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Time course gene expression in the one-carbon metabolism network using HepG2 cell line grown in folate-deficient medium

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Abstract

The integrated view of the expression of genes involved in folate-dependent one-carbon metabolism (FOCM) under folate deficiency remains unknown. Dynamics of changes in the transcriptional expression of 28 genes involved in the FOCM network were evaluated at different time points (0, 2, 4, 6, 12, 24 and 48 h) in human hepatoma HepG2 cell line. Combined experimental and computational approaches were conducted for emphasizing characteristic patterns in the gene expression changes produced by cellular folate deficiency.

Bivariate analysis showed that folate deficiency (0.3 nmol/L of folate vs. 2.27 μmol/L in control medium) displayed rapid and coordinated regulation during the first 2 h with *differential* expression for *hRfc1* (increased by 69%) and *Ahcy* (decreased by 437%). Density analysis through the time points gave evidence of differential expression for five genes (*Ahcy, Cth, Gnmt, Mat1A, Mtrr* and *hRfc1*). Differential expression of *Ahcy, Gnmt, Mat1A and Mtrr* was confirmed by time-series analysis gene expression. We also found a marked differential expression of *Mtrr*. Qualitative analysis of genes allowed identifying four clusters of gene that was coexpressed. Two of these clusters were consistent with specific metabolic functions as they associated genes involved in the remethylation (*Mthfr* and *Mtrr*) and in the transmethylation (*Dnmt*1and *Dnmt3B*) pathways.

The study shows a strong influence of folate status on *Mtrr* transcription in HepG2 cells. It suggests also that folate deficiency produces transcription changes that particularly involve the clusters of genes related with the remethylation and the transmethylation pathways.

Keywords: Folate; Gene expression; One-carbon metabolism; Nutrigenomics

Abbreviations: AHCY, (*S*)-adenosylhomocysteine hydrolase; AdoMet, adenosylmethionine; AdoHcy, adenosylhomocysteine; BHMT, betaine homocysteine methyltransferase; CBS, cystathionine betasynthase; COB, cobalamin-dependent enzyme; CTH, cystathionine gamma-lyase; DHFR, dihydrofolate reductase; DNMT, DNA-(5-cytosine)-methyltransferases; FOL, folate cycle pathway; FR, folate receptor; FTR, folate transport protein; FTCD, formiminotransferase cyclodeaminase; GAMT, guanidinoacetate methyltransferase; GART, phosporibosylglycinamide formyltransferase; phosphoribosylglycinamide synthase, phosphoribosylaminoimidazole synthase; GLCL, gammaglutamylcysteine synthase; GNMT, glycine *N*-methyltransferase; GSS, glutathion synthase; MAT, adenosylmethionine transferase genes; MS, methionine synthase or methyltetrahydrofolatehomocysteine methyltransferase (MTR); MSR, methionine synthase reductase or methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR); MTHFD, methylenetetrahydrofolate dehydrogenase, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTHFS, 5,10-methylenetetrahydrofolate synthase 5-formyltetrahydrofolate cycloligase; PEMT, phosphatidylethanolamine *N*-methyltransferase; hRFC, human reduced folate; SAA, sulfur-containing amino acids; SHMT, serine hydroxymethyltransferase; TMT, *trans*-methylation reaction; TS, tymidylate synthase.

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1. Introduction

Folate-mediated one-carbon metabolism (FOCM) is a network of interrelated biochemical reactions that involve successive transfer of one-carbon groups to a wide variety of biochemical molecules such as DNA, proteins and lipids. It also plays a central role in the remethylation of homocysteine (Hcy) and the synthesis of sulfur-containing amino acid (methionine, cysteine, glutathione). Folate deficiency is also associated with apoptosis [1] and cancer such as uterin cervix [2,3], lung and breast [4–6], and the colorectal one [7–10]. In addition, enzymes in the FOCM pathways such as dihydrofolate reductase (DHFR) and thymidilate synthase (TS) are targets of anti-cancer drugs [11–13]. Moreover, DNA-(5-methylcytosine)-methyltransferase (DNMT) and folate receptor (FR) are candidates as targets for chemotherapy [14,15]. Understanding of the FOCM network is thus a significant research topic in fundamental biology, oncology, aging and public health. Nevertheless, the integrated view of the FOCM gene regulation under folate deficiency remains unknown.

The description of how subcellular processes are coordinately regulated and how genes are coexpressed in response to folate deficiency may open new perspectives in association studies in nutrigenomics and nutrigenetics related with folate metabolism and liver diseases. Indeed, experimental and population studies have shown that nutritional deficiency in 5-methyltetrahydrofolate, the methyl donor for Hcy remethylation, influences the pathogenesis of alcohol-related liver diseases [16]. In addition, hepatic steatosis may be associated with genetic disorders of Hcy metabolism [17]. This has been reported in severe hyperhomocysteinemia due to deficiency for the cystathionine β-synthase (CBS) gene or to a genetic disruption of the remethylation and transmethylation pathways [18–20].

In this study, we focus on genes involved in FOCM (Fig. 1). We have investigated the transcription patterns of genes involved in FOCM using the human hepatocarcinoma HepG2 as cellular model. Those patterns have been analyzed using computational approaches that might lead to new biological insights of FOCM in folate deficiency condition. Our study provides data that may serve in the evaluation of gene–nutrient and gene–gene interactions involved in the association between one-carbon metabolism and liver diseases.

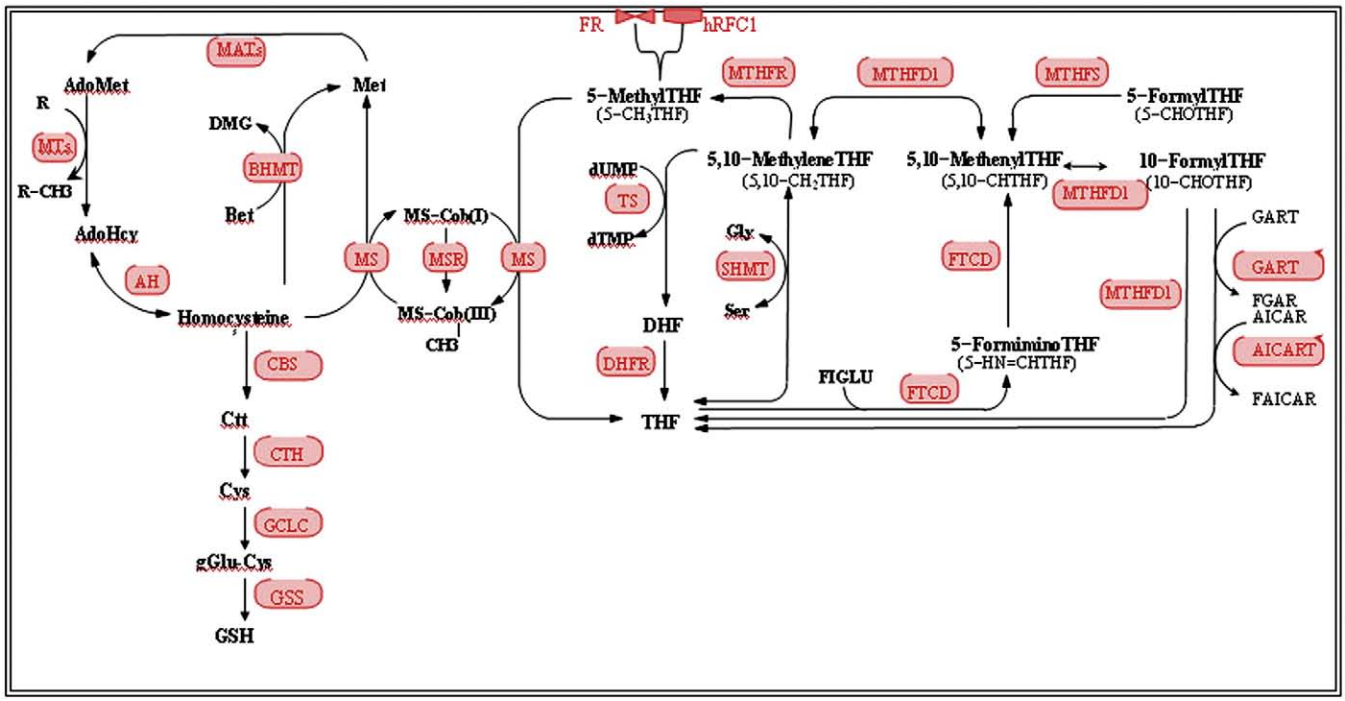

Fig. 1. Folate-derived one-carbon metabolism network. AH: (*S*)-adenosylhomocysteine hydrolase; Ado: adenosine; AICAR(T): 5-phosphoribosyl-5-aminoimidazole-4-carboxamide (transformylase); BHMT: betaine homocysteine methyltransferase; CBS: cystathionine beta-synthase; CTH: cystathionine gamma-lyase; DHFR: dihydrofolate reductase; FR: folate receptor; FTCD: formiminotransferase cyclodeaminase; GART: phosporibosylglycinamide formyltransferase, phosphoribosylglycinamide synthase, phosphoribosylaminoimidazole synthase; GLCLC: gamma-glutamylcysteine synthase; GSS: glutathion synthase; MATs: adenosylmethionine transferase genes that include MATA1, MAT2A and MAT2B; MS: methionine synthase or methyltetrahydrofolate-homocysteine methyltransferase; MSR: methionine synthase reductase; MTs: methyltrasferase [including DNMTs: DNA-(5-cytosine)-methyltransferases; GAMT: guanidinoacetate methyltransferase; FIGLU: formiminoglutamate; GNMT: glycine *N*-methyltransferase; PEMT: phosphatidylethanolamine *N*-methyltransferase]; MTHFD: methylenetetrahydrofolate dehydrogenase NADP+ dependent, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase; MTHFR: 5,10-methylenetetrahydrofolate reductase; MTHFS: 5,10-methylenetetrahydrofolate synthase 5-formyltetrahydrofolate cycloligase; hRFC: human reduced folate carrier (solute carrier family 19, member 1, SLC19A1); SHMT: serine hydroxymethyltransferase; TS: tymidylate synthase.

2. Material and method

2.1. In vitro studies

2.1.1. Materials

Minimum Essential Medium Eagle (MEM), either lacking or containing folic acid (2.27 μm), and phosphate-buffered saline (PBS) were purchased from Invitrogen (Invitrogen, Paisley, Scotland, UK). Dialyzed fetal bovine serum (14-801F) and trypsin were from Cambrex (Cambrex Bio Science, Verviers, Belgium). HepG2 cell line (human hepatocellular carcinoma cells) was ordered from ECACC (No. 85011430).

2.1.2. Cell culture and treatments

HepG2 cells were maintained as monolayer culture in complete MEM (folate-repleted medium), supplemented with 20% fetal bovine serum and maintained at 37°C in a humidified 5% CO_2 incubator. The medium was changed every 2 days. For the experiment, 60% to 80% confluent HepG2 cells grown in complete MEM were first trypsinized and then replated at a concentration of 250,000 cells/25-cm^2 culture flasks after cell counting (0.5 ml of cell suspension in 10 ml of Z-Pak Isotron II dilient, Beckman Coulter, Villepinte, France) according to the manufacturer's instruction. Twenty-four hours later and after growth medium removal, adhering cells were gently washed in PBS. One flask was immediately used for total RNA extraction and referred to as T0 or initial time point. In all other flasks, cells were grown in either control experimental medium, i.e., complete MEM (CEM), or in a folic acid-depleted media (FDM), both depleted of antibiotics. Exogenous folate source was minimized using dialyzed fetal bovine serum at 4% in both media. Finally, folate concentration was 0.3 nmol/L in FDM and 2.27 μmol/L in CEM. Cells were harvested after 2 h (T2), 4 h (T4), 6 h (T6), 12 h (T12), 24 h (T24) or 48 h (T48) of incubation in either medium. Experiments were run in duplicates for each time point.

2.1.3. Total RNA extraction and reverse transcription

After removing the medium, cells were lysed in each flask with Trizol reagent (Life Technologies, Faraday, CA, USA) and total RNA extracted following the manufacturer's instruction. Aliquots were transferred in RNase-free 1.5-ml microtube and stored at −80°C until processed for gene quantitation. One microgram of RNA was transcribed to cDNA using the Sensiscript RT kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instruction.

2.1.4. Assay designs and relative quantitative real-time PCR

The expression levels of mRNA for selected genes implicated in FOCM (Table 1) were measured in each sample by real-time PCR using human sequence-based TaqMan Gene Expression Assays on the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). TaqMan Gene Expression Assay IDs are listed in Table 1. All TaqMan Gene Expression Assays have been designed by the Applied Biosystems genome-aided primer and probe design pipeline (https://products.appliedbiosystems.com). According to the company, design parameters such as %GC content, melting temperature and amplicon length are optimized and designed to hybridize across exon junctions to ensure high amplification efficiency. Two replicates were run for each gene in a 96-well format plate. Probes contained a 6-carboxy-fluorescein phosphoramidite (6FAM dye) label at the 5′-end of the gene and a minor groove binder and nonfluorescent quencher at the 3′-end. Real-time PCR reactions were carried out using 100 ng of cDNA for each reaction, 12.5 μl of 2× TaqMan PCR master mix (Applied Biosystems), 1.25 μl of 2× TaqMan Gene Expression in a final volume of 25 μl. Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 40 repeats of 15 s at 95°C, and 1 min at 60°C. An endogenous control gene (β-actin, Cat No. 4326315E Applied Biosystems) and one no-template control were also run in duplicate in each plate. The use of β-actin as a control RNA was relevant in this study because we observed that its expression was not modulated by the absence of folic acid.

2.1.5. Absolute quantification of Fr and hRfc1

The folate receptor (*Fr*) and the human reduced folate carrier (*hRfc1*) gene expression measurement were performed by absolute quantitative RT-PCR on the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems) using an alternative method developed in our laboratory, based on a bacterial artificial chromosome vector. Briefly, primers and fluorogenic probes were designed with Primer Express Software version 2 (Applied Biosystems) and obtained from the same company. In order to avoid amplification of the target gene in genomic DNA, the probes spanned the junction between two exons, covered by the forward and reverse primers. Quantitative PCR was performed with 100 ng of cDNA as described above. A negative control containing distilled water was always included. Standard curves for quantification of each transcript were realized using plasmid dilutions ranging from 10^2 to 10^7 copies and allowed the determination of copy number for each transcript.

2.1.6. Gene expression calculation

Gene expression was quantified using the comparative threshold cycle method [21]. Such a method estimated the gene expression as follows: $\Delta C_t = (C_{t \text{ for target gene}} - C_{t \text{ for } \beta\text{-actin}})$, where C_t is the number of cycles it takes for a gene sample to reach the level where the rate of amplification is the greatest during the exponential phase. Gene expression levels were calculated as mean of the 'fold change' in gene expression relative to initial value at time point T0 using the $2^{-\Delta\Delta Ct}$ method [22], where $\Delta\Delta C_t = \Delta C_{t\ (TX)} - \Delta C_{t\ (T0)}$. Time TX represents any time point (X) and T0 represents the initial time point beginning the experiment for each gene. The log $(2^{-\Delta\Delta Ct})$ value of genes at T0 is 0. Genes are considered to be down-regulated for log $(2^{-\Delta\Delta Ct}) < 0$ and up-regulated for log $(2^{-\Delta\Delta Ct}) > 0$.

Table 1
List and references of selected genes of folate transport and metabolism

Protein name [a]	Gene name	Metabolic function [b]	Gene Bank Accession	Locus	AB Assay ID [c]
AHCY	*Ahcy*	SAA	NM_000687	20q11.22	Hs00426322_m1
BHMT	*Bhmt*	SAA	NM_001713	5q12.3	Hs00156084_m1
CBS	*Cbs*	SAA	NM_000071	21q22.3	Hs00163925_m1
CTH	*Cth*	SAA	NM_001902	1p31.1	Hs00266453_m1
DHFR	*Dhfr*	FOL	NM_000791	5q11.2	Hs00758822_s1
DNMT1	*Dnmt1*	TMT	NM_001379	19p13.2	Hs00945871_m1
DNMT2	*Dnmt2*	TMT	NM_004412	10p15.1	Hs00189402_m1
DNMT3A	*Dnmt3A*	TMT	NM_175629	2p23	Hs00602456_m1
DNMT3B	*Dnmt3B*	TMT	NM_175848	20q11.2	Hs00171876_m1
DNMT3L	*Dnmt3L*	TMT	NM_013369	21q22.3	Hs00203536_m1
FOLR	*Fr*	FTR	NM_000802	11q13.5	Hs01124177_m1
FTCD	*Ftcd*	FOL	NM_006657	21q22.3	Hs00198409_m1
GAMT	*Gamt*	TMT	NM_000156	19p13.3	Hs00164681_m1
GART	*Gart*	FOL	NM_000819	21q22.12	Hs00531926_m1
GCLC	*Gclc*	SAA	NM_018960	6p12	Hs00155249_m1
GNMT	*Gnmt*	TMT	NM_018960	6p12	Hs00219089_m1
GSS	*Gss*	SAA	NM_000178	20q11.2	Hs00609286_m1
MAT1A	*Mat1A*	SAA	NM_000429	10q23.1	Hs00166395_m1
MAT2A	*Mat2A*	SAA	NM_005911	2p11.2	Hs00428515_g1
MAT2B	*Mat2B*	SAA	NM_000254	5q34	Hs00165188_m1
MTHFD1	*Mthfd1*	FOL	NM_005956	14q23.3	Hs00602830_m1
MTHFR	*Mthfr*	FOL	NM_005957	1p36.22	Hs00195560_m1
MTHFS	*Mthfs*	FOL	NM_006441	15q23	Hs00197574_m1
MS	*Mtr*	COB	NM_000254	1q43	Hs00165188_m1
MSR	*Mtrr*	COB	NM_024010	5p15.2	Hs00242867_m1
PEMT	*Pemt*	TMT	NM_148172	17p11.2	Hs00540979_m1
SLC19A1	*Rfc1*	FTR	NM_003056	21q22.3	Hs00161870_m1
SHMT	*Shmt1*	FOL	NM_004169	17p11.2	Hs00244618_m1
TS	*Ts*	FOL	NM_001071	18p11.32	Hs00426591_m1

[a] AHCY: (*S*)-adenosylhomocysteine hydrolase; Ado: adenosine; BHMT: betaine homocysteine methyltransferase; CBS: cystathionine beta-synthase; CTH: cystathionine gamma-lyase; DHFR: dihydrofolate reductase; DNMT (1, 2, 3A, 3B and 3L): DNA-(5-cytosine)-methyltransferases; FR: folate receptor; FTCD: formiminotransferase cyclodeaminase; GAMT: guanidinoacetate methyltransferase; GART: phosporibosylglycinamide formyltransferas, phosphoribosylglycinamide synthase, phosphoribosylaminoimidazole synthase; GLCL: gamma-glutamylcysteine synthase; GNMT: glycine *N*-methyltransferase; GSS: glutathion synthase; MAT (1A, 2A and 2B): adenosylmethionine transferase genes; MS: methionine synthase or methyltetrahydrofolate-homocysteine methyltransferase; MSR: methionine synthase reductase; MTHFD: methylenetetrahydrofolate dehydrogenase NADP+ dependent, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase; MTHFR: 5,10-methylenetetrahydrofolate reductase; MTHFS: 5,10-methylenetetrahydrofolate synthase 5-formyltetrahydrofolate cycloligase; PEMT: phosphatidylethanolamine *N*-methyltransferase; RFC: reduced folate carrier (solute carrier family 19, member 1, SLC19A1); SHMT: serine hydroxymethyltransferase; TS: tymidylate synthase.

[b] Metabolic functions are folate transport (FTR); folate cycle pathway (FOL); cobalamin-dependent enzymes (COB); sulfur-containing amino acid synthesis (SAA); *trans*-methylation reaction (TMT).

[c] AB Assay ID: Applied Biosystems references of identification for primers and probes.

2.2. Computational approaches

2.2.1. Statistical analysis

Statistical analysis was performed with JMP software (SAS Institute, Cary, NC, USA) for testing the distribution of gene expression. Gene expression data (or fold change) are presented as mean±S.E.M. Statistical significance was estimated with Student's *t* test. A P value <.05 was considered significant. Bivariate normal distribution analyses were used for statistical evaluations of gene expression between CEM and FDM at different time points with JMP software. Pairwise correlation scores were computed (scripts written in MatLab) to compare the time series of gene expression in CEM and FDM conditions. A high correlation coefficient between two time series indicates that genes display similar expression in both media.

2.2.2. Qualitative analysis

Another MatLab script has been developed for the qualitative analysis of gene expression. Gene expressions were studied based on their variations (increase or decrease) but not on their absolute values. More precisely, we interpreted the sign of the gene expression variation in function of time. Such an approach shows genes that are qualitatively coexpressed despite folate conditions and emphasizes distinct qualitative behaviours. We focused our analysis on gene "cluster" identification.

3. Results

3.1. Quantitative analysis

From the 29 initially selected genes (Table 1), we constituted groups of genes related to metabolic function:

two gene codes for folate transport proteins (Table 1): *Fr* and *hRfc1*; nine codes for enzymes involved in folate cycle pathway: *Dhfr, Ftcd, Gart, Mthfd1, Mthfr, Mthfs, Shmt1, Ts*; two codes for cobalamin-dependent enzymes: *Ms* and *Msr*; nine codes for enzymes involved in sulfur-containing amino acid synthesis: *Ahcy, Bhmt, Mat1A, Mat2A, Mat2B, Cbs, Cth, Gclc, Gss*; and eight codes for *trans*-methylation reaction enzymes: *Dnmt1, Dnmt2, Dnmt3a, Dnmt3B, Dnmt3l, Gamt, Gnmt* and *Pemt*. From all these initially selected genes only the *Dnmt3l* expression levels have been found to be extremely low. Consequently, this gene was excluded from the study. We first analyzed the kinetics of global gene expression level as mean±S.E. of the fold change calculated by log $2^{-\Delta\Delta Ct}$ in CEM and in FDM (Table 2). Fold change of gene expressions in the FDM was significantly lower ($P<.05$) than the level in CEM at T2 (0.22±0.16 vs. 1.13±0.91), T4 (0.77±0.10 vs. 1.77±0.19), T6 (−0.04±0.18 vs. 0.68±0.13) and T48 (0.45±0.21 vs. 1.93±0.17). We did not find any significant difference between CEM and FDM at T12 and T24. There was a transient decrease in the fold change at T6 in both medium. Frequency of down-regulated genes in FDM at T2 was 28.6% (8/28) compared to 3.6% in CEM. The frequency was increased to 71.4% at T6 in FDM compared to 14.3% in CEM. No gene was down-regulated in CEM at T12 and at T24 and T48. In Fig. 2, we explored gene expression by using density ellipse curve based on bivariate normal distribution (i.e., 97% of data is inside the ellipse), attempting to discriminate candidate genes according to their sensitivity for folate deficiency. As shown in the figure, 97% of the ellipse covers genes, except *hRfc1* (gene 2) and *Ahcy* (3) at T2; *Mtrr* (22) at T4; *hRfc1* (2), *Cth* (6) and *Mtrr* (22) at T6; *Mat1A* (18) at T12; *Gnmt* (16) and *Mat1A* (18) at T24; and *Gnmt* (16) at T48. *hRfc1* and *Ahcy* have been found to be differentially expressed within the first 2 h with increased fold change by 69% for *hRfc1* and decreased fold change by 437% for *Ahcy*. For correlation coefficient of gene expression analysis, Fig. 3 illustrates the results of the comparison time-series gene expression depending on the folate deprivation. Correlation score equal to 1 indicates a perfect similarity between gene expression despite folate conditions. We considered that 97% similarity was a threshold discriminating genes owning similar expression from the others. Genes that displayed differential modification of their expression patterns were *Fr, Ahcy, Dnmt1, Dnmt3B, Gnmt, Mat1A* and *Mtrr*.

3.2. Qualitative analysis

Results in Fig. 4 show that 14 genes did not present qualitative modifications of their expressions: *Pemt, Gamt, Dnamt3A, Dnmt2, Mat2B, Mat2A, Gclc, Ts, Shmt, Mthfd1, Gart, Ftcd, Dhfr* and *Mtr*. Note here that the absence of modification of qualitative gene expression pattern did not imply any variation in gene expression such as amplification of gene expression. This analysis of gene behavior with MatLab allowed identifying four clusters of gene that followed similar qualitative expression patterns: Cluster 1 (*Mthfr* and *Mtrr*), Cluster 2 (*Bhmt* and *Mthfs*), Cluster 3 (*Gss*, *Cth* and *hRfc1*) and Cluster 4 (*Dnmt3B* and *Dnmt1*). *Mthfr* and *Mtrr* in Cluster 1 were both coexpressed and displayed down-regulation from 2 to 4 h. They respectively code for a folate cycle enzyme and a cobalamin-dependent enzyme, which are both key enzymes for Hcy remethylation. *Bhmt* and *Mthfs* in Cluster 2 displayed up-regulation from 24 to 48 h. They respectively code for an enzyme involved in the sulfur-containing amino acid and folate cycle. In Cluster 3, *Gss*, *Cth* and *hRfc1* displayed down-regulation from 24 to 48 h. The first two genes code for enzymes involved in the Hcy transulfuration pathway; *hRfc1* codes for folate transport. In Cluster 4, *Dnmt3B* and D*nmt1* were coexpressed with an increase in their expression between 12 and 24 h. Both genes code for two DNA methyltransferases of the transmethylation pathway.

4. Discussion

Many factors, such as *trans*-regulators, feedback inhibition systems, promoter 5-cytosine (CpG islands) methylation or epigenetic changes in chromatin, are known to play a major role in gene expression [23–30]. An intuitive understanding of the dynamics of gene expressions is difficult. As a consequence, using computational analysis is helpful for describing unambiguously the structure of regulatory systems, predicting their behavior in a systemic way and identifying candidate genes as markers of cellular response

Table 2
Mean fold change ($\log_2^{-\Delta\Delta Ct}$) and S.E. at different time points; lowest and highest values; and frequency of DGF [a] in CEM and FDM

	CEM	FDM	*P* value	CEM			FDM		
	Mean±S.E.	Mean±S.E.		Lowest	Highest	DGF% [a]	Lowest	Highest	DGF% [a]
T0	0	0	–	–	–	–	–	–	–
T2	1.13±0.91	0.22±0.16	<.05	−0.10	2.05	3.6	−1.90	3.38	28.6
T4	1.77±0.19	0.77±0.10	<.05	−0.27	3.45	3.6	−0.92	1.66	13.6
T6	0.68±0.13	−0.04±0.18	<.05	−0.99	2.49	14.3	−2.17	3.88	71.4
T12	3.25±0.33	3.17±0.28	NS	0.14	6.56	0	0.09	5.90	0
T24	2.29±0.17	1.93±0.19	NS	0.37	3.74	0	−0.52	3.52	3.6
T48	1.93±0.17	0.45±0.21	<.05	0.32	4.35	0	−1.38	3.25	32.4

Test of comparison between mean±S.E. of gene expression in CEM and FDM at each time point.
[a] Down-regulated gene frequency (%).

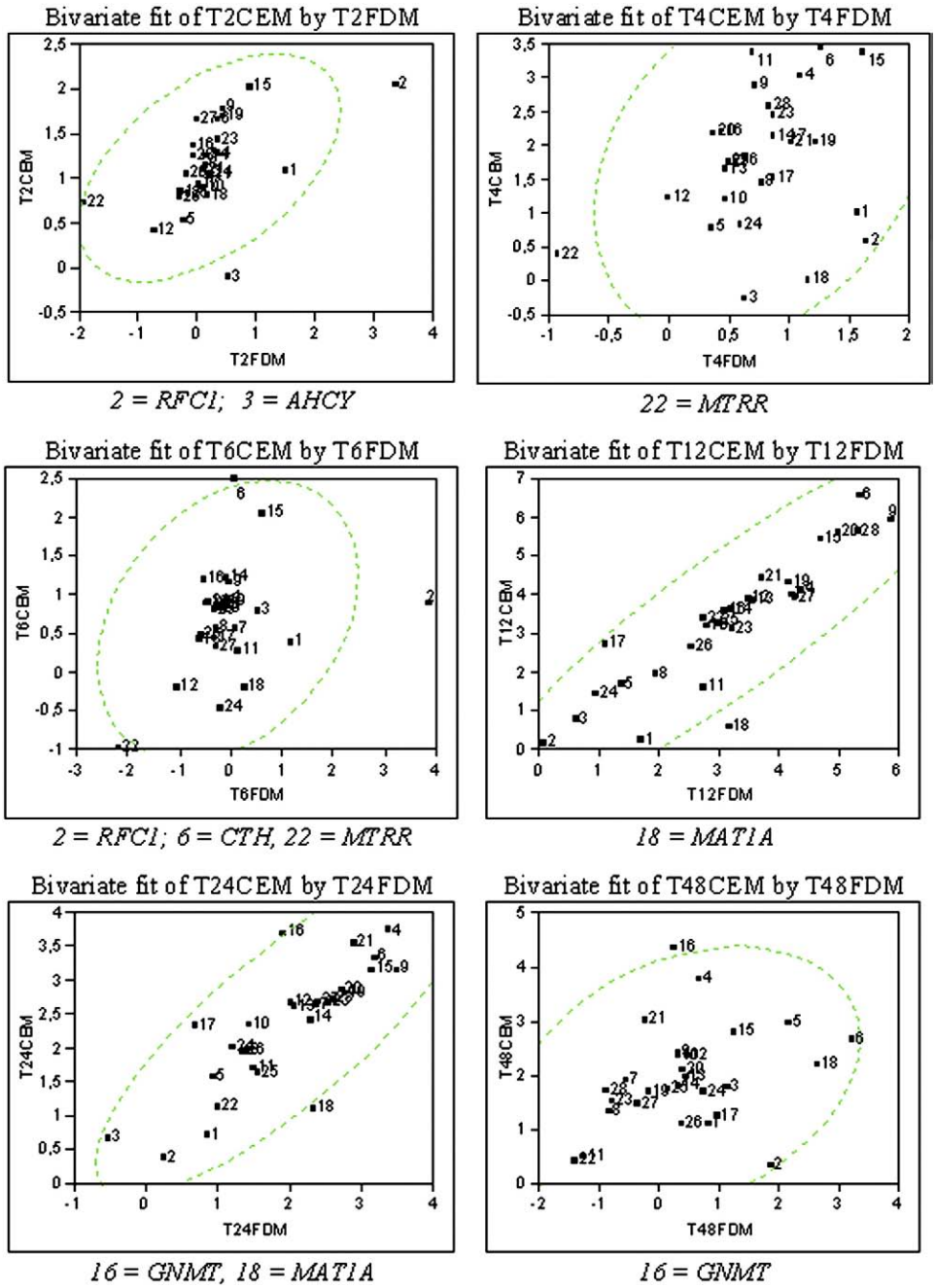

Fig. 2. Bivariate density analysis for the difference between CEM and FDM at different time points. Ellipse corresponds to 95% of tolerance. *hRfc1* (gene 2) and *Ahcy* (3) at T2; *Mtrr* (22) at T4; *hRfc1* (2), *Cth* (6) and *Mtrr* (22) at T6; *Mat1A* (18) at T12; *Gnmt* (16) and *Mat1A* (18) at T24; *Gnmt* (16) at T48 displayed significant differences when using 97% of distribution between CEM and FDM.

to environmental stimuli. Our study was limited to the decrypting of the folate deficiency effect on genes involved in FOCM and attempted to determine, by using computational analysis, specific genes based on their behaviors as gene markers. We have studied 23 enzyme reactions and two transport systems (Fig. 1). Results based on HepG2 cell line showed that transcriptional response to folate deprivation occurred rapidly, with differential expression first appearing within 2 h (Fig. 2). The percentage of negatively expressed genes at T2 was 28.6% in FDM compared to 3.6% in CEM (Table 2). The percentage increased to 71.4% throughout the 6 h of experiment. An increased transcriptional level of all genes was observed for cells cultivated in CEM from T12 to T48. The decrease of gene expression in FDM as well as in CEM at T6 may be associated with HepG2 cells' kinetics of division or doubling time. The difference in gene expression observed in CEM and FDM cells was induced by folate deficiency rather than by serum depletion, as both conditions were tested in 4% serum, although cells were initially grown for 24 h in complete medium containing 20% serum. Cell

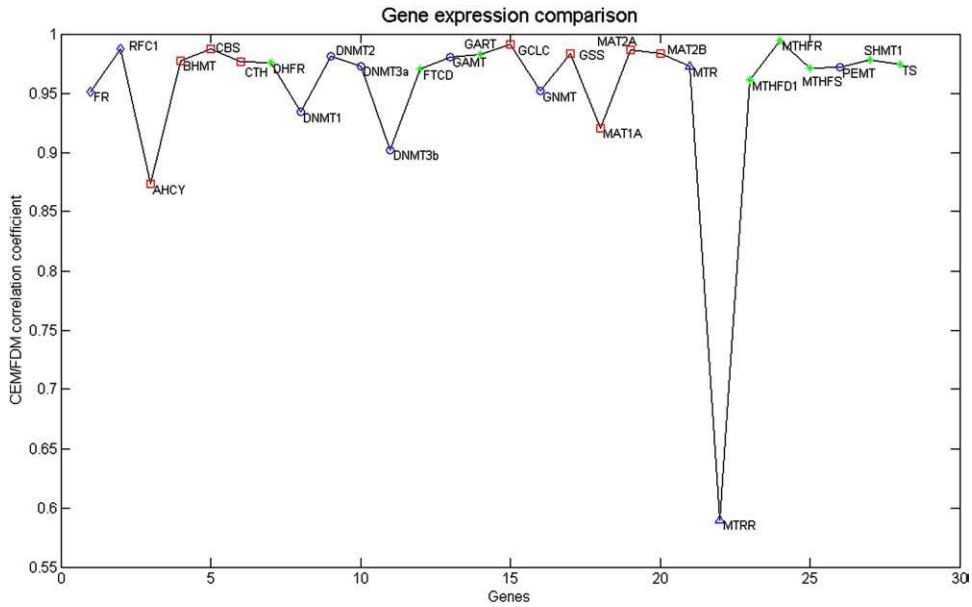

Fig. 3. Time-series analysis: correlation coefficient of gene expressions across the time. Each dot represents a specific gene. Correlation score equal to 1 indicates a perfect similarity between gene expression despite folate conditions. Each gene is associated with a class that emphasizes the localisation of the reaction encoded by the gene within the one-carbon metabolism graph.

counting was performed and no significant differences in cell growth rate were observed between CEM and FDM media at different time points. Cell numbers counted at 72 h were 201,000+2000 cells per 25 cm^2 in CEM and 202,000+2000 cells per 25 cm^2 in FDM. The correlation coefficient analysis across the time allowed identifying seven genes. Among these identified genes, four (*Ahcy, Gnmt, Mat1A* and *Mtrr*) were identified by bivariate analysis, rendering both approaches as an interesting tool to be developed. Sensitivity of these genes to folate deficiency is not well known.

To go a step beyond simple recording of fold changes of gene expression levels in this biological system, we combined experimental data and computational analyses. Fig. 3 shows that *Mtrr* displayed a marked time-series expression. It is worthy to note that, in this experiment, among all genes *Mtrr* was the only gene down-regulated at

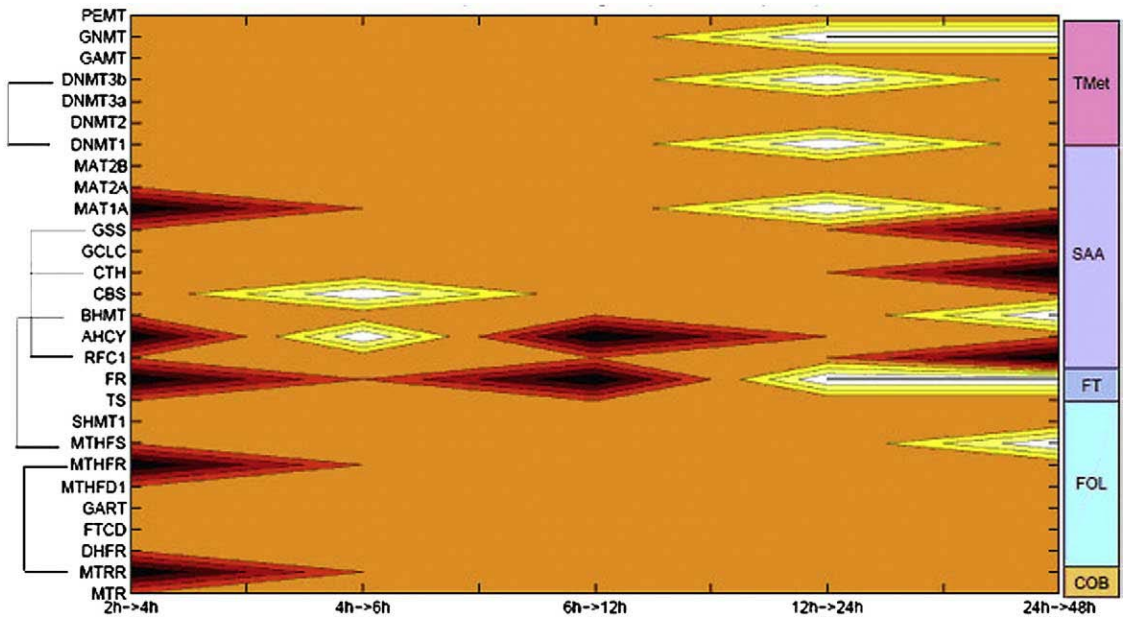

Fig. 4. Qualitative expression of genes. There are four clusters of coexpressed gene as follows: DNMT3B and DNMT1; MTHFR and MTRR; CTH, GSS and RFC1; BHMT and MTHFS. Note that 14 genes do not present expression modification.

T4 in FDM (Table 2). With the qualitative expression of genes (Fig. 4), results showed that *Mthfr* and *Mtrr* were coexpressed (Cluster 1). Coexpression analysis is of interest, because this tool can be used to interpret the effect of a perturbation in the folate-mediated one-carbon genetic network. *Mtrr* encodes for the MSR enzyme which is responsible for the reductive activation of MS-Cob(II). Mechanistically, methionine synthase, which contains a cobalamin (Cob) cofactor, catalyzes the methyl group transfer from methyltetrahydrofolate to cob(I)alamin producing MS-Cob(III)-CH_3 and utilizes the methyl group to methylate Hcy to form methionine. Approximately once in every thousand turnovers, the cob(I)alamin cofactor of methionine synthase becomes oxidized by molecular oxygen forming the inactive cob(II)alamin form [31,32]. MSR provides the electron that, along with transfer of a methyl group from AdoMet, returns methionine synthase to the active methylcobalamin form. Reductive methylation is the mechanism by which AdoMet together with an electron reactivates the enzyme after oxidative inactivation. MSR acts also as a chaperone of MS [33]. It therefore acts as an intermediary methyl carrier, but is unfortunately poorly studied. MTHFR and MSR are critical enzymes responsible for the biosynthesis of methionine, the precursor for methylation reaction, and the regeneration of tetrahydrofolate for nucleotide biosynthesis. Alterations therefore in the expression of both enzymes could have important effects on DNA methylation and DNA instability. With regard to our results, folate deficiency may strengthen MSR oxidative inactivation. AdoMet is the universal donor of the methyl group and an allosteric inhibitor for MTHFR [34]. Perhaps results from this approach (Fig. 4) point to a discrete contribution of AdoMet in the FOCM gene regulation network. An integration of gene expression data into the metabolic model may be useful for a deep illustration.

The behavior of *Dnmt3B* and *Dnmt1* (Cluster 4) is particularly interesting to analyze from a metabolic point of view. DNMT1 enzyme, which maintains the methylation pattern during DNA replication, is also involved in *de novo* DNA methylation [35]. DNMT3B enzyme is known as *de novo* methyltransferase and has preferred target sites, which are different from DNMT1 [36]. In this study, as HepG2 cells were under growing condition and consequently under dynamic division, we may suppose that the need of the –CH_3 group for DNA methylation may be crucial for *de novo* methylation. In the understanding of cancerisation for instance, functional cooperation of *de novo* methyltransferase DNMT3B and maintenance methyltransferase DNMT1 has been found by Rhee et al. [37] and Peyrin-Biro et al. [38] to be essential in almost all of the methylation processes in the colorectal cancer cell line HCT116. The relevance of other clusters is not well known. Additional studies based on the interaction between *Gss, Cth* and *hRfc1* or between *Bhmt* and *Mthfs* would provide insights into the interplay of folate deficiency in genetic networks with implication in biomedicine applications. Regarding MTRR and MTHFR, our findings also suggest interest in the study of the impact of MSR-66A>G associated with MTHFR-677C>T SNPs in liver diseases.

In conclusion, our study has outlined a strong susceptibility of *Mtrr* and other genes of the remethylation and transmethylation pathways to changes in transcription under folate cellular deprivation. Identified clusters by qualitative expression analysis suggest investigating further gene–gene interactions in the influence of folate/Hcy genetic determinant in pathologies. Our approaches for describing gene expression dynamics will allow testing new hypotheses for understanding some of the cellular metabolic mechanisms underlying folate deficiency.

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