AZD1080, a specific inhibitor of GSK-3β, inhibits stemness and malignancies in osteosarcoma cancer stem-like cells

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Abstract. Osteosarcoma is the most common primary bone cancer, primarily affecting children and young adults. Cancer stem cells (CSCs), a subpopulation presenting stemness, critically influence prognosis and promote recurrence and metastasis. Due to the crucial role of glycogen synthase kinase-3 beta (GSK-3β) in maintaining stemness, it is considered as an important target for drug development. The aim of the present study was to evaluate the inhibitory effect of AZD1080, a GSK-3β inhibitor, on osteosarcoma CSCs. AZD1080 treatment clearly inhibited sphere formation in U2OS and 143B cells and dissociated spheres in CSCs derived from U2OS and 143B; in both processes, stemness markers OCT4 and SOX2 were markedly decreased, without affecting cell proliferation or apoptosis. AZD1080 treatment inhibited phosphorylation of GSK-3β and its downstream regulated genes, including HEY1, HES1, CyclinD1 and β-catenin. It was also observed that GSK-3β activity was critical for the inhibitory effects of AZD1080 treatment on sphere formation and stemness. Moreover, GSK-3β knockdown inhibited sphere formation and invasion capacity, indicating that AZD1080 exerts inhibitory roles in a GSK-3β-dependent manner. Taken together, the results showed AZD1080 as a specific inhibitor of CSC stemness, without cytotoxicity, and indicated it was a promising therapeutic agent that targeted GSK-3β signaling in osteosarcoma.

Introduction

Osteosarcoma is the most common solid bone cancer, primarily affecting children and young adults. It most frequently occurs in long bones, especially the distal femur and proximal tibia, characterized by tumors that produce bone-like substances, manifesting as a soft tissue mass with both osteolytic and osteoblastic bone destruction. Histologically, osteosarcoma can be classified into three types: Osteoblastic, chondroblastic and fibroblastic (1,2). Osteosarcoma is highly invasive, with ~20% of patients experiencing metastasis, commonly to the lungs, but also locally to other parts of the bone. Osteosarcoma cells are similar to osteoblasts in their ability to produce osteoid, suggesting that osteosarcoma originates from osteoblasts or osteoprogenitor cells. Additionally, its histological variability, with regions of osteoblastic, chondroblastic, or fibroblastic cells (3), suggests that the cell of origin for osteosarcoma may be a multipotent cell. Therefore, osteosarcoma was previously considered a 'differentiation defect disease', originating from mesenchymal stem cells or osteoprogenitor cells, caused by genetic and epigenetic disruptions in the osteoblast differentiation pathway (4,5). However, with the proposal of the 'cancer stem cell hypothesis', increasing research has demonstrated the existence of cancer stem cells (CSCs) in osteosarcoma (6). This cell subpopulation plays a crucial role in tumor drug resistance, recurrence and metastasis and has become a new therapeutic target for developing drugs.

AZD1080 is an effective and selective small molecule inhibitor of GSK3, first reported to be synthesized by AstraZeneca in 2013 (7,8). Its high-resolution X-ray crystal structure confirms that AZD1080 can target and inhibit GSK3β by binding to the ATP pocket and *in vitro* studies have shown strong selectivity for GSK3β. Initially, AZD1080 was reported as a treatment for Alzheimer's disease, but as GSK3β became a popular key target for cancer research, AZD1080 began to be studied as an anti-tumor drug. In 2016, Chen *et al* (9) found that AZD1080 could inhibit the proliferation, invasion, migration and lamellipodia formation of ovarian cancer cells and induce G_1 arrest in a concentration-dependent manner. AZD1080 also markedly downregulates the expression of glycogen synthase kinase-3 beta (GSK-3β), CDK2, CDK1, cyclin D1, MMP9 and Bcl-xL at both mRNA and protein levels (9). In 2021, Chandra *et al* (10) reported the therapeutic effects of GSK3β inhibitors, including AZD1080, on breast and ovarian cancer. In 2023, Zhang *et al* (11) described that AZD1080 could be used as a nanodrug targeting the tumor microenvironment, potentially helping to overcome lung cancer resistance to programmed cell death protein 1/programmed death-ligand 1 blockade.

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GSK3β has been one of the hot targets in tumor-related research in recent years, while also being highly controversial. A number of researchers have detected activation and overexpression of GSK3β in clinical tumor samples and both GSK3β inhibitors and gene silencing can lead to significant tumor growth inhibition (12). However, some argue that caution should be exercised when considering GSK3β inhibition as a tumor treatment approach. This is because β-catenin, as a classic phosphorylation substrate of GSK-3β, causes GSK-3β to function as a tumor suppressor in the Wnt signaling pathway (8). It is evident that GSK3β has a dual nature in tumors. GSK3β is a multifunctional monomeric protein involved in various cellular functions in tumors, including differentiation, survival, glycogen metabolism, protein synthesis, immune response and cell death (13). It is a core component of a number of pathways mediated by insulin/insulin receptor, insulin-like growth factor (IGF)/IGF receptor (IGFR), Wnt/β-catenin, Hedgehog, nuclear factor κB (NF-κB) and transforming growth factor-β (TGF-β) signaling pathways (13). To date, ~40 proteins involved in numerous cellular processes have been clearly confirmed as GSK-3β substrates, with >500 proteins proposed as GSK-3β substrates (13). GSK-3β can regulate gene expression by activating or inhibiting certain transcription factors such as β-catenin, NF-κB, nuclear factor of activated T cells, cAMP response element-binding protein, c-Jun and activator protein-1 (14). GSK-3β is a key element in the PI3K and Wnt signaling pathways and is also one of the earliest identified substrates of the oncogenic kinase AKT. In fact, GSK-3β, as a negative regulator of Wnt/β-catenin, is involved in the regulation of various protein destruction complexes (8). In addition, GSK3β plays a critical role in several pro-oncogenic pathways, including Wnt/β-catenin, Hedgehog, Notch and c-Myc-mediated signaling (15). Accumulating evidence suggests that the abnormal activity and expression of GSK3β contribute to the progression of various cancer types and their resistance to pharmacological or radiological therapies (16-20). Notably, GSK3β induces Akt activation in phosphatase with tensin homology (PTEN)-deficient tumors, while the accumulation of β-catenin and the Notch Intracellular Domain (NICD) facilitates the synergistic interaction between Notch and Wnt signaling, thereby enhancing the stemness of CSCs (20).

The present study explored the potential mechanisms of AZD1080 as a specific inhibitor of GSK-3β in regulating osteosarcoma stem cells. It also noted the regulatory role of GSK-3β in maintaining stemness and malignant behavior of osteosarcoma stem cells. It is hoped that the present study can provide new insights for clinical treatment of poor prognosis caused by cancer stem cells.

Materials and methods

Cell culture and cancer stem cells enrichment. The human osteosarcoma cell lines 143B and U2OS were obtained from ATCC. 143B cells were cultured in α-MEM containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics (penicillin, 100 U/ml, streptomycin, 100 μg/ml; Gibco; Thermo Fisher Scientific, Inc.). U2OS cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and penicillin-streptomycin (5,000 U/ml; Gibco; Thermo Fisher Scientific, Inc.). Cells were propagated at 37˚C with 5% CO_2 and 100% humidity. The cells were routinely passaged every 2-3 days when they reached ~80% confluence. Cancer stem cells were enriched by serum-free culture. Cells were plated in ultra-low attachment plates at a density of 2,500 cells/ml in RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with B27 supplement (Invitrogen; Thermo Fisher Scientific, Inc.), 20 ng/ml human epidermal growth factor (EGF; Invitrogen; Thermo Fisher Scientific, Inc.) and 20 ng/ml human basic fibroblast growth factor (bFGF; Invitrogen; Thermo Fisher Scientific, Inc.). Following culture for 2 weeks, colonies with a diameter ≥50 μm were regarded as sarcospheres and quantified by inverted phase contrast microscopy. Spheres were dissociated and re-plated to form the next generation of spheres every 14 days.

Transfection. Transfection of U2OS and 143B cells with small interfering (si)RNA (100 nM) was performed using Viromer (Lipocalyx GmbH) according to the manufacturer's instructions. siRNA oligonucleotides were purchased from MilliporeSigma. The following siRNA sequences were used: GSK-3β (5'-CUCAAGAACUGUCAAGUAATT-3'), negative control (5'-UAGCGACUAAACACAUCAA-3'). The transfection complex was co-incubated with the cells for 24 h at 37˚C. The culture medium was changed after transfection, and subsequent experiments and detection were performed 48 h later.

AZD1080 treatment. The 143B and U2OS OS stem cells were treated with 10 μmol/l AZD1080 and the formation and differentiation of spheres observed. The effect of AZD1080 on stemness of OS stem cells was observed by comparing with OS stem cells without AZD1080.

CCK-8 assay. 143B and U2OS OS stem cells ($3x10^3$ cells/well) were plated in 96-well plates. At 24 h incubation with AZD1080, CCK-8 reagents were added and incubated for another 2 h. The absorbance at 450 nm using a Multiskan Spectrum microplate reader represented the number of viable cells.

Flow cytometry. U2OS and 143B OS stem cells were treated with AZD1080. The cells were then washed with PBS and fixed with 70% ice-cold ethanol at 4˚C overnight. After fixation, the cells were stained with propidium iodide at room temperature in the dark for 10 min. The cell cycle was analyzed by flow cytometry (FACSCalibur; BD Biosciences). The data were analyzed using CellQuestPro™ v.6.0 software (Becton, Dickinson and Company).

For cell apoptosis detection, the apoptotic rate of the two OS cell lines was analyzed using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime Institute of Biotechnology). The distribution of viable, early apoptotic, late apoptotic and necrotic cells was detected using a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using CellQuestPro™ v.6.0 software. The apoptotic rate was calculated as the percentage of early and late apoptotic cells.

Transwell assay. Matrigel was rapidly added to the upper chamber of the Transwell insert and this was transferred

to a 37˚C incubator for 1-2 h. To assess invasion potential, Matrigel-coated Transwell was used. AZD1080-treated cells were seeded in the upper chamber of the Transwell insert. The migration capacity over the Matrigel and the membrane towards the bottom chamber containing 10% FBS was examined. Cells were fixed in 4% paraformaldehyde solution at room temperature for 20 min, and stained with 0.1% crystal violet at room temperature for 10 min and counted.

Colony formation assay. The colony formation capability of U2OS and 143B OS stem cells in soft agar media was investigated. Briefly, 100 single-cell suspension of U2OS/143B OS stem cells were resuspended in 0.8 ml growth media containing 0.3% low melting temperature agarose and were plated in triplicate on 24-well plate over a base layer of 0.8 ml growth media containing 0.6% low melting temperature agarose. The plates were incubated for 14-15 days until colonies were formed and colonies with a diameter >50 μm were counted.

Western blotting. Proteins were extracted with Protein Lysis Buffer (Mammalian Cell Lysis Kit; MCL1; MilliporeSigma). Lysates were centrifuged at 10,000 x g at 4˚C for 10 min and supernatants collected. Protein concentrations were assessed using the BCA Kit. Cell lysates containing 40 μg protein were separated on a 10% SDSPAGE gel and then transferred on polyvinylidene difluoride membranes using a Trans Blot Turbo (Bio-Rad Laboratories, Inc.). Membranes were blocked in a solution of Tris buffered saline containing 0.05% Tween-20 and 5% skimmed milk for 1 h at room temperature. Primary antibodies were incubated overnight at 4˚C. Primary antibodies used were anti-Hes1 (ab108937; 1:500; Abcam), anti-Hey1 (ab154077; 1:500; Abcam), anti-GSK3β (ab32391; 1:500; Abcam), anti-PTEN (ab267787; 1:500; Abcam), anti-MMP-2 (ab92536; 1:500; Abcam), anti-MMP-9 (ab76003; 1:500; Abcam), anti-E-cadherin (ab314063; 1:500; Abcam), anti-Vimentin (ab92547; 1:500; Abcam) and anti-GAPDH (ab8245; 1:2,000; Abcam). Horseradish peroxidase-conjugated secondary antibodies (GB23301 and GB23303; 1:3,000; Wuhan Servicebio Technology Co., Ltd.) were incubated for 2 h at room temperature. Finally, membranes were developed using an enhanced chemiluminescence substrate (G2161; Wuhan Servicebio Technology Co., Ltd.). The Bio-Rad Gel Doc XR+ Gel Imaging System Software Image Lab (version 6.1; Bio-Rad Laboratories, Inc.) was used for analysis.

Reverse transcription-quantitative (RT-q)PCR. TRIzol® (Thermo Fisher Scientific, Inc.) was directly added to the culture dish to lyse the cells (U2OS and 143B) after remove of growth media. The cell density for RNA extraction was ~5x10^6/ml. RNA extraction using the RNeasy Mini Kit (Qiagen, Inc.), generation of cDNA with Superscript III reverse transcription Kit (Invitrogen; Thermo Fisher Scientific, Inc.) and PCR using the SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the ABI Prism 7900TM Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). RNA extraction, cDNA synthesis and qPCR were performed according to the manufacturer's protocols. Experiments were performed in triplicate using three independent cDNAs. Cycling conditions were: Denaturation at 94˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec for 40 cycles followed by 5 min at 72˚C. Primer sequences are below: GSK-3β (F:5'-CAAGCGTGAGACTCGTGATCCTTC-3', R:5'-AGGCTGCTGCACCGGCTTGCAGCT-3'); MMP2 (F:5'-CCCACTGCGGTTTTCTCGAAT-3', R:5'-CAAAGGGGTATCCATCGCCAT-3'); MMP9 (F:5'-GGGAGACGCCCATTTCG-3', R:5'-CGCGCCATCTGCGTTT-3'); PTEN (F:5'-TGGATTCGACTTAGACTTGACCT-3', R:5'-GGTGGGTTATGGTCTTCAAAAGG-3'); GAPDH (F:5'-GAAGGTGAAGGTCGGAGT-3', R:5'-GAAGATGGTGATGGGATTTC-3').

The relative expressions (defined as fold change) of the target genes ($2^{-\Delta\Delta Cq}$) were normalized to the endogenous GADPH references (ΔCq) (21).

Statistical analysis. All experiments were performed in triplicate with three independent biological replicates. Random design studies were analyzed using an unpaired t-test. For comparisons of more than two groups, one-way analysis of variance with the Bonferroni post hoc test was applied. All tests were two-sided. GraphPad Prism 10.0 (Dotmatics) was used for all statistical analyses. All results are presented as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AZD1080 exerts a stemness inhibitory effect on osteosarcoma-derived CSCs. Considering that AZD1080 exerts an inhibitory effect on tumors, including ovarian cancer (9), its regulatory role on osteosarcoma-derived CSCs was explored. First, U2OS and 143B cells were cultured in serum-free medium for 5-10 days, obtaining non-adherent cell spheres capable of forming spheres (Fig. 1A). It was found that the expression levels of OCT4 and SOX2 were markedly increased in CSCs (Fig. 1B), indicating successful enrichment of CSCs.

AZD1080 is a specific inhibitor of GSK-3β activity (9), so 10 μmol/l AZD1080 was co-incubated with CSCs for 1-3 days and no significant effect on cell viability was found (Fig. 1C); flow cytometry analysis of the cell cycle also showed that AZD1080 treatment did not affect the cell cycle distribution of U2OS CSCs and 143B CSCs (Fig. 1D); meanwhile, AZD1080 treatment had no significant effect on the apoptosis rate (Fig. 1E). Notably, after co-culturing 10 μmol/l AZD1080 with CSC spheres for 1-3 days, the spheres showed disintegration and partial adherent growth phenotype (Fig. 2A) and the levels of OCT4 and SOX2 markedly decreased due to AZD1080 treatment (Fig. 2B). To further identify whether AZD1080 affects the formation process of CSC spheres, 10 μmol/l AZD1080 was added to serum-free medium to culture adherent cells and observed the formation of spheres. As shown in Fig. 3A, without addition of AZD1080, cell spheres gradually formed within 1-4 days; while after adding AZD1080, although cells continued to grow adherently, the formation of spheres was completely inhibited. AZD1080 treatment markedly decreased OCT4 and SOX2 (Fig. 3B). These results indicated that 10 μmol/l AZD1080 specifically inhibited the formation and enrichment of osteosarcoma CSCs without affecting cell proliferation ability, exerting a specific stem cell inhibitory activity.

In addition, AZD1080 disrupted existing spheres (Fig. 2A) by inhibiting pathways critical for CSC adhesion and maintenance (for example by downregulating OCT4/SOX2 and Notch

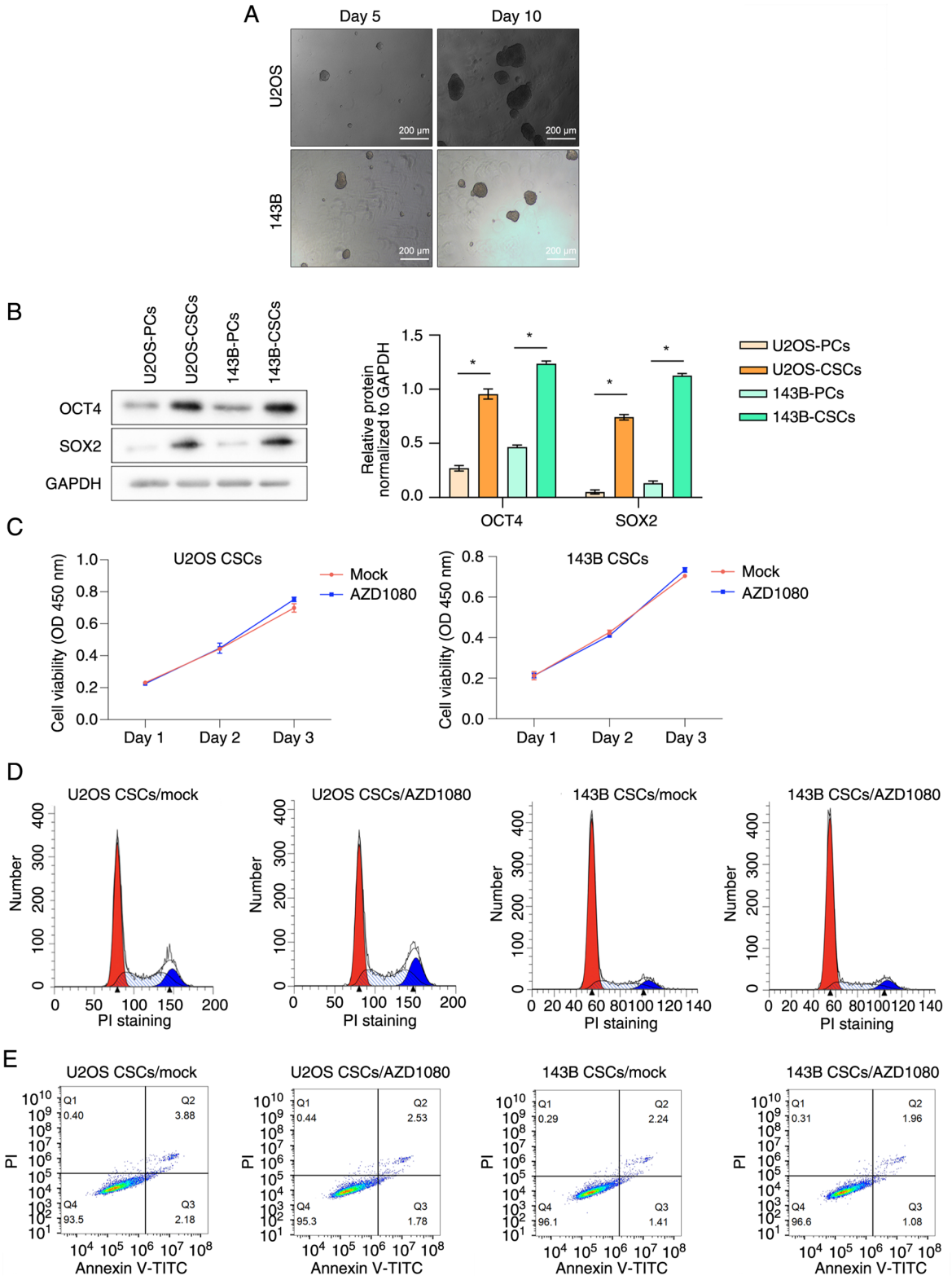

Figure 1. AZD1080 presented no obvious effect on proliferation or apoptosis in CSCs derived from U2OS and 143B. (A) Following culture in SFM for 5 and 10 days, cell morphology was observed. (B) After 5 days of culture in SFM, cells were collected and total protein was employed for western blot analysis for OCT4 and SOX2. *P<0.05, vs. PCs group. After 1, 2 or 3 days of co-culture with 10 μmol/l AZD1080, cell viability was detected by performing (C) CCK-8 assay, (D) cell cycle distribution was detected by flow cytometry assay following PI staining and (E) cell apoptosis was detected by performing flow cytometry assay following FITC/PI double staining. CSCs, cancer stem cells; PCs, parental cells; SFM, serum-free culture medium.

targets). Simultaneously, it prevented new sphere formation (Fig. 3A) by blocking early stages of CSC aggregation and stemness acquisition. The two effects were mediated through GSK-3β inhibition, which destabilized CSC niche signaling.

AZD1080 markedly inhibits the invasion and tumor formation capacity of CSCs. The malignant behavior of cancer stem-like cells is a critical cause of poor prognosis (22). Although 10 μmol/l AZD1080 showed no effect on proliferation of

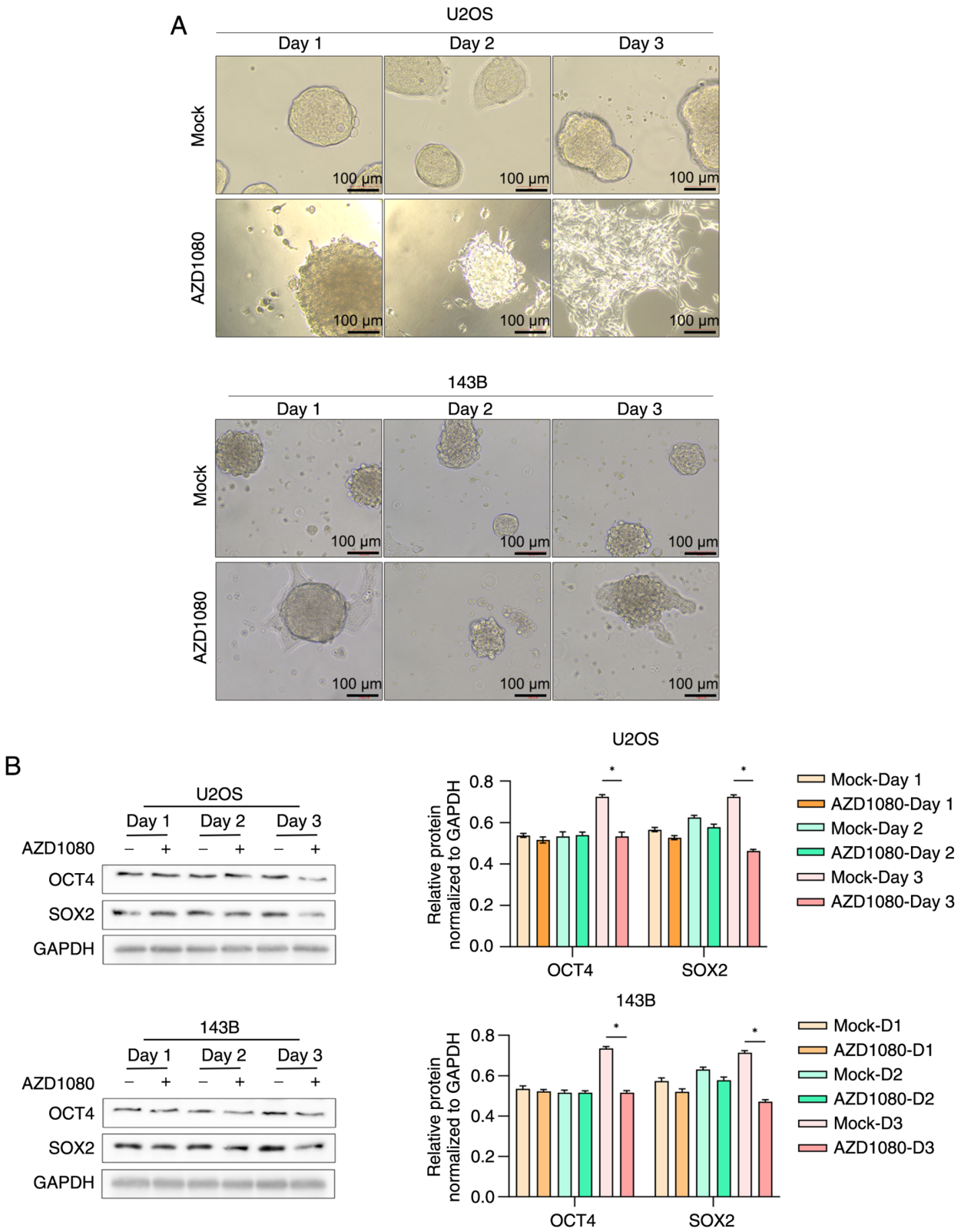

Figure 2. Addition of AZD1080 promoted dissociation of spheres and cell attachment. (A) 100 Spheres >50 μm in diameter were co-cultured with or without 10 μmol/l AZD1080 for 1-3 days in SFM and sphere morphology and attachment capacity were observed. (B) Total protein of CSCs co-cultured with or without 10 μmol/l AZD1080 for 1, 2 or 3 days was collected and analyzed by western blotting to detect OCT4 and SOX2. *P<0.05, vs. mock group. CSCs, cancer stem cells.

osteosarcoma cells, its effect on invasion and tumor formation capacity was subsequently examined. After 24 h pretreatment with AZD1080, the invasion of CSCs was assessed using a Transwell assay. As shown in Fig. 4A, compared with the untreated group (mock), the AZD1080-treated group exhibited markedly reduced invasion. The present study then examined the effect of AZD1080 on the tumor-forming capacity of CSCs *in vitro*. As expected, AZD1080 markedly inhibited

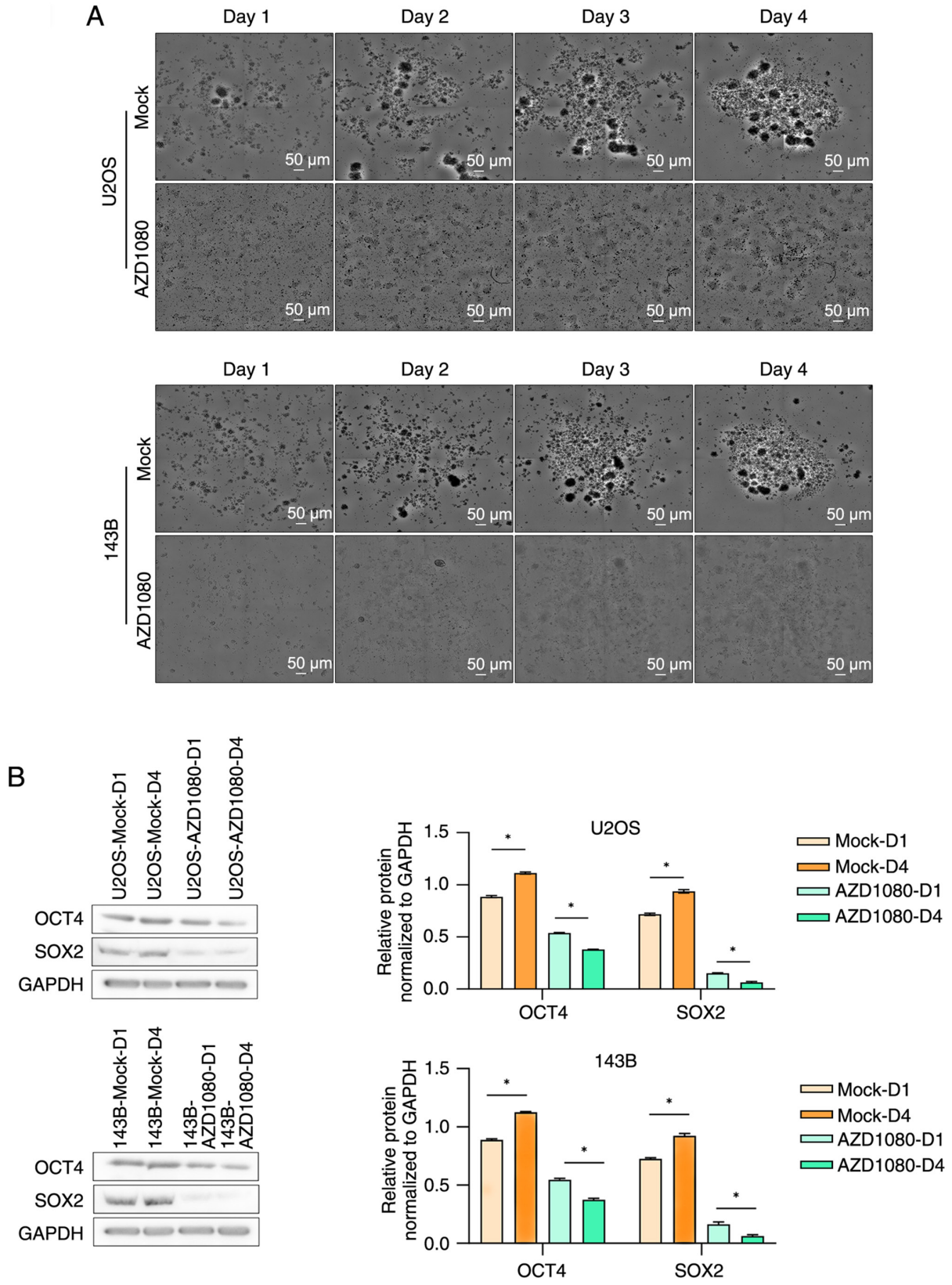

Figure 3. Addition of AZD1080 inhibited sphere formation without affecting cell attachment. (A) AZD1080 (10 μmol/l) was added in SFM for U2OS and 143B culture for 1-4 days and cell morphology and sphere formation were imaged. (B) Total protein of cells co-cultured with or without 10 μmol/l AZD1080 for 1 and 4 days was collected and analyzed by western blotting to detect OCT4 and SOX2. *P<0.05, vs. mock group. SFM, serum-free culture medium.

the colony-forming ability of CSCs in soft agar (Fig. 4B). These results suggested that AZD1080 may inhibit malignant behavior by suppressing cancer stem cell stemness. Thus, AZD1080 inhibited invasion and colony formation specifically in osteosarcoma CSCs, highlighting its therapeutic potential against CSC-driven malignancy.

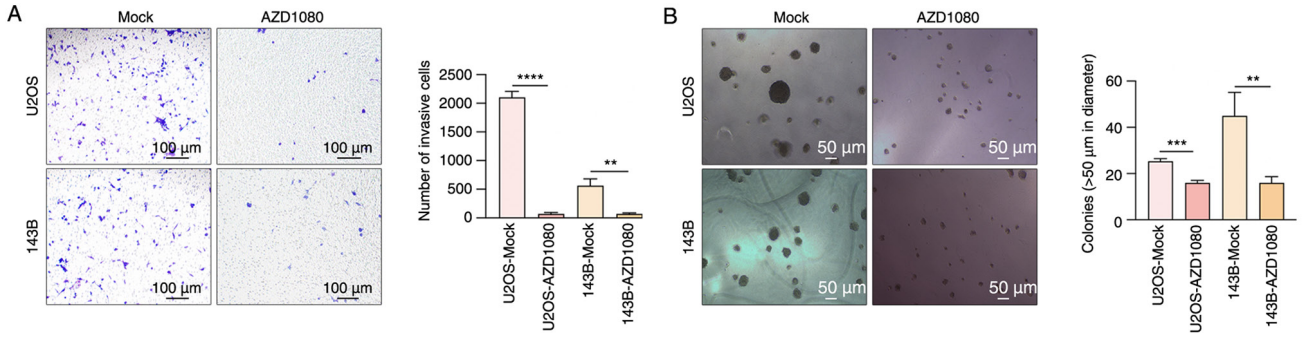

Figure 4. Addition of AZD1080 inhibited invasion and tumor formation in soft agar of CSCs derived from U2OS and 143B. Spheres were collected and dissociated to obtain single cells of CSCs, which were then co-cultured with 10 μmol/l AZD1080 for 24 h. (A) The invasion capacity of single cells of CSCs were detected by performing Transwell assay. (B) Tumor formation assay was measured in soft agar. **P<0.01, ***P<0.001, ****P<0.0001 vs. mock group. CSCs, cancer stem cells.

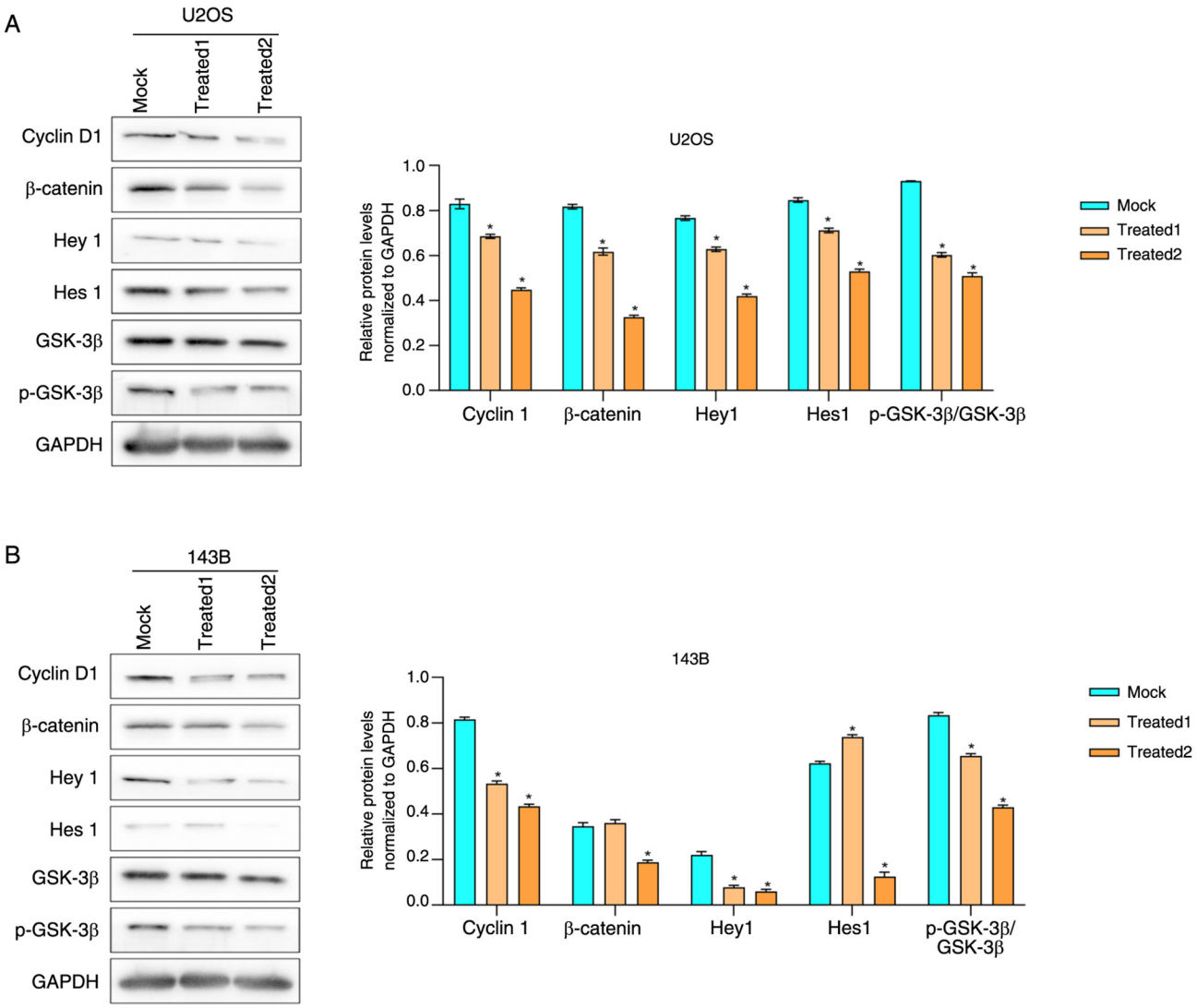

Figure 5. AZD1080 inhibited GSK-3β phosphorylation and its downstream regulated signaling. Spheres were collected and dissociated to obtain single cells of CSCs, which were then co-cultured with 10 μmol/l AZD1080 for 24 h. Total protein was collected to detect GSK-3β total protein and phosphorylated protein, including its downstream regulated proteins, CYCLIND1, β-catenin, HEY1, HES1 in (A) U2OS CSCs and (B) 143B cells. *P<0.05 vs. mock group. GSK-3β, glycogen synthase kinase-3β; CSCs, cancer stem cells.

AZD1080 inhibits GSK3β and its downstream signaling. GSK3β is an established regulator of the Notch signaling pathway (23,24), presenting essential regulatory role on Notch1 in osteosarcoma cells (25). Thus, the target genes of Notch1, including HES1 and HEY1, were detected. As presented in Fig. 5A and B, AZD1080 treatment markedly

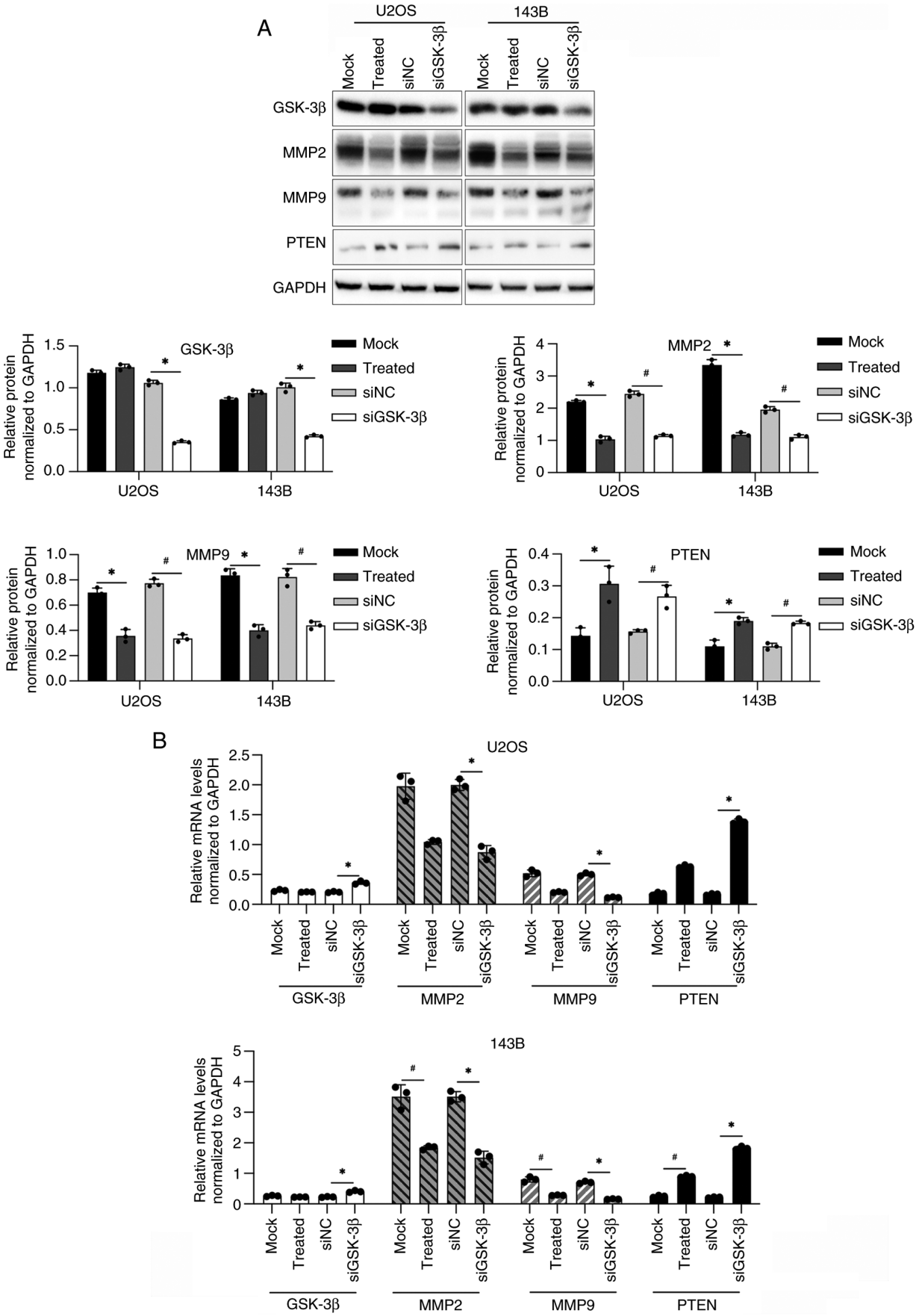

Figure 6. AZD1080 inhibited PTEN signaling via regulating GSK-3β activity. (A) After AZD1080 treatment for 24 h, or GSK-3β knockdown for 48 h, total protein was collected from CSCs derived from U2OS and 143B and western blot was performed to detect GSK-3β, MMP2, MMP9 and PTEN. *P<0.05, vs. mock group; #P<0.05, vs. siNC group. (B) Following AZD1080 treatment or GSK-3β knockdown, mRNA levels including GSK-3β, MMP2, MMP9 were measured by performing reverse transcription-quantitative PCR. *P<0.05, vs. mock group; #P<0.05, vs. siNC group. PTEN, phosphatase with tensin homology; GSK-3β, glycogen synthase kinase-3β; si, small interfering; NC, negative control.

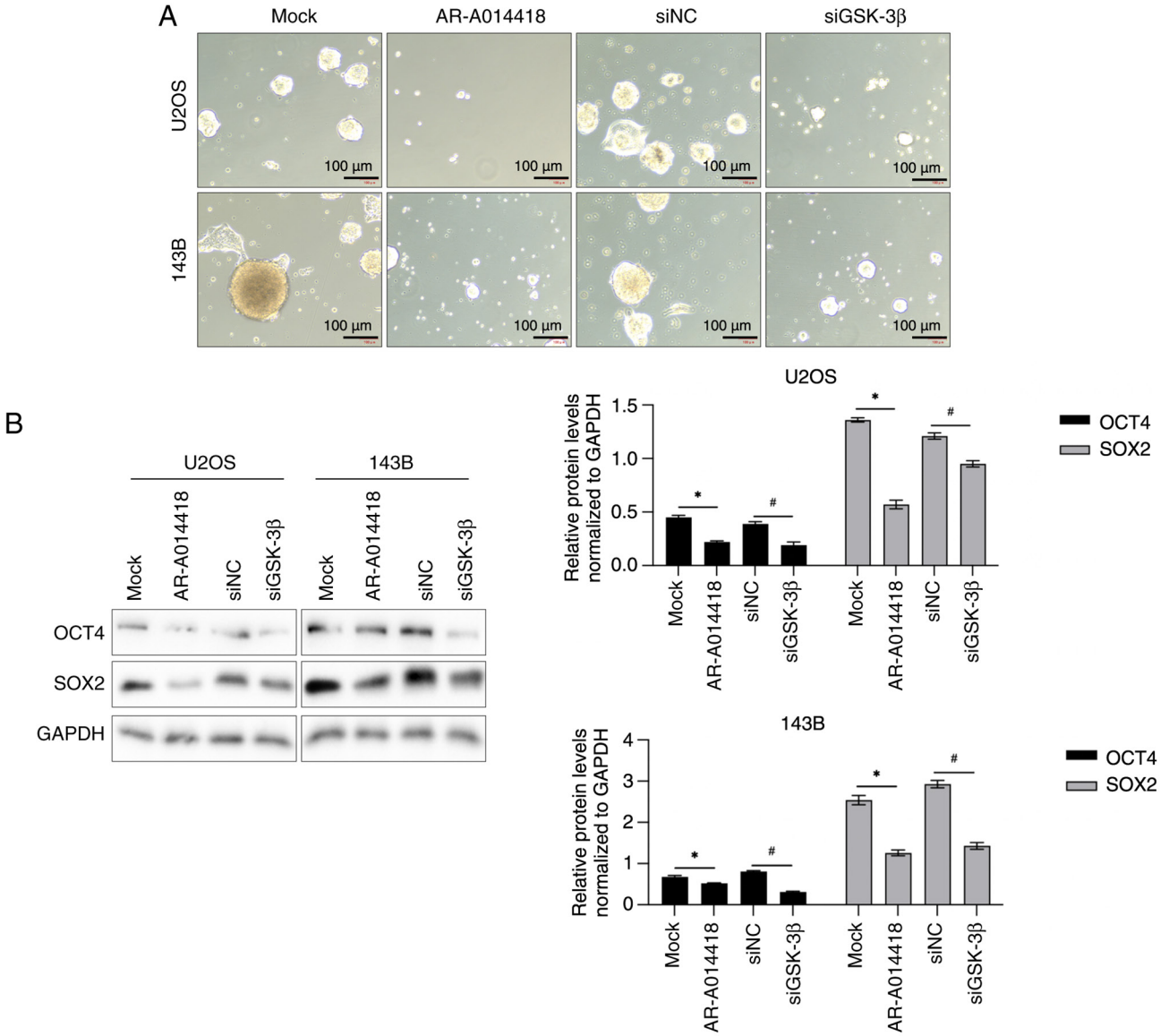

Figure 7. GSK-3β is critical for stemness maintenance in CSCs derived from osteosarcoma. (A) Following 10 μmol/l AR-A014418 treatment for 24 h, or GSK-3β knockdown for 48 h, U2OS or 143B cells were cultured in SFM for 10 days and sphere formation was observed. (B) Following 24 h culture in SFM, total protein was obtained to detect OCT4 and SOX2. *P<0.05, vs. mock group; #P<0.05, vs. siNC group. GSK-3β, glycogen synthase kinase-3β; CSCs, cancer stem cells; SFM, serum-free culture medium.

decreased phosphorylated GSK3β without affecting GSK3β total protein. HES1 and HEY1 levels were decreased markedly by AZD1080 treatment, indicating AZD1080 inhibited GSK3β and its downstream signaling.

PTEN is reported as a tumor suppressor gene, which is inhibited by GSK3β post-transcriptionally in osteosarcoma cells by inhibiting MMP-2 and MMP-9 (26,27). Based on the results showing that AZD1080 inhibits invasion, the present study further evaluated the effects of AZD1080 on PTEN, MMP-2 and MMP-9. As presented in Fig. 6A and B, AZD1080 treatment markedly decreased MMP2 and MMP9, transcriptionally and post-transcriptionally, meanwhile AZD1080 treatment increased PTEN transcriptionally and post-transcriptionally. To confirm whether GSK-3β is a key regulator of MMP2, MMP9 and PTEN, siRNA targeting GSK-3β mRNA was employed to knockdown GSK-3β expression. After knocking down GSK-3β efficiently, MMP2, MMP9 and PTEN were modified (Fig. 6A and B). All these results indicated that AZD1080 inhibits GSK3β phosphorylation and thus regulates its downstream signaling.

GSK3β is crucial for maintaining stem cell stemness and invasive ability. To further confirm whether the GSK3β activity inhibited by AZD1080 is critical for maintaining stemness and invasive ability, a GSK3β inhibitor, AR-A014418 or siGSK-3β, was employed to block GSK3β's regulatory effect and then observed the enrichment of CSCs. The results showed (Fig. 7A) that both AR-A014418 and siGSK-3β significantly inhibited sphere formation; meanwhile, the expression of stemness-related factors markedly decreased (Fig. 7B), indicating that GSK3β's regulatory activity is crucial for stemness. Meanwhile, the changes in invasive ability after treatment with AR-A014418 or siGSK-3β were also examined. As

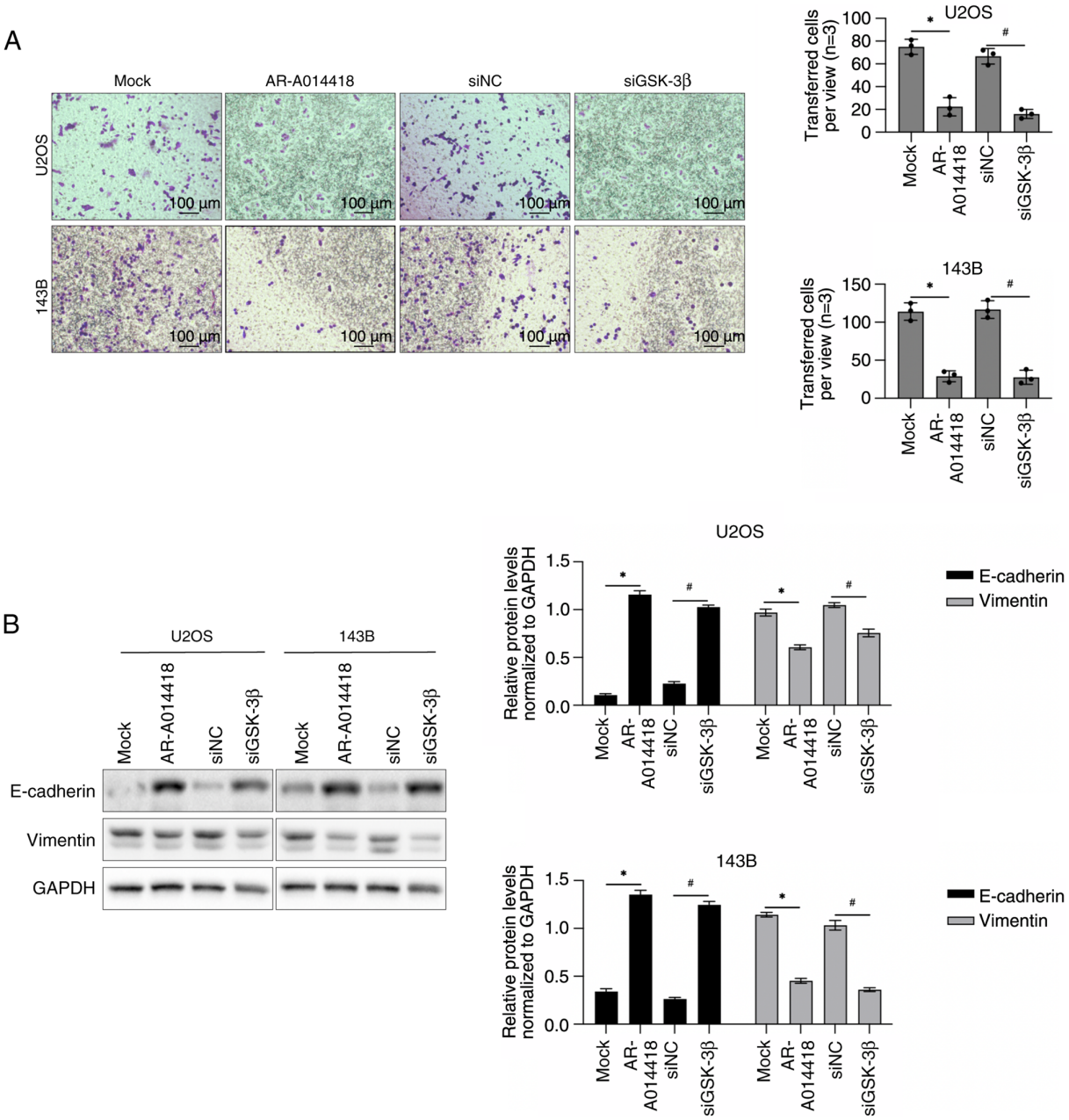

Figure 8. GSK-3β is critical for invasion capacity. (A) Following 10 μmol/l AR-A014418 treatment for 24 h, or GSK-3β knockdown for 48 h, CSCs derived from U2OS or 143B were used to detect invasion capacity. *P<0.05, vs. mock group; #P<0.05, vs. siNC group. (B) Total protein was obtained to detect E-cadherin and Vimentin. *P<0.05, vs. mock group; #P<0.05, vs. siNC group. GSK-3β, glycogen synthase kinase-3β; CSCs, cancer stem cells; si, small interfering; NC, negative control.

expected, both AR-A014418 and siGSK-3β markedly inhibited the invasive ability (Fig. 8A). The present study also examined changes in EMT-related markers, including E-cadherin and Vimentin. The results showed (Fig. 8B) that AR-A014418 or siGSK-3β markedly increased the expression of E-cadherin and decreased the expression of Vimentin, indicating that the EMT process was inhibited.

Discussion

The present study investigated the direct effects of the GSK-3β inhibitor AZD1080 on osteosarcoma stem cells. It was found that AZD1080 had no significant effect on the viability and proliferation of osteosarcoma cells, but specifically inhibited the stemness and invasive ability of tumor stem cells. The results also showed that AZD1080 had a significant inhibitory effect on the downstream signaling pathways of GSK-3β, indicating that GSK-3β is the direct target of AZD1080's stemness inhibition activity.

While the present study focused on 10 μM AZD1080 [a concentration validated in prior studies for GSK-3β inhibition (8,9,28)], there is a need for dose- and time-response experiments. Future work will explore these parameters, but the current data robustly demonstrated AZD1080's efficacy at this concentration. Although the current sphere dissociation and re-plating assays (Figs. 2 and 3) provided preliminary evidence of CSC self-renewal inhibition, there are limitations to this analysis. More rigorous functional assays will be added

in future studies, for example serial sphere formation and limiting dilution assays.

In the meantime, to refine this research it is planned to: i) Re-establish GSK-3β activity for example by overexpressing a constitutively active form to confirm that the observed effects are specifically due to its inhibition by AZD1080. ii) Perform experiments across a range of AZD1080 concentrations and multiple time points to determine the minimum effective dose and to map the temporal dynamics of CSC inhibition. iii) Overexpress GSK-3β or employ a constitutively active mutant in cells treated with AZD1080 to confirm that the observed effects (reduced sphere formation, decreased stemness marker expression, diminished invasion) are directly due to GSK-3β inhibition. iv) Complement the current Transwell assays with additional methods, such as wound healing or 3D invasion assays, to provide a more comprehensive evaluation of cell motility and invasiveness.

In recent years, GSK-3β has attracted attention in an increasing number of tumors. However, since GSK-3β also plays a role in normal cells, it has certain limitations in the targeted therapy of CSCs. This requires that the selected drug has good CSCs targeting, otherwise it is easy to produce off-target effects. It is noteworthy that AZD1080 showed strong CSCs targeting in the present results, which makes AZD1080, as a GSK-3β inhibitor targeting CSCs, able to evade normal cells and directly target to inhibit the stemness of CSCs.

In osteosarcoma, it is considered that high levels of GSK3β may be an important cause of osteosarcoma induction and its exacerbation (29). CSCs, as the most special cell population in tumors, have always been a very special presence in tumor research. The Notch signaling pathway, as another of the three classical stemness pathways of CSCs, also plays a significant role in osteosarcoma (30). It has been reported that Notch signaling is also associated with increased aldehyde dehydrogenase (ALDH) activity in osteosarcoma cells, which can lead to a highly invasive metastatic phenotype in mouse osteosarcoma cells (31). ALDH is one of the important markers of osteosarcoma tumor stem cells and it is hypothesized that this highly invasive metastatic phenotype may originate from the enhanced stemness of osteosarcoma tumor stem cells caused by Notch activation. The present study also showed that AZD1080 treatment inhibited the downstream target genes of Notch1; it inhibited the stemness of tumor stem cells and also inhibited invasive ability. The present study also found that another small molecule inhibitor of GSK-3β, AR-A014418, also has the effect of inhibiting tumor stem cell stemness and invasive ability. Although there are a number of small molecule inhibitors of GSK-3β, it does not mean that they all have the potential to be therapeutic drugs, because selectively inhibiting the target protein in tumor stem cells without affecting the function of the pathway in normal cells is the key to reducing drug side effects. Therefore, whether AZD1080 can be used as a potential therapeutic drug still needs more discussion on side effects.

Studies have shown that the Notch signaling pathway is closely related to the drug resistance, invasiveness and metastatic potential of osteosarcoma. This association has been confirmed in various experimental models (human/mouse osteosarcoma cell lines and *in vivo* mouse/dog models and patient samples) (32,33). These studies also indirectly confirm the hypothesis that osteosarcoma models with Notch activation have higher drug resistance and metastatic potential compared with normal osteoblasts or non-metastatic osteosarcoma cell lines. This may be evidence of enhanced stemness of osteosarcoma tumor stem cells and indicates that high expression levels of Notch signal-related genes or proteins are associated with the invasion and metastasis of osteosarcoma tumors and closely affect the survival of osteosarcoma patients.

Currently, there are no reports on the relationship between GSK3β and Notch signaling pathways in osteosarcoma, especially on their relationship in osteosarcoma tumor stem cells. The Notch pathway is usually associated with inhibition of cell differentiation (34) and the detachment and nuclear translocation of its intracellular domain (NICD) activates the Notch pathway. GSK3β phosphorylation in the Ser/Thr-Pro-Ser/Thr region stabilizes NCID in Notch 1 (22,35) and NCID in Notch 3 (36) and inactivates it in Notch2 (37). Reducing Notch1 recycling is also an example of GSK3β's negative regulation of this pathway (24). It can be seen that the association between GSK3β and the Notch signaling pathway is very close, which may provide new thoughts for the clinical treatment of osteosarcoma.

Finally, the results of our present study are based on *in vitro* experiments only, due to various conditions. This leads to some limitations in the present study. Nevertheless, the results provided scientific evidence for the therapeutic potential of AZD1080. Follow-up studies are planned to conduct an *in vivo* xenograft model study to further demonstrate the efficacy and safety of AZD1080.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

PG and KD designed the experiments. PG, ZL and HG are responsible for cell culture and malignant analysis. XH, CZ and BW were responsible for data analysis. KD wrote the manuscript. KD and PG confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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