Impact Of Folate Depletion On Expression Of Folate Metabolizing Enzymes

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IMPACT OF FOLATE DEPLETION ON EXPRESSION OF FOLATE METABOLIZING ENZYMES

by

WU YIZHEN

THESIS

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Approved by:

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INTRODUCTION:

Folate is the generic term for a large family of similar chemicals. As picture 1 shows, native folates can have different oxidation state. Since native folates can have many structural forms of folyl-coenzymes, it can be difficult to elucidate how the folate derivatives interconvert and how these folate derivatives function in folate biochemistry. ([Mark Lucock, et al, 2000])

Human cannot synthesize folate, so, we need to consume a variety of dietary sources for the uptake of folates. Yeast extracts such as Marmite, liver, kidney, leafy green vegetables, and citrus fruit are the rich sources containing folates. On the other hand, if we consume large amounts of moderate sources of the folates, such as bread, potatoes, and dairy products, it will actually provide a
significant contribution to the total folate intake, especially since the fortification of these foods with supplemental folate began in 1998. (Mark Lucock, et al, 2000)

Food folates exist mainly as 5-methyltetrahydrofolate (5-methyl-H4PteGlun) and formyltetrahydrofolate (formyl-H4PteGlun) (Ratanasthien K, et al, 1977). However, 5-Methyl-H4PteGlun is the predominant food folate compared with formyl-H4PteGlun (Stokstad ELR, et al, 1967; Butterworth CE, et al, 1963) and it will be easily oxidized to 5-methyl-5,6-dihydrofolate (5-methyl-5,6-H2PteGlun) (Donaldson KO, et al, 1962). So that 5-methyl-5,6-H2PteGlun can also represent a substantial amount of the total food folate (Ratanasthien K, et al, 1977).
picture 2: Folate-depend one-carbon metabolism pathway.

As picture 2 shows, folates carry one-carbon units that exist at various levels of oxidation for three major metabolic roles: synthesis of thymidylate, methionine, and purine, conversion of serine to glycine, and homocysteine remethylation cycle. Since these reactions involve various electron transfer steps, specific folate metabolism enzyme and coenzymes such as FADH$_2$ and NADPH will be necessary. (Mark Lucock, et al, 2000)

*Purine and Pyrimidine Nucleotide Biosynthesis:*

The most important roles of folic acid derivatives may be synthesis of purine
and pyrimidine nucleotides. To synthesize the purine ring, 10-formyl-H4PteGlu will donate one-carbon unit to aminoimidazole-4-carboxamide ribonucleotide (AICAR) and glycinamide ribonucleotide (GAR) respectively, and 10-formyl-H4PteGlu will become H4PteGlu1. (Mark Lucock, et al, 2000) The enzymes responsible for this reaction are AICAR transformylase and GAR transformylase. On the other hand, to synthesize the pyrimidine nucleotides, deoxyuridylate monophosphate (dUMP) will be methylated by thymidylate synthase (TS) which is rate limiting enzyme in the elaboration of DNA to form thymidylate monophosphate (TMP), and 5,10-methylene-H4PteGlu as a donor will be converted to H2PteGlu in this reaction. (Mark Lucock, et al, 2000)

The Interconversion of Serine and Glycine:

Serine hydroxymethyltransferase (SHMT) is the enzyme responsible for reversible interconversion of serine and glycine, and this reaction requires vitamin B6 as a cofactor:

$$\text{H4PteGlu} + \text{serine} + \text{NAD}^+ \rightleftharpoons \text{5,10-methylene-H4PteGlu} + \text{glycine} + \text{H2O}.$$  

Actually, 5,10-methylene-H4PteGlu is at the branch point for three important pathways (Green JM, et al, 1988), because 5,10-methylene-H4PteGlu can be converted to 5-methyl-H4PteGlu by 5,10MTHFR; and then with the help of B12-dependent MetSyn, homocysteine (Hcy) can be converted to methionine while the 5-methyl-H4PteGlu was converted to H4PteGlu (Banerjee RV, et al,
1990); also with the enzymes of TS, 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methylenetetrahydrofolate cyclohydrolase, 5,10-methylene-H4PteGlu can be used to synthesize the DNA thymine and purine, respectively.

The Homocysteine Remethylation Cycle: Folate-Dependent de Novo Methionine

Biosynthesis:

From picture 2, we can see that homocysteine (Hcy) connects two important pathways and is regulated by several enzymes. One is the biosynthesis of methionine from Hcy which is activated when the S-adenosylmethionine (SAM) level drops. The other pathway is the transulphuration from Hcy to cystathionine which is activated by elevated level of SAM (Selhub J, et al, 1992).

Before Hcy enters the remethylation cycle, 5,10-methylene-H4PteGlu will first be reduced to 5-methyl-H4PteGlu by 5,10MTHFR, which in vivo is irreversible (Green JM, et al, 1988). Then while converting 5-methyl-H4PteGlu to the H4PteGlu by Vitamin B12-dependent methionine synthase (MetSyn) and methionine synthase reductase (MSR) which reductively activates MetSyn, then Hcy will be converted to methionine (Banerjee RV, et al, 1990; Leclerc D, et al, 1998). When methionine is activated by ATP, the methionine can be converted to the methyl donor SAM by the enzyme methionine adenosyltransferase. SAM is an essential molecule since a variety of important biomolecules such as adrenalin, phosphatidylcholine, and carnitine are methylated by it and it can
regulate both remethylation and transsulphuration pathways (Green JM, et al, 1988). After the methylation process, SAM is converted to S-adenosylhomocysteine (SAH), and then SAH will be hydrolyzed back to Hcy by S-Adenosylhomocysteine hydrolase to recommence a new remethylation cycle (Finkelstein JD, et al, 1974).

When the Hcy is converted to cystathionine by transsulphuration reaction, the thiol group in Hcy will condense with serine by cystathionine \( \beta \)-synthase in which vitamin B6 works as a cofactor. Once it enters the transsulphuration pathway, Hcy will no longer serve as a precursor for methionine biosynthesis and the cystathionine will be further hydrolyzed to cysteine and \( \alpha \)-ketobutyrate by another B6-dependent enzyme, \( \gamma \)-cystathionase. (Mark Lucock, et al, 2000)
Picture 3: The role of the proteins coded by studied genes in folate metabolism. (*Min shena, et al, 2005*)

As we know folate is involved in one-carbon transfer pathways. The folate influences the thymidine monophosphate (dTMP) and the purine nucleotides synthesis, consequently affect the DNA synthesis and repair. Through the one-carbon pathway, the folate level also influences the DNA methylation, consequently affect the gene expression. The picture 3 illustrate the genes relate
to folate metabolic pathways and I will explain the function of these genes in the following content:

**Reduced folate carrier (RFC)** is the primary cellular transport system for the intake of reduced folates and folic acid antagonists (i.e., antifolates) including methotrexate (MTX), which functions as a bidirectional anion exchanger, taking up folate cofactors and exporting various organic anions, including thiamine pyrophosphate. *(Nunez MI, et al, 2012)*

**Thymidine kinase (TK)** is an ATP-thymidine 5'-phosphotransferase which can be found in most living cells. It is present in two forms in mammalian cells, TK1 and TK2. *(Xu Y, et al, 2012)*

Thymidine kinase catalyses the reaction:

- Thd + ATP → TMP + ADP

- **Thd**: deoxythymidine, **ATP**: adenosine 5’-triphosphate, **TMP**: deoxythymidine 5’-phosphate a, **ADP**: adenosine 5’-diphosphate.

Since thymidine kinases is part of the unique reaction chain to introduce deoxythymidine into the DNA, it plays important role in the synthesis of DNA and thereby in cell division. *(Xu Y, et al, 2012)*

**Serine hydroxymethyltransferase 1 (SHMT1)** is a pyridoxal phosphate-containing enzyme that catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methylene-tetrahydrofolate:
H4PteGlu + serine + NAD+ \rightleftharpoons 5,10-

methylene-H4PteGlu + glycine + H2O.

And then the 5,10-methylenetetrahydrofolate from this reaction will serve as a key component for synthesis of methionine, thymidylate, and purines in the cytoplasm. (Weiner AS, et al, 2012)

Serine hydroxymethyltransferase 2 (SHMT2) is the mitochondrial form of a pyridoxal phosphate-dependent enzyme that catalyzes the reversible reaction of serine and tetrahydrofolate to glycine and 5,10-methylene tetrahydrofolate in mitochondrial. The SHMT2 actually is primarily responsible for glycine synthesis which has been suggested to be the primary source of intracellular glycine. (Hebbring SJ, et al, 2102)

Gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase) (GGH) is the gene that codes for an enzyme that catalyzes the hydrolysis of folylpoly-gamma-glutamates and antifolylpoly-gamma-glutamates to give 5-methyl-tetrahydrofolate by the removal of gamma-linked polyglutamates and glutamate.( Silva IH, et al, 2013)

Folylpolyglutamate synthase (FPGS) is the gene that encodes the enzyme that catalyzes the ATP-dependent addition of glutamate moieties to folate and convert them to polyglutamate derivatives, in which the tetrahydrofolate is the enzyme's major substrate.
ATP + tetrahydropteroyl-(gamma-Glu)(n) + L-glutamate = ADP + phosphate + tetrahydropteroyl-(gamma-Glu)(n+1).

So that, this enzyme has been playing an important role in retaining both cytosolic and mitochondrial folylpolyglutamate in appropriate concentrations which is essential for the survival of proliferating cells. (Christoph DC, et al, 2012)

Thymidylate synthase (TYMS) is the gene that encodes the enzyme that catalyzes the conversion of deoxyuridylate to deoxythymidylate by methylation using 5,10-methylenetetrahydrofolate (methylene-THF) as a one-carbon unit cofactor. This enzyme establishes and maintains the dTMP (thymidine-5-prime monophosphate) pool which is critical for DNA replication and repair. (Christoph DC, et al, 2012)

Dihydrofolate reductase (DHFR) is the gene that encodes the enzyme that converts dihydrofolate into tetrahydrofolate which is an important substrates required for the de novo synthesis of purines, thymidylic acid, and certain amino acids. (Milic V, et al, 2012)

Methylenetetrahydrofolate reductase (NAD(P)H) (MTHFR) is the protein encoded by this gene that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is a one-carbon unit co-substrate for homocysteine remethylation to methionine.
This enzyme is regulated by the concentration of SAM and dihydrofolate, when the level of SAM increased, the activity of MTHFR will be inhibited. (Trifonova EA, et al, 2012)

Methylenetetrahydrofolate dehydrogenase 1 (NADP+ dependent) (MTHFD1) is the gene that encodes an enzyme that has three distinct enzymatic activities: The 5,10-methylenetetrahydrofolate dehydrogenase convert 5,10-methylenetetrahydrofolate to 5,10-methenyltetrahydrofolate; the 5,10-methenyltetrahydrofolate cyclohydrolase convert 5,10-methenyltetrahydrofolate to 10-formyltetrahydrofolate; and the 10-formyltetrahydrofolate synthetase convert 10-formyltetrahydrofolate to tetrahydrofolate. This enzyme can be used to recycle the 5,10-methylenetetrahydrofolate back to tetrahydrofolate which is an essential substrates for de novo synthesis of methionine, thymidylate, and purine. (Zampieri BL, et al, 2012)

Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) is the gene that encodes a mitochondrial bifunctional enzyme which has both methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase activities. The enzyme functions as a homodimer and requires magnesium and inorganic phosphate to works as cofactor, which will lead to the formation of enzyme-magnesium complex allowing the binding of NAD+.
(Zampieri BL, et al, 2012)
5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) (also known as cobalamin-dependent methionine synthase) is the gene that encodes the enzyme that catalyzes the conversion of homocysteine to methionine with vitamin B12 work as cofactor and also converted 5-methyltetrahydrofolate to tetrahydrofolate. (Weiner AS, et al, 2012)

5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) is the protein encoded by this gene. As we know methionine is an essential amino acid in protein synthesis and one-carbon metabolism, and it is synthesized by the enzyme methionine synthase which might eventually become inactive since the vitamin B12 cofactor will oxidize it during the synthesis process. The MTRR can regenerate a functional methionine synthase via reductive methylation. (Zhou D, et al, 2012)

Cystathionine-beta-synthase (CBS) is the gene that encodes the enzyme that catalyzes the conversion of homocysteine to cystathionine with serine as co-substrate and it is the first step in the transsulfuration pathway. This enzyme will be activated by elevated level of SAM and uses vitamin B6 as a cofactor. (Pey AL, et al, 2013)
Purpose of this study:

This subject is significant and important for thesis study because it could tell us how the cells will regulate their folate metabolism enzymes when they are under the stress of folate depletion. **Our hypothesis is the MEF cells will regulate their folate metabolism enzymes in different ways compared with BNL CL.2 cells when they suffer folate depletion.**

In order to test our hypothesis we will inspect the changes in gene expression of 15 genes which are related to the folate metabolism in two different cell lines. One is MEF cell line and the other one is BNL-C2 cell line. These two cell lines will be both raise in full folate medium and folate depletion medium. And then we will isolate the RNA from these two cell lines and make the cDNA from it to run the real-time PCR to figure out the difference of gene expression change level between the MEF and BNL-CL2 cell lines when they suffer the folate depletion condition.
MATERIALS AND METHODS:

Tissue Cultures:

Transformed SV40 T-antigen mouse embryonic fibroblasts (MEFs) were derived from the embryos (ung+/+) of the ung moused developed by Endres et al. [Endres M, 2004]. BNL CL. 2 were normal liver cells derived from BALB/c mice. These cells were grown in either folate added standard DMEM (GIBCO BRL, Grand Island, NY, USA) containing 4.5g/L glucose, 4mg/L folic acid, glutamax, glutamine, 10% dialyzed fetal bovine serum or customized folate-free DMEM (GIBCO BRL) media supplemented with 10% dialyzed fetal bovine serum, glutamax and glutamine. Each of the growth media was supplemented with 1% penicillin-streptomycin and both MEF and BNLCL.2 cells were incubated at 37°C in 10% CO2. For getting the folate depleted cells, at first, folate-free media will be supplied with low amount of thymidine and adenosine so that the cells can survive. And then, the percentage of T/A will be reduced stepwise over time, 1X being the starting concentration. The MEF cells and BNL CL2 cells were both passaged three times for each T/A concentration until 0% T/A was achieved. Before each passage, the cells were grown until they became 75% confluent.
**Harvesting Cells:**

The flasks of cells were first visualized under the microscope to determine if the cells were 75% confluent. The old media from the cells was removed and the flasks were washed with 5 ml of pre-warmed 1X PBS-EDTA. Once the 1X PBS-EDTA was removed, 2 ml of trypsin was added. The flasks were then incubated for approximately 2-3 minutes at 37°C in order to detach the cells from the bottom of the flask. The cells were again viewed under the microscope to ensure that the cells were lifted off the flask. To stop the trypsin from working, 3 ml of complete growth media was added. Using a serological pipette, the cells were pipette up and down several times for the cells to separate from each other. The cells along with the added complete media were transferred into a 15 ml conical tube for washing. The cells were centrifuged for 5 minutes at 1300 rpm at 4°C. The media was carefully removed without disturbing the white visible pellet on the bottom of the conical tube and 5 ml of 1X PBS was added to the pellet. The cells were again centrifuged at 1300 rpm for 5 minutes at 4°C and the 1X PBS was carefully removed without removing the pellet. Then we will add the 1ml Isol-RNA Lysis Reagent to the pellet and mix well to isolate the RNA in next step.
Doubling Times:

Calculations:

\[
\begin{align*}
\text{Live conc. cell count} \times 2 \ (1:2 \ \text{trypan blue dilution}) &= \# \text{ of cells/ml} \\
\text{EX}: \ 2.56 \times 10^5 \text{cells/ml} \times 2 &= 512,000 \ \text{cells/ml} \\
\end{align*}
\]

Must be consistent throughout each passage when performing doubling time

\[
\frac{\# \text{ of cells to be plated}}{\# \text{ of cells/ml}} = \frac{\text{amt of cells in ml or } \mu l \text{ to be plated}}{\text{for next doubling time passage}}
\]

\[
\text{EX}: \frac{250,000 \ \text{cells}}{512,000 \ \text{cells/ml}} = 0.4822 \ \text{ml or } 488 \ \mu l \ \text{of cells to be plated in order to grow } 250,000 \ \text{cells in the next passage}
\]

Total \# \text{ of cells/flask} = (\# \text{ of cells/ml}) \times 2 \ (\text{trypan blue dilution}) \times \text{total volume}

Picture 4: The calculation of doubling time.

The MEF cells and BNL CL 2 cells were examined daily under a light microscope, observing the morphology, density of the cells, and color of the media. Once the cells became 75% confluent, the 10 mm petri dishes containing the cells were carefully removed from the incubator. Then, we will harvest the cells as above shows. Once a single cell suspension was achieved, the cells were transferred into a 15-ml conical tube. Trypan blue (10\(\mu l\)), which is rejected by live cells but accumulates in dead cells, was transferred into a 1.5 ml eppendorf tube along with 10\(\mu l\) of cells and counted in the TC 10 Automated Cell counter. The total number of cells/ml was then calculated as picture 4 shows and used to
determine the amount of cells to plate for the next passage. And we can obtain the doubling time of MEF cells and BNL CL2 cells easily by the equation:

\[ T_d = (t_2 - t_1) \times \frac{\log(2)}{\log\left(\frac{q_2}{q_1}\right)}. \]

\( T_d \) is the doubling time, \( t_1 \) is the time we passage the cells, \( t_2 \) is the time we harvest the cells, \( q_1 \) is the number of cells we plated at first, \( q_2 \) is the total number of cells after we harvested.
**RNA Isolation:**

Once the cell were harvested, the whole RNA will be isolated using Isol-RNA Lysis Reagent (5 Prime Inc, Gaithersburg, MD). First, we will let samples incubate in Isol-RNA Lysis Reagent (5 Prime Inc, Gaithersburg, MD) for 5 minutes at room temperature. Then, spin the samples at 12,000g for 10 minutes at 4°C, so that DNA will pellet down and we can collect supernatant. After that, to each sample, we add 0.2 ml of chloroform for every 1 ml of Isol-RNA Lysis Reagent (5 Prime Inc, Gaithersburg, MD) used and shake vigorously for 15 seconds and incubate at room temperature for 2-3 minutes. After incubation, we centrifuge samples at 12,000 x g for 10 minutes at 4°C. The 4 degree spins are essential for proper phase separation. And then we transfer the aqueous phase to a fresh tube. The aqueous phase is the colorless upper phase that corresponds to ~60% of the volume of Isol-RNA Lysis Reagent (5 Prime Inc, Gaithersburg, MD) used. Then we add 0.5 ml isopropanol into samples and mix by pipetting. Also, we incubate them at room temperature for 10 minutes and centrifuge for 10 minutes at 12,000 x g at 4°C. After that we remove the supernatant from each sample. A pellet should be observed at the bottom of the tube. Then we wash the Pellet with 1 ml of 75% ethanol for every 1 ml of Isol-RNA Lysis Reagent (5 Prime Inc, Gaithersburg, MD) used and mix samples by flicking and inverting the tubes or by vortexing. The pellet should come loose from the bottom of the tube, but remain intact. And then we centrifuge at 7500 x g for 5 minutes at 4°C. After that, we need to remove all of the ethanol from each tube, but not to
disrupt the pellet and redissolve the pellet by adding 30 ul of RNase free water. If the pellet is not dissolved, then we incubate them at 55-60°C for ~10 minutes with the interval of every 2 minutes. Finally, we need to quantify RNA samples using Nanodrop, so that we can made the cDNA in next step.
cDNA Synthesis:

After the isolation of whole RNA from cells, we can synthesis cDNA from the RNA we isolated. First, we need to Prepare the reverse transcription reaction mix by combining the following components of the RT-PCR kit (Perkin Elmer, Waltham, MA) in a sterile 1.5 ml microcentrifuge tube on ice.

Master Mix

- Nuclease-free water (Perkin Elmer, Waltham, MA) 5 µl
- 5x Reaction Buffer (Perkin Elmer, Waltham, MA) 4µl
- MgCl₂ (Perkin Elmer, Waltham, MA) 4µl
- dNTP (Perkin Elmer, Waltham, MA) 1µl
- Reverse Transcriptase (Perkin Elmer, Waltham, MA) 1µl

We need to prepare sufficient mix to allow 15 µl for each cDNA synthesis reaction to be performed and vortex them gently to mix and keep them on ice prior to dispensing into the reaction tubes. After that, we aliquot 15 µl of the reverse transcription reaction mix to each reaction tube on ice and be careful to prevent cross-contamination. Then, we thaw the experiment RNA and return any unused portion to the freezer as soon as aliquots are taken. On ice, we combine the experimental RNA (500ng-1000ng) and the cDNA random primer in nuclease-free water for final volume of 5 µl per RT reaction.

- Reaction primer = 1 µl
- RNA = 1-3 µl (depending on concentration)
• dH₂O = ____ (certain volume to make total = 5 µl)
• Total = 5 µl

Then, we close each tube of RNA tightly and place the tubes into a preheated 70°C heat block for 5 min. After that, we immediately chill the samples on ice for 5 min and then spin each tube for 10 seconds in microcentrifuge to collect the condensate. Then we add 5 µl of RNA and primer mix to the reaction tubes containing RTase reaction mix left on ice in early step. We cap the tubes and centrifuge for 1 min to mix the samples and then place the reaction tubes in the thermocycler (Eppendorf North America, NY) and set parameters:

• 5 min @ 25°C
• 60 min @ 42°C
• 15 min @ 70°C
• Hold @ 4°C
• Can store at -20°C

And then, we purify the cDNA samples with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and quantify the cDNA using NANO-DROP (Eppendorf North America, NY)
Gene Cloning:

Once we synthesis the cDNA, we can amplify the gene fragments we want from the cDNA samples with specific design primers. And then we will insert the gene fragment we targeted into a well designed plasmid called pCRII-topo (Life Technologies, Grand Island, NY) that has a single 3' overhanging nucleotide (a thymidine).

...GAATTCCGCCCTT AGGGCGAATTC...

...CTTAAGCGGGGA TTCCCGGTAAAG...

This vector cannot easily reclose since the 3' overhanging ends are incompatible (both being T bases) to each other. On the other hand, it is perfect at accepting PCR products made with Taq polymerase (Life Technologies, Grand Island, NY). Since Taq polymerase (Life Technologies, Grand Island, NY) has a limited "terminal transferase" activity which can add a single 3' overhanging adenosine to its products of synthesis:

NNNNNN...NNNNNNNA

ANNNNNNN...NNNNNN

And then we will transform the recombinant plasmids to the competent cells and spread them on the LB agar plates with X-gal and Ampicillin on it. Since the plasmid contains the antibiotic gene on it, only the cell harbor the plasmids
can survive on the ampicillin contained agar plates. Also, since we using the blue-white screening system to distinguish the colonies (clones) of transgenic cells from those that contain the parental vector, we will select the white colonies to raise them in LB broth overnight and then isolate the plasmids for standard curves later.
Real Time RT - PCR:

Once total RNA was isolated from the MEF and BNL CL.2 cells, 2μg of isolated RNA was used to synthesize cDNA using random hexamer primers and an RT-PCR kit (Perkin Elmer, Waltham, MA). The newly synthesized cDNA was then purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). A LightCycler real time PCR machine (Roche, Indianapolis, IN) was used to quantify the transcripts. Each PCR reaction consisted of 2μL of purified cDNA, 4mM MgCl2, 0.5μM each of sense and antisense primers, and 2μL of FastStart DNA master SYBR Green I enzyme-SYBR reaction mix (Roche). The parameters for all of the amplifications are detailed in Table 1. All the transcripts were normalized to the housekeeping gene, RPL-4.

Table 1 Real Time PCR parameters for all amplifications.

<table>
<thead>
<tr>
<th></th>
<th>Duration</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturing step</td>
<td>10 min</td>
<td>99°C</td>
</tr>
<tr>
<td>Denature</td>
<td>10 sec</td>
<td>96°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>10 sec</td>
<td>62°C</td>
</tr>
<tr>
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Table 2: Primer sequences in quantitative real time RT-PCR

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<th>Gene</th>
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<th>Anti-sense primer 5’-3’</th>
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<td>Reverse Primer</td>
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Folate Microbiology Assay:

Folate was measured from the MEF and BNL CL.2 cells using the folate microbiological assay as described by Home et al [Home DW, 1988]. This widely used microbiological assay measures folic acid derivatives in serum as well as other biological samples [Home DW, 1988]. The folinic acid (calcium salt) [(6-ambo)-5-HCO-H4PteGlu] was prepared in water at a concentration of 6 mM (6×10^6 fmol/μl) and diluted to 6×10^3 fmol/μl, which was then diluted to 60 fmol/μl and eventually to a final dilution of 2b fmol/μl (working solution). The single strength folic acid casei medium was prepared by dissolving 9.4 gram of the powder folic acid casei medium and 50 mg of sodium ascorbate (Vitamin C) into 100 ml of diH2O. The mixture was then filtered through a 0.22-μm sterile syringe filter. The working buffer was prepared by dissolving 3.2 gram of sodium ascorbate into 19 ml of diH2O and adding 1 ml potassium phosphate buffer (1mol/l, pH 6.1). The mixture was then sterilized through a 0.22-μm sterile syringe filter. Lastly, the L. casei inoculum was prepared. One vial of lyophilized Lactobacillus casei, stored at -80°C when first received, was suspended in 1 ml of the medium to grow overnight at 37°C. From this inoculum, 0.25 ml was added to the remaining 199 mL of medium and incubated for approximately 18 hours at 35°C. The mixture was then cooled down in an ice bath. An equal volume of cold (4°C) sterile glycerol (800ml/L) was added to the mixture. From the mixture, 2ml aliquots were prepared and stored at -80°C. Once the L. casei inoculum was incubated overnight at 37°C,
the OD was measured and diluted to an OD value of 0.5 (the standard OD value of the *L. casei* inoculum). The microtiter plate was then set up as shown in Table 3. A multiple pipette was used to add the working buffer mixture (8 μl/sample) and media (150 μl/sample) along with either autoclaved or sterile water. As shown in Table 4, 2 fmol/μl of folate with the corresponding volume was added to each designated well on the plate. Due to folate being light sensitive, the lights were turned off during the procedures of this assay. Using a multiple pipette, 20 μl of *Lactobacillus casei* (OD=0.05) was added to the standards and samples for the exception of one blank. Autoclaved or sterile water was added around the well of the microtiter plate to create enough humidity whereas parafilm was used to cover the plate in order to keep the humidity within the microtiter plate. The plate was also covered with oil film to prevent the degrading of folate from light. The plate was incubated overnight at 37°C for approximately 18 hours. Before the plate was read at an absorbance of approximately 570-635 using a Dynatech Model MR600 reader, the pellet of the bacteria was resuspended using a pipette.

Table 3: Folate assay microtiter plate setup

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<tr>
<th>Folate (fmol)</th>
<th>1 standard</th>
<th>2 standard</th>
<th>3 Standard</th>
<th>4 blank</th>
<th>5 sample</th>
<th>6 s</th>
<th>7 s</th>
<th>8 s</th>
<th>9 s</th>
<th>10 s</th>
<th>11 s</th>
<th>12 s</th>
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<tbody>
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<td></td>
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Table 4: Folate assay reaction mixture

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RESULT & DISCUSSION:

Figure 1: Absence of Folate in media induces folate depletion in MEF cells. (*p value < 0.01.) Folate levels in MEFS cells was measured using the microbiological assay as described in methods. The level of folate is very low (near 0) when they are grown under folate depletion condition which resulted in almost 100% reduction in folate level.

Figure 2: Absence of Folate in media induces folate depletion in BNL CL.2 cells. (*p value < 0.01.) Folate levels in BNL CL.2 cells was measured using the microbiological assay as described in methods. The level of folate is very low (near 10) when they under the folate depletion condition which resulted in greater than 90% reduction in folate level.
Figure 3: Absence of Folate in media induces CBS/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. CBS transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the CBS expression was elevated more than 2 fold in MEF cell lines under folate depletion condition.

Figure 4: Absence of Folate in media induces CBS/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. CBS transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the CBS expression was elevated around 2 fold in BNL CL.2 cell lines under folate depletion condition.
Figure 5: Absence of Folate in media induces GGH/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. GGH transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the GGH expression was elevated more than 2 fold in MEF cell lines under folate depletion condition.

Figure 6: Absence of Folate in media induces GGH/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. GGH transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the GGH expression was elevated more than 2 fold in BNL CL.2 cell lines under folate depletion condition.
Figure 7: Absence of Folate in media induces MTR/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. MTR transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the MTR expression was elevated 2 fold in MEF cell lines under folate depletion condition.

Figure 8: Absence of Folate in media induces MTR/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. MTR transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the MTR expression was elevated around 2 fold in BNL-CL.2 cell lines under folate depletion condition.
Figure 9: Absence of Folate in media induces RFC/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. RFC transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell line, the RFC expression were elevated in MEF cell lines under folate depletion condition.

Figure 10: Absence of Folate in media depress RFC/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. RFC transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell line, the RFC expression were depressed in BNL CL.2 cell lines under folate depletion condition.
Figure 11: Absence of Folate in media induces TYMS/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. TYMS transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the TYMS expression was elevated more than 2 fold in MEF cell lines under folate depletion condition.

Figure 12: Absence of Folate in media induces TYMS/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. TYMS transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the TYMS expression was elevated more than 2 fold in BNL CL.2 cell lines under folate depletion condition.
Figure 13: Absence of Folate in media induces FPGS/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. FPGS transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell line, the FPGS expression was elevated in MEF cell lines under folate depletion condition.

Figure 14: Absence of Folate in media did not influence FPGS/RPL4 expression in BNL CL.2 cells. cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. FPGS transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell line, the FPGS expression did not significantly change much in BNL CL.2 cell lines under folate depletion condition.
Figure 15: Absence of Folate in media induces MTHFD1/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. MTHFD1 transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell line, the MTHFD1 expression was elevated in MEF cell lines under folate depletion condition.

Figure 16: Absence of Folate in media did not influence MTHFD1/RPL4 expression in BNL CL.2 cells. cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. MTHFD1 transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell line, the MTHFD1 expression did not change much in BNL CL.2 cell lines under folate depletion condition.
Figure 17: Absence of Folate in media depress MTHFD2/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. MTHFD2 transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the MTHFD2 expression was depressed in MEF cell lines under folate depletion condition.

Figure 18: Absence of Folate in media depress MTHFD2/RPL4 expression in BNL CL.2 cells. (*p value < 0.05.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. MTHFD2 transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the MTHFD2 expression was depressed in BNL CL.2 cell lines under folate depletion condition.
Figure 19: Absence of Folate in media induces MTHFR/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. MTHFR transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the MTHFR expression was elevated 2 fold in MEF cell lines under folate depletion condition.

Figure 20: Absence of Folate in media induces MTHFR/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. MTHFR transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the MTHFR expression was elevated in BNL-CL.2 cell lines under folate depletion condition.
Figure 21: Absence of Folate in media induces MTRR/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. MTRR transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the MTRR expression was elevated in MEF cell lines under folate depletion condition.

Figure 22: Absence of Folate in media induces MTRR/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. MTRR transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the MTRR expression was elevated in BNL CL.2 cell lines under folate depletion condition.
Figure 23: Absence of Folate in media induces DHFR/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. DHFR transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the DHFR expression was elevated more than 2 fold in MEF cell lines under folate depletion condition.

Figure 24: Absence of Folate in media induces DHFR/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL.2 cells as described in method. DHFR transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the DHFR expression was elevated more than 2 fold in BNL CL.2 cell lines under folate depletion condition.
Figure 25: Absence of Folate in media induces SHMT1/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. SHMT1 transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the SHMT1 expression was elevated almost 2 fold in MEF cell lines under folate depletion condition.

Figure 26: Absence of Folate in media induces SHMT1/RPL4 expression in BNL CL.2 cells. (* p value < 0.05.) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. SHMT1 transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the SHMT1 expression was elevated in BNL-CL.2 cell lines under folate depletion condition.
Figure 27: Absence of Folate in media depress SHMT2/RPL4 expression in MEF cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from MEF cells as described in method. SHMT2 transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the SHMT2 expression was depressed in MEF cell lines under folate depletion condition.

Figure 28: Absence of Folate in media depress SHMT2/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.) cDNAs were prepared from RNA isolated from BNL CL.2 cells as described in method. SHMT2 transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the SHMT2 expression was depressed in BNL CL.2 cell lines under folate depletion condition.
**Figure 29:** Absence of Folate in media induces TK/RPL4 expression in MEF cells. (*p value < 0.01.*) cDNAs were prepared from RNA isolated from MEF cells as described in method. TK transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the TK expression was elevated more than 2 fold in MEF cell lines under folate depletion condition.

**Figure 30:** Absence of Folate in media induces TK/RPL4 expression in BNL CL.2 cells. (*p value < 0.01.*) cDNAs were prepared from RNA isolated from BNL CL2 cells as described in method. TK transcript levels were determined by real time RT-PCR analysis and normalized to RPL4. Data are presented relative to expression in folate added group. Compared with full folate added cell lines, the TK expression was elevated in BNL CL.2 cell lines under folate depletion condition.
Figure 31: Doubling time of MEF cells and BNL CL2 cells in full folate media. (*p value < 0.05.) MEF cells and BNL CL2 cells are raised in full folate media. After we harvested the cell, we will calculate the doubling time of the two cell line respectively as the method described before. Compared with MEF cell line, the doubling time of BNL CL2 cell line was increased nearly 2 fold.

Figure 32: Doubling time of MEF cells and BNL CL2 cells in folate depleted media. (*p value < 0.05.) MEF cells and BNL CL2 cells are raised in folate depleted media. After we harvested the cell, we will calculate the doubling time of the two cell line respectively as the method described before. Compared with MEF cell line, the doubling time of BNL CL2 cell line was increased more than 2 fold.
From Figures 1 and 2, we can be sure that the MEF cell line and BNL CL.2 cell line we used are truly folate depleted. This allows us to directly connect the gene expression results to folate status.

From the figure 3 to figure 30, we see that with the exception of RFC, FPGS, SHMT1 and MTHFD1, most of the folate metabolizing genes (TK, SHMT2, GGH, TYMS, DHFR, MTHFD2, MTHFR, MTR, MTRR and CBS) have the similar expression patterns in response to folate depletion condition. Such as the gene expressions of CBS, GGH, MTR, TYMS, MTHFR, MTRR, DHFR, and TK are all significantly elevated when the two cell lines suffered the folate depletion condition; while the gene expressions of MTHFD2 and SHMT2 are significantly depresses when they suffered the folate depletion. But since our hypothesis is the MEF cells will regulate their folate metabolism enzymes in different ways compared with BNL CL.2 cells when they suffer folate depletion, we will focus on explaining the results that shows different change level between the two cell lines.

The first one that shows different change level is the RFC gene (figure 9 and figure 10), the RFC expression was significantly elevated in MEF cell lines while the RFC expression was depressed in BNL CL2 cell line. As we have talked before, RFC is the primary cellular transport system for the intake of reduced folates and folic acid antagonists (i.e., antifolates) including methotrexate (MTX), which functions as a bidirectional anion exchanger, taking up folate cofactors and exporting various organic anions, including thiamine
pyrophosphate (Nunez MI, et al, 2012). So, the RFC expression was elevated in MEF cell line might be due to the cell needing to take up more folate from the environment to fulfill their needs of replication and growth. But the RFC expression was depressed in BNL CL2 cell line might be due to the cell already adapt the environment with depleted folate, they don't need so much transporters to intake the folate from outside to save some energy. And the BNL CL2 cells are trying to maintain the homeostasis of folate concentration inside the cell by growing slowly and less replication.

The second one that shows different change level is the FPGS gene (figure 13 and figure 14), the FPGS expression was significantly elevated in MEF cell lines while the RFC expression didn't has much change in BNL CL2 cell line. As we have known that FPGS was the gene encodes the enzyme that catalyzes the ATP-dependent addition of glutamate moieties to folate and convert them to polyglutamate derivatives, in which the tetrahydrofolate is the enzyme's major substrate.

\[
\text{ATP + tetrahydropteroyl-(gamma-Glu)(n) + L-glutamate = ADP + phosphate + tetrahydropteroyl-(gamma-Glu)(n+1).}
\]

So that, this enzyme has been playing an important role in retaining both cytosolic and mitochondrial folylpolyglutamate in a appropriate concentrations which is essential for the survival of proliferating cells(Christoph DC, et al, 2012). So, the FPGS expression was significantly elevated in MEF cell line
might due to the cell try to maintain the concentration of folate and folate derivatives inside the cytosol or mitochondria, because the MEF cells are replicate really fast and frequently, they need lots of folate derivatives to synthesize the thymine and purine which make the DNA. But the FPGS expression didn't change much in BNL CL2 cell line might be due to the role of liver as the central organ to maintain folate homeostasis, they will release the folylpolyglutamate to the plasma and let them circulate to other organs where needs the folate derivatives. Since the liver cells don't need much folate derivatives to support their replication and growth, they may not need to urge themselves to maintain the concentration of folate and folate derivatives inside the cytosol or mitochondria by elevating the FPGS expression even under the folate depletion condition.

The third gene differentially regulated in the two cell lines is MTHFD1 (figure 15 and figure 16). MTHFD1 expression was significantly elevated in MEF cell lines while the MTHFD1 expression didn't change in BNL CL2 cell line. As we talked before, MTHFD1 encodes an enzyme that has three distinct enzymatic activities: The \textit{5,10-methylenetetrahydrofolate dehydrogenase} convert 5,10-methylenetetrahydrofolate to 5,10-methenyltetrahydrofolate; the \textit{5,10-methenyltetrahydrofolate cyclohydrolase} convert 5,10-methenyltetrahydrofolate to 10-formyltetrahydrofolate; and the \textit{10-formyltetrahydrofolate synthetase} convert 10-formyltetrahydrofolate to tetrahydrofolate. This enzyme can be used to recycle the
5,10-methylenetetrahydrofolate back to tetrahydrofolate which is an essential substrates for de novo synthesis of methionine, thymidylate, and purine (Zampieri BL, et al, 2012). The reason why the MTHFD1 expression was significantly increased in MEF cell line might be due to MEF cells are growing faster and replicating themselves more frequently than BNL-CL2 cells. So, the MEF cells will need more tetrahydrofolate for the synthesis of methionine, thymidylate, and purine to make the DNA. But the BNL-CL2 grows more slowly, so that they might not need to elevate the MTHFD1 expression to get the tetrahydrofolate.

The fourth gene differentially regulated in the two cell lines is SHMT1 (figure 25 and figure 26). In MEF cell lines, SHMT1 expression was significantly elevated around 2 fold compared with full folate cells; while in the BNL CL2 cell line, SHMT1 expression just increase slightly. As we introduced before, SHMT1 is a pyridoxal phosphate-containing enzyme that catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methylene-tetrahydrofolate:

\[ \text{H4PteGlu} + \text{serine} + \text{NAD}^+ \rightleftharpoons 5,10\text{-methylene-H4PteGlu} + \text{glycine} + \text{H2O}. \]

And then the 5,10-methylenetetrahydrofolate from this reaction will serve as a key component for synthesis of methionine, thymidylate, and purines in the cytoplasm. (Weiner AS, et al, 2012) The reason why the SHMT1 expression was
significantly increased in MEF cell line might still be due to MEF cells are
growing faster and replicating themselves more frequently than BNL-CL2 cells. And since the 5,10-methylene-tetrahydrofolate is at the branch point of three important pathways, which leading to the synthesis of methionine, thymidylate, and purine, the MEF cells will need more of 5,10-methylene-tetrahydrofolate for DNA synthesis. But the BNL-CL2 grows more slowly, so that they might not need to elevate the SHMT1 expression as much as MEF cells did.

Also, from figures 31 and 32, we can be sure that MEF cells are growing much faster than BNL CL2 cells no matter in full folate media or in folate depleted media, which match what we explained before. So, we can draw a conclusion that at least the rapidly growing cells such as MEF cells need more 5,10-methylene-tetrahydrofolate and tetrahydrofolate inside the cell for DNA synthesis or cell growth could be one of the reasons to explain why the RFC, FPGS, SHMT1 and MTHFD1gene are differentially regulated in the two cell lines when they both under folate depletion condition.
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ABSTRACT

IMPACT OF FOLATE DEPLETION ON EXPRESSION OF FOLATE METABOLIZING ENZYMES

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Folate is a water-soluble vitamin B that plays a critical role in the de novo nucleotide synthesis, DNA repair, DNA methylation, and cellular growth. By involved in the one-carbon metabolic pathways, folate influences the thymidine monophosphate (dTMP) and the purine nucleotides synthesis, consequently affect the DNA synthesis and repair. Through the one-carbon pathway, the folate level also influences the DNA methylation, consequently affect the gene expression.

In this study, we inspected the change of gene expression of 14 genes which are related to the folate metabolism in two different cell lines. One is MEF cell line and the other one is BNL-C2 cell line. So that we could figure out the difference of gene expression change level between the MEF and BNL-CL2 cell lines when they suffer the folate depletion condition.
From the result we got, we can know that except from RFC, FPGS, SHMT1 and MTHFD1, other 11 genes all have the similar gene expression change level between the MEF and BNL-CL2 cell lines when they suffer the folate depletion condition. The different gene expression of RFC between the two cell lines might due to liver needs to release the folate derivatives to other body part where dividing cell regularly such as skin cells. The difference of FPGS between two cell lines might due to liver is the central organ for folate homeostasis, the gene expression in liver won't change much even the cells are suffering folate depletion condition. However, skin cells needs large amount of folate or its derivatives to support the cell division or cell growth, when the skin cells suffer the folate depletion condition, skin cells might increase the gene expression of FPGS to reestablish the folate homeostasis. The difference of MTHFD1 and SHMT1 between two cell lines might due to liver is not the organ replicate cells so regularly like the skin cells, when the skin cells suffer the folate depletion condition, skin cells might increase the gene expression of MTHFD1 and SHMT1 to get more tetrahydrofolate and 5,10-methylene-tetrahydrofolate which are substrates for de novo synthesis of methionine, thymidylate and purine.
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