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Research Article

Activation and Role of Stat3 in Human Primary Osteosarcoma

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Abstract

Osteosarcoma is the most common primary bone tumor in children and adolescents, and numerous patients either are not sensitive to chemotherapy or develop drug resistance to current chemotherapy regimens. Therefore, it is necessary to develop several potentially useful therapeutic agents. We investigated the potential for targeting signal transducer and activator of transcription-3 (Stat3) to improve the therapeutic outcome. In this study, we initially showed that Stat3 is over-activated in a variety of human osteosarcoma cell lines and clinical samples (20/25cases). Moreover, we evaluated the therapeutic response of targeting Stat3 using shRNA or Stat3 inhibitor (napabucasin). Inhibiting Stat3 delayed tumor growth *in vitro* by inducing the apoptosis. We have utilized chemotherapy regimen to enrich stem cells in osteosarcoma and found Stat3 was further activated in the enriched stem cells. Next studies suggested napabucasin could decrease the sarcosphere formation and target stem cells in osteosarcoma; Downregulation of stemness markers was observed after U2OS were treated with napabucasin by qRT-PCR. Further investigation found that napabucasin inhibited osteosarcoma growth in vivo without severe side effects. In conclusion, our data demonstrate that Stat3 inhibition has antitumor potential and napabucasin is an effective inhibitor of osteosarcoma stem cells.

Keywords: Napabucasin; Osteosarcoma; Stat3; Stem cell

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in childhood and adolescence. Despite aggressive treatment, about a third of affected patients die of their disease [1]. Current treatment with neoadjuvant chemotherapy followed by surgical resection and additional adjuvant chemotherapy results in a 5-year-survival rate of 60–70% for people with non-metastatic osteosarcoma treated with combinations of methotrexate, cisplatin, doxorubicin and ifosfamide [2,3]. Survival rates have improved little over the past 30 years, now and there is a continued need for new therapeutic approaches for further improvement of OS patient prognosis [4]. Recently, the use of molecular-targeted cancer therapy has been receiving attention for various tumors, because of several potential advantages in features such as drug metabolism and accumulation, optimum doses, and side effects, over conventional anticancer agents [5]. Molecular-targeted therapy is currently favored as a replacement for conventional OS therapies.

Signal transducer and activator of transcription 3 (Stat3) acts as a point of convergence for several oncogenic signaling pathways and is persistently activated in numerous tumors, which is well known for promoting tumor cell survival and proliferation [6,7]. Besides, a number of the immunosuppressive factors, such as VEGF, produced by tumor cells in a Stat3-dependent manner are also angiogenic factors. A role of constitutively activated Stat3 in tumor cells in promoting tumor angiogenesis and metastasis has been documented [6]. We analyzed the expression of Stat3 in several osteosarcoma cells and several specimens from osteosarcoma patients, and in most of them we observed Stat3 activation. We therefore hypothesized that Stat3 inhibition may have anti-osteosarcoma effects.

The main purpose of this study was to evaluate whether STAT3 could be a good target for treating osteosarcoma. The importance of this study is that treatment with anti-STAT3 drugs directed against this specific target, which is aberrantly activated only in tumors, may be more effective and have fewer side effects than conventional therapy. Our findings show that inhibition of STAT3 with short hairpin RNA (shRNA) suppresses osteosarcoma.

Results

p- Stat3 is highly expressed and activated in human osteosarcoma

Stat3 has been detected in a variety of human cancer cell lines and tissue sections, including human colon, breast, and cancers, and was associated with poor prognosis [8-10]. To evaluate the activitation of stat3 in osteosarcoma, we firstly analyzed the expression of p-Stat3 and Stat3 in osteosarcoma cell lines by

western blotting. As shown in (Figure1 A)., Stat3 was expressed in osteoblast cell line (hfob) and osteosarcoma cell lines (U2OS, MG63, HOS and 143B), but the expression of p-Stat3 is higher in osteosarcoma cell lines compared with osteoblast cell line. Besides, the level of p-Stat3 is much higher in relative high-grade cell line 143B, which has higher metastatic ability than its parental cell line HOS. Next, we further examined p-Stat3 expression in primary human osteosarcoma samples. Clinical samples from 25 cases of osteosarcoma patients treated with standard protocol were collected for IHC, and most of them were grade IIB according to Ennecking staging system. From Figure1 B, we found that osteosarcoma tissues expressed p-Stat3 in both cytoplasm and nucleus.

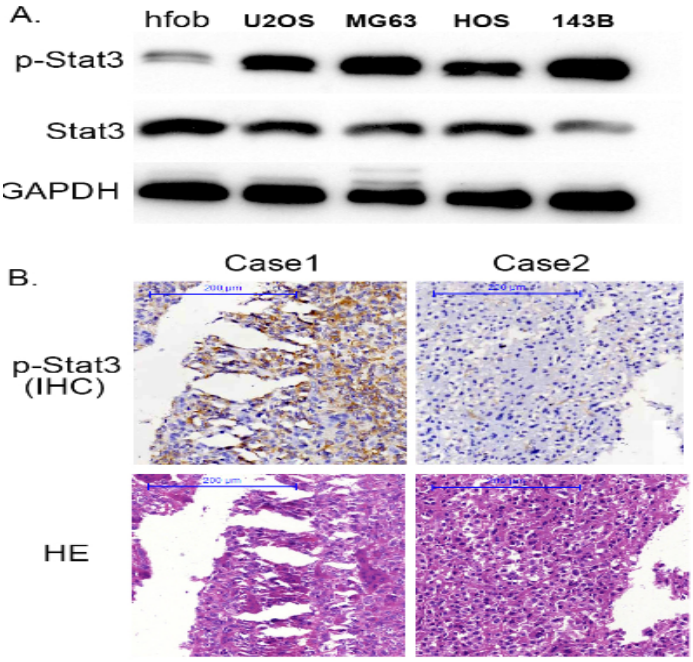

Figure: 1 p- Stat3 is highly expressed and activated in human osteosarcoma.

A: Protein expression of p-Stat3 in osteoblast cell line (hfob) and human osteosarcoma cell lines; **B:** Immuno- histochemical analysis was performed on 3-uM sections from paraffinembedded tissues of 25 patients with osteosarcoma using the primary antibodies against p-Stat3 and HE stain. Among them, twenty of the evaluable specimens (80%) expressed p-Stat3 (13 moderate to high expression and 7 low); only five did not express p-Stat3. Taken together, these initial findings demonstrated that p-Stat3 is both highly expressed and functional in human osteosarcoma cell lines, thereby providing a rationale for examining the effect of stat3 blockade on the osteosarcoma pro-tumorigenic phenotype.

Stat3 blockade inhibits the growth of osteosarcoma and induces cell apoptosis *in vitro*

Targeting Stat3 has shown promising therapeutic effects for human cancers that display activated Stat3, such as neuroblastoma [11,12]. We also found that p-Stat3 was over-expressed in an array of osteosarcoma cell lines and clinical samples. To further confirm the finding that Stat3 activity is associated with osteosarcoma tumorigenesis, we investigated whether alteration of the levels of active Stat3 could modulate growth and clonogenicity of osteosarcoma cell lines. We performed a Stat3 knockdown in cells, which expressed decreased p-Stat3, and the effect on osteosarcoma cell growth was investigated by MTT assays and colony formation. MTT assay demonstrated there is a decrease in osteosarcoma cell proliferation after knockdown Stat3 since the 2nd day, including U2OS and 143B. The number of colonies also significantly reduced in U2OS and 143B cells with decreased p-Stat3 level. These results indicate that reduction of Stat3 expression impairs clonogenicity and tumorigenicity of osteosarcoma cell lines *in vitro*.

To further confirm the antitumor potential of inactivating Stat3, we detected the effects of Stat3 inhibitor Napabucasin which is a newly found small molecule with the ability to inhibit gene transcription of Stat3, which was able to induce cell death and suppress cancer stemness properties. To explore the effect of Napabucasin on osteosarcoma cells, 4 cell lines were treated with Napabucasin in dose-dependent for 48 hours. The calculated IC50 values range from 4.68 to 12.17 uM. Moreover, Napabucasin suppressed the proliferation of U2OS, MG63 and 143B in dose- and time-dependent manner. At the concentration as low as 2.5uM, Napabucasin inhibited the proliferation after 3 days of treatment (Figure 2F). Significant inhibition of cell growth was observed after 1 day at the concentration of 10uM. These results indicate that Napabucasin exhibits antiproliferative capacity against osteosarcoma cells *in vitro* and Stat3 is critical for cell proliferation.

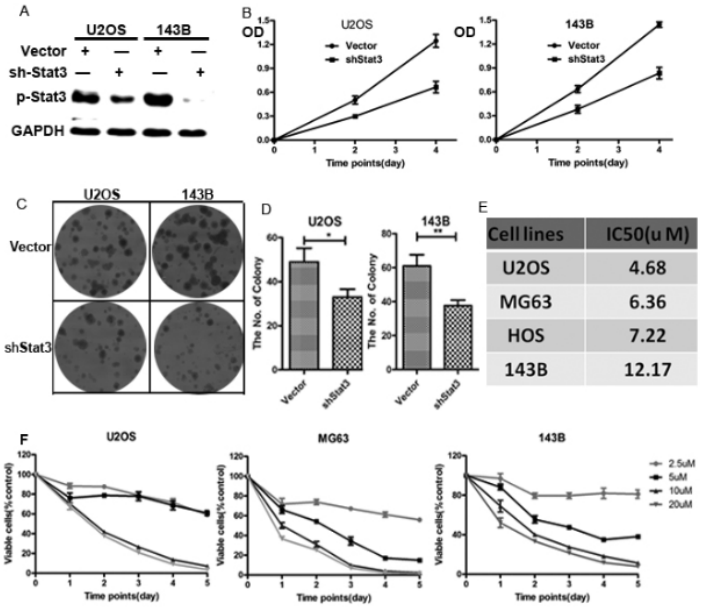

Figure 2: Stat3 inhibition delays the growth of osteosarcoma cells *in vitro*.

A: Western blotting analysis of U2OS and 143B cells transfected with shRNA targeting Stat3; **B:** MTT analysis of the growth of two groups of negative control and knockdown of Stat3 in U2OS and 143B cells; **C:** Colony formation analysis of the cells in B; **D:** The IC50 of Stat3 inhibitor (napabucasin) on osteosarcoma cell lines, **E:** U2OS, MG63, HOS and143B by MTT; **F:** The effect of napabucasin on viability of osteosarcoma cell, including U2OS, MG63 and 143B cells treated with different concentrations as measured MTT assays.

Inhibition of Stat3 shows the potential of antiproliferation in osteosarcoma. Therefore, we sought to determine whether treatment with Napabucasin could induce osteosarcoma cells apoptosis. U2OS and 143B were treated with Napabucasin for 48hours at different concentrations, cell apoptosis of osteosarcoma cells induced by Napabucasin was further analyzed by flow cytometry assays for analyzing the portion of apoptotic cells. Annexin V/PI staining of these cells demonstrated a significant increase in apoptotic cells following Napabucasin treatment (Figure 3A Annexin V and PI double-positive fraction). Both early apoptotic cells (Annexin V single positive) and late apoptotic cells (Annexin V and PI double-positive fraction) were obviously increased in Napabucasin treated U2OS and 143B cells. caspase 3 activity assays were also performed by extracting cellular protein from Napabucasin -treated U2OS and 143B cells, which indicate that caspase 3 activity was statistically significantly increased (eg, caspase-3 activity in U2OS and 143 cells treated for 48 hours with 10umol/L were about 2.34 and 1.95-fold compared with control, in untreated cells). Conclusively, all the above results indicated that Napabucasin induce the cytotoxicity by apoptosis in osteosarcoma cells.

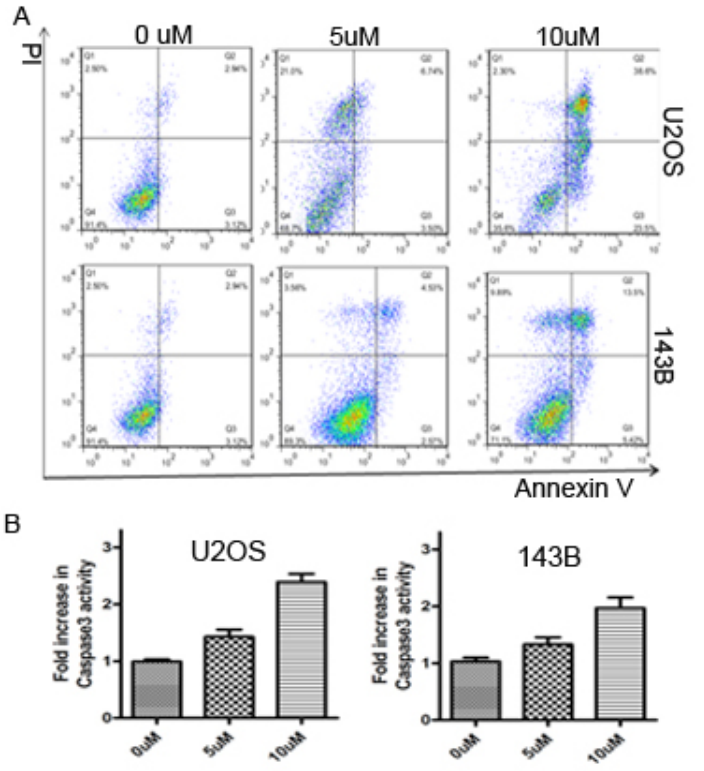

Figure 3: Stat3 inhibitor induces the apoptosis of osteosarcoma cells.

A: U2OS and 143B were treated with napabucasin (10uM) for 48 hours, then the cells were double stained with propidium iodide (PI) and Annexin V and analyzed using flow cytometry to evaluate the apoptosis; **B:** caspase-3 activity assays were performed in U2OS and 143 cells after treatment with napabucasin.

Stat3 inhibitor decreases the osteosphere formation in human osteosarcoma

Recent studies indicate that short exposure to the chemotherapy drug could enrich osteosarcoma stem cells, which exhibit an increased ability to form clones and sarcospheres [13]. However, drugs or compounds that selectively target osteosarcoma stem cells have not been explored thus far. Some evidences show that Stat3 is over-activated in cancer stem cell and is a potential target for deleting the cancer stem cell, such as in pancreatic cancer, prostate cancer [14]. Recently, napabucasin has been identified as a selective inhibitor of prostate cancer stem cells, but its role in the inhibition of osteosarcoma stem cell remains to be determined [15]. Thus, to test whether napabucasin could target osteosarcoma stem cells, we determined the ability of the control and napabucasin treated cells to form osteospheres.

Firstly, we treated U2OS and 143B cells with MTX (100 ng/mL) for 5 days to compare the ability of the MTX treated

cells and their parental cells to generate sarcospheres. As shown in (Figure 4 A&B), MTX treated cells exhibited increased sarcosphere formation compared to parental cells. Both the cell number in single sarcosphere and the total number of sarcosphere are increased. The fold change in MTX-treated U2OS is 2.25, and the fold change in MTX-treated 143B is about 1.80. Besides, we detected the Stat3 activation in MTX treated U2OS and 143B cells, and found that Stat3 is over-activated in MTX-treated cells compared with their parental cells. Thus, we wanted to next determine whether Stat3 inhibitor could target osteosarcoma stem cells. Our results indicated that napabucasin could obviously reduce the ability of osteosphere formation in MTX-treated U2OS and 143B osteosarcoma cells compared with DMSO (Fold change: 2.58 in MTX-treated U2OS cells, 2.70 in MTX-treated 143B cells). OCT4, NANOG, and SOX2 have been defined as the core pluripotent TFs in embryonic stem cells, as well as the markers of cancer stem cells, which are required for self-renewal in many cancers[16]. Therefore, we investigated whether napabucasin could affect the expression of Sox2, OCT4, and NANOG. We found that the mRNA was down-regulated in napabucasin-treated cells compared with control. Above all, the findings indicated that Stat3 may play an important role in maintaining osteosarcoma stem cell characters, and napabucasin is an effective inhibitor for targeting osteosarcoma stem cells.

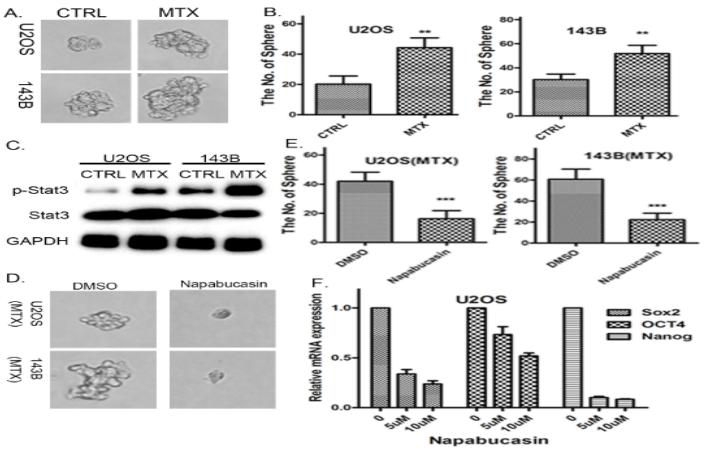

Figure 4: Stat3 inhibitor decreases the sphere formation and suppresses the expression of stem cell markers in osteosarcoma cells.

A&B. Morphology and the numbers of sarcospheres from osteosarcoma cell lines U2OS and 143B treated with MTX; C. The expression of p-Stat3 in osteosarcoma cell lines U2OS and 143B treated with MTX; D&E. Morphology and the numbers of sarcospheres from the cell lines in A. treated with napabucasin. F. The mRNA expression of stem cell markers, including SOX2, OCT4 and Nanog in U2OS treated with napabucasin.

Stat3 inhibition suppressed the tumor growth on osteosarcoma mouse xenograft models

The *in vitro* data prompted us to test the anti-osteosarcoma activity of Stat3 inhibition in mouse xenograft models that $5X10^5$ 143B cells in 200uL PBS were injected into nude mice subcutaneously. Then, the mice were randomly separated into two groups (control group and napabucasin treatment group). The napabucasin treatment groups were treated i.p. with napabucasin at 40 mg/kg. Tumor measurements and body weights were recorded over the courses. Treatment with napabucasin induced significant tumor growth inhibition (see Figure 5A), as compared to the control groups. As shown in (Figure 5B and C), the average tumor weights at the termination of the study were as follows: control group, 0.97 g; napabucasin group, 0.43 g; the mean volume of tumors were: control group, 972.8mm^3; napabucasin group, 480.2 mm^3; In addition, the average body weight of the mice in the two groups was not significantly different (Figure 5D). Our results demonstrate that Stat3 inhibitor napabucasin could suppresse the tumor growth of osteosarcoma in vivo without obvious side effect.

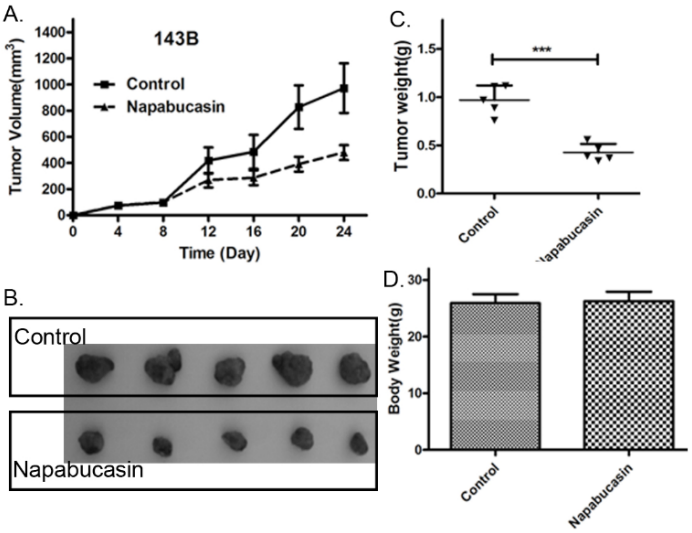

Figure 5: Stat3 inhibition suppressed the tumor growth on osteosarcoma *in vivo*.

A: Examination of tumor volumes to evaluate the effect of napabucasin on 143B cells in a xenograft model; **B:** Xenografts excised from the tumor-bearing mice in A at day 24; **C:** Weights of the xenografts from B at day 24; E, The body weight of tumor-bearing mice in B at day 24.

Discussion

Different chemotherapy regimens, including Methotrexate (MTX), Cisplatin (CDP), Doxorubicin (ADM), and Ifosfamide (IFO) have been used for the treatment of osteosarcoma since the 1970s, which improved the prognosis greatly [17]. Since then, the survival rates were not improved obviously. Besides, chemotherapy resistance is a common problem that can significantly diminish clinical outcomes. Because current chemodrugs for the treatment of osteosarcoma mainly target proliferating tumor cells, which can dramatically reduce tumor bulk but have minimal cytotoxicity on osteosarcoma stem cells. Therefore, it is necessary to develop

several potentially useful therapeutic agents for overcoming the challenge.

In this study, we show that Stat3 is over-activated in a variety of human osteosarcoma cell lines compared with osteoblast cell line (hfob), sustains osteosarcoma cell growth and the character of stem cell. In the clinical samples, we also found the increased activation of Stat3 in clinical osteosarcoma tissues. Stat3 acts as a suppressor of apoptosis in a number of cell lines and inhibition of Stat3 delays tumor growth *in vitro* and *in vivo* by inducing apoptosis in tumor cells. Recent studies have found that Stat3 is a promising target against cancer stem cell, such as in prostate cancer. We enriched osteosarcoma stem cells by chemotherapy, which were over-expressed p-Stat3. We also demonstrated that Stat3 inhibitor decreases the osteosphere formation, which might represent a novel strategy for the treatment of osteosarcoma.

Stat3 are a family of transcription factors that are activated by membrane-bound receptors and that subsequently translocate to the nucleus to promote the functional genes expression. In particular, Stat3 is a transcription factor that can promote oncogenesis and it is commonly activated in cancer as well as in tumor-associated myeloid cells [18-21]. P-STAT3 causes transcriptional activation of downstream genes involved in various processes, such as cell proliferation, differentiation, survival, and angiogenesis. Besides, Stat3 is a convergence point for many signaling pathways. Stat3 is required to maintain tumor NF-kB activity efforts which play have critical roles in the cell survival of osteosarcoma [22,23]. In our results, we demonstrated that Stat3 maintains the growth and survival of osteosarcoma cells. Therefore, efforts are ongoing to develop anticancer drugs that target Stat3. Napabucasin is a newly found small molecule with the ability to inhibit gene transcription of Stat3, which was able to suppress cancer stemness properties and induce cell death, which inhibited the expressions of stemness markers and kill stemness-high cancer cells isolated from several kinds of tumors [15,24-27]. In our study, our results showed that napabucasin not only inhibited cell proliferation, cell survival, colony formation ability, and tumorigenic potential of osteosarcoma cells, and suppressed osteosphere formation. Additionally, the mRNA expressions of stem cell markers in osteosarcoma cells were decreased after treatment with napabucasin. These results suggest the combination of napabucasin and chemotherapy may have complementary and additive antitumor effects on osteosarcoma. More importantly, napabucasin seemed to have no adverse effect on hematopoietic or other normal adult stem cell [15,28]. To our knowledge, these are the first data to show the effect of napabucasin on osteosarcoma stem cells. In summary, our results show that Stat3 is over-activated in cells and clinical samples, and play the key role in maintaining the character of osteoarcoma stem cell. Stat3 inhibition using shRNA and the inhibitor, napabucasin significantly inhibits the progression and tumorigenesis of osteosarcoma cells *in vitro* and *in vivo*. Future studies should focus on exploring the potential mechanisms of napabucasin, for it could be vital in clinical use. Our findings suggest napabucasin might be a novel and effective way to kill the osteosarcoma cells, especially the stem cells.

Materials and methods

Reagents

Napabucasin (2-acetylnaphtho[2,3-b]furan-4,9-dione) was purchased from Selleckchem. Methotrexate (MTX) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-liumbromide (MTT) and were purchased from Sigma (St. Louis, MO). Antibodies against stat3 and phosphorylated-stat3 were from Cell Signaling Technology (Danvers, MA). Antibodies against GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Human osteosarcoma cell line U2OS, MG63, HOS, 143B were cultured according to the instructions from American Type Culture Collection (ATCC). All of the cell lines were grown in Dulbecco's modified Eagle medium (Invitrogen, Grand Island, NY) with 10% FBS (Invitrogen) at 37°C and 5% CO_2.

Cell viability assay

Cell viability was measured using MTT assay. Briefly, osteosarcoma cells were seeded in 96-well plates at a density of 4,000 cells per well. They were treated with different concentrations of Napabucasin for the indicated times, and the cell viability was measured by MTT assays. IC50 values were calculated using GraphPad Prism software.

Apoptosis assays

Cells were seeded at 50% confluency in six-well plates, and after overnight incubation, culture media were replaced with fresh media containing either diluent control or Napabucasin. After 24-hour incubation, was evaluated. The cells were harvested, washed twice with PBS, and resuspended in binding buffer (500 uL, 1–5 $X10^5$ cells). Annexin V and PI (500ul each) were then added to the cells, and the mixture was incubated for 15 minutes in the dark at room temperature. The drug-induced apoptosis were analyzed using a Cytomics FC500 flow cytometer (Beckman Coulter).

Sphere formation assay

Osteosarcoma sphere cells cultured in DMEM/F12 (Invitrogen, Carlsbad, CA) culture medium supplemented with N2 medium (Invitrogen, Carlsbad, CA), human EGF (10 ng/ml, PeproTech, Rocky Hill, NJ), human bFGF (10 ng/ml, PeproTech, Rocky Hill, NJ) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) were treated with either diluent control or Napabucasin in six-well ultra-low attachment plates (Corning Inc., Corning, NY). After culture for 2 weeks, the spheres were counted under a light microscope.

Transduction of shRNA

Cells were transfected with shRNA using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The shRNA against human Stat3 targets the sequence:CCGGGCACA ATCTACGAAGAATCAACTCGAGTTGATTCTTCGTAGATT-GTGCTTTTTG in PLKO.1.

RNA extraction and quantitative real-time PCR

RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed to produce cDNA using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit (Thermo). Real-time PCR amplification was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) on Hard-Shell PCR Plates (Bio-Rad).

Caspase-3 activity assay

The osteosarcoma cells were treated with either diluent control or Napabucasin, and caspase-3 activity assays were performed according to the manufacturer's instructions (Calbiochem, Billerica, MA). Briefly, cellular protein was extracted from treated osteosarcoma cells and the protein concentrations were tested. Then, 10 µL of caspase-3 substrate was added to 30 µg of extracted cellular protein, and after incubation for 2 hours, caspase-3 activity was measured at 405 nm with a microtiter plate reader as recommended in the manufacturer's instructions.

Western blot analysis

The treated cells were collected and washed in cold PBS twice. Then, cells were lysed in lysis buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% (m/v) NP-40, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 0.1 mM NaF and 1.0 mM dithiothreitol). The lysates were clarified by centrifugation at 4℃ for 15 min at 13,000×g. The concentration of protein in the supernatants was measured with the Bradford assay. Then equal amounts of protein were resolved on a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a PVDF membrane (Millipore, Boston, MA, USA). Membranes were blocked for 1h at room temperature with 5% defatted milk (BioRad) and incubated with primary antibody overnight at 4℃. Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the enhanced chemiluminescence system (Pierce, USA).

Immunohistochemical Staining

The phosphorylated-stat3 primary antibody was diluted in 1 : 50. The arrays were deparaffinized in heat oven for 2 hours at 55℃ followed by serial xylene washes. They were rehydrated in graded alcohols, and subjected to antigen retrieval using citrate buffer (pH 6) PT module set for 20 min at 97℃. The slides were incubated with 10% normal goat serum at room temperature for 30 min to reduce nonspecific reaction. Subsequently, the slides were incubated with rabbit monoclonal antibody against phosphorylated-stat3 overnight at 4℃ in a humidified container. After washing with PBS three times, the tissue slides were treated with a non-biotin horseradish peroxidase detection system according to manufacturer's instructions (Dako).

In vivo therapeutic studies

The animal experiments were approved by the Institutional Review Board of Zhejiang University. The 143B cells ($5X10^5$ in 200 uL PBS) were injected near the scapula of the nude mice (SLAC Animal Center, Shanghai, China). Four days after the cell injection, the mice were randomly separated into control group or napabucasin group. Napabucasin (40mg/kg) or PBS was injected intraperitoneally q3d. Tumor length and width were measured with a caliper every 4 days, and tumor volume was calculated using the formula V = 1/2 X $width^2$ X length. Body weights were recorded every 4 days. Mice were humanely euthanized at the 24^{th} day.

Statistical analysis

SPSS 20.0 software (SPSS Inc, Chicago, USA.) was used to perform the statistical analysis. All results were given as the mean ± Standard Deviation (SD) of three independent experiments, and the differences between groups were evaluated using Student t test. P values that were less than 0.05 were considered to connote statistical significance.

Funding

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