AWARD NUMBER: W81XWH-13-1-0315

TITLE: Targeting MUC1-mediated tumor-stromal metabolic interaction in Triple-negative breast cancer

PRINCIPAL INVESTIGATOR: Pankaj Singh, PhD

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, NE 68198-7696

REPORT DATE: November 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Targeting MUC1-mediated tumor-stromal metabolic interaction in Triple-negative Breast Cancer

Pankaj Singh, PhD
E-Mail: Pankaj.singh@unmc.edu

Universidade of Nebraska Medical Center
Omaha, NE 68198-7696

Mucin1 (MUC1), a glycoprotein is aberrantly overexpressed in TNBC and facilitates growth and metastasis of triple negative breast cancer (TNBC) cells. This occurrence can be partially attributed to MUC1 interaction with hypoxia-inducible factor alpha (HIF1α), a key regulator of glycolysis. We previously observed that ectopic overexpression of MUC1 increased glucose uptake, lactate secretion and enhanced the expression of glycolytic enzymes. Therefore we hypothesized that MUC1 stabilizes HIF1α to facilitate metabolic reprogramming. In the present study we examined the effect of MUC1 expression on cancer cell metabolism of TNBC cell lines. MUC1 was ectopically overexpressed in the MDA-MB231 cell line and stably knocked down in the MDA-MB468 and BT-20 cell lines. Results indicate that MUC1 expression altered the expression of several metabolic genes. Furthermore, untargeted global metabolomic profiling identified metabolite alterations in which MUC1 expression modulates cancer cell metabolism to facilitate growth properties of TNBC cells. Thus our results support the notion that MUC1 serves as a metabolic regulator in TNBC, facilitating metabolic reprogramming that influences growth of TNBC.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>4</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>4</td>
</tr>
<tr>
<td>3. Overall Project Summary</td>
<td>4-13</td>
</tr>
<tr>
<td>4. Key Research Accomplishments</td>
<td>13</td>
</tr>
<tr>
<td>5. Conclusion</td>
<td>14</td>
</tr>
<tr>
<td>7. Inventions, Patents and Licenses</td>
<td>14</td>
</tr>
<tr>
<td>8. Reportable Outcomes</td>
<td>14</td>
</tr>
<tr>
<td>9. Other Achievements</td>
<td>14</td>
</tr>
<tr>
<td>10. References</td>
<td>15</td>
</tr>
<tr>
<td>11. Appendices</td>
<td>15</td>
</tr>
</tbody>
</table>
INTRODUCTION
Breast cancer, the second leading cause of cancer deaths in women, is the most common cancer among North American women, accounting for nearly 1 in 3 cancer cases diagnosed in the U.S. women [1, 2]. Triple negative breast cancer (TNBC) subtype accounts for approximately 15-25% of all breast cancer cases and has an increased incidence of metastasis, high recurrence within 1-3 years and a high mortality rate [3]. Therefore, identifying factors that facilitate tumor growth and metastases have the potential to serve as novel molecular targets for breast cancer therapy. MUC1, a glycoprotein associated with chemoresistance, is aberrantly overexpressed in over 90% of early TNBC lesions [4-6]. Much of the oncogenic potential role of MUC1 can be attributed to the participation of the small, cytoplasmic tail of MUC1 (MUC1 CT) in signal transduction and transcriptional events, facilitating growth and metastasis[6-9]. Oncogenic potential can also be attributed to MUC1 ability to interact and stabilizes hypoxia-inducible factor alpha (HIF1α), a key regulator of glycolysis [10]. As metastasis is the leading cause of cancer related deaths, this process relies on cooperation between the tumor cells and their surrounding stromal, establishing a reactive tumor microenvironment. Stromal cells can serve as a sink for the end-products of aerobic glycolysis (i.e, lactate) and provide a source of metabolites (i.e., pyruvate) to support to support cancer growth, invasion and metastasis [9]. Hence our overall research focus is to investigate how signaling through MUC1 facilitates hypoxia-dependent and independent metabolic cross-talk between epithelial and stromal components in TNBC; thus facilitating tumor growth and metastasis. Additionally, we will examine if co-targeting MUC1 and HIF1α will block epithelial-stroma metabolic cross-talk, diminish chemoresistance and reduce tumor growth and metastasis in TNBC. Findings from the proposed study may identify MUC1 as a novel therapeutic target for breast cancer, particularly for the TNBC subtype.

KEYWORDS: cancer metabolism, glycolysis, mucin1, pentose phosphate pathway, triple negative breast cancer

OVERALL PROJECT SUMMARY

Methodology
Cell Culture
MDA-MB231 (Neo and MUC1) and MDA-MB468 (NEO and shMUC1) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a humidified atmosphere at 37°C with 5% CO2 under atmospheric oxygen condition (20%).

MTT Cell Viability Assay
The viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT (Sigma-Aldrich, St. Louis, MO)] to formazan. Cells were seeded at 3 × 10^4 cell/well in 96-well plates in a final volume of 100 μl, incubated overnight and then incubated for 72 hours under indicated conditions. Twenty microliters of MTT reagent (diluted in culture media) was added to each well and cells were incubated for 4 hr at 37°C. The MTT formazan precipitate was then dissolved in 100 μl of DMSO, and the absorbance was measured at 570 nm using a Cytation 3, plate reader (Biotek, USA).
Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed in 384 Well Optical Reaction Plates (Applied Biosystems) using SYBRGreen PCR Master Mix (Roche). Reactions were carried out on an ABI 7500 thermocycler (Applied Biosystems). All samples were amplified in duplicate and quantification of the expression level of each gene was calculated using the delta delta CT method, normalized to $\beta$-actin. Data presented as fold change relative to the control cells (Neo).

Cell Proliferation and Kinetics

For cell proliferation assay cells were seeded at a density of $5 \times 10^3$ cells/well in 96-well plates in triplicates. After 72 h, cells were incubated with 20 $\mu$l of 3-(4,5-dimethyltetrazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT (Sigma-Aldrich, St. Louis, MO) (5mg/ml) solution at 37 °C for 4 h and followed by the addition of 100 $\mu$l of DMSO. The plates were read at 570 nm using a benchmark microplate reader (Biotek, Cytation 3, Winooski, VT). For cell kinetics cells were seeded at 5,000 cells/well in a 12 well plate then collected by trypsinization and counted at the indicated time point.

Liquid chromatography and tandem mass spectrometry for polar metabolites

Cells cultured for 24 h were rinsed with PBS then frozen on dry ice for metabolite extraction. Extracts were collected by the addition of 80% methanol on dry ice followed by plates were maintained at -80°C for 10 min and lysed using cell scrapers. Lysates were collected in polyproyl tubes and centrifuged at 3400 rpm at 4°C for 10 min to remove the precipitates. Pellets were suspended in 200 $\mu$l of LC-MS grade water and centrifuged to collect the water-soluble supernatants. The combined supernatants were concentrated for 1h using speed vacuum concentrator followed by lyophilization for 2h using Freezone (-105°C) lyophilizer (Labconco). Lyophilized concentrates were suspended in equal volumes of LC-MS grade water and 10 $\mu$l were utilized for LC-MS/MS using multiple reaction monitoring (MRM) method described previously [11]. Data acquisition was carried out using Analyst™1.6 software (AB SCIEX) and peaks were integrated with Multiquant™ (AB SCIEX). Peak areas were normalized with the respective protein concentrations and the resultant peak areas were subjected to Metaboanalyst.2 for relative quantification analyses.

Statistical Analysis: Nonparametric Tukey tests were used to compare differences between cell lines. A p-value of 0.05 or less will be deemed significant.

Results

MUC1 expression induces metabolic alterations in TNBC cells

To examine the role that MUC1 plays in metabolic alterations in TNBC, MUC1 was ectopically overexpressed in MDA-MB-231 cells and stably knocked down in MDA-MB-468 cells. The altered MUC1 expression levels ranged from a 5-fold increase in MDA-MB-231 cells and a 10-20-fold decrease in MDA-MB-468 cells compared to control cells respectfully (data not shown). As previously shown from principle component analysis (PCA), polar metabolites distinctly separated the cells in a MUC1 expression-dependent manner, suggesting that altered MUC1 expression induced a significant change in the metabolite profile of the TNBC cells (Fig 1 A, C). Furthermore, heat maps of unsupervised hierarchical clustering indicated an overall metabolic distinction between control and experimental cells, evident by cells segregated into tight clusters (Fig 1 B, D).
Figure 1. Principle component analysis (PCA) model. Analysis was performed using MetaboAnalyst 2.0 on differentially expressed polar metabolites of the control (red) and experimental (green or blue) groups. Circles represent 95% confidence interval for similarities in metabolite profiles. (A,C) Principle component analysis (PCA) plots generated from LC-MS/MS data of cell metabolites. (B,D) Heat map of metabolites generated from the normalized-mean peak intensities for each metabolite identified from triplicate sets, from 0% (red) to 100% (green).
Metabolite expression profiles in TNBC cells

To better understand the role of MUC1 in TNBC metabolism, we subjected individual metabolites to pathway enrichment analyses using Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways. These analyses identified highly significant enrichment of multiple pathways. Figs 2-3 identify the top 10 affected pathways, ordered by impact factor. Nitrogen metabolism and Aminoacyl-tRNA biosynthesis were most significantly altered in MDA-MB-231 closely followed by Glycine, serine, and threonine metabolism (Fig 2). Aminoacyl-tRNA biosynthesis, D-Glutamine and D-glutamate metabolism, Nicotinate and nicotinamide metabolism, and Nitrogen metabolism were amongst the redundant pathways identified in MDA-MB-468 (Fig 3). Nitrogen metabolism and D-Glutamine and D-glutamate metabolism pathways were filtered out as potential target pathways for MDA-MB-231 and MDA-MB-468 with several individual metabolites significantly altered within each pathway (Fig 4).

Figure 2. MUC1 alters MDA-MB-231 metabolism. (A) Summary of pathway analysis, with circles representing matched pathways. The color and size of each circle are based on the p-value and pathway impact value, respectively. (B) Summary results of metabolic pathways altered. Total is the total number of compounds in the pathway; Hits is the matched number from the user uploaded data; FDR is the p-value adjusted using False Discovery Rate; the Impact is the pathway impact value calculated from pathway topology analysis.
Figure 3. MUC1 alters MDA-MB-468 metabolism. (A, C) Summary of pathway analysis, with circles representing matched pathways. The color and size of each circle are based on the p-value and pathway impact value, respectively. (B, D) Summary results of metabolic pathways altered. Total is the total number of compounds in the pathway; Hits is the matched number from the user uploaded data; FDR is the p-value adjusted using False Discovery Rate; the Impact is the pathway impact value calculated from pathway topology analysis.
Figure 4. MUC1 alters TNBC metabolism. Representation of (A) D-Glutamine and D-glutamate metabolism and (B) Nitrogen metabolism pathways. Orange indicates the KEGG metabolites altered by MUC1 expression. Blue represents the KEGG metabolite map numbers in the same pathway that do not appear to be altered.
MUC1 regulates glutamine metabolism in TNBC cells

Glucose (Glc) and glutamine (Gln) have been shown to play a role in promoting cancer growth, participating in energy formation and redox homeostasis [12]. Previously reported results showed that MUC1 overexpression significantly increased glucose and glutamine uptake in MDA-MB-231 cells and MUC1 knockdown reduced glucose and glutamine uptake in MDA-MB-468 cells (Fig 5 A-B). These results indicate that MUC1 can facilitate the uptake of glucose and glutamine as a carbon source. Therefore we focused on D- Glutamine and D-glutamate metabolism. The effect of altered MUC1 expression on the dependence of TNBC on Gln was then investigated (Fig 6). As previously reported MDA-MB-231 cells exhibited glutamine dependency [13] but glutamine deprivation had no effect on MDA-MB-468 cells (Fig 6A). Cell viability assays revealed that MUC1 expression altered cell glutamine dependency compared to control cells. Results showed that glutamine dependency increased with MUC1 overexpression in MDA-MB-231 cells. Glutamine dependency was further examined using the aminotransferase inhibitor, aminooxyacetate (AOA) in complete and low Gln conditions. MUC1 expression altered cell survival in a dose-dependent manner with increased cytotoxicity with MUC1 overexpression in MDA-MB-231 cells and decreased cytotoxicity with MUC1 knockdown in MDA-MB-468 cells (Fig 6 B-C). To further examine the role of MUC1 in glutamine metabolism, a panel of genes regulating glutamine metabolism was examined. A significant alteration in mRNA expression of \textit{SLC1A5}, \textit{SLC38A2}, \textit{GOT1}, \textit{GLS2}, \textit{GOT2}, \textit{OGDHII}, and \textit{CMYC} was observed as a result of altered MUC1 expression (Figure 7). No change in mRNA levels of \textit{GLS1} was observed in either cell line. These results collectively indicate that altered MUC1 expression effects Gln dependency of TNBC.

---

**Figure 5. MUC1 regulates glucose and glutamate in TNBC cells.** (A) glucose uptake was determined by performing [3\textmu Ci $[^3\text{H}]$-2DG uptake assay and (B) glutamine uptake was determined by performing 3\textmu Ci $[^3\text{H}]$-glutamine uptake. Bars represent counts normalized with cell number and plotted relative to control. *p<0.05, **p<0.01 and ***p<0.001
Figure 6. MUC1 alters glutamine dependency in TNBC. (A) Growth of TNBC cells (72h) incubated with complete or low glutamine (0.3 mM) cell media. *p<0.05, **p<0.01 vs. complete, #p<0.05 vs. low glutamine (B-C) Cell viability of cells (72h) incubated with indicated concentrations of AOA. *p<0.05, **p<0.01 and ***p<0.001
Discussion
In summary, our results support the notion that MUC1 serves as a metabolic regulator in TNBC and a potential mechanism by which MUC1 contributes to the metabolic process involved breast cancer. Glucose and glutamine serve as the main carbon sources in proliferating cells, and uptake of both nutrients is directed by growth factor signaling[14]. Untargeted global metabolomic profiling identified metabolite alterations in which MUC1 expression modulates cancer cell metabolism to facilitate growth properties of TNBC cells (Figure 1). Pathway enrichment analyses using Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways identified Nitrogen metabolism and D-Glutamine and D-glutamate metabolism pathways as potential target pathways (Figures 2-4). Observed alterations in glucose and glutamine uptake (Figure 5) suggest MUC1 facilitates the utilization of both glucose and glutamine as carbon sources to maximize ATP production in TNBC. Next, examining expression of genes regulating glutamine metabolism revealed MUC1 facilitated alteration in a number of the genes regulating this process (Figure 7). Furthermore, the use of aminotransferase inhibitor AOA suggests MUC1 expression regulates glutamine dependency (Figure 6). This data demonstrates that treatment of TNBC cells exhibiting high expression of MUC1 with AOA is associated with increase cell death and may serve as a therapeutic option, particularly in MUC1 overexpressing TNBC (Figure 8).
**KEY RESEARCH ACCOMPLISHMENTS**

- Determined MUC1 regulates metabolite levels in TNBC cells
- Determined glucose and glutamine uptake is altered by MUC1 expression
- Determined alterations of key genes regulating glutamine metabolism facilitated by MUC1
- Determined MUC1 alter glutamine dependency
- Determined AOA interferes glutamine to enhance cytotoxicity in a MUC1-dependent manner

*Figure 8. Regulation of glutamine metabolism by MUC1 in TNBC.* MUC1 regulates glutamine metabolism by increasing transcription of genes regulating glutaminolysis. As a result carbon flux to glutamiolysis is enhanced. AOA interferes with glutaminolysis, thereby hindering cell growth. Abbreviations in the figure include the following: acetyl coenzyme A (AcCoA), α-ketoglutarate (α-KG), solute carrier family 1 member 5 (SLC1A5), tricarboxylic acid cycle (TCA cycle), transcription factor (TF) and electron transport chain (ETC).
CONCLUSION
In conclusion, the data presented here establishes a role of MUC1 in metabolic reprogramming in TNBC. Presented data suggest that MUC1 facilitates metabolic reprogramming of TNBC to promote cell growth, which can in part be due to the interaction of MUC1 with HIF1α. PLS-DA indicates clustering into two separate groups that can be metabolically differentiated. In addition, pathway analysis indicates that MUC1 is involved multiple metabolic pathways, altering the expression of several metabolites. Our findings highlight the potential of targeting MUC1 in TNBC.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press: Nothing to report
(2) Peer-Reviewed Scientific Journals: Nothing to report
(3) Invited Articles: Nothing to report

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.
Poster presentation, “MUC1 serves as metabolic regulator in triple negative breast cancer”. Presented at the American Association for Cancer Research (AACR) Annual meeting, New Orleans, LA April 16-20, 2016

INVENTIONS, PATENTS AND LICENSES: Nothing to report

REPORTABLE OUTCOMES: Nothing to report

OTHER ACHIEVEMENTS: Nothing to report

For each section, 4 through 9, if there is no reportable outcome, state “Nothing to report.”
REFERENCES: