The *Min* (Multiple Intestinal Neoplasia) Mutation: Its Effect on Gut Epithelial Cell Differentiation and Interaction with a Modifier System

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Abstract. *Min* is a fully penetrant dominant mutation that leads to the development of multiple intestinal adenomas throughout the duodenal-to-colonic axis. *Min*/+ C57BL6/J mice have an average life-span of 120 d. Multi-label immunocytochemical studies of these lesions demonstrate patches of differentiated enterocytes, and scattered enteroendocrine, goblet and Paneth cells. Expression of endogenous marker genes within these differentiated cells can be directly correlated with the position occupied by the adenoma along the duodenal-to-colonic axis and mirrors the regional differentiation of the normal gut epithelium. The presence of multiple lineages in adenomas together with their retention of spatial information suggests that tumorigenesis in *Min*/+ mice may be initiated in a multipotent stem cell normally located at the base of intestinal crypts. To study the time-dependent properties of these tumors, genetic conditions were sought in which *Min*/+ animals could survive for up to 300 d. *Min* is fully penetrant in hybrids with either AKR/J or MA/MyJ. However, the hybrids demonstrate a reduction in the number of intestinal adenomas. Preliminary backcross analysis is consistent with a single major modifier locus unlinked to *Min* in both the AKR/J and MA/MyJ strains. The increased lifespan of the hybrid animals is also associated with the development of invasive tumors. New tumors do not arise continuously over the lifespan of these animals; instead all adenomas appear to be established by 100 d of age or sooner. These studies indicate that the *Min*/+ mouse is a powerful model system for analyzing the mechanisms that establish and maintain a balance between proliferation and differentiation in the continuously renewing gut epithelium and for an assessment of the multi-step hypothesis of intestinal neoplasia.

The mouse intestinal epithelium undergoes perpetual renewal of its four principal terminally differentiated cell types: the polarized absorptive enterocyte, the mucus-producing goblet cell, a complex population of enteroendocrine cells, and the defensin/lysozyme containing Paneth cell which is thought to function as part of the biological barrier to bacterial translocation across the gut (reviewed in Gordon, 1989). Renewal and differentiation are rapid, extraordinarily well organized in several spatial dimensions, and dependent upon multipotent stem cells that are functionally anchored at the base of intestinal crypts (Cheng and Leblond, 1974). In the adult small intestine, 6–10 crypts, each composed of ∼250 cells, surround each villus. [^{3}H]Thymidine labeling studies indicate that 150 cells, located in the middle portion of each crypt, pass through the cell cycle every 12 h resulting in the generation of 300 new cells/crypt/d (reviewed in Potten and Loeffler, 1990). These cells undergo a bipolar migration. ∼12 cells emerge from each crypt per hour and are translocated in coherent vertical bands up the adjacent villus (Cheng and Leblond, 1974; Potten and Loeffler, 1990; Schmidt et al., 1985*b*). Differentiation of enterocytes, goblet and enteroendocrine cells occurs during this upward migration which is completed in 3 d when the cells are exfoliated into the gut lumen at the apical extrusion zone of the villus. Each villus contains a steady state level of ∼3,500 surface epithelial cells and sheds ∼1,400 cells/day resulting in a net loss of 2×10^8 cells/small bowel/d (Potten and Loeffler, 1990). Paneth cells differentiate during a downward journey to the base of the crypt where they reside for ∼3 wk before they are removed by mechanisms yet to be fully defined. Epithelial cell migration and differentiation are also closely coupled in the mouse colon. As cells emerge from the colonic crypt, they form a hexagonal shaped cuff that surrounds the crypt orifice. This cuff represents the homolog of the small intestinal villus (Schmidt et al., 1985*b*).

Normal (Cheng and Leblond, 1974; Winton et al., 1988; Griffiths et al., 1988; Winton et al., 1989; Winton and Ponder, 1990; Schmidt et al., 1990), chimeric (Ponder et al., 1985; Schmidt et al., 1985*a*,*b*, 1988), and transgenic (Cohn et al., 1991) mice have been used to characterize the stem cell organization of the gut epithelium. The receptor for the *Dolichos biflorins* agglutinin lectin encoded by the *Dlb-1* gene located on chromosome 11 (Uitderdyk et al., 1986) has been a particularly useful lineage marker for analyzing the activity and biological properties of these stem cells. Inbred

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strains with the *Dlb-1*^{b} allele will bind the lectin in their crypt and villus epithelial cells whereas *Dlb-1*^{a}/*Dlb-1*^{a} mice are DBA-negative (Schmidt et al., 1984, 1985*a*,*b*). Analysis of adult aggregation chimeras formed from *Dlb-1*^{b} and *Dlb-1*^{a} homozygous embryos indicated that each adult small intestinal crypt is monoclonal: crypts contain either wholly DBA-positive cellular populations or wholly negative populations – but never mixed populations (Ponder et al., 1985). In contrast, studies of the clonal organization of neonatal chimeric gut revealed that, when crypts begin to form immediately after birth, they are polyclonal. They subsequently undergo conversion to monoclonality within the first 2 wk after birth by an unknown mechanism that has been termed purification (Schmidt et al., 1988).

An in vivo assay that detects somatic mutations at the *Dlb-1* locus in (C57BL/6J × SWR) F1 mice that are *Dlb-1*^{a}/*Dlb-1*^{b}, has been used to characterize the stem cell organization of the mouse gut in greater detail (Winton et al., 1988; Winton and Ponder, 1990). The amplified clonal descendants of mutated stem cells can be identified using histochemical methods as unstained groups of cells in crypts or on (polyclonal) villi that are otherwise DBA-positive. The rate of spontaneous mutation defined by this assay appears to be remarkably low: 0.5–13.5 mutations per 10^{5} small intestinal crypts (or 10^{4} villi) per year in untreated animals (O'Sullivan et al., 1991; Winton et al., 1991). Treatment of such mice with ethylnitrosurea (ENU) (Winton et al., 1988; Winton and Ponder, 1990) or gamma irradiation (Winton et al., 1989) results in the accumulation of monophenotypic, DBA-negative crypts, suggesting that each small intestinal crypt may ultimately be renewed by a single stem cell.

This view of the clonal organization of intestinal crypts is independently supported by studies using the X chromosome–linked polymorphic enzymes glucose-6-phosphate dehydrogenase (Griffiths et al., 1988) and phosphoglycerate kinase (Ponder et al., 1985; Inoue et al., 1988), as lineage markers and by analyses of transgenic mice containing fatty acid binding protein/human growth hormone fusion genes (Sweetser et al., 1988*a*,*b*; Cohn et al., 1991; Roth et al., 1991*b*).

Stem cells are generally defined by their high capacity for self-renewal. Their potential for asymmetric division yields one daughter that remains a stem cell (thus guaranteeing self-maintenance) while the other undergoes commitment to a differentiation program. Given the continuous and rapid renewal of the mouse intestinal epithelium, the multipotent small intestinal stem cell can be further characterized by its enormous division potential. Estimates suggest that over the 3-yr lifespan of a laboratory animal, each stem cell may undergo 1,000 divisions (Potten and Loeffler, 1990). Another feature is its ability to sustain geographic differences in the differentiation program of its clonal descendants depending upon its location along the duodenal-to-colonic axis. The molecular mechanisms that sustain these regional differences are not known. However, experiments involving transplantation of fetal gut, harvested before cytodifferentiation of its epithelium, into the subcutaneous tissues of syngeneic or nude adult mouse recipients reveal that the isografts undergo appropriate region-specific development in the absence of luminal contents (e.g., Rubin et al., 1991). This observation suggests that (*a*) the lumen is not the source of positional information and (*b*) stem cells are programmed with "spatial memory" before the appearance of the epithelial monolayer and the formation of a mature crypt-to-villus axis.

The normal mouse small intestine is able to establish a balance between proliferation and differentiation and to maintain it in the absence of neoplastic transformation. If transformation occurs in a lineage descendant, its rapid migration would be expected to result in its removal from the epithelial population unless the initiation event simultaneously enhanced the probability of self-renewal and functional anchorage. Alternatively, the already anchored stem cell may itself be involved in the initiation of gut neoplasia. A corollary to this latter hypothesis is that gut neoplasms should arise from single crypts and be monoclonal (Schmidt and Mead, 1990). This expectation is supported by several studies of mouse and human adenomas and carcinomas (Oomen et al., 1984; Ponder and Wilkinson, 1986; Fearon et al., 1987; Griffiths et al., 1989).

Current models for the origins of human colorectal carcinomas indicate that they arise as a consequence of a number of genetic mutations (Vogelstein et al., 1989; Fearon and Vogelstein, 1990). This multistep model envisions that progression from premalignant villus adenomas→more dysplastic adenomas→frank adenocarcinoma with subsequent invasion is associated with the accumulation of lesions that affect oncogenes and several tumor suppressor genes. Although the sequence may be variable, mutation or loss of genes at the familial adenomatosis polyposis (FAP) locus on chromosome 5 (i.e., the mutated in colon cancer [MCC] and adenomatous polyposis coli [APC] genes) (Bodmer et al., 1987; Leppert et al., 1987; Kinzler et al., 1991*a*,*b*; Groden et al., 1991; Joslyn et al., 1991) may lead to the development of small, monoclonal adenomas. Progression may reflect the accumulation of mutations in certain oncogenes, such as *K-ras* (Forrester et al., 1987; Bos et al., 1987; Vogelstein et al., 1988; Farr et al., 1988), as well as deletion or mutation of the deleted colon carcinoma (DCC) gene on chromosome 18 and the p53 locus on chromosome 17 (Baker et al., 1989; Nigro et al., 1989; Fearon et al., 1990). DCC is normally expressed in the colonic epithelium and encodes a member of the cell adhesion glycoprotein family (Fearon et al., 1990). Its loss or mutation may affect cell–cell and cell–extracellular matrix interactions. p53 is a 375-residue nuclear phosphoprotein that can suppress cellular transformation in oncogene cooperation assays, possibly through dominant negative effects (Jenkins et al., 1984; Parada et al., 1984; Eliyahu et al., 1989; Finlay et al., 1989; Marshall, 1991; Milner and Metcalf, 1991).

Additional information about how cell-specific, region-specific and developmental stage–specific expression of genes are regulated in the gut is critical for deriving the answers to several important questions concerning intestinal epithelial tumorigenesis. (*a*) Why does the incidence or type of cancer vary as a function of position along the duodenal-to-colonic axis? (*b*) Are stem cells the site of initiation of the transformation? (*c*) To what extent are the normal pathways for commitment and differentiation retained in these neoplasms and (*d*) can they be modulated in vivo?

Recently, a mouse model of intestinal neoplasia has been identified (Moser et al., 1990). Germline mutagenesis of C57BL/6J (B6) males by ENU followed by mating to AKR/J (AKR) females yielded a pedigree carrying an autosomal

dominant mutation that is fully penetrant, producing multiple intestinal adenomas throughout the length of the gastrointestinal tract. *Min* (multiple intestinal neoplasia) mice have been maintained by crossing *Min*/+ males with B6 females. B6-*Min*/+ mice develop anemia by 60 d of age. Their average lifespan is 119 ± 31 d (Moser et al., 1990). The average total number of tumors in duodenal, jejunal, ileal, and colonic segments is 29 ± 10.

In this present study, we have defined cellular differentiation within the tumors of *Min*/+ mice as a function of both position along the duodenal-to-colonic axis and age. A survey was conducted using molecular markers specific for enterocytes, enteroendocrine, goblet and Paneth cells. The products of the homologous, but unlinked, mouse liver and intestinal fatty acid binding protein genes (*Fabpl* and *Fabpi*; Sweetser et al., 1987) were used as enterocytic markers for two reasons. First, both genes are expressed as enterocytes emerge from the crypt, creating a distinctive crypt-to-villus distribution of their mRNA and protein products (Sweetser et al., 1988*a*,*b*). Second, each establishes and maintains regional differences in their expression along the duodenal-to-colonic axis from the time of initial cytodifferentiation of the gut in late gestation through adulthood (Roth et al., 1991*a*; Rubin et al., 1991; Cohn et al., 1991). *Fabpl* transcription is confined to the small intestine (and hepatocytes). The highest steady-state levels of its mRNA occur in proximal jejunum, progressively failing as one proceeds to the terminal ileum. No mRNA is detectable in cecum or colon. Expression of *Fabpi* is confined to the intestine: the distribution of its mRNA and protein products along the cephalo-caudal axis is similar to *Fabpl* with the exception that it is also transcribed in the cecum and proximal colon (Sweetser et al., 1988*a*,*b*; Cohn et al., 1991; Rubin et al., 1991). Thus, an analysis of *Fabpi* and *Fabpl* expression in gut neoplasms allows simultaneous assessment of their ability to support differentiation and their capacity for retaining positional information. A similar logic was used when selecting the enteroendocrine cell population. While enteroendocrine cells represent <1% of the gut epithelial cells, they provide sensitive markers for its spatial differentiation. At least 15 different cell types have been identified based on their principal neuroendocrine product. Each cell type has a distinctive distribution along the crypt-to-villus and/or duodenal-to-colonic axes (Roth et al., 1990). Multiple label immunocytochemical studies (Roth et al., 1990; Roth and Gordon, 1990; Roth et al., 1991*a*; Rubin et al., 1991) have disclosed complex interrelationships between these populations that suggest, in certain cases, sequential expression of different endocrine products during their migration/differentiation along the crypt-to-villus axis. It is possible to exploit the complexity of the gut's enteroendocrine cell population to infer the biological properties of this multipotent stem cell (e.g., spatial memory, and regulation of commitment/differentiation of its descendants). Analysis of enteroendocrine populations represented in adenomatous lesions distributed along the proximal-to-distal axis of the gut in B6-*Min*/+ mice offered the promise not only of assessing their ability to retain positional information but also determining whether they are initiated in the stem cell (Roth et al., 1991*b*) or in its descendants. The presence of goblet cells within the tumors was assessed with histochemical stains for mucin. Because Paneth cells normally arise during a downward migration/differentiation to the crypt base, their presence or absence from these lesions should provide additional insights not only about the cellular target in which the *Min* mutation initiates tumor formation but also about the capacity of Paneth cells to arise in the absence of a well organized crypt-to-villus axis. (This latter point also applies to all of the cellular markers studied.)

Finally, genetic studies have revealed an unlinked modifier locus or loci that affect(s) the number of tumors that develop in *Min* mice and allowed us to examine the biological properties of these lesions in older animals. Our data suggest that the *Min* mouse represents a powerful model system for investigating the events involved in tumor formation and progression.

Materials and Methods

Mice

All mice were bred at the McArdle Laboratory for Cancer Research from C57BL6/J (B6), AKR/J (AKR) or MA/MyJ (MA) mice that were purchased from the Jackson Laboratory (Bar Harbor, ME). The Min pedigree is maintained by crossing *Min*/+ males with B6 females. F1 animals were produced by crossing B6 *Min*/+ males (N4-N10) with AKR, MA or (AKR × MA) F1 females. The backcross progeny were produced by mating F1 *Min*/+ mice of either sex with B6 mice. These matings were set up before determining the genotype of the F1 parent with respect to *Min*. The genotype was then established retrospectively by progeny test and by examination of the intestine of the F1 animals. Only the progeny from F1 mice shown to carry *Min* were included in the backcross set.

Tumor Scoring

Anemia provides an early and sensitive phenotypic marker of *Min* (Moser et al., 1990). Anemic mice were identified by pallor or by low hematocrit. Animals were killed by CO_2 asphyxiation when moribund or at 300 d of age. The intestinal tract from duodenum to colon was removed. Sections 4-cm in length were harvested from duodenum, jejunum, and ileum (see Sweetser et al., 1988*a* for a description of how these regions are operationally defined), opened longitudinally, and washed in PBS. The entire colon was treated in an identical manner. Tumors were counted under the dissecting microscope (at 10× magnification). The smallest scorable tumors were ~0.5 mm in diameter.

Immunocytochemical Methods

Unopened duodenal, jejunal, ileal, and colonic segments were fixed overnight in Bouin's fluid, transferred to 70% ethanol and subsequently embedded in paraffin. Alternatively, fixed tissues were incubated overnight in PBS containing sucrose (10% wt/vol) and frozen for cryostat sectioning. 5-μm-thick paraffin sections or 10-μm-thick frozen sections were prepared from adenomas randomly harvested from each segment as well as normal appearing epithelium (n = six 131-140-d-old B6-*Min*/+ mice; and two 304-d-old F1 mice from AKR × B6-*Min*/+ and MA × B6-*Min*/+ intercrosses). Paraffin sections were treated with xylene and rehydrated in graded ethanols, H_2O, and PBS. Cryostat sections were briefly brought to room temperature and rehydrated with PBS. Rehydrated sections were then preincubated for 30 min in PBS containing 0.2% nonfat powdered milk, 2% BSA, and 0.3% Triton X-100. Primary antisera were diluted in this buffer and incubated with the sections at 4°C overnight. After a series of PBS washes, sections were incubated for 1 h with gold-labeled goat antirabbit serum, subsequently washed with PBS and water and bathed in silver enhancement solution using a protocol supplied by the manufacturer (Amersham Corp., Arlington Heights, IL). Silver precipitation was monitored under the light microscope and the reaction terminated by a series of water washes. Sections were lightly counterstained with hematoxylin.

Alternatively, antigen-antibody complexes were detected with fluorescent-labeled secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Methods for multiple labeling using simultaneous immunofluorescence or sequential immunogold silver/immunofluorescence

staining have been described in earlier publications (Roth et al., 1990; Roth and Gordon, 1990).

The following antisera were used for these studies (their final dilutions for immunogold silver staining [IGSS] and sources are listed in parentheses): rabbit antirat liver fatty acid binding protein (L-FABP, 1:1,000, Sweetser et al., 1988*a*), rabbit antirat intestinal fatty acid binding protein (I-FABP, 1:1,000, Sweetser et al., 1988*b*), rabbit antihuman lysozyme (1:1,000, Dako Corp., Santa Barbara, CA), rabbit or goat antiserotonin (1:2,000, Incstar Corp, Stillwater, MN), rat antiserotonin (1:2,000, Eugene Tech, Allendale, NJ) rabbit antisubstance P (1:2,000, Roth and Gordon, 1990), rabbit antigastric inhibitory peptide (GIP, 1:1,000, Peninsula Laboratories, Belmont, CA), rabbit antiglucagon-like peptide 1 (GLP-1, residues 1-19, 1:1,000, Peninsula), rabbit anticholecystokinin (CCK, residues 1-39, 1:1,000, Peninsula), rabbit antineurotensin (1:1,000, Incstar), rabbit antipeptide tyrosine tyrosine (PYY, TRQR43 from C. J. Evans, UCLA, 1:2,000), rabbit antisomatostatin (1:1,000, Incstar), and rabbit antipancreatic polypeptide (PP, 1:1,000, Dako). The immunostaining characteristics and unique specificities of these sera are demonstrated in previous reports (Roth et al., 1990; Roth and Gordon, 1990).

Goblet cells were identified with two separate histochemical stains for mucin: (*a*) mucicarmine and (*b*) periodic acid-Schiff (PAS) stain with α-amylase pretreatment to remove glycogen.

Results

B6-Min/+ mice develop intestinal tumors throughout the duodenal-to-colonic axis. These tumors appear as adenomatous polyps with moderate dysplasia, mild nuclear atypia, and frequent mitotic figures (Fig. 1 *A*). Surveys of 28 lesions harvested from six animals ranging in age from 131 to 140 d revealed no obvious morphologic differences between lesions as a function of their position along the cephalo-caudal axis. No invasive adenocarcinomas were identified in these six mice.

Fabpl and Fabpi Expression in Intestinal Tumors Is Affected by Their Position along the Duodenal-to-Colonic Axis

A minority of the cells in serially sectioned, small intestinal neoplasms harvested from six B6-*Min*/+ mice reacted with L-FABP antibodies. L-FABP-immunoreactive cells were found in patches distributed among histologically identical L-FABP-negative cells. Fig. 1, *B* and *C* show results that are representative of those obtained from 18 serially sectioned adenomas. By contrast, L-FABP-positive cells were not present in any of the colonic lesions in the three animals we surveyed. Patches of I-FABP-immunoreactive cells were also encountered in 8 of 10 small intestinal adenomas that were serially sectioned and appeared similar to L-FABP-positive patches (data not shown). However, unlike *Fabpl*, *Fabpi* was also expressed in proximal colonic, but not distal colonic, adenomas ($n = 3$ animals). Thus, although these proteins accumulated in only a small subpopulation of cells, their patterns of expression in small and large intestinal adenomas appear to recapitulate the normal regional specificity of *Fabpi* and *Fabpl* transcription in the adult mouse intestine. It is important to note that immunostaining of normal-appearing duodenal, jejunal, ileal, and colonic epithelium in six B6-*Min*/+ mice indicated that the *Min* mutation had no effect on the distribution of L-FABP and I-FABP along the crypt-to-villus and duodenal-to-colonic axes when compared to their distributions in three normal littermates (data not shown).

Surveys of Enteroendocrine Populations in B6-Min/+ Mouse Intestinal Adenomas

Serotonin-immunoreactive cells are distributed throughout the length of the mouse intestinal tract and represent the largest enteroendocrine cell population in the normal adult mouse gut (Roth et al., 1990). Serotonergic cells were present in all 18 adenomas examined independent of their location along the cephalo-caudal axis (Fig. 1 *E*). Of the 11 enteroendocrine cell types examined (see below), this type was encountered most frequently. The number of serotonin-immunoreactive cells in a given adenoma ranged from one to two cells per section to ∼5% of all tumor cells/section. Unlike L-FABP-positive and I-FABP-positive enterocytes, these enteroendocrine cells were diffusely scattered throughout the lesion and not clustered in discrete patches of contiguous cells or in discrete regions of adenomas.

Substance P-immunoreactive cells are found throughout the normal mouse small intestine and proximal colon but are rare in the distal colon (Roth et al., 1990; Roth and Gordon, 1990). Immunocytochemical studies indicated that substance P cells represented <1% of the cells/section in 18 of the 20 serially sectioned small intestinal and proximal colonic tumors we examined. No substance P-immunoreactive cells were detected in the distal colonic lesions of three ∼140-d-old B6-*Min*/+ animals.

In normal B6 mouse intestine (and in regions of normal appearing gut in B6-*Min*/+ animals), substance P was frequently colocalized with serotonin in the same cell. Coexpression of these neuroendocrine products is normally influenced by cellular location along the crypt-to-villus axis (Roth et al., 1990; Roth and Gordon, 1990). Substance P-immunoreactive cells in the adenomas typically contained serotonin (Fig. 1, *D–F*). Since there are many more serotonin-immunoreactive cells than substance P-immunoreactive cells in these lesions, most serotonin-positive cells were substance P-negative.

Secretin-immunoreactive cells are normally limited to the small intestinal villus (Roth et al., 1990). Previous studies revealed complex interrelationships between substance P-, serotonin- and secretin-immunoreactive cells (Roth and Gordon, 1990). The percentage of cells that coexpress substance P and serotonin, or serotonin and secretin is affected by their location along the crypt-to-villus axis. These observations suggested a migration-dependent differentiation program that involves sequential expression of substance P, serotonin, and secretin (Roth and Gordon, 1990), and indicate that the three cell types are sensitive markers of differentiation along this axis. No secretin cells were found in any of the 11 serially sectioned tumors examined that arose in intestinal segments which normally contain this enteroendocrine cell population. This includes adenomas that contain cells which express substance P or serotonin alone or those that coexpress both of these neuroendocrine products.

The representation of other enteroendocrine cell types in tumors reflects their distribution along the duodenal-to-colonic axis of the normal gut and the site of origin of the tumor. For example, neurotensin-positive cells are present throughout the entire intestine of normal adult B6 mice (Roth et al., 1990). Rare (<1%) neurotensin-immunoreactive cells were found in both small and large bowel tumors ($n = 3$ of 8 tumors). Gastric inhibitory peptide (GIP)-, gastrin-, and

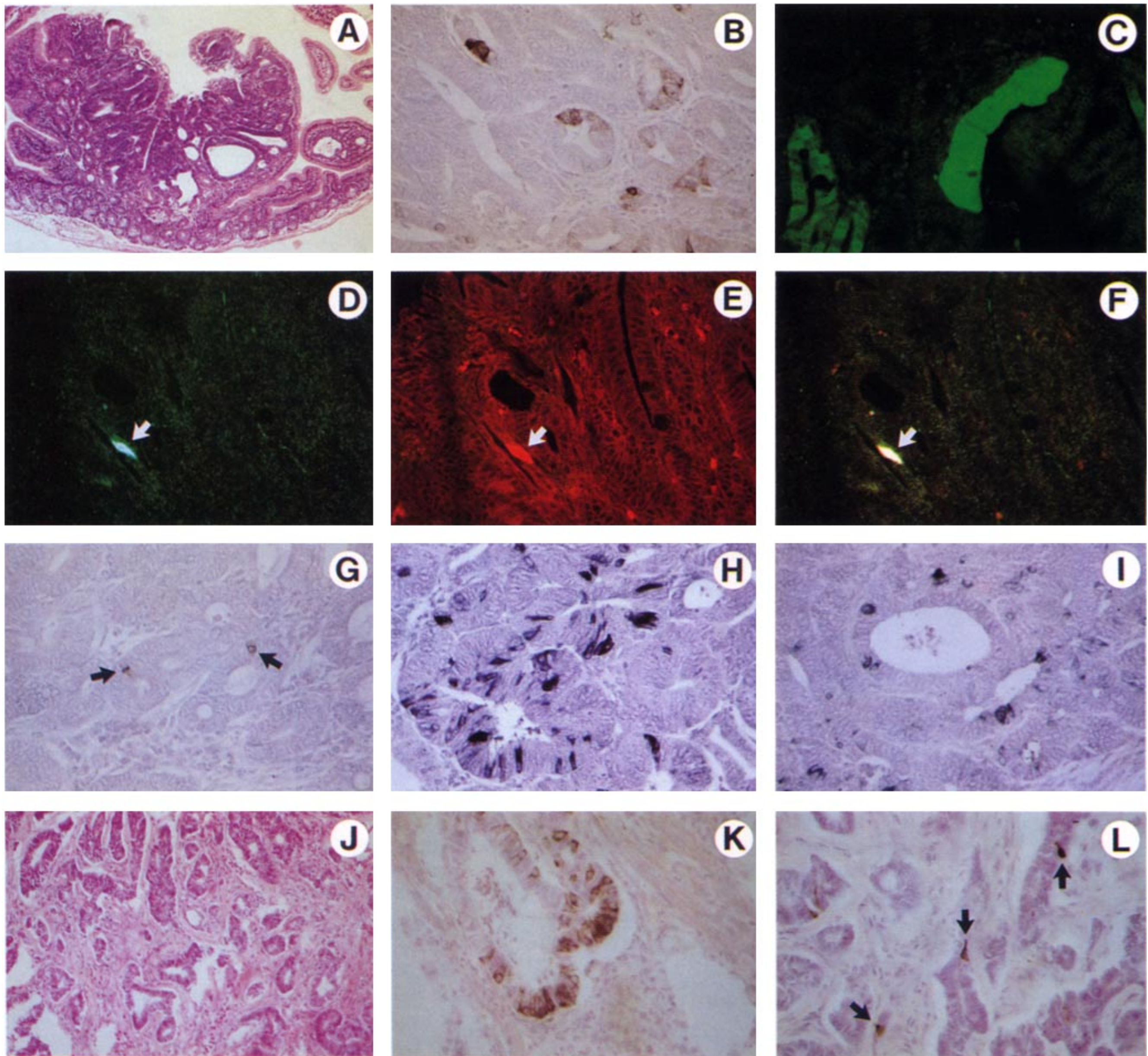

Figure 1. Immunocytochemical studies of cellular differentiation in *Min* intestinal neoplasms. (*A*) Hematoxylin and eosin-stained section from the proximal jejunum of a B6-*Min*/+ mouse shows a typical adenoma protruding into the intestinal lumen (100×). Adenomas arising in the small intestine frequently contained L-FABP–immunoreactive cells. Small patches of L-FABP containing cells in two separate jejunal adenomas are seen in *B* and *C*. L-FABP was detected with a rabbit anti-L-FABP antiserum and either the immunogold silver staining (IGSS) method (*B*) or with fluorescein-labeled second antibodies (*C*). L-FABP cells occurred scattered individually throughout the tumor, as in *B*, and/or in contiguous groups of intensely labeled cells, as in *C*. *Min*+ adenomas frequently contained enteroendocrine cell subpopulations characteristic of the intestinal segment in which they arose and complex enteroendocrine cell interrelationships appeared to be maintained. *D–F* demonstrate the colocalization of substance P and serotonin immunoreactivity within a jejunal adenoma. The tumor tissue was first incubated with rabbit antisubstance P serum and detected with IGSS, followed by rat antiserotonin serum which was detected with β-phycoerythrin–labeled donkey antirat serum. A substance P–immunoreactive cell is seen in *D*, visualized with reflected light-polarization microscopy. A single serotonin-immunoreactive cell is seen in *E*. Double exposure of the section in *F* clearly shows the colocalization of substance P and serotonin in this cell. Colonic tumors contained enteroendocrine cell subpopulations appropriate for this intestinal segment. A section from a distal colonic adenoma immunostained for PYY shows two immunoreactive cells in *G*. This cell type is largely confined to the colon and tumors arising in the small intestine did not contain PYY-immunoreactive cells. Lysozyme immunoreactive cells were found in adenomas throughout the intestine. Small intestinal tumors contained relatively frequent, intensely labeled cells (*H*) while large intestinal tumors had fewer and weaker stained cells (*I*). (*B–I*, 400×). Small intestinal segments from two 304-d-old MA × AKR (*Min*+) mice showed invasive adenocarcinomas. A hematoxylin and eosin-stained section from a jejunal tumor demonstrates a moderately differentiated adenocarcinoma extending into the intestinal muscularis (*J*, 200×). Enterocytic, enteroendocrine, goblet and Paneth cell differentiation was found in the adenocarcinomas. *K* shows a patch of L-FABP immunoreactive cells and *L* several serotonin cells in the neoplasm invading into the muscularis (400×). Lysozyme-positive Paneth cells and mucin-containing goblet cells were also detected in these tumors (data not shown).

cholecystokinin (CCK)-immunoreactive cells are normally restricted to the small intestine (Roth et al., 1990). Rare (<1%) GIP-, gastrin- and CCK-positive cells were noted in small bowel adenomas (three of seven, one of six, and five of six tumors, respectively) but not in colonic lesions (n = 4 tumors examined for each polypeptide). Glucagon-like peptide-1 (GLP-1) cells are more abundant in normal colonic epithelium compared to small intestinal epithelium (Roth et al., 1990). In contrast, peptide tyrosine tyrosine (PYY) cells are rare in the small intestine but are present in moderate numbers in the normal colon (Roth et al., 1990). Similarly, pancreatic polypeptide (PP) immunoreactive enteroendocrine cells are essentially confined to the normal distal colon (Roth et al., 1990). The representation of each of these cell types (i.e. GLP-1, PYY and PP) in B6-*Min*/+ neoplasms harvested from duodenum, jejunum, ileum, proximal, and distal colon was appropriately position dependent (e.g., see Fig. 1 *G*; n = 2–6 adenomas/segment).

Mucin-Containing Goblet Cells

In all 28 tumors examined, PAS positive (after α amylase pretreatment) and mucicarmine-positive cells were identified (data not shown). These cells had the typical histologic appearance of mucin-containing goblet cells and were found scattered throughout the tumors as individual or small groups of cells.

Surveys of Lysozyme-producing Cells

In normal B6 mice, Paneth cells are located at the base of small intestinal crypts. Lysozyme-positive Paneth cells are much less frequent in colonic crypts. This asymmetric distribution was recapitulated in B6-*Min*/+ neoplasms harvested along the duodenal-to-colonic axis. Fig. 1 *H* illustrates the frequency of lysozyme immunoreactive cells found in 19 small intestinal tumors we surveyed (≤10% of neoplastic cells). In all these adenomas, lysozyme-positive cells were more numerous than the most abundant enteroendocrine cell subpopulation (i.e., those that were serotonin-positive) but always less populous than L-FABP-positive cells and the undifferentiated cells. Staining was also noted in colonic lesions but was both less intense (Figure 1 *I*) and involved fewer cells.

Identification of Genetic Backgrounds That Can Modify the B6–Min/+ Phenotype

We attempted to identify genetic conditions that would alter the temporal pattern of expression of the Min phenotype. We reasoned that if such alterations were to lengthen the lifespan of the affected animals, it might be possible to assess the time course of tumor formation and progression under controlled conditions. We therefore tested the phenotype of *Min*/+ mice hybrid between B6 and the inbred strains AKR or MA. Male *Min*/+ mice from the B6 line were crossed with females from these strains and the resulting F1 generation animals were killed when moribund or at 300 d of age. The intestines were removed and scored for tumors. The tumors were counted in four regions: the duodenum, jejunum, ileum, and colon. In each set of F1 *Min*/+ mice, the number of tumors was greatly decreased and the average lifespan was greatly increased compared to the B6 *Min*/+ mice (Table I). The average total tumor number for the hybrid *Min*/+ animals was 7 for the (MA × B6) F1 and 6 for the (AKR × B6) F1 as compared with 29 for B6 *Min*/+ mice. In each cross, ∼50% of the F1 generation (AKR, 13 of 28 animals; MA, 9 of 14 animals) developed tumors, indicating that *Min* is fully penetrant in the hybrid animals. This was more fully substantiated by progeny testing of eight of the tumor-free (AKR × B6) F1 mice by crossing with B6 mice. In no case did any of these phenotypically normal mice give rise to tumor-bearing progeny. These analyses indicate that there are alleles carried by these two strains, AKR and MA, that can act dominantly to modify the action of *Min* resulting in a decreased number of tumors.

Most of the hybrid animals in the first set were not scored until 300 d of age. To analyze the time course of tumor formation, another series of (AKR × B6-*Min*/+) F1 mice was produced and sacrificed at 100 or 175 d of age and examined for tumors. Again, close to 50% of the animals had tumors (13 of 27 animals at 100 d; 12 of 21 animals at 175 d), indicating that even at 100 d the tumor phenotype of *Min* is fully penetrant on hybrid background. The tumor multiplicities of the *Min*/+ F1 animals sacrificed at 100 or 175 d of age were indistinguishable from those of the F1 mice killed at 300 d of age (Table I). As tested by the Wilcoxon Rank Sum Test, the only significant difference in the tumor multiplicities at different ages was between 104 and 175 d ($P = 0.04$). Therefore, new tumors seem not to arise continually over the lifespan of the animals. Rather, it seems that most tumors are established before 100 d of age.

Characterization of the Tumors in F1 Animals

The tumors present in the hybrid animals were typically adenomas with the phenotypic characteristics of the B6 *Min*/+ lesions described above. However, the longer lifespan of these animals afforded us the opportunity to assess tumors in animals over 300 d of age. Since the time course analysis described above indicated that most of the tumors are present before 100 d of age, the tumors in these older animals had been growing for over 200 d. In contrast to younger mice, surveys of two such 304-d-old mice disclosed focally invasive adenocarcinomas in the small intestine (Fig. 1 *J*). These adenocarcinomas showed moderate degrees of cellular anaplasia and nuclear atypia. As in the case of the adenomas, they contained patches of L-FABP-positive and I-FABP-positive cells, a variety of enteroendocrine populations, mucin-containing goblet cells, and lysozyme-immunoreactive cells (Fig. 1, *K* and *L*). The position-dependent occurrence of these cells paralleled that noted in adenomatous lesions present in the same F1 animal and in the younger B6-*Min*/+ mice described above. These analyses indicate that, given time, some of the adenomas can progress to adenocarcinomas and yet retain residual differentiative capacity.

Initial Genetic Characterization of the Modifier System

To ascertain whether this modifier system maps to the *Min* locus and to investigate its genetic complexity, a backcross analysis was performed. F1 *Min*/+ animals from each set were crossed to B6 animals to produce a segregating backcross generation. Animals were killed when moribund or at 300 d of age. Each intestinal tract was then scored for the presence of tumors as described for the F1 sets.

Table I. The Number of Tumors Is Suppressed in Min/+ F1 Hybrids

Background	Age ± 1 SD	Number of mice		Average number of tumors among tumor-bearing mice ± SD		
		Total	Tumor-bearing	In small intestine	In colon	Total
	d					
B6	118 ± 26	68	29	24 ± 7.2	5 ± 4.3	29 ± 7.5
MAxB6	349 ± 102	14	9	6 ± 3.1	1 ± 1.7	7 ± 3.7
AKRxB6	293 ± 52	28	13	5 ± 4.8	0.9 ± 1.0	6 ± 4.7
AKRxB6	175 ± 4	21	12	7 ± 5.1	0.8 ± 1.3	8 ± 4.8
AKRxB6	104 ± 1	27	13	3 ± 1.8	0.6 ± 1.0	4 ± 2.6

The AKR backcross set consisted of 154 animals, 77 of which were scored as *Min*/+. The MA backcross set included 148 animals, 83 of which were *Min*/+. There were no differences in the average total tumor number owing to the sex of the Fl *Min*/+ parent or the sex of the backcross animal. Also, equal numbers of *Min*/+ females and males were recovered. The average total tumor multiplicities for the AKR and MA backcross generations, 16 ± 12 and 16 ± 11, fell between the Fl and B6 *Min*/+ numbers (see Table I). The distributions of tumor numbers for the backcross, Fl and B6 *Min*/+ mice are shown in Fig. 2. The distributions are broad, encompassing both parental distributions. These data are consistent with the presence of a single modifier locus with major effects that are unlinked to *Min*.

Discussion

Phenotypic analysis of the multiple intestinal neoplasia mutation has indicated that the adenomatous lesions in B6-*Min*/+ mice contain a complex population of undifferentiated and differentiated cells. The presence of enteroendocrine, enterocytic, goblet, and Paneth cells within a given tumor, together with the fact that expression of gene products within these cells is appropriate for the position occupied by the tumor along the duodenal-to-colonic axis, suggest that these adenomas may arise from a single, mutated, multipotent stem cell in a crypt. Further genetic analysis has disclosed a single, major modifier locus unlinked to *Min* in AKR and MA strains. This modifier results in a reduction in number of adenomas and an increase in lifespan. Older hybrid *Min*/+ animals develop focally invasive adenocarcinomas. These observations indicate that the Min mouse (*a*) defines a single gene (*Min*) that can affect the normal balance between proliferation and differentiation in the gut epithelium; (*b*) provides an opportunity for further characterization of multipotent intestinal stem cells; and (*c*) together with the modifier system represents an opportunity to define steps that can lead to the evolution of intestinal neoplasia.

Is the Multipotent Crypt Stem Cell the Site of Initiation of Tumorigenesis in Min Mice?

The presence of multiple differentiated cell types in human colorectal and mouse intestinal neoplasms has been described in several reports (Kirkland, 1986; 1988; van den Ingh et al., 1986; Carroll et al., 1990; Oomen et al., 1984). Although they represented only a minor fraction of the total cellular population, their existence suggests the possibility that these lesions arose from a progenitor cell and that the capacity to differentiate along a number of pathways was retained during initiation and progression of the neoplasm. The ability of such lesions to generate multiple lineages has also been reproduced in culture: several cell lines derived from human colonic adenocarcinomas (e.g., HT-29 and HRA-19) are able to produce enteroendocrine, goblet, and enterocyte-like descendants (Pinto et al., 1982; Huet et al., 1987).

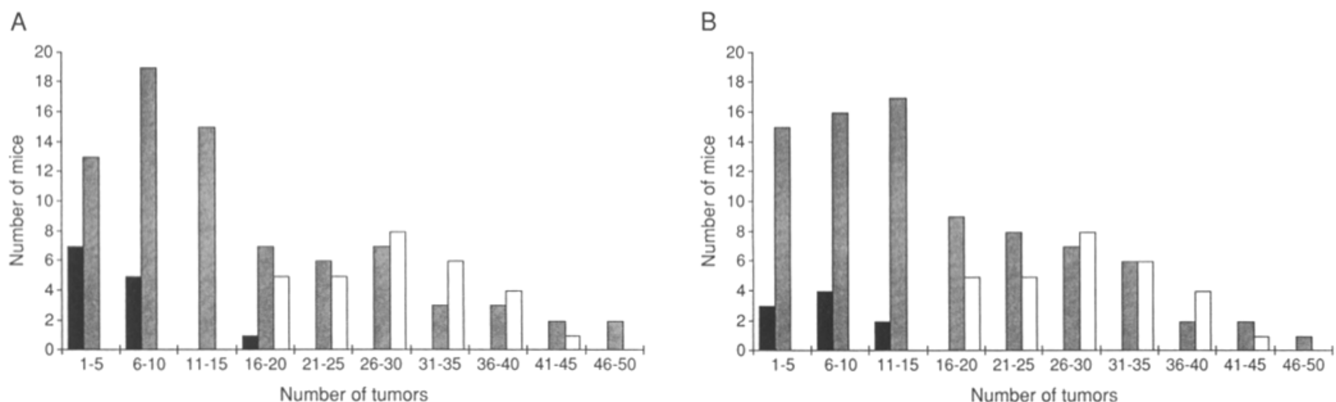

Figure 2. Tumor multiplicity in *Min*/+ mice from the AKR and MA backcrosses. To analyze the system of modifiers in the AKR (*A*) and MA (*B*) strains, hybrid *Min*/+ animals were backcrossed to B6 mice. The crosses were done in each parental orientation. Mice resulting from such backcrosses were scored for *Min* and tumor counts were performed. The average number of tumors for mice in each generation is given in the text. The distributions of tumor number for the *Min*/+ mice from each backcross generation (*hatched bars*) are compared with those of the *Min*/+ Fl hybrid parents (*solid bars*), and B6-*Min*/+ mice (*open bars*).

Finding multiple, differentiated cell types in Min adenomas and the retention of position-dependent differences in enterocytic and enteroendocrine differentiation programs within these lesions is fully consistent with initiation within a multipotent stem cell. If a stem cell is the site of initiation of tumorigenesis, then it can already provide the potential for multiple differentiation programs, spatial memory (see Introduction) as well as functional anchorage and an enormous capacity for renewal. The chance that a single germline mutational event would yield such a complex constellation of biological properties in any other cell located along the crypt-to-villus axis seems improbable. Given the rapid migration of cells along the axis, it also seems implausible that multiple independent or interdependent somatic events could accumulate to yield the necessary array of proposed properties of the initiated cell. It remains to be seen whether the multipotential stem cell population that we believe may serve as the source of the Min adenomas corresponds precisely to any normal intestinal stem cell population, and whether the growth of the adenoma is clonal or, as in neonatal intestinal epithelium, polyclonal.

The Modifier System of the Min Phenotype

The incidence of intestinal tumors due to the action of *Min* is reduced in mice of Fl hybrid genetic backgrounds containing a contribution from AKR or from MA. The resistance factor(s) of AKR and MA are either codominant or fully dominant. We do not yet know whether the sensitive B6 background represents the null allele(s) of resistance factor(s), or whether it contributes codominant susceptibility determinants. Further genetic analysis, including mapping of the modifier system, is necessary to assess fully its genetic structure in AKR and MA, and its formal mode of action. We can state unambiguously, however, that the modifier system is not encoded entirely at the *Min* locus. If it were, resistance would segregate from *Min* in the backcross progeny and all *Min*/+ animals would match the B6-*Min*/+ mice in tumor multiplicities.

A number of instances of parental imprinting have been reported recently for genetic disorders in the human, including heritable cancer syndromes (Reik, 1989; Hall, 1990). We have, therefore, asked whether the action of the modifier system shows evidence for parental imprinting. Neither the sex of the Fl parent nor the sex of the backcross progeny was correlated with tumor multiplicity. Therefore, no evidence has arisen for an imprintable component of the modifier system.

Susceptibility to Min-induced Tumor Formation vs. Age

The modifier system has permitted the study of tumor formation and progression over an extended age range in hybrid *Min*/+ animals. This analysis indicates that most, if not all, tumors are initiated before 100 d of age. Thus, the probability of tumor formation is not constant with age; *Min*-induced neoplasms seem to be juvenile lesions. In principle, the staging of *Min*-induced tumors can reflect changes in the expression of the mutant *Min* gene and/or changes in the susceptibility of the target cell population. More precise investigations of the staging of tumor formation are called for, in particular to know whether there is a window of susceptibility at the neonatal stage during which time crypt lineages are polyclonal (Schmidt et al., 1988). One must keep in mind in this context that even on a sensitive background, the number of *Min*-induced tumors is very small in comparison to the total number of stem cells in the intestinal tract; tumor formation is a low probability event.

Given time, *Min*-induced tumors can progress to more invasive forms. Such invasiveness may be an intrinsic property of the undifferentiated cell population comprising the early adenomas, or it may depend upon further somatic mutational events that occur during proliferation of non-invasive adenoma stem cells. The Min mouse strain permits investigation of the process of progression by providing a uniform background upon which defined environmental or genetic modifications can be introduced.

Prospectus

In this report, we have described a further refinement of the genetic system of the mouse defined by the dominant mutant allele *Min*, multiple intestinal neoplasia. The modifier system is used experimentally to permit the observation of *Min*-induced tumors over a longer time period than that afforded on the B6 background. The system is not yet characterized in genetic detail, nor at the molecular level.

A number of issues are raised by the studies reported here. In surveys of B6- or hybrid-*Min*/+ adenomas, the majority of cells did not react with lineage specific markers. What are these cells? Do they represent an inappropriate expansion of a normal class of cells – e.g., one which normally resides in the crypt such as its transit cell population (see Potten and Loeffler, 1990)? Given the dynamic balance maintained between proliferation and differentiation in the normal crypt, would the expansion of such a cellular compartment represent an unbalanced proliferation or the elimination of a maturation process? Transgenes may represent a powerful tool to characterize further this predominant, undifferentiated cellular population present in *Min* adenomas. As noted above, *Fabpl* is a sensitive marker of enterocytic differentiation. Linkage of nucleotides −4,000 to +21 or −596 to +21 of the rat L-FABP gene to several reporters produces a pattern of reporter mRNA and protein accumulation along the duodenal-to-ileal axis which mimics that of the intact endogenous *Fabpl* gene but produces inappropriate (precocious) expression in the crypt where *Fabpl* is normally silent (Sweetser et al., 1988*a*; Hansbrough et al., 1991). Crossing *Min* heterozygotes with L-FABP/reporter transgenics will afford an opportunity to assess whether transgene expression is supported in the seemingly undifferentiated cells of *Min* adenomas and thereby operationally define similarities to crypt epithelial cells. Investigating growth factor and growth factor receptor gene expression in the adenomatous lesions and the normal appearing crypts of *Min*/+ mice, and in the crypts of wild type mice may provide clues about the mechanisms that establish and maintain the balance between proliferation and differentiation along the crypt-to-villus axis. Finally, what are the molecular identities of the *Min* locus and the modifier system? What are the normal physiologic functions and mechanisms of action of the wild type *Min* allele and its modifiers? Progress both in the molecular genetics of the mouse (e.g., Todd et al., 1991) and in the biological analysis of the intestinal epithelium promise to bring answers to these questions.

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