

Inhibition of the JAK2/STAT3 signaling pathway exerts a therapeutic effect on osteosarcoma

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Abstract. Osteosarcoma (OS) is the most common type of malignant bone tumor. Despite aggressive multimodal treatments, including surgical resection, chemotherapy and adjunctive immunotherapies, patients with OS with high-grade malignancy have a poor five-year survival rate that has remained unchanged over the past two decades, highlighting the urgent requirement for novel therapeutic approaches. Signal transducers and activators of transcription 3 (STAT3) has been implicated as an oncogene and therapeutic target in a variety of neoplastic diseases. The aim of the present study was to determine whether inhibition of the janus kinase 2 (JAK2)/STAT3 pathway by FLLL32, a specific JAK2/STAT3 inhibitor, is able to provide a potential therapy for OS. FLLL32 inhibited OS cell growth *in vitro* and delayed OS growth in an OS xenograft nude mouse model. STAT3 knockdown by short hairpin RNA delayed OS formation *in vivo*. Thus, the JAK2/STAT3 pathway is important in OS formation. Efficacy of the FLLL32 pharmacological inhibitor in delaying OS growth suggests that targeting JAK2/STAT3 may be a potential therapeutic strategy for patients with OS.

Introduction

Osteosarcoma is the most common type of bone sarcoma. It affects ~560 infants and adolescents annually in the United

States (1). There are two peaks in incidence, the first is during the pubertal skeletal growth spurt (15-19 years old) and the second is during old age (75-79 years old). The latter is attributed to the sarcomatous transformation of Paget's disease of bone and other benign bone lesions (1). Twenty percent of patients with osteosarcoma (OS) present with clinically detectable metastases, with micrometastases presumed to be present in a number of the remaining patients (2).

The management of patients with OS is complicated. Current treatments typically include preoperative chemotherapy, surgical resection, postoperative chemotherapy and adjunctive immunotherapy (2,3). Limb-salvage procedures with wide surgical margins that aim to completely remove all clinically detectable tumors surgically are the mainstay of surgical intervention (1). The use of intensive chemotherapeutic agents, such as high-dose methotrexate with leucovorin rescue, adriamycin, cisplatin, ifosfamide and cyclophosphamide has improved relapse-free survival rates in patients with localized extremity tumors from <20% with surgery only, to ~70% (1-3). However, unfortunately the efficacy and toxicity of these agents limits their use (4). Despite aggressive multimodal treatment, patients with synchronous and metachronous metastatic disease with local relapse and nonresectable primary disease have a poor 5-year survival rate, which has remained unchanged over the past two decades (3). This highlights the urgent requirement for novel, innovative therapeutic approaches, such as targeted therapies (5,6) and interferon-mediated immunotherapy (7).

Signal transducers and activators of transcription 3 (STAT3) is a latent transcription factor that participates in the transcriptional activation of apoptosis and cell cycle progression (8). STAT3 regulated genes are involved in invasiveness, proliferation, angiogenesis, lymphangiogenesis and inflammation. It has been implicated as an oncogene in a variety of neoplastic diseases as well as OS (8,9). A study demonstrated that mice lacking STAT3 undergo incomplete gestation (10). Dysregulation of STAT3 has been implicated as a key participant in tumor cell survival, proliferation and metastasis (8). A constitutively active mutant of STAT3 has been demonstrated to transform fibroblasts and induce tumor formation in nude

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mice and OS transformation (11). STAT3 activation (phosphorylation) has been demonstrated in a subset of human OS tissues, and human and canine OS cell lines (12,13), and OS stem like cells (9,14). Gibbs *et al* (14) showed the existence of a small subpopulation of self-renewing bone sarcoma cells that are capable of forming suspended spherical clonal colonies (also termed sarcospheres) in anchorage-independent serum-starved conditions. These bone sarcoma cells express activated STAT3 (14). Murase *et al* (9) demonstrated that a side population in OS cell lines induce tumorigenesis in nude mice. STAT3 is often correlated with a malignant tumor phenotype and STAT3 expression in OS can be used as a prognostic predictor. The high level of STAT3 protein is associated with poor tumor differentiation and presentation of metastasis (15). Moreover, the 5-year overall and relapse-free survival rates for patients with OS with high STAT3 expression are lower than those for patients with low STAT3 expression (15). In addition, the status of STAT3 protein expression was an independent prognostic factor for disease-free survival and overall survival (12,16).

Materials and methods

Cell culture. The 143.98.2 OS cell line was obtained from American Type Culture Collection (ATCC CRL-11226™; Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum and 1% penicillin/streptomycin (GE Healthcare Life Sciences, Logan, UT, USA) at 37°C with 5% CO₂. For drug treatment, inhibitors were added 2 h after plating cells. FLLL32 was provided by Dr James Fuchs (17). NSC74859 was purchased from Selleckchem (Houston, TX, USA). The study was approved by the ethics committee of the Second Affiliated Hospital of Soochow University (Suzhou, China).

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. The MTS assay was performed as described previously (18). Briefly, OS cells were plated in quadruplicate for each dose of STAT3 inhibitor on 96-well plates at a density of 500 cells/well in serum-containing growth medium. Plates were incubated at 37°C and 5% CO₂. Two hours after plating, cells were treated with carrier (0.1% dimethyl sulfoxide), FLLL32 or NSC 74859 at 1, 10, 100, 1,000 or 10,000 nM. Proliferation was quantified four days following the addition of drug by an MTS assay using a Cell Titer 96 Proliferation kit (Promega Corporation, Madison, WI, USA). Absorbance was read at 490 nm in a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Immunohistochemistry and histology. Tissue was embedded in paraffin and blocks were cut to generate 6-μm sections. Deparaffinized sections were stained with either hematoxylin and eosin or incubated overnight at 4°C with the following antibodies: Polyclonal rabbit anti-human NCL-Ki67p antibody (1:1,000; #KI67P-CE; Leica Microsystems, Inc., Buffalo Grove, IL, USA) or monoclonal rabbit anti-human pSTAT3 (Tyr705) (1:1,000; #9145; Cell Signaling Technology, Inc., Danvers, MA, USA). The following day, sections were incubated in horseradish peroxidase (HRP)-labeled secondary

antibody and staining was detected by DAB (DAKO, Herndon, VA, USA). Sections were viewed with a Axiovert 200 inverted microscope (Carl Zeiss, Thornwood, NY, USA) equipped with a digital imaging system.

Western blot analysis. Tumor proteins were extracted using extraction buffer (20 mM NaPO₄, 150 mM NaCl, 2 mM MgCl₂, 0.1% Nonidet P-40, 10% glycerol, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 μM phenylalanyl oxalate, 10 mM okadaic acid, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml tosyl-L-phenylalanine chloromethyl ketone and 10 μg/ml N^α-tosyl-L-lysine chloromethyl ketone). Protein concentration was estimated using Coomassie® Plus Protein Assay Reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (40 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4-20% or 16% tris-glycine gel (Invitrogen Life Technologies, Carlsbad, CA, USA) and electrotransferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk+0.1% Tris-buffered saline with Tween-20 to minimize nonspecific binding. The pSTAT3 antibody; monoclonal rabbit anti-human STAT3 (1:1,000; #4904) and cleaved caspase 3 (Asp175) (1:2,000; #9664) antibodies; and polyclonal rabbit anti-human β-actin antibody (1:10,000; #4967) (Cell Signaling Technology, Inc.) were used. Binding of the HRP-conjugated secondary antibody to the membrane was visualized using a chemiluminescent detection system (Amersham, Arlington Heights, IL, USA). Anti-β-actin was used as a loading control. At least three different tumor lysates were analyzed for each antigen.

Lentiviral infection. 143.98.2 OS cells were infected at 60% confluence with human shSTAT3 (TRCN0000353630) or non-target control (pLKO.1-puro-non-target control, cat. no. SHC016; Sigma-Aldrich, St. Louis, MO, USA). Lentiviral particles were incubated with OS cells in the presence of polybrene (8 μg/ml; Sigma-Aldrich) daily for 2 days, followed by selection in puromycin (2 μg/ml; Sigma-Aldrich) for at least five days. Surviving cells were collected for xenograft injection.

Mouse xenograft. Female athymic nude (nu/nu) mice (Harlan Laboratories, Harlan, IN, USA) were maintained in a pathogen-free environment with free access to food and water, 12 h on/off light cycle, 22°C temperature, and 21-22% oxygen. A total of 143.98.2 OS cells (2×10⁶) were injected in a total volume of 150 μl 30% Matrigel (BD Biosciences, San Jose, CA, USA) into the flanks of 5- to 6-week-old mice; right flanks were injected with shSTAT3 cells and left with non-target control cells. Tumor volume was calculated as $L \times W^2 (\pi/6)$, where L is the longest diameter and W is the width. For the drug treatment xenograft, 143.98.2 OS cells were injected only into the right flanks of nude mice. Tumors were dissected 1 h after the final dose was administered and immediately flash frozen in liquid nitrogen for biochemical analysis or fixed in 4% paraformaldehyde for histological analysis.

In vivo drug treatment. FLLL32 was dissolved in 12.5% alcohol+12.5% cremaphor (Sigma-Aldrich) by boiling for

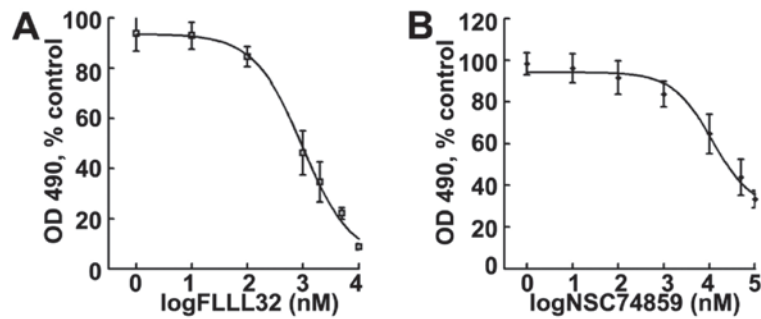


Figure 1. Inhibition of JAK2/STAT3 decreases osteosarcoma cell growth *in vitro*. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt assay demonstrated a dose-dependent decrease in proliferation of cells in response to (A) FLLL32 and (B) NSC74859. JAK2, janus kinase 2; pSTAT3, phosphorylated signal transducers and activators of transcription 3 transcription factor.

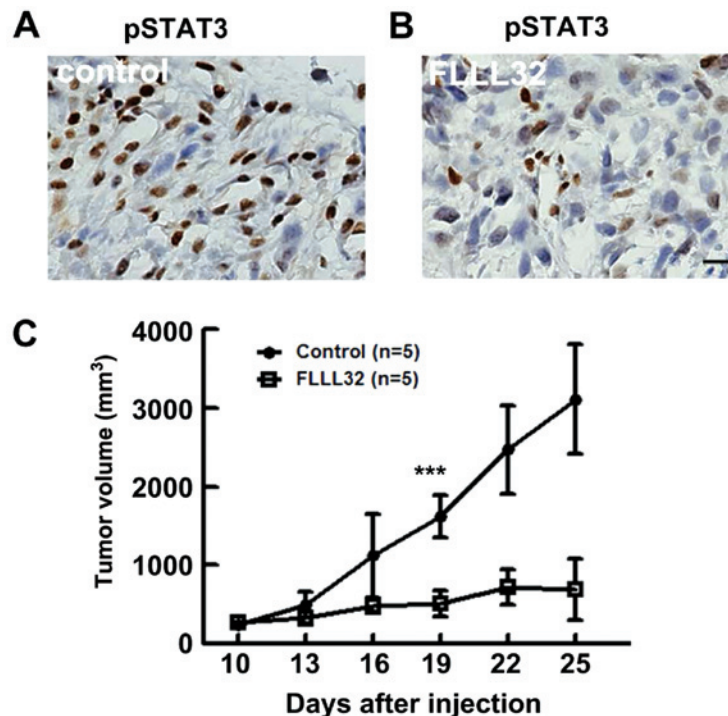


Figure 2. JAK2/STAT3 inhibitor, FLLL32, delays mouse OS growth *in vivo*. Representative images of immunostaining of pSTAT3 expression in (A) vehicle control- and (B) FLLL32-treated OS xenografts. pSTAT3 is in DAB (brown), blue is hematoxylin counterstain. Scale bar, 50 μ m. (C) Inhibitory effect of FLLL32 on 143.98.2 mouse OS growth in nude mice. A significant difference in the tumor size was observed in mice in the FLLL32 (200 mg/kg) daily treatment group (n=5) compared with vehicle treated controls (n=5) (P<0.001) ***P<0.001 vs. control group. JAK2, janus kinase 2; pSTAT3, phosphorylated signal transducers and activators of transcription 3 transcription factor; OS, osteosarcoma.

15 min. FLLL32 was prepared fresh every other day. FLLL32 (200 mg/kg) or vehicle (12.5% alcohol+12.5% cremaphor) were administered to tumor bearing nude mice daily for 3 weeks by intraperitoneal injection in 200-300 μ l based on mouse weight. Mice were weighed twice a week in order to monitor potential side effects of weight loss. The treated mice were sacrificed with 100% CO₂ for 5 min and cervical dislocation subsequent to 15 consecutive days of treatment, when the tumor size reached >10% of the mouse body weight.

Statistical analysis. All statistical analyses were performed by unpaired, two-tailed Student's t-test using Microsoft Excel 2000 software (Microsoft Corporation, Redmond, WA, USA). P<0.05 was considered to indicate a significant difference.

Results

JAK2/STAT3 inhibition prevents OS cell proliferation *in vitro*. To determine whether the JAK2/STAT3 pathway is involved in OS cell proliferation, a dose-response analysis of FLLL32, a specific JAK2/STAT3 inhibitor on a human 143.98.2 OS cells, was conducted. After 4 days of treatment, FLLL32 decreased cell survival in a dose-dependent manner. The average IC₅₀ was ~500 nM (Fig. 1A). The effects of another STAT3 inhibitor, NSC74859, on the same human 143.98.2 OS cell line were then observed. NSC74859 showed similar effects as FLLL32, decreasing human OS cell survival (Fig. 1B). These results suggest that JAK2/STAT3 mediated STAT3 signaling contributes to osteosarcoma cell proliferation *in vitro*. JAK2/STAT3 inhibition prevents OS cell growth.

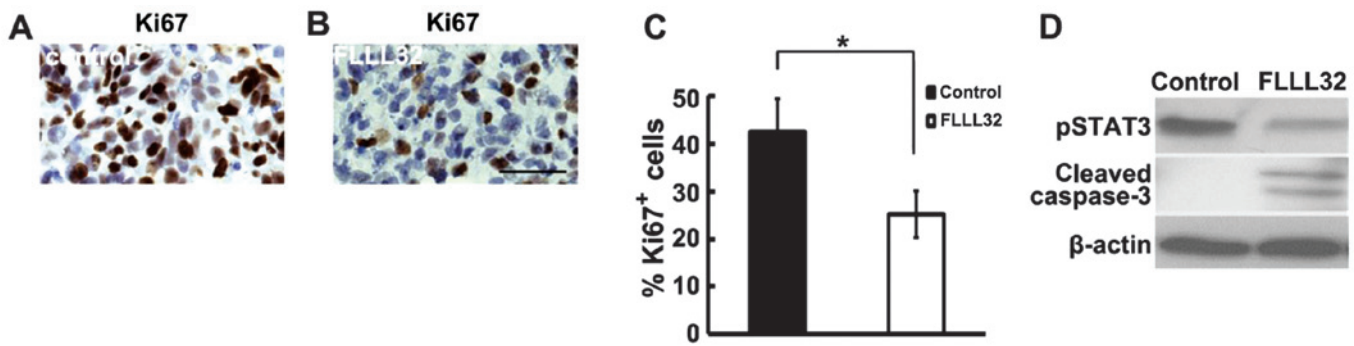


Figure 3. FLN32 inhibited OS cell proliferation and induced cell apoptosis *in vivo*. Representative images of immunostaining of Ki67 expression in (A) vehicle control- and (B) FLN32-treated OS xenografts. Ki67 is in DAB (brown), blue is hematoxylin counterstain. Scale bar=50 μ m. (C) Quantification of Ki67. $P<0.05$, compared with control; $n=5$ for each group. (D) Western blot analysis of pSTAT3 and cleaved caspase 3 in OS xenografts following treatment with FLN32. β -actin was used as a loading control. pSTAT3, phosphorylated signal transducers and activators of transcription 3 transcription factor; OS, osteosarcoma.

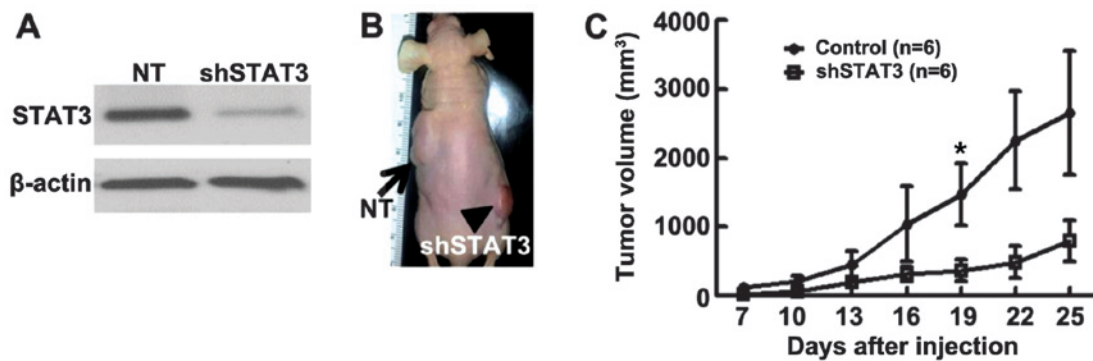


Figure 4. Genetic inhibition of STAT3 prevents the growth of 143.98.2 OS xenograft in nude mice. (A) Western blot analysis confirming knockdown of STAT3 in human 143.98.2 OS cells infected with shRNA-STAT3 lentivirus. The control was a non-target shRNA. (B) Athymic nude mice were injected subcutaneously with shRNA STAT3 (shRNA)-expressing 143.98.2 OS cells in right flank (black arrowhead), and non-target shRNA-expressing cells in the left flank (NT, black arrow). Tumor growth was detectable on both sides. The image of the tumor was captured at day 25. (C) Tumor volume was measured twice weekly, and mice were sacrificed at day 25. Tumor volume calculations demonstrate that shSTAT3 inhibits the growth of 143.98.2 OS xenografts ($n=6$) compared with the non-target ($n=6$). $P<0.05$ vs. control group. STAT3, signal transducers and activators of transcription 3 transcription factor; shRNA, short hairpin RNA; OS, osteosarcoma.

Pharmacological inhibition of JAK2/STAT3 delays OS xenograft growth in vivo. It was then analyzed whether blocking the JAK2/STAT3 pathway may affect growth in a human 143.98.2 OS xenograft model. Nude mice with average OS tumors ~ 150 mm³ ($n=5$) were treated for 10 days after cell transplantation with FLN32 (200 mg/kg) daily via intraperitoneal injection. All mice survived the treatment period without significant weight loss. Following sacrifice, immunostaining was performed to determine pSTAT3 levels at the end of the experiment with or without FLN32 treatment. The pSTAT3 level was significantly lower in the treatment group compared with that of vehicle controls, indicating that the drug was effective at this time point (Fig. 2A and B). Tumor volume measurements demonstrated that FLN32 decreased OS growth significantly compared with vehicle controls ($P<0.001$) (Fig. 2C).

Cell proliferation and cell death were then analyzed in paraffin sections of excised OSs by Ki67 staining and western blot analysis, respectively. There was a significant difference in the percentage of Ki67⁺ proliferating cells in FLN32-treated OS ($n=5$) compared with vehicle controls ($n=5$, $P<0.05$) (Fig. 3A-C). Western blot analysis demonstrated decreased pSTAT3 and increased cleaved caspase 3 in FLN32-treated OS xenografts as compared with vehicle control (Fig. 3D). The

results suggest that FLN32 delayed OS xenograft growth by inhibiting cell proliferation and inducing cell apoptosis.

STAT3 deficiency delayed OS formation in vivo. With any chemical inhibitor, there is the potential for lack of specificity. Therefore, to confirm the role of STAT3 in OS formation, 143.98.2 OS cells were infected with a lentivirus encoding shRNA targeting STAT3. A non-targeting shRNA with the same backbone served as a control. Western blot analysis confirmed that the STAT3 level was decreased following infection of OS cells with this vector (Fig. 4A). MTS assay of shSTAT3 expressing cells revealed decreased numbers of viable cells, but in contrast to cells exposed to JAK/STAT3 inhibitors, cleaved caspase-3 was not detectable, suggesting that these shSTAT3 infected cells were not dying by apoptosis (data not shown). sh-non-target control 143.98.2 OS cells were injected subcutaneously into the left side of nu/nu (nude) mice, and shSTAT3 transduced 143.98.2 OS cells to the right side of nu/nu mice ($n=6$ for each group). Tumor volumes were measured every 3 days starting at 7 days after transplantation. Tumor growth was detected on both sides of the animals (Fig. 4B, control: black arrow; shSTAT3: black arrowhead). However, the left side, which received the non-target shRNA-expressing OS cells, exhibited significant

tumor growth compared with the right side, which received the same number of shSTAT3-expressing OS cells (Fig. 4C). Mice were sacrificed at day 25, when volumes of control non-target tumors reached allowable limits. These results confirm a key role for STAT3 in OS tumor formation in xenografts.

Discussion

The present study demonstrated that the JAK2/STAT3 inhibitor, FLLL32, inhibited human OS cell growth *in vitro* as well as delayed human OS xenograft growth *in vivo*. Genetic knockdown of STAT3 by shSTAT3 significantly delayed OS xenograft tumor formation *in vivo*. These data support a vital role of the JAK2/STAT3 pathway in OS formation.

The data indicate that STAT3 activation (phosphorylation) contributes to the survival and proliferation of OS cells, and is crucial in the development and progression of OS by promoting cell proliferation and protecting against apoptosis, providing a potential promising molecular target for gene therapy in human OSs. Similar results were detected using a STAT3 inhibitor S31-201 (19). Current OS chemotherapies that prolong life do not specifically target the OS initiating cells (OS stem-like cells), such as CD271⁺ OS cells. These cells possess the self-renewal, differentiation and proliferation properties of stem cells. They also overexpress Nanog, Oct3/4 and STAT3 and are often resistant to traditional radiation chemotherapies (20). The results of the present study indicated that STAT3 may be important to the CD271⁺ tumor initiating cells in OS, and blockade of the STAT3 pathway may provide a promising target for removing these OS initiating cells that potentially contribute to OS formation.

The partial delay in tumor growth following treatment with FLLL32 suggests that drug combination may synergize the therapeutic effects. The blockade of STAT3 protein signaling can be achieved by various means, including dominant-negative mutants, antisense methods, inhibition of upstream signaling, phosphotyrosyl peptides, the double-stranded DNA decoy method and RNA interference (21-24). Testing different combinations of these treatments is required to determine an effective therapeutic strategy.

Overall, the present data demonstrated that STAT3 appears to govern the propagation of OS initiating cells. Current cancer therapies that prolong life do not specifically target cancer-initiating cells, and these cells are often resistant to traditional radiation and chemotherapies. Targeted inhibition of STAT3 in these OS initiating cells provides a novel approach for the treatment of OS.

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