KO MAT2A to further inhibit hypomorphic PRMT5 in MATP-deficient TNBC cells for metastasis suppression in vitro and in vivo

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Abstract

Triple-negative breast cancer is one of the common types with the lack of three joint receptors, ER, PR, and HER2, which leads to inadequate responses for current breast cancer targeting therapy. Previous studies have reported that MTAP deletion confers enhanced sensitivity for PRMT5 inhibition, and inhibition of PRMT5 suppresses cell growth by decreasing arginine methylation of eIF4E and FGFR3. The KO of MAT2A will reduce the level of SAM, which is the substrate for arginine methylation, to inhibit PRMT5 and reduce cell proliferation. This study investigates the comparison of effects for MAT2A KO in MATP-deficient and MTAP+/+ TNBC cells in vivo and in vitro..

Keywords: Protein arginine methyltransferase V (PRMT5), Methylthioadenosine (MTA), Methylthioadenosine phosphorylase (MATP), Methionine adenosyltransferase II alpha (MAT2A), Triple-negative breast cancer (TNBC), S-adenosylmethionine (SAM), eIF4E, FGFR3

1. INTRODUCTION

Triple-negative breast cancer (TNBC) is a specific subtype of breast cancer that lacks or expresses a low level of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Compared to other types of breast cancer, TNBC has higher invasiveness and higher metastatic potential. The 5-year relative survival rate from diagnosis of the localized stage is over 90%. However, around 25% of those cases will relapse with distant metastasis with a 12% 5-year survival rate. Because of the absence of three common receptors, the targeted therapies for TNBC are limited and inefficient. Therefore, a more suitable treatment for TNBC is needed to be developed. Surprisingly, methylthioadenosine phosphorylase (MTAP) is a frequent loss in breast cancer cells, informing a potential therapy for TNBC MTAP-deficient tumor cells.

MTAP is a critical rate-limiting enzyme in the methionine and adenine salvage pathway, and it is located proximate to tumor suppressor genes, CSKN2A, in DNA sequence. Therefore, MTAP could be frequently deleted with the tumor suppressor gene CSKN2A in the cancer cell. Based on the previous research, the loss of MTAP lead to the accumulation of MTAP substrate MTA intracellularly. MTA is a SAM-competitive inhibitor of PRMT5, which negatively regulates PRMT5 activity by binding to the PRMT5 substrate binding site. PRMT5 is an oncogene that plays an essential role in cancer cell survival and proliferation. It facilitates the transfer of the methyl group from SAM to arginine residues of histone H3 and H4 to regulate the transcription of the relative gene. To overcome the increasing inhibitor MTA, the cancer cell needs to produce more PRMT5 to sustain normal cell activity. Hence, a higher PRMT5 expression level is required for normal cell proliferation in MTAP-deficient cancer cells. The hypomorphic PRMT5 by MTAP loss enhances the genetic dependency of PRMT5 in cancer cells. One of the most important methods for treating cancers is controlling the genetic dependency of cancer cells. Hence, the enhancing dependency of PRMT5 of MTAP deficient cancer cells reveals the potential vulnerability of TNBCs.

Oncogene FGFR3 and eIF4E are oncogenes that play an essential role in cell proliferation and thus promote tumorigenesis. PRMT5 regulates the expression of FGFR3 and eIF4E via the methylation of histone on promoters. The downregulation of MTAP suppresses the expression of those oncogenes and thus inhibits cell proliferation and promotes apoptosis.

Methionine adenosyltransferase II alpha (MAT2A) produces PRMT5 substrate SAM for methylation activity. The knockdown of MAT2A results in reduced substrate SAM concentrations. As the accumulated SAM-competitive inhibitor MTA inhibits PRMT5 from binding to SAM in MTAP-deficient cells, the further downregulating SAM level by MAT2A KO should have a significant inhibition effect on PRMT 5 catalytic methylation activity and thus suppresses cell proliferation. Therefore, to test the hypothesis that the inhibition of PRMT5 in MTAP-deficient TNBC cells by MAT2A KO will have a stronger antitumor effect through MAT2A/PRMT5/eIF4E&FGFR3 axis compared to $MTAP^{+/+}$ TNBC

cells, a systematic experiment is designed. This paper focuses on investigating the effect of inhibition of PRMT5 by knockdown of MAT2A in MTAP-deficient TNBC and $MTAP^{+/+}$ TNBC cells *in vitro* and in *vivo*.

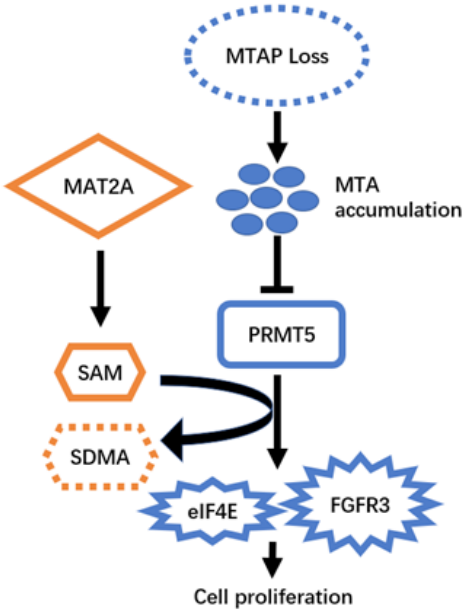

Figure 1: MAT2A/PRMT5/eIF4E&FGFR3 axis. This figure shows the vulnerability of PRMT5 in MTAP loss cells and the potential mechanism for further inhibition by MAT2A KO.

2. Methods

2.1 Cell culture

MTAP-deficient human triple-negative breast cancer cells (MDA-MB231) and $MTAP^{+/+}$ human triple-negative breast cancer cell line (MDA-MB435) will be maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin and streptomycin at 37°C in 5% CO_2 humidified environment.

2.2 Animal model

Eight-week-gold female mice with 25 g in weight will be used for the study. The cultured MDA-MB231 and MDA-MB435 will be harvested and re-suspended in vitro. The animals will be separated into two groups and injected with 50 µl of the cell suspension to produce MDA-MB231 and MDA-MB435 xenografted models, respectively.

2.3 CRISPR/Cas9 genome editing

SgRNA that targets ACGAGGCGTTCATTGAGGAG GGG of MATA generated from previous research will be used. The sgRNA will be cloned into Addgene Plasmid 104994 to produce lenti-SpCas9-Puro-MAT2A.

Each cell line and xenograft model will be divided into three groups: (1) negative control: nonsense targeting (transduced with lenti-SpCas9-2A-Puro（empty vector）); (2) KO MAT2A (transduced with lenti-SpCas9-Puro-MAT2A); (3) Positive control: nonsense targeting with Taxol treatment.

2.4 Flow cytometry

The newly synthesized DNA will be targeted by Invitrogen Click-iT EdU Flow Cytometry Assay Kits to estimate cell proliferation referencing to ThermoFisher Scientific. This assay will be applied to samples collected from each cell line after the treatment.

2.5 Real-time quantitative PCR

Total RNA of cells for each cell line will be quantified using NanoDrop ND-2000 spectrophotometer according to Thermo Fisher Scientific. The extracted RNA will be converted to cDNA by reverse transcription by PrimeScript 1st strand complementary DNA synthesis kit. Real-time quantitative RT-PCR will be carried out by IQ SYBR offered by Bio-Rad Laboratories. The expression level of MTAP, MAT2A, PRMT5, eIF4E, and FGFR3 will be determined. cDNA will be amplified using the primers according to Integrated DNA Technology. The RT-qPCR result will be performed using the comparative ΔΔCt method following the manufacturer's instructions.

2.6 Western Blot

Protein will be extracted through cell lysis with FLAG-IP lysis buffer (50 mM tris, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol). The prepared sample will be mixed with SDS-PAGE 2x Sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 0.02% bromophenol blue) to run SDS-PAGE gel electrophoresis. The gels from SDS-PAGE will be soaked in western blotting buffer (25 mM tris, 192 mM glycine, 20% ethanol) for 5 min and assembled to run western blot transfer. The membrane will then be incubated with a blocking agent, primary antibody, and secondary antibody, according to Thermo Fisher Scientific.

2.7 Statistical Analysis

Statistical analysis will be performed using the student's T-Test on GraphPad Prism®, and p values of less than 0.05 will be considered statistically significant.

3. Results

With the combination of three possible results (decrease, greatly decrease, or no change) for every three measurements (Cell proliferation, Histone methylation, oncogene expression), 27 possible results are predicted. There will be 27 possible results for each cell line, *in vivo*

or *in vitro*. 531,441 possible result is expected for three measurements applying on $MTAP^{+/+}$ and $MTAP^{-/-}$ cell lines, *in vivo* or *in vitro*

Possible results :27^4=531,441

As the number of total possible results is too large to discuss, we focus on only 7 possible results in this study. Those possible results of interest are picked and predicted with a higher possibility referring to the previous study. Seven possible results are predicted and summarized in Table 1.

Table 1. Possible results

	Result 1	Result 2	Result3	Result4	Result5	Result6	Result7
Cell proliferation							
$MTAP^{+/+}$ in vivo	+	-	++	+	-	+	-
$MTAP^{-/-}$ in vivo	++	++	++	+	-	++	-
$MTAP^{+/+}$ in vitro	+	-	++	+	-	-	-
$MTAP^{-/-}$ in vitro	+	++	++	+	-	-	-
Histone methylation							
$MTAP^{+/+}$ in vivo	+	-	++	-	+	+	-
$MTAP^{-/-}$ in vivo	++	++	++	-	++	++	-
$MTAP^{+/+}$ in vitro	+	-	++	-	+	-	-
$MTAP^{-/-}$ in vitro	++	++	++	-	++	-	-
eIF4E & FGFR3 expression							
$MTAP^{+/+}$ in vivo	+	-	++	-	+	+	-
$MTAP^{-/-}$ in vivo	++	++	++	-	++	++	-
$MTAP^{+/+}$ in vitro	+	-	++	-	+	-	-
$MTAP^{-/-}$ in vitro	++	++	++	-	++	-	-

"+" represents a decrease ("++" represents a more significant decrease).

"-" represents not significantly different from the negative control.

3.1 Possible Results 1: Applying MAT2A KO inhibits the TNBC cell proliferation in both $MTAP^{+/+}$ cell line (MDA-MB435), $MTAP^{-/-}$ cell line (MDA-MB231), and cell lines from MDA-MB231 xenografted models and MDA-MB435 xenografted models with the downregulation of histone methylation, eIF4E, and FGFR3 expression level, while the inhibition effect on $MTAP^{-/-}$ TNBC cell is more significant than $MTAP^{+/+}$ TNBC cell.

CRISPR/Cas9-MAT2A model knockout the MAT2A in all cell samples, inhibiting histone methylation. The expression level of eIF4E and FGFR3 decreases, and the proliferation of cell samples is inhibited. The less intensive band responding to methylated histone on Western blot gel and smaller relative mRNA expression value for both eIF4E and FGFR3 will be observed on $MTAP^{-/-}$ cell line sample compared to the $MTAP^{+/+}$ cell line sample. The anti-tumorigenesis effect is more critical in MTAP deficient TNBC cells.

3.2 Possible Results 2: Applying MAT2A KO inhibits the TNBC cell proliferation in MTAP-/- cell line (MDA-MB231) and cell line from MDA-MB231 xenografted models with the downregulation of histone methylation, eIF4E, and FGFR3 expression level, but not MTAP+/+ (MDA-MB435) cell line, and MDA-MB435 xenografted models.

CRISPR/Cas9-MAT2A model knockout the MAT2A in $MTAP^{-/-}$ cell line sample, inhibiting histone methylation. The weaker band of methylated histone on gel and smaller relative mRNA expression values for both eIF4E and FGFR3 will be observed on $MTAP^{-/-}$ cell line sample compared to the control group. A similar band for methylated histone and no difference in relative mRNA expression value compared to the control group will be observed on the $MTAP^{+/+}$ cell line sample.

3.3 Possible Results 3: Applying MAT2A KO inhibits the TNBC cell proliferation in both MTAP+/+ (MDA-MB435), MTAP-/- cell line

(MDA-MB231), and cell lines from MDA-MB231 xenografted models and MDA-MB435 xenografted models with the downregulation of histone methylation, eIF4E, and FGFR3 expression level, while the inhibition effect is similar in both cell lines.

CRISPR/Cas9-MAT2A model knockout the MAT2A in all cell samples, inhibiting histone methylation. The expression level of eIF4E and FGFR3 is decreased, and the proliferation of cell samples is inhibited. The weak bands responding to methylated histone on gel and smaller relative mRNA expression values for both eIF4E and FGFR3 will be observed on $MTAP^{-/-}$ and $MTAP^{+/+}$ cell line samples.

3.4 Possible Results 4: Applying MAT2A KO inhibits the TNBC cell proliferation in MTAP+/+ (MDA-MB435), MTAP-/- cell line (MDA-MB231), and cell lines from MDA-MB231 xenografted models and MDA-MB435 xenografted models with no change of histone methylation, eIF4E, and FGFR3 expression level.

CRISPR/Cas9-MAT2A model knockout the MAT2A in all cell samples, inhibiting cell proliferation. The reduced cell proliferation will be observed in the flow cytometry result for all cell samples, while the similar bands for methylated histone and no difference in relative mRNA expression value compared to the control group will be indicated.

3.5 Possible Results 5: Applying MAT2A KO has no effect on cell proliferation in MTAP+/+ (MDA-MB435), MTAP-/- cell line (MDA-MB231), and cell lines from MDA-MB231 xenografted models and MDA-MB435 xenografted models, but downregulates histone methylation, eIF4E, and FGFR3 expression level.

CRISPR/Cas9-MAT2A model knockout the MAT2A in all cell samples, inhibiting histone methylation indicated by the weak band for methylated histone. The expression level of eIF4E and FGFR3 is decreased, indicated by a small relative mRNA expression value. However, flow cytometry results show no effect on cell proliferation.

3.6 Possible Results 6: Applying MAT2A KO inhibits the TNBC cell proliferation in MTAP+/+ (MDA-MB435) and MTAP-/- cell line (MDA-MB231) in vivo with downregulation of histone methylation, eIF4E, and FGFR3 expression level, but not cell lines from MDA-MB231 xenografted models and MDA-MB435 xenografted models.

CRISPR/Cas9-MAT2A model knockout the MAT2A in the *in vitro* cell samples, inhibiting histone methylation. The expression level of eIF4E and FGFR3 is decreased, and the proliferation of cell samples is inhibited. However, the MAT2A KO has no effect on *in vivo* cell sample. A similar band is observed on the gel for in vivo sample as the control group.

3.7 Possible Results 7: Applying MAT2A KO has no effect on cell proliferation in both MTAP+/+ (MDA-MB435), MTAP-/- cell line (MDA-MB231), and cell lines from MDA-MB231 xenografted models and MDA-MB435 xenografted models, and no change in histone methylation, eIF4E, and FGFR3 expression level.

MAT2A knockout in all cell samples does not affect cell proliferation, histone methylation, EIF4E expression, and FGFR3 expression. The experiment results for all cell lines are similar to the negative control.

4. Discussion

A previous study reveals that MTAP loss leads to accumulated MTA, which inhibits PRMT5 catalyzed histone methylation for oncogene expression. The cancer cell often expresses more PRMT5 to offset the low enzyme activity for proliferation, which confers heightened susceptibility to further inhibition of PRMT5. To test the difference in the preclinical antitumorigenic effect of PRMT5 inhibition in MATP-deficient and $MATP^{+/+}$ TNBC cells, this study KO MAT2A using CRISPR/cas9 in $MATP^{-/-}$ and $MATP^{+/+}$ TNBC cells, as well as $MATP^{-/-}$ and $MATP^{+/+}$ xenografted mice.

Possible results 1 fully supports the hypothesis that MAT2A KO targets the vulnerability of MTAP deficient TNBC cells. The MAT2A KO can effectively inhibit PRMT5 by reducing the concentration of the substrate SAM for EIF4E and FGFR3 histone methylation and $MATP^{-/-}$TNBC cell is more sensitive to PRMT5 inhibition than the $MATP^{+/+}$ TNBC cell. A further study investigating the intracellular concentration of SAM should be done for conforming to the pathway. To improve the experiment, the $MATP^{wt}$ and $MATP^{-/-}$ isogenic clone TNBC cells should be designed and used to minimize the variable. This could be achieved by using CRISPR-induced MATP knockout MDA-MB435 cell and wildtype MDA-MB435 cell. For further investigation of the preclinical effect, the same experiment would be done on Formalin fixed-paraffin embedded TNBC samples.

Possible results 2 fully support the hypothesis that MTAP

loss produces potential vulnerability of triple-negative breast cancer cells. However, compared to the possible results 1, the antitumor effect is lost in this case. We believe that this is because $MTAP^{+/+}$ TNBC cell may recruit another pathway to remedy the MAT2A KO, therefore the inhibition of PRMT5 by MAT2A KO in the $MTAP^{+/+}$ TNBC cell is a failure. However, the MTAP deficient TNBC cell is more sensitive to the PRMT5 inhibition, hence, the inhibition of PRMT5 by MAT2A KO is successful through the remediation of another pathway is processed. Further studies investigating the potential remediation mechanism of MAT2A KO should be done. The measurement of intracellular SAM level would be used to reveal the potential remediation mechanism.

Possible results 3 partially support the hypothesis. The MAT2 KO will inhibit cell growth by inhibiting PRMT5 activity. However, the same antitumorigenic effect on both cell lines is likely caused by the solid anti-tumor effect of MAT2A KO. The lacked specificity inhibition effect may reveal that inhibition of PRMT5 by MAT2A KO has a large side effect. The MAT2A KO would inhibit all cell growth instead of inhibiting $MATP^{-/-}$ cell specificity. Further studies investigating the preclinical side effects of MAT2A KO should be done. If the negative control also shows reduced cell proliferation, the antitumorigenic effect on both cell lines might be caused by CRISPR techniques itself instead of MAT2A KO by CRISPR. Other techniques, such as micro-RNA knockdown, should be used to avoid the influence of techniques.

Possible results 4 contradicts the hypothesis. This result is likely caused by the failure of inhibition of PRMT5. The antitumor effect by MAT2A KO may be caused by inhibition of methionine-mediated DNA synthesis through the SAM/mTORC1/S6K1/CAD pathway rather than PRMT5 inhibition. A further experiment investigating the intracellular phosphorylated S6K1, phosphorylated CAD, and SAM levels would be used to verify the potential pathway. This result may be also caused by off-target CRISPR/cas9 modification. A further investigation is required to check if Cas9 protein cleaves some off-target sites rather than the MAT2A gene.

Possible results 5 contradicts the hypothesis. The unlike possible results 5 is likely caused by experiment errors in measuring the cell proliferation. Further cell proliferation and apoptosis measurements should be applied.

Possible results 6 contradicts the hypothesis. Possible results 6 may be caused by unsuccessful gene editing on xenograft models. To improve the experiments that CRISPR/cas9 induced MAT2A KO before the xenograft model was constructed should be performed instead of direct genome editing in xenograft models. The different xenograft models construct would be used to exclude the potential systematic errors.

Possible results 7 contradicts the hypothesis. The unlike possible results 7 is likely to be caused by the off-target of the CRISPR/Cas9 modification. Future studies should use other experiments like siRNA transfection to knockdown MAT2A to verify the potential reason.

5. Conclusion

This study investigates the antitumor effect of PRMT5 inhibition by MAT2A KO in MTAP wt and MTAP deficient TNBC cells. The research results of this study will indicate whether or not the PRMT5 inhibition in MTAP deficient TNBC cell has a stronger antitumor effect than it in MTAP wt TNBC cell. The MTA accumulated in MTAP-deleted TNBC cells creates a hypomorphic PRMT5 state that is more sensitive to further PRMT5 inhibition. In this study, the effect of MAT2A KO, which inhibits the production of the substrate SAM for PRMT5 methylation activity is investigated to provide a potential therapy for MTAP-deficient TNBC.

Since the target therapies for TNBC is inefficient because of the lack of three common receptors, the vulnerability of PRMT5 in MTAP-deficient TNBC would be considered for therapeutic targeting. As PRMT5 is required for T-cell survival and proliferation, a novel strategy improvement is required to reduce the side effects. Exploiting MTAP loss combines with PRMT5 inhibition as a treatment for triple-negative blast cancer would be investigated in the future study.

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