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14. ABSTRACT

The most significant finding in this year of research has been that plasmid AHCY_13A containing a miRNA sequence targeted towards the enzyme S-adenosylhomocysteinase knocks down AHCY protein in HEK 293T cells and does have effects on cancer cell function. We look forward to investigating and characterizing these effects in the coming year.

15. SUBJECT TERMS
Folate, breast cancer, dihydrofolate reductase, DNA methyltransferase, methylenetetrahydrofolate reductase, S-adenosylhomocysteinase

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Table of Contents

Introduction: This training grant focuses on the effects of perturbing one carbon and folate metabolism on epigenetic regulation, breast cancer cell phenotype and breast cancer cell function. The **objective** of the project is to understand the relationships between one carbon metabolism, DNA synthesis, DNA methylation and gene expression within the context of breast cancer. Our hypothesis is that the inhibition of key enzymes in one carbon metabolism: dihydrofolate reductase (DHFR), methylenetetrahydrofolate reductase (MTHFR), S-adenosylhomocysteinase (AHCY) and DNA methyltransferase 1 (DNMT1) by miRNA or drugs will decrease breast cancer cell growth, modulate cell migration, and decrease the ability of the cell to survive anoikis by increasing expression of hypermethylated genes including transcription factors.

Body: The original tasks outlined in the proposal were to characterize the effects on global and gene specific methylation and gene expression in human breast carcinoma (MCF7, MDA-MB231) and mouse mammary tumor cells (Met-1, DB-7) of the following:

a. Folate deficiency

b. 5-aza-2'-deoxycytidine (ADC) exposure

c. siRNA directed against DNA methyltransferase 1 (DNMT1)

d. siRNA directed against MBD2 protein

In the next couple of paragraphs the original proposed task is in bolded text while the modified task is in italicized text followed by our rationale for making the change.

Work in four cell lines: MCF7 and MDA-MB-231 (human) and Met1 and DB-7 (mouse). *We are currently working in the two human cell lines MCF7 and MDA-MB-231.* Making cells folate deficient proved to be difficult (see #2 for more information), so we decided to make tetracycline inducible stable knockdowns targeting four key enzymes in one carbon metabolism instead. The amount of work involved in doing this is much greater than that originally proposed, and therefore we decided to concentrate on making the stably transduced cell lines using the human cell lines first, with the thought that we could repeat our successes in the mouse cell lines if time and funding allowed for it. To date we have generated all the plasmids required for making the stable cell lines and are in the process of screening the miRNA containing plasmids in HEK 293T cells for adequate protein knockdown. We are also in the process of selecting MDAMB231 clones that express non-leaky TetR systems.

Test effects of folate deficiency on global and gene specific DNA methylation and gene expression. *Achieving sufficient folate deficiency was difficult without affecting*

cell growth and replication. We decided to take a different approach and inhibit four enzymes involved in one carbon and folate metabolism specifically dihydrofolate reductase (DHFR), methylenetetrahydrofolate reductase (MTHFR), DNA methyltransferase 1 (DNMT1) and S-adenosylhomocysteinase (AHCY). We are in the process of creating tetracycline inducible, stably transduced cell lines that express miRNA against each of the four enzymes when the cell line is treated with tetracycline. Once the cell lines are made we will study the effects of depletion of protein expression on breast cancer cell growth, apoptosis, replication, migration, ability to survive anoikis, DNA methylation (global and gene specific) and concentrations of one carbon metabolites (S-adenosylmethionine, S-adenosylhomocysteine and homocysteine) (Table 1).

Effect of DNMT1 inhibition by 5'-aza deoxycytidine (ADC) on global and gene specific DNA methylation and gene expression. *We plan to inhibit DNMT1 with both ADC and lentiviral mediated transduction of miRNA against DNMT1.* To date we have treated MCF7 cells with ADC and examined gene expression levels using mRNA microarray analysis.

Effect of MBD2 inhibition on gene expression. *Since we are no longer testing the effects of folate deficiency we decided to focus more closely on inhibiting enzymes more closely related to folate and one carbon metabolism i.e. DHFR, MTHFR and AHCY.* We decided to inhibit DHFR using the drug methotrexate as well as lentiviral mediated transduction of miRNA against DHFR. We wanted to measure the inhibition of DHFR activity by both drug and miRNA and spent time trying to develop an assay to measure DHFR activity. We tried two approaches and were unsuccessful both times so we are focusing on lentiviral mediated inhibition of DHFR which can be assessed easily using western blots.

Once the tetracycline inducible stably transduced cell lines are made, we will perform the assays outlined in Table 1 to assess changes in breast cancer cell function and phenotype.

Table 1: Assays to assess breast cancer cell function and phenotype after inhibition of DHFR, MTHFR, AHCY or DNMT.

Measured Endpoint	Assay
Cell proliferation	Trypan blue exclusion assay and MTT assay
Cell apoptosis	TUNEL assay and caspase 3 staining
Cell migration	Scratch assay
Anoikis	Propidium iodide staining followed by flow cytometry
Differential gene expression	mRNA microarray
Gene-specific promoter methylation	Methylation specific PCR or pyrosequencing

Key Research Accomplishments:

We have completed synthesis of plasmid constructs required to make stable cell lines. The tetracycline inducible stably knocked down cell lines are designed using a modified two plasmid pLenti6 lentiviral system from Invitrogen. The first plasmid constitutively expresses the Tet Repressor protein and has zeocin resistance in mammalian cells (Figure 1a). The second plasmid expresses a fluorescent mCherry marker and miRNA sequence designed to knockdown DHFR, DNMT1, MTHFR or AHCY under the control of two tet operon sites (Figure 1b). In the stable cell line, Tet Repressor protein binds to the Tet operon sites preventing expression of the miRNA sequence. Tet repressor protein has a higher affinity for doxycycline than it does for the Tet operon sites, so when doxycycline is added to the media, expression of the miRNA sequence is de-repressed and target protein expression is knocked down.

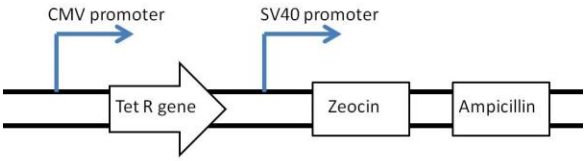


Figure 1a: The Tet Repressor plasmid, pLenti6/TR/zeocin, expresses the Tet Repressor protein and has mammalian zeocin resistance and bacterial ampicillin resistance.

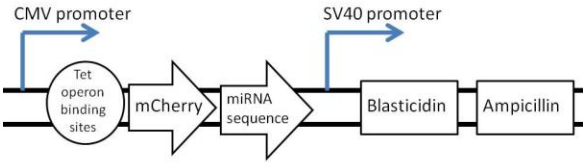


Figure 1b: Tet repressor protein binds to the Tet operon binding sites and controls expression of mCherry fluorescent protein and miRNA sequence. (pL6.3/CMV/mCherry/blasticidin/miRNA)

Seven miRNA containing plasmids are currently being screened in HEK 293T cells to determine their effectiveness at knockdown of the target protein (Fig 2a-2h). pLenti6/TR/zeocin is being cloned into MDAMB231 cells as this report is being submitted (Fig 3).

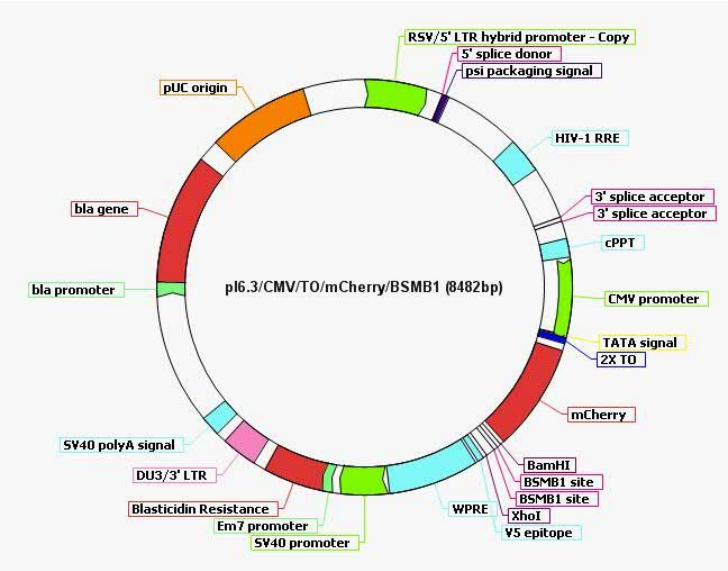
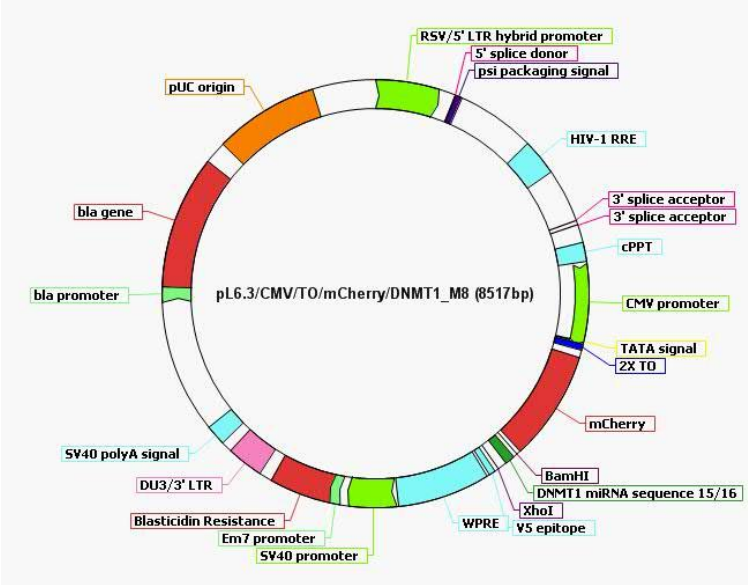


Figure 2a: miRNA sequences were ligated into this pLenti6.3/TO/mcherry backbone using the BSMB1 restriction sites.

Figure 2b: pLenti6.3/CMV/TO/mCherry backbone with miRNA sequence targeting DNMT1 ligated in.



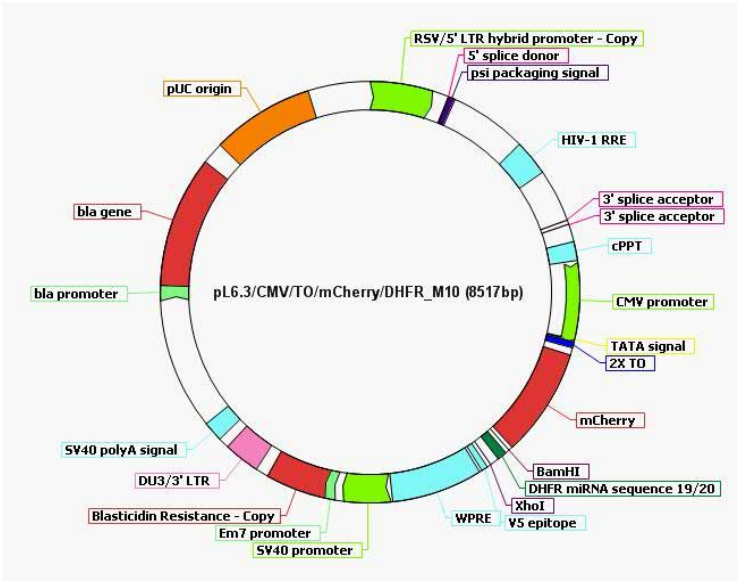
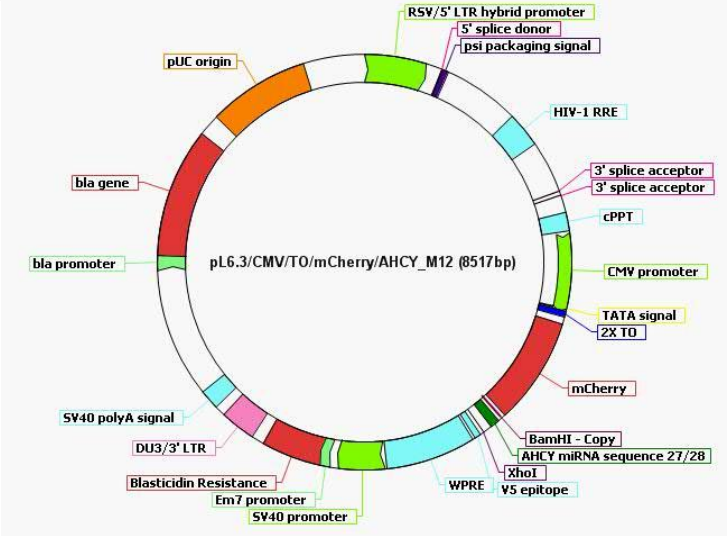


Figure 2c: pLenti6.3/CMV/TO/mCherry backbone with miRNA sequence targeting DHFR ligated in.

Figure 2d: pLenti6.3/CMV/TO/mCherry backbone with miRNA sequence targeting AHCY ligated in.



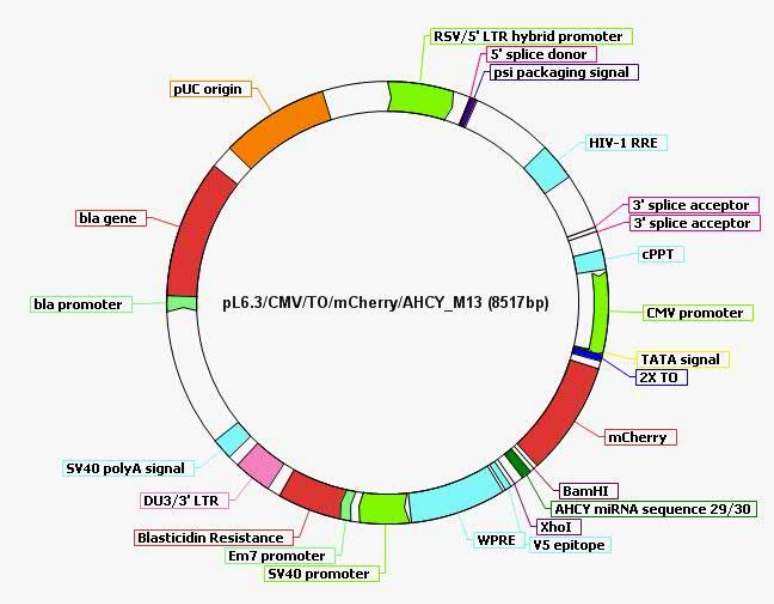
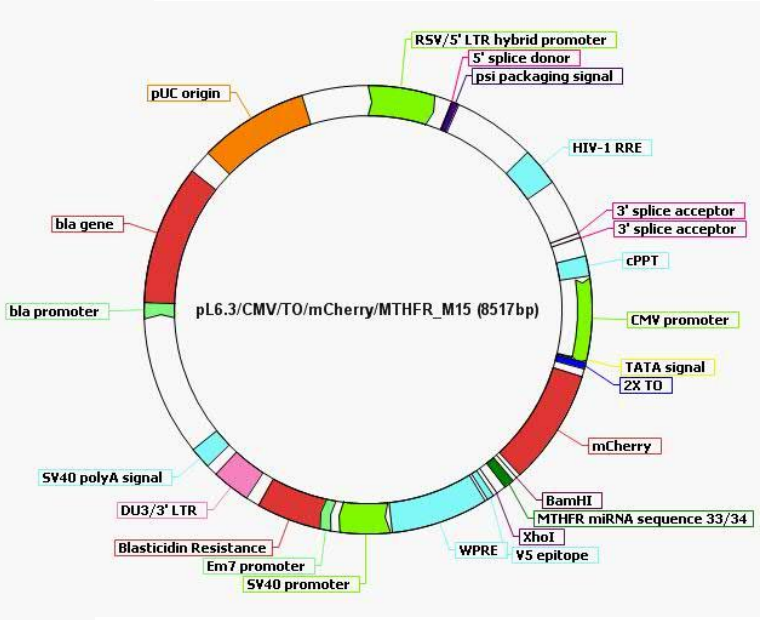


Figure 2e: pLenti6.3/CMV/TO/mCherry backbone with an miRNA sequence targeting AHCY ligated in.

Figure 2f: pLenti6.3/CMV/TO/mCherry backbone with miRNA sequence targeting MTHFR ligated in.



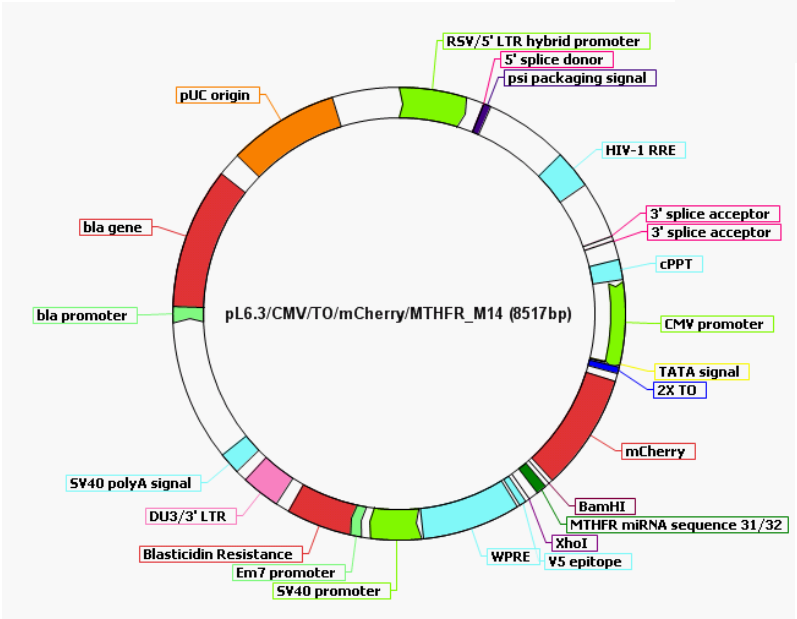


Figure 2g: pLenti6.3/CMV/TO/mCherry backbone with miRNA sequence targeting MTHFR ligated in.

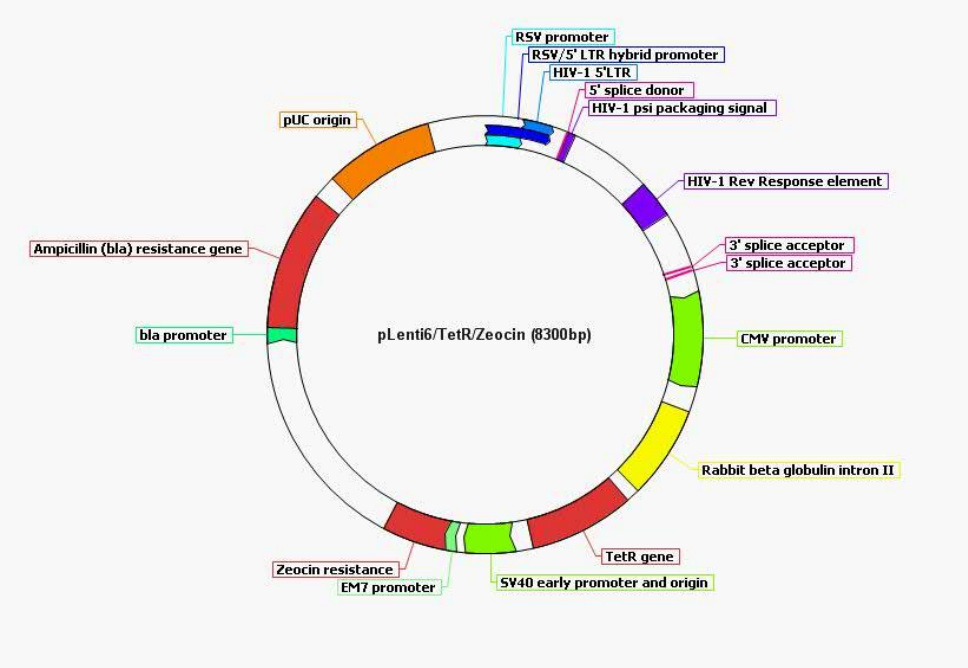
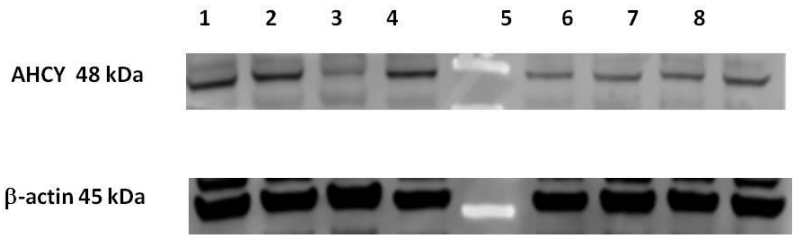


Figure 3: pLenti6/TetR/Zeocin expresses the Tet Repressor protein that binds to the Tet operon sites on plasmid pL6.3/CMV/TO/mCherry and prevents expression of mCherry and the miRNA sequence.

We transiently transfected the plasmids into HEK 293T cells to determine whether target protein knockdown is lethal to cells and to identify miRNA sequences that produce the greatest knockdown of the target protein. The optimal incubation time for maximum knockdown of the target protein was identified by doing time course experiments. Plasmid was transiently transfected into HEK293T cells at 50% confluency and incubated for 24, 48, 72 or 96 hours before being harvested. The cell lysates from this experiment have been collected and we will be running them on western blots in the coming weeks. We do have the results from the plasmids AHCY_M13 and AHCY_M12. The results of knockdown of AHCY in HEK293T cells, 48 h post transfection, done in triplicate show that plasmid AHCY_M13 has a greater knockdown effect on protein levels than AHCY_M12 (Figure 4).



1. mCherry only **2.** Scrambled siRNA
3.Expt #1 miRNA 13A for AHCY **4.** Expt #1 miRNA 12A for AHCY **5.** Expt #2 miRNA 13A for AHCY
6. Expt #3 miRNA 13A for AHCY **7**. Expt #2 miRNA 12A for AHCY **8.**Expt #3 miRNA 12A for AHCY

Figure 4: Plasmid AHCY_M13 is more effective at knocking down expression of AHCY than plasmid AHCY_M12.

We were unsuccessful in developing a reliable assay to measure DHFR activity from cell lysate. We tried to measure DHFR activity in two ways. First we used a DHFR activity kit from Sigma Aldrich (cat no. CS0340) to measure DHFR activity in MCF7 and MDAMB231 cell lysates. The assay measures NADH removal (absorbance at 340 nm) as DHFR reduces dihydrofolate to tetrahydrofolate. The kit comes with a supply of purified DHFR and is used mainly for screen compounds for anti-DHFR activity. We first verified that the activity of purified DHFR decreased as the concentration of methotrexate in the reaction was increased (Figure 5).

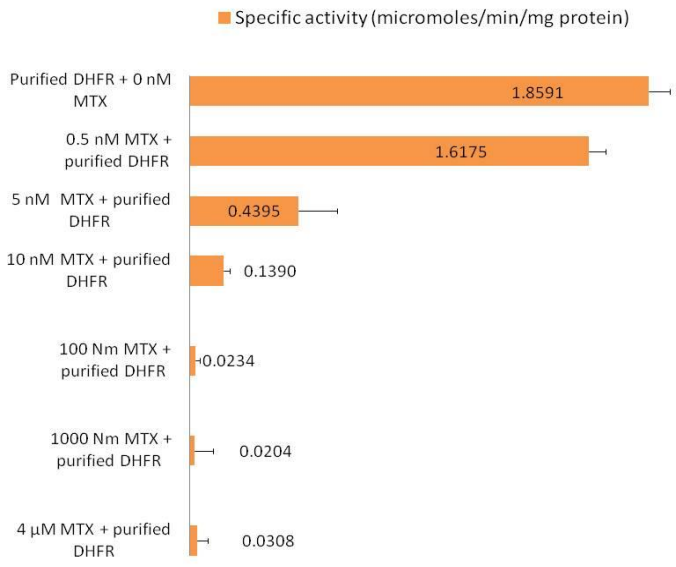


Figure 5: DHFR activity decreases as the concentration of methotrexate (MTX) increases.

Next, we measured the activity of DHFR found in MCF7 cell lysate. The activity of DHFR in MCF7 cell lysate (0.00058 μmole/min/g protein) was low compared to the activity of the purified DHFR provided with the kit (1.86 μmole/min/g protein) and was even lower than the activity of the purified DHFR inhibited by 4 μM methotrexate (0.03082 μmole/min/g protein), the concentration supposed to completely inhibit DHFR activity (Figure 6).

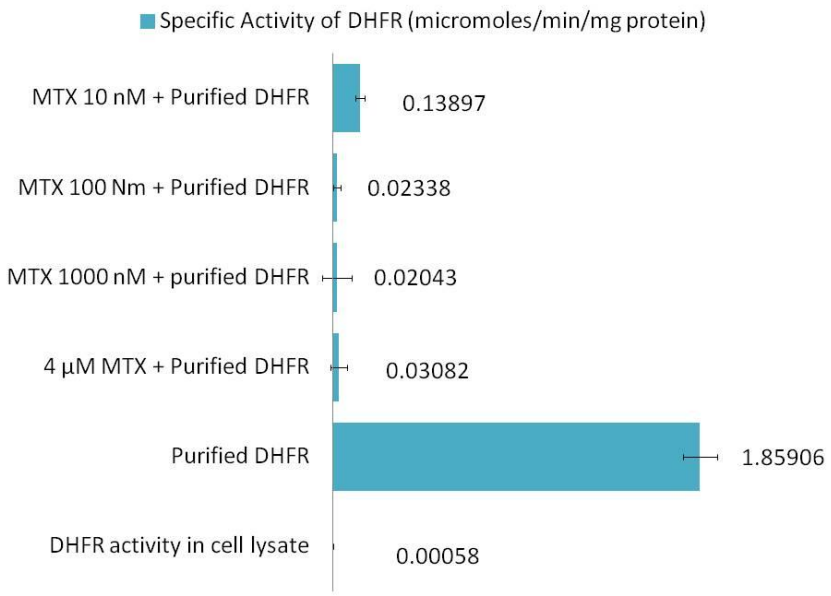


Figure 6: DHFR activity in cell lysate is very low (0.00058 μmole/min/mg protein) compared to the activity of the purified DHFR enzyme provided by the kit (1.86 μmole/min/mg protein).

At this point we believed the assay was not sensitive enough to detect changes in activity of DHFR within cell lysates. To confirm this, we incubated MCF7 and MDAMB231 cells for 24 h with a range of methotrexate concentrations previously used in the literature to inhibit DHFR and then measured DHFR activity in the cell lysates (Figures 7 & 8).

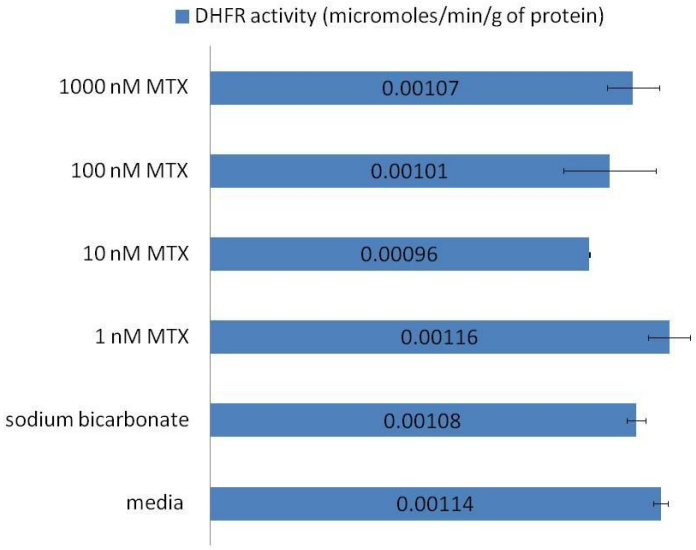


Figure 7: There is no significant difference in the activity of DHFR MCF7 cells incubated with methotrexate for 24 hours.

Cells were also incubated in just media or media + the equivalent volume of sodium bicarbonate used to dissolve the methotrexate to control for any changes due to the addition of sodium bicarbonate.

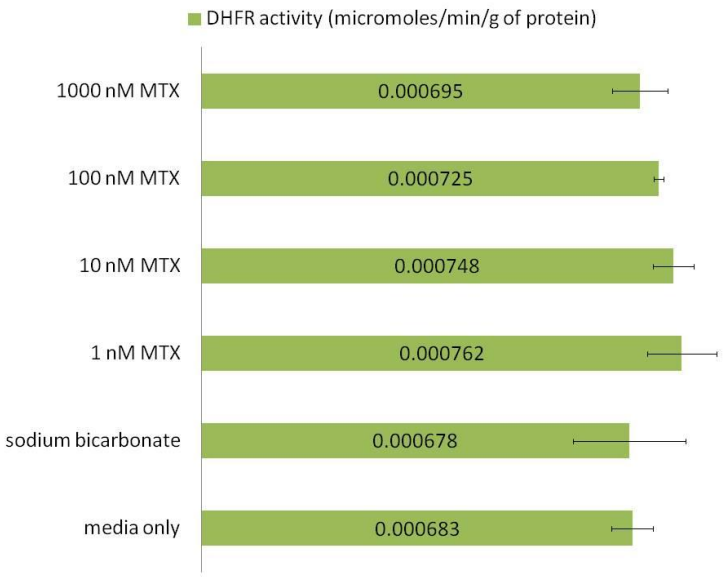


Figure 8: DHFR activity is not significantly different in MDAMB231 control cells vs. MDAMB231 cells incubated with methotrexate for 24 hours.

The results of both experiments showed no significant differences in DHFR activity thus we concluded that the kit from Sigma Aldrich was not sensitive enough to detect differences in DHFR activity in cell lysate.

Next we tried to develop an HPLC-based fluorescent assay to detect the accumulation of tetrahydrofolate (THF) in cell lysate samples as a way of measuring DHFR activity (Gao et al. 2009). Briefly purified DHFR or cell lysates were incubated with Dihydrofolic acid and NADH for 20 min at 37°C and the reaction stopped with 0.3M trichloroacetic acid. Tetrahydrofolate standards were prepared fresh for each HPLC run. The initial concentration of tetrahydrofolate was determined by its absorbance at 298 nm (molar extinction coefficient in 0.1M pH7.5 phosphate buffer is 22000 M^{-1} CM^{-1}). Standards and samples were run on a Luna C18; 4.6x250mm, 5 µm reverse phase column protected with a guard column and tetrahydrofolate was eluted at 7 min and detected by UV fluorescence (excitation 295 nm, emission 365 nm).

We started by doing test runs of the THF standards to make sure the results were consistent and reproducible. Unfortunately we were unable to produce a consistent standard curve. We had problems with the THF peak splitting on some runs and not others and with the peak eluting at 8.5 min instead of 7 min on some runs. Further, there was some reduction of Dihydrofolic acid to tetrahydrofolate occurring in reactions where there was no enzyme present. The inability to reproduce the THF standard curve

and trying to troubleshoot the problems with the peak splitting was time consuming and ultimately, my mentors and I, decided that I should focus primarily on cloning and producing the tetracycline inducible stably knocked down cell lines and return to this assay if time and funding permitted.

Cancer tumor antigens are expressed when MCF7 cells are treated with ADC for 72 hours. We treated cells with ADC for 72 hours, collected RNA and conducted mRNA microarray expression analysis. Genes were considered statistically significant if they had increased or decreased expression by at least 2 fold or higher. The cancer tumor antigens listed in Table one were a group of proteins that had significantly increased gene expression. Literature on these proteins suggests that they are potential targets for cancer immunotherapies. These preliminary results show that ADC treatment has potential to be used as an adjuvant therapy for breast cancer. We hypothesize that the increase in gene expression is due in part to de-methylation of the promoter regions of these genes. We are in the process of confirming this by evaluating the methylation status of the gene promoter regions using bisulfite conversion and pyrosequencing to quantify gene promoter methylation with and without ADC treatment.

Table 2: mRNA microarray analysis of ADC treated MCF7 cells shows an increase in expression of the cancer tumor antigen proteins.

Gene Name	Fold Change
G antigen (GAGE) proteins 2, 4, 5, 6, 7, 7B, 12C, 12D, 12E, 12G, 12H	17.8
G antigen 1-7, 7B, 8, 11	25.6
G antigen 7	18.4
Melanoma antigen (MAGE) family A 8	17.1
Melanoma antigen family A 9, 9B	98.4
Melanoma antigen family A 12	5.0
Melanoma antigen family A 11	6.6
P antigen family member 2 (prostate associated)	39.9
Cancer testis antigen proteins CT45 1-6	35.3
Cancer testis antigen proteins CT45 1-6	28.6

Reportable Outcomes:

Abstract published and poster presentation (see Appendix) at the Era of Hope conference 2011.

Conclusion: The original research plan has been modified in several ways due to difficulties encountered while conducting the proposed experiments. The main concept of perturbing 1-C metabolism remains the same, but the methods used to perturb the metabolic pathway are different. We are developing tetracycline inducible stably transduced MCF7 and MDA-MB-231 cells expressing miRNA against one of the following enzymes: dihydrofolate reductase, methylenetetrahydrofolate reductase, DNA methyltransferase 1 and S-adenosylhomocysteinase instead of targeting DNMT1, and MBD2 with siRNA in four different cell lines. Instead of making cells folate deficient by lowering the amount of folate in the media, we are using miRNA targeted to DHFR and MTHFR to create a perturbation that mimics folate deficiency. We have completed making the plasmids and are currently cloning them into MCF7 and MDAMB231 cells. We are also following up on the methylation status of the cancer tumor antigens which increase expression when MCF7 cells are treated with ADC. We will continue to conduct functional assays throughout the screening and cloning process so that we gather as much information as we can about what the knockdown of DHFR, MTHFR, DNMT1 and AHCY does to breast cancer cell phenotype, function and DNA methylation.

References:

Gao, L, Chalupsky K, Stefani E, Cai H. Mechanistic insights into folic acid-dependent vascular protection: Dihydrofolate reductase (DHFR)-mediated reduction in oxidant stress in endothelial cells and angiotensin II-infused mice: A novel HPLC-based fluorescent assay for DHFR activity. Journal of Molecular and Cellular Cardiology 47 (2009), p.752-760.

Appendix 1: Abstract submitted to Era of Hope 2011

Era of Hope 2011 Poster Session: P11 Cellular Metabolism *Jump to* Sessions Index *502 Jump to* Authors Index
Poster P11-8 BC093366-3276

EPIGENETIC MECHANISMS OF FOLATE NUTRITION IN BREAST CANCER

Rebecca Carmen Lobo[1], David L. Boucher[1], Jane Qian Chen[1], Ralph Green[2], Joshua W. Miller[1], and Alexander D. Borowsky[1]

University of California, Davis[1] and University of California, Davis Medical Center [2] One Carbon Metabolism

In 1998 the U.S. government implemented mandatory fortification of cereal grain products with folic acid to decrease the incidence of neural tube defects (NTDs). While this action was successful in lowering NTD incidence, recent epidemiological studies are highlighting a possible association between excess folic acid and cancer incidence. Current research on the effects of excess folic acid on breast cancer initiation and progression are inconclusive.

DNA methylation is a form of epigenetic regulation that results in chromatin remodeling and ultimately gene silencing. It is essential for cellular differentiation and function. Aberrant DNA methylation is a characteristic of cancer cells, including mammary tumors. The B vitamin folate is required for the synthesis of purines, thymidine, and S-adenosylmethionine (SAM), the methyl donor for DNA methylation. DNA methyltransferase 1 maintains methylation patterns and catalyzes the transfer of the methyl group from SAM to a cytosine residue.

Our objective is to understand the relationships between folate, one-carbon metabolism, DNA methylation, and gene expression within the context of breast cancer. We hypothesize that modulation of folic acid levels or inhibition of key enzymes in one-carbon metabolism (dihydrofolate reductase [DHFR], methylenetetrahydrofolate reductase [MTHFR], DNA methyltransferase 1 [DNMT1], or adenosylhomocysteinase [AHCY]) will affect breast cancer cell growth.

Working with two parent breast cancer cell lines, MCF7 and MDA MB 231, we have developed tetracycline-inducible, stably transfected cell lines that have one of the enzymes listed (DHFR, MTHFR, DNMT1, or AHCY) knocked down. We will compare the effects of knocking down enzyme expression in this way to the effects of treating breast cancer cells with methotrexate or excess folic acid. We will measure changes in cell growth, migration, survival of anoikis, global and gene-specific DNA methylation changes, as well as one-carbon metabolite levels. Folate metabolism is important in DNA synthesis and methylation. The U.S. population is frequently exposed to twice or more the recommended dietary allowance of folic acid, and there is a need to determine the effects of excess folic acid on cancer cell proliferation, which can serve as a surrogate of cancer progression.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-10-1-0235.

Epigenetic Mechanisms of Folate Nutrition in Breast Cancer

Rebecca C. Lobo[1], David L. Boucher[2], Jane Q. Chen[3], Ralph Green[1], Joshua W. Miller[1], Alexander D. Borowsky[3]

Department of Nutrition[1]; Department of Biomedical Engineering[2]; Medical Pathology and Laboratory Medicine[3], University of California, Davis

Introduction

Cancer cells have aberrant DNA methylation patterns characterized by global hypomethylation of the genome with gene-specific hypermethylation.
Cytosines in CpG dinucleotides can be methylated to form 5'-methylcytosines. In most cases, the methylation of CpG dinucleotides in the promoter region of a gene results in changes in chromatin structure and ultimately modification (usually down regulation) of gene transcription. Known and suspected tumor suppressor genes are often silenced in cancer cells while oncogenes that should be silenced are expressed.

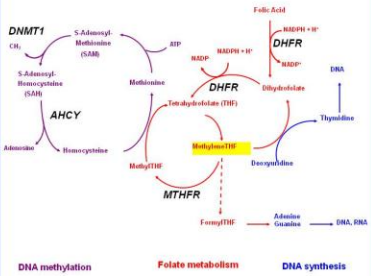


Figure 1. One-Carbon Metabolism

Both DNA synthesis and DNA methylation are needed for cancer cell survival. A second distinguishing feature of cancer cells is that they are hyper-proliferative. Replicating cells require both deoxynucleotide triphosphates (dNTPs) and a supply of methyl groups to successfully reproduce methylation patterns.

Methyl groups are cycled through the one-carbon (1-C) metabolic pathway for two main purposes: generation of dNTPs and methylation of proteins and DNA bases (CpG dinucleotides). Folate is one of the major co-factors necessary for the successful cycling of 1-C units through the pathway. Folate metabolism remains at the center of the pathway with the supply of methylenetetrahydrofolate being divided between both DNA methylation and dNTP synthesis (Figure 1).

Objective

To understand the relationships between folate, one-carbon metabolism, DNA methylation and gene expression within the context of breast cancer.

Hypothesis

The inhibition of key enzymes in one-carbon metabolism:

dihydrofolate reductase (DHFR)
methylenetetrahydrofolate reductase (MTHFR)
DNA methyltransferase 1 (DNMT1)
adenosylhomocysteinase (AHCY)

by miRNA will decrease breast cancer cell growth, modulate cell migration, and ability of the cell to survive anoikis by increasing gene expression of hyper-methylated genes.

Methods

To study one-carbon metabolism in the context of breast cancer we chose to create tetracycline inducible, stably transduced cell lines expressing miRNA against each of four proteins: DHFR, MTHFR, DNMT1 or AHCY.

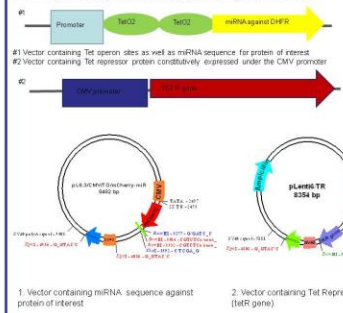
We chose to create the inducible cell lines using MCF7 and MDA-MB-231 parent cell lines (Table 1).

Characteristic	MCF7	MDA-MB-231
Estrogen Receptor (ER)	ER $^+$	ER $^-$
Progesterone Receptor (PR)	PR $^+$	PR $^-$
Her2	Her2 $^-$	Her2 $^-$

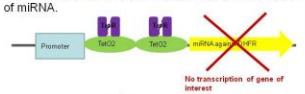
Table 1: Comparison of characteristics of breast cancer cells

Methods

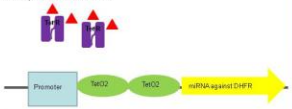
How the tetracycline inducible cell system works



Without tetracycline Tet repressor protein (Tet R) binds to the Tet operon sites (TetO2) preventing transcription of miRNA.



When tetracycline (▲) is added to the system the Tet R protein binds to it with greater affinity than it binds to the Tet operon sites. This allows transcription of miRNA.

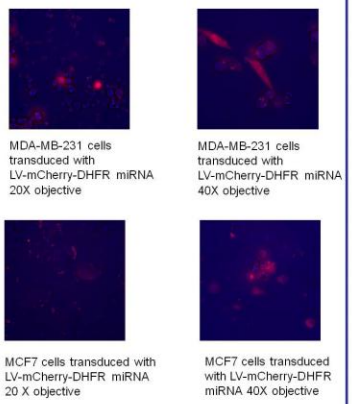


The miRNA produced binds to mRNA of the protein of interest targeting it for destruction. Over time this causes a knockdown in expression of the protein of interest.

Table 2: Functional assays to be performed on tetracycline inducible stably transduced cell lines

Measured Endpoint	Assay
Cell Proliferation	Trypan blue exclusion assay and MTT assay
Cell Apoptosis	TUNEL assay and caspase 3 staining
Cell Migration	Scratch assay
Anoikis	Propidium iodide staining followed by flow cytometry
1-C metabolites ([S-Adenosylmethionine], [S-Adenosylhomocysteine] and [Homocysteine])	High performance liquid chromatography
Gene expression	mRNA microarray
Gene specific promoter methylation	Bisulfite sequencing or pyrosequencing

Preliminary Results



MDA-MB-231 cells transduced with LV-mCherry-DHFR miRNA 20X objective

MDA-MB-231 cells transduced with LV-mCherry-DHFR miRNA 40X objective

MCF7 cells transduced with LV-mCherry-DHFR miRNA 20 X objective

MCF7 cells transduced with LV-mCherry-DHFR miRNA 40X objective

Current & Future Directions

To date we have successfully transduced both MCF7 and MDA-MB-231 cells with LV-mCherry-DHFR miRNA and LV-mCherry-AHCY miRNA.

Future endeavors include:

- Transduction of MCF7 and MDA-MB-231 cells with LV-mCherry-DNMT1 miRNA and MTHFR miRNA
- Cloning and selection of clones containing operating tetracycline inducible systems
- Transduction of tetracycline inducible MCF7 and MDA-MB-231 clones with LV-DHFR miRNA, LV-AHCY miRNA, LV-MTHFR miRNA and LV-DNMT1 miRNA
- Functional assays as described in the methods section (Table 2)

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