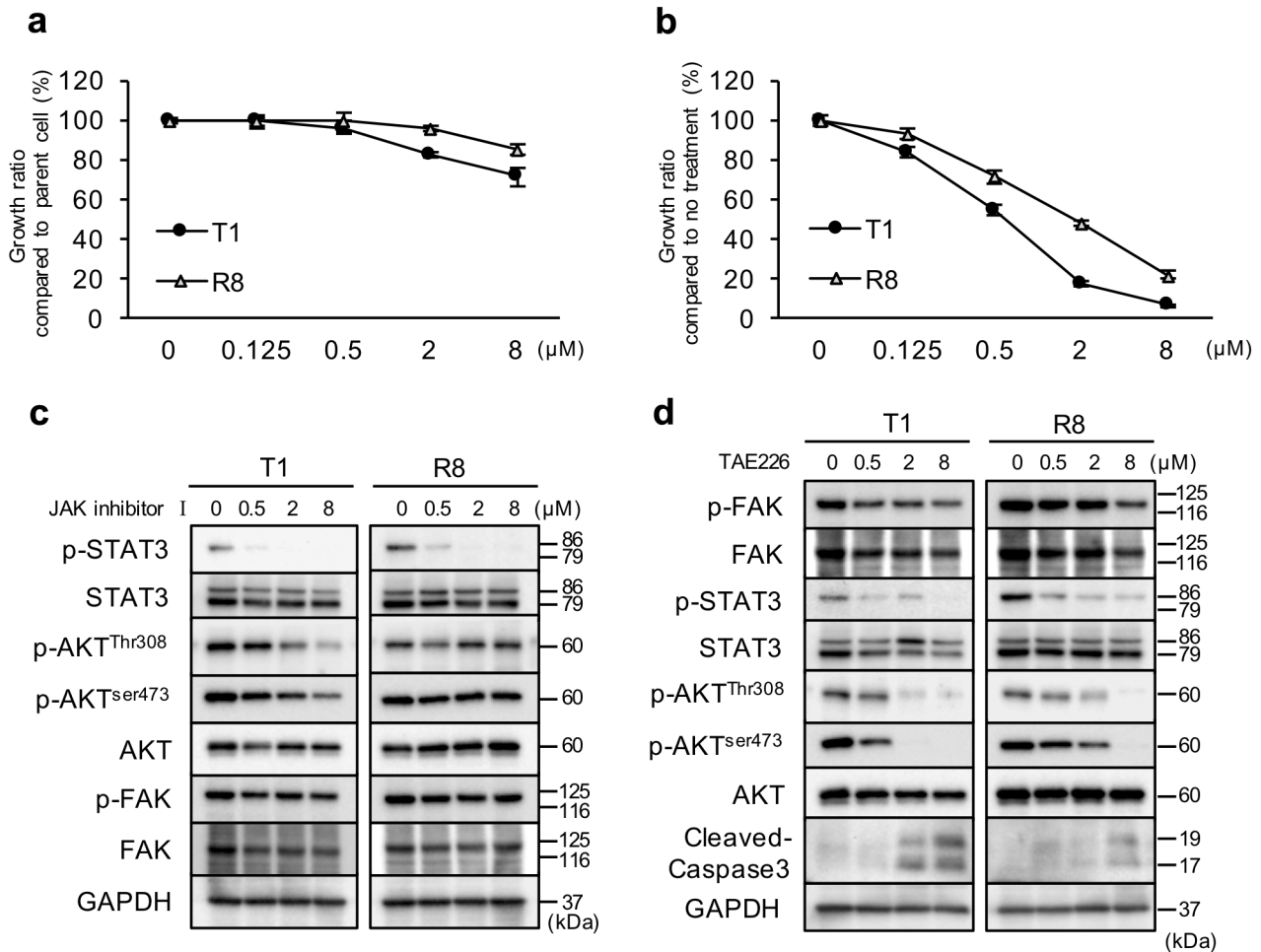ive control) at an MOI of 10–160. **b–d** Western blotting analyses showing the expression of the indicated proteins in GIST-T1 and -R8 cells 48 h after infection with AdSOCS1 or AdLacZ at an MOI of 10–160 (**b**) or 40 (**c, d**)

elongin BC-containing E3 ubiquitin-ligase complex through the conserved SOCS box to promote the degradation of target proteins, such as FAK. SOCS1 promoted polyubiquitination and degradation of FAK in an SOCS box-dependent manner and inhibited FAK-dependent signaling events [25]. In this study, we demonstrated that SOCS1 overexpression was associated with a reduction in the levels of phosphorylated and total FAK and phosphorylated AKT. This effect was clear in both imatinib-sensitive and -resistant GIST cells. Furthermore, inhibition of the FAK pathway through

the use of an FAK inhibitor or RNA interference decreased AKT phosphorylation, suggesting a cross-talk between the FAK and AKT/PI3 K pathways. Importantly, our results indicate that, besides the KIT signaling, the activation of the FAK pathway is also critical in both GIST imatinib-sensitive and -resistant cells. On the other hand, inhibition of the JAK/STAT pathway did not have anti-proliferative effects enough compared to FAK inhibitor. Therefore, we considered that the clinical effect of STAT 3 inhibitor on GIST patients might not be expected.

Fig. 3 a Caspase-3/7 activity was determined using luminescence-based assay 48 h after infection with AdSOCS1 or AdLacZ at an MOI of 10. **b** Expression of cleaved-caspase3 72 h after infection with AdSOCS1 or AdLacZ at 160MOI

Fig. 4 Cell proliferation was determined 72 h after treatment of GIST-T1 and -R8 cells with the JAK inhibitor I (**a**) or a FAK inhibitor (**b**). Western blotting analyses showing the expression of the indicated proteins in GIST-T1 and -R8 cells 24 h after treatment of GIST-T1 and -R8 cells with the JAK inhibitor I (**c**) or a FAK inhibitor (**d**)

a

b
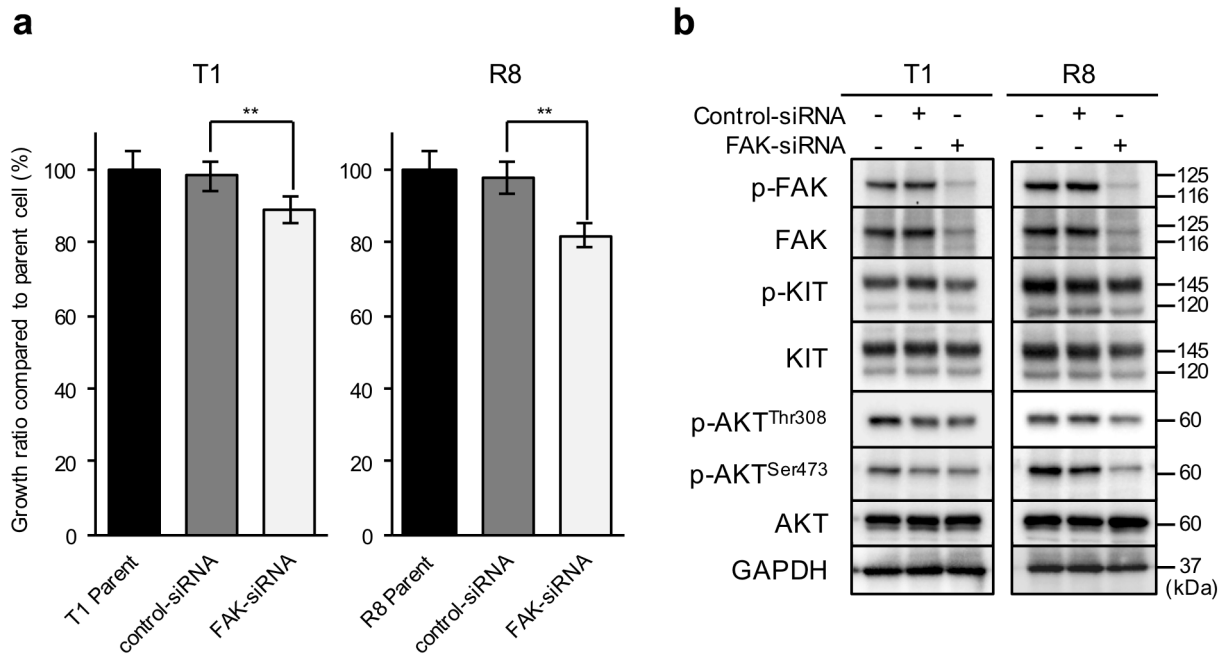
Fig. 5 **a** Cell proliferation in GIST-T1 and -R8 cells 72 h after FAK-RNA interference. **b** Western blotting analysis showing the expression of the indicated proteins in GIST-T1 and -R8 cells 72 h after FAK-RNA interference

Almost all patients with sporadic advanced or metastatic GIST are treated with imatinib as the first choice [6, 7]. Although this drug has revolutionized treatment options and significantly improved the clinical outcomes for patients with GIST, there remain several problems. A subset of GIST cases may lose their susceptibility to imatinib, which depends on the specific *KIT* mutations [26]. Moreover, imatinib-resistant GISTs often show secondary *KIT* mutations [27]. Approximately 15% of the patients show primary imatinib resistance, while more than 80% of the patients have acquired resistance [6]. In this study, we demonstrated that SOCS1 overexpression has antitumor effects other than the inhibition of KIT signaling. Therefore, SOCS1 gene therapy might potentially be beneficial in patients whose KIT signaling is not sufficiently inhibited by treatment with tyrosine kinase inhibitors. Furthermore, patients with GIST cannot discontinue the use of the drug even if complete response is obtained, because it would inevitably lead to re-progression and disease relapse [28]. We have previously reported that SOCS1 strongly induces apoptosis via suppression of anti-apoptotic proteins in various cancers [10–13]. Because SOCS1 overexpression induces apoptosis in both GIST cell lines used in this study, we hypothesize that it might be possible to kill GIST cell that are viable after imatinib therapy by SOCS1 gene therapy. Therefore, SOCS1 gene therapy may represent a new treatment for GISTs, thanks to its anti-tumor effects. Moreover, the co-treatment with imatinib and SOCS1 gene therapy might improve clinical outcome because of the different antitumor effects of these therapies.

There are some limitations associated with the use of SOCS1 gene therapy in this study. First, we used GIST-T1(imatinib-sensitive) and GIST-R8 (imatinib-resistant) which were established from GIST-T1 with continuous imatinib exposure in our laboratory. Although our results using two cell lines might not be enough to confirm definite conclusion, GIST cell lines are generally difficult to establish and making drug-resistant cell lines is even more difficult to establish from drug-sensitive ones. We considered that some data accumulation including animal experiments might be needed to apply clinical trial for SOCS1 gene therapy as a new GIST treatment in the future. Secondary, we used a replication-defective recombinant adenoviral vector, capable to growth and multiply in 293 cells only. Therefore, AdSOCS1 therapy would have its therapeutic effects only in the area of the tumor injected with the virus particles. Although this therapy might be difficult to adapt to GIST patients with multiple distant metastasis such as peritoneal metastasis, imatinib-resistant GIST patients who often showed a single or a small number of resistant lesions, particularly liver metastasis, might benefit from this therapy through endoscopic or percutaneous administration of the viral particles. Moreover, we believe that SOCS1 gene therapy has the advantage that local injection can reduced side effects compared to systemic treatment and perform tumor-specific treatment. Regarding SOCS gene therapy, we are scheduled to begin human clinical trials and the treatment efficacy and safety results are awaited. In addition, we have reported that SOCS1 overexpression has antitumor effect by inhibiting

various cell growths' signaling including to MAPK [10–15]. Since the FAK inhibition of AdSOCS1 might be only one of the antitumor mechanisms against GIST, we need to further investigate the multiple inhibitory effect of SOCS1 gene therapy in GIST.

In conclusion, SOCS1 gene therapy, obtained by using an adenoviral vector, suppresses proliferation and induces apoptosis in imatinib-sensitive and -resistant GIST cells. SOCS1 overexpression suppresses the JAK/STAT and FAK/PI3 K pathways, while it does not affect KIT. In addition, the suppression of the FAK/PI3 K pathway has a significant antitumor effect in imatinib-resistant GIST cells. We consider that more data accumulation is expected to lead to the successful clinical application of SOCS1 gene therapy for GIST treatment.

Compliance with ethical standards

Human rights statement All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions.

Animal studies This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of interest No author has conflicts of interest that could potentially influence the described research.

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