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14. ABSTRACT

My postdoctoral research focuses on alteration of folate metabolism in breast cancer. The connection between folate metabolism and breast cancer has long been recognized. Paradoxically, we know relatively little about the basic science of folate metabolism in the context of breast cancer. We do not yet know whether folate metabolism is altered in breast cancer compared to normal mammary tissues, and whether this alteration can contribute to breast cancer pathogenesis. These are important questions to be addressed in my research. Our central hypothesis is that either direct mutations of folate metabolic enzymes, or altered signal transduction pathways in breast cancer (such as PI3K pathway hyperactivation), leads to altered folate metabolism, which in turn contributes to the uncontrolled cell growth seen in breast cancer. In this fellowship period (Year 1), I have been investing in developing key methodologies that are essential for studying alteration of folate metabolism in breast cancer. Notably, I have successfully developed and validated a robust HPLC method that permits quantitative assessment of intracellular folates and capturing of the dynamics of their interconversions upon various stimuli, thus paving the way for future endeavors in testing specific hypotheses. I have presented part of this project in the poster session of the 2014 FASEB "Folic Acid, Vitamin B12, and One-Carbon Metabolism" conference. We hope that by gaining basic knowledge of folate metabolism, we can build a firm foundation for rational design of therapeutics and for early detection and diagnosis of breast cancer.

15. SUBJECT TERMS
Folic Acid, Metabolism, Breast Cancer, Signal Transduction, Growth Factor

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Introduction

My postdoctoral research focuses on alteration of folate metabolism in breast cancer. The connection between folate metabolism and breast cancer has long been recognized; for example, folate antagonists such as methotrexate have been used in breast cancer chemotherapy for more than 60 years. Paradoxically, we know relatively little about the basic science of folate metabolism in the context of breast cancer. We do not yet know whether folate metabolism is altered in breast cancer compared to normal mammary tissues, and whether this alteration can contribute to breast cancer pathogenesis. These are important questions to be addressed in my research. In particular, I am intrigued by the possibilities that either direct mutations of folate metabolic enzymes, or altered signal transduction pathways in breast cancer (such as PI3K pathway hyperactivation), may lead to altered folate metabolism, which may in turn contribute to the uncontrolled cell growth seen in breast cancer. We believe that by gaining basic knowledge of folate metabolism, we can build a firm foundation for rational design of therapeutics and for early detection and diagnosis of breast cancer. My mentor, Dr. Lew Cantley, is an expert in signal transduction and cancer metabolism. His laboratory provides the ideal environment in which I may attain success in deconvoluting the complex relationship between signal transduction and folate metabolism in breast cancer. This postdoctoral fellowship provides me with the unique opportunity to pursue these research ideas and ultimately establish my own independent breast cancer research program.

Key Words

Folic Acid, Metabolism, Breast Cancer, Signal Transduction, Growth Factor

Overall Project Summary

Current Objectives:

The objectives are to understand whether folate metabolism is altered in breast cancer compared to normal mammary tissues, and whether this alteration can contribute to breast cancer pathogenesis. The central hypothesis is that either direct mutations of folate metabolic enzymes, or altered signal transduction pathways in breast cancer (such as PI3K pathway hyperactivation), leads to altered folate metabolism, which in turn contributes to the uncontrolled cell growth seen in breast cancer.

Results, Progress and Accomplishments

Probing the Interactions between Wild-type and Mutant ALDH1L1 in vitro (SOW Tasks 1 and 2)

These two tasks are now completed. I have co-expressed MBP-tagged wild-type ALDH1L1 with Flag-tagged wild-type, E901K, A870T, or C707A ALDH1L1 in both 293T and baculovirus-Sf21 insect cell systems and subsequently purified the wild-type-mutant complexes using anti-Flag affinity chromatography. Fig. 1 showed that the mutant is expressed at very similar levels to that of wild-type, thus mimicking the *in vivo* conditions in which usually a single copy of ALDH1L1 gene was found to be mutated.

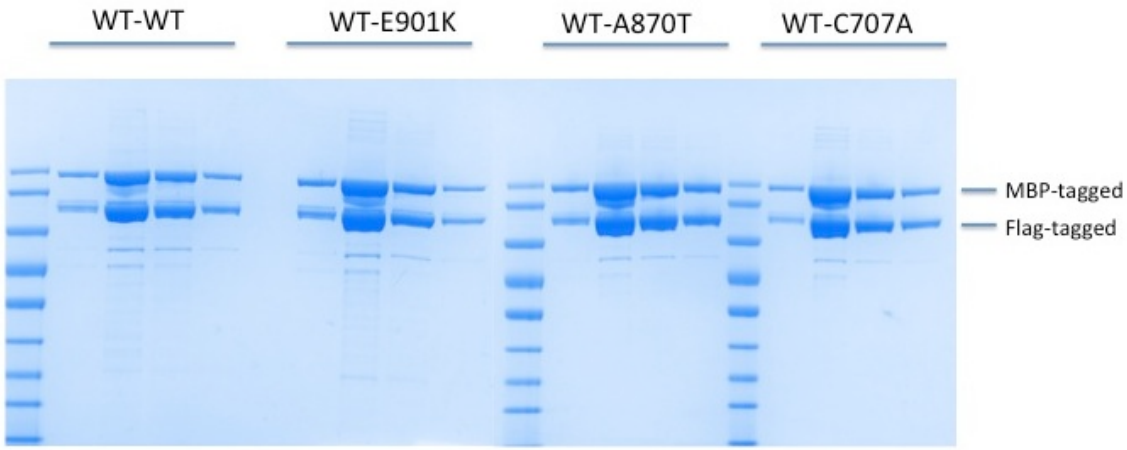

Fig. 1. Co-expression and purification of MBP-tagged wild-type ALDH1L1 associated with Flag-tagged wild-type, E901K, A870T, or C707A mutant ALDH1L1.

Subsequently I went on to test the 10-formyl-THF dehydrogenase activities of these wild-type-mutant complexes compared to the wild-type enzyme. Surprisingly, the presence of mutant ALDH1L1 showed little effect on the overall activities of the complexes (Fig. 2), suggesting that the wild-type enzyme may help stabilize the interface interactions even though the E901K and A870T mutations tend to disrupt the interactions between subunits. Native-PAGE of these complexes provided further evidence that the subunit interactions were largely unperturbed in these wild-type-mutant ALDH1L1 complexes (data now shown).

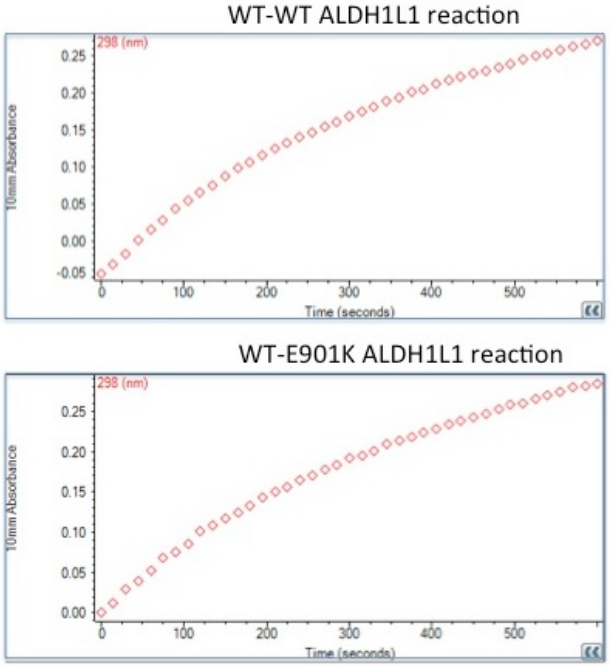

Fig. 2. 10-formyl-THF dehydrogenase activity assay. The reaction was monitored by a UV spectrophotometer at 298 nm. 298 nm is the characteristic absorbance of THF product of this reaction. The results showed that the wild-type-E901K mutant complexes catalyze this reaction at a similar rate to wild-type enzyme. Similar reactions were obtained for other wild-type-mutant complexes (data not shown).

Successful Development of Key Methodology that Permits Quantitative Assessment of Intracellular Folates (SOW Task 3a)

A crucial aspect of my research is to be able to quantitate the individual folate species and to capture the dynamics of their interconversions upon various stimuli. However, there are two major hurdles in folate analysis. One is that most intracellular folates (THF and 10-formyl-THF in particular) are prone to oxidation and degradation. A second hurdle is that intracellular folates all have a polyglutamate tail of variable chain lengths [1]. As a result, there are at least a dozen of individual folate species, and this presents an enormous challenge for chromatographic resolution of folates. During this training period, I noted this gap in methodology in the folate metabolism field and thus directed my efforts to filling in this gap.

I have successfully developed a method that permits quantitative assessment of intracellular folates. To prevent oxidation of intracellular folates, 0.2 % (w/v) sodium ascorbate and 0.2 M β-mercaptoethanol were included in the lysis buffer. To circumvent complications arising from the polyglutamate tails, I expressed and purified a hydrolytic enzyme, γ-glutamyl-hydrolase (GGH), from 293T cells (Fig. 3), and then used this recombinant enzyme to cleave the polyglutamate tails of intracellular folates. Finally, an ion-pair reversed phase HPLC was used to resolve individual folate monoglutamates. As shown in Fig. 4**,** all major intracellular ^{3}H-labeled folates from MCF7 cells are well resolved by HPLC, with negligible amounts of degradation products present. Importantly, the 10-formyl-THF peak was converted entirely to the THF peak upon incubation of the MCF7 cellular extract with recombinant ALDH1L1 (which is known to catalyze such a reaction), confirming the identity of the 10-formyl-THF peak.

(A) **(B)**

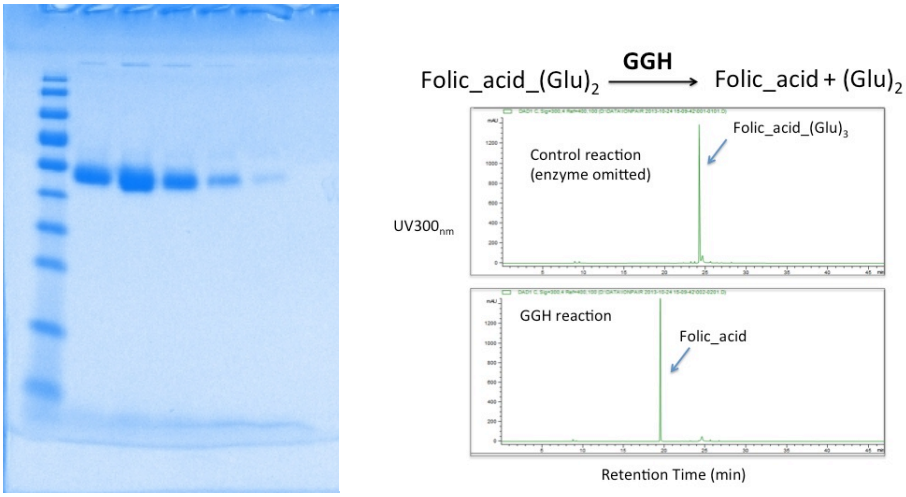

Fig 3. (A) Expression and purification of rat γ-glutamyl-hydrolase (GGH) from 293T cells. **(B)** Purified GGH is catalytically active.

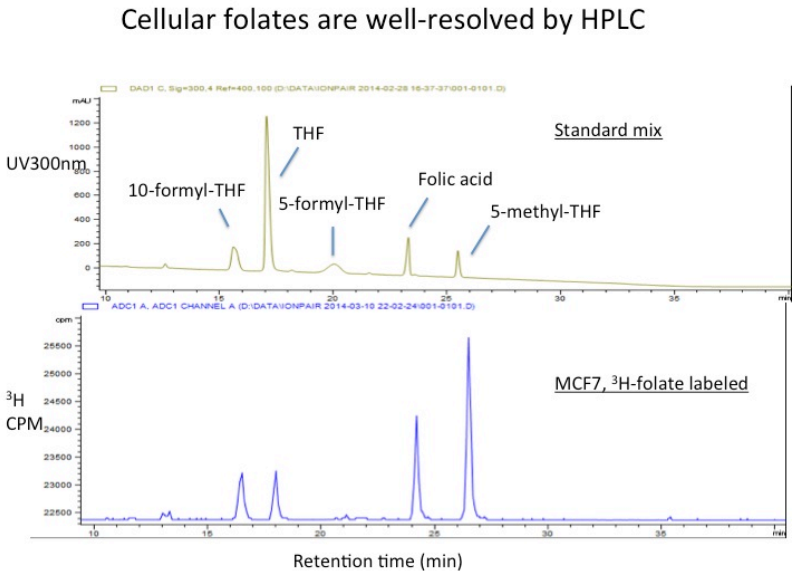

Fig 4. **[Top]** HPLC analysis of folate standards. As the standards were not radiolabeled, they were detected by their characteristic UV absorbance at 300 nm. **[Bottom]** HPLC analysis of intracellular folates from MCF7 cells labeled with ^{3}H-folic acid.

Validation of the HPLC Method for Folate Analysis

To validate this HPLC method, I focused on a few well-studied problems, made predictions of folate distribution changes based on extensive literature evidence, and then applied this HPLC method to see if the expected results are obtained. Listed below are a few such tests, the results of which confirmed the robustness and sensitivity of this HPLC method.

<u>"5-methyl-THF trap"</u>
It is well established that when methionine is absent in the culture medium or when methionine synthesis is impaired due to vitamin B12 deficiency (vitamin B12 is a cofactor in methionine synthase), 5-methyl-THF becomes by far the most abundant folate species (For ref., see [2]). Mechanistically, S-adenosyl-methionine is a potent allosteric inhibitor of MTHFR, the enzyme that catalyzes the irreversible conversion of 5,10-methylene-THF to 5-methyl-THF. Accordingly, a drop in methionine concentration

(and consequently a drop in S-adenosyl-methionine concentration) relieves this inhibition, and causes an irreversible conversion of all other folates into 5-methyl-THF.

As shown in Fig. 5 , upon methionine deprivation for 1hr, 5-methyl-THF accounts for >90% of all folates in MDA-MB-468 triple negative breast cancer cells, exactly as predicted.

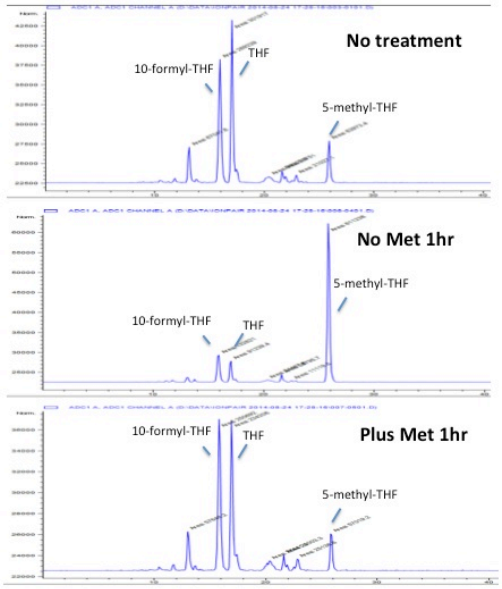

Fig. 5: [Top] Folate distribution of MDA-MB-468 with no treatment. [Middle] Folate distribution of MDA-MB-468 cells with methionine deprivation for 1 hr. [Bottom] Folate distribution of MDA-MB-468 with medium change for 1 hr (control).

Serine is a major one-carbon donor

Serine is known to be the major one-carbon donor for cells [1]. It follows that acute withdrawal of serine from the culture medium would deprive cells of the one-carbon source, leaving the unsubstituted THF as the predominant species. Indeed, this is what we have observed (Fig. 6).

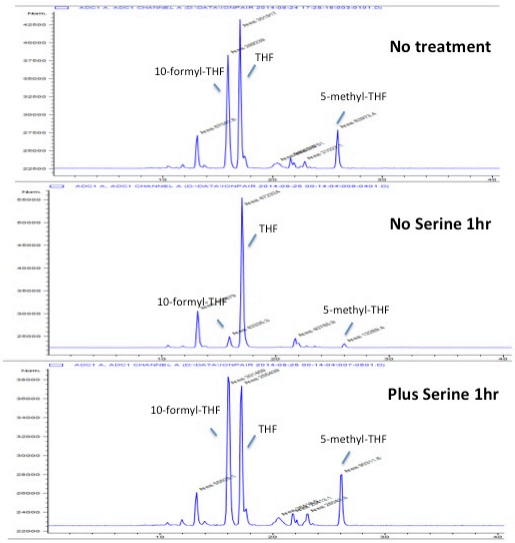

Fig. 6: [Top] Folate distribution of MDA-MB-468 with no treatment. [Middle] Folate distribution of MDA-MB-468 cells with serine deprivation for 1 hr. [Bottom] Folate distribution of MDA-MB-468 with medium change for 1 hr (control).

Mitochondrial one-carbon pathway fuels cytosolic 10-formyl-THF production

Extensive evidence in the literature suggests that the mitochondrial one-carbon pathway fuels cytoplasmic purine and dTMP synthesis by generating in the mitochondria formate which then enters the cytoplasm [3]. A pertinent prediction is that mitochondrial one-carbon deficiency should result in a decreased 10-formyl-THF production. We have recapitulated mitochondrial one-carbon deficiency by knocking down SHMT2 or MTHFD1L, the first or last enzyme respectively of the mitochondrial one-carbon pathway, in MCF7 and MDA-MB-468 breast cancer cell lines. As expected, the ratio of 10-formyl-THF to THF is greatly diminished in the knockdown cells compared to the control cells (Fig. 7).

Surprisingly, we also observed that an unknown folate metabolite accumulated to a significant extent in these knockdown cells. Addition of sodium formate to the culture medium both normalizes the ratio of 10-formy-THF to THF and prevents the accumulation of the unknown folate metabolite. These results not only confirmed a critical role of mitochondrion-generated formate in sustaining a healthy 10-formyl-THF : THF ratio for optimal growth, but also raised the question as to whether there exists an alternative cytosolic one-carbon pathway, in which the unknown folate metabolite is an intermediate. My current efforts are directed towards elucidating the chemical structure of the unknown folate metabolite, and probing the kinetics of one-carbon fluxes upon acute withdrawal of nutrients.

Fig. 7 (A)

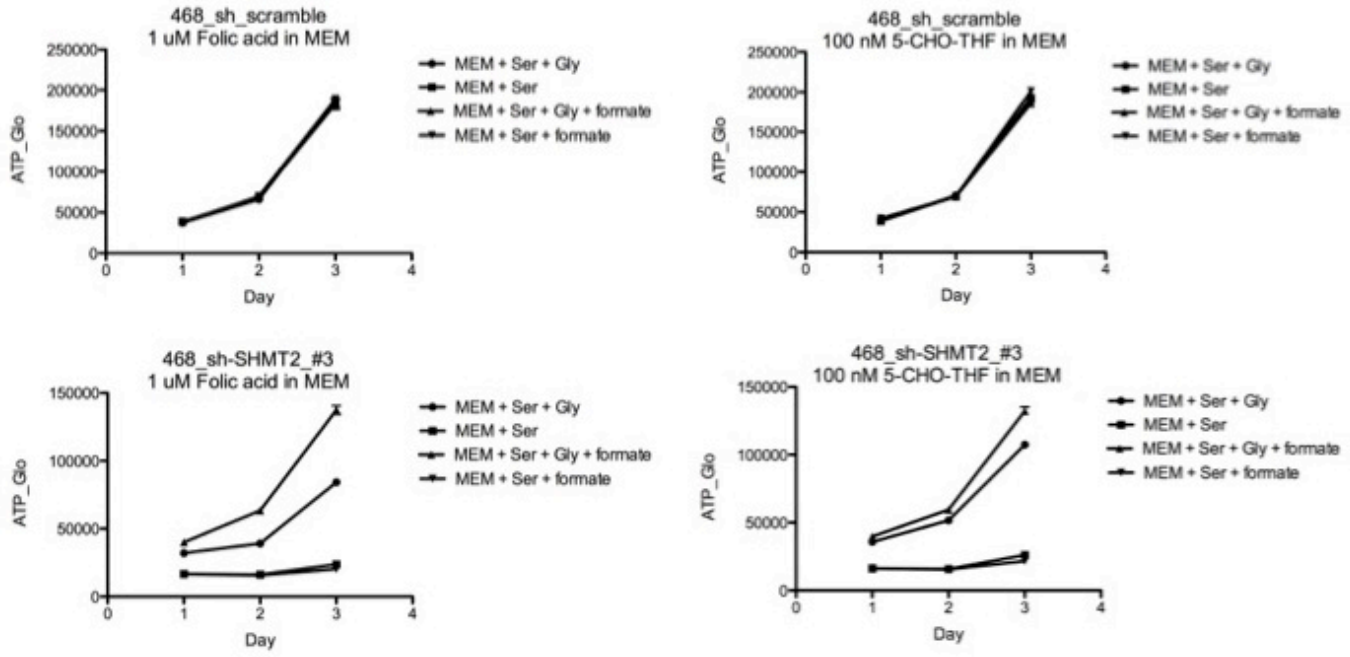

Fig. 7 (B)

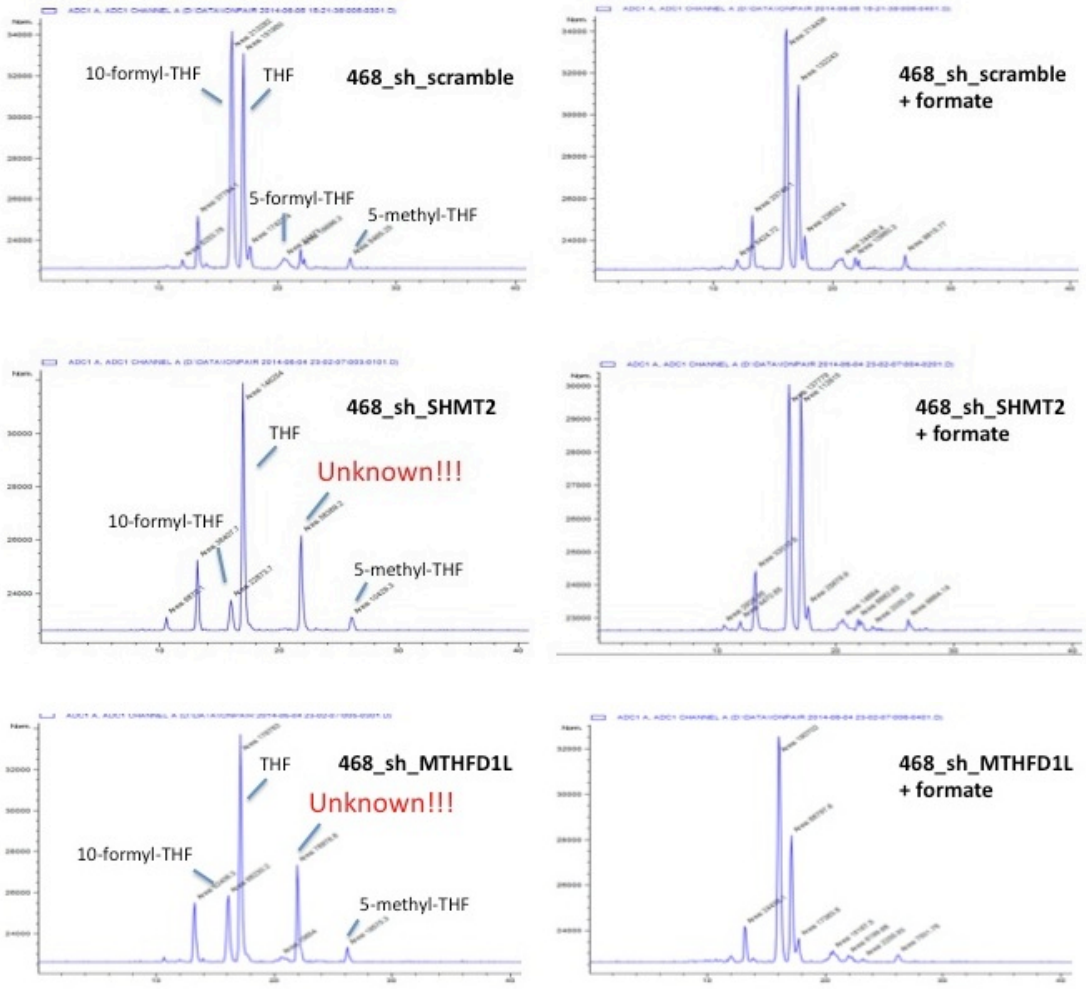

Fig 7: **(A)** Knocking down SHMT2 or MTHFD1L in MDA-MB-468 cells causes glycine auxotrophy; formate does not rescue glycine auxotrophy, but stimulates growth in the presence of glycine. **(B)** Mitochondrial one-carbon deficiency results in a diminished 10-formyl-THF : THF ratio.

Successful Development and Application of Radiolabeling and Dilution Assays for de novo Biosynthesis of DNA/RNA Nucleotides (SOW Task 3c)

As one-carbon/folate metabolism lies immediately upstream of *de novo* biosynthesis of nucleotides, measurement of *de novo* nucleotide synthesis represents a crucial functional readout of any perturbations of folate metabolic fluxes. I have successfully developed an assay for this purpose (Fig. 8). When this method was applied to study the effects of knocking down SHMT2 or MTHFD1L in MDA-MB-468 breast cancer cells on *de novo* nucleotide biosynthesis, the results obtained (Fig. 8) fit well with the folate profiling results shown in the previous section (Fig. 7).

Fig. 8

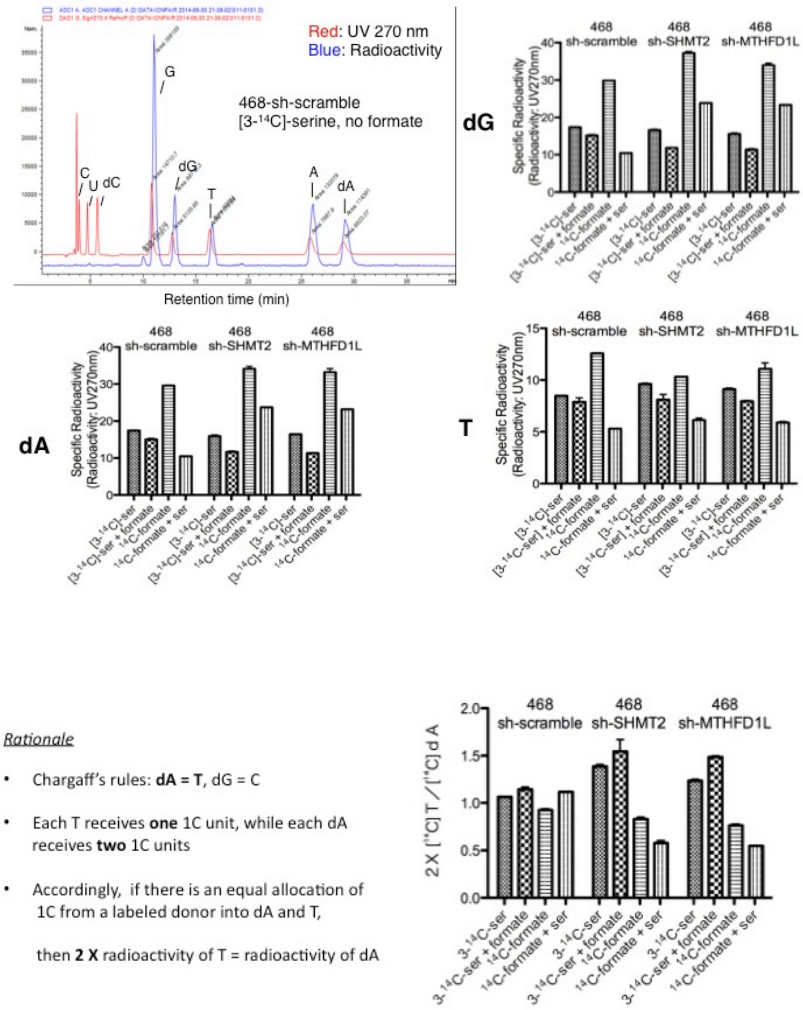

Fig. 8: These experiments demonstrated that the mitochondrial one-carbon pathway is crucial for *de novo* purine synthesis and fit with folate profiling results in Fig. 7. DNA/RNA was extracted from cells using phenol/chloroform, and hydrolyzed into nucleosides using nuclease P1 and alkaline phosphatase. The nucleosides were analyzed by reversed-phase HPLC with both diode array and radioactivity detection.

Regulation of Folate Metabolism by Growth Factor Signaling

With the key methodology successfully developed, I am currently working at the exciting front of understanding how growth factor signaling regulates folate metabolism. In the first set of experiments, the folate profiles of MDA-MB-468 breast cancer cells under exponential growth and serum-starved conditions were compared. As shown in Fig. 9, strikingly, whereas 5-methyl-THF was a minor species under exponential growth conditions, it became the most prominent species upon serum starvation for 24 hr.

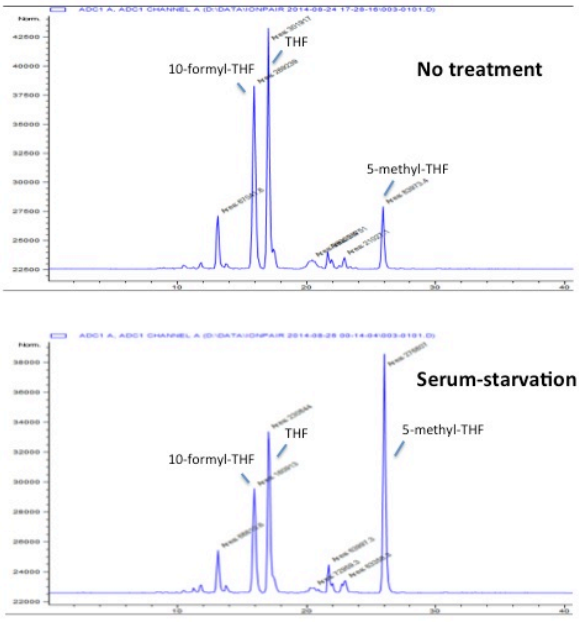

Fig. 9: [Top] Folate distribution of MDA-MB-468 under exponential growth conditions. [Middle] Folate distribution of MDA-MB-468 cells under serum-starvation for 24 hr.

One likely explanation is that growth factor signaling regulates the metabolic enzyme that generates 5-methyl-THF, namely methylene-tetrahydrofolate reductase (MTHFR). Indeed, it is reported that MTHFR is phosphorylated at multiple serine/threonine residues at its N-terminus [4]. Importantly, it is shown that some of these phosphorylation sites inhibit MTHFR enzyme activity. Based on the protein sequence, we noted that GSK3 is likely one of kinases that phosphorylates this region. This prediction is based on our lab's extensive experience in identifying protein kinase substrate specificity using peptide library approaches [5]. Since GSK3 is a direct target of AKT, we have hypothesized that activation of the PI3K/AKT signaling pathway will cause MTHFR dephosphorylation via phosphorylation and inhibition of GSK3.

I have confirmed the phosphorylation of MTHFR in a few breast cancer cell lines including MCF7, MDA-MB-468, and MDA-MB-231. In addition, I have utilized the newly developed phos-tag gel to better resolve the multiply phosphorylated species. (The next exciting experiments are to examine MTHFR phosphorylation status upon PI3K, ATK, and GSK3 inhibition, or upon insulin or EGF stimulation. Meanwhile, I will probe the fluxes of the one-carbon metabolic pathways under identical conditions. Ultimately, we hope to tie in the changes in folate metabolic fluxes with the changes in the phosphorylation status of MTHFR, in order to tease out the mechanisms by which growth factor signaling regulates folate metabolism.

Key Research Accomplishments

- Successful Development and Validation of a Key HPLC Method that Permits Quantitative Assessment of Intracellular Folates

- Successful Development and Application of Radiolabeling and Dilution Assays for de novo Biosynthesis of DNA/RNA Nucleotides

- Poster Presentation at the 2014 FASEB "Folic Acid, Vitamin B12, and One-Carbon Metabolism" conference

- Collaboration with Dr. Dean Appling's group at UT-Austin. Dr. Dean Appling's group will provide me with Mthfd1l knockout MEFs, which will be extremely useful tools for this project.

Conclusion

The project is progressing well. The key methodologies developed in this fellowship period (Year 1) have paved the way for future efforts of testing exciting hypotheses regarding how folate metabolism is altered in breast cancer. Our new data strongly suggest that growth factor signaling regulates folate metabolism. As dysregulated growth factor signaling is often a driving event in breast carcinogenesis, our future efforts will be directed towards elucidating the mechanisms by which growth factor signaling regulates folate metabolism, and how these pathways are dysregulated in breast cancer. The results of these experiments will have the potential to reveal novel therapeutic targets for breast cancer.

Opportunities for Training

- I have presented part of this project in the poster session of the 2014 FASEB "Folic Acid, Vitamin B12, and One-Carbon Metabolism" conference.

- I have initiated collaboration with Dr. Dean Appling of UT-Austin, who is an expert in folate/one-carbon metabolism. Dr. Dean Appling's group will provide me with Mthfd1l knockout MEFs, which will be extremely useful tools for this project.

Publications, Abstracts, and Presentations

Abstract for the 2014 FASEB "Folic Acid, Vitamin B12, and One-Carbon Metabolism" conference.

Dissecting folate metabolism in glycine auxotrophs with impaired mitochondrial one-carbon pathway

Yuxiang Zheng and Lewis C. Cantley
Weill Cornell Medical College, New York, NY

A deficiency in the mitochondrial one-carbon pathway is known to cause glycine auxotrophy in mammalian cells. An additional phenotype is a dependence on exogenous formate for optimal growth, consistent with the view that the mitochondrial pathway fuels cytoplasmic purine and dTMP synthesis by generating formate in the mitochondria which then enters the cytoplasm. Here, we sought to unravel the mechanistic basis for the aforementioned phenotypes of mitochondrial one-carbon deficiency. To this end, we developed a method for cellular folate analysis (based on the methods of Horne and of Chabner), in which cells are labeled by ^{3}H-folic acid or ^{3}H-folinic acid, the polyglutamate tail of intracellular folates cleaved by recombinant gamma-glutamyl hydrolase, and the resulting folate monoglutamates resolved by HPLC. Using this method, we found that the 10-formyl-THF : THF ratio is greatly diminished when SHMT2 or MTHFD1L, the first or last enzyme of the mitochondrial one-carbon pathway respectively, is knocked down in MCF7 and MDA-MB-468 breast cancer cell lines. Surprisingly, an unknown folate metabolite accumulated to a significant extent in these knockdown cells. Addition of sodium formate to the culture medium both normalizes the ratio of 10-formyl-THF to THF and prevents the accumulation of the unknown folate metabolite. These results not only confirmed a critical role of mitochondrion-generated formate in sustaining a healthy 10-formyl-THF: THF ratio for optimal growth, but also raised the question as to whether there exists an alternative cytosolic one-carbon pathway, in which the unknown folate metabolite is an intermediate. Our current efforts are directed towards elucidating the chemical structure of the unknown folate metabolite, and probing the kinetics of one-carbon fluxes upon acute withdrawal of nutrients.

Inventions, Patent and Licenses

N/A

Reportable Outcomes

N/A

Other Achievements

N/A

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Appendices

N/A