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TITLE: Homocysteine Is an Oncometabolite in Breast Cancer, Which Promotes Tumor Progression and Metastasis

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<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b> The hypothesis in this project is that homocysteine is an oncometabolite in breast cancer. We propose to test this hypothesis with three specific aims: (1) Investigate using two different mouse models of spontaneous breast cancer (MMTV-HRAS mouse and MMTV-PyMT mouse) whether <i>Mthfr</i> is silenced through DNA methylation and as a result the levels of the oncometabolite homocysteine are elevated in tumors; (2) Investigate whether homocysteine promotes breast cancer progression and lung metastasis by comparing the disease process in MMTV-HRAS and MMTV-PyMT mice on two different genetic backgrounds: <i>Mthfr</i> <sup>+/+</sup> and <i>Mthfr</i> <sup>-/-</sup> . Investigate the ability of homocysteine to induce TGF-β, ANGPTL4, and MMP-9 in breast cancer cell lines and to disrupt the barrier function of lung microvascular endothelial cells; (3) Investigate using breast cancer cell lines whether over expression of MTHFR or exposure to N <sup>5</sup> -methyltetrahydrofolate decreases cell proliferation in vitro and suppresses tumor growth in xenografts in vivo.						
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## Introduction

There were five tasks proposed for this reporting period in the approved Statement of Work.

TASK 1: Generation of MMTV-HRAS/Mthfr<sup>-/-</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> transgenic mice.

TASK 2: Monitor tumor incidence, metastasis and survival time in MMTV-HRAS/Mthfr<sup>+/+</sup> and MMTV-HRAS/Mthfr<sup>-/-</sup> mice and PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mice.

TASK 3: Collection of tumor tissues and blood samples from MMTV-HRAS/Mthfr<sup>+/+</sup> and MMTV-HRAS/Mthfr<sup>-/-</sup> mice and PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mice and measure homocysteine level.

TASK 4: Analyze the role of homocysteine on expression of TGF- $\beta$ , ANGPTL-4 and MMP-9 in breast cancer cell lines.

TASK 5: Analyze the influence of homocysteine and ANGPTL-4 on the permeability of lung microvascular endothelial cells.

## Keywords

Homocysteine, Oncometabolite, Breast cancer, Methylenetetrahydrofolate reductase, Mouse models of breast cancer, Tumor progression, Metastasis to the lung, Breast cancer cell lines, Lung endothelial cells

## Accomplishments

Task 1. Generation of MMTV-HRAS/Mthfr<sup>-/-</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> transgenic mice.

This task has been completed. This involves intercrossing of MMTV-HRAS transgenic mice with Mthfr<sup>-/-</sup> mice to generate the mouse lines with two different genetic backgrounds: MMTV-HRAS/Mthfr<sup>+/+</sup> and MMTV-HRAS/Mthfr<sup>-/-</sup>. It also involves intercrossing of MMTV-PyMT transgenic mice with Mthfr<sup>-/-</sup> mice to generate the mouse lines with two different genetic backgrounds: MMTV-PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup>.

We have successfully generated sufficient number of these mouse lines necessary for the proposed studies.

TASK 2: Monitor tumor incidence, metastasis and survival time in MMTV-HRAS/Mthfr<sup>+/+</sup> and MMTV-HRAS/Mthfr<sup>-/-</sup> mice and MMTV-PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mice.

We have completed the proposed studies with MMTV-PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mice as the appearance of breast tumors occurs relatively early in MMTV-PyMT mice. The studies with MMTV-HRAS/Mthfr<sup>+/+</sup> and MMTV-HRAS/Mthfr<sup>-/-</sup> mice are still ongoing as tumors appear in MMTV-HRAS mice at a much later age than in MMTV-PyMT mice (~8 months versus ~3 months).

We monitored the appearance of tumors in mammary glands in MMTV-PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mice. The average age at which tumors appeared in MMTV-PyMT/Mthfr<sup>+/+</sup> mice was  $91 \pm 4$  days. In contrast, tumors appeared at an earlier age in MMTV-PyMT/Mthfr<sup>-/-</sup> mice ( $68 \pm 4$  days) (Fig. 1). The difference between the two values was statistically significant ( $P < 0.01$ ). The tumors were allowed to grow in both groups and when the mice became moribund, they were killed and the tumor tissues were collected and processed for immunostaining, preparation of protein lysates for western blotting, and preparation of RNA for qPCR. Lungs were perfused with India ink and Fekete solution to visualize metastatic nodules. We found more metastatic nodules in

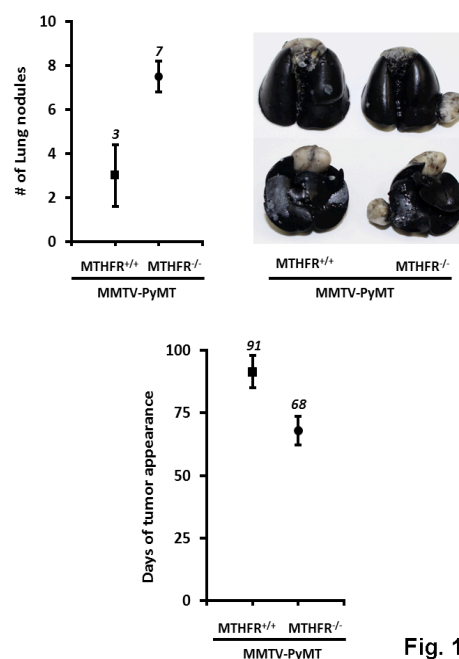


Fig. 1<sub>3</sub>

MMTV-PyMT/Mthfr<sup>-/-</sup> mice than in MMTV-PyMT/Mthfr<sup>+/+</sup> mice (Fig. 1). On an average, there were 3 nodules in MMTV-PyMT/Mthfr<sup>+/+</sup> mouse lungs in contrast to 7 nodules in MMTV-PyMT/Mthfr<sup>-/-</sup> mice (Fig. 1).

**TASK 3:** Collection of tumor tissues and blood samples from MMTV-HRAS/Mthfr<sup>+/+</sup> and MMTV-HRAS/Mthfr<sup>-/-</sup> mice and PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mice and measure homocysteine level.

We have completed the proposed studies with MMTV-PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mice; the studies with MMTV-HRAS/Mthfr<sup>+/+</sup> and MMTV-HRAS/Mthfr<sup>-/-</sup> mice are still ongoing. We collected the tumors from MMTV-PyMT/Mthfr<sup>+/+</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mice and measured the tissue levels of the amino acid homocysteine. The tissue concentration of homocysteine in breast tumors obtained from MMTV-PyMT mice on intact Mthfr genetic background was 13.4 ± 2.3 μM. This value was not significantly different from the tissue levels of this amino acid in non-cancerous mammary glands obtained from MMTV-PyMT mice at 2 months of age when there were no visible tumors (11.7 ± 3.2 μM). However, the levels of homocysteine were significantly higher in tumors obtained from MMTV-PyMT transgenic mice on Mthfr-null background (93.6 ± 11.9 μM). Thus, there was a ~7-fold increase in the tissue levels of homocysteine in breast tumors in MMTV-PyMT transgenic mice when on Mthfr-null background than on Mthfr<sup>+/+</sup> background.

We will perform similar analyses for homocysteine levels from tumors obtained from MMTV-HRAS transgenic mice when the experiments are terminated in these mouse lines.

The occurrence of similar levels of homocysteine in breast tumors versus normal mammary gland with intact Mthfr came as a surprise because we expected downregulation of Mthfr in mammary tumors so as to generate the oncometabolite homocysteine. This however was not the case. We therefore investigated the expression profile of MTHFR in normal human mammary epithelial cell lines and human breast cancer cell lines to determine if the expression of the enzyme is different in normal cells versus cancer cells. We complemented these studies by assessing the expression of Mthfr in breast tumors obtained from three different mouse models of spontaneous breast cancer (MMTV-HRAS-Tg, MMTV-Neu-Tg, and MMTV-PyMT-Tg) and in matched non-cancerous mammary glands (see below under TASK 4).

**TASK 4:** Analyze the role of homocysteine on expression of TGF-β, ANGPTL-4 and MMP-9 in breast cancer cell lines.

Literature evidence indicates a role for mutations in MTHFR that affect the function of the enzyme in increased risk for breast cancer, but there is no data as to what happens to MTHFR expression in breast cancer. Therefore, we first examined the expression levels of mRNA for this enzyme in breast cancer using three different approaches (Fig. 2). First, we used MCF10A cells (a human mammary epithelial cell line) in four different stages of neoplastic phenotype: MCF10A1 (normal cell), MCF10A2 (transfected with the oncogene HRAS), MCF10A3 (cell line isolated from the primary site of tumor that arose from xenografting MCF10A2 in mammary fat pad of nude mice), and MCF10A4 (cell line isolated from lung metastasis of the tumor that arose from xenografting MCF10A2 in mammary fat pad of nude mice). We found that MTHFR mRNA levels did not decrease, but actually increased in MCF10A cells as the cells become neoplastic and metastatic as a result of HRAS. We then examined the expression levels of MTHFR mRNA in normal mammary epithelial cell lines (MCF10A, MCF12A, and HMEC) and breast cancer cell lines (ER-positive: MCF7, T47D, ZR75.1, and MB361; ER-negative: MB231, HCC1937, BT20, and MB453). Again, we found the expression levels to increase in cancer cell lines compared to normal cells. Next, we examined the expression levels of Mthfr mRNA in breast tumors from three different mouse models of spontaneous breast cancer (MMTV-Neu, MMTV-HRAS, and MMTV-PyMT) and compared the expression levels with those in normal mammary gland. The expression levels increased in tumor tissues compared to normal tissue.

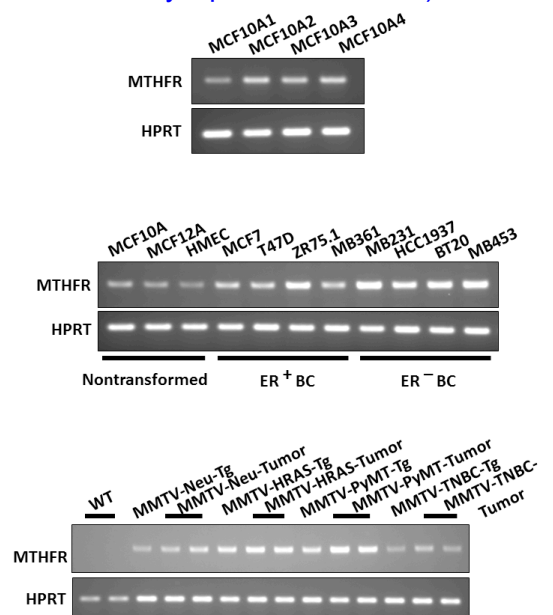


Fig. 2

We cultured the human breast cancer cell lines MCF7 and MB231 in the presence (500  $\mu$ M) and absence of homocysteine for three passages (chronic exposure to homocysteine in the form of homocysteine thiolactone, HTL) and then prepared total RNA from the cells to assess the expression of TGF- $\beta$ , ANGPTL-4, and MMP-9 by RT-PCR. We found that treatment with homocysteine enhanced the expression of all three genes that are involved in tumor promotion (TGF- $\beta$ ) and metastasis (ANGPTL-4 and MMP-9) in both cell lines (Fig. 3; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

**TASK 5:** Analyze the influence of homocysteine and ANGPTL-4 on the permeability of lung microvascular endothelial cells.

Lung is the one of the primary sites for breast cancer metastasis, and it is believed that the permeability of lung microvascular endothelial cells plays a critical role in metastasis by allowing the circulating cancer cells to seed the lung parenchymal tissue. ANGPTL-4 has been shown to increase the permeability of lung endothelial cells and this protein is expressed at higher levels in breast cancer cells. Our data show that exposure of breast cancer cells to homocysteine increases the expression of ANGPTL-4. Therefore, we hypothesized that homocysteine promotes lung metastasis of breast cancer by increasing the expression of ANGPTL-4 in tumor cells and possibly also in lung endothelial cells, consequently increasing the permeability of these cells. To test this hypothesis, we examined the effects of homocysteine and recombinant ANGPTL-4 on the permeability of lung microvascular endothelial cells in vitro. For this, we used two different experimental approaches. First, we used the Electric Cell-substrate Impedance Sensing (ECIS) system to measure the impedance of the monolayers of lung endothelial cells with and without exposure to homocysteine or recombinant ANGPTL-4. The impedance is inversely proportional to permeability of the monolayer. With this system, we found homocysteine (Fig. 4A) as well as ANGPTL-4 decreased the impedance of these cells, thus indicating increased permeability of the monolayer (Fig. 4B). In the second approach, we cultured the endothelial cells on a porous filter in a Transwell culture system in the presence or absence of homocysteine or ANGPTL-4 and periodically monitored the transcellular electrical resistance (TER). We found this resistance to decrease by  $35 \pm 6$  % ( $N = 6$ ;  $p < 0.01$ ) when the cells were cultured in the presence of homocysteine (250  $\mu$ M) and ANGPTL-4 (50  $\mu$ g/ml). The decrease in TER is an indication of increased permeability. We corroborated this data with the transcellular transfer of the macromolecule FITC-dextran, which increased by  $24 \pm 4$  % and  $38 \pm 7$  %, respectively in cells exposed to homocysteine (250  $\mu$ M) and ANGPTL-4 (50  $\mu$ g/ml). In both cases, the difference was statistically significant ( $p < 0.01$ ).

### Synopsis of the experiments completed

- The generation of MMTV-PyMT/Mthfr<sup>+/+</sup>, MMTV-PyMT/Mthfr<sup>-/-</sup>, MMTV-PyMT/Mthfr<sup>+/-</sup> and MMTV-PyMT/Mthfr<sup>-/-</sup> mouse lines has been accomplished.
- The hypothesis that deletion of Mthfr would promote breast cancer growth and lung metastasis has been validated for MMTV-PyMT mice.
- The association between Mthfr deletion and elevated levels of homocysteine in breast tumors has been established.
- There was a surprising finding that breast tumors themselves do not alter the expression of MTHFR expression.

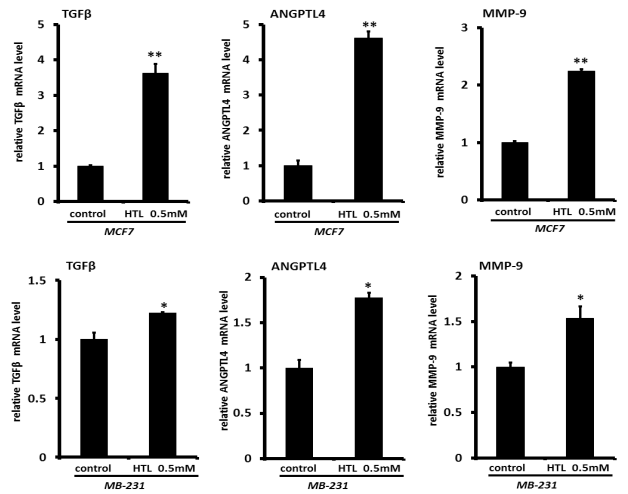
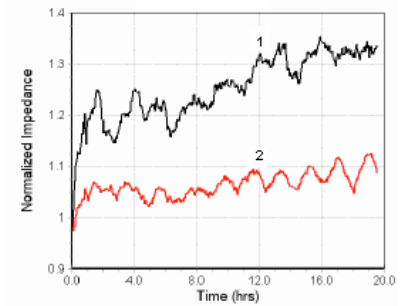
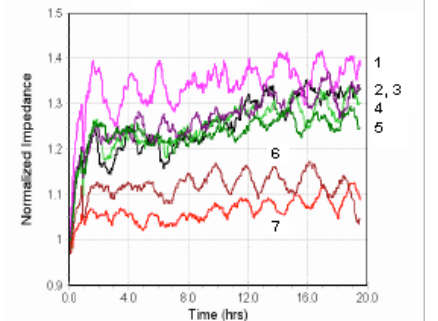


Fig. 3



1, Control  
2, Homocysteine (250 micromolar)

Fig. 4A



1, Control  
Tracings 2-7 represent a dose-response study for recombinant ANGPTL4 in the range of 1-50 microgram/ml (1, 2.5, 5, 10, 25, 37.5, and 50  $\mu$ g/ml)

Fig. 4B

- Homocysteine and ANGPTL-4 render lung microvascular endothelial cells more permeable, thus potentially facilitating the transfer of circulating tumor cells across the blood vessel into lung parenchymal tissue for effective seeding, thus facilitating lung metastasis.

## **Impact**

Elevated circulating levels of homocysteine are widely considered as a risk factor for cardiovascular disease because of the deleterious effects of this amino acid on vascular endothelial cells. We initiated the current project with the hypothesis that homocysteine is also an oncometabolite for breast cancer that promotes tumor growth at the primary site and also promotes metastasis of the cancer in the lung. Our studies with MMTV-PyMT mice have now established that this hypothesis is indeed true. Deletion of Mthfr in mice increases the tissue levels of homocysteine in mammary gland. Even though this elevated homocysteine levels are not sufficient in itself to initiate tumorigenesis, the increase in the levels of this amino acid due to deletion of the enzyme Mthfr promotes tumor growth at the primary site, accelerates the process of carcinogenesis, and facilitates lung metastasis in a spontaneous mouse model of breast cancer. These findings suggest that monitoring the circulating levels of homocysteine in breast cancer patients might be of prognostic value to identify the patients who are likely to exhibit an aggressive tumor growth and increased risk for lung metastasis.

## **Changes/Problems**

We have completed almost all of the tasks that were approved for year 2 (Year 1 at TTUHSC) of the project period. We will continue with the other tasks that have been approved for year 3 (Year 2 at TTUHSC).

There are no changes in project goals or experimental approaches.

## **Products**

The data on the promotion of breast cancer growth and lung metastasis by Mthfr deletion will be prepared for publication once the experiments with MMTV-HRAS are completed.

## **Participants and other Collaborating Institutions**

Texas Tech University Health Sciences Center

## **Special Reporting Requirements**

None

## **Appendices**

None