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Genetics

Apc^{Min}, a mutation in the murine *Apc* gene, predisposes to mammary carcinomas and focal alveolar hyperplasias

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ABSTRACT **Apc^{Min} (*Min*, multiple intestinal neoplasia) is a point mutation in the murine homolog of the *APC* gene. *Min*/+ mice develop multiple intestinal adenomas, as do humans carrying germ-line mutations in *APC*. Female mice carrying *Min* are also prone to develop mammary tumors. *Min*/+ mammary glands are more sensitive to chemical carcinogenesis than are +/+ mammary glands. Transplantation of mammary cells from *Min*/+ or +/+ donors into +/+ hosts demonstrates that the propensity to develop mammary tumors is intrinsic to the *Min*/+ mammary cells. Long-term grafts of *Min*/+ mammary glands also gave rise to focal alveolar hyperplasias, indicating that the presence of the *Min* mutation also has a role in the development of these lesions.**

Mutations in the human *APC* gene have been shown to be involved in both sporadic and familial colon cancer. Individuals carrying germ-line mutations in the *APC* gene are at risk for the development of adenomatous colon polyps that can progress to cancer (1, 2). In some families there is also an increased risk for desmoid tumors, small intestine tumors, mandibular osteomas, or retinal dysplasias (3).

The *APC* gene is expressed in most tissues that have been surveyed (4, 5), in contrast to the narrow spectrum of tissues overtly predisposed to neoplasia by mutations in this gene. A limited spectrum of neoplastic transformation is also observed for other broadly expressed genes in which mutations can predispose to cancer, such as *RB* (6), *P53* (7), and *NF* (8). To understand more deeply the relationship between the pattern of *APC* gene expression and the pattern of neoplasia induced by germ-line or somatic defects in the *APC* gene, we focused on two questions: (*i*) Is the action of the mutated *APC* gene autonomous to the tumor cell lineage or does it involve intercellular interactions? (*ii*) What other factors—genetic, developmental, and environmental—influence the spectrum of *APC*-induced neoplasms?

We have been investigating neoplasia in mice that carry Apc^{Min} (*Min*), a nonsense mutation at codon 850 of *Apc*, the murine homolog of the *APC* gene (9). This mutation is analogous to that seen in humans with familial adenomatous polyposis. C57BL6/J (B6) mice that carry *Min* develop numerous intestinal tumors at an early age and rarely survive beyond 150 days of age (10). This mouse model can be used to explore experimentally the range of tissues made susceptible to tumorigenesis by this *Apc* mutation and to examine the question of the cellular autonomy of *Min* action in tumor formation.

While establishing and maintaining the Min pedigree on the B6 background, we noted that *Min*/+ females occasionally developed mammary tumors. No mammary tumors were seen in +/+ females from this pedigree. The possibility that a second distinct pathway of neoplasia is induced in mice by the *Min* mutation deserves serious investigation.

One obstacle to the analysis of mammary carcinogenesis in *Min*/+ mice is the short life span because of the intestinal tumors. Therefore, we have investigated whether the incidence of mammary tumors in *Min*/+ mice can be increased by treatment with chemical carcinogens. Separately, we have transplanted mammary cells from *Min*/+ animals into histocompatible recipients (11); this has allowed us to follow the fate of the mammary tissue for a longer period of time, free from the complication of intestinal neoplasia.

MATERIALS AND METHODS

Mice. All mice were either purchased from The Jackson Laboratory or bred in the McArdle Laboratory. The Min pedigree is maintained by crossing C57BL/6J (B6) females with B6.*Min*/+ males. Mutant animals were identified first by the development of anemia and later at necropsy by scoring the intestines for tumors as described (10). The animals used in these experiments were from the N14 to N18 backcross generation onto B6. At the time of these experiments, molecular typing for the *Min* nonsense allele was not yet established. Retrospectively, we know that the intestinal tumor phenotype is correlated with the *Min*/+ genotype to a very high degree of confidence (9). Hybrid F_1 animals were obtained by crossing females from the AKR/J (AKR) or CAST/Ei (CAST) strains with B6.*Min*/+ males. Backcross animals were produced by crossing *Min*/+ F_1 animals of either sex with B6 mates. Mammary tumors were removed either at autopsy or by resection, fixed in buffered 10% (vol/vol) formalin for at least 24 h, and processed for histological analysis. Animals were killed by CO_2 asphyxiation. For resection of tumors, animals were anesthetized with Nembutal.

Spontaneous Mammary Tumors. Animals developing spontaneous mammary tumors were identified when visible tumors were noted during regular screening of the mice to identify *Min*/+ animals.

***N*-Ethyl-*N*-nitrosourea (ENU) Injections.** Animals were given ENU at 50 mg/kg (body weight) (Sigma) by intraperitoneal (i.p.) injection (12). The injections were performed in a high-velocity chemical hood, after which animals were housed in the hood for 24 h to allow for the spontaneous decay of ENU, transferred to clean boxes, and returned to the animal rooms. ENU-treated mice were observed weekly for signs of anemia or mammary tumors. Animals were killed when moribund or 65 days after ENU injection. At necropsy, intestines and mammary glands were examined visually for the presence of tumors.

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Abbreviations: *Apc*/*APC*, adenomatous polyposis coli gene—murine/human; ENU, *N*-ethyl-*N*-nitrosourea; DMBA, 7,12-dimethylbenz[*a*]anthracene.

Transplantation and Chemical Carcinogenesis. Mammary cell transplantation was performed when the recipient (B6 × AKR)F_1 females were 4–5 weeks of age. Abdominal mammary glands were collected into serum-free medium from 50- to 70-day-old females from the B6.*Min*/+ strain. The genotype at the *Apc* locus of the mammary donors was determined by examination of the intestines for tumors. Animals with intestinal tumors were scored as *Min*/+; tumor-free animals were scored as +/+ (see ref. 10). The mammary glands from donors of each genotype, +/+ or *Min*/+, were pooled on ice, enzymatically treated, and processed to produce a monodispersed epithelial cell suspension as described (13). Recipient females were injected with 2.5×10^4 mammary cells in each of two sites in the white intrascapular fat pad. Any one animal received either *Min*/+ or +/+ cells.

At 5 weeks after transplantation, animals from the set that received the *Min*/+ cells and from the set that received the +/+ cells were each randomly assigned to one of three treatment groups: 7,12-dimethylbenz[*a*]anthracene (DMBA), ENU, or control. DMBA at 1 mg (Eastman Kodak) in 0.2 ml of sesame oil was delivered by intubation weekly for 6 weeks. ENU was delivered by a single i.p. injection at a dose of 200 mg/kg (body weight). Untreated animals were followed as controls.

The untreated and ENU-treated mice received a graft of a whole pituitary gland from a B6 female under the left kidney capsule 8–9 weeks after cell transplantation (3–4 weeks after ENU treatment). DMBA-treated mice received pituitary grafts 3 weeks after the final DMBA treatment.

At 1- to 2-week intervals for 52 weeks, all mice were palpated for the presence of tumors at both the transplant site and the 10 intact *in situ* mammary glands of the host animals. Whenever possible, tumors were resected and the animals were kept for further observation. Moribund animals were sacrificed, and complete necropsies were performed. Any tumors or growths were recorded and fixed in buffered 10% formalin, embedded, sectioned, and stained for histological evaluation. Intrascapular fat pads without tumors were removed, whole-mounted, and scored for mammary development (14).

Statistical Analysis. The proportion of tumors arising under different conditions was modeled with a generalized linear model (15) assuming binomial variability as implemented in the GLIM computer package (available from the Royal Statistical Society of London). Differences between the treatment conditions were tested by comparing the change in scaled deviance due to that predictor variable with the χ^2 reference distribution.

Estimates of the median time to first tumor were obtained from the product limit estimate of the survival curve (16). The logarithmic rank test was used to test the effects of treatment in the survival curves (16). All calculations were carried out in the SAS statistical analysis program (17).

RESULTS

Spontaneous Tumors. In five B6 *Min*/+ females that developed mammary tumors, the average age at detection of the tumors was 114 ± 25 days. Because the average life span of *Min*/+ mice on the B6 background is only ≈120 days (10), perhaps the tumor incidence would rise if the life span were increased. Therefore, we screened longer-lived hybrid *Min*/+ animals (18) for mammary tumors. These hybrids were obtained from backcrosses of (AKR × B6)F_1 *Min*/+ and (CAST × B6)F_1 *Min*/+ animals with B6 mates. The average life span of the *Min*/+ females was 197 ± 96 days in the AKR backcross and 227 ± 101 days in the CAST backcross. The numbers of backcross animals developing mammary tumors are shown in Table 1. The average ages of tumor detection in these mice were 127 ± 32 days for the AKR backcross animals and 207 ± 108 days for the CAST backcross animals. All of the animals developing mammary tumors in the two backcrosses were scored as carrying *Min*, based on the presence of intestinal tumors or by genotyping (9). No animals scored as wild-type developed mammary tumors, even though the average life span for these female mice was 345 ± 23 days in the AKR backcross and 354 ± 107 days in the CAST backcross. The effect of *Min* on the number of hybrid and backcross mice that developed mammary tumors is highly significant ($P < 0.0001$).

Table 1. Spontaneous mammary tumors

Background	Genotype	Tumor-bearing/ total, no./no.
(AKR × B6)F_1	*Min*/+	1/5
	+/+	0/7
(AKR × B6) *Min*/+ × B6	*Min*/+	3/39
	+/+	0/42
(CAST × B6) *Min*/+ × B6	*Min*/+	9/79
	+/+	0/53

Female mice were checked for mammary tumors at regular intervals and at necropsy. Tumors were confirmed by histological evaluation. *Min* genotype was scored on the basis of intestinal phenotype and then confirmed by DNA-based genotyping (9).

***In Situ* Carcinogenesis.** To determine whether the number of B6 *Min*/+ mice that developed mammary tumors could be increased, we injected animals with ENU at 10–13 or 32 days of age. ENU is known to be a broadly acting and effective carcinogen (19) and somatic mutagen (20) in the mouse and, therefore, might be expected to increase the tumor incidence if secondary somatic mutations are involved. The mice were killed when moribund or 65 days after injection and examined for intestinal and mammary tumors. As shown in Table 2, 37.5% of the *Min*/+ females treated at 10–13 days of age developed mammary tumors, with an average age of detection of 65 ± 6 days. No mammary tumors were seen in any of the +/+ females or in the *Min*/+ mice injected with ENU at 32 days of age. The number of younger ENU-treated animals developing mammary tumors is significantly higher than all other groups ($P = 0.0001$).

Transplantation and Chemical Carcinogenesis. Experiments similar to those described for ENU were attempted with multiple exposures to DMBA, but the *Min*/+ animals did not tolerate the treatment regimen. Therefore, to study the effect of DMBA on *Min*/+ mammary glands and to increase observation time, mammary glands were transplanted from *Min*/+ and +/+ B6 littermates into normal (B6 × AKR)F_1 recipients. Such transplantation experiments also tested whether the effects of the *Min* mutation are intrinsic to the mammary gland parenchyma.

Mammary gland development was observed at all graft sites. Treatment with DMBA or ENU significantly increased the number of *Min*/+ grafts developing tumors compared

Table 2. Effect of ENU on mammary tumor formation

Age at treatment, days	Treatment	Genotype	Mammary tumor-bearing/total, no./no.
10–13	ENU	*Min*/+	18/48
		+/+	0/50
	Control	*Min*/+	0/15
		+/+	0/21
32	ENU	*Min*/+	0/9
		+/+	0/13

B6-*Min*/+ females were injected with ENU (50 mg/kg) between 10 and 13 days or at 32 days of age. Animals were checked weekly for signs of mammary tumors and were sacrificed when moribund or 65 days after treatment. Mammary glands were scored for tumors at necropsy. All mammary tumors were confirmed by histological evaluation.

with the untreated animals with *Min*/+ grafts ($P = 0.002$) and the treated animals with +/+ grafts ($P = 0.001$) (Table 3). The effects of the two treatments on the *Min*/+ grafts were not significantly different from each other ($P = 0.759$). In contrast to ENU treatment, DMBA treatment induced tumors both in the +/+ grafts and in the *in situ* mammary glands of the (B6 × AKR)F_1 hosts ($P = 0.001$). The numbers of hosts with *in situ* tumors after DMBA treatment did not differ significantly between recipients of *Min*/+ (10/29) and +/+ (11/28) grafts ($P = 0.718$).

Compared with ENU, DMBA had an effect on both the time to first tumor observation in the *Min*/+ grafts ($P < 0.001$) and the life span of the hosts, regardless of the genotype of the graft. Most of the untreated and ENU-treated animals survived to the end of the experiment (52 weeks), but most of the DMBA-treated animals had died by 28 weeks after treatment.

Upon examination of histological sections from whole mounts of the grafted mammary glands from the untreated and the ENU-treated animals, focal alveolar hyperplasias were observed in many of the *Min*/+ grafts (Table 3). Whole mounts of intrascapular fat pads could be prepared only from nontumor bearing grafts. Hyperplasias were more frequent in the ENU-treated *Min*/+ grafts than in the untreated *Min*/+ grafts ($P = 0.0137$). No examples of hyperplasia were noted in the grafts of animals treated with DMBA or in any of the +/+ grafts. The *in situ* glands of the hosts were not examined in whole-mount preparations.

Histological Analysis. Histological evaluation was performed on tumors arising spontaneously or after chemical carcinogenesis. All tumors were classified as mammary carcinomas and usually contained areas of adenocarcinoma and adenoacanthoma (21) (Fig. 1 *A* and *B*). This was true whether the tumors were of spontaneous origin or chemically induced. The hyperplastic foci noted in the longer-lived graft recipients were encapsulated and were surrounded by normal mammary tissue (Fig. 1*C*).

We also examined four spontaneously arising tumors for evidence of viral particles. Parts of each of the four tumors were fixed for electron microscopy and sectioned. No evidence of viral particles was found (C. Sattler, McArdle Laboratory, personal communication).

DISCUSSION

These results demonstrate that female mice carrying the *Min* allele of the *Apc* gene have an increased risk of mammary hyperplasia and neoplasia. This susceptibility was most apparent on hybrid genetic backgrounds where the *Min*/+ animals have a reduced intestinal tumor load and an increased life span (18). On each of the two hybrid backgrounds screened, ≈10% of *Min*/+ females developed spontaneous mammary tumors. This low penetrance for mammary neoplasia contrasts with the 100% penetrance for intestinal adenomas in these mice. Most of the mammary tumors were evident after 100 days of age. The incidence of spontaneous mammary tumors in the B6 *Min*/+ females is lower than that observed for the hybrid animals, perhaps because of the shorter life span on the B6 background. Alternatively, modifier alleles such as those that decrease the intestinal tumor number in the hybrid animals (18) may be acting directly to increase the susceptibility to mammary neoplasia.

The B6 and AKR strains are not susceptible to spontaneous mammary tumors (22), and in our colony we have not noted spontaneous mammary tumors in any +/+ females from any of the strains used in these studies. Nearly all of the mice that developed mammary tumors were virgin animals, indicating that the hormonal stimulation of pregnancy and lactation is not necessary for *Min*-induced mammary tumor development. Whether hormonal stimulation could increase the incidence of tumors in *Min*/+ mice has not yet been tested.

We tested the effects of chemical carcinogens on the incidence of mammary tumors in B6 *Min*/+ mice to decrease the latency of tumor development. ENU was used because it is known to be a potent point mutagen in the mouse and also because it does not induce mammary tumors at high frequency in wild-type mice with similar doses and exposure times (19). When ENU is administered to mice between 10 and 13 days of age, the *Min*/+ females show an increased incidence of mammary tumors relative to untreated *Min*/+ females and to ENU-treated +/+ siblings. After ENU treatment, mammary tumors arose very rapidly, with visible tumors present an average of 55 days after treatment. No tumors were observed in any of the animals that were treated at 32 days of age and then followed for 65 days. Similarly, tumors were not seen in the untreated *Min*/+ animals sacrificed at 75 days of age, as would be expected from the lack of tumors seen in young B6 *Min*/+ females in the Min pedigree. This sensitivity of the mammary gland of the prepubertal *Min*/+ female mice to ENU-induced tumors warrants further investigation. These results indicate that the presence of the *Min* defect alone is not sufficient for efficient induction of mammary tumors by ENU within the time period studied. The sensitivity of the younger *Min*/+ mice could be related to the developmental state of the mammary gland at the time of treatment or to the number of cell cycles the mammary cells pass through after ENU treatment.

ENU treatment of the *Min*/+ mice stimulated tumor formation only in the mammary and intestinal tissue (E.M.M. and A.R.M., unpublished observations), the same tissues that are the targets for spontaneous *Min*-induced neoplasia. Therefore, ENU amplifies an existing potential in these

Table 3. Mammary transplants—neoplasia and hyperplasia

Treatment	Genotype	Tumor-bearing/total, no./no.	Median time to tumor, weeks	Hyperplastic/tumor-free grafts, no./no.	Median time to death, weeks
Control	*Min*/+	2/28	46.5	5/26	52
	+/+	0/26	NA	0/26	52
DMBA	*Min*/+	10/29	12.0	0/19	21
	+/+	1/28	14.0	0/27	23.5
ENU	*Min*/+	10/26	23.5	9/16	52
	+/+	0/25	NA	0/25	52

Mammary glands were collected from B6-*Min*/+ or +/+ siblings. The genotype of the donor was inferred by scoring of the intestine for tumors. *Min*/+ or +/+ mammary cell preparations were isolated and injected into the intrascapular fat pad of +/+ (AKR × B6)F_1 female recipients. Animals were sacrificed when moribund or 52 weeks after chemical treatments. Whole mounts of tumor-free intrascapular fat pads containing the mammary transplants were scored for mammary development. Abnormal regions were scored histologically for evidence of neoplasia or hyperplasia. NA, not applicable.

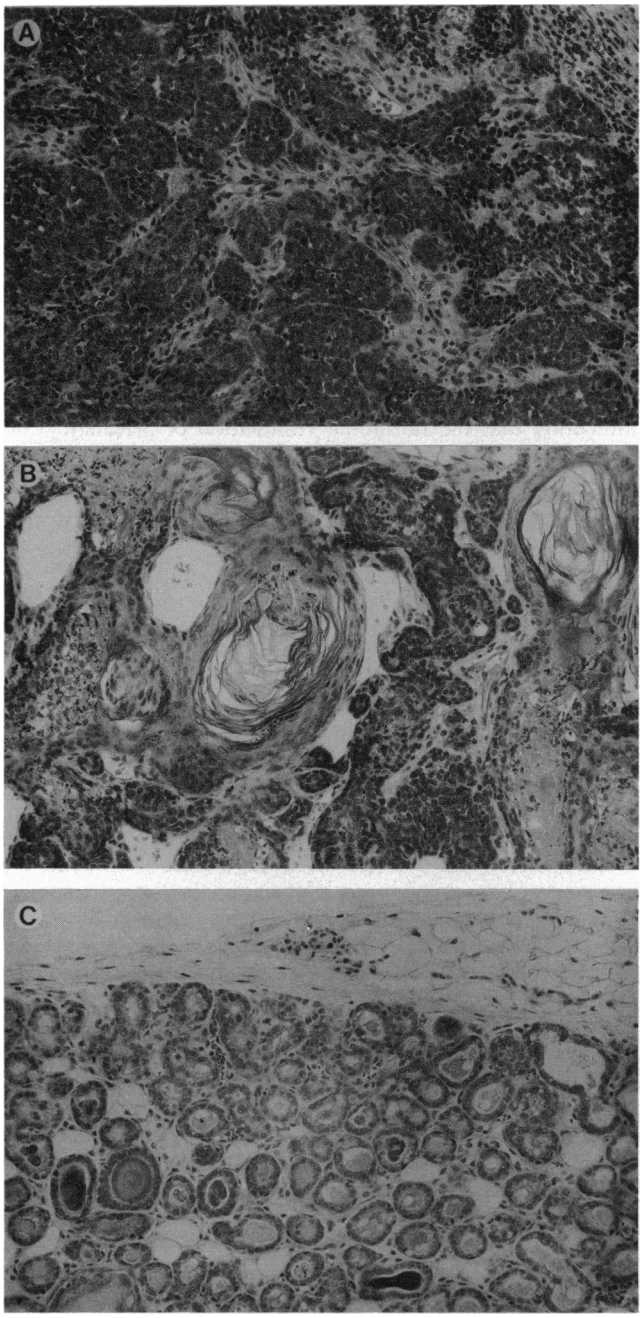

FIG. 1. (*A* and *B*) Photomicrographs of two regions of a mammary tumor from a 131-day-old (CAST × B6) × B6 female. (*A*) Region of adenocarcinoma. (*B*) Region of adenoacanthoma. (*C*) Section from a hyperplastic nodule from a graft of B6 *Min*/+ cells into an ENU-treated (B6 × AKR)F_1 host female. The entire lesion measured 10 × 5 mm. The edge of the lesion is encapsulated and surrounded by normal mammary tissue.

animals over the short time period studied and does not create a new target tissue.

The transplant procedure itself did not appear to alter the susceptibility to tumor formation in the *Min*/+ or +/+ mammary tissue, because two tumors arose in 28 untreated *Min*/+ grafts and none were observed in the untreated +/+ grafts. Both ENU and DMBA increased the incidence of tumor formation in the *Min*/+ grafts. For ENU this effect was specific to the *Min*/+ grafts since no tumors were seen in either the +/+ grafts or the *in situ* mammary glands of the host animals. This result indicates that the susceptibility to mammary tumor development observed in young ENU-treated animals was not specific to the chronological age of the mammary cells, since the transplanted cells were removed from older animals. However, the transplanted cells must proliferate and redifferentiate to establish the graft, perhaps replicating the conditions present in the glands of the younger ENU-treated mice. In addition, the earliest tumors in the grafts were observed at 15 weeks after ENU treatment, a much longer period of observation than for *Min*/+ animals that were treated with ENU.

In contrast to ENU, DMBA is known to be an effective mammary carcinogen in mice (23, 24). After DMBA treatment, tumors developed with a short latency in transplanted glands of each genotype. However, the *Min*/+ grafts gave rise to tumors at a significantly higher rate than the grafts from +/+ sibs. The rate of tumor development in the *Min*/+ grafts was also higher than that of the *in situ* glands, taking into consideration the fact that each mouse has 10 *in situ* glands, but only one transplanted gland. Tumors developed in the *in situ* glands of hosts carrying *Min*/+ grafts at the same frequency as in hosts carrying +/+ grafts, indicating that the genotype of the graft did not exert a systemic effect. The latency of the DMBA-induced tumors was much shorter than that of the tumors arising after ENU treatment or in the untreated group. In fact, most of the DMBA-treated animals had died before tumors were noted in animals of the other groups. Therefore, it is possible that the incidence of DMBA-induced tumors would increase further if the treatment did not shorten the life span of the host animals. The life spans of the DMBA-treated animals were not related to the genotype of the graft, indicating that the toxic effect of the DMBA was unrelated to tumor formation in the grafts.

We are intrigued by the focal alveolar hyperplasias in the *Min*/+ grafts of animals from the untreated and ENU-treated groups. Hyperplasias were noted only in animals that survived to the end of the experiment and, therefore, may be age- or time-dependent phenomena. Thus, the lack of hyperplastic nodules in the DMBA-treated animals may be related to the short survival time of these mice relative to the control or ENU-treated mice. Since no B6-*Min*/+ mice survive to 52 weeks of age, we do not know whether the development of hyperplasias is related to the transplant procedure. Mammary hyperplasias have not been noted in any of the longer-lived hybrid *Min*/+ animals, suggesting either that the genetic background affects the tendency for hyperplasia or that hyperplasia is generated by the transplant procedure or by exposure to high prolactin levels from the pituitary graft. The hyperplasias were focal, indicating that *Min* itself is not sufficient to induce hyperplasia and that further somatic events are required. The incidence of hyperplasia was increased by ENU treatment, but only in the *Min*/+ grafts. This result indicates that ENU treatment is not sufficient to induce mammary hyperplasias in the absence of *Min* and that ENU can increase the susceptibility of *Min*/+ mammary cells to hyperplasia.

The transplant studies allow us to conclude that the action of the *Min* mutation in mammary tumor formation in the *Min*/+ female mouse is intrinsic to the mammary gland and not mediated by systemic effects. This inherent susceptibility can be increased by treatment with known mutagens, indicating that further somatic mutational events are involved in the development of the mammary tumors. Whether these further events involve any of the genes known to be involved in mammary tumor formation in mice or humans remains to be tested. The identification of the further somatic events required for mammary neoplasia in these mice might aid in understanding the decreased penetrance of the mammary neoplasia relative to the intestinal neoplasia in Min mice. Is the same array of genes involved in neoplasia in these two tissues or are there tissue-specific pathways?

The fact that the *Min* mouse exhibits two distinct pathways of neoplasia gives an opportunity to investigate whether each pathway reflects a unique combination of genetic lesions. This information will help us to understand the basis for the narrowing of the spectrum of neoplasia below the spectrum of tissue expression of a tumor suppressor gene. Also, it may help us to understand why the spectrum of tumors seen in animals and humans with inherited mutations in tumor suppressor genes differs from the variety of tumors in which somatic lesions in these genes are found. Note for example that somatic mutations in *APC* are frequently found in human pancreatic cancer (29). This understanding of combinatorial genetics will in turn help us to understand why the spectrum of neoplasms elicited by a defect in a tumor suppressor gene may differ between mouse and human (25–28).

The observation of hyperplastic lesions in the mammary transplants from *Min*/+ animals in the untreated and ENU-treated group suggests that *Min* also predisposes to hyperplasia. If the hyperplastic foci can lead to tumor formation, they may represent an intermediate step on an alternative pathway to *Min*-induced mammary tumorigenesis.

Like mutations of the *APC* gene in humans, the *Min* mutation confers a susceptibility to intestinal neoplasia. However, increased susceptibility to mammary neoplasia has not been reported to be a feature of the human syndromes associated with mutations in *APC*. The development of mammary tumors in *Min*/+ female mice may reflect a functional difference between the mouse and human gene products. Alternatively, the increased risk of mammary tumors in humans due to germ-line mutations in *APC* may be too small to be noted in most families, given the background rate of mammary tumors in the human population. However, loss of heterozygosity at *APC* may be involved in mammary cancers that do develop in humans carrying mutations in *APC*. *APC* mutations or allele loss may also play a role in human mammary tumors from patients not carrying germ-line mutations. A screen for such events at the *APC* locus in human mammary tumors would help to resolve this issue.

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