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***SPRR1A* is a potential therapeutic target for osteosarcoma: *in vitro* and *in vivo* evaluations using generated artificial osteosarcoma cancer stem cell-like cells**

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Abstract. Cancer stem cells (CSCs) have been implicated as critical mediators in the progression, chemoresistance and metastatic capabilities of diverse malignancies, including osteosarcoma (OS). The authors have succeeded in generating CSC-like cells (MG-OKS) from the OS cell line MG-63 by transducing defined factors. A significant increase in small proline-rich protein 1A (SPRR1A) expression, a cross-linked envelope protein in keratinocytes, was observed in MG-OKS cells. Therefore, SPRR1A could be involved in

tumor initiation, growth and poor OS progression. However, its specific role in OS remains unclear. The present study aimed to evaluate the role of SPRR1A in OS both *in vitro* and *in vivo* using MG-OKS cells. Three experimental groups were established: MG-OKS cells transfected with SPRR1A small interfering (si)RNA (siMG-OKS), untransfected MG-OKS cells and MG-OKS cells transfected with scrambled siRNA (scMG-OKS) as controls. SPRR1A expression, morphological changes, cell proliferation and migration were assessed in these groups. RNA sequencing was performed to examine the genetic changes caused by SPRR1A suppression. To evaluate tumorigenicity *in vivo*, cells from each group were subcutaneously transplanted into the backs of nude mice. Tumor volume and Ki-67 expression were assessed and compared among the three groups at four weeks post-transplantation. The siMG-OKS group exhibited altered cell morphology, reduced cell proliferation and decreased migratory abilities *in vitro*. RNA sequencing revealed suppression of genes involved in cell adhesion in the siMG-OKS group. Furthermore, the *in vivo* tumorigenicity of siMG-OKS was lower than that of the other two experimental groups. These findings suggest that SPRR1A is one of the key cell adhesion-related molecules involved in OS progression, potentially serving as a therapeutic target for this refractory tumor. However, further research is needed to fully elucidate the mechanisms by which SPRR1A influences OS pathogenesis and to explore its clinical potential.

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Abbreviations: CSC, cancer stem cell; EGFP, enhanced green fluorescent protein; MD, migration distance; NGS, next-generation sequencing; OS, osteosarcoma; PBS, phosphate-buffered saline; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; scMG-OKS, scrambled siRNA-transfected MG-OKS; siMG-OKS, MG-OKS cells transfected with SPRR1A siRNA; SE, standard error; TPM, transcripts per million; SPRR1A, small proline-rich protein 1A; FAK, focal adhesion kinase; CDK2, cyclin-dependent kinase 2; CDKN1B, cyclin-dependent kinase inhibitor 1B; Skp2, S-phase kinase-associated protein-2; CCND1, cyclin D

Key words: *SPRR1A*, OS, CSC, tumor progression, focal adhesion

Introduction

Osteosarcoma (OS) represents the predominant form of primary malignant bone neoplasms, and predominantly

affects children and adolescents, with a second peak in their 60s (1). Advances in combination chemotherapy and limb-sparing surgery have improved the 5-year survival rates of patients with OS. However, patients with non-metastatic OS have recently reached a plateau in survival rates of ~70%, whereas those with either metastatic or recurrent OS remain <30% (2,3). Therefore, elucidating the mechanisms underlying disease progression, chemoresistance and metastasis is crucial (4).

Cancer stem cells (CSCs) are considered to be a major factor in the treatment failure for malignant tumors, contributing to issues such as chemotherapy resistance and distant metastasis (5,6). Therefore, exploring therapeutic methods against CSCs is essential for improving the outcomes of patients with malignant tumors. However, CSCs represent only a small fraction of the cell population, making their collection for therapeutic research challenging (7). Previously, the authors have succeeded for the first time in artificially generating CSC-like cells from the MG-63 OS cell line through transduction with defined gene sets, including octamer-binding transcription factor 3/4 (OCT3/4), Krüppel-like factor 4 (KLF4) and SRY-box transcription factor 2 (SOX2) (8). These cells were named 'MG-OKS'. Notably, MG-OKS cells showed significantly enhanced CSC properties, including reduced proliferation rates, elevated chemoresistance, enhanced spheroid formation capacity and increased migratory potential.

The expression of several specific genes increased in MG-OKS cells; among the genes whose expression is increased in MG-OKS cells, the present study focused on small proline-rich protein 1A (SPRR1A). SPRR1A is present in normal skin and esophagus (9,10). This protein serves as a structural component of the cornified envelope with a barrier function, and is widely acknowledged as a marker for terminal squamous cell differentiation (11).

Increased SPRR1A expression has been reported in some types of non-squamous cell carcinomas; however, it is not usually observed in normal non-squamous tissues. SPRR1A expression is a possible prognostic factor for colorectal cancer (12), pancreatic ductal adenocarcinoma (10) and diffuse large B-cell lymphoma (13). The expression of molecules that are not expressed in the native cancer lineage is generally associated with poor prognosis (14-16). Thus, it was hypothesized that SPRR1A, whose expression is upregulated in MG-OKS, may play a role in tumor initiation, growth and poor prognosis of OS. However, the role of SPRR1A in OS remains unknown. The present study aimed to evaluate the role of SPRR1A in OS both *in vitro* and *in vivo* using our newly generated artificial MG-OKS cells.

Materials and methods

Cell lines and culture conditions. MG-63, a human OS cell line, was procured from RIKEN BRC through the National Bio-Resource Project of the MEXT. Cells were maintained in a medium consisting of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all from MilliporeSigma) at 37°C in a 5% CO₂ humidified atmosphere. For retrovirus production, Plat-A packaging cells (Cosmo Bio Co., Ltd.) were cultured in the presence of 1 mg/ml puromycin and

10 mg/ml blasticidin (both from Thermo Fisher Scientific, Inc.).

Generating CSC-like cells from the OS cell line. A modified retroviral transduction protocol (17) was employed to generate CSC-like cells from the MG-63 line. Specifically, the polycistronic retroviral vector pMXs encoding OCT3/4, KLF4 and SOX2 (pMXs-OKS) (18) was used for reprogramming, while pMXs-enhanced green fluorescent protein (EGFP) (19) served as the control vector. Retroviral vectors (pMXs-OKS or pMXs-EGFP) were transfected into Plat-A cells (in DMEM containing 10% FBS without antibiotics) to produce retroviral particles. CSC-like cells were generated by introducing pMXs-OKS into MG-63 and named 'MG-OKS', and those generated by introducing the control pMXs-EGFP were named 'MG-GFP'. The Institutional Review Board of Kobe University approved the study protocol.

Gene expression analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The RNeasy Mini Kit (cat. no. 74104; Qiagen, Inc.) was used to extract total RNA from cultured cells. cDNA synthesis was performed using the High-capacity cDNA Transcription kit (cat. no. 4368814; Applied Biosystems; Thermo fisher Scientific, Inc.) according to the manufacturer's protocol. RT-qPCR was carried out on an ABI Prism 7500 Sequence Detection System (Applied Biosystems; Thermo fisher Scientific, Inc.) using SYBR Green Master Mix (Applied Biosystems; Thermo fisher Scientific, Inc.) under the following conditions: One cycle at 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. The relative mRNA expression of the transgenes OCT3/4, KLF4 and SOX2, along with the established CSCs marker genes CD24, CD26 and CD133, as previously reported (20,21), as well as SPRR1A, were evaluated. Pre-designed primers (Invitrogen; Thermo Fisher Scientific, Inc.) were used to assess the relative mRNA expression of OCT3/4, KLF4, SOX2, CD24, CD26, CD133 and SPRR1A. The primer sequences are listed in Table I. The 2^{-ΔΔC_q} method (22) was used for quantification, with β-actin as the normalization control.

Knockdown of SPRR1A through siRNA transfection. MG-OKS were seeded in a 6-well plate 24 h before transfection to achieve 70-80% confluency on the day of transfection. Before transfection, 7.2 µl of Lipofectamine™ RNAiMAX Transfection Reagent (cat. no. 13778075; Invitrogen; Thermo Fisher Scientific, Inc.) and 1.2 µl of Silencer™ Pre-Designed siRNA SPRR1A or scramble siRNA (as negative control; Invitrogen; Thermo Fisher Scientific, Inc.) was diluted in 240 µl of Opti-MEM™ I Reduced Serum Medium (Gibco; Thermo Fisher Scientific, Inc.). The transfection complexes were incubated at 25°C for 5 min and then added dropwise to each well. Following a 24-h incubation at 37°C in a CO₂ incubator, the medium was replaced with a fresh complete culture medium, and the cells were incubated for an additional 24 h. The efficiency of SPRR1A knockdown was assessed by RT-qPCR and immunoblot analyses. Cells with confirmed SPRR1A inhibition were used for subsequent experiments. MG-OKS cells transfected with SPRR1A siRNA were named 'siMG-OKS', and those transfected with scramble siRNA were named 'scMG-OKS'.

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Target gene	Primer sequence (5'-3')
hOCT3/4	S: CCCCAGGGCCCCATTTTGGTACC A/S: ACCTCAGTTTGAATGCATGGGAGAGC
hSOX2	S: TTCACATGTCCCAGCACTACCAGA A/S: TCACATGTGTGAGAGGGGCGAGTGTGC
hKLF4	S: CATGCCAGAGGAGCCCAAGCCAAAGAGGGG A/S: CGCAGGTGTGCCTTGAGATGGGA ACTCTTT
CD24	S: TGCTCCTACCCACGCAGATT A/S: GGCCAACCCAGAGTTGGAA
CD26	S: CAAATTGAAGCAGCCAGACA A/S: CACACTTGAACACGCCACTT
CD133	S: TGGGGCTGCTGTTTATTATTCT A/S: TGCCACAAAACCATAGAAGATG
SPRR1A	S: CACCCCAAAGTGCCTGAG A/S: TTCTGCTTGGTCTTCTGCTG
β -actin	S: GATCATTGCTCCTCCTGAGC A/S: ACATCTGCTGGAAGGTGGAC

SPRR1A, small proline-rich protein 1A; OCT3/4, octamer-binding transcription factor 3/4; KLF4, Krüppel-like factor 4; SOX2, SRY-box transcription factor 2; S, sense; A/S, antisense.

Assessment of cell proliferation using the WST-8 assay. The proliferative capacity of the cells was assessed using the WST-8 assay. A total of 2 days after siRNA transfection, cells were seeded into 96-well plates at a density of 5×10^3 cells/well in a volume of 100 μ l culture medium. Subsequent to a 24-h incubation, 10 μ l of the Cell Counting Kit-8 solution (Dojindo Laboratories, Inc.) was introduced into each well and incubated for an additional 2 h at 37°C in a 5% CO₂ environment. The optical density was then quantified at 450 nm employing a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc.).

Assessment of cell migration using the wound healing assay. A total of 2 days after siRNA transfection, cells were seeded into 6-well culture plates at a density of 2×10^5 cells per well in 2 ml of culture medium and cultured until reaching 80% confluence. A linear scratch was introduced in the cell monolayer using a 200- μ l pipette tip to create an artificial wound. The plates were then washed with phosphate-buffered saline (PBS; Takara Bio, Inc.) to eliminate detached cells and maintained in DMEM with 2% FBS (serum-reduced culture media) for 24 h. Wound healing was monitored at 0 and 24 h, and images were acquired using a microscopy system (BZ-X710 Microscope and BZ-X Viewer, BZ-X Analyzer imaging system; Keyence Corporation). The migration distance (MD) for each experimental group was quantified using the following equation: MD=width 0 at h-width 24 at h (where width 0 at h represents the width of the wound at 0 h and width 24 at h represents the

width of the wound at 24 h). The MD value of the MG-OKS cell population served as a reference for calculating the relative cell migration ability, which was determined using the formula: Relative cell migration ability=MD (siMG-OKS) or MD (scMG-OKS)/MD (MG-OKS) (23).

Immunoblot analysis. Cell lysis was prepared using the Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc.). Soluble proteins were isolated by centrifugation at 20,000 x g for 15 min at 4°C. Protein concentration was determined using BCA protein assay kit (cat. no. 23227; Thermo Fisher Scientific, Inc.). Equal quantities of protein (15 μ g) were combined with electrophoresis sample buffer and boiled for 5 min before loading onto a 10.0-20.0% polyacrylamide gel. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred onto polyvinylidene difluoride membranes (MilliporeSigma). The membranes were blocked with 5% non-fat dry milk (Bio-Rad Laboratories, Inc.) in Tris-buffered saline with 10% Tween-20 for 30 min at 25°C and then incubated overnight at 4°C with primary antibodies diluted in CanGet Signal Solution 1 (Toyobo Life Science). Following washing steps, the membranes were probed with horseradish-peroxidase-conjugated secondary antibodies (1:5,000; cat. no. NA934; GE Healthcare Dharmacon, Inc.) in the CanGet Signal Solution 2 (Toyobo Life Science) for 30 min at 25°C. Protein bands were visualized using SuperSignal West Femto enhanced chemiluminescent substrate (Thermo Fisher Scientific, Inc.), and chemiluminescence reactions were detected using a Chemilumino analyzer Las-3000 mini (FUJIFILM Wako Pure Chemical Corporation). To ensure equal protein loading, the membranes were stripped and re-probed with a mouse anti-human β -actin antibody (1:5,000; cat no: A5441; MilliporeSigma). The primary antibody used in the present study was a rabbit anti-human SPRR1A antibody (1:1,000; cat. no. NBP2-93464; Novus Biologicals, LLC). Quantification of protein expression was performed by measuring band intensities using ImageJ software version 1.53t (National Institutes of Health).

In vivo xenograft tumor model. Male BALB/c nude mice (5 weeks-old, n=18, body weight range: 19-22 g) were obtained from CLEA Japan and housed under specific pathogen-free (SPF) conditions. The mice were provided with a pathogen-free laboratory diet and allowed to drink autoclaved water *ad libitum*. The housing environment was maintained at a temperature of 25°C with a relative humidity of 50-60% and a 12/12-h light/dark cycle. All animal experiments were conducted in accordance with the guidelines set forth by the Japanese Physiological Society for the care and use of laboratory animals. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Kobe University (approval no. P230401; date, 2023-02; Kobe, Japan) and adhered to the university's animal experimentation regulations. For the xenograft tumor model, a suspension of 2×10^6 cells in 200 μ l of serum-free PBS (Takara Bio, Inc.) was subcutaneously injected into the dorsal flank region of each mouse (n=6 for each group). Tumor growth was monitored for 4 weeks post-transplantation, and tumor volume was calculated using the formula: $0.5 \times (\text{length}) \times (\text{width})^2$ (8).

At the experimental endpoint, all tumors were surgically excised, and morphometric analyses were performed by two blinded examiners. Humane endpoints were established and strictly followed, which included: Xenograft tumor volume exceeding 10% of the animal's body weight, tumor diameter surpassing 20 mm, tumor-induced weight loss exceeding 20%, or signs of immobility, inability to eat, ulceration, infection, or necrosis. All mice reached the predefined study endpoints and were humanely euthanized by cervical dislocation under deep anesthesia induced by an intraperitoneal injection of pentobarbital sodium (100 mg/kg body weight). Confirmation of death was based on the absence of heartbeat and the presence of pupil dilation.

Immunofluorescence staining of frozen tumor sections. A total of 4 weeks post-cell transplantation, the mice were humanely euthanized, and the tumor tissues were collected for histological analyses. Frozen tumor sections with a thickness of 10 μ m were prepared using a cryostat. Immunofluorescence staining was performed to assess the proliferative activity of the tumor cells. The sections were incubated overnight at 4°C with a primary rabbit polyclonal antibody targeting human Ki-67 (1:100; cat. no. NB500-170; Novus Biologicals, LLC) diluted in CanGet Signal immunostain solution A (cat. no. NKB-501; Toyobo Life Science). Following washing steps, the sections were incubated with the secondary antibody, Alexa-Fluor 647 goat anti-rabbit IgG (2:1,000; cat. no. A-27040; Invitrogen; Thermo Fisher Scientific, Inc.) diluted in PBS (Takara Bio, Inc.) for 1 h at 25°C. Finally, nuclear staining was achieved using the 4',6-diamino-2-phenylindole solution (1:5,000; MilliporeSigma) diluted in PBS (Takara Bio, Inc.) for 15 min at 25°C. The proliferative activity of the tumor tissues was then assessed using a fluorescence microscope (BZ-X710 Microscope and BZ-X Viewer, BZ-X Analyzer Imaging System; Keyence Corporation). The percentage of Ki-67-positive tumor cells, was quantified in four randomly selected microscopic fields per section using ImageJ software version 1.53t (National Institutes of Health). Two blinded examiners performed all the studies.

Transcriptome profiling by RNA sequencing. Total RNA was isolated from cultured cells two days post-siRNA transduction using an RNeasy Mini Kit (cat. no. 74104; Qiagen, Inc.) according to the manufacturer's protocol. RNA quality and concentration were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.) to measure concentration (MG-OKS, 309.5 ng/ μ l; siMG-OKS, 296.3 ng/ μ l; scMG-OKS, 304.4 ng/ μ l) and purity (A260/280; MG-OKS, 2.07; siMG-OKS, 2.1; scMG-OKS, 2.07). Total RNA samples derived from MG-OKS, siMG-OKS and scMG-OKS cells were submitted to Macrogen, Inc. Library preparation using the TruSeq Stranded mRNA LT Sample Prep Kit (cat. no. RS-122-2101; Illumina, Inc.) was performed by Macrogen, Inc. Paired-end RNA sequencing was subsequently conducted on an Illumina NovaSeq 6000 System (Illumina, Inc.), with a read length of 101 bp for both forward and reverse directions (5'-3'). The obtained reads were aligned to the human transcriptome (hg38) reference sequences utilizing Strand next-generation sequencing (NGS) software version 3.1.1. (Strand Life Sciences; <https://www.strand-ngs.com/>).

The aligned reads were normalized to transcripts per million (TPM), and the resulting normalized counts were standardized to a value of 1. Log2-transformed TPM values were utilized to compare gene expression levels among MG-OKS, siMG-OKS and scMG-OKS cells. Scatter plots were employed to visualize gene expression data, and pathway analyses were conducted using WikiPathways database (<https://wikipathways.org/>) within the Strand NGS software platform (<https://www.strand-ngs.com/>). Z-scores were calculated to identify differentially expressed genes by subtracting the overall mean gene intensity from the normalized intensity of each gene and dividing the result by the standard deviation (SD) of all measured intensities, as described by the equation: $Z\text{-score} = (\text{intensity} - \text{mean intensity}) / \text{SD}$ (24).

Statistical analyses. Statistical analyses were carried out using the EZR statistical software version 1.54 (Saitama Medical Centre, Jichi Medical University; <http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html>; Kanda, 2012). Data were expressed as the mean $\pm$ standard error of the mean. One-way analysis of variance (ANOVA) followed by Tukey-Kramer test was employed to assess statistical differences among multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of MG-OKS cells. The properties of MG-OKS were first examined to identify if they were consistent with previous studies (8). The results revealed that MG-OKS cells exhibited significantly higher transcript levels of OCT3/4, KLF4 and SOX2 compared with both MG-63 and MG-GFP cells ($P < 0.05$, $n = 3$) (Fig. S1A). These findings suggested that the reprogramming process successfully induced the expression of the desired transcription factors in MG-OKS cells.

Additionally, the expression of CSC markers previously reported in various cancers, including CD24, CD26 and CD133 (20,21), was evaluated. RT-qPCR analysis demonstrated that MG-OKS cells displayed significantly elevated mRNA levels of CD24, CD26 and CD133 compared with MG-63 and MG-GFP cells ($P < 0.05$, $n = 3$) (Fig. S1B). These results indicated that MG-OKS cells possess a gene expression profile consistent with CSC-like properties.

Furthermore, morphological assessment of the cells revealed distinct differences between MG-OKS cells and their MG-63 and MG-GFP counterparts. MG-OKS cells exhibited elongated cell bodies with protrusions, a feature not found in MG-63 and MG-GFP cells (Fig. S1C).

Alteration of cell morphology by knockdown of SPRR1A in MG-OKS cells. The efficiency of knockdown of SPRR1A by siRNA transfection was evaluated by performing RT-qPCR and immunoblot analysis. The expression of the SPRR1A gene was significantly reduced by 56 and 61% in siMG-OKS cells compared with MG-OKS and scMG-OKS cells, respectively ($P < 0.05$, $n = 7$) (Fig. 1A). Immunoblot analysis quantified by band intensity using ImageJ software showed a 25 and 23% reduction in SPRR1A protein levels compared

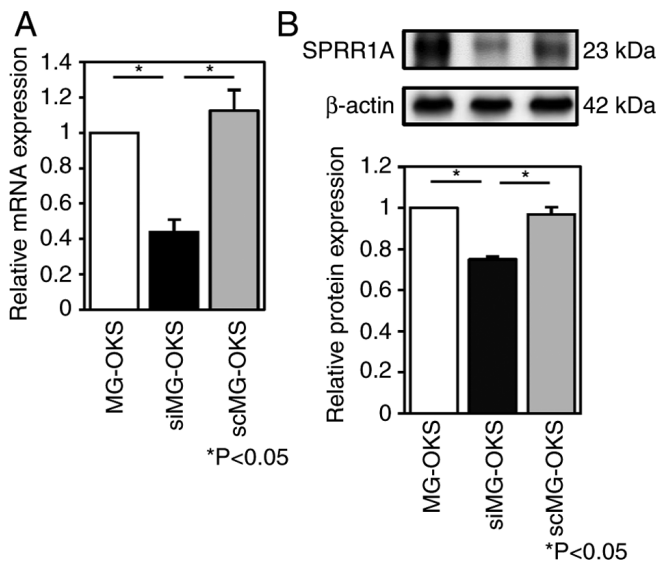


Figure 1. siRNA-induced SPRR1A knockdown. (A) Reverse transcription-quantitative PCR of SPRR1A. The mRNA expression levels of SPRR1A in each group were normalized against β -actin levels and the mRNA expression level of MG-OKS cells was set to 1. Data are expressed as the mean value $\pm$ SE (n=7). * $P < 0.05$, one-way ANOVA with Tukey's test. (B) SPRR1A protein expression was examined using immunoblot analysis (upper). The graph shows protein expression quantified using ImageJ software (lower). The protein expression levels of SPRR1A in each group were normalized against β -actin levels and the protein expression level of MG-OKS cells was set to 1. Data are expressed as the mean value $\pm$ SE (n=4). * $P < 0.05$, one-way ANOVA with Tukey's test. si-, small interfering; SPRR1A, small proline-rich protein 1A; SE, standard error; sc-, scrambled.

with MG-OKS and scMG-OKS, respectively ($P < 0.05$, n=4) (Fig. 1B). No significant differences in SPRR1A gene expression or protein levels were observed between MG-OKS and scMG-OKS.

Morphological changes were assessed microscopically, and the protrusions observed in MG-OKS (8) were decreased in siMG-OKS. By contrast, they were observed in scMG-OKS as in MG-OKS (Fig. 2A).

SPRR1A knockdown significantly impairs proliferation and migration of MG-OKS cells. Having established the efficient knockdown of SPRR1A in MG-OKS cells, it was sought to investigate the functional consequences of reduced SPRR1A expression on cell proliferation and migration, two critical processes involved in cancer progression and metastasis.

To assess the impact of SPRR1A knockdown on cell proliferation, the WST-8 assay was performed. The siMG-OKS group revealed a 79 and 78% reduction in cell proliferation at 48 h after siRNA transfection compared with the MG-OKS and scMG-OKS groups, respectively ($P < 0.05$, n=6) (Fig. 2B). There were no significant differences in cell proliferation between the MG-OKS and scMG-OKS groups.

Wound healing assays were performed to assess how SPRR1A knockdown affects cell migration ability. siMG-OKS group demonstrated a 70 and 69% reduction in cell migration ability compared with MG-OKS and scMG-OKS, respectively ($P < 0.05$, n=3) (Fig. 2C and D). There were no significant differences in cell migration ability between the MG-OKS and scMG-OKS groups.

SPRR1A knockdown results in the suppressed tumorigenicity of MG-OKS cells in vivo. To investigate the impact of SPRR1A knockdown on tumorigenicity, a xenograft mouse model was employed. MG-OKS, siMG-OKS and scMG-OKS cells were subcutaneously transplanted into the dorsal flank region of immunodeficient nude mice. A total of 4 weeks after cell transplantation, tumor volumes were compared, and immunofluorescence staining was performed. The transplanted xenografts showed 100% engraftment in all mice. Tumor volume 4 weeks after cell transplantation was significantly smaller in the siMG-OKS group than in the MG-OKS and scMG-OKS groups (MG-OKS, 0.20 ± 0.032 cm³; siMG-OKS, 0.072 ± 0.007 cm³; scMG-OKS, 0.17 ± 0.027 cm³) ($P < 0.05$, n=6) (Fig. 3A and B). There was no significant difference in the tumor volume between the MG-OKS and scMG-OKS groups. Immunofluorescence staining of implanted tumors revealed significantly decreased Ki-67 positivity, a proliferation marker (25,26), in siMG-OKS group compared with MG-OKS and scMG-OKS groups (MG-OKS, $36 \pm 2.7\%$; siMG-OKS, $11 \pm 1.8\%$; scMG-OKS, $37 \pm 1.7\%$) ($P < 0.05$, n=6) (Fig. 4A and B). There was no significant difference in Ki-67 positivity between MG-OKS and scMG-OKS groups. In addition, RT-qPCR and immunoblot analyses revealed no significant differences in SPRR1A expression among the three groups (Fig. S2A and B). Furthermore, immunostaining also showed similar SPRR1A expression patterns across all groups (Fig. S2C and D).

RNA sequencing identifies several downregulated genes and associated pathways. To analyze the genetic changes induced by the knockdown of SPRR1A, RNA sequencing was performed. Scatter plots were used to visualize the comparison of commonly altered genes between MG-OKS and scMG-OKS cells against siMG-OKS cells. A total of 961 genes were downregulated <2 -fold in siMG-OKS cells compared with MG-OKS cells (blue dots), whereas 728 genes were downregulated <2 -fold in siMG-OKS cells compared with scMG-OKS cells (blue dots) (Fig. 5A). Among these genes, 452 were commonly downregulated in siMG-OKS (Fig. 5B), and 11 pathways were identified using pathway analysis, including 'Interleukin-4 and Interleukin-13 signaling', 'Extracellular matrix organization, Vitamin D Receptor Pathway', and 'Focal Adhesion PI3K-Akt-mTOR signaling pathway'. Each of the detected pathways was associated with a $P < 10^{-5}$ (Fig. 5C). A heatmap was used to visualize the changes in individual genes. The results revealed decreased expression of several focal adhesion-related genes, including focal adhesion kinase (FAK) (Fig. 5D), and altered expression levels of several cell cycle-related genes, including S-phase kinase-associated protein-2 (Skp2), cyclin-dependent kinase 2,4 (CDK2,4), cyclin D (CCND1) and cyclin-dependent kinase inhibitor 1B (CDKN1B) (Fig. 5E).

Discussion

CSCs are postulated to be pivotal contributors to the unfavorable prognosis observed in patients with refractory OS. Moreover, these cells are hypothesized to be instrumental in the etiology of disease recurrence and metastatic progression (5). The authors have previously generated artificial CSC-like cells

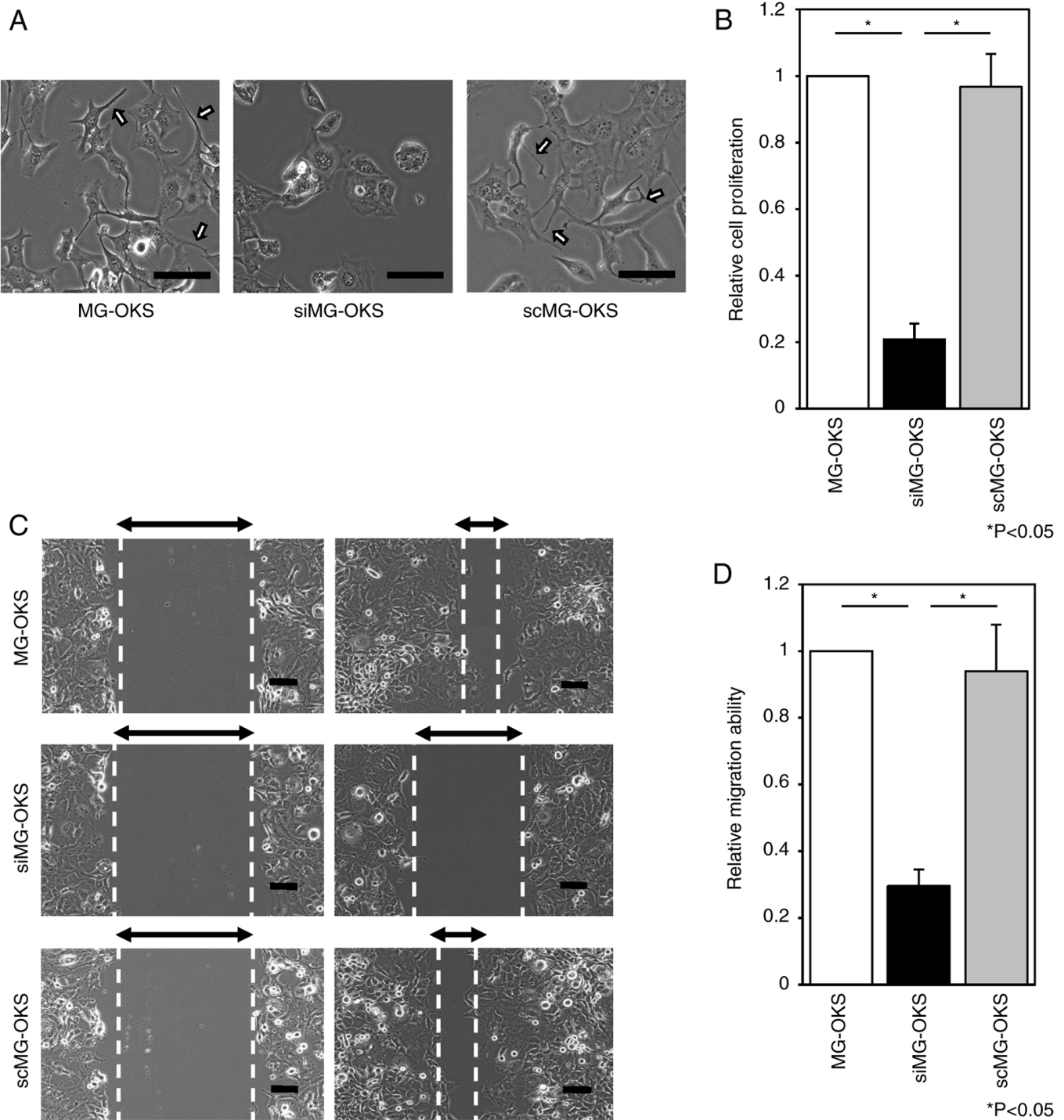


Figure 2. Effects of siRNA-induced SPRR1A knockdown in MG-OKS. (A) SPRR1A inhibition led to distinct morphological changes (arrows). Scale bars, 100 μ m. (B) The proliferation of MG-OKS cells was set to 1. Data are expressed as the mean value $\pm$ SE. (n=6) *P<0.05, one-way ANOVA with Tukey's test. (C) Representative phase contrast images of wound healing assays at 0 and 24 h for each group. (D) The relative cell migration ability of each group, with the cell migration ability of the MG-OKS group set to 1. Data are expressed as the mean value $\pm$ SE (n=3). *P<0.05, one-way ANOVA with Tukey's test. si-, small interfering; SPRR1A, small proline-rich protein 1A; SE, standard error; sc-, scrambled.

from one of the OS cell lines to elucidate the role of CSCs in OS and it was found that these cells exhibit upregulation of SPRR1A (8). Previously, an association between SPRR1A and poor prognosis was reported in several cancers (10,12), and although SPRR1A expression levels have been associated with several cancers, the signaling pathways involved in its induction and downstream effects on cellular behavior and gene expression are poorly understood (27). In particular, no studies

have investigated the role of SPRR1A in OS, and its relevance remains unclear.

In the present study, the knockdown of SPRR1A expression reduced cellular protrusion formation and gene expression associated with cell adhesion, including FAK, and significantly decreased migration ability. Cell adhesion and migration are intricate, dynamic processes comprising multiple stages. The process is regulated by FAK, a

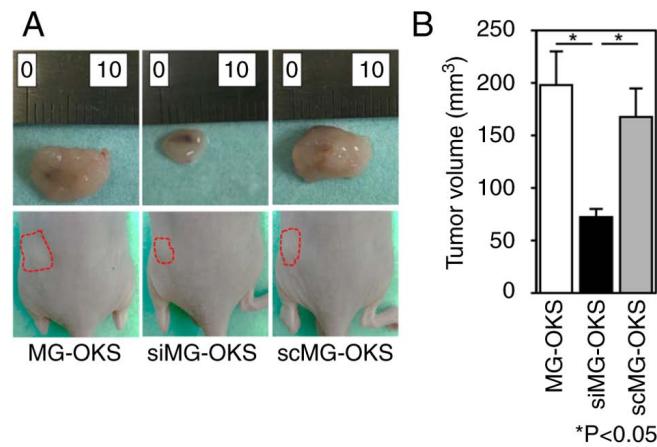


Figure 3. *In vivo* tumorigenicity after cell transplantation. (A) Representative images of immunodeficient nude mice after 4 weeks of subcutaneous cell transplantation in each group (lower panel) and tumors after resection (upper panel). (B) Tumor volume in each group after 4 weeks of subcutaneous cell transplantation (n=6 for each group). Data are expressed as the mean value $\pm$ standard error (n=6). *P<0.05, one-way ANOVA with Tukey's test. si-, small interfering; sc-, scrambled.

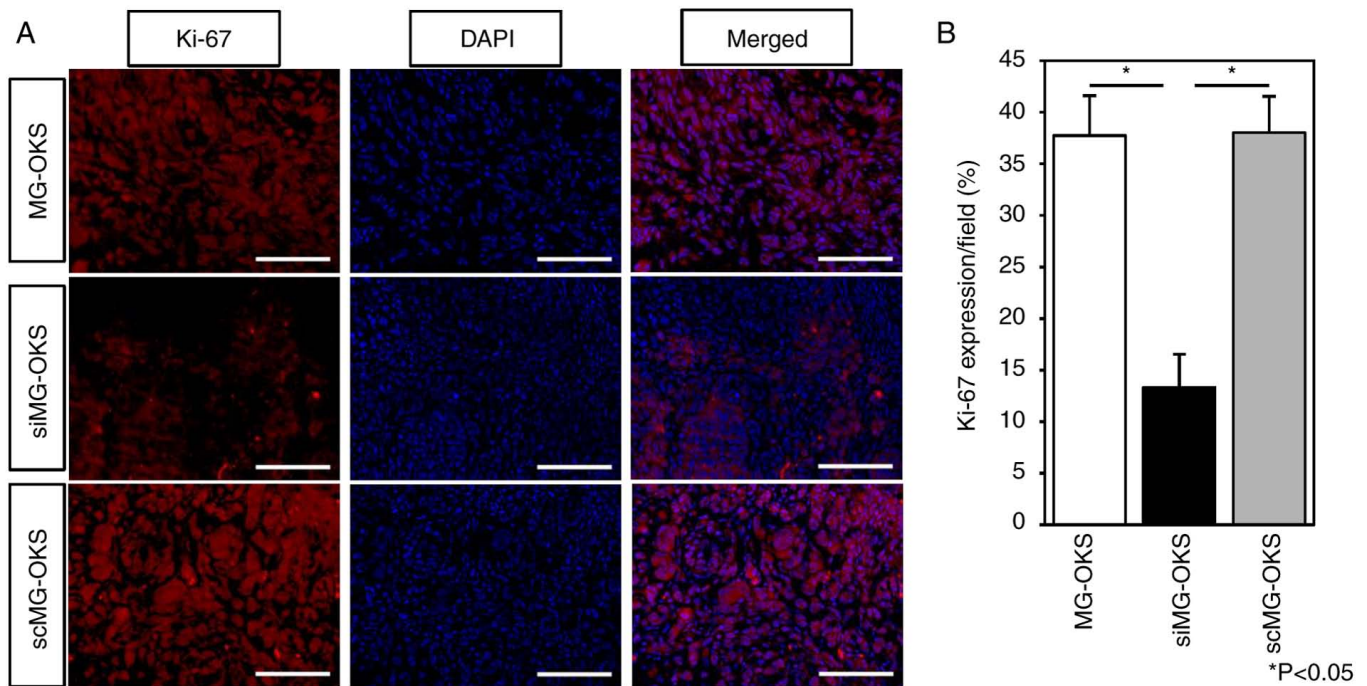


Figure 4. Immunofluorescence staining of Ki-67. (A) Histology of xenografts obtained from the transduced and MG-OXS cells. Representative images of immunofluorescence were shown. Tumors derived from siMG-OXS cells were negative for Ki-67 (red). Nuclei were stained with blue fluorescent 4',6-diamino-2-phenylindole. Scale bars, 100 μ m. (B) A total of 4 fields were randomly selected and positive areas were assessed using ImageJ software. Data are expressed as the mean value $\pm$ standard error (n=6). *P<0.05, one-way ANOVA with Tukey's test. si-, small interfering; sc-, scrambled.

non-receptor tyrosine kinase overexpressed in several types of tumors; FAK has been reported to be related to the engagement of cell adhesion molecules and assembly of focal adhesions, ultimately controlling cell migration (28). Maziveyi *et al* (29) reported that inhibition of focal adhesion in breast cancer cells leads to reduced adhesion to the extracellular matrix, decreased formation of protrusions, and impaired migration ability (29). Taken together, the results of the present study suggested that downregulation of genes related to focal adhesion, such as FAK, induces reduced cellular protrusion formation and cell migration ability.

On the other hand, the current results revealed that the knockdown of *SPRR1A* in MG-OXS significantly reduced cell proliferation. As aforementioned, *SPRR1A* knockdown also exhibited reduced cell adhesion in siMG-OXS. In addition, our sequencing data showed decreased FAK, Skp2 and CDK2 and increased CDKN1B in siMG-OXS. Benaud and Dickson (30) reported that decreased cell adhesion in mammary epithelial cells correlates with G1 phase arrest via CDK2 inhibition due to increased CDKN1B. It has also been reported that inactivation of FAK by decreased cell adhesion inhibits the expression of Skp2, which is responsible for CDKN1B degradation (31,32). Collectively, the present results suggested that the increase

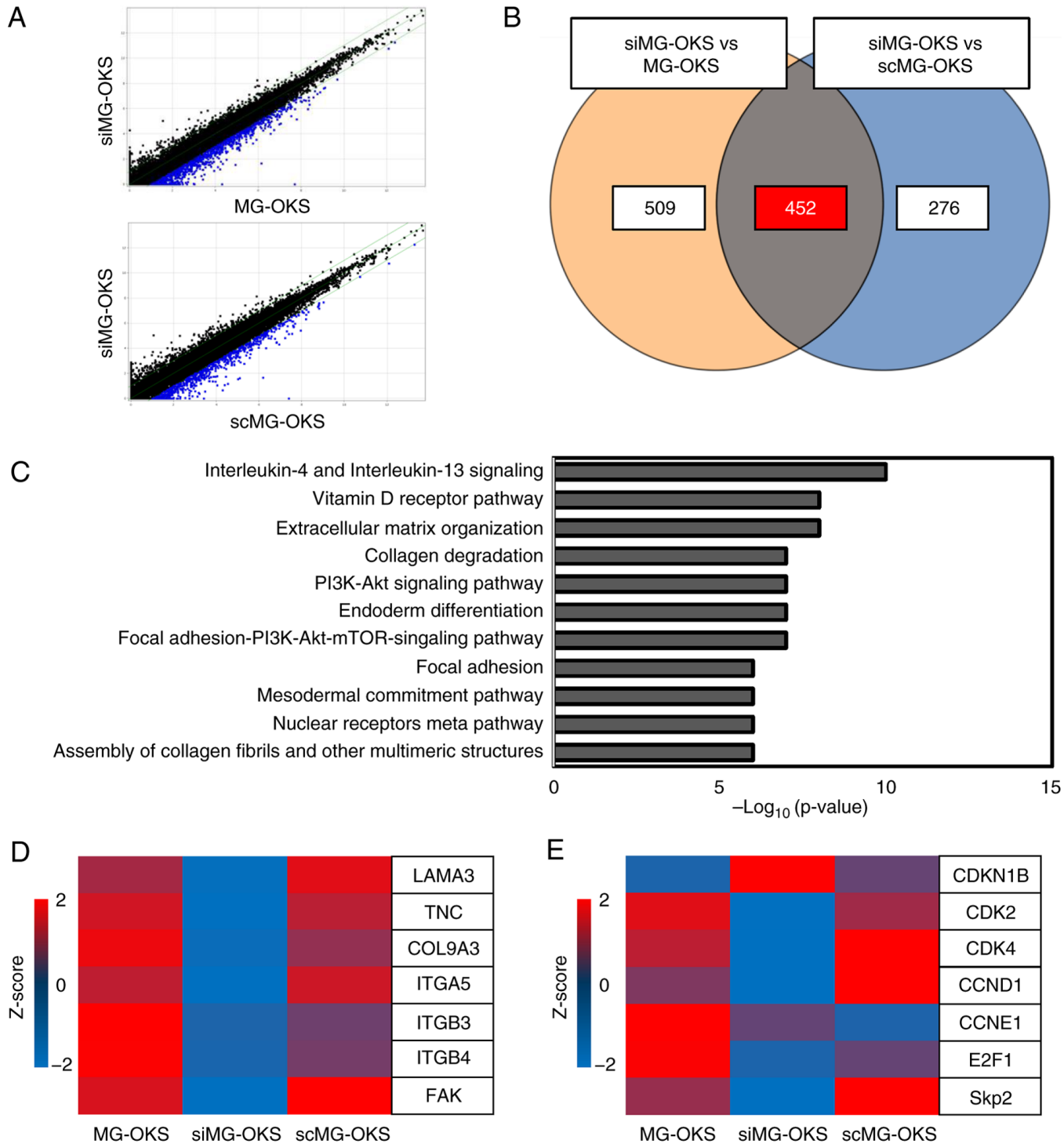


Figure 5. RNA sequencing of MG-OXS, siMG-OXS, and scMG-OXS cells. (A) Scatter plots showing ≥ 2 -fold downregulated genes (blue dots). (B) Venn diagram showing the number of the 2-fold downregulated genes in the comparison between siMG-OXS vs. MG-OXS (left), siMG-OXS vs. scMG-OXS (right), and the number of differentially expressed genes in both comparisons (center). (C) The list of 11 pathways with $P < 10^{-5}$. (D and E) Expression heat maps of genes associated with (D) cell adhesion and (E) cycle. Gene signal intensities were normalized and converted to Z-scores. si-, small interfering; sc-, scrambled; FAK, focal adhesion kinase; Skp2, S-phase kinase-associated protein-2; CDK2,4, cyclin-dependent kinase 2,4; CCND1, cyclin D; CDKN1B, cyclin-dependent kinase inhibitor 1B.

in CDKN1B in siMG-OXS cells inhibits CDK2, leading to reduced cell proliferation. Moreover, it has been reported that the inhibition of the PI3K/AKT/mTOR pathway involves the inhibition of CCND1 and CDK4 (33), and inhibition of CCND1 and CDK4 leads to cell cycle arrest (34). Collectively, the sequencing data generated in the present study suggest that

cell cycle inhibition contributes not only to CDK2 but also to CDK4 inhibition, and that reduced cell adhesion should induce decreased cell proliferation through multiple pathways rather than a single pathway.

The results of *in vivo* studies indicated that siMG-OXS xenografts had fewer Ki-67 positivity than MG-OXS

xenografts. High expression of Ki-67 has been reported to be associated with decreased 5-year survival and increased distant metastases in patients with OS (26,35). Given the decreased number of Ki-67 positivity, the results suggested reduced malignant potential in the siMG-OKS cells. The present results are consistent with previous studies, reporting that lower SPRR1A expression is associated with lower malignancy in colorectal and pancreatic ductal carcinomas (10,12).

The present study presents several noteworthy limitations that warrant consideration in the interpretation of results and design of future investigations. Firstly, the transient nature of siRNA-mediated SPRR1A knockdown via lipofection may have resulted in diminished gene suppression effects by the time of evaluation, as evidenced by the absence of significant differences in SPRR1A expression in xenograft samples (Fig. S2A-D). Despite this constraint, the current findings suggest that SPRR1A knockdown likely exerts its influence during the early stages of tumor formation. The observation of significant differences in tumor tissues with respect to tumorigenicity, despite the temporary nature of SPRR1A suppression, is particularly intriguing and warrants further elucidation. Furthermore, the potential for off-target effects inherent to siRNA-mediated knockdown cannot be overlooked. While commercially available siRNA sequences designed to minimize off-target effects were utilized, the possibility of unintended gene silencing or activation of cellular stress responses cannot be entirely excluded. In an attempt to address this concern, rescue experiments were preliminarily performed by utilizing a plasmid vector to overexpress SPRR1A in siMG-OKS cells. These experiments showed partial restoration of cell proliferative capacity (data not shown), suggesting complex interactions with downstream signaling pathways that warrant further investigation. To address these technical limitations, future studies should consider employing more stable knockdown methods, such as shRNA, to investigate the long-term effects of SPRR1A inhibition, particularly in *in vivo* studies.

Secondly, the substantial heterogeneity characteristic of OS and the limitations of our cell line-based approach present challenges. The enormous heterogeneity observed in OS is demonstrated by the identification of multiple pathways (14 driver genes) in exome sequencing studies, which increases the complexity of effective therapeutic strategies for OS, reflected clinically as a refractory and relapsing disease (36). The utilization of a single OS cell line (MG-63) and its derivative MG-OKS cells in the present investigation potentially constrain the generalizability of our findings and may not comprehensively encompass the intricate complexities inherent to the human tumor microenvironment. While studies employing patient-derived OS samples offer superior representation of tumor heterogeneity and microenvironment effects (37), it is pertinent to acknowledge that such models are constrained by the limited availability of primary chemo-naïve OS specimens (38).

Thirdly, further mechanistic studies are necessary to fully elucidate SPRR1A's specific regulatory roles in the identified pathways and genes. Additionally, the absence of clinical correlation data limits the immediate translational impact of the present findings.

Fourthly, the current study focused principally on primary tumor growth and did not address the potential role of SPRR1A in OS metastasis. Given the clinical significance of metastatic disease in OS, future research utilizing appropriate metastasis models is crucial to comprehensively understand SPRR1A's function in disease progression.

Despite these limitations, the present study is the first to address the function of SPRR1A in OS using newly generated artificial OS CSC-like cells, which provides crucial clues for the development of novel OS treatment strategies that target CSCs.

In conclusion, it is significant that the present study is the first to provide evidence that SPRR1A is one of the key cell adhesion-related molecules involved in OS progression, and further elucidation of the underlying pathophysiology and the exploration and identification of SPRR1A inhibitors may contribute to the development of OS therapeutic approaches from a different perspective.

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Availability of data and materials

The datasets generated in the present study may be requested from the corresponding author. The data generated in the present study may be found in the Gene Expression Omnibus under accession number GSE268670 or at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE268670>.

Authors' contributions

TMi, NF and SF collected and analyzed data and wrote the manuscript. HH, NF, RS, YN, TK, TT, SY, YM, KK, YH, SH, ToMa and TaMa collected and/or assembled data. TMi, NF and SF confirm the authenticity of all the raw data. MK-A performed data analysis and interpretation. NF, TaA, RK and ToA conceived/designed, collected and/or assembled data, performed data analysis and interpretation, and provided final approval of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments involving animals were conducted in accordance with the Japanese Physiological Society Guidelines for the care and use of laboratory animals. The Institutional Animal Care and Use Committee of Kobe University approved the study protocol (approval no. P230401; date, 2023-02; Kobe, Japan). The research adhered to the animal experimentation regulations of Kobe University, Japan.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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