Glycogen synthase kinase 3β promotes osteosarcoma invasion and migration via regulating PTEN and phosphorylation of focal adhesion kinase

Running title: GSK3β involved in osteosarcoma metastasis

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Abstract

Aim: Typical features of human osteosarcoma are highly invasive and migratory capacities. Our study aimed to investigate the roles of glycogen synthase kinase 3β (GSK3β) in human osteosarcoma metastasis.

Methods: GSK3β expressions in clinical osteosarcoma tissues with or without metastasis were examined by immunohistochemical staining. The expressions of GSK3β, p-GSK3β\textsuperscript{Ser9}, and p-GSK3β\textsuperscript{Tyr216} in human osteoblast cells (hFOB1.19) and human osteosarcoma cells (MG63, SaOS-2 and U2-OS) were detected by western blotting. The GSK3β activity was measured by non-radio isotopic in vitro kinase assay. Migration and invasion abilities of MG-63 cells treated with small-molecular GSK3β inhibitors were respectively examined by monolayer-based wound-healing assay and transwell assay. The mRNA expressions of GSK3β, matrix metalloproteinase-2 (MMP-2), MMP-9, phosphatase with tensin homology (PTEN), and focal adhesion kinase (FAK) were detected after siRNA transfection for 72 h. Meanwhile, protein expressions of GSK3β, FAK, p-FAK\textsuperscript{Y397}, PTEN, MMP-2, and MMP-9 were measured by western blotting.

Results: Clinical osteosarcoma tissues with metastasis showed higher GSK3β expressions. MG63 and U2-OS cells which were easy to occur metastasis showed significantly higher expressions and activities of GSK3β than SaOS-2 cells. Inhibition of GSK3β with small-molecular GSK3β inhibitors in MG63 cells significantly attenuated cell migration and invasion. These effects were associated with reduced expressions of MMP-2 and MMP-9. Moreover, increased PTEN and decreased p-FAK\textsuperscript{Y397} expressions were observed following GSK3β knock-down by siRNA transfection.

Conclusion: GSK3β might promote osteosarcoma invasion and migration via pathways associated with PTEN and phosphorylation of FAK.

Keywords: Human osteosarcoma; Metastasis; Glycogen synthase kinase 3β
Introduction

Invasion and metastasis of human osteosarcoma often occur in the early stages, and the lungs are the most distant metastatic sites, accounting for about 90% of all metastases (1). Until now, few reliable predictors have been identified to guide the choice or intensity of the human osteosarcoma therapy (2). Though neoadjuvant chemotherapy could improve the survival rate of patients with osteosarcoma in last decades, invasive nature and pulmonary trend metastasis still remain to be the main reasons for the failure of osteosarcoma therapy (3). Thus, revealing the signaling pathways involved in the metastatic process of human osteosarcoma has become an important aspect for developing novel effective therapeutic aimed at improving the survival rate of osteosarcoma (4).

Glycogen synthesis kinase 3β (GSK3β) plays an important role in cell growth, proliferation and cell migration, and it is widely concerned in the field of oncology. GSK3β is a serine/threonine protein kinase and has emerged as a key enzyme in regulating several important cellular signaling pathways via phosphorylating its substrates (5). Nowadays, the developed small molecule inhibitors of GSK3β have been used to treat type II diabetes and Alzheimer disease in clinical (6-8). Under normal physiological conditions, GSK3β negatively regulates cell survival and proliferation through phosphorylating multiple oncogene proteins (e.g. β-catenin) and carcinogenic transcription factors (e.g. c-Myc), which result in their ubiquitin degradation and inactivation. Thus, GSK3β is typically supposed as a tumor-suppressor gene (9). Our previous work demonstrated that the over-expression and abnormal activation of GSK3β in gastrointestinal cancer cells inhibit the apoptosis, and promote cell survival and proliferation (10). In addition, reducing the activity or expression level of GSK3β inhibits the survival and proliferation of gastrointestinal cancer cell, and induced cell apoptosis. Therefore, GSK3β has potentially to be a novel therapeutic target for gastrointestinal cancer (10,11).

In the study of Cai et al., GSK3β inhibitor treatment results in the decrease of cell survival and proliferation rate, indicating that GSK3β may be associated with the occurrence of osteosarcoma (12). Tang et al. reported that over-expressing GSK3β in osteosarcoma cell significantly improved the colony formation, and increased the tumor formation rate. Importantly, they demonstrated that the abnormal activation of GSK3β promoted the growth of osteosarcoma tumor (13). Those findings revealed that GSK3β plays an important role in the tumor genesis of osteosarcoma, but the underlying molecular mechanisms of GSK3β regulating the metastasis and invasion of osteosarcoma
still remains unknown.

Here, we set out to explore the underlying molecular mechanisms of GSK3β in the metastasis of osteosarcoma via small molecular inhibitors and siRNA knock down analysis.

Materials and methods

Ethics statement

Written informed consents were obtained from all of the patients with osteosarcoma before the samples were collected from surgery. This study was approved by the Medical Ethics Committee of Harbin Medical University Cancer Hospital.

Immunohistochemical staining of clinical samples

The collected osteosarcoma tissue specimens from patients with primary metastatic osteosarcoma (n = 24) and patients with non-metastatic osteosarcoma (n = 16) were fixed in 10% formalin and embedded in paraffin. The lung metastasis was diagnosed by computed tomography (CT) imaging. Expression levels of GSK3β in primary osteosarcoma tissues with or without metastasis were examined by standard immunohistochemical staining (14). Immunohistochemical staining was carried out by the avidin-biotin method using an ABCComplex/HRP kit (Waitai, China). The tissue sections were deparaffinized, antigen retrieved by microwave and blocked of non-specific immunoreactions. Next, tissue sections were incubated with primary antibodies against GSK3β (1:100; BD Biosciences). For the negative control, primary antibodies were replaced by non-immune mouse IgG (Abcam). After rinsing in phosphate-buffered saline, tissue sections were then incubated with biotinylated secondary antibodies (1:2000; Abcam). Subsequently, the nuclei were counterstained with hematoxylin. The quantitative analysis of GSK3β expression levels in tumor samples of patients with primary metastatic osteosarcoma and patients with non-metastatic osteosarcoma was performed by TissueFachs software (TissueGnostics GmbH).

Cell lines and cell culture

Human osteosarcoma cell lines (MG-63 and SaOS-2) and normal human osteoblast cell line (hFOB1.19) were purchased from the American Type Culture Collection (ATCC). Human osteosarcoma cells and osteoblast cells were cultured in DMEM and DMEM/Ham’s F12 mediums (Gibco, Thermo Fisher Scientific) respectively, supplemented with 10% fetal bovine serum. Another human osteosarcoma cell line U2-OS was obtained from the Human Cancer Cell Line Bank located
in the Cancer Research Institute of Kanazawa University. U2-OS cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Western blotting

The total cellular protein was extracted from cultured cells using lysis buffer (Sigma-Aldrich, St. Louis, MO) containing a mixture of protease and phosphatase inhibitors (Sigma-Aldrich). The total cellular protein samples were loaded onto sodium dodecyl sulfate (SDS) polyacrylamide gels and then transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences, USA). After the membranes were blocked in blocking solution, the blots were incubated overnight at 4°C with primary antibodies. The primary antibodies were against GSK3β (1:1,000; BD Biosciences), and its fractions phosphorylated at the serine⁹ residue (p-GSK3βSer⁹; 1:1,000; Cell Signaling Technology) and the tyrosine²¹⁶ residue (p-GSK3βTyr²¹⁶; 1:1,000; BD Biosciences); β-catenin (1:1,000; BD Biosciences) and its fractions phosphorylated at S33, S37, and/or threonine 41 (T41) residues (p-β-cateninS33/37/T41); and β-actin (1:4,000; Abcam); FAK (1:2,000; Cell Signaling Technology); p-FAKY³⁹⁷ (1:2,000; Invitrogen); PTEN (1:2,000; Cell Signaling Technology); MMP-2 and MMP-9 (1:3,000; Abcam). Specific horseradish peroxidase (HRP)-conjugated secondary antibodies were used for following incubations overnight at 4°C. Immunoblotting signals were measured by the CS analyzer (ATTO, Japan).

Non-radio isotopic in vitro kinase assay (NRIKA)

The non-radio isotopic in vitro kinase assay developed in our laboratory (10) was used to detect GSK3β activity in cultured cells. It uses a sequential combination of immunoprecipitations to isolate GSK3β in cellular protein extracted from cultured cells, an in vitro kinase reaction that uses recombinant β-catenin protein (substrate) and non-radio isotopic adenosine triphosphate, followed by immunoblotting to detect p-β-cateninS33/37/Thr⁴¹.

Cell migration and invasion abilities

Migration and invasion abilities of MG-63 cells were respectively examined by monolayer-based wound-healing assay and transwell assay according the description of Ayako et al. (14). Briefly, confluent monolayers of MG-63 cells in the presence of dimethyl sulfoxide (DMSO, control), or small-molecule GSK3β inhibitors (SB-216763, Sigma-Aldrich; AR-A014418,
Calbiochem) at indicated concentrations (5, 10, 15 or 20 μmol/L) were scratched mechanically with a micropipette tip to create wound cell-free zone. The extent of cell migration was measured by the ability to close the artificially created gap. The gap distance between the wound edges was monitored at three fixed time points from 6 to 24 hours by a CCD camera (AxioCam MRm, Zeiss) connected to a phase-contrast microscope (Axiovert 40 CFL, Zeiss).

MG-63 cell invasion ability was examined by the transwell assays using matrigel-coated 24-well double chamber system (BD BioCoat™ Matrigel™ Incubation Chamber, BD Bioscience). MG-63 cells were suspended in serum-free medium containing DMSO or small-molecule GSK3β inhibitors (SB-216763, AR-A014418; 20 μmol/L). Approximately 2×10⁴ cells were applied to the upper chamber pairing with the lower chamber suspended in medium containing 10% fetal bovine serum (as a chemo-attractant) and DMSO or small-molecule GSK3β inhibitors (SB-216763 or AR-A014418; 20 μmol/L). Cells on the lower side of the chamber were fixed and stained with Diff-Quick Kit (Symex) after incubation for 24 h. In each assay, the total number of cells per high-power microscopic field on the lower side of the matrigel coated chamber was counted and scored for invading cells. Meanwhile, the mRNA expressions of GSK3β, MMP-2 and MMP-9 in MG63 cells under different treatments were measured by semiquantitative reverse transcription-PCR (RT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to monitor the efficiency of RT-PCR. The primers for GSK3β, MMP-2, and MMP-9 were designed and synthesized by Shanghai GenePharma.

**Small interfering RNA (siRNA) transfection**

Small interfering RNA (siRNA) specific to human GSK3β (target sequence, 5′-GCUCCAGAUCAUGAGAAAGCUAGAU-3′; GSK3β Validated Stealth RNAi) and negative control siRNA (Stealth RNAi Negative Control Low GC duplex) were purchased from Invitrogen. MG63 cells were transfected with 20 nmol/L of either GSK3β-specific or negative control siRNA by using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's instructions. Effects of RNA interference on expression of GSK3β were determined by RT-PCR and western blotting, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to monitor the efficiency of RT-PCR. The primers for GSK3β, MMP-2, MMP-9, PTEN, and FAK for RT-PCR were designed and synthesized by Shanghai GenePharma (Shanghai, China).
(Supplementary table 1). After siRNA transfection for 72 h, the protein expressions of GSK3β, FAK, p-FAK<sup>Y397</sup>, PTEN, MMP-2, and MMP-9 were analyzed by western blotting as described above.

**Statistical analysis**

Statistical analyses were performed using the SPSS software (version 16.0; SPSS Inc., Chicago, IL). All experiments were repeated at least three times to calculate the mean and standard deviation (SD). The Student’s t-test was conducted for normally distributed data. A p-value < 0.05 was regarded as statistically significant.

**Results**

**Expressions of GSK3β in primary human metastatic or non-metastatic osteosarcoma tissues**

The expressions of GSK3β in tissue samples of 24 patients with primary metastatic osteosarcoma and 16 patients with non-metastatic osteosarcoma were detected by the immunohistochemical staining. The immunohistochemistry staining results showed that the percentage of GSK3β positive cells in the tumor samples of patients with primary metastatic osteosarcoma was markedly higher than that of patients without metastasis (67.8 ± 5.8 % vs. 24.8 ± 3.6 %, p < 0.001; Figure 1).

**Expressions, phosphorylation and activity of GSK3β in human osteosarcoma and osteoblast cells**

Compared to normal human osteoblast cells (hFOB1.19), the human osteosarcoma cells (MG63, SaOS-2 and U2-OS) showed higher protein expression levels of GSK3β and active form of GSK3β (p-GSK3β<sup>Tyr216</sup>), while lower levels of inactive form of GSK3β (p-GSK3β<sup>Ser9</sup>) (Figure 2). Furthermore, the MG63 and U2-OS cells which were easy to occur metastasis showed significantly higher levels of GSK3β and active GSK3β (p-GSK3β<sup>Tyr216</sup>), and lower levels of inactive (p-GSK3β<sup>Ser9</sup>) compared to SaOS-2 cells (Figure 2).

In order to explore the activity of phosphorylating β-catenin, substrates of GSK3β, in human osteosarcoma cells and non-tumor cells, we performed the NRIKA analysis in these cells. The human osteosarcoma cells exhibited stronger phosphorylating β-catenin activity compared to normal osteoblast cells hFOB1.19 (Figure 3). More specifically, the MG63 and U2-OS cells showed significantly higher activity of phosphorylating β-catenin when compared to SaOS-2 cells.

**Effects of GSK3β inhibitors on MG63 cell migration and invasion**

To address the relationship of GSK3β with tumor metastasis, we tested the effects of
small-molecular GSK3β inhibitors, AR-A014418 and SB-216763, on MG-63 cell migration and invasion. The wound healing assay showed that both of the two GSK3β inhibitors (AR-A014418 and SB-216763) significantly reduced the migration of MG-63 cells on a concentration depend manner compared to the DMSO treated cells (Figure 4A). In addition, AR-A014418 exhibited more effective inhibition on MG63 cell migration than SB-216763. The Transwell assay showed that both of the two GSK3β inhibitors significantly inhibited the invasion of MG63 cells (Figure 4B). The number of invasion cells when treated with AR-A014418 was significantly lower than treated with SB-216763 (Figure 4C).

The transcriptional activity of GSK3β inhibited by AR-A014418 and SB-216763 were also analyzed by RT-PCR. Both of the AR-A014418 and SB-216763 significantly inhibited the transcriptional activity of GSK3β. AR-A014418 exhibited more significant inhibition of GSK3β than SB-216763 (Figure 4D). Meanwhile, the AR-A014418 significantly inhibited the expressions of MMP-2 and MMP-9 (Figure 4D). The protein expression levels of GSK3β were also significantly suppressed by AR-A014418 and SB-216763 according to western blotting analysis (Figure 4E).

*Changes in the metastasis related mRNA and proteins following GSK3β knock-down*

Moreover, GSK3β was knocked down by using RNA interference experiments. Consistent with the results of treatments with GSK3β inhibitors, GSK3β specific siRNA significantly reduced the invasion ability of MG63 cells (Figure 5 A, B). The mRNA and protein expression levels of extracellular matrix enzyme MMP-2 and MMP-9 were also significantly reduced in MG-63 cells (Figure 5C, D). Meanwhile, the GSK3β knock-down significantly increased the expressions of PTEN in the mRNA level and protein level (Figure 5 C, D). Though knocking down of GSK3β in MG63 cells did not significantly affect the total FAK expression, the protein expression of p-FAK_Y397 was reduced (Figure 5 C, D).

*Discussion*

Although many studies have showed the pathogenic mechanism of osteosarcoma, few were focus on the underlying molecular mechanisms about metastasis of osteosarcoma (3). Shimozaki *et al.* recently demonstrated a critical role for GSK3β in sustaining survival and proliferation of osteosarcoma (15). However, the roles of GSK3β in the process of invasion and metastasis of osteosarcoma are still not clear. In our study, the expression of GSK3β in osteosarcoma tissues from
patients with metastasis is higher than that without metastasis. MG63 and U2-OS cells which were easy to occur metastasis showed significantly higher levels of GSK3β and active GSK3β (p-GSK3β^{Tyr216}), and lower levels of inactive (p-GSK3β^{Ser9}) compared to SaOS-2 cells. Furthermore, we found the activities of GSK3β in MG63 and U2-OS cells were higher than that in SaOS-2 cells. These findings indicated that active GSK3β is correlated with human osteosarcoma metastasis.

In our study, inhibition of GSK3β with small-molecular GSK3β inhibitors significantly reduced the migration and invasion ability of osteosarcoma cells. Meanwhile, the expressions of MMP-2 and MMP-9 were also reduced after treatments of small-molecular GSK3β inhibitors. MMP-2 and MMP-9 are highly expressed in osteosarcoma tissues, and they play important roles in the invasion and metastasis of tumor cells (16,17). Thus, inhibition of GSK3β may suppresses osteosarcoma cell migration and invasion by reducing the expressions of MMP-2 and MMP-9.

Moreover, the invasion ability of osteosarcoma cells following GSK3β knock-down by siRNA transfection for 72 h was obviously inhibited. The mRNA and protein expression levels of MMP-2 and MMP-9 were also significantly reduced. Phosphatase with tensin homology (PTEN) is a newly discovered tumor suppressor gene whose protein stability directly affects the migration of cells (18,19). Furthermore, GSK3β regulates the stability of PTEN via regulating the phosphorylation of PTEN, resulting in the ubiquitination and degradation of the PTEN (20). The treatment of MG63 cells with GSK3β siRNA transfection significantly increased the expression of PTEN in the mRNA level and protein level. The abnormal expression and phosphorylation levels of FAK in sarcoma is also reported to affect tumor cell migration and cell adhesion (21,22). GSK3β regulates the phosphorylation of FAK, and GSK3β inhibitors reduce the phosphorylation level of the FAK (23,24). Knocking down of GSK3β in MG63 cells did not significantly affect the total FAK in mRNA and protein, but reduced the p-FAK^{Y397}, suggesting an involvement in the mechanism of GSK3β-mediated tumor metastasis. It has been reported that the secretions of MMP-2 and MMP-9 are negatively correlated with PTEN, while are positively correlated with FAK (25,26). Therefore, it could be speculated that GSK3β might promote the osteosarcoma metastasis by reducing the stability of PTEN and increasing the phosphorylation of FAK, and then accelerate the secretions of MMP-2 and MMP-9.

**Conclusion**
In summary, our study revealed that inhibition of GSK3β might suppress the osteosarcoma invasion and migration via the pathways associated with PTEN and phosphorylation of focal adhesion kinase, followed by reduced expression of MMP-2 or MMP-9.

**Abbreviations**

- glycogen synthase kinase 3β (GSK3β)
- matrix metalloproteinase-2 (MMP-2)
- phosphatase with tensin homology (PTEN)
- focal adhesion kinase (FAK)
- computed tomography (CT)
- American Type Culture Collection (ATCC)
- sodium dodecyl sulfate (SDS)
- Small interfering RNA (siRNA)

**Competing interests:** The authors declare no conflict of interest.

**Authors' contributions**

WM and LYK participated in the design of this study, and they both performed the statistical analysis. HWY and JJB carried out the study and collected important background information. CYS and GFQ drafted the manuscript. All authors read and approved the final manuscript.

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Figure legends

**Figure 1.** Measurements of GSK3β in tissue samples collected from patients with primary metastatic osteosarcoma and non-metastatic osteosarcoma by immunohistochemical staining. (A) Microscopy images of positive areas of GSK3β in tissue samples of non-metastatic and metastatic osteosarcoma. (B) Percentage of GSK3β positive cells in tissue samples of non-metastatic and metastatic osteosarcoma. **p < 0.01, compared with non-metastatic osteosarcoma.

**Figure 2.** Protein expressions and phosphorylation of GSK3β in human osteosarcoma cells and normal human osteoblast cells. (A) The GSK3β, phosphorylated at the serine9 residue (p-GSK3βSer9; inactive form) and the tyrosine216 residue (p-GSK3βTyr216; active form) in protein extracts from three human osteosarcoma cells (MG63, SaOS-2 and U2-OS) and normal human osteoblast cells (hFOB1.19) were detected by western blotting. (B) Relative expression of GSK3β in hFOB1.19, MG63, SaOS-2 and U2-OS cells. (C) Relative expression of p-GSK3βSer9 in hFOB1.19, MG63, SaOS-2 and U2-OS cells. (D) Relative expression of p-GSK3βTyr216 in hFOB1.19, MG63, SaOS-2 and U2-OS cells. ** p < 0.01, compared with hFOB1.19 cells; # p < 0.05, ## p < 0.01, compared with SaOS-2 cells.

**Figure 3.** Detection of GSK3β activity by non-radio isotopic in vitro kinase assay (NRIKA) in three human osteosarcoma cells (MG63, SaOS-2 and U2-OS) and normal human osteoblast cells (hFOB1.19). (A) GSK3β activity to phosphorylate its substrate (β-cateninHis) was demonstrated by expression of the phosphorylated β-cateninHis (p-β-cateninSer33/37/Thr41) in the test lanes (T) and in the condition of little or no expression of p-β-cateninSer33/37/Thr41 in the negative control reaction (NC). The amount of GSK3β and the presence of β-cateninHis (substrate) in the NRIKA reaction were monitored by immunoblotting with mouse monoclonal antibodies to GSK3β and β-catenin, respectively. Levels of β-cateninHis and its phosphorylated fraction (p-β-cateninSer33/37/Thr41) in each reaction were measured by densitometric analysis of immunoblotting signals using a CS analyzer. (B) Relative expression of p-β-catenin in hFOB1.19, MG63, SaOS-2 and U2-OS cells. ** p < 0.01, compared with hFOB1.19 cells; ## p < 0.01, compared with SaOS-2 cells.

**Figure 4.** Effects of small molecule GSK3β inhibitors on the migration, invasion and related protein
expressions of MG63 cells. (A) Concentration effects on MG63 cells migration detected by wound-healing assay in the presence of DMSO, SB-216763 (5, 10, 15, or 10 μmol/L) or AR-A014418 (5, 10, 15 or 10 μmol/L). (B) Invading MG63 cells through matrigel-coating transwell after the treatments with DMSO, SB-216763 (20 μmol/L) or AR-A014418 (20 μmol/L). (C) Statistic analysis for invasion ability of MG63 under treatments with DMSO, SB-216763 (20 μmol/L) or AR-A014418 (20 μmol/L). (D) The mRNA expressions of GSK3β, MMP-2 and MMP-9 in MG63 cells treatments with DMSO, SB-216763 (20 μmol/L) or AR-A014418 (20 μmol/L). (E) The protein expressions of GSK3β in MG63 cells treatments with DMSO, SB-216763 (20 μmol/L) or AR-A014418 (20 μmol/L). * p < 0.05, compared with DMSO; ** p < 0.01, compared with DMSO.

Figure 5. Changes in the invasion ability, gene, and protein expressions of MG63 cells following GSK3β knock-down by siRNA transfection for 72 h. (A) Invading MG63 cells through matrigel-coating transwell after siRNA transfection. (B) Statistic analysis for invasion ability of MG63 cells under siRNA transfection. (C) The mRNA expressions of GSK3β, MMP-2, MMP-9, PTEN, and FAK in MG63 cells. (D) The protein expression of GSK3β, FAK, p-FAK\textsuperscript{Y397}; PTEN, MMP-2, and MMP-9 in MG63 cells. ** p < 0.01, compared with negative control.
A

10 x

40 x

Non-metastatic

Metastatic

B

Immunohistochemical staining

Percentage of GSK3β positive cells (%)

Metastatic

Non-metastatic

**
A

|    | hFOB | MG63 | SaOS-2 | U2-OS |
|----|------|------|--------|-------|
| NC |      |      |        |       |
| T  |      |      |        |       |

\( \text{p-} \beta \text{-catenin}^{\text{Ser33/37Thr41}} \)
\( \text{GSK3}\beta \)
\( \beta \text{-catenin}^{\text{His}} \)

B

**Non-radio isotopic in vitro kinase assay**

Relative expression of p-\( \beta \)-catenin

| Cell lines | hFOB | MG63 | SaOS-2 | U2-OS |
|------------|------|------|--------|-------|
|            |      |      |        |       |

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*This image contains scientific data and graphs related to the expression of p-\( \beta \)-catenin and GSK3\( \beta \) in different cell lines (hFOB, MG63, SaOS-2, U2-OS). The data is presented in the form of a Western blot and a bar graph. The Western blot shows the expression levels of p-\( \beta \)-catenin and GSK3\( \beta \) in each cell line, while the bar graph compares the relative expression of p-\( \beta \)-catenin across the same cell lines. The graphs indicate significant differences in expression levels between the control (NC) and treated (T) conditions.*
A. The number of invasion cells was measured under different treatments with DMSO, AR-A014418, and SB216763 at 5μM, 10μM, 15μM, and 20μM.

B. Images showing the effect of DMSO, AR-A014418, and SB216763 at 4× and 10× magnification.

C. Transwell assay results showing the number of invasion cells.

D. RT-PCR analysis of GSK3β, MMP2, and MMP9 expression levels under different treatments.

E. Western blot analysis showing the relative expression levels of GSK3β and β-actin.
**A** Negative control | GSK3β siRNA

**B** Transwell assay

- The number of invasion cells
- Treatments
  - Negative control
  - GSK3β siRNA

**C** RT-PCR

| Genes     | Relative mRNA expression levels |
|-----------|---------------------------------|
| GSK3β     | ![Graph showing mRNA expression levels for GSK3β](image) |
| MMP2      | ![Graph showing mRNA expression levels for MMP2](image) |
| MMP9      | ![Graph showing mRNA expression levels for MMP9](image) |
| PTEN      | ![Graph showing mRNA expression levels for PTEN](image) |
| FAK       | ![Graph showing mRNA expression levels for FAK](image) |

**D**

- Relative expression levels for p-FAK Y397, FAK, PTEN, MMP2, MMP9, GSK3β, β-actin
- Negative control
- GSK3β siRNA

**Notes:**
- **p-FAK Y397**
- **FAK**
- **PTEN**
- **MMP2**
- **MMP9**
- **GSK3β**
- **β-actin**