Action of *Min* and *Mom1* on Neoplasia in Ectopic Intestinal Grafts[1]

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Abstract

Mice heterozygous for *Min*, a mutant allele of *Apc*, develop adenomas throughout the intestinal tract. Tumor multiplicity in Min mice is influenced by genetic modifier loci. Previously, we mapped one of these modifier loci, *Mom1*, to distal mouse chromosome 4. *Mom1* is a semidominant modifier of both tumor size and multiplicity in Min mice. Recent evidence suggests that *Mom1* may encode a secretory phospholipase, *Pla2g2a*. Pla2g2a is expressed in a variety of cell types and seems to be involved in inflammatory responses and bacterial defense mechanisms. Here, we determine whether *Min* and *Mom1* act in a tissue-autonomous fashion using ectopic intestinal isografts. Within the small intestinal grafts, both *Min* and *Mom1* act in a tissue-autonomous manner. There is no evidence that either *Min* or *Mom1* has a systemic effect on tumor development. However, within the colonic grafts, the Min phenotype does not appear to be autonomous; the development of colonic tumors in Min mice seems dependent on factors beyond the *Min* genotype of the colonic epithelium. Micro-environmental factors, such as digestive secretions, dietary components, or intestinal flora, may be critical factors contributing to the development of *Min*-induced colonic tumors. However, these factors are not required for the action of *Min* or *Mom1* within the small intestine.

Introduction

A number of genetic factors are known to play major roles in determining risk for the development of intestinal cancer. For example, germline mutation of the *Apc*[4] gene predisposes both humans and mice to the development of spontaneous adenomas of the intestinal tract (1–3). We have shown previously that *Min* is a nonsense mutation affecting residue 850 in the 2845-amino acid mouse Apc protein (3). In the C57BL/6J (B6) strain, *Min*/+ mice develop, on average, a total of 30 adenomas in the scored regions of the intestinal tract (4). On the inbred B6 background, the *Min* mutation is 100% penetrant. However, on the AKR genetic background, the *Min* mutation is incompletely penetrant, with *Min*/+ mice developing, on average, a total of 0.1 tumor in the scored regions of the intestinal tract (5). The differences between B6 *Min*/+ mice and AKR *Min*/+ mice indicate that genetic factors regulate both the penetrance and the expressivity of the Min phenotype.

We have mapped one of these genetic factors, *Mom1*, to a region of distal mouse chromosome 4 (6). *Mom1* is a semidominant modifier of both tumor multiplicity and size.[5] Analysis of tumor diameters in mice of varying ages suggests that *Mom1* may affect tumor multiplicity by influencing the net growth rate of *Min*-induced intestinal adenomas.[5] Recently, the *Pla2g2a* gene has been proposed as a candidate for *Mom1* (7). Pla2g2a is expressed and secreted by a variety of cell types, including Paneth cells, one of the differentiated cell types within the intestine (8–9). Pla2g2a, a secreted molecule, is predominantly known for its role in promoting inflammatory responses through arachidonic acid metabolites such as prostaglandins and leukotrienes (10). Recently, Pla2g2a has been shown to have direct bactericidal properties (11).

Mapping data suggest that *Mom1* may be a complex locus, consisting of at least two tightly linked genes.[6] This mapping is consistent with the hypothesis that *Pla2g2a* is part of the *Mom1* locus.[6] Interestingly, two additional genes encoding phospholipases, *Pla2g2c* and *Pla2g5*, have recently been shown to be tightly linked to *Pla2g2a* on distal mouse chromosome 4 (12). It is possible, therefore, that *Mom1* represents a complex of phospholipase genes.

Environmental factors also may be key determinants of risk for the development of intestinal cancer. Epidemiological studies have suggested that diets high in fat and low in bulk fiber correlate with increased risk for colorectal cancer in humans (13–14). Experiments involving intestinal carcinogenesis in laboratory rodents have further suggested that the specific type of fat and fiber in the diet represents an important variable (15–17). Studies with Min mice on varying diets

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[4] The abbreviations used are: *Apc*, adenomatous polyposis coli; *Min*, multiple intestinal neoplasia; *Mom1*, modifier of Min 1; *Pla2g2a*, *Pla2g2c*, and *Pla2g5*, nonpancreatic secretory phospholipase groups IIA, IIC, and V, respectively; ROSA, reverse orientation splice acceptor; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

[5] K. A. Gould, W. F. Dietrich, N. Borenstein, E. S. Lander, and W. F. Dove. *Mom1* is a semi-dominant modifier of intestinal adenoma size and multiplicity in *Min*/+ mice, Genetics, in press.
[6] K. A. Gould, C. Luongo, A. R. Moser, M. K. McNeley, N. Borenstein, A. Shedlovsky, W. F. Dove, K. A. Hong, W. F. Dietrich, and E. S. Lander. Genetic evaluation of candidate genes for the *Mom1* modifier of intestinal neoplasia in mice, Genetics, in press.

and under varying conditions of husbandry suggest that environmental components can modulate the number and distribution of polyps within the intestine (18–19).

Bacteria within the intestine have also been implicated in playing an important role in intestinal tumorigenesis. However, experiments with chemical carcinogenesis protocols have produced conflicting results. Bacteria within the intestine appear to enhance the effects of some chemical carcinogens, such as dimethylhydrazine (20). However, with other carcinogens, such as azoxymethane, the lumenal bacteria seem to have a protective function, reducing the carcinogenic effect (20). When bacteria do not participate in metabolic activation of the carcinogen, they may instead serve as a competitive substrate for the damaging effects of the carcinogen within the lumen.

The interaction of genetic and environmental factors may play a pivotal role in the relative risk for intestinal cancer. These interactions are complex, and we are just beginning to assess them. Both *Min* and *Mom1* provide excellent opportunities to investigate this question. In the case of *Mom1,* the locus may exert its effect on tumor multiplicity by modulating an environmental factor. Alternatively, a particular environmental factor may have a differential effect on animals of different *Mom1* genotypes. This question is particularly interesting in light of the possibility that *Mom1* may encode a secretory phospholipase involved in inflammation and microbial defense mechanisms (7). Thus, the effect of *Mom1* may be mediated through an interaction with external stimuli.

To begin to address the mechanism of action of *Min* and *Mom1* on tumor formation in Min mice, we have used intestinal isografts. In this procedure, fetal intestines are transplanted to a s.c. site on the back of a histocompatible host animal (21). In this ectopic site, the intestines become highly vascularized and develop relatively normally. Isografts contain normal crypt and villus architecture as well as all four differentiated cell types of the intestinal epithelium (22–24). Grafted intestines are capable of absorbing amino acids, glucose, and fat (24). Grafts even demonstrate spontaneous, unidirectional peristalsis, indicating the presence of normal smooth musculature and innervation (24).

Intestinal isografts have been used to show that many features of the intestine are already programmed within the tissue prior to birth. Analysis of intestinal isografts has revealed that enzyme expression patterns within the gut are developmentally programmed and are not induced in response to dietary changes from the neonatal to postweaning period (25–26).

Here, we use intestinal isografts to determine whether the actions of *Min* and *Mom1* are preserved when the intestine is placed in an ectopic site. The grafted intestines differ from *in situ* intestines (the intact intestines in their normal position in the abdominal cavity) with respect to exposure to ingested material, digestive secretions, and intestinal flora. Therefore, using intestinal isografts, we have begun to investigate the influence of these factors on the development of *Min*-induced neoplasia and the ability of *Mom1* to influence tumor multiplicity.

Results

Use of the *ROSA11* Transgene. In our standard protocol, to score tumors in the *in situ* intestine, the intestine is slit lengthwise, rinsed in buffer to remove contents, and laid flat with the lumenal surface facing up. Initial experiments involving the grafting of B6 *Min*/+ fetal intestines revealed that the grafted intestines were convoluted rather than straight. As a result, the grafted intestines were difficult to lay flat. This situation, complicated further by their small size, made it extremely difficult to enumerate tumors within the grafts by our routine scoring technique. To circumvent these problems, we sought conditions that would permit the visualization of small tumors against an irregular background.

We found that use of the *lacZ* transgenic line, ROSA11, eliminated difficulties in analyzing grafted intestines. The ROSA11 line was generated in promoter trap experiments in ES cells (27). Expression of the randomly integrated, promoterless reporter gene, a *lacZ*-neo^r fusion, is driven by an anonymous promoter. The insertion site of the fusion gene in the ROSA11 line is unknown. Mice from the ROSA11 line have previously been reported to be homozygous-viable and to express *lacZ* ubiquitously within the embryo (27). We observed that in adult ROSA11 mice, *lacZ* expression was not ubiquitous. Within the epithelium of the small intestine, β-galactosidase activity was detected in cells within the crypt but not in the villus (Fig. 1*A*). A similar pattern was observed in the colon: *lacZ* activity was detectable in the basal region of the colonic crypts, the region analogous to the small intestinal crypt, and there was no detectable *lacZ* activity in the upper region of colonic crypts or in the surface epithelium (Fig. 1*B*).

By crossing mice from the ROSA11 line with B6 Min mice, we obtained progeny heterozygous for both *ROSA11* and *Min*. The *Min*-induced intestinal adenomas in these mice also expressed *lacZ*. This suggests that the tumors in Min mice are probably derived from the stem cells or proliferative cells within the crypt. This staining pattern provided a very simple method for enhanced visualization of tumors. Because tumors in *ROSA11*/+ *Min*/+ mice stain an intense blue, they are very easy to identify against the unstained (beige) background of villi or colonic epithelium (Fig. 1*C*).

It was important to determine whether the ROSA11 strain carried any alleles that modify the Min phenotype. ROSA11 was generated on a 129/Sv genetic background, which is known to carry alleles, unlinked to *Mom1,* that modify the Min phenotype.[5] To eliminate these alleles, we made ROSA11 congenic on B6 by backcrossing *ROSA11*/+ males to B6 females for 13 generations. To determine whether any 129 modifier loci were still present in the congenic line because of linkage to the *ROSA11* insertion site, we compared the phenotypes of *Min*/+ progeny produced in the ROSA11 × Min cross. *Min*/+ mice that were heterozygous for *ROSA11* had an average tumor multiplicity of 23. This value did not differ significantly from the average of 25 tumors in their *Min*/+ siblings that did not carry *ROSA11* (two-sided $P = 0.3$; Fig. 2). This indicates that the ROSA11 line does not carry any linked modifier alleles derived from the 129/Sv ES cell line.

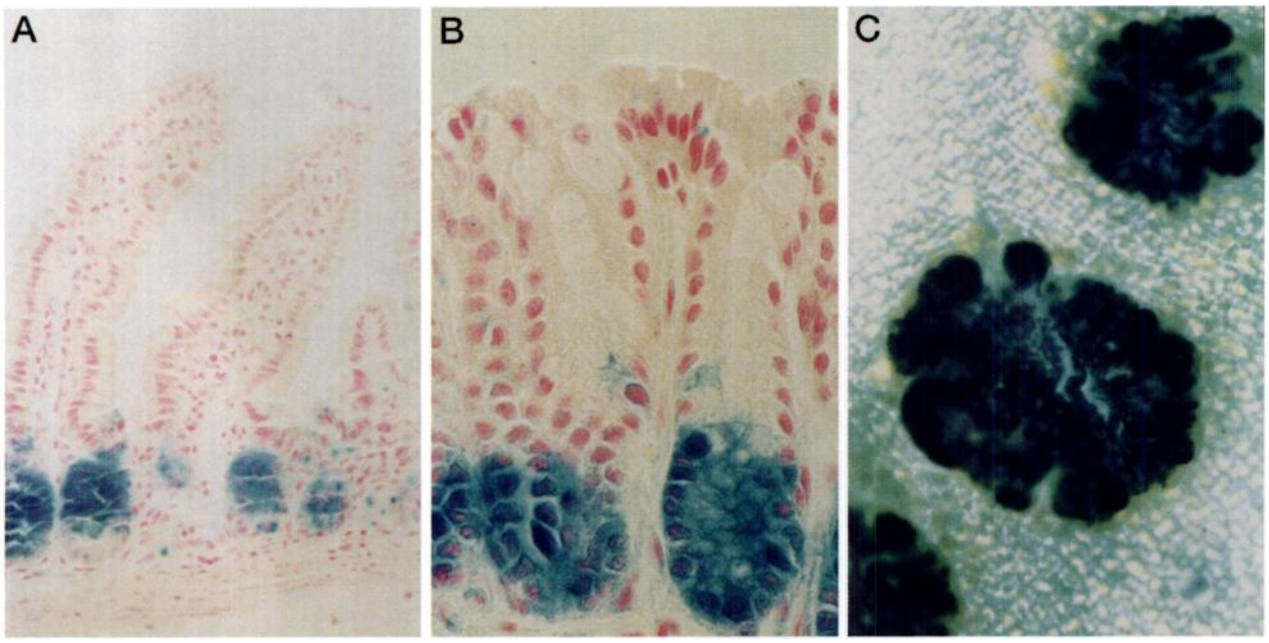

Fig. 1. X-Gal staining in the intestinal epithelium and intestinal tumors in ROSA11 mice. Histological analysis of the epithelium of the small intestine (×400; *A*) and colon (×1000; *B*) is shown. After whole-mount staining with X-Gal, tissue was sectioned and counterstained with Nuclear Fast Red. Tumors show strong X-Gal staining in Min mice carrying ROSA11 (×30; *C*).

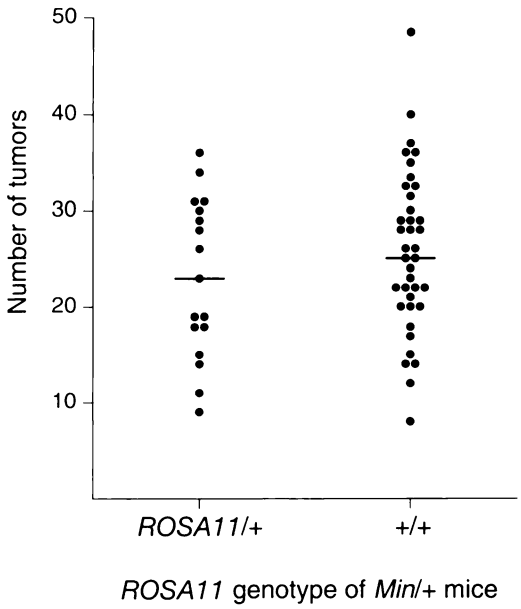

Fig. 2. Tumor multiplicity in the *ROSA11*/+ × *Min*/+ cross. The scatter plot shows the distribution of tumor multiplicities in *Min*/+ mice produced in the *ROSA11*/+ × *Min*/+ cross. The horizontal bar indicates the position of the mean.

Microbiological Status of the Grafts. One of the important microenvironmental differences between grafted and *in situ* intestines is the microbiological status. Bacteria normally enter the digestive tract during parturition. After birth, bacteria continue to colonize the neonatal gut, and by the post-weaning period, the normal balance of intestinal flora is established. By contrast, grafted intestines are generated from fetuses derived by sterile hysterectomy and are surgically implanted by aseptic techniques. The intestinal isografts showed no overt signs of bacterial contamination on the macroscopic or histological level (data not shown). However, to confirm that the grafts were indeed sterile, we cultured the internal contents of a subset of grafts ($n = 4$) under conditions in which aerobic and anaerobic microorganisms could be detected. After 7 days of culture, no growth was observed in any culture (data not shown). These results suggest that the lumenal environment of the grafted intestines is free of microorganisms.

Action of *Min* within grafts. To determine whether tumors form in *Min*/+ intestinal grafts, we transplanted pieces of small intestine and the entire colons from fetuses produced by crossing B6 *Min*/+ females to a *ROSA11* homozygous male. Host animals were B6 males at 60 days of age. Grafts were removed after 100 days and processed (see "Materials and Methods").

No tumors were observed in any (0 of 30) of the +/+ small intestinal or colonic grafts in B6 hosts. This result is consistent with the hypothesis that the graft procedure does not promote *Min*-independent tumor formation. No tumors were observed in the intestines of the B6 (non-*Min*) host animals receiving *Min*/+ or +/+ grafts. This result indicates that expression of the *Min* allele in the graft does not have a systemic effect leading to the development of tumors in non-*Min* tissues.

Tumors were observed in all (17 of 17) B6 *Min*/+ small intestinal grafts in B6 hosts. Because the sizes of the grafts varied significantly, the tumor multiplicity was calculated per unit area (cm^2). Grafts averaged 1.08 cm^2 in size and showed

Table 1 Tumor multiplicity data from *Min*/+ small intestinal isografts

For each set of grafts, the average tumor multiplicity ± SD is given. The number of isografts in each group is given in parentheses.

Mom1 genotype of host	Average tumor multiplicity (per cm^2)	
	Mom1 genotype of grafts	
	$Mom1^{B6/B6}$	$Mom1^{AKR/B6}$
$Mom1^{B6/B6}$	7.7 ± 5.4 (17)	1.9 ± 1.5 (6)
$Mom1^{AKR/AKR}$	5.1 ± 4.3 (17)	ND[a]

[a] ND, not determined.

an average of 7.7 tumors/cm^2 (Table 1). This indicates that *Min*/+ cells within tissues other than the intestine are not required for the development of intestinal tumors in *Min*/+ mice. The presence of *Min*/+ intestinal tissue in the graft is sufficient to predispose the graft to the development of tumors within it.

The intestinal grafts were also examined for the presence of cystic crypts, another lesion observed in the small intestines of *Min*/+ mice. Cystic crypts appear to result from crypts that have been sealed off and have become lined with abnormal, often anaplastic, cells (28). As these lesions are derived from crypt cells, cystic crypts in *ROSA11*/+ *Min*/+ mice do express *lacZ* (data not shown). Although a large cystic crypt may be the same size as a small adenoma, these two lesions can be distinguished at both the gross morphological and the histological level. Cystic crypts, primarily located in the proximal one-third of the intestine, can be detected in 96% of B6 *Min*/+ mice (28). On the B6 background, *Min*/+ mice develop, on average, 6.4 cystic crypts (28). Previous studies have suggested that cystic crypts may not be precursors of intestinal adenomas but rather represent a distinct, nonneoplastic end point (28). Cystic crypts were detected in 81% of the *Min*/+ small intestinal grafts. The grafts carried, on average, 9.7 cystic crypts/cm^2.

The average of 7.7 tumors/cm^2 within the B6 *Min*/+ small intestinal grafts in B6 hosts is significantly higher than the average of 3.3 tumors/cm^2 observed within scored regions of the *in situ* small intestines of *Min*/+ mice ($P = 3.5 \times 10^{-3}$). Similarly, the average of 9.7 cystic crypts/cm^2 within the grafts was significantly higher than the average of 0.81 cystic crypts/cm^2 observed within scored regions of the *in situ* small intestines of *Min*/+ mice ($P = 9 \times 10^{-3}$). These observations may be due to differences in architecture between the grafted and *in situ* intestines. Alternatively, it is possible that these results reflect an important microenvironmental difference between grafted and *in situ* intestines.

No tumors were observed in the 16 B6 *Min*/+ colonic grafts from B6 hosts. These grafts represent a total epithelial surface area of 4.8 cm^2. This area is equivalent to 1.4 *in situ* colons. On the basis of the average of 5.5 tumors per *in situ* colon in *Min*/+ mice at 100 days of age, we expected to observe a total of 7.9 tumors in the colon tissue of these grafts. The failure to detect even a single colonic adenoma in the grafts is significantly different from the expected result (Poisson; $P = 3.7 \times 10^{-4}$). This suggests that within the colon, microenvironmental factors may be essential for the development of adenomas in *Min*/+ mice. One possibility is that the lumenal contents of the *in situ* colon, bacterial metabolites and components of the fecal material, may act to initiate and/or promote tumor development.

Nonsystemic Action of $Mom1^{AKR/AKR}$. To determine whether the effect of *Mom1* is systemic, we transplanted small intestine and colon from fetuses produced by crossing B6 *Min*/+ females with a *ROSA11* homozygous male. Host animals were B6.$Mom1^{AKR/AKR}$ males at 60 days of age. Grafts were removed after 100 days and processed for analysis as above.

The average surface area of *Min*/+ small intestinal grafts in $Mom1^{AKR/AKR}$ hosts was 1.04 cm^2. This value did not differ significantly from the average size of 1.08 cm^2 observed in the B6 hosts. This result indicates that the *Mom1* genotype of the host has no effect on final graft size. Similarly, the average of 5.1 tumors/cm^2 in the 17 *Min*/+ grafts in the $Mom1^{AKR/AKR}$ hosts did not differ significantly from the average of 7.7 tumors/cm^2 in grafts in the B6 ($Mom1^{B6/B6}$) hosts ($P = 0.09$; Table 1). This result strongly indicates that *Mom1* does not act in a systemic fashion to reduce tumor multiplicity.

In this experiment, it was possible to use *Min*/+ mice as hosts. In previous experiments with B6 hosts, this was not possible because the average life span of the B6 *Min*/+ mice is approximately 120 days of age. The hosts must remain healthy and viable for 160 days, because the grafts, transplanted when the hosts are 60 days of age, are maintained for 100 days. The *Min*/+ mice homozygous for $Mom1^{AKR}$ have a much longer life span than B6 *Min*/+ mice and remain healthy throughout the duration of the experiment. Therefore, some of the $Mom1^{AKR/AKR}$ hosts described above were themselves heterozygous for *Min*. The average of 4.4 tumors/cm^2 in the nine B6 *Min*/+ grafts in $Mom1^{AKR/AKR}$ *Min*/+ hosts did not differ significantly from the average of 5.7 tumors/cm^2 in the eight B6 *Min*/+ grafts in $Mom1^{AKR/AKR}$ +/+ hosts ($P = 0.43$; Fig. 3). Similarly, the genotype of the grafts (*Min*/+ or +/+) had no detectable effect on the tumor multiplicity of the $Mom1^{AKR/AKR}$ *Min*/+ hosts: 7.7 tumors on average in the nine recipients of *Min*/+ grafts compared with an average of 6.8 tumors in the nine recipients of +/+ grafts ($P = 0.37$).

Cystic crypts were also observed in the *Min*/+ small intestinal grafts in $Mom1^{AKR/AKR}$ hosts, averaging 4.5/cm^2. This value was not significantly different from that observed in the small intestinal grafts in B6 hosts ($P = 0.09$). Previously, we had not investigated the ability of *Mom1* to influence cystic crypt multiplicity in the *in situ* intestines of Min mice. Therefore, we determined cystic crypt multiplicity in age-matched control mice of differing *Mom1* genotypes. We found that the average cystic crypt multiplicity of 7.0 in the *in situ* small intestines of B6.$Mom1^{B6/B6}$ *Min*/+ mice does not differ significantly from the average multiplicity of 6.2 in B6.$Mom1^{AKR/AKR}$ *Min*/+ mice ($P = 0.25$). Thus, there is no evidence from the grafted or *in situ* intestines to suggest that the *Mom1* genotype influences cystic crypt multiplicity.

As in the grafts in B6 hosts, no tumors were observed in the *Min*/+ colonic grafts in $Mom1^{AKR/AKR}$ hosts. In total, 14 *Min*/+ colonic grafts in $Mom1^{AKR/AKR}$ hosts were analyzed. These grafts represent a total epithelial surface area of 3.43

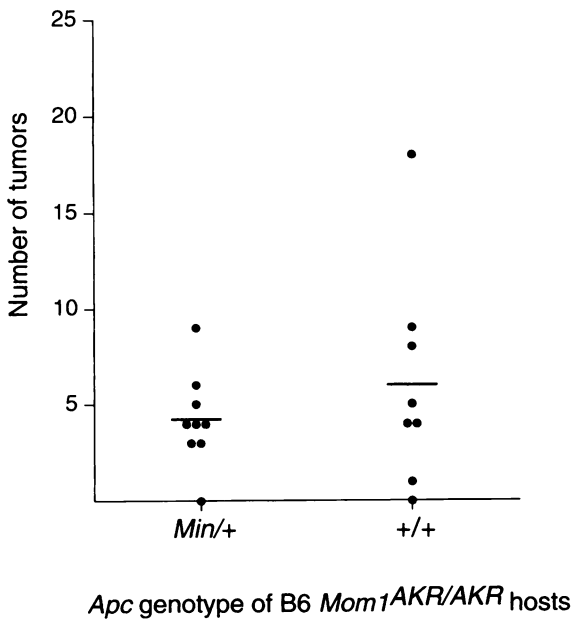

Fig. 3. The effect of the *Apc* genotype of the host on tumor multiplicity in the *Min*/+ grafts. The scatter plot shows the distribution of tumor multiplicities in *Min*/+ grafts in $Mom1^{AKR/AKR}$ *Min*/+ and $Mom1^{AKR/AKR}$ +/+ hosts. The horizontal bar indicates the position of the mean.

cm^2. This area is equivalent to 1.02 *in situ* colons. Under the hypothesis that, as in the small intestinal isografts, the *Mom1* genotype of the host has no effect on tumor multiplicity in the colonic grafts, we expected to observe a total of 5.7 tumors in the colon tissue of these grafts. The failure to detect even a single colonic adenoma in the grafts was significantly different from this expected result (Poisson; $P = 3.3 \times 10^{-3}$). However, because no tumors have been detected in the previous 16 *Min*/+ colonic grafts in B6 hosts, the failure to observe tumors in these 14 colonic grafts in $Mom1^{AKR/AKR}$ hosts cannot be attributed to an effect of $Mom1^{AKR}$. In addition, analysis of small intestinal isografts revealed no evidence for a systemic effect of *Mom1*. In experiments described thus far, no tumors were observed in a total of 30 colonic grafts. The surface area of these 30 grafts is equivalent to 2.95 *in situ* colons. On this basis, we expected to observe a total of 13.6 tumors from the 30 colonic grafts. Failure to observe even a single tumor in these 30 colonic grafts is highly significant (Poisson; $P = 1.24 \times 10^{-6}$). These results support the hypothesis that microenvironmental factors may be critical for the development of colonic tumors in *Min*/+ mice.

Action of $Mom1^{AKR/AKR}$ within Grafts. Because *Mom1* apparently does not act in a systemic fashion, we established another series of grafts to determine whether a reduction in tumor multiplicity is observed when the grafted *Min*/+ intestine also carries $Mom1^{AKR}$. For this experiment, we transplanted segments of small intestine and the entire colon from fetuses produced by crossing B6.$Mom1^{AKR/AKR}$ *Min*/+ females to a *ROSA11* homozygous male. Host animals were B6 males at 60 days of age. Grafts were removed after 100 days and processed as above.

The average size of the B6.$Mom1^{AKR/B6}$ *Min*/+ small intestinal grafts was 1.41 cm^2. These grafts were not significantly larger than *Min*/+ small intestinal grafts in B6 hosts ($P = 0.06$). However, these B6.$Mom1^{AKR/B6}$ *Min*/+ small intestinal grafts carried an average of 1.9 tumors/cm^2. This value is significantly lower than the average of 7.7 tumors/cm^2 observed in the $Mom1^{B6/B6}$ *Min*/+ small intestinal grafts in B6 hosts ($P = 3.3 \times 10^{-3}$; Table 1). This difference suggests that tumor multiplicity in Min mice is influenced by the *Mom1* genotype of the intestinal tissue itself. However, these results cannot distinguish between cell-autonomous and non-cell-autonomous action of *Mom1* within the intestinal epithelium. If *Mom1* does encode a secreted molecule, it must be produced within the intestinal epithelium and act in a paracrine or autocrine manner.

Within the *in situ* intestine, heterozygosity for $Mom1^{AKR}$ correlates with a 2-fold decrease in tumor multiplicity relative to $Mom1^{B6}$ homozygotes.[5] By contrast, in the grafts, heterozygosity for *Mom1* seems to have a stronger effect, resulting in a 4-fold decrease in tumor multiplicity relative to grafts homozygous for $Mom1^{B6}$. The effect of *Mom1* in the grafted intestines is significantly higher than the expected 2-fold effect ($P = 4 \times 10^{-3}$). The reason for this difference in the magnitude of the effect of *Mom1* between grafted and *in situ* intestines is unclear.

Interestingly, the multiplicity of cystic crypts was also reduced in the B6.$Mom1^{AKR/B6}$ *Min*/+ small intestinal grafts. These grafts contained an average of 0.7 cystic crypt/cm^2. This value is significantly lower than the 9.7/cm^2 observed in the B6.$Mom1^{B6/B6}$ *Min*/+ small intestinal grafts ($P = 8 \times 10^{-3}$). This result is surprising, because *Mom1* genotype does not appear to affect cystic crypt multiplicity in the *in situ* intestinal tract.

No tumors were observed in the 6 B6.$Mom1^{AKR/B6}$ *Min*/+ colonic grafts. Because no tumors have been detected in any of the previous 30 *Min*/+ colonic grafts, the failure to observe tumors in these six colonic grafts cannot be attributed to an effect of $Mom1^{AKR}$.

Discussion

The set of observations from this and other work that needs discussion is summarized in Table 2. Each of the experiments summarized in this table uses congenic mouse lines to characterize the effects of *Min* and *Mom1* individually. Congenic strains have been an essential component of the investigation into the mechanism of action of these loci. If, instead, one attempts an analysis of the effects of multiple genetic factors at the same time, any differences in the mechanism of action of these factors would result in contradictory observations. These circumstances would prevent the precise determination of the mechanism of action of any single factor.

Tumors and cystic crypts develop in *Min*/+ small intestinal isografts. The development of *Min*-induced lesions within the small intestine is a function of the *Min*/+ genotype of cells within that tissue and does not require that the *Min* mutation be present in cells of other tissues. This indicates that the *Min* mutation acts in a tissue-autonomous fashion. The density of tumors and cystic crypts within the grafted small

Table 2 Summary of effects of *Min* and *Mom1* on tumor and cystic crypt development in *in situ* and grafted intestines

For each case, the effect of *Min* or $Mom1^{AKR/B6}$ is indicated by a Y (Yes) or an N (No). In each case where it is relevant, the fold increase or decrease is indicated by an arrow in parentheses.

	Tumor multiplicity		Cystic crypt multiplicity	
	Min	$Mom1^{AKR/B6}$	*Min*	$Mom1^{AKR/B6}$
Small intestine				
Host	Y[a]	Y(↓2×)[b]	Y[c]	N
Graft	Y(↑2×)	Y(↓4×)	Y(↑1.5×)	Y(↓14×)
Colon				
Host	Y[a]	Y(↓2×)[b]	N[c]	NA[d]
Graft	N	ND[e]	NA	NA

[a] From Moser *et al.* (4).
[b] From Dietrich *et al.* (6) and Gould[5].
[c] From Shoemaker *et al.* (28).
[d] NA, not applicable.
[e] ND, not determined.

intestines is higher than that observed in the *in situ* intestine (Table 2). It remains to be determined whether this result reflects an unidentified structural or architectural difference between the grafted *versus in situ* tissue or whether it reflects a difference in the microenvironment of the two tissues. The difference in lesion density seems not simply to arise from differences in mitotic index, because grafted intestines are reported to have a decreased mitotic index relative to *in situ* intestines (29). It is possible that some element of the normal intestinal microenvironment may act to reduce tumor and cystic crypt multiplicity. Alternatively, some aspect of the microenvironment of the grafts may enhance the multiplicity of these lesions.

Analysis of tumor multiplicities in B6 *Min*/+ small intestinal grafts in $Mom1^{B6/B6}$ and $Mom1^{AKR/AKR}$ hosts indicates that *Mom1* does not act in a systemic fashion to affect tumor multiplicity (Table 2). Comparison of tumor multiplicities in *Min*/+ small intestinal grafts carrying $Mom1^{AKR}$ or $Mom1^{B6}$ indicates that the *Mom1* genotype within the *Min*/+ intestine does influence tumor multiplicity (Table 2). Taken together, these results indicate that the local presence of the $Mom1^{AKR}$ allele within the intestine, but not other tissues, is necessary and sufficient to observe an effect of $Mom1^{AKR}$ on tumor multiplicity. In $Mom1^{AKR/AKR}$ *Min*/+ hosts, no effect of *Mom1* was observed on tumor multiplicity in the *Min*/+ small intestinal grafts. However, in these same hosts, the effect of *Mom1* on the hosts' intestines was clear, because these mice had a low average tumor multiplicity (Table 2).

From our analysis of small intestinal isografts, we have drawn a number of conclusions about the mode of action of *Mom1*. Our results can also be interpreted under the specific hypothesis that *Mom1* is *Pla2g2a*. These conclusions would also apply to the two linked phospholipase genes, *Pla2g2c* and *Pla2g5*, if they are, as hypothesized, components of *Mom1*. Under this hypothesis, the tissue autonomy of *Mom1* would indicate that expression of *Pla2g2a* by the Paneth cells but not by neutrophils, mast cells, platelets, or fibroblasts would be required for the *Mom1* effect on tumor multiplicity. Although Pla2g2a is a secreted molecule and high serum levels of Pla2g2a are present in a number of disease states, Pla2g2a would not act in a systemic fashion to influence tumor multiplicity in Min mice (30). Because the grafts do not contain ingested material, any effect of Pla2g2a on tumor multiplicity could not be mediated through direct metabolism of dietary lipids. Finally, the effect of *Mom1* on tumor multiplicity is observed in the isografts despite the fact that the grafts are sterile. This indicates that any effect of *Pla2g2a* on tumor multiplicity would not be dependent on its bactericidal properties (11).

The effects of both *Min* and $Mom1^{AKR}$ appear to be autonomous to the small intestine at the level of the whole tissue. However, these experiments do not assess autonomy of action at the cellular level. The data do indicate that neither *Min* nor *Mom1* has a systemic effect on tumor formation. Therefore, if either of these genes is nonautonomous at the cellular level, it would involve a short range of action, acting in a paracrine or autocrine manner within the intestine.

Within the *in situ* intestine, heterozygosity for $Mom1^{AKR}$ correlates with a 2-fold decrease in tumor multiplicity relative to $Mom1^{B6}$ homozygotes.[5] However, in the grafts, heterozygosity for *Mom1* seems to have a stronger effect, resulting in a 4-fold decrease in tumor multiplicity relative to grafts homozygous for $Mom1^{B6}$. The reason for this difference in the magnitude of the effect of *Mom1* between grafted and *in situ* intestines is unclear. One possible explanation for this observation is that the molecule encoded by *Mom1* has an extended half-life or greater local concentration in the grafted intestines. Perhaps in the absence of the normal intestinal contents moving rapidly through the small intestine, the observed effect of $Mom1^{AKR}$ is enhanced. This hypothesis can also explain the fact that *Mom1* seems to influence cystic crypt multiplicity within the grafted small intestines but not in *in situ* intestines.

Tumors did not form in any (0 of 36) *Min*/+ colonic grafts in either *Min*/+ or +/+ hosts. This result suggests that the development of tumors in the colon of *Min*/+ mice may not simply depend on the *Min* genotype of either host or graft tissue of the animal. It is possible that *Min*, although acting autonomously within the colon, is insufficient to produce tumors in this tissue in the absence of some external factor. Further investigation is required to determine what factor(s) is missing in the grafted colons. Analysis of Min mice under germ-free conditions may be useful in distinguishing between the potential effects of colonic flora and ingested

material. It is also possible that the failure of any tumors to form in the colonic grafts is due to an unidentified structural or architectural difference between the grafted *versus in situ* colons.

There are published examples of circumstances in which colonic flora play a vital role in determining susceptibility to chemically induced tumors. A particularly striking example of this is cycasin, the β-glucoside of methylazoxymethanol. Cycasin, a potent intestinal carcinogen in conventionally housed rats, has no effect on the colons of germ-free animals. This is due to the absence of bacteria in the colon needed to cleave the glucosidic bond to produce the proximal carcinogen, methylazoxymethanol (31).

Because no tumors were observed in the 16 B6 *Min*/+ colonic grafts in B6 hosts, it was not possible to determine whether *Mom1* acts in a systemic or tissue-autonomous fashion with respect to the colon. It is formally possible that the mode of action of *Mom1* within the colon is different from that observed in the small intestine. Therefore, although *Mom1* clearly acts in a tissue-autonomous fashion in the small intestine, we cannot assume that this will also apply to the colon.

Materials and Methods

Mice. All mice were bred at McArdle Laboratory for Cancer Research. The B6 *Min*/+ mice were obtained from our B6-Min colony. The B6.*Mom1*$^{AKR/AKR}$ *Min*/+ and +/+ mice were obtained from our B6.*Mom1*^{AKR} congenic line, which is segregating for *Min*. This B6.*Mom1*^{AKR} congenic line carries a 35-cM region of distal mouse chromosome 4, between the markers *D4Mit9* and *D4Mit180*, from the AKR strain.[5] This region contains all three phospholipase genes mapped to distal chromosome 4 (12). Mice were genotyped to identify carriers of the *Min* mutation with a PCR-based assay described previously (6). The B6 mice were obtained from our breeding colony derived from B6 mice obtained from The Jackson Laboratory (Bar Harbor, ME). The ROSA11 mice were obtained from our B6.ROSA11 congenic line derived from a single *ROSA11* heterozygous male provided by G. Friedrich and P. Soriano (Baylor University, Houston, TX). The *ROSA11* heterozygotes and homozygotes were identified by progeny testing in crosses with B6 mice.

Preparation and Implantation of Intestinal Isografts. Isografts were prepared and implanted as in Rubin *et al.* (32). Briefly, timed pregnant females were sacrificed at day 15 of gestation by injection of a lethal dose of Nembutal. Fetuses were removed by Cesarean section and placed individually in wells of a Linbro culture dish containing RPMI 1640 (pH 7.4) on ice. The gastrointestinal tract of each fetus was removed by dissection in medium on ice. The remaining tissue from each fetus was frozen in liquid N_2 for subsequent genotypic analysis. The stomach was dissected away from the intestine, and the proximal 1.5 cm (of ~2 cm total) of small intestine was excised and used for grafting. The cecum was then dissected away, and the entire colon was used for grafting.

Graft recipients were B6 or B6.*Mom1*^{AKR} male mice approximately 60 days of age. Mice were anesthetized with Nembutal. An incision was made along the dorsal midline, and the s.c. fascia was exposed. Grafts were implanted by drawing up the fetal intestine (with medium) into an 18-gauge needle attached to a 1-cc syringe. The needle was inserted between the fascia and the skin, and the intestine was slowly injected into this site. The ends of the fetal intestine were attached to the fascia with 7–0 proline suture. The incision was closed with surgical clips. Grafts were removed 100 days after implantation.

Graft Staining. Upon removal, grafts were opened along the longitudinal axis. The contents of the grafts (mucus and debris of sloughed epithelial cells) were removed by rinsing in PBS. Grafts were laid flat on bibulous paper and fixed for 30 min in 0.2% glutaraldehyde. After fixation, grafts were washed and stained with X-Gal as in Sanes *et al.* (33). After staining, grafts were postfixed overnight in 10% neutral buffered formalin. Formalin was then cleared by three overnight incubations in 70% ethanol.

Counting of Tumors and Cystic Crypts in Grafts. After clearing in ethanol, tumors and cystic crypts in grafted intestines were enumerated with a dissecting microscope at ×30. Grafts were measured on the same microscope at ×10.

Counting of Tumors and Cystic Crypts in *in Situ* Intestines. Intestines were scored for tumors by a modification of a method described previously (4). In this method, 4-cm segments from the proximal, middle, and distal small intestine (roughly corresponding to the duodenum, jejunum, and ileum), and the entire colon were laid flat on bibulous paper and fixed overnight in 10% neutral buffered formalin. Formalin was then cleared by three overnight incubations in 70% ethanol. After clearing in ethanol, tumors and cystic crypts in *in situ* intestines were enumerated with a dissecting microscope at ×30. Segments were measured on the same microscope at ×10.

Genotyping Grafts. DNA from each fetal donor was isolated using a previously described protocol designed for DNA isolation from frozen spleen.[6] However, in this case we used fetal tissue frozen at the time of sacrifice as a source of DNA; 2 μl of DNA was then used in a PCR assay that permits identification of carriers of the *Min* mutation (6).

The genotypes of all tumor-free grafts typed by the above method as *Min*/+ were confirmed by a quantitative PCR-based assay. This was essential, because the mothers of all fetuses were heterozygous for *Min*. Contamination of the fetal tissue used for genotype analysis with maternal blood or tissue could result in a false positive. Therefore, for retyping, a piece of the graft itself was excised with a sterile scalpel blade. DNA was isolated from this tissue by a modification of Goelz *et al.* (34). Briefly, tissue samples were placed in a conical bottom 1.5-ml microfuge tube in 500 μl of TE9 [500 mM Tris, 20 mM EDTA, and 10 mM NaCl (pH 9.0)] containing 1% SDS and 500 μg/ml proteinase K. Samples were incubated at 60°C overnight with occasional maceration with a sterilized pestle. The DNA was extracted once with phenol/chloroform, once with chloroform, and then precipitated with 2 volumes of 100% ethanol. The DNA was resuspended in 25 μl of deionized water. Grafts were genotyped by amplification of 2 μl of this DNA, as described previously (35). The products of this PCR were quantitated on a phosphorimager. From this quantitation, all retyped samples were confirmed to be *Min*/+ grafts.

Statistical Analysis. The Wilcoxon rank sum test was used for all statistical analyses except, as indicated in the text, when the Poisson distribution was used. For the Wilcoxon rank sum test, one-sided *P*s are given, except where indicated otherwise.

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