Osteosarcoma-initiating cells show high aerobic glycolysis and attenuation of oxidative phosphorylation mediated by LIN28B

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Abstract
Osteosarcoma (OS) is a highly malignant bone tumor and the prognosis for non-responders to chemotherapy remains poor. Previous studies have shown that human sarcomas contain sarcoma-initiating cells (SIC), which have the characteristics of high tumorigenesis and resistance to chemotherapy. In the present study, we characterized SIC of a novel OS cell line, screened for SIC-related genes, and tried to regulate the proliferation of OS by metabolic interference. Initially, we established a new human OS cell line (OS13) and isolated clones showing higher tumorigenesis as SIC (OSHIGH) and counterpart clones. OSHIGH cells showed chemoresistance and their metabolism highly depended on aerobic glycolysis and suppressed oxidative phosphorylation. Using RNA-sequencing, we identified LIN28B as a SIC-related gene highly expressed in OSHIGH cells. mRNA of LIN28B was expressed in sarcoma cell lines including OS13, but its expression was not detectable in normal organs other than the testis and placenta. LIN28B protein was also detected in various sarcoma tissues. Knockdown of LIN28B in OS13 cells reduced tumorigenesis, decreased chemoresistance, and reversed oxidative phosphorylation function. Combination therapy consisting of a glycolysis inhibitor and low-dose chemotherapy had antitumor effects. In conclusion, manipulation of glycolysis combined with chemotherapy might be a good adjuvant treatment for OS. Development of immunotherapy targeting LIN28B, a so-called cancer/testis antigen, might be a good approach.

KEYWORDS
glycolysis, osteosarcoma, sarcoma stem cell, testis antigen, Warburg effect

1 INTRODUCTION
Osteosarcoma is the most common primary malignant bone tumor in children and adolescents. Most osteosarcoma cases are high-grade and some are resistant to conventional chemotherapy. Therefore, the development of novel treatments is needed.

Previous studies have shown that several human cancers have a small subpopulation of cells called cancer stem-like cells or SICs. These cells have the ability to self-renew and differentiate into various cell types, which is a hallmark of cancer stem cells. Osteosarcoma is one of the most aggressive bone tumors, and it is characterized by a high rate of local recurrence and distant metastasis. Despite advances in chemotherapy, the prognosis for patients with osteosarcoma remains poor, particularly for those who are resistant to chemotherapy.

In this study, we characterized SIC of a novel OS cell line, screened for SIC-related genes, and tried to regulate the proliferation of OS by metabolic interference. Initially, we established a new human OS cell line (OS13) and isolated clones showing higher tumorigenesis as SIC (OSHIGH) and counterpart clones. OSHIGH cells showed chemoresistance and their metabolism highly depended on aerobic glycolysis and suppressed oxidative phosphorylation. Using RNA-sequencing, we identified LIN28B as a SIC-related gene highly expressed in OSHIGH cells. mRNA of LIN28B was expressed in sarcoma cell lines including OS13, but its expression was not detectable in normal organs other than the testis and placenta. LIN28B protein was also detected in various sarcoma tissues. Knockdown of LIN28B in OS13 cells reduced tumorigenesis, decreased chemoresistance, and reversed oxidative phosphorylation function. Combination therapy consisting of a glycolysis inhibitor and low-dose chemotherapy had antitumor effects. In conclusion, manipulation of glycolysis combined with chemotherapy might be a good adjuvant treatment for OS. Development of immunotherapy targeting LIN28B, a so-called cancer/testis antigen, might be a good approach.
cancer-initiating cells (CIC), which are characterized by self-renewal capacity, differentiation potential, and cancer-initiating ability. 2, 3 Sarcoma-initiating cells (SIC) with features similar to those of CIC have also been observed in sarcomas. SIC are thought to play a role in recurrence and metastasis, and it is therefore important to develop a treatment strategy targeting SIC for sarcomas. 4, 5

Cancer metabolism has recently attracted much attention. 5 The metabolic plasticity of cancer is involved in its progression, drug resistance, and metastasis. 6 Cancer cells generate an enormous amount of energy for their survival by glycolysis in an aerobic micro-environment, the so-called Warburg effect. 7 Aguilar et al reported that glycolysis is essential for CIC; 8 however, the role of cell metabolism in CIC and SIC remains unknown.

In the present study, we established a new osteosarcoma cell line (OS13) and isolated clones based on higher and lower growth capacity (OS HIGH and OS LOW cells, respectively). OS HIGH cells had the characteristics of SIC. Using next-generation sequencing, we identified LIN28B, which was expressed at a higher level in OS HIGH cells than in OS LOW cells. Silencing of LIN28B changed the metabolism of osteosarcoma and induced the loss of SIC characteristics.

2 | MATERIALS AND METHODS

Mice were maintained and experimented on in accordance with the guidelines of the ethics committee of Sapporo Medical University School of Medicine, Animal Experimentation Center (permit number 15-070). Any animal found to be unhealthy or sick was promptly killed. The study was approved by the Institutional Review Board of Sapporo Medical University. Written informed consent was obtained from all patients according to the guidelines of the Declaration of Helsinki.

2.1 | Establishment of cell lines

The biopsy specimen of a conventional osteosarcoma in the distal femur of a 15-year-old girl was minced and cultured with Iscove's Modified Dulbecco's Medium (IMDM; Gibco BRL), containing 10% FBS in a 5% CO 2 incubator. After 1 year of continuous passages, a cell line was established and designated OS13.

2.2 | Cell lines and culture

Human osteosarcoma cell lines (OS2000, KIKU, OS13, HOS, U2OS, and HuO9), and one human bone malignant fibrous histiocytoma cell line (MFH03) were used. OS2000, KIKU, and MFH03 were established in our laboratory. 9-11 The other cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank and from the ATCC. OS2000 and MFH03 cells were cultured in IMDM containing 10% FBS and the others were cultured in DMEM (Sigma-Aldrich) containing 10% FBS in a 5% CO 2 incubator.

2.3 | Clonal sphere formation assay after limiting dilution

OS13 cells were seeded in a flat-bottom 96-well culture plate under the condition of limiting dilution. Subsequently, the sphere-forming ability of each clone was assessed as follows. Clonal cells were plated at 500 cells/well in six-well ultra-low attachment plates (Corning Inc.) and cultured in serum-free IMDM with 10 ng/mL recombinant human epidermal growth factor, 10 ng/mL human basic fibroblast growth factor, 1% penicillin and streptomycin, and 2% B-27 supplement (Life Technologies Corp.). On day 8, numbers of colonies were counted.

2.4 | Xenograft model

Mice had free access to food and water and were housed in sterile cages containing wood shavings and bedding under a 12-h light/dark cycle with a controlled room temperature. Cells (1 × 10 5, 1 × 10 3, and 1 × 10 2) were suspended in 100 μL PBS and mixed with Matrigel (BD Biosciences) in a 1:1 volume ratio. This mixture was s.c. injected into the backs of 4-week-old non-obese diabetic/scid IL2γ null (NSG) mice (male and female, NOD.Cg-Prkd+/+Il2γtm1Wjl/SzJ; The Jackson Laboratory) under anesthesia using isoflurane inhalation. Tumor sizes were evaluated three times per week for 7 weeks. For the in vivo treatment model, sarcoma cells (1 × 10 5 cells) were suspended in 50 μL PBS and mixed with Matrigel in a 1:1 volume on day 0. This mixture was s.c. injected into the backs of the NSG mice (4 to 20 weeks old, male and female) under anesthesia. The mice were randomly assigned to experimental groups. Tumor size was evaluated three times per week for 7 weeks. From day 0, Adriamycin (Sandoz) was given i.p. once a week for 3 weeks. Additionally, from day 0, glycolysis inhibitors (2-deoxyglucose [2-DG] and dichloroacetate [DCA]) were given i.p. three times per week for 6 weeks. No anesthesia was needed to give the drugs. At the endpoint, mice were killed by cervical dislocation under anesthesia.

2.5 | Chemoresistance assay

Effects of a chemotherapy agent on the high clone and low clone cells were evaluated using CCK-8 (Dojindo) according to the manufacturer's protocol. The clone cells were plated at 15,000 cells/well in a 96-well plate with 100 μL culture medium. After 24 hours, the culture medium was replaced and Adriamycin was directly added at various concentrations. After 24-h treatment, the cells were incubated with 10 μL CCK-8 reagent for 4 hours at 37°C in a 5% CO 2 incubator. Absorbance at 450 nm was measured with a Bio-Rad ELISA reader (Bio-Rad).

2.6 | Reverse transcription-PCR

Total RNA was extracted from clone cells using the RNasy Mini Kit (Qiagen) with DNase I treatment and reverse-transcribed using
SuperScript III (Invitrogen) according to the manufacturer’s protocol. RT-PCR was carried out with Taq DNA polymerase (Qiagen) using forward primer 5’-AGCCCCTTGGATATTCCAGTC-3’ and reverse primer 5’-AATGTGAATTCCACTGGTTCTCCT-3’ for LIN28B. The PCR mixture was denatured at 94°C for 2 minutes, followed by 30 cycles at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Quantitative PCR was carried out using a StepOne Real-Time PCR system (Applied Biosystems). LIN28B (Hs01013729_m1), SOX2 (Hs01053049_s1), POU5F1 (Hs00999632_g1), and NANOG (Hs04260366_g1) primers and probes were designed using the TaqMan Gene Expression assay (Applied Biosystems). Human normal tissue panels (Human MTC panels I and II; Takara Bio, Inc.) were used as normal tissue. Each experiment was carried out in triplicate and normalized to the GAPDH gene as an internal control. miRNAs were extracted using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s protocol. Mature let-7a and let-7g were quantified using a predesignated TaqMan MicroRNA Assay (Applied Biosystems).

2.7 | Next-generation sequencing

Total RNAs were extracted from cells using the RNeasy Mini Kit followed by sequencing with Illumina Hiseq 2500 and analyzed by Hokkaido System Science Co., Ltd. Gene ontology enrichment analysis was carried out for differentially expressed genes using DAVID, the database of the National Institute of Allergy and Infectious Diseases.

2.8 | Immunohistochemistry

Immunohistochemical staining was done using formalin-fixed paraffin-embedded sections of biopsy specimens from 40 sarcoma patients as previously described. A rabbit anti-lin28B polyclonal antibody (ab71415; Abcam) was used at a 1:200 dilution. Reactivity of the anti-lin28B antibody was determined by the staining pattern of the nuclei and cytosol of tumor cells. Cases were graded as follows: negative (<5% positive cells), low (5%–50% positive cells), and high (>50% positive cells).

2.9 | LIN28B knockdown

LIN28B shRNA in a lentiviral plasmid (TRCN0000122599) and control shRNA (SHC002V) were used according to the manufacturer’s protocol (Sigma-Aldrich). Briefly, OS13 cells were infected with lentiviral transduction particles at a MOI of 1.0 in the presence of polybrene (8 ng/µL). Infected cells were selected by a puromycin antibiotic prior to the analysis.

2.10 | Western blotting

Cells were suspended in ice-cold RIPA buffer for 10 minutes. The lysates were mixed with 2 x sample buffer and boiled for 5 minutes. Lysates were separated on 10% SDS-PAGE and transferred to PVDF membranes (Immobilon-P Transfer Membranes; Merck). The membranes were incubated primarily with rabbit anti-LIN28B monoclonal antibody (ab191881; Abcam) or mouse anti-β-actin monoclonal antibody (clone AC-15; Sigma-Aldrich) for 1 hour at room temperature. Membranes were then stained with a peroxidase-labeled secondary antibody and visualized with an ECL detection system (GE Healthcare).

2.11 | Metabolic analysis

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined by a Seahorse XF24 analyzer (Agilent Technologies, Inc.). Cells were plated at 15,000 cells/well in XF24-well plates. The next day, the cells were washed and equilibrated with an assay medium for 30 minutes at 37°C in a CO₂-free incubator. Initial measurements of OCR and ECAR were carried out before the addition of glucose. Subsequent measurements were done after 25 mM glucose had been added into the wells through injection ports. Three types of reagent were subsequently added to evaluate mitochondrial respiration capacity. Oligomycin (Agilent Technologies, Inc.) is an inhibitor of oxidative phosphorylation (OXPHOS); flavin adenine dinucleotide (FCCP; Agilent Technologies, Inc.) is an accelerator of the electron transport chain by perforation of the inner mitochondrial membrane; and rotenone and antimycin-A (Agilent Technologies, Inc.) are inhibitors of the mitochondrial NADH dehydrogenase/complex.

2.12 | Aerobic glycolysis inhibition

Sphere formation assays were carried out in culture media with either no glucose or a high concentration of glucose. Serum-free DMEM (Nacalai Tesque, Inc.) containing 25 mM glucose was used as the high glucose medium. 2-DG (Sigma-Aldrich) and DCA (Sigma-Aldrich) were used at final concentrations of 10 mM each.

2.13 | Measurement of ATP production

ATP levels were measured using a Cellno ATP Assay Kit (Toyo B-Net Co., Ltd) according to the manufacturer’s protocol. Prior to measurement, cells were cultured in IMDM medium with or without reagents for 45 minutes. Luminescence intensity was measured using an Infinite M1000 Pro plate reader (Tecan).

2.14 | Statistical analysis

For comparisons, we used the unpaired t test in JMP software (SAS Institute Inc.). Where relevant, figures indicate statistical parameters, including the value of n, means ± SD, and statistical significance.
3 | RESULTS

3.1 | Establishment of the osteosarcoma cell line OS13

A tumor cell culture was maintained for 1 year and designated OS13. Biopsy specimens showed the presence of pleomorphic cells with atypical nuclei in neoplastic bones (Figure S1A). Karyotype analysis of OS13 showed multiple numerical and structural chromosomal aberrations (Figure S1B). Subcutaneous inoculation of OS13 cells into the NSG mice resulted in the formation of malignant tumors. Histologically, the xenografted tumors consisted of pleomorphic cells; however, no neoplastic bone was seen (Figure S1C).

3.2 | Identification of a clone that showed higher tumorigenicity as SIC

Previously, we isolated SIC from sarcoma cell lines using the side population and ALDEFLUOR assays based on activity of the drug

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efflux ATP-binding cassette ABCG2 and aldehyde dehydrogenase activity, respectively. However, we could not separate the populations of SIC and non-SIC using these methods. Therefore, we attempted to establish a single cell clone by limiting dilution to separate SIC and non-SIC among OS13 bulk cells. Sphere-formation ability of the resultant 54 clones was subsequently assessed to isolate clones showing higher and lower tumorigenesis. Surprisingly, most of the clones showed higher tumorigenesis (Figure S2), suggesting that OS13 bulk cells contained a very large proportion of SIC. Finally, we selected clones with higher tumorigenicity as SIC (OS\textsuperscript{HIGH}) and clones with lower tumorigenicity as non-SIC (OS\textsuperscript{LOW}). As shown in Figure 1A–C, the OS\textsuperscript{HIGH} cells formed many large spheres, whereas the OS\textsuperscript{LOW} cells formed only a few small colonies.

To assess tumorigenesis in vivo, 1 × 10\textsuperscript{2} to 1 × 10\textsuperscript{4} clone cells were injected into the NSG mice. Tumor formation was observed in two of three mice that received 10\textsuperscript{2} OSHIGH cells but in only one of three mice that received 10\textsuperscript{2} OSLOW cells (Figure 1D). Following injections of more than 10\textsuperscript{3} cells, OS\textsuperscript{HIGH} cells formed a larger tumor mass than that formed by OS\textsuperscript{LOW} cells (Figure 1E,F). OS\textsuperscript{HIGH} cells also showed higher resistance to Adriamycin, a key drug used in chemotherapy for osteosarcoma (Figure 1G). These results indicated that OS\textsuperscript{HIGH} cells and OS\textsuperscript{LOW} cells have characteristics of SIC and non-SIC, respectively. However, OSHIGH cells showed expression levels of stem cell-related genes including SOX2, NANOG, and POU5F1 that were similar to those in OS\textsuperscript{LOW} cells (Figure 1H).

### 3.3 OS\textsuperscript{HIGH} cells were dependent on aerobic glycolysis and attenuation of mitochondrial function

Oxygen consumption rate and ECAR were determined to assess the metabolic features of OSHIGH and OSLOW cells. OCR was significantly decreased in OSHIGH cells compared with OSLOW cells (Figure 2A). In contrast, both OSLOW and OSHIGH cells were highly dependent on aerobic glycolysis (Figure 2B). After injections of glucose, OCR of OSLOW cells increased, whereas that of OSHIGH cells decreased (Figure 2C). ECAR rapidly increased in both OSLOW and OSHIGH cells (Figure 2D). Next, the activity of mitochondria was assessed (Figure 2E,F). Treatment of the cells with oligomycin, which partially downregulates mitochondrial respiration, resulted in a decrease in the OCR of OSLOW cells and a slight decrease in that of OSHIGH cells. OCR of both clones increased in response to treatment with

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FCCP and decreased in response to treatment with rotenone and antimycin-A, which completely inhibited mitochondrial respiration. OS\textsuperscript{LOW} cells responded to the reagents, but OS\textsuperscript{HIGH} cells showed only slight changes in OCR. ECAR of both clones increased similarly in response to the reagents. These results suggested that OS\textsuperscript{LOW} cells have functional mitochondria. Therefore, OS\textsuperscript{HIGH} might be more...
dependent than OS\textsuperscript{LOW} cells on glycolysis for the generation of ATP. In contrast, OS\textsuperscript{LOW} cells use both aerobic glycolysis andOXPHOS in mitochondria.

### 3.4 | Identification of LIN28B as a SIC-related gene

We carried out gene expression profiling of both clones using RNA-sequencing. Numerous genes were found to be upregulated in OS\textsuperscript{HIGH} cells compared with OS\textsuperscript{LOW} cells. Gene ontology enrichment analysis showed that the gene signature of OS\textsuperscript{HIGH} cells was significantly related to nucleosome organization or cell reproduction (Table S1). We selected candidate representative markers of OS\textsuperscript{HIGH} cells by the following procedure. First, we excluded genes expressed in human normal tissues based on NCBI gene data and confirmed the expression status by RT-PCR. Next, we selected genes expressed in OS\textsuperscript{HIGH} cells at higher levels than in OS\textsuperscript{LOW} cells. Among several candidates, we focused on LIN28B, which has been described as an oncogene in various human cancers.\textsuperscript{15} mRNA of LIN28B was highly expressed in OS13 bulk cells and in OS\textsuperscript{HIGH} cells (Figure 3A) and showed specific expression in the testis and placenta among normal human tissues (Figure 3B,C). LIN28B was not detected in human normal tissues other than the testis and placenta. Therefore, LIN28B could be categorized as a so-called cancer/testis antigen.\textsuperscript{3} LIN28B was also expressed in several sarcoma cell lines (Figure 3D). Expression status of LIN28B protein in 40 sarcoma biopsy specimens was also assessed. Of the 40 specimens, 35 (87.5%) were graded as either low or high staining. High-grade staining was observed in five of eight (62.5%) conventional osteosarcomas (Figure 3E, Tables S2 and S3).

### 3.5 | LIN28B is associated with sarcoma-initiating ability and the inhibition of mitochondrial function

To determine whether LIN28B has an effect on the tumorigenesis of OS13 cells, we inhibited LIN28B expression using shRNA. Expression of mRNA and protein of LIN28B was decreased in short hairpin LIN28B (shLIN28B) cells (Figure 4A,B). LIN28B
knockdown cells showed decreased in vitro sphere formation (Figure 4C). Expression levels of mature let-7a and let-7g were markedly restored (Figure 4D) and resistance to Adriamycin decreased (Figure 4E). shLIN28B cells showed expression levels of stem cell-related genes similar to those in short hairpin control (shControl) cells (Figure 4F).

We then addressed the metabolic status of shLIN28B cells. In the presence of glucose, OCR of shLIN28B cells markedly increased compared with that of shControl cells (Figure 4G). shLIN28B cells showed mitochondrial function and glycolysis similar to that of OS13 cells. These results suggested that knockdown of LIN28B might switch the metabolic status of OS13 cells to that of OS13 cells.

3.6 | Manipulation of aerobic glycolysis reduced tumorigenesis of OS13 cells and enhanced chemotherapeutic effect

Because the proliferation of both OS13 and OSLOW cells depended on glycolysis and LIN28B might inhibit OXPHOS by upregulation of PDK1, we assessed the sphere-formation ability of OS13 cells after treatment with the glycolysis inhibitors 2-DG and DCA. Both inhibitors reduced the number of spheres in shControl and bulk OS13 cells (Figure 5A, B and Figure S3A, B). Moreover, shLIN28B cancelled the inhibitory effect of DCA. These results suggested that the inhibition of glycolysis and the recovery of mitochondrial function decreased the sphere-formation ability of OS13 cells expressing LIN28B. Cell viability after treatment with 2-DG and DCA was greatly decreased in OS13 and MFH03 cells (Figure 5C, D). The other glycolysis inhibitors showed inhibitory effects on cell viability (Figure S4A, B). This effect might be because the metabolism of sarcoma cells is highly dependent on glycolysis. Decreases of ATP production after exposure to glycolysis inhibitors were not dependent on LIN28B expression (Figure S5A, B).

3.7 | Glycolysis inhibitors enhanced chemotherapeutic effect and suppressed tumorigenesis in sarcoma cells

Osteosarcoma-initiating cells are resistant to chemotherapy through LIN28B expression and depend on glycolysis to maintain tumor-initiating ability (Figures 4E and 5A). To sensitize osteosarcoma-initiating cells to chemotherapy, we assessed the effects of combination therapy that consisted of glycolysis inhibitors and Adriamycin, a key chemotherapy drug for osteosarcoma.

OS13 cells were initially treated with Adriamycin with or without 2-DG in vitro. Results showed that 2-DG could enhance the effects of low-dose Adriamycin on OS13 cells (Figure 6A). Similar results to those in OS13 cells were also observed in MFH03 cells (Figure 6B). DCA also increased the effects of Adriamycin, although the effects were limited (Figure 6C, D).

Finally, we assessed the in vivo effects of glycolysis inhibitors and Adriamycin, assuming an adjuvant setting, in OS13 cells. Compared with the PBS control group, the 2-DG group did not show significant antitumor effects. In addition, rapid growth of the tumor was observed in the DCA group (Figure 6E–H). Low-dose Adriamycin (1 mg/kg) alone did not show an antitumor effect on OS13 in vivo; however, a combination of Adriamycin and 2-DG showed additional effects on the low-dose Adriamycin and 2-DG groups (Figure 6I–L). Most mice that received Adriamycin at the middle dose (3 mg/kg) and high dose (10 mg/kg) died as a result of toxicity before the treatment ended (Figure S6).
In the present study, a novel osteosarcoma cell line (OS13) was established and characterized as follows: (i) OS\textsuperscript{HIGH} cells derived from OS13 bulk cells showed higher levels of in vitro and in vivo tumorigenesis and chemoresistance; (ii) the metabolic status of OS\textsuperscript{HIGH} cells highly depended on glycolysis but not on OXPHOS; (iii) LIN28B was identified as a SIC-related protein and expressed in 85% of sarcoma primary tissues; (iv) knockdown of LIN28B led to an increase in mitochondrial respiration; and (v) manipulation of aerobic glycolysis combined with chemotherapy decreased in vitro and in vivo tumorigenesis of OS13 cells.

Using next-generation sequencing of OS\textsuperscript{HIGH} and OS\textsuperscript{LOW} cells, we identified LIN28B as a candidate SIC-related gene. The LIN28 family is a family of RNA-binding proteins that regulate the degradation of let-7 miRNAs and play key roles in many human malignancies.\textsuperscript{15,17} A LIN28 homolog, LIN28B, was first reported to be overexpressed in hepatocellular carcinoma (HCC).\textsuperscript{18} LIN28B expression promotes cancer development by suppression of the expression of let-7 miRNAs in several human cancers including colon cancer,\textsuperscript{19} HCC,\textsuperscript{16,20} B-cell lymphoma,\textsuperscript{21} breast cancer,\textsuperscript{22} and multiple myeloma.\textsuperscript{23}

LIN28B mRNA was found to be expressed in various sarcomas, but was not detectable in normal organs other than the testis and placenta. In the testis, LIN28B protein was expressed in spermatogonia and spermatocytes but not in spermatids and sperm. This expression profile is the so-called “cancer stem cell/testis antigen” pattern and is an ideal target for immunotherapy using peptide vaccination or adoptive cell transfer of genetically engineered exogenous T-cell receptor-expressing T cells (TCR-T cells).\textsuperscript{3,24}

The molecular mechanisms underlying attenuation of resistance to Adriamycin by knockdown of LIN28B remain unclear. However, LIN28B is known to mediate paclitaxel resistance through p21 and Rb expression and let-7 miRNA inhibition, which increase RAS expression.\textsuperscript{25,26} These mechanisms might confer resistance to Adriamycin through LIN28B expression in sarcoma cells.

In the present study, we showed that aerobic glycolysis was enhanced and mitochondrial function was impaired in OS\textsuperscript{HIGH} cells expressing LIN28B as SIC. Ma et al reported that LIN28B regulates aerobic glycolysis through downregulation of let-7 miRNAs, upregulation of PDK1 and suppression of OXPHOS.\textsuperscript{16} Aguilar et al reported that glycolysis is essential for CIC.\textsuperscript{8} These findings also indicate that SIC of osteosarcoma might require predominant aerobic glycolysis, the so-called Warburg effect.

We inhibited aerobic glycolysis using two glycolysis inhibitors. DCA is an inhibitor of PDK1 that regulates the phosphorylation of pyruvate dehydrogenase (PDH) and increases glucose uptake. PDH promotes the entry of pyruvate into the mitochondrial tricarboxylic acid cycle and suppresses production of lactate. 2-DG is a glycolysis inhibitor that inhibits the activity of the first enzyme in the glycolysis pathway and totally blocks aerobic glycolysis. We found that DCA treatment decreased sphere-formation ability and cell viability through restored mitochondrial capacity. In contrast, ATP levels and tumorigenesis of OS13 cells were greatly decreased by 2-DG treatment. These results suggest that most OS13 cells depend on the glycolysis pathway for cell proliferation regardless of the fact that glycolysis yields a smaller amount of ATP compared with mitochondrial OXPHOS. This possibility is supported by the results of a previous study showing that cancer cells require not only rapid ATP synthesis but also various resultant metabolites generated by glycolysis for proliferation.\textsuperscript{7} For example, the accumulation of glycolytic intermediates promotes the pentose phosphate pathway, resulting in the generation of NADPH and ribose-5-phosphate. Ribose-5-phosphate is essential for the biosynthesis of nucleic acids.\textsuperscript{27}

In the in vivo study, a combination of 2DG and low-dose Adriamycin showed antitumor effects against xenografted OS13 cells. These results are comparable with the in vitro study and suggested that glycolysis inhibitors might be useful for patients receiving chemotherapy for osteosarcoma.

In conclusion, we showed that LIN28B plays important roles in enhanced glycolysis in osteosarcoma-initiating cells. Use of glycolysis inhibitors combined with chemotherapy might be a novel therapeutic approach for patients with osteosarcoma. Thus, the development of immunotherapy targeting LIN28B appears to be a good approach.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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REFERENCES

1. Posthuma-DeBoer J, Witlox MA, Kaspers GJ, van Royen BJ. Molecular alterations as target for therapy in metastatic osteosarcoma: a review of literature. *Clin Exp Metastasis*. 2011;28:493-503.

2. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer*. 2008;8:755-768.

3. Hirohashi Y, Torigoe T, Tsukahara T, Kanaseki T, Kochin V, Sato N. Immune responses to human cancer stem-like cells/cancer-initiating cells. *Cancer Sci*. 2016;107:12-17.

4. Genadry KC, Pietrobono S, Rota R, Linardic CM. Soft tissue sarcoma cancer stem cells: an overview. *Front Oncol*. 2018;8:475.

5. Scott DA, Richardson AD, Filipp FV, et al. Comparative metabolic flux profiling of melanoma cell lines: beyond the Warburg effect. *J Biol Chem*. 2011;286:42626-42634.

6. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer*. 2004;4:891-899.

7. Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. *Cell Metab*. 2016;23:27-47.

8. Aguilar E, Marin de Mas I, Zodda E, et al. Metabolic reprogramming and dependencies associated with epithelial cancer stem cells independent of the epithelial-mesenchymal transition program. *Stem Cells*. 2016;34:1163-1176.

9. Nabeta Y, Kawaguchi S, Sahara H, et al. Recognition by cellular and humoral autologous immunity in a human osteosarcoma cell line. *J Orthop Res*. 2003;8:554-559.

10. Wada T, Uede T, Ishii S, Matsuyama K, Yamawaki S, Kikuchi K. Monoclonal antibodies that detect different antigenic determinants of the same human osteosarcoma-associated antigen. *Cancer Res*. 1998;58:2273-2279.

11. Tsukahara T, Kawaguchi S, Ida K, et al. HLA-restricted specific tumor cytolyis by autologous T-lymphocytes infiltrating metastatic bone malignant fibrous histiocytoma of lymph node. *J Orthop Res*. 2006;24:94-101.

12. Tsukahara T, Kawaguchi S, Torigoe T, et al. Prognostic significance of HLA class I expression in osteosarcoma defined by anti-pan HLA class I monoclonal antibody, EMR8-5. *Cancer Sci*. 2006;97:1374-1380.

13. Murase M, Kano M, Tsukahara T, et al. Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas. *Br J Cancer*. 2009;101:1425-1432.

14. Emori M, Tsukahara T, Murase M, et al. High expression of CD109 antigen regulates the phenotype of cancer stem-like cells/cancer-initiating cells in the novel epithelioid sarcoma cell line ESX and is related to poor prognosis of soft tissue sarcoma. *PLoS ONE*. 2013;8:e84187.

15. Balzeau J, Menezes MR, Cao S, Hagan JP. The LIN28/let-7 Pathway in Cancer. *Front Genet*. 2017;8:31.

16. Ma X, Li C, Sun L, et al. Lin28/let-7 axis regulates aerobic glycolysis and cancer progression via PDK1. *Nat Commun*. 2014;5:5212.

17. Zhang J, Ratanasirinawoot S, Chandrasekaran S, et al. LIN28 regulates stem cell metabolism and conversion to primed pluripotency. *Cell Stem Cell*. 2016;19:66-80.

18. Guo Y, Chen Y, Ito H, et al. Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma. *Gene*. 2006;384:51-61.

19. Wang T, He Y, Zhu Y, et al. Comparison of the expression and function of Lin28A and Lin28B in colon cancer. *Onco Targets*. 2016;7:79605-79616.

20. Viswanathan SR, Powers JT, Einhorn W, et al. LIN28 promotes transformation and is associated with advanced malignancies. *Nat Genet*. 2009;41:843-848.

21. Chang TC, Zeitels LR, Hwang HW, et al. Lin-28 transactivation is necessary for Myc-mediated let-7 repression and proliferation. *Proc Natl Acad Sci USA*. 2009;106:3384-3389.

22. Piskounova E, Polytarchou C, Thornton JE, et al. LIN28A and LIN28B inhibit let-7 microRNA biogenesis by distinct mechanisms. *Cell*. 2011;147:1066-1079.

23. Manier S, Powers JT, Sacco A, et al. The LIN28B/let-7 axis is a novel therapeutic pathway in multiple myeloma. *Leukemia*. 2017;31:853-860.

24. Tsukahara T, Hirohashi Y, Kanaseki T, et al. Peptide vaccination therapy: Towards the next generation. *Pathol Int*. 2016;66:547-553.

25. Lv K, Liu L, Wang L, et al. Lin28 mediates paclitaxel resistance by modulating p21, Rb and Let-7a miRNA in breast cancer cells. *PLoS ONE*. 2012;7:e40008.

26. Kumar MS, Erkeland SJ, Pester RE, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci USA*. 2008;105:3903-3908.

27. Yu L, Chen X, Wang L, Chen S. The sweet trap in tumors: aerobic glycolysis and potential targets for therapy. *Onco Targets*. 2016;7:38908-38926.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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