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Apc$^{Min/+}$ Mouse Model of Colon Cancer: Gene Expression Profiling in Tumors

Daniel Leclerc, Liyuan Deng, Jacquetta Trasler, and Rima Rozen*

Departments of Human Genetics, Pediatrics and Biology, McGill University—Montreal Children's Hospital, Montreal, Quebec, Canada H3H 1P3

Abstract The Apc$^{Min/+}$ mouse is a popular animal model for studies of human colon cancer, but the molecular changes associated with neoplasia in this system have only been partially characterized. Our aim was to identify novel genes involved in tumorigenesis in this model. RNA from intestinal adenomas and from pre-neoplastic small intestine were prepared from six Apc$^{Min/+}$ mice. The tumor transcriptomes were analyzed with high-density oligonucleotide microarrays representing ~12,000 probe sets; we compared their profiles with those of matched pre-neoplastic intestine. Stringent analysis revealed reproducible changes for 98 probe sets representing 90 genes, including novel observations regarding 50 genes whose involvement in this mouse model has never been reported. In addition to the expected changes in growth regulatory genes, the altered gene products could be assigned to four functional groupings that should enhance tumorigenesis: metabolic changes that would result in a high rate of glycolysis, alterations in enzymes involved in reactive oxygen species or carcinogen metabolism, cytoskeletal elements, and proteins involved in tumor invasion or angiogenesis. A fifth group consisted of expression changes that might restrict tumor progression, suggesting that the adenomatous state reflects a balance of pro- and anti-tumorigenic factors. Since many of the altered genes had not previously been reported to be involved in any tumorigenic processes, our observations provide a host of new candidates for potential modulation to prevent or treat intestinal neoplasia. Supplementary material for this article can be found at http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/v93.html. J. Cell. Biochem. 93: 1242–1254, 2004.

Key words: colorectal cancer; DNA microarrays; familial adenomatous polyposis; Min mouse; tumor transcriptome

Colorectal cancer is a leading cause of cancer and cancer-related mortality worldwide [Pitari et al., 2003], with approximately 300,000 new cases and 200,000 deaths in Europe and the USA each year. Colon cancer is known to progress from normal tissue to adenoma and carcinoma through accumulation of genetic alterations [Kinzler and Volgelstein, 1996]. The identification of tumor-specific targets for diagnosis, therapeutics or even prevention has been a principal goal for many investigators.

Mutations in the adenomatous polyposis coli (*APC*) tumor suppressor gene occur very early in the transformation process and are found in a majority of sporadic colorectal tumors as well as in familial adenomatous polyposis (FAP), a hereditary form of colon cancer [Kinzler and Volgelstein, 1996; MIM no. 175100]. Patients with dominantly inherited FAP develop hundreds to thousands of colorectal tumors. If not removed, some of the lesions inevitably progress to carcinomas. However, for tumor initiation, additional mutations are apparently required. Studies in FAP have suggested that the rate-limiting step for tumor formation is a somatic mutation in the wild-type *APC* allele inherited from the unaffected parent.

A mutant mouse, *Apc*$^{Min/+}$, was identified with multiple intestinal neoplasia in 1990 [Moser et al., 1990]. These mice rarely survive beyond 120 days due to intestinal tumors that are typical exophytic adenomas; invasive carcinomas occur less frequently. They have a mutated *Apc* gene, similar to the defect in FAP

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*Correspondence to: Rima Rozen, Montreal Children's Hospital Research Institute, 4060 St. Catherine Street West, Room 200, Montreal, Quebec, Canada H3Z 2Z3.
E-mail: rima.rozen@mcgill.ca

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patients and in many sporadic cancers. This animal model mimics the rapid development of adenomatous polyps that affects humans with germ-line inactivation of one *APC* gene. Apc^{+} allelic loss occurred in all 30 lesions examined by Levy et al. [1994], who observed inactivation even in the earliest recognizable phase of tumors. This suggests that the alteration of the second *Apc* allele is an early event in tumorigenesis. The $Apc^{Min/+}$ mouse has previously been shown to be a robust indicator of tumorigenesis, with changes in tumor number observed in response to a variety of agents.

Although the $Apc^{Min/+}$ mice have been very useful in colon cancer studies, the molecular events involved in neoplasia have only been partially characterized. Traditional laboratory approaches are often limited in scope and, therefore, biased in the pathways that are assessed. Proliferation is a well-defined feature of the cancer phenotype, but many other cellular processes may be affected including metabolic changes, cell–cell, and cell–matrix interactions, invasion and motility, programmed cell death and angiogenesis. Clearly, such a multifaceted problem requires a global approach to quantify gene expression changes in addition to the more traditional gene-by-gene approaches. We, therefore, employed microarray analysis to profile gene expression changes associated with tumor formation.

MATERIALS AND METHODS

Biological Samples

Animal experimentation was approved by the Animal Care Committee of the Montreal Children's Hospital. Details on breeding, genotyping and polyp analysis can be found in Trasler et al. [2003]. We used male $Apc^{Min+/-}$ mice that were 91-day-old. At this age, the mice have weights that are similar to those of the congenic strain C57BL/6J (data not shown). Polyps observed in the small intestine were carefully dissected to exclude as much of the non-adenomatous tissue as possible. Adenomas smaller than 1 mm and larger than 2 mm were omitted. Six RNA preparations from tumors were compared to six RNA preparations from pre-neoplastic intestine (normal flat mucosa). Tumor and normal intestine RNA pairs were always from the same mouse. Details on diets and genotypes are provided in Table II. Total RNA was isolated with the Trizol reagent method (Invitrogen, Burlington, Canada) and treated with ribonuclease-free DNAse (Invitrogen) as recommended. RNA concentration was determined by absorbance at 260 nm, and integrity was confirmed by denaturing agarose gel electrophoresis.

Microarray and Data Analysis

Microarray experiments were performed at the "McGill University and Genome Québec Innovation Centre," using the Affymetrix (Santa Clara, CA) Murine Genome U74Av2 GeneChip containing 12,488 probe sets. Synthesis of cDNA and labeled cRNA, array hybridization, and scanning were performed according to standard Affymetrix protocols. Resultant data were treated using two different methods. Method no. 1: data from independent experiments were normalized and analyzed as we previously reported [Chen et al., 2002]. We considered as "significant changes" only those involving genes with an average change greater than 2.5-fold, with changed intensities of 200 U or greater and having the higher intensity values qualified as "Present" by the Affymetrix MAS software, using recommended default settings for detection, P-value cut-off ($P = 0.04$). We report only the probe sets with the above changes for all six tumor/normal intestine pairs described above. Method no. 2: raw data were processed for background adjustment, quantile normalization, and summarization using RMA (log scale Robust Multichip Averaging) [Irizarry et al., 2003]. Resultant probe level summaries were reported to behave as a more reliable set of data than the default expression measure provided by Affymetrix MAS software [Irizarry et al., 2003]. Our 12 microarray hybridizations were not all performed at the same time. They were performed as three groups of four DNA chips, where the four chips represent two normal tissues and two tumors. For this reason, the analysis was independently evaluated on these three groups. The LPE algorithm (Local Pooled Error) [Jain et al., 2003] was used to identify differentially expressed genes within each group. LPE analysis is based on statistical significance and provides valid comparisons when relatively small numbers of replicates are available. It also avoids potential flaws in fold-change based approaches [Jain et al., 2003]. Probe sets harbouring P-values < 0.01 and $z > 4$ or $z < -4$ were retained. A list of differentially expressed genes that appear in

the three groups was compiled. This generated a list of 398 probe sets (data not shown) that retained all 98 probe sets identified using Method no. 1 above. Genes represented by these 98 probe sets will be discussed. The primary data have been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession nos. GSM12501-GSM12512 and GSE784.

Validation of Microarray Data With RT-PCR

To validate the results of microarray analysis, seven representative genes, deemed to be valuable on the basis of the microarray results, were subjected to RT-PCR analysis using six normal intestine/tumor pairs of RNA. RNAs were reverse-transcribed (Superscript II, Invitrogen) after random hexamer priming according to the manufacturer's recommendations. For every amplicon, a control reaction without reverse transcriptase was performed for each RNA sample to verify that the subsequent PCR did not proceed from contaminating genomic DNA. RT-PCR was carried out with the specific primers described in Table I. Reactions were performed as recommended for *Taq* polymerase (Invitrogen), using 0.4 μg of each primer in a 50 μl reaction, except for *Gapdh* amplification, where 0.075 μg of each primer was used. PCR amplification was performed as follows: 94°C for 3 min, then 20–37 cycles of 94°C for 30 s, annealing for 30 s at the temperature indicated in Table I, 72°C for 1 min. The PCR products from different cycles were loaded on acrylamide gels and visualized by ethidium bromide. The expression of the housekeeping gene *Gapdh* was assessed using the same protocol and used to normalize for slight variances in input cDNA. Quantitative analysis was performed using the BioRad (Mississauga, Canada) GelDoc system and Quantity One software, as recommended by the manufacturer. The procedures were validated in prior studies by PCR amplification of different concentrations of cDNAs (data not shown). For every sample, the number of PCR cycles was varied to ensure that data were generated from amplicons obtained within the exponential phase. For *Retnlb*, the level of expression was greatly increased in tumors compared to normal intestine. In this particular case, the relative amount of message in normal intestine and tumor could not be deduced from comparing amplicons obtained after the same number of cycles. Therefore, the efficiency of amplification for tumor RNA template was judged from intensities obtained for successive cycles in order to extrapolate the relative amount of amplicon at higher cycle numbers, for comparison with normal tissue samples.

Immunohistochemistry

Tissues were fixed for 12 h at 4°C in fresh 4% paraformaldehyde and specimens were embedded in paraffin. Paraffin sections (6-μm) were processed for anti-claudin-4 immunostaining, by the avidin/biotin conjugate (ABC) immunoperoxidase procedure using Vectastain Elite kit (Vector, Burlingame, CA) according

TABLE I. Strategies for Generating Specific Amplicons for RT-PCR Analysis of Representative Genes With Altered Expression in Tumors

Gene	Primers[a]	Annealing temp (°C)	Size (bp)
Retnlb	5′-GCTCTCAGTCGTCAAGAGCCTAA-3′ 5′-GCCACAAGCACATCCAGTGACAA-3′	60	108
Pla2g2a	5′-CCTGGAGAAAAGTGGATGTGGTA-3′ 5′-CGTTTCTGACAGGAGTTCTGGTT-3′	62	102
Serpinb5	5′-GATGAGTCCACAGGCCTGGAGAA-3′ 5′-GACTTTGGCATTGGCCATGGTA-3′	60	96
Lama3	5′-GTCTTGATCCACATCGCAAGTCAA-3′ 5′-GTCCCACCTGCCTCACTGTTCAT-3′	60	98
Lamb3	5′-CGAGCCTCATCTACCTGTGGATT-3′ 5′-GAGGCAGCCTGGAGTCACACTT-3′	60	100
Lamc2	5′-GATGGCTCCAGGCAAGACACTT-3′ 5′-CCAGTGACTGCTTGGATGTTCAT-3′	60	82
Dnmt1	5′-GGGTCTCGTTCAGAGCTG-3′ 5′-GCAGGAATTCATGCAGTAAG-3′	55	201
Gapdh	5′-CAGGAGCGAGACCCCACTAACAT-3′ 5′-GTCAGATCCACGACGGACACATT-3′	60	506

[a]For each gene, the first primer corresponds to sense sequence, and the second one is the antisense oligonucleotide used for PCR.

to manufacturer's instructions. Unmasking of possible blocked antigenic binding sites was performed by microwave heat treatment-mediated antigen retrieval. In short, deparaffinized and re-hydrated sections were immersed in boiling 0.01 M sodium citrate buffer (pH 6.0) for 10 min, and slowly cooled down to room temperature for 20 min. Serum (10%) was used to block non-specific binding sites. Incubation with 1/100 primary antiserum [polyclonal; raised in rabbit (Lab Vision Corporation, Fremont, CA)] was performed overnight at 4°C. In the final step, the sections were counterstained with hematoxylin. Other chemicals were of the highest purity available. All slides were examined by light microscopy.

RESULTS AND DISCUSSION

Our results and discussion will focus on the 50 genes whose involvement in the biology of the $Apc^{Min/+}$ mouse model has not previously been reported. To facilitate interpretation, these genes were classified into functional groups (Table II). Because of space limitations, the reader is invited to refer to Supplementary Tables A–C, that accompany the article online (http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/v93.html), for alternative gene names and supplementary information on these 50 genes as well as 40 other genes that also showed reproducible changes in our study as well as that of a previous report [Paoni et al., 2003].

Validation of Oligonucleotide Microarray Assay Using RT-PCR

A panel of seven genes was analyzed by RT-PCR, to validate the conclusions obtained from microarray analysis (Fig. 1). Quantitative estimates from RT-PCR and microarrays were similar. We confirmed upregulation of *Retnlb*, which constitutes a positive control given the previous observation by Steppan et al. [2001] in $Apc^{Min/+}$ mice. In normal intestine, the product of this gene, RELMβ, is a goblet cell-specific protein [He et al., 2003]. When Moser et al. [1992] examined 28 $Apc^{Min/+}$ mouse tumors, they found goblet cells in all of them, scattered throughout the tumors as individual cells or as small groups of cells. Our observations and those of Steppan et al. [2001] are insufficient for determining whether the increased expression of Retnlb in tumors is restricted to goblet cells or whether other cell types show high expression of this gene in tumors. *Pla2g2a* has been described as an important modifier of tumorigenesis in $Apc^{Min/+}$ mice; we obtained independent observations on increased expression in tumors. The marked increase of *Serpinb5* is in agreement with reported changes in human colonic adenomas [Song et al., 2002]. Our observations support the trimeric nature of Ln-5, which has been debated ((http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/v93.html) Table C), since we confirmed the simultaneous upregulation of *Lama3*, *Lamb3*, and *Lamc2*.

Because tumorigenesis in $Apc^{Min/+}$ mice is influenced by activity of DNA methyltransferase 1 (Dnmt1) [Cormier and Dove, 2000; Trasler et al., 2003], we questioned whether *Dnmt1* expression was altered in tumors compared to pre-neoplastic intestine in our study. The absolute call for probe set 101445_at, specific for *Dnmt1*, suggests an increased *Dnmt1* expression in tumors compared to normal intestine (GEO accession no. GSE784). Our RT-PCR data (Fig. 1) confirmed these observations. These findings are consistent with previous reports on up-regulation of *DNMT1* in various types of tumors [Nagai et al., 2003].

Functions or Pathways That are Altered in Tumors

Glucose metabolism. One of the most common biochemical phenotypes of highly proliferative, poorly differentiated cancer cells, including those derived from colon, is their propensity to sustain high rates of glycolysis [Warburg, 1956], their primary energy source, under nonaerobic and aerobic conditions ("Warburg effect"). Our observations for $Apc^{Min/+}$ tumors, on six genes encoding five enzymes (*HexII*, *G6pc* and *G6pt1*, *Fbp1*, *Gdc-1*, *Aldob*) (Table II and Table A), correlate well with a shift in metabolism away from gluconeogenesis, toward glycolysis.

A significant rise in glycolytic enzyme activities and a simultaneous fall in gluconeogenic enzyme activities, namely glucose-6-phosphatase (G-6-Pase, genes *G6pc* and *G6pt1*) and fructose-1,6-biphosphatase (*Fbp1*, see Table A), were found in mammary carcinoma bearing rats [Sujatha and Sachdanandam, 2002]. Such changes were also observed in renal carcinoma for G-6-Pase and fructose-1,6-biphosphatase, in addition to a diminished activity of α-glycerol-3-phosphate dehydrogenase, another enzyme involved in gluconeogenesis [Balabanov et al.,

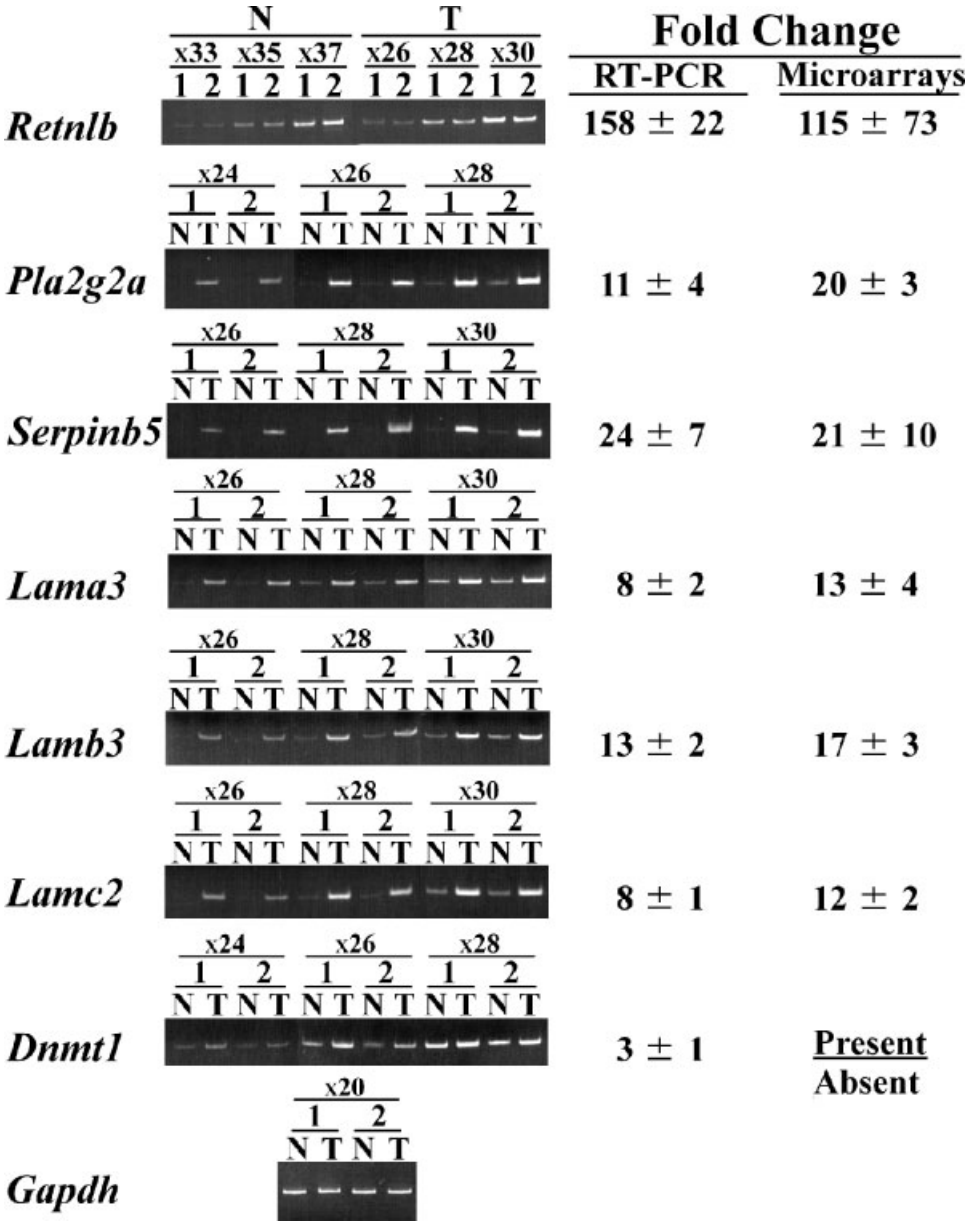

Fig. 1. Determination of relative mRNA levels by RT-PCR in tumors and normal intestine, for seven representative genes. Total RNA was isolated from normal (N) or tumor (T) tissue and mRNAs were amplified by reverse transcription/PCR before analysis on polyacrylamide gels as described in ''Materials and Methods.'' Representative examples are shown for RNA prepared from two different mice, labeled ''1'' and ''2.'' Amplified genes are indicated on the left. Cycle numbers are indicated above the pictures. Amplicons were quantitated by densitometry and the numbers at the right of the pictures summarize the results obtained from six paired tumor/normal tissues and are expressed as fold change ± standard error. RNA from four pairs had also been used in the microarray analyses. Two other pairs of RNA preparations were from mice that had not been used for microarray analyses. The altered expression deduced for the same genes from microarray data (also for six paired samples) is shown for comparison.

2001]. Based on earlier reports, McCarty [2001] proposed that the low glycerol-3-phosphate dehydrogenase (*Gdc-1*) in most cancers may contribute to their inefficient use of glycerol. Decreased expression of aldolase B (*Aldob*) was shown in human hepatocellular carcinoma [Kinoshita and Miyata, 2002].

Glucose-6-phosphatase, a key enzyme in glucose homeostasis, is a multicomponent system located in the endoplasmic reticulum that catalyzes the terminal step in gluconeogenesis. We observed a reduction in gene expression of both of the two subunits of G-6Pase. To our knowledge, this is the first study showing a

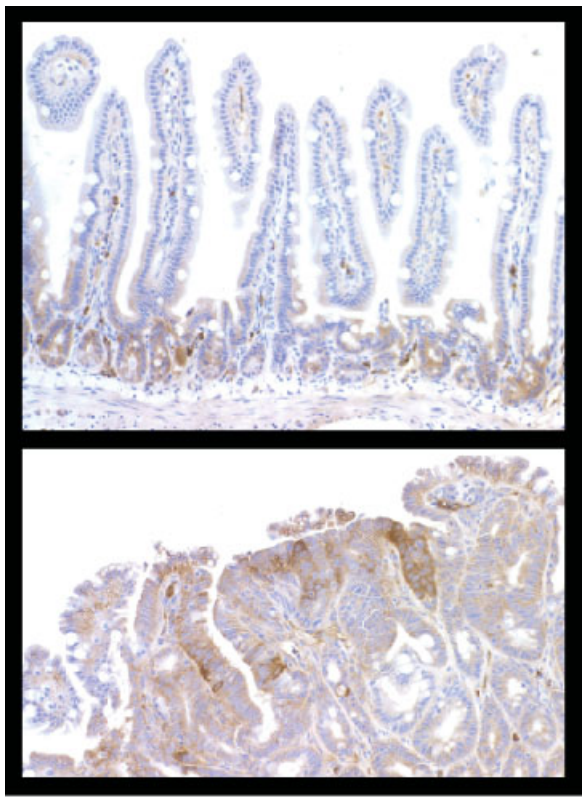

Fig. 2. Immunohistochemical examination of claudin-4 in normal intestine (**top**) and in an adjacent tumor (**bottom**). Stronger staining of tumor is evident. Original magnification 20×. Negative controls, in which the primary antibody was absent, were processed in parallel; no positive staining was observed (data not shown).

concomitant decrease of expression of these two genes in neoplasia.

Aldolase B (*Aldob*) is one of three aldolase isozymes. In oocytes and early embryos that have a high rate of glycolysis, aldolase A and C mRNA are abundant. In contrast, aldolase B, adapted to dietary fructose metabolism and gluconeogenesis, occurs only at a residual level [Kajita et al., 2000]. The reduction in *Aldob* expression in tumors suggests an adjustment towards high glycolytic activity.

Enzymes involved in reactive oxygen species (ROS) or carcinogen metabolism. The product of the *Cyba* gene ($p22^{phox}$) associates with *Cybb*-encoded protein ($gp91^{phox}$) to form cytochrome b-558 (also called β-558 or cytochrome b245), which is the membrane component of NADPH oxidase. It functions as the final electron transporter in the oxidation of NADPH; its upregulation results in the generation of ROS, such as O_2^- and H_2O_2. ROS generated in autocrine fashion by this enzyme may play a role in signaling malignant melanoma growth [Brar et al., 2002].

Detoxification capacity influences the susceptibility of tissues to cancer development. Several enzyme systems are involved in detoxification of ROS and cooperate to protect the organism against reactive, potentially carcinogenic, agents. Oxidative metabolism (phase I metabolism), carried out by the cytochrome P450 (CYP) enzyme family, initiates biotransformation of compounds that do not possess functional groups suitable for conjugation and can generate toxic and mutagenic intermediates. Following CYP-dependent oxidative activation, conjugation enzymes (phase II metabolism), including glutathione transferases, generally decrease the reactivity of these intermediates by linkage reactions that facilitate their excretion. Epoxide hydroxylase serves in a similar manner by catalyzing the addition of water to epoxides, thereby generating dihydrodiols of lower reactivity. Five genes that showed decreased expression in tumors are involved in detoxification of endogenous or foreign carcinogens: *Cyp3a16*, *Gstt1*, *Gsta4*, *Gstm6*, and *Ephx2*.

Among genes that were downregulated in tumors, the most striking change (12-fold reduction) was observed for *Cyp3a16*, encoding a P450 enzyme. Several P450 sequences can be deduced from data recently available from human and mouse genome projects, but, to our knowledge, the identity of the human ortholog of *Cyp3a16* has not yet been addressed. From thorough comparison of murine and human sequences, we deduced that its human ortholog protein is CYP3A4 (also called CYP3A, GenBank P08684). Nakamura et al. [2002] observed that the message encoding CYP3A/CYP3A4, was considerably lower in the colon cancer cell line Caco-2 compared to human duodenal enterocytes. Its involvement in cancer may also be related to the metabolism of retinoids, since CYP3A4 is a major CYP enzyme catalyzing oxidative conversion of retinol to retinal [Chen et al., 2000].

Gstt1 (GST Theta 1), *Gsta4* (GST Alpha 4), and *Gstm6* (GST Mu 6) are glutathione *S*-transferases (GST) genes. The observed reduction in GSTs in Barrett's epithelium may contribute to the increased cancer risk in this tissue [van Lieshout et al., 1999]. RT-PCR analysis has shown reduced levels of *GSTT1*

TABLE II. Fifty Genes* With Altered Expression in $Apc^{Min/+}$ Tumors

Category	Gene	Acc._No	Probe set[a]	Gene product	Fold change[b]
Glucose metabolism	*HexII*	Y11666	94375	Hexokinase 2	6 ± 1
	G6pc	U00445	103333	Glucose-6-Ptase, catalytic	−6 ± 1
	G6pt1	AF080469	97430	Glucose-6-Ptase, transport protein 1	−5 ± 1
	Gdc-1	M25558	92592	Alpha glycerol-3-P dehydrogenase	−3.5 ± 0.4
	Aldob	AI527354	101532_g	Aldolase B	−3.7 ± 0.4
ROS or carcinogen metabolism	*Cyba*	AW046124	97013_f	Cytochrome b-245, alpha polypeptide	5 ± 1
	Cyp3a16	D26137	101638_s	Cytochrome P450, 3a16	−12 ± 3
	Gstt1	X98055	95019	Glutathione S-transferase, theta 1	−5 ± 1
	Gsta4	L06047	96085	Glutathione S-transferase, alpha 4	−6 ± 1
	Gstm6	AI326397	104637	Glutathione S-transferase, mu 6	−6 ± 2
	Ephx2	Z37107	93051	Epoxide hydroxylase	−5 ± 1
Cytoskeleton	*Tmsb10*	AI852553	98129	Thymosin beta-10	3.1 ± 0.2
	Capg	X54511	160106	Capping protein (actin filament)	7 ± 1
	Macmarks	X61399	97203	Myristoylated alanine-rich C kinase	8 ± 1
	Capn5	Y10656	102316	Calpain 5	3.8 ± 0.3
	Ck7	AA755126	97920	Cytokeratin 7	18 ± 3
Tissue remodeling and angiogenesis	*Spock2*	AI844853	104375	Testican-2	13 ± 5
	Cldn4	AB000713	101410	Claudin 4	4.7 ± 0.5
	Gjb3	X63099	104232	Gap junction membrane protein beta 3	7 ± 1
	Lrg	AW230891	97420	Leucine-rich alpha-2-glycoprotein	9 ± 1
	Ly6d	X63782	160553	Lymphocyte antigen 6 complex, locus D	5 ± 1
	Mcpt1	X68803	94728_f, 94729_r	Mast cell protease 1	69 ± 23
		M57401	94735_s		
	Cma2	M68899	95806_f	Chymase 2, mast cells	17 ± 4
	Angl	U72672	95228_f	Angiogenin-like	3.4 ± 0.5
	Mtf	M26071	97689	Tissue factor	8 ± 2
Changes that may restrict tumor progression	*Gpx2*	X91864	99810	Glutathione peroxidase 2	6 ± 1
	Actn1	AA867778	92280	Actinin, alpha 1	4.0 ± 0.5
	Igfbp4	X76066	101571_g	Insulin-like growth factor binding protein 4	5 ± 1
	Sfn	AF058798	96704	Stratifin	2.9 ± 0.1
	Slc2a2	X15684	103357	GLUT2	−6 ± 1
	Slc2a5	AI606956	160839	GLUT5 transporter	−8 ± 2
	Fabp6	U00938	99977	Fatty acid binding protein 6 (gastrotropin)	75 ± 13
	Cck	X59520	96055	Cholecystokinin	−5 ± 1
	Serpinb5	U54705	100034	Maspin	21 ± 10
Growth factors, related molecules, and cell cycle	*RegIIIγ*	D63362	96064	Regenerating gene III gamma	7 ± 1
	Fgfrp	U04204	100884	Fibroblast growth factor regulated protein	7 ± 1
	Cks1	AB025409	97468	CDC28 protein kinase 1	3.0 ± 0.2
Others	*Mylc2a*	AA839903	100403	Myosin light chain, regulatory A	5.9 ± 0.2
	Vegp	U46068	160446	von Ebner minor salivary gland protein	35 ± 11
	Pglyrp	AF076482	104099	Peptidoglycan recognition protein	5.1 ± 0.4
		AV092014	162475_f		
	Anxa10	AJ238978	92494	Annexin A10	55 ± 20
	Ip3r1	X15373	94977	Inositol 1,4,5-triphosphate receptor 1	−7 ± 1
	Dao1	D10210	103602	D-amino acid oxidase	−6 ± 1
	Adh1	M22679	94906	Alcohol dehydrogenase 1	−4 ± 1
	Srb1	U37799	100095	Scavenger receptor class B1	−6 ± 1
	Pla2g12	AI845798	104343_f	Phospholipase A2, group XII-2	6 ± 1
	—	AW494830	104737	EST, similar to rat 2c9 gene	60 ± 36
	—	AI852760	103744	EST	6 ± 1
	—	AI265638	103714	EST (putative reductase)	−3.5 ± 0.3
	—	AI850090	96634	EST	−7 ± 2

*Listed according to the order of appearance in "Results and Discussion."
[a]Description of probe sets can be obtained from www.affymetrix.com, adding the suffix "_at" to probe IDs listed in this table.
[b]Estimate of the fold induction of each gene, ±standard error (n = 6). Three mice were $Apc^{Min/+}/Dnmt1^{+/+}$ and the three others were $Apc^{Min/+}/Dnmt1^{+/-}$. The three mice of each genotype were under one of the three following diets. Diet no. 1 is the control diet described in Sibani et al. [2002]; this diet has the required amount of folate for rodents (2 mg/kg). Diet no. 2 is a modification of amino acid-defined diet TD 99366 (Harlan Teklad, Madison, WI) with methionine adjusted at 3.3 g/kg, vitamin mix 10g/kg, menadione sodium bisulfite 50 mg/kg and succinylsulfathiazole 10 g/kg; this diet also has the required amount of folate for rodents. Diet no. 3 is identical to Diet no. 2, except for the folic acid concentration that was 7 times lower (0.3 mg/kg).

transcripts in human colon and pancreas tumors, but increased levels in lung and ovary tumors [Elek et al., 2000]. Interestingly, the *GSTT1* gene is absent from a significant proportion of the population. Chen et al. [1996] reported a frequency of 46% of the *GSTT1* null genotype among myelodysplastic syndrome (MDS) cases, compared to 16% among controls, resulting in a 4.3-fold increased risk of MDS. The authors suggested that the mechanism of the association might be decreased detoxification of environmental or endogenous carcinogens. In addition, women with the *GSTT1* null genotype were found to have a 3-fold increased risk of breast cancer [Matheson et al., 2002]. The GST Mu phenotype (immunoreactive Mu

protein) is lost in a significant proportion of neoplastic colorectal adenocarcinomas, but not in adjacent colonic epithelial cells [Barker et al., 2002]. The protective role of the glutathione biotransformation system against colorectal carcinogenesis was reviewed by Grubben et al. [2001].

Ephx2 encodes the cytosolic epoxide hydroxylase (soluble epoxide hydroxylase, sEH). Epoxides include highly electrophilic species with a pronounced potential to react with DNA. The hydrophobicity of epoxides is decreased by sEH, resulting in less reactive, more polar, and therefore excretable substances. The cytosolic epoxide hydroxylase decreased mutagenicity of epoxide-containing compounds in the Ames' assay [Beetham et al., 1993]. De Waziers et al. [1991] observed a decrease in epoxide hydroxylase and CYP3A activities in human colorectal polyps, and proposed that they could represent pretumoral markers.

Proteins of the cytoskeleton. Studies of malignantly transformed cells have shown a direct association between disorganization of the cytoskeleton and tumorigenicity (Table B). Tmsb10 (thymosin beta-10) is a major actin monomer sequestering factor, overexpressed in colon, breast, ovarian, and uterine carcinomas, and in germ cell tumors [Santelli et al., 1999]. Capg is a member of the gelsolin/villin family and regulates actin polymerization in response to changes in the concentration of calcium. Increased expression has been reported in tumors and cell lines (Table B). Macmarks is a member of the MARCKS family (myristoylated alanine-rich C kinase), which contains major substrates for protein kinase C (PKC). These proteins act in the control of modeling the actin cytoskeleton by PKC and calcium/calmodulin-dependent signals [Jonk et al., 1994].

Capn5 (calpain 5) is a member of the calpain family (as is Capn2, see Table A) and of intracellular calcium-dependent cysteine proteases. E-cadherin is functionally inactivated through calpain-mediated proteolysis during the tumorigenic progression of prostate cancer [Rios-Doria et al., 2003]. E-cadherin, which is inactivated in many adenocarcinomas, binds to β- and γ-catenin, which in turn bind to α-catenin; this complex physically associates with the actin cytoskeleton.

Keratins compose the intermediate filaments of epithelial cells. Although cytokeratin 7 (*CK7*) is usually not expressed at high levels in intestine or in colon carcinoma, its expression has been found to be more frequent in high-grade than in low-grade carcinoma [Park et al., 2002].

Tumor invasion and angiogenesis. Although few tumors in $Apc^{Min/+}$ mice initially exhibit an invasive phenotype, it is relevant to discuss genes related to invasion and metastasis, since the increased lifespan of these mice by the *Mom1* modifier is known to be accompanied by the development of invasive tumors [Moser et al., 1992]. Changes in expression of proteins that are involved in intercellular contacts or adhesion may contribute to the invasion and migration of tumors. *Spock2* (sparc/osteonectin, cwcv, and kazal-like domains proteoglycan 2) encodes a matricellular protein involved in tissue remodeling although its role is not well-established (Table B). Upregulation of *Cldn4* (claudin 4), a member of a group of proteins involved in cell–cell contacts, has been reported in several cancer types (Table B). We performed immunohistochemical evaluation of claudin-4 protein expression in paraffin-embedded tissue samples for normal intestine and adjacent tumors (Fig. 2). The tumors revealed stronger expression of claudin-4 than normal tissue. Immunolabeling was noted in membranous and cytoplasmic regions, consistent with previous observations in ovarian [Rangel et al., 2003] and pancreatic cancer [Nichols et al., 2004]. This is the first study of increased expression of claudin-4 mRNA or protein in $Apc^{Min/+}$ mouse tumors.

Connexin-31 (*Gjb3*), a member of the gap junction gene family, is highly expressed in malignant choriocarcinoma cells, as well as invasive trophoblast cells [Winterhager et al., 1996]. Trophoblast invasion during embryo implantation resembles tumor cell invasion in some aspects. Lrg (leucine-rich α2-glycoprotein) can bind extracellular matrix proteins such as laminin, which may modulate cell adhesion locally [Saito et al., 2002].

The role of the lymphocyte antigen 6 complex, locus D (*Ly6d*), is not well characterized, but it showed increased expression in carcinomas (Table B). It controls important interaction parameters between endothelial cells and cancer cells [Eshel et al., 2002].

Transformed malignant cells produce a variety of lytic enzymes that degrade the extracellular matrix and allow cancer cells to invade tissues, lymphatic channels, and the vascula-

ture. Mcpt1 is a chymase (chymotrypsin-like protease) expressed only at the mucosal mast cell stage [Huang et al., 1991]. Tumor growth depends on angiogenesis and mast cell infiltration may contribute to tumor angiogenesis and tumor invasion [Takanami et al., 2000]. The increase in expression for *Mcpt1* was particularly striking, among the highest changes observed in our study (69 ± 23). We also observed an increased signal (17 ± 4) for *Cma2* (chymase 2, mast cells) that has a sequence very similar to *Mcpt1*. It is a gene of the same family of proteases, from the same region of mouse Chr14.

Angiogenesis is one of the most important biological features that is closely related to carcinogenesis, tumor growth, and tumor metastasis. Angiogenin is a potent blood vessel inducing protein that was originally purified from conditioned media of the human colon carcinoma cell line HT-29, and serum angiogenin concentration in colorectal cancer patients correlates with cancer progression [Shimoyama et al., 1999]. We observed an increased amount of *Angl* (angiogenin-like) mRNA in tumors. Mouse tissue factor (*Mtf*) expression is related to the metastatic potential of colorectal cancer. Correlation between TF expression and microvessel density in human colorectal cancer has been observed, supporting the proposed link to angiogenesis [Nakasaki et al., 2002].

Changes that may restrict the progression of tumors. We observed a number of alterations that may reflect mechanisms for restricting progression of adenomas. They may be part of cellular defense systems against the developing tumor; an understanding of these types of host mechanisms may also lead to novel therapeutic approaches.

Excess ROS can induce toxicity, mutations and ultimately cancer. Products of oxidative stress are deactivated by the involvement of multiple enzymes, including glutathione peroxidases. Increased expression of glutathione peroxidases, such as *Gpx2*, (and *Gpx5*, Table A), may relate to the increased oxidative stress in tumors.

Since high expression of α-actinin is correlated with loss of tumorigenicity (Table B), it is likely that the increase in *Actn1*, as we observed in tumors, is a compensatory mechanism to limit the tumor growth.

A majority of human colon cancers express IGFBP4, although this protein functions as an inhibitory protein for colon cancer cells [Dai et al., 1997]. The *Sfn* gene (stratifin) normally acts as a tumor suppressor (Table B).

The SLC2 family of facilitated hexose transporters play important roles in glucose homeostasis [Uldry and Thorens, 2004]. GLUT2, encoded by *Slc2a2*, is a glucose transporter in the basolateral membrane of intestinal epithelium. Decreased expression was reported in human islet cell tumors [Seino et al., 1993] and in renal basophilic cell tumors [Ahn et al., 1993]. *Slc2a5* encodes GLUT5, a fructose transporter that also transports glucose, normally found in the apical and lateral membranes of intestinal epithelial cells. It is highly expressed in vivo in human breast cancer but is absent in normal breast tissue [Zamora-Leon et al., 1996]. We observed decreased expression of these two transporters, which should limit glucose availability and tumor growth. Fabp6 (fatty acid binding protein 6), the Na^+-dependent bile acid transporter from small intestine, is part of the lipocalin family. Its increased expression may also reflect an effort to fight the tumor since it decreases exposure of the colorectal epithelium to bile acids that promote tumor growth (see Table B).

Downregulation of the *Cck* (cholecystokinin) gene product has been reported in human prostate cancer. Since CCK peptide is expected to stimulate growth of normal and neoplastic tissue [Jensen, 2002], the observed decrease may be part of a response mechanism to limit tumor growth. We observed a dramatic increase in expression of maspin (*Serpinb5*), a serine protease inhibitor. The literature is unclear regarding an increase or decrease of expression of this gene in different cancers (Table B). Song et al. [2002] reported decreased expression of Maspin from colonic adenoma to metastatic carcinomas, suggesting a tumor suppressive or a metastasis suppressive function.

Growth factors, related molecules, and cell cycle. It is not surprising that a number of growth factors or cell cycle regulators are altered in this tumor model. We identified changes in *RegIIIγ*, *Fgfrp*, and *Cks1* (discussed in Table B) that had not been previously reported. We also observed consistent changes in other growth-regulating genes (Table A) that were also identified in Paoni et al. [2003].

Others. Nine genes and four ESTs that could not be assigned to the afore-mentioned biological pathways are discussed in Table B.

General Comments

We investigated the modification of the intestinal transcriptome of $Apc^{Min+/-}$ mice by tumorigenesis. "Transcriptome" refers to mRNAs expressed by a genome in a given tissue at a given time. The tumoral transcriptome was analyzed by microarrays and compared to the transcriptome of normal intestine from the same mouse. When thousands of genes in two microarray data sets are evaluated simultaneously, the probability of detecting false positives rises sharply. Therefore, the number and design of replicates in microarray experiments determine the degree of accuracy of the data. Our design involved 6 replicates (12 samples analyzed as 6 paired tumor/normal intestine RNAs). The 98 probe sets that we report (Table II and supplementary material) represent very robust changes—more than 2.5-fold differences—observed in the 6 pairs. Given this robustness, we chose to confirm the changes in the microarrays for only a limited number of genes, by RT-PCR. Since the RT-PCR results were quantitatively similar to those from microarrays, since our microarrays identified differential expression of some genes that were already known to be affected in tumors from $Apc^{Min/+}$ mice or in colon cancer-related samples (see supplemental Tables), and since many of the genes were known to play roles in cellular proliferation or tumor development, we conclude that our results are likely to be very accurate.

Since loss of APC increases transcriptionally active β-catenin/TCF-4 complexes, it would be interesting to examine how changes observed in $Apc^{Min/+}$ mice relate to expression changes in mice lacking *Tcf-4* [mouse model described by Korinek et al., 1998]. Until data derived from this other mouse model become available, it is interesting to note that blockage of the Wnt cascade in human colorectal cancer cells using a dominant-negative version of TCF-4 [van de Wetering et al., 2002] also increases expression of claudin-4 (for which we observed increases in expression by microarray analysis and by immunohistochemistry).

The increased expression of a connexin gene may have implications for the *HMPS*/*CRAC1* locus (hereditary mixed polyposis syndrome/colorectal adenoma and carcinoma). Jaeger et al. [2003] reported that this locus maps to an interval on human chromosome 15q13-q14, based on studies of a large Ashkenazi family. Daley et al. [2003] showed that this syndrome is also important in other ethnic groups. Based on current physical maps (www.ensembl.org), we noticed that the gene *CX36*/*GJA9*/*CXA9* (connexin 36) maps to the interval reported by both groups, very close to the peak at D15S1007 reported by Jaeger et al. [2003]. This gene is a strong homolog of *GJB3*/*CX31* (connexin 31), the ortholog of *Gjb3* identified in the present study. We suggest that *CX36*/*GJA9*/*CXA9* should be examined as a candidate gene for *HMPS*/*CRAC1*.

Paoni et al. [2003] recently reported their analyses of $Apc^{Min/+}$ mice, using laser capture microdissection of tumors. However, they filtered their microarray data with lower stringency than that in our analysis, since some changes considered to be significant in their report were not systematically observed in all their biological replicates and/or involved changes as low as 1.5-fold when normal tissue and adenoma were compared. This would likely have contributed to the higher number of genes for which they observed differential expression between normal intestine and adenomas. Forty of the 90 genes that were shown to be differentially expressed in our analysis (see Table A) were also reported by Paoni et al. [2003]. The 50 genes that we report in Table I were not reported by these authors, and are novel observations. Genes expressed at low levels (including some "housekeeping genes") may be below the threshold of detection for RNA preparations that are technically more difficult to isolate, such as those obtained through laser capture. Consequently, the integrity of RNA preparations or other technique-related variations may explain why some genes in the glycolytic pathway, associated with the well-characterized Warburg effect, were detected in our study but absent from the list obtained by the microdissection study. Furthermore, it is possible that some of the changes that we observed could not be detected by microdissection-based methods if they involved the periphery of the tumors or heterogeneous areas. Complex paracrine effects, which would be observed using our methodology, could have a major impact on tumor development.

In summary, our observations should help in deciphering the complex circuitry involved in the formation or development of tumors in $Apc^{Min/+}$ mice. Our data confirm the regulation of a series of genes previously demonstrated

to play crucial roles in tumorigenesis. More importantly, the majority of genes found to be modulated in our study had never been reported to be regulated in this mouse model of colon cancer, and some of the expression changes had never been described in any tumor model. Although the precise involvement and interactions of these gene products in the formation/progression of adenomas needs to be evaluated, our data provide useful new information that will contribute to the understanding of colorectal tumor formation and progression.

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