Transcriptional profiling of the transition from normal intestinal epithelia to adenomas and carcinomas in the APC\textsuperscript{Min/+} mouse

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There is considerable evidence implicating mutations in the adenomatous polyposis coli (APC) gene in the development of colorectal cancer. APC encodes a protein that functions as a tumor suppressor (20, 22) and is one component of a multimeric complex composed of APC, GSK-3b, conductin, and ß-catenin (2, 31). This complex facilitates the ubiquitin-mediated degradation of ß-catenin (30, 34), a protein that plays an essential role in cell adhesion and nuclear signaling. APC also directly inhibits the transcriptional transactivation induced by the complex of ß-catenin with the transcription factor Htcf-4 (18). Germ-line mutations in the APC gene are linked to the syndrome familial adenomatous polyposis (FAP) (9, 15, 17, 27). Individuals with FAP have multiple intestinal adenomas that progress to carcinomas if left untreated. Approximately 85% of sporadic colorectal cancers also contain mutations in APC (13).

A model system useful in the study of FAP and APC-related colorectal cancer is the APC\textsuperscript{Min/+} mouse (26, 33). These mice have a heterozygous, dominant mutation in the APC gene and, like individuals with FAP, develop intestinal adenomatous polyps that progress to carcinomas. Despite the phenotypic difference that the tumors in the APC\textsuperscript{Min/+} mice are predominately located in the small intestine and not the colon, the model has reasonable fidelity to the human experience. Tumor number in APC\textsuperscript{Min/+} mice is increased by a high-fat diet (26), an association also implicated in sporadic colorectal cancer in humans. Furthermore, a number of different nonsteroidal anti-inflammatory drugs were shown to inhibit tumorigenesis in this model (1, 3, 11, 12, 24), a finding consistent with human epidemiological (35) and clinical trial results (7, 19, 28, 32). Thus the APC\textsuperscript{Min/+} mouse is a valuable research tool for studying the biological pathways involved in APC-mediated tumorigenesis and for screening potential chemotherapeutic agents.

Although it is clear that loss in APC-mediated tumor suppression is linked to enhanced ß-catenin signaling, little is known concerning the biological pathways that translate those signals into malignant epithelial cell proliferation. To address this issue, we used a combination of laser capture microdissection and DNA microarrays to characterize the changes in gene expression that accompany the progression from normal intestinal epithelia to adenomas and carcinomas in the APC\textsuperscript{Min/+} mouse.

MATERIALS AND METHODS

Mice and tissue processing. The protocol used in the study was approved by the University of Notre Dame Institutional
Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and adheres to the principals set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996. Male wild-type (WT) and APC\textsuperscript{Min+} mice (5 wk old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were fed a standard rodent diet and kept on a 12:12-h light/dark cycle. Mice were weighted twice weekly, and those with excessive weight loss and/or bleeding were killed and omitted from the study. At 90 days of age, the mice were injected with a lethal dose of rodent cocktail (10 mg/g), and their intestines were removed. The ileum was divided in two, opened, and gently cleaned. Each ileum half was embedded in Tissue-Tek (Sakura Finetek, Torrance, CA), fast frozen using dry ice, and stored at −80°C.

Cryosections (8 µm) were cut from the frozen ilea, mounted on double frosted slides (Fisher Scientific, Fair Lawn, New Jersey), and stored at −80°C. Every 5th section was stained with 1% toluidine blue for histological evaluation.

**Histological evaluation of adenomas and carcinomas.** Adenomas were evaluated according to the grade of dysplasia. Only adenomas showing low or moderate dysplasia were chosen. Lesions with low dysplasia showed slightly modified mucosal architecture. Glands displayed budding and branching, crypt lengthening, serration, and cystic changes. Enlarged cells displayed minimal mucin production. Homogeneous nuclei, rounded or ovoid with pseudostriatification in the superficial portion of the enlarged glands, were observed. Large adenomas (>1.5 mm) and those exhibiting severe dysplasia were omitted. Severe dysplasia was defined as a high architectural disruption of the glandular component, crowding and prominent cellular atypia, mucin absent, pseudostratification, cigar shaped, pleomorphic and hyperchromatic nuclei, increased mitosis, and prominent nucleoli. Carcinomas were evaluated based on severity of dysplasia (>50% severe dysplasia) and histological evidence of disruption of the muscularis mucosae, early invasion to submucosa and muscular layers. The amount of stromal desmoplasia, presence of inflammation, necrosis, and increased thickening of the intestinal wall were also considered in the diagnosis.

**Laser capture microdissection.** Cryosections were stained with the HistoGene kit (Arcturus, Mountain View, CA) following the manufacturer's protocols. Epithelial cells from WT crypts and from APC\textsuperscript{Min+} tumors were identified by a pathologist familiar with this model and captured using the PixCell Ie Laser Capture Microdissection system and Capture HS caps (Arcturus). A 7.5-µm diameter laser spot was used with 60 mW of laser power for a single-fire duration of 1.3 ms to select cells. Approximately 30,000 laser firings/sample were used, employing several slides. Six different biological replicates were used for WT samples and five each for APC\textsuperscript{Min+} normal-appearing ilea, APC\textsuperscript{Min+} adenomas, and carcinomas. Individual animals were used for each biological replicate, but cells harvested from multiple tumors within each animal were pooled to obtain enough starting RNA.

**Gene expression analysis.** Total RNA was isolated using the PicoPure RNA Isolation kit (Arcturus). Samples were processed through two rounds of amplification using the RiboAmp RNA Amplification kit (Arcturus) with one modification. In the second round of amplification, the Enzo RNA Transcript Labeling kit (Enzo Biochem, Farmingdale, NY) was used for synthesis of biotin-labeled cRNA. The yield of labeled cRNA was 70.4 ± 18.3 µg.

Labeled cRNA from each sample (15 µg) was individually hybridized to a MG-U74Av2 GeneChip probe array (Affymetrix, Santa Clara, CA). These probe arrays enable 12,488 sequences to be surveyed simultaneously for differential expression. GeneChips were run and analyzed as previously described (14). To compare data between GeneChips, global scaling was performed on the individual microarrays, using Affymetrix MicroArray (MAS 5.0) software, and the target intensity was set to 500. No further qualifications were placed on signal data. To minimize false-positive results, a conservative, statistical approach was used to identify differentially expressed transcripts between two groups (14). This analysis was performed within the Affymetrix Data Mining Tool 3.0. The methodology is based on the Mann-Whitney pair-wise comparison test, and has been previously validated to produce a very low frequency of false-positive results by one of the study investigators (N. F. Paoni) studying changes in myocardial gene expression in a rodent model of heart failure (14). In the validation study every gene identified as differentially expressed by microarray analysis at a confidence level of \( P \leq 0.05 \) was independently evaluated on the same RNA samples using real-time, RT-PCR (Taq-Man), the most stringent validation possible. Twenty-four of the 25 genes tested were found to be differentially expressed by TaqMan (\( P \leq 0.05 \)), and the \( P \) value of the 25th gene was 0.07, proving the microarray analytical technique to be highly reliable. The same data analysis procedure was subsequently found to produce dependable results when applied to the transcriptional profiling of the time course of skeletal muscle regeneration in a rodent model of hind limb ischemia (29). Changes in gene expression with a \( P \leq 0.05 \) were included in the gene lists. Visualization of gene expression levels was performed using GeneSpring software (Silicon Genetics; Redwood City, CA). The primary data from each sample microarray has been deposited in the Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/geo/). The series accession number is GSE4422.

**RESULTS**

Transcriptional profiles of APC\textsuperscript{Min+} adenomas and carcinomas are closely related. Laser capture microdissection was used to obtain samples highly enriched in epithelial cells from ileum crypts in WT mice, epithelial cells from crypts in normal-appearing regions of APC\textsuperscript{Min+} ilea, and from adenomas and carcinomas in the ilea of APC\textsuperscript{Min+} mice (Fig 1). Biotin-labeled cRNA obtained from the samples was individually hybridized to Affymetrix GeneChip probe arrays. Differential gene expression was determined between wild-type epithelial cells (WTEC) and APC\textsuperscript{Min+} normal epithelial cells, WTEC, and adenomas; WTEC and carcinomas; and adenomas and carcinomas as described in the MATERIALS AND METHODS. No changes in gene expression were found between WTEC and epithelial cells harvested from normal-appearing regions of APC\textsuperscript{Min+} ilea. We found 223 known genes differentially expressed between adenomas and WTEC; 163 were induced and 60 repressed. The comparison of carcinoma to WTEC yielded 189 induced and 68 repressed genes. Nine genes were found to be differentially expressed in carcinomas relative to adenomas, three were induced, and six were repressed. There was substantial overlap in the data sets obtained from adenomas and carcinomas with 173 genes found on both lists. This is likely an underestimate of the similarity in the data sets since in many cases when differential expression was

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Fig. 1. Representative tissue sections of ilea before and after laser capture microdissection (LCM). A–D: sections of ilea stained with toluidine blue; normal mucosa at 4× original magnification (A); adenoma with enlarged lumen glands and mild glandular disorganization, 4× magnification (B); early adenocarcinoma showing glandular invasion of muscularis mucosae and submucosa, 4× magnification (C) and 10× magnification (D). E and F: wild-type ileum before (E) and after (F) capture of epithelial cells by LCM (10× magnification). G and H: APCMin+/− adenoma before (G) and after (H) capture (4× magnification). I and J: APCMin+/− carcinoma before (I) and after (J) capture (4× magnification).
significant in only one tumor type there was a tendency for differential expression in the other.

The gene expression profiles of the 307 genes differentially expressed between WTEC and adenomas or carcinomas are shown in Fig. 2 for all of the study groups. The data show the reproducibility of the expression patterns of the biological replicates within each study group. These data also show the similarity in expression patterns between the two control groups, WTEC and the normal-appearing intestinal epithelial cells from the APCMin/+ mice, and between the two APCMin/+ tumor types, adenomas and carcinomas. The striking contrast in expression patterns for these genes between WTEC and APCMin/+ tumors is also evident. The gene list, organized into functional categories and containing an estimate of the fold induction/repression of each gene, can be viewed in the GEO database using accession number GSE422.

Differential gene expression between WTEC and adenomas and carcinomas for genes that mediate cell growth. It is impossible to discuss all of the gene expression data in a single article, so this report will focus on biological pathways and individual molecules that may influence tumor growth. Altered gene expression for members of several important growth-regulating pathways were observed in the tumors from APCMin/+ mice (Fig.3). Induced genes included members of the insulin-like growth factor (IGF) axis (IGF binding proteins 1 and 5); the epidermal growth factor receptor ligand, amphiregulin; the dominant-negative helix-loop-helix protein, inhibitor of DNA binding 1; group IIa phospholipase A2 and the phospholipase inhibitor annexin A1, which are involved in arachidonic acid metabolism; and members of the interleukin-1 (IL-1) axis (IL-1 receptor-like 1 and IL-1 receptor antagonist). The vitamin D receptor, a negative regulator of tumor growth, was repressed in both tumor types. Tumors had altered transcript abundance for genes important in apoptosis (Fig.4). There was a reversal in the expression pattern of three caspases in tumor epithelium compared with WTEC. Caspase 6, which had low expression in WTEC, was induced in adenomas and carcinomas, whereas the normally high transcript levels of caspases 1 and 7 were reduced in the tumors. Two closely related factors thought to be pro-apoptotic also showed opposite patterns of gene expression in WTEC vs. tumors. Cell death-inducing DNA fragmentation factor effector A had low expression in WT epithelia and was induced in adenomas and carcinomas,
whereas cell death-inducing DNA fragmentation factor effector B was highly expressed in WT cells and repressed in the tumors. These findings together with the other changes in gene expression in this functional cluster are consistent with there being a substantial disruption in the normal apoptotic pathways in the tumor epithelium.

The increase in cell proliferation in the tumors is associated with increased protein synthesis and transport, and we found changes in mRNA abundance for several proteins involved in these processes (Fig.5).

Prominent in this group were three members of the ras oncogene family, RAB 3D, RAB 6, and RAB 11a, which are involved in protein transport and intracellular signaling and were induced in tumors compared with WTEC. We also found evidence of possible altered protein catabolism in the tumors. The expression levels of the proteasome subunits beta types 8, 9, and 10, and ubiquitin-specific protease 2 were repressed in the tumors compared with WTEC. These changes could affect protein degradation rates and allow growth promoting transcription factors and growth factors/receptors to have longer residence times in tumor epithelial cells compared with WTEC.

Tumor growth can lead to hypoxia, and we found a four- to sixfold induction of hypoxia inducible factor 1α in the tumors, which can promote angiogenesis. There was also induction of members of the glycolytic pathway (glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase), which could enable tumor growth in low-oxygen environments.

Genes differentially expressed between adenomas and carcinomas. A direct comparison of gene expression between adenomas and carcinomas revealed nine genes differentially expressed (Fig.6). Some may potentially contribute to the malignant growth of the carcinomas. The genes segregated into two groups. One was induced in adenomas relative to WTEC, but then returned to more normal levels in the carcinomas (calbindin-D9K, transient receptor potential cation channel, SRY-box containing gene 17, glutathione peroxidase 5, mast cell protease 2, and small proline-rich protein 2F). The other group showed a low level of induction in the adenomas relative to WTEC, but then further induction in carcinomas [IGF binding protein 5, serine (or cysteine) proteinase inhibitor, and serum amyloid A3].
DISCUSSION

In general, we found a great deal of similarity in the gene expression profiles of epithelial cells isolated from adenomatous polyps and carcinomas. This finding is consistent with the theory that carcinomas in APCMin/+ mice progress from adenomas. Compared with WTEC, the transcripts induced/repressed in the tumor epithelium provide several insights into the increased growth potential of these cells. First, there are several changes in transcript abundance for members of important growth-regulating pathways. One is the IGF system. The IGF pathway is important in cellular proliferation and apoptosis, and data from in vitro and in vivo studies suggest that overexpression of members of the IGF axis may play an important role in a number of malignancies (25). There is considerable evidence that this axis influences colon carcinogenesis (8). The activities of IGFs are mediated by binding proteins, and the induction of IGF binding proteins 1 and 5 in adenomas and carcinomas indicates that these factors may be important in mediating the IGF responses. These binding proteins may also play a role in the malignant growth of the carcinomas. IGF binding protein 5 expression was significantly increased in carcinomas vs. adenomas, and the opposite trend was observed for IGF binding protein 1 (Fig. 3).

Amphiregulin gene expression was induced in both tumor types. It was recently shown that blocking EGF-like growth factors transforming growth factor-α, amphiregulin, and CRIPTO-1, using antisense mixed-backbone oligonucleotides, inhibited the growth of GEO human colon carcinoma cells in vitro and growth of GEO tumor xenographs in nude mice (5). Thus amphiregulin may be important for promoting tumor growth in this model system.

Forced expression of Id-1 ("inhibitor of DNA binding-1") in the epithelium of the small intestine in mice is associated with adenoma formation (36), and we found Id-1 to be induced in both adenomas and carcinomas.

An unexpected finding was the induction of the group IIa phospholipase A2 in adenomas and carcinomas (7.0- and 8.3-fold, respectively). The group IIa phospholipase, also called "modifier of Min-1" (Mom1), functions as a negative regulator of tumor growth in APCMin/+ mice (4, 6, 23). The C57BL/6J APCMin mouse strain used in this study is homozygous for the Mom1-sensitive allele, a frame-shift mutation caused by a T insertion in exon 3 of the group IIa phospholipase gene that results in little or no intestinal mRNA expression of the enzyme under normal conditions (16). However, it is known that mice carrying the mutated gene can show induction of group IIa phospholipase under extreme conditions (16), and this likely explains our observations. However, since the group IIa phospholipase gene product is defective in C57BL/6 mice, it should not have a negative impact on tumor growth in this model.

In addition to the induction of pathways that promote cell growth, we observed repression of negative growth-regulating systems. The vitamin D receptor was downregulated in the tumors, and the IL-1 converting enzyme, caspase 1, was repressed while IL-1 receptor antagonist was induced. There was altered gene expression for elements of the apoptotic and protein catabolism systems, which also normally function in negative growth regulation. Thus the transcriptional profiles of the tumors showed a marked shift in the balance of growth promoting and inhibiting pathways toward cell growth.

Among the significant findings of this study were the differences in gene expression between adenomas and carcinomas. Some may contribute to the increased growth potential of carcinomas. One example is the difference in IGF binding protein expression discussed above. Our data also indicate that two growth-inhibiting pathways were induced in adenomas relative to WTEC and that these were substantially lost in the transition to carcinomas. Several experimental findings indicate an association between high calcium intake and reduced risk of colorectal cancer, although the mechanism is largely unknown (21). Calbindins are calcium binding proteins that enhance transcellular calcium transport (10), Calbindin-D9K expression was highly induced in adenomas relative to WTEC, but returned to near normal levels in carcinomas. A similar expression pattern was observed for the transient receptor potential cation channel, which is also involved with calcium transport and signaling. These findings suggest that changes in calcium homeostasis may be an important determinant in the transition from adenomas to carcinomas.

We also observed that transcript abundance for SRY box containing gene 17, Sox17, was increased in adenomas relative to WTEC, but decreased again in carcinomas. In Xenopus, Sox17 was shown to bind β-catenin and inhibit its TCF-mediated signaling activity (37). Since excessive β-catenin signaling is the underlying mechanism of carcinogenesis in the APCMin/+ mice, the loss of this inhibitory pathway could significantly impact the growth potential of carcinomas.

This paper focuses on a subset of the genes identified in the study that may influence tumor growth. We also identified genes in other important functional groups including transcription regulation, signal transduction, transport, metabolism and biosynthesis, cell adhesion, and proteases and protease inhibitors. By studying the gene lists, a fairly extensive picture emerges of how tumor epithelium in vivo differs from its normal counterpart. What is especially valuable about this data set is that these changes can be either directly or indirectly traced to a deficiency in the tumor suppressor APC. Future studies will be necessary to elucidate the intricacies of how these molecular events are interrelated and whether similar mechanisms function in colon carcinogenesis in humans.

As is the case with all data sets derived from DNA microarray analyses, it is important to address the reliability of the results. In this study RNA was prepared from five or six separate biological replicates per group that were individually hybridized to GeneChip probe arrays. A conservative statistical approach was
then used to identify differentially expressed transcripts between the groups. The methodology is based on the Mann-Whitney pair-wise comparison test and has been previously validated to produce a very low frequency of false-positive results by one of the study investigators (N. F. Paoni) (14).

The data in Fig. 2 illustrate the reproducibility of the expression patterns within the groups for the 307 genes differentially expressed between tumors and WTEC in this study. The data also show the high degree of similarity between the related groups WTEC/epithelial cells isolated from uninvolved regions of \( \text{APC}^{\text{Min}}/\text{ ilea} \), and adenomas/carcinomas. Of the 223 genes identified as differentially expressed between WTEC and adenomas, 173 or 77.6% of them were also independently found to be differentially expressed between WTEC and carcinomas, with \( p \leq 0.05 \) in each case. The odds that both events happened by chance would be \(<1\) in 400. In many other cases, if a gene reached statistical significance on one gene list, then there was also a trend for similar expression on the other (see Figs. 3–6). Furthermore, the fact that no differences in gene expression were found between WTEC and epithelial cells isolated from normal-appearing regions of the \( \text{APC}^{\text{Min}}/\) small bowel is consistent with there being a very low level of false positives generated in the study. Although, for the reasons stated above, we believe the gene list to be reliable, we have not confirmed the expression of all of the genes on the list. Investigators interested in exploring results from this study should independently confirm the differential expression of genes of specific interest on a case-by-case basis.

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