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Manipulation of the mouse germline in the study of *Min*-induced neoplasia

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The Min mouse, generated by random germline mutagenesis, carries a mutation in the mouse homolog of APC and is a model of inherited human intestinal tumorigenesis. To identify other genes in the pathway(s) of intestinal tumorigenesis, genes that modify the Min phenotype have been sought. Several have been identified, including Mom1 *and the genes for the 5-cytosine DNA methyltransferase and the DNA mismatch repair factor Msh2.* Min*-dependent tumorigenesis also occurs in mammary glands, the pancreas, and the body wall. The Min mouse has therefore become a model for tumorigenesis in a variety of organs. Identifying modifiers of its phenotype will help in piecing together the pathways of tumorigenesis in each of these tissues.*

Key words: Apc / intestinal / Min / Mom1 / tumorigenesis

Abbreviations not defined in text: 129, 129/SvJ; AKR, AKR/J; APC, adenomatous polyposis coli; B6; BALB, BALB/cByJ; BTBR, BTBR/Pas; CAST, *M. m. castaneus*; cDNA, complementary DNA; DBA, DBA2/J; *scid*, severe combined immune deficiency; SWR, SWR/J.

IN THE DECADE since the induction of the *Min (multiple intestinal neoplasia*) mutation, the Min mouse has become a powerful tool for the study of the early steps of intestinal cancer. The penetrance of its tumor phenotype, the speed with which the tumors develop, and the large number that form are properties that make the Min mouse useful in the identification of other genes involved in tumorigenesis. The search among natural and induced mutations for these 'modifier of Min' (Mom) loci involved in *Min*-dependent tumorigenesis is under way.

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Discovery of *Min*

In 1986, the original Min mutant emerged from a phenotypic screen following germline mutagenesis of mice with ethylnitrosourea (ENU).[1] ENU causes a variety of base changes, including transitions and transversions, in the mouse germline; it is the most potent known mouse mutagen.[2-5] By 1985, it had been shown to cause forward mutations in specific genes at a rate of up to 1 in 700.[6] Our laboratory's first phenotypic screens confirmed the effectiveness of ENU-induced germline mutagenesis, yielding one set of recessive alleles affecting phenylalanine metabolism and another set causing embryonic lethality.[7-9] Additional mutagenesis yielded an intriguing surprise: an anemic mouse that ran in circles.

This anemic circler was bred to wild-type animals, to follow the inheritance of its phenotypes.[1] Anemia and circling were inherited independently, indicating that they were caused by separate mutations (another example of the potency of germline mutagenesis by ENU). Dissection of the anemic mice revealed that the anemia was probably due to the presence of multiple intestinal neoplasms, for which the mutation was named.[1] These mice developed tumors within a few weeks of birth, and the phenotype was fully penetrant.[1] The Min phenotype resembles familial adenomatous polyposis (FAP), an inherited human disease in which patients generally develop hundreds of intestinal tumors by age 30 due to a mutation in the *APC* gene.[10-12] This resemblance led to the identification of the *Min* mutation as a nonsense mutation in the mouse homolog, *Apc*.[3] Mutation of the *APC* tumor suppressor gene is now known to be an early step in the development of many, if not all, types of intestinal tumors, whether sporadic or inherited (see refs 13, 14).

Intestinal biology and tumorigenesis

Most intestinal cancers arise from the single epithelial layer that lines the intestine. This layer contains four

differentiated cell types that are produced from stem cells embedded in the intestinal crypts (Figure 1). The epithelial tissue is highly proliferative: in mice, its cells are renewed about every three days. A small fraction of cells in the crypt also undergo programmed cell death, especially in response to DNA damage.[15]

According to one model, four to six mutations are required for the development of intestinal cancer.[16-18] Genetic changes that correlate with the establishment of these cancers include loss or mutation of both alleles of *APC* and *p53*, as well as activation of *Ki-RAS*. (The gene *deleted in colorectal cancer (DCC)*, previously included in such lists, now appears not to be deleted frequently.[19]) These changes correlate with distinct stages of tumor progression. For example, *APC* is mutated in tumors of every stage, while *p53* is frequently mutated in human tumors that have become invasive (Figure 2). Other changes that correlate with colorectal cancer include: global DNA hypomethylation, regional hypermethylation, reduced expression of E-cadherin, and overexpression of c-myc, cyclooxygenase-2, and ligands of the epidermal growth factor receptor (EGFR), including transforming growth factor α (TGFα), amphiregulin and cripto.[20-25]

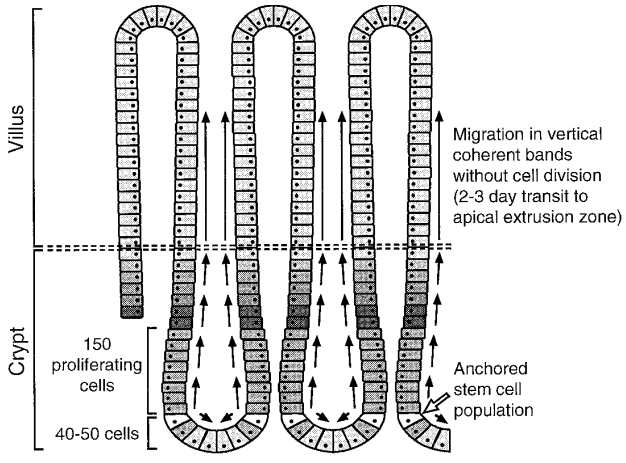

Figure 1. Diagram of the mouse small intestine. The four cell types found in the intestine are derived from stem cells and migrate in the directions indicated by arrows. This figure is a redrawing of Figure 1 in ref 117. Reproduced from The Journal of Cell Biology, 1989, Vol. 108, pp 1187–1194 by copyright permission of the Rockefeller University Press and with the Author's permission.

The *Apc* molecule

The *APC* gene encodes a large (2844 amino acid) multi-domain polypeptide, highly conserved between humans and mice (90% at the amino acid level).[3,11,26] Immunoprecipitation and yeast two-hybrid analyses have revealed that the APC molecule binds a variety of partners: other APC molecules, the human homolog of Drosophila discs large (DLG), β-catenin, microtubules, and an uncharacterized protein named EB1.[27-34] These interactions, as well as cell culture and transgenic studies, suggest that APC might be involved in cellular adhesion, migration, signaling and proliferation[35-41] (see also ref 42).

The Min phenotype

B6-Min mice, which carry the Apc^{Min} mutation in heterozygous form on a C57BL/6J (B6) background, develop an average of more than 50 tumors within 90 days of birth. These mice appear to be most susceptible to tumor formation very early in life: somatic mutagenesis is most effective at inducing tumors in B6-Min mice during the first two weeks after birth.[43] In untreated mice, tumors develop throughout the intestine, but they are more frequent in the small intestine than in the large. Some adenomas reach several millimeters in diameter, but they do not become invasive, presumably because of the short lifespan of these mice. On the B6 genetic background, *Min*/+ mice rarely live beyond 150 days of age.[1] This premature death is apparently due to secondary effects, including anemia and intestinal blockage.[1]

In B6-Min mice, the intestinal phenotype of the *Min* mutation is dominant and fully penetrant at the organismal level: all *Min*/+ progeny develop multiple intestinal tumors.[3] However, the *Min* allele may act recessively at the cellular level: every tumor analysed from untreated B6-Min mice has lost the wild-type *Apc*

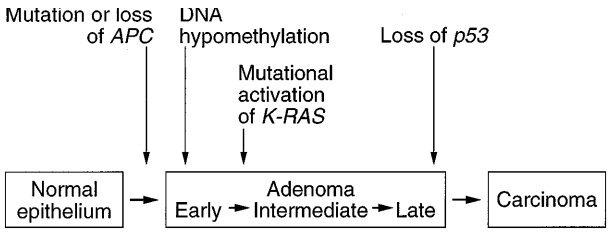

Figure 2. Model of colorectal cancer development. Revised from ref 23.

allele, suggesting that the wild-type allele suppresses tumorigenicity.[44,45] This observation and similar observations in humans suggests that *Apc* is a classical tumor suppressor gene.[13]

Apc loss of function versus altered function

While the wild-type *Apc* allele is likely to be a classical tumor suppressor at the cellular level, some mutant alleles might represent more than simple loss of wild-type function: they might either interfere with the wild-type function or have an increased or novel activity that is oncogenic.[13] Support for this idea comes from the observation that the *Min* mutation and the great majority of germline and somatic *APC* mutations in the human cause premature termination of the polypeptide.[13,14] These truncated proteins, if stable, would retain the N-terminal portion of the protein required for homodimerization, but would lack regions that bind other factors, such as microtubules, DLG, and EB1.[27,29,46] Though not all *APC/Apc* truncation mutations are likely to create alleles with altered function, some are suspect. These include germline mutations that cause truncation in a central region of the protein (aa 1286–1513) and are associated with a particularly severe form of FAP.[13] Cell culture studies also suggest that some truncated APC molecules may inhibit the function of the wild-type APC; one transgenic study suggests they might not.[47,48]

It is theoretically possible that tumor formation in FAP patients requires a truncated APC molecule. Arguing against this is the observation that human germline mutations that delete the entire *APC* gene still predispose their carriers to tumors.[49,50] However, it is possible that the wild-type allele is truncated in tumors formed in these patients. Ideally, one could generate a mouse homozygous for a germline deletion of *Apc* and determine whether this complete loss of function leads to tumor formation. However, mice homozygous for an *Apc* deletion would probably not survive embryogenesis: homozygosity for the *Min* mutation, for example, leads to early embryonic lethality.[51] One method of circumventing this early embryonic lethality would be to induce deletion of *Apc* specifically in neonates. This might be accomplished by emerging techniques that allow regulated Cre/lox recombination (see ref 52). Analysis of homozygous *Apc* loss would involve creating a mouse in which both *Apc* alleles are flanked by lox sites, and in which a *Cre* gene is expressed late in development or in the first weeks of life, when mice are particularly susceptible to tumor induction.[43] Expression of Cre would then result in the deletion of both copies of *Apc*.

Phenotypes: *Min* versus *FAP*

As noted above, B6-Min mice and FAP patients share significant traits: germline mutations (frequently truncations) in *APC/Apc* lead to the formation of multiple intestinal adenomas in which the wild-type allele of *APC/Apc* is mutated or lost. However, mouse and human *APC/Apc* mutants differ in several other respects: (1) Min mice develop mainly tumors of the small intestine, while FAP patients develop mainly tumors of the large intestine;[1,10] (2) about 10% of B6-Min females develop mammary tumors, but there is no known correlation between FAP and breast cancer;[53] (3) Up to 93% of FAP patients develop one or more additional complications, such as bone and body wall tumors, skin cysts and enlarged pigmented retinal cells, but these lesions have not been detected in untreated B6-Min mice.[10]

Some of these differences between B6-*Min* mice and FAP patients are due to differences in genetic background: Min mice develop extra-intestinal lesions seen in FAP patients when carrying mutations in genes other than *Apc* (see below). Genetic background might also affect APC-dependent tumorigenesis among humans. Humans display dramatic variation in the severity of the intestinal phenotype and in the frequency of non-intestinal phenotypes, such as bone and body wall (desmoid) cancers.[10] Although this variation appears to be due in part to differences among *APC* mutations (see ref 13), that explanation is not sufficient: dramatic phenotypic differences have been seen even among patients with the same *APC* mutation.[54,55] The identification of modifiers in mice, where small effects can be detected because of the homogeneity of genetic background and environment, should promote the understanding of human intestinal tumors. Knowledge of modifers may also help in the future to assess an FAP patient's risk for developing severe intestinal and non-intestinal disease.

Modifiers of *Min* (*Mom* loci)

The large number of tumors that form in Min mice allows the detection of quantitative modifier genes.

Table 1. Mom1 resistance and sensitivity alleles among inbred strains

Cross	*Mom1*$^{X/B6}$	*Mom1*$^{B6/B6}$	Mom1 phenotype of strain X
B6×(AKR×B6-*Min*/+)	11.7	22.0	Mom1^R
B6×(MA×B6-*Min*/+)	8.2	23.6	Mom1^R
B6×(CAST×B6-*Min*/+)	11.3	16.7	Mom1^R
B6×(SWR×B6-*Min*/+)	8.5	19.1	Mom1^R
B6×(DBA×B6-*Min*/+)	14.0	29.0	Mom1^R
B6×(BALB×B6-*Min*/+)	18.5	27.9	Mom1^R
B6×(BTBR×B6-*Min*/+)	40.0	38.4	Mom1^S
B6×(129×B6-*Min*/+)	27.9	32.9	Mom1^S

X denotes the non-B6 allele in each cross (e.g. AKR). B6 *Min*/+ mice develop, on average, 30 tumors in the regions counted; the B6 strain is *Mom1*^{S}. Animals from different crosses were scored at different ages. Data were compiled from refs 57,58.

The first to be identified was a naturally occurring allelic variant among mouse strains, named *Modifier of Min 1*, or *Mom1*.[56,57] When B6-Min mice were crossed with eight other strains (AKR, MA, CAST, 129, BTBR, BALB, SWR, DBA), all but one (BTBR) were shown to carry a dominant modifier of Min.[56-58] The F1 progeny of crosses between these strains and B6 Min developed fewer tumors, on average, than B6-Min mice.[56-58] Backcross analyses were performed to map genes responsible for this dominant effect. For example, to identify AKR modifiers that reduce tumor number, (AKR X B6-Min)F_1 *Min*/+ mice were crossed to B6 animals.[57] In such a cross, the resulting backcross progeny receive, on average, one quarter AKR-derived alleles, with each animal receiving a different set. By following the inheritance of AKR and B6 genomic markers in these backcross progeny, the inheritance of particular regions of the AKR genome could be correlated with decreased tumor load and, thereby, with inheritance of the dominant resistance modifier of *Min*. Such analysis mapped *Mom1* to distal chromosome 4.[57] AKR, MA, CAST, SWR, DBA and BALB carry tumor-resistance alleles of *Mom1*; B6, BTBR and 129 carry susceptibility alleles (Table 1).[57,58] Strain 129 carries at least one dominant modifier unlinked to *Mom1*.[58] AKR, MA, CAST and SWR carry dominant modifiers in addition to *Mom1*.[57,58] AKR carries recessive resistance alleles as well as dominant ones; together, these reduce adenomas to an average of less than one per mouse in a strain in which the *Min* mutation has been extensively backcrossed to AKR[57,59] (A.R. Shoemaker, unpublished data).

To confirm the location of *Mom1*, and to analyse it in the absence of other modifiers, a 35 cM segment of AKR chromosome 4 was introduced in a B6 genetic background through multiple generations of backcrossing.[60] The process of eliminating AKR alleles not linked to this region was accelerated by using markers to identify those backcross animals that inherited the fewest AKR alleles. (For a detailed discussion of the theoretical and practical aspects of this 'marker-assisted backcross' method, see refs 60-63).

The resulting B6 animals carrying the AKR *Mom1* region (B6.*Mom1*^{AKR}) were used to show that *Mom1* is a semidominant modifier of Min. One copy of the *Mom1*^{AKR} tumor-resistance allele reduces tumor number about two-fold; two copies reduce tumor number four-fold.[60] *Mom1* also reduces tumor size in a semidominant manner.[60] Analysis of the number and size of tumors in animals of various ages suggests the effect on tumor size may be due to an effect on the net growth rate of adenomas.[60]

To facilitate the identification of the gene encoded by *Mom1*, a fine-structure map of the *Mom-1* interval was generated. B6.*Mom1*^{AKR} animals were bred to B6 animals. Progeny that carried new recombinations in the distal region of chromosome 4 were mated to Min mice, and the Mom1 phenotype was assessed.[58] Inheritance of AKR alleles in the interval between markers *D4Mit54* and *D4Mit284* correlated with a *Mom1* resistance phenotype (Mom1^R), indicating that *Mom1* lies within this approximately 4 cM interval.[58] Interestingly, an intermediate phenotype is seen in some animals that carry a recombination within this interval. This suggests that *Mom1* might be a cluster of linked genes with additive effects on tumor number.[58]

Markers in the *Mom1* region are not lost in Min-induced tumors from (AKR X B6) *Min*/+ F1 mice. This indicates that *Mom1* may not act as a classical tumor suppressor.[58] If *Mom1*^{R} encodes a secreted factor that suppresses tumors, this failure to lose the *Mom1* region in tumors might not be surprising: loss of the allele for a secreted factor from the tumor lineage may not confer a growth advantage, because the factor might reach the tumor from surrounding cells.[64] Alternatively, the *Mom1*^{S} allele may be dominantly oncogenic at the cellular level. Yet another possibility is that *Mom1* is lost but its flanking markers are not.

Recently, a strong candidate for at least the proximal component of *Mom1* has emerged: the gene for secretory phospholipase 2 group 2a (*Pla2g2a*), which cleaves fatty acids from the sn2 position of phospholipids and is expressed predominantly in cells at the base of small intestinal crypts.[58,65-67] All six strains known to carry tumor-resistance alleles of *Mom1*

encode full-length Pla2g2a; all three strains known to carry sensitive alleles of *Mom1* bear a frame-shift mutation that creates a premature stop codon and a protein with apparently little or no phospholipase activity.[58,65,68] In addition, *Pla2g2a* maps within the 4 cM region that encompasses *Mom1*.[58,65]

The conclusive identification of *Mom1* is fraught with the difficulties that accompany the identification of genes initially identified by polymorphisms within the species, such as *Bcg* (which confers resistance to parasitic infection in mice) and *BRCA1* (which suppresses breast cancers in humans). In these positional cloning efforts, investigators invested years in mapping the genes genetically and physically, only to be left with several candidate genes that bore multiple polymorphisms.[69-73] For example, the gene considered most likely to be responsible for the Bcg phenotype, based on tissue specificity, bore multiple amino acid variations in each strain analysed.[71] Targeted mutation of the gene, *Nramp*, was required to confirm that it causes the Bcg phenotype.[72]

Similarly, the *Pla2g2a* polymorphism is one of many differences among inbred strains expected to lie in the *Mom1* region. Indeed, an amino acid-altering mutation in a second candidate gene, *Rap1Gap*, correlates perfectly with Mom1 phenotype among the nine inbred strains.[58] This perfect correlation between *Rap1Gap* and *Pla2g2a* genotypes suggests that this region of chromosome 4 in these inbred strains is derived from only two ancestral chromosomes. This, in turn, implies that other natural variants in the *Mom1* region are likely to correlate perfectly with Mom1 phenotype among inbred strains. High-resolution mapping can help exclude candidates: a single fine-structure recombinant reveals *Rap1Gap* to be proximal to *Mom1*.[58] Similarly, further mapping of the *Mom1* regions should either strengthen *Pla2g2a*'s candidacy, or exclude it. The ever-increasing number of markers available for mapping and the accumulation of mapped cDNAs will make this approach even more powerful. However, final confirmation or rejection of the hypothesis that the proximal portion of *Mom1* is *Pla2g2a* will require the creation of a transgenic mouse that expresses *Pla2g2a* appropriately in an otherwise sensitive background, or of a targeted *Pla2g2a* mutation in an otherwise resistant background. The most informative transgenic *Pla2g2a* would be expressed in intestinal crypts, at a level comparable to that of the endogenous gene. However, the best test of *Pla2g2a*'s candidacy would be the construction of either a 'knock-in' mutation, in which the mutant *Pla2g2a* present in $Mom1^S$ strains is corrected through homologous recombinations with an intact copy, or of a knockout mutation involving the reverse process.

Pla2g2a is a neighbor to two other secretory phospholipases in the *Mom1* region of mouse chromsome 4.[74-76] The presence of this cluster of related genes has led to the hypothesis that one or both of the other phospholipases, *Pla2g2c* and *Pla2g5*, might comprise part of *Mom1*. This hypothesis is currently being tested.

The mechanism by which Pla2g2a might suppress tumor growth is not obvious. Overexpression of human PLA2G2A from a transgene in mice causes hyperplasia of skin cells.[77] Since hyperplasia is a step in tumor formation, Pla2g2a's ability to induce it suggests that it would increase, rather than decrease, tumor number.[78] Pla2g2a might also affect tumorigenesis through its release of the fatty acid arachidonate. Again, this might be expected to enhance tumor development rather than prevent it, because arachidonic acid can be metabolized by cyclooxygenase to yield the mutagen malondialdehyde, and it can participate in the creation of other carcinogens.[79] In addition, arachidonic acid can be metabolized to yield prostaglandins, and drugs that inhibit prostaglandin synthesis (such as piroxicam and sulindac) lead to a reduction in tumor number in mice and humans.[80-84] However, prostaglandins can play contradictory roles. For example, they can both activate and inhibit the immune response.[85,86] Pla2g2a is also bactericidal, and might thereby prevent the formation of mutagenic compounds by intestinal flora.[87] However, recent analyses of sterile intestinal grafts suggest that bacteria play no major role in the ability of *Mom1* to influence *Min*-induced tumor formation in the small intestine.[88]

Human modifiers of *APC*

Do humans carry genes that modify the *APC* mutant phenotype? The search for human modifiers of FAP has begun with an analysis of human chromosomal region 1p35–36, syntenic with the *Mom1* region of the mouse genome.[89] FAP patients were grouped according to the severity of the intestinal tumor phenotype, and genomic markers were used to determine whether the inheritance of a particular part of chromosome 1 correlated with disease severity. The results suggest that chromosome 1p35–36 carries a modifier, but it does not appear to act as a simple dominant resistance factor.[89] This human modifier

may (or may not) be a *Mom1* homolog. If *Mom1* is the gene identified in this analysis, its failure to behave as a simple dominant may be due to genetic background and environmental factors, or to a lack of the equivalent of the mouse alleles among the patients studied.

Another study asked specifically whether the human homologs of the cluster of phospholipases, *PLA2G2A*, *PLA2G2C* and *PLA2G5*, affect attenuated adenomatous polyposis coli, a less severe form of FAP, in humans.[90] Again, patients were grouped into three classes, based on the severity of their disease. Sequencing of all four exons of each gene revealed no polymorphisms that correlated with disease severity.[90] If one or all of these phospholipases are *Mom1*, we are left with three scenarios: (1) *Mom1* does not alter the tumor phenotype in humans; or (2) the relevant mutations are not in exons; or (3) the appropriate groups of patients with varied disease severity have not yet been analysed.

Chromosome 1p35–36 is frequently deleted in human intestinal tumors, suggesting the presence of a tumor suppressor.[91] This tumor suppressor is not likely to be the human homolog of *Mom1*: neither the *Mom1* region nor *Pla2g2a* in particular appears to be lost in *Min*-induced tumors in the mouse.[58] In addition, recent analysis suggests that, although *PLA2G2A* is lost in about 31% of human colon cancers, the *PLA2G2A* coding region incurs no intragenic mutation in the majority of tumors.[92] These results suggest that the putative tumor suppressor gene at 1p35–36 identified by somatic deletion is distinct from, but linked to, *PLA2G2A*.

Modification of *Min* through loss of function and gain of function

The search for modifiers of *Min* in the mouse began with the analysis of natural variants, but it has expanded to include targeted mutations that cause loss of function and transgenes that act as gain-of-function mutations, as these have become available. As described above, loss of *APC*, *p53*, global hypomethylation, regional hypermethylation and the overexpression of cyclooxygenase-2, EGFR, TGFα, cripto, amphiregulin, and c-myc have each been implicated in the progression of intestinal tumors. To determine whether these changes actually promote intestinal tumorigenesis or simply correlate with it, mutants that carry germline mutations in some of these potential modifiers have been crossed to B6-Min.

p53 modification of *Min*

p53, which functions in genome surveillance and apoptosis, is often lost or mutated in the later stages of intestinal cancer progression in humans.[23,93] Loss of *p53* has not been observed to affect the development or progression of intestinal adenomas in *Min*/+, $p53^{-/-}$ mice.[64,94] One explanation for this might be that the adenomas that form in Min mice do not develop to a stage at which p53 affects tumorigenesis in the intestine.[64,94]

The combination of the *Min* mutation and *p53* loss of function leads to the development of pancreatic cancers (in at least 83% of animals)[94] and a lesion often seen in FAP patients — body wall fibromas (in 100% of animals; W.F. Dove, R. Halberg, D. Katzung, and L. Donehower, work in progress). These results lend strength to the hypothesis that genes other than *APC* are involved in the generation of the many non-intestinal FAP phenotypes, and indicate that *p53* is a genetic modifier of *Min*.

Modification of *Min* through epigenetic change: methylation

Global hypomethylation and regional hypermethylation correlate with tumorigenesis. To test the effect of methylation on tumorigenesis directly, mice carrying a targeted mutation in the gene that encodes the DNA methyltransferase responsible for methylating CpG islands, the 5-cytosine methyltransferase (*Dnmt*), were crossed to Min mice.[95] Surprisingly, $Dnmt^{+/-}$ mice display global genomic demethylation, but develop 2.5-fold fewer tumors than $Dnmt^{+/+}$, *Min*/+ mice.[95] (Note: homozygous deletion of *Dnmt* causes embryonic lethality.[96] For this mutant, as for *Min* itself, the creation of an allele that could be inducibly deleted in the adult would be very informative.) Treatment of adult *Min*/+ mice with 5aza-2'-deoxycytidine (5azadC), which covalently inactivates methyltransferase and reduces methylation, reduces tumor number six-fold. Together, *Dnmt* and 5azadC synergistically reduce tumor number: *Dnmt*–/–, *Min*/+ mice treated with 5azadC develop 60-fold fewer tumors than untreated *Min*/+ heterozygotes.[95]

The reason for this reduced tumor number is not clear. One explanation is that the regional DNA hypermethylation observed in tumors reduces the expression of tumor suppressors, and that a lack of methyltransferase prevents the initial hypermethylation.[95] Another explanation is that methylated cyto-

sines can be deaminated, causing transition mutations through replication errors; a reduction in methylation would make such mutations less frequent.[95]

DNA mismatch repair modification of *Min*

In the past few years, mutations in genes that repair DNA base-pair mismatches have been correlated with predisposition to hereditary non-polyposis colon cancer (HNPCC; see ref 97). Mice homozygous for a targeted disruption of one of these genes, *msh2*, develop intestinal tumors after about five months.[98] These tumors (fewer than 10 per animal) differ from *Min*/+ tumors in their morphology, which suggests that *msh2*$^{-/-}$ and *Min*/+-derived tumors develop along different paths.[98] However, these pathways do not appear to be completely independent: *msh2*$^{-/-}$-derived tumors also appear to undergo mutation at *Apc*, as shown by a decrease in staining by antibodies that recognize the carboxy terminus of Apc.[98] If mutation at *Apc* were a requirement for the initiation of tumors in *msh2*$^{-/-}$ mice, then *msh2* and the *Min* mutation might be expected to act synergistically. Indeed, 1-3 months-old *msh2*$^{-/-}$, *Min*/+ mice developed 3.5-fold more tumors than *msh2*$^{+/+}$, *Min*/+ animals .[99] *msh*$^{-/-}$, *Min*/+ mice also developed detectable tumors more quickly.[99] Antibody staining revealed a lack of full-length Apc in these tumors; however, genotyping revealed little loss of heterozygosity in the *Apc* region. One possible explanation of this difference is that wild-type *Apc* undergoes internal mutation owing to the mismatch repair deficiency in these *msh2*$^{-/-}$, *Min*/+ mice.[99] Such mutations have been detected in the *APC* genes of HNPCC patients.[100,101]

scid fails to modify *Min*

The immune system has long been invoked as a suppressor of tumorigenesis. A variety of mice that have defective immune systems are available. The *scid* mutation, for example, prevents proper VDJ recombination and leads to severe immune deficiency. To determine whether the immune system is involved in the development of *Min*-induced tumors, *Min*/+, *scid*$^{-/-}$ mice were analysed.[103] Surprisingly, the *scid* mutation does not detectably affect tumor formation in Min mice.[103] Analysis of the effects of the several other available immune-deficient mice, such as those that carry the *nu*, *beige*, *xid*, and others that carry targeted mutations, should help determine whether the immune system plays any role in the formation of intestinal tumors.[104]

Other potential *Mom* loci

Cyclooxygenase-2 (Cox-2), which is involved in prostaglandin synthesis and may regulate cell adhesion and apoptosis, has been implicated in colon cancer by two observations: its overexpression has been detected in colon cancers, and drugs (non-steroidal anti-inflammatory agents, such as piroxicam and sulindac) that inhibit it suppress intestinal tumor formation in Min mice and humans[105,106]; see ref 107. Does a *Cox-2* loss-of-function mutation mimic this suppression of tumorigenesis? Recently, Oshima and colleagues addressed this question and found that homozygous disruption of *Cox-2* reduced tumor number about seven-fold in animals carrying a targeted mutation in *Apc*.[102] *Cox-2* therefore appears to be a modifier of this targeted allele of *Apc*, and may also be a modifier of *Min*.

Avoiding embryonic lethality in the search for *Moms*

A number of knockout mutations that might affect intestinal tumor formation in Min mice cause embryonic lethality in homozygous form. This lethality prevents the analysis of *Min*/+ mice homozygous for the mutation of interest. However, *Min*/+ mice heterozygous for the mutation might survive to be analysed and reveal the gene's role in tumorigenesis. This approach, used successfully in the analysis of methyltransferase deficiency, requires that intermediate levels of a potential modifier have a detectable effect. In other words, these modifiers might be either dominant or dosage-sensitive. A growing number of dosage-sensitive genes, including *Dmnt* and *Mom1*, have been identified.[109] Another method, noted above, is the creation of an adult mosaic mouse using inducible, site-specific recombination. A related solution is the creation of a chimeric mouse. In both mosaic and chimeric mice, tissue that is wild-type for the mutation being tested might be intermingled with mutant tissue; this wild-type tissue could influence the mutant phenotype. Yet another solution involves tissue grafting: removing the intestine from fetuses of interest (e.g. *Min*/+, *X*$^{-/-}$), and grafting them ectopically into a healthy adult recipient.[110] Such grafted

intestines appear to be good models of resident intestines: wild-type grafted intestines develop apparently normal architecture and vascular and lymph systems, and small intestinal grafts carrying the *Min* mutation develop tumors.[88,111]

A number of other targeted mutations await analysis. These include transgenes and knockouts of the genes for growth factors that are implicated in the development of human intestinal cancers. In particular, the EGFR ligands TGFα, amphiregulin and cripto are frequently overexpressed[21,22] (see also ref 20). In cultured cells, overexpression of TGFα alone transforms Rat-1 fibroblasts; TGFα and EGFR together induce transformation of NIH3T3 cells.[112,113] *In vivo*, overexpression of TGFα from a transgene induces tumor formation in mammary glands and in the liver.[114-116] Although TGFα overexpressed in the intestine does not lead to tumor formation, it does lead to hyperproliferation of intestinal cells.[115] Such an increase in cell number is the earliest morphological change detected in human colorectal cancer.[78] By mating Min mice to those carrying a TGFα transgene, one can determine whether overexpression of TGFα promotes tumorigenesis in the intestine *in vivo*. Such analyses of other factors correlated with tumorigenesis (c-myc, amphiregulin, and crypto) would also be very informative.

Studies of such transgenic gain-of-function (overexpression) alleles would determine whether the factors expressed can promote tumorigenesis. A complementary approach would be to analyse loss-of-function alleles (targeted disruptions) of the same genes, to determine whether they are required for tumorigenesis.

Sorting the modifiers

Modifiers of Min are beginning to accumulate; now they need to be assembled into pathways of tumorigenesis. Genetically, two genes can be determined to be acting at the same step, in sequential steps, or in independent steps based on their single and double mutant phenotypes.[118] However, this approach requires that gene products have completely lost their function, that each mutation have a unique phenotype, and that a combination of mutations in separate pathways have a phenotype that differs from that of each mutation alone. In the many cases where these conditions cannot be met, molecular biological and biochemical approaches can be used to determine pathways of tumorigenesis. For example, analysis of gene expression can reveal modifiers that are overexpressed or deleted early in tumorigenesis, and are therefore assumed to act earlier than modifiers expressed or deleted later (see ref 23). Such analysis suggests that *Cox2*, which is expressed in the smallest colon adenomas analyzed (but not in normal tissue), acts early in tumor development.[102] Analysis of gene function can also strongly suggest a pathway: for example, *Dnmt* would be a likely upstream modifier of a tumor suppressor gene regulated by methylation. Such combined genetic, molecular biological, and biochemical analyses of modifiers should help elucidate the pathways by which *Min*-induced tumors develop.

Conclusion

Random mutagenesis combined with observant screening yielded the Min mouse, which carries a mutation in the *Apc* tumor suppressor gene. The Min mouse has been a powerful model for the study of inherited intestinal tumorigenesis. Its value is derived in part from its combination, unique among mouse models of cancer, of rapid tumor development and a close resemblance to human disease. The Min mouse now appears to be a model of additional cancers, including sporadic intestinal, as well as breast, pancreatic and body wall cancers. The generation of Min mice that carry both natural and induced mutations at other loci has led to the identification of several modifiers of the Min phenotype, including *Mom1, p53* and *Dnmt*. Analysis of these modifiers and others yet to be identified should reveal critical steps in the pathways of tumorigenesis.

Note: For a more extensive recent discussion of many of the topics raised in this review, as well as some that could not be addressed in this space, see ref 42.

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Note added in proof

The *beige* mutation has now been shown to have no detectable effect on *Min*-induced tumorigenesis (Cancer Research, March 1, 1997).