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Mutation at the folate receptor 4 locus modulates gene expression profiles in the mouse uterus in response to periconceptional folate supplementation ☆

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ABSTRACT

Periconceptional supplementation of folic acid to the diet of women is considered a great success for a public health intervention. Higher folate status, either by supplementation, or via the mandatory fortification of grain products in the United States, has led to significant reduction in the incidence of neural tube defects. Besides birth defects, folate deficiency has been linked to a variety of morbidities, most notably to increased risk for cancer. However, recent evidence suggests that excess folate may be detrimental — for birth defect incidence or in the progression of cancer. How folate mediates beneficial or detrimental effects is not well understood. It is also unknown what molecular responses are elicited in women taking folate supplements, and thus experience a bolus of folate on top of the status achieved by fortification. To characterize the response to a periconceptional regimen of supplementation with folinic acid, we performed gene expression profiling experiments on uterus tissue of pregnant mice with either wildtype alleles or targeted disruption at the folate receptor 4 locus. We observed that, depending on the genetic background, folinic acid supplementation affects expression of genes that contribute to lipid metabolism, protein synthesis, mitochondrial function, cell cycle, and cell activation. The extent of the response is strongly modulated by the genetic background. Finally, we provide evidence that folinic acid supplementation in the mutant paradigm affects histone methylation status, a potential mechanism of gene regulation in this model.

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1. Introduction

1.1. Background

Periconceptional folate supplementation has been a public health success story for the prevention of neural tube defects [1]. The seminal discovery by Smithells [2] was that the incidence of birth defects such as spina bifida was associated with low folate status. Subsequently, it was demonstrated that providing women who wish to become pregnant with folate supplements was correlated with a significantly lower incidence of neural tube defects [3], without any obvious detrimental effects [4]. In the last 15 years, many countries adopted the recommendation to encourage folate uptake in the periconceptional period as means of birth defect prevention. The use of folate supplements, and the introduction of mandatory fortification of grain products with folic acid in the United States in 1998, have resulted in significantly higher folate levels in serum and red blood cells [1]; these higher folate levels are credited with the concomitant reduction of birth defect incidence [5] in the last decade.

The mechanism by which folate prevents neural tube defects is still a mystery. Folate is a carrier for single-carbon moieties, with crucial roles for nucleotide synthesis, amino acid synthesis, as well as methylation reactions in the cell. Consequences of folate deficiency are best characterized in cancer research, where it is thought that low folate levels lead to insufficient nucleotide supply, increased incorporation of uracil into DNA, increased strand breaks, impaired DNA repair mechanisms, accumulation of mutations, and overall genome instability, a hallmark of neoplastic transformation [6,7]. Furthermore, imbalances in DNA methylation were observed, which are also thought to contribute to genome instability. Therefore, low folate status is considered to be a risk for the development of neoplasms, implying that folate fortification may alleviate that risk and lead to beneficial effects for public health beyond prevention of birth defects.

Recently, concerns have arisen that high folate status may be associated with consequences that are not unequivocally advantageous [8]. Folate can act as a mitogen for cancer cells [9], and thereby stimulate the growth of pre-existing neoplasms. In patients with a history

of colorectal adenoma, folic acid was associated with greater risk for multiple adenomas and non-colorectal cancer [10]. Tumors originating from ovarian and prostate cancer express high levels of folate receptors, indicating that those cells are exceptionally adept at scavenging folate to promote their proliferation. In fact, this feature has been exploited in the design of targeted chemotherapeutic agents [11], as well as in the invention of molecular tools to visualize folate receptor-expressing tumors during surgery [12]. High folate status clearly constitutes a concern with respect to the progression of such tumors. In the developing rodent embryo, evidence has recently been presented that very high folate levels in the dam can lead to an increase in birth defect frequency [13]. Furthermore, in some paradigms of genetic neural tube defect susceptibility, higher folate appeared to be detrimental and lead to higher neural tube defect incidence compared to lower folate status [14]. Together, these data suggest that high folate consumption may not be entirely beneficial.

On the molecular level, folate status has been associated with gene expression [15]. Initially, folate-dependent differences in gene expression levels were attributed to epigenetic events due to the presumed effects of folate on DNA methylation [16,17]. Yet, methylation reactions shape other domains of the epigenome besides DNA methylation, and evidence has arisen that changes in gene expression occur in response to folate deficiency or supplementation without affecting DNA methylation [18,19]. Many of these studies focused on cancer. However, it is reasonable to assume that the highest folate levels would be found in women who, because they want to become pregnant and prevent potential birth defects, take preconceptional folate supplements in addition to folate intake from fortified foods. The molecular consequences of such a bolus of folate, for instance on the female reproductive tract, are not well understood. The primary interface between mother and developing embryo is the endometrium of the uterus, where the nutritional interaction is established during the process of implantation, a crucial step for reproductive success. Whether folate modulates this interaction is currently unclear. Therefore, we decided to investigate the effects of preconceptional folate supplementation on the female reproductive tract, focusing on the uterus at the time of implantation. Using a mouse model system, we demonstrate a folate effect on gene expression in the uterus, and a broader response in mice lacking folate receptor 4, and suggest an effect on histone methylation.

2. Materials and methods

2.1. Folate supplementation and tissue preparation

Purina 5001 (LabDiet, Inc.) was used as diet for all mice. Female mice of the C57BL/6 strain (or from a strain carrying the Folr4 mutation on the C57BL/6 genetic background) were injected intraperitoneally with folinic acid at a dose of 12.5 mg/kg body weight, or with saline as control. Dams received two injections, at 10 and 3 days before mating, respectively. Dams were mated to C57BL/6 males; noon of the day when presence of a vaginal plug could be detected was designated day 0.5 of gestation. At 3.5 days of gestation, mice were euthanized, uterus tissue was dissected, and either used to extract RNA, or processed for histology. RNA was extracted using TRIzol (Invitrogen Life Technologies), quantified on a NanoDrop ND-1000 spectrophotometer, and stored at −80 °C for further analysis.

2.2. Mice lacking Folr4

Mice with a mutation in the Folr4 gene were generated via homologous recombination in mouse embryonic stem cells. For this purpose, a construct was made where the first two coding exons of the gene were flanked by loxP recombination sites, followed by the gene for neomycin resistance and third loxP site. Electroporation of the construct, selection of ES cell clones with the correct constellation at the Folr4 gene, and generation of chimeric mice were done as

described [20]. Homozygous mice carrying the floxed locus were viable. To generate a null mutation, homozygous Folr4FL mice were crossed to mice expressing Cre recombinase in the oocyte [21], resulting in heterozygous offspring with one allele of the Folr4 gene exhibiting a deletion of the first two coding exons. PCR analysis of the locus produced an amplicon of 413 bp indicative of the deletion, compared to an amplicon of 1365 bp from the wildtype allele. Mice with this Folr4 null allele were backcrossed to the C57BL/6 genetic background for more than 10 generations. Heterozygous mice were used to generate the homozygous Folr4 mutant female mice used in this study. All procedures involving animals were approved by the Institutional Animal Care and Use Committee.

2.3. Gene expression profiling

Affymetrix Mouse 430 2.0 microarrays were used to perform gene expression profiling experiments as described previously [22]. We processed three RNA samples from wildtype C57BL/6 dams, four from wildtype C57BL/6 dams treated with folinic acid, three from Folr4 mutant dams, as well as four from Folr4 mutant dams supplemented with folinic acid for array hybridization. All samples were obtained from individual animals. Tissue dissection and RNA extraction were performed on the same day, and all further processing, including array hybridizations, was done in parallel to ensure technical consistency between samples. Arrays were normalized as described [22], and array data were processed to identify differential gene expression using CyberT software [23]. We used the implementation of the software available at http://cybert.ics.uci.edu/, employing a Bayes-regularized t-test after applying variance stabilized normalization [24]. Annotation for molecular function was performed using GO terms, with manual curation based on MGI (http://www.informatics.jax.org/) and GeneCards (http://www.genecards.org/). Results from probes for non-coding RNAs, or probes where gene identity was missing, were omitted. Data for one probe per gene were retained for the final list. When a gene was represented by more than one probe, duplicates were eliminated based on criteria of high p-value, low expression and low fold-change. In the very few cases where duplicates displayed opposite directions of change for the same gene, the gene was removed from consideration. Pathway analysis was performed using the DAVID (http://david.abcc.ncifcrf.gov/) [25] suite of tools; adjusted p-values given in the Results section (p_{adj}) refer to p-values obtained after Benjamini–Hochberg correction. Visualization of expression differences was done in Microsoft Excel. Lists of folate- and genotype-responsive probes are presented in Supplementary Table 1. All microarray analyses reported here have been deposited to GEO (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE36974).

2.4. Expression measurements

Validation of expression measurements for selected genes was performed by real-time quantitative PCR on an Applied Biosystems 7900 system as described previously [26]. Expression measurements were generated using the gene for DNA polymerase subunit epsilon 4 as the reference gene for normalization [26]. Primer sequences are listed in Supplemental Table 2. Three replicate assays per sample were performed, and statistical evaluation of PCR data was done via two-tailed t-test based on at least n = 4 samples; samples included those used for microarray as well as independently generated samples.

2.5. Histology

Paraffin-embedded tissue sections [27] or cryosections [28] were processed for in situ hybridization with digoxigenin-labeled antisense riboprobes as described previously. For immunohistochemistry with antibodies against histone H3 lysine K4 trimethylation (Millipore, Billerica, MA), frozen uterus tissue was sectioned at 16 μm, fixed in 1% formaldehyde in PBS, washed, blocked in PBS containing 2% goat

serum and 0.2% BSA, incubated with primary antibody over night at 4 °C, washed in PBS, and developed with goat-anti rabbit-Alexa 594 secondary antibody (Invitrogen, Carlsbad, CA). Brightfield images were recorded on a Leica Z16 macroscope, whereas fluorescent images were captured on a Leica TCS SP5 confocal microscope, using the same instrument settings for all specimens, respectively.

3. Results

The focus of folate supplementation before and at onset of pregnancy has always been on pregnancy outcome and prevention of birth defects. Very little attention, however, has been paid to molecular responses to folate in the expecting mother, where the consequences of a boost in folate levels are not well understood. To gain insights into the molecular consequences of preconceptional folate supplementation on female reproductive tissues, we conducted an experiment where mice received two injections of folinic acid (a stable folate derivative) at 10 and 3 days before mating, respectively. Considering that implantation is a crucial step in reproductive success, we focused on uterus tissue at 3.5 days after mating, the time when the blastocyst reaches the uterus. With the main emphasis on the response to folinic acid, we processed uterus tissue for transcription profiling via microarrays, and analyzed (i) gene expression in the uterus of wildtype mice in response to folinic acid supplementation, (ii) gene expression in the uterus in response to

folinic acid in the presence of a genetic liability in form of a mutation at the gene for folate receptor 4, and (iii) explored to what extent supplementation with folinic acid may be able to reverse expression changes brought about by the Folr4 deficiency.

3.1. Identification of folate-responsive genes in peri-implantation mouse uterus

The response of mouse uterus tissue to folinic acid supplementation is shown in Fig. 1A, based on statistical significance in a Bayes-regularized t-test ($p < 0.05$). We found a moderate magnitude of the transcriptional response to folate supplementation before pregnancy, with 188 gene probes showing a folate-dependent 1.5-fold or higher increase in expression level, and 246 gene probes exhibiting a decrease of expression. Lowering the fold-change cutoff to 1.2-fold in either direction (using the same statistical test), we detected a total of 830 gene probes that change expression in response to folate, with 406 probes showing increase, and 424 probes showing a decrease in expression levels. This suggested that the response to folate in this paradigm is comprised by relatively small changes of expression levels for a moderately large number of genes. Genes of the folate cycle were unaffected, as were genes encoding methyltransferases. We chose five genes (2 up- and 3 down-regulated) that represented different levels of expression for validation of the array-based
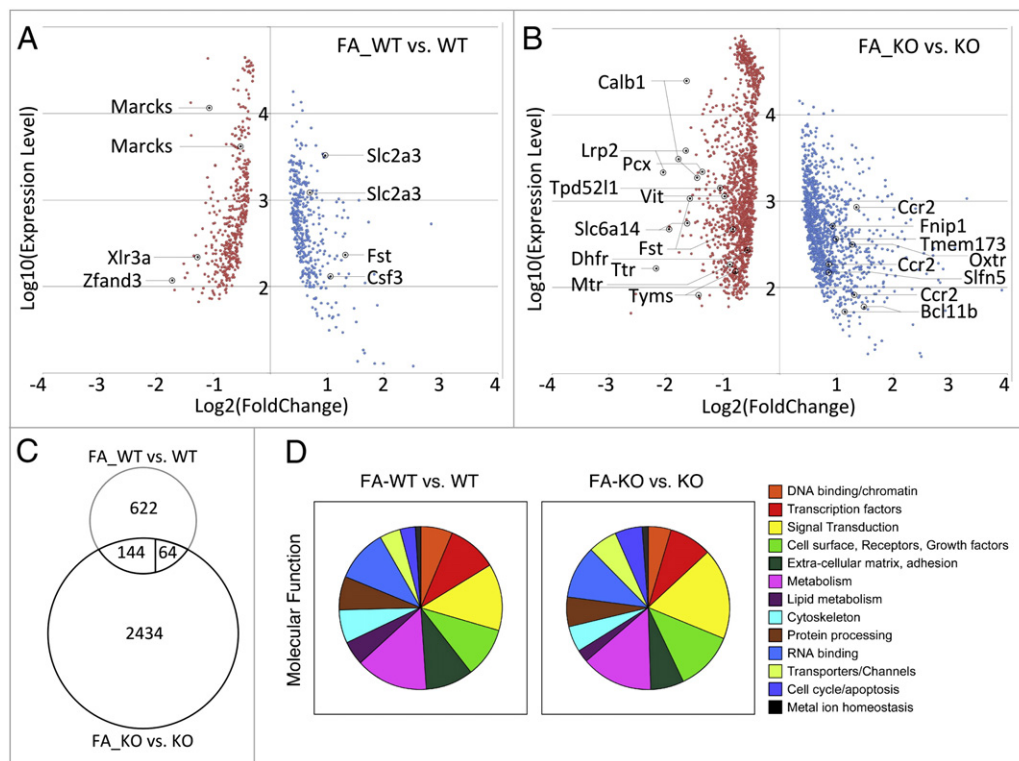

Fig. 1. Transcriptional responses to folate supplementation in the uterus. A. Comparison of gene expression profiles in normal (WT) and folinic acid (FA) supplemented normal (FA_WT) mice revealed a moderate response consisting of predominantly small changes: at a 1.2-fold or greater difference between average expression levels in each group, 830 probes were altered (406 with increased expression level, 424 with decreased expression level); at a 1.5-fold or greater difference, this number was reduced to 434 probes (188 increased, 246 decreased), whereas at 2-fold or greater change, only 89 gene probes were detected (47 increased, 42 decreased). Of the 830 gene probes, 4 passed a Benjamini–Hochberg correction ($p_{adj} < 0.1$). All probes with a p-value below 0.05 in a Bayes-regularized t-test are shown, with their respective expression level on the array plotted against the observed fold change. Marked gene probes correspond to those genes validated by quantitative PCR (see Table 1). B. Comparison of gene expression profiles in Folr4-mutant (KO) and folinic acid (FA) supplemented mutant (FA_KO) mice demonstrated a more vigorous transcriptome response: at a 1.2-fold or greater difference between average expression levels, 2642 probes were altered (1177 expression levels increased, 1465 expression levels decreased); at a 1.5-fold or greater difference, this number was reduced to 1786 probes (885 increased, 901 decreased), whereas at 2-fold or greater change, only 451 gene probes were detected (299 increased, 152 decreased). Of the 2642 gene probes, 448 passed a Benjamini–Hochberg correction ($p_{adj} < 0.1$). All probes with a p-value below 0.05 in a Bayes-regularized t-test are shown in the plot. Marked gene probes correspond to those genes validated by quantitative PCR (see Tables 1 and 3). C. Comparison of the response to folinic acid supplementation between wildtype and Folr4 mutant tissue. Only 208 gene probes are common between the two responses; out of those, 144 change expression in a fashion concordant between the two genotypes, whereas 64 show altered expression in a discordant manner. D. Annotation of differentially expressed genes by molecular function comprising the transcriptional response to folinic acid supplementation in either wildtype (FA_WT vs. WT) or Folr4 mutant (FA_KO vs. KO) tissue. Note the large segments for metabolism (including oxidative phosphorylation and mitochondrial function), protein processing (including localization), and RNA binding (including ribonucleoprotein complexes).

expression changes by qPCR. In all cases, we were able to confirm the direction of the expression change with statistical significance as determined by a t-test (Table 1); the assay for Zfand3 did not reach statistical significance.

In order to determine which biological pathways may be affected by folate supplementation, we used the genes derived with greater than 1.2-fold change as well as statistical significance. Annotation for molecular function of the products encoded by differentially expressed genes revealed (Fig. 1D) that molecules involved in metabolism (11.4%), signal transduction (10.7%), RNA binding (8.5%), transcriptional regulation (8.3%), as well as receptors and growth factors (8.1%) comprised the largest groups. Using the DAVID suite of tools (http://david.abcc.ncifcrf.gov/) [25] for further functional annotation, we analyzed gene groups with increased or decreased expression separately; the results are summarized in Table 2, section A. Among the genes with increased expression, we found enrichment for the gene ontology term 'lipid metabolic process'. In the group of genes with decreased expression, the gene ontology terms 'ribonucleoprotein complex', 'mRNA metabolic process' and 'structural constituent of ribosome' were detected. Together, these results suggest that folinic acid supplementation decreases the expression of components of the protein synthesis mechanism in cells of the uterus, and may increase lipid metabolism.

3.2. Identification of folate-responsive genes in Folate receptor 4-deficient mice

Interestingly, we observed a much broader transcriptional response to folinic acid supplementation in uteri derived from mice with a deletion of the Folr4 gene (Fig. 1B). Applying the same criteria as before for wildtype dams (more than 1.2-fold change in either direction, $p < 0.05$), we detected 2642 gene probes in Folr4 mutants that responded to folate, with 1177 probes increasing, and 1465 probes decreasing in their expression levels. 451 probes showed more than 2-fold change; 299 of those showed increased expression in response to folate supplementation, whereas 152 responded with a decrease. The qPCR validation of the expression changes of nine

Table 1
Gene expression analyses by RT-PCR.

Representative genes at different expression levels were chosen to validate the microarray results. For the comparison of wildtype vs. folinic acid-supplemented wildtype, 5 genes are shown. We observed concordant expression changes in the array and the qPCR, although not all PCR assays reach statistical significance in a t-test. For the comparison of mutant vs. folinic acid-supplemented mutant, 6 genes are shown that represent differentially regulated genes at various expression levels according to the array data. Furthermore, three genes of the folate pathway were among the genes of the folinic acid response: Dhfr, Mtr, and Tyms all show decreased expression in response to folinic acid in the mutant.

Gene symbol	Microarray		qRT-PCR	
	Fold change	p-value	Fold change	p-value
FA_WT vs. WT				
Csf3	2.07	0.001	2.15	0.029
Marcks	−2.11	0.000	−1.34	0.027
Scl2a3	1.95	0.002	2.02	0.032
Xlr3a	−2.42	0.000	−2.49	0.020
Zfand3	−3.29	0.000	−1.17	0.051
FA_KO vs. KO				
Bcl11b	2.80	0.002	5.00	0.009
Calb1	−3.41	0.000	−5.05	0.024
Lrp2	−4.13	0.000	−5.95	0.044
Oxtr	2.41	0.000	2.64	0.053
Slc6a14	−3.85	0.000	−7.97	0.023
Ttr	−4.49	0.000	−8.37	0.001
Dhfr	−1.48	0.029	−1.53	0.049
Mtr	−1.83	0.012	−1.91	0.030
Tyms	−2.12	0.000	−2.49	0.005

Table 2
Summary of top-scoring categories in DAVID pathway analysis.

Pathway analysis was performed using the DAVID (http://david.abcc.ncifcrf.gov/) [25] suite of tools; adjusted p-values (p_{adj}) refer to p-values obtained after Benjamini–Hochberg correction. All analyses were performed with the default settings and classification stringency at "medium".

Increase			Decrease		
Go term	p_{adj}	Genes	Go term	p_{adj}	Genes
A: Folinic acid-supplemented wildtype vs. wildtype					
Lipid metabolic process	0.011	29	Ribonucleoprotein complex	4.3×10^{-06}	30
			Structural constituent of ribosome	2.5×10^{-05}	11
			mRNA metabolic process	6.4×10^{-04}	20
			Vasculature development	0.016	15
B: Folinic acid-supplemented Folr4 mutant vs. Folr4 mutant					
Immune system process	1.9×10^{-26}	115	Structural constituent of ribosome	2.5×10^{-59}	83
Leukocyte activation	1.5×10^{-09}	38	Cell cycle	1.5×10^{-10}	83
T cell activation	1.3×10^{-06}	23	m-phase	2.3×10^{-10}	51
Regulation of immune response	2.2×10^{-09}	35	Mitochondrion	2.6×10^{-10}	141
Cytokine binding	4.8×10^{-06}	20	Oxidative phosphorylation	0.064; 5.8×10^{-14}	20
			Mitochondrial lumen	3.5×10^{-03}	22
C: Folr4 mutant vs. wildtype					
Structural constituent of ribosome	1.7×10^{-21}	36	Defense response	8.7×10^{-05}	25
Mitochondrion	0.1	33	Immune response	1.8×10^{-07}	31
D: Folinic acid-supplemented Folr4 mutant vs. Folr4 mutant compared to Folr4 mutant vs. wildtype					
Immune system process	6.6×10^{-06}	24	Structural constituent of ribosome	2.4×10^{-29}	36
			Mitochondrion	0.036	33
			Glutathione transferase activity	0.0054	6

genes representing different levels of expression on the array is shown in Table 1.

Among genes of the folate cycle, we find Dhfr, Mthfd1, Mthfd1l, Mthfd2, Mthfr, Mtr, Shmt1, Slc46a1, and Tyms mildly deregulated; only Tyms and Mthfd2 showed more than a 2-fold expression change. qPCR analysis of Dhfr, Mtr and Tyms confirmed direction and magnitude of the expression change initially observed in the microassay experiment. Notably, we also detected downregulation of Lrp2 (Megalin), which can bind soluble folate receptors; this change in expression was apparent on the array as well as in qPCR assays. We observed moderate deregulation of genes encoding methyltransferases, but no coordinate change in one direction: Ezh1, Mettl7a1, Mll5, Pcmtd1, Pcmtd2, Setd3, Suv420h1, Suv420h2, and Tgs1 exhibited an average fold change of 1.51; and Alkbh8, Dnmt1, Lcmt1, Mtr, N6amt2, Nsd1, Prmt1, Setdb1, and Trmt112 an average fold change of −1.51.

Similar to the situation observed in wildtype mice, the folate response in Folr4 mutant uterus was primarily composed of moderate magnitude changes for a large group of genes. Fig. 1D shows that the distribution of encoded products follows a similar pattern as in the comparison of supplemented to unsupplemented wildtype females, although genes encoding 'signal transduction' molecules (15.2%) were enriched compared to wildtype. Groups comprising 'metabolism' (11.7%), 'receptors and growth factors' (9.8%) and 'RNA binding' (8.7%) were similarly represented, but in absolute number, there were 3–4 fold more genes responding to folinic acid in the Folr4 mutants.

We obtained further annotation information using DAVID tools, analyzing the groups of up- and down-regulated genes separately (Table 2, section B). Among genes with increased expression in response to folinic acid, gene ontology terms related to immune response were highly enriched. In the group of genes that exhibit a decrease in expression as response to folinic acid supplementation in the mutant, the term 'structural constituent of ribosome' was strongly represented, paralleling the supplementation response in wildtype mice. However, considering individual gene identities, we noted relatively little concordance in the response to folinic acid between wildtype and Folr4 mutants (Fig. 1C): only 208 gene probes were found to overlap between the two supplementation paradigms, and of these, only 144 showed a concordant direction of change.

Interestingly, strong associations to the GO terms 'cell cycle', 'M-phase', 'mitochondrion', 'oxidative phosphorylation' and 'mitochondrial lumen' were found among the genes with decreased expression. Together, this suggested that folinic acid supplementation in Folr4 mutant mice, in addition to eliciting a much broader transcriptomic response, may also lead to a dampening of cellular processes such as mitosis, possibly through decreased functionality of ribosomes which would affect protein synthesis, and to a reduction in mitochondrial functionality which could affect energy homeostasis of cells. The intriguing finding of extensive differences in the folinic acid response prompted us to investigate in more detail the differences between tissues from unsupplemented wildtype and mutant females.

3.3. Uterus gene expression modulated by a mutation in the gene for folate receptor 4

Folr4 is expressed in a restricted pattern in cells of the endometrium in uterus tissue obtained from a wildtype mouse (Fig. 2A and B). The Folr4 mutation deletes the first two coding exons of the Folr4 gene. Homozygous Folr4 mutant mice can be recovered from matings of heterozygote mice at Mendelian ratio. There was no evidence of birth defects, nor of overt morphological differences in the uterus at the time of isolation. Allowing a minimum fold-change of 1.2-fold in either direction at $p < 0.05$ in a Bayes-regularized t-test, we detected 992 gene probes with altered expression when comparing Folr4 mutant to wildtype: 606 with increase, and 386 with decrease of expression (Fig. 2C). At 2-fold change, 87 probes were detected, 40 with increase and 47 with decrease of expression. Such a moderate response is consistent with the fact that Folr4 is not expressed throughout the uterus, but limited to the endometrium and solitary cells in the uterine parenchyma. Other folate receptors and folate pathway genes were not changed in their expression. Using DAVID, we find that the gene ontology term 'structural constituent of ribosome' is significantly enriched in the group of genes with increased expression, whereas the terms 'defense response' and 'immune response' were found to be enriched in the group of genes with decreased expression in the Folr4 mutant. Results from the pathway analyses are summarized in Table 2 section C.

This suggested that the lack of Folr4 may have a rather moderate effect on the development of uterus tissue, consistent with the absence of overt morphological differences in uterus tissue of mutant dams compared to wildtype. Furthermore, the decreased expression of genes associated with the term 'immune response' and 'defense response' in the mutant may be related to the expression of Folr4 on regulatory T-cells [29].

3.4. Reversal of genotype-dependent changes by folate supplementation

When analyzing genotype-mediated differential gene expression in Folr4 mutant dams, we noted that 562 gene probes (representing 451 genes) were also responsive to folinic acid supplementation (Fig. 3A), changing their expression levels between normal mutant and supplemented mutant. Intriguingly, except for 2 genes, all those gene probes show a statistically significant discordant response in the two comparisons: 359 of 360 probes (representing 279 genes) with increased expression in the unsupplemented mutant compared to wildtype displayed a decrease of expression in the folinic acid-supplemented mutant compared to the unsupplemented mutant (Fig. 3B). Likewise, 201 of 202 probes (representing 172 genes) with decreased expression in the mutant when compared to wildtype exhibited an increase of expression in the folinic acid supplemented mutant uterus. The two genes that do not conform are Cxcl17 and Pdgfd. In total, approximately 57% of the gene probes showing changes in expression attributable to genotype also respond to folinic acid supplementation in the mutant; notably, the magnitude of
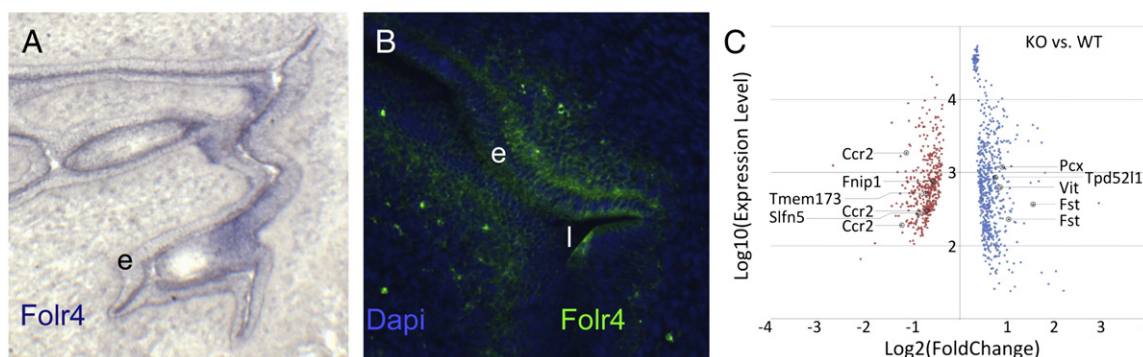

Fig. 2. Expression of Folr4 in the uterus and altered gene expression in Folr4 mutant uterus. A. Expression of Folr4 mRNA in the uterus at 3.5 days of gestation as visualized by in situ hybridization. Folr4 mRNA was detected in cells of the endometrium. B. Expression of Folr4 protein visualized by immunohistochemistry. Similar to the in situ hybridization, Folr4 protein expression (green) is limited to the endometrium. In addition a few solitary cells in the uterine parenchyma were detected, suggesting that the antibody is more sensitive than the DIG-labeled riboprobe under these conditions; e, endometrium; l, lumen of the uterus. C. Transcriptome analysis comparing gene expression in uterus tissue from Folr4 mutant mice (KO) to uterus tissue of wildtype mice (WT). At a 1.2-fold or greater difference between average expression levels in each group, 992 probes were altered (606 with increased expression level in the mutant, 386 with decreased expression level); at a 1.5-fold or greater difference, this number was reduced to 567 probes (305 increased, 262 decreased), whereas at 2-fold or greater change, only 87 gene probes were detected (40 increased, 47 decreased). Of the 992 gene probes, 20 passed a Benjamini–Hochberg correction ($p_{adj} < 0.1$). All probes with a p-value below 0.05 in a Bayes-regularized t-test are shown, with their respective expression level on the array plotted against the observed fold change. Marked gene probes correspond to those genes examined by quantitative PCR.
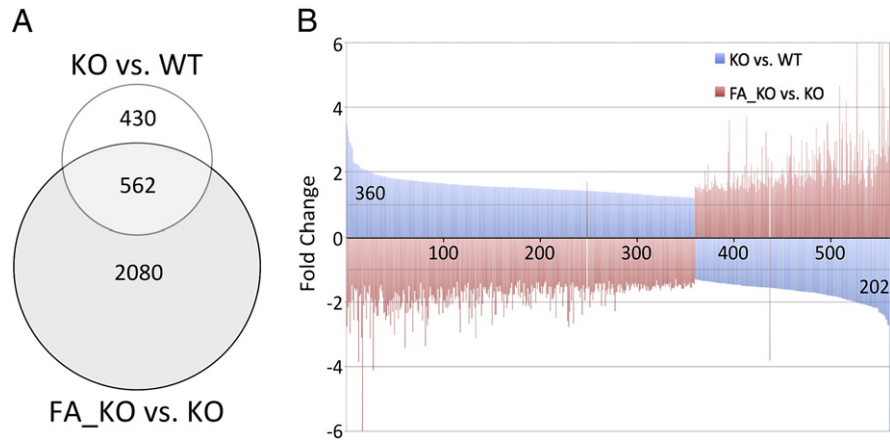
Fig. 3. Folinic acid can correct changes in expression caused by the Folr4 mutation. A. Comparison of statistically significant responses (p < 0.05 in a Bayes-regularized t-test) in Folr4$^{-/-}$ and normal dams (KO vs. WT, 992 probes), and in FA-supplemented Folr4$^{-/-}$ and non-supplemented Folr4$^{-/-}$ dams (FA_KO vs. KO, 2642 probes), respectively, revealed 562 probes that responded under both conditions. B. These 562 probes showed discordant directions for the changes in expression levels when compared between the two conditions: 360 probes show increased expression in the mutant vs. the wildtype, whereas 202 show decreased expression. These probes exhibit the opposite expression change when comparing the supplemented mutant to the mutant (except for 2 probes), indicating that FA can, in part, rectify the effect of the Folr4 mutation. Y-axis: fold-change between average expression levels for any given gene in each comparison.

changes imposed by the mutation is comparable to the magnitude of changes induced by folinic acid in the mutant: absolute fold-changes for each gene are highly similar, yet, the direction of change is back towards levels comparable to unsupplemented wildtype.

Interestingly, gene ontology terms 'structural constituent of ribosome' and 'mitochondrion', which were associated with increased gene expression levels due to mutant genotype, are now found in the group of genes with decreased expression in response to folinic acid. Conversely, the term 'immune system process' attached to genes with decreased expression level due to mutant genotype, but these are now found reversed to normal by folinic acid supplementation (Table 2, section D).

We sought to confirm this finding of inverse direction of change for selected genes by measuring expression levels by quantitative real-time PCR. Eight genes were chosen for this analysis: Fst, Pcx, Tpd52l1, and Vit are four genes that were up-regulated in the mutant compared to wildtype, whereas Ccr2, Fnip1, Slfn5, and Tmem173 represent four genes with decreased expression in the mutant (Fig. 2C). In the microarray experiment, all eight genes showed a reversal of the initial expression levels in the mutant by folinic acid supplementation. In the validation study (Table 3), we were able to confirm the initial observations: Fst, Pcx, Tpd52l1, and Vit expression levels were increased in the mutant compared to the wildtype, and Ccr2, Slfn5, Fnip1, and Tmem173 levels were decreased, although the comparison between groups did not

reach statistical significance for the latter two genes. As in the microarrays, opposing direction of changes in expression of these genes were found when comparing folinic acid-supplemented mutant samples to non-supplemented mutant samples: supplementation decreased Fst, Pcx, Tpd52l1, and Vit, and increased Ccr2, Fnip1, Slfn5, and Tmem173. Together, the results demonstrate the reversal of mutation-dependent molecular alterations by folinic acid supplementation.

3.5. Histone methylation patterns in Folr4 mutant uterus

Finally, to explore potential mechanisms for folate-dependent gene regulation, we investigated whether the status of histone H3 lysine K4 trimethylation (H3K4me3), a histone modification typically associated with promoters of actively transcribed genes, would be responsive to folinic acid supplementation. To this end, we stained cryosections of uterus tissue with an antibody specific for trimethylation at lysine 4 of histone 3 (H3K4me3); due to the stronger transcriptomic response to supplementation, we chose uterus tissue from mutant dams in this study and compared supplemented mutant to non-supplemented mutant. We observed a signal for H3K4me3 in uterus tissue from a non-supplemented Folr4 mutant mouse (Fig. 4A) in many, but not all, cell nuclei on a representative section of uterus tissue. In parallel, staining for H3K4me3 in uterus tissue from a folinic acid-supplemented mutant revealed a brighter signal in nearly all cell nuclei of a section (Fig. 4B).

Table 3
Folinic acid-mediated reversal of gene expression changes in Folr4 mutant mice.

We observed 562 gene probes that appear to reverse the expression change elicited by the Folr4 mutation when supplemented with folinic acid. Eight genes were chosen for validation by qPCR: four genes that were initially up-regulated in the mutant vs. the wildtype, yet showed a decrease in expression in folinic acid-supplemented mutants, and four genes with the opposite pattern of gene expression changes: reduced expression in mutant compared to wildtype, and increased expression in folinic acid supplemented mutant compared to unsupplemented mutant. In all cases, the direction of the expression change could be confirmed by qPCR, although not all qPCR assays reached statistical significance in a t-test.

| Gene symbol	Microarray				qRT_PCR			
	KO vs. WT		FA_KO vs. KO		KO vs. WT		FA_KO vs. KO	
	Fold change	p-value	Fold change	p-value	Fold change	p-value	Fold change	p-value
Fst	2.92	0.042	−2.98	0.048	3.31	0.018	−3.28	*0.130*
Pcx	1.85	0.009	−2.56	0.035	2.13	0.042	−5.03	0.026
Tpd52l1	1.67	0.008	−2.07	0.003	1.62	0.023	−1.49	*0.296*
Vit	1.82	0.041	−1.96	0.037	1.68	0.032	−2.04	0.003
Ccr2	−2.19	0.017	2.55	0.012	−1.98	0.002	2.27	0.028
Fnip1	−1.47	0.046	1.91	0.024	−1.40	*0.118*	1.34	0.036
Slfn5	−1.84	0.042	1.81	0.047	−1.53	0.021	1.24	*0.361*
Tmem173	−1.65	0.009	2.49	0.025	−1.30	*0.156*	1.87	0.042
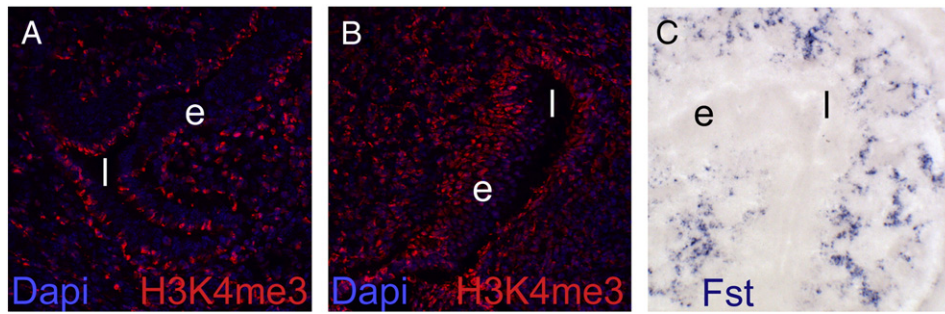
Fig. 4. Histone methylation responses to folinic acid supplementation. A. Cryosection of a Folr4$^{-/-}$ uterus stained with an antibody against trimethylated histone H3 lysine 4 (H3K4me3, red). B. Folr4$^{-/-}$ uterus after supplementation with folinic acid (FA), stained, processed and imaged in parallel under identical conditions as in A. Stronger and more widely distributed staining indicates that folinic acid stimulates histone methylation in the uterus, in a pattern that appears broader than the expression pattern of Folr4. Sections in A, and B were counterstained with Dapi to reveal nuclei. C. Expression pattern of Follistatin (Fst) in uterus tissue at 3.5 days of gestation occurs in a pattern different from the expression of Folr4. Fst was never detected in the endometrium, but appears to be restricted to uterine glands.

This suggests that supplementation with folinic acid leads to a stimulation of H3K4 trimethylation levels in uterus tissue, with strong representation in the endometrium. As the H3K4me3 staining pattern, which included the domain of Folr4 expression, also contained a majority of Folr4 non-expressing cells in the uterus, the altered histone methylation status may contribute to the altered gene expression levels in response to folinic acid.

4. Discussion

4.1. Transcriptional effects of folate supplementation in the mouse uterus

In this study, we demonstrate that preconceptional supplementation with folinic acid alters the transcriptome of uterus tissue in a mouse model. The overall responses were moderate in magnitude of expression changes, which may be related to the fact that we measured transcriptome responses seven days after the last injection of folinic acid. We find that components of RNA processing and protein synthesis pathways respond with decreased gene expression. Genes in these categories were also downregulated by folinic acid in mice carrying the Folr4 mutation, suggesting that under our experimental conditions, folinic acid may have a dampening effect on fundamental cellular processes. Such an action would be contrary to what one might expect in light of the notion that folate can act as a mitogen in cancer cells [9,30,31]. Our histological observations provided no indication for the notion that folate acts as a mitogen in the uterus: cell density (as detected by nuclear staining with DAPI) did not differ between folinic acid-supplemented and unsupplemented specimen (data not shown). Yet, given that we investigated one time point only, this aspect warrants further investigation.

4.2. Identification of mitochondrial pathways as targets of folate supplementation

In Folr4 mutants, the uterine response to folinic acid supplementation involved a much larger number of genes, and this broader response contained, in top scoring categories, genes associated with immune function, cell cycle regulation, and mitochondrial function. Pathways related to immune function were increased by folinic acid in the mutant, whereas genes associated with cell cycle and mitochondrial function were decreased by supplementation. The link to mitochondrial function is in line with a recent report on gene expression profiles in response to dietary folate status in mice that were heterozygous for a mutation in Lrp6, a gene encoding a Wnt co-receptor [30]. In that study, genes for oxidative phosphorylation and mitochondrial function were found to respond to folate, both in embryonic crania at 9.5 days of gestation, as well as in adult liver. Our

experiments, in Folr4 mutant mice, now provide further support for the idea that mitochondrial biology may be affected by folate. Folates themselves have been considered to be antioxidants [31], and as such could potentially influence levels of oxidative stress, a factor that has been implicated in birth defect etiology. Notable from our results is that in mutant tissue, folinic acid supplementation downregulated the expression of genes encoding components of the respiratory chain, suggesting that high doses of folinic acid may negatively affect mitochondrial energy metabolism, and possibly mitochondrial function in general.

4.3. Maternal genotype modulates the response to folate supplementation

Transcriptional responses to external stimuli, such as the presence or absence of a micronutrient, are highly dependent on the status of the cell in question: pre-existing conditions, such as transcription factor repertoire, or epigenetic states, shape the capability of the cell to respond. The experiment conducted with Folr4 mutant mice underscores this notion. Under identical conditions of folinic acid concentration and schedule of supplementation, the Folr4-deficient mutants exhibited a much broader transcriptional response to folate compared to wildtype mice (see Fig. 1C). Yet, when we compared the group of genes affected by folinic acid in the wildtype to the genes affected by folinic acid in the mutant, we found that the overlap between the responses was small, indicating that the mutant genotype fundamentally alters the transcriptome response to supplementation. This result in the mutant paradigm indicates that a significant interaction occurred between folate supplementation and genotype in the mutant female tissue. Thus, our work extends the cases of folate-dependent gene–environment interactions to uterus.

Interestingly, the much broader transcriptional response in Folr4 mutants occurs even though the majority of cells in the wildtype uterus do not express Folr4 (see Fig. 2), indicating that other transporters are involved. Uterus tissue also expresses folate receptors Folr1 and Folr2, as well as the folate transporters Slc46a1 (proton-coupled folate transporter) and Slc19a1 (reduced folate carrier). Slc19a1, specifically, is ubiquitously expressed, which should permit folate entry into nearly every cell, particularly so under folate supplementation conditions. The wide distribution of the H3K4me3 signal in the supplemented mutant uterus underscores this interpretation, as it indicated that almost the entire tissue is capable of responding to folinic acid.

Under the theoretical assumption that the targeted disruption of the Folr4 gene has cell-autonomous effects, the parsimonious prediction would be that in the mutant, molecular responses to the genetic manipulation would be restricted to cells in the uterus that normally

express only Folr4. One would further expect that cells negative for Folr4 respond similarly to folinic acid supplementation, regardless whether they are in a wildtype or mutant animal; considering that the majority of cells in uterus are Folr4 negative, one might even expect the overall tissue responses to be quite similar. However, our results clearly demonstrate that this is not the case: quantity (number of genes) and quality (types of genes and associated pathways) of the response are altered by Folr4 deletion. This prompts our conclusion that the Folr4 mutation has non-cell-autonomous effects on neighboring cells, and that these effects also alter the response to folinic acid supplementation. Intriguingly, more than 50% of the genes affected by Folr4 deficiency in the mutant (Fig. 3A) respond to folinic acid supplementation, providing evidence that the Folr4 mutation and folinic acid supplementation converge on some pathways common to both paradigms. The most prominent pathways in our analysis are related to immune function and mitochondrial biology (Table 2 section D).

Additionally, it is conceivable that the Folr4 disruption, through biological action at earlier stages in development of females, may affect the uterine tissue cell-non-autonomously. Support for this possibility comes from genes like Follistatin: Fst responds to genotype and supplementation in opposing fashion, yet it is expressed in cells outside of the Folr4 expression domain (Fig. 4C), constituting an example for non-cell-autonomous regulation of expression by genotype. Taken together, our results strongly suggest that the initial lack of Folr4 predisposes cells outside of the Folr4 expression domain to mount a broader transcriptomic response than in the wildtype. Thus, genotype has a strong modifying effect on the response to folate supplementation. While the underlying mechanism for cell-non-autonomous actions of Folr4 remains to be investigated, we conclude that preexisting conditions, such as e.g. genetic liabilities, or susceptibilities introduced by environmental exposure, can exert a significant influence on size and repertoire of the response to folate supplementation. Thus, responses to folate can be modulated, and may depend on pre-existing conditions such as genetic, nutritional, or metabolic susceptibilities.

Furthermore, the notion that responses to folate are strongly modulated by genetic factors is underscored by the recent finding that folate status in mouse mutants of neural tube defect genes is not unequivocally beneficial: higher folate status relieved the neural tube defect burden in some of these mutant mouse strains, but aggravated it in others [14], demonstrating that the response to folate was highly dependent on the genetic background. In contrast to the mouse strains utilized in that study, homozygous Folr4 mutant embryos of both sexes develop normally and do not display neural tube defects; thus, they are clearly unsymptomatic with respect to neural tube defects. Yet, the significant modulation of the response to folinic acid by the Folr4 genotype raises the question how many other 'phenotypically unsymptomatic' genetic variations or mutations could have the potential to modulate the response to folate. The answer to this question has considerable implications for folate supplementation and fortification in humans. While functional consequences of human DNA variation are often milder than what is observed in inbred mouse models with targeted gene disruptions, it still stands to reason that folate also interacts with the genetic background in the human population, and may thus elicit distinct molecular responses in different individuals. If this were the case, health benefits from folate may vary by individual genetic constitution as well. Therefore, the concept that folate is unequivocally beneficial may deserve critical re-evaluation.

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Acknowledgements

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