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The Na+/K+ ATPase Inhibitor Ouabain Attenuates Stemness and Chemoresistance of Osteosarcoma Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCE 1 Weixiong Guo*
BCD 1 Bo Wei*
DEF 2 Tingting Chen
DEF 3 Xiaotao Xu
BC 3 Feiling Ruan
AFG 1 Min Xiang

1 Department of Orthopedics, The Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong, P.R. China
2 Department of Cardiovascular, The Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong, P.R. China
3 Department of Operation Room, The Affiliated Hospital of Guangdong Medical University, Zhanjiang, Guangdong, P.R. China

* Weixiong Guo and Bo Wei contributed equally

Corresponding Author: Min Xiang, e-mail: xiangmin_x@yeah.net
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Background: The purpose of this study was to explore the effects of the Na+/K+ ATPase inhibitor ouabain in regulating osteosarcoma (OS) cell stemness.

Material/Methods: Western blot, qPCR, sphere-forming analysis, DNA methylation analysis, and Ca^{2+} concentration detection were performed to evaluate the stem-like traits of cells and ouabain-induced effects and related mechanisms on OS cell stemness. Cell viability assessment was performed to evaluate the effect of ouabain on OS cell chemosensitivity.

Results: Ouabain reduced the ALDH1 activity, the expression of critical stemness regulators, sphere size and number, and migration, invasion, and adhesion ability, but had little effects on cell viability. Additionally, the intracellular Ca^{2+} concentration and methylation level of the critical stemness regulators were higher in OS cells than in spheres formed by OS cells. Mechanistic studies revealed that ouabain leads to DNA methylation of stemness markers through increasing intracellular Ca^{2+} concentration. Notably, inhibition of Ca^{2+} channel or DNA methylation rescued the inhibition of ouabain on OS cell stemness. Additionally, ouabain enhances cisplatin sensitivity of OS cells, which is involved in Ca^{2+} channel and DNA methylation.

Conclusions: This work provides a potential compound for treating OS patients, especially OS patients with chemoresistance.

MeSH Keywords: Chemotherapy, Adjuvant • Colony-Forming Units Assay • Osteosarcoma

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Background

Osteosarcoma (OS) is a highly malignant bone tumor that often occurs in children and adolescents under 25 years old [1]. It is often found in the metaphysis of long bones and is often accompanied by lung metastasis in the early stage [1]. With traditional treatment strategies, including radical limb salvage surgery, pre-operative neoadjuvant chemotherapy and post-operative multi-drug combination chemotherapy, the 5-year survival rate is only 50–70% for patients with small primary tumors and their treatment is far from ideal, perhaps due to the complex mechanisms contributing to OS occurrence [2]. Therefore, it is necessary to elucidate the mechanism of OS occurrence.

Previous studies have shown that OS is composed of a variety of tumor cell subsets with different functions [3]. Only a small number of OS cells have the characteristics of cancer stem cells (CSCs), such as self-renewal and multi-directional differentiation, which enable OS stem cells to differentiate into different types of cancer cells [4]. Additionally, OS stem cells play a decisive role in the proliferation, invasion, recurrence, and metastasis of OS. These studies suggest that the occurrence of osteosarcoma may be closely related to the existence of OS stem cells and that OS progression could be slowed by targeting OS stem cells. For example, a previous study indicated that the chemoresistance of OS is driven by OS stem cell-like cells, and this process is contributed to by the TGFβ-miR-499a-SHKBP1 pathway [5]. The long non-coding RNA (lncRNA) THOR increases OS stem cell-like cell progression via enhancing the mRNA stability of a stemness critical regulator SOX9 [6]. lncRNA hypoxia-inducible factor-2α (HIF-2α) promoter upstream transcript (HIF2PUT) suppresses OS stem cell-like cell progression by downregulating HIF2 expression [7]. DNA methylation refers to the process of transferring methyl to a specific base (cytosine C); this process is catalyzed by DNA methyltransferase. Methylation of various tumor-suppressor genes or oncogenes plays an important role in the occurrence and development of OS. For example, a previous study identified a quinoline-based DNA methyltransferase inhibitor as a possible adjuvant in OS therapy [8]. The methylation of PTEN promoter mediated by DNMT3B and G9a in human dental pulp mesenchymal stem cells promotes osteogenesis [9]. The m6A methyltransferase METTL3 promotes OS cell progression by downregulating the m6A level of LEF1 and activating Wnt/β-catenin pathway [10]. A recent study indicated that circulating tumor cells (CTCs) with stem cell-like traits shape DNA methylation to enable metastasis seeding, and this process could be rescued by the Na+/K+ ATPase inhibitor ouabain through enhancing intracellular Ca^{2+} concentration [11]. Therefore, we speculated that ouabain could attenuate OS cell stemness by regulating intracellular Ca^{2+} concentration and thus DNA methylation.

We found that the intracellular Ca^{2+} concentration and the methylation level of the critical stemness regulators were decreased in spheres formed by OS cells. The Na+/K+ ATPase inhibitor ouabain decreased OS cell stemness, as evident by decreasing the expression of stemness regulators, ALDH1 activity, sphere-forming capacity, and migration, invasion, and adhesion abilities, and this process was rescued by inhibition of Ca^{2+} channel or DNA methylation.

Material and Methods

Cell culture and reagents

OS cell lines MG63 and U2OS cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPIM 1640 medium (Biological Industries, Kibbutz Beit Haemek, Israel) with 15% fetal bovine serum (FBS, Biological Industries) with in a humidified atmosphere with 5% CO_2 at 37°C. The Na+/K+ ATPase inhibitor ouabain was purchased from MedChem Express (Monmouth Junction, NJ), and concentrations of 5 nM, 10 nM, and 20 nM were used in this work. Azacitidine (5-AzaC) and verapamil were purchased from Selleck Chemicals and the concentrations of 50 nM and 5 μM were used, respectively.

Quantitative real-time PCR (qCR)

The process of RNA extraction was performed using RNA Isolator Total RNA Extraction Reagent (Vazyme, Nanjing, China). Then, cDNA was reversely synthesized and qPCR was performed using the Hifair™ III One Step RT-qPCR Probe Kit (YEASEN). qPCR was constructed on the StepOne Plus PCR system. $2^{-\Delta\Delta ct}$ method was used to calculate the relative expression levels of transcripts.

Western blot

Cells were lysed and whole protein was extracted using RIPA lysis buffer (Beyotime, Beijing, China). The BCA Protein Quantification Kit (Tiangen, Beijing, China) was used to measure the protein concentration. The procedure was performed following the protocols detailed in a previous work [12].

Sphere-forming analysis

The procedure was performed as detailed in a previous work [13].

ALDH1 activity analysis

ALDH1 activity was examined using ALDH Activity Assay Kit (Colorimetric) (Abnova, Taipei, China).

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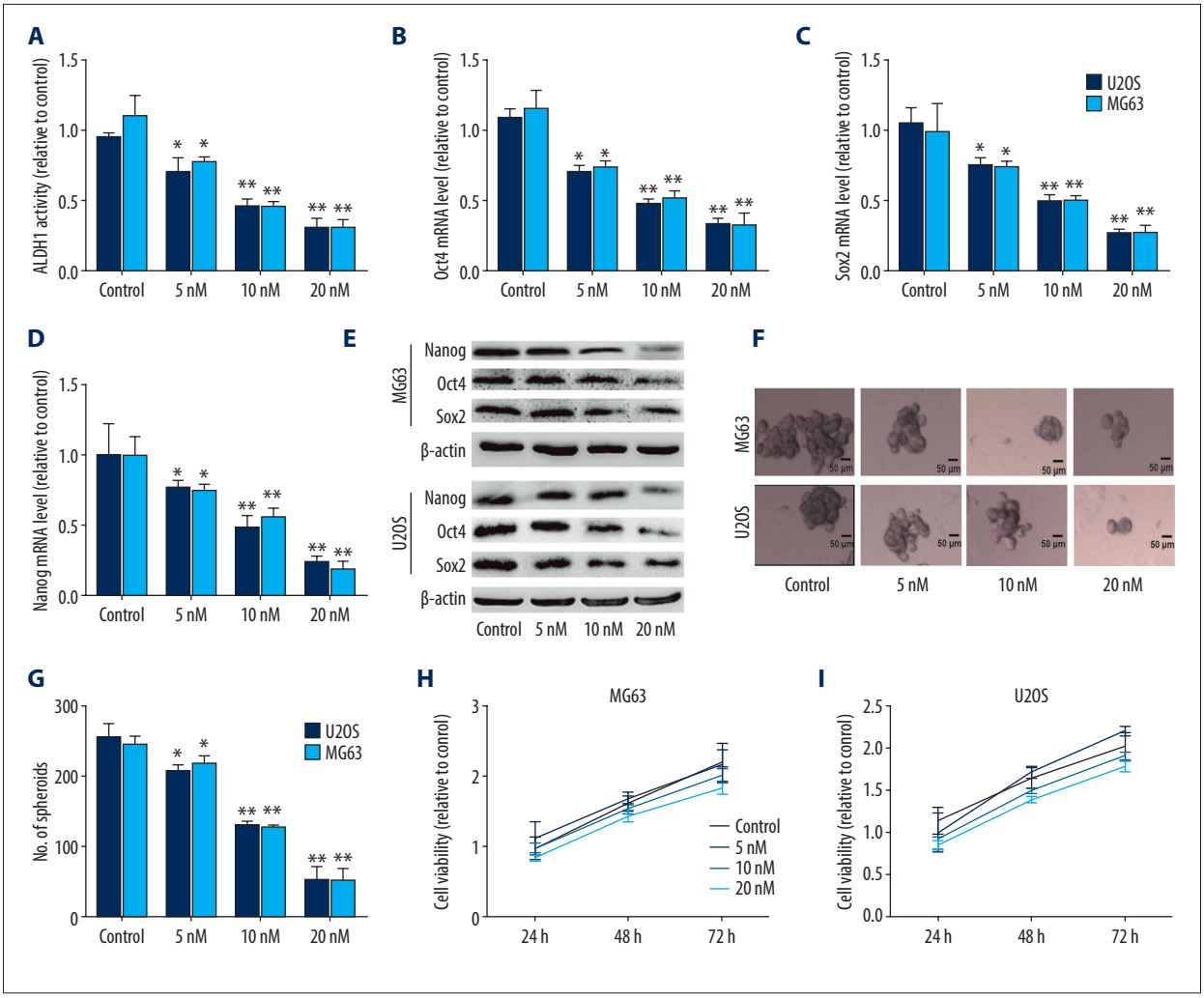

Figure 1. Ouabain reduces OS cell stemness but has little effects on cell viability. (**A**) The activity of ALDH1 was measured in OS cells treated with different concentrations of ouabain. (**B–D**) QPCR analysis on the mRNA levels of critical stemness regulators in OS cells with ouabain treatment. (**E**) Western blot analysis of the protein levels of critical stemness regulators in OS cells treated with ouabain. (**F**) Sphere size was evaluated in OS cells with ouabain treatment. (**G**) Sphere number was determined in OS cells with ouabain treatment. (**H, I**) Cell viability was assessed in OS cells with ouabain treatment. ** P<0.01 *vs.* control.

Cell viability analysis

Single-cell suspension (100 µl/well) was incubated in 96-well plates for 12–24 h. Then, different concentrations of ouabain were added to medium. After 24 h, 48 h, and 72 h, we added 10 µl CCK-8 solution (MedChem Express) to each well for another 1.5-h incubation. The absorbance at 450 was determined for cell viability.

Transwell migration and invasion assay

The procedure for cell migration and invasion assay was performed as detailed in a previous work [14]. For invasion ability detection, the upper chambers were pre-coated with Matrigel (Thermo Fisher Scientific) 24 h later for migration assay and 48 h later for invasion assay.

Cell adhesion assay

The 96-well plates were pre-coated with Matrix gel (Thermo Fisher Scientific), and blocked using 2.5% BSA. OS cells were suspended and seeded into the 96-well plates at a concentration of 10^6 cells/well in 200 µl serum-free medium supplemented with ouabain treatment. After 1 h, the non-adherent cells were washed and the CCK8 assay was performed to determine the number of adherent cells.

Intracellular Ca^{2+} measurement

For detecting Ca^{2+} concentration, Fluo-3-AM (Powder), Cell Permeant (YEASEN, Shanghai, China; Cat # 40703ES50) was used. Briefly, cells were washed 3 times with HBSS (hanks

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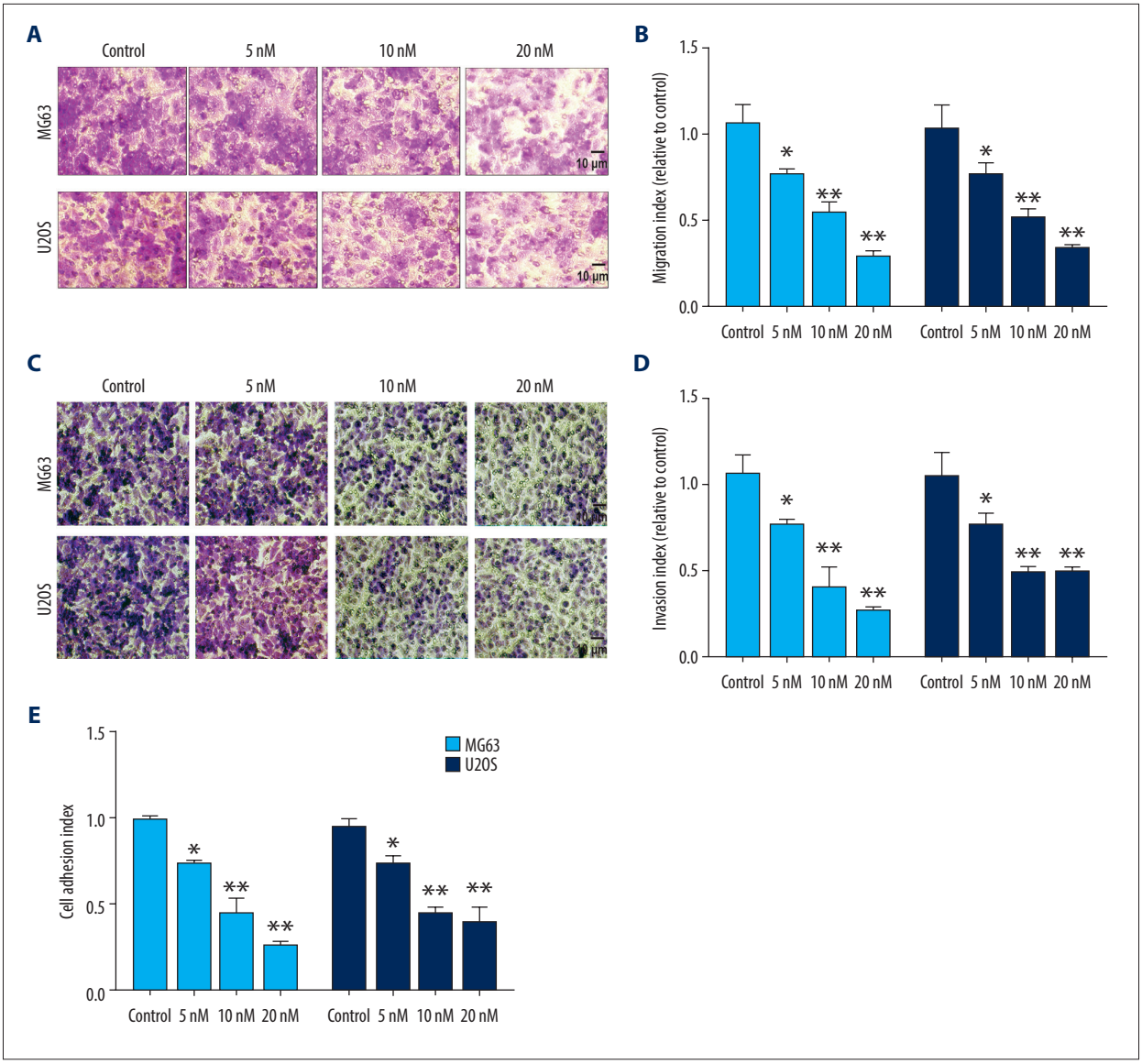

Figure 2. Ouabain inhibits OS cell migration, invasion, and adhesion ability. (**A, B**) The migration ability was evaluated in OS cells with ouabain treatment. (**C, D**) The invasion ability was determined in OS cells with ouabain treatment. (**E**) Cell adhesion capacity was measured in OS cells treated with ouabain. * P<0.05, ** P<0.01 *vs.* control.

balanced salt solution) solution. Fluo-3-AM working fluid (5 μM) was added to the cells with 20% Pluronic F-127 (Sigma, Cat # P2443), and the amount of added Fluo-3-AM working fluid was based on the coverage of the cells. Cells were cultured at 37°C for 15–60 min, then the cells were washed 3 times with HBSS solution to remove the residual Fluo-3-AM working fluid. HBSS solution was added to cover the cells. After incubating at 37°C for 20–30 min, the plates were scanned using the Operetta High Content Imaging System (Perkin Elmer), and Fluo-3 intensity was calculated using the Columbus Image Data Storage and Analysis System (Perkin Elmer).

DNA methylation analysis

MethPrimer (*http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi*) was used to analyze the CpG islands on Sox2, Oct4, and Nanog promoter using the method previously published [15]. Briefly, the PureLink™ Pro 96 Genomic DNA Purification Kit (Thermo Fisher Scientific) was used to extract genomic DNA. Genomic DNA was treated with sodium bisulfite using the CpGenome DNA Modification Kit (Merck Millipore, Billerica, MA, USA). PCR Primers were designed to expand Sox2, Oct4, and Nanog promoter with nuclear CpG island region or not.

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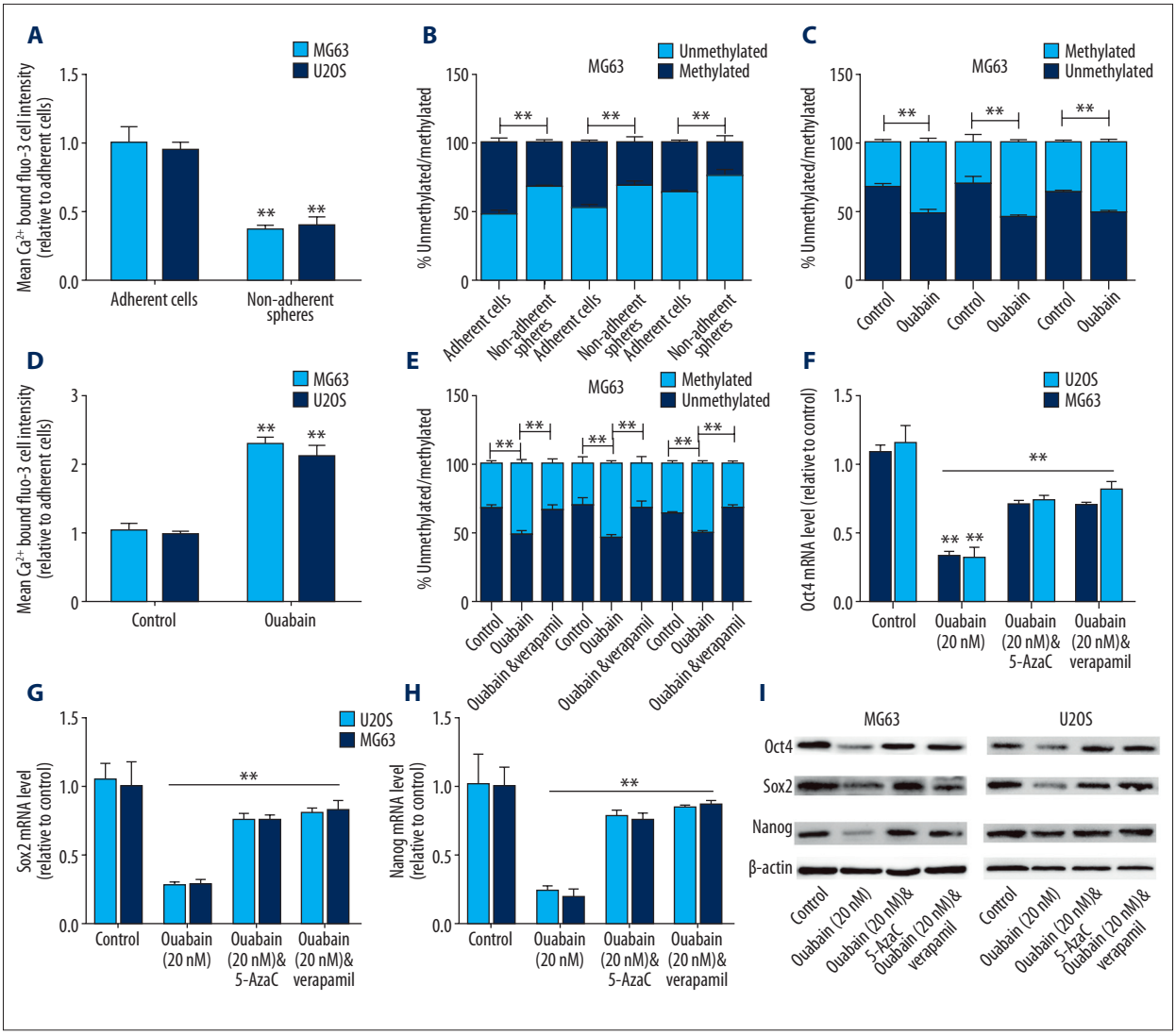

Figure 3. Ouabain leads to DNA methylation of stemness markers through increasing intracellular Ca^{2+} concentration. (**A**) The Ca^{2+} concentration was determined in OS adherent cells and OS non-adherent spheres. (**B**) The methylation levels of stemness regulators were determined in MG63 adherent cells and non-adherent spheres. (**C**) The methylation levels of stemness regulators were detected in MG63 cells treated with or without ouabain. (**D**) The Ca^{2+} concentration was measured in OS cells treated with or without ouabain. (**E**) The methylation levels of stemness regulators were detected in MG63 cells with ouabain plus verapamil treatment or not. (**F–H**) The mRNA levels of stemness regulators were determined in OS cells with ouabain, ouabain plus 5-AzaC, and ouabain plus verapamil treatment. (**I**) The protein levels of stemness regulators were measured in the OS cells described in (**F**). ** P<0.01 *vs.* control.

Statistical analysis

All data are expressed as the mean±SEM, where the mean represents number of independent experiments (n≥3). Statistical analysis was performed using Prism7 (GraphPad software). The *t* test was used for analyzing the datasets with only 2 groups. Differences between groups were analyzed using one-way ANOVA with the Tukey-Kramer post hoc test. P value less than 0.05 was considered significant.

Results

Ouabain reduces OS cell stemness but has little effect on cell viability

We first investigated the effects of ouabain on OS cell stemness and found that ouabain significantly decreased ALDH1 activity in OS cells in a concentration-dependent manner (Figure 1A). Moreover, the expression of critical stemness regulators (Oct4, sox2 and Nanog) was decreased by ouabain in OS

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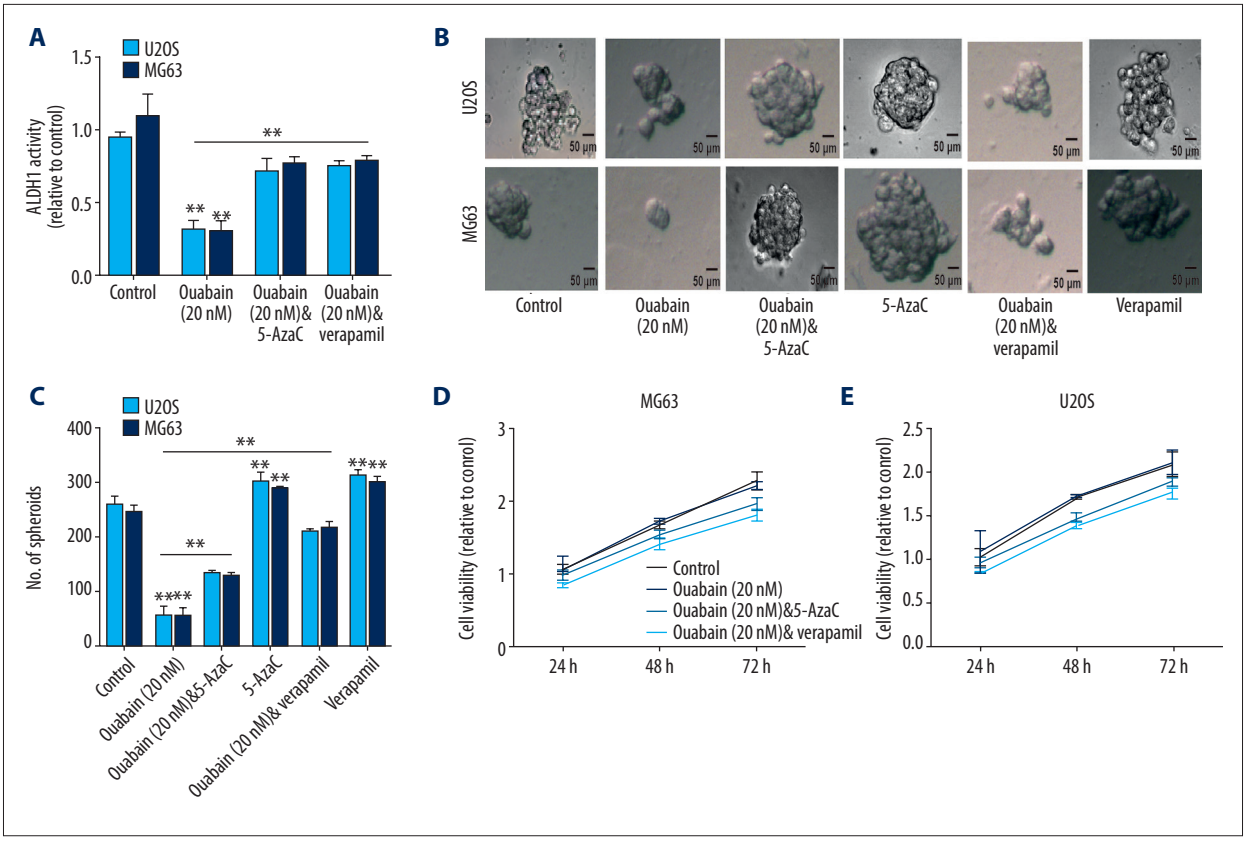

Figure 4. Inhibition of DNA methylation or blocking calcium channel rescues ouabain-mediated inhibition on OS cell stemness. (**A**) Analysis on ALDH1 activity was performed in OS cells treated with ouabain, ouabain plus 5-AzaC, and ouabain plus verapamil. (**B, C**) Sphere size and number were examined in OS cells treated with ouabain, ouabain plus 5-AzaC, and ouabain plus verapamil. (**D, E**) Cell viability was determined in OS cells treated with ouabain, ouabain plus 5-AzaC, and ouabain plus verapamil. ** P<0.01 *vs.* control.

cells (Figure 1B–1E). Additionally, both the sphere size and number were reduced by ouabain (Figure 1F, 1G). Notably, the viability of OS cells was decreased slightly, but the difference was not significant (Figure 1H, 1I). These results demonstrate that ouabain can reduce OS cell stemness.

Ouabain inhibits OS cell migration, invasion, and adhesion ability

Since stem-like cells result in tumor cell metastasis, we further examined the effects of ouabain on OS cell migration and invasion. OS cell migration, invasion, and cell adhesion abilities were reduced by ouabain treatment (Figure 2A–2E).

Ouabain leads to DNA methylation of stemness markers through increasing intracellular Ca^{2+} concentration

DNA methylation plays a critical role in regulating gene expression. A recent study found that inhibition of Na+/K+ ATPase and intracellular increase of calcium level negatively affects the stemness of circulating tumor cells [11]. Therefore, we hypothesized that the intracellular Ca^{2+} concentration and its mediated DNA methylation are involved in ouabain-mediated regulation of the expression of stemness regulators (Oct4, sox2, and Nanog). We tested this by measuring the intracellular Ca^{2+} concentration and methylation level of the critical stemness regulators and found that the intracellular Ca^{2+} concentration and methylation level of the critical stemness regulators were higher in OS cells than in OS cells-formed spheres, which exhibit stem cell-like traits [6] (Figure 3A, 3B). As expected, the methylation level of stemness regulators was remarkably increased in OS cells treated with ouabain (Figure 3C). To gain more insights into the mechanisms contributing to ouabain in regulating OS cell stemness, we tested whether ouabain regulates calcium level in OS cells, finding that the intracellular Ca^{2+} level was increased in OS cells with ouabain treatment (Figure 3D). Notably, inhibition of Ca^{2+} channel using verapamil decreased the methylation level induced by ouabain treatment (Figure 3E). Additionally, inhibition of methylation or Ca^{2+} channel rescued the suppressive effect of ouabain on stemness regulator expression (Figure 3F–3I).

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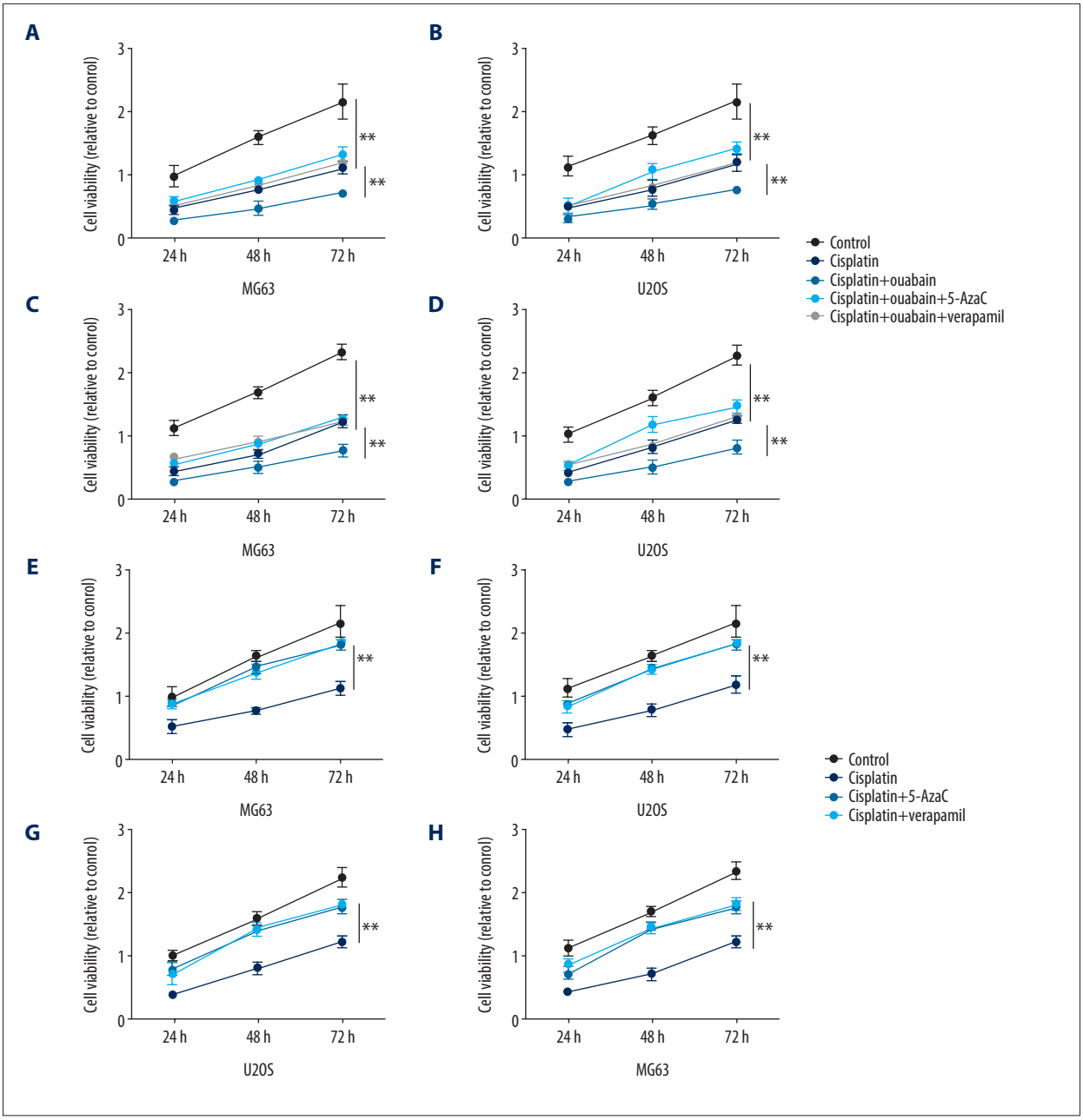

Figure 5. Ouabain enhances cisplatin sensitivity of OS cells. (**A–D**) Cell viability analysis was performed in OS cells treated with cisplatin or methotrexate, cisplatin or methotrexate+ouabain, cisplatin or methotrexate+ouabain+5-AzaC, and cisplatin or methotrexate+ouabain+verapamil. (**E–H**) Cell viability assay was carried out in OS cells treated with cisplatin or methotrexate, cisplatin or methotrexate+5-AzaC, and cisplatin or methotrexate+verapamil. ** P<0.01 *vs.* control.

Inhibition of DNA methylation or blocking calcium channel rescues ouabain-mediated inhibition on OS cell stemness

We explored whether inhibition of DNA methylation using Azacitidine (5-AzaC) or blocking calcium channel could rescue the decreased stemness caused by ouabain treatment. As expected, both 5-AzaC and verapamil rescued the decreased ALDH1 activity caused by ouabain treatment in OS cells (Figure 4A). Additionally, ouabain-induced reduction of sphere-forming ability was partially reversed by 5-AzaC and verapamil treatment, as evident by the rescue of sphere size and number (Figure 4B, 4C). Notably, 5-AzaC and verapamil alone increased the spheroid-forming ability (Figure 4B, 4C) but had little effect on OS cell viability (Figure 4D, 4E).

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Ouabain enhances cisplatin sensitivity of OS cells

Since tumor cells with stem-like traits are resistant to chemotherapy, we finally examined the effects of ouabain on cisplatin and methotrexate sensitivity of OS cells. As shown in Figure 5A–5D, the sensitivity of cisplatin or methotrexate was enhanced by ouabain, and this effect was rescued by 5-AzaC and verapamil treatment. Importantly, the sensitivity of cisplatin or methotrexate was attenuated by 5-AzaC and verapamil treatment (Figure 5E–5H). Therefore, our results suggest that Na+/K+ ATPase inhibitor ouabain attenuates the stemness and chemoresistance of OS cells through increasing intracellular Ca^{2+} concentration and the consequent facilitation of DNA methylation remodeling of critical stemness regulators such as Oct4, sox2, and Nanog.

Discussion

Our study identified the DNA methylation events in the critical stemness regulators (Oct4, Sox2, and Nanog) of OS cells. We found that different status of DNA methylation of stemness regulators between OS-adherent cells and non-adherent spheres, and thus a lower level of DNA methylation of stemness regulators in spheres and higher level in adherent cells. Additionally, the Na+/K+ ATPase inhibitor ouabain reduced OS cell stemness through increasing intracellular Ca^{2+} concentration and thus the DNA methylation of master stemness regulators. This is the first study demonstrating the effects of Na+/K+ ATPase inhibitor on OS cell stemness.

Oct4, sox2, and Nanog are active in normal embryonic stem cells, and, apart from contributing to stem cell renewal and proliferation, they also facilitate CSCs progression [16]. Many studies have shown that targeting these master stemness regulators can reduce tumor progression. For example, targeting Oct4/sox2 can reduce the quiescent stem-like cell pool and thus inhibit glioma progression [17]. The transcription factor NF-YA maintains sox2-positive cervical cancer stem cells through specifically promoting sox2 activation [18]. Consistent with the above, in OS progression, sox2 is required for tumor development and cell proliferation and knockdown of sox2 inhibits its OS cell migration and invasion [19]. Additionally, Oct4 promotes OS progression through regulating lncRNA AK055347 expression [20]. However, a recent work indicates that silencing of these master stemness regulators (Oct4, Sox2, Klf4, c-Myc, or Nanog) is insufficient to avoid the formation of teratomas, but can affect their differentiation potential [21], meaning that different stemness regulators might have different roles in different tumors by different mechanisms.

Here, we focused on exploring the more detailed mechanisms contributing to OS cell stemness. Interesting, a recent work showed that CTCs display stem cell-like traits, which can be reduced by the Na+/K+ ATPase inhibitor ouabain, so we speculated that ouabain might attenuate OS cell stemness. As expected, upon inhibition of Na+/K+ ATPase, intracellular Ca^{2+} level was upregulated. Notably, the increased Ca^{2+} concentration promoted the methylation of master stemness regulators (Oct4, Sox2, and Nanog) because verapamil, an inhibitor of Ca^{2+} channel, can rescue this effect. Notably, the elevation of Ca^{2+} concentration has been confirmed to promote OS cell apoptosis [22], and the antidepressant mirtazapine can exert cytotoxicity on OS cells through inducing cytosolic Ca2+ elevation [23]. In this work, we first measured the intracellular Ca^{2+} concentration in adherent OS cells and non-adherent OS spheres and found that Ca^{2+} concentration is remarkably higher in OS spheres, indicating that Ca^{2+} channel might have a critical role in OS cell stemness. We assessed whether combined use of activator of Ca^{2+} channel and Na+/K+ ATPase inhibitor could exert a greater inhibition on OS cell stemness than that of single use. Finally, we further confirmed that the Na+/K+ ATPase inhibitor ouabain enhances chemotherapeutic effects dependent on DNA methylation and Ca^{2+} channel. *In vivo* experiments are needed to confirm this.

Conclusions

This work provides key insights into the biology of OS stem cells and highlights the connection between OS cell stemness and DNA methylation or Ca^{2+} channel. We concluded that ouabain attenuates the stemness and chemoresistance of OS cells through increasing intracellular Ca^{2+} concentration and the consequent facilitation of DNA methylation remodeling of critical stemness regulators such as Oct4, sox2, and Nanog. Thus, the Na+/K+ ATPase inhibitor ouabain might be able to inhibit OS occurrence and reduce OS spread.

Conflict of interest

None.

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