

Depleting HIF-1 α attenuates the progression of osteosarcoma, but tumorigenicity is sustained through HIF-independent pathways

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Abstract. Poor survival of patients with osteosarcoma means that novel therapeutic targets are needed. A previously developed osteosarcoma mouse model revealed that HIF-1 target genes are upregulated under anchorage-independent growth conditions. HIF-1 α is highly expressed at the hypoxic invasion front *in vivo*. Knockout of HIF-1 α attenuates cell growth under hypoxic and non-adherent conditions *in vitro*, as well as growth of primary and metastatic osteosarcoma in C57BL/6 mice, suggesting key roles for HIF-1 α in osteosarcoma progression. However, tumors with a rich vasculature develop in the absence of HIF-1 α . Thus, the HIF-independent survival pathways on which HIF-KO clones depend needs to be identified. The present study revealed that expression of glycolysis-related genes, which are targets of HIF, decreased in HIF-KO clones, but the sensitivity of each clone to inhibitors varied: Some were less sensitive than HIF wild-type cells under hypoxic conditions. Compound screening revealed that the pathways upon which KO clones depend for survival differ. Indeed, inhibiting the mitochondrial electron transport chain, PI3K or

mTOR further reduced growth of KO clones under hypoxic conditions, although one clone was less sensitive to these treatments and retained high proliferation capacity under hypoxic conditions. This clone was extremely sensitive to inhibition of the mevalonate synthesis pathway, suggesting that this might be the mechanism underlying resistance to HIF-targeted therapies. Thus, although HIF-1 is an attractive therapeutic target for osteosarcoma, it is necessary to identify and inhibit heterogenous HIF-independent pathways upon which individual tumor cells rely.

Introduction

Osteosarcoma is the most common type of primary tumor of the bone in childhood and adolescence (1). Adoption of neoadjuvant treatment regimens that combine surgery and chemotherapy have improved prognosis; however, survival rates for patients with metastatic osteosarcoma remain at ~30% (2,3). Therefore, identification of novel therapeutic targets and development of alternative options are required.

Our previous study described development of a mouse model of osteosarcoma by overexpressing c-MYC in bone marrow stromal cells derived from Ink4a/Arf-null mice (4). When inoculated into C57BL/6 syngeneic mice, these highly tumorigenic cells, designated accelerated bone and tumor formation (AXT) cells, rapidly formed osteoid-rich primary tumors and metastatic lesions that pathologically mimic human osteosarcoma (5,6).

Anoikis is a type of cell death induced by detachment from the extracellular matrix (ECM) (7,8). Highly malignant tumor cells can escape anoikis, enabling them to grow without the ECM and develop into metastatic lesions. Consistent with their high metastatic capacity *in vivo*, AXT cells also proliferate in non-adherent cultures, and under these conditions, the expression levels of downstream target molecules of hypoxia inducible factor-1 (HIF-1) are considerably upregulated.

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The HIF-1 complex, which comprises HIF-1 α and HIF-1 β subunits, is a key regulator that enables malignant cells to survive and continue to grow under hypoxic microenvironmental conditions (9-11). HIF-1 α transcriptionally activates multiple target genes and interacts with several molecules to promote progression, metastasis and therapeutic resistance of various types of malignancy, including osteosarcoma. Analysis of human osteosarcoma specimens suggests that the rate of positive staining for HIF-1 α in human osteosarcoma specimens is 34-88% (12), and high expression of HIF-1 α is associated with clinical stage (12,13), metastasis (12), overall survival (12,14) and resistance to treatment (14,15), making it a potential prognostic predictor of poor prognosis. Several *in vivo* experimental studies demonstrate that HIF-1 α promotes osteosarcoma progression and metastasis directly (16-19). Thus, HIF-1 α and its associated pathways may be promising therapeutic targets for osteosarcoma. However, the stage of osteosarcoma development at which HIF-1 α is expressed, and the role it plays, is unclear. In particular, experimental verification of the validity and problems of HIF-targeting therapy for osteosarcoma remains unsolved. Using a syngeneic mouse model of osteosarcoma to conduct depletion of HIF-1 α and subsequent *in vitro* and *in vivo* analyses may provide clues to resolving these issues.

In the present study to clarify the role of HIF-1 α in the progression of osteosarcoma, HIF-1 α was knocked out in AXT cells using CRISPR-Cas9 with two different target sequences to produce three HIF-1 α -knockout (KO) clones. Using HIF-KO cells, cell growth and cell cycle status was evaluated *in vitro* and the effects of HIF-1 α depletion *in vivo* on primary tumor formation, metastasis, tumor angiogenesis and gene expression changes was assessed.

Materials and methods

Reagents. Roxadustat (25 μ M; 27 h), buparlisib (1 μ M; 2 days) (MedChemExpress), antimycin A (1 μ M; 2 days), oligomycin (1 μ M; 2 days) (Abcam), rotenone (1 μ M; 2 days), mevalonate (200 μ M; 17 h or 2 days) (MilliporeSigma), BAY-876 (3.3-30.0 μ M), everolimus (1 or 5 μ M, 19.5 h or 2 days), temsirolimus (1 or 5 μ M; 19.5 h or 2 days), shikonin (1 μ M; 2 days), PT2385 (10 μ M; 19.5 h), TC-S7009 (10 μ M; 19.5 h) (Selleck Chemicals), simvastatin (0.5-3.0 μ M; 4.5, 6, 14, 17, 21.5, 25 h or 2 days), atorvastatin (1-3 μ M; 2 days) and metformin (1-5 mM; 17 h or 2 days) (Tokyo Kasei Kogyo) were reconstituted in dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical Corporation). 2-deoxy-D-glucose (3.3-30.0 mM; 2 days; Tokyo Chemical Industry Co., Ltd.), Farnesyl Diphosphate (FPP; 50 μ M; 2 days) and Geranylgeranyl Diphosphate (GGPP; 50 μ M; 2 days; Echelon) were dissolved in water. Reagents were applied to AXT, control (Mock), or HIF-KO cells at the final concentrations for the desired duration shown in parentheses at 5% CO₂ at 37°C.

Establishment of HIF-1 α -KO (HIF-KO) cells using CRISPR-Cas9. Mouse osteosarcoma AXT cells were immortalized cells previously established by overexpressing c-MYC in bone marrow stromal cells derived from *Ink4a/Arf*-null mice (4). The gRNA sequences targeting the HIF-1 α exon 3 (bHLH domain) and exon 5 (Oxygen-Dependent Degradation

Domain; ODDD) were searched using CHOPCHOP (version 3; <http://chopchop.cbu.uib.no/>; SCR_015723) (20) and two sequences were used: #1, 5'-AGATGTGAGCTCACA TTGTGGGG-3' for KO1-1 and 1-2; and #2, 5'-GCTAACAGATGACGGCGACATGG-3' for KO2 (the protospacer adjacent motif (PAM) is underlined). The oligos were annealed and ligated into the lentiCRISPRv2-puro vector (Addgene_98290; Addgene, Inc.), and digested with *BsmBI* according to the protocol provided by the Zhang lab (<https://www.addgene.org/crispr/zhang/>). The Cas9 is integrated in the lentiCRISPRv2-puro vector. After confirmation of the inserted sequence, the lentiCRISPRv2-puro vectors were co-transfected along with the psPAX2 (cat. no. 12260; Addgene, Inc.) and pCMV-VSV-G (Addgene_8454; Addgene, Inc.) vectors into Lenti-X 293T cells (CVCL_4401; Takara Bio) using FuGENE HD (Promega Corporation) to generate infectious lentivirus. The empty lentiCRISPRv2-puro vector was used to establish control (Mock) cells. The virus-containing medium was added to AXT cells, and infected cells were selected with puromycin. To obtain knockout cells, puromycin-resistant cells were subjected to single-cell cloning.

Cell culture. Human osteosarcoma U2OS (cat. no. HTB-96) and SaOS-2 (cat. no. HTB-85) cell lines were purchased from American Type Culture Collection. These cells were authenticated by examination of *in vitro* growth characteristics and morphological properties provided evidence of correct cell identity. AXT cells, HIF-1 α -knockout AXT cells or human SaOS-2 and U2OS cells were cultured under 5% CO₂ at 37°C in Iscove's Modified Dulbecco's Medium (IMDM; Nacalai Tesque, Inc.) or McCoy's 5A medium (Thermo Fischer Scientific, Inc.), respectively, supplemented with 10% FBS (MilliporeSigma). For non-adherent cell culture, 6 cm ultra-low-attachment surface dishes (Corning, Inc.) were used.

Cell proliferation assay. AXT cells, including HIF-1 α -knockout AXT cells were treated with trypsin at 37°C for 2 min, collected and washed with serum-free medium, and then transferred to 96-well cell culture plates (1 $\times$ 10³ cells per well in 50 μ l of IMDM supplemented with 10% FBS). Ultra-low-attachment surface plates (Corning Inc.) were used for non-adherent culture. Cells were incubated for 1 h at 37°C before addition of 50 μ l of the corresponding medium supplemented with agents at twice the desired final concentrations: Buparlisib (1 μ M), antimycin A (1 μ M), oligomycin (1 μ M), rotenone (1 μ M), mevalonate (200 μ M), BAY-876 (3.3-30.0 μ M), everolimus (1 μ M), temsirolimus (1 μ M), shikonin (1 μ M), simvastatin (0.5-3.0 μ M), atorvastatin (1-3 μ M), metformin (1-5 mM), 2-deoxy-D-glucose (3.3-30.0 mM), FPP (50 μ M) and GGPP (50 μ M). Hypoxic conditions were created by placing oxygen absorbers in an airtight bag, and adjusting ventilation to maintain a constant 2% oxygen concentration using an oxygen monitor. (Bionix culture kit; Sugiyamagen Co., Ltd.). After incubation for 2 days, cell viability was measured using a Cell Titer Glo assay kit (Promega Corporation). Assays were performed in at least triplicate, and data are expressed as the mean $\pm$ SD relative (fold-change) to the corresponding control value for cells incubated in the absence of the indicated agents.

Reverse transcription-quantitative PCR (RT-qPCR). Extraction of total RNA from cultured cells, RT and qPCR were performed using the NucleoSpin RNA kit, PrimeScript reverse transcriptase (Takara Bio, Inc.) and Thunderbird SYBR qPCR mix (Toyobo Co., Ltd.). The sequences of the PCR primers are listed in Table SI. To prepare samples from the mouse tissue, the left lung was suspended in lysis buffer (NucleoSpin RNA kit) and disrupted using a BioMasher (Nippi, Inc.). To evaluate circulating tumor cells (CTCs), total RNA was extracted from 200 μ l blood obtained from mice by cardiac blood sampling after euthanasia using a NucleoSpin RNA blood kit (Takara Bio, Inc.). Since GFP is expressed by AXT cells, tumor cells were quantitated based on the level of *Gfp* mRNA expression relative to *Actb* mRNA expression. Thermal cycler StepOne (Thermo Fisher Scientific, Inc.) was used for qPCR analysis with the 2step protocol; 60 sec at 95°C, then 40 cycles of 15 sec at 95°C and 60 sec at 60°C with the melting and dissociation curve process. The fold change of gene expression was determined using the relative quantification $2^{-\Delta\Delta C_q}$ method (21).

Western blot analysis. Cell lysates from Mock and HIF-KO cells were prepared with 2x Laemmli sample buffer (Bio-Rad Laboratories, Inc.) supplemented with 5% β -mercaptoethanol or radioimmunoprecipitation assay (RIPA) buffer (Nacalai Tesque, Inc.) supplemented with protease and phosphatase inhibitor cocktails (Nacalai Tesque, Inc.). Protein concentrations were determined using a BCA assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 μ g per lane) were used, and western blot analyses were conducted according to standard semidry transfer procedures using 5–20% gradient precast polyacrylamide gels (ATTO Corporation) and PVDF membranes (ATTO Corporation). Membranes were blocked with 5% skim milk (Nacalai Tesque, Inc.) for 1 h at room temperature and subsequently incubated overnight at 4°C with the primary antibodies listed in Table SII. After washing 4 times, the membranes were incubated with HRP-conjugated secondary antibodies (1:3,000; listed in Table SII) for 1 h at room temperature. Detection procedures were performed using a Clarity Western ECL substrate (Bio-Rad Laboratories, Inc.) and Amersham Hyperfilm ECL (Cytiva). Signal intensities of bands were quantitated with ImageJ software (version 1.49; National Institutes of Health) (22).

GTP-RhoA assay. Mock and HIF-KO cells were treated with or without 1 μ M simvastatin for 4.5 h at 37°C. Cells were lysed with a magnesium-containing buffer (Rho activation assay kit; cat. no. 17-294 MilliporeSigma) and GTP-RhoA was isolated from each lysate containing 1,183 μ g of protein with beads conjugated with a GST fusion protein containing the Rho binding domain of Rhotekin. A part of the same lysate was used for evaluation of total RhoA.

Cell cycle analysis. Cells were trypsinized, washed with PBS and fixed with 70% ethanol for ≥ 48 h at -20°C. Subsequently, the cells were washed twice with ice-cold PBS, and stained with PBS containing 10 μ g/ml propidium iodide and 20 μ g/ml RNase A (MilliporeSigma). The DNA content of at least 10,000 singlet cells was analyzed by flow cytometry (FACSVerse; BD Biosciences).

Cholesterol measurement. Total cholesterol was extracted from 1×10^6 Mock and HIF-KO cells treated with or without 1 μ M simvastatin for 10 h at 37°C (n=3) by the traditional Bligh-Dyer method using acetate, methanol and chloroform (23). In the final step, the dried chloroform layer was dissolved in 50 μ l of isopropanol and quantified using a cholesterol measurement kit (LabAssay Cholesterol; FUJIFILM Wako Pure Chemical Corporation).

Animal care. All animal care and procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals at Hoshi University, as adopted by the Institutional Animal Care and Use Committee on Animal Research of Hoshi University (approved no. P24-091). A total of 81 mice were used in this study; 7-week-old female syngeneic C57BL/6J mice (Sankyo Labo Service Corporation) (total number, 39; weight ~18 g) or 42 12-week-old C57BL/6 SCID mice (strain no. 001913; The Jackson Laboratory; 29 female mice; weight ~21 g and 13 male mice; weight ~25 g). Mice were housed under specific pathogen-free conditions in ventilated cages (floor area 501 cm²; five mice per cage) with ALPHA-dri bedding (Shepherd Specialty Papers) and fed a standard chow diet with access to water *ad libitum*. Animals were inspected daily to ensure that they were not distressed during the experiments. Rooms were temperature controlled at 22°C and kept on a 12-h light/dark cycle. All procedures were performed under inhalational anesthesia using Narcobit-E (Natsume Seisakusho Co., Ltd.) with isoflurane (FUJIFILM Wako Pure Chemical Corporation) at a concentration of 4% for induction and 2% for maintenance. Before analysis, the mice were euthanized by intraperitoneal injection of a lethal dose (100 mg/kg) of pentobarbital sodium (Tokyo Kasei Kogyo Co., Ltd.). After administering pentobarbital, mortality was confirmed by both respiratory arrest (arrest of the chest wall movement) and no response to a toe pinch. The chest cavity was then opened to confirm cardiac arrest and death.

Tumor xenograft model. To establish tumor xenografts, AXT cells (including Mock and HIF-KO cells) suspended in 50 μ l of IMDM or 50 μ l of Matrigel (Corning, Inc.) were injected subcutaneously into the flanks of 39 7-week-old female syngeneic C57BL/6J mice (Sankyo Labo Service Corporation) or 42 12-week-old C57BL/6 SCID mice (strain no. 001913; The Jackson Laboratory), respectively. Unless otherwise specified, 1×10^6 cells were transplanted per site. Mice were assigned randomly into experimental groups. For blinding purposes, researchers performing the cell inoculation were aware of group assignments, while outcome assessment was conducted by other, blinded researchers. No exclusion criteria were set and data from all mice were presented. The criteria for endpoints were as follows: i) The mean tumor diameter was not >20 mm; ii) the combined tumor burden was <15% of body weight (10-week-old mice had a body weight of ~20 g); iii) there was no ulceration, infection or necrosis of the tumor; iv) body weight loss was >20% of the baseline weight. No mice reached the endpoint criteria in the present study. Daily inspection confirmed that growing tumors did not meet the endpoint criteria. The major and minor axes of the tumors were measured, and the estimated tumor weight was calculated from the following formula, with reference to

the guidelines of Washington State University (<https://iacuc.wsu.edu/documents/2017/12/tumor-burden-guidelines.pdf/>): estimated tumor weight (mg)=tumor volume (mm³)=d² x D/2, where d and D are the shortest and longest diameters in mm, respectively. The volume of tumors with the maximum diameter in each mouse was listed in Table SIII. After euthanasia, tumors were collected, imaged and weighed for data collection. None of the mice unexpectedly died during the study.

Gene set enrichment analysis (GSEA) of adherent vs. non-adherent cells. Total RNA was collected from AXT cells cultured under adherent or non-adherent conditions for 21 h at 37°C. Comparison of these cells was performed using a microarray (Takara Bio, Inc.). GSEA (24,25) was performed using the fgsea package (26) in R software (version 4.3.1) (27), based on gene expression data obtained from microarray analysis. Genes were ranked according to the log₂-fold-change in expression between non-adherent cells and adherent cells (n=1 each) and this ranked list was used for GSEA. Gene sets were obtained from the Molecular Signatures Database (28). An enrichment plot for the 'QI_HYPOXIA' gene set (29) was generated using the plotEnrichment function in fgsea.

GSEA and visualization of significantly associated genes. Using total RNA collected from mice inoculated with Matrigel embedded Mock and HIF-KO cells, RNA-seq was performed by Rhelixa, Inc. Reads obtained from next generation sequencing were mapped to the mouse reference genome (GRCm38) using HISAT2 (version 2.1.0) (30). Transcript-level read counts were quantified using featureCounts (SCR_012919; version 1.6.3) (31). Transcripts with low expression were also included in the analyses. GSEA was performed using the fgsea package (26), with genes ranked by log₂-fold-change in expression between HIF-1 α knockout samples (n=3; KO1-1, KO1-2 and KO2) and Mock controls (n=1). Enrichment results with a false discovery rate (FDR) <0.2 were visualized using the plotGseaTable function in fgsea. To examine the expression patterns of genes identified as enriched by GSEA, a heatmap was generated using the pheatmap package (SCR_016418) (32), annotated with their corresponding gene sets. RNA-seq datasets in this paper are available on the Gene expression omnibus site (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE307480>). The accession number is GSE307480.

Immunohistochemistry. Tumors were collected, fixed with 4% paraformaldehyde at room temperature for 2 days and embedded in paraffin. Deparaffinized and hydrophilized tissue sections (5- μ m thickness) were put into citrate buffer (0.01 mol/l, pH 6.0) and heated in an oven at 90°C for 1 h for antigen retrieval. After a brief wash with PBS, tissue sections were put into 3% H₂O₂-methanol at room temperature for 8 min for inactivation of intrinsic peroxidase activity. After a brief wash with PBS, tissue sections were blocked with 3% bovine serum albumin (BSA; FUJIFILM Wako Pure Chemical Corporation)-PBS at room temperature for 1 h and subsequently incubated with the primary antibodies listed in Table SII diluted with 1.5% BSA-PBS at 4°C overnight. After washing with PBS 3 times, samples were treated with a horseradish peroxidase (HRP)-conjugated Histofine simple stain kit (1:1 dilution, listed in Table SII;

Nichirei Biosciences, Inc.) as the secondary antibody at room temperature for 1 h. After washing with PBS, staining was developed using the 3,3'-diaminobenzidine substrate (Impact DAB; Vector Laboratories, Inc). Then Mayer's hematoxylin solution (FUJIFILM Wako Pure Chemical Corporation) was used for nuclear staining at room temperature for 1 min. For H&E staining, deparaffinized and hydrophilized sections were stained with hematoxylin solution for 4 min at room temperature, washed with water and stained with 1% eosin Y solution (FUJIFILM Wako Pure Chemical Corporation) for 2 min at room temperature.

Hypoxic regions were evaluated using a rabbit IgG polyclonal anti-pimonidazole antibody (cat. no. PAb2627, NPI Inc.); mice were injected intraperitoneally with pimonidazole hydrochloride (60 mg/kg; NPI Inc.) 1 h before tumor collection. For quantitative analysis of blood vessels, CD31 immunostaining was observed under a BZ-X800 multifunctional microscope (Keyence Corporation). Microvessel density was quantified as the CD31-positive area normalized to the total solid tumor area using BZ-X800 Analyzer exe 1.1.2.4 software (Keyence Corporation).

Statistical analysis. Unless indicated otherwise, quantitative data are expressed as the mean $\pm$ SD relative to the control value and data were analyzed by one-way analysis of variance (ANOVA) with the Dunnett's post hoc test. For the angiogenesis analysis (Fig. 5D), the data did not meet the assumption of normality using the Shapiro-Wilk test, therefore, differences among groups were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Shapiro-Wilk test to assess normality was applied only for angiogenesis quantitation. All analyses were performed using GraphPad Prism 9 (Dotmatics). P<0.05 was considered to indicate a statistically significant difference (*P<0.05, **P<0.01, ***P<0.005 and NS, not significant). All assays were performed at least in triplicate. To evaluate the magnitude of differences in tumor weight between groups, effect sizes were calculated for each comparison between the control (Mock) and knockout groups (KO1-1, KO1-2 and KO2) using Hedges'g (33), a bias-corrected standardized mean difference. Effect sizes and their 95% confidence intervals (not shown) were estimated with the cohen.d function in the effsize R package (34), with missing values removed listwise. Group means and standard deviations were calculated from the available numeric observations.

Results

Osteosarcoma cells upregulate downstream targets of HIF-1 α under non-adherent conditions. The ability of adherent cells to grow under non-adherent anchorage-independent conditions is one of the hallmarks of malignancy (7,8). Our previous study developed mouse osteosarcoma AXT cells that proliferate both under adherent and non-adherent conditions (Fig. 1A) (35). Analysis of metabolite levels suggested that non-adherent conditions mimic the *in vivo* environment more closely when compared with adherent normal culture conditions (35). Therefore, therapeutic approaches that inhibit the mechanisms or molecules enabling non-adherent cell growth might exert an anti-tumor effects on osteosarcoma *in vivo*.

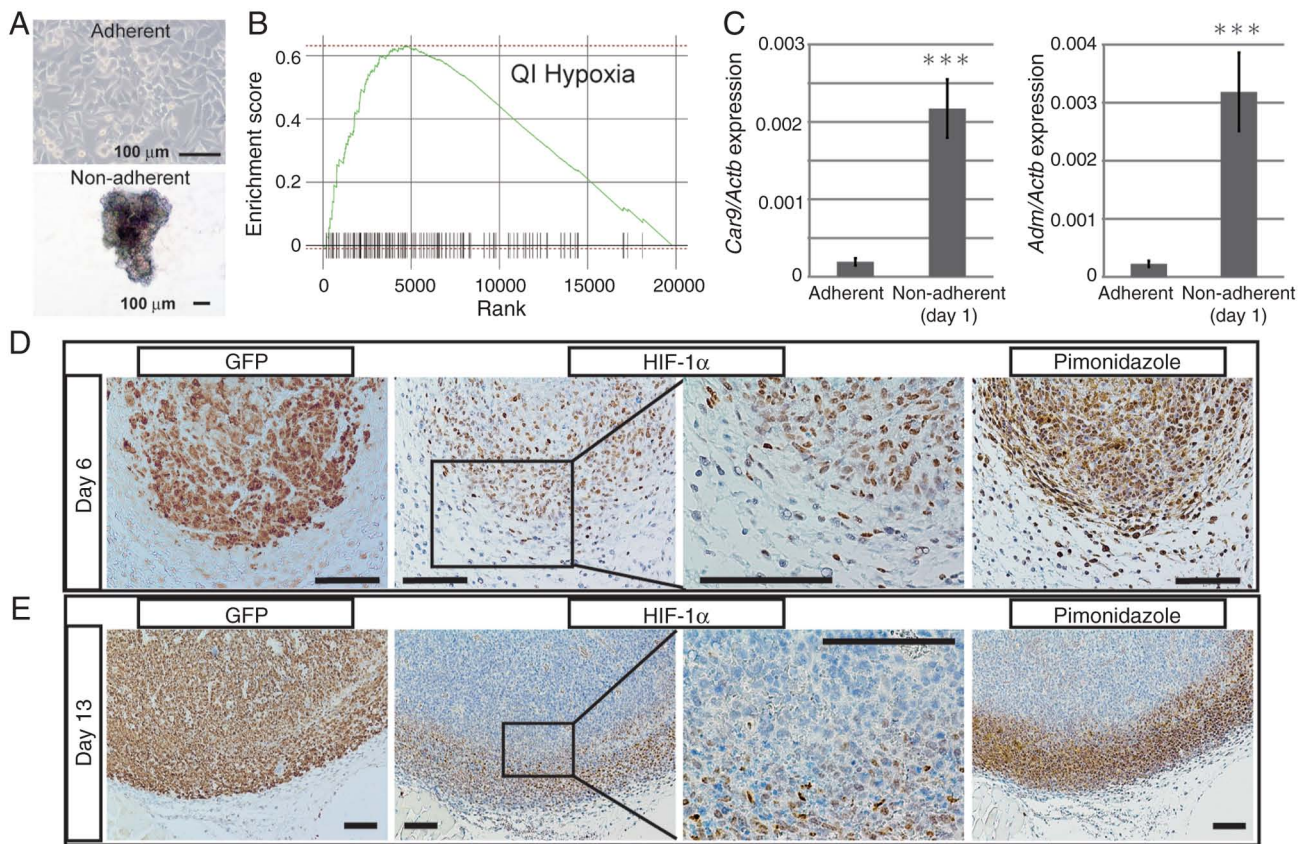


Figure 1. Expression of HIF-1α in osteosarcoma. (A) Bright-field microscopy of AXT cells cultured under adherent or non-adherent conditions. (B) The gene set 'QI_HYPOXIA' was enriched significantly among the upregulated genes in non-adherent cells. (C) Reverse transcription-quantitative PCR analysis of *Car9* and *Adm* mRNA in AXT under adherent or non-adherent conditions. Data are normalized to the corresponding levels of *Actb* mRNA, and are shown as the mean $\pm$ SD of triplicate values. Immunohistochemical staining of GFP, HIF-1α and reductively activated pimonidazole in serial sections of osteosarcoma derived from AXT cells in C57BL/6J mice (n=1 each) at (D) Day 6 and (E) Day 13 post-inoculation. Scale bar, 100 μm. HIF, hypoxia inducible factor. AXT, Accelerated bone and tumor formation. *** $P < 0.005$.

GSEA was used to analyze changes ($\log_2$ -fold) in expression of genes by adherent and non-adherent AXT cells. Only 'QI_HYPOXIA', which comprises genes upregulated under hypoxic conditions in prostate cancer cells (29), was enriched significantly in non-adherent cells (normalized enrichment score=2.05, FDR <0.001; Fig. 1B). These results suggest that hypoxia-related transcriptional programs may be more activated in non-adherent cells than in adherent cells. Expression of carbonic anhydrase 9 (*Car9*) and adrenomedullin (*Adm*), both transcriptional targets of HIF-1α, in AXT cells was higher under non-adherent conditions when compared with adherent conditions (Fig. 1C). Similarly, expression of HIF-1α transcriptional target genes *NDRG1* and *ADM* by human osteosarcoma cell lines Saos2 and U2OS was higher under non-adherent conditions compared with adherent conditions (Fig. S1A and B).

In vivo expression of HIF-1α in osteosarcoma was analyzed by examining serial sections of tumors derived from AXT cells. AXT cells express GFP, which allows direct detection of tumor areas (Fig. 1D and E). In small tumors on Day 6 post-inoculation, the nucleus of the majority of tumor cells was positive for HIF-1α. Hypoxic areas in tissues were visualized by administering pimonidazole, which is reductively activated in hypoxic cells and forms stable covalent adducts with thiol (sulphydryl) groups within amino acids (36). On Day 6

post-implantation, the majority of tumor cells were positive for reductively activated pimonidazole, which was detectable by a specific antibody, indicating that the entire tumor was hypoxic at Day 6 (Fig. 1D). At 13 days post-implantation, HIF-1α was highly expressed in peripheral tumor areas in which osteosarcoma cells proliferate and infiltrate the surrounding tissue. Importantly, areas with high expression of HIF-1α were hypoxic (Fig. 1E). These results suggest that hypoxic regions emerge during growth and progression of osteosarcoma *in vivo*, and that HIF-1α may play an important role.

Knocking out HIF-1α attenuates cell growth under hypoxic and non-adherent conditions. AXT cells, in which HIF-1α was knocked out using CRISPR-Cas9 with two different target sequences, were used to evaluate the role of HIF-1α in growth of osteosarcoma. Two KO clones (KO1-1 and KO1-2) and one clone (KO2) were obtained using target sequences #1 and #2, respectively (Fig. 2A). Each cell type had a different morphology. KO1-1 cells were similar to control (Mock) cells, whereas KO1-2 cells were round and KO2 cells were larger when compared with the Mock, KO1-1 and KO1-2 cells. In Mock cells, HIF-1α protein was detectable under normoxic conditions, and addition of the HIF-PH inhibitor roxadustat (37) led to accumulation of HIF-1α. By contrast, no HIF-1α protein was detected in KO cells (Fig. 2B). Expression

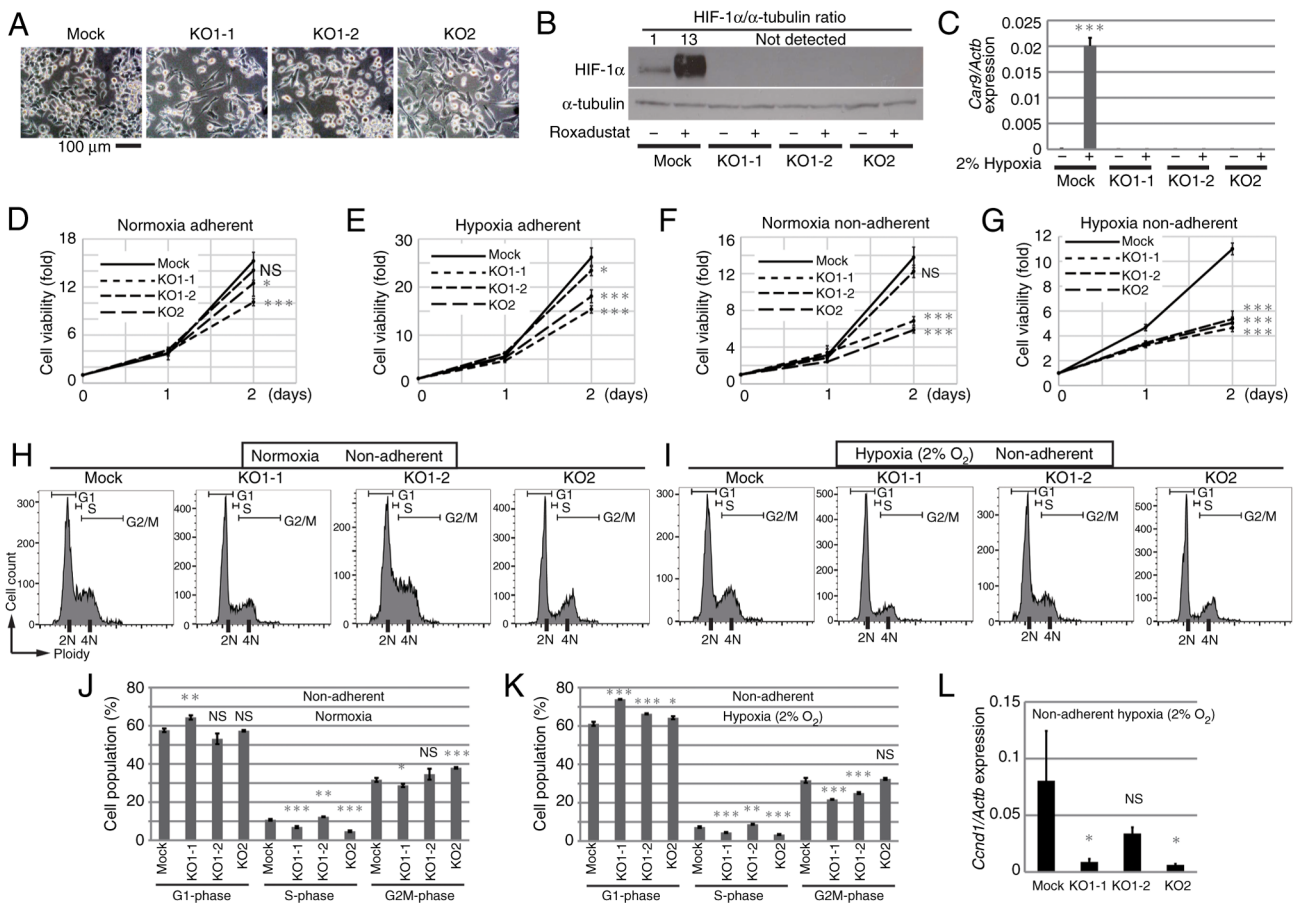


Figure 2. Attenuation of cell growth in HIF-1 α -knockout cells *in vitro*. (A) Morphology of control (Mock) and HIF-1 α -knockout AXT (HIF-KO, KO1-1, 1-2 and KO2) cells. (B) Western blot analysis of HIF-1 α expression in Mock and HIF-KO cells in the presence/absence of 25 μ M roxadustat for 27 h. The relative (fold) values of HIF-1 α against the corresponding control value normalized to the intensities α -tubulin bands are shown. HIF-1 α bands of HIF-KO cells were not detected. (C) Reverse transcription-quantitative PCR analysis of *Car9* in Mock and HIF-KO cells under normoxic or hypoxic (2% O₂) conditions for 20 h. Data are normalized to the corresponding levels of *Actb* mRNA, and are shown as the mean $\pm$ SD of triplicate values. Growth of Mock and HIF-KO cells under (D) normoxia and adherent conditions, (E) hypoxia and adherent conditions, (F) normoxia and non-adherent conditions and (G) hypoxia and non-adherent conditions. The ratio relative to the value for Day 0 was calculated for each data point (n=4; D-G). Flow cytometry analysis of DNA content in Mock and HIF-KO cells under (H) normoxia and non-adherent conditions and (I) hypoxia and non-adherent conditions for 31 h. (J) The population of each cell fraction in (H) are shown (n=3). (K) The population of each cell fraction in (I) are shown (n=3). (L) Reverse transcription-quantitative PCR analysis of *Ccnd1* in Mock and HIF-KO cells under non-adherent or hypoxic (2% O₂) conditions for 21 h (n=3). HIF, hypoxia inducible factor; KO, knockout. *P<0.05, **P<0.01, ***P<0.005 and NS, not significant.

of *Car9*, a transcriptional target of HIF-1 α , was upregulated significantly in Mock cells under hypoxic (2% O₂) conditions compared with normoxic conditions (Fig. 2C). By contrast, no increase in *Car9* was observed in HIF-KO cells under hypoxic conditions. The effects of HIF-1 α depletion on cell proliferation were evaluated under adherent and non-adherent culture conditions, as well as under normoxic and hypoxic conditions. Under adherent and normoxic conditions, KO cells tended to proliferate slightly more slowly when compared with Mock cells (Fig. 2D). Under hypoxic conditions, the difference was more pronounced, although the growth rate of KO cells still remained high (Fig. 2E). Notably, the proliferation rate of KO1-2 was almost equivalent with that of Mock cells. Under non-adherent conditions, proliferation of KO1-1 and KO2 cells was suppressed significantly, while proliferation of KO1-2 cells was similar to that of Mock cells (Fig. 2F). There were differences between the tumor cell clones, suggesting that the intracellular mechanism in KO1-2 cells allowed proliferation even in the absence of HIF-1 α . Under non-adherent hypoxic conditions, proliferation of all knockout cells was lower

when compared with that of Mock cells (Fig. 2G). Cell cycle analysis showed that under normoxic and adherent conditions, there was no reduction in the number of S-phase KO cells compared with Mock cells, and no significant induction of apoptosis (Fig. S2A-D). As non-adherent cells were fragile and the majority of cells were physically damaged during sample preparation, only live cells were gated and analyzed (Figs. S2E, 2H and I). Consistent with cell growth under non-adherent normoxic conditions, the S-phase population of KO1-1 and KO2 cells, but not KO1-2 cells, was lower when compared with that of Mock cells (Fig. 2H and J). The S-phase population of Mock cells was lower under non-adherent hypoxic conditions when compared with under normoxic conditions (Fig. 2J and K). The S-phase population of KO1-1 and KO2 cells was smaller when compared with that of Mock cells, whereas the S-phase population of KO1-2 was the same as that of Mock cells, while the number of cells in G2/M was lower when compared with that of Mock cells (Fig. 2I and K). Consistent with these results, expression of mRNA encoding Cyclin D1 in KO1-1 and KO2 under non-adherent hypoxic culture was

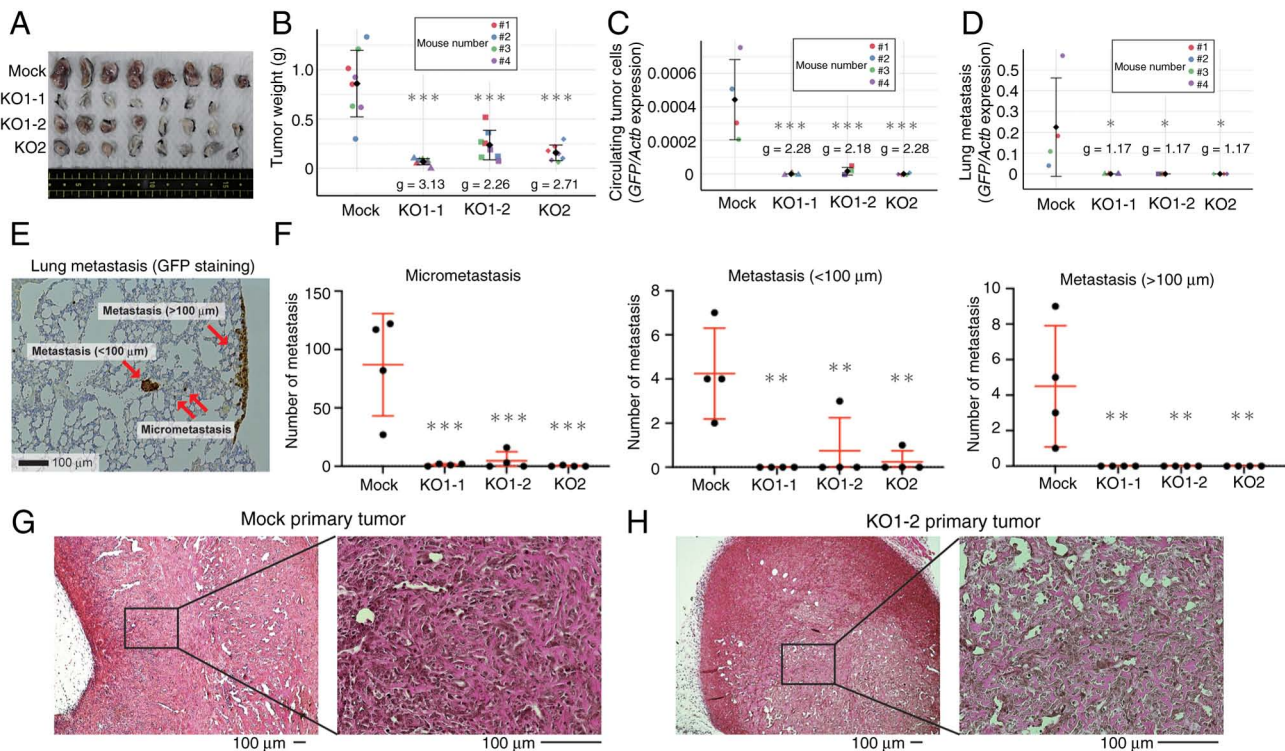


Figure 3. Depletion of HIF-1 α decreases tumorigenicity of osteosarcoma cells. (A) A macroscopic image of primary tumors excised 21 days after subcutaneous and bilateral inoculation of 1×10^6 cells per site into C57BL/6J mice (n=4 each). (B) Jittered scatter plots show tumor weight distributions for individual mice. Colors correspond to animal ID and point shapes indicate group identity. Black points and vertical bars represent the group mean and standard deviation, respectively. Effect sizes (Hedges' g) are annotated for each knockout group. Reverse transcription-quantitative PCR analysis of *GFP* mRNA in (C) whole blood and (D) the left lung from all mice in the group inoculated with the indicated cells. Effect sizes (Hedges' g) are annotated for each knockout group. (E) Representative images of lung metastatic lesions stained with GFP. The arrows indicate lung metastases classified according to whether the major axis is $>100 \mu\text{m}$ or whether they represent tiny metastases consisting of a few cells. (F) The number of metastases present throughout a randomly sliced section was counted. Data represent the mean value from the right lungs of all four mice. (G, H) H&E staining of representative primary tumors from mice inoculated with the (G) Mock or (H) KO cells. HIF, hypoxia inducible factor; KO, knockout. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$.

significantly lower when compared with that in Mock cells, while that in KO1-2 tended to be lower when compared with that in Mock cells, although the difference was not significant (Fig. 2L).

These results suggest that HIF-1 α supports proliferation under non-adherent hypoxic conditions *in vitro*; however, the mechanism used by KO1-2 cells might maintain high proliferation even in the absence of HIF-1 α .

Depleting HIF-1 α decreases tumorigenic activity *in vivo*. AXT cells form osteoid-rich tumors, a characteristic essential for the definitive diagnosis of osteosarcoma, as well as lung metastasis in C57BL/6 mice (4-6). In the present study, HIF-KO cells were transplanted into mice to evaluate tumorigenicity. The weight of the primary KO cell tumors 21 days after subcutaneous implantation of 1×10^6 cells was significantly lower when compared with that of Mock tumors (Fig. 3A and B). No tumors developed at the transplantation site in one mouse inoculated with KO1-1, suggesting that the suppression of tumorigenic activity *in vivo* is stronger when compared with that of cell growth *in vitro*.

After forming primary tumors, AXT cells spontaneously develop hematogenous metastatic lesions. Because AXT cells express GFP, the amount of lung metastases and CTCs in the blood can be quantified by measuring the expression level of GFP (4,5,35). Such measurements revealed that the number

of lung metastases and CTCs was reduced significantly after KO of HIF-1 α (Fig. 3C and D). Further evaluation of lung metastases by immunohistochemical staining for GFP also showed a significant reduction of lung metastasis; in particular, there were no lesions with a diameter $>100 \mu\text{m}$ in HIF-KO cell-inoculated mice (Fig. 3E and F). Importantly, tumors derived from HIF-KO cells also contained osteoids, and there were no histological changes suggestive of osteosarcoma after knockout of HIF-1 α (Figs. 3G and H, S3A and B).

Altering inoculation conditions does not rescue the reduced tumorigenicity induced by HIF-1 α KO. Accumulating evidence indicates that hypoxic environments affect tumor immunity (38,39), and that suppression of HIF-1 α in tumor cells activates tumor immunity (40,41). Therefore, tumor cells were transplanted into SCID mice to examine whether tumor immunity is involved in the regression of HIF-1 α KO tumors. Similar to transplantation into C57BL/6 mice, transplantation into SCID mice did not restore tumorigenic and metastatic activity completely (Fig. 4A-D). Thus, it seems that tumor immunity mediated by T cells and B cells is not the main suppressor of tumor formation by HIF-KO cells. Next, whether further improvements in the transplant environment increased tumor cell engraftment was tested. For this purpose, Matrigel was used to provide a basement membrane to prepare the microenvironment from the time of transplantation (42).

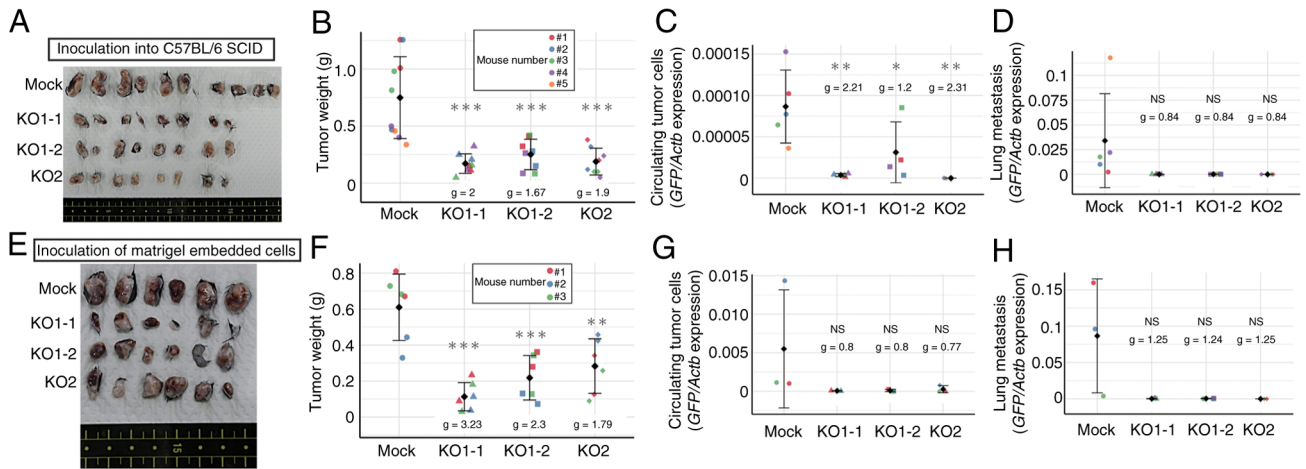


Figure 4. Tumorigenicity of HIF-KO cells under different transplantation conditions. (A) Macroscopic image of excised primary tumors 20 days after subcutaneous and bilateral inoculation of 1×10^6 cells per site into C57BL/6 SCID mice (Mock, $n=5$; HIF-KO, $n=4$). (B) Weight of primary tumors. Reverse transcription-quantitative PCR analysis of *GFP* mRNA in (C) whole blood specimens and in (D) the left lungs from all mice in the group. (E) Macroscopic image of primary tumors excised 21 days after subcutaneous and bilateral inoculation of Matrigel embedded cells (1×10^6 per site) into C57BL/6J mice ($n=3$ each). (F) Weight of primary tumors shown. Reverse transcription-quantitative PCR analysis of *GFP* mRNA in (G) whole blood specimens and in (H) the left lungs from all mice in the group. HIF, hypoxia inducible factor; KO, knockout. * $P<0.05$, ** $P<0.01$, *** $P<0.005$ and NS, not significant.

Studies show that co-injection with Matrigel increases tumor formation by cancer cell lines and enhances engraftment of primary human epithelial cancer cells in immunocompromised mice (43,44); however, in the present study, KO cells failed to form tumors and metastases as efficiently as Mock cells (Fig. 4E-H).

The small group sizes of *in vivo* experiments is a limitation in the present study. However, the knockout groups demonstrated reduced tumor growth compared with the Mock controls. The effect size estimates consistently indicate reduced tumor growth in all knockout groups relative to the Mock control, suggesting that HIF-1 α plays an important role in tumorigenesis and progression of osteosarcoma *in vivo*.

Osteosarcoma develops a rich vasculature even in the absence of HIF-1 α . To clarify the functions of HIF-1 α , its expression in tumors immunohistochemically was evaluated over time. At 2 days after subcutaneous implantation of Mock-derived tumors, high nuclear expression of HIF-1 α was observed throughout areas of dense viable cells (except the interior, which harbored GFP-negative dead cells), whereas KO tumors did not express HIF-1 α (Fig. 5A). At 21 days post-implantation, the interior of the tumor was filled with viable cells, with notable bone formation and HIF-1 α was highly expressed in the peripheral areas in which cells were proliferating rapidly (Fig. 5B). Again, no expression of HIF-1 α was observed in KO cell tumors. Analysis with pimonidazole suggested that areas with high HIF-1 α expression were hypoxic (Fig. 1D and E). The pattern of hypoxic areas appearing mainly around the tumor periphery was the same in Mock- and HIF-KO-derived tumors (Fig. S4A), suggesting that oxygen supply inside the tumor was not disrupted by HIF-1 α loss. Therefore, tumor angiogenesis was evaluated. Staining for CD31, a marker of vascular endothelial cells, suggested that the inside of the tumor was vascularized (Figs. 5C and S4A). Quantification of CD31 positive areas indicated that the KO1-2 cell-derived tumors had abundant blood vessels at a level comparable to that of the mock-derived tumors, while tumors derived from KO2

are less vascularized when compared with those from Mock cells (Figs. 5D and S4B). Consistent with the emerging pattern of hypoxic areas, vascularization tended to be more prevalent within the tumor when compared with its periphery. These findings suggest that global tumor oxygen supply is not severely disrupted by HIF-1 α deletion. Expression of vascular endothelial growth factor A (VEGF-A), a transcriptional target of HIF-1 α , increased in Mock cells under hypoxic conditions *in vitro*, but not in KO cells (Fig. 5E); however, unlike *Car9* (Fig. 2C), expression of *Vegfa* was not negligible in KO cells (Fig. 5E). To analyze gene expression in tumor cells *in vivo*, each cell type (embedded in Matrigel) was implanted subcutaneously into mice, harvested on the next day and total RNA was extracted (Fig. 5F). Expression of *Vegfa* in Mock cells was ~2-fold higher when compared with in KO cells, but robust expression was still detected in KO cells (as observed *in vitro*; Fig. 5G). Regarding an alternative driver of VEGF-A regulation, HIF-2 α was expressed at significantly lower levels when compared with HIF-1 α in Mock and HIF-KO cells as assessed by RT-qPCR analysis (Fig. S5A). Furthermore, under hypoxic conditions, the addition of HIF-2 α inhibitors, PT2385 or TC-S7009, had little effect on *Vegfa* expression (Fig. S5B). STAT3 activation differed between HIF-KO clones, making it unlikely to be a common molecular mechanism for increasing *Vegfa* in HIF-KO cells (Fig. S5C). Notably, treatment of mTOR inhibitors, temsirolimus or everolimus, under hypoxia reduced the expression level of *Vegfa* in all HIF-KO cells, although temsirolimus did not significantly affect that in KO1-2 cells (Fig. S5D). Therefore, mTOR pathway is partially responsible for maintaining the basal *Vegfa* expression in the absence of HIF-1 α . Importantly, it was suggested that non-tumor cells may be involved in angiogenesis *in vivo*. Immunostaining of α -smooth muscle actin (α SMA), a useful marker for a part of fibroblasts including cancer-associated fibroblasts (45), revealed α SMA-positive cells comprising various cells including tumor cells, fibroblastic cells and CD31-positive cells, likely representing mature vascular endothelial cells (Fig. S5E). In addition, F4/80-positive macrophages were abundantly accumulated at the

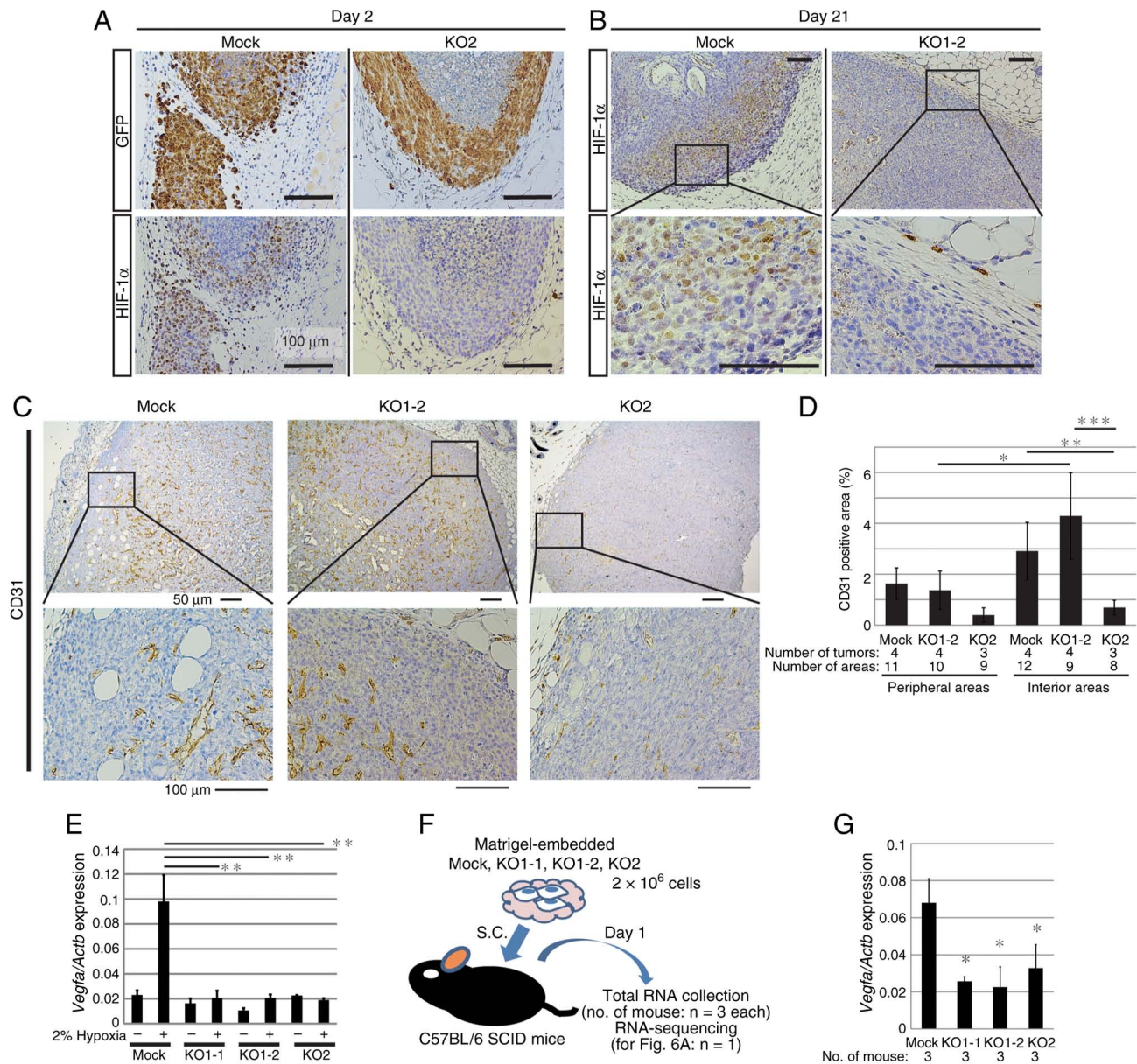


Figure 5. HIF-1 α is highly expressed at the hypoxic invasion front. Immunohistochemical staining of GFP and HIF-1 α in serial sections of osteosarcoma derived from Mock and HIF-KO cells at day (A) 2 and (B) 21. Scale bar, 100 μ m. (C) Immunohistochemical staining of CD31 in osteosarcoma derived from Mock and HIF-KO cells. (D) Vascularization was quantified based on immunohistochemistry as CD31-positive area in peripheral (40x) and interior (20x) solid tumor regions. One tumor derived from KO2 was too small to evaluate. Statistical significance was evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison test. (E) Reverse transcription-quantitative PCR analysis of *Vegfa* in Mock and HIF-KO cells under non-adherent normoxic or hypoxic (2% O₂) conditions for 21 h (n=3 each). (F) Scheme for RNA extraction. Cells were harvested 1 day after subcutaneous implantation of each clone (2x10⁶ cells per site) into 3 locations per mouse. (G) Reverse transcription-quantitative PCR analysis of *Vegfa* mRNA in Mock and HIF-KO cells collected as in (F) (n=3 each). HIF-KO, hypoxia inducible factor knockout. *P<0.05, **P<0.01 and ***P<0.005.

tumor periphery and within the tumor (Fig. S5F). These stromal cells may support the angiogenesis of HIF-KO-derived tumors *in vivo*.

These results indicate that HIF-1 α -independent expression of VEGF-A in osteosarcoma cells may be sufficient to form vasculature to support tumor growth, even though the HIF-KO tumors are smaller than those formed by Mock cells.

HIF-KO cells show differing dependence on glycolysis for growth. To explore changes in gene expression upon depletion of HIF-1 α *in vivo*, RNA-seq analysis was carried out. GSEA revealed that genes included in the QI_HYPOXIA gene

set were enriched among those downregulated in HIF-KO samples relative to Mock (Fig. S6) which is consistent with the GSEA findings from the comparison between non-adherent and adherent cells (Fig. 1B). In addition to hypoxia-related genes, several gene sets associated with muscle function or anion transport and carbohydrate catabolism were downregulated in HIF-1 α KO samples compared with the Mock control (Figs. 6A and S6). By contrast, few gene sets contained genes with high upregulation ratios in KO cells, and no gene set with significantly increased expression was extracted. In addition, there were no common genes whose expression levels in KO cells were >2 fold when compared with those in Mock

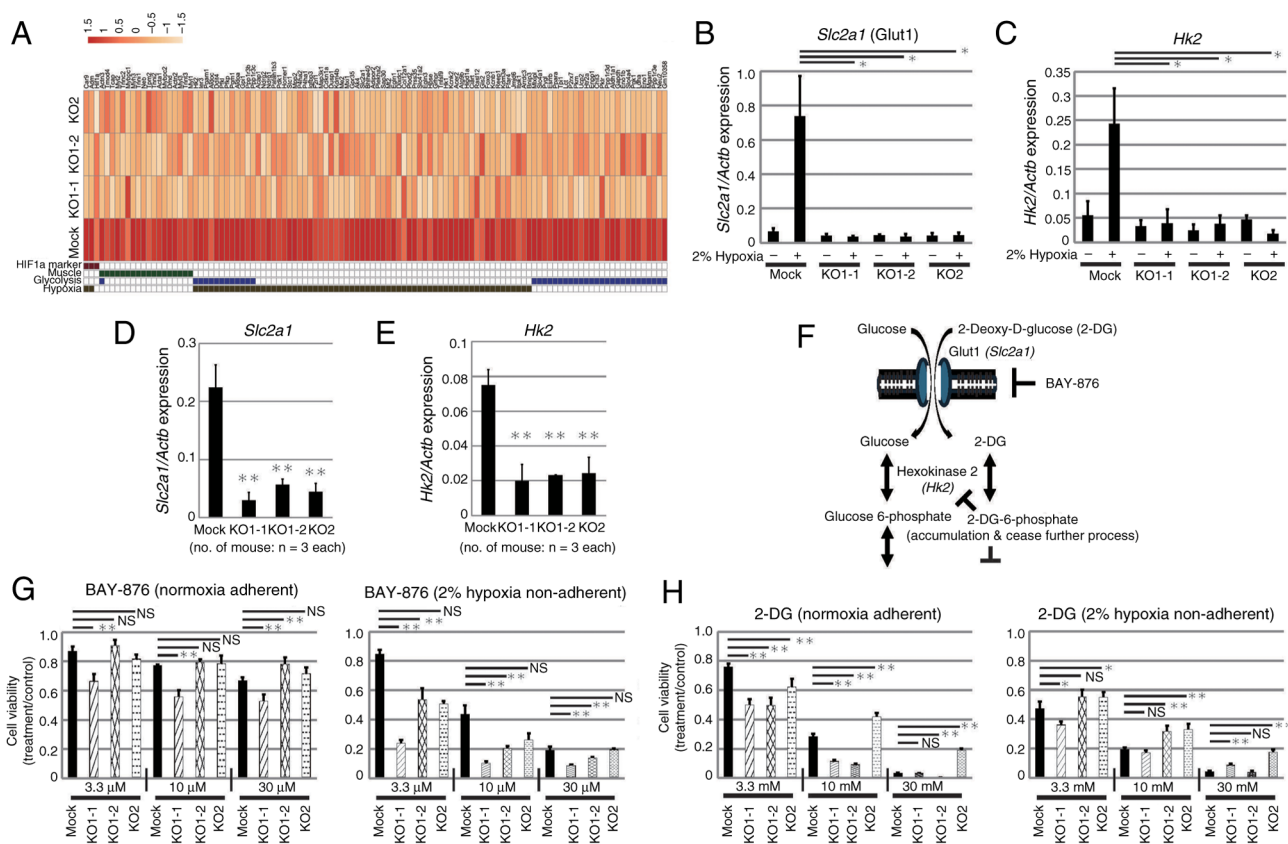


Figure 6. Changes in glycolysis-related molecules by depletion of HIF-1 α . (A) Gene set enrichment heatmap of genes in the QI_HYPOXIA, REACTOME_STRIATED_MUSCLE_CONTRACTION and GOBP_CARBOHYDRATE_CATABOLIC_PROCESS sets shows reduced expression of these genes in HIF-KO samples when compared with that in the Mock control (n=1 each). Reverse transcription-quantitative PCR analysis of (B) *Slc2a1* and (C) *Hk2* mRNA in Mock and HIF-KO cells under adherent in normoxic or hypoxic (2% O₂) conditions for 20 h (n=3). Reverse transcription-quantitative PCR analysis of (D) *Slc2a1* and (E) *Hk2* mRNA collected as in Fig. 5F (n=3). (F) Scheme showing how BAY-876 and 2-Deoxy-D-glucose (2-DG) affect Glut1 and Hexokinase function. Viability of Mock and HIF-KO cells treated for 2 days with the indicated concentrations of (G) BAY-876 or (H) 2-DG. Assays were performed in quadruplicate, and data are expressed as mean $\pm$ SD relative (-fold) to the corresponding control value for cells incubated in the absence of these agents. HIF-KO, hypoxia inducible factor knockout. *P<0.05 and **P<0.01.

cells. Although depleting HIF-1 α suppressed tumor formation significantly *in vivo*, it failed to eliminate tumors completely (Figs. 3 and 4). Thus, treatments in addition to HIF-1 α depletion would be required to overcome osteosarcoma, and elucidation of the survival pathways on which KO cells depend in the absence of HIF-1 α was attempted.

Expression of glucose transporter 1 (Glut1 coded by *Slc2a1*), which is involved in glucose uptake and hexokinase 2 (*Hk2*), an enzyme involved in the early stage of glycolysis, increased significantly in Mock cells under hypoxic culture, but not in KO cells (Fig. 6B and C). In addition, *in vivo* expression levels of these genes in cells collected as described in Fig. 5F, was significantly increased in Mock cells when compared with that in KO cells (Fig. 6D and E). Therefore, the functions of these molecules were inhibited and the effects on proliferation under normoxia and hypoxia were analyzed (Fig. 6F). The sensitivity of all cells to BAY-876, a specific inhibitor of Glut1, was higher under hypoxic conditions, and proliferation was suppressed in a dose-dependent manner, suggesting that hypoxic cells are more dependent on glycolysis when compared with normoxic cells (Fig. 6G). KO cells tended to be more sensitive when compared with Mock cells to low concentrations of BAY-876. KO1-1 cells were the most sensitive, suggesting a dependency on glycolysis. 2-Deoxy-D-glucose (2-DG) is taken up into

cells (as is glucose) and phosphorylated by hexokinase 2; however, the subsequent reactions do not proceed, resulting in accumulation within the cell and inhibition of glycolysis (46). Dose-dependent inhibition of proliferation was observed for all cells under both normoxic and hypoxic conditions (Fig. 6H). Notably, KO1-1 cells tended to be more sensitive to 2-DG compared with Mock, KO1-2 and KO2 cells while KO2 cells were less sensitive (as in the case of BAY-876). These results indicate that Glut1 and HK2 function during proliferation of HIF-KO cells, and that sensitivity to inhibition varies among clones, suggesting that the clones have differing levels of dependence on glycolysis for survival.

The pathways upon which osteosarcoma cells rely to survive in the absence of HIF-1 α are heterogeneous. To clarify the pathways upon which HIF-1 α KO cells depend for survival, the effects of various compounds on proliferation was analyzed. The sensitivity of KO cells to mitochondrial electron transport inhibitors antimycin A, rotenone and oligomycin was higher under normoxic conditions when compared with under hypoxic conditions (Fig. 7A), indicating that the cells were more dependent on mitochondria for survival under normoxia. Growth inhibition induced by these inhibitors was weaker in Mock cells and KO1-2 cells when compared with that in

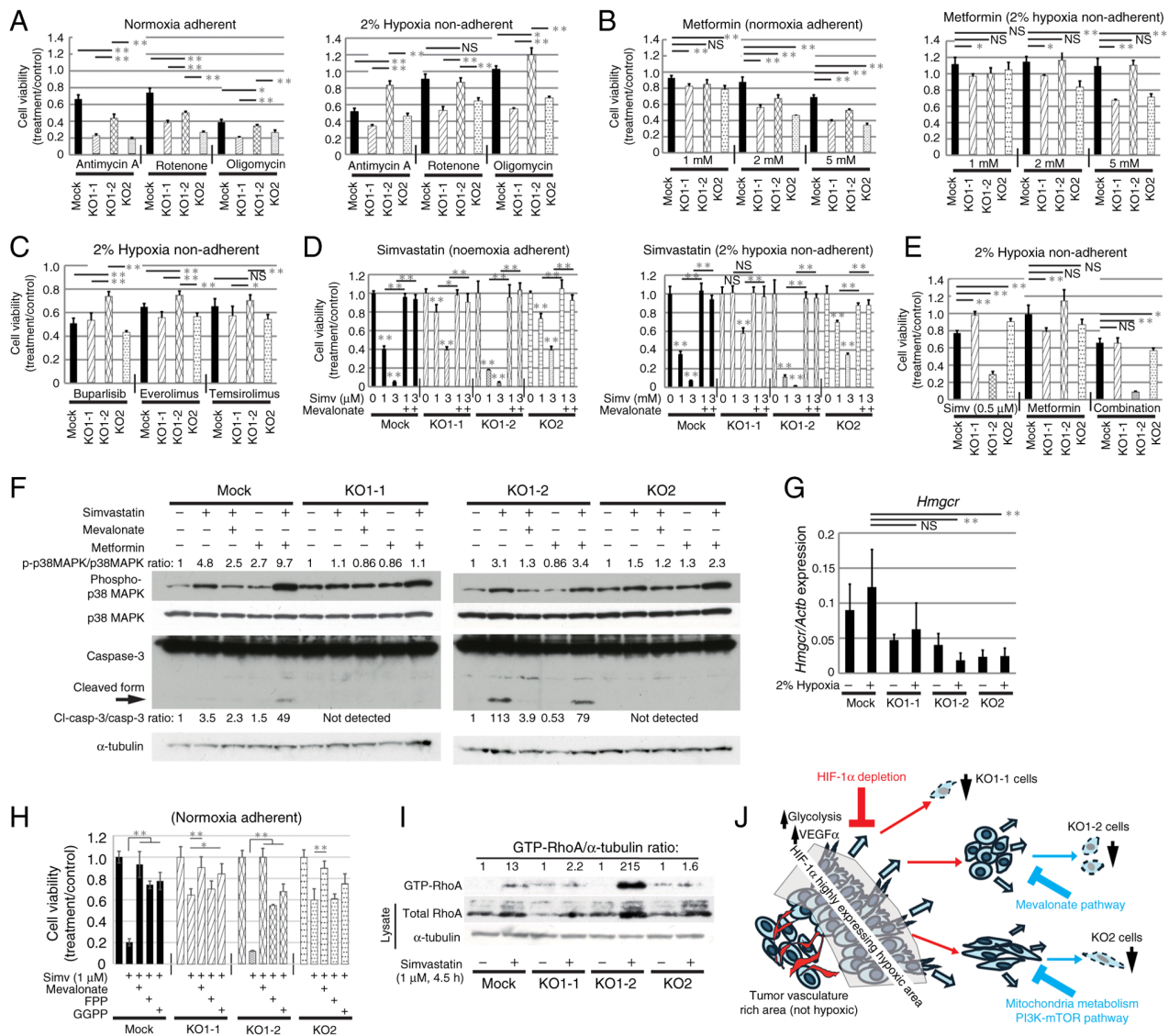


Figure 7. Different survival pathways on which osteosarcoma cells rely in the absence of HIF-1 α . Viability of Mock and HIF-KO cells treated for 2 days with 1 μ M of (A) the indicated reagents or (B) metformin. Assays were performed in quadruplicate, and data are expressed as the mean $\pm$ SD relative (-fold) to the corresponding control value for cells incubated in the absence of these reagents. (C) Viability of Mock and HIF-KO cells treated for 2 days with 1 μ M of the indicated reagents. Assays were performed in quadruplicate. (D) Viability of Mock and HIF-KO cells treated for 2 days with simvastatin. Cells were also co-treated with mevalonate (200 μ M) and simvastatin. Assays were performed in quadruplicate. (E) Viability of Mock and HIF-KO cells treated for 2 days with 0.5 μ M simvastatin or 2 mM metformin. Cells were also co-treated with metformin plus simvastatin. Assays were performed in quadruplicate. (F) Western blot analysis to detect expression of the indicated molecules in Mock and HIF-KO cells cultured for 17 h in the presence/absence of 0.5 μ M simvastatin, 200 μ M mevalonate and 2 mM metformin under adherent normoxic conditions. Arrows indicate the cleaved active form of Caspase 3. The relative (fold) values of p-p38 or cleaved-Caspase3 against the corresponding control value normalized to the intensities of p38 or Caspase3 bands are shown. (G) Reverse transcription-quantitative PCR analysis of *Hmgcr* mRNA levels in Mock and HIF-KO cells cultured for 20 h under adherent normoxic or hypoxic conditions (n=3). (H) Viability of Mock and HIF-KO cells treated for 2 days with 1 μ M simvastatin alone or co-treated with 200 μ M mevalonate, 50 μ M FPP or 50 μ M GGPP under adherent normoxic conditions. Assays were performed in quadruplicate. (I) Western blot analysis of GTP-RhoA and RhoA in Mock and HIF-KO cells treated with 1 μ M simvastatin for 4.5 h. GTP-RhoA was isolated with beads conjugated with a GST fusion protein containing the Rho binding domain of Rhotekin that specifically binds to GTP-RhoA. A part of the same lysate was used for evaluation of total RhoA. The relative (fold) values of GTP-RhoA against the corresponding control value normalized to the intensities of α -Tubulin bands are shown. (J) Proposed model for the heterogeneity of pathways relied on by osteosarcoma cells after HIF-1 α depletion. HIF-KO, hypoxia inducible factor knockout. *P<0.05 and **P<0.01.

KO1-1 and KO2 cells. A similar trend was observed when metformin was added. Under hypoxic conditions, treatment with up to 5 mM metformin did not inhibit growth of Mock or KO1-2 cells, but did inhibit that of KO1-1 and KO2 cells (Fig. 7B). These findings suggest that Mock and KO1-2 cells are less dependent on the mitochondrial electron transport chain for survival under hypoxia when compared with KO1-1 and KO2 cells. KO1-2 cells were less sensitive to the PI3K inhibitor buparlisib and the mTOR inhibitors everolimus

and temsirolimus, when compared with Mock, KO1-1 and KO2 cells (Fig. 7C). In addition, the sensitivity of KO1-2 to shikonin, which inhibits Pkm2 (47), was lower when compared with that of Mock, KO1-1 and KO2 cells (Fig. S7A), suggesting that survival of KO1-2 cells depends on a different mechanism.

Inhibition of the mevalonate synthesis pathway markedly and dose-dependently suppressed the proliferation of KO1-2 cells under both normoxic and hypoxic conditions (Figs. 7D and S7B). Growth inhibition by simvastatin or

atorvastatin was prevented by addition of mevalonate, indicating that this inhibition is specific to the mevalonate synthesis pathway. Treatment with statins suppressed growth of all cells, but the sensitivity of KO1-1 and KO2 was relatively low. Our previous study demonstrated that combined administration of a statin and metformin inhibited growth and induced apoptosis of osteosarcoma cells (6). Therefore, the present study analyzed the sensitivity of each cell clone to co-treatment with simvastatin and metformin under hypoxic conditions (Fig. 7E). Co-treatment of Mock and KO1-2 cells, whose proliferation was not suppressed by metformin alone, with simvastatin decreased proliferation. By contrast, KO1-1 and KO2 cells, which were less sensitive to simvastatin than Mock and KO1-2 cells, showed synergistic growth suppression when co-treated with metformin. Similar synergistic inhibition was observed upon combined treatment with atorvastatin and metformin (Fig. S7C). Our previous study suggests that simvastatin induces apoptosis in osteosarcoma cells via activation of p38MAPK and AMPK (6). The present study found that simvastatin increased phosphorylation of p38MAPK in Mock and KO1-2 cells, which was abolished by addition of mevalonate, indicating that this phosphorylation is mevalonate-dependent (Fig. 7F). Activation of AMPK became more pronounced with higher concentrations and longer treatment of simvastatin, and the intensity of activation was stronger in KO1-2 and Mock cells (Fig. S7D and E). In KO1-2 cells treated with 0.5 μ M of simvastatin alone for 17 h, expression of cleaved Caspase 3 was detected (Fig. 7F), suggesting induction of apoptosis. Co-treatment with simvastatin and metformin increased phosphorylation of p38MAPK in all cells. Notably, cleaved Caspase 3 was detected in Mock and KO1-2 cells after treatment with simvastatin and metformin, which is consistent with the high sensitivity of both cells to simvastatin (Fig. 7E and F).

Expression levels of HMG-CoA reductase (*Hmgcr*) and synthase (*Hmgcs*) enzymes, which are key components to the mevalonate synthesis pathway, did not vary significantly between cells under normoxic and hypoxic conditions. The protein level of HMGCR did not change with simvastatin treatment (Figs. 7G and S7F and G). Furthermore, the present study found no significant differences in expression between cells after transplantation (Fig. S7H and I; samples prepared as in Fig. 5F). Sensitivity to statins may change with forced expression of HMGCR. The present study attempted to establish HMGCR-highly expressing KO1-2 cells using lentivirus but this could not be achieved (data not shown). Subsequently, the present study analyzed the other molecular changes in each cell type following the treatment of simvastatin. The amount of cholesterol in the cells tended to be slightly lower in Mock and KO1-2 cells and decreased similarly in all cells with the addition of simvastatin (Fig. S7J). Growth inhibition by simvastatin was significantly prevented by co-treatment of FPP or GGPP, although the rescue was not as complete as with mevalonate (Fig. 7H). FPP and GGPP are required for post-translational prenylation of small GTPases of the Ras and Rho families (48,49). The highly sensitive KO1-2 cells showed markedly increased intracellular accumulation of GTP-RhoA when compared with the Mock, KO1-1 and KO2 cells with 4.5 h of simvastatin treatment (Fig. 7I). This finding was consistent with the changes observed in the parent AXT cell

line when simvastatin caused apoptosis (6). Thus, abnormalities in signals involved in the prenylation pathway such as differences in intracellular GTP-RhoA accumulation, might be more strongly associated with simvastatin sensitivity than differences in cholesterol synthesis. To clarify the mechanisms underlying the emergence of cells with different sensitivity to simvastatin, the parental AXT cells were subjected to limiting dilution, single cell cloning was performed and the sensitivity to simvastatin was evaluated (Fig. S7K). The results showed that the sensitivity to simvastatin differed between clones, indicating that heterogeneity pre-existed in the parental AXT cells.

Collectively, these data suggest that HIF-1 α -independent survival pathways differ among KO clones and that the mevalonate synthesis pathway may support cell growth under hypoxic conditions in the absence of HIF-1 α (Fig. 7J).

Discussion

The findings of the present study suggest that HIF-1 α plays a key role in rapid progression of osteosarcoma *in vivo*. The effect of HIF-1 α depletion was more pronounced *in vivo* than *in vitro* culture, suggesting that the *in vivo* environment is more hostile to cancer cells in terms of oxygen and nutritional conditions when compared with *in vitro*, and that the function of HIF-1 α is important *in vivo*. Reduction of tumorigenicity persisted even after changing the transplantation conditions. When transplanted into immune-competent mice, tumorigenesis of KO1-1 cells was particularly poor, with one mouse showing no tumor formation after inoculation. By contrast, when inoculated into SCID mice, KO1-1 cells formed tumors readily, showing a tendency toward increased tumorigenicity. Reduced growth *in vivo* after HIF-1 α depletion may be advantageous in that it allows anti-tumor immune responses to eliminate tumor cells; thus, HIF-1 α may be a potential therapeutic target for osteosarcoma. However, eradication of tumors that form even in the absence of HIF-1 α is necessary for treatment to be completely successful. Notably, even after HIF-1 α depletion, rich vascularization can occur and the non-negligible basal expression of *Vegfa* was observed in the present study. The findings suggest that HIF-1-independent mechanisms, such as activation of cell intrinsic signals in cancer cells (for example, activation of the mTOR pathway) and the tumor microenvironment, are involved in the regulation of *Vegfa* expression and vascular formation. Therefore, although HIF-targeted therapy is expected to suppress tumor vasculature formation, this strategy may be ineffective in some cell types.

Previously, drugs that target HIF specifically are being used clinically to treat cancer types that depend on HIF for survival (50). In 2023, the HIF-2 α -targeting drug belzutifan was approved by the US Food and Drug Administration for use in patients with advanced renal cell carcinoma (51). Notably, a mutation that interferes with drug binding and precludes HIF-2 complex dissociation has been identified as the cause of resistance to the HIF-2 inhibitor PT2385 (52). Moreover, even when HIF inhibitors suppress the function of HIF, tumor progression can still occur, as shown in the present study in the osteosarcoma model. Analysis of HIF-1 α knockout clones revealed that individual cells have different properties. The results of compound screening revealed that each of the three clones depended on

a different pathway for survival. The difficulty is that in the absence of HIF-1 α , the pathway on which survival depends cannot be easily identified. Furthermore, in clinical settings, these clones may be mixed within osteosarcoma tumors.

The reasons why such heterogeneity exists, and its emerging mechanisms are important issues. To adapt to the environment and survive various stimuli, malignant tumor cells plastically change their phenotype through the acquisition of genetic mutations and epigenetic modifications (for example, drug-tolerant persister cells) (53). Single-cell RNA-seq is a definitive method providing useful information for solving this problem. However, due to several constraints, it was difficult to perform this for the present study. In addition, the present study acknowledges other limitations including that the group sizes of *in vivo* experiments were small owing to the ethical and feasibility constraints. The transcriptome analysis was conducted on only one sample (n=1), and statistical analysis was not possible. Therefore, the graph shows the fold change using lenient thresholds for the purpose of screening to identify variable genes. The assessment of metabolic flux using labeled precursors is also required as a future direction to elucidate the mechanism of differential mevalonate dependence between HIF-KO clones.

Finally, the data suggested that although targeting HIF-1 is a potentially attractive therapy for osteosarcoma, to achieve a complete cure, it may be necessary to identify and inhibit pathways that tumor cells rely on for survival in the absence of HIF-1.

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Availability of data and materials

The data 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 GSE307480 or at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE307480>.

Authors' contributions

TS contributed to conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing-original draft and project administration, AK contributed to conceptualization, resources, data curation, formal analysis, validation, investigation, visualization, methodology

and writing-original draft. TT contributed to resources, data curation, software, formal analysis, validation, investigation, visualization, methodology and writing-original draft. AS contributed to data curation, software, formal analysis, validation, investigation and visualization. HN, HH, SH, YT, HM and YF contributed to resources, data curation, conducting experiments and contributing to the design of experimental models. AM and HS contributed to conceptualization, resources, data curation, funding, supervision, validation, methodology and project administration. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

All animal care and procedures were performed in accordance with the guidelines of Hoshi University, and the present study was approved by the Committee on Animal Research of Hoshi University (approval no. P24-091).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Fletcher CDM, Bridge JA, Hogendoorn PCW and Mertens F: Tumors of soft tissue and bone. In: WHO Classification of tumors. 4th edition. IARC Press, Lyon, 2013.
2. Gill J. and Gorlick R: Advancing therapy for osteosarcoma. *Nat Rev Clin Oncol* 18: 609-624, 2021.
3. Kager L, Tamamyan G and Bielack S: Novel insights and therapeutic interventions for pediatric osteosarcoma. *Future Oncol* 13: 357-368, 2017.
4. Shimizu T, Ishikawa T, Sugihara E, Kuninaka S, Miyamoto T, Mabuchi Y, Matsuzaki Y, Tsunoda T, Miya F, Morioka H, *et al*: c-MYC overexpression with loss of Ink4a/Arf transforms bone marrow stromal cells into osteosarcoma accompanied by loss of adipogenesis. *Oncogene* 29: 5687-5699, 2010.
5. Shimizu T, Sugihara E, Yamaguchi-Iwai S, Tamaki S, Koyama Y, Kamel W, Ueki A, Ishikawa T, Chiyoda T, Osuka S, *et al*: IGF2 preserves osteosarcoma cell survival by creating an autophagic state of dormancy that protects cells against chemotherapeutic stress. *Cancer Res* 74: 6531-6541, 2014.
6. Kamel WA, Sugihara E, Nobusue H, Yamaguchi-Iwai S, Onishi N, Maki K, Fukuchi Y, Matsuo K, Muto A, Saya H and Shimizu T: Simvastatin-induced apoptosis in osteosarcoma cells: A key role of RhoA-AMPK/p38 MAPK signaling in antitumor activity. *Mol Cancer Ther* 16: 182-192, 2017.
7. Paoli P, Giannoni E and Chiarugi P: Anoikis molecular pathways and its role in cancer progression. *Biochim et Biophys Acta* 1833: 3481-3498, 2013.
8. Schafer ZT, Grassian AR, Song L, Jiang Z, Gerhart-Hines Z, Irie HY, Gao S, Puigserver P and Brugge JS: Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature* 461: 109-113, 2009.
9. Semenza GL: Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3: 721-732, 2003.
10. Pouyssegur J, Dayan F and Mazure NM: Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 441: 437-443, 2006.
11. Bertout JA, Patel SA and Simon MC: The impact of O₂ availability on human cancer. *Nat Rev Cancer* 8: 967-975, 2008.
12. Luo D, Ren H, Zhang W, Xian H, Lian K and Liu H: Clinicopathological and prognostic value of hypoxia-inducible factor-1 in patients with bone tumor: A systematic review and meta-analysis. *J Ortho Surg Res* 14: 1-12, 2019.

13. Yang QC, Zeng BF, Dong Y, Shi ZM, Jiang ZM and Huang J: Overexpression of hypoxia-inducible factor-1 α in human osteosarcoma: Correlation with clinicopathological parameters and survival outcome. *Jpn J Clin Oncol* 37: 127-134, 2007.
14. Ren H, Zhang YH, Li HY, Xie T, Sun LL, Zhu T, Wang SD and Ye ZM: Prognostic roles of hypoxia-inducible factor-1 α expression in osteosarcoma: A meta-analysis. *Onco Targets Ther* 9: 1477-1487, 2016.
15. Chen Y, Yang Y, Tuan Z, Wang C and Shi Y: Predicting chemosensitivity in osteosarcoma prior to chemotherapy: An investigational study of biomarkers with immunohistochemistry. *Oncol Lett* 3: 1011-16, 2012.
16. El-Naggar AM, Veinotte CJ, Cheng H, Grunewald TGP, Negri GL, Somasekharan SP, Corkery DP, Tirode F, Mathers J, Khan D, *et al*: Translational activation of HIF-1 α by YB-1 promotes sarcoma metastasis. *Cancer Cell* 27: 682-697, 2015.
17. Zhang Y, Cheng H, Li W, Wu H and Yang Y: Highly-expressed P2X7 receptor promotes growth and metastasis of human HOS/MNNG osteosarcoma cells via PI3K/Akt/GSK3 β /b-catenin and mTOR/HIF1 α /VEGF signaling. *Int J Cancer* 145: 1068-1082, 2019.
18. Xu WN, Yang RZ, Zheng HL, Jiang LS and Jiang SD: NDUFA4L2 regulated by HIF-1 α promotes metastasis and epithelial-mesenchymal transition of osteosarcoma cells through inhibiting ROS production. *Front Cell Dev Biol* 8: 515051, 2020.
19. Luo P, Zhang YD, He F, Tong CJ, Liu K, Liu H, Zhu SZ, Luo JZ and Yuan B: HIF-1 α -mediated augmentation of miRNA-18b-5p facilitates proliferation and metastasis in osteosarcoma through attenuation PHF2. *Sci Rep* 12: 10398, 2022.
20. Labun K, Montague TG, Krause M, Torres Cleuren YN, Tjeldnes H and Valen E: CHOPCHOP v3: Expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res* 47: W171-W174, 2019.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
22. Schneider CA, Rasband WS and Eliceiri KW: NIH image to ImageJ: 25 Years of image analysis. *Nat Methods* 9: 671-675, 2012.
23. Bligh EG and Dyer WJ: A rapid method for total lipid extraction and purification. *Can J Biochem Physiol* 37: 911-917, 1959.
24. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, *et al*: PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34: 267-73, 2003.
25. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES and Mesirov JP: Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102: 15545-15550, 2005.
26. Korotkevich G, Sukhov V and Sergushichev A: Fast gene set enrichment analysis. *bioRxiv*, New York, 2019. <http://biorxiv.org/content/early/2016/06/20/060012>
27. Core Team: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2023. <https://www.R-project.org/>
28. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP and Tamayo P: The molecular signatures database (MSigDB) hallmark gene set collection. *Cell Syst* 1: 417-425, 2015.
29. Qi J, Nakayama K, Cardiff RD, Borowsky AD, Kaul K, Williams R, Krajewski S, Mercola D, Carpenter PM, Bowtell D and Ronai ZA: Siah2-dependent concerted activity of HIF and FoxA2 regulates formation of neuroendocrine phenotype and neuroendocrine prostate tumors. *Cancer Cell* 18: 23-38, 2010.
30. Kim D, Paggi JM, Park C, Bennett C and Salzberg SL: Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* 37: 907-915, 2019.
31. Liao Y, Smyth GK and Shi W: FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30: 923-930, 2014.
32. Kolde R: pheatmap: Pretty Heatmaps. R package version 1.0.12, 2019. <https://CRAN.R-project.org/package=pheatmap>
33. Hedges LV: Distribution theory for glass's estimator of effect size and related estimators. *J Educ Stat* 6: 107-128, 1981.
34. Torchiano M: effsize: Efficient Effect Size Computation. R package version 0.8.1, 2020. doi:10.5281/zenodo.1480624
35. Shimizu T, Kimura K, Sugihara E, Yamaguchi-Iwai S, Nobusue H, Sampetean O, Otsuki Y, Fukuchi Y, Saitoh K, Kato K, *et al*: MEK inhibition preferentially suppresses anchorage-independent growth in osteosarcoma cells and decreases tumors in vivo. *J Orthop Res* 39: 2732-2743, 2021.
36. Cobb LM, Nolan J and Butler SA: Distribution of pimonidazole and RSU 1069 in tumour and normal tissues. *Br J Cancer* 62: 915-918, 1990.
37. Hoppe G, Yoon S, Gopalan B, Savage AR, Brown R, Case K, Vasanthi A, Chan ER, Silver RB and Sears JE: Comparative systems pharmacology of HIF stabilization in the prevention of retinopathy of prematurity. *Proc Natl Acad Sci USA* 113: E2516-E2525, 2016.
38. Palazon A, Tyrakis PA, Macias D, Velica P, Rundqvist H, Fitzpatrick S, Vojnovic N, Phan AT, Loman N, Hedenfalk I, *et al*: An HIF-1 α /VEGF-A axis in cytotoxic T cells regulates tumor progression. *Cancer Cell* 32: 669-683.e5, 2017.
39. Cowman SJ and Koh MY: Revisiting the HIF switch in the tumor and its immune microenvironment. *Trends Cancer* 8: 28-42, 2022.
40. Lequeux A, Noman MZ, Xiao M, Van Moer K, Hasmim M, Benoit A, Bosseler M, Viry E, Arakelian T, Berchem G, *et al*: Targeting HIF-1 α transcriptional activity drives cytotoxic immune effector cells into melanoma and improves combination immunotherapy. *Oncogene* 40: 4725-4735, 2021.
41. Salman S, Meyers DJ, Wicks EE, Lee SN, Datan E, Thomas AM, Anders NM, Hwang Y, Lyu Y, Yang Y, *et al*: HIF inhibitor 32-134D eradicates murine hepatocellular carcinoma in combination with anti-PD1 therapy. *J Clin Invest* 132: e156774, 2022.
42. Passaniti A, Kleinman HK and Martin GR: Matrigel: History/background, uses, and future applications. *J Cell Commun Signal* 16: 621-626, 2022.
43. Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM and Morrison SJ: Efficient tumor formation by single human melanoma cells. *Nature* 456: 593-598, 2008.
44. Pretlow TG, Delmoro CM, Dilley GG, Spadafora CG and Pretlow TP: Transplantation of human prostatic carcinoma into nude mice in matrigel. *Cancer Res* 51: 3814-3817, 1991.
45. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL and Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF1/CXCL12 secretion. *Cell* 121: 335348, 2005.
46. Dey S, Murmu N, Mondal T, Saha I, Chatterjee S, Manna R, Haldar S, Dash SK, Sarkar TR and Giri B: Multifaceted entrancing role of glucose and its analogue, 2-deoxy-D-glucose in cancer cell proliferation, inflammation, and virus infection. *Biomed Pharmacother* 156: 113801, 2022.
47. Chen J, Xie J, Jiang Z, Wang B, Wang Y and Hu X: Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. *Oncogene* 30: 4297-4306, 2011.
48. Gao J, Liao J and Yang G: CAAX-box protein, prenylation process and carcinogenesis. *Am J Transl Res* 1: 312-325, 2009.
49. Hori Y, Kikuchi A, Isomura M, Katayama M, Miura Y, Fujioka H, Kaibuchi K and Takai Y: Post-translational modifications of the C-terminal region of the rho protein are important for its interaction with membranes and the stimulatory and inhibitory GDP/GTP exchange proteins. *Oncogene* 6: 515-522, 1991.
50. Jonasch E, Donskov F, Iliopoulos O, Rathmell WK, Narayan VK, Maughan BL, Oudard S, Else T, Maranchie JK, Welsh SJ, *et al*: Belzutifan for renal cell carcinoma in von Hippel-Lindau disease. *N Engl J Med* 385: 2036-2046, 2021.
51. Chen AH and Grana AK: Belzutifan's role in the treatment landscape of clear cell renal cell carcinoma. *Pharmacotherapy* 45: 356-366, 2025.
52. Courtney KD, Ma Y, Diaz de Leon A, Christie A, Xie Z, Woolford L, Singla N, Joyce A, Hill H, Madhuranthakam AJ, *et al*: HIF-2 complex dissociation, target inhibition, and acquired resistance with PT2385, a first-in-class HIF-2 inhibitor, in patients with clear cell renal cell carcinoma. *Clin Cancer Res* 26: 793-803, 2020.
53. De Conti G, Dias MH and Bernards R: Fighting drug resistance through the targeting of drug-tolerant persister cells. *Cancers (Basel)* 13: 1118, 2021.

