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EDITED BY

Erhu Zhao,
Southwest University, China

REVIEWED BY

George Bayliss,
Brown University, United States
Longfei Deng,
Southwest University, China

*CORRESPONDENCE

Robert M. Hoffman
all@anticancer.com
Yasunori Tome
yastome@med.u-ryukyu.ac.jp

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Reversion of methionine addiction of osteosarcoma cells to methionine independence results in loss of malignancy, modulation of the epithelial-mesenchymal phenotype and alteration of histone-H3 lysine-methylation

Yusuke Aoki^{1,2,3}, Qinghong Han¹, Yasunori Tome^{3*},
Jun Yamamoto^{1,2}, Yutaro Kubota^{1,2}, Noriyuki Masaki^{1,2},
Koya Obara^{1,2}, Kazuyuki Hamada^{1,2}, Justin D. Wang⁴,
Sachiko Inubushi⁵, Michael Bouvet², Steven G. Clarke⁶,
Kotaro Nishida³ and Robert M. Hoffman^{1,2*}

¹AntiCancer Inc, San Diego, CA, United States, ²Department of Surgery, University of California San Diego, La Jolla, CA, United States, ³Department of Orthopedic Surgery, Graduate School of Medicine, University of the Ryukyus, Nishihara, Japan, ⁴School of Medicine, California University of Science and Medicine, Colton, CA, United States, ⁵Department of Surgery, Kobe University, Kobe, Japan, ⁶Department of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, CA, United States

Methionine addiction, a fundamental and general hallmark of cancer, known as the Hoffman Effect, is due to altered use of methionine for increased and aberrant transmethylation reactions. However, the linkage of methionine addiction and malignancy of cancer cells is incompletely understood. An isogenic pair of methionine-addicted parental osteosarcoma cells and their rare methionine-independent revertant cells enabled us to compare them for malignancy, their epithelial-mesenchymal phenotype, and pattern of histone-H3 lysine-methylation. Methionine-independent revertant 143B osteosarcoma cells (143B-R) were selected from methionine-addicted parental cells (143B-P) by their chronic growth in low-methionine culture medium for 4 passages, which was depleted of methionine by recombinant methioninase (rMETase). Cell-migration capacity was compared with a wound-healing assay and invasion capability was compared with a transwell assay in 143B-P and 143B-R cells *in vitro*. Tumor growth and metastatic potential were compared after orthotopic cell-injection into the tibia bone of nude mice *in vivo*. Epithelial-mesenchymal phenotypic expression and the status of H3 lysine-methylation were determined with western immunoblotting. 143B-P cells had an IC₅₀ of 0.20 U/ml and 143B-R cells had an IC₅₀ of 0.68 U/ml for treatment with rMETase, demonstrating that

143B-R cells had regained the ability to grow in low methionine conditions. 143B-R cells had reduced cell migration and invasion capability *in vitro*, formed much smaller tumors than 143B-P cells and lost metastatic potential *in vivo*, indicating loss of malignancy in 143B-R cells. 143B-R cells showed gain of the epithelial marker, ZO-1 and loss of mesenchymal markers, vimentin, Snail, and Slug and, an increase of histone H3K9me3 and H3K27me3 methylation and a decrease of H3K4me3, H3K36me3, and H3K79me3 methylation, along with their loss of malignancy. These results suggest that shifting the balance in histone methylases might be a way to decrease the malignant potential of cells. The present results demonstrate the rationale to target methionine addiction for improved sarcoma therapy.

KEYWORDS

malignancy, methionine addiction, Hoffman effect, methionine-independent revertant, epithelial-mesenchymal phenotype, histone-methylation, osteosarcoma

Introduction

Cancers were originally described as methionine dependent, as it was thought that the cancer cells lost the ability to synthesize methionine (1–3). However, we showed that cancer cells make normal or greater-than-normal amounts of methionine from homocysteine, but still require large amounts of exogenous methionine in order to grow and survive, unlike normal cells, due to excess transmethylation reactions (4–10). Our seminal studies of methionine addiction of cancer cells (4–9) were confirmed by Wang, et al., who showed that tumor-initiating cells were highly methionine-addicted (11). We further characterized methionine addiction of cancer cells (12–16). Thus, cancer cells must have much larger-than-normal amounts of methionine in order to survive and proliferate, which is described as methionine addiction. Other teams are now using the term “methionine addiction of cancer” (11, 17, 18). We have previously shown that osteosarcoma cells, including 143B, Saos-2, MNNG-HOS, and U-2OS, are methionine-addicted, and 143B is the most methionine addicted among these osteosarcoma cell lines (19). However, the linkage of the methionine addiction and malignancy is incompletely understood.

Methionine-independent revertant cells, isolated from methionine-addicted parental cancer cells in low-exogenous methionine conditions (20), have reduced malignancy (13, 15, 16, 21, 22), suggesting that reversion of methionine addiction of cancer cells to methionine independence is related to malignancy itself. We and Borrego et al. have previously shown that methionine-independent revertant cells have reduced growth ability in soft agar (16, 21, 22). We have also shown methionine-independent revertant cells

have lost tumorigenicity in subcutaneous-xenograft mouse models (13, 16) and lost metastasis in xenograft mouse models (15).

It was previously reported that methylation of histone H3 lysine marks in tumor-initiating cells was increased compared to non-tumor-initiating cells (11). Our previous studies also showed that histone-H3 lysine-methylation was increased in cancer cells compared to normal cells (12, 23), and it is further increased in high-malignancy variants selected from parental cancer cells, compared to the parental cells (16). We also previously reported that the status of the histone-H3 lysine-methylation in methionine-addicted cancer cells is unstable during methionine restriction, using recombinant L-methionine α -deamino- γ -mercapto-methane lyase (rMETase) (24), at concentrations which arrest cancer-cell proliferation (12, 14). These results suggest that the status of the histone-H3 lysine-methylation might be associated with malignancy of cancer cells. However, the molecular relationship of methionine addiction and malignancy is incompletely understood.

In osteosarcoma, the 5-year survival rate for patients who have metastases, mostly lung metastasis, is about 30%, while the 5-year survival rate for overall osteosarcoma patients is about 60% (25). In the metastatic processes, epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) are thought to be required for cell plasticity (26). Recently, EMT/MET has been studied in osteosarcoma (27–37) and other types of sarcoma (38–45), as well as carcinomas, since the theory that sarcoma cells can reside in an intermediate phenotype between epithelial and mesenchymal has been widely accepted (46–48).

In the present report, we show that reversion of methionine addiction of osteosarcoma cells to methionine-independence results in loss of malignancy, modulation of the epithelial-

mesenchymal phenotype, and alteration of histone-H3 lysine-methylation. The present results provide unique clues for further understanding of the fundamental basis of cancer.

Materials and methods

Cell culture

The 143B human osteosarcoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM; #10-103-CV, Corning Inc., Corning, NY, USA), supplemented with 1 IU/ml penicillin/streptomycin (#15-240-062, Thermo Fisher Scientific, Waltham, MA, USA) and 10% fetal bovine serum (FBS; Access Biologicals, Vista, CA, USA).

Recombinant methioninase production

Recombinant methioninase (rMETase) is a tetramer, with each monomer having a 172-kDa molecular weight. The procedure for the production of rMETase, from recombinant *E. coli* with the *Pseudomonas putida* gene, has been previously reported (24).

Depletion of methionine in the culture medium with rMETase

Normal methionine-containing DMEM with/without different amount of rMETase, 0 U/ml; 0.125 U/ml; 0.25 U/ml; 0.5 U/ml; 1 U/ml; 2 U/ml; and 4 U/ml, was incubated at 37°C for 3 h. The concentration of methionine in each medium was determined with an HPLC (Hitachi L-6200A Intelligent pump, Hitachi, Ltd., Tokyo, Japan). Experiments were performed twice.

Selection for methionine-independent revertant osteosarcoma cells

Methionine-independent revertant 143B osteosarcoma cells (143B-R) were selected by the following procedure, modified from previous reports (13, 15, 16): methionine-addicted parental 143B osteosarcoma cells (143B-P) were cultured in normal medium with increasing concentrations of rMETase (0.75-1.6 U/ml) for 3 weeks, followed by culture in normal medium for 3 weeks and passage. This procedure was repeated 4 times.

rMETase sensitivity assay

Each 143B-P and 143B-R cells were cultured in 96-well plates (1.0×10^3 cells/well) in normal DMEM (100 μ l/well) and incubated at 37°C overnight. Each cell was treated with normal medium or different concentration of rMETase, at 37°C for 72 h, as follows: 0 U/ml; 0.025 U/ml; 0.05 U/ml; 0.1 U/ml; 0.2 U/ml; 0.4 U/ml; 0.8 U/ml; and 1.6 U/ml. After the treatment period, WST-8 reagent (10 μ l) (#CK04, Dojindo laboratory, Kumamoto, Japan) was added to each well and the plate was additionally incubated at 37°C for 1 h. Absorbance at 450 nm was measured, and rMETase-sensitivity cell-survival curves and half-maximal inhibitory concentration (IC_{50}) were obtained, as previously described (14). Experiments were performed twice in triplicate.

Wound healing assay

Each 143B-P and 143B-R cells were cultured in 6-well plates in normal medium and incubated at 37°C overnight. Wounds were made by scratching the monolayers with a 200 μ l pipette tip. The plates were washed with phosphate-buffered saline (PBS; #MB1039-1X, BioPioneer Inc., San Diego, CA, USA) twice to remove floating cells, followed by incubation at 37°C. The wound areas were measured by light microscopy (Olympus IX70, Olympus Corporation, Tokyo, Japan) at four and eight hours after making the wound. Experiments were performed twice in triplicate.

Invasion assay

24-well Transwell inserts (#3422, Corning Inc.) were coated with 100 μ l of Matrigel Matrix (200 μ g/ml) (#354234, Corning Inc.) for each well, in advance. 143B-P or 143B-R cells were cultured in the upper chambers of the insert (2.5×10^4 cells/well) with 250 μ l FBS-free medium, with the lower chambers filled with normal medium, containing 10% FBS, followed by incubation at 37°C for 18 h. The inserts were removed and washed with PBS twice, followed by fixation with 100% methanol (#33900HPLC, Pharmco, Brookfield, CT, USA) for 20 min and staining with 0.5% crystal violet (#C0775-25G, Sigma-Aldrich, Burlington, MA, USA) for 15 min. The inner side of the inserts was wiped with cotton swabs. The remaining cells were counted under a light microscopy (Olympus IX70, Olympus Corporation). Experiments were performed twice in triplicate.

Western immunoblotting

143B-P or 143B-R cells were cultured in 25 cm² cell-culture flasks in normal DMEM for more than 5 days. Cells were then lysed to extract total proteins, using RIPA Lysis and Extraction buffer (#89900, Thermo Fisher Scientific) with 1% Halt Protease Inhibitor Cocktail (#87786, Thermo Fisher Scientific). Extraction of total histones used an Epiquik Total Histone Extraction Kit (#OP-0006-100, Epigentek, Farmingdale, NY, USA). Western immunoblotting for total proteins and histones was performed as follows: Total proteins or histones were loaded onto 7.5% or 12% SDS-PAGE gels, for electrophoresis, respectively. After separation of proteins or histones by electrophoresis, they were transferred to 0.45 μm or 0.2 μm polyvinylidene difluoride (PVDF) membranes (#GE10600023 or #GE10600021, respectively, GE Healthcare, Chicago, IL, USA). The membranes were blocked with Bullet Blocking One for Western Blotting (#13779, Nakalai Tesque, Inc., Kyoto, Japan). Anti-ZO-1 antibody (1:1,000, #8193, Cell Signaling Technology, Danvers, MA, USA); anti-vimentin antibody (1:1,000, #5741, Cell Signaling Technology); anti-Snail antibody (1:1,000, #3879, Cell Signaling Technology); anti-Slug antibody (1:1,000, #9585, Cell Signaling Technology); anti-beta actin antibody (1:1,500, #20536-1-AP, Proteintech, Rosemont, IL, USA); anti-H3K4me3 antibody (1:1,000, #9751, Cell Signaling Technology); anti-H3K9me3 antibody (1:1,000, #13969, Cell Signaling Technology); anti-H3K27me3 antibody (1:1,000 #9733, Cell Signaling Technology); anti-H3K36me3 antibody (1:1,000, #4909, Cell Signaling Technology); anti-H3K79me3 antibody (1:1,000, #74073, Cell Signaling Technology); anti-H3 antibody (1:1,500, #17168-1-AP, Proteintech) were used. Beta-actin or total histone-H3 were used as internal loading controls. Horseradish-peroxidase-conjugated anti-rabbit IgG (1:20,000, #SA00001-2, Proteintech) was used as the secondary antibody. The signals were detected with a UVP ChemStudio (Analytik Jena, Upland, CA, USA), enhanced by Clarity Western ECL Substrate (#1705061, Bio-Rad Laboratories, Hercules, CA, USA). The protein signals were normalized to those of beta-actin, the histone signals were normalized to those of total histone-H3, for relative quantification. Experiments were performed three times.

Animals

Female athymic nu/nu nude mice (4-6-week-old) (AntiCancer Inc., San Diego, CA, USA) were used for the present study. All mice were bred and maintained in a barrier facility with high efficacy particulate air (HEPA) -filtered rack under standard conditions (12 h light/dark cycles). The care and use of animals was reviewed and approved under Assurance Number A3873-1, with The Institutional Animal Care and Use

Committee (IACUC) protocol, based on the National Institutes of Health (NIH) Guide for the Care and Use of Animals, approved. A cocktail of anesthetics and analgesics [ketamine (20 mg/kg) (#11695-0702-1, Henry Schein, Inc., Melville, NY, USA), xylazine (15.2 mg/kg) (#59399-111-50, Akorn Operating Company LLC, Lake Forest, IL, USA), acepromazine maleate (0.48 mg/kg) (#0010-3827-01, Boehringer Ingelheim GmbH, Ingelheim, Germany)] was used for all surgical procedures to minimize animal distress.

Osteosarcoma orthotopic xenograft mouse model

143B-P or 143B-R cells [2.5×10^5 cells/5 μl PBS and 5 μl Matrigel Matrix (#354234, Corning Inc.)] were injected with 28G syringes (#329461, BD, Franklin Lakes, NJ, USA) into the left proximal tibia of ten mice each. The procedure was performed as described in previous reports (49, 50). Tumor size was measured twice a week with calipers and calculated with the following formula: tumor volume (mm³) = length (mm) × width (mm) × width (mm) × 1/2. All mice were sacrificed four weeks after cell injection. Tumor and lung samples were obtained to examine spontaneous metastases in the lungs macroscopically and with subsequent hematoxylin and eosin (H&E) staining.

H&E staining

From each mouse with a primary tumor in the tibia at necropsy, tumor tissue and lung tissues were obtained and fixed with 10% formalin (#SF99-20, Thermo Fisher Scientific) for 48 h, followed by embedding in paraffin (#PARA2, Cancer Diagnostics, Inc., Durham, NC, USA). The tissues were then sectioned, deparaffined, and rehydrated. H&E staining was performed according to standard protocols.

Statistical analysis

The Student's t-test was performed to statistically analyze the means between two groups, and the Fisher's exact test was performed to statistically evaluate the metastatic potential of 143B-P and 143B-R cells, with JMP pro ver. 15.0.0 (SAS Institute, Cary, NC, USA). IC₅₀ values were obtained with ImageJ ver. 1.53a (National Institutes of Health, Bethesda, MD, USA). Bar graphs show the mean and error bars show standard deviation of the mean. A probability value ≤ 0.05 was defined as a statistically-significant difference.

Results

Methionine-independent revertant osteosarcoma cells were selected from methionine-addicted parental osteosarcoma cells in low-methionine medium

rMETase decreased the level of methionine in the culture medium, in a concentration-dependent manner (Figure 1A). 143B-R cells were selected from 143B-P cells in low methionine media after 4 cycles of selection. 143B-R cells were more normal-fibroblast-like, becoming larger and more bipolar and aligned in direction than 143B-P cells, which is consistent with previous reports of other methionine-independent revertant cells (21) (Figure 1B). 143B-R cells became resistant to methionine restriction, induced by rMETase, compared to 143B-P, with the following IC_{50} : 143B-P: 0.20 U/ml; 143B-R: 0.68 U/ml ($P < 0.001$), which is consistent with previous reports of other methionine-independent revertant cells from various cancer types (15, 20, 22, 51) (Figures 1C, D).

Methionine-independent revertant osteosarcoma cells had reduced cell migration and invasion capacity

A wound healing assay and cell-invasion assay were performed to compare the cell migration and invasion capacity of 143B-R cells and 143B-P cells. 143B-R cells showed significantly decreased cell migration ($P < 0.01$) and invasion ($P = 0.018$) capacity, compared to 143B-P (Figure 2), indicating that methionine-independent 143B-R cells lost malignancy *in vitro*.

Methionine-independent revertant osteosarcoma cells had reduced tumor growth and lose metastatic potential *in vivo*

To compare the metastatic potential of 143B-P and 143B-R cells *in vivo*, orthotopic xenograft mouse models, in which 143B-P or 143B-R cells were injected in the proximal tibia, were

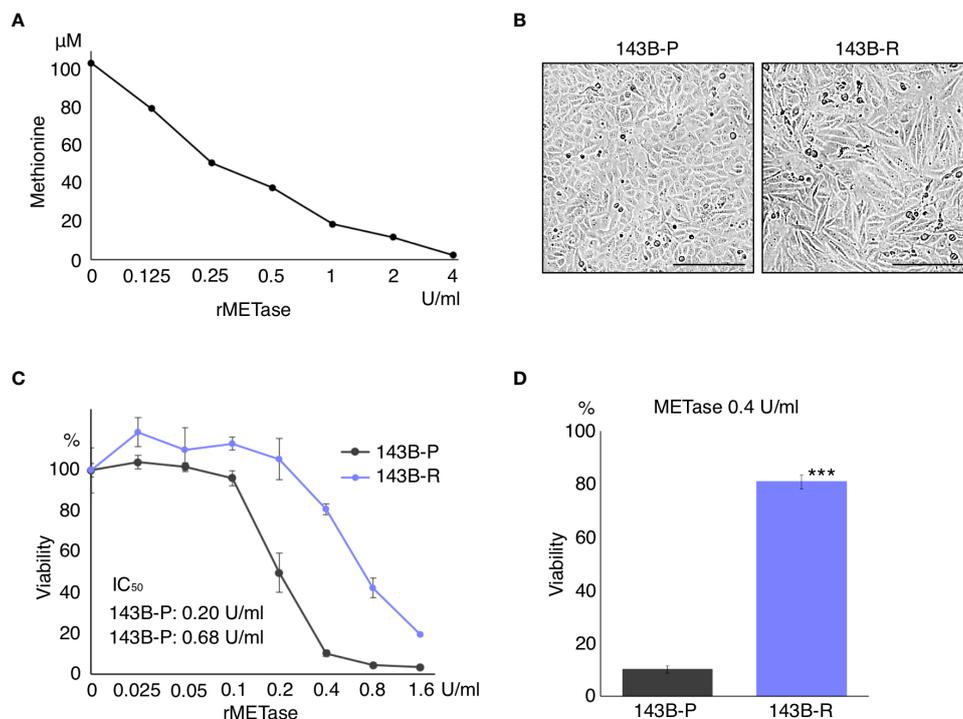


FIGURE 1

Methionine-independent revertant osteosarcoma cells become less-sensitive to methionine restriction. (A) Dose-dependent methionine depletion of DMEM effected by rMETase. The methionine level in DMEM was measured 3 h after rMETase was added. (B) Morphology of 143B-P and 143B-R cells. Scale bar in photomicrographs: 250 μm. Magnification: 100×. (C, D) Methionine-restriction sensitivity of 143B-P and 143B-R cells with rMETase. 143B-P: methionine-addicted parental 143B osteosarcoma cells, 143B-R: methionine-independent 143B osteosarcoma cells. *** $P < 0.001$.

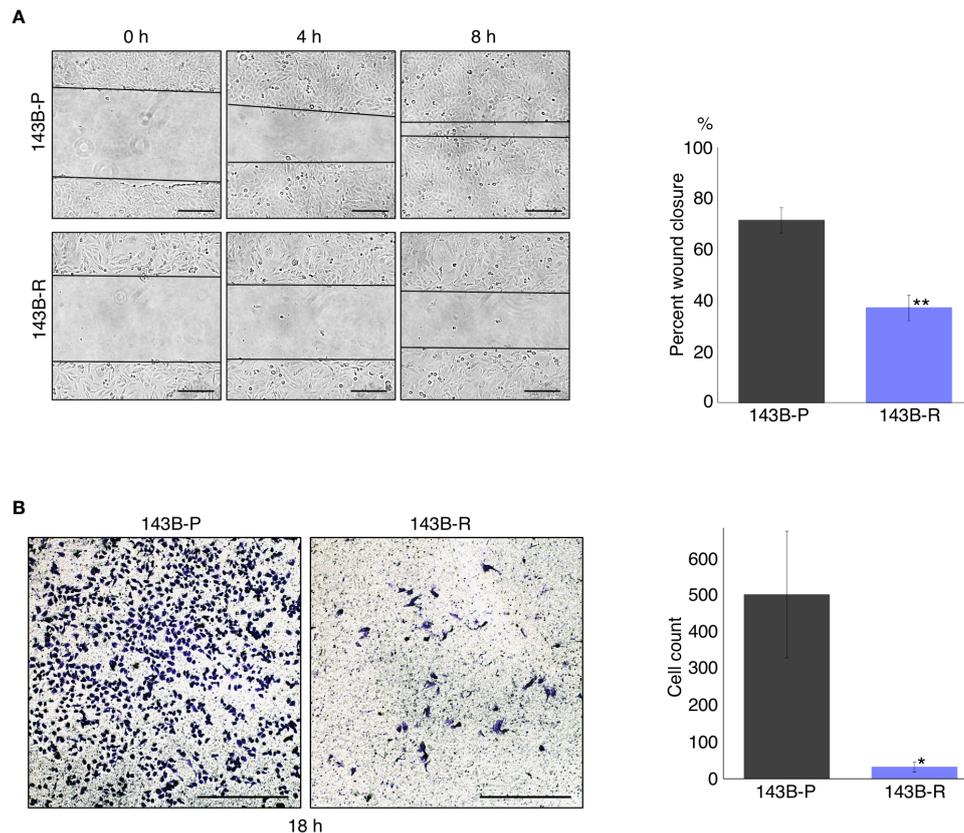


FIGURE 2

Methionine-independent revertant osteosarcoma cells have reduced cell migration and invasion capacity. **(A)** Cell migration capacity of 143B-P and 143B-R cells, in the wound-healing assay. Scale bar in photomicrographs: 250 μ m. Magnification: 100 \times . **(B)** Cell-invasion capacity of 143B-P and 143B-R cells, with the transwell assay. Scale bar in photomicrographs: 250 μ m. Magnification: 100 \times . 143B-P: methionine-addicted parental 143B osteosarcoma cells, 143B-R: methionine-independent 143B osteosarcoma cells. * $P < 0.05$, ** $P < 0.01$.

established and spontaneous lung metastasis from the tibia was examined. The 143B-R tumor size was significantly smaller than the 143B-P tumor size ($P = 0.034$), although both of them formed primary tumor tissue at the same ratio, 7 out of 10 mice (Figures 3A–C). Histological differences of the primary tumor tissues in 143B-P and 143B-R were not demonstrated with H&E staining (Figure 3D). Four different lung samples were then randomly obtained from each of the 7 mice with primary tumor. The lungs were examined for metastases from the primary tibia tumor. Macroscopically, 4 out of 7 lungs in 143B-P-injected mice had metastatic lesions, in contrast, no macroscopic metastatic lesions were seen in 143B-R mice (Figure 3E). Histological analysis demonstrated that 143B-P cells formed spontaneous lung metastases in 5 out of 7 mice, in contrast, 143B-R cells formed no metastases ($P = 0.021$) (Figure 3E). These results indicate that methionine-independent 143B-R cells lost malignancy *in vivo*, as well as *in vitro*.

Expression of an epithelial marker was increased and the expression of mesenchymal markers were decreased in methionine-independent revertant osteosarcoma cells

We then performed western immunoblotting to compare the gene expression levels related to the epithelial-mesenchymal phenotype in 143B-R cells and 143B-P cells. 143B-R cells showed gain of the epithelial marker, ZO-1 ($P = 0.012$) and loss of mesenchymal markers, vimentin ($P < 0.001$), Snail ($P < 0.001$), and Slug ($P < 0.001$), compared to 143B-P cells (Figure 4). These results indicate that EMT is, at least in part, related to the metastatic process in osteosarcoma, as well as carcinoma cells, which is consistent to previous reports (27–37, 46–48), and also suggest that the modulation of epithelial-mesenchymal phenotype may, at least in part, be related to the

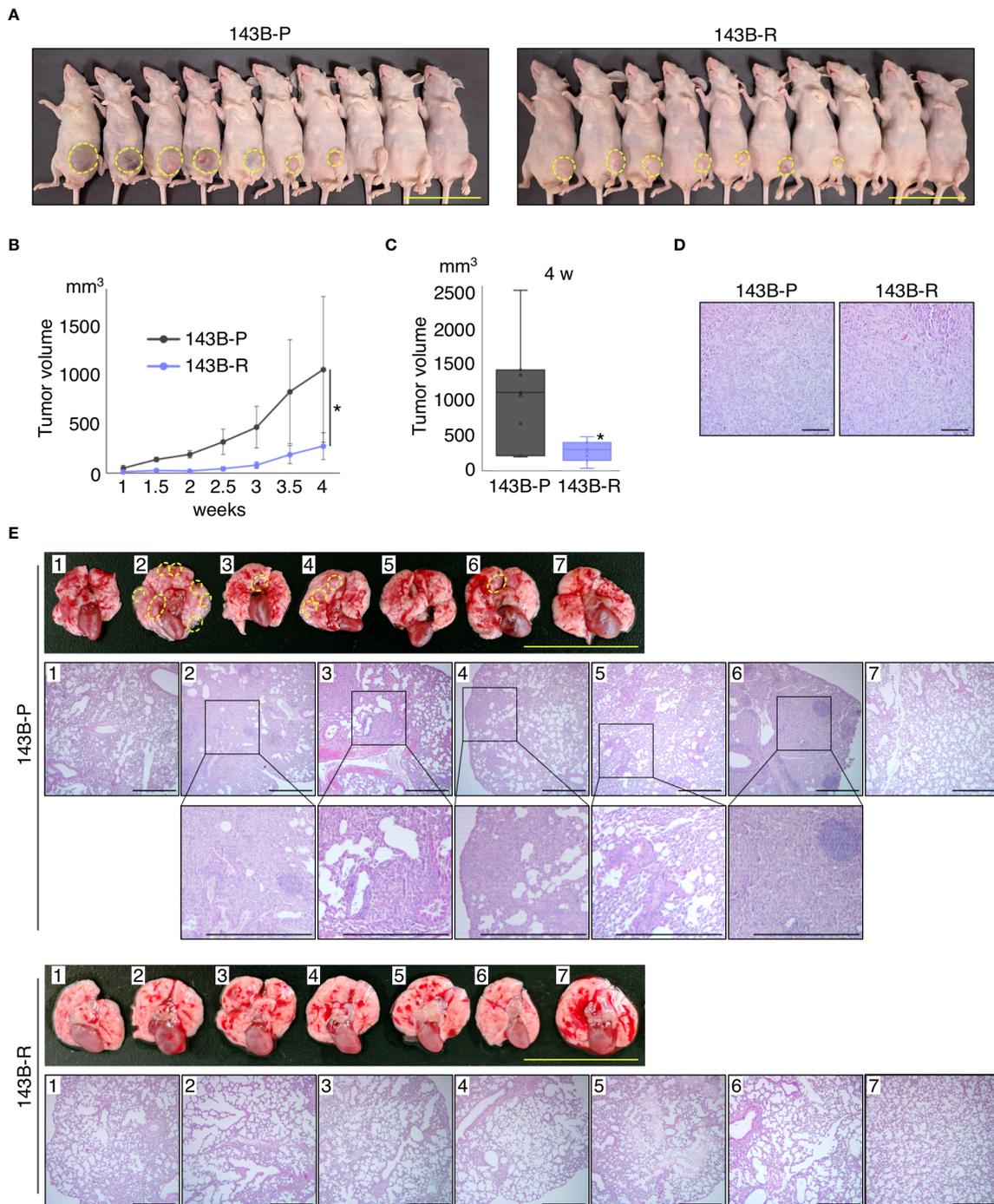


FIGURE 3 Methionine-independent revertant osteosarcoma cells have reduced tumor growth and lost metastatic potential, in orthotopic xenograft mouse models. **(A–C)** Tumor growth in the orthotopic xenograft mouse models of 143B-P or 143B-R cells. Dashed yellow lines show the edge of the tumor tissue. Scale bar: 50 mm. * $P < 0.05$. **(D)** Representative photomicrographs of H&E-stained primary tumor tissues in the tibia of 143B-P and 143B-R cells. Scale bar: 100 μ m. Magnification: 100x. **(E)** Spontaneous lung metastases from the tibia of 143B-P and 143B-R cells. Dashed yellow lines show metastatic lesions in the lung. Scale bar in photographs: 25 mm. Scale bar in photomicrographs: 500 μ m. Magnification of photomicrographs: 40x. 143B-P: methionine-addicted parental 143B osteosarcoma cells, 143B-R: methionine-independent 143B osteosarcoma cells.

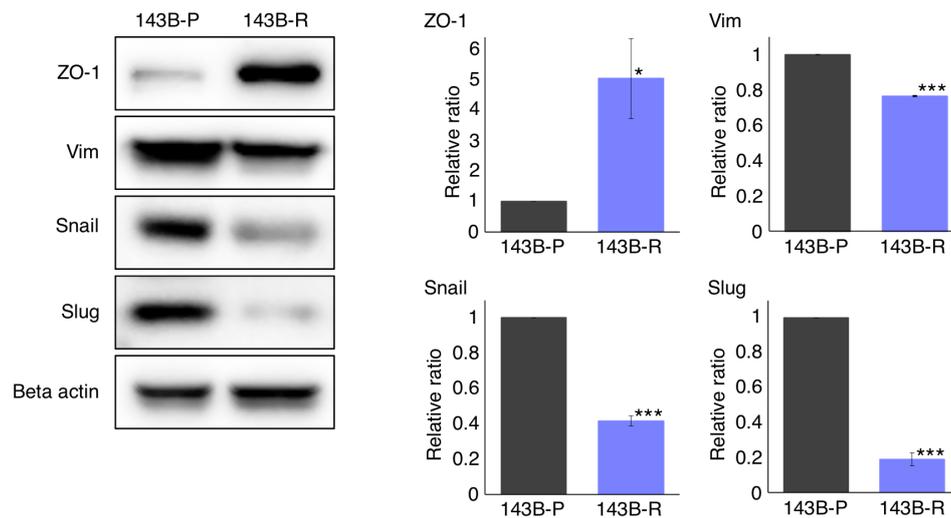


FIGURE 4

The expression of epithelial and mesenchymal markers in 143B-P and 143B-R cells, demonstrated with western immunoblotting. 143B-P: methionine-addicted parental 143B osteosarcoma cells, 143B-R: methionine-independent 143B osteosarcoma cells. * $P < 0.05$, *** $P < 0.001$.

relationship of methionine addiction and malignancy of osteosarcoma cells.

The status of histone-H3 lysine-methylation was significantly altered in methionine-addicted parental osteosarcoma cells and methionine-independent revertant osteosarcoma cells

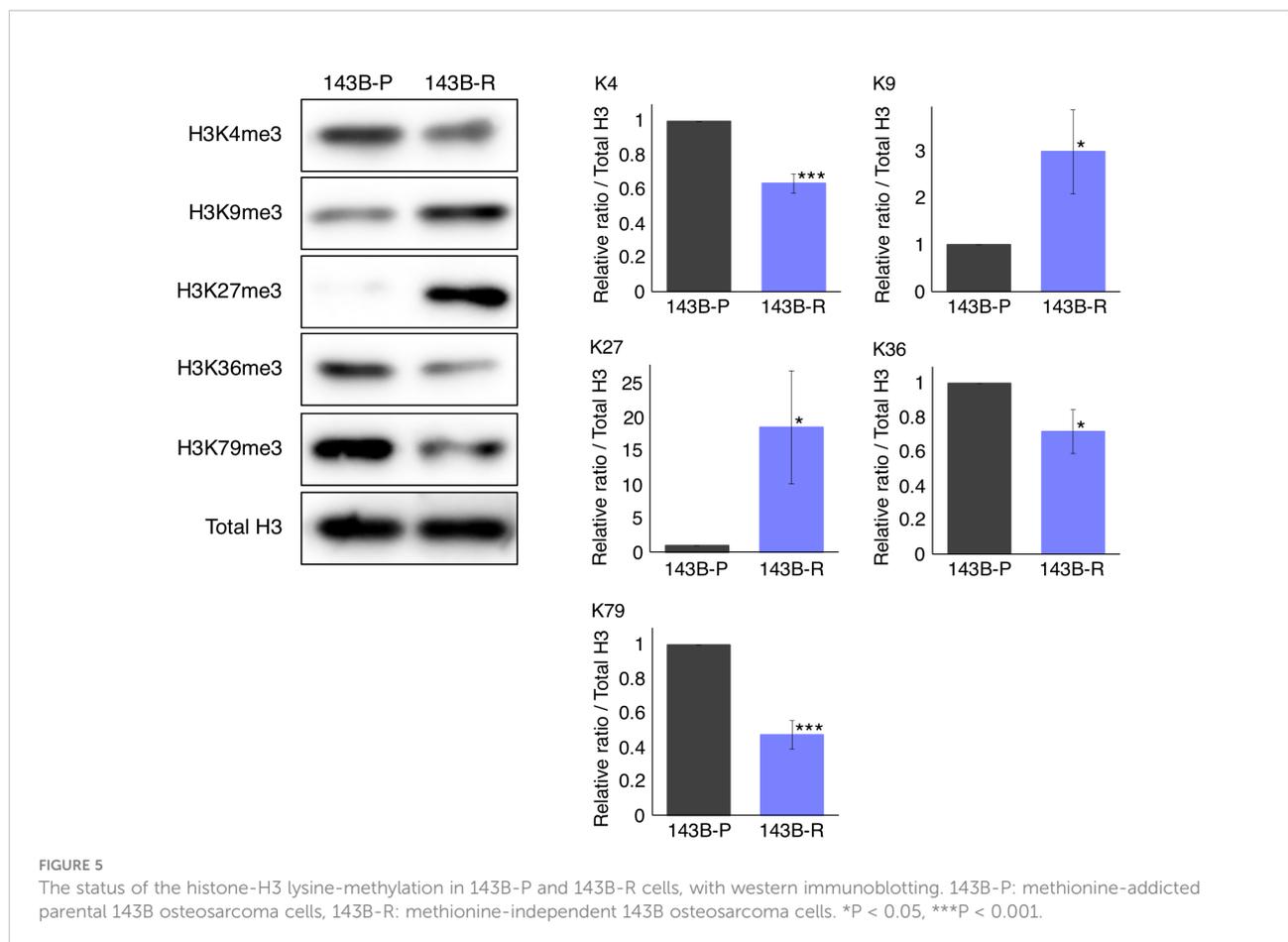
To compare the status of histone-H3 lysine-methylation in 143B-R and 143B-P, western immunoblotting was performed. The levels of histone H3K9me3 and H3K27me3 were increased ($P = 0.035$, $P = 0.042$, respectively) and the levels of histone H3K4me3, H3K36me3, and H3K79me3 were decreased ($P < 0.001$, $P = 0.036$, $P < 0.001$, respectively) in 143B-R cells, compared to 143B-P cells (Figure 5). These results suggest that unbalanced histone-H3 lysine-methylation status may be involved in the relationship of methionine addiction and malignancy of osteosarcoma cells.

Discussion

The present study showed that revertant 143B-R cells, selected from parental 143B-P cells long-term in low-methionine media, became more resistant to methionine restriction than 143B-P cells. 143B-R cells had reduced cell

migration and invasion capacity *in vitro* and reduced tumor growth and loss of metastatic potential *in vivo*, indicating they lost malignancy, which is consistent with other methionine-independent revertant cells selected from other methionine-addicted cancer types (13, 15, 16, 21, 22). These results strongly support the concept that methionine addiction is closely related to malignancy.

In the present study, histone H3K9me3 and H3K27me3 were increased, and histone H3K4me3, H3K36me3, and H3K79me3 were decreased, in 143B-R cells. Histone H3K9me3 and H3K27me3 are involved in gene repression and histone H3K4, H3K36, and H3K79 are involved in gene promotion (52–57). Although which of the histone-H3 lysine-methylation-modifications are directly involved in specific gene-modifications is still uncertain (58, 59), the results of the present study suggest that shifting the balance in histone methylases might be a way to decrease the malignant potential of cells, and the loss of malignancy of the methionine-independent revertant cells may be related to regulation of specific gene-expression changes *via* these histone-H3 lysine-mark modifications. The present study suggests that histone-H3 lysine-methylation may be involved in the relationship of malignancy and methionine addiction, as previously reported (15, 16). Although, in the present study, the western immunoblotting clearly shows the changes in histone-H3 lysine-methylation when 143B-P cells revert to 143B-R cells, future experiments will further investigate changes in histone-H3 lysine-methylation in 143B-P and 143B-R cells in IHC experiments. The mechanism of how the changes in the methylation of the histone-H3 lysine marks, including H3K4me3, H3K9me3, H3K27me3, H3K36me3, and H3K79me3,



affects properties of sarcoma will be investigated in future studies.

The histone-H3 lysine-trimethylation, including H3K4me3, H3K9me3, H3K27me3, H3K36me3, and H3K79me3, were all reduced in methionine-independent revertant cells of methionine-addicted HCT116 colon cancer cells and H460 lung cancer cells (15). In the present study, selection for 143B-R cells was performed more strictly and for a longer period than in previous studies, with 4 cycles of the following selection procedure repeated: cells were cultured in methionine depleting medium until almost all the cells died, followed by rescue of any remaining live cells in normal medium and subsequent passage. Moreover, 143B osteosarcoma cells are much more sensitive to methionine restriction than HCT116 colon cancer cells and H460 lung cancer cells (IC₅₀ of rMETase: 143B: 0.20 U/ml; HCT116: 0.71 U/ml; H460: 1.14 U/ml), suggesting 143B cells are more methionine-addicted than HCT116 colon-cancer cells or H460 lung-cancer cells (12). These differences might have contributed to selection of 143B-R cells with different histone-H3 lysine methylation changes from the revertant cells of HCT116 or H460 cells.

In the present study, 143B-R cells showed gain of an epithelial marker, ZO-1, and loss of mesenchymal markers, vimentin, Snail, and Slug, also contributing to the benign characteristics of 143B-R cells, which formed no spontaneous metastases in the lungs, in contrast to 143B-P cells. These results suggest that 143B-R cells have lost their metastatic potential, to which the modulation of epithelial-mesenchymal phenotype and alteration of histone-H3 lysine-methylation may be related. Changes in histone-H3 lysine-methylation, including H3K4me3, H3K9me3, H3K27me3, H3K36me3, and H3K79me3, are associated with epithelial-mesenchymal phenotype changes (60–62). Borek, almost 60 years ago, suggested unbalanced methylation is a basis of cancer (63). The present results support Borek's hypothesis.

The present study demonstrated that reversion of methionine addiction of cancer cells to methionine-independence results in loss of malignancy, and showed the modulation of the epithelial-mesenchymal phenotype and unbalanced histone-H3 lysine-methylation status are involved in the relationship of methionine addiction and malignancy. Methionine addiction of cancer is known as the Hoffman Effect (64). Future studies will focus more on the relationship of the

modulation of the epithelial-mesenchymal phenotype and histone-H3 lysine-methylation status to methionine addiction and malignancy, by upregulating or blocking the changes we have observed.

Data availability statement

Datasets are available on request: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee Assurance Number A3873-1.

Author contributions

YA, YT and RMH were involved in study conception and design. YA, QH, JY, YK, NM, KO, KH, and JW were involved in acquisition of data. YA, YT, JY, YK, NM, KO, SI, MB, SC, KN, and RMH analyzed and interpreted data. YA, YT, and RMH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

QH is an employee of AntiCancer Inc. YA, JY, YK, NM, KO, KH, and RMH are or were unsalaried associates of AntiCancer Inc.

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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