*Review Article*

# Multistep carcinogenesis of the colon in *Apc*$^{Min/+}$ mouse

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**Colon cancer arises through different histological stages representing different genetic and epigenetic alterations. The *Apc*$^{Min/+}$ mouse has a point mutation at the *Apc* gene, and it is considered to be a model for human familial adenomatous polyposis. Our previous studies have revealed the presence of a number of intramucosal microadenomas in the colons of *Apc*$^{Min/+}$ mice, in which only a few macroscopic tumors were recognized. These observations suggest that there are two distinct stages for colon carcinogenesis in *Apc*$^{Min/+}$ mouse, and the *Apc*$^{Min/+}$ mouse is regarded as a good model to study multistage colon carcinogenesis. A number of genes that modify intestinal tumorigenesis have been identified using *Apc* mutant mice combined with other mutant mice. It has become apparent that epigenetic modification strongly affects intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. We herein describe the different stages of colon tumorigenesis and their modifiers, and discuss the possible application of *Apc* mutant mice in order to better understand the molecular mechanisms of multistage carcinogenesis in the large bowel of humans. (*Cancer Sci* 2007; 98: 6–10)**

## *Apc*$^{Min/+}$ and *Apc* mutant mice

The loss of *APC* function has been proven to play a pivotal role in colorectal carcinogenesis.[1] *APC* is now recognized as a recessive tumor suppressor gene, and inactivation of both alleles is necessary for tumor formation. The mutant mouse lineage is considered to be predisposed to multiple intestinal neoplasms (Min) and is thus regarded as one of the models for colorectal tumorigenesis. Originally, this lineage was established from an ethylnitrosourea-treated C57BL/6 male mouse, and its phenotype is an autosomal dominant trait.[2] The dominant mutation is known to be located in *Apc*, the mouse homolog of the human *APC* gene, resulting in truncation of the gene product at amino acid 850.[3] Although homozygous *Apc*$^{Min/Min}$ mice die as embryos, *Apc*$^{Min/+}$ mice develop multiple intestinal neoplasias in their intestinal tracts within several weeks of birth. It is also known that most of the intestinal tumors in mice that are heterozygous for a mutant allele of *Apc* have lost *Apc* function by LOH, especially in mice with a C57BL/6 genetic background.[4] Using gene targeting strategies in ES cells, several lines of genetically engineered *Apc* mutant mice, including mice with conditional inactivating alleles, have been established.[5–7] Although the onset, severity and location of tumors vary among these lines, they all develop multiple tumors in their intestine.

A number of the genes that modify intestinal tumorigenesis have been identified using *Apc* mutant mice. Deletions in the genes related to arachidonic acid metabolism, such as *Ptgs2*, *cPLA*[2] and *EP2*, have been shown to suppress intestinal tumorigenesis,[8–11] whereas deletions in genes related to genomic stability increases tumorigenesis in *Apc* mutant mice.[12,13] Such experiments greatly expand our understanding of intestinal tumorigenesis while also helping to clarify the molecular mechanisms of carcinogenesis in organs other than the intestine.

## Small intestine and colon

The intestinal tract consists of the small intestine (duodenum, jejunum and ileum) and the large intestine or colon. There are a number of anatomical and physiological differences between the small intestine and the colon. Developmentally, the midgut forms the distal part of the duodenum, all of the small intestine, cecum, appendix and the ascending and transverse colon, whereas the hindgut develops the remaining colon and rectum.

In the small intestine, the stem and progenitor compartment is believed to reside in crypts. Stem cells in the small intestine are suggested to be located on the fourth or fifth position from the bottom of the crypts and then differentiate into four different cell types: absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Enterocytes, enteroendocrine cells and goblet cells occupy the villi whereas the Paneth cells reside at the bottom of the crypts and secrete antimicrobial agents.[14] In contrast, the mucosa of the colon has a flat surface epithelium instead of villi and consists only of a crypt compartment. The proliferative stem and precursor cells in the colon occupy the bottom two-thirds of the crypts, whereas differentiated cells constitute the surface epithelium and top third of the crypts. The stem and progenitor cells in the colonic crypts differentiate into enterocytes, enteroendocrine cells and goblet cells, whereas Paneth cells are absent in the colon under normal physiological conditions.

Given such differences between the small intestine and colon, it should be noted that the major location of intestinal tumors in *Apc* mutant mice is the small intestine, whereas most human bowel cancers tend to arise in the colon. In addition, there is also evidence indicating that tumor formation in the colon does not always correlate with that in the small intestine in *Apc*$^{Min/+}$ mice. For example, *Apc*$^{Min/+}$;*BubR1* compound mice develop tumors preferentially in the colon, whereas they demonstrate fewer tumors in the small intestine than control *Apc*$^{Min/+}$ mice.[15] These findings imply that tumorigenesis in the colon is different from that in the small intestine. It is therefore important to differentiate between tumorigenesis in the small intestine and large intestine.

## Small dysplastic crypts as pretumoral lesions in the colon of *Apc*$^{Min/+}$ mouse

Colon carcinogenesis is regarded as a multistep event with genetic and epigenetic alterations. In humans, the adenomatous polyposis coli, β-catenin, Ki-*ras* oncogene and *p53* genes are thought to play important roles at different stages of colorectal carcinogenesis.[1,16,17] In previous studies, we have shown the presence of small dysplastic crypts in the colonic sections of

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Abbreviations: *Apc*/*APC*, adenomatous polyposis coli; ES, embryonic stem; LOH, loss of heterozygosity.


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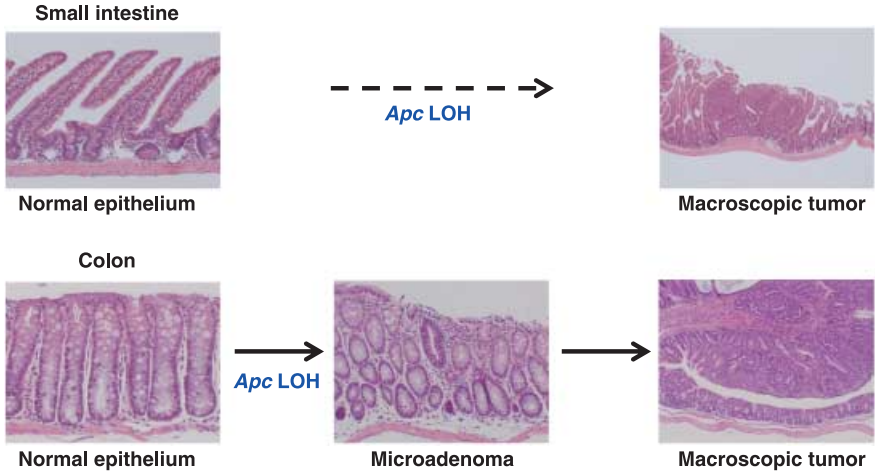


**Fig. 1.** Intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. A previous study indicated that the process of tumorigenesis in the small intestine is different from that in the colon. There are two histologically distinguishable lesions in the colon of the *Apc*$^{Min/+}$ mouse.

rodents that are treated with colon-specific carcinogens.[18,19] These small dysplastic crypts were shown to harbor frequent β-catenin mutations, which are also detectable in most colon tumors.[20] Because of the accumulation of β-catenin protein, such crypts were designated as β-catenin accumulated crypts (BCAC). A series of studies have demonstrated that BCAC are likely to be direct precursor lesions of carcinogen-induced colon tumorigenesis.[21]

We revealed the presence of a number of intramucosal microadenomas in the colon of *Apc*$^{Min/+}$ mice.[22] Micoradenomas in the colon of *Apc*$^{Min/+}$ mice consisted of one to six dysplastic crypts. Importantly, such microadenomas in the colon were found to have lost the remaining allele of *Apc*, thus indicating a loss of the *Apc* function to have already occurred in such crypts.[22] Accordingly, it seems to be reasonable to apply Knudson's 'two-hit' theory to the formation of the microadenomas.[23] In agreement with the presence of *Apc* LOH, the accumulation of β-catenin is observed in all microadenomas in the colon of the *Apc*$^{Min/+}$ mouse, suggesting that microadenomas are identical lesions to BCAC.[21] Together with the fact that colon tumors in *Apc*$^{Min/+}$ mice harbor frequent *Apc* LOH and the accumulation of β-catenin, microadenomas may therefore be direct precursors of colon tumors in *Apc*$^{Min/+}$ mice (Fig. 1).

Aberrant crypt foci (ACF) were first described by Bird *et al.*[24] and a number of studies, including a molecular analysis, have emphasized the significance of ACF as preneoplastic lesions in colon carcinogenesis.[25] Therefore, ACF are now used to evaluate potential chemopreventive agents against colon carcinogenesis.[26,27] Nevertheless, there is increasing evidence indicating the lack of any correlation between tumor development and the formation of ACF.[28,29] Interestingly, previous observations showed a lack of classical ACF in the surface of the colonic mucosa of *Apc*$^{Min/+}$ mice with a number of miroadenomas,[21,30] thus suggesting that microadenomas are independent lesions of ACF. K-*ras* mutations are recognized in the majority of classical ACF in rats and humans,[31] indicating that K-*ras* mutations are closely associated with the formation of ACF. Consistent with this notion, mouse strains carrying oncogenic alleles of K-*ras* develop ACF in the colon.[32] These findings suggest the activation of Wnt and K-*ras* pathways to be responsible for the formation of BCAC and ACF, respectively. Interestingly, the contribution of BCAC (or microadenomas in *Apc*$^{Min/+}$ mouse) and ACF to colon carcinogenesis was suggested by examining the phenotype of *Apc*$^{Min/+}$ mice and mice carrying oncogenic alleles of K-*ras*. It is important to note that *Apc*$^{Min/+}$ mice develop colonic tumors as well as microadenomas, whereas activated K-*ras* mice develop only ACF but no tumors in their colon.[32] It seems to be true that the activation of Wnt signaling is important as an initiating event and BCAC are direct precursors of colon tumors. The significance of ACF with a K-*ras* mutation in premalignant lesions remains to be resolved (Fig. 2). It is possible that activated K-*ras* may cause oncogenic stress, which may thus induce cells to initiate apoptosis, eventually leading to the elimination of this cell population.

## Two distinct stages for colon tumorigenesis in the *Apc*$^{Min/+}$ mouse: micoradenomas and macroscopic tumors

In *Apc*$^{Min/+}$ mice, the number of colonic microadenomas per area was higher than that of adenomatous lesions in the small intestine, suggesting that the loss of *Apc* occurs frequently in colonic crypts as well as in the epithelium of the small intestine.[22] Despite the frequent development of microadenomas, the number of macroscopic colonic tumors is much less than that of small intestinal tumors. Interestingly, the size of microadenomas in the colon of *Apc*$^{Min/+}$ mice does not increase with time, suggesting that microadenoma itself is a self-limiting lesion (Y Yamada and H Mori, unpublished data). Based on the multistep carcinogenesis theory in the colon, the findings indicate that there are at least two distinct stages for colon tumorigenesis in *Apc*$^{Min/+}$ mice and additional events are thus required for transition from microadenomas to macroscopic tumors (Fig. 1). In contrast to colonic lesions, the adenomatous lesions in the small intestine show various sizes, and the mean size of the lesions is significantly larger than in the colon.[22] As the loss of *Apc* is also involved in the earliest lesions in the small intestine,[5] it is possible that, in the small intestine, the loss of function of *Apc* results in the formation of microadenomas that could develop directly into intestinal tumors by aging (Fig. 1). These findings may explain why the *Apc*$^{Min/+}$ mouse develops intestinal tumors preferably in the small intestine, again suggesting that the mechanisms of tumorigenesis involved in the small intestine may differ from those in the colon.

## Alterations required for transition from microadenomas to macroscopic tumors in the colon

It remains unclear which event is mainly responsible for the transition from microadenomas to macroscopic tumors in the colon of *Apc*$^{Min/+}$ mice. For example, *p53*, K-*ras* and B-*raf* mutations or microsatellite instabilities, which are observed frequently in human colon cancers, are not detectable in colon tumors of *Apc*$^{Min/+}$ mice (Y Yamada and H Mori, unpublished data). In our previous study using a rat model, specific β-catenin mutations at the residues that regulate β-catenin levels directly


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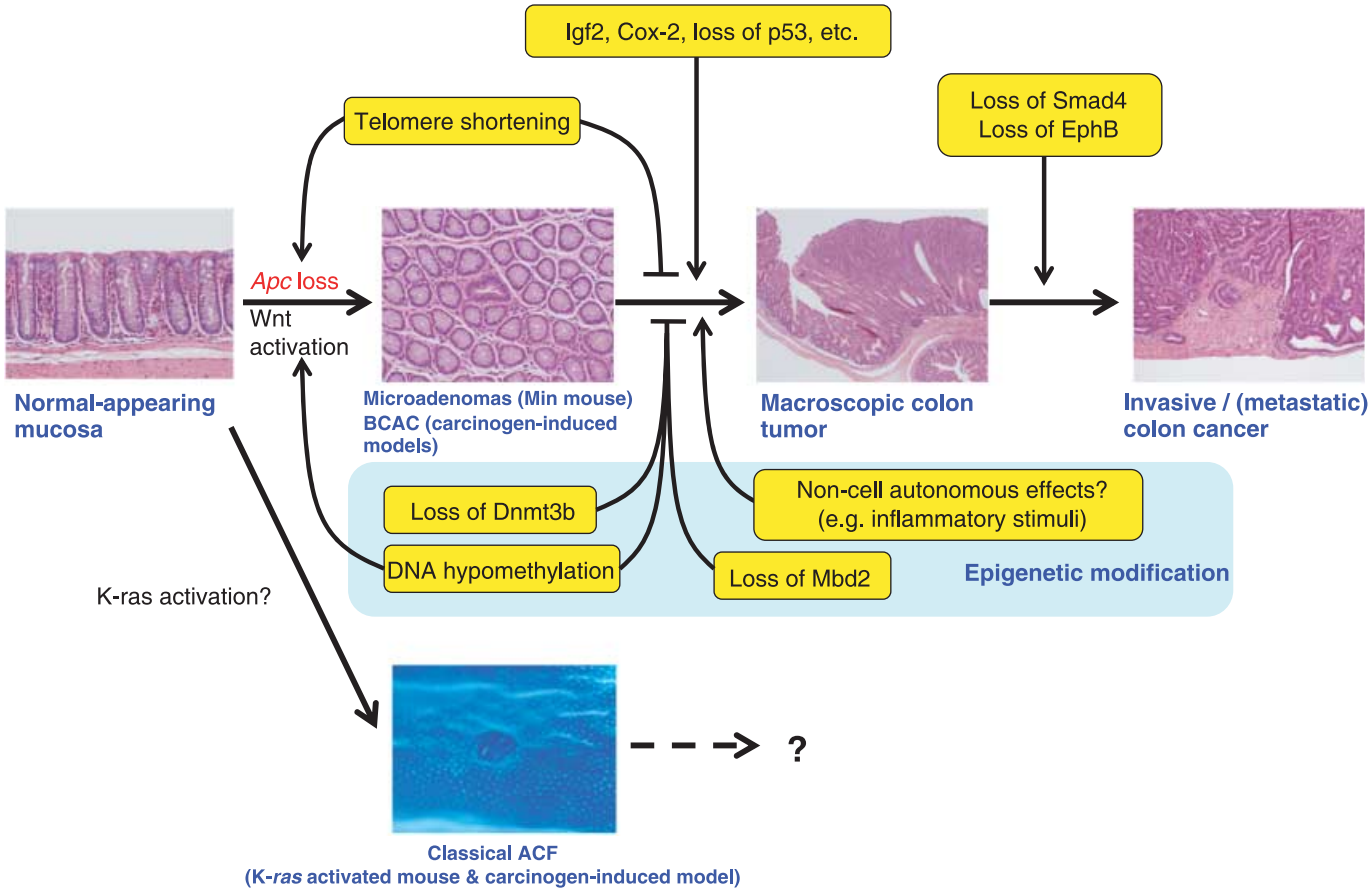


**Fig. 2.** A model for multistage colon carcinogenesis in mice and its possible modifiers. Genetically engineered mice have shown a number of modifiers for different stages of colon carcinogenesis.

were selected from a wide spectrum of mutations during the development of colon tumors from intramucosal small lesions.[20] This finding suggests that an increased level of oncogenic β-catenin is therefore required for tumor development in the colon, and activation of the β-catenin/Tcf pathway, which is involved in not only the initiation stages but also the promotion stages. Consistent with this assumption, the expression of nuclear β-catenin has been shown to correlate with the size of colon neoplasms in humans.[33] In addition, a recent study indicated that β-catenin/Tcf signaling can be further activated by upstream signals regardless of any constitutive activation of the pathway by downstream mutations in colon cancer cells.

Together with the critical contribution of the microenvironment (stem cell niche) in maintaining the self-renewal of stem cells,[34] recent findings that cancer-associated fibroblasts play a significant role in the tumor promotion of breast cancer[35] have shed some new light on the involvement of the non-cell autonomous effect on tumor formation. In *Apc* mutant mice, fibroblasts and endothelial cells adjacent to neoplastic cells are reported to express COX-2, which is suggested to enhance tumorigenesis[36] and to be a molecular target of cancer prevention.[37] Furthermore, several immune-deficient mice with accompanying colonic inflammation are reported to develop proliferative lesions that occasionally have the potential to progress into adenocarcinomas.[38] A recent study has also shown that the inflammatory stimuli induced by dextran sodium sulfate strongly promotes colon tumorigenesis in the $Apc^{Min/+}$ mouse.[39] Importantly, these findings suggest the existence of a non-cell autonomous effect on colorectal carcinogenesis.

## Effects of epigenetic modifications on intestinal tumorigenesis in the $Apc^{Min/+}$ mouse

Changes in DNA methylation status are one of the most common molecular alterations in human neoplasia.[40] The role of aberrant hypermethylation in the silencing of tumor suppressor genes has been well documented.[40] In contrast, the functional significance of genome-wide DNA hypomethylation is still unclear, although this alteration has been reported in a wide variety of human cancers.[41,42] In colon carcinogenesis, DNA hypomethylation has been observed in both adenomas and adenocarcinomas,[42] suggesting that such hypomethylation is associated with the early stages of carcinogenesis.

Dnmt1 has been demonstrated as a maintenance DNA methyltransferase that is essential to maintain global DNA methylation levels,[43,44] and therefore Dnmt1 hypomorphic mice express global DNA hypomethylation.[45,46] It was shown recently that Dnmt1 hypomorphic mice develop an increased number of microadenomas, whereas a significant reduction in the number of macroscopic colonic tumors in $Apc^{Min/+}$ mice was also revealed.[46] Such observations indicate that the forced reduction of DNA methylation levels has dual effects on intestinal carcinogenesis, suggesting that global DNA hypomethylation can promote early events in tumorigenesis. Because microadenomas in DNA hypomethylated mice harbor frequent *Apc* LOH,[46] the increased incidence of microadenomas may be attributable to an elevated rate of loss of the wild-type *Apc* allele, which is also consistent with previous studies demonstrating DNA hypomethylation-dependent LOH events.[47,48]


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Although the precise mechanism by which the reduced DNA methylation level suppresses the transition from microadenomas to macroscopic tumors remains to be elucidated, a recent study showed the targeted deletion of *Dnmt3b*, de novo DNA methyltransferase, to suppress colon tumorigenesis in *Apc*$^{Min/+}$ mice, whereas no such effect was observed on the formation of microadenomas.[49] DNA hypermethylation accompanied by aberrant gene silencing has been shown to be associated with neoplastic progression in many tumors, including colon cancers.[50,51] Dnmt3b is involved in regional DNA methylation[52] and therefore, it is expected to play a role in DNA hypermethylation in cancers. Because site-specific DNA hypermethylation at several genes has also been reported in the intestinal tumors of *Apc*$^{Min/+}$ mice,[53] it is possible that such regional hypermethylation plays an important role in the transition from microadenomas to macroscopic tumors. In addition, mice lacking DNA methyl-binding protein (MBD2) also consistently develop a smaller number of intestinal tumors in *Apc*$^{Min/+}$ mice.[54]

Biochemical evidence indicates that DNA methylation is one component of a wider epigenetic program that includes other postsynthesis modifications of chromatin, and site-specific DNA hypermethylation is associated with the inactive states of chromatin.[55] It is interesting to note that *Dnmt3b* is just involved in the transition of microadenomas to macroscopic tumors but it is not necessary to maintain tumor cell growth.[49] Site-specific DNA hypermethylation may play a role in the fixation of silenced chromatin, and once tumor growth is initiated, Dnmt1 may maintain such heterochromatic states. This notion is also consistent with the decreased tumor formation in *Apc*$^{Min/+}$ mice with Dnmt1 hypomorphs. A global understanding of how DNA hypermethylation is associated with transcriptional repression, which is also linked with chromatin structure, is therefore necessary to elucidate the molecular mechanisms by which DNA hypomethylation suppresses the transition from microadenomas to macroscopic tumors.

## Progression to invasive colon cancer

Most tumors in the *Apc*$^{Min/+}$ mouse are benign adenomas and do not demonstrate either aggressive invasion or metastasis, which are critical characteristics of human cancers.[56] Although a loss of p53 function is considered to play important a role in the conversion of adenomas to adenocarcinomas in humans,[16] *Apc*$^{Min/+}$;*p53*$^{-/-}$ mice indicate no evidence of malignant transformation.[57] However, some genes are suggested to be molecular determinants involving tumor invasions in *Apc* mutant mice. *Smad4*$^{+/-}$;*Apc*$^{\Delta 716/+}$ mice develop larger tumors, although the tumor number does not change in comparison with *Apc*$^{\Delta 716/+}$ mice.[58] In addition, *Ephb3*$^{-/-}$;*Apc*$^{Min/+}$ mice consistently showed larger colorectal polyps than their control littermates.[59] Around half of *Ephb3*$^{-/-}$;*Apc*$^{Min/+}$ animals develop carcinomas that invade the muscle layer, which is thus a feature of malignancy that is not present in *Ephb3*$^{+/+}$;*Apc*$^{Min/+}$ tumors. Although species-specific requirements for cellular transformation have been suggested,[60] *Apc* mutant mice are useful models for investigating malignant transformation in colon carcinogenesis. Further analyses are thus needed to determine how intestinal cancer cells metastasize to the liver and/or lung, and to establish practical mouse models for cancer metastasis.

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