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Polyamine Enzymes as Potential Targets for Cancer Therapeutics to Mitigate Diabetic Triple Negative Breast Cancer Advancement

Hadassha Tofilau
University of Nebraska at Kearney, dasshtofilau@gmail.com

POLYAMINE ENZYMES AS POTENTIAL TARGETS FOR CANCER THERAPEUTICS TO MITIGATE DIABETIC TRIPLE NEGATIVE BREAST CANCER ADVANCEMENT

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Hadassha Melania Nofoato'o Tofilau

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THESIS ACCEPTANCE

Acceptance for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the degree Master of Science, University of Nebraska at Kearney.

Supervisory Committee

Name	Department
	Biology
	Chemistry
Pawan Kumar Mishra	CIP, UNMC

Surabhi Chandra

Supervisory Committee Chair

06/21/2022

Date

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ABSTRACT

Breast cancer (BC) is the second leading cause of death in women. Co-existence of diabetes and BC can be lethal, leading to higher mortality than BC alone. TNBC is highly refractive, heterogenous, and resistant to most chemotherapies. Polyamines play a role in cell growth and are elevated in cancer, though their role in diabetic TNBC has not been explored. We hypothesized that high glucose/diabetic conditions increase TNBC cell proliferation through modification of enzymes in the polyamine pathway and that miR-133a would decrease TNBC cell proliferation. For this study, MDA-MB-231 and MCF-10A cell lines were both treated with normal (5mM) and high glucose (25mM) concentrations and effects on polyamine levels, polyamine enzyme, and cell proliferation were monitored. Polyamine levels were assayed using reverse phase HPLC, cell proliferation assessed using a fluorescence-based assay, and polyamine enzyme expression was monitored at mRNA and protein levels using RT-PCR and Western Blots, respectively. Polyamine inhibitor, alpha-difluoromethylornithine (DFMO, 5mM), was used in combination with glucose treatments. Further, miR-133a was used alongside glucose treatments. Under diabetic/high glucose conditions, putrescine levels were elevated in MDA-MB-231 cells, which correlated with higher cell proliferation. Polyamine enzyme activity of ODC increased (from ~75 pMolCO_2/hr/mg protein to ~150 pMolCO_2/hr/mg protein) and SMOX decreased (from ~0.4 pMolH_2O_2/min/mg protein to ~0.06 pMolH_2O_2/min/mg protein). DFMO reduced cell proliferation, however re-supplementation of spermidine and spermine restored cell proliferation, indicating cytostatic action. Interestingly, miR-133a significantly decreased cell proliferation under

both normal and diabetic conditions. Cell proliferation increased with high glucose in MCF-10A cells also. Our study suggests that diabetic conditions advance TNBC and can induce cancerous states in breast epithelial tissue. Polyamine enzymes play an active role in this process, and further miR-133a shows promise and should be explored to determine its role in targeting the polyamine pathway to mitigate diabetic TNBC advancement.

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ABBREVIATIONS

TNBC, triple negative breast cancer; DM, diabetes mellitus; Type 2 DM, T2DM; Ornithine Decarboxylase, ODC; Spermidine synthase, SPDS; Spermine synthase, SPMS; Spd/Spm N1-acetyltransferase, SAT1; Spermine oxidase, SMOX, Acetylated polyamine oxidase, AcPAO; S-adenosylmethionine synthase, SAMS; S-adenosylmethionine decarboxylase, AdoMetDC; alpha-difluoromethylornithine, DFMO; microRNAs, miRs

I. Introduction

1.1 Triple Negative Breast Cancer

Breast Cancer is the most commonly diagnosed cancer and the second leading cause of death in women worldwide as well as in the United States (U.S.) [1]. As of January 2022, over 3.8 million women have a history of breast cancer in the U.S. [2]. Further, 1 in 8 U.S. women and 1 in 833 U.S. men will develop breast cancer [2]. Amongst the different subtypes of breast cancer, the highly refractive and metastatic Triple Negative Breast Cancer (TNBC) accounts for 15-20% of all breast cancer patients and contributes to the maximum number of deaths in women with breast cancer [3-5].

Breast cancer is subdivided into five molecular categories based on gene expression: Luminal A, Luminal B, Her2/neu overexpression, normal-like and basal-like [6]. Roughly 75% of the basal-like molecular subtype of breast cancer is TNBC which carries the poorest prognosis. This is partially attributed to it being highly refractive, heterogenous, and resistant to most chemotherapies [7]. It is considered triple negative because it lacks the estrogen receptor, progesterone receptor, and the Human Epidermal Growth Factor 2 (HER2) receptor, thus making it difficult to treat with traditional hormonal therapies [4, 5]. Individuals diagnosed with TNBC are typically premenopausal under the age of 40 and are of Latin or African American descent [1].

TNBC can be further distinguished into a variety of subgroups: basal-like 1, basal-like 2, mesenchymal, mesenchymal stem-cell like, immunomodulatory, and luminal androgen receptor [4, 8]. The heterogenic nature of TNBC makes it difficult to have targeted therapies. So far, there is a lack of mono-chemotherapeutic options that target

TNBC at the molecular level. Furthermore, there are risk factors for TNBC that are still not known. This concern has led to research that focuses on determining factors that may increase or contribute to a TNBC diagnosis.

With the advancements in healthcare and early diagnoses, there is a 2.6% chance that an individual will die from breast cancer [6, 9]. However, the complications and long-term implications are lasting and include bone loss and osteoporosis, blood clots, peripheral neuropathy, as well as heart problems [10]. Risk factors that increase the likelihood of being diagnosed with breast cancer include aging, endogenous hormones such as increased estradiol, alcohol consumption, high-fat diets as well as diabetes mellitus [11-15].

2. Diabetes mellitus and Breast Cancer

According to the American Diabetes Association, of the 34.2 million Americans with diabetes mellitus (DM) [16], Type 2 DM (T2DM) accounts for 90% of those diagnosed with DM [17]. T2DM is caused by a combination of genetic and environmental factors such as a sedentary lifestyle, obesity, high-fat diets, as well as impairment of pancreatic β cells [18]. T2DM is also associated with a number of complications such as cardiovascular disease and has been known to increase the likelihood that an individual would have neuropathy, nephropathy, and even increased incidence of cancers such as pancreatic cancer, colorectal cancer, and breast cancer [19-21].

Over the years, a link between T2DM and breast cancer has been investigated. Risk factors associated with breast cancer overlap with risk factors for DM: obesity, high

fat diets, advanced age, as well as a sedentary lifestyle [22]. A key characteristic of DM is hyperglycemia, and it has been shown that hyperglycemia can influence cancer growth and proliferation thereby worsening cancer prognosis [23, 24]. Individuals with Type 2 DM (T2DM) are at an increased risk of being diagnosed with breast cancer; approximately a 16-26% increase [20, 25, 26]. In these instances, it has been reported that those diagnosed with T2DM as well as breast cancer or diabetic breast cancer, have a higher mortality rate than those who have only one or the other [15, 20, 27]

2.1 TNBC and Diabetes: Are there linking Factors?

Previous studies have shown that breast cancer incidence is worsened by diabetes. However, the relationship between diabetes and TNBC remains unclear [20, 25, 26] . Literature on the topic is conflicting with some studies suggesting that there is no correlation between diabetes and TNBC [23, 28]. Ma et al. on the other hand, reported that in the People's Republic of China, T2DM was a risk factor that increased the incidence of metastasis in individuals with early-stage TNBC as well as increased the likelihood that TNBC would recur [29]. The conflicting reports could be the result of differences in study sizes (i.e. ranging from about 50-100,000), and it highlights a significant gap in the body of knowledge related to this topic. All of the studies that constitute what we know about the potential relationship between diabetes and TNBC were meta-analyses and did not consider the molecular and cellular mechanisms of the diseases.

2.2 Metabolic Signaling Pathways in Breast Cancer and Diabetes Mellitus

While T2DM and breast cancer have been causally linked, the cellular and molecular mechanisms have yet to be fully understood. One metabolic pathway found to be associated with both T2DM and breast cancer is the hyperinsulinemia-insulin receptor signaling pathway [30-32] . Insulin receptors (IRs) regulate glucose, lipid, as well as protein metabolism in tissues[31]. Breast cancer cells have been shown to overexpress IRs which allows the cancer cells to bind to insulin which are present in elevated amounts due to diabetic conditions[33]. Insulin binding to its receptors in breast cancer cells results in the activation of downstream signaling insulin receptor substrates IRS-1 or IRS-1/4 that promotes the activation of the RAS/RAF/MEK/ERK MAP Kinase pathway [33]. This pathway is responsible for cell proliferation, differentiation, metastasis and metabolism in cells, making its over-activation in cancer cells a cause for concern [34]. Other pathways that have been found to link the breast cancer and T2DM include the mTOR signaling pathway [35] , pro-inflammatory pathways that increase chronic inflammation [36], and the insulin and IGF-1 receptors signal transduction pathways [37].

Polyamines have been found to have insulin-like characteristics such as inhibition of lipolysis and facilitating transport of glucose [38, 39]. Previous studies in mouse models show that polyamines could reverse the effects of hyperglycemia. Specifically, the polyamine spermine plays a role in stimulating insulin release [40, 41]. Interestingly, individuals with DM have decreased polyamine enzymes, however, patients with either

only breast cancer or a comorbidity of DM and breast cancer have increased polyamine enzyme content [42].

Evidence in the literature has suggested that glucose metabolism impairment has a negative effect on cancer therapeutics [43, 44]. Hyperglycemia has been shown to be a link between breast cancer and T2DM, however, the role that increased glucose in the bloodstream has on cancer cell growth and proliferation remains unclear [32]. The pathways involved and the roles they play in influencing this behavior are an area of active research. Metabolic dysregulation is a hallmark of diabetes and cancer. Thus, polyamines, which are impacted in both conditions, are important molecules to explore.

3. Polyamines

Polyamines are aliphatic polycations that play an important role in cell growth and development [45, 46]. They also play a role in regulating gene transcription and translation [47]. There are three primary polyamines produced in mammals: putrescine, spermidine, and spermine [48-50]. The primary sources of mammalian polyamines are ingestion of food, cellular synthesis, as well as generation by the microbiome in the gut [45, 46]. Polyamine levels are tightly regulated through cellular processes, however, conditions such as chronic kidney disease, Alzheimer's disease, Parkinson's disease as well as cancer have elevated polyamine content [51, 52] .

3.1 Polyamines as Biomarkers for Breast Cancer

Dysregulations in the polyamine biosynthesis pathway, specifically, elevated polyamine content has been linked with a variety of cancers such as colorectal, prostate, pancreatic, skin as well as breast cancer [53-57]. In breast cancer, it has been reported

that intracellular polyamine amounts were higher than in normal breast tissue, a 3- to 6-fold increase [58, 59]. Further, TNBC cells have been documented to have even higher intracellular amounts of polyamines than other subtypes [60, 61].

Polyamines are responsible for promoting the interactions of transcription factors such as NF-kappaB and estrogen receptors with their corresponding response elements, thus resulting in proliferation of malignant and invasive breast cancer tumor cells, as well as estrogen-negative tumor cells [62, 63]. Ornithine decarboxylase, the rate-limiting enzyme of polyamine biosynthesis, has been documented to be increased in breast cancer [42, 64]. Furthermore, it has been shown that inhibition of polyamine synthesis and uptake causes a decrease in TNBC cell proliferation [61]. This supports the idea that targeting the polyamine biosynthesis pathway in breast cancer cells would be an effective chemotherapeutic option.

3.2 Polyamine Metabolism

Polyamines are synthesized from the amino acids arginine, ornithine, and methionine, and rely on unique enzymes to harness each amino acid pool separately [65, 66]. As shown in **Figure 1**, the predominant pathway of polyamine biosynthesis begins with arginine. Arginine is converted via arginase to ornithine, which is further metabolized by ornithine decarboxylase (ODC) to produce the first polyamine, putrescine. ODC is the rate limiting enzyme of the polyamine biosynthesis pathway and is tightly regulated at the transcriptional and post-transcriptional level [67, 68]. Next, spermidine synthase converts putrescine to spermidine, followed by the conversion of spermidine to spermine by the enzyme spermine synthase.

Working in parallel to the primary pathway of polyamine biosynthesis is the secondary pathway through methionine cycle, which predominantly occurs in the liver [69, 70]. Methionine is shuttled as S-adenosyl methionine via S-adenosylmethionine synthase (SAMS). This is further metabolized to decarboxylated S-adenosylmethionine through S-adenosylmethionine decarboxylase. The decarboxylated S-adenosylmethionine can replenish polyamine pools through spermidine synthase (for spermidine) and spermine synthase (for spermine).

3.3 Polyamine Catabolism

While non-reversible, it is possible to recycle spermine and spermidine through the action of N-acetyltransferase intermediates [45, 71]) (**Figure 1**). Spermine can be converted to spermidine through the action of spermine oxidase (SMOX). Alternatively, spermine or spermidine can be converted to lower polyamines through a two-step reaction involving spermidine/spermine N1-acetyltransferase (SAT1) and acetylpolyamine oxidase (AcPAO). This is an important part of polyamine catabolism because SAT1 is the primary driving force for maintaining intracellular polyamine pools [45, 72]. When intracellular polyamines are depleted, SAT1 upregulates polyamine biosynthesis [72]. Oxidative deamination of polyamines by Cu^{2+} dependent diamine oxidase plays an important role in terminal catabolism of polyamines [71].

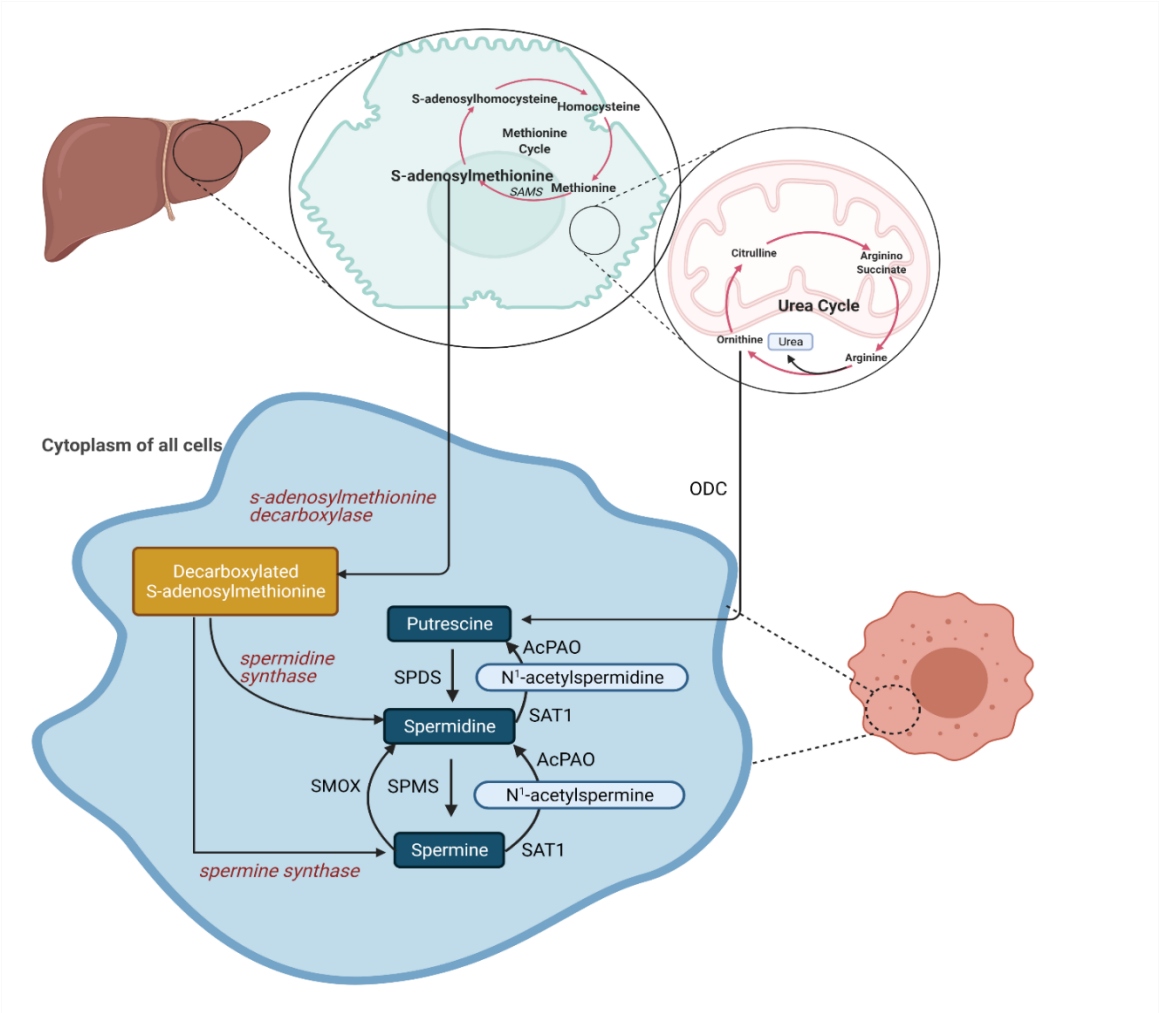


Figure 1. Depiction of primary and secondary pathway of polyamine biosynthesis pathway. This figure shows the amino acids involved in the polyamine biosynthesis pathway along with the enzymes involved in the primary pathway: Ornithine Decarboxylase (ODC), Spermidine synthase (SPDS), Spermine synthase (SPMS), Spd/Spm N1-acetyltransferase (SAT1), Spermine oxidase (SMOX), Acetylated polyamine oxidase (AcPAO) and the secondary pathway (S-adenosylmethionine synthase (SAMS), S-adenosylmethionine decarboxylase (AdoMetDC)) and the products that result from the pathway. Created with BioRender.com.

3.4 Potential Cancer Therapeutics for Polyamine Inhibition

While there are many polyamine inhibitors being explored, the most widely used which shows promise is alpha-difluoromethylornithine (DFMO) [73]. DFMO is an irreversible inhibitor of ODC as shown in **Figure 2** [73-75]. It has been used to effectively reduce cell growth and proliferation successfully in some breast cancer cell lines such as MCF-7, and MDA-MB-435 [75, 76]. It is also an effective cancer therapeutic agent in combination with other chemotherapeutic drugs against some cancers [74]. However, DFMO as a mono-chemotherapeutic agent exerts a cytostatic effect rather than a cytotoxic effect as shown in previous studies [64, 76, 77]. Thus, other potential cancer therapeutics to target the polyamine biosynthesis pathway are needed.

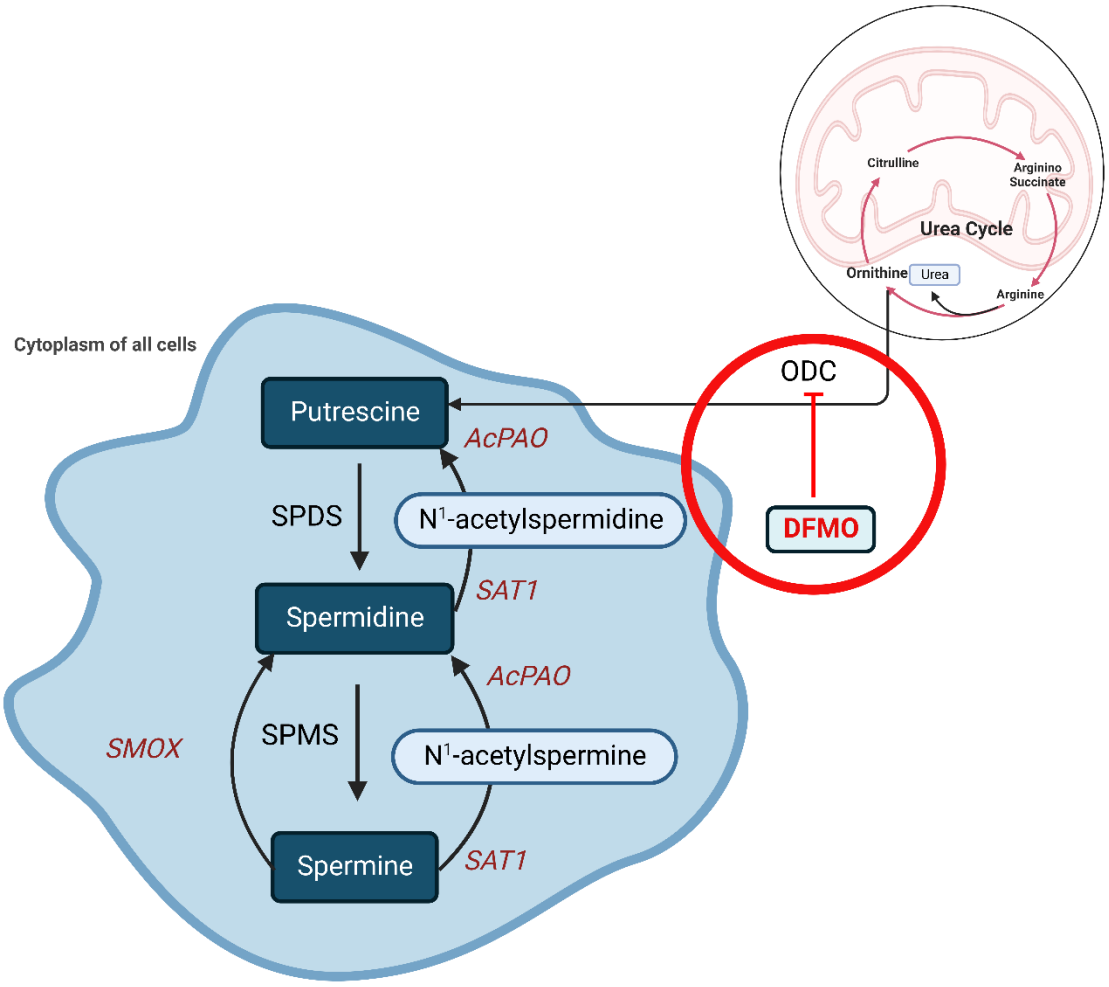


Figure 2. DFMO target in the polyamine pathway. This is a schematic of difluoromethylornithine (DFMO) inhibition of ornithine decarboxylase (ODC) (circled in red) in the polyamine biosynthesis pathway. Created with BioRender.com.

3.5 Specific microRNAs as Potential Polyamine Inhibitors for Chemotherapy

microRNAs (miRs) are small non-coding RNAs approximately 19-25 nucleotides long known for their role as post-transcriptional regulators [78, 79]. There are two ways in which miRs modulate gene expression: RNA activation, where they target promoter elements at the 5' untranslated region and cause gene transcription or RNA interference, where they target the 3' untranslated region and cause mRNA degradation resulting in repression of translation [80]. miRs have been used as a potential cancer therapeutic throughout recent years and play a role in gene-silencing and mRNA degradation of potential targets [79]. They also have the ability to act as tumor suppressors [81]. In particular, miR-133a was first found in mice but has since been linked with cancers like breast cancer [81].

miR-133a has been linked as a potential miR involved in the regulation of polyamines in diseases such as diabetic cardiomyopathy and is known to be anti-hypertrophic [39]. In breast cancer specifically, miR-133a plays a role in regulating the cell cycle and proliferation of breast cancer and can act as a tumor suppressor [81, 82]. This makes miR-133a a potential candidate for breast cancer treatment, and especially for diabetic breast cancer.

Recently, DM has been identified as a disease associated with miR dysregulation [81, 83, 84] . miRNAs play a role in altering gene expression that is seen in impaired β cells, as well as tissues resistant to insulin that could potentially be involved in the development of DM [83]. In particular, it is reported that there is a five-fold decrease in

miR-133a in individuals with T2DM [83]. In breast cancer cell lines MCF-7 and MDA-MB-231, miR-133a has been found to be downregulated as well [85].

Studies in the last ten years have shown that miR-133a suppresses breast cancer tumor growth in mice [86]. It has been shown to effectively target the EGFR/AKT/ERK signaling pathways [81, 87]. While its role in affecting other cellular pathways has been documented, its effect on the polyamine biosynthesis pathway remains unclear. Further, the effect that miR would have on TNBC under diabetic conditions has yet to be explored. miRs have the ability to modulate both transcriptional and post-transcriptional activities of genes making them ideal for targeting the polyamine biosynthesis pathway. Since it is downregulated in both T2DM and TNBC cell lines, it has the potential to be an effective therapeutic against both diseases simultaneously.

The purpose of this study is to fill in the gaps in the knowledge regarding polyamine metabolism and diabetic TNBC. This involves establishing a causal link between polyamines and glucose, the molecular mechanisms involved, and the implications these associations may have. It also seeks to enhance diabetic breast cancer therapeutics by utilizing miRs for the inhibition of the polyamine biosynthesis pathway.

4. Specific Aims and Hypotheses

There are two primary objectives for this study. The first is to determine potential targets in the polyamine biosynthesis pathway for the action of miR-133a. The second objective is to treat with DFMO and miR-133a to determine their efficacy in targeting polyamine metabolism in diabetic triple negative breast cancer cells, focusing specifically on cell proliferation. The hypotheses are as follows: (1) Potential targets in the polyamine

biosynthesis pathway are involved with diabetic TNBC proliferation. (2) miR-133a will reduce TNBC cell proliferation and has the potential to be an effective regulator of the polyamine biosynthesis pathway.

5. Key Findings

Our study shows that polyamine enzymes are dysregulated under high glucose conditions in TNBC and play a key role in TNBC cell proliferation. Anabolic enzyme ODC is elevated while catabolic enzyme SMOX is downregulated in the primary polyamine pathway. Elevation of ODC caused an increase in the polyamine putrescine which directly correlated with an increase in cell proliferation in TNBC cells when treated with high concentrations of glucose. MCF-10A, normal-like breast epithelial, also showed marked elevation in cell proliferation. The polyamine inhibitor, DFMO, decreased cell proliferation. However, when polyamines putrescine, spermidine, and spermine were added back, cell proliferation was restored. Use of miR-133a was shown to effectively decrease cell proliferation under both normal and high glucose conditions in TNBC cells, indicating its potential as a diabetic TNBC therapeutic.

6. Significance of Study

While much devotion has been given to understand the growth and proliferation of breast cancer, research that documents the molecular mechanisms that may be involved, along with the potential therapeutics remains scarce. Studies into the molecular biology behind breast cancer have found that polyamine levels are elevated in breast cancer tissue. However, the role that polyamines play in diabetic breast cancer remains a relatively unknown area of study. Understanding how polyamines function in diabetic

triple negative breast cancer may give insight as to how its growth can be mitigated. Inhibition of parts of the pathway that yield polyamines may give more clarity as to how polyamines function in diabetic breast cancer and fill the gaps in the knowledge in this area of study. Furthermore, this is the first study in which miRs are shown to target cell proliferation and have the potential to target polyamine metabolism in diabetic breast cancer.

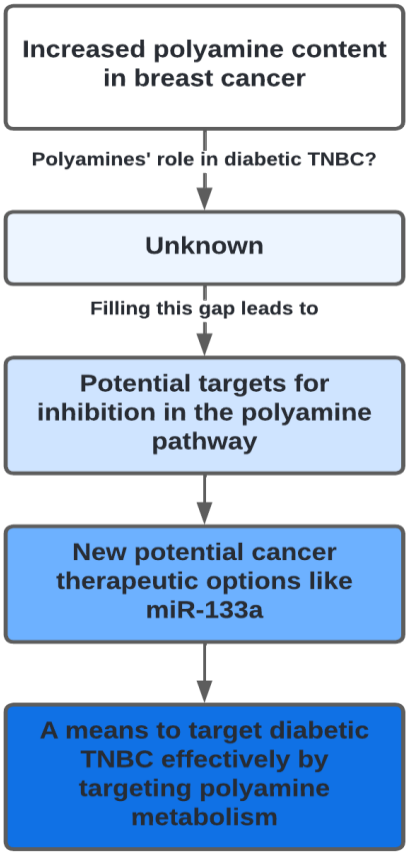


Figure 3. Schematic of study significance and its broader implications for healthcare. This figure shows the gap-in-knowledge the study seeks to fill in regard to the relationship between polyamines in diabetic TNBC. It further shows how findings could lead to a targeted approach for mitigating diabetic TNBC advancement. Created with Lucidchart.

II. Materials and Methods

1. Materials

1.1 Reagents and Supplies

Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, 1:1), phenol red-free DMEM, OptiMEM™ Reduced Serum Medium, Lipofectamine™ 3000 Transfection Reagent, horse serum, penicillin/streptomycin, GlutaMAX, PrestoBlue® cell viability reagent, RestoreTM Western blot stripping buffer and modified-Lowry assay kit were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Fetal bovine serum (FBS) was obtained from American Type Culture Collection (Manassas, VA, USA). Glucose, insulin, hydrocortisone, and cholera toxin as well as polyamines spermidine and spermine were obtained from Millipore Sigma (Milwaukee, WI, USA).

Cell lysis buffer, protease inhibitor cocktail, primary antibodies against ornithine decarboxylase (ODC), GAPDH, progesterone receptor, estrogen receptor, spermidine/spermine acetyltransferase (SSAT1), and secondary anti-rabbit IgG HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against spermine oxidase (SMOX) were purchased from Millipore Sigma (Milwaukee, WI, USA). Alpha-difluoromethylornithine (DFMO) was obtained from Bachem Americas Inc (Torrance, CA, USA). Putrescine was purchased from MP Biomedicals (Solon, OH, USA). Mini-PROTEAN® TGX Stain-FreeTM gels, Everyblot Blocking Buffer and other supplies for Western blot were purchased from Bio-Rad (Richmond, CA, USA). RT-PCR supplies, forward and reverse primers of ODC, SAT1,

and SMOX were obtained from Integrated DNA Technology (Coralville, IA, USA). SYBR® Green Supermix was purchased from Quantabio (Beverly, MA, USA).

1.2 Cell Lines and Cell Culture Conditions

All cell lines: Non-tumorigenic mammary epithelial cells (MCF-10A ref# CRL-10317), and late-stage human breast adenocarcinoma cell line (MDA-MB-231 ref# CRM-HTB-26D), were obtained from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in a humidified 37°C incubator with 5% CO_2. For non-tumorigenic mammary epithelial cells, MCF-10A, were grown in DMEM/F12 supplemented with 5% horse serum, 20ng/mL EGF, 0.5µg/mL hydrocortisone, 100ng/mL cholera toxin, 10µg/mL insulin, 100IU/mL penicillin, and 100µg/mL streptomycin. MDA-MB-231 was grown in DMEM/F12 and supplemented with 5% fetal bovine serum, 1% GlutaMax, 100IU/mL penicillin, and 100ug/mL streptomycin.

1.3 Authentication of Cell Lines and Primers

Validation of cell lines was routinely performed by checking for receptor expression or absence (triple negative cell line) using Western blot technique. MDA-MB-231 and MCF-10A which lack the estrogen, progesterone and Her2 receptors were analyzed against MCF-7 breast cancer cell line which expresses all three receptors. Primer specificity was monitored using positive controls for specific gene expression as well as melt curve in qPCR to assess any primer-dimer formation.

2. Methods

2.1 Treatment Groups

Two different glucose concentrations were used as part of this study: 5mM represents the normal glucose treatment, and 25mM represents the high glucose treatment. These correspond to fasting conditions in the cells and diabetic glucose concentrations, respectively in humans (23250919). To account for osmolality, 20mM of mannitol was used as the negative control for the study.

2.2 Polyamine Analysis

Each cell line was grown and treated in T-175 flasks. The cells were then trypsinized and 2 x 10^6 cells were pelleted and placed in freshly prepared 1x SSAT buffer (5mM HEPES pH=7.2, 1mM DTT). The samples were stored at -80°C until analysis. To determine intracellular polyamine concentrations, precolumn dansylation, and reversed phase high-performance liquid chromatography methods were utilized. Protein samples were extracted and sent for analysis at Johns Hopkins University or University of Nebraska-Lincoln. Each of the groups were treated after 8h of plating the cells and incubated for 48h post-treatment. The time point of treatment was determined based on the active phase of cell growth (7632663; 11289306).

2.3 Polyamine Enzyme Activity

For ODC, SAT1, and SMOX activity assays, cells were grown in T-175 flasks and treated. Following 48h incubation, cells were lysed using buffer composed of 25mM Tris HCl pH7.5, 0.1mM EDTA, and 2.5mM DTT. Samples were stored at -80°C and shipped to Johns Hopkins University for analyses. ODC enzymatic activity was

expressed as pmol of CO_2/h per mg of protein while SMOX enzymatic activity was expressed as pmol of H_2O_2/min per mg of protein, and SAT1 was expressed as pmol of n^1-acetylspermidine/min per mg of protein.

2.4 Polyamine Metabolic Enzyme Expression

Polyamine metabolic enzyme expression was monitored using qPCR, and Western Blot. Briefly, MDA-MB-231 cells were seeded at a density of 3.0 x 10^5 cells per well in 6-well plates. After an 8h incubation period, cells were treated with 5mM glucose and 25mM glucose respectively and left to incubate for 48 h. Following incubation, samples were extracted and prepared as described below.

2.4.1 qPCR for mRNA Expression

2.4.1.1 RNA Isolation

RNA isolation was conducted using the RNeasy Mini Kit (Qiagen Cat. No. 74104). Following 48h incubation, media was aspirated, and cells were rinsed twice with 1 mL cold PBS per well. Procedure followed was as per manufacturer's protocol. Buffer RLT (350µL) and 70% cold ethanol was added to each well, cells scraped, and all the content was transferred to RNeasy spin columns placed in a 2 mL collection tubes. Following this, 700µL Buffer RWI was added to the RNeasy spin columns. Each sample was then centrifuged at 8000 x g for 15s using the Sorvall Legend Micro 21 centrifuge (Thermo Scientific) and the flow-through was discarded. After centrifugation, 500 µL Buffer RPE was added to each RNeasy spin column and each sample was centrifuged at 8000 x g for 15s. The flow-through was discarded and 500 µL Buffer RPE was added to each RNeasy spin column. Samples were centrifuged at 8000 x g for 2 min and the flow-

through discarded. For the drying step after washing, each sample was centrifuged at 8000x g for 1 min. RNeasy spin columns were placed in new 1.5 mL collection tubes and had 30 µL of RNase-free water added directly to the spin column membrane. Samples were centrifuged at 8000x g for 1 min for RNA elution. Quantification of RNA concentration (ng/µL) was done using the NanoDrop One (ThermoFisher Scientific).

2.4.1.2 cDNA Synthesis

cDNA synthesis was conducted using the Quantabio qScript cDNA SuperMix (Cat No. 95048-025) using the manufacturer's protocols. 1µg RNA per sample was added in 0.2 mL microtubes, 4µL of the qScript cDNA SuperMix for a total volume of 20µL. Samples were incubated as follows: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, hold at 4°C using Bio-Rad T100 Thermal Cycler (Richmond, CA, USA). cDNA was then stored at -20°C.

2.4.1.3 qPCR

A master mix was prepared such that each sample received 1µL of the forward primer, 1µL of the reverse primer, 1µL of RNase free water, and 5µL of SYBR® Green Supermix. A reaction mix was then set up using 2µL cDNA and 8µL master mix for each sample in a MicroAmp® Optical 8-Cap strip for a final volume of 10µL for each reaction. Primers used are given in **Table 1** with actin serving as the endogenous control. Each reaction was performed in triplicate at 95°C/5 min (Stage 1); 95°C/30 sec, 60°C/30 sec, and 72°C/30 sec (Stage 2, 40 cycles) using QuantStudio 5 Real-Time PCR system. This was followed by a melt cycle that consisted of a stepwise increase in temperature from 60 to 99°C. Results were analyzed using Design and Analysis software Applied

Biosystems by Thermo Fisher Scientific ver. 2.6.0, and fold change was calculated using $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer Sequences used for qPCR of MDA-MB-231 cells.

Primers	Forward	Reverse
ODC	5'TCTGATGACGAAGATGAG3'	5'GGTTTAGGTCTCTTTTGC3'
SAT 1	5'ATCTAAGCCAGGTTGGAATG3'	5'GCACTCCTCACTCCTCTGTT3'
SMOX	5'CGCAGACTTACTTCCCCGGC'	5'CGCTCAATTCCTCAACCACG3'
Actin	5'GACGACATGGAGAAAATC3'	5'ACTCCTGCTTGCTGATCCAC3'

2.5 Western Blot for Protein Expression

2.5.1 Sample Preparation

Following 48h incubation, media was aspirated from the cells and rinsed twice with 1 mL cold PBS per well. Lysis buffer master mix composed of 1x lysis buffer (stock 10x from Cell Signaling) and 1x protease inhibitor (stock 100x from Millipore Sigma) was added to each well at 100µL per well. Cells were scraped and contents transferred to 1.5 mL centrifuge tubes and incubated on ice for 10 min. Following this, samples were centrifuged (Eppendorf Centrifuge 5417R) for 10 min at 10,000 rcf. The supernatant was transferred to new 1.5 mL tubes.

Protein concentration was estimated using BCA method kit (Thermofisher, Cat. No. 23225). Sample buffer needed and protein sample were added to each tube for a total volume of 50µL per sample and boiled for 5 min at 100°C. Samples were the cooled and loaded into the gel.

2.5.2 Protein Separation by Gel Electrophoresis

Samples (50-80μg/well) were loaded on 4-20% Tris-HCl gel (Cat. No. 4568094), and the gel was left to run at 210 V for 20 min. Following this, proteins were transferred onto a nitrocellulose membrane using the Trans-blot® Turbo™ Transfer System by Bio-Rad. After transfer process, membrane was incubated in Bio-Rad Everyblot Blocking Buffer (Cat. No. 12010020) for 5 min on a shaker at room temperature. Primary antibodies were prepared in Bio-Rad Everyblot Blocking Buffer according to manufacturer's protocols for estrogen-alpha receptor (1:1000), progesterone receptor (1:000), GAPDH (1:5000), SAT1 (1:1000), and SMOX (1:500). The membrane was incubated in primary antibody on a shaker overnight at 4°C. Following overnight incubation, the membrane was washed twice and incubated in anti-rabbit IgG secondary antibody (1:5000) for 1h using a shaker at room temperature. The membrane was washed then incubated in a 2mL substrate solution composed of 1:1 dilution Bio-Rad Clarity™ Western ECL Subtrate Luminol/enhancer solution and Peroxide solution for 5 min. The membrane was imaged using the Bio-Rad ChemiDoc Touch Imaging system.

GAPDH was used as a loading control. Following imaging of membrane, the membrane was washed then incubated in stripping buffer for 15 min. The membrane was then washed three times with wash buffer for 10 min, 5 min, and 5 min. Following this, it was blocked with Bio-Rad Everyblot Blocking Buffer for 5 min. The membrane was incubated overnight on a shaker at 4°C with GAPDH. The following day, protocol for secondary antibody addition and imaging was followed.

2.5.3 Cell Proliferation Assay with Polyamine Inhibition and Re-supplementation

Cells were seeded in 96-well plates at a density of 8,000 cells per well and left to incubate for 8h to allow it to adhere. Following this, the cells were treated at 90µL per well with five replicates of each treatment. For re-supplementation, DFMO, and each polyamine (putrescine, spermidine, and spermine) along with 1mM aminoguanidine were added with either 5mM or 25mM glucose to treat cells. Aminoguanidine was added to prevent extracellular oxidation by bovine serum oxidase (30138353). Proliferation was monitored 48h post-treatment using PrestoBlue dye. To conduct this, 10µL of dye was added to each well and the plate incubated for 10 min at 37°C. Fluorescence was monitored at 560/600nm (excitation/emission) using a microplate reader as well as the Gen5 software (BioTek Synergy 2; Winooski, VT, USA). **Figure 4** shows a simplified depiction of the experimental timeline. Readings for blank wells containing media only with no cells were subtracted from all treated wells during analyses. Percent change was calculated relative to the 5mM glucose control group.

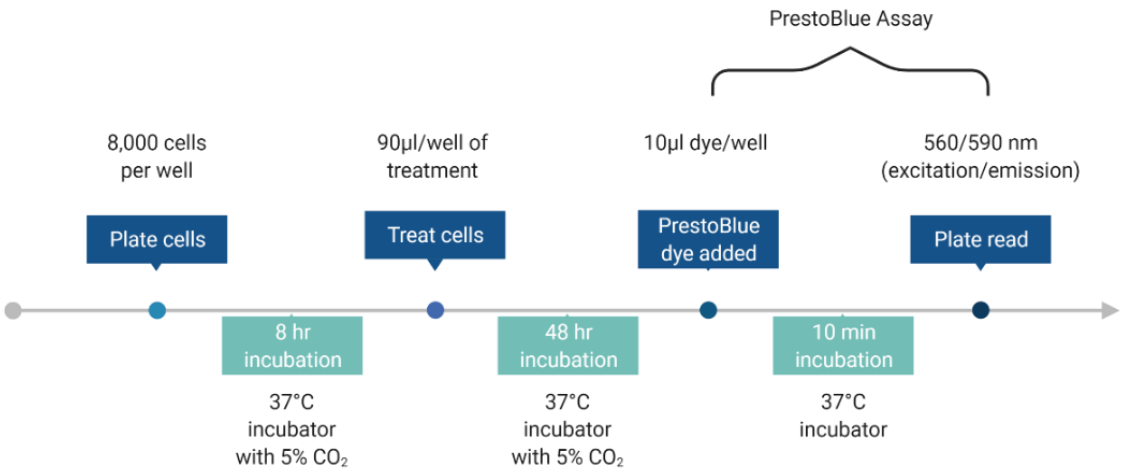


Figure 4. Timeline for Cell Proliferation and Polyamine Inhibition and Re-supplementation. This figure depicts the protocol used for polyamine inhibition and re-supplementation and the timeframe in which the protocol was conducted. Created with BioRender.com.

2.6 Transfection and Cell Proliferation

Cells were seeded in 96-well plates at a density of 20,000 cells per well and left to incubate for 24h to allow it to reach 70-90% confluency prior to treatment. Following this, the cells were transfected. Transfection protocol was optimized using manufacturer's protocols. Briefly, 100ng/uL of miR-133a was diluted in OptiMEM™ Reduced Serum Medium for a total volume of 20 µL. Lipofectamine™ 3000 Transfection Reagent was diluted separately in OptiMEM™ Reduced Serum Medium for a total volume of 40 µL. Diluted miR and diluted Lipofectamine were mixed together at a 1:1 ratio for a total volume of 20 µL and left to incubate for 5 min at room temperature. Non-targeting RNA dilution and treatment preparation was the same as the protocol for miR-133a treatment. Following the 5min incubation, 10 µL of the RNA-lipid complex was added to each well and 35 µL OptiMEM™ reduced serum medium for a total of 45µL per well with three replicates of each treatment. After transfection (4h), each well received 45µL of 10mM or 50mM glucose concentrations to achieve a final concentration of 5mM and 25mM glucose, respectively. Control wells received 45µL of OptiMEM™ reduced serum medium and 45µL of 10mM or 50mM glucose concentrations to achieve a final concentration of 5mM and 25mM glucose, respectively. miR-lipid complex was allowed to remain in the cells for the entire period of treatment (48hr). Proliferation was monitored as described earlier.

2.7 Statistical Analyses

All values were expressed as mean ± SEM. Results were analyzed using the Graph Pad software (Prism 5.0) and show the results of at least three experiments that are performed in replicates. Statistical comparison between more than two different groups were conducted using one-way ANOVA followed by Tukey's test. Differences are considered statistically significant at $p \leq 0.05$.

III. Results

1.1 Polyamine Analyses

Polyamine content has been shown to be elevated in breast cancer cells [53-57]. Analyses of intracellular polyamine content for MDA-MB-231 and MCF-10A showed that overall polyamine content for MDA-MB-231 was higher than MCF-10A under both normal (5mM Glu) and high (25mM Glu) glucose conditions (**Table 2**). In normal glucose conditions, putrescine showed a ten-fold increase, spermidine was elevated as well, and spermine showed a two-fold increase in MDA-MB-231 compared to MCF-10A. The trend noted under normal conditions between MDA-MB-231 cells and MCF-10A is the same trend noted under high glucose conditions as well. In MDA-MB-231 cell line specifically, putrescine showed marginal increase (indicated with an *), whereas spermidine and spermine did not show any significant change when comparing cells treated with normal and high glucose.

Table 2. Polyamine levels in MDA-MB-231 and MCF-10A under normal and high glucose conditions.

Cell Lines		**Polyamines (nmol/10^6 cells)**		
	Treatment	**Putrescine**	**Spermidine**	**Spermine**
MDA-MB-231	5mM Glu	0.51±0.12	4.22±1.32	1.18±0.42
	25mM Glu	0.69±0.13*	4.61±1.58	1.02±0.38
MCF-10A	5mM Glu	0.05±0.01	3.60±0.07	0.65±0.04
	25mM Glu	0.03±0.02	3.28±1.86	0.74±0.46

1.2 MDA-MB-231 Polyamine Metabolic Enzyme Expression: qPCR

In the MDA-MB-231 cell line, polyamine enzyme expression was shown to be variable. Under high glucose conditions, ODC activity was trending towards a decrease in mRNA expression (**Figure 5a**). This was also shown to be the case for catabolic polyamine enzymes SAT1 (**Figure 5b**) and SMOX (**Figure 5c**), with both enzymes trending towards a decrease in expression. Taken together, the data shows that while not significant, mRNA expression under high glucose conditions are trending towards a decrease in expression.

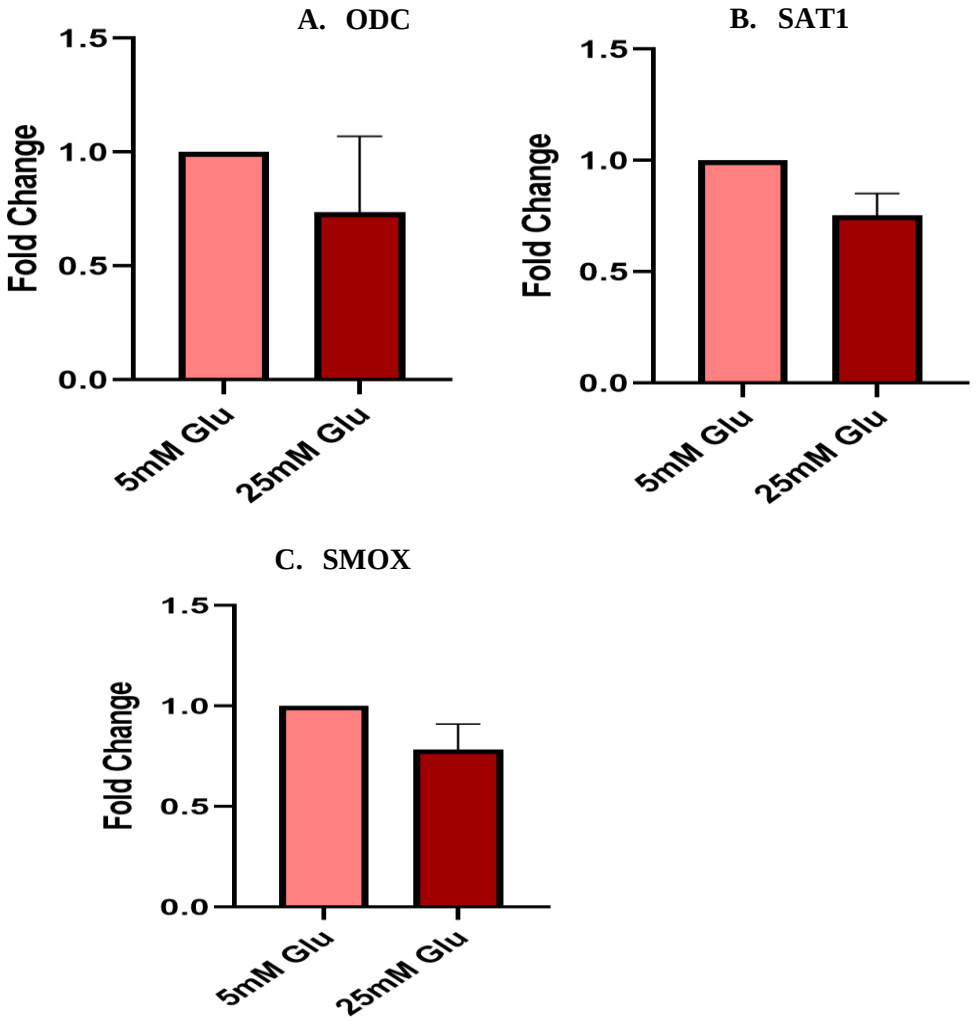


Figure 5. Polyamine enzymes mRNA expression in MDA-MB-231. mRNA Expression of polyamine enzymes (A) ornithine decarboxylase (ODC), (B) spermidine/spermine n1-acetyltransferase (SAT1) and (C) spermine oxidase (SMOX) under normal (5mM Glu) and high (25mM Glu) glucose conditions. Samples were run in triplicate and experiments repeated for a total of three times. Values are mean ± SEM (n=3).

1.3 MDA-MB-231 Polyamine Metabolic Enzyme Expression: Western Blot

The western blot showed that SMOX showed up around 37 kD (**Figure 6**). This was confirmed by using the A2A-HEK, human embryonic kidney cell line, which was used by the manufacturer, to confirm SMOX presence. GAPDH was used as a loading control to normalize the amount of protein loaded per sample. Further, based on the blot, there is visual confirmation that there is a decrease in SMOX protein under high glucose conditions. Thus showing that protein expression of the polyamine catabolic enzyme SMOX trended towards a decrease under high glucose conditions. SAT1 and ODC protein expressions were not detectable.

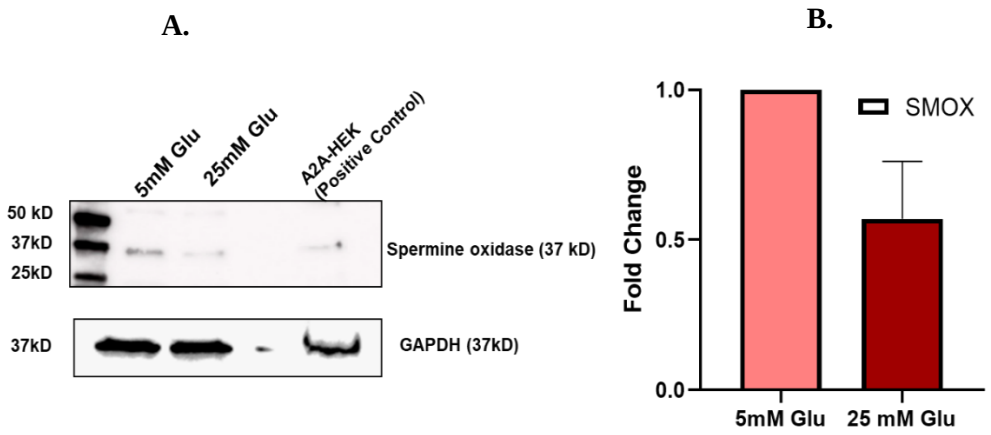


Figure 6. SMOX protein expression in MDA-MB-231 cells under normal and high glucose conditions. (A) MDA-MB-231 Western blot analysis of polyamine catabolic enzyme spermine oxidase (SMOX) (B) Fold change of high glucose (25mM Glu) conditions when compared to normal glucose (5mM Glu) conditions. Experiments were repeated for a total of three times. Values are mean ± SEM (n=3).

1.4 Polyamine Enzyme Activity

When comparing both cell lines under normal conditions, ODC activity is significantly higher ($p < 0.05$) in MDA-MB-231 than in MCF-10A (Figure 2). Under high glucose conditions, both cell lines showed significant increase in the enzyme activity of ODC ($p < 0.001$). In MDA-MB-231 cells, ODC activity changed from approximately 75 $pMolCO_2$/hr/mg protein to approximately 150 $pMolCO_2$/hr/mg protein (**Figure 7a**) and in MCF-10A there was a change from roughly 25 $pMolCO_2$/hr/mg protein to 110 $pMolCO_2$/hr/mg protein with high glucose.

Interestingly, under normal conditions, SAT1 enzyme activity is downregulated in MDA-MB-231 compared to MCF-10A. under high glucose conditions (**Figure 7b**). The trend in MDA-MB-231 cells was similar to that of ODC; there was an increase from approximately 3 n^1-acetylspermidine pMol/mg protein/min to 4.5 n^1-acetylspermidine pMol/mg protein/min with high glucose treatment. In MCF-10A, however, the increase was more significant, an elevation from 5pMol/min mg protein to nearly 11.5 pMol/min mg protein.

SMOX enzyme activity was found to be higher under normal glucose conditions in the MDA-MB-231 cell line compared to the MCF-10A cell line (**Figure 7c**). Under high glucose conditions, enzyme activity of SMOX was downregulated significantly in MDA-MB-231 decreasing from about 0.4 $pMolH_20_2$/min/mg protein to roughly 0.06 $pMolH_20_2$/min/mg protein, though no significant change was noted in SMOX activity in MCF-10A.

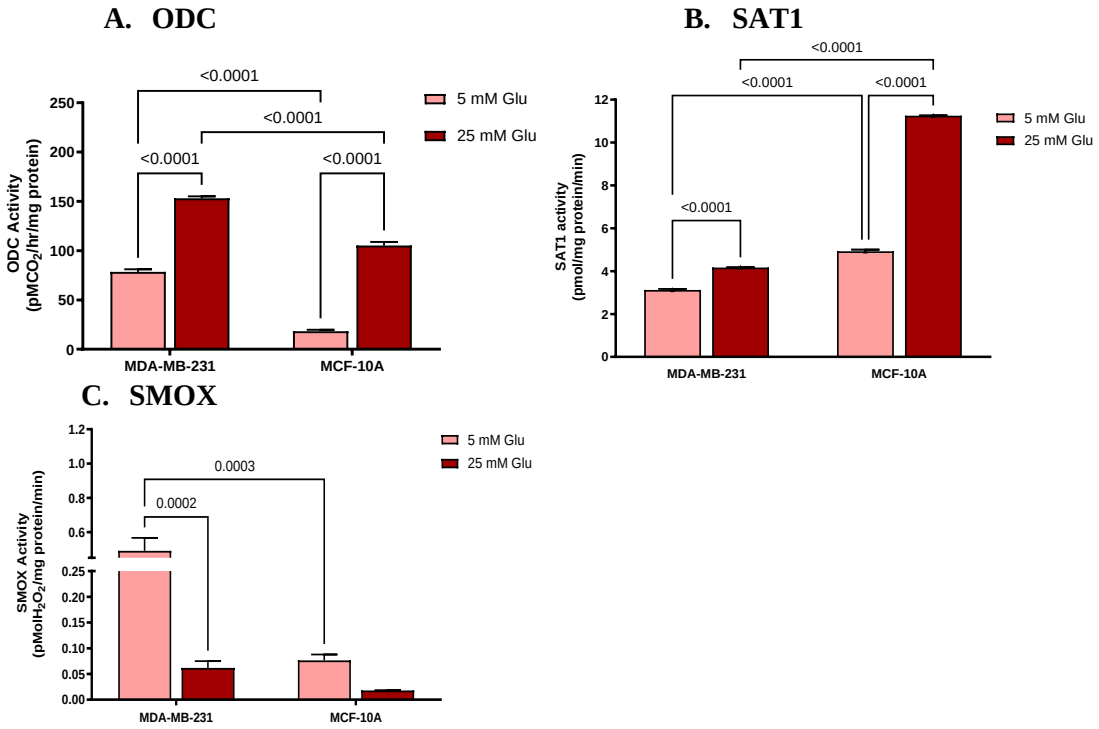


Figure 7. Polyamine Enzyme Activity in MDA-MB-231 and MCF-10A cells under normal and high glucose conditions. Enzyme activities of ornithine decarboxylase (ODC), (B) spermidine/spermine n1-acetyltransferase (SAT1) and (C) spermine oxidase (SMOX) in MDA-MB-231 TNBC cell line (left) and MCF10A normal-like breast epithelial cell line (right) under low (5mM Glu) and high (25mM Glu) glucose conditions. Samples were run in triplicate and experiments repeated for a total of three times. Values are mean ± SEM (n=3).

1.5 Polyamine Inhibition in MDA-MB-231

It has previously been reported that MDA-MB-231 showed an increase in cell proliferation when treated with high glucose [64]. Findings reported here support this with MDA-MB-231 cells undergoing a 150% increase in cell proliferation in high glucose conditions (**Figure 8**). Treatment with the polyamine inhibitor DFMO, showed a significant decrease in cell proliferation. Cell proliferation with DFMO and high glucose showed about 50% decrease in cell proliferation compared to cells treated under high glucose conditions only. Our findings indicated that DFMO may be effective against decreasing cell proliferation in MDA-MB-231 under high glucose conditions.

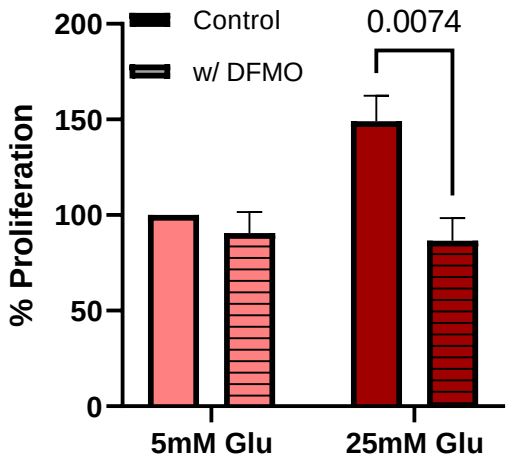


Figure 8. Effect of DFMO on cell proliferation under normal and high glucose conditions in MDA-MB-231. MDA-MB-231 cells under normal glucose conditions (5mM Glu) treated with only 5mM Glu and a combination of 5mM Glu and the polyamine inhibitor, DFMO (left). Cells were also treated with only 25mM Glu and a combination of 25mM Glu and the polyamine inhibitor, DFMO (right). Samples were run in triplicate and experiments repeated for a total of four times. Values are mean ± SEM (n=4).

1.6 MDA-MB-231 Polyamine Inhibition and Re-supplementation

For our current study, we explored the effect of adding back the polyamines, putrescine, spermidine, and spermine while also treating with DFMO. Following treatment with DFMO, a decrease in cell proliferation under high glucose conditions is noted but no change observed under normal glucose conditions. When re-supplementing with the polyamine putrescine, no change was seen in cell proliferation (**Figure 9a**). Re-supplementation with spermidine resulted in a significant increase in cell proliferation ($p<0.05$) under high glucose conditions following inhibition (**Figure 9b**). Re-supplementation with spermine also showed a similar trend with spermidine re-supplementation. Spermine re-supplementation resulted in an increase in cell proliferation under high glucose conditions (**Figure 9c**). Taken together, the data shows that the polyamine inhibitor, DFMO, exerts a cytostatic effect on MDA-MB-231 cells.

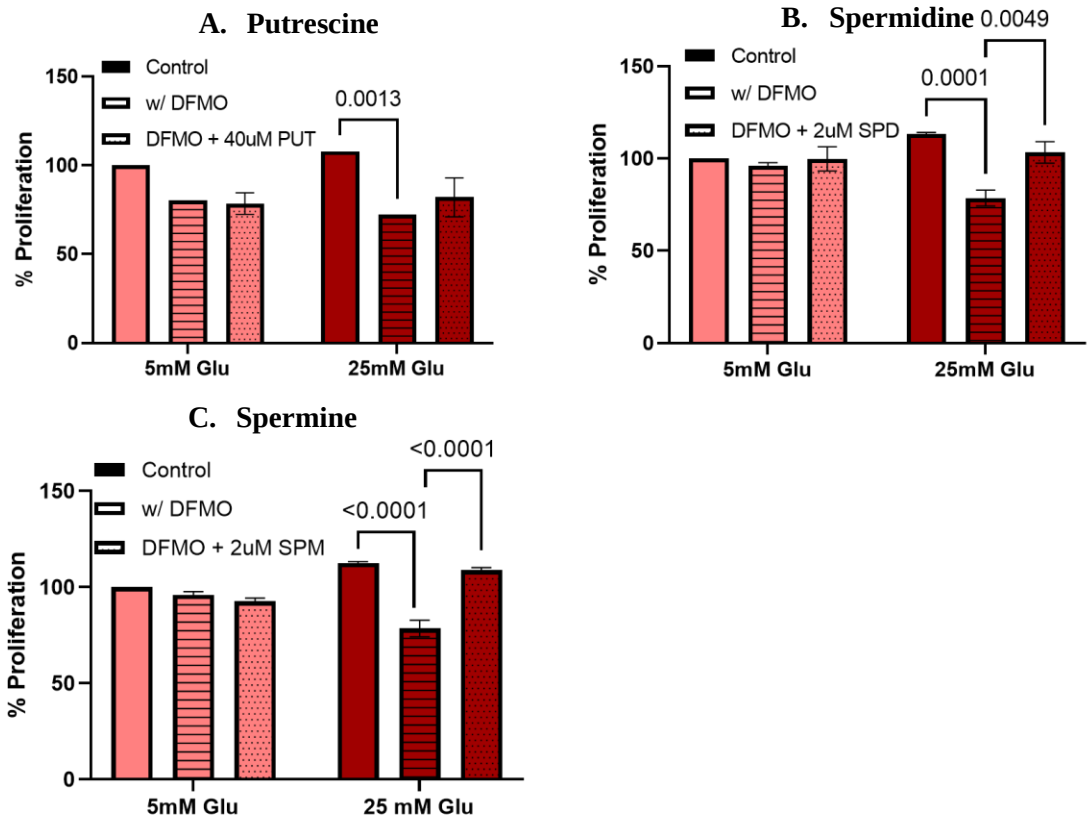
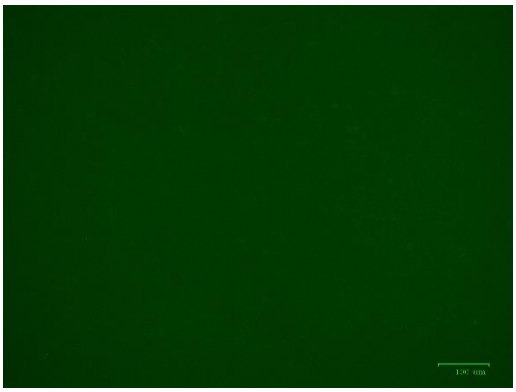


Figure 9. MDA-MB-231 Cell Proliferation following polyamine inhibition with DFMO and polyamine re-supplementation. (A) Putrescine (B) Spermidine (C) Spermine re-supplementation under normal (5mM Glu) and high (25mM Glu) glucose conditions. Samples were run in triplicate and experiments repeated for a total of four times. Values are mean ± SEM (n=4).

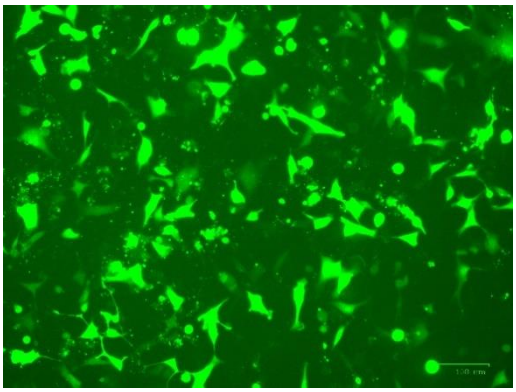
1.7 miR-133a Transfection

Cells were plated and transfected with miR-133a that had been tagged with a GFP protein. After 24 and 48 h, cells were evaluated for successful transfection by fluorescence. Cells treated with miR-133a fluoresced green while cells treated only with normal and high glucose or normal and high glucose with non-targeting silent RNA did not fluoresce under the microscope (**Figure 10**).

A. Negative Control



B. Normal Glucose



C. High Glucose

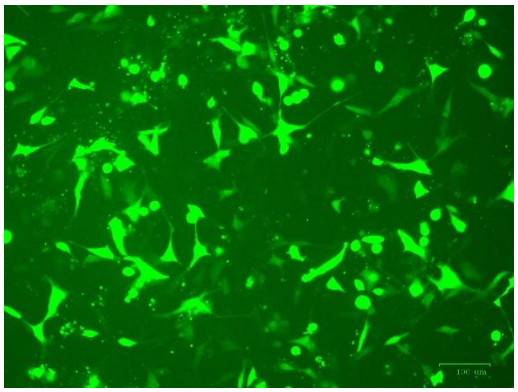


Figure 10. MDA-MB-231 transfection with GFP-Tagged miR-133a. MDA-MB-231 cells treated with (A) normal glucose only and miR-133a under (B) normal and (C) high glucose conditions under fluorescent microscopy after 48hr treatment.

1.8 miR-133a and MDA-MB231 Cell Proliferation

Treatment with miR-133a showed that under normal and low glucose conditions miR-133a significantly decreased cell proliferation (**Figure 11**). Treatments with non-targeting silent RNA also showed a decrease in cell proliferation under both normal and high glucose concentrations. Under high glucose concentrations, there was a clear difference in efficacy of miR-133a treatments versus non-targeting silent RNA treatments in terms of cell proliferation. Cells treated with miR-133a more significantly decreased cell proliferation ($p<0.0001$) than cells treated with the non-targeting silent RNA ($p<0.05$). Taken together, this data shows that miR-133a impacts cell proliferation in MDA-MB-231 cells in both normal and high glucose states.

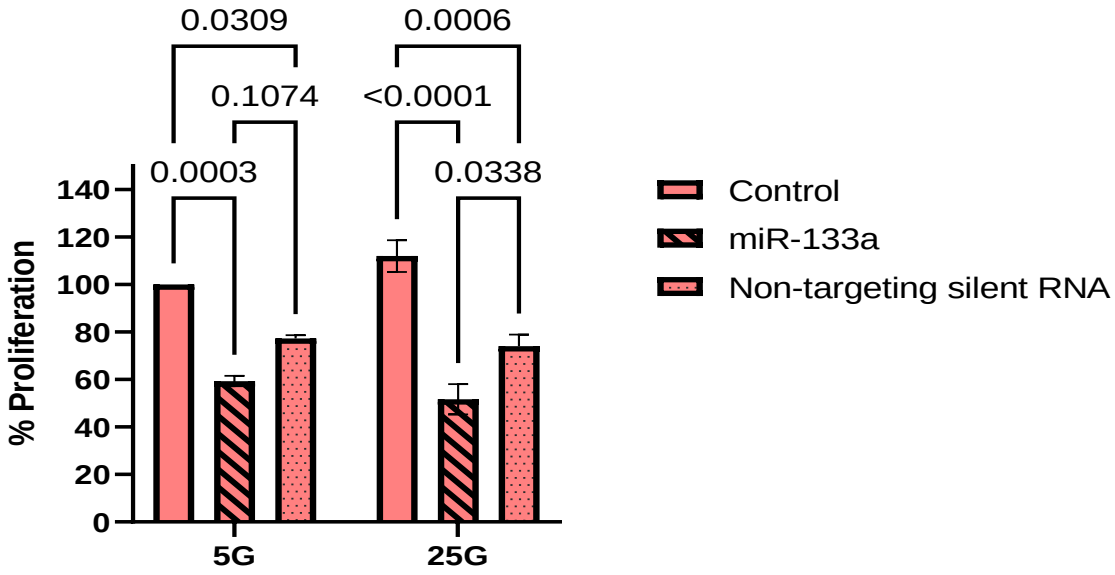


Figure 11. Cell proliferation of MDA-MB-231 cells treated with miR-133a. MDA-MB-231 treated with miR-133a and non-targeting silent RNA showing a decrease in cell proliferation under both normal and high glucose conditions. Samples were run in triplicate and experiments repeated for a total of three times. Values are mean ± SEM (n=3).

1.9 RNA-RNA Binding Prediction and Interaction of miR-133a and ODC

It has been shown that miR133a is predicted to have a strong binding affinity for ODC (**Figure 12**). Taken together with previously shown data, this shows that miR133a has the potential to bind to ODC and inhibit cell proliferation. The binding prediction of the microRNA and the target mRNA sequence revealed a hybridization energy of −15.86 kcal/mol in exon 2 position 77 to position 94 with two mismatching nucleotides at position 87 and 88 (**Figure 12a, Figure 12b**). The negative hybridization energy indicates that strong binding can occur between ODC and miR-133a thus making it a potential cancer therapeutic to modulate the expression of ODC. [39]

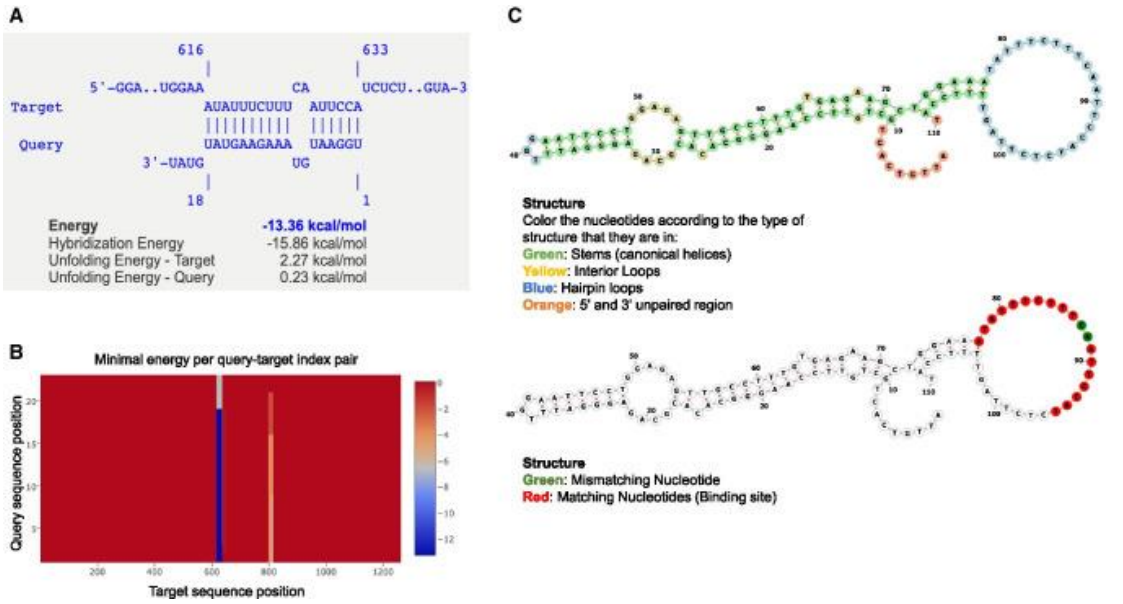


Figure 12. RNA-RNA binding prediction and interaction of hsa-miR-1-3p and ODC.

RNA-RNA binding prediction was performed using the IntaRNA online tool (http://rna.informatik.uni-freiburg.de) to measure the hybridization energy between ODC1 (NM_001287189.2) and the hsa-miR-1-3P (MI0000651). A secondary structure analysis was performed using Forna (Kerpedjiev P, Hammer S, Hofacker IL, 2015). Forna (force-directed RNA): Simple and effective online RNA secondary structure diagrams. Bioinformatics 31(20):3377–9.) server in Vienna's RNA lab tool to reveal the binding position of the microRNA in the target sequence. The top structure of C represents color-coded mRNA secondary structure of ODC1 Exon 2. The nucleotide colors represent the type of structure they form, green: stems (canonical helices), red: multiloops (junctions), yellow: interior loops, blue: hairpin loops, orange: 5′ and 3′ unpaired region. The bottom structure represents the binding site of the microRNA, the green color represents mismatching Nucleotide, while the red color represents matching nucleotides at the binding site. [39]

IV. Discussion

The hypotheses for this study were: (1) Potential targets in the polyamine pathway are involved with diabetic TNBC cell proliferation, and (2) miR-133a will reduce TNBC cell proliferation through the inhibition of polyamine biosynthesis pathway. These hypotheses were assessed through two specific aims: (1) determine polyamine biosynthesis and/or catabolic enzymes dysregulated with diabetic TNBC cell proliferation, and (2) use chemotherapeutic agents and miRNA mimics against diabetic TNBC cells to determine their potential in targeting polyamine metabolism.

Results reported here show that the TNBC cell line, MDA-MB-231, has elevated basal polyamine content compared to the normal-like breast epithelial cell line MCF-10A. Further, under high glucose conditions, MDA-MB-231 exhibits an increase in the first polyamine, putrescine when compared with normal glucose. With respect to polyamine enzymes, high glucose exposure elevated the biosynthetic enzyme, ornithine decarboxylase (ODC) while downregulating the catabolic enzyme, spermine oxidase (SMOX), which would correlate to higher polyamine concentrations in MDA-MB-231. In addition, high glucose treated TNBC cells exhibit marked increase in cell proliferation.

Upon inhibiting the polyamine biosynthesis pathway using the ODC inhibitor DFMO, a decrease in cell proliferation was noted in the high glucose treatment group, though DFMO displayed only a cytostatic effect. Interestingly, transfection of MDA-MB-231 cells with miR-133a remarkably suppressed cell proliferation under both normal glucose and high glucose conditions. Furthermore, genomic interaction data reveals strong binding of miR-133a to ODC gene, indicating miR-133a could be a potential

therapeutic option for diabetic TNBC regulated through polyamines. Taken together, our results provide strong evidence that support our proposed hypotheses.

1.1 Aim 1: Determine polyamine biosynthesis and/or catabolic enzymes dysregulated with diabetic TNBC cell proliferation

It has been well documented in previous literature that polyamine content is elevated in a variety of cancers including breast cancer [53-57]. In DM, polyamines have been shown to improve the action of insulin [45]. Spermidine and spermine specifically are known regulators of the insulin signaling pathway [88]. Further, the catabolic enzyme SAT1 exerts a protective effect in DM by improving glucose tolerance [89]. Results of our current study support previous literature on the relationship between breast cancer and polyamines by showing that the TNBC cell line MDA-MB-231 has elevated intracellular polyamine content (5- to 10-fold) compared to the normal-like breast epithelial, MCF-10A cell line under normal glucose. Putrescine was the only polyamine which showed a marginal increase under high glucose in TNBC cells. This can be attributed to the fact that the intracellular polyamine content is already elevated in TNBC cells under normal conditions, thus further increases in polyamine content with high glucose is not as evident. While the relationship between polyamines and DM, along with polyamines and breast cancer has been explored, there are few studies that have studied polyamines with both DM and breast cancer. Our study is unique in investigating polyamines as a potential link between DM and TNBC.

Intracellular source of polyamines includes biosynthesis, cellular uptake, and synthesis by intestinal flora [45, 46]. In this study, we aimed to explore the cellular

biosynthesis pathway. It was observed that mRNA expression of key polyamine enzymes ODC, SAT1, and SMOX were not markedly affected under high glucose in TNBC cells. However, activity of polyamine synthesis enzyme, ODC, was significantly increased, while activity of catabolic enzyme, SMOX, was significantly decreased upon high glucose exposure. This data suggests that polyamine pathway is affected in diabetic TNBC, which could attribute to TNBC advancement. Interestingly, SAT1 (catabolic enzyme) activity was also upregulated. This is similar to previous report that indicated that ODC and SAT1 are upregulated in breast cancer tissues, and SMOX is downregulated compared to non-tumor tissues [54]. SAT1 is the primary polyamine enzyme active in maintaining the intracellular polyamine pools [45, 72]. SAT1 upregulation suggests that it is working to restore intracellular polyamine pools back to their original levels which indicates an increase in the polyamines spermine and spermidine. Further, SAT1 converts spermine into spermidine and spermidine into putrescine. The lack of change in the spermidine and spermine levels could be accounted for by the decrease in SMOX, and an increase in SAT1 activity. Further, the secondary pathway feeds into the spermidine and spermine polyamine pools keeping their levels from changing, while the upregulation of ODC contributes to an increase in the putrescine levels. Therefore, an increase in SAT1 and ODC enzyme activity could account for why putrescine is markedly elevated in TNBC under high glucose compared to normal conditions.

There is a clear discrepancy between mRNA expression and activity of polyamine enzymes. While mRNA expression does not change with glucose treatments, activity of

these polyamine enzymes shows variable trends accounting for the increase in polyamines. According to literature, this discrepancy can be explained by the fact that polyamine enzyme activity may be regulated post-transcriptionally [90]. It is also important to note that this does not consider contributions made by the secondary pathway that feeds into the primary polyamine biosynthesis pathway (**Figure 1 from Introduction**). From these results, we can infer that polyamine enzymes ODC and SMOX could be potential targets in the polyamine biosynthesis pathway for the action of cancer therapeutics. ODC is the key rate-limiting enzyme of the polyamine biosynthesis pathway while SMOX is part of the catabolic pathway responsible for the catalytic oxidation of spermine to spermidine, and a source of ROS-induced damage to DNA [91, 92]. Moreover, SMOX is considered to be a local death inducer in breast cancer, and low expression is a potential biomarker of breast cancer [92]. Further, literature has shown that another polyamine enzyme associated with the catabolic pathway, acetylated polyamine oxidase (AcPAO), is downregulated in breast cancer tissues compared to non-tumorigenic tissues and could potentially be another target to explore in the pathway [54]. This enzyme was not tested in the present study but would be a target of interest in the future.

1.2 Aim 2: Use chemotherapeutic agents and miRNA mimics against diabetic TNBC cells to determine their potential in targeting polyamine metabolism.

Inhibition of polyamine synthesis has been approached extensively though there is a lack of an effective chemotherapeutic. ODC inhibitor, DFMO, has shown the most promising effect, however, it exhibits a cytostatic instead of cytotoxic effect [74]. The

inefficacy of DFMO also arises from its poor availability, and compensatory increase in polyamine uptake and metabolism in tumor environment [93]. Currently approved use of DFMO has been in combination with NSAIDs for treatments of skin cancer as well as other cancers such as colon cancer [94, 95]. We have shown that in diabetic TNBC cells DFMO is effective in reducing cell proliferation but not under normal glucose conditions. This could potentially be due to the fact that ODC is tightly regulated under normal conditions at transcriptional, post-transcriptional, and translational levels [67, 68].

A previous study by our lab showed this trend as well and supports our current findings [64]. To evaluate whether DFMO is indeed inducing cytotoxicity, polyamines (putrescine, spermidine and spermine) were re-supplemented exogenously while inhibiting with DFMO. It was observed that cell proliferation which was decreased with DFMO retracted to the non-DFMO levels in high glucose state. The effect was more evident with spermidine and spermine supplementation although putrescine supplementation did not reverse the DFMO induced inhibition of proliferation. This suggests that DFMO exerts a cytotoxic effect on TNBC cells under high glucose conditions upstream of the pathway (ODC inhibition to decrease putrescine) and causes a cytostatic effect downstream of the pathway, as re-supplementation of spermidine and spermine restores cell growth. Thus, DFMO is not an effective inhibitor of the polyamine biosynthesis pathway under diabetic conditions. This aspect of DFMO inhibition and polyamine re-supplementation under diabetic conditions has not been explored previously, thus making our study unique.

The inefficiency of DFMO has led to the search for more efficient chemotherapeutic options. Over the years, there has been an increased interest in the use of microRNA (miR) and miR mimics as potential cancer therapeutic options [96, 97]. While there are many miRs of interest, miR-133a has been known to function as a tumor suppressor in breast cancer thus making it a potential cancer therapeutic to mitigate diabetic TNBC cell proliferation [82]. miR-133a is found to be downregulated in both DM and breast cancer, thus suggesting that its upregulation could help in mitigating the progression of both diseases [81, 98, 99]. Our results show that TNBC cells treated with miR-133a show decreased cell proliferation under both normal and high glucose conditions compared to negative control siRNA. Further, our study showed that miR-133a hindered cell proliferation under normal conditions whereas DFMO did not. This indicates that miR-133a has the potential to be more effective than DFMO as a mono-therapeutic option. While there are no studies on the effect of miR-133a on TNBC under diabetic conditions, there are studies that have shown that miR-133a suppressed tumor growth and prevented cell proliferation in breast cancer [81, 100].

We have shown the RNA-RNA binding prediction for ODC and miR-133a which indicates a strong binding affinity between the two thus indicating that miR-133a could potentially regulate the polyamine biosynthesis pathway. Future studies should explore the effect of miR-133a on polyamine enzymes identified in this study as potential targets. So far, we can infer that though DFMO and miR-133a are both potential candidates for inhibiting TNBC cell proliferation under diabetic conditions, DFMO is merely cytostatic and its inhibitory effects on cell proliferation can be reversed whereas miR-133a has the

potential to bind to and modulate the behavior of mRNA of target genes of the polyamine biosynthesis pathway [81].

1.3 Summary

As previously mentioned, there are few studies that look at the role of polyamines in diabetic TNBC. Our study helps provide insight into the topic and propose potential roles of polyamines in diabetic TNBC and potential cancer therapeutics that may be beneficial. Further, the results of our study indicated a dysregulation in polyamine content in triple negative cells, either cancerous or non-cancerous under high glucose conditions. This could be an important biomarker in identifying individuals at risk of getting breast cancer and may also suggest that individuals with diabetes who have triple negative breast epithelial cells (non-cancerous) could have an increased likelihood of being diagnosed with TNBC in their lifetime.

While we have managed to elucidate the role of the polyamine pathway in diabetic TNBC progression, time constraints prevented us from exploring the secondary pathway that feeds into the primary one. Further, optimization of the transfection protocol took longer than anticipated, thus making it difficult to explore the direct effect that miR-133a had on polyamine metabolism and catabolism. However, by exploring this topic, we have added to the literature that could potentially contribute to a targeted therapeutic option to mitigate and treat individuals with this comorbidity of DM and TNBC.

1.4 Conclusion and Future Directions

In this study we have shown that high glucose conditions increase cell proliferation of TNBC via the polyamine biosynthesis pathway by altering the enzymes,

ODC and SMOX. Further, DFMO is not a very effective inhibitory agent for the polyamine biosynthesis pathway in diabetic TNBC cells because it exerts a cytostatic effect which can be reversed when polyamines are re-introduced. miR-133a decreases cell proliferation of diabetic TNBC cells and has the potential to regulate ODC and target the polyamine biosynthesis pathway.

Currently, there is a lack of targeted therapeutics for TNBC, and current strategies have not been very effective. Further, the rate at which individuals are being diagnosed with TNBC as well as the predicted number of individuals estimated to have T2DM is alarming. Therefore, novel approaches to mitigate this comorbidity are necessary. In the future, studies should focus on identifying the specific ways by which miR-133a influences the regulation of the polyamine biosynthesis pathway focusing specifically on the targets identified in this study. Our study focused on the biosynthesis and catabolic pathways; future studies should consider the role of the secondary pathway and explore it for potential targets. Additionally, there are three sources of polyamines, we explored one, the other two ways should also be explored to further elucidate the role of polyamines in diabetic TNBC advancement. Finally, studies should also explore how miR-133a effects cells *in vivo* as opposed to *in vitro*.

V. LITERATURE CITED

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