Myh Deficiency Enhances Intestinal Tumorigenesis in Multiple Intestinal Neoplasia (Apc\textsuperscript{Min/−/−}) Mice

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ABSTRACT

Monallelic APC and biallelic MYH (homolog of Escherichia coli mutY) germ-line mutations are independently associated with a strong predisposition to colorectal adenomas and carcinoma in humans. Whereas mice heterozygous for mutant Apc develop intestinal tumors, mice homozygous for mutant Myh do not show increased tumor susceptibility. We analyzed the phenotype of Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−} mice and found that they developed significantly more adenomas in the small intestine than did Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/+} or Apc\textsuperscript{Min/+}/Myh\textsuperscript{+/−} mice (median 231 versus 151 versus 152). In the large bowel, Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−} mice showed significant increases in the number of aberrant crypt foci. In addition, Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−} mice developed an increased number of mammary tumors. Molecular analyses suggested that at least 19% of intestinal tumors from Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−} mice had acquired intragenic Apc mutations rather than allelic loss. Consistent with a defect in base excision repair, three intragenic Apc mutations in polyps without allelic loss from Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−} mice were shown to be G:C to T:A transversions which resulted in termination mutations rather than allelic loss. We reasoned that, despite the data above, Myh deficiency enhances intestinal tumorigenesis in Apc\textsuperscript{Min/+} mice. The excess small-bowel adenomas in Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−} mice, therefore, appear to be a model of MYH-associated polyposis in humans.

INTRODUCTION

Familial adenomatous polyposis is a rare, autosomal dominant disease characterized by hundreds or thousands of colorectal adenomas, resulting in a near 100% lifetime risk of colorectal cancer. In addition, familial adenomatous polyposis patients often develop extra-colonic features, including upper gastrointestinal tumors, congenital hypertrophy of the retinal pigment epithelium (CHRPE), desmoid tumors and dental abnormalities. Familial adenomatous polyposis is caused by germ-line mutations in the adenomatous polyposis coli (APC) gene, and somatic APC mutations are found in up to 80% of sporadic colorectal cancers. Both familial adenomatous polyposis and sporadic tumors usually harbor biallelic APC mutations, resulting in constitutive activation of the Wnt signaling pathway by up-regulating the level of β-catenin (1, 2).

Patients with MYH (homolog of Escherichia coli mutY)-associated polyposis have a phenotype similar to patients with mild familial adenomatous polyposis (3, 4). However, the disease shows autosomal recessive inheritance. MYH-associated polyposis accounts for ~30% of patients with 15 to 100 adenomas, and ~10% of APC mutation-negative patients with classical polyposis (>100 adenomas; ref. 4). Colorectal cancer associated with MYH-associated polyposis appears to have a prevalence similar to that associated with familial adenomatous polyposis (5).

MYH-associated polyposis is caused by biallelic germ-line mutations in the base excision repair gene MYH (6). Base excision repair is important in repairing oxidative DNA damage originating as a byproduct of normal cellular metabolism or from extrinsic sources. The most frequent mutagenic lesion is 7,8-dihydro-8-oxoguanine (8-oxo-G). 8-oxo-G can mispair with adenine during DNA replication and cause G:C to T:A transversions. The products of three human base excision repair genes, MTH1, OGG1, and MYH, act together to prevent 8-oxo-G-induced mutagenesis. In the nucleotide pool, MTH1 hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP (7–9); OGG1 detects and removes 8-oxo-G generated in situ or incorporated into DNA during replication (10–13); and MYH, an adenosine-specific DNA glycosylase, scans the daughter strand after DNA replication and removes adenosines mispaired with 8-oxo-G (14–16). MYH deficiency leads to an increased frequency of G:C to T:A mutations in genes such as APC and K-ras in MYH-associated polyposis tumors (17, 18).

In contrast to MYH-associated polyposis patients, MYH-deficient mice do not seem to show increased tumor susceptibility. Mice homozygous for a loss-of-function Myh allele (Myh\textsuperscript{−/−}) showed no difference from wild-type littermates in terms of survival after 14 months or of tumor incidence after 15 to 17 months (19). However, Myh and Ogg1\textsuperscript{−/−}“double knockout” mice displayed a marked increase of lymphomas and lung and ovarian tumors. Myh\textsuperscript{−/−} mice have been shown to accumulate 8-oxo-G at an increased rate in the liver when compared with wild-type mice, although no age-dependent accumulation was detected in brain, small intestine, spleen, or kidney (20).

We aimed at developing a mouse model for MYH-associated polyposis. We reasoned that, despite the data above, Myh deficiency might have some subtle effect on the intrinsic mutation rate that was insufficient to cause macroscopic tumor development on an otherwise wild-type genetic background. Because most colorectal tumors from MYH-associated polyposis patients seem to harbor biallelic APC mutations (17, 18), we crossed Myh-deficient mice with multiple-intestinal-neoplasia (Apc\textsuperscript{Min/+}) mice, which carry a nonsense mutation at codon 850 of the Apc gene. Apc\textsuperscript{Min/+} mice are predisposed to multiple adenomas, primarily in the small intestine. In addition, these mice have an increased risk of developing mammary tumors, desmoids, and epidermoid cysts (21). Because homozygosity for the Min allele is embryonic-lethal, we analyzed animals of the six remaining genotypes (Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/+}, Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−}, Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/+}, Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−}, Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/+}, and Apc\textsuperscript{Min/+}/Myh\textsuperscript{−/−}) for the development of intestinal tumors, cystic crypts of the small intestine and aberrant crypt foci (ACFs) of the colon.

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MATERIALS AND METHODS

Mouse Breeding. Animals were bred at Cancer Research United Kingdom Laboratories (South Mimms, United Kingdom). Experiments were conducted in full accordance with the United Kingdom Animal Procedures Act 1986. The C57BL/6J-ApcMin/+ mice were originally a gift from A. R. Moser (Mc Ardle Laboratory, University of Wisconsin, Madison, WI). The 129/C57BL/6J-Myh<sup>-/-</sup> mice have been described previously (19). Male Apc<sub>Min</sub>/+ mice were bred with female Myh<sup>-/-</sup> mice. Of the F<sub>1</sub> generation, male Apc<sub>Min</sub>/+Myh<sup>-/-</sup> mice were intercrossed with Apc<sup>+/+</sup>/Myh<sup>-/-</sup> females. Only the F<sub>2</sub> offspring were included in the analyses because these are expected to share equivalent contributions of the genetic background from the two strains, with the only systematic difference between the groups of mice being Myh itself and, inevitably, small regions flanking this gene. Our three study groups were Apc<sub>Min</sub>/+Myh<sup>-/-</sup>, Apc<sub>Min</sub>/+Myh<sup>+/+</sup>, and Apc<sub>Min</sub>/+Myh<sup>-/-</sup> mice; our three control groups were Apc<sup>-/-</sup>/Myh<sup>-/-</sup>, Apc<sup>+/+</sup>/Myh<sup>-/-</sup>, and Apc<sup>-/-</sup>/Myh<sup>-/-</sup> mice. Animals of the six groups arose at the expected Mendelian frequencies (P = 1, χ<sup>2</sup> test).

Genotyping. DNA was extracted from tail snips by using standard methods. All of the animals were genotyped for Apc and Myh with three-primer, allele-specific PCR assays (19, 22); all founder mice were homozygous for the sensitive allele of Mom1 (22).

Counts of Tumors, Cystic Crypts, and Aberrant Crypt Foci. Mice were sacrificed at 110 to 130 days by CO<sub>2</sub> asphyxiation. The intestinal tract was removed and divided into four segments consisting of three equal lengths of small intestine (proximal, middle, and distal) and the whole of the large intestine. Each segment was flushed with PBS, opened longitudinally, and spread out on filter paper. The samples were fixed in Carnoy’s medium for 3 hours and stored in 70% EtOH. Before analysis, preparations were stained with 0.2% methylene blue for 3 minutes and were washed in PBS for 20 minutes. Tumors were counted with a dissecting microscope at ×3 magnification, and categorized according to size with an inoculating loop with an inner diameter of 1 mm and an outer diameter of 2 mm. Cystic crypts in the small intestine and ACFs in the large intestine were counted at ×3 and ×5 magnification, respectively. Criteria used to identify ACFs were dark-blue staining, increased crypt size, and flat appearance in the boundary of the mucosal area. All of the counts were done in duplicate by an observer (O. M. S.) blinded to the genotype of the mice. A subset of cases was counted by a second observer (C. T.) to confirm the first observer’s results.

Histology. After tumor counting, the proximal small-bowel segment from five animals per study group (Apc<sub>Min</sub>/+Myh<sup>-/-</sup>, Apc<sub>Min</sub>/+Myh<sup>+/+</sup>, and Apc<sub>Min</sub>/Myh<sup>-/-</sup>) was rolled up, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). In addition, selected large tumors of the small and large intestine were processed as well as all detected mammary tumors. To examine the histology of colonic ACFs, serial transverse sections were prepared at 4 μm. All samples were analyzed by a histopathologist (G. S.) blinded to the genotype of the mice.

DNA Extraction from Tumor and Normal Tissue. Tumors were dissected from the methylene blue-stained, whole-mount preparations with a dissecting microscope at ×3 magnification. A fresh scalpel was used for each sample, and care was taken to avoid the surrounding normal epithelium. In total, 134 small-bowel and 64 colonic tumors from 12 Apc<sub>Min</sub>/+Myh<sup>-/-</sup> mice, 147 small-bowel and 21 colonic tumors from 16 Apc<sub>Min</sub>/+Myh<sup>+/+</sup> mice, and 167 small-bowel and 16 colonic tumors from 13 Apc<sub>Min</sub>/+Myh<sup>-/-</sup> mice were collected. Tail tissue and normal bowel epithelium were also sampled. DNA was extracted from these samples with standard methods.

Loss of Heterozygosity (Allelic Loss) Analysis at Apc and Myh. Loss of heterozygosity (LOH) at Apc was analyzed with a fluorescence–single-strand conformational polymorphism (F-SSCP) assay that distinguished the wild-type and Min alleles (Fig. 1) with dye-labeled primers: Apc<sub>LOH</sub> forward (5’-TACGGTATTGCCCAGCTCTT-3’) and Apc<sub>LOH</sub> reverse (5’-TGCAAGAC-CTCGTGTITTIGATGA-3’). Each 25-μL PCR reaction contained 1× PCR buffer (Perkin-Elmer, Boston, MA), 2 mmol/L MgCl<sub>2</sub>, 200 μmol/L dNTPs, 200 μmol/L concentration of each primer, 30 ng of genomic DNA, and 1 unit of TaqDNA polymerase (Quagen). PCR conditions consisted of 95°C for 5 minutes, 35 cycles of 95°C/55°C/72°C for 1 minute each, and a final step at 72°C for 10 minutes. The resulting PCR products were run at 18°C through a 50-cm capillary on the ABI 3100 and analyzed with Genotyper 2.5 software (Perkin-Elmer Applied Biosystems). Allelic loss was considered present if the relative ratio of normal-to-tumor peak area was <0.5 or >2, thereby allowing for contaminating normal tissue. The validity of the F-SSCP-LOH assay had been established with serial dilutions of Apc wild-type and Min alleles, and results were confirmed for selected samples by sequencing tumor DNA alongside normal DNA (details not shown). Loss of the wild-type Myh allele was analyzed with the same allele-specific PCR assay as for the genotyping, in which a 262-bp product is diagnostic of the wild-type allele and a 376-bp product is diagnostic of the targeted allele. The common forward primer was fluorescently labeled. PCR products were run on the ABI 3100 and allelic loss scored as above.

Mutation Analysis. Primer pairs amplifying coding regions and exon-intron boundaries were designed for Apc exons 5 to 14, Myh exons 2 to 15, and K-ras exon 1. Apc codons 652 to 1648 were covered by 10 overlapping primer pairs. Details and PCR conditions are available from the authors. Apc and Myh were screened by F-SSCP analysis. In addition, areas of Apc rich in T/AGAA sequences that are particularly susceptible to G:C→T mutation to chain termination codons in humans (codons 894-1357 and 1545–1648 encompassing 44 such sites) were analyzed by direct DNA sequencing. For F-SSCP analysis, PCR products were run at 18°C and 24°C on the ABI 3100 and were analyzed with Genotyper 2.5 software. For tumor samples showing aberrant SSCP bands, the relevant region of the gene was sequenced directly from a new PCR product alongside normal DNA.

RESULTS

Myh Deficiency Enhances Tumor Formation in the Small Intestine of Apc<sub>Min</sub>/+ Mice. In the small intestine, Apc<sub>Min</sub>/+Myh<sup>+/+</sup>, Apc<sub>Min</sub>/+Myh<sup>-/-</sup>, and Apc<sub>Min</sub>/+Myh<sup>-/-</sup> F<sub>2</sub> offspring developed median numbers of 151, 152, and 231 polyps, respectively (Table 1; Fig. 2). Thus, tumor multiplicity in the small intestine was increased by...
were grouped according to size, it was evident that neither tended to have a high cystic crypt count (In general, animals with a high polyp count in the small intestine...s).

<table>
<thead>
<tr>
<th>Segment</th>
<th>Apc&lt;sup&gt;Min&lt;/sup&gt;/Myh&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Apc&lt;sup&gt;Min&lt;/sup&gt;/Myh&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Apc&lt;sup&gt;Min&lt;/sup&gt;/Myh&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small bowel</td>
<td>Adenomas 151, 62–285 (161 ± 58)</td>
<td>152, 25–345 (164 ± 92)</td>
<td>231, 68–336 (228 ± 84)</td>
</tr>
<tr>
<td></td>
<td>Proximal 17, 6–35 (21 ± 8)</td>
<td>22, 6–57 (25 ± 16)</td>
<td>38, 17–82 (44 ± 21)</td>
</tr>
<tr>
<td></td>
<td>Middle 65, 22–105 (62 ± 23)</td>
<td>62, 5–128 (60 ± 34)</td>
<td>101, 21–131 (85 ± 35)</td>
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<tr>
<td></td>
<td>Distal 84, 30–150 (78 ± 33)</td>
<td>68, 13–184 (79 ± 47)</td>
<td>99, 30–156 (99 ± 36)</td>
</tr>
<tr>
<td>Cystic crypts</td>
<td>13, 3–90 (20 ± 24)</td>
<td>7, 0–116 (24 ± 32)</td>
<td>28, 1–171 (46 ± 50)</td>
</tr>
<tr>
<td>Large bowel</td>
<td>Adenomas 1, 0–12 (2 ± 3)</td>
<td>1, 0–6 (2 ± 2)</td>
<td>1, 0–5 (2 ± 1)</td>
</tr>
<tr>
<td></td>
<td>ACFs 20, 4–52 (23 ± 13)</td>
<td>15, 3–48 (17 ± 12)</td>
<td>43, 21–96 (48 ± 22)</td>
</tr>
</tbody>
</table>

NOTE: Values are medians and ranges. Means ± SD are given in parentheses. The three study groups had similar male-to-female ratios (8:7, 11:14, and 6:9 for Apc<sup>Min</sup>/Myh<sup>+/+</sup>, Apc<sup>Min</sup>/Myh<sup>+/−</sup>, and Apc<sup>Min</sup>/Myh<sup>−/−</sup> mice, respectively).

No adenomas or cystic crypts were found in the small intestine of any of 15 animals from each of the three control groups (Apc<sup>+/+</sup>/Myh<sup>+/+</sup>, Apc<sup>+</sup>/Myh<sup>+/−</sup>, and Apc<sup>−/−</sup>/Myh<sup>-/−</sup> mice).

**Myh Deficiency Increases ACF Number in the Colon of Apc<sup>Min</sup>/Myh<sup>-/−</sup> Mice.** Apc<sup>Min</sup>/Myh<sup>-/−</sup>, Apc<sup>−/−</sup>/Myh<sup>-/−</sup>, and Apc<sup>−/−</sup>/Myh<sup>-/−</sup> mice all developed a median of one polyp in the colon (P = 0.18 for Apc<sup>Min</sup>/Myh<sup>-/−</sup> versus Apc<sup>−/−</sup>/Myh<sup>-/−</sup>; P = 0.16 for Apc<sup>Min</sup>/Myh<sup>-/−</sup> versus Apc<sup>-/−</sup>/Myh<sup>-/−</sup>, Mann–Whitney U test; Table 1; Fig. 1). In addition, adenoma sizes did not differ significantly between Apc<sup>Min</sup>/Myh<sup>-/−</sup>, Apc<sup>-/−</sup>/Myh<sup>-/−</sup>, and Apc<sup>-/−</sup>/Myh<sup>-/−</sup> mice, with 82% (19 of 23), 60% (28 of 47), and 88% (21 of 24), of lesions being >2 mm in size, respectively (P = 0.06 for Apc<sup>-/−</sup>/Myh<sup>-/−</sup> versus Apc<sup>-/−</sup>/Myh<sup>-/−</sup>; P = 0.70 for Apc<sup>-/−</sup>/Myh<sup>-/−</sup> versus Apc<sup>-/−</sup>/Myh<sup>-/−</sup>, Fisher’s Exact test).

ACFs are putative premalignant lesions that develop in the colon of Apc<sup>Min</sup>/Myh<sup>-/−</sup> mice (25–27) and that can be induced by chemical carcinogens (28, 29). ACFs were counted for the same samples that were also scored for colonic adenomas. Apc<sup>Min</sup>/Myh<sup>-/−</sup>, Apc<sup>-/−</sup>/Myh<sup>-/−</sup>, and Apc<sup>-/−</sup>/Myh<sup>-/−</sup> mice all developed a median of one polyp in the colon (P = 0.18 for Apc<sup>Min</sup>/Myh<sup>-/−</sup> versus Apc<sup>-/−</sup>/Myh<sup>-/−</sup>; P = 0.16 for Apc<sup>Min</sup>/Myh<sup>-/−</sup> versus Apc<sup>-/−</sup>/Myh<sup>-/−</sup>, Mann–Whitney U test; Table 1; Fig. 1). In all of the animals, ACFs occurred predominantly in the distal colon. Histopathological analysis of selected ACFs revealed mild dysplasia in all of the cases (Fig. 3). Surprisingly, ACF numbers were not associated with colonic tumor numbers, which suggested that...
there is no simple stochastic progression of ACF to adenoma ($R^2 = 0.0054, P = 0.61$, linear regression analysis).

No tumors or ACFs were found in the colonies of any of 15 animals from each of the three control groups (Apc^+/+/, Myh^+/+, Apc^+/+/, Myh^-/-, and Apc^-/-/Myh^-/-).

Adenomas from Myh-Deficient Apc^Min/+ Mice Show No Evidence of Progression to Carcinoma. Histopathological analysis of 8 small-bowel and 8 large-bowel tumors from Apc^Min/+/, Myh^+/+, 21 small-bowel and 17 large-bowel tumors from Apc^Min/+/, Myh^-/-, and 30 small-bowel and 17 large-bowel tumors from Apc^Min/+/, Myh^-/- mice revealed that all were adenomatous polyps and none displayed evidence of malignant change. Thus, similar to tumors from Myh wild-type and heterozygous mice, tumors from Myh-deficient Apc^Min/+ mice did not appear to progress to carcinoma by 120 to 130 days of age. In addition, no differences were apparent in the histology of normal intestinal mucosa between the three study groups.

Myh-Deficient Apc^Min/+ Mice Develop an Increased Frequency of Mammary Gland Tumors. Mammary gland tumors were detected in 0 (0%) of 16 Apc^Min/+/, Myh^+/+ tumors, 3 (37%) of 83 Apc^Min/+/, Myh^-/- tumors, and 4 (27%) of 95 Apc^Min/+/, Myh^-/- mice. The increase in mammary gland tumors was significant in Myh-deficient as compared with Myh wild-type and heterozygous mice ($P = 0.028$, Fisher’s Exact test). All mammary gland tumors showed squamous cell differentiation, and one, from a Myh-deficient mouse, had invaded into the surrounding fat.

Small-Bowel Tumors from Apc^Min/+/, Myh^-/- but Not from Apc^Min/+/, Myh^+/+ Mice Show a Reduced Frequency of Allelic Loss at Apc. Tumor formation in Apc^Min/+ mice has been shown to involve somatic inactivation of the wild-type Apc allele (30, 31). On a C57BL/6J background, this generally occurs by allelic loss. If the increased number of small-bowel tumors in Apc^Min/+/, Myh^-/- mice reflected a failure to repair oxidative DNA damage, a corresponding proportion of the tumors from these mice were expected to harbor truncating somatic mutations rather than allelic loss at Apc. We found that 118 (93%) of 127 tumors from Apc^Min/+/, Myh^-/- mice and 87 (89%) of 98 tumors from Apc^Min/+/, Myh^-/- mice had lost the wild-type Apc allele, compared with 121 (81%) of 150 tumors from Apc^Min/+/, Myh^-/- mice (Fig. 1; $P < 0.001$, for Myh-deficient mice versus Myh wild-type and heterozygous mice, Fisher’s Exact test).

These results are consistent with the notion that whereas the growth of most polyps in Apc^Min/+/, Myh^-/- mice is initiated because of loss of wild-type Apc, as in Apc^Min/+/, Myh^-/- mice, the significant excess of tumors occurs because absent Myh activity leads to “second hits” that are distinct from allelic loss. The fraction of tumors that retained the Apc wild-type allele in Myh-deficient mice (~19%) was, however, lower than the observed increase in tumor number (~53%). This difference may be the result of chance but may also reflect a requirement for mutation in one or more additional genes for macroscopic polyp development. Alternatively, Myh deficiency may have some other effect in addition to increasing the intrinsic mutation rate.

Analysis of large-bowel polyps from each genotypic class revealed no significant difference in the frequency of allelic loss at Apc. We found that 6 (100%) of 6 tumors from Apc^Min/+/, Myh^+/+, 18 (100%) of 18 tumors from Apc^Min/+/, Myh^-/-, and 15 (94%) of 16 tumors from Apc^Min/+/, Myh^-/- harbored loss of the wild-type Apc allele ($P = 1.0$ for Apc^Min/+/, Myh^-/- versus Apc^Min/+/, Myh^+/+, Fisher’s Exact test).

G:C to T:A Truncating Somatic Mutations Occur in Adenomas from Myh-Deficient Apc^Min/+ Mice. All 29 small-bowel tumors without allelic loss at Apc from Apc^Min/+/, Myh^-/- mice were examined for somatic mutations in Apc. Mutations were detected in 3 (10%) polyps. All three changes were G:C to T:A transversions (2× nucleotide (nt) 3919G>T, nt4754C>A) resulting in “stop” codons (2× E1307X, S1585X). The sequence context around the E1307X mutations was AAAGAGA (Fig. 4), the reverse of the consensus motif (underlined) identified in human MYH-associated polyposis tumors. No somatic Apc mutations were detected in eight and nine small-bowel tumors without allelic loss from Apc^Min/+/, Myh^+/+ and Apc^Min/+/, Myh^-/- mice, respectively.

Adenomas from Myh-Deficient Apc^Min/+ Mice Do Not Harbor K-ras Mutations. Activating K-ras mutations occur in ~50% of sporadic colorectal adenomas in humans (32, 33), and tumors from MYH-associated polyposis patients show an increased frequency of K-ras mutations as compared with tumors from familial adenomatous polyposis patients (18, 34). In addition, about 75% of lung tumors from Myh and Ogg1 double-knockout mice harbor G to T mutations in K-ras codon 12 (19). However, we found no K-ras mutation in 20 intestinal tumors from Apc^Min/+/, Myh^-/- nor in 40 tumors from Apc^Min/+/, Myh^-/- mice (35).

Adenomas from Myh-Heterozygous Apc^Min/+ Mice Do Not Show Somatic Mutations or Allelic Loss at Myh. In our study, Apc^Min/+/, Myh^-/- mice did not show an increase in polyp number or size as compared with Apc^Min/+/, Myh^-/- mice, providing little evidence for a role of Myh heterozygosity in tumor development. However, it has been suggested in humans that inactivation of the wild-type Myh allele in heterozygotes is selected in intestinal tumorigenesis (36). We, therefore, screened adenomas from Apc^Min/+/, Myh^-/- mice for somatic mutations and allelic loss at Myh. Only 2 (1%) of 137 tumors showed allelic loss at Myh, but in one of these cases, LOH targeted the inherited mutant rather than the wild-type allele. A total of 82 tumors were subsequently screened for somatic mutations in Myh exons 2 to 15, but no changes were detected. Taken together, these data do not support the hypothesis that inactivation of the wild-type Myh allele is selected in tumors from Apc^Min/+/, Myh^-/- mice.

FIG. 4. Somatic G to T transversion mutation in Apc resulting in a termination codon (E1307X) identified in two small-bowel adenomas from Apc^Min/+/, Myh^-/- mice. Forward sequence from tumor DNA (A) and cloned mutant allele (B).
creased adenomatous polyp number in the small bowel. In addition, *Myh* deficiency increased the frequency of mammary tumors. The magnitude of the increase in tumor number in the small intestine was correlated with the fraction of tumors that failed to show loss of the wild-type *Apc* allele. This association is consistent with studies on tumors from *Apc<sup>Min/+</sup>*/Myh<sup>-/-</sup>* mice with deficiencies in the base excision repair protein Mbd4 or the MMR proteins Mlh1, Msh2, or Pms2 (24, 37–40). In addition, G:C→T:A somatic *Apc* mutations were found in polyps from *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* mice, as expected for defective base excision repair. Taken together, these data strongly suggest that the loss of *Myh* function enhances tumorigenesis in *Apc<sup>Min/+</sup>* mice.

Our finding of exclusively G:C→T:A somatic mutations in polyps from *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* mice (and no such mutations in polyps from *Apc<sup>Min/+</sup>/Myh<sup>+/+</sup>* and *Apc<sup>Min/+</sup>/Myh<sup>+/−</sup>* mice) provides good evidence to show that this is the mechanism that causes the excess of tumors, as it is in humans (6, 17). Previous studies have shown somatic G:C→T:A *Apc* mutations to be very rare in tumors from *Apc<sup>Min/+</sup>* mice (40, 41), we detected truncating somatic *Apc* mutations in a minority of *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* tumors without allelic loss, a similarly low mutation frequency to that reported in almost all previous studies in which *Apc<sup>Min/+</sup>* mice have been crossed with animals defective in DNA repair (24, 37, 40). A highly plausible explanation for the low frequency of somatic, truncating *Apc* mutations is that adenomas from *Apc<sup>Min/+</sup>* mice are polyclonal, the contribution of each mutant allele to the tumor DNA too small to be detected in most cases (42).

Detailed examination of both tumor size and histology failed to reveal any evident of tumor progression in the small bowel of *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* mice by 120 to 130 days of age. These data are consistent with studies on tumors from *Apc<sup>Min/+</sup>* mice with deficiencies in Mlh1, Msh2, or Pms2 (24, 38, 39) and suggest that *Myh* deficiency has a larger effect on tumor initiation than on progression in the small bowel. *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* mice showed a more pronounced increase in tumor number in the proximal and middle segments than in the distal segment of the small bowel. A similar pattern is observed in MYH-associated polyposis patients who sometimes develop duodenal polyps (4). MMR-deficient *Apc<sup>Min/+</sup>* mice, by contrast, show a uniform increase in the number of small-bowel polyps (24). The finding in *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* mice and MYH-associated polyposis patients may reflect variation in the level of oxidative stress throughout the small bowel, although it could also reflect variation in the level or activity of *Myh* protein or site-specific differences in functional redundancy.

Heterozygous *Myh* deficiency had no detectable effect on tumor development in *Apc<sup>Min/+</sup>* mice or the frequency of allelic loss at *Apc*. Furthermore, inactivation of the wild-type *Myh* allele was very rare in these mice. Although these data must be interpreted with some caution because of the possible polyclonal origin of polyps in the mouse small intestine, they are consistent with our findings, in sporadic colorectal cancers from humans, that failed to detect somatic mutations or allelic loss at *MYH* (43).

Besides intestinal adenomas, *Apc<sup>Min/+</sup>* mice are predisposed to two types of lesions of unknown neoplastic potential, cystic crypts of the small intestine and ACFs of the colon. Cystic crypt multiplicity is increased in *Apc<sup>Min/+</sup>* mice deficient in Mlh1 or treated with chemical carcinogens (23, 24). Although our data were suggestive of a similar effect in *Myh*-deficient *Apc<sup>Min/+</sup>* mice, the increase did not reach statistical significance. In contrast, *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* mice showed a significant, ~2-fold, increase in the number of ACFs, supporting a role of *Myh* deficiency in promoting the development of these lesions.

In conclusion, our data suggest that *Myh* deficiency leads to an increase in the intrinsic mutation rate. This increase is insufficient to cause intestinal tumorigenesis in our *Myh*-deficient mice on an otherwise wild-type genetic background but is made manifest when mice who already harbor the first step of tumorigenesis (*Apc* mutation) are studied. *MYH* deficiency may have a more profound effect on the mutation rate in humans, who are subjected to greater levels of oxidative stress in the intestine than are the laboratory mouse. Although we did not detect somatic K-ras mutations, we believe that the excess adenomas in *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* mice are a useful model of MYH-associated polyposis, allowing studies of molecular mechanisms or the evaluation of therapeutic agents. Furthermore, suppression of loss of the wild-type *Apc* allele, for example, by crossing *Apc<sup>Min/+</sup>/Myh<sup>-/-</sup>* mice onto the AKR genetic background (44), may produce a mouse model of MYH-associated polyposis in which most polyps develop as a result of defective base excision repair.

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Myh Deficiency Enhances Intestinal Tumorigenesis in Multiple Intestinal Neoplasia (Apc<sup>Min/+</sup>) Mice

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