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Investigation of the molecular response to folate metabolism inhibition☆

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Abstract

We investigated the molecular response to folate metabolism inhibition by exposing human lymphoblast cell lines to the methionine adenosyltransferase inhibitor cycloleucine. We carried out microarray analysis on replicate control and exposed cells by examining 47,000 transcripts on the Affymetrix HG U133 plus 2.0 arrays. We identified 13 genes that we considered reliable responders to cycloleucine treatment: chemokine receptor 3 (CXCR3), prostaglandin-endoperoxide synthase 2, growth arrest-specific 7, reduced folate carrier, klotho beta, early growth response 1, diaphanous homolog 3, prostaglandin D2 synthase (PGDS), butyrophilin-like 9, low-density lipoprotein receptor-related protein 11, chromosome 21 orf15, G-protein-coupled receptor 98 (GPR98) and cystathionine-beta-synthase (CBS). We further demonstrated that four of these genes, CXCR3, PGDS, GPR98 and CBS, consistently responded to cycloleucine treatment in additional experiments over a range of concentrations. We carried out gene-specific DNA methylation analysis on five genes, including CBS, and found no evidence that DNA methylation changes were mediating the gene expression changes observed. Pathway analysis of the microarray data identified four pathways of relevance for response to cycloleucine; the immune response NF-AT signaling pathway was the most statistically significant. Comparison with other gene expression studies focusing on folate deficiency revealed that gene products related to immune cells or the immune response is a common theme. This indicates that apart from their role in the immune response, it is likely that these gene products may also have a role to play in the cellular response to folate status.

Keywords: Folate; Cycloleucine; Microarray; Methylation; Immune; CBS

1. Introduction

The folate status of an individual is associated with a number of common diseases and birth defects including colon cancer [1] and neural tube defects [2]. The disease association of this essential B vitamin relates to its function as a 1-C donor in a number of enzyme-catalyzed reactions that ultimately supply nucleotide synthesis and methylation (Fig. 1). Folate status has an inverse relationship with homocysteine, i.e., when an individual's folate status is low, his or her homocysteine level tends to be high [3]. High homocysteine levels are also associated with common disease particularly cardiovascular events [4] and cognitive function [5]. However, whether high homocysteine itself is pathogenic or simply a biomarker has not been decisively demonstrated.

The question of why folate status matters in terms of disease risk and development is thought to relate to nucleotide supply (particularly pyrimidine synthesis), uracil misincorporation and *S*-adenosylmethionine (SAM) availability for methylation reactions [6]. While it is clear that folate/homocysteine levels are considered important factors in disease risk and progression, the molecular mechanism of the cellular response to this remains to be elucidated. A number of studies have begun to address this by examining gene expression patterns in both animal models and cultured human cells. Some of these studies have focused on a folate deficiency model in normal human cells. Among these, Courtemanche et al. [7] examined 695 genes in primary human lymphocytes, while Katula et al. [8] examined 17,000 genes in human fibroblasts. The molecular response to folate deficiency has also been investigated in human cancer cell lines. Jhaveri et al. [9] examined 2008 genes in nasopharyngeal epidermoid carcinoma KB cells, while Novakovic et al. [10] examined just 96 apoptosis-specific genes in colon cancer cells. Gene expression profiling in folate-deficient rodent cancer models has also been investigated by Crott et al. [11] using Affymetrix U34A microarrays in rat colonic mucosa, Garcia-Crespo et al. [12] in *Mthfr* mutant mice and MacFarlane et al. [13] in *Mthfd1* mutant mice. Apart from simple folate deficiency gene expression profiling, Bliek et al. [14] identified folate responsive genes by adding 5-methyltetrahydrofolate to cultured folate-deficient orofacial cleft cell lines using Affymetrix HG U133 plus 2.0 GeneChips. An alternative approach to investigating the molecular response to folate deficiency is to inhibit specific enzymes of the folate metabolic pathway. Kager et al. [15] used this approach to investigate the response of the dihydrofolate reductase

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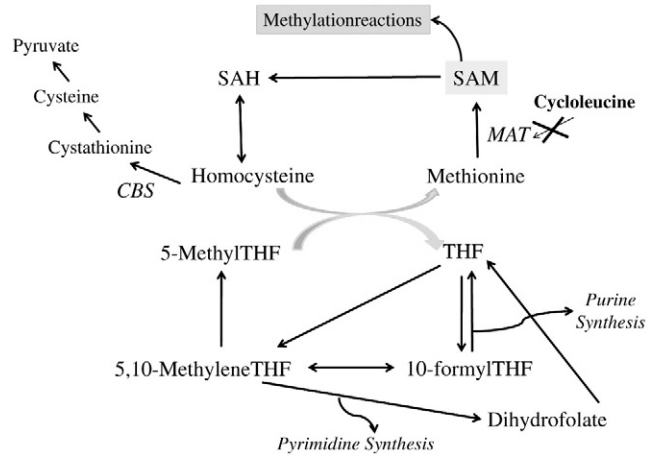

Fig. 1. A simplified view of cytoplasmic folate metabolism. SAH=*S*-adenosylhomocysteine, THF=tetrahydrofolate. Not all enzymes are included for clarity. SAM is the methyl donor for methylation reactions, including DNA methylation, and is enzymatically converted from L-methionine by MAT. Cycloleucine inhibits MAT activity resulting in lowered SAM levels. CBS is involved in the transsulfuration pathway by converting homocysteine to cystathionine. The folate metabolites 5-10-methylene-THF and 10-formyl-THF supply the 1-Cs for pyrimidine and purine synthesis, respectively.

inhibitor methotrexate (Mtx) to subtypes of acute lymphoblastic leukemia (ALL) employing Affymetrix HG U133A GeneChips.

A full assessment of the global gene expression response of cells to folate status requires an analysis of the entire human transcriptome. Although Kager et al. [15] and Bliek et al. [14] carried out such an analysis, these studies focused on folate responses to Mtx in ALL patients and orofacial cleft lymphoblasts, respectively. We sought to assess the entire human transcriptome by employing Affymetrix HG U133 plus 2.0 arrays in normal human lymphoblast cell lines that have been exposed to an established folate enzyme inhibitor, i.e., cycloleucine. We identified a number of potential genes and pathways that respond to inhibition of folate metabolism and compared these to other studies. Of the genes that we identified, we demonstrate that four genes were consistent in their response, and these included cystathionine-beta-synthase (CBS); prostaglandin D2 synthase, hematopoietic (PGDS); chemokine (C-X-C motif) receptor 3 (CXCR3) and G-protein-coupled receptor 98 (GPR98). However, we found no evidence that gene-specific DNA methylation changes played a significant role in mediating some of the gene expression changes that we observed.

2. Materials and methods

2.1. Cell culture: cycloleucine inhibition

2.1.1. Microarray cell culture experiment

Coriell lymphoblast cell line 17158 (Coriell Institute for Medical Research, Camden, NJ, USA) was cultured as recommended in RPMI 1640 with 10% fetal bovine serum and 1% penicillin–streptomycin (10,000 U:10 mg/ml) at 37°C with 5% CO_2. The cells were confirmed as free from mycoplasma contamination by routine testing using a polymerase chain reaction (PCR)-based assay. A series of preliminary experiments were carried out where the concentration and length of cellular exposure to cycloleucine (Sigma-Aldrich, UK) were assessed. Concentrations ranging from 0 to 20 mM over a 72-h time course were examined for an impact on cell proliferation rate. Cells were counted using the Cell Proliferation Kit I (MTT) (Roche, UK). A concentration of 10 mM cycloleucine over a 24-h period was found to have no major impact on the cell proliferation rate of the cells compared to controls (data not shown). Cells for the microarray experiment were treated with 10 mM cycloleucine [dissolved in phosphate-buffered saline (PBS)] for 24 h. Control cells were treated with just PBS using the same volume. Control and experimental cells were carried out in replicates of five, giving a total of 10 T_{25} flasks.

2.1.2. Confirmatory cell culture experiment

The 17158 cell line was cultured under similar conditions as described for the 'Microarray Cell Culture Experiment' except that experimental cells were cultured over a series of cycloleucine concentrations over an extended time course, i.e., 0, 5, 7.5 and 10 mM of cycloleucine for 24, 48 and 72 h. Each concentration and time point were carried out in replicates of six: one triplicate set for RNA extraction and the second triplicate set for DNA extraction.

2.2. Gene expression analysis using Affymetrix HG U133 plus 2.0 GeneChips

RNA was extracted using Qiagen RNeasy kit (Cat. No. 74104, UK) and Qiagen Qiashredders (Cat. No. 79654, UK). DNase 1 treatment to remove potential contaminating genomic DNA was carried out by 'on-column' treatment as described in the Qiagen manual. RNA quality was verified by measurement of A_{260}/A_{280nm} ratios using a Thermo Scientific NanoDrop 1000 and by resolution on a 1% agarose gel. All further processing of RNA was carried out by Almac Diagnostics Ltd. (Northern Ireland) where further quality control checks were carried out prior to processing. The gene expression profiles of all 10 samples were generated following hybridization to individual Affymetrix HG U133 plus 2.0 GeneChips. A hierarchical clustering and principal component analysis of the 10 individual profiles indicated that four of the samples (two controls and two treated) displayed an atypical or variable expression profile in the control or cycloleucine groups. These samples were excluded from further analysis. The signals for each gene across the remaining replicate GeneChips were merged to give a summarized and normalized intensity value for the control and treated groups. To select genes that were changing significantly between the control and cycloleucine-treated cells, the data set was subjected to a number of filtering steps based on intensity values, background fluorescence, signature *P* value, fold change and statistical significance (Student's *t* test). The intensity filter removes genes where no transcript is likely to be present in the sample based on the fluorescence detected. The lower the *P* value, the more confidence that the transcript is present (stringent *P* value=.001; less stringent *P* value=.05). The background filter removes genes that are affected by background fluorescence fluctuations (stringent threshold=3×average standard deviation of background noise over all arrays; less stringent threshold=2×-average standard deviation of background noise over all arrays). The fold change filter removes genes based on the fold change between experimental and control conditions. The coefficient of variation (CV) of the background noise level is used to estimate a suitable fold change estimate (stringent=3×CV with a floor of 1.5; less stringent=2×CV with a floor of 1.3). The signature filter was based on the gene intensity ratio between the two conditions(stringent *P* value=.001; less stringent *P* value=.05). The final filter was based on the statistical significance of differential gene expression based on a *t* test with a *P* value of .001. Gene lists were divided into three groups based on stringency of statistical significance, i.e., 'stringent' and 'less stringent' and an 'on/off' list. The final stringent list consists of those genes that passed background intensity, fold change and signature filters with the stringent criteria and the differential expression *t* test. The less stringent list consisted of those genes that passed the intensity, background, fold change and signature filters applying the less stringent criteria as described above. The 'on/off' list consists of those genes where there is confidence that the gene is switched on in one condition and switched off in the other. The 'on/off' list was based on the intensity *P* values were 'on'≤.001 and 'off'≥.001, and genes were further filtered based on the confidence associated with the differential expression, i.e., the signature *P* value with a threshold of .001. The processing and analysis of the microarray data are compliant with the Minimum Information About A Microarray Experiment standard as described in Brazma et al. [16]. Pathway analysis was performed by Almac Diagnostics Ltd. using MetaCore software.

2.3. Reverse transcription-quantitative PCR (RT-qPCR)

RNA was extracted from each culture flask and was subjected to DNase I treatment either by 'on-column' treatment with DNase I (Qiagen) as described for the microarray analysis or by treatment subsequent to elution using DNase I as recommended by the manufacturer (Invitrogen). Elimination of potential genomic DNA contamination was verified using a robust PCR assay flanking an intron following cDNA synthesis. RNA was synthesized to cDNA using Superscript II (Invitrogen, UK) and a combination of oligo(dT) and random hexamers according to the manufacturer's instructions. All RT-qPCR assays were designed using Probefinder software and the Universal Probe Library system (Roche, UK), were intron spanning and were performed using Probes Master Mix and the Lightcyler 480 instrument (Roche, UK) as recommended by the manufacturer. All primers and probes utilized for each assay are available on request. The PCR efficiencies for each assay were assessed using the pooled sample of the experimental cDNAs. A panel of reference genes (endogenous control) was tested, and 40S ribosomal protein S13 (RPS13) was identified as the most appropriate for this analysis, i.e., did not show a response to cycloleucine treatment. The assays were carried out in duplicate incorporating 'minus Superscript' and PCR negative controls. Relative expression was calculated using the Relative Quantification software employing the E-Method. All expression ratios are relative to 0 mM cycloleucine, and each cDNA sample was performed in duplicate with a standard deviation between replicates of <0.3 Cp.

2.4. Gene-specific DNA methylation analysis by methylation-sensitive–high-resolution melting analysis (MS-HRM)

DNA was extracted from the 'Confirmatory Cell Culture Experiment' using the DNeasy Blood and Tissue kit (Qiagen, UK). DNA was eluted in 100 μl sterile H_2O and treated with 5 μl RNase cocktail (5 U/μl, Ambion) at room temperature for 20 min. DNA was visualized following electrophoresis on a 1% agarose gel and quantified on a Thermo Scientific NanoDrop 1000. MS-HRM assays were designed and performed as recommended by Wojdac et al. [17] for the following genes: adenosine deaminase, RNA-specific, B1 (ADARB1); endothelin receptor type B (EDNRB); CBS; forkhead box P4 (FOXP4) and runt-related transcription factor 1 (acute myeloid leukemia aml1 oncogene) (RUNX1). Specific regulatory regions of each gene were chosen for analysis based on the likelihood that they were subject to DNA methylation modifications. This included the presence of a CpG island and/or reported as methylated in the literature. The size of PCR product for each gene is as follows: ADARB1, 181 bp; EDNRB, 148 bp; CBS, 123 bp; FOXP4, 133 bp; RUNX1, 167 bp. The region and primer sequences are available in Supplementary Information (Table S1). MS-HRM analysis employs bisulfite treatment of both unknown and standard curve DNA samples with melting curve analysis. In our hands, the Qiagen Epitect Bisulfite kit outperformed the EZ DNA methylation kit (Zymo research) in terms of ease of use, conversion efficiency and quality of methylation profile. Methylated and unmethylated control DNAs (Millipore) were bisulfite treated, and a range of methylation concentrations was used for the standard curve. Routinely, 100%, 50%, 25%, 5% and 0% methylated samples were adequate to obtain a suitable standard curve. All assays were performed in duplicate on the Roche Lightcycler 480 instrument using the Lightcycler HRM Master Mix (Roche, UK) using previously optimized conditions for each gene assay. A standard curve was included on every plate.

3. Results

3.1. Microarray analysis identifies novel responders to folate metabolism inhibition

3.1.1. Gene lists

Comparison of the microarray profiles of control versus cycloleucine [inhibits methionine adenosyltransferase (MAT)]-treated cells

Table 1
Differentially expressed genes identified from microarray analysis of cycloleucine-treated cells compared to controls

Gene symbol	Gene name	Affy probe IDs	Microarray fold change
		Stringent gene list	
CXCR3	Chemokine (C-X-C motif) receptor 3	207681_at/217119_s_at	−3.28/−2.23
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	204748_at	−2.81
GAS7	Growth arrest-specific 7	211067_s_at	−2.72
SLC19A1	Solute carrier family 19 (folate transporter), member 1	1555952_at	−2.59
KLB	Klotho beta	244276_at	−2.22
EGR1	Early growth response 1	201694_s_at	−2.14
DIAPH3	Diaphanous homolog 3 (*Drosophila*)	232596_at	−2.05
SNX5	Sorting nexin 5	223666_at	−1.93
GM2A	GM2 ganglioside activator	215891_s_at	−1.92
LOC645676	Hypothetical LOC645676	1554057_at	−1.87
IL7R	Interleukin 7 receptor	226218_at	−1.81
RGS2	Regulator of G-protein signaling 2, 24 kDa	202388_at	−1.81
SAMHD1	SAM domain and HD domain 1	204502_at	−1.78
HIST1H2AJ	Histone cluster 1, H2aj	208583_x_at	−1.77
AA77752	CDNA: FLJ22539 fis, clone HRC13227	227491_at	−1.75
AI220427	Transcribed locus	230696_at	−1.70
SPC24	SPC24, NDC80 kinetochore complex component, homolog (*S. cerevisiae*)	235572_at	−1.69
PPIL5	Peptidylprolyl isomerase (cyclophilin)-like 5	242154_x_at	−1.66
LRRC41	Leucine rich repeat containing 41	215765_at	−1.62
HAAO	3-Hydroxyanthranilate 3,4-dioxygenase	205657_at	−1.62
AA868461	Transcribed locus	229549_at	−1.61
HPDL	4-Hydroxyphenylpyruvate dioxygenase-like	229332_at	−1.59
RGS1	Regulator of G-protein signaling 1	216834_at	1.67
AI916498	–	230589_at	1.70
TRIM73	Tripartite motif-containing 73	1554250_s_at	1.75
AW341473	Prostate-specific P775P mRNA sequence	243241_at	1.76
PSEN2	Presenilin 2 (Alzheimer disease 4)	211373_s_at	1.76
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	201925_s_at	1.80
CASZ1	Castor zinc finger 1	243386_at	1.82
EDNRB	Endothelin receptor type B	204271_s_at	1.88
ADCY1	Adenylate cyclase 1 (brain)	235049_at	1.93
TGOLN2	Trans-golgi network protein 2	1554608_at	1.97
BTNL9	Butyrophilin-like 9	230992_at	2.21
LRP11	Low-density lipoprotein receptor-related protein 11	225060_at	2.25
C21orf15	Chromosome 21 open reading frame 15	1558589_at	3.06
GPR98	G-protein-coupled receptor 98	224275_at	3.08
		On/off gene list	
PGDS	Prostaglandin D2 synthase, hematopoietic	206726_at	−7.23
AI823546	Transcribed locus	231150_at	−6.55
C1orf161	Chromosome 1 open reading frame 161	1553333_at	−4.01
AI653327	Transcribed locus	237764_at	−3.76
ADARB1	Adenosine deaminase, RNA-specific, B1 (RED1 homolog rat)	234799_at	−3.53
ZNF93	Zinc finger protein 93	1569240_at	−3.29
BC036426	CDNA clone IMAGE:4799031	1570160_at	2.75
LOC645513	Similar to septin 7	239556_at	3.33
CABYR	Calcium binding tyrosine-(Y)-phosphorylation regulated	219928_s_at	3.35
ECHDC3	Enoyl coenzyme A hydratase domain containing 3	219298_at	3.41
AL832806	–	1558385_at	3.45
DFNB31	Deafness, autosomal recessive 31	47553_at	3.78
LOC646522	Hypothetical LOC646522	237662_at	3.89
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	206580_s_at	4.27
LOC157503	Hypothetical protein LOC157503	230692_at	4.86
SLC35D2	Solute carrier family 35, member D2	233325_at	9.92

Table 2
Pathway analysis of microarray data

Map	Cell process	*P* value
Immune response_NF-AT signaling and leukocyte interactions	Cytokine and chemokine mediated signaling pathway, immune response	.0076
Regulation of lipid metabolism_regulation of lipid metabolism via LXR, NF-Y and SREBP	Transcription	.0181
Development_hedgehog and PTH signaling pathways participation in bone and cartilage development	Response to extracellular stimulus	.0241
Development_EDNRB signaling	G-protein-coupled receptor protein signaling pathway, response to hormone stimulus, response to extracellular stimulus	.0335

identified a list of differently expressed genes. The 'stringent' gene list consisted of genes that passed a number of filters with a final statistical significance of <.001. This list consists of 36 genes which varied in fold difference ranging from −3.28 to 3.08 (Table 1). The cutoff fold difference was set at 1.5-fold. The 'on/off' gene list is based on intensity of the gene-specific signal in the experimental and control culture conditions. The 'on/off' list consists of 16 genes (Table 1). However, apart from PGDS and EFEMP2, the remaining genes on the on/off list had a relatively low-intensity signal in both control and treated samples (data not shown) and, therefore, require independent validation.

3.1.2. Pathway analysis

Interpretation of microarray data is often enhanced by carrying out pathway analysis, i.e., the identification of differentially expressed genes that are common to a specific pathway. This often points to the key pathway that may be of importance for a particular cellular response. However, in the case of our microarray analysis; the stringent gene list consisted of just 36 genes. This is an insufficient gene number to carry out such an analysis. To overcome this problem, we relaxed the *P* value cutoff for the stringent list from .001 to .01. This resulted in 246 genes available for pathway analysis but with an increased probability of inclusion of a higher number of false positives. The pathway analysis identified four biological pathways that were statistically significant (Table 2).

3.1.3. Validation

We selected 11 genes for further validation in order to test the robustness of our microarray data. Genes were selected based on their fold differential expression value in order to get a representation of both up-regulated and down-regulated genes across the range of the stringent, less stringent gene lists or the 'on/off' list. All genes were assessed by RT-qPCR in the same RNA stock that was utilized in the microarray analysis. The RT-qPCR analysis validated 7 out of the 11 genes (Table 3). The four genes that did not validate included two genes where the RT-qPCR assay failed (EFEMP2, CAV1). The other two genes (TFEC, NFATC1) simply did not validate. The microarray fold difference for TFEC was 1.61, with a similar fold difference for NFATC1 of −1.42. However, LCK with a fold difference of −1.41 did validate. This suggests that within this data set, fold differences of −1.4 to 1.6 are not consistently reliable and need to be validated independently.

3.2. Novel responders to cycloleucine treatment focus on 13 reliable genes

Our microarray analysis has identified a range of genes that are responding to inhibition of MAT (Fig. 1). Our validation analysis described above indicated that the most reliable gene expression changes are those that displayed a fold difference of at least 2.0 and were derived from the stringent gene list. In addition to these genes from the stringent list, RT-qPCR-validated genes from the less stringent or on/off lists also form part of our reliable gene list (Table 4). Reliable down-regulated genes include CXCR3, prostaglandin-endoperoxide synthase 2 (PTGS2), growth arrest-specific 7 (GAS7), solute carrier family 19 (or reduced folate carrier) (SLC19A1), klotho beta (KLB), early growth response 1 (EGR1), diaphanous homolog 3 (DIAPH3), PGDS and CBS. Reliable up-regulated genes include butyrophilin-like 9 (BTNL9), low-density lipoprotein receptor-related protein 11 (LRP11), chromosome 21 open reading frame 15 (C21orf15) and GPR98.

3.3. Confirmation of CXCR3, GPR98, PGDS and CBS as responders to folate metabolism inhibition in a cycloleucine dose response experiment

The difficulty with analyzing gene expression patterns is to identify those changes that consistently respond to a particular treatment. Microarray analyses tend to only capture those gene expression changes that occur within a single experiment. We examined the gene expression pattern of the seven genes from the microarray experiment that were validated by RT-qPCR in a confirmatory cell culture experiment. This second cell culture experiment involved the same cell line used for the microarray experiment, but this time, cells were exposed to a range of cycloleucine concentrations (0, 5, 7.5 and 10 mM) for 24 h (the

Table 3
Microarray validation analysis by RT-qPCR of selected genes from the microarray gene lists

Gene	Gene name	Gene list	RT-qPCR[a]
CXCR3	Chemokine (C-X-C motif) receptor 3	Stringent/−3.28, −2.23	**−5.02**
PGDS	Prostaglandin D2 synthase, hematopoietic	On–off/−7.23	**−3.76**
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	On–off/4.27	Failed
GPR98	G-protein-coupled receptor 98	Stringent/3.08	**2.05**
CBS	Cystathionine-beta-synthase	Less stringent/1.80	**1.98**
TFEC	Transcription factor EC	Less stringent/1.61	−1.4
EDN1	Endothelin 1	Less stringent/−2.36	**−2.3**
EDNRB	Endothelin receptor type B	Stringent/2.37	**2.23**
CAV1	Caveolin 1, caveolae protein, 22 kDa	Less stringent/1.69	Failed
LCK	Lymphocyte-specific protein tyrosine kinase	Less stringent/−1.43	**−1.41**
NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	Less stringent/-1.42	-1.10

[a] Fold changes of validated genes are shown in bold.

Table 4
Reliable genes that respond to cycloleucine treatment

Gene	Gene name	Fold change[a]
CXCR3	Chemokine receptor 3	−3.28/−2.23
PTGS2	Prostaglandin-endoperoxide synthase 2	−2.81
GAS7	Growth arrest-specific 7	−2.72
SLC19A1	Solute carrier family 19 or Reduced Folate Carrier	−2.59
KLB	Klotho beta	−2.22
EGR1	Early growth response 1	−2.14
DIAPH3	Diaphanous homolog 3	−2.05
PGDS	Prostaglandin D2 synthase, hematopoietic	−3.76*
BTNL9	Butyrophilin-like 9	2.21
LRP11	Low-density lipoprotein receptor-related protein 11	2.25
C12orf15	Chromosome 21 open reading frame 15	3.06
GPR98	G-protein-coupled receptor 98	3.08
CBS	Cystathionine-beta-synthase	1.98*

[a] Microarray fold changes are given for all genes except those denoted by a * which refers to the validated RT-qPCR value.

microarray experiment involved 10 mM cycloleucine for 24 h). The results of this analysis showed that just four out of the seven genes showed a similar expression change to the microarray analysis (Table 5).

3.4. Gene-specific DNA methylation analysis of selected differentially expressed genes: ADARB1, CBS, EDNRB, RUNX1 and FOXP4

The treatment of cells with the MAT inhibitor cycloleucine will result in a reduction in the methyl group donor SAM. Similarly, SAM levels are also likely to drop in the presence of low folate. SAM provides the methyl group required for a variety of methylation reactions within the cell including DNA methylation. DNA methylation is just one of a plethora of mechanisms that are used to control gene expression, and an impact on DNA methylation is thought to be one of the mechanisms by which folate status influences health and disease. In terms of gene-specific expression, DNA methylation of specific CpGs in the promoter region of some genes is thought to have an influence in switching genes on or off. However, deciphering which CpGs are relevant and the percentage methylation required to influence this process is far from clear. Moreover, not all genes are subject to methylation changes in their regulatory regions. We examined the DNA methylation patterns of specific genes that showed changes in gene expression from the cycloleucine microarray experiment described here or an additional folate/riboflavin deficiency microarray experiment (data not shown) *and* had a recognizable CpG island. This included ADARB1, EDNRB, CBS, FOXP4 and RUNX1. All genomic regions were examined by MS-HRM in control (0 mM) and 10 mM cycloleucine for 24, 48 and 72 h as described in the 'Confirmatory Cell Culture Experiment.' ADARB1, EDNRB, CBS and FOXP4 all displayed 0% DNA methylation under both control and cycloleucine treatment conditions. RUNX1 did exhibit DNA methylation in both control and cycloleucine-treated samples ranging from 0% to 25%. The percentage methylation was similar in control and treated samples at 24 and 72 h but exhibited 25% in control versus 0% in treated samples at 48 h. However, these data did not correlate with changes in gene expression observed in the microarray analysis.

Table 5
RT-qPCR fold differences of seven validated genes in confirmatory cycloleucine experiment

Gene	Confirmatory cell culture[a]			Microarray[a,b]
	5 mM	7.5 mM	10 mM	10 mM
CBS	**1.99**	**7.99**	**4.08**	**1.98**
CXCR3	**−1.61**	**−1.49**	**−3.6**	**−5.02**
EDN1	1.61	1.23	1.4	−2.3
EDNRB	−12.5	−14.3	−14.3	2.23
GPR98	**1.91**	**2.35**	**2.13**	**2.05**
PGDS	**−1.6**	**−2.2**	**−1.58**	**−3.76**
LCK	−1.17	−1.41	1.14	−1.41

[a] All fold differences are normalized to RPS13 endogenous control and relative to 0 mM cycloleucine.
[b] The values in this column are based on RT-qPCR values on RNA samples shared with the microarray analysis.

4. Discussion

We exposed cells to the MAT inhibitor cycloleucine [18] and analyzed the response at the transcriptional level by microarray analysis. MAT catalyzes the production of SAM and occurs in three forms in mammals [19]. MAT II is the nonhepatic form and is ubiquitously expressed. Inhibition of MAT following exposure to cycloleucine results in disruption of methionine metabolism and a reduction in SAM levels (Fig. 1). We identified a list of genes and potential pathways (Tables 1 and 2) that responded to methionine metabolism disruption in our microarray experiment. The challenge with all microarray experiments is to identify those genes that have biological relevance. We pinpoint 13 genes that we consider reliable (Table 4) and observed consistent responses with four genes in additional cell culture experiments (Table 5).

The four genes that consistently responded to cycloleucine in additional cell culture experiments include two G-protein-coupled receptors, *CXCR3* (down-regulated) and *GPR98* (up-regulated). CXCR3 binds three chemokine ligands: CXCL10 (IP-10), CXCL9 (MIG) and CXCL11 (ITAC). Chemokine receptors and their ligands have traditionally been associated with regulation of the immune response and in autoimmune diseases but more recently appear to have a more diverse role. Chemokines are now known to play an equally important role in development and physiology of the nervous system [20]. CXCR3 specifically has been implicated in cancer and in atherosclerosis [21,22]. Interestingly, CXCL9 (a ligand for CXCR3) was identified as differentially expressed in the mouse *Mthfd1* deficiency model mentioned above [13]. GPR98, also known as very large G-protein-coupled receptor 1, is a calcium binder. It is important for normal development of the central nervous system. Mutations within GPR98 have been associated with Usher's syndrome type II and familial febrile seizures. However, the exact ligand that binds GPR98 remains to be identified [23–25]. The third of our four consistent genes is PGDS, which is an essential enzyme for producing prostanoids in the immune system and mast cells [26]. Mice null for the murine equivalent have a more severe inflammatory response [26]. Our fourth consistent responder is, not surprisingly, CBS, showing up-regulation to cycloleucine treatment in both the microarray experiment and in the confirmatory cell culture experiment. Prudova et al. [27] previously described how CBS protein levels were regulated by SAM through stabilization of the enzyme. CBS is thought to regulate cysteine synthesis through the transsulfuration pathway by protein instability when methionine is restricted, i.e., through lack of SAM binding. When methionine is plentiful, CBS protein levels are up-regulated due to a higher level of SAM. This study observed no impact on CBS mRNA levels in response to cycloleucine treatment with subsequent transfer from a methionine restricted to a methionine-supplemented media, although the less sensitive method of Northern blotting was used. Our treatment of cells with cycloleucine would restrict SAM levels (see Fig. 1), but the methionine supply would not be compromised. Our observation of consistent up-regulation of CBS mRNA in the presence of cycloleucine, i.e., low SAM levels (in the presence of methionine), is in contrast to that observed at the protein level by Prudova et al. [27]. This indicates that SAM may also up-regulate CBS at the transcriptional level when methionine is plentiful. However, the molecular mechanism for this requires further investigation.

We also assessed whether reduction in the levels of the methyl-donor SAM would result in changes in gene expression due to changes in gene-specific DNA methylation patterns. We analyzed the promoters of five genes (ADARB1, EDNRB, CBS, FOXP4 and RUNX1) that demonstrated gene expression changes in response to the cycloleucine microarray experiment or in response to reduced levels of folate or riboflavin (data not shown). We saw no evidence that the gene expression changes observed for these five genes were being mediated by DNA methylation. However, we do acknowledge that we only examined one section of the promoters of these genes and additional regions of the promoters should be examined before DNA methylation changes can be definitively ruled out.

We compared our microarray data to previous studies that have also investigated folate-responsive gene expression patterns. We noted that SLC19A1 (or reduced folate carrier) (Table 4) also showed down-regulation in response to folate deficiency in the study by Courtemanche et al. [7]. SLC19A1 was also differentially expressed in different subtypes of ALL in response to Mtx [15]. Although another folate receptor, folate receptor α, is known to up-regulate in response to folate deficiency [9] and in cancer cells [28], we did not observe a significant response to cycloleucine in our microarray experiment. We also noted that genes encoding interleukin receptors and chemokine ligands/receptors were not only a feature of our data but were also differentially expressed in a folate deficiency rat [11] and human fibroblast study [8] plus a mouse *Mthfd1* deficiency model [13]. Moreover, pathway analysis of our microarray data identified an immune response pathway as significant (Table 2). Therefore, it appears that changes in expression of components of the immune response are a feature of folate metabolism disruption and indicate a possible alternative role for these gene products.

In summary, the genes that we identified from our microarray analysis and subsequent validation experiments are likely to be important for mediating the cellular response to a block in methionine metabolism. Our four confirmed responders to cycloleucine treatment with subsequent lowered SAM levels include two enzymes (CBS and PGDS) and two G-protein-coupled receptors (CXCR3 and GPR98). Low B vitamin status, particularly low folate status, also affects methionine metabolism, and therefore, these results will have relevance for understanding the complex relationship between low folate status and increased risk of a number of birth defects and adult diseases. The consistent finding between our study and others is that elements of immune cells/response appear to be particularly relevant for the cellular response to folate status.

Supplementary materials related to this article can be found online at doi:10.1016/j.jnutbio.2011.10.006.

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