Silencing of galectin-3 represses osteosarcoma cell migration and invasion through inhibition of FAK/Src/Lyn activation and β-catenin expression and increases susceptibility to chemotherapeutic agents

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Abstract. Galectin-3 is involved in tumor cell proliferation, adhesion, angiogenesis and metastasis. Galectin-3 promotes β-catenin/Wnt signaling, and β-catenin-related oncogenesis has been frequently reported in osteosarcoma. However, the correlation between galectin-3 and β-catenin signaling in osteosarcoma is poorly defined. We hypothesized that galectin-3 may control the migration and invasion of cancer cells and that silencing of galectin-3 would therefore, suppress motility in osteosarcoma cells. In the present study, we show that galectin-3 silencing in cultured human osteosarcoma cells had decreased cell migration and invasion capabilities; reduced the expression and activation of FAK, Src, Lyn, PI3K/Akt, ERK1/2 and β-catenin, which are key mediators of invasion; inhibited the expression and secretion of VEGF, MCP-1, IL-8, IL-6, MMP2/9 and phospho-Stat3; and potentiated sensitivity to cisplatin. Our results suggest that galectin-3 may be a feasible therapeutic target for osteosarcoma.

Introduction

Galectin-3 (Gal-3) is a chimera-type member of the carbohydrate-binding protein family with high affinity for β-galactosides (1). Gal-3 is located in the nucleus, cytosol, mitochondria, cell surface and extracellular space and is also secreted. Gal-3 has been shown to be involved in extracellular interactions between the cell surface and extracellular matrix glycoproteins and glycolipids, and intracellular interactions between nuclear and cytoplasmic proteins to regulate signaling pathways (2). Gal-3 contributes to various cellular processes, including cell growth, malignant transformation, metastasis, angiogenesis, adhesion, migration and drug resistance (3,4). Gal-3 expression in pancreatic cancer and gastric cancer cells increases cell migration and invasion (5,6). Gal-3 in breast cancer cells greatly enhances adhesion to endothelial cells (7). Overexpression of Gal-3 through the introduction of Gal-3 cDNA increases proliferation of oral tongue squamous cell carcinoma (TSCC) cells and induces invasion and epithelial-to-mesenchymal transition (EMT) (8). Gal-3 also promotes β-catenin/Wnt signaling and induces cyclin D1 and c-Myc in TSCC and breast cancer cells (8,9).

β-catenin is a multi-functional protein that plays a key role at adherent junctions and in Wnt signaling, and deregulation of β-catenin is associated with the development of various human malignancies (10). Binding of canonical Wnts to frizzled (Fz) receptor and low-density lipoprotein 5 or 6 (LRP5/6) co-receptors leads to inhibition of β-catenin phosphorylation and subsequent translocation into the nucleus and activates the expression of Wnt-responsive genes (11). Wnt signaling increases osteoprogenitor cell proliferation and prevents apoptosis (12,13). Based on gene expression profiling of human osteosarcoma, deregulated Wnt signaling is also involved in high metastatic rate and poor long-term survival (14).

Osteosarcoma (OS) is the most extensive malignant bone tumor, occurring primarily in children and adolescents and accounting for ~35% of all cases of bone cancer and the survival rate of OS patients with localized disease is ~65% (15). However, despite significant progress in OS treatment in recent years, because of the high rate of metastasis and relapse and the poor response to combination chemotherapy and radiation therapy, this disease has a poor prognosis and the

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survival rate of patients with metastasis is ~25% (16). Despite aggressive chemotherapeutic treatment strategies, metastatic lesions develop rapidly and the molecular mechanisms that are involved in osteosarcoma growth and metastasis are not fully understood.

EMT is a major phenotype of cancer metastasis and invasion. Wnt/β-catenin and focal adhesion signaling pathways (FAK, Src and paxillin) cooperatively regulate the overall process of EMT (17). Mechanical loading on osteocyte also activates the β-catenin signaling pathway by a mechanism involving nitric oxide, focal adhesion kinase and the Akt signaling pathway (18). Increased β-catenin-mediated activity has been frequently reported in osteosarcoma (19,20). However, the correlation between Gal-3 and β-catenin signaling in OS remained unclear. In the present study, we provide the first demonstration that Gal-3 is implicated in the regulation of migration and invasion of OS cells subjected to expression of β-catenin. We also focus on discovering the molecular mechanism underlying OS metastasis.

Materials and methods

Cell culture. The human OS cell line HOS was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) and antibiotics under a humidified atmosphere with 5% CO_2.

Chemicals. Cisplatin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile dimethyl sulfoxide (DMSO). PD98059 was purchased from Calbiochem (San Diego, CA, USA). LY294002 was purchased from Cell Signaling Technology (Beverly, MA, USA).

Transfection with small interfering RNA (siRNA). Experimentally verified human Gal-3-siRNA duplex, FAK-siRNA duplex, and negative control-siRNA were obtained from Bioneer Corp. (Daejeon, Korea) and siRNA targeting human β-catenin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transiently transfected by electroporation under optimized conditions. Briefly, cells were electroporated with 300 nM siRNA in serum-free medium in a 0.4-cm electroporation cuvette using the Bio-Rad Gene Pulser Xcell System (Bio-Rad Laboratories, Hercules, CA, USA).

Cell proliferation assay. Cell proliferation was measured using the AlamarBlue assay. After 24-h transfection, cells were plated in triplicate in 96-well plates at a density of $5x10^3$ cells/well. After incubation for 24 h, AlamarBlue (Serotec Ltd., Kidlington, Oxford, UK) was added (10% by volume) to each well and relative fluorescence was determined 9 h later using a SpectraMax M2e Multi-Detection Microplate Reader (Molecular Devices, Sunnyvale, CA, USA; excitation, 530 nm; emission, 590 nm). Relative fluorescence unit (RFU) values were expressed as mean ± standard deviation (SD) of three determinations.

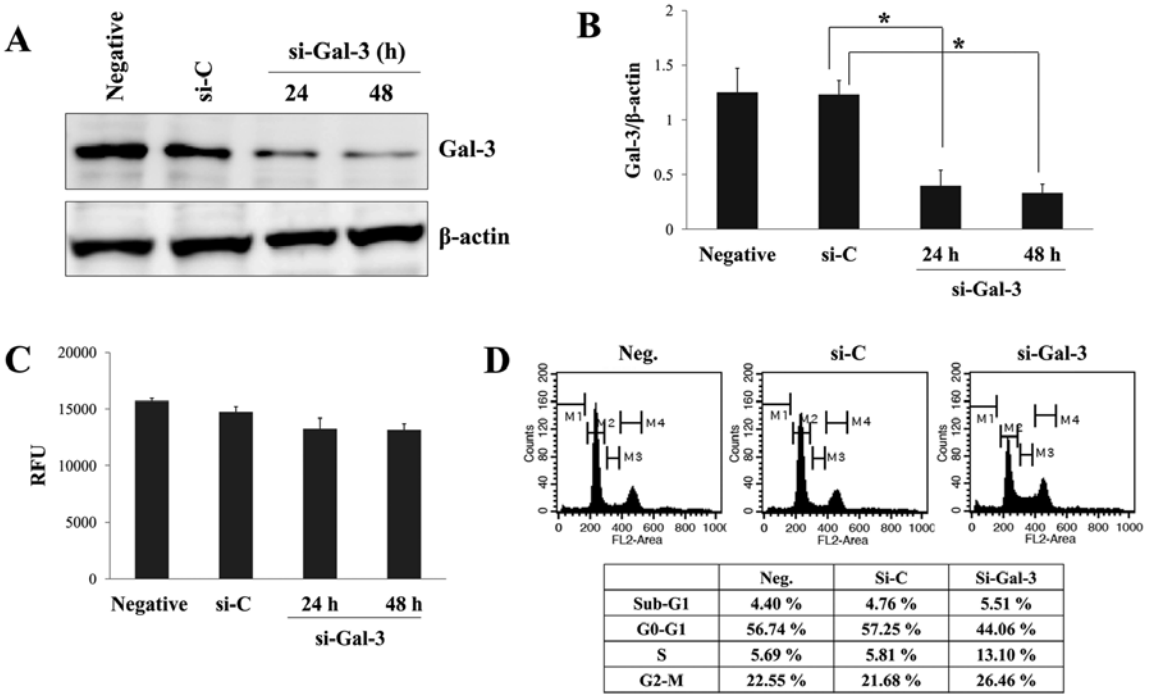
Migration and invasion assay. EBV-infected HCECs transendothelial migration assay was performed using CytoSelect™ tumor transendothelial migration assay kit (Cell Biolabs, Inc., San Diego, CA, USA), according to the manufacturer's protocol. Migrated cells were measured the fluorescence (RFU) by microplate reader. The invasion assay was determined using the CultreCoat 96 Well Medium BME Cell Invasion Assay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. Invaded cells were stained with calcein AM and quantified by microplate reader.

Wound healing assay. Wound healing assays were used to measure the migration ability of bone cancer cells. Transfected HOS cells were plated in 6-well plates. After the cell layers had reached confluence, we inflicted a uniform wound in a straight line in each monolayer using a 200-μl micropipette tip and washed the plates with PBS to remove all cell debris. The cells were cultured in 5% CO_2 at 37˚C and images were taken at 0 and 48 h after scratching using an inverted phase contrast microscope at x100 magnification.

ELISA. Concentrations of IL-6 and IL-8 in the culture supernatants were quantified using a single cytokine ELISA kit (Single Analyte ELISArray; Qiagen, Hilden, Germany). VEGF and Gal-3 were quantified using a single cytokine ELISA assay kit from R&D Systems. The data are expressed as the average of biological replicates ± SD.

Analysis of apoptosis and cell cycle by flow cytometry. The percentage of human HOS cells undergoing apoptosis was determined by flow cytometry using FITC-labeled Annexin V (BD Biosciences, San Diego, CA, USA) and 7-amino-actinomycin D (7-AAD; BD Biosciences). After transfection for 24 h, cells were treated with 1, 2, 5 or 10 μM cisplatin for 24 h and then harvested, rinsed with PBS, and incubated with Annexin V and 7-AAD in Annexin V binding buffer at room temperature for 15 min in the dark. For cell cycle analysis, cells were harvested after 36-h transfection, washed twice with PBS (2% FBS), fixed with 70% cold aqueous ethanol, and stored at 4˚C for at least 1 h. Cell pellets were stained with staining solution containing RNase A (10 mg/ml) and propidium iodide (PI; 2 mg/ml) in PBS for 30 min in the dark at room temperature. The percentage of apoptotic cells and the DNA content were measured using a FACSCalibur flow cytometer (BD Biosciences) equipped with the CellQuest Pro software (BD Biosciences).

Real-time PCR. Total RNA was extracted using an RNeasy Mini kit (Qiagen) according to the manufacturer's protocol and transcribed into cDNA using oligo(dT) (Bioneer) and reverse transcriptase (Bioneer). mRNA levels were quantified using an ECO Real-Time PCR system (Illumina, Inc., San Diego, CA, USA) and a SYBR-Green Master Mix kit (Takara, Tokyo, Japan) with the following specific primer sets: Gal-3 (upstream primer, 5'-CCA AAG AGG GAA TGA TGT TGC C and downstream primer, 5'-TGA TTG TAC TGC AAC AAG TGA GC); IL-6 (upstream primer, 5'-GTG TTG CCT GCT GCC TTC CCT G and downstream primer, 5'-CTC TAG GTA TAC CTC AAA CTC CAA); IL-8 (upstream primer, 5'-ATG ACT TCC AAG CTG GCC GTG GCT and downstream primer, 5'-TCT CAG CCC TCT TCA AAA ACT TCT C); VEGF (upstream primer, 5'-AGG AGG GCA GAA TCA TCA



	Neg.	Si-C	Si-Gal-3
Sub-G1	4.40 %	4.76 %	5.51 %
G0-G1	56.74 %	57.25 %	44.06 %
S	5.69 %	5.81 %	13.10 %
G2-M	22.55 %	21.68 %	26.46 %

Figure 1. Gal-3 expression and the effect of Gal-3 knockdown on proliferation of human osteosarcoma cells. (A) Gal-3 expression in HOS cells. Cells were transfected with either Gal-3-siRNA or control-siRNA for 24 and 48 h or left untreated (negative control). Total proteins were extracted for western blotting with anti-Gal-3 antibody. β-actin was used as a loading control. (B) Protein quantification by densitometry (mean ± SD, n=3) revealed >77% knockdown of Gal-3 in cells transfected with Gal-3-siRNA. Results were normalized to β-actin values. *P<0.05. (C) Effect of Gal-3 silencing on proliferation of HOS cells as measured by the AlamarBlue assay. The RFU levels of the HOS cells are shown (mean ± SD, n=3). Cells were transfected with the indicated reagent (negative, control-siRNA or Gal-3-siRNA) and then cultured in 96-well microplates for 24 or 48 h. The RFU levels were detected using a microplate reader. (D) Effect of Gal-3 silencing on cell cycle distribution in HOS cells as measured by PI staining. Cell cycle distribution was analyzed and presented as diagrams (upper panel) and a graph (lower panel). Results are representative of three independent experiments.

CG and downstream primer, 5'-CAA GGC CCA CAG GGA TTT TCT); MMP2 (upstream primer, 5'-TGG CAA GTA CGG CTT CTG TC and downstream primer, 5'-TTC TTG TCG CGG TCG TAG TC); MMP9 (upstream primer, 5'-TGC GCT ACC ACC TCG AAC TT and downstream primer, 5'-GAT GCC ATT GAC GTC GTC CT); STAT3 (upstream primer, 5'-ACC TGC AGC AAT ACC ATT GAC and downstream primer, 5'-AAG GTG AGG GAC TCA AAC TGC). A specific primer set for β-actin (upstream primer, 5'-ATC CAC GAA ACT ACC TTC AA and downstream primer, 5'-ATC CAC ACG GAG TAC TTG C) was used as a control. The relative amount of mRNA was calculated using the arithmetic formula $2^{-\Delta\Delta Cq}$, where ΔCq is the difference between the threshold cycle of a given target cDNA and an endogenous reference cDNA.

Immunoblotting. Cells were washed in PBS and lysed in NP-40 buffer (Elpis Biotech, Daejeon, Korea) supplemented with a protease inhibitor cocktail (Sigma-Aldrich). To address phosphorylation events, an additional set of phosphatase inhibitors (Cocktail II; Sigma-Aldrich) was added to the NP-40 buffer. Protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL, USA). Proteins (10 μg/sample) were resolved by SDS-PAGE and transferred to nitrocellulose (Millipore Corp., Billerica, MA, USA). The membranes were blocked with 5% skim milk and standard western blot analysis was performed. Chemiluminescence was detected using an ECL kit (Advansta Corp., Menlo Park, CA, USA) and the multiple Gel DOC system (Fujifilm). Primary antibodies (Abs) against the following proteins were used: MMP2, MMP9, caspase-3, caspase-9, PARP, β-actin, phospho-FAK (Tyr^{397}), phospho-FAK (Tyr^{925}), FAK, phospho-Src (Tyr^{416}), Src, phospho-Lyn (Tyr^{507}), Lyn, phospho-Stat3 (Tyr^{705}), Stat3, phospho-PI3K p85 (Tyr^{458}), PI3K p85, phospho-Akt (Ser^{473}), and Akt (Cell Signaling Technology); Gal-3 and VEGF (Santa Cruz Biotechnology). Data were analyzed using ImageJ 1.38 software.

Confocal microscopy. Cells were permeabilized with permeabilization buffer (0.1% saponin in PBS) and incubated with primary Ab against Gal-3, β-catenin, phospho-Src, phospho-Lyn and phospho-FAK for 30 min on ice, followed by incubation with FITC-conjugated secondary Ab for 20 min. The nucleus was stained with PI (BD Biosciences) for 10 min. Cells were mounted in a Dako fluorescent mounting medium and observed by confocal laser scanning microscope (Carl Zeiss) at x400 magnification. Images were acquired using confocal microscopy software release 3.0 (510META; Carl Zeiss).

Statistical analysis. Data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using one-way analysis of the variance (ANOVA). P-values <0.05 were considered statistically significant.

Results

Effect of Gal-3 silencing on proliferation and cell cycle distribution of human osteosarcoma cells. Many previous

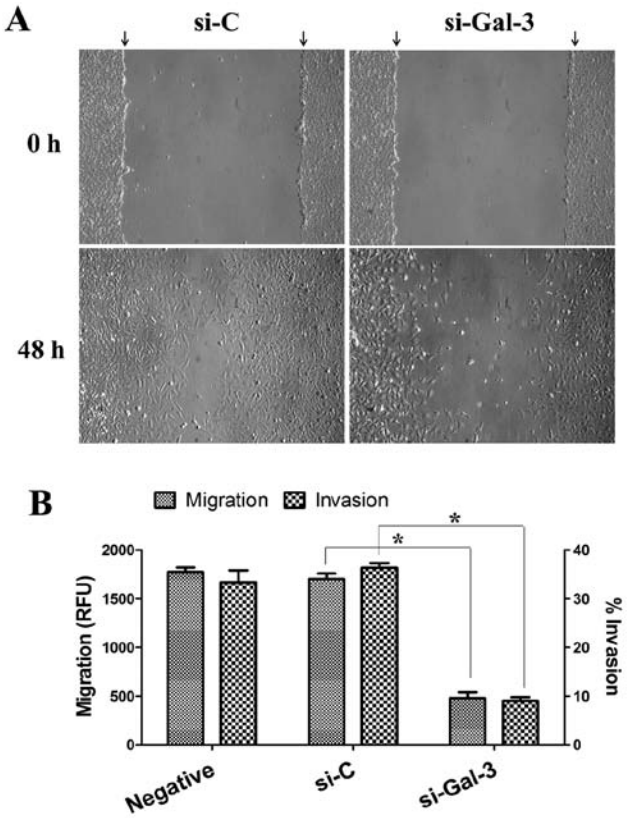


Figure 2. Effect of Gal-3 silencing on motile properties of HOS cells. After transfection with either Gal-3-siRNA or control-siRNA for 24 h, cells were tested for motility. (A) Cell motility was impaired by Gal-3 silencing as measured by a wound healing assay. Cells transfected with either Gal-3-siRNA or control-siRNA were wounded (0 h) and maintained for 48 h in complete medium. Arrows indicate the edges of the wounds. Wound closure (measured after 48 h) was slower in cells transfected with Gal-3-siRNA than in those transfected with control-siRNA. (B) The migratory capacity of HOS cells was suppressed by silencing of Gal-3, as determined by a Transwell migration assay kit. RFU is the relative fluorescence unit. Gal-3 silencing inhibited invasiveness, as detected by a BME cell invasion assay kit as described in Materials and methods. *P<0.002. Each value is the mean ± standard deviation of 3 determinations.

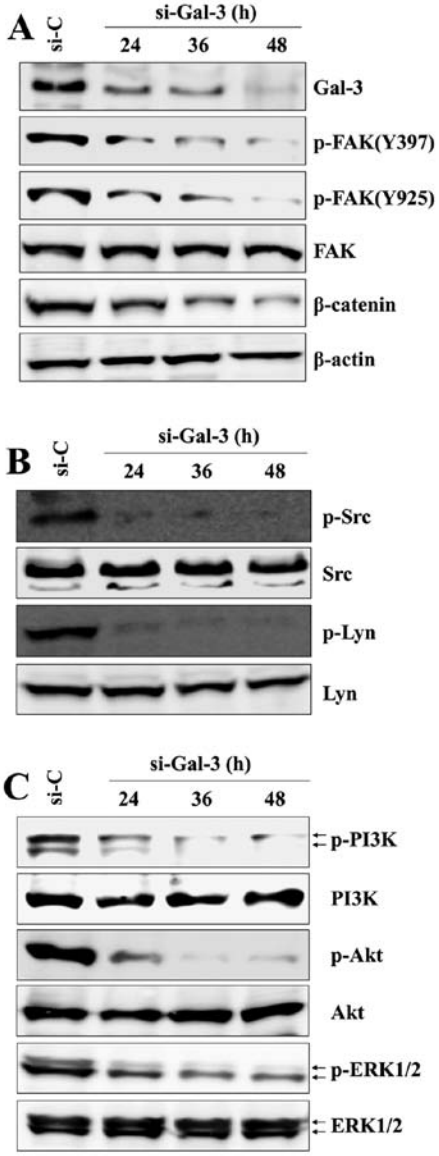


Figure 3. Gal-3 silencing decreases the expression of β-catenin and suppresses the activation of FAK/Src family and ERK/Akt. After transfection with Gal-3-siRNA or control-siRNA for the indicated times, the levels of total and phosphorylated proteins were detected by western blotting with Abs to (A) Gal-3, p-FAK (Tyr397 and Tyr925), FAK, β-catenin, (B) p-Src, Src, p-Lyn, Lyn, and (C) p-PI3K, PI3K, p-Akt, Akt, p-ERK1/2 and ERK1/2. β-actin was used as a loading control. Results are representative of three independent experiments.

studies have implied that Gal-3 is involved in growth, angiogenesis, migration and invasion in various cancers (3,4). To determine the potential role of Gal-3 in OS progression, we inhibited Gal-3 expression in the HOS cell line using a siRNA system, which is a powerful tool for investigating gene function. We first confirmed that HOS cells expressed high levels of Gal-3 by RT-PCR (data not shown) and western blot analysis (Fig. 1A) analyses. Significant inhibition of Gal-3 expression at the protein level was detected 24 and 48 h after transient silencing of Gal-3 with specific Gal-3-siRNA, whereas control-siRNA had little effect on Gal-3 expression and there was no noticeable difference between untreated control cells and control-siRNA transfected cells (Fig. 1A and B). At 48 h after transfection, protein levels of Gal-3 were reduced by ~77% in cells transfected with Gal-3-siRNA. We examined whether Gal-3-siRNA affected proliferation and cell cycle distribution of HOS cells. Contrary to our expectations, there was no significant difference in proliferation between cells transfected with Gal-3-siRNA or control-siRNA (Fig. 1C). Although treatment with Gal-3-siRNA slightly affected cell cycle distribution, Gal-3 silencing did not cause great changes in the distribution compared with control-siRNA (Fig. 1D). These data suggested that Gal-3 silencing does not block the proliferation of HOS cells completely but slightly contributes to the growth and cell cycle.

Gal-3 silencing suppresses migration and invasion of osteosarcoma cells. In addition to proliferation, increased migration and invasion are crucial features defining cancer progression and inhibition of these functions may be a possible target for anticancer therapy. Gal-3 expression in pancreatic cancer and gastric cancer cells increase cell migration and invasion (5,6). We therefore characterized the role of Gal-3 in the migration

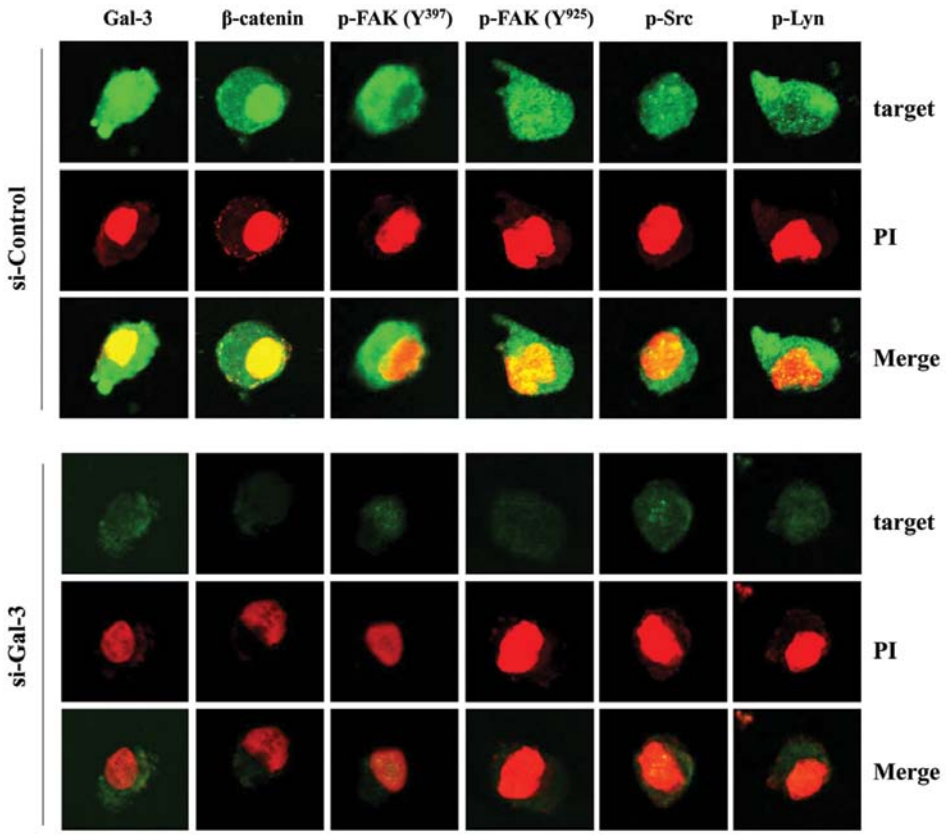


Figure 4. Subcellular distribution of p-FAK (Tyr397 and Tyr925), p-Src, p-Lyn and β-catenin in HOS cells after Gal-3 silencing. After transfection with either Gal-3- or control-siRNA for 48 h, cells were permeabilized with 0.1% saponin in PBS. Intracellular staining was performed using Abs against p-FAK (Tyr397 and Tyr925), p-Src, p-Lyn, β-catenin and Gal-3. The nucleus was stained with PI. Cells were observed under a confocal microscope (magnification, x400). Green fluorescence indicates Gal-3, β-catenin, p-FAK (Tyr397 and Tyr925), p-Src or p-Lyn and red fluorescence indicates the nucleus.

and invasion of OS cells. Wound healing and migration assays were performed to examine the effect of Gal-3 silencing on cell motility. The time required for wound closure in HOS cells with silenced Gal-3 was significantly longer than that required for corresponding control cells (Fig. 2A), suggesting the involvement of Gal-3 in cell migration. As shown in Fig. 2B, Transwell migration assays showed that the migration capacity was reduced ~3.5-fold in Gal-3-siRNA transfected cells compared with the control-siRNA group (Gal-3-siRNA, 479±60 RFU; control-siRNA, 1.701±56 RFU). Furthermore, we found that Gal-3 silencing also suppressed invasiveness of the HOS cells; cell invasion was decreased ~4-fold in Gal-3-silenced cells compared with control-siRNA cells (Gal-3-siRNA, 9.027±0.466%; control-siRNA, 36.324±0.719%). These results indicate that Gal-3 mainly regulates OS cell migration and invasion at least *in vitro* in our experimental systems.

Gal-3 silencing decreases β-catenin expression and suppresses activation of FAK/Src/Lyn and Akt/ERK. Next, we investigated the molecular mechanism by which Gal-3 mediates the motility of HOS cells. Recent studies have shown that Gal-3 is closely associated with β-catenin/Wnt signaling in several tumor types (8,9). The PI3K/Akt and ERK1/2 pathways are key signaling pathways involved in the migratory behavior or invasiveness of various tumors (21). ERK1/2 controls cytoskeletal reorganization and focal adhesion turnover through the activation of specific cytoskeletal and focal adhesion proteins, such as FAK and paxillin (22). First, we investigated the potential signaling molecules underlying Gal-3-mediated β-catenin expression, focusing on FAK and Src family tyrosine kinases (SFKs). Gal-3 silencing markedly reduced β-catenin expression and the phosphorylation of FAK at Y397 and Y925 residues in a time-dependent manner (Fig. 3A) and decreased phosphorylation of Src and Lyn, downstream signaling molecules of FAK, time-dependently (Fig. 3B). To further investigate the signaling pathway that mediates β-catenin expression, we assessed the expression of PI3K/Akt and ERK1/2 by western blotting. We observed that the phosphorylation levels of PI3K/Akt and ERK1/2 were strongly reduced in a time-dependent manner after Gal-3 silencing (Fig. 3C). In contrast, cells transfected with control-siRNA exhibited little or no change in phospho-PI3K/Akt or phospho-ERK1/2. Notably, after transfection with Gal-3-siRNA, decreases in phospho-FAK/Src/Lyn and phospho-Akt/ERK1/2 occurred within 24 h and a subsequent decrease in β-catenin expression was detected at 36 h (Fig. 3). These results imply that the reduction in phosphorylation of these tyrosine kinases occurs before the downregulation of β-catenin after silencing Gal-3 in HOS cells. We also observed changes in the subcellular distribution of these molecules after depletion of Gal-3 using confocal microscopy. As indicated in Fig. 4, the levels of phospho-FAK, phospho-Src, phospho-Lyn, and β-catenin in Gal-3-silenced cells were significantly attenuated compared

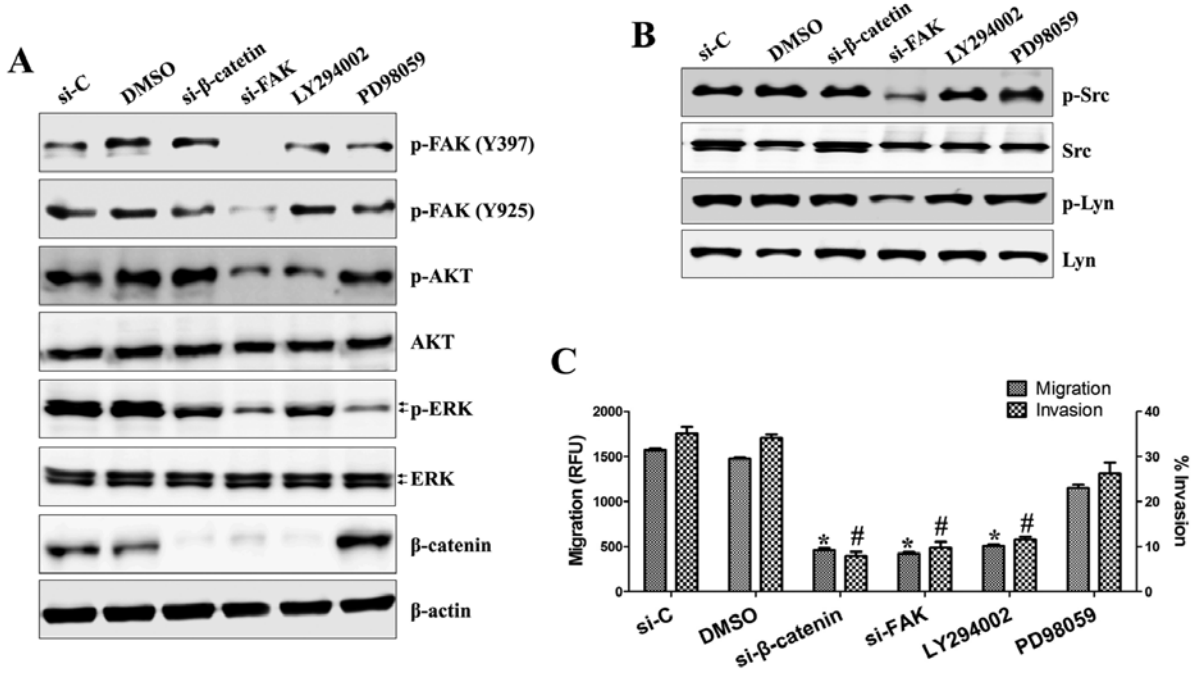


Figure 5. FAK, Src, Lyn, Akt and β-catenin, but not ERK1/2, are key mediators of osteosarcoma cell migration and invasion. Cells were transfected with siRNA specific for β-catenin, FAK or control-siRNA for 36 h or treated with LY294002 (25 μM) or PD98059 (20 μM) for 36 h as indicated. (A and B) Total protein was extracted from cell lysates and subjected to western blotting for phospho-FAK (Tyr397 and Tyr925), phospho-Akt, phospho-ERK1/2, β-catenin, phospho-Src, phospho-Lyn and β-actin. (C) The migratory capacity and invasiveness of HOS cells was inhibited by the knockdown of β-catenin, FAK and Akt, but not ERK1/2, as detected by Transwell migration assay kit and BME cell invasion assay kit. The procedure is described in detail in Materials and methods. *P<0.002. #P<0.001. Each value is the mean ± SD of three determinations.

with control-siRNA cells. In particular, Gal-3 and β-catenin were distributed in the cytoplasm and nuclei of the control-siRNA group (Fig. 4), suggesting co-localization of these proteins.

The FAK/Src-Lyn/Akt-ERK axis induces β-catenin and promotes migration and invasion of osteosarcoma cells. To clarify the order of Gal-3-mediated events and confirm whether these signals are essential for Gal-3-mediated β-catenin regulation in HOS cells, we used β-catenin-siRNA, FAK-siRNA, the PI3K/Akt inhibitor LY294002 and the ERK1/2 inhibitor PD98059. After treatment of HOS cells with these inhibitors for 36 h, we analyzed protein expression by western blotting. As shown in Fig. 5A and B, treatment with FAK-siRNA inhibited the activation of Akt, ERK1/2, Src and Lyn and expression of β-catenin, whereas LY294002 treatment suppressed only β-catenin expression. However, inhibition of ERK1/2 or β-catenin had no effect on these proteins. We next examined whether these signals directly contribute to cell motility. As shown in Fig. 5C, there was a dramatic inhibition of migration in cells with silenced β-catenin, FAK or Akt compared with cells treated with control-siRNA or DMSO (459±25, 422±17, 507±19 RFU vs. 1569±18, 1472±16 RFU, respectively), whereas the ERK1/2 inhibitor PD98059 (1148±39 RFU) had little effect on cell migration. Invasion was also suppressed by treatment with β-catenin-siRNA, FAK-siRNA or LY294002 (7.914±0.671, 9.767±0.794 and 11.539±0.473%, respectively), but not by PD98059 (26.243±1.764%), compared with control-siRNA or DMSO-treated cells (35.108±0.849 and 34.051±0.543%). These results suggest that FAK leads to the activation of Src and Lyn and then induces the activation of Akt and ERK1/2.

Expression of matrix metalloproteinases and proinflammatory cytokines is mediated by Gal-3 through the FAK and Akt/ERK signaling pathway. Gal-3 has a proinflammatory function in lung and liver fibrotic disease (23,24) and mediates a number of important signaling molecules, including IL-8, IL-6, MMPs and Stat3 (25). FAK has been reported to regulate cell migration and invasion by promoting pro-inflammatory cytokines, and MMP-mediated matrix degradation has been implicated in FAK- and β-catenin-induced migration and invasion (22). We therefore examined whether proinflammatory expression of cytokines and MMPs was controlled by Gal-3 signaling. We observed a remarkable decrease in MMP2 and MMP9 expression in Gal-3-silenced cells (Fig. 6A and B). Gal-3 silencing also suppressed the secretion and expression of VEGF, MCP-1, IL-8 and IL-6 (Fig. 6A and C) and abolished the phosphorylation of Stat3 compared with control-siRNA (Fig. 6A and B). The secretion levels of VEGF, MCP-1, IL-8 and IL-6 were suppressed in cells treated with FAK-siRNA, β-catenin-siRNA, LY294002 or PD98059 (Fig. 6D). Expression of both MMP2 and MMP9 was blocked in cells treated with FAK-siRNA, β-catenin-siRNA or LY294002, whereas PD98059 had little effect (Fig. 6E). Stat3 activation was inhibited in cells treated with FAK-siRNA, β-catenin-siRNA, LY294002 or PD98059 (Fig. 6E). These data suggest that Gal-3-mediated HOS cell migration and invasion is achieved through the activation of FAK, which regulates expression of β-catenin and MMP2/9 and the production of proinflammatory cytokines through PI3K/Akt and ERK1/2 signaling.

Gal-3 silencing sensitizes osteosarcoma cells to cisplatin. The potential effect of Gal-3 silencing on drug-induced apoptosis

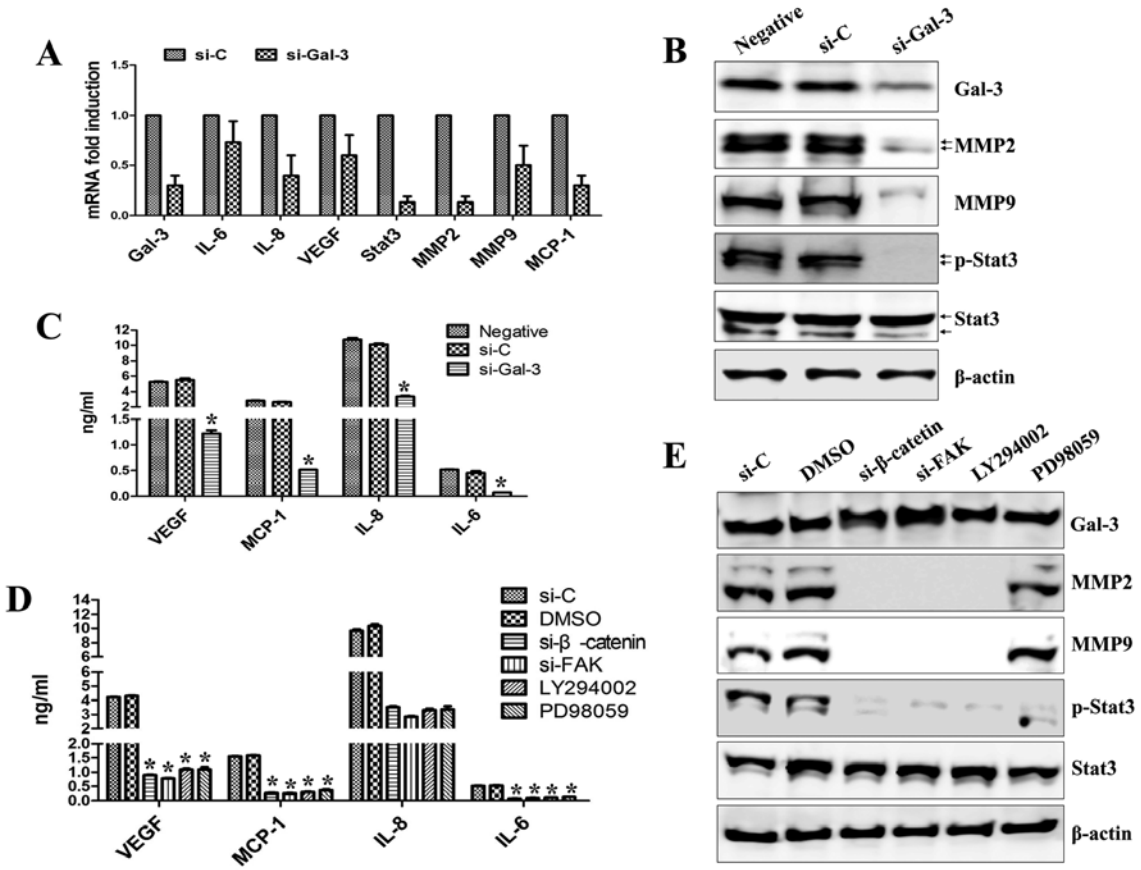


Figure 6. Gal-3 silencing inhibits MMP expression and cytokine secretion in HOS cells. Cells were transfected with siRNA specific for Gal-3, β-catenin, FAK or control-siRNA for 36 h or treated with LY294002 (25 μM) or PD98059 (20 μM) for 36 h as indicated. (A) Total RNA was extracted from cell lysates and subjected to real-time PCR for Gal-3, MMP2, MMP9, Stat3, IL-6, IL-8, VEGF, MCP-1 and β-actin mRNA expression. *P<0.05. Each value is the mean ± SD of three determinations. (B) The effect of knockdown of Gal-3 on the secretion of VEGF, MCP-1, IL-6 and IL-8. (C) The effect of knockdown of Gal-3 on the expression of Gal-3, MMP2, MMP9 and Stat3. (D) The effect of knockdown of β-catenin, FAK, Akt or ERK1/2 on the secretion of VEGF, MCP-1, IL-6 and IL-8. (E) The effect of knockdown of β-catenin, FAK, Akt or ERK1/2 on the expression of Gal-3, MMP2, MMP9 and Stat3. (C and E) The levels of total and phosphorylated proteins were detected by western blotting. β-actin was used as a loading control. (B and D) After transfection, VEGF, MCP-1, IL-8 and IL-6 concentrations in the culture supernatants were quantified by ELISA. *P<0.05. Each value is the mean ± SD of three determinations.

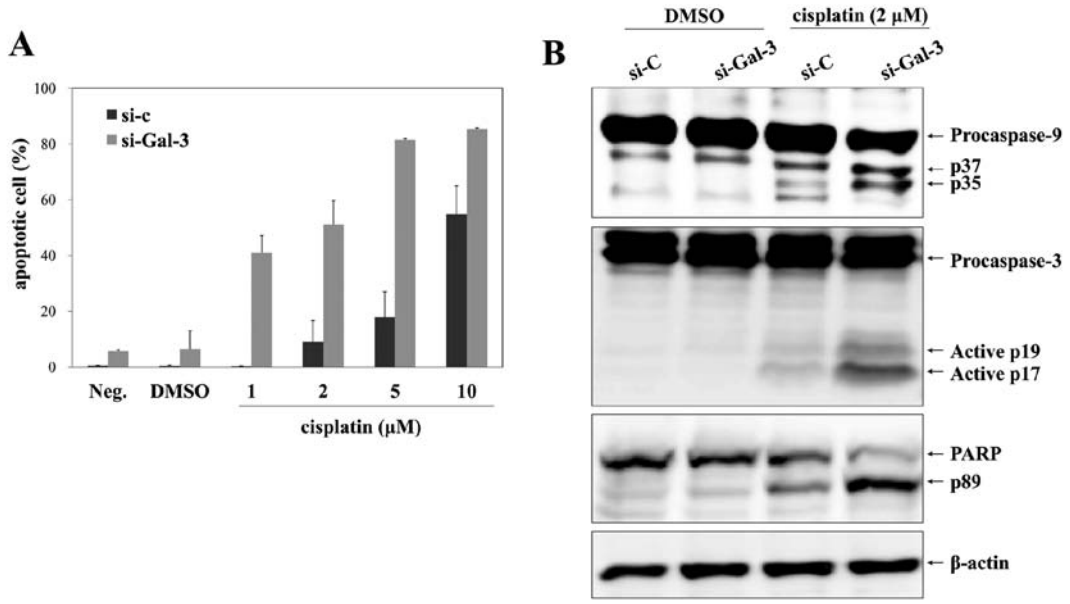


Figure 7. Gal-3 silencing sensitizes osteosarcoma cells to cisplatin. (A) Cells were transfected with Gal-3-siRNA or control-siRNA for 24 h, and then exposed to cisplatin (1, 2, 5 and 10 μM) for 24 h. Cell apoptosis was detected by the Annexin V/7-AAD assay. (B) Cells were transfected with Gal-3-siRNA or control-siRNA for 24 h, and then exposed to 2 μM cisplatin for 24 h. Western blot analysis of active casapse-9, caspase-3 and PARP cleavage was performed to determine the apoptotic response. β-actin was used to normalize protein content. Results are representative of three independent experiments.

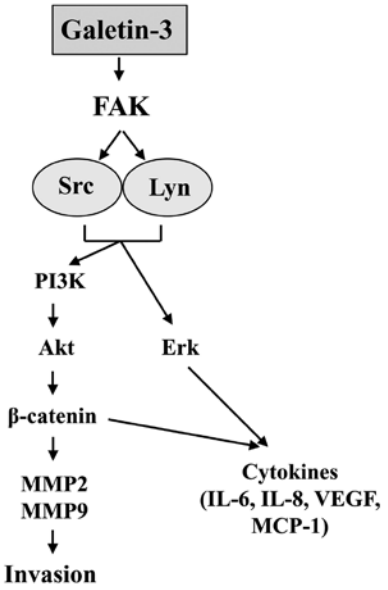


Figure 8. Schematic diagram showing the intracellular signaling pathway by which Gal-3 induces migration and invasion of HOS cells. Gal-3 induces FAK-mediated Src/Lyn and Akt/ERK activation, leading to expression of β-catenin and MMP2/9. As a result, the secretion of proinflammatory cytokines is enhanced. Silencing of Gal-3 expression using specific target siRNA suppresses osteosarcoma cell invasion.

in HOS cells was analyzed by Annexin V/7-AAD staining. After transfection for 24 h, cells were exposed to cisplatin or DMSO for a further 24 h. Cisplatin treatment of cells transfected with control-siRNA did not have a great effect on cell apoptosis up to concentrations of 5 μM, and treatment with 2 μM cisplatin induced apoptosis in only 9.06±7.55% of cells (Fig. 7A). Noatbly, transfection with Gal-3-siRNA dramatically increased the extent of apoptosis induced by 2 μM cisplatin to 51.15±8.46%, whereas treatment with Gal-3-siRNA alone had little effect on apoptosis (Fig. 7A). Moreover, combination treatment with Gal-3-siRNA and cisplatin clearly elicited the activation of caspase-9 and caspase-3 and the cleavage of PARP, whereas these signals were barely detectable in the control-siRNA group (Fig. 7B).

Discussion

The migration and invasion of cancer cells are important processes in metastasis. Therefore, inhibition of the invasion and metastasis of cancer cells has been considered a feasible strategy for the treatment of malignancy. Upregulation of Gal-3 has been shown to be a potential metastatic factor in a variety of human cancers (3,4) and rat OS (26). However, the role of Gal-3 in OS in humans remains obscure. The results of the present study implicate a role of Gal-3 in regulating the migration and invasion of OS cells through the activation of FAK and Src/Lyn. In particular, we provide the first evidence that Gal-3 regulates Lyn kinase. To understand how Gal-3 regulates OS progression, we used a siRNA system for Gal-3 silencing. Silencing of Gal-3 inhibited the activation of PI3K/Akt, ERK1/2, FAK, Src and Lyn and suppressed MMP2 and MMP9 expression. Our results suggest a novel mechanism by which Gal-3 contributes to the acquisition of the OS metastatic phenotype by increasing Lyn expression.

Overexpression of Gal-3 has been reported in multiple types of human tumors, including thyroid, pancreatic cancer, hepatocellular carcinoma, gastric, colon, breast, prostate cancer, head and neck squamous cell carcinomas and glioma (27). However, the role of Gal-3 in osteosarcoma progression is not clear. Recent studies have shown that Gal-3 is closely associated with β-catenin in the invasion of various cancer cells (8,9), although the relationship between galectin-3 and Wnt signaling in OS remained unclear. We observed that Gal-3 silencing significantly diminished the expression of β-catenin in HOS cells (Figs. 3A and 4), suggesting that Gal-3 expression activates β-catenin/Wnt signaling in OS.

Recent studies have shown that Gal-3 activates the K-Ras/RAF1/ERK pathway, resulting in migration of colon cancer cells (28), and the functions of Gal-3 include binding to cell adhesion molecules and suppression of cell-cell and cell-matrix interactions (3). Diverse reports have shown that targeting the PI3K/Akt signaling pathway results in downregulation of tumor invasion and tumorigenesis in malignant cancer cells, including OS (29,30). However, our results showed that Gal-3-induced phospho-ERK1/2 did not influence the invasion and migration of HOS cells (Fig. 5C and D).

FAK and SFKs, including Src, Lyn, Fyn, Lck, Hck, Fgr, Blk and Yes, are non-receptor tyrosine kinases that are activated in response to stimulation by various cellular factors (31,32). Expression of FAK and SFKs is increased in a variety of tumors and contributes to the regulation of tumor progression, adhesion, motility, angiogenesis, invasion and metastasis (32,33). FAK activation is a central factor in different signaling transduction cascades including the PI3K/Akt, ERK1/2 and p38-MAPK pathways (22). Abnormal activation of Lyn has been implicated in the progression and migration of various tumors, including prostate and breast cancer (34,35). Inhibition of Lyn using siRNA was recently shown to significantly suppress tumor growth and decrease lung metastases in Ewing's sarcoma (36). Although some studies have linked Src and Lyn to Ewing's sarcoma, there are no studies demonstrating the involvement of FAK, Src and ERK in Gal-3-mediated invasion in OS.

Recent studies have reported a correlation between Gal-3 and FAK expression; Gal-3 triggered FAK activation in HUVEC cells (37) and was required for stabilization of FAK in thyroid cancer (38). In the present study, we found that depletion of FAK by specific siRNA reduced the phosphorylation level of Src, Lyn, Akt and ERK1/2 (Fig. 5A and B). The data suggest that FAK might be the upstream modulator of SFKs, Akt and MAPKs and directly or indirectly interacts with Src, Lyn, Akt and ERK1/2 in OS. It is possible that Gal-3 directly or indirectly induces the activation of FAK, Src, Lyn, Akt and ERK1/2, leading to cell invasion (Fig. 8).

Activation of PI3K/Akt enhances MMP expression and facilitates tumor metastasis (21). IL-8, IL-6 and VEGF are directly regulated by Wnt signaling and influence cancer cell migration and invasion (39). MMPs play a crucial role in cancer cell invasion and metastasis by breaking down the extracellular matrix to expedite the penetration of cancer cells (40). MMP2 and MMP9 have been proposed as significant downstream targets of the β-catenin signaling pathway (41). MMP2 and MMP9 are commonly overexpressed in OS tissue and their activation has been implicated in tumor invasion

and metastasis in OS (42). We found that silencing of Gal-3 or β-catenin suppressed the secretion of IL-8, IL-6, VEGF and MCP-1 and reduced expression of MMP2, MMP9 and phospho-Stat3 compared with cells transfected with control-siRNA (Fig. 6). Therefore, we conclude that the β-catenin signaling pathway regulates MMPs and proinflammatory cytokines in HOS cells.

Although standard chemotherapeutic drugs such as cisplatin, doxorubicin, cyclophosphamide, methotrexate, ifosfamide and bleomycin have improved the efficiency of OS therapy, uncontrolled tumor expansion and metastasis are key factors responsible for the poor prognosis of OS (43). The knockdown of Gal-3 in HOS cells also enhanced the efficacy of cisplatin to induce apoptosis through activation of the caspase pathway. The PI3K/Akt pathway is well known to be a major cell survival pathway in many cancers, including OS (31,44). In the present study, we observed that Gal-3 and FAK directly activate the downstream effectors Src, Lyn, Akt and ERK1/2. These data suggest that combination treatment with Gal-3 blocking and cisplatin might be a new treatment for advanced or metastatic OS.

In conclusion, the present study demonstrates that Gal-3-mediated migration and invasion of human OS cells is induced by the activation of Akt and ERK1/2 through FAK and Src/Lyn signaling pathways. We propose that Gal-3 might be a feasible therapeutic target for OS.

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