

Effects of dietary folate and aging on gene expression in the colonic mucosa of rats: implications for carcinogenesis

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Folate depletion and aging are risk factors for colorectal cancer. We investigated the effects of folate status and aging on gene expression in the rat colon. Young (weanling) and older (12 month) rats were fed folic acid depleted (0 mg/kg) and supplemented (8 mg/kg) diets for 20 weeks. Gene expression was measured in colonic mucosal scrapings ($n = 3$ per group) using oligonucleotide arrays (Affymetrix U34A). Folate depletion induced the up-regulation of immune-related genes, urokinase and inducible nitric oxide synthase and the down-regulation of adhesion molecules (protocadherin-4, nidogen and integrin α V) and vascular endothelial growth factor in young rats. The abbreviated response to depletion in old rats (62 changes versus 136 in the young) included up-regulation of caspase-2 and deleted in colon cancer. Gene expression changes due to aging were more abundant in folate depleted than supplemented rats (38 versus 119 genes, respectively). In folate-deficient rats, aging induced the down-regulation of immune-related genes, urokinase, p53, insulin-like growth factor binding protein-3 and vav-1 oncogene. In folate supplemented rats, aging induced the down-regulation of vascular endothelial growth factor and caspase-2. Lower expression of adhesion molecules and higher expression of urokinase with folate depletion in young rats may indicate that cell detachment and migration, cancer-related processes, may be modulated by folate status. An age-related decline in p53 and IGF-BP3 expression was only observed in folate depleted animals, indicating that folate supplementation may reduce the risk for age-associated cancers by suppressing deleterious changes in the expression of certain genes.

Introduction

Folate is essential for the synthesis, repair and methylation of DNA, processes that are central to maintaining the integrity of

Abbreviations: 0-FA, 0 mg/kg folic acid; 8-FA, 8 mg/kg folic acid; DCC, deleted in colon cancer; ECM, extracellular matrix; H&E, hematoxylin and eosin; IELs, intra-epithelial lymphocytes; IGF-BP3, insulin-like growth factor binding protein 3; iNOS, inducible nitric oxide synthase; MHC major histocompatibility complexes; MMP-16, matrix metalloproteinase 16; TCR, T cell receptor; TIMP-2, tissue inhibitor of metalloproteinase 2; VEGF, vascular endothelial growth factor.

the genome. Folate depletion is reported to cause various genetic and epigenetic aberrations in mammalian cells, such as uracil misincorporation (1,2), genomic (3) and p53-specific (4) DNA breaks and genomic (5) and p53-specific (4) hypomethylation. These deleterious genetic and epigenetic changes are thought to underlie the inverse relationship between blood folate status and the risk for colorectal cancer. Indeed, it is estimated that people with the highest intakes of folate have a 30–40% lower risk for colorectal cancer than those with the lowest intakes (6). It is appropriate to study aging in conjunction with folate status in regard to colon carcinogenesis because the elderly often have impaired assimilation of dietary folate (7), colons of elder animals are particularly sensitive to folate depletion (8) and aging is perhaps the strongest risk factor for colorectal cancer, with the risk increasing from 0.07 to 0.87 to 4.0% in people aged 0–39, 40–59 and 60–79, respectively (9).

Deleterious genetic and epigenetic changes due to inadequate folate may promote or accelerate carcinogenesis by changing the expression of genes involved in the regulation of the cell cycle, cell death and other critical processes, such as DNA repair. Folate deficiency may affect gene expression by disturbing DNA methylation, inducing DNA breaks within the gene, inducing gene deletions and by inducing gene amplification. DNA methylation is an important method of gene silencing (10) and folate depletion is reported to cause genomic hypomethylation in humans (5) and in cell culture (11) and p53 hypomethylation in rats (4). In addition, folate depletion may induce gene amplification *in vitro* (12), probably via breakage–fusion–bridge cycles, a random process initiated by double-stranded DNA breakage (1,13). Although several mechanisms by which folate depletion disrupts DNA integrity are well characterized, the down-stream effects of these molecular changes are less clear. Particularly, we know very little of the gene pathways through which folate depletion may enhance colorectal carcinogenesis.

Cell culture studies utilizing northern blotting and RT–PCR to study the abundance of specific mRNA transcripts show that folate depletion causes an up-regulation of folate receptor α (12), folate-binding protein (14) and metallothionein (15). Furthermore, Kim *et al.* (16) reported that p53 expression in the rat colon is reduced by severe folate depletion and increased above basal levels with supplementation and plasma and mucosal folate concentrations are positively correlated with adenomatous polyposis coli mRNA levels.

Gene array technology has been used extensively to profile the gene expression patterns of various cancers, however, the use of gene arrays to study the effect of nutritional intervention, and more specifically folate depletion, is very much in its infancy. To date, only one published study has investigated the effect of folate status on mass gene expression (17). Human nasopharyngeal epidermoid carcinoma cells were grown in medium containing either 2 mM (replete) or 2–10 nM (deplete) folic acid and gene expression was analyzed using cDNA

arrays. Of the approximately 2000 genes studied, five genes were up-regulated and three genes down-regulated in response to folic acid depletion. Among these was H-cadherin, a cell adhesion molecule commonly down-regulated in cancers, which showed a 2.5-fold reduction in gene expression. This down-regulation was associated with hypermethylation of a CpG island in the first exon and intron of the H-cadherin gene.

We therefore aimed to study the effects of dietary folate depletion in combination with aging on gene expression in the rat colon using oligonucleotide probe arrays which probe for almost 9000 transcripts. These studies further our understanding of how folate depletion and aging disturb the cellular milieu and predispose to colorectal neoplasia.

Materials and methods

Details of the animal protocol are reported elsewhere (8). Briefly, weaning ($n = 16$) and middle aged (12 month old, $n = 16$) male Sprague–Dawley rats were fed amino acid-defined diets containing 0 (0-FA) or 8 mg/kg folic acid (8-FA). Water was supplied *ad libitum* and diet consumption was matched to the group consuming the lowest amount within each age group. In the absence of any added sulfonamides in the diet, this regimen has been established to produce a moderate, not severe, degree of folate depletion (8). After 20 weeks on the diet, rats were anesthetized in a carbon dioxide breathing chamber and the abdomen was opened. Plasma and colonic samples were obtained before the animals were killed by cutting the aorta. The colorectum was removed, opened longitudinally on an ice-cold glass plate and washed with ice-cold saline. A small section of the transverse colon was immediately fixed in formalin and embedded in paraffin within 24 h. The tissue was then sectioned, mounted on a glass microscope slide and stained with hematoxylin and eosin (H&E). The remaining colon was gently scraped with a glass microscope slide to remove the mucosa as previously described (18). Mucosal scrapings were divided into several aliquots before being frozen in liquid nitrogen and stored at -70°C .

Colonic sections were examined for variations in epithelial and submucosal lymphocyte populations in a blind fashion. An experienced pathologist quantified the presence of intra-epithelial lymphocytes (IELs) per high power (400 \times) field of view. An average of 10 randomly selected fields across the section was taken. Furthermore, the presence of lymphocytes in the lamina propria was semi-quantitatively assessed using a rating of 1–3 after visual inspection of the entire section. No obvious lesions or abnormalities in cell distribution or morphology were detected in any of the sections.

Total RNA was isolated from an aliquot of colonic mucosa using TRIzol reagent (Invitrogen, San Diego, CA). RNA samples were randomly selected from 3 animals per group (total = 12) and 5 μg total RNA was prepared for gene expression analysis by oligonucleotide array (U34; Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Hybridized, stained and washed probe arrays were scanned using a laser scanner (Affymetrix). The fluidics station and scanner were controlled by MicroArray Suite 5.0 software (Affymetrix).

Abundances of urokinase and GAPDH transcripts were analyzed using RT–PCR. Primer sequences and product lengths were as follows: GAPDH forward, accatcaccattctccagga; GAPDH reverse, agccttccatggtggtgaa (109 bp); urokinase forward, caatgctcacagatccgatgc; urokinase reverse, gccaatgg-cacatagcacca (103 bp). One nanogram of total RNA was reverse transcribed and then 2.5 or 5 μl of the RT product was amplified by PCR (both using a SYBR Green RT–PCR kit; Applied Biosystems, Foster City, CA) using an ABI7700 sequence detector system controlled by SDS version 1.7a (Applied Biosystems). Dissociation curves were generated for each assay using Dissociation Curve software version 1.0 (Applied Biosystems) to ensure that non-specific amplification did not occur. All PCR products were analyzed by gel electrophoresis to ensure that the amplicon was of the anticipated length. Primers were designed using Primer Express version 2.0 software (Applied Biosystems) and synthesized by the Tufts University core facility. BLAST searches were performed on primer sequences using NCBI to ensure primer specificity.

Fluorescence intensity data from gene array experiments were exported to MS Excel and then into SAS version 8.02 (SAS Institute, Cary, NC) for analysis. Differences between groups were tested using two-way ANOVA followed by Tukey's post-test and significance was accepted at $P < 0.05$. Genes with a 1.5-fold or greater change in expression are reported. Fold changes are discussed with the 8-FA (supplemented) and young groups being the reference or control groups for the up- or down-regulation in the 0-FA

Table I. Intra-epithelial lymphocytes in H&E stained colonic sections

	0 mg/kg FA	8 mg/kg FA
Young	8.1 \pm 1.9	6.7 \pm 0.7
Old	4.9 \pm 1.0	5.4 \pm 1.5

Data are expressed as number of IELs per high power field (400 \times). $n = 3$ per group. Data are means \pm SEM. ANOVA $P = 0.41$.

Table II. Number of genes changing for each comparison

2-way ANOVA $P < 0.05$	Tukey's post-test $P < 0.05$ (no fold change restrictions)	$P < 0.05$ and ≥ 1.5 -fold change	Total		
			Down	Up	
487	I. Young 0-FA ^a versus Young 8-FA	162	136	84	52
	II. Old 0-FA ^a versus Old 8-FA	69	62	21	41
	III. Young 0-FA ^a versus Old 0-FA	140	119	71	48
	IV. Young 8-FA ^a versus Old 8-FA	60	38	25	13

^aReference value for comparison.

(depleted) or old groups (see Table III). Data regarding the presence of IELs and RT–PCR were also analyzed by two-way ANOVA using SAS software.

Results

Microscopy

The number of IELs present is shown in Table I. There was no significant difference in the number of IELs per high power field ($P = 0.41$). Semi-quantitative analysis of the lamina propria also failed to detect any differences in the presence of lymphocytes (data not shown). No overt pathologies or lesions were present and all sections appeared normal and healthy.

Probe array analysis

The number of genes meeting the criteria for a significant change is shown in Table II. FA depletion induced more than twice the number of changes in expression in young animals than old animals (comparison I versus II). Similarly, the observed difference in gene expression between age groups was >2 -fold greater under conditions of FA depletion than supplementation (comparison III versus IV). Thus age appears to have a large impact on the colon's response to folate depletion and folate depletion magnifies the extent of expression differences between age groups.

The names and data for the majority of changed genes are reported in Tables III and IV. Only annotated genes are reported. Genes considered to be of low relevance are omitted for the sake of brevity, but the entire list of genes whose expression was altered is available as Supplementary Tables 1–4 (see Supplementary material).

Folate effect in the young. In the comparison of FA intakes in the young, 84 genes were down-regulated and 52 genes up-regulated in response to depletion. Of particular interest was the up-regulation of several immune cell-specific transcripts, including T cell receptor (TCR) α , δ and ζ chains (3.7- to 13.5-fold) and subunits of major histocompatibility

Table III. Effect of dietary folic acid on colonic gene expression in young and old rats

Folate effect in young rats								
GenBank no.	Affymetrix probe ID	Gene description		Young- 0 mg/kg FA	Young- 8 mg/kg FA	Fold change	ANOVA <i>P</i>	Tukey <i>P</i>
U75412	U75412cds_at	Anti-idiotype immunoglobulin M heavy chain gene	I	9.4 ± 1.0	1.9 ± 0.3	+4.9	0.027	0.022
U76836	U76836_g_at	T-cell receptor (CD3) alpha 2 chain	I	61.8 ± 15.1	16.5 ± 7.0	+3.7	0.029	0.039
X53430	X53430_at	T-cell receptor (CD3) delta chain	I	160.4 ± 64.9	11.9 ± 1.8	+13.5	0.030	0.048
D13555	D13555_at	T-cell receptor (CD3) zeta chain	I	55.3 ± 15.2	13.3 ± 2.3	+4.1	0.012	0.022
X78985	X78985cds_s_at	CD5 antigen	I	51.0 ± 11.4	16.7 ± 1.7	+3.0	0.011	0.029
X03015	X03015_at	CD8 antigen alpha-chain	I	71.7 ± 16.6	21.2 ± 2.1	+3.4	0.003	0.013
D29646	D29646_at	CD38 antigen	I	114.6 ± 8.1	53.8 ± 12.8	+2.1	0.005	0.008
AI045440	rc_AI045440_at	CD43 (Sialophorin/leukosianin)	I	48.2 ± 10.1	15.8 ± 1.7	+3.1	0.008	0.018
AF009133	AF009133_at	CD94 antigen	I	21.9 ± 6.0	6.9 ± 0.4	+3.2	0.016	0.034
M10094	M10094_at	MHC Ib (truncated)	I	9.4 ± 1.7	3.2 ± 0.5	+2.9	0.010	0.015
M15562	M15562_at	MHC II alpha chain RT1.D alpha (u)	I	814.5 ± 253.7	227.7 ± 15.4	+3.6	0.024	0.048
M15562	M15562_g_at	MHC II alpha chain RT1.D alpha (u)	I	2170.6 ± 534.7	829.1 ± 56.3	+2.6	0.020	0.035
Z49761	Z49761_at	MHC II alpha chain	I	55.9 ± 5.7	26.9 ± 4.9	+2.1	0.000	0.005
AA818894	rc_AA818894_at	Proteoglycan peptide core protein		57.5 ± 11.2	18.9 ± 2.9	+3.0	0.008	0.009
AF083269	AF083269_g_at	Actin-related protein complex 1b		542.1 ± 59.8	339.6 ± 38.0	+1.6	0.020	0.029
U77038	U77038_g_at	Protein tyrosine phosphatase non-receptor type 6		187.7 ± 30.6	87.7 ± 13.2	+2.1	0.012	0.032
X63434	X63434_at	Urokinase	A	26.7 ± 3.1	12.7 ± 1.7	+2.1	0.006	0.008
U67908	U67908_at	Chymase 1	A	82.6 ± 12.9	24.6 ± 3.4	+3.4	0.002	0.003
X14674	X14674_at	Protamine 2		15.0 ± 1.1	7.2 ± 1.7	+2.1	0.032	0.029
D10729	D10729_s_at	Proteasome subunit RC1		234.3 ± 35.8	125.2 ± 6.4	+1.9	0.013	0.026
D10757	D10757_at	Low molecular mass polypeptide 2		85.6 ± 8.1	51.9 ± 4.7	+1.6	0.013	0.019
D10757	D10757_g_at	Low molecular mass polypeptide 3		187.5 ± 18.2	109.5 ± 8.3	+1.7	0.003	0.009
D17370	D17370_at	Cystathionine gamma-lyase		131.7 ± 23.3	56.3 ± 9.7	+2.3	0.017	0.033
U75689	U75689_s_at	Deoxyribonuclease I-like 3		32.9 ± 3.0	5.3 ± 2.8	+6.2	0.000	0.000
U03699	U03699	Nitric oxide synthase 2 (inducible)	C	8.8 ± 2.0	2.0 ± 0.5	+4.4	0.038	0.043
U39476	U39476_at	Vav 1 oncogene	C	28.2 ± 2.7	14.2 ± 2.1	+2.0	0.008	0.018
E13573	E13573cds_s_at	Death protein 5	C	29.7 ± 1.2	10.8 ± 4.6	+2.8	0.039	0.029
X76489	X76489cds_at	CD9 antigen	I	260.4 ± 30.7	484.8 ± 27.3	-1.9	0.007	0.006
X61654	X61654_at	CD63 antigen	I	1152.8 ± 57.4	1966.9 ± 74.5	-1.7	0.001	0.001
AJ005023	AJ005023_at	MHC Ib alpha chain	I	6.1 ± 1.9	17.1 ± 0.8	-2.8	0.008	0.019
AJ001713	AJ001713_at	Myosin-rhogap		8.6 ± 0.5	16.6 ± 2.1	-1.9	0.017	0.015
M28654	M28654cds_at	Rat myosin heavy chain		7.7 ± 1.9	30.2 ± 3.0	-3.9	0.017	0.016
AJ002556	AJ002556_s_at	Microtubule-associated protein 6		1.9 ± 0.1	5.0 ± 0.8	-2.6	0.007	0.005
L28818	L28818cds_at	Involucrin	A	0.9 ± 0.3	4.7 ± 1.0	-5.0	0.038	0.036
M15797	M15797_at	Nidogen (entactin)	A	0.8 ± 0.0	1.5 ± 0.1	-1.9	0.000	0.001
L02896	L02896_at	Glypican 1	A	44.6 ± 3.9	89.7 ± 9.5	-2.0	0.005	0.007
S58528	S58528_at	Integrin alpha V subunit	A	7.0 ± 1.0	11.9 ± 1.2	-1.7	0.025	0.031
U35371	U35371_at	Axonal-associated cell adhesion molecule	A	1.6 ± 0.5	7.7 ± 0.5	-4.7	0.007	0.007
D31854	D31854_s_at	Subtilisin - like endoprotease	A	6.4 ± 0.8	12.0 ± 1.2	-1.9	0.011	0.033
D63886	D63886_s_at	Matrix metalloproteinase 16	A	2.1 ± 0.7	17.5 ± 2.2	-8.3	0.002	0.002
M11794	M11794cds#2_f_at	Metallothionein	A	790.7 ± 66.6	1592.4 ± 92.5	-2.0	0.027	0.018
M61142	M61142_at	Metalloendopeptidase	A	22.9 ± 1.9	36.8 ± 1.5	-1.6	0.007	0.005
S72594	S72594_s_at	Tissue inhibitor of metalloproteinase 2	A	401.1 ± 72.4	834.0 ± 118.0	-2.1	0.043	0.039
AB004276	AB004276_s_at	Protocadherin 4	A	5.3 ± 0.9	18.4 ± 1.6	-3.5	0.041	0.028
U86635	U86635_at	glutathione S-transferase mu 5		64.1 ± 12.7	153.5 ± 10.6	-2.4	0.007	0.007
U86635	U86635_g_at	glutathione S-transferase mu 5		63.7 ± 14.2	155.8 ± 7.6	-2.4	0.004	0.003
AB012234	AB012234_g_at	NF1-X1 protein		47.3 ± 8.5	117.3 ± 9.1	-2.5	0.050	0.045
AB012235	AB012235_at	NF1-X1 protein		10.9 ± 2.1	40.1 ± 1.9	-3.7	0.019	0.012
D12769	D12769_at	Kruppel-like factor 9		107.6 ± 9.5	195.4 ± 13.7	-1.8	0.001	0.001
D12927	D12927_at	Transcription elongation factor S-II		16.0 ± 1.9	26.4 ± 2.2	-1.7	0.006	0.007
L20913	L20913_s_at	Vascular endothelial growth factor	C	4.3 ± 1.3	13.4 ± 1.0	-3.1	0.032	0.047
AA799560	rc_AA799560_at	N-myc downstream-regulated gene 2	C	184.0 ± 42.2	370.1 ± 14.8	-2.0	0.014	0.028
AI071299	rc_AI071299_at	TGF-β inducible early growth response protein 1	C	43.6 ± 8.0	93.3 ± 10.8	-2.1	0.022	0.014
S77492	S77492_i_at	Bone morphogenetic protein 3	C	53.4 ± 17.3	132.2 ± 18.5	-2.5	0.039	0.028
AB015432	AB015432_s_at	Tumor-associated protein 1	C	50.9 ± 1.4	76.1 ± 5.6	-1.5	0.032	0.023
S79820	S79820_g_at	Hepatic leukemia factor-alpha isoform	C	0.8 ± 0.0	1.6 ± 0.1	-2.0	0.017	0.033
Z38067	Z38067exon_g_at	c-Myc exon 2	C	28.6 ± 3.4	60.6 ± 3.0	-2.1	0.028	0.019
L13151	L13151cds_at	Ras GTPase-activating protein 1	C	15.4 ± 1.6	25.0 ± 2.6	-1.6	0.036	0.040
AF042713	AF042713_at	Neurexophilin 3		6.2 ± 0.6	13.6 ± 1.7	-2.2	0.016	0.046
M92340	M92340_at	Interleukin 6 signal transducer		12.0 ± 5.2	35.5 ± 7.3	-3.0	0.049	0.039
D42148	D42148_at	Growth potentiating factor		64.7 ± 6.9	156.0 ± 33.5	-2.4	0.045	0.033
U75899	U75899mRNA_g_at	Heat shock 27 kDa protein 2		38.3 ± 5.2	73.8 ± 9.3	-1.9	0.042	0.049
AF077354	AF077354_g_at	Heat shock 70 kDa protein 4		86.6 ± 0.9	132.9 ± 9.4	-1.5	0.006	0.007
AI045249	rc_AI045249_at	Heat shock 70 kDa protein 8		27.2 ± 2.1	52.9 ± 4.3	-1.9	0.012	0.008
U30186	U30186_at	DNA-damage inducible transcript 3		36.5 ± 5.4	64.6 ± 6.2	-1.8	0.009	0.031

Table III. Continued

Folate effect in old rats								
GenBank no.	Affymetrix probe ID	Gene description		Old-0 mg/kg FA	Old-8 mg/kg FA	Fold change	ANOVA <i>P</i>	Tukey <i>P</i>
AF072892	AF072892_s_at	Chondroitin sulfate proteoglycan 2 (versican)		10.5 ± 2.7	2.8 ± 1.0	+3.8	0.018	0.030
J00692	J00692_at	Alpha-actin		160.4 ± 8.0	96.0 ± 11.8	+1.7	0.000	0.002
D90404	D90404_at	Cathepsin C		187.9 ± 19.3	125.3 ± 3.0	+1.5	0.026	0.019
J04215	J04215_at	Integrin-binding sialoprotein	A	3.6 ± 1.0	0.5 ± 0.1	+7.6	0.008	0.010
M15797	M15797_at	Nidogen (entactin)	A	1.5 ± 0.0	0.9 ± 0.1	+1.6	0.000	0.004
U50044	U50044cnds_at	von Willebrand factor		5.5 ± 0.8	1.5 ± 0.6	+3.7	0.013	0.023
D38072	D38072_at	Protein tyrosine phosphatase, non-Rc type 12		32.5 ± 2.5	20.1 ± 1.1	+1.6	0.023	0.016
U09307	U09307_at	Protein tyrosine phosphatase, non-Rc type 11		7.0 ± 0.4	1.5 ± 0.3	+4.6	0.008	0.008
rc_AA892562	rc_AA892562_g_at	Dyskeratosis congenita 1		52.4 ± 3.1	33.0 ± 6.1	+1.6	0.038	0.049
E03424	E03424cnds_s_at	GTP cyclohydrolase 1		40.8 ± 8.9	10.2 ± 0.8	+4.0	0.027	0.018
X65190t	X65190mRNA_s_at	Cytochrome b5 reductase		31.2 ± 7.0	11.3 ± 0.6	+2.8	0.036	0.049
S79676	S79676_s_at	Interleukin 1β converting enzyme		464.1 ± 79.9	241.1 ± 25.3	+1.9	0.025	0.040
U17253	U17253_at	Ngfi-A binding protein 1		18.1 ± 1.2	10.6 ± 0.5	+1.7	0.012	0.015
AF025671	AF025671_s_at	Caspase-2	C	36.8 ± 5.6	18.7 ± 4.0	+2.0	0.026	0.048
U68725	U68725_at	Deleted in colorectal cancer	C	6.8 ± 1.2	0.9 ± 0.2	+7.6	0.001	0.002
L00124	L00124_at	Elastase 2	A	28.5 ± 6.9	51.2 ± 3.8	-1.8	0.019	0.029
AB016532	AB016532_at	Period homolog 2		63.5 ± 6.4	95.0 ± 6.9	-1.5	0.001	0.012
L08812	L08812_at	Transcription factor EC		3.9 ± 0.2	15.6 ± 3.4	-4.0	0.018	0.019

I, genes involved in immunity.

A, genes involved in cell adhesion and extracellular matrix remodeling.

C, genes potentially involved in carcinogenesis.

Data are absolute fluorescence (means ± SEM). *n* = 3 per group.

complexes (MHC) I and II (2.1- to 3.6-fold), urokinase (2.1-fold) and inducible nitric oxide synthase (iNOS) (4.4-fold) with FA supplementation.

Several genes that are associated with cell-extracellular matrix (ECM) interactions and tissue remodeling were down-regulated with depletion. These included the adhesion molecules protocadherin-4 (3.5-fold), nidogen (1.9-fold) and integrin α V (1.7-fold), the ECM-digesting proteases matrix metalloproteinase 16 (MMP-16) (8.3-fold) and metalloendopeptidase (1.6-fold) and the angiogenesis promoter vascular endothelial growth factor (VEGF) (3.1-fold). In addition, tissue inhibitor of metalloproteinase 2 (TIMP-2) (2.1-fold), three heat shock proteins (HSP 27 kDa protein 2 and 70 kDa proteins 4 and 8) (1.5- to 1.9-fold) and c-Myc (2.1-fold) were down-regulated (Table III).

Folate effect in the old. In old rats, FA depletion altered the expression of fewer genes than in the young, with only 21 genes down-regulated and 41 up-regulated. Among the up-regulated genes were caspase-2 (2.0-fold) and deleted in colon cancer (DCC) (7.6-fold). Except for nidogen, which was down-regulated in the young and up-regulated in the old animals, there was no overlap in which genes underwent expression changes in young and old rats as a result of FA depletion (Table III).

Aging effect during folate depletion. Older age caused the up-regulation of 48 genes and down-regulation of 71 genes in 0-FA rats. Among the down-regulated genes were several immune cell-specific transcripts including CD antigens (5, 8, 36, 38, 43, 45, 74 and 94) (1.6- to 5.0-fold), TCR α , β , δ and ζ chains (3.5- to 14.1-fold) and subunits of MHC I and II (2.6- to 5.2-fold). Several ECM-interacting genes were also down-regulated, including caveolin 3 (5.7-fold), mast cell protease (2.0-fold), chymase 1 (2.0-fold), urokinase (1.8-fold), integrin β 7 (2.4-fold) and glycosylation-dependent cell adhesion molecule (4.6-fold). In addition, p53 (1.7-fold), insulin-like

growth factor binding protein 3 (3.2-fold) and vav 1 oncogene (1.9-fold) were down-regulated.

Up-regulated genes included DCC (5.9-fold) and nidogen (1.8-fold) (Table IV).

Aging effect during folate supplementation. The aging effect, in terms of numbers of genes changing, was lower in the 8-FA group, with only 13 genes up-regulated and 25 genes down-regulated. Among these changes was the down-regulation of caspase-2 (2.0-fold) and VEGF (3.3-fold). α -Actin was up-regulated in both the 8-FA (1.8-fold) and 0-FA (3.2-fold) groups, whereas nidogen was up-regulated in the 8-FA (1.8-fold) and down-regulated in the 0-FA (1.6-fold) groups with advancing age (Table IV).

RT-PCR analysis

Urokinase expression was analyzed by RT-PCR and normalized for GAPDH expression. Gene array analysis showed that urokinase expression was approximately 2-fold lower in young 0-FA than young 8-FA and old 0-FA colons ($P < 0.05$). This effect was confirmed by RT-PCR, however, it did not reach statistical significance (Figure 1). It should be noted that small changes in expression are difficult to detect by RT-PCR because a 2-fold difference in expression translates to only a one cycle difference in C_t (ΔC_t) because the PCR product theoretically doubles every cycle during the exponential phase of the PCR. Perhaps more relevant to the concordance of the RT-PCR and gene array data was the fact that both end points were significantly and negatively correlated ($r^2 = 0.54$, $P = 0.007$, $n = 12$).

Discussion

Using oligonucleotide probe arrays we have identified several groups of genes that are sensitive to dietary FA depletion and aging in the colonic mucosa of rats. Both age and folate status had marked effects on gene expression in this organ. Age has

Table IV. Effect of aging on colonic gene expression in folate depleted and supplemented rats

Aging effect in folate depleted rats								
GenBank no.	Affymetrix probe ID	Gene description		Young- 0 mg/kg FA	Old- 0 mg/kg FA	Fold change	ANOVA <i>P</i>	Tukey <i>P</i>
AA801174	rc_AA801174_at	Ig rearranged mu-chain C region	I	290.6 ± 83.8	47.5 ± 21.9	-6.1	0.018	0.021
L11025	L11025_at	T-cell receptor (CD3) alpha chain	I	8.6 ± 1.9	2.4 ± 0.3	-3.5	0.040	0.047
X14319	X14319cds_g_at	T-cell receptor (CD3) beta chain	I	199.3 ± 70.6	32.7 ± 10.4	-6.1	0.030	0.046
X53430	X53430_at	T-cell receptor (CD3) delta chain	I	160.4 ± 64.9	11.4 ± 1.9	-14.1	0.030	0.048
D13555	D13555_at	T-cell receptor (CD3) zeta chain	I	55.3 ± 15.2	11.2 ± 0.6	-4.9	0.012	0.017
X78985	X78985cds_s_at	CD5 antigen	I	51.0 ± 11.4	12.3 ± 3.4	-4.1	0.011	0.016
X03015	X03015_at	CD8 antigen alpha-chain	I	71.7 ± 16.6	20.6 ± 3.7	-3.5	0.003	0.012
D10587	D10587_g_at	CD36 antigen	I	103.4 ± 6.9	63.4 ± 4.6	-1.6	0.000	0.002
D29646	D29646_at	CD38 antigen	I	114.6 ± 8.1	58.6 ± 11.4	-2.0	0.005	0.013
AI045440	rc_AI045440_at	CD43 (Sialophorin/leukosianin)	I	48.2 ± 10.1	10.9 ± 3.6	-4.4	0.008	0.008
M25823	M25823_s_at	CD45 antigen	I	59.7 ± 14.3	19.7 ± 4.2	-3.0	0.025	0.024
X13044	X13044_at	CD74 antigen	I	1196.8 ± 362.5	297.2 ± 55.0	-4.0	0.032	0.040
X13044	X13044_g_at	CD74 antigen	I	1496.1 ± 509.8	301.4 ± 15.6	-5.0	0.037	0.046
AF009133	AF009133_at	CD94 antigen	I	21.9 ± 6.0	5.5 ± 0.6	-4.0	0.016	0.022
M10094	M10094_at	MHC Ib (truncated)	I	9.4 ± 1.7	3.2 ± 1.0	-3.0	0.010	0.014
M15562	M15562_at	MHC II alpha chain RT1.D alpha (u)	I	814.5 ± 253.7	204.9 ± 30.0	-4.0	0.024	0.040
M15562	M15562_g_at	MHC II alpha chain RT1.D alpha (u)	I	2170.6 ± 534.7	828.0 ± 99.0	-2.6	0.020	0.035
X53054	X53054_at	MHC II RT1.D beta chain.	I	98.3 ± 28.3	22.0 ± 2.2	-4.5	0.024	0.029
X53054	X53054_g_at	MHC II RT1.D beta chain.	I	377.4 ± 114.2	90.9 ± 5.2	-4.2	0.030	0.038
X56596	X56596_s_at	MHC II RT1.B-1 beta-chain	I	243.7 ± 63.0	77.3 ± 2.9	-3.2	0.035	0.037
Z49761	Z49761_at	MHC II RT1.Ma	I	55.9 ± 5.7	10.8 ± 0.8	-5.2	0.000	0.000
X52082	X52082cds_s_at	T-cell alloantigen RT6.1	I	16.1 ± 4.4	4.6 ± 0.7	-3.5	0.020	0.033
AJ001184	AJ001184_at	Linker for activation of T cells	I	106.7 ± 29.3	32.8 ± 3.9	-3.3	0.026	0.033
AA818894	rc_AA818894_at	Proteoglycan peptide core protein	I	57.5 ± 11.2	24.1 ± 1.7	-2.4	0.008	0.020
M34253	M34253_at	Interferon regulatory factor 1	I	98.1 ± 9.1	61.6 ± 5.4	-1.6	0.018	0.037
M55050	M55050_at	Interleukin 2 receptor beta chain	I	52.7 ± 5.6	25.3 ± 4.8	-2.1	0.011	0.021
AI043968	rc_AI043968_g_at	Caveolin 3	A	4.0 ± 0.6	0.7 ± 0.2	-5.7	0.047	0.037
S44606	S44606_s_at	Integrin beta 7	A	53.3 ± 1.7	21.9 ± 2.8	-2.4	0.001	0.001
U67908	U67908_at	Chymase 1	A	82.6 ± 12.9	29.4 ± 1.1	-2.8	0.002	0.006
U67915	U67915_at	Mast cell protease 1	A	423.8 ± 35.9	212.8 ± 26.5	-2.0	0.039	0.045
X63434	X63434_at	Urokinase	A	26.7 ± 3.1	15.2 ± 2.0	-1.8	0.006	0.023
L08100	L08100_at	Glycosylation dependent cell adhesion molecule 1	A	96.3 ± 17.0	20.9 ± 6.1	-4.6	0.032	0.022
X03468	X03468_at	Apolipoprotein A-II		400.8 ± 71.7	166.4 ± 11.3	-2.4	0.029	0.026
D10757	D10757_g_at	Low molecular mass polypeptide 2		187.5 ± 18.2	108.4 ± 14.0	-1.7	0.003	0.008
M37941	M37941mRNA_s_at	Adenosine monophosphate deaminase 1		7.5 ± 1.0	3.3 ± 0.4	-2.3	0.011	0.010
X62086	X62086mRNA_s_at	Cytochrome P450 3A1		186.9 ± 17.0	84.8 ± 26.2	-2.2	0.018	0.034
U77038	U77038_g_at	Protein tyrosine phosphatase, non-receptor type 6		187.7 ± 30.6	84.4 ± 18.5	-2.2	0.012	0.028
L19180	L19180_at	Protein tyrosine phosphatase, receptor type D		120.7 ± 23.8	16.3 ± 1.2	-7.4	0.041	0.028
AI072089	rc_AI072089_at	Casein kinase II alpha chain		34.1 ± 3.1	18.2 ± 3.9	-1.9	0.011	0.029
AI227887	rc_AI227887_g_at	Cell division cycle 42 homolog		717.6 ± 23.7	448.7 ± 59.2	-1.6	0.004	0.003
U75689	U75689_s_at	Deoxyribonuclease gamma (I-like 3)	C	32.9 ± 3.0	12.7 ± 3.3	-2.6	0.000	0.003
L26267	L26267_at	Nuclear factor kappa B p105 subunit	C	302.5 ± 26.7	186.6 ± 13.4	-1.6	0.002	0.005
AI009405	rc_AI009405_s_at	Insulin-like growth factor-binding protein 3	C	15.5 ± 3.3	4.8 ± 0.8	-3.2	0.025	0.043
U39476	U39476_at	Vav 1 oncogene	C	28.2 ± 2.7	14.6 ± 1.1	-1.9	0.008	0.021
X13058	X13058_at	Tumor protein p53 (Li-Fraumeni syndrome)	C	158.8 ± 14.8	95.6 ± 5.7	-1.7	0.006	0.007
AJ005023	AJ005023_at	MHC class Ib alpha chain	I	6.1 ± 1.9	18.7 ± 0.8	+3.0	0.008	0.009
X52713	X52713_at	Myxovirus (influenza virus) resistance 3	I	11.6 ± 2.8	34.4 ± 1.0	+3.0	0.002	0.003
AA799389	AA799389_at	Rab3B protein		4.9 ± 2.3	17.1 ± 1.4	+3.5	0.002	0.002
J00692	J00692_at	Alpha-actin		49.5 ± 4.7	160.4 ± 8.0	+3.2	0.000	0.000
M28654	M28654cds_at	Myosin heavy chain		7.7 ± 1.9	26.2 ± 5.7	+3.4	0.017	0.042
J04215	J04215_at	Integrin-binding sialoprotein (bone sialoprotein II)	A	0.8 ± 0.2	3.6 ± 1.0	+4.5	0.008	0.018
M15797	M15797_at	Nidogen (entactin)	A	0.8 ± 0.0	1.5 ± 0.0	+1.8	0.000	0.001
L02896	L02896_at	Glypican 1	A	44.6 ± 3.9	79.9 ± 7.1	+1.8	0.005	0.027
X65190	X65190mRNA_s_at	Cytochrome b5 reductase		11.2 ± 2.2	31.2 ± 7.0	+2.8	0.036	0.047
M98826	M98826mRNA_g_at	Phosphorylase kinase gamma		1.5 ± 0.3	7.7 ± 1.3	+5.2	0.013	0.017
U09307	U09307_at	Protein tyrosine phosphatase non-receptor type 11		2.4 ± 0.9	7.0 ± 0.4	+2.9	0.008	0.023
U67137	U67137_at	Guanylate kinase associated protein		2.7 ± 0.7	11.8 ± 2.1	+4.3	0.037	0.049
D12927	D12927_at	Transcription elongation factor S-II		16.0 ± 1.9	25.3 ± 1.2	+1.6	0.006	0.013
U17253	U17253_at	Ngfi-A binding protein 1		11.0 ± 0.1	18.1 ± 1.2	+1.6	0.012	0.021
S79820	S79820_g_at	Hepatic leukemia factor-alpha isoform		0.8 ± 0.0	1.6 ± 0.3	+2.0	0.017	0.033
U41853	U41853_at	Oxygen regulated protein (150kD)		15.6 ± 3.2	28.0 ± 1.8	+1.8	0.048	0.035
U68725	U68725_at	Deleted in colorectal cancer	C	1.2 ± 0.3	6.8 ± 1.2	+5.9	0.001	0.003

Table IV. *Continued*

Aging effect in folate supplemented rats

GenBank no.	Affymetrix probe ID	Gene description		Young- 8 mg/kg FA	Old- 8 mg/kg FA	Fold change	ANOVA <i>P</i>	Tukey <i>P</i>
K00512	K00512_at	Myelin basic protein		10.3 ± 1.2	3.5 ± 0.4	-3.0	0.005	0.011
AF020210	AF020210_s_at	Dynamin 1-like		51.8 ± 3.0	31.6 ± 3.9	-1.6	0.019	0.033
U35371	U35371_at	Axonal-associated cell adhesion molecule	A	7.7 ± 0.5	2.9 ± 1.4	-2.7	0.007	0.025
M15797	M15797_at	Nidogen (entactin)	A	1.5 ± 0.1	0.9 ± 0.1	-1.6	0.000	0.003
D63886	D63886_s_at	Matrix metalloproteinase 16	A	17.5 ± 2.2	9.1 ± 2.5	-1.9	0.002	0.048
X52583	X52583cds_s_at	Nuclear pore glycoprotein P62		14.6 ± 1.3	3.1 ± 1.7	-4.7	0.026	0.019
D14568	D14568_at	Protein phosphatase 3 regulatory subunit B		50.3 ± 3.9	27.8 ± 1.3	-1.8	0.039	0.047
U76557	U76557_at	O-linked N-acetylglucosamine transferase		42.8 ± 2.9	26.2 ± 2.9	-1.6	0.017	0.011
AB017140	AB017140_g_at	Homer 1 (neuronal immediate early gene)		43.1 ± 3.8	28.0 ± 1.6	-1.5	0.011	0.015
L02121	L02121_at	Cyclin-dependent kinase 5		35.9 ± 4.5	17.6 ± 2.5	-2.0	0.034	0.045
L20913	L20913_s_at	Vascular endothelial growth factor	C	13.4 ± 1.0	4.0 ± 1.0	-3.3	0.032	0.041
AF025671	AF025671_s_at	Caspase-2	C	38.2 ± 2.1	18.7 ± 4.0	-2.0	0.026	0.033
J00692	J00692_at	Alpha actin		52.7 ± 5.8	96.0 ± 11.8	+1.8	0.000	0.022
AB016532	AB016532_at	Period homolog 2		54.8 ± 1.5	95.0 ± 6.9	+1.7	0.001	0.003

I, Genes involved in immunity.

A, Genes involved in cell adhesion and extracellular matrix remodelling.

C, Genes potentially involved in carcinogenesis.

Data are absolute fluorescence (means ± SEM). *n* = 3 per group.

previously been shown to have a significant impact on both molecular end points in the colon (19) as well as on its cell biology (20), and our observations delineate the broad array of changes that may underlie many of the alterations in the biology of the older colon. We find it particularly remarkable that alterations in folate status modified the expression of a set of genes in the older colon that was very distinct from the set of genes altered in the young colon, underscoring the profound changes in the colon due solely to the effect of age. In fact, only one gene (nidogen) changed in both young and old rats in response to dietary folate depletion.

Apart from the lack of overlap in genes changing due to folate depletion in young and old rats, young rats exhibited a more vigorous response to FA depletion (136 and 62 changed genes, respectively). This result seems somewhat counter-intuitive, since old rats have higher colonic proliferation rates (21) and require more dietary folate to maintain similar tissue folate levels (8) than the young. Nonetheless, the broader response to FA depletion in the young rats may be related to a higher resistance to cancer in young than old colons.

An earlier study of FA depletion in cell culture using cDNA arrays reported that H-cadherin was down-regulated 2.5-fold in FA depleted cells (17). We observed a similar response with protocadherin-4, another cadherin involved in cell-cell adhesion, which was down-regulated 3.5-fold in 0-FA compared with 8-FA rats in the young but not old. Furthermore, in young rats we observed that integrin (α V subunit), nidogen and involucrin, molecules involved in cell adhesion to the ECM or basement membrane, were all between 1.7- and 5-fold down-regulated in the 0-FA compared with 8-FA colons. In concert with the down-regulation of adhesion-related transcripts, young 0-FA rats had a 2-fold up-regulation of urokinase compared with young 8-FA rats. Urokinase expression in the old rats does not appear to be sensitive to FA depletion. Therefore, the apparently higher urokinase expression in young 0-FA than old 0-FA rats (26.7- and 1.2-fold, respectively) may simply be a function of the presence of a folate effect in the young and the absence of a similar effect in the old. Urokinase (urinary plasminogen activator) is consistently

over-expressed in cancers and is involved in ECM degradation, both directly and indirectly, and in stimulating angiogenesis, cell proliferation and migration (22). Together these changes may indicate that colonic cells from young 0-FA rats have an enhanced capacity to detach from their environment and invade, characteristics necessary for tumorigenesis. Nevertheless, the observed changes are not entirely consistent with traditional concepts of carcinogenesis. For example, VEGF and MMP-16, which are associated with angiogenesis and ECM digestion, respectively, were down-regulated with depletion in young colons. Macroscopic tumor expansion, angiogenesis and invasion, features of cancers associated with VEGF (23) and MMPs (24), are phenomena related to later stages of carcinogenesis and therefore the counter-intuitive changes in expression observed here may be non-relevant.

We also detected up-regulation of iNOS due to FA depletion in young colons. Elevated iNOS expression has been detected in cancerous versus normal human colonic mucosa (25,26) and also in rat intestinal cells treated *in vitro* with 1,2-diacyl-sn-glycerol, a tumor-promoting agent (27). During colorectal tumorigenesis iNOS enzyme activity is reported to be highest in adenomas and declines with advancing tumor stage to a level similar to that of normal tissue during metastasis (28). It is possible that the increase in iNOS expression may be partially explained by elevated homocysteine concentrations in the young 0-FA group compared with the young 8-FA group [25.9 ± 9.5 and 3.4 ± 0.6 μ M in plasma (8)]. Support for this hypothesis is provided by Woo *et al.* (29), who reported that treatment of macrophages with 0.05–0.1 mM homocysteine caused a significant increase in NO production and iNOS mRNA and protein.

It is tempting to hypothesize that the up-regulated iNOS expression we detected, and hence increase in NO production, may be linked to changes in the MMP and TIMP enzymes as described above. Although there are no reports on an effect of NO on MMP-16 and TIMP-2 specifically, NO is known to regulate the expression of various other members of the MMP and TIMP families (30). In addition, NO may explain the

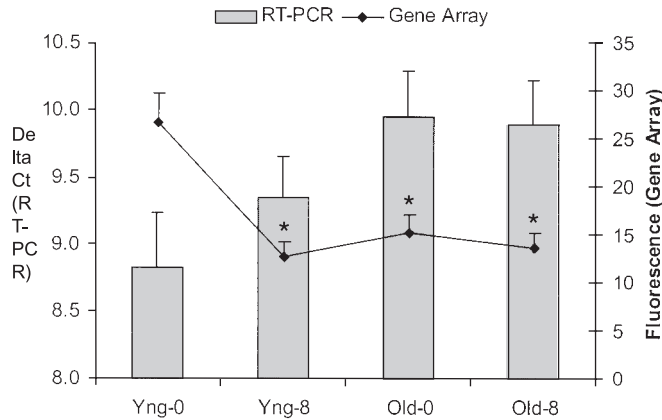


Fig. 1. Effect of FA depletion and aging on urokinase (X63434) expression. Analysis by gene array and RT-PCR. Data are means \pm SEM. $n = 3$ and $n = 6$ per group for gene array and RT-PCR, respectively. RT-PCR data corrected for GAPDH expression. RT-PCR ANOVA $P = 0.11$. * $P < 0.05$ versus young 0-FA.

observed urokinase up-regulation in young 0-FA colons, since treatment of coronary venular endothelial cells with the NO donor sodium nitroprusside is reported to increase urokinase activity in a dose-dependent fashion (31).

Zhu and Melera (15) have previously shown that metallothionein, a metal-binding protein, is up-regulated in FA depleted Chinese hamster lung fibroblasts *in vitro*, affording cells a growth advantage. In our system, however, we observed a 2-fold down-regulation of metallothionein due to FA depletion in young but not old rats. This discrepancy may be due to differences between the *in vitro* and *in vivo* situation, particularly in the severity of folate depletion, or due to tissue- or species-specific differences.

Several immune-related transcripts were also up-regulated due to FA depletion in young colons and, in fact, constitute the largest functional group of changed genes. This change in expression cannot be explained by quantitative changes in colonic lymphocyte populations since no significant differences were detected in the presence of intraepithelial (Table II) or lamina propria lymphocytes (data not shown). The cell-mediated immune system may play an important role in monitoring the appearance of neoplastic cells and destroying them (32) and it is tempting to speculate that the changes in immune transcripts are the result of an increased appearance of abnormal preneoplastic cells.

It is particularly interesting that in both young and old rats, FA depletion did not affect the expression of any folate-related (including folate-binding, folate-dependent and folate-metabolizing proteins) genes (data not shown). It is possible that in this model the abundance of folate-related proteins is regulated by post-translational mechanisms or because the moderate depletion induced in our system was insufficient to induce detectable changes in the abundance of folate-related transcripts. In support of this, Zhu *et al.* (12) report that despite stable mRNA levels, folate receptor protein expression is inversely related to medium folate concentrations in fibroblasts *in vitro*.

Our data show that p53 expression is reduced due to aging, but only in the 0-FA group. This result indicates that FA supplementation can suppress the age-related decline in p53 expression and fits well with the hypothesis that FA supplementation may reduce the risk of age-associated cancers, by

reducing or preventing deleterious changes in gene expression. Studies from this laboratory have previously shown that severe FA deficiency decreases p53 expression in the colonic mucosa of rats (16). In the current study no differences in p53 abundance were observed between the 0-FA and 8-FA groups, however, the previous study utilized a more severe model of folate deficiency induced by succinylsulfathiazone addition to the diet. An age-related decline in p53 transcript has been reported for the rat colon (33), however, some studies were unable to detect these changes (34,35), possibly due to differences in the FA content of rat chow since we only observed an age-related decline in p53 in 0-FA rats.

Similarly, insulin-like growth factor binding protein 3 (IGF-BP3) mRNA was more abundant in young 0-FA than old 0-FA rats, an effect suppressed by FA supplementation. IGF-BP3 mRNA has previously been shown to decline with age in the rat colon (34–36). It is possible that this decline may be mediated by p53, because p53 is reported to stimulate IGF-BP3 production (37). IGF-BP3 sequesters IGF-1 and thereby inhibits DNA synthesis and proliferation (37). A decline in IGF-BP3 and subsequent increase in IGF-1 bioavailability with age support the hypothesized increased cancer risk in old 0-FA than young 0-FA rats because lowered IGF-BP3 and elevated IGF-1 are associated with carcinogenesis (38,39).

Several changes detected in this study are in agreement with the literature [e.g. p53 and IGF-BP3 (33–36)] and add weight to the validity of using gene arrays to measure gene expression in this system. Furthermore, we verified urokinase expression changes by RT-PCR and the results support those obtained by gene array analysis (Figure 1). Although many changes in our data seem to fit well with the hypothesis that aging and FA depletion, at least in synergy, increase the risk for carcinogenesis, various changes seem counter-intuitive and expose our lack of knowledge of cellular processes and their perturbation.

One limitation of this study is the use of colonic mucosal scrapings rather than a homogeneous population of colonocytes. Mucosal scrapings yield a heterogeneous population of cells primarily containing colonocytes but with some lymphocytes, macrophages, endothelial cells and smooth muscle cells as well. However, alternative approaches have their own distinct limitations, since protocols for the isolation of homogeneous populations of colonocytes require enzymatic digestion and or agitation and centrifugation for between 30 and 90 min (40,41), conditions that would no doubt stress the colonocytes sufficiently to artificially perturb gene expression patterns. Similarly, cell cultures of colonocytes have an architecture that is vastly different from the reality of the *in vivo* milieu, producing its own artifacts. Our data set has been validated both *in silico*, whereby the expression changes of several transcripts parallel previous reports in the literature (29,33–36), and also using real-time RT-PCR for the urokinase transcript.

We have identified changes in gene expression related to ECM remodeling and cell attachment as novel end points of FA depletion that are related to cancer and possibly other degenerative diseases. Previously the cell adhesion molecule H-cadherin has been identified as being responsive to FA concentrations (17), however, this is the first report of the effects of FA depletion in combination with aging on mass gene expression in an *in vivo* model. Of particular importance is the finding of increased urokinase expression with FA depletion in the young. As discussed, urokinase is frequently over-expressed in cancers and plays an important

role in migration and invasion (22). Our data support those of other groups who show age-related declines in p53 and IGF-BP3 expression, however, this is the first report of a suppression of these changes due to FA supplementation.

Supplementary material

Supplementary material can be found at <http://www.carcin.oupjournals.org>

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