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Studies of neoplasia in the Min mouse [1]

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Contents

Abbreviations: 129, 129/SvJ (or 129/J or 129/Sv or 129/Sv-Pas); AAPC, attenuated adenomatous polyposis coli; ACF, aberrant crypt foci; AKR, AKR/J; APC, adenomatous polyposis coli; *arm*, *armadillo*; 5-aza-dC, 5-aza-2′-deoxycytidine; B6, C57BL/6J; BALB, BALB/cByJ; BTBR, BTBR/Pas; CAST, *M. m. castaneus*; cDNA, complementary DNA; CHRPE, congenital hypertrophy of the retinal pigment epithelium; *Cox*, cyclooxygenase; DBA, DBA2/J; *Dcc*, deleted in colorectal carcinomas; *DLG*, discs large gene; DMBA, 7,12-dimethylbenz[*a*]anthracene; *Dnmt*, DNA methyltransferase; dUTP, deoxyuridine triphosphate; dpc, days post-coitum; ENU, *N*-ethyl-*N*-nitrosourea; FAP, familial adenomatous polyposis; *GSK 3β*, glycogen synthase kinase 3β; HNPCC, hereditary nonpolyposis colorectal cancer; LOD, logarithm of the odds; MA, MA/MyJ; *Mcc*, mutated in colorectal cancer; MCR, mutation cluster region; *Min*, multiple intestinal neoplasia; *Mom1*, modifier of *Min* 1; mRNA, messenger RNA; *Msh2*, *mutS* homolog 2; NSAID, nonsteroidal anti-inflammatory drugs; PCR, polymerase chain reaction; *Pla2g2a*, phospholipase A2; *scid*, severe combined immunodeficiency; SSLP, simple sequence length polymorphism; SWR, SWR/J; *Wnt1*, wingless/int1 homolog

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1. Introduction

The study of human disease has been greatly facilitated by the use of animal model systems. Recent advances in the understanding of the genetics and biology of the mouse have made this species a particularly useful experimental organism. Mutant strains of mice have largely come from three sources: naturally occurring variation [1–3]; phenotypic screening following germline mutagenesis [2,4,5]; and, more recently, targeted mutation of cloned genes [6–8]. *N*-ethyl-*N*-nitrosourea (ENU), the most potent known germline mutagen in the mouse, has been used to induce germline mutations resulting in mouse models for a variety of human disorders [4,5,9–11]. It was through phenotypic screening following ENU mutagenesis that the Min mutant mouse was discovered [12]. The Min mouse has proven to be a very useful model for studying human intestinal cancer.

Apc^{Min} (*Min* = multiple intestinal neoplasia) is an autosomal dominant mutation that predisposes mice to develop adenomas throughout the intestinal tract [12,13]. On the C57BL/6J (B6) genetic background, *Min*/+ mice develop, on average, more than 50 tumors throughout the entire length of the intestinal tract and rarely live past 150 days of age [12]. Since all intestinal tumors in B6 *Min*/+ mice are benign adenomas, the premature death of these animals is associated with secondary effects of tumor growth, including severe, chronic anemia and intestinal blockage [12].

Familial adenomatous polyposis (FAP) is a human cancer syndrome in which affected individuals develop as many as several thousand intestinal adenomas, often by the second decade of life [14,15]. If not removed, some of these adenomas will develop into carcinomas [15]. FAP results from germline mutation of the adenomatous polyposis coli (*APC*) tumor suppressor gene located on human chromosome 5 [16–19]. The phenotypic similarities between FAP patients and Min mice led us to examine whether the Min phenotype is due to germline mutation of the mouse *Apc* gene. Genetic mapping localized *Min* to the region of mouse chromosome 18 that also carries *Apc* [20]. Sequence analysis of the entire 8535 bp *Apc* cDNA identified a nonsense mutation in *Min*/+ mice that results from a T/A → A/T transversion at nucleotide 2549 (codon 850) of *Apc* [13]. The average intestinal tumor multiplicity in Min mice has remained relatively constant during extensive back-

crossing to B6 (currently N > 35 generations). Since the 19th backcross generation, selection for each generation has been based on genotyping for the single base pair *Min* mutation and not on phenotype. Thus, it is unlikely that persisting ENU-induced mutations at other loci are contributing to the Min phenotype.

The phenotypic and genetic similarities between Min mice and humans with FAP make Min an excellent animal model for addressing many fundamental questions associated with intestinal neoplasia. Some of the important issues include: What is the role of somatic mutation of *Apc* in intestinal tumor formation and what mutational mechanisms are involved? What are the consequences of *Apc* mutations on growth and development in other tissues? What other genes are involved in tumor formation and/or progression in the intestine (and other tissues), and what are their modes of action? And finally, can Min mice be used to identify and assess pharmacologic agents for the treatment and prevention of human cancer?

2. APC and human intestinal cancer

The development of intestinal cancer involves progression through a series of distinct morphological stages [21–25]. Analysis of specific genetic alterations at each of these morphological stages in humans has led to the development of a model implicating the accumulation of mutations in several tumor suppressor genes and oncogenes in tumor formation and progression [22,25,26]. In FAP patients, somatic mutation of the wild-type allele of *APC* is the most frequently detected somatic event associated with tumor formation [27–37]. In addition, somatic mutation of *APC* has been shown to occur frequently in cases of sporadic colon cancer, as well as in hereditary non-polyposis colon cancer (HNPCC), a familial cancer syndrome resulting from inherited defects in DNA mismatch repair [30,31,34,36,38–43]. These results point to loss of normal APC function as a very early, if not initiating, event in intestinal tumor formation.

2.1. Potential APC functions

A diagram of putative APC binding domains and a summary of known *APC* mutations is presented in Fig. 1. While it remains unclear precisely how disruption of APC function can promote tumor formation, intriguing possibilities have been raised by the recent discovery that APC may be an important molecule in regulating cell adhesion. This hypothesis is based primarily on evidence that APC can bind to and regulate β-catenin [44–48]. β-catenin is one member of a family of intracellular catenins that regulate cell-cell adhesion between epithelial cells, in part through interactions with E-cadherin [49–53].

Immunoprecipitation experiments have demonstrated that three imperfect 15 amino acid repeats located between residues 1020 and 1169 of APC are involved in a constitutive binding interaction with β-catenin in human cells (Fig. 1) [47,48]. APC can also trigger a post-translational down-regulation of β-catenin that appears to be mediated by binding of β-catenin to a second region of APC [44]. This binding domain consists of several 20 amino acid repeats located between amino acids 1342 and 2075 of APC [44,46]. APC-mediated regulation of β-catenin levels may be further controlled by the activities of Wnt1 and glycogen synthase kinase 3β_(GSK 3β) (Fig. 2) [54,55]. These findings have led to a tentative model implicating *APC* in a *Wnt1* signal transduction pathway (Fig. 2) [45,54–57]. However, since *Wnt1* does not appear to be expressed in the intestine, it is unclear whether this pathway is fundamentally important in regulating intestinal tumorigenesis.

Both APC and E-cadherin bind to a series of 42 amino acid repeat motifs within β-catenin that have been termed *arm* motifs owing to their original discovery in the Drosophila homolog of β-catenin, *armadillo* [58–60]. Interestingly, APC contains seven arm repeats between amino acids 453 and 766 [17,18,59]. Catenin binding to APC may, therefore, be further regulated by interactions between *arm* repeats and catenin binding sites within APC [57]. APC and E-cadherin form similar but independent complexes with β-catenin [55,57,58]. However, both β-catenin and E-cadherin appear to exist in large excess relative to APC in at least some types of epithelial cells [57,61]. This argues against a direct competition by APC for β-catenin binding of E-cadherin, unless APC levels are locally elevated [61]. Näthke et al. [61] have found elevated levels of APC in regions of epithelial cell membranes that are ac-

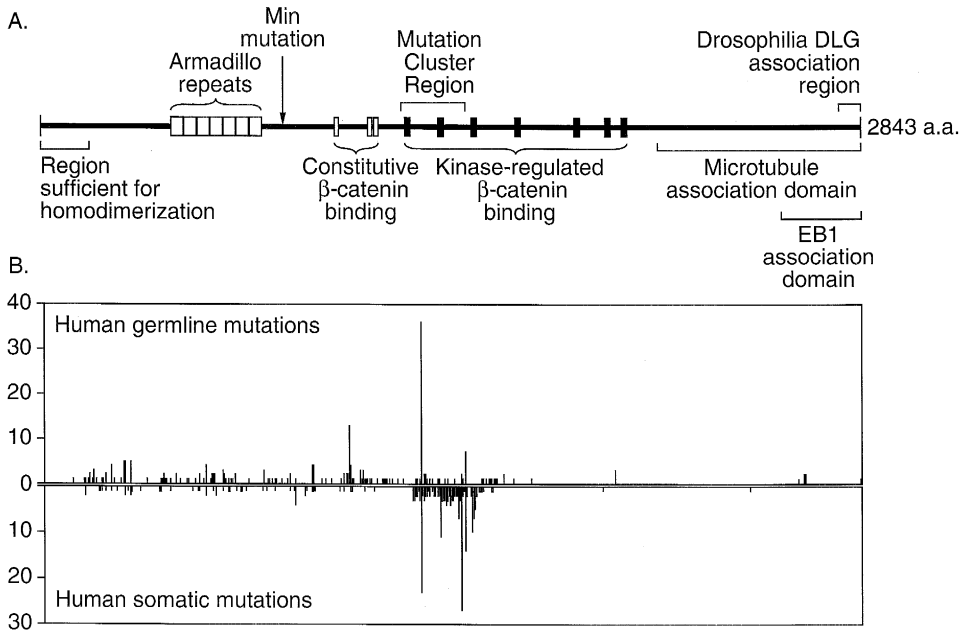

Fig. 1. Potential APC functional domains and location of *APC* mutations. The locations of potential APC functional domains are indicated. The amino acids of APC corresponding to each domain are as follows. APC homodimerization, 1 to 171. Armadillo repeat region, 453 to 766. Constitutive β-catenin binding, 1020 to 1169. Kinase regulated β-catenin binding, 1342 to 2075. Microtubule association domain, 2143 to 2843. EB1 association domain, 2560 to 2843. DLG association domain, 2771 to 2843. The mutation cluster region covers codons 1286 to 1513. The *Min* mutation is located at codon 850 of *Apc*. B. Human germline *APC* mutations are shown above and human somatic *APC* mutations are shown below the *X* axis, respectively. The *X* axis coresponds to the map shown in A. Mutation data were compiled from references [28,31,72,75,76,78].

tively involved in cell migration. Furthermore, these authors observed high levels of Apc protein at the crypt/villus border, where normal cell migration is needed for cellular exit from the crypts of the small intestine. Thus, in these circumstances APC may indeed successfully compete with β-catenin for E-cadherin binding.

The amino terminus of APC has been shown to mediate APC homodimerization [62,63]. The first 45 amino acids of APC are necessary and the first 171 amino acids sufficient for this interaction [63]. Moreover, immunoprecipitation analysis of extracts from human colorectal cancer cell lines known to express both full-length and truncated forms of APC suggests that the products of wild-type and some mutant forms of the gene may be able to associate in vivo [63].

Three distinct interactions have been described for the carboxyl terminus of APC. When overexpressed in colon cancer cell lines, the final 700 amino acids of APC can interact with microtubules [64,65]. Yeast two-hybrid analysis indicates that amino acids 2560–2843 of APC can bind a protein of unknown function called EB1 [66]. Recently, two-hybrid studies have also shown that the final 72 amino acids of APC are sufficient for binding to the human homolog of the *Drosophila discs large gene* (*DLG*) [67]. While the functional significance of these three carboxyl domains is unclear, binding of APC to microtubules and DLG may also regulate cell adhesion. DLG is thought to be a component of tight junctions in mammalian cells [68]. Intriguingly, it has been reported that a trimeric complex of DLG, APC, and β-catenin can be immunoprecipitated from mouse brain cells [67]. The interaction of APC with microtubules has been hypothesized to mediate signals from β-catenin/cadherin to the cytoskeleton [57,64,65].

These studies implicate *APC* in cell adhesion processes. This possibility is particularly important in light of the fact that the intestinal epithelium is a tissue of extremely rapid cell proliferation and migration [69–71]. It seems plausible that perturbation of cell adhesion homeostasis would have serious impli-

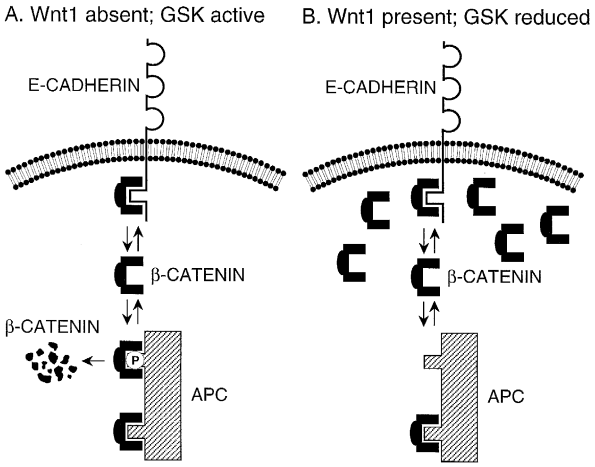

Fig. 2. Potential role of APC in a Wnt1 signal transduction pathway. The interactions between APC, β-catenin, and E-cadherin as well as the potential regulation of these interactions by GSK 3β and Wnt1 are shown. In the absence of Wnt1, GSK 3β is postulated to be active. GSK 3β phosphorylation of APC enhances binding of β-catenin to the 20 aa repeats located between amino acids 1342 and 2075 of APC. Binding in this region of APC leads to β-catenin degradation. β-catenin also binds constitutively to the 15 aa repeats located between 1020 and 1169 of APC. Wnt1 activity is believed to reduce GSK 3β activity. Under these conditions β-catenin does not bind to the 20 aa repeats of APC, and therefore β-catenin degradation is prevented. Thus, intracellular levels of β-catenin increase. Note that β-catenin can still binds in the 15 aa repeat region of APC. Modified from [54], with permission.

cations for growth regulation in this tissue. However, it is also conceivable that *APC* is involved in other processes that influence neoplasia. In this context, it is of considerable interest to determine how specific mutations of *APC* affect these processes.

2.2. APC mutations

Extensive data on the nature of *APC* mutations exist, with several hundred germline and somatic *APC* mutations identified (Fig. 1) [27,28,30–32,34]. The *APC* open reading frame encodes a 2843 amino acid protein that is organized into 15 coding exons [17,18]. The vast majority ($>95\%$) of both germline and somatic *APC* mutations result in premature truncation of the polypeptide [28,30,31,34]. Germline mutations are distributed throughout the 5′ half of the gene, although mutations at either codon 1061 or 1309 account for about 35% of known germline mutations [28,31,57,72,73]. Somatic mutations, by contrast, are heavily concentrated in a mutation cluster region (MCR) located between codons 1286 and 1513 [28,30,31,74].

The relationship between *APC* mutations and FAP phenotypes has led to provocative hypotheses about potential functions of mutant *APC* alleles. The preponderance of truncation mutations implies a possible dominant negative role for truncated APC polypeptides. The high frequency of mutations in the MCR and the more severe polyposis associated with germline MCR mutations suggest that mutations occurring in the MCR may be more efficient at inducing tumor formation than mutations elsewhere in the gene [57,74–76]. Dominant negative function of mutant polypeptides might, therefore, result from deleterious activity of the homodimerization and/or constitutive β-catenin-binding domains located in the first 1500 amino acids of APC. However, germline mutation prior to codon 157 often results in an attenuated adenomatous polyposis coli (AAPC) phenotype, characterized by reduced polyp number and later age of onset [75,77,78]. The explanation for this apparent discrepancy is unclear but may involve production of functional protein due to reinitiation of APC transcripts.

Other data from studies of human intestinal tumorigenesis also do not easily fit either a neomorphic or antimorphic dominant negative model of mutant APC function. Several cases of apparent germline deletion of *APC* have been reported that result in severe polyposis [79–82]. Western blot analyses of cell lines with *APC* mutations between codons 232 and 1338 demonstrated that mutations upstream of codon 715 may produce unstable products [83].

The presence of extracolonic manifestations in FAP, such as desmoid tumors, epidermoid cysts, osteomas, lipomas, and congenital hypertrophy of the retinal pigment epithelium (CHRPE) may also be correlated with the location of the *APC* mutation [75,84]. Specifically, CHRPE has been reported to occur more frequently in FAP patients with germline mutation after approximately codon 500 of *APC*, while desmoids, osteomas, and epidermoid cysts are commonly associated with germline mutation after codon 1400 [75,84–86]. However, CHRPE, osteomas, and epidermoid cysts are also seen in patients with germline deletion of *APC* [81,87]. Thus, as for

the intestinal tumor phenotype, factors in addition to the location of the germline *APC* mutation are likely to influence the occurrence of extracolonic manifestations in FAP.

Numerous studies have found *APC* mutations in a small proportion of a variety of sporadic human tumor types. Somatic mutation or *APC* allele loss have been reported for pancreatic [88], esophageal [89], and stomach tumors [90,91], as well as in hepatoblastoma [92], and small-cell lung carcinoma [93]. The Min mouse provides an excellent model system for determining if *Apc* is involved in regulating tumorigenesis in other tissues. Indeed, experiments with Min have further implicated *Apc* mutation as contributing to pancreatic, desmoid, and mammary tumorigenesis (see Sections 3.4 and 5.5).

Hypotheses about the relationship between *APC* alleles and disease phenotype must also take into account the action of environmental factors and genetic modifier loci, as well as ascertainment bias. Since most human studies involve genetically heterogeneous populations, the function of APC may be affected by segregating modifier genes. In addition, human studies can be biased owing to an increased likelihood of identifying more severely affected families. For example, individuals with a germline *APC* deletion that develop a large number of intestinal tumors, perhaps due to the action of modifier loci that enhance the phenotype, are more likely to be identified than less severely affected individuals with a similar *APC* deletion. A major advantage of using mouse models to study the genetics of intestinal cancer is the ability to control the genetic background experimentally. In addition to the Min mouse, two other mutant *Apc* strains now exist, each with a different germline *Apc* mutation [94,95]. The variability in the phenotypes of each of these mutant strains provides the opportunity to explore these questions in detail (see Section 7).

3. Phenotypic comparison of Min and FAP

The human and mouse *APC/Apc* genes are 86 and 90% identical at the nucleotide and amino acid levels, respectively [13]. As mentioned, *Min* is a nonsense mutation at codon 850 of *Apc*. The protein product of the Apc^{Min} allele would contain the homodimerization domain as well as the *armadillo* repeat region but would lack all other known APC binding domains (Fig. 1). This truncated polypeptide product is expressed in both normal intestinal epithelial cells and tumor cells from B6 *Min/+* mice (Fig. 3).

A comparison of the phenotypes associated with heterozygosity for germline mutations in *APC/Apc* for humans and Min mice is shown in Table 1.

3.1. Cellular characteristics of intestinal tumors

B6 *Min/+* mice and FAP patients share several phenotypic characteristics, most notably the presence of multiple intestinal tumors. In *Min/+* mice, most of these tumors occur in the small intestine, whereas in humans, tumors of the colon tend to be much more prevalent [12,14,15,24,96]. Immunohistochemical analyses of cell types in intestinal tumors from *Min/+* mice indicate that these tumors are comprised of differentiated and undifferentiated cells [97].

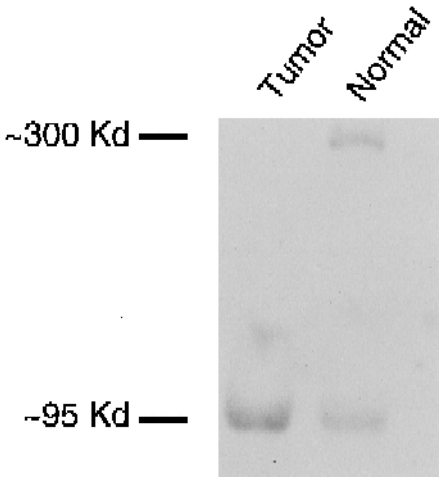

Fig. 3. Analysis of Apc expression in the mouse intestine. 100 μg of total protein isolated from normal colon tissue or from a pooled set of colon tumors from a B6 *Min/+* mouse was analyzed by Western blot according to the protocol in [83]. Apc expression was detected with a monoclonal antibody specific for the first 29 amino acids of APC (Oncogene Science, Cambridge, MA). Approximate molecular weights are based on molecular weight standards run in the same gel. The products of both the Apc^{+} and Apc^{Min} alleles are detected in normal tissue. Consistent with allele loss analyses (see Section 4.1), only the product of the Apc^{Min} allele is detected in tumor tissue. Similar results have been observed by others (Li-Kuo Su and Riccardo Fodde, pers. commun.).

Table 1
Comparison of phenotypes for mice and humans with germline *Apc / APC* mutations

Mouse (*Min / +*)	Human
Multiple colon adenomas	Multiple colon adenomas (100–1000 s)
Small intestine adenomas	Small intestine adenomas
Cystic intestinal crypts [a]	
Epidermoid cysts [a]	Epidermoid cysts
Desmoid tumors [a,b]	Desmoid tumors
Mammary adenoacanthoma [c]	Gastric polyps
	Osteomas of skull and mandible
	Hypertrophy of retinal pigment epithelium
	Abnormal dentition
	Lipomas

[a] See Section 3.3; [b] see Section 5.5; [c] see Section 3.4.

The normal intestinal epithelium of both mice and humans is comprised of four differentiated cell types: absorptive enterocytes; mucus-producing goblet cells; enteroendocrine cells; and Paneth cells [98–100]. The progenitor cell for these terminally differentiated cell types is believed to be a multipotent stem cell located near the base of the crypts of Lieberkühn [69,71,101–104]. The frequency of the differentiated cell types shows a characteristic pattern along the duodenal-to-colonic axis of the intestine [105–107]. Tumors from *Min / +* mice contain cells that express cell differentiation markers characteristic of each of these four cell types, and the expression pattern is appropriate for the location of a given tumor along the duodenal-to-colonic axis [97]. However, the majority of cells in these tumors do not express any of the cell differentiation markers examined and, therefore, probably represent undifferentiated cells. This observation is similar to what is observed for human colorectal adenomas and for intestinal tumors seen in other mouse systems [108,109]. The presence of a complex mixture of cell types in intestinal tumors suggests that these tumors are initiated in a multipotent stem cell and that some of the cells comprising the adenomas retain the ability to differentiate.

3.2. Timing of intestinal tumor formation in Min mice

Work from a number of laboratories has led to an understanding of tissue development in the mouse intestine [110–118]. Analysis of chimeric mice indicates that intestinal crypts in adult mice are clonal units, whereas early in postnatal development intestinal crypts are polyclonal [116,117]. During the second and third weeks of life, crypt clonality is established through a process known as crypt purification [117]. Other important changes that occur in the mouse intestine during the first several weeks of life include: a significant increase in crypt number though a process of crypt fission; changes in microfloral status; and changes in immunocyte levels [110,114,118–121].

Analysis of intestinal tumor multiplicity in 100 to 300 day-old (AKR/J (AKR) × B6)F_1 *Min / +* suggests that tumor number does not significantly increase over time [97]. To examine the timing of intestinal tumor initiation in B6 *Min / +* mice, we treated the mice with ENU at various ages and determined the intestinal tumor multiplicity 65 days after treatment [122]. ENU was used as the mutagen because it is known not to be a strong intestinal carcinogen and it is a direct-acting alkylating agent that avoids complications from differential metabolic activation [123,124]. The results of these experiments indicate that intestinal tumors in B6 *Min / +* mice are more likely to be initiated during the first several weeks of life [122]. ENU treatment of *Min / +* mice at 5 to 14 days of age increased tumor multiplicity 3.8-fold over untreated mice, whereas treatment between 20 and 35 days of age resulted in only a 1.6-fold increase in tumor multiplicity. Intestinal tumor number also remains relatively constant in untreated B6 *Min / +* mice that range in age from 67 to 97 days. This result suggests that tumors are initiated early in life in untreated as well as ENU-treated *Min / +* mice. There are several possible explanations for these results. The polyclonal nature of intestinal crypts in young mice may mean that there is a larger population of target cells per crypt that can acquire a tumor-initiating mutation prior to 14 days of age. It is also conceivable that a mutation acquired before crypt fission could be passed on to subsequent generations of crypts, potentially leading to the formation of multiple tumors. Other biological differences between the intestines of younger and older mice may also contribute to the enhanced sensitivity to tumor induction seen for treatment before 14 days of age.

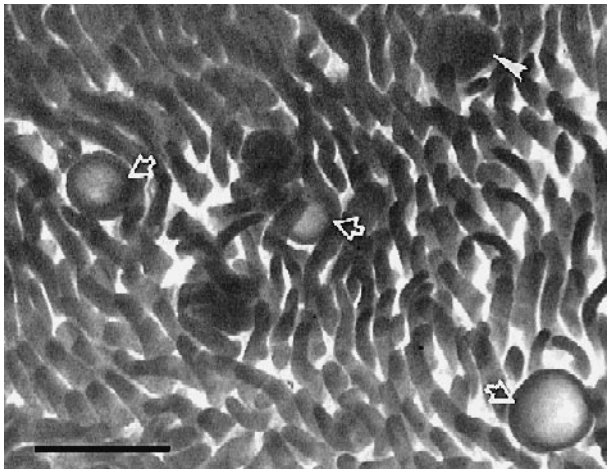

Fig. 4. Cystic crypts and intestinal tumors in the small intestine of an ENU-treated B6 *Min/+* mouse. A photograph of the small intestine of a B6 *Min/+* mouse treated with ENU at 17 days of age is shown. Several cystic crypts are indicated by arrows, and a small adenoma is indicated by an arrowhead. The black bar indicates 1.0 mm. Reproduced from [122] with permission.

An important, yet unresolved, issue concerning intestinal tumorigenesis is the clonal origin of tumors. Analysis of human intestinal tumors has led to conflicting evidence about tumor clonality [125,126]. Experimental analyses with mouse models such as Min may help resolve this question.

3.3. Other lesions observed in Min mice

Somatic treatment of *Min/+* mice with ENU also dramatically affects the formation of a distinct type of intestinal lesion, the cystic crypt. Cystic crypts are intestinal crypts that have become sealed off and are lined with abnormal cells that are often anaplastic [122]. Cystic crypts can be distinguished from tumors by their smaller size, more rounded structure, and their often transparent appearance in whole-mount preparations (Fig. 4). The developmental fate of these lesions is unclear. However, the relationship between age at ENU treatment and cystic crypt multiplicity is distinct from that seen for intestinal tumors. In contrast to the adenoma induction, the cystic crypt multiplicity for mice treated with ENU at 30–35 days of age was at least as high as for mice treated at all younger ages. In addition, cystic crypts are found almost exclusively in the proximal half of the small intestine, and no cystic crypts have been found in the colon. These results suggest that cystic crypts may not be preneoplastic lesions.

Two extracolonic manifestations associated with FAP in humans, epidermoid cysts and desmoid tumors, also occur in *Min/+* mice under certain conditions ([96,122]; A.R.M., W.F.D., D. Katzung, unpublished data). Epidermoid cysts are seen only rarely in untreated *Min/+* mice. However, somatic ENU treatment has a dramatic effect on the development of these lesions. Sixty percent (40/67) of B6 *Min/+* mice treated with ENU before 25 days of age developed epidermoid cysts, primarily located in the skin of the back [122]. Desmoid tumors, a significant post-operative complication in FAP patients, are not commonly seen in untreated or ENU-treated B6 *Min/+* mice. Genetic factors that can influence the multiplicity of these fibromatoses in *Min/+* mice are beginning to be defined (see Section 5.5). CHRPE is also not seen in untreated B6 *Min/+* mice (D. Alberts, personal commun.).

3.4. Female Min mice are prone to mammary tumorigenesis

One significant phenotype of the Min mouse that has not yet been documented in FAP patients is enhanced susceptibility to mammary tumor formation. Approx. 5% of B6 *Min/+* females spontaneously develop a single mammary adenoacanthoma [127]. Both the incidence and multiplicity of mammary tumors are dramatically increased in *Min/+* females by somatic treatment with ENU [127,128]. The most dramatic effect is seen in females treated with a single dose of ENU between 25 and 35 days of age. In one study, 82% of B6 *Min/+* females treated at 25 to 35 days of age developed mammary tumors within 65 days after treatment (A.R.M., unpublished data). The fact that ENU treatment of B6 Apc^{+}/Apc^{+} (+/+) females did not lead to mammary tumor formation demonstrates the necessity for the Apc^{Min} allele in this neoplastic pathway [127]. Transplantation of mammary tissue from *Min/+* females into +/+ hosts demonstrated that tumor susceptibility is intrinsic to *Min/+* mammary tissue. When B6 *Min/+* or B6 +/+ mammary cells were transplanted into the intrascapular fat pad of wild-type hosts, *Min/+*, but not +/+, transplants were

highly susceptible to tumor formation after treatment of the hosts with either ENU or 7,12-dimethylbenz[*a*]anthracene (DMBA), a potent mammary carcinogen [127]. Microscopic analyses have revealed the presence of more subtle abnormalities in *Min/+* mammary glands, even without ENU treatment. Distention of mammary ducts, keratin cyst formation in the proximal duct of cervical glands, and focal alveolar hyperplasias have been observed ([127]; A.R.M., unpublished data). These findings suggest that *Min/+* mice may be useful for the study of events involved in neoplastic transformation of the mammary gland.

3.5. The Min / Min phenotype

Embryos homozygous for the *Min* mutation fail early in development [129]. *Min/Min* conceptuses are able to form blastocysts and undergo uterine implantation at the same rate as *Min/+* or *+/+* embryos. However, shortly after implantation, at approx. 6.5 days post-coitum (dpc), *Min/Min* embryos demonstrate severe defects in primitive ectoderm development. By 10.5 dpc, approx. 25% of decidual swellings from *Min/+* intercrosses are abnormally small, with their major embryonic remnants consisting of trophoblastic giant cells. In light of the recent evidence suggesting that APC may regulate cell adhesion, it is interesting to note that some similarities exist between *Min/Min*, *β-catenin −/−*, and *E-cadherin −/−* embryos. The primary defect in all three of these mutants seems to involve disruption of cellular interactions rather than intrinsic cell lethality [129–132].

4. Somatic genetics of tumor formation in Min mice

Numerous studies of human intestinal adenomas have shown that at least 50% of tumors from FAP patients have lost or mutated the remaining wild-type *APC* allele [27–31,33–37]. Mutation of both *APC* alleles is also commonly observed in sporadic colon tumors [28,30,34–36,133]. In addition, somatic *APC* mutations have been found in some dysplastic aberrant crypt foci (ACF) in humans [134,135]. ACF are clusters of one to several abnormal intestinal crypts and have been hypothesized to be preneoplastic lesions [136]. These results suggest a classical tumor suppressor function for *APC* and indicate that loss of normal APC function may initiate tumor formation. The failure to find somatic mutation of *APC* in 100% of adenomas may reflect difficulties in detecting mutations in heterogeneous tumor tissue samples and/or in finding more subtle mutations that could, for example, affect *APC* expression. However, it should be noted that the mathematical two-hit model initially proposed by Knudson does not require that the two mutations occur in the two alleles of a single autosomal gene [137]. An alternative explanation for the failure to find somatic *APC* mutations in intestinal tumors could involve second-site non-complementation [96].

4.1. Apc^+ allele loss in intestinal adenomas from Min mice

Analysis of intestinal adenomas in B6 *Min/+* mice revealed that all of the tumors examined had lost the wild-type *Apc* allele [138,139]. In order to minimize normal cell contamination that could hinder studies of allelic loss, DNA was prepared from sectioned tumors, carefully avoiding regions of the tumor that appeared to contain a high percentage of normal cells [139]. A quantitative PCR assay was then used to determine *Apc* allelic status (Fig. 5). Analysis of 47 adenomas from B6 *Min/+* mice showed extensive Apc^+ loss in all cases, relative to normal tissue controls [139]. A similar finding of 100% Apc^+ allelic loss in B6 *Min/+* adenomas was reported by Levy et al. [138], using an allele-specific ligase chain reaction.

What is the mechanism of Apc^+ loss? By analyzing simple sequence length polymorphism (SSLP) markers along the length of several mouse chromosomes in adenomas from $(AKR \times B6)F_1$ *Min/+* mice, we found that, on this genetic background, the entire chromosome 18 homolog that carries the wild-type *Apc* allele is lost [139]. One possible explanation for this result is that hemizygosity at other chromosome 18 loci may enhance the probability of adenoma formation in *Min/+* mice. In this regard, it is important to note that *Mcc* (mutated in colorectal cancer) and *Dcc* (deleted in colorectal carcinomas), two other genes possibly involved in colon cancer in

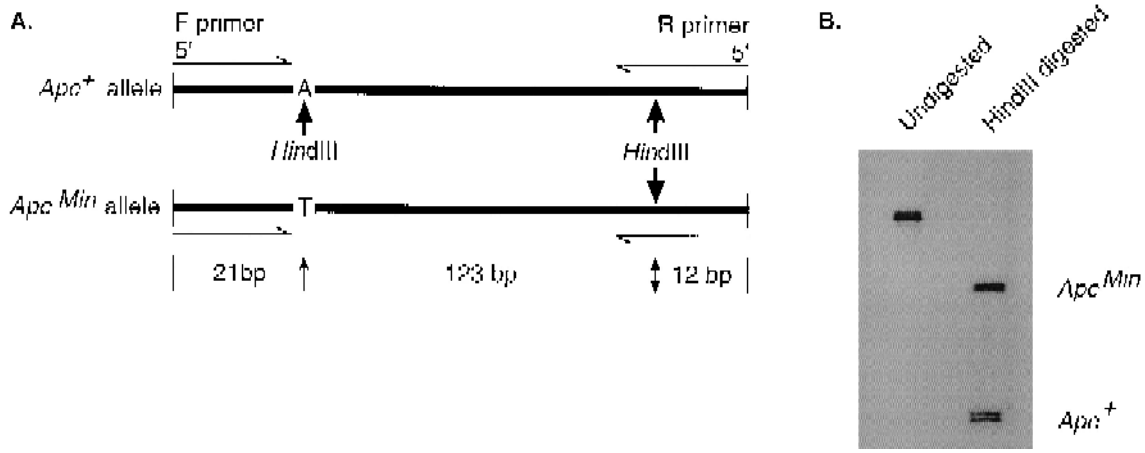

Fig. 5. *Apc* locus PCR-based assay. Schematic diagram showing the differences in the *Hin*dIII digestion patterns for the PCR products generated from the Apc^{+} and Apc^{Min} alleles. In this assay, PCR primers that flank the *Min* mutation are used. Amplification of the Apc^{+} allele results in 155 bp product containing two *Hin*dIII sites, while the 155 bp product from the Apc^{Min} allele contains only one restriction site. The A (deoxyadenosine) and T (deoxythymidine) nucleotides represent the single base pair alteration between Apc^{+} and Apc^{Min} on the B6 background. Autoradiogram of a denaturing acrylamide gel showing the undigested and *Hin*dIII-digested PCR products generated in the assay. The ratios of Apc^{+}/Apc^{Min} is determined by quantitative assessment of ^{32}P-labeled products. Reproduced from [139] with permission.

humans, are also located on mouse chromosome 18 [20,140].

4.2. Alternative somatic mutational mechanisms involved in intestinal tumor formation in Min mice

The chromosomal loss associated with spontaneous intestinal tumor formation in *Min*/+ mice contrasts with the somatic *APC* truncation mutation mechanism that predominates in human tumor formation. Do any conditions exist in which the somatic mutational mechanism involved in adenoma formation in *Min*/+ mice more closely resembles that seen in human tumor formation? Several possibilities have begun to emerge. Seven of 51 intestinal tumors from γ-irradiated $(AKR \times B6)F_1$ *Min*/+ animals contained deletions encompassing the Apc^{+} allele, but retained *Mcc* and/or *Dcc* [141]. In addition, Apc^{+} loss was not observed in four of 55 tumors from these irradiated animals. The genetic background of *Min*/+ mice also affects the mechanism of adenoma formation. In contrast to B6 *Min*/+ and $(AKR \times B6)F_1$ *Min*/+ animals, three of nine tumors from (*M.m. castaneus* (CAST) × B6)F_1 *Min*/+ and one of 25 tumors from (129/SvJ (129) × B6)F_1 *Min*/+ mice did not show Apc^{+} loss [141]. These results were obtained by quantitative PCR analyses of *Apc* and SSLP allelic ratios. Thus, it is certainly possible that more subtle mutations in *Apc* and/or other loci exist in these tumors. Recent data indicate that at least 25% of ENU-induced intestinal tumors in B6 *Min*/+ mice have acquired somatic truncation mutations in *Apc* [142]. Surprisingly, some ENU-induced intestinal tumors also appear to demonstrate Apc^{+} allele loss. In contrast, at least 12% of the tumors from ENU-treated mice did not contain any detectable somatic *Apc* mutation [142].

The development of a conditionally immortalized cell line from the normal intestinal epithelium of B6 *Min*/+ mice by SV40 has led to some interesting ideas concerning neoplastic transformation mediated by Apc^{Min} and Ha-*Ras* [143,144]. Clones from this *Min*/+ cell line that also express an exogenous copy of activated Ha-*Ras* have been shown to form colonies in soft agar. In addition, these cells were able to induce tumor formation in nude mice within 17 days of injection [143]. In contrast, cells from an Apc^{+}/Apc^{+} line that expressed the same activated *Ras* allele were not able to form colonies in soft agar and did not form tumors in nude mice until 90 days after injection. Intriguingly, analysis of one of the

tumors from the *Min/+* cell line suggested that these tumors may be able to form without loss of the remaining Apc^{+} allele [143]. Further investigations are needed to determine if intragenic *Apc* mutations and/or mutations at other loci are involved in this neoplastic process.

These in vitro studies, suggesting cooperation between Apc^{Min} and activated *Ras* in neoplastic transformation, seem to contrast with the in vivo results of Kim et al. [145]. In a study involving crosses of B6 *Min/+* mice to several transgenic lines, the presence of an activated K-*ras* allele was not found to increase intestinal tumor number or progression in *Min/+* mice [145]. However, the interpretation of these results is complicated by the fact that the transgene promoter used in these experiments is not known to lead to expression in the intestinal stem cell.

In further contrast to the studies with immortalized cell lines, recent results from our laboratory indicate that activation mutations of either K- or Ha-*ras* are uncommon in intestinal tumors from *Min/+* mice [142].

5. Genetic modification of the Min phenotype

As discussed earlier, phenotypic variation in FAP is believed to be influenced, in part, by the location of a given germline *APC* mutation. However, a great deal of phenotypic variability is often observed within FAP families, where affected individuals carry the same germline *APC* mutation [78,146,147]. Two additional sources of phenotypic variation in FAP include environmental and genetic differences between individuals. Identification of genetic modifier loci can be extremely difficult in human populations due to environmental and genetic heterogeneity. The Min mouse can be used in controlled genetic analyses to identify loci that can influence the expressivity (and possibly even the penetrance) of neoplastic processes.

5.1. Mom1

Initial studies involving crosses of B6 *Min/+* mice to AKR, as well as to several other inbred strains, indicated that these F_1 mice showed a decrease in average intestinal tumor number and an increased lifespan relative to B6 *Min/+* animals [97]. For example, $(AKR \times B6)F_1$ *Min/+* mice average 6.0 ± 4.7 intestinal tumors and can survive for up to one year. This change represents a significant decrease in tumor number from the average of approx. 30 tumors seen in the scored regions of B6 *Min/+* intestines [12,97].

In our laboratory, intestinal tumors are usually scored from four representative regions of the intestinal tract. Specifically, we score tumors in 4-cm segments from the proximal, medial, and distal small intestine, as well as the entire large intestine. These four segments represent one-third to one-half the length of the entire intestinal tract. We have found that, for most experiments, this method of scoring provides an accurate sampling of intestinal tumor multiplicity. (See references [12] and [122] for a detailed description of this tumor scoring protocol.)

The reduced intestinal tumor number of $(AKR \times B6)F_1$ *Min/+* mice demonstrates that the AKR strain carries alleles that act dominantly or semi-dominantly to reduce tumor number in *Min/+* mice. To examine this effect further, *Min/+* F_1 animals were crossed to B6 mice to produce a segregating backcross generation. Tumor multiplicity in this backcross population had a roughly bimodal distribution, indicating that a small number of segregating loci were influencing tumor number [148]. Analysis of the variance in the $(AKR \times B6)F_1$ *Min/+* $\times$ B6 backcross population gave an estimate of 1.8 unlinked genetic factors controlling this quantitative trait, thus suggesting that genetic mapping of a modifier locus might be feasible. By genotyping 110 animals from the $(AKR \times B6)F_1$ *Min/+* $\times$ B6 backcross set with SSLP markers located throughout the genome, evidence for a modifier locus on distal mouse chromosome 4 was obtained. This locus has been named *Mom1* for Modifier of *Min* 1. Analysis of backcross sets with two other inbred strains (CAST and MA/MyJ (MA)) indicated that the *Mom1* region on chromosome 4 also influenced tumor multiplicity in these crosses. The combined LOD score for the genetic mapping from these three strains was greater than 14.

Detailed analyses of *Mom1* are complicated by the fact that most inbred strains of mice that carry a resistance allele at *Mom1*, such as AKR, also carry additional, unlinked modifier alleles [148,149]. Therefore, we have generated a strain in which the chromosomal region containing the AKR allele of

Mom1 (*Mom1*^{A}) has been transferred onto the sensitive B6 background [149]. Analyses utilizing this B6 Mom1^{A} strain and *Min*/+ mice have shown that *Mom1* is a semi-dominant modifier of both intestinal tumor multiplicity and size in *Min*/+ mice [149,150]. B6 *Min*/+ mice that are heterozygous for *Mom1* (*Mom1*$^{A/B}$) have twofold fewer tumors than mice homozygous for the B6 allele of *Mom1*. *Min*/+ *Mom1*$^{A/A}$ mice have fourfold fewer tumors than *Min*/+ *Mom1*$^{B/B}$ mice. Intestinal tumor multiplicity in *Min*/+ *Mom1*$^{A/B}$ mice also increased from an average of 10.7 for mice sacrificed at 80 days, to 14.9 at 120 days, and 15.3 at 200 days. The average maximal tumor diameter for small intestinal adenomas from 120-day-old *Min*/+ mice was reduced from 2.16 mm (*Mom1*$^{B/B}$) to 1.57 mm (*Mom1*$^{A/B}$) to 1.41 mm (*Mom1*$^{A/A}$). Thus, *Mom1* affects total intestinal tumor multiplicity and net tumor growth rate in *Min*/+ mice [150]. Interestingly, *Mom1* affects tumor number but not tumor size in the large intestine [150].

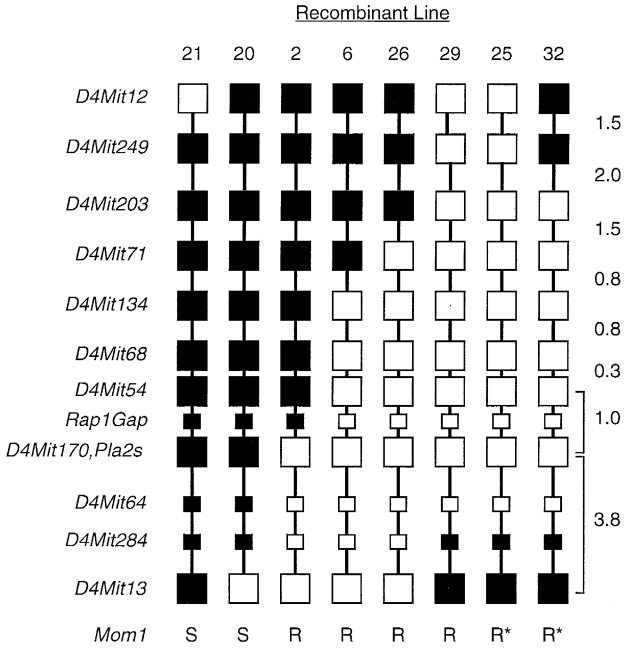

Fig. 6. Genotypes of recombinant lines in the *Mom1* region. For each recombinant line, open squares indicate positions where each line is heterozygous AKR/B6, and filled squares indicate positions at which each line is homozygous B6. Small squares indicate a gene or marker that is known to map within the interval shown, but the precise position within the interval is not known. For each line, the deduced Mom1 phenotype is classified as sensitive (S), resistant (R), or partially resistant (R*). The genetic distance (in cM) for each interval is given on the right of the figure. The genetic distances are based on 400 meioses in an AKR×B6 cross.

The secretory phospholipase A2 gene (*Pla2g2a*) has been proposed as a candidate for *Mom1* [151]. This claim is based on three observations. First, *Pla2g2a* maps to the same 15 cM region of mouse chromosome 4 as *Mom1*. Second, there is concordance between *Pla2g2a* genotype and Mom1 phenotype in four inbred strains. Finally, high levels of *Pla2g2a* mRNA are observed in the intestines of the resistant strains AKR, CAST, and MA, but only very low levels of *Pla2g2a* mRNA are detected in the intestines of the sensitive B6 strain [151]. Sequence analysis of *Pla2g2a* revealed that the B6 allele of this gene contains an A/T insertion in exon 3 leading to premature termination [151]. In contrast, the resistant AKR, CAST, and MA strains were shown to carry a functional *Pla2g2a* allele. By analyzing the *Pla2g2a* genotype of seven other strains, MacPhee et al. [151] predicted that the C3H/HeJ (C3H), CBA/J (CBA), and DBA/2J (DBA2) strains would demonstrate the Mom1 resistance phenotype while the P/J, A/J, C58/J, and 129/SvJ would show Mom1 sensitivity. The authors presented tumor multiplicity data for (P/J×B6)F_1 *Min*/+ animals to support the claim that the P/J strain exhibits Mom1 sensitivity. However, analysis of segregating backcross populations is necessary to assess the effect of individual loci, such as *Mom1*, on tumor multiplicity (see discussion below).

Further analysis of *Pla2g2a* as a candidate for *Mom1* has come from the explicit comparison of the *Pla2g2a* genotype and the Mom1 phenotype in five additional inbred strains, as well as by the creation of a high resolution genetic map of the *Mom1* region of chromosome 4 (Fig. 6) [149]. In order to examine the Mom1 phenotype of a given inbred strain, segregating backcross populations are generated by mating *Min*/+ F_1 mice from each original cross back to the B6 strain. Each backcross animal is then typed for polymorphic SSLP markers in the *Mom1* region. This *Mom1* genotype is then compared with the intestinal tumor multiplicity for each animal. Strains are classified as carrying a resistance allele at *Mom1* if the mice heterozygous for the *Mom1* region show a

significantly reduced tumor number in comparison with the mice that inherit two B6 alleles at *Mom1*. By these criteria, SWR/J (SWR), DBA2, and BALB/cByJ (BALB) were shown to carry a *Mom1* resistance allele, while the strains 129/Sv-Pas and BTBR/Pas (BTBR) carry a *Mom1* sensitivity allele [149]. Although there is one report suggesting that hybrids between B6 *Min*/+ and 129/Sv mice do not show a decrease in tumor multiplicity (see Section 5.3), in our laboratory (129/Sv-Pas × B6)F_1 *Min*/+ mice do exhibit reduced tumor number [149]. Through analysis of backcross populations this strain was classified as *Mom1* sensitive, thus indicating that the reduced tumor multiplicity is due to the action(s) of other, as yet unidentified, loci. We also have evidence that *Min*/+ F_1 hybrids between B6 and the 129/SvJ and 129/J strains show reduced tumor multiplicity [A.R.M. and C.L., unpublished data].

Analysis of the *Pla2g2a* genotype demonstrated SWR, DBA2, and BALB to be *Pla2g2a*$^+$, while 129/Sv-Pas and BTBR carry the same frameshift mutation in *Pla2g2a* as B6 [149]. Thus, the *Pla2g2a* genotype and the Mom1 phenotype show 100% concordance in nine inbred strains [149,151]. Although discordance for even one strain could disprove a candidate, concordance, even among nine strains, does not constitute proof. The *Pla2g2a* mutation in the B6, 129, and BTBR strains most likely arose in a common ancestor and has subsequently been preserved in these three strains. The concordance between *Mom1* and *Pla2g2a* could thus be the result of linkage disequilibrium between these two loci [152]. Linkage disequilibrium can be seen for another gene that maps to the *Mom1* region, *Rap1GAP*. *Rap1GAP*, a key regulator of the RAP1 G-protein, shows 100% concordance between genotype and Mom1 phenotype in the same nine strains analyzed for *Pla2g2a* [149].

In order to learn more about the *Mom1* locus and to test the *Pla2g2a* and *Rap1GAP* candidates more thoroughly, a high-resolution genetic map of the *Mom1* region has been generated (Fig. 6) [149]. By crossing the B6 Mom1$^{A/B}$ strain to B6, eight lines recombinant in the *Mom1* interval were generated that were, in turn, crossed to B6 *Min*/+ mice. Analysis of genotype with SSLP markers in the *Mom1* region and tumor phenotype in these lines further limited *Mom1* to an approx. 4 cM region between the SSLP markers *D4Mit54* and *D4Mit284* (Fig. 6). By determining the *Pla2g2a* and *Rap1GAP* genotypes of each of these lines, it was shown that *Pla2g2a* remained recombinationally inseparable from *Mom1*. However, one phenotypically resistant recombinant line (*Mom1*$^{A/B}$) was homozygous for the B6 allele of *Rap1GAP*, thus eliminating *Rap1GAP* as a candidate for *Mom1* [149].

Most intriguingly, two of eight recombinant lines showed an intermediate phenotype with respect to intestinal tumor number [149]. One of these lines (Rec 32) is a derivative of the other (Rec 25); it is likely that they share a common distal recombination breakpoint in the *Mom1* region (Fig. 6). The intermediate phenotype of these lines raises the possibility that *Mom1* is a complex locus comprised of multiple genes that modify tumor number in *Min*/+ mice [149].

How does this result affect the candidacy of *Pla2g2a* for *Mom1*? It has recently been shown that two other phospholipases, *Pla2g2c* and *Pla2g5*, are tightly linked to *Pla2g2a* in both mice and humans [153,154]. *Mom1*-mediated reduction in intestinal tumor multiplicity may thus be due to the combined effect of multiple phospholipase gene products. It will be important to determine if there is any correlation between genotype at *Pla2g2c* and/or *Pla2g5* and Mom1 phenotype in *Min*/+ mice. Preliminary studies in humans have not identified any phospholipase mutations associated with sporadic colon cancer or AAPC [155,156]. However, a recent report suggests that a modifier locus for human FAP may be present on human chromosome 1p35-36, the region syntenic with the *Mom1* region of mouse chromosome 4 [157].

It is presently unclear which of the proposed biochemical functions of *Pla2g2a* could account for inhibition of intestinal tumor multiplicity and growth. As Pla2g2a is a secreted molecule that is produced by the Paneth cells at the base of intestinal crypts, one might expect *Pla2g2a* to be capable of non cell-autonomous action [149,151,158]. Loss of heterozygosity may not be required for tumor formation with negative regulators of tumorigenesis that act in this fashion [77,149,159]. Consistent with this hypothesis, we have recently shown that neither the *Pla2g2a* locus nor the *Mom1* region undergoes allele loss in intestinal tumors from *Min*/+ mice [149].

A better understanding of the functions of both

Apc and *Mom1* has been achieved by investigating the tissue autonomy of these two loci. Through the use of intestinal grafts, *Apc* and *Mom1* have been shown to function in a tissue autonomous fashion within the mouse small intestine [158]. In these studies, segments of small and large intestine were obtained from 15 day-old embryos and transplanted to a subcutaneous site on the backs of 60 day-old histocompatible host animals. Such intestinal grafts show fairly normal development, including relatively normal tissue organization, nutrient absorption capability, and unidirectional peristalsis [160–162].

The tumor number in these small intestinal isografts is affected by the Apc^{Min} and *Mom1* genotype of the graft, but not by the genotype of the host [158]. To correct for the differential size of the grafts relative to the size of in situ intestines, tumor multiplicity was calculated as the number of tumors per cm^2 of intestinal tissue. All the small intestinal grafts from B6 *Min*/+ donors in B6 Apc^{+}/Apc^{+} (+/+) hosts developed tumors, irrespective of the host genotype at *Mom1*. The average number of tumors/cm^2 in B6 *Min*/+ small intestinal grafts in $Mom1^{A/A}$ hosts is not significantly different from the average for the B6 *Min*/+ grafts in $Mom1^{B/B}$ hosts. In addition, the average number of tumors per cm^2 in the *Min*/+ grafts in $Mom1^{A/A}$ hosts is not influenced by the host *Apc* genotype (*Min*/+ vs. +/+). However, the average number of tumors/cm^2 in *Min*/+ $Mom1^{A/B}$ grafts in B6 +/+ hosts is significantly lower than the average for *Min*/+ $Mom1^{B/B}$ grafts in B6 +/+ hosts [158]. The genotype of the graft did not influence the tumor multiplicity of the host animals in any of these experiments [158]. These results show that both Apc^{Min} and *Mom1* act autonomously in the graft tissue from the small intestine, rather than systemically. Interestingly, the effects of both *Min* and *Mom1* on tumor multiplicity in these small intestinal grafts were stronger than the effects seen in the in situ small intestine [158]. The explanation for this effect may involve the unique architecture and environment of the intestinal grafts. As these grafts are closed structures, the enhanced effect of *Mom1* might be due to prolonged exposure to *Mom1* gene product(s) relative to in situ intestines. In addition, these intestinal grafts have been shown to be free from colonization by microflora [158]. The enhanced effect of *Mom1* in the grafts could, therefore, result from improved *Mom1*-mediated tumor inhibition in a germ-free environment.

No tumors were observed in a total of 36 *Min*/+ colon grafts in either *Min*/+ or +/+ hosts [158]. Thirty of these 36 grafts were *Min*/+ $Mom1^{B/B}$, while the other 6 were *Min*/+ $Mom1^{A/B}$. This result demonstrates that tumorigenesis in *Min*/+ colon grafts differs from tumorigenesis in *Min*/+ small intestinal grafts. Further investigation is required to identify the cause(s) for this difference. Since no colon tumors were observed in any of the *Min*/+ colon grafts, it was not possible to assess the tissue autonomy of *Mom1* in the large intestine.

The *Mom1* genotype does not affect cystic crypt multiplicity in the in situ small intestine [158]. Neither *Mom1* nor *Apc* genotype of the host animal affects the average number of cystic crypts per cm^2 in *Min*/+ small intestinal grafts. However, the average number of cystic crypts/cm^2 in *Min*/+ $Mom1^{A/B}$ grafts is significantly lower than the average for *Min*/+ $Mom1^{B/B}$ grafts [158].

These results have important implications for the consideration of *Pla2g2a* as a *Mom1* candidate. Two possible mechanisms for tumor inhibition by this phospholipase include the action of *Pla2g2a* in the metabolism of dietary lipids and the involvement of *Pla2g2a* in bactericidal processes [151,158]. The results from the intestinal graft experiments demonstrate that *Mom1* can lead to reduced tumor multiplicity in intestinal tissue in the absence of ingested material or microbes. Thus, if *Pla2g2a* does confer resistance to intestinal neoplasia, it must exert this effect via mechanisms that are at least partially independent of lipid metabolism and/or bactericidal activity.

The ultimate assessment of *Pla2g2a* as a *Mom1* candidate can be achieved by creating mouse strains with the appropriate targeted mutation and/or properly expressed transgene of *Pla2g2a*. Crosses of these strains to the Min strain can provide definitive proof for or against this *Mom1* candidate and, if substantiated, could also provide insight into the apparently complex nature of *Mom1*. Proper interpretation of these experiments will require assessment of any unlinked modifier alleles that may be present in the strain(s) used. In addition, since *Mom1* acts semidominantly and may be a complex locus, analy-

sis of transgenic lines will require determination of transgene copy number and expression pattern.

5.2. The AKR Min strain

The full extent to which the intestinal phenotype of *Min*/+ mice can be modified by genetic background is observed in a mouse strain created by backcrossing the B6 *Min* allele onto the AKR strain (currently N > 8). In one set of 23 AKR *Min*/+ mice, only nine animals developed any intestinal tumors by 150 days of age. The average tumor multiplicity for these 23 mice was 0.5 ± 0.8, as scored for the entire length of the intestinal tract [[128]; A.R.S., A.R.M., unpublished data]. Therefore, on the AKR background, not only is the expressivity of the *Min* mutation reduced dramatically, but the penetrance is reduced to 39% (9/23) from the 100% seen in B6 *Min*/+ mice. This result shows that AKR allele(s) at loci in addition to *Mom1* can influence the intestinal tumor phenotype of *Min*/+ mice. The map position(s) and identity of these loci are unknown. It is unclear what combination of genetic and environmental factors leads to the variability seen for tumor multiplicity in FAP. Comparison of the intestinal phenotypes of B6 *Min*/+ and AKR *Min*/+ mice demonstrates that differences in modifier alleles alone can account for extreme differences in tumor multiplicity in mice.

5.3. Modification of Min by DNA methyltransferase

Another approach for identifying modifiers of Min involves crossing *Min*/+ mice with strains carrying targeted mutations in previously cloned genes. One of the most intriguing result obtained from this type of experiment has come from a cross of *Min*/+ mice to a DNA methyltransferase (*Dnmt*)-deficient strain [163]. $Dnmt^{S/+}$ mice carry a targeted mutation in the mammalian DNA (cytosine-5) methyltransferase gene that leads to an approx. 50% decrease in DNA methylation levels [163–165]. Analysis of the effect of homozygosity for the *Dnmt* mutation on intestinal tumor multiplicity is not possible, as $Dnmt^{S/S}$ mice die *in utero* [163–165]. Because $Dnmt^{S/+}$ mice carry one functional allele of this methyltransferase, the authors also applied a pharmacological approach to study the effects of methylation status on intestinal neoplasia in *Min*/+ mice. This was achieved by treatment of *Min*/+ mice with the potent DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC). The effects of genetic and pharmacological manipulation of DNA methylation levels on tumor multiplicity were dramatic. Heterozygosity at the *Dnmt* locus reduced tumor multiplicity by a factor of 2.5 in *Min*/+ mice. Treatment of *Min*/+ $Dnmt^{+/+}$ mice with 5-aza-dC reduced intestinal tumor multiplicity by a factor of 5.7. By combining 5-aza-dC treatment with heterozygosity at *Dnmt*, intestinal tumor number in *Min*/+ mice was decreased by a factor of 57 [163]. The stronger effect seen by treatment with 5-aza-dC relative to $Dnmt^{S/+}$ status is consistent with the observation that chronic treatment with 5-aza-dC results in a more pronounced decrease in DNA methylation than does inactivation of one *Dnmt* allele [163]. This finding contrasts with the observation that global DNA hypomethylation is correlated with enhanced intestinal tumor formation [166]. Hence, the results obtained with the *Min*/+ and $Dnmt^{S/+}$ animal models suggest a need to re-examine the role of DNA methylation in intestinal tumorigenesis.

The *Dnmt* mice used in these experiments were generated on a 129/Sv background [164,165]. In contrast to results from our laboratory using the 129/Sv-Pas, 129/J, and 129/SvJ strains, these authors did not see reduced tumor multiplicity in $(129 \times B6)F_1$ $Dnmt^{+/+}$ *Min*/+ animals relative to B6 *Min*/+ mice [163]. It is presently unclear whether this is due to differences in the 129 substrains used and/or differences in conditions of animal husbandry between research facilities.

By comparing tumor multiplicities in *Min*/+ mice treated with 5-aza-dC beginning either in the first or eighth week of life, Laird et al. [163] provided preliminary evidence that methylation status can affect the initiation stage of tumor formation in *Min*/+ mice. Intestinal tumor multiplicity in B6 *Min*/+ mice treated weekly for the first 14 weeks of life was reduced by a factor of 5.7 relative to untreated *Min*/+ mice, whereas treatment from week 8 to week 14 after birth apparently did not have any effect on tumor number. In light of our experiments demonstrating that intestinal tumors in *Min*/+ mice are predominantly initiated during the first several weeks of life, this finding is consistent with methylation

status affecting the initiation or early development of tumors [122].

There are two strong hypotheses for how methylation status could regulate oncogenesis. The first hypothesis states that methylation levels can affect gene expression and thus alter the expression of critical tumor suppressor genes and/or oncogenes [163,167–169]. The second hypothesis suggests that methylation contributes to neoplastic transformation by affecting somatic mutation rates at 5-methylcytosine residues [163,167–169]. Spontaneous deamination of 5-methylcytosine at CpG dinucleotides is known to lead to C/G → T/A transitions and is believed to be at least partly responsible for high mutation rates at CpG sites [169]. Laird et al. [163] argue that reduction of somatic point mutation rate is the most likely explanation for suppression of tumor formation in hypomethylated *Min/+* mice. However, tumors from *Min/+* mice showed similar rates of Apc^+ allele loss, regardless of *Dnmt* genotype or 5-aza-dC treatment. From this observation, the authors suggest that intestinal tumor formation in *Min/+* mice must require an event(s) in addition to somatic Apc^+ loss and that methylation status controls the somatic mutation rate at this other locus (or loci). Experiments addressing the effect of methylation status on mutation rate in vivo may clarify this situation.

5.4. Modification of Min by DNA mismatch repair

Germline mutation of several DNA mismatch repair genes is associated with hereditary nonpolyposis colorectal cancer (HNPCC) in humans [39–41,170–172]. Mice that are homozygous for a targeted null mutation in the *Msh2* (*mutS* homolog 2) gene develop lymphomas, skin tumors, and a small number of intestinal tumors by one year of age [173,174]. *Min/+* $Msh2^{-/-}$ mice develop approx. 340 adenomas throughout the entire length of the intestinal tract by three months of age [175]. This tumor number represents a 3.4-fold increase over the number of intestinal adenomas in *Min/+* $Msh2^{+/+}$ mice reported by this group.

Min/+ $Msh2^{-/-}$ mice were also reported to develop more colonic aberrant crypt foci (ACF) than *Min/+* $Msh2^{+/+}$ mice [175]. However, the relationship between elevated ACF and increased tumor number in these mice is unclear. The most dramatic increase in intestinal tumor number in *Min/+* $Msh2^{-/-}$ mice was observed in the small intestine, whereas ACF are exclusively located in the colon [136,175].

More intriguing is the finding that the *Msh2* genotype seems to affect the mechanism of somatic *Apc* mutation in intestinal tumor formation in *Min/+* mice [175]. While all 27 adenomas examined by quantitative PCR from *Min/+* $Msh2^{+/+}$ mice had lost the wild-type *Apc* allele, only 5 of 34 tumors from *Min/+* $Msh2^{-/-}$ lost the Apc^+ allele. Immunohistochemical analysis of 15 intestinal tumors from *Min/+* $Msh2^{-/-}$ mice demonstrated a lack of staining with an antibody specific for the carboxy-terminal 20 amino acids of APC. This result suggests that somatic truncation mutation of the wild-type *Apc* allele rather than *Apc* allele loss occurs in intestinal tumors from *Min/+* $Msh2^{-/-}$ mice.

No evidence for enhanced intestinal tumor progression was observed in *Min/+* $Msh2^{-/-}$ versus *Min/+* $Msh2^{\pm}$ or +/+ mice [175]. However, the decreased lifespan of the *Min/+* $Msh2^{-/-}$ mice may have hindered these analyses.

Genetic analysis of *Min/+* mice has thus far yielded three loci — *Mom1*, *Dnmt*, and *Msh2* — each of which can significantly modify intestinal neoplasia in *Min/+* mice. For *Mom1* and *Dnmt*, modification of intestinal tumorigenesis is fully dependent on *Apc* genotype. The value of learning more about how these modifiers function, and of identifying other modifiers, is clearly significant.

5.5. Other genetic modifiers of the Min phenotype

The importance of the *p53* tumor suppressor gene in a variety of neoplastic processes, including intestinal cancer, is well documented [25,151,176–178]. Extensive data from human studies indicate that loss of *p53* function is correlated with later stages of intestinal tumor progression, most likely the adenoma-to-carcinoma transition [125,179]. Two independent studies involving crosses of *p53*-deficient mice with *Min/+* mice have indicated that *p53* deficiency does not strongly influence intestinal tumor multiplicity in Min mice [159,180]. Determination of whether *p53* affects intestinal tumor progression in *Min/+* mice is complicated by the fact that

both *Min*/+ and *p53*$^{-/-}$ mice are short-lived [12,180,181].

However, the combination of *Min* and *p53* deficiency has been shown to affect tumor formation in other tissues. In the report of Clarke et al. [180], 83% of *p53*$^{-/-}$ *Min*/+ mice developed either preneoplastic foci (61%) or acinar cell adenocarcinoma (22%) of the pancreas. These adenocarcinomas were shown to have lost the wild-type *Apc* allele, thus implying a tumor suppressor function for *Apc* in the pancreas. Our laboratory has recently found that *p53* deficiency can also dramatically increase desmoid tumor multiplicity in *Min*/+ mice (W.F.D., D. Katzung, R. Halberg, L. Donehower, unpublished data). Desmoid tumors are also frequently observed in longer-lived hybrid *Min*/+ mice, particularly in multiparous females (A.R.M., unpublished data).

Genes that influence DNA repair and/or immunosurveillance capabilities are believed to be important in controlling neoplastic processes in many tissue types [39,182–185]. Hence, it is somewhat surprising that no evidence for increased intestinal tumor multiplicity or progression is observed in *Min*/+ mice that are also homozygous for the severe combined immunodeficiency (*scid*) gene [183]. *scid* mice are defective in double-strand DNA break repair and are immunocompromised owing to a deficiency in V(D)J recombination [183,186]. Thus, *scid*-mediated defects in double strand DNA break repair and/or B and T cell-mediated immunosurveillance do not contribute to intestinal tumorigenesis in B6 *Min*/+ mice.

6. Pharmacological modification of Min

An ultimate goal of cancer research is to apply basic research findings to the treatment and prevention of human disease. The use of Min mice to study agents for possible chemopreventive and/or chemotherapeutic treatment allows for a potential interface between basic research and clinical application.

6.1. NSAIDs

The methyltransferase inhibitor 5-aza-dC is an example of a pharmacological agent that can significantly inhibit adenoma formation in *Min*/+ mice [163]. However, the high toxicity of this drug makes it an impractical chemopreventive agent for human use. A potentially more useful class of anti-intestinal cancer agents are the nonsteroidal anti-inflammatory drugs (NSAIDs). While NSAIDs can also have significant toxicity, these effects are generally much less severe than for 5-aza-dC, and therefore these drugs have more clinical potential [187–190]. Several studies have indicated that NSAIDs can inhibit intestinal tumor formation and/or induce tumor regression in both animals and humans [187,188,190,191]. The mechanism of this inhibition is not known, but regulation of prostaglandin biosynthesis and/or apoptosis has been proposed [188,189,192–196]. NSAIDs such as piroxicam and sulindac have been reported to inhibit the activity of cyclooxygenase (Cox) enzymes [188,189,193]. Cyclooxygenases are important in the formation of prostaglandins and thromboxanes from arachidonic acid [188]. These eicosanoids are an important class of biosignaling molecules. Overexpression of cyclooxygenase has been observed in human colon tumors as well as in cell lines exhibiting reduced apoptotic capability [196,197].

Piroxicam and sulindac have been shown to reduce significantly the tumor multiplicity of *Min*/+ mice. In the experiments with piroxicam, B6 *Min*/+ mice were given the drug continually, via the diet, beginning at 30 days of age, and tumor multiplicity was then assessed 6 weeks later [194]. Treatment with the highest dose of piroxicam (200 ppm) led to a reduction in tumor number by a factor of 8. Interestingly, this effect seemed limited to the small intestine, as no difference in colon tumor number was seen between treated and untreated *Min*/+ mice. However, since the average tumor number in the colons of untreated B6 *Min*/+ mice was very low in these experiments (0.6 ± 0.3), an effect of piroxicam in the colon would have been difficult to detect. The fact that tumor multiplicity was reduced even though treatment was not begun until 30 days of age suggests that piroxicam affects tumor promotion and/or maintenance rather than tumor initiation [122].

Reduction of intestinal tumor number in *Min*/+ mice has also been reported in two studies with sulindac. In the study of Boolbol et al. [192], B6 *Min*/+ mice were given sulindac at a dose of approx. 160 ppm in the drinking water beginning at 5–6 weeks of age. Treatment with sulindac for ap-

prox. 9 weeks reduced average tumor multiplicity to 0.1 relative to the average of 11.9 tumors seen in untreated *Min/+* littermates. Since sulindac treatment was begun at 5–6 weeks of age, the results presented here are again more consistent with an effect on tumor outgrowth or possibly tumor regression, rather than tumor initiation.

To explain these results, these authors examined the intestinal expression of cyclooxygenase-2 (*Cox-2*), as well as the levels of enterocyte apoptosis, in untreated B6 mice (*Min/+* and *+/+*) versus sulindac-treated B6 *Min/+* mice. Interestingly, tumor-free regions of intestinal epithelium from the small intestine of untreated B6 *Min/+* mice appeared to express higher levels of *Cox-2* than normal intestinal tissue from *+/+* littermates [192]. This result might be due to a non-autonomous action of the tumors. Alternatively, elevated *Cox-2* expression may represent a heterozygous phenotype of Apc^{Min}. Similarly, Williams et al. [198] found elevated levels of Cox2 mRNA and protein in intestinal tumors from untreated *Min/+* mice. Treatment of B6 *Min/+* mice with sulindac reduced *Cox-2* expression to levels similar to those observed in *+/+* mice [192]. It is unclear whether this reduction of Cox-2 is responsible for reduced tumor multiplicity or is an independent effect.

The authors suggest that the level of enterocyte apoptosis is reduced in normal intestinal epithelial tissue of *Min/+* mice relative to *+/+* controls and that this effect is reversed by sulindac treatment [192]. However, while this result was observed when apoptosis was measured by immunoperoxidase analysis, the outcome was less clear when analyzed by terminal transferase-mediated dUTP nick end-labeling [192]. Also, apoptosis was compared in enterocytes that are predominantly located within the intestinal villi [192]. A more meaningful examination of the role of apoptosis in regulating tumorigenesis requires comparisons of apoptotic rates for cells within the crypts.

It is unclear why the average tumor multiplicity for the control animals in this sulindac experiment, which were scored for tumors along the entire length of the intestine, was dramatically lower ($\sim$12 tumors/animal) than most other reported values ($\sim$50 tumors/animal) [122,163]. This discrepancy may be due to differences in diet or other environmental factors. Clearly, a better understanding of the various environmental factors that influence intestinal neoplasia is needed.

In another study, tumor multiplicity was reduced by a factor of 2–3 in *Min/+* mice given sulindac in either the drinking water or the feed beginning at approx. 30 days of age [199]. Interestingly, a slightly stronger effect was observed when treatment was begun prenatally by treating pregnant females. This suggests that NSAIDs may be able to inhibit tumor formation and/or induce tumor regression. Alternatively, tumor growth may be more effectively inhibited by early administration of this drug. The results of these three studies lend further evidence that NSAIDs can have valuable chemopreventive and/or chemotherapeutic potential and support further investigation into their mode(s) of action.

6.2. Other pharmacological modifiers of Min

Protease inhibitors have shown promise as chemopreventive agents for treatment of several tumor types [200]. The Bowman-Birk protease inhibitor (BBI) has been reported to inhibit carcinogen-induced colon, liver, lung, esophageal, and oral epithelial tumors [200]. Interestingly, BBI appears to be capable of tumor inhibition even when applied many days after carcinogen exposure, and chemosuppression is maintained after ending BBI treatment [200].

Treatment of B6 *Min/+* mice with BBI leads to a 40% reduction in total tumor multiplicity [201]. This effect was observed for treatment with either 0.1 or 0.5% BBI in the diet, beginning prenatally. Although the mechanism(s) of tumor suppression by protease inhibitors is not known, these results support further investigations of this class of drugs as intestinal tumor chemopreventive agents.

7. Animal models in the future

The study of any genetic process is greatly enhanced by the availability of multiple mutant alleles of the gene(s) of interest. The recent development of two additional mouse strains with targeted *Apc* muta-

tions will contribute to investigations of the role of *Apc* in neoplasia. Mouse strains with targeted mutations of *Apc* leading to termination at codons 1638 (*Apc*1638N) and 716 (*Δ*716) have been reported [94,95]. Mice with the 1638N mutation develop only about five intestinal tumors, while *Δ*716 mutant mice develop as many as 250 intestinal tumors [94,95]. Tumors in both strains were found throughout the length of the intestinal tract, but the majority of tumors in the *Δ*716 strain were found in the small intestine. The wild-type *Apc* allele is lost in intestinal tumors from mice of both mutant strains [94,95]. Tumor histopathology seems to differ between the *Δ*716 strain and the other two *Apc* mutant strains. In particular, a large proportion of intestinal tumors in the *Δ*716 mice were classified as microadenomas. These lesions show a striking histological similarity to the cystic crypts seen in *Min*/+ mice [95,122]. It may, therefore, be inappropriate to classify these neoplasms as adenomas.

The differences in these three *Apc* mutant strains offer the potential for examining the role of different alleles of *Apc* in tumorigenesis, both alone and in combination with modifier genes. Do *Mom1, Dnmt,* and/or *Msh2* affect intestinal neoplasia in the *Δ*716 and/or 1638N strains? Are there other modifiers unique to these *Apc* mutant strains? Finally, what is the effect of $Apc^{\Delta 716}$ and/or *Apc*1638N on tumorigenic processes in other tissues?

To investigate possible dominant negative effects of mutant Apc polypeptides, transgenic lines overexpressing two different mutant *Apc* alleles have been created [202]. In these studies, transgenic lines that overexpress Apc truncated at either codon 716 or 1287 were generated. Expression of either mutant allele in the presence of two wild-type *Apc* alleles did not lead to intestinal tumor formation in either strain. While this result argues against a neomorphic dominant negative activity for these two mutant *Apc* alleles, several caveats must be considered. The mice analyzed in these experiments were either chimeric or hybrids of the B6 and 129 strains [202]. This raises the possibility that modifier alleles prevented tumor formation. In addition, since this transgene was expressed in the presence of two wild-type alleles of *Apc*, the mutant phenotype could be obscured if the dominant negative *Apc* allele acted as an antimorph.

The phenotypic similarities between Min mice and familial human intestinal cancer expand the opportunities for experimental investigation of this disease. The importance of *APC* and several other genes in intestinal tumorigenesis has been gleaned from human studies. Experiments with human cells have led to important advances in understanding how APC may function. The advantages provided by experimental manipulation of the mouse should allow for further analysis of the role of *Apc* and other genes in neoplasia. Support for somatic mutation of *APC* as an intestinal tumor-initiating event has been provided by analyses of *Apc* mutant mice. Experimental manipulations with Min mice have also begun to shed light on the timing of intestinal tumor initiation. One application of these results is a better understanding of how pharmacological agents influence intestinal tumorigenesis. Human studies have also hinted at a role for *APC* in controlling neoplasia in other tissues. Rigorous tests of potential tumor suppressor function for *Apc* in the pancreas, mammary gland, and other tissues are more feasible in animal model systems.

Examination of genetic and pharmacological modulation of DNA methylation in the Min mouse has led to a re-examination of how this epigenetic process affects oncogenesis. Furthermore, the ability to do controlled genetic experiments in a mouse system enhances the ability to identify novel genes involved in the regulation of neoplasia. *Mom1* is an example of a gene that may represent a new class of tumor regulating genes. Biological manipulations such as tissue grafting and the creation of chimeric animals allow for examination of how genes such as *Apc* and *Mom1* function. A better understanding of gene function is critical in order to devise strategies for the management of cancer. For example, there are important advantages for chemopreventive/chemotherapeutic agents that can function in a non-autonomous fashion. The development of animal models is also crucial for developing and testing pharmacological agents for disease treatment. The ability of NSAIDs to impede intestinal tumorigenesis has been corroborated by analyses with the Min mouse. The challenge that now exists is to use this animal model to help elucidate the mechanisms responsible for this tumor inhibition. Work with Min and other mutant mouse strains will no doubt continue to help in the understanding and, hopefully, treatment of human disease.

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