Suppressor of cytokine signalling-1 induces significant preclinical antitumor effect in malignant melanoma cells

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Abstract: Malignant melanoma is the most aggressive form of skin cancer, responsible for the majority of skin cancer-related deaths. Metastatic melanoma is resistant to surgery, radiation or chemotherapy, and an effective therapy has not yet been established. Our study investigated the therapeutic potential of the suppressor of cytokine signalling-1 (SOCS-1), an endogenous inhibitor of the intracellular cytokine signalling pathway, for treating melanoma. Adenovirus vectors encoding the SOCS-1 gene were used to overexpress SOCS-1 in three melanoma cell lines (G361, SK-MEL5 and SK-MEL28). In G361 and SK-MEL5, overexpression of SOCS-1 significantly reduced cell proliferation and induced apoptosis in vitro and in vivo. Furthermore, we indicated that the antiproliferative effect of SOCS-1 correlated not only with decreased levels of the activation of signal transducer and activator of transcription (STAT)3 but also with increased levels of p53 expression and phosphorylation. These findings indicate the potential for clinical use of SOCS-1 for melanoma treatment.

Key words: apoptosis – JAK/STAT – malignant melanoma – p53 – suppressor of cytokine signalling-1

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Introduction

Melanoma is the leading cause of skin cancer-related deaths and is notorious for its resistance to therapy. The global incidence of melanoma is dramatically increasing, with about 200,000 new cases and about 65,000 melanoma-associated deaths every year (1,2). The regulatory pathways involved in melanoma development are associated mainly with the activation of tumor growth signals, for example the Ras–Raf–MEK–ERK signal, the PI3K–AKT mammalian target of rapamycin (mTOR) signal or the Janus kinase (JAK) signal transducer and activator of transcription (STAT) signal, and the inactivation of tumor suppressor signals, for example the p14ARF–p53 signal or the p16-Rb signal (3–6).

Recently, BRAF and MEK kinase inhibitors have been shown to extend survival time in patients with BRAF-mutant melanoma; in addition, immunotherapy with anticytotoxic T-lymphocyte antigen–4 antibodies and antiprogrammed cell death protein 1 antibodies has been shown to prolong survival in patients with advanced melanoma (1,7,8). Despite the advances that these novel treatments have made, treatment responses are often limited by the development of drug resistance (9,10). Therefore, researchers should focus on signalling pathways involved in melanoma growth other than the Ras–Raf–MEK–ERK signal.

In melanoma, it has been reported that STAT3 is constitutively activated in clinical specimens, and the phosphorylated STAT3 (Tyr705) expression has been immunohistologically detected in thick melanomas (>1.0 mm) and metastases (11). In addition, it has also been reported that the presence of melanoma cells with an elevated expression of phosphorylated STAT3 (p-STAT3) is associated with poor relapse-free survival in AJCC stage I/II (12).

In addition, JAK inhibitor has been shown to inhibit the growth of melanoma cells via induction of apoptosis in vitro (13). Therefore, we suggest that the JAK-STAT signal is a promising therapeutic target for melanoma.

The signalling pathway that comprises JAKs and STAT proteins is important for relaying signals from various cytokines outside the cell to inside, for example interleukin (IL)-6, IL-11, interferon-γ, granulocyte colony-stimulating factor and leukaemia inhibitory factor (14). In these cytokines, IL-6 is reported to play an important role in the progression of melanoma (15–17). Binding of a cytokine to its cognate receptor induces receptor dimerization, which initiates the activation of JAKs. The activated JAKs phosphorylate the receptor cytoplasmic domain, which creates docking sites for SH2-containing signalling proteins, including STATs (18,19). Activated STAT dimmers then translocate to the cell nucleus and act as transcription factors by binding to specific response elements in the promoter of their target genes (14).

Suppressor of cytokine signalling (SOCS) family proteins are important negative regulators of proinflammatory cytokine signals. They suppress the JAK-STAT signalling pathway by inhibiting JAK activity. There are eight members in the SOCS family: CIS and SOCS 1–7; the first member of this family, SOCS-1, was cloned in 1997 (20,21). The SOCS-1 protein has a central SH2 domain, an aminoterminal domain of variable length and sequence and a 40–amino acid module on the carboxy-terminal region known as the SOCS box (22,23). SOCS-1 has been shown to directly bind to JAK2 and inhibit its catalytic activity (18,24). Although SOCS-1 has an ability to regulate several tumor-related signals, SOCS-1 gene in various cancers including melanoma was found to be fre-
quently silenced due to hypermethylation in the CpG islands of the promoter region (25–27). This is likely one of the causes of constitutively activated STATs in these cancers.

There are some reports suggesting that the overexpression of SOCS-1 inhibits not only the JAK/STAT signal but also the other signals concerned with cell growth signals. For example, SOCS-1 suppresses STAT3 and p38 mitogen-activated protein kinase (MAPK) activation in gastric cancer cell proliferation (28). It also suppresses focal adhesion kinase (FAK) and epidermal growth factor receptor (EGFR) and activates p53 in non-small cell lung cancer cell proliferation (29). In these ways, SOCS-1 targets multiple signalling pathways. Therefore, SOCS-1 can be a candidate for therapy to which tumor cells cannot easily develop resistance.

In this study, we used melanoma cell lines G361, SK-MEL5 and SK-MEL28 to investigate the possibility of applying SOCS-1 gene transduction in melanoma therapies. Moreover, we studied the mechanisms of the SOCS-1 antitumor effects in detail.

Materials and methods

Cell lines

Human melanoma cell lines (A2058, G361, SK-MEL5 and SK-MEL28) were obtained from Japanese Collection of Research Bioresources (Osaka, Japan). The details are described in the Supporting Information.

Reagents

JAK inhibitor I was purchased from Calbiochem (La Jolla, CA, USA). Anti-IL-6R monoclonal antibody was obtained from Chugai Pharmaceutical Co. (Tokyo, Japan). pVAX-hp53 was provided by Dr. Yasufumi Kaneda (Osaka University, Osaka, Japan).

Preparation of adenoviruses

The replication-defective recombinant adenoviral vector expressing the mouse SOCS-1 gene and LacZ gene was prepared and used as described previously (28).

Real-time PCR analysis

Total RNA was prepared from cells using an RNAsasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNAs were synthesized from 100 ng of each total RNA preparation using a Quantitect Reverse Transcription Kit (Qiagen), all according to the manufacturer’s instructions. The details are described in the Supporting Information.

X-gal staining

The transduction efficiency of adenoviral vectors was assessed by means of X-gal staining. The details are described in the Supporting Information.

Cell viability assay

Details experimental procedures describing cell viability assay can be found in the Supporting Information.

SDS-PAGE and Western blot analysis

Whole-cell protein extract was prepared from melanoma cells in RIPA buffer [10 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, a phosphatase inhibitor cocktail and a protease inhibitor cocktail (both from Nacalai Tesque and both at a concentration of 1×)], followed by centrifugation (14,000 × g, 4°C, 15 min). Extracted proteins were resolved on SDS-PAGE and transferred to an ImmobilonTM PVDF Transfer Membrane (Millipore, Bedford, MA, USA). The details are described in the Supporting Information.

Cell cycle assay

Details experimental procedures describing cell cycle assay can be found in the Supporting Information.

Small interfering RNA transfection

Commercial p53 and STAT3 small interfering RNA (siRNA) were obtained from Qiagen (Hilden, Germany). Cells were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer’s instructions. The details are described in the Supporting Information.

Co-immunoprecipitation

Cells were lysed in an ice-cold 1× IP buffer. After clearing of the lysate, anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the Dynabeads® M-270 Epoxy in the Dynabeads® Co-Immunoprecipitation Kit, followed by overnight incubation at 37°C. The details are described in the Supporting Information.

Deletion mutant

HA-tagged SOCS1ΔSH2 was produced by cutting HA-tagged SOCS-1 with restriction enzymes (PpuMI and Bpu1102I) and treating it using the DNA Blunting Kit (TaKaRa), according to the manufacturer’s instructions.

Immunoprecipitation of HA-tagged protein

Cells were lysed in an ice-cold 1× IP buffer. After clearing, the lysate was added to EZViewTM Red Anti-HA Affinity Gel (Sigma-Aldrich, St. Louis, MO) and incubated by end-over-end mixing for 1 h, according to the manufacturer’s instructions. Captured protein complexes were separated, washed and eluted using influenza hemagglutinin (HA) peptide (Sigma-Aldrich) and analysed by Western blotting.

Mouse xenograft model

All animal experiments were conducted according to the National Institute of Biomedical Innovation (Osaka, Japan) institutional ethical guidelines for animal experimentation. Female ICR nu/nu mice, 4–5 weeks of age, were obtained from Charles River Japan (Yokohama, Japan). For subcutaneous xenograft experiments, ICR nu/nu mice were inoculated subcutaneously in the flank with 4 × 10⁵ cells in a total volume of 100 μl of PBS. When the tumor sizes reached approximately 100 mm³, 4 × 10⁸ plaque-forming units (pfu) of either AdSOCS1 or AdLacZ were injected intratumorally twice a week for 3 weeks. Tumor volumes were calculated weekly using the following formula: tumor volume (mm³) = length × width × height.

Immunohistochemistry

Subcutaneously implanted tumors were harvested and paraffin embedded for immunohistochemical analysis using anti-SOCS-1 antibody (Abcam, Cambridge, MA, USA) with Dako ChemMate Envision Kit (Dako Copenhagen, Denmark). The images were visualized using an optical microscope (FX100; OLMPUS, Tokyo, Japan).

TUNEL assay

TUNEL assay (with DAPI nuclear counterstaining) for apoptosis was conducted using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA), according to the manufacturer’s instructions. The images were visualized using a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan).

Statistical analysis

Data are shown as the mean ± SD for the number of experiments indicated. In the experiments of the xenograft model, data are shown as the mean ± SEM. To test for statistically significant differences between two groups, unpaired Student’s t-test was used.
For comparisons among three or more groups, the values were analysed via one-way ANOVA followed by Scheffe comparisons. Differences were considered significant at \( P < 0.05 \).

**Results**

**Overexpression of SOCS-1 inhibits the growth of G361 and SK-MEL5 cells**

We first measured the IL-6 levels in 24-h culture supernatants in melanoma cell lines, G361, SK-MEL5, SK-MEL28, and A2058; a malignant pleural mesothelioma cell line, H226, was used as a positive control. As shown in Fig. S1a, G361, SK-MEL5, and A2058 were identified as cell lines with moderately high IL-6 secretion, but SK-MEL28 was identified as a cell line with little secretion. Next, we examined the constitutively activated level of STAT3 downstream of the IL-6/JAK/STAT3 signalling pathway in melanoma cell lines by Western blotting. As shown in Fig. S1b, high levels of p-STAT3(Tyr705) were detected in SK-MEL5, moderate levels were detected in G361 and A2058, and low levels were detected in SK-MEL28. We then tested the effect of anti-IL-6R monoclonal antibody treatment. As shown in Fig. S1c, anti-IL-6R monoclonal antibody did not inhibit the cell growth in G361, SK-MEL5 and SK-MEL28 cells. These results suggest that cell proliferation and constitutive phosphorylation of STAT3 in G361, SK-MEL5 and SK-MEL28 cells occur independently of IL-6 signalling and that signalling pathways other than IL-6 may be involved in cell proliferation of these cells.

The JAK/STAT signalling pathway is negatively regulated by SOCS-1 in normal cells. However, SOCS-1 gene is reported to be silenced in various cancer cells including melanoma cells. In accordance with this, real-time PCR analysis revealed that all melanoma cell lines examined (G361, SK-MEL5, SK-MEL28 and A2058) expressed very low levels of SOCS-1 and failed to upregulate SOCS-1 expression in response to INF-\(\gamma\), whereas human PBMC upregulated SOCS-1 expression in response to IFN-\(\gamma\) (Fig. S1d).

Next, to investigate the potential of SOCS-1 as a therapy for malignant melanoma, we used a replication-defective recombinant adenoviral vector to introduce SOCS-1 in G361, SK-MEL5, and SK-MEL28 melanoma cells, in which different levels of p-STAT3 (Tyr705) were detected (Fig. S1b). G361, SK-MEL5, and SK-MEL28 cells were infected with AdLaCZ at the indicated multiplicity of infection (MOI) and stained with X-gal 24 h after infection; then we confirmed sufficient transduction efficiency of the adenovirus vector at an MOI of 40 (Fig. S2a). AdSOCS-1 significantly inhibited the cell growth in G361 and SK-MEL5, but not significantly in SK-MEL28 (Fig. 1a). Moreover, AdSOCS-1 induced attenuation of p-STAT3(Tyr705) in a dose-dependent manner in G361 and SK-MEL5, in which the p-STAT3(Tyr705) expression level is moderately high (Fig. S2b).

**Overexpression of SOCS-1 increases apoptosis and induces G0/G1 cell cycle arrest in G361 and SK-MEL5 cells**

Next, we investigated the mechanisms by which AdSOCS-1 inhibited cell growth. Two cell lines, G361 and SK-MEL5, in which AdSOCS-1 significantly inhibited the cell growth, were used. The
 initiation of apoptosis is characterized by specific activation of the caspase family of proteases. Caspase-3 activation is known to play a role during the apoptosis process. To demonstrate whether overexpression of SOCS-1 induces apoptosis, caspase-3 activity was assessed. As shown Fig. 1b, overexpression of AdSOCS-1 significantly increased caspase-3 activity in a dose-dependent manner in G361 and SK-MEL5 as compared with AdLacZ-treated cells. In addition, we investigated the expression levels of anti-apoptotic proteins, Mcl-1 and Bcl-xl. As shown in Fig. 1c, each was decreased by the overexpression of SOCS-1 in both cells. These findings indicate that the overexpression of SOCS-1 induced apoptosis in G361 and SK-MEL5 cells.

In addition to apoptosis, cell cycle regulation is an important mechanism for the inhibition of tumor cell growth. To investigate the effect of SOCS-1 on cell cycle regulation, we infected G361 and SK-MEL5 cells with AdSOCS-1 and then analysed cell cycle distribution via flow cytometry. When G361 and SK-MEL5 cells were treated with AdSOCS-1, the G0/G1 cell population significantly increased, and the S cell population significantly decreased more than in the cells treated with AdLacZ. In the G2/M cell population, only G361 cell population was significantly decreased (Fig. 1d).

**Overexpression of SOCS-1 increases phosphorylation and expression levels of p53**

The constitutive activation of the JAK/STAT signalling pathway is associated with melanoma cell growth. To examine the effects of JAK inhibitor on growth suppression, G361 and SK-MEL5 cells were exposed to 0–1.0 μM JAK inhibitor I. We confirmed that p-STAT3(Tyr705) expression levels were completely inhibited at 1 μM in both cells (Fig. S3a). As shown in Fig. 2a, 1 μM JAK inhibitor I inhibited melanoma cell growth by 20–30%. Moreover, we confirmed that JAK inhibitor I decreased the anti-apoptotic protein levels, suggesting that JAK inhibitor I also induces apoptosis (Fig. S3b). To examine whether STAT3 plays a role in G361 and SK-MEL5 cell proliferation, melanoma cells were transfected with STAT3 siRNA or negative (control) siRNA and cell proliferation assays were performed. The effective knockdown of STAT3 in melanoma cells was confirmed by Western blot analysis (Fig. S3c).

When cells were transfected with STAT3 siRNA, the cell growth was significantly inhibited compared with those transfected with control siRNA (Fig. 2b). This result indicated that the growth of these melanoma cells was at least partially dependent on STAT3. Next, to clarify the difference between JAK inhibitor I and SOCS-1, we investigated the signalling pathway associated with melanoma cell growth. We assessed several signalling pathways other than the JAK-STAT signalling pathway involved in melanoma cell growth, such as Ras–Raf–MEK–ERK signal, PI3K–AKT–mTOR signal, and the tumor suppressor signals (such as the p14-p53 signal or the p16-Rb signal). The results confirmed that phosphorylation and expression levels of p53 were upregulated by infection with AdSOCS-1 at an MOI of 40, but not by treatment with 1.0 μM JAK inhibitor I (Fig. 2c). No other signals except for p53 signal which were affected by AdSOCS-1 were detected (data not shown).

**Figure 2. Overexpression of SOCS-1 increases phosphorylation and expression levels of p53**

(a) Cells were cultured in DMEM medium containing 10% FBS and were exposed to 0–1.0 μM JAK inhibitor I. After a 72-h culture, viable cell numbers were counted with the WST-8 assay. Figures show the growth ratio of JAK inhibitor I-infected cells, which was calculated by a percentage of the growth of non-treated cells. Each value represents the average ± SD of the hexaplicate. (b) G361 and SK-MEL5 cells were transfected with STAT3 siRNA or negative control siRNA. After a 96-h culture, viable cell numbers were counted with the WST-8 assay. When cells were transfected with STAT3 siRNA, the cell growth was significantly inhibited compared with those transfected with negative control siRNA. After a 96-h culture, viable cell numbers were counted with the WST-8 assay. When cells were transfected with STAT3 siRNA, the cell growth was significantly inhibited compared with those transfected with negative control siRNA. After a 96-h culture, viable cell numbers were counted with the WST-8 assay. When cells were transfected with STAT3 siRNA, the cell growth was significantly inhibited compared with those transfected with negative control siRNA. (c) Cells were cultured in DMEM medium containing 10% FBS and were exposed to 1.0 μM JAK inhibitor I, AdLacZ or AdSOCS-1, at an MOI of 40. After incubation for 48 h, proteins were extracted and expression levels of p53 and its downstream p21 were assessed by Western blot analyses. * and ** indicate P-value < 0.05 and < 0.01, respectively.
SOCS-1 regulates p53 expression

To assess the association between SOCS-1 and p53, G361 and SK-MEL5 cells were transfected with either AdSOCS-1 or AdLacZ (as the control) at an MOI of 40. Subsequently, we detected SOCS-1 in the co-immunoprecipitate of a p53-specific antibody (Fig. 3a). This demonstrated that SOCS-1 forms a complex with p53 and may regulate p53 expression level by interacting with p53.

Next, using COS7 cells, we investigated the domain in SOCS-1 that is required for the formation of a complex with p53. COS7 cells were co-transfected with pVAX-hp53 and pcDNA3.1 (empty vector), pcDNA3.1-HA-tagged SOCS-1 (HA-SOCS-1), or pcDNA3.1-HA-tagged SOCS-1 (D)SH2 (HA-SOCS-1 (D)SH2) (Fig. S4). We detected p53 in HA-tagged immunoprecipitates using EZview™ Red Anti-HA Affinity Gel only when cells were transfected with a HA-SOCS-1. This result suggests that the SH2 domain of SOCS-1 is important to form a complex with p53 (Fig. 3b).

Next, we assumed that the upregulation of p53 by SOCS-1 could contribute to the inhibition of G361 and SK-MEL5 cell proliferation. Therefore, melanoma cells were transfected with p53 siRNA or negative control siRNA and combined with cells either infected with AdSOCS-1 or AdLacZ, then cell proliferation assays were performed. When cells were infected with AdLacZ, the cell growth was not significantly different in cells transfected with p53 siRNA or negative control siRNA. However, when cells were infected with AdSOCS-1, the cell growth inhibition effect was significantly attenuated in cells transfected with p53 siRNA or negative control siRNA. This result indicated that SOCS-1 inhibited the cell growth at least in part by upregulating p53 signal. Moreover, we confirmed that p53 siRNA did not affect p-STAT3 levels in G361 and SK-MEL5 cells (Fig. 3d). Therefore, these results, together with the finding in the Fig. 2b, suggest that SOCS-1 inhibits melanoma cell growth by independently regulating JAK/STAT signal and p53 signal.

SOCS-1 exhibits antitumor activity in a melanoma xenograft model

We also evaluated the therapeutic effect of an AdSOCS-1 injection on the growth of melanoma cells in vivo. We established a xenograft model of ICR nu/nu mice in which G361 and SK-MEL5 cells were subcutaneously implanted. Injection of AdSOCS-1 vector (4 × 10^8 pfu/100 μl) intratumorally twice a week significantly suppressed tumor growth compared with control AdLacZ injection (Fig. 4a). The Western blot analysis of each tumor tissue indicated that SOCS-1 was overexpressed and that p-STAT3 (Tyr705) levels were inhibited in tissues from AdSOCS-1-injected mice (Fig. 4b). By immunohistochemical staining analysis and TUNEL
staining, the expression of SOCS-1 and apoptosis was stronger in tissues from AdSOCS-1-injected mice than in those from AdLacZ-injected mice (Fig. 4c and d). SOCS-1 positive cells account for about 80–90% in tissues from AdSOCS-1-injected mice, whereas about 0–5% in tissues from AdLacZ-injected mice (Fig. 4c). These results suggest that the overexpression of SOCS-1 induced apoptosis in melanoma cells in vivo.

Discussion

In this study, we focused on investigating the impact of the JAK/STAT signal in melanoma and the inhibition of this signal as an effective antitumor therapy. The signalling pathway that comprises JAKs and STAT proteins is important for relaying signals from various cytokines (14). IL-6, one of these cytokines, is reported to be associated with faster cell growth progression in melanoma (15–17). However, IL-6 blockage failed to show a growth inhibitory effect against melanoma cells in vitro. In this study, JAK inhibitor I or knockdown of STAT3 demonstrated a growth inhibitory effect in vitro. These results suggest that the cell proliferation in G361 and SK-MEL5 cells occurs independently of the IL-6 signalling pathway; therefore, other cytokines that activate JAKs may be involved in melanoma cell proliferation, similar to the cell lines of gastric cancer as previously reported by our group (28).

Upon induction, SOCS-1 suppresses JAK/STAT signalling pathway, but is frequently silenced in melanoma cell lines (25). Next, we showed that the overexpression of SOCS-1 inhibited the proliferation of G361 and SK-MEL5 malignant melanoma cells via inhibition of JAK/STAT signalling pathway in a dose-dependent manner. In SK-MEL28, the overexpression of SOCS-1 failed to inhibit the cell proliferation markedly. This may be explained by the finding that the constitutively activated level of STAT3 in SK-MEL28 is very low. As for the mechanisms to inhibit the cell proliferation by the overexpression of SOCS-1 in G361 and SK-MEL5, we demonstrated the occurrence of apoptosis and cell cycle regulation. STAT3 has been reported to mediate the transcription of some anti-apoptotic proteins (30,31). In our data, the overexpression of SOCS-1 induced a decrease in the expression of anti-apoptotic proteins, Mcl-1 and Bcl-xl, and significantly increased caspase-3 activity in a dose-dependent manner. In studies of cell cycle regulation, stable SOCS-1-transfected melanoma cells have been reported to lead to the G1/S transition defect, a secondary blockage in mitosis and accumulation of cells in the metaphase (32). Our study also shows that the overexpression of SOCS-1 induced the G0/G1 arrest in G361 and SK-MEL5 cells (Fig. 1e). The disruption G2/M transition may differ among cell lines.

SOCS-1 is a JAK/STAT signal inhibitor, and so it is necessary to compare the effect of JAK inhibitor with that of AdSOCS-1. JAK inhibitor I, as well as AdSOCS-1, induced apoptosis and inhibited cell growth. However, AdSOCS-1, but not JAK inhibitor...
I, upregulated the expression and phosphorylation levels of p53, which is important for the transmission of the suppressor signal. This finding is reasonable because the activation of p53 can also lead to cell cycle arrest, DNA repair and apoptosis (33). Parrillas et al. (32) also described that SOCS-1 upregulated the expression levels of p53 using a melanoma cell line, BLM. However, BLM has been reported to harbour a p53 mutation (34). To enhance the p53 function by SOCS-1, p53 should be wild type. In fact, G361 and SK-MEL5 cell lines which we used in in vitro and in vivo studies have wild type p53. Overexpression of SOCS-1 has been reported to inhibit the proliferation of gastric cancer cell line via the suppression of p38 MAPK activity, in addition to its JAK/STAT signal activity (28). SOCS-1 also inhibits the proliferation of non-small-cell lung cancer cell line via the suppression of FAK and EGFR and the activation of p53 (29). Therefore, it is likely to be different among cell lines which signals other than the JAK/STAT3 pathway were inhibited by the overexpression of SOCS-1.

Collectively, SOCS-1 appears to be a promising candidate for therapy, which does not easily promote cellular resistance. Although we also investigated several signalling pathways associated with melanoma cell growth, such as the Ras-Raf-MEK-ERK signal and the PI3K-AKT-mTOR signal, SOCS-1 did not affect other signalling pathways associated with melanoma cell growth besides the p53 signal. The reason for the failure of SOCS-1 to suppress MAPK signalling in G361 and SK-MEL5 may be attributed to the mutation and constitutively activation in BRAF in G361 and SK-MEL5 (35). Moreover, only SK-MEL28 has a mutation of p53 (36,37). Therefore, low levels of constitutive activation of STAT3, as well as mutation of p53 in SK-MEL28, may be the reason why SOCS-1 does not inhibit cell proliferation significantly.

In melanoma, it has been reported that STAT3 is constitutively activated in clinical specimens, and p-STAT3(Tyr705) expression has been detected immunohistologically in thick melanoma (>1.0 mm) and melanoma metastases (11). Moreover, although inactivating mutation or allelic loss of TP53 is common in human cancers (38), the TP53 locus is intact in the majority (>95%) of melanomas and the incidence of point mutation or allelic loss of TP53 is very low (39,40). Given these circumstances, SOCS-1 is a potentially effective therapy in melanoma.

In our data, phosphorylation of p53 at Ser15 and Ser392, as well as expression levels of p53, was upregulated in G361 and SK-MEL5 by the overexpression of SOCS-1. Phosphorylation of p53 at 15 occurs after DNA damage; this leads to a reduced interaction between p53 and its negative regulator, the oncoprotein MDM2 (41). MDM2 inhibits p53 accumulation by targetting it for ubiquitination and proteasomal degradation, and the interference between p53 and MDM2 promotes both the accumulation and the activation of p53 in response to DNA damage (40,41). Phosphorylation of p53 at Ser392 has been reported to be induced by DNA damage. It plays a role in the activation of the sequence-specific DNA binding of p53, and probably also in the stabilization of the p53 tetramer, which is crucial for its activity and influences the transcriptional activation of p53 (42).

It has been reported that the mechanism of p53 activation by SOCS-1 involves a direct interaction between the SH2 domain of SOCS-1 and the N-terminal transactivation domain of p53 (43). Our study also showed that the SH2 domain in SOCS-1 forms a complex with p53. Therefore, SOCS-1 interacts with p53 and may enhance p53 stability, which enhances the interaction of p53 with other proteins that promote p53 protein degradation. We also demonstrated that AdSOCS-1-mediated inhibition of melanoma cell proliferations was attenuated by the knockdown of p53 by siRNA. This may be because p53 induced a stronger antitumor effect caused by increased stability induced by SOCS-1.

In conclusion, we demonstrated the antitumor effect and mechanisms of SOCS-1 against G361 and SK-MEL5 melanoma cells both in vitro and in vivo. New molecular targets or combined therapy in malignant melanoma now need to be identified to help more patients. SOCS-1 targets multiple signals and is easy to inject intratumorally. We hope that these findings may lead to the successful clinical application of SOCS-1 for melanoma.

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Author contributions
T.N. designed the research study. N.T. conducted the research, analysed the data and wrote the manuscript. S.S. conducted the research and analysed the data. R.N. conducted the preparation of adenoviruses. T.O. supported the production of deletion mutant. M.F., A.T., H.M., T.K., I.K. and T.N. contributed to interpretation of data and revised the manuscript critically.

Conflict of interests
The authors have no conflict of interest.

Supporting Information
Additional supporting data may be found in the supplementary information of this article.

Figure S1. Anti-IL-6R monoclonal antibody does not inhibit the growth of melanoma cells, G361 and SK-MEL5.

Figure S2. Overexpression of SOCS-1 induces attenuation of p-STAT3(Tyr705) in a dose-dependent manner in G361 and SK-MEL5.

Figure S3. JAK inhibitor I also induces apoptosis.

Figure S4. The structural map of SOCS-1 and SOCS-1ASH2, which deleted the functional region of the SH2 domain

Appendix S1. Material and Methods.

References
SOCS-1 suppresses malignant melanoma growth
