Ca^{2+}-activated K^{+} channel \( \text{K}_{\text{Ca}}^{1.1} \) as a therapeutic target to overcome chemoresistance in three-dimensional sarcoma spheroid models

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Abstract

The large-conductance Ca^{2+}-activated K^{+} channel \( \text{K}_{\text{Ca}}^{1.1} \) plays a pivotal role in tumor development and progression in several solid cancers. The three-dimensional (3D) in vitro cell culture system is a powerful tool for cancer spheroid formation, and mimics in vivo solid tumor resistance to chemotherapy in the tumor microenvironment (TME). \( \text{K}_{\text{Ca}}^{1.1} \) is functionally expressed in osteosarcoma and chondrosarcoma cell lines. \( \text{K}_{\text{Ca}}^{1.1} \) activator-induced hyperpolarizing responses were significantly larger in human osteosarcoma MG-63 cells isolated from 3D spheroid models compared with those from adherent 2D monolayer cells. The present study investigated the mechanisms underlying the upregulation of \( \text{K}_{\text{Ca}}^{1.1} \) and its role in chemoresistance using a 3D spheroid model. \( \text{K}_{\text{Ca}}^{1.1} \) protein expression levels were significantly elevated in the lipid-raft-enriched compartments of MG-63 spheroids without changes in its transcriptional level. 3D spheroid formation downregulated the expression of the ubiquitin E3 ligase FBXW7, which is an essential contributor to \( \text{K}_{\text{Ca}}^{1.1} \) protein degradation in breast cancer. The siRNA-mediated inhibition of FBXW7 in MG-63 cells from 2D monolayers upregulated \( \text{K}_{\text{Ca}}^{1.1} \) protein expression. Furthermore, a treatment with a potent and selective \( \text{K}_{\text{Ca}}^{1.1} \) inhibitor overcame the chemoresistance of the MG-63 and human chondrosarcoma SW-1353 spheroid models to paclitaxel, doxorubicin, and cisplatin. Among several multidrug resistance ATP-binding cassette transporters, the expression of the multidrug resistance-associated protein MRP1 was upregulated in both spheroids and restored by the inhibition of \( \text{K}_{\text{Ca}}^{1.1} \). Therefore, the pharmacological inhibition of \( \text{K}_{\text{Ca}}^{1.1} \) may be an attractive new strategy for acquiring resistance to chemotherapeutic drugs in the TME of \( \text{K}_{\text{Ca}}^{1.1} \)-positive sarcomas.
1 | INTRODUCTION

Potassium (K⁺) channels play a critical role in the proliferation, apoptosis, migration, adhesion, and metastasis of cancer cells by controlling cell volumes and Ca²⁺ signaling. The large-conductance Ca²⁺-activated K⁺ channel KC₄.1.1 (also known as BK, BKCa, Maxi-K, and Slo1) encoded by the KCNA1 gene has been implicated in tumor development and progression in breast, prostate, cervical, renal, and colorectal cancers and glioma, by promoting the driving force of Ca²⁺ influx through voltage-independent Ca²⁺ channels. The amplification of KCNA1 correlates with a high tumor stage and poor prognosis in breast cancer, and has potential as a tumor grade-associated marker of prostate cancer. The functional diversity of KC₄.1.1 is due to the alternative splicing of the KC₄.1.1 α subunit and its kinetic modulation by the auxiliary β (β1-4) and γ (γ1-4) subunits. KC₄.1.1 is distributed in "lipid rafts," which are cholesterol-enriched nanodomains, in cancer cells and contributes to the fine-tuning of cancer-associated functions, such as proliferation, migration, and metastasis.

Bone sarcoma accounts for more than 10% of all sarcomas. Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents. Chemotherapy is an important strategy for the treatment of patients with high-grade OS. Chondrosarcoma (CS) is the second most common primary malignancy of bone and is resistant to both chemotherapy and radiation. There are currently no promising drugs available for CS. KC₄.1.1 is functionally expressed in human OS and CS cell lines; however, limited information is currently available in its therapeutic significance in the tumor microenvironment (TME).

The hypoxic TME promotes cancer progression and survival. The overexpression of hypoxia-inducible factor (HIF)-1α has been shown to correlate with a poor patient prognosis, and promotes the resistance of solid tumors to chemotherapies. HIF-1α also promotes cancer stemness. Three-dimensional (3D) in vitro cancer spheroid models mimic the TME of human solid tumors, and are an efficient tool for investigating metastasis, invasion, chemoresistance and radioresistance, and stemness. Histone deacetylases (HDACs) are classified into 4 groups: classes I, II, III, and IV, and HDAC3 and sirtuin 1 (SIRT1), belonging to classes I and III, respectively, regulate hypoxia-induced metastasis and chemoresistance in a wide range of cancer types. NAD⁺-dependent SIRT1 is downregulated by decreased NAD⁺ levels under hypoxic TME. Furthermore, low SIRT1 levels predict poor survival and metastasis in breast cancer.

Recent studies have implicated ion channels in chemoresistance to cisplatin (CIS), doxorubicin (DOX), and paclitaxel (PAC). ABC transporters play a major role in the acquisition of chemoresistance, to which the multidrug resistance-associated protein

MRP1 is a key contributor. MRP1 is upregulated through the nuclear factor erythroid 2-related factor 2, NRF2 signaling pathways, that play an essential role in poor prognosis and chemoresistance in cancer.

A recent study has reported that antiandrogen-induced KC₄.1.1 protein degradation was mediated by the ubiquitin E3 ligases, F-box and WD repeat domain-containing (FBXW7), and murine double minute (MDM2) in a KC₄.1.1-expressing breast cancer cell line. FBXW7 is a tumor suppressor gene and its downregulation has been shown to promote tumor stemness by preventing the protein degradation of stemness regulators, such as c-Myc and Sox2. A previous study has demonstrated that the loss of FBXW7 increased chemoresistance, and showed that cancer spheroid formation-mediated chemoresistance was positively associated with the hypoexpression of FBXW7.

The objective of the present study was to elucidate the mechanisms underlying the post-translational modification of KC₄.1.1 and the therapeutic significance of its functions in the TME using a 3D spheroid culture system in KC₄.1.1-expressing sarcoma cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Paxilline (PAX; Cayman Chemical), NS1619 (Abcam), DOX hydrochloride (FUJIFILM Wako Pure Chemical), PAC (FUJIFILM Wako Pure Chemical), CIS (FUJIFILM Wako Pure Chemical), DiBAC₄(3) (DOJINDO), Fura-2 AM (DOJINDO), and siRNA for FBXW7 (Thermo Fisher Scientific) were purchased. HDAC/SIRT inhibitors (vorinostat and NCO-01) were provided by Prof. Takayoshi Suzuki (Osaka Univ., Osaka, Japan). The other chemicals used in the present study were from Sigma-Aldrich, FUJIFILM Wako Pure Chemical, and Nacalai Tesque unless otherwise stated.

2.2 | Cell culture

The human OS cell line MG-63 and CS cell line SW-1353 were purchased from the RIKEN Cell Bank. The lines were cultured in D-MEM medium (FUJIFILM Wako Pure Chemical) supplemented with 10% fetal bovine serum (Sigma-Aldrich Japan) and penicillin (100 units/mL)/streptomycin (100 μg/mL) mixture (FUJIFILM Wako Pure Chemical). All cells were cultured in a humidified atmosphere containing 5% CO₂ in air at 37°C. Flat-bottomed dishes and plates (Corning) were used in the two-dimensional (2D) cell culture. The PrimeSurface® system (Sumitomo Bakelite) was used for 3D spheroid formation. Cell suspensions of MG-63 and SW-1353 were
seeded onto a PrimeSurface 96 U plate at 10^4 cells/well and then cultured for 3-4 d.

2.3 | RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was isolated from cancer cells by the conventional acid guanidium thiocyanate-phenol-chloroform method. The concentration and quality of RNA were confirmed using a microvolume spectrophotometer, NanoDrop One (Thermo Fisher Scientific). Reverse transcription was performed using ReverTra Ace (ToYoBo) with random hexanucleotides. Quantitative real-time PCR was performed using the Luna Universal qPCR Master Mix (New England Biolabs Japan) and the Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific). PCR primers of human origin are listed in Table S1. The relative expression levels were calculated using the 2^(-ΔΔCt) method and normalized to β-actin (ACTB).  

2.4 | Western blots

Lipid raft-enriched protein fraction lysates were extracted using the ULTRARIPA kit for Lipid Rafts (BioDynamics Laboratory) in accordance with the manufacturer’s instructions. Whole cell lysates were extracted using RIPA buffer. Equal amounts of protein were subjected to SDS-PAGE and immunoblotting with anti-KCa1.1 polyclonal (rabbit; approximately 100 kDa; APC-021, Alomone Labs), anti-FBXW7 polyclonal (rabbit; app. 70 kDa; AS872, ABclonal), anti-SIRT1 polyclonal (rabbit; app. 130 kDa; Medical & Biological Laboratories, MBL), anti-HDAC3 polyclonal (rabbit; app. 50 kDa; H-99, SCB), anti-MRP1 polyclonal (rabbit; app. 250 kDa; bs-0657R, Bioss Antibodies), and anti-ACCTB monoclonal (mouse; 43 kDa; 6D1, MBL) antibodies, and were then incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (Merck Millipore). The resulting images were analyzed using an Amersham Imager 600 (GE Healthcare Japan). The optical density of the protein band signal relative to that of the ACTB signal was calculated using ImageJ software (v.1.42, NIH), and protein expression levels in the vehicle control were then expressed as 1.0.

2.5 | Measurement of the membrane potential and intracellular Ca^{2+} concentrations by fluorescence indicators

The membrane potential was measured using the fluorescence voltage-sensitive dye DiBAC_4(3), as previously reported.  

In the cell cycle distribution analysis, isolated cells were fixed overnight in ice-cold 70% ethanol. Fixed cells were stained with PBS containing propidium iodide (10 μg/mL) and DNase-free RNase (0.1 mg/mL). Stained cells were subjected to a flow cytometric analysis (FACSCanto II, BD Biosciences) acquiring at least 10 000 events. Data were analyzed using BD FACSDiva software.

2.6 | Cell viability assay

A cell viability assay was performed in accordance with our previous study.  

In the immunochemistry assay, fixed and non-permeabilized cells were stained with an anti-KCa1.1 polyclonal antibody (rabbit; extracellular, APC-151, Alomone Labs) followed by an Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific), and then analyzed by flow cytometry (FACSCanto II, BD Biosciences).

2.7 | Statistical analysis

Statistical analyses were performed using Statistical software XLSTAT (version 2013.1). To assess the significance of differences between the 2 groups and among multiple groups, unpaired/paired Student t tests with Welch correction or Tukey tests were used. Results with a P-value of less than .05 were considered to be significant. Data were presented as means ± SEM.
RESULTS

3.1 | \( \text{K}_\text{Ca}^{1.1} \) activity in isolated cells from MG-63 and SW-1353 spheroids

Previous studies have reported that the OS and CS cell lines, MG-63 and SW-1353, functionally express \( \text{K}_\text{Ca}^{1.1} \). 3D spheroid model generation from these cell lines was performed using the ultra-low attachment cultureware, PrimeSurface 96 U. At 4 d after cell seeding, spheroidal aggregates of cancer cells were formed in both cell lines (Figures 1A and 2A). Hyperpolarizing responses induced by the \( \text{K}_\text{Ca}^{1.1} \) activator, NS1619, were significantly larger in acutely isolated MG-63 cells from 3D spheroids (\( n = 19 \)) than in those from 2D adherent cell monolayers (\( n = 18; P < .01 \); Figure 1B,C).

Pretreatment with the selective \( \text{K}_\text{Ca}^{1.1} \) blocker, PAX (5 \( \mu \text{mol/L} \)) significantly reduced NS1619-induced hyperpolarizing responses (approximately 80%, \( n = 11 \)). We then examined the gene and protein expression levels of \( \text{K}_\text{Ca}^{1.1} \) in MG-63 cells using real-time PCR and western blot assays. The expression level of the \( \text{K}_\text{Ca}^{1.1} \) protein with a molecular weight of approximately 100 kDa was increased in MG-63 spheroids (\( n = 4 \) for each, \( P < .01 \); Figure 1D,E), without changes in that of the \( \text{K}_\text{Ca}^{1.1} \) transcript (Figure 1G). Consistent with our previous findings, 27 the band signal specific for \( \text{K}_\text{Ca}^{1.1} \) almost completely disappeared following the pretreatment with the primary antibody with excess antigen (not shown). The results of the flow cytometric analysis using the Alexa Fluor 488-conjugated antibody against the extracellular domain of human \( \text{K}_\text{Ca}^{1.1} \) indicated a significantly higher mean fluorescence intensity in MG-63 cells.

**FIGURE 1** Comparison of \( \text{Ca}^{2+} \)-activated \( \text{K}^{+} \) channel \( \text{K}_\text{Ca}^{1.1} \) expression and activity between human OS MG-63 cells cultured as 2D monolayers and 3D spheroids. A, Phenotypic properties of MG-63 cells cultured with ultra-low attachment PrimeSurface 96 U plates (upper panel: day 0, lower panel: day 4). Brightfield images were obtained with the Axios Observer Z1 microscope system (Carl Zeiss). Bars show 50 \( \mu \text{m} \). B, Time course of the voltage-sensitive fluorescent dye imaging of \( \text{K}_\text{Ca}^{1.1} \) activator (1 \( \mu \text{mol/L} \) NS1619)-induced hyperpolarizing responses in isolated cells from 2D monolayers (“2D”) and 3D spheroids (“3D”) of MG-63. The fluorescence intensity of DiBAC\(_4\)(3) before the application of NS1619 is expressed as 1.0. Images were measured every 5 s. C, Summarized results of NS1619-induced hyperpolarizing responses in cells isolated from at least 3 different batches in each group. Cell numbers used in experiments are shown in parentheses. The values for fluorescence intensity were obtained by measuring the average for 1 min (12 images). D, \( \text{K}_\text{Ca}^{1.1} \) protein expression in the lipid raft-enriched protein lysates of both groups. Blots were probed with anti-\( \text{K}_\text{Ca}^{1.1} \) (approximately 100 kDa, upper panel) and anti-ACTB (43 kDa, lower panel) antibodies. E, Summarized results were obtained as the optical density of \( \text{K}_\text{Ca}^{1.1} \) and ACTB band signals. After compensation for the optical density of the \( \text{K}_\text{Ca}^{1.1} \) protein band signal with that of the ACTB signal, the \( \text{K}_\text{Ca}^{1.1} \) signal in “2D” was expressed as 1.0 (\( n = 4 \) for each). F, Fixed, non-permeabilized MG-63 cells were stained with an Alexa 488-fused anti-\( \text{K}_\text{Ca}^{1.1} \) (extracellular) antibody, and mean fluorescence intensities were measured using flow cytometry. Their values in “2D” were expressed as 1.0 (\( n = 4 \) for each). G, Real-time PCR examination of the \( \text{K}_\text{Ca}^{1.1} \) transcript in both groups (\( n = 4 \) for each). Expression levels were shown as a ratio compared with ACTB. Results are expressed as means ± SEM. **\( P < .01 \) vs 2D.
Conversely, NS1619-induced hyperpolarizing responses were observed in acutely isolated SW-1353 spheroids (n = 18); however, similar responses were also found in those from 2D monolayers (n = 13; P > .05; Figure 2B,C). Corresponding to these results, no significant differences were observed in the expression levels of KCa1.1 transcripts and proteins between 2D- and 3D-cultured SW-1353 cells (n = 4 for each, P > .05; Figure 2C-E). These results indicated that (a) KCa1.1 activity was high in both spheroids and (b) the decrease in KCa1.1 protein degradation or increase in the localization of the plasma membrane lipid rafts of KCa1.1 may be involved in enhancing KCa1.1 activity in MG-63 spheroids. The transcriptional expression levels of other KCa channels (KCa2.x and KCa3.1) and the auxiliary β and γ subunits of KCa1.1, which affect channel kinetic properties, were very low (Figures S1 and S2).

3.2 Downregulation of the ubiquitin E3 ligase, FBXW7 in MG-63 spheroids

To identify candidates involved in the protein degradation of KCa1.1 in MG-63 cells (see Section 1), we compared the expression levels of FBXW7, MDM2, and cereblon (CRBN) between the 2D monolayers and 3D spheroids of both sarcomas.27,30 As shown in Figure 3A-C, the expression levels of FBXW7 transcripts and proteins were significantly decreased by spheroid formation in MG-63 cells.
Conversely, the expression levels of FBXW7 were significantly lower in the 2D monolayers of SW-1353 cells, and no significant changes in the expression levels of FBXW7 were noted in SW-1353 spheroids (Figure 3D-F). In both spheroids, a significant increase in the expression levels of MDM2 and CRBN (Figure S3) was observed, suggesting a negligible contribution to the regulation of KCa1.1 activity in both spheroids. The siRNA-mediated suppression of FBXW7 (Figure 4A) induced an increase in the expression level of the KCa1.1 protein by preventing FBXW7-mediated KCa1.1 protein degradation \((P < .01; \text{Figure 4B,C})\). These results strongly suggest that the decrease in FBXW7 expression is associated with the increase in KCa1.1 activity in MG-63 spheroids.

### 3.3 Overcoming chemoresistance by KCa1.1 inhibition in both sarcoma spheroids

Cancer spheroid formation mimicking the TME promotes resistance to chemotherapy drugs, which is referred to as “chemoresistance.” PAC, DOX, and CIS are the most widely used chemotherapy drugs. As shown in Figures 5, 3D spheroids of MG-63 and SW-1353 cells exhibited chemoresistance to PAC, DOX, and CIS \((n = 5 \text{ for each, } P < .01)\). To investigate whether KCa1.1 is involved in the acquisition of chemoresistance, both sarcoma spheroids were pre-incubated with the selective KCa1.1 inhibitor PAX \((10 \mu \text{mol/L})\) for 24 h. As shown in Figure 6, sensitivity to chemotherapy drugs was significantly, but not completely, recovered by the PAX treatment \((n = 5 \text{ for each, } P < .01)\).

ABC transporters are responsible for drug resistance. Therefore, we initially examined the expression levels of MDR1 (ABCB1), MRP1 (ABCC1), MRP2 (ABCC2), and BCRP1 (ABCG2), possible target molecules transporting chemotherapy drugs, using real-time PCR. The expression levels of MRP1 transcripts were significantly higher in the 3D spheroids of both cells than in 2D monolayers \((n = 4 \text{ for each, } P < .01; \text{Figure 7A,D})\). The other ABC transporters were rarely expressed in any group, with expression levels of less than 0.002 in arbitrary units. Corresponding to the results obtained on transcriptional expression, the expression levels of MRP1 proteins were significantly increased in the 3D spheroids of both cells \((n = 4 \text{ for each, } P < .01 \text{ and } .05 \text{ vs 2D in MG-63 and SW-1353, respectively; Figure 7B,C,E,F})\).
FIGURE 4 Effects of the siRNA-mediated inhibition of FBXW7 on expression levels of the K_{Ca1.1} protein in 2D-cultured MG-63 cells. A, Real-time PCR examination of FBXW7 transcripts in control siRNA (si-cont) and FBXW7 siRNA (si-FBXW7)-transfected 2D monolayers of MG-63 cells (n = 4 for each). Expression levels are shown as a ratio compared with ACTB. Protein expression of K_{Ca1.1} in si-cont and si-FBXW7 groups. Blots were probed with anti-K_{Ca1.1} (approximately 100 kDa) and anti-ACTB (43 kDa) antibodies (B). Summarized results were obtained as the optical density of K_{Ca1.1} band signals (C). After compensation for the optical density of the K_{Ca1.1} protein band signal with that of the ACTB signal, the optical density in "si-cont" was expressed as 1.0 (n = 4 for each). Results are expressed as means ± SEM. **P < .01 vs si-cont.

FIGURE 5 Effects of chemotherapeutic reagents on the viability of 2D- and 3D-cultured MG-63 and SW-1353 cells. Effects of the treatment with 100 nmol/L paclitaxel (PAC), 1 μmol/L doxorubicin (DOX), and 10 μmol/L cisplatin (CIS) for 48 h on the viability of 2D- and 3D-cultured MG-63 (A-C) and SW-1353 (D-F) cells using the WST-1 assay. Cell viability in "2D" was expressed as 1.0. Results are expressed as means ± SEM. **P < .01 vs 2D.
Consistent with the \( \text{K}_{\text{Ca}}1.1 \) inhibition overcoming chemoresistance (Figure 6), the expression levels of MRP1 transcripts and proteins were recovered by treatment with PAX for 24 h in both spheroids (\( n = 4 \) for each, \( P < .01 \); Figure 8). No changes were observed in the transcriptional expression levels of the other ABC transporters following the PAX treatment in both spheroids (less than 0.002 in arbitrary units).

### 3.4 HDAC-mediated epigenetic regulation of FBXW7 in sarcoma spheroids

In the present study, the mechanisms underlying the downregulation of FBXW7 in spheroid formation have not yet been elucidated (Figure 3). FBXW7 is epigenetically modified in cancer cells.\(^{32,33}\) Furthermore, previous studies have reported that, among the 18 members of class I-IV HDACs, HDAC3, SIRT1, and SIRT2, belonging to classes I and III, were regulated in the TME of a wide range of cancer types.\(^{18,19}\) As shown in Figure 9, the expression levels of HDAC3 and SIRT1 transcripts and proteins were significantly decreased in MG-63 spheroids (\( n = 4 \) for each, \( P < .01 \)). The expression levels of SIRT2 transcripts were significantly lower compared with those of HDAC3 and SIRT1 transcripts, and no significant differences were noted between 2D and 3D (Figure 9C). To clarify the involvement of HDAC3 and/or SIRT1 in the expression of FBXW7 in MG-63 cells, we examined the effects of treatments with the HDAC inhibitors vorinostat (10 \( \mu \text{mol/L} \) for class I, II, and IV HDAC subtypes) and NCO-01 (50 \( \mu \text{mol/L} \) for class III SIRT1/2) for 24 h on the expression levels of FBXW7 in the 2D monolayers of MG-63 cells (Figure 9D).

**Figure 6** Effects of the treatment with the \( \text{K}_{\text{Ca}}1.1 \) inhibitor, PAX on chemoresistance acquired in MG-63 and SW-1353 spheroids. Effects of the treatment with 100 nmol/L PAC, 1 \( \mu \text{mol/L} \) DOX, and 10 \( \mu \text{mol/L} \) CIS for 48 h on cell viability in vehicle- and PAX-treated MG-63 (A-C) and SW-1353 (D-F) spheroids. The viability in the untreated cells with PAX was expressed as 1.0. Results are expressed as means ± SEM. **\( P < .01 \) vs vehicle control.
In 2D monolayers, the expression levels of FBXW7 were significantly decreased by NCO-01, but not vorinostat (Figure 10A). These results suggested that FBXW7-mediated KCa1.1 protein degradation was regulated by SIRT1 in MG-63 cells. We then examined the effects of HDAC inhibitor treatments on KCa1.1 protein expression in MG-63 cells. Consistent with these results, the expression levels of the KCa1.1 protein were increased by NCO-01, but not vorinostat in 2D-cultured MG-63 cells (n = 4 for each, \( P < .05 \); Figure 10B, C). No significant changes in the expression level of the KCa1.1 transcript by the SIRT1 inhibition were observed in 2D-cultured MG-63 cells (Figure 10D). These results suggested that FBXW7 is a downstream target of SIRT1 and that the SIRT1-FBXW7-KCa1.1 axis plays a pivotal role in poor prognosis and chemoresistance in KCa1.1-expressing solid cancers.

Potential involvement of NRF2 in KCa1.1 inhibition-induced MRP1 downregulation and overcoming chemoresistance in sarcoma spheroids

MRP1 was upregulated in sarcoma spheroids (Figure 7), and was mostly recovered by the KCa1.1 inhibition (Figure 8). However, the underlying mechanisms remain unclear. Activation of the transcription factor NRF2 contributed to the acquisition of chemoresistance through the upregulation of MRP1 in cancer spheres.\(^{34,35}\) As shown in Figure 11, the expression levels of NRF2 transcripts were increased by spheroid formation in both sarcomas (n = 4 for each, \( P < .01 \) vs 2D; Figure 11A,G), and were reversed by KCa1.1 inhibition (10 \( \mu \text{mol/L PAX} \)) for 24 h (n = 4 for each, \( P < .01 \) vs PAX; Figure 11B,H). Treatment with the NRF2 inhibitor ML385 (10 \( \mu \text{mol/L} \)) for 48 h decreased MRP1 expression levels in both sarcoma spheroids (n = 4 for each, \( P < .01 \) vs vehicle control; Figure 11E,K). Heat shock factor 1 (HSF1), which controls chemoresistance in cancer,\(^{36}\) was also increased by spheroid formation in both sarcomas (n = 4 for each, \( P < .01 \) vs 2D; Figure 11C,I), and this was reversed by KCa1.1 inhibition (10 \( \mu \text{mol/L PAX} \); n = 4 for each, \( P < .01 \) vs PAX; Figure 11D,J). In contrast with NRF2, the treatment with the HSF1 inhibitor KRRB11 (10 \( \mu \text{mol/L} \)) for 48 h did not affect MRP1 expression levels in either sarcoma spheroid (n = 4 for each, \( P > .05 \) vs vehicle control; Figure 11F,L). No significant changes were observed in the expression levels of the MRP1, NRF2, and HSF1 transcripts following PAX treatment in KCa1.1 negative-human prostate cancer PC-3 cells and human colorectal cancer HCT-116 cells (Figure S5). These results
suggested that NRF2 is a critical transcriptional regulator of MRP1 in sarcoma spheroids, and appears to play a pivotal role in the overcoming of chemoresistance by the inhibition of \( \text{K}_{\text{Ca}1.1} \).

**4 | DISCUSSION**

Cancer spheroid models constructed by 3D culture systems are widely utilized in cancer research. They exhibit physiologically relevant cell-cell and cell-matrix interactions, gene expression, and signaling pathway profiles that reflect in vivo tumors under hypoxic TME, and are applicable to in vitro chemosensitivity or chemoresistance assays.\(^{37-39}\) Cancer spheroids exhibit growth arrest in the \( G_0 \) phase, similar to that in the quiescent region of TME. Riedl et al.\(^{40}\) reported that the number of cells in the \( S \)-phase was significantly lower in the 3D spheroids of various types of cancers than in 2D monolayers. In the present study, the cell cycle of MG-63 and SW-1353 spheroids was arrested at the \( G_1/S \) checkpoint (Figure S6). Additionally, we observed a positive correlation between hyperpolarization responses and \( [\text{Ca}^{2+}]_i \) increases induced by the application of \( \text{K}_{\text{Ca}1.1} \) activator in isolated cells from MG-63 and SW-1353 spheroids (Figure S7). The abundant expression of \( \text{K}_{\text{Ca}1.1} \) in multiple cancer types has been attracting increasing interest in cancer research due to its crucial role in cancer-associated functions such as proliferation and metastasis. In the present study, human OS MG-63 cells and CS SW-1353 cells abundantly expressed \( \text{K}_{\text{Ca}1.1} \) transcripts (Figures S1 and S2). However, the pathophysiological roles of \( \text{K}_{\text{Ca}1.1} \) in cancer spheroids mimicking TME have not yet been elucidated. The main results of the present study are as follows:

(i) \( \text{K}_{\text{Ca}1.1} \) activity was larger in MG-63 cells isolated from 3D spheroids than in those from 2D monolayers, together with a decrease in \( \text{K}_{\text{Ca}1.1} \) protein degradation, but not transcriptional repression (Figure 1).

(ii) SIRT1-mediated FBXW7 downregulation caused a decrease in \( \text{K}_{\text{Ca}1.1} \) protein degradation by spheroid formation in MG-63 cells (Figures 3, 4, 9, and 10).

(iii) \( \text{K}_{\text{Ca}1.1} \) inhibition overcame acquired chemoresistance to PAC, DOX, and CIS by spheroid formation (Figures 5 and 6).
(iv) Upregulation of MRP1 through NRF2 signaling pathways was associated with the acquisition of chemoresistance in both sarcoma spheroids, and K<sub>Ca1.1</sub> inhibition suppressed NRF2-mediated MRP1 expression (Figures 7, 8, and 11).

Collectively, the present results demonstrated that K<sub>Ca1.1</sub> is a key modulator of chemoresistance in sarcoma cells, indicating that targeting of K<sub>Ca1.1</sub> is a promising therapeutic strategy for overcoming chemoresistance.

The pluripotency-associated ubiquitin E3 ligase FBXW7 is a well known stemness regulator, and its downregulation is associated with an increase in stemness factors. We previously demonstrated that FBXW7 was a key contributor to antiandrogen-induced K<sub>Ca1.1</sub> protein degradation in breast cancer cells. As shown in Figure 1, the upregulated expression of the K<sub>Ca1.1</sub> protein in lipid raft fractions was observed in MG-63 spheroids, which increased K<sub>Ca1.1</sub> activity. Furthermore, FBXW7 was abundantly expressed in the 2D monolayers of MG-63 cells (Figure 3A-C), and the upregulated expression of the K<sub>Ca1.1</sub> protein was observed following siRNA-mediated FBXW7 inhibition (Figure 4). The expression level of FBXW7 was significantly lower in 2D monolayers of SW-1353 cells than in those of MG-63 cells (Figure 3D). Previous studies have reported that the ubiquitin E3 ligases MDM2 and CRBN also contributed to K<sub>Ca1.1</sub> protein degradation and regulated its activity. As shown in Figure S3A,C, the expression level of MDM2 was increased in MG-63 and SW-1353 spheroids. The amplification of MDM2 is associated with poor prognosis in cancer patients, and its overexpression decreased survival rates and contributed to the acquisition of chemoresistance to DOX and CIS. Similar to MDM2, the expression of CRBN was increased by spheroid formation in both cells (Figure S3B,D). These results strongly suggested that FBXW7 is a main contributor to K<sub>Ca1.1</sub> protein degradation in MG-63 spheroids, possibly in SW-1353 ones.
The mechanisms underlying FBXW7 hypoexpression in MG-63 spheroids were examined. The class I and class III HDACs, HDAC3 and SIRT1 regulate hypoxia-induced metastasis and chemoresistance in a wide range of cancer types. SIRT1 was previously shown to be downregulated in the hypoxic TME, resulting in poor prognosis and chemoresistance in some cancers. As shown in Figure 9, decreased SIRT1 was detected in MG-63 spheroids. The decrease observed in FBXW7 expression was elicited by treatment with the SIRT1/2 inhibitor NCO-01 (50 μmol/L), but not the pan-HDAC inhibitor vorinostat (10 μmol/L) in 2D-cultured MG-63 monolayers. Expression levels were shown as a ratio to ACTB. Protein expression of KCa1.1 in protein lysates of the vehicle-, vorinostat (10 μmol/L), and NCO-01 (50 μmol/L)-treated 2D monolayers of MG-63 cells. Blots were probed with anti-KCa1.1 (approximately 100 kDa) and anti-ACTB (43 kDa) antibodies. Summarized results were obtained as the optical density of KCa1.1 band signal. After compensation for the optical density of the KCa1.1 protein band signal with that of the ACTB signal, optical density in the vehicle control was expressed as 1.0 (n = 4 for each). Results are expressed as means ± SEM. *P < .05, **P < .01 vs vehicle control.

Figure 10 Decreased expression of FBXW7 and increased protein expression of KCa1.1 by SIRT1 inhibition in 2D-cultured MG-63 cells. Real-time PCR examination of FBXW7 (A) and KCa1.1 transcripts (D) in vehicle-, vorinostat (10 μmol/L)-, and NCO-01 (50 μmol/L)-treated, 2D-cultured MG-63 monolayers. Expression levels were shown as a ratio to ACTB. Protein expression of KCa1.1 in protein lysates of the vehicle-, vorinostat (10 μmol/L)-, and NCO-01 (50 μmol/L)-treated 2D monolayers of MG-63 cells. Blots were probed with anti-KCa1.1 (approximately 100 kDa) and anti-ACTB (43 kDa) antibodies (B). Summarized results were obtained as the optical density of KCa1.1 band signal (C). After compensation for the optical density of the KCa1.1 protein band signal with that of the ACTB signal, optical density in the vehicle control was expressed as 1.0 (n = 4 for each). Results are expressed as means ± SEM. *P < .05, **P < .01 vs vehicle control.
of MRP1 functions is mainly a reduction in intracellular drug accumulation in the plasma membrane. The high expression of \( K_{Ca} \text{1.1} \) was associated with the upregulation of MRP1, resulting in the acquisition of chemoresistance (Figure 5). Furthermore, pharmacological blockade of \( K_{Ca} \text{1.1} \) decreased the expression of MRP1 in both sarcoma spheroids (Figure 6). Therefore, the inhibition of \( K_{Ca} \text{1.1} \) is a promising therapeutic strategy for reversing chemoresistance in solid cancers. MRP1 is upregulated through the Notch1-NRF2 signaling pathways. \(^{25,47-51}\) Li et al.\(^{47}\) showed that Notch1 was associated with MRP1 in OS. \(^{51}\) In the present study, NRF2 was upregulated in both sarcoma spheroids (Figure 11A,G) and was suppressed by treatment with the \( K_{Ca} \text{1.1} \) inhibitor (Figure 11B,H). In addition, the NRF2 inhibitor suppressed MRP1 expression in the 3D spheroids of both cells (Figure 11E,K). We examined the expression levels of Notch1 in 2D and 3D spheroids. However, its expression levels were low (less than 0.01 in arbitrary units) and no significant changes

**FIGURE 11** Increased expression of NRF2 transcripts decreases by inhibition with \( K_{Ca} \text{1.1} \), and effects of NRF2 and HSF1 inhibitors on the expression of MRP1 transcripts in sarcoma spheroids. Real-time PCR examination of NRF2 and HSF1 in 2D- and 3D-cultured MG-63 (A, C) and SW-1353 cells (G, I) and in vehicle- and PAX (10 \( \mu \text{mol/L} \))-treated (24 h) MG-63 (B, D) and SW-1353 spheroids (H, J). Real-time PCR examination of MRP1 in vehicle- and ML385 (10 \( \mu \text{mol/L} \))-treated (24 h) MG-63 (E), and SW-1353 (K) spheroids and in vehicle- and KRIIB11 (10 \( \mu \text{mol/L} \))-treated (24 h) MG-63 (F) and SW-1353 spheroids (L). Expression levels are shown as a ratio compared with ACTB. Results are expressed as means \( \pm \) SEM. \(^{**P < .01}\) vs “2D” or the vehicle control.
were found. Mun et al.45 showed that HSF1 contributes to chemoresistance. HSF1 was also upregulated in both sarcoma spheroids (Figure 11C,1) and was suppressed by pretreatment with the KCa1.1 inhibitor (Figure 11D,1). However, no significant changes were observed in MRP1 expression by treatment with the HSF1 inhibitor in both sarcoma spheroids (Figure 11F,1). Therefore, KCa1.1 appears to be an important regulator of NRF2-mediated MRP1 expression in KCa1.1-expressing sarcoma cells in vivo.

FBXW7 plays a prominent role in the induction of chemoresistance in cancer cells because its downregulated expression in several cancers resulted in the acquisition of chemoresistance,52,53 however the underlying mechanisms have not yet been elucidated. The present results suggested that FBXW7 hypoexpression-induced enhancements in KCa1.1 activity may, at least partly, contribute to FBXW7-mediated chemoresistance.

In conclusion, the present results suggested that KCa1.1 serves as a critical chemoresistance-related molecule in human OS and CS cells. Chemoresistance is a significant factor associated with poor outcomes in sarcoma patients. Increased KCa1.1 levels upregulated MRP1, which reduced intracellular drug accumulation in the plasma membrane, in sarcoma spheroids. The inhibition of KCa1.1 may be a promising therapeutic strategy for reversing chemoresistance in human OS and CS. Mitochondrial ion channels are emerging as new oncological targets. A recent study focused on the crucial involvement of mitochondrial K+ channels in chemoresistance in cancer cells.54,55 Further studies are needed to elucidate the contribution of mitochondrial KCa1.1 to the mechanisms underlying chemosensitivity or chemoresistance in solid cancer.

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The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
SO designed the study and wrote the initial draft of the manuscript. SO, JK, KE, MM, and HK contributed to data collection, analyses, and interpretation. EE and TS synthesized and quantitatively estimated HDAC/SIRT inhibitors. All authors critically reviewed the manuscript, approved its final version, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

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SUPPORTING INFORMATION
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