

# Extent and Instability of Trimethylation of Histone H3 Lysine Increases With Degree of Malignancy and Methionine Addiction

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**Abstract.** *Background/Aim:* Methionine addiction is a fundamental and general hallmark of cancer, termed the Hoffman effect. Methionine addiction is due to excessive use of and dependence on methionine by cancer cells. In the present report, we correlated the extent of methionine addiction and degree of malignancy with the amount and stability of methylated histone H3 lysine marks. *Materials and Methods:* We established low- and high-malignancy variants from a parental human pancreatic-cancer cell line and compared their sensitivity to methionine restriction and histone H3 lysine methylation status. *Results:* A low-malignancy, low-methionine-addiction revertant of the parental pancreatic-cancer cell line had less methylated H3K9me3 and was less sensitive to methionine restriction effected by recombinant methioninase (rMETase) than the parental cell line. A high-malignancy variant of the pancreatic cancer cell line had increased methylated H3K9me3 and was more sensitive to methionine

restriction by rMETase with regard to inhibition of proliferation and to instability of histone H3 lysine methylation than the parental cell line. Orthotopic malignancy in nude mice was reduced in the low-methionine-addiction revertant and greater in the high-malignancy variant than in the parental cell line. *Conclusion:* The present study indicates that the degree of malignancy is linked to the extent of methionine addiction and the level and instability of trimethylation of histone H3, suggesting these phenomena are linked as a fundamental basis of oncogenic transformation.

Methionine addiction (1) is a property of all types of cancer, termed the Hoffman effect (2-4). Methionine addiction of cancer has intense current interest, especially in the rapidly expanding field of diet and cancer (5-8). Methionine addiction is due to excess use of methionine by cancer cells for transmethylation reactions, resulting in dependence on exogenous methionine, despite high levels of endogenous synthesis of methionine (1, 9-11).

The Hoffman effect may be greater than the Warburg effect as seen in comparison of methionine- and glucose-based positron-emission tomography (PET) imaging of cancer (12). Methionine restriction results in a block in the late S/G<sub>2</sub> phase of the cell cycle only in cancer cells, where they become sensitized to DNA-synthesis inhibitors (13-15). Excess transmethylation reactions in cancer cells results in overmethylation of trimethylated histone H3 lysine marks (16-18). Histone H3 overmethylation in cancer cells, but not normal cells, is unstable under methionine restriction (17). Rare methionine-independent revertants, which were selected by methionine restriction, are able to grow in conditions with low methionine or with precursors of methionine in place of methionine (18-20). The methionine-independent revertants lose overmethylation of trimethylated-histone H3 marks, as well as their malignancy (18, 20, 21).

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**Key Words:** Methionine addiction, methionine dependence, methionine restriction, transmethylation, histone H3 lysine, overmethylation, low-methionine-addiction revertant, high-methionine addiction variants, malignancy.

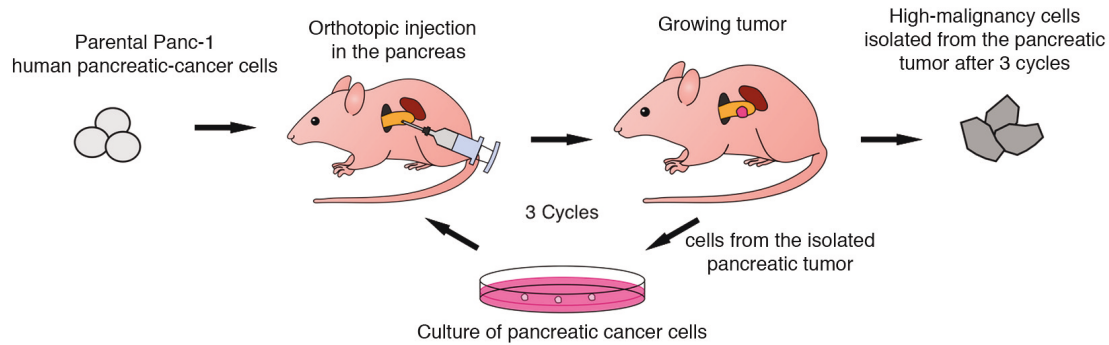


Figure 1. Diagram of the establishment of Panc-1-M cells through orthotopic-passage.

In the present report, we established a high-malignancy, highly methionine-addicted variant and a low-malignancy non-methionine-addicted revertant from the same parental methionine-addicted cancer cell line, in which we demonstrate that the extent of methionine addiction is linked to the degree of malignancy and to the level and instability of trimethylation of histone H3 lysine marks.

## Materials and Methods

**Cell culture.** Panc-1 human pancreatic cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and stably transduced to express green fluorescent protein (GFP) as previously described (21). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin/streptomycin.

**Recombinant methioninase production.** Methioninase [L-methionine  $\alpha$ -deamino- $\gamma$ -mercapto-methane lyase (rMETase)] is a pyridoxal-phosphate-dependent tetramer with each monomer having a molecular weight of 172 kDa. The methioninase gene was previously derived from *Pseudomonas putida* (22), and cloned in *Escherichia coli* (22, 23). The production procedure, including fermentation, heating step, polyethylene-glycol precipitation and DEAE-Sephrose chromatography are described in our previous publication (23).

**Isolation of low-methionine-addiction revertant cancer cells.** Rare low-methionine-addiction revertant Panc-1 cells (Panc-1-R) were selected by resistance to rMETase (18).

**In vivo isolation of high-malignancy variants.** Isolation of high-malignancy variants of Panc-1 (Panc-1-M) was performed as previously described by orthotopic passage three times in nude mice (Figure 1), which resulted in cells with high malignancy (24).

**Wound healing assay.** Cells were cultured in 6-well dishes ( $8 \times 10^5$  cells/well) and cell-free gaps were made by scratching a monolayer of 80% confluent cells with micro pipet tips. After washing the dishes three times with medium, the cells were incubated and the gap areas were measured at 12, 24 and 36 hours after scratching (25).

**Soft agar colony-formation assay.** In 6-well dishes, 2.5 ml of 0.7% agar in complete DMEM with 10% FBS and 100 IU/ml penicillin/streptomycin were placed as a lower layer. Then 5,000 cells were suspended in 2 ml of 0.3% agar in complete DMEM with 10% FBS and 100 IU/ml penicillin/streptomycin and layered on top of the bottom layer. Cells were cultured for 14 days and resulting colonies were stained with 0.01% crystal violet and counted (20).

**Efficacy of rMETase on viability of cancer cells in vitro.** Cells were cultured in 96-well plates ( $2 \times 10^3$  cells/well) and treated with rMETase (0.05 U/ml to 6.4 U/ml) for 96 hours. A Cell-Counting Kit-8 (Dojindo, Kumamoto, Japan) was used to construct growth curves and half-maximal inhibitory concentrations ( $IC_{50}$ ) of r-METase were calculated as described previously (17).

**Immunoblotting.** Anti-H3K9me1 (1:1,000, #14186; Cell Signaling Technology, Danvers, MA, USA); anti-H3K9me2 (1:1,000, #4658; Cell Signaling Technology) or anti-H3K9me3 (1:1,000, #13969; Cell Signaling Technology) were used as primary antibodies. Total histone H3 (1:5,000, 17168-1-AP; Proteintech, Rosemont, IL, USA) was used as a loading control. Previously-described techniques were used for histone extraction, immunoblotting and signal detection (17, 26-28).

**Determination of tumorigenicity of parental cancer cells, low-methionine-addiction revertants and high-malignancy cancer cells in an orthotopic mouse model.** Nude-mouse (AntiCancer Inc, San Diego, CA, USA) studies were performed with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol following the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1 (26).

Panc-1 cells, Panc-1-R cells and Panc-1-M cells ( $0.5 \times 10^6$  or  $1 \times 10^6$  cells/50  $\mu$ l PBS) were injected orthotopically into the pancreas of five nude mice and grown for 42 days, at which point the tumor weight was measured.

**Statistical analyses.** JMP PRO ver. 15.0.0 (SAS Institute, Cary, NC, USA) was used for statistical analysis. Comparisons between groups of used the Mann-Whitney *U*-test. Error bars on the graphs represent the standard error of the mean. A probability value of  $p < 0.05$  was defined as statistically significant.

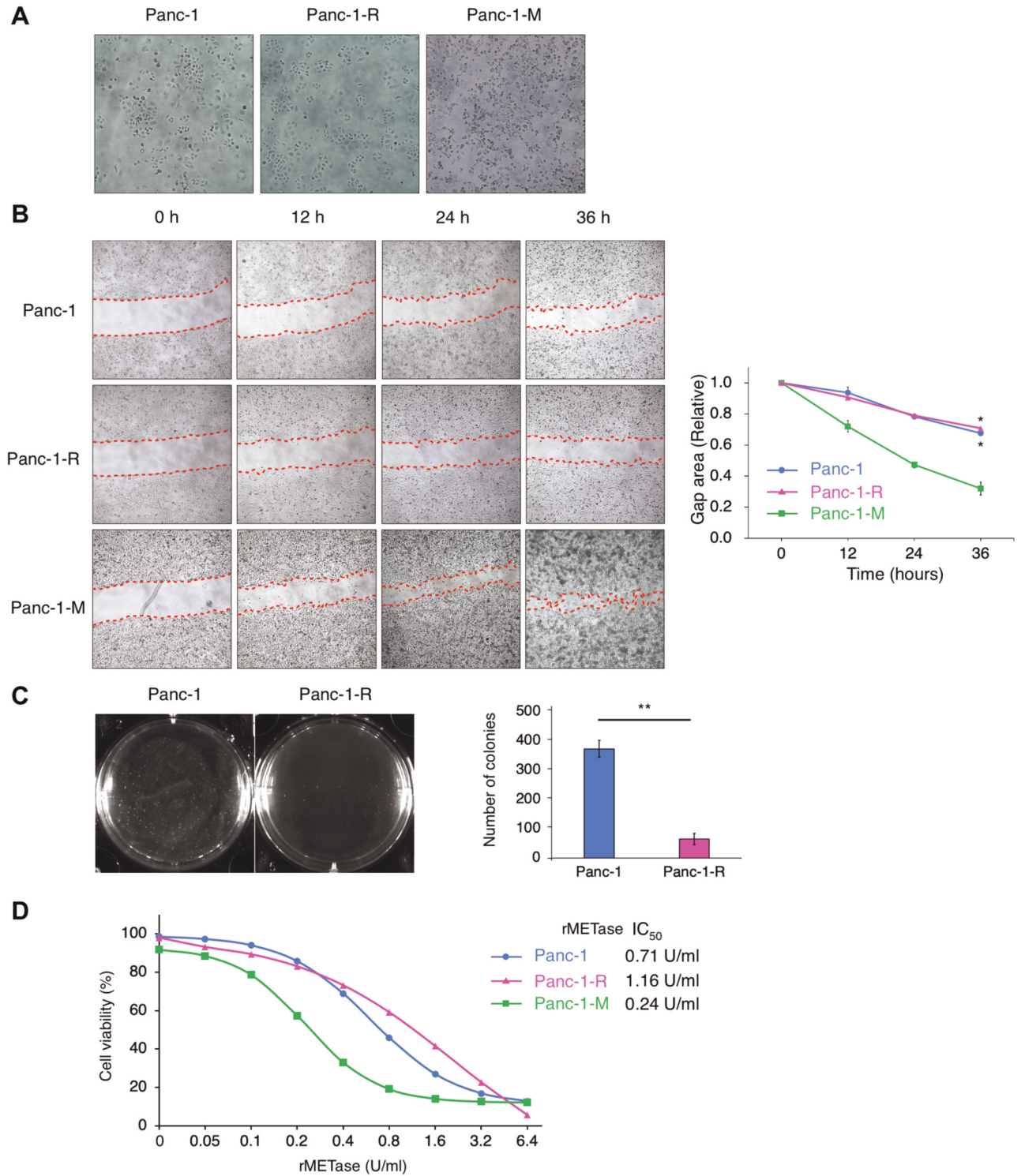


Figure 2. Comparison of malignancy between parental Panc-1 cells, low-methionine-addiction revertant Panc-1-R cells and high-malignancy Panc-1-M cells *in vitro*. A: Morphology of parental Panc-1 cells, Panc-1-R cells and Panc-1-M cells ( $\times 100$ ). B: Wound-healing assay in Panc-1 cells, Panc-1-R cells and Panc-1-M cells. Left: Representative images at 0, 12, 24 and 36 hours after the wounding scratch was made ( $\times 40$ ). Right: Graph of the relative gap area (mean $\pm$ SEM,  $n=3$ ). \*Significantly different from Panc-1-M cells at  $p=0.001$ . C: Colony-formation assay of parental Panc-1 cells and Panc-1-R cells. Left: Representative image at 14 days of culture. Right: Bar graph of the number of colonies at 14 days of culture (mean $\pm$ SEM,  $n=3$ ). \*\* $p<0.0001$ ). D: Sensitivity to recombinant methioninase (rMETase). Parental Panc-1 cells, Panc-1-R cells and Panc-1-M cells were cultured for 96 hours with different concentrations of rMETase ( $n=3$ ).  $IC_{50}$ : Half-maximal inhibitory concentration.



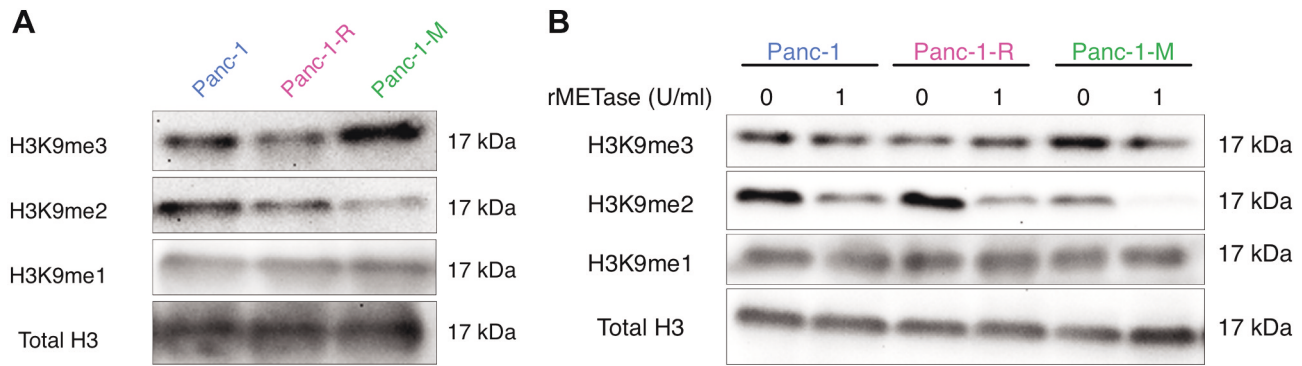


Figure 3. Comparison of histone H3 lysine methylation between parental Panc-1 cells, low-methionine-addiction revertant Panc-1-R cells and high-malignancy Panc-1-M cells *in vitro*. A: Immunoblot of H3K9me1, H3K9me2 and H3K9me3 in Panc-1, Panc-1-R and Panc-1-M cells. B: Immunoblot of H3K9me1, H3K9me2 and H3K9me3 in parental Panc-1 cells, Panc-1-R cells and Panc-1-M cells treated with (1) or without (0) 1 U/ml of rMETase.

## Results

*In vitro* malignancy characteristics and methionine addiction are elevated in orthotopically-passaged cancer cells and are reduced in low-methionine-addiction revertants. We first compared malignancy characteristics between parental Panc-1 cells, Panc-1-R low-methionine-addiction revertant and orthotopically-passaged Panc-1-M cells *in vitro*. There was no apparent difference of morphology between parental Panc-1 cells and Panc-1-R cells. In contrast, the morphology of the Panc-1-M cells changed from round to spindle shape (Figure 2A). There was no difference in the speed of wound healing between parental cells and Panc-1-R cells. In contrast, the speed of wound healing was faster in Panc-1-M cells compared to parental Panc-1 cells and Panc-1-R cells ( $p=0.001$ ) (Figure 2B). The number of the colonies formed in soft agar was significantly lower in Panc-1-R cells compared to parental Panc-1 cells ( $p<0.0001$ ) (Figure 2C). These results indicate that malignancy was elevated in the orthotopic-passaged Panc-1-M cancer cells and reduced in Panc-1-R cells compared to parental Panc-1 cells *in vitro*.

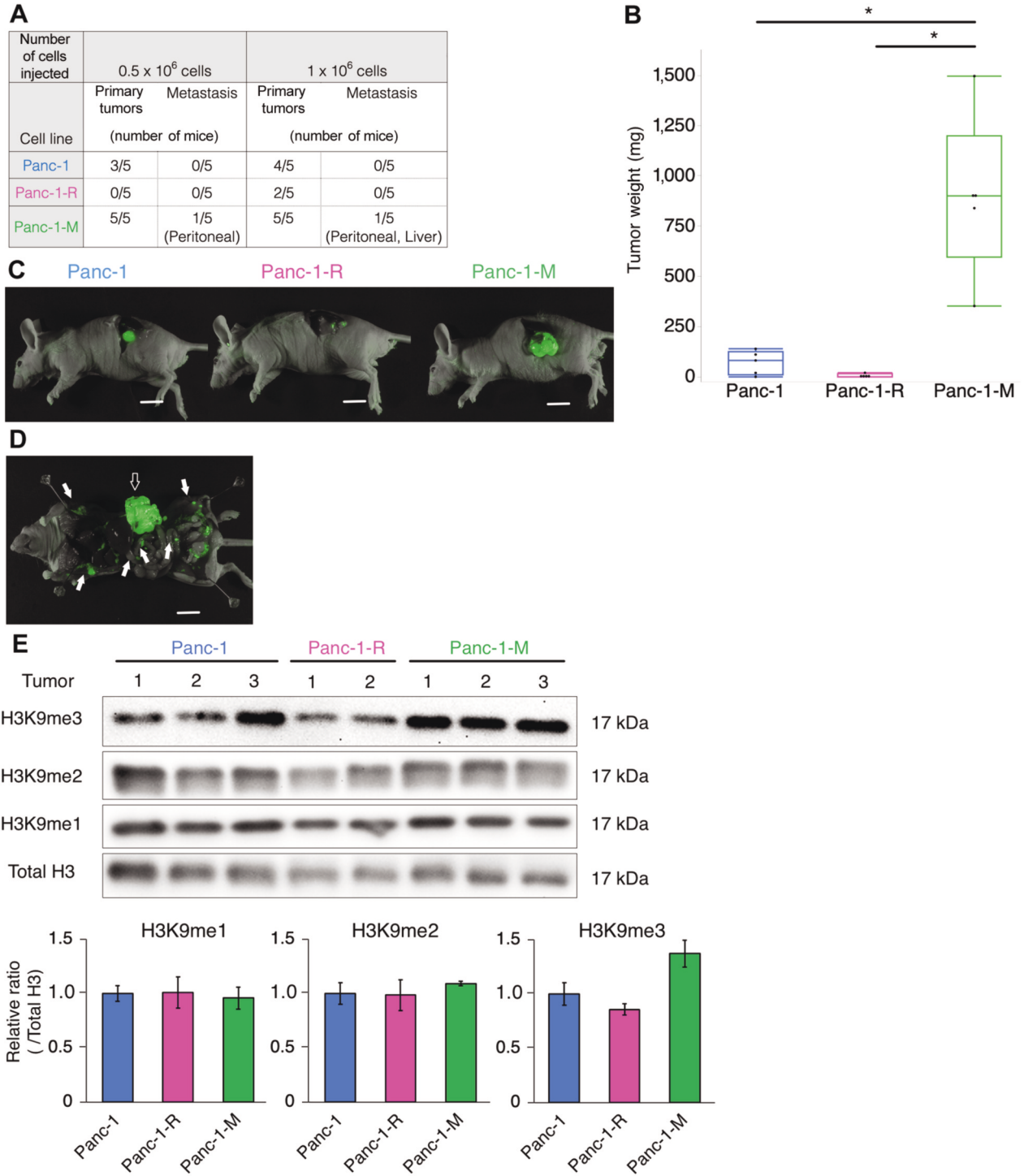
To evaluate the methionine addiction of parental Panc-1 cells, low-malignancy Panc-1-R cells and high-malignancy Panc-1-M cells, their sensitivity to rMETase was compared. The half-maximal inhibitory concentration ( $IC_{50}$ ) of rMETase was higher in Panc-1-R cells and lower in Panc-1-M cells than parental Panc-1-cells respectively ( $IC_{50}$ : Panc-1: 0.71 U/ml; Panc-1-R: 1.16 U/ml; Panc-1-M: 0.24 U/ml) (Figure 2D). These results indicate that the higher the malignancy of the cells, the more they are addicted to methionine as indicated by the increased sensitivity to methioninase.

*Methionine addiction is linked to malignancy and the overmethylation of H3K9me3 in vitro*. We then compared the histone methylation status of H3K9 marks between parental

Panc-1, Panc-1-R and Panc-1-M cells cultured *in vitro*. The level of H3K9me3 was elevated in Panc-1-M cells and was reduced in Panc-1-R cells compared to parental Panc-1 cells (Figure 3A). The level of H3K9me2 was reduced in high-malignancy Panc-1-M cells compared to parental Panc-1 cells and Panc-1-R cells. There was no apparent difference in the levels of H3K9me1 between the three types of the cells.

We also compared the stability of histone methylation status under rMETase action. The level of H3K9me3 was reduced by rMETase in parental Panc-1 cells and Panc-1-M cells. In contrast, the level of H3K9me3 was not altered by rMETase in Panc-1-R cells (Figure 3B). The level of H3K9me2 was reduced in all three cell lines by rMETase. The level of H3K9me1 was not apparently altered by rMETase in any of the cell lines. These results indicate that methionine addiction is linked to malignancy and unstable overmethylation of H3K9me3 *in vitro*.

*Methionine addiction and the level of H3K9me3 are linked to degree of tumorigenicity*. To compare the *in vivo* malignancy of parental Panc-1 cells, low-methionine-addiction revertant Panc-1-R cells and high-malignancy, high methionine-addicted Panc-1-M cells, the tumorigenicity and metastatic capability of these cells was compared in an orthotopic xenograft mouse model. Panc-1-M cells formed tumors in 5/5 nude mice when  $0.5 \times 10^6$  cells were injected, compared to 3/5 mice with tumors from Panc-1 and 0/5 mice with tumors from Panc-1-R (Figure 4A). The mean tumor weight was significantly higher in Panc-1-M tumors compared to Panc-1 and Panc-1-R tumors after injection of  $1 \times 10^6$  cells in the nude-mouse pancreas ( $p<0.001$ ) (Figure 4B and C). Only the Panc-1-M cells formed metastases (Figure 4D). Immunoblotting showed that the level of H3K9me3 was lower in Panc-1-R tumors and higher in Panc-1-M tumors than in parental Panc-1 tumors (Figure 4E). These results indicate that the extent of methionine addiction is linked to the degree of malignancy and to overmethylation of H3K9me3 *in vivo*.



## Discussion

The present report increases our understanding of the relationship of the methionine addiction of cancer, first discovered in 1976 by one of us (RMH) (1), malignancy and histone H3 lysine methylation. A crucial aspect of the present study was to isolate both low-malignancy and high-malignancy variants of the pancreatic cancer cell line Panc-1. The low-malignancy variant was selected by resistance to methioninase, and thereby the isolated variant developed low-methionine addiction and was termed a ‘methionine-independent revertant’ of methionine-addicted cells (17-20). The high-malignancy variant was isolated by several passages of the parental cells orthotopically from nude mouse to nude mouse. The low-methionine-addiction revertant Panc-1-R lost tumorigenicity at  $0.5 \times 10^6$  cells /mouse; the parental cells were intermediate in tumorigenicity and the high-malignancy Panc-1-M cells had 100% tumorigenicity and only Panc-1-M was able to form metastases. The methioninase- sensitivity studies showed that methionine addiction is linked to the degree of malignancy in the present report. Breillout *et al.*, more than 30 years ago, found that cancer cells selected for increasing malignancy required more methionine (29). The present study, which isolated both low- and high-malignancy variants of Panc-1, has shown that methionine addiction is linked to malignancy. Wang *et al.* also showed that tumor-initiating cells are more addicted to exogenous methionine than non-tumor-initiating cancer cells (7), confirming our original results (1).

Our recent study showed that methionine-independent revertants isolated from methionine-addicted cancer cells lose the overmethylation of trimethylated histone H3 marks, and their malignancy (18). We had also shown earlier that low-methionine-addicted revertants lost malignancy characteristics (20, 21). Raboni *et al.* confirmed that methioninase treatment results in loss or reduction of histone lysine marks in methionine-addicted cancer cells (4). The present study is the first in which the histone methylation status and stability, and malignancy were compared between three types of malignancy variants which have different dependence on methionine.

The present study demonstrates that methylation of H3K9me3 increases with malignancy as well as with methionine addiction starting from low-malignant low-methionine-addiction revertant cells to high-malignancy high-methionine-addiction cells. These results suggest that methionine addiction is linked to overmethylation of H3K9me3 and both are linked to malignancy, suggesting methionine addiction and overmethylation are a fundamental basis of oncogenic transformation (14, 27-29).

## Conflicts of Interest

JY, YA, SI, KH, YT and RMH are or were unsalaried associates of AntiCancer Inc. QH is an employee of AntiCancer Inc. The Authors declare that there are no potential conflicts of interest.

## Authors' Contributions

JY and RMH designed and performed experiments and wrote the paper; YA, SI, QH, KH, YT, KM, RM, MB and SGC gave technical support and conceptual advice. Writing, review, and/or revision of the article: JY, IE and RMH.

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