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CONSTRUCTION OF A MOUSE MODEL OF COLON CANCER

by

Amin Fazeli

B.S., Biology, University of California, Los Angeles

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Signature of Author .....
Department of Biology
May 29, 1997

Certified by .....
Professor Robert A. Weinberg
Thesis Supervisor

Accepted by .....
Professor Richard Young
Chairman of the Biology Graduate Committee

DEDICATION

I dedicate this study to my brother, Amir, to my parents, Dr. Hassan Fazeli and Ms. Razieh Tayebi and to Robert A. Weinberg, my mentor and my advisor, who believed in me and stood by me.

ABSTRACT

In 1990 a team of scientist at Johns Hopkins proposed that human colon cancer is caused by a succession of defined genetic alterations including mutation of the *Adenomatous Polyposis Coli* (*APC*) tumor suppressor gene, reduced DNA methylation, mutation of the N- or Ki-*ras* proto-oncogene, inactivation of the *Deleted in Colorectal Cancer* (*DCC*) gene, and mutation of the *p53* tumor suppressor gene. To test the veracity of this genetic model of colon cancer, the technique of gene targeting was used to create genetic alterations in mice that are similar to those observed in human colonic tumors and their effects on the biology of normal and neoplastic intestine was examined.

In the first part of this study, mice were created that are constitutionally heterozygous for a mutation of the mouse homologue of the *APC* gene and were found to be predisposed to intestinal adenomas. In the second part, these mice were used to ask whether DNA hypomethylation plays an early and causative role in the formation of intestinal tumors. These findings indicate that, contrary to the prediction of the Hopkins model, reducing DNA methylation in cells of intestine suppresses the formation of adenomas. In the third part, the effect of inactivation of the *Deleted in Colorectal Cancer* (*DCC*) gene on formation and progression of intestinal adenomas was tested. The findings failed to provide support for a tumor suppressor function for *Dcc*, but showed that *Dcc*-deficient mice did have defects in the formation of commissures in the spinal cord and the brain that were strikingly similar to those observed in mice deficient in *netrin-1*, an axonal chemoattractant. In the last part, the effects of inactivation of the *p53* tumor suppressor gene on the incidence of apoptotic cell death was examined. The findings suggest that wild type *p53* retards the progression of benign colonic adenoma to malignant carcinomas by mechanism(s) other than the promotion of apoptosis.

Thesis Advisor: Robert A. Weinberg

Title: Professor, Department of Biology, M.I.T.
Member, Whitehead Institute for Biomedical Research

BIOGRAPHICAL NOTE

Amin Fazeli

Education

Massachusetts Institute of Technology Cambridge, MA
- Candidate for Doctor of Philosophy (Ph.D.), February 1997
- Members of Thesis Committee: Dr.'s Robert A. Weinberg (Thesis Advisor), Richard O. Hynes, Rudolf Jaenisch, & Philip A. Sharp
- Presented research findings in universities & colleges (Amherst, Harvard, Johns Hopkins, Stanford & Yale) and major international conferences (Frederick, FASEB, American Association for Cancer Research and Cold Spring Harbor Symposium)
- Teaching Assistant, MIT Courses on Experimental Biology & Human Physiology

Yale University School of Medicine New Haven, CT
- Candidate for Doctor of Medicine (M.D.), June 1999

University of California at Los Angeles (UCLA) Los Angeles, CA
- Bachelor of Science (B.S.) in Biology, June 1987

Awards/Honors

- Stanley J. Sarnoff Fellow in Cardiovascular Sciences, 1988-9
- Graduated with College Honors, B.S. degree, UCLA, June 1987

Selected Publications

- **Fazeli, A.**, Dickinson, S. L., Hermiston, M., Tighe, III, R. V., Steen, R. G., Small, III., C. G., Stoeckli, E. T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R. T., Gordon, J. I., Tessier-Lavigne, M., and Weinberg, R. A. (1997). Phenotype of mice lacking functional *Deleted in Colorectal Cancer* gene (*DCC*). ***Nature*** 386, 796-804.

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Background
- Born in Yazd, Iran

ACKNOWLEDGMENTS

I would like to thank Stephanie L. Dickinson for being my partner in my studies for the past three years.

I thank Dr. Roderick Bronson (Tufts University School of Veterinary Medicine, Boston) for his kind help in the histological analysis of the intestinal adenomas.

The work described in each chapter was performed in collaboration with several investigators. In particular, in Chapter 1, mice with $Apc^{del\ 8}$ mutation were created and analyzed by Shigeto Miura and Tetsuo Noda in Japan. $Apc^{Min/+}$ and $Apc^{Min/Min}$ ES cells were generated by Martina Klemm. Stephanie Dickinson, Clayton G. Small, III and Helen Rayburn provided extensive technical assistance. For the experiments described in Chapter 2, 5-aza-dC injections, DNA methylation (Figure 2) and the allelic loss analyses (Figure 5) were performed by Laurie Jackson-Grusby and Peter Laird. $Dnmt^{S}$ mice were created by En Li when he was in Rudolf Jaenisch's laboratory at the Whitehead Institute. For the work described in Chapter 4, Immunohistochemical analysis of the postnatal intestines were performed by Michelle Hermiston in Jeffrey I. Gordon's laboratory in Washington University. Helen Rayburn helped with the generation of $Dcc^{-/-}$ chimeric mice. The protocol for whole-mount immunostaining of embryonic spinal cord was optimized by Kazuko Keino-Masu. The dye-I tracing experiments were performed by Esther T. Stoeckli in Marc Tessier-Lavigne's laboratory at University of California in San Francisco. For the work described in Chapter 5, Robert G. Steen helped extensively with genotyping of mice and Stephanie L. Dickinson with the analysis of apoptotic cells.

In this text whenever I write "we", I am referring to the collection of the scientist who contributed to the studies of a given chapter and to whom I am indebted. They are Stephanie Dickinson, Shigeto Miura, Clayton G. Small, III, Helen Rayburn, Roderick T. Bronson, Tyler Jacks, Martina Klemm, Earlene M. Schmitt, Yusuke Nakamura, Tetsuo Noda, and Robert A. Weinberg for Chapter 2, Peter W. Laird, Laurie Jackson-Grusby, Stephanie Dickinson, W. Edward Jung, En Li, Robert A. Weinberg, and Rudolf Jaenisch for Chapter 3, Robert A. Weinberg, Stephanie L. Dickinson, Michelle L. Hermiston, Robert V. Tighe, III, Robert G. Steen, Clayton G. Small, III, Esther T. Stoeckli, Kazuko Keino-Masu, Masayuki Masu, Helen Rayburn, Jonathan Simons, Roderick T. Bronson, Jeffrey I. Gordon and Marc Tessier-Lavigne for Chapter 4, and Robert G. Steen, Stephanie L. Dickinson, Dolores Bautista, William F. Dietrich, Roderick T. Bronson, Robert S. Bresalier, Eric S. Lander, Jose Costa and Robert A. Weinberg for Chapter 5.

Chapter 3 was published in the scientific Journal *Cell* and Chapter 4 is *in press* in the British scientific Journal *Nature* as a full article. Chapters 2 & 5 are being prepared for publication in scientific journals. To the extent that these chapters were prepared for publication elsewhere, Rudolf Jaenisch, Peter Laird and Laurie Jackson-Grusby have made extensive contribution to writing Chapter 3, Marc Tessier-Lavigne and Jeffrey I. Gordon to Chapter 4 and Robert Weinberg to all chapters.

I also wish to thank Drs. Kenneth Kinzler and Bert Vogelstein for their gifts of human *APC* probes and anti-APC antibodies and helpful suggestions in the course of generating and analyzing the Apc^{8} mutation. I also wish to acknowledge Ms. Karen Johnson and Drs. David Hill and Steven F. Dowdy for their help in Apc protein analysis. I am grateful to Jessica Dausman and Ruth Curry for help in maintenance of the mouse colony for studies on DNA methylation and colon cancer. I thank

Robert Steen and Dr. William Dietrich for their help in setting up the initial *Apc*^{Min} genotype assays. I also wish to thank Bradly C. Ekstrand and Eric Fearon for help in generating the data in Figure 1C of Chapter 4. I also wish to thank Elvie Taylor for technical assistance. I would like to thank Tito Serafini for considerable advice on brain anatomy, V. Lee and M. Yamamoto for antibodies, and D. Shehu and J. Mak for technical assistance. I wish to thank Dr. Tyler Jacks for his gift of a *p53* mouse and the DNA probe used for LOH analysis at the mouse *p53* locus. I would like to thank Dr.'s. C. Richard Boland and Andrew Feinberg for the gift of tumors and for helpful discussions.

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ABBREVIATION USED

APC	Adenomatous polyposis coli gene
Apc	mouse homologue of the *APC* gene
Apc Min	The Min allele of the *Apc* gene
5-aza-dC	DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine
B6	C57BL/6 mouse strain
B6 <-> 129/Sv	Mice chimeric for 129/Sv and C57BL/6 strains
BrdU	5'-bromo-2'-deoxyuridine
Cb	cerebellum
C/EBPa	CCAAT enhancer-binding protein
C. elegans	*Caenorhabditis elegans*
CpG	Cytidine-Guanine dinucleotides
CTB	CholeraToxin B subunit
d	dorsal root ganglion
DBA	*Dolicos biflorus* agglutinin
DCC	*Deleted in Colorectal Cancer*
Dnmt	mammalian DNA (cytosine-5) methyltransferase enzyme
$Dnmt^N$	N allele of Dnmt
$Dnmt^S$	S allele of Dnmt
DPC4	*Deleted in Pancreatic Cancer-4* gene
drez	dorsal root entry zone
E(number)	embryonic day (E0 = day of vaginal plug)
EF	embryonic fibroblasts
ES	embryonic stem cells
ENU	Alkylating agent Ethylnitroso Urea
FAP	Familial adenomatous polyposis
FITC	fluorescein isothiocyanate- conjugated
G418	Drug Geniticin
GS	Gardner's syndrome (GS)
HaC	habenular commissure
H&E	hematoxylin and eosin
HGD	high grade dysplasia)
Kb	Kilobase pairs
kDa	Kilodalton
KO vector	Knock out Vector
LGD	low grade dysplasia
LOH	Loss of heterozygosity
mc	motor column
mg	milligram
Min	Multiple intestinal neoplasia (mice heterozygous for the Apc^{Min} mutation)
ml	milliliter
mM	millimolar
Mom-1	Modifier of Min-1 locus
NCAM	neural cell adhesion molecule
neo	neomycin transfersae gene
netrin-1	Gene encoding the Netrin-1 protein
NF-M	Neurofilament-M
p53	*p53* tumor suppressor gene
PAS	Periodic Acid Schiff
PBS	phosphate-buffered saline

PC	posterior commissure
PCR	Polymerase Chain Reaction
PHT	(PBS / 1% heat-inactivated goat serum / 0.1% Triton X-100)
PN	pontine nuclei
P28	postnatal day 28
RT	Reverse transcription
SAM	methyl donor S-adenosyl methionine
TdT	Terminal deoxytransferase enzyme
TS	Turcot's syndrome
TUNEL	Terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labeling assay
UEA1	*Ulex europaeus* agglutinin type I lectin
µg	microgram
µl	microliter
µm	micrometer
UNC	Mutations causing an uncoordinated phenotype in flat worm *Caenorhabditis elegans*,
v	ventricle
W/V	Weight/volume

NOMENCLATURE

In referring to genes and proteins, I have used the nomenclature that is widely used by those in the field. However, the system of nomenclature described below was used when possible.

The name of species are italicized (e.g. *Caenorhabditis elegans* and *Drosophila melanogaster*). The abbreviation for the human gene is capitalized for all letters but only the first letter of the abbreviation for the mouse gene is capitalized but all the subsequent letters are lower case (e.g. human *APC* gene versus mouse *Apc* gene). Whereas genes are italicized, the same abbreviation referring to the protein product of the gene is not and is used for both species (e.g. APC protein for both human and mouse). For a targeted mutation of a gene in mouse that is shown to be a loss-of-function mutation (or a null mutation) the (-) symbol in superscript is used to refer to the null allele (e.g. Apc^{-}, $p53^{-}$ or Dcc^{-}). One exception to this rule is the *netrin-1* mutation which is a leaky or hypomorphic mutation and yet is called *netrin-1*$^{-}$. In some instances, the name used by the creators of the allele are used (e.g. Apc^{Min}, Apc^{δ} or $Dnmt^{S}$).

In *C. elegans*, all letters in the abbreviation for the protein are capitalized while the gene is not but is italicized (e.g. UNC-40 protein encoded by the *unc-40* gene).

CHAPTER 1

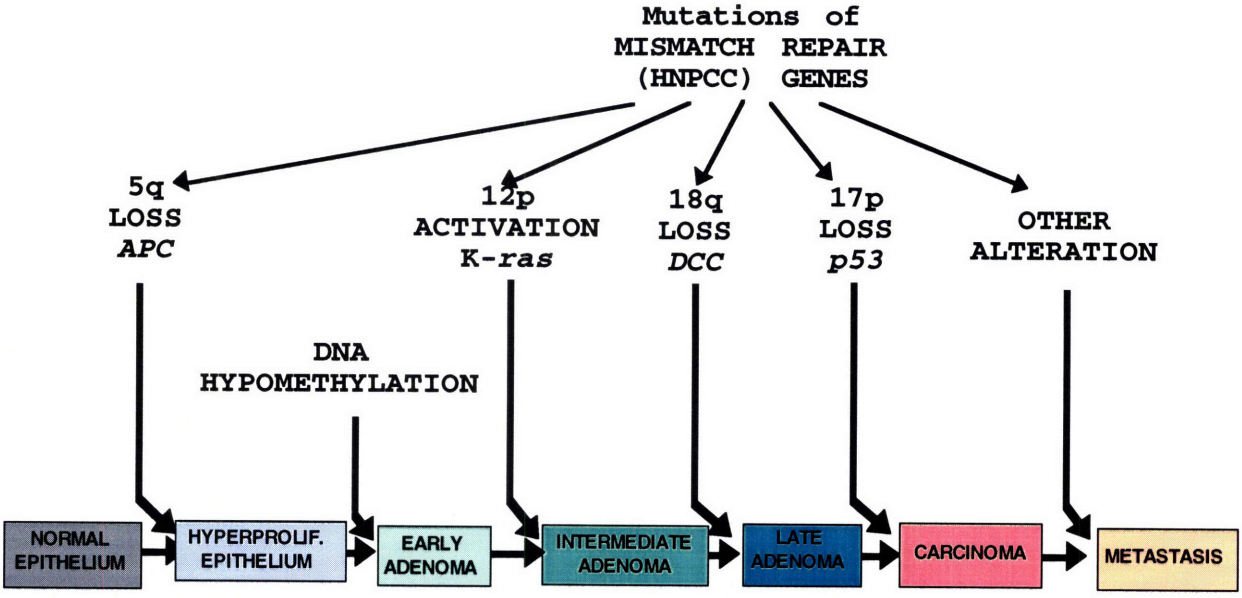
Genetic Model Of Human Colon Cancer

In 1990, a team of scientist at Johns Hopkins University proposed a genetic model for colon cancer (Fearon and Vogelstein, 1990) that was largely based on the data regarding the chromosomal sites of and the frequency of loss of heterozygosity in colorectal tumors . Loss of heterozygosity (LOH) represents a process often used by developing tumor cell clones to eliminate both copies of a tumor suppressor gene(Cavenee et al., 1983). Initially, one gene copy is inactivated by mutation; thereafter the second, still intact gene copy is discarded, frequently accompanied by duplication of the chromosomal arm carrying the mutant allele.

According to this genetic model, human colon cancer is caused by a succession of defined genetic alterations and these alterations include inactivation of the *Adenomatous Polyposis Coli* (*APC*) tumor suppressor gene on chromosome 5q, reduced DNA methylation, mutation of the N- or Ki-*ras* proto-oncogene, inactivation of the *Deleted in Colorectal Cancer* (*DCC*) gene on 18q, and loss of the *p53* tumor suppressor gene on 17p (Figure 1). More recent findings have suggested that mutations of components of the DNA mismatch repair system may help increase the rate of progression leading to colonic tumors by increasing the rate of mutations of *APC*, *DCC* and *p53* (for a review see Kinzler and Vogelstein, 1996).

Figure 1

Fearon and Vogelstein's genetic model for formation of colon cancer. Human colon cancer is proposed to develop by a succession of defined genetic alterations: inactivation of the *Adenomatous Polyposis Coli* (*APC*) tumor suppressor gene on chromosome 5q, reduced DNA methylation, mutation of the N- or Ki-*ras* proto-oncogene, inactivation of the *Deleted in Colorectal Cancer* (*DCC*) gene on 18q, and loss of the *p53* tumor suppressor gene on 17p.


Mutations of
MISMATCH REPAIR
(HNPCC) GENES
5q
LOSS
APC
12p
ACTIVATION
K-ras
18q
LOSS
DCC
17p
LOSS
p53
OTHER
ALTERATION
DNA
HYPOMETHYLATION
NORMAL EPITHELIUM
HYPERPROLIF. EPITHELIUM
EARLY ADENOMA
INTERMEDIATE ADENOMA
LATE ADENOMA
CARCINOMA
METASTASIS

Mutations of *APC* are important for the formation of most colon carcinomas, in that mutant alleles of this gene, created by somatic mutations, are found in 90% of the non-familial or sporadic cases of this disease (Adams et al., 1993; Alvarez et al., 1988). Mutations of *APC* are found in the same frequency in early-stage adenomas and in advanced adenocarcinomas, indicating that the loss of APC function plays an important role in an early step in colon cancer progression (Adams et al., 1993). Inheritance of a mutant allele of the *APC* gene located on chromosome 5q21-22 causes the familial adenomatous polyposis (FAP) disease, a dominantly inherited form of intestinal cancer that is characterized by the formation of hundreds to thousands of benign polyps in the colon and rectum by the third or fourth decade of life (1, 2).

Reductions in the global level of DNA methylation are also proposed to be an early step in the formation of colonic tumors because both early adenomas and advanced carcinomas show a similar decrease in the global level of DNA methylation compared to cells of normal colon (Feinberg et al., 1988; Gama-Sosa et al., 1983). To the extent that these changes in DNA methylation are thought to contribute to the oncogenic process, it has been proposed that their effects are mediated through changes in the expression levels of proto-oncogenes and tumor-suppressor genes. One model proposes that the commonly observed global DNA hypomethylation in human tumors is of selective advantage to the tumor cell through facilitated proto-oncogene expression (Feinberg and Vogelstein, 1983) (Hanada et al., 1993; Rao et al., 1989; Vorce and Goodman, 1991). In the alternative model, high expression of the DNA methyltransferase is thought to drive hypermethylation of specific loci such as tumor-suppressor genes or genes specific to differentiated cell types (Baylin et al., 1991; El-Deiry et al., 1991; Greger et al., 1989; Issa et al., 1994; Issa et al., 1993; Kautiainen and Jones, 1986; Ohtani-Fujita et al., 1993; Silverman et al., 1989). There is, however, no direct evidence that either mechanism is operative in colon carcinogenesis.

The *K-ras* proto-oncogene is also mutated in 50% of intermediate adenomas (Bos et al., 1987; Vogelstein et al., 1988) . LOH of 18q appears to play a part in a relatively early step during the development of about 30% of colorectal tumors when small adenomas (polyps) progress to

more dysplastic, larger, intermediate adenomas (Barlow, 1993). In the advanced adenomas - the immediate precursors of carcinomas - the incidence of 18q LOH approaches 65% (Alhonen et al., 1987; Allen et al., 1988; Barlow, 1993). Consistent with the possibility that 18q carries a colonic tumor suppressor gene are chromosome transfer experiments demonstrating that one normal copy of chromosome 18 is sufficient to suppress the tumorigenicity of human colon carcinoma cell lines (Bartolucci et al., 1989; Barton et al., 1991). The *DCC* gene has been identified as the target of LOH on chromosome 18q. However, experiments to date have failed to provide conclusive proof that *DCC* is a tumor suppressor gene. For instance, in one study, analysis of the *DCC* alleles retained in 60 human colonic tumors affected by 18q LOH led to the discovery of mutant alleles in only 2 tumors (Cho et al., 1994). Thus, the *DCC* gene represents an anomaly to the common observation that both alleles of tumor suppresor genes are inactivated during tumor pathogenesis.

LOH of the *p53* gene in human colonic adenomas coincides with the appearance of highly dysplastic cells within less dysplastic, intermediate grade adenomas (Boehm and Drahovsky, 1983). Such highly dysplastic adenomatous cells are presumably the immediate precursors of colon carcinomas. At this transition point, one allele of the *p53* gene acquires a point mutation that converts it to a dominant-negative allele of *p53* (Hollstein et al., 1991; Nigro et al., 1989). Subsequently, the second allele of *p53* gene is deleted just before the adenoma is transformed into a carcinoma (Ohue et al., 1994).

The present study was designed to examine the role of three proposed colonic tumor suppressor genes *APC*, *DCC* and *p53* in the initiation and progression of colonic adenomas by mutating their counterparts in mice. In addition, in order to assess the role of DNA hypomethylation in colon cancer, the effect of reduced DNA methylation on the formation of intestinal adenomas was examined in mice through partial inactivation of their DNA methylatransferase, the enzyme that is responsible for the bulk of the methylation of the cytosine residue in CpG dinucleotides.

Techniques of gene targeting (Capecchi, 1989) were used to generate mice that are constitutionally heterozygous for a targeted mutation in genes implicated in *APC* and *DCC*. Mice

carrying germ-line mutations in the DNA methyltransferase gene and the *p53* gene had been created prior to this study (Jacks et al., 1994; Li et al., 1993; Li et al., 1992) using the same techniques. Briefly, embryonic stem (ES) cells are transfected with a fragment of DNA that is homologous to the gene of interest. A mutation that would disrupt the function of the endogenous gene is introduced into the gene by homologous recombination. ES cells heterozygous for the targeted allele are then used to generate chimeric mice. If heterozygous cells contribute the germ cells, the chimeric mice generate progeny that are constitutionally heterozygote.

Figure 2

Targeted mutagenesis in mice. Embryonic stem (ES) cells are transfected with a fragment of DNA that is homologous to the gene of interest. A mutation that would disrupt the function of the endogenous gene is introduced into the gene by homologous recombination. ES cells heterozygous for the targeted allele are then used to generate chimeric mice. If heterozygous cells contribute the germ cells, the chimeric mice generate progeny that are constitutionally heterozygote.

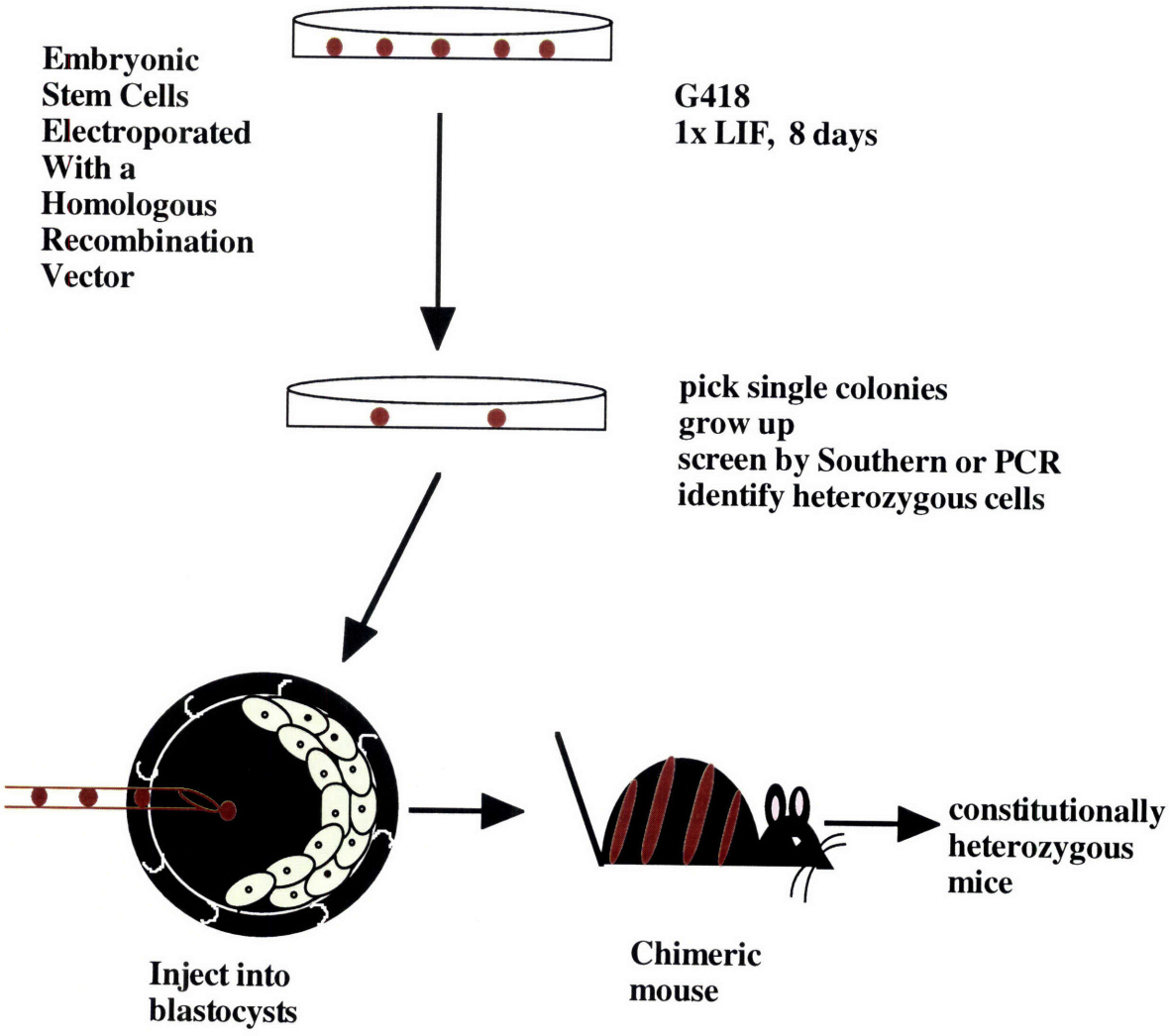
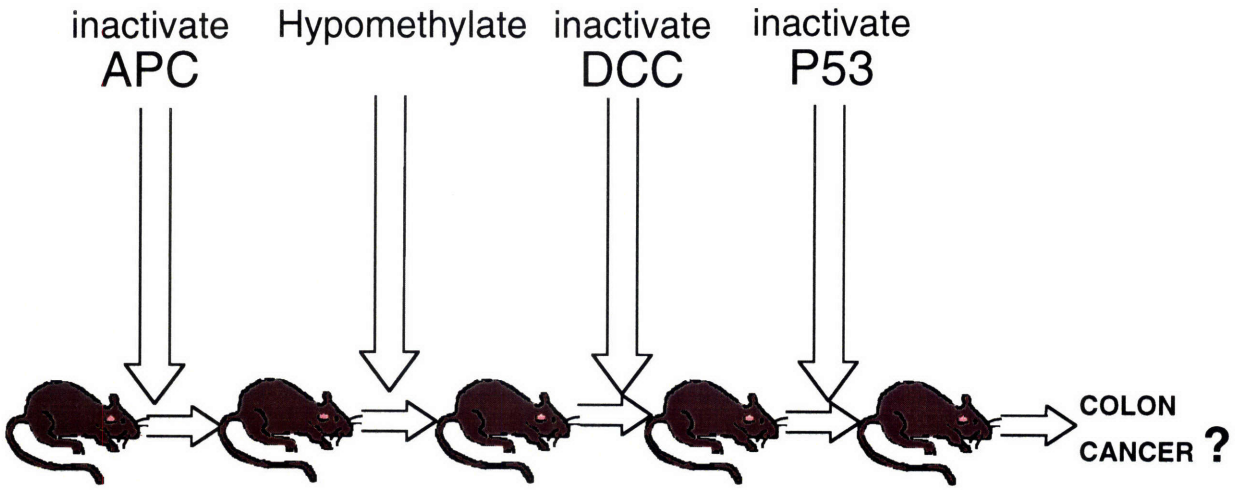

Embryonic
Stem Cells
Electroporated
With a
Homologous
Recombination
Vector
G418
1x LIF, 8 days
pick single colonies
grow up
screen by Southern or PCR
identify heterozygous cells
Inject into
blastocysts
Chimeric
mouse
constitutionally
heterozygous
mice

Figure 3

Construction of a mouse model for colon cancer. In this study, techniques of gene targeting are used to generate mice that are constitutionally heterozygous for a targeted mutation in genes implicated in *APC* and *DCC*. Mice carrying germ-line mutations in the DNA methyltransferase gene and the *p53* gene had been created prior to this study. The effects on intestinal adenoma formation and progression of inactivating the *Apc*, *Dcc*, DNA methyltransferase gene, and *p53* genes are examined in this study.


inactivate
APC
Hypomethylate
inactivate
DCC
inactivate
P53
COLON
CANCER ?

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CHAPTER 2

Attenuated Adenomatous Polyposis And Facial Osteomas in a Mouse with a Hypomorphic Allele of *Apc*

Introduction

Familial adenomatous polyposis (FAP) is a dominantly inherited form of intestinal neoplasia that is caused by the inheritance of a mutant allele of the human *Adenomatous Polyposis Coli* (*APC*) gene (Bodmer et al., 1987; Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). The FAP disease is characterized by the development of large numbers of adenomatous polyps (adenomas or polyps) in the intestine by 20-30 years of age; most of these polyps are found in the colon (Bulow, 1990). Gardner's syndrome (GS) and Turcot's syndrome (TS) are two variant forms of the FAP disease that are distinguished by the additional presence of extracolonic tumors (Hamilton et al., 1995; Nishisho et al., 1991). The GS patients frequently develop facial osteomas, fibromas of various tissues, epidermoid cysts in skin and desmoid tumors (Bulow, 1990; Gregory and Ho, 1992; Gurbuz et al., 1994; Leppard and Bussey, 1975; Leppard, 1974) while the TS patients are often afflicted by medulloblastomas (Hamilton et al., 1995; Mori et al., 1994). No correlation exists between specific mutant alleles of *APC* and the extracolonic manifestations of the FAP disease listed above, suggesting the existence of other genetic elements that influence the formation of these non-colonic tumors (Hamilton et al., 1995; Nishisho et al., 1991).

Mutations of *APC* are also important for the formation of sporadic colon carcinomas, in that mutant alleles of this gene, created by somatic mutations, are found in 90% of the non-familial cases of this disease (Miyoshi et al., 1992; Powell et al., 1992). Mutations of *APC* are found in the same frequency in early-stage adenomas and advanced adenocarcinomas, indicating that the loss of APC function plays an important role in an early step in colon cancer progression (Powell et al., 1992).

The APC protein encodes a 312 kilodalton (Kd) cytoplasmic protein which has a series of heptad repeats in its N-terminal 900 amino acids, seven armadillo repeats between amino acids 453 and 766, several beta-catenin binding sites in its middle third, and microtubule binding sites in its C-terminus (Polakis, 1995). The N-terminal 171 amino acids of APC are sufficient for homo-oligomerization of this protein *in vitro* although the minimal oligomerization domain of APC may

be smaller (Joslyn et al., 1993; Su et al., 1993). The large majority of *APC* mutations (>95%) are chain-terminating mutations that result in the deletion of the C-terminal domains of the encoded protein (Miyoshi et al., 1992).

Genetic studies have revealed a correlation between the site of mutation in the *APC* gene and the severity of polyposis. One class of mutations are chain-terminating mutations in the third and the fourth exons of *APC*, and affect the first 156 codons of the *APC* reading frame (Spirio et al., 1993; Su et al., 1993). These mutations cause attenuated polyposis (AAPC) (Spirio et al., 1993). In families carrying these alleles, some carriers develop no polyps even by the age of 40 while others may display as many as one hundred polyps (Spirio et al., 1993). The majority of *APC* mutations belong to a second class that cause classical polyposis characterized by the formation of hundreds to thousands of adenomas (Nagase et al., 1992). The boundary between these two classes of mutations is very small: there are only ten codons that separate the most downstream *AAPC* mutation from the most 5' mutation in a classical FAP family (Fodde et al., 1992; Spirio et al., 1993; Spirio et al., 1992).

Two mechanistic models have been proposed to account for the difference in the severity of the alleles that cause the attenuated and the classical polyposis (Spirio et al., 1993). One model states that the *AAPC* alleles are true nulls and that the classical polyposis alleles, in contrast, function in a dominant-negative fashion (Bourne, 1991; Spirio et al., 1993; Su et al., 1993). Thus, the truncated APC proteins of classical polyposis patients may form a complex with and compromise the functioning of co-expressed wild-type APC protein, resulting in a phenotypic effect even in heterozygous colonic epithelial cells.

Studies to date have provided conflicting evidence on the mechanism of action of such truncated gene products. For example, truncated APC proteins like the protein encoded by the Apc^{Min} allele caused by chain termination at codon 850 in the mouse *Apc* gene that in heterozygous configuration ($Apc^{Min/+}$) predisposes mice to multiple intestinal adenomas have been shown to override a G_0/G_1 block imposed by wild type APC and cause the entry of cultured NIH 3T3 cells into the S phase of the cell cycle (Baeg et al., 1995). Other evidence in favor of the

dominant-interfering hypothesis comes from recent experiments showing that the Apc^{Min} allele, co-expressed with wild-type allele in heterozygous intestinal cells, cooperates with the activated v-Ha-*ras* gene to promote tumorigenesis in nude mice (D'Abaco et al., 1996).

Yet other observations argue against a dominant-negative mode of action of C-terminally truncated APC proteins. Prominent among these observations is the report of three patients who carried deletions of chromosome 5q21 encompassing the entire *APC* gene and developed classic familial polyposis (Herrera et al., 1986; Joslyn et al., 1991; Lindgren et al., 1992). Another observation that would seem to militate against the dominant-negative mode of action comes from studies of mice, which have shown that the wild type allele of *Apc* gene is lost in nearly 100% of the intestinal adenomas in the Min mice and other mice that carry C-terminal truncations of *Apc* (Fodde et al., 1994; Laird et al., 1995; Levy et al., 1994; Luongo et al., 1994; Oshima et al., 1995). This outcome can be interpreted in several ways. For example, the Apc^{Min} allele may in heterozygous configuration reduce but not eliminate the wild type function; the residual wild type function may then be lost through deletion of the wild type allele, conferring even growth advantage on intestinal epithelial cells. Finally, ectopic expression of truncated APC in the mouse intestine failed to result in any pathology (Oshima et al., 1995). This result is also subject to several interpretations. For instance, the introduced mutant *Apc* allele may not have been expressed in the proper cells and in proper amounts to trigger polyp formation.

Another explanation for the low number of polyps in attenuated polyposis families was proposed by Varesco *et al.*, who observed that mutation of an intronic splice donor site results in the skipping of exon 9, reduced levels of *APC* transcript, and a late onset polyposis in an FAP family (Varesco et al., 1994). Although the effect of this and other *AAPC* mutations on the expression of APC protein is not known, these investigators have proposed that inefficient splicing could delay the onset of of polyposis (Varesco et al., 1994). Here we describe a germline mutation in the mouse homologue of the *APC* gene (*Apc*) that drastically reduces but does not completely eliminate the expression of APC protein. This mutation causes attenuated polyposis and supports the model of Varesco *et al.* that the expression of small amounts of APC protein reduces the

severity of polyposis and a delay in the onset of disease. Moreover, the phenotype of the embryos homozygous for this mutation connects the role of APC in differentiation with its role in tumoriogenesis.

Materials and methods

Construction of *Apc*^{8} and *Apc*$^{del\ 8}$ Targeting Vectors

The *Apc*^{8} targeting vector was constructed by inserting fragments of murine *Apc* gene extending from intron 7 to intron 8 (isolated from a 129Sv mouse genomic DNA library) into the plasmid pPNT (Tybulewicz et al., 1991). The regions of homology consist of a 9.5 Kb and a 3.4 Kb fragments. The *pgk-neo* -polA expression cassette was inserted at the Pml-I site in the eighth exon of *Apc* leading to the generation of a new Eco RV restriction site within the eighth exon of a targeted gene. The HSV-*tk* cassette (also under the control of the *pgk* promoter) was inserted adjacent to the sequences from the eighth *Apc* intron. The HSV-*tk* and *pgk-neo* -polA cassettes are transcribed in the same transcriptional orientation as the *Apc* gene. Southern analysis on Eco-RV-digested DNA from *Apc*$^{8/+}$ mice using a probe from intron 8 displays an *Apc*^{8}-specific 5.0 Kb restriction fragment in addition to the 6.2 Kb fragment corresponding to the wild-type allele.

The *Apc*$^{del\ 8}$ targeting vector was constructed by introducing a termination codon at the second codon of exon 8 by oligonucleotide insertional mutagenesis. The remaining sequences of the eighth exon and a 0.9 Kb portion of intron 8 were deleted and replaced by the *pgk-neo* -polA expression cassette. The *neo* and the HSV-*tk* cassettes were inserted in the same orientation as the *Apc*. The presence of the targeted *Apc*$^{del\ 8}$ allele in the genomic DNA was detected by Southern analysis on Hind III-digested genomic DNA using a probe from intron 7. This probe detected a 4.2 Kb band in the heterozygous cells in addition to the wild-type 7.0 Kb fragment.

Generation of Heterozygous Mice

Electroporation of *Apc*^{8} and *Apc*$^{del\ 8}$ KO vectors into 129Sv D3 and J1 ES cells respectively, subsequent drug selection (180 micrograms of G418 per ml of medium) and Southern blottings were done using standard procedures. Chimeric mice were generated using techniques described in *Teratocarcinomas and Embryonic Stem Cells* (Robertson, 1987). Chimeric animals were crossed with 129Sv inbred mice and the progeny were progenies were screened for the *Apc* mutation. The heterozygous progeny of this cross were inbred 129Sv.

Tumor Analysis

After euthanization, the entire gastrointestinal tract of a mouse was removed, dissected along the cephalo-caudal axis, and washed in phosphate buffer saline. Adenomas were at least twice as wide as a villus and at most 5-6 millimeters in diameter and were readily visible in the intestines upon inspection under a dissecting microscope. Adenomas were removed surgically, fixed in Bouin's fixative, dehydrated in graded solutions of alcohol, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E).

For LOH analysis, adenomas were removed surgically and DNA was prepared from them using standard methods. The facial osteomas were freed from the normal surrounding tissue under a dissecting microscope and DNA was prepared from the tumor. Southern blot analysis for the *p53* locus was performed as previously described (Jacks et al., 1994).

All mice in this study were maintained in the facilities of the Whitehead Institute for Biomedical Research and were fed *ad libitum*. Their diet consisted of Agway Prolab Rat, Mouse and Hamster 3000 chow, which has a crude protein content of at least 22%, a crude fat content of at least 5% and a crude fiber content of at most 5%.

Mouse Genotyping

Apc^{8} mice were genotyped by PCR using the primers (*Apc* Code: 5'AGCTTCAGAGTGGCCTA3', *Apc* Rev: 5'ACCTTTGTCCCCAGATG3' and *Neo* 18.5: 5'TCCTCGTGCTTTACGGTATC3'; one cycle of 1 minute (min) 95°C; followed by 30 cycles of 1 min 95°C, 1 min 50°C, 2 min 72°C; followed by one cycle of 5 min 72°C).

C57BL/B6 *Apc*$^{Min/+}$ mice were purchased from the Jackson laboratory (Bar Harbor, Maine) and crossed to 129 sv mice. 129 sv/B6 Min pups were genotyped by PCR (one cycle of 2 min 94°C; followed by 35 cycles of 1 min 94°C, 2 min 55°C, 3 min 72°C; followed by one cycle of 5 min 72°C) using oligos specific for the *Apc*^{Min} (5'^{2532}TGAGAAAGACAGAAGTTA^{2549}3') and the wild type allele (5'^{2241}GCCATCCCTTCACGTTAG^{2258}3') and a universal downstream primer (5'^{2859}TTCCACTTTGGCATAAGGC^{2841}3') (Luongo et al., 1994).

P53 mice were generated in the laboratory of R.A.W. (Jacks et al., 1994). Mice were genotyped for the *p53* locus using *p53*X6S (5'TTATGAGCCACCCGAGGT3'), *p53*X7AS (5'TATACTCAGAGCCGGCCT3') and *Neo* 18.5 primers using the conditions used for the *Apc*^{8} PCR.

ENU Mutagenesis

ENU was injected intraperitoneally and according to the protocol of Moser *et al.* (Moser et al., 1993).

Generation of Homozygous Mutant and Control Embryonic Fibroblasts

Heterozygous inbred 129Sv *Apc*$^{8/+}$ mice were intercrossed and embryos were extracted at E11.5. Cells were extracted from the embryos using the techniques in *Teratocarcinomas and Embryonic Stem Cells* (Robertson, 1987) and genotyped for *Apc*^{8} by PCR.

Generation of Homozygous Mutant Embryonic Stem Cells

Homozygous $Apc^{8/8}$ ES cells were generated from 129Sv $Apc^{8/+}$ ES cells using the techniques of Mortensen *et al.*(Mortensen et al., 1992) . 10^4or 10^5 heterozygous cells were plated on gelatinized 10-cm dishes and 24 hours later medium containing 400 to 600 micrograms of G418 per ml of medium was added to the cells. In each dish, on average ten colonies survived two weeks of culturing in medium containing G418. These colonies were picked, passaged and genotyped. Ten percent of the picked colonies had lost the wild type allele of *Apc* and showed only the mutant DNA fragment upon Southern analysis (data not shown). We assumed that these colonies had lost the wild type *Apc* and duplicated the Apc^8 allele and its *pgk-neo*-polA cassette and thus became resistant to high concentrations of G418 by virtue of having two copies of the *neo*. The colonies that survived high G418 but remained heterozygous served as the control for the homozygous mutant cells. The homozygous mutant or the control heterozygous ES cells were injected into C57BL/6 blastocysts to generate chimeric mice.

C57BL/6 $Apc^{Min/Min}$ ES cells were generated using the protocols in *Teratocarcinomas and Embryonic Stem Cells* (Robertson, 1987).

Protein Analysis

Western blottings were performed using the FE9 anti-N-terminal APC monoclonal antibody (Oncogene Science) and using 3% low melting agarose gels according to the protocol of Smith *et al.* (Smith et al., 1993). Total cellular protein was extracted from cells using two buffers: a denaturing buffer (125 millimolar (mM) Tris-HCl PH 6.8, 20% V/V Glycerol, 2% W/V SDS, 2% V/V betamercaptoethanol, 0.001% W/V bromophenol Blue) or a non-denaturing buffer (50 mM Tris-HCl PH 7.5; 100 mM NaCl, 1% NP40, 1 mM PMSF, 1 microgram/ml Aprotinin and Leupeptin each). APC protein was readily detectable in the agarose westerns performed on the denatured extracts but the existence of SDS in the buffer was incompatible with the standard

protein assays used to quantify the concentration of protein. On the other hand, the use of the non-denaturing buffer allowed us to measure the concentration of protein in the extracts but prevented the detection of APC protein using the FE9 antibody. Thus for each western blot, the experiment was done in two parts. The cells in each preparation were counted and protein was extracted from one half using the non-denaturing buffer and from the second half using an equal volume of denaturing buffer. The protein concentration was measured in the non-denaturing preparation and the volume of the extract that contained 5 milligrams of total protein was determined. Then, an equal fraction of the denatured preparation was western blotted. This method of quantitation was relatively accurate because equal fractions of the two preparations contained equal amounts of the retinoblastoma protein used as internal standard (data not shown). The ratio of the amount of APC protein in the lanes corresponding to wild-type and the $Apc^{8/8}$ mutant cells was determined using a Computing Densitometer (Model 300A, Molecular Dynamics).

RNA Analysis

Reverse transcription (RT) using random hexamer oligos and PCR were performed using standard protocols. Primers used in the RTPCR analyses on the total cellular RNA in the sense orientation were *Apc* exon 1 ($5'^{67}$CTTCGACAAGAGCTAGAAG$^{85}3'$), *Apc* exon 3 ($5'^{279}$CTACGGAAGTCGGGAAGG$^{296}3'$), *Apc* exon 6 ($5'^{644}$GAATAGCCAGGATCCAGC$^{661}3'$), *Apc* exon 7 ($5'^{727}$TCATCTCAGAGCAGGCAT$^{744}3'$), *Apc* exon 9 ($5'^{1023}$CTGTATATCCATGCGGCAG$^{1041}3'$) and *Apc* exon 9a ($5'^{1249}$ACCTGTTGGGAGTGGCAG$^{1267}3'$). The primers in the antisense orientation were *Apc* exon 5 ($5'^{548}$GTCATGTCTGTCTGTAAGG$^{530}3'$), *Apc* exon 9 ($5'^{1041}$CTGCCGCATGGATATACAG$^{1023}3'$)' *Apc* exon 10 ($5'^{1328}$ATGCTCAACAGGAGCTGG$^{1310}3'$) and *Apc* exon 14

(5'^{1756}CGCTTTTGAGGGTTGATTC^{1738}3'). Sequencing of the PCR reaction products was performed according to the protocol of Sarkar *et al.* (Sarkar et al., 1993).

Results

Construction of *Apc*$^{8/+}$ mice

To create a mutation altering the murine homologue of the *APC* gene (*Apc*), we constructed a vector that targets the eighth exon of this gene which spans codons 278-311 (Fig. 1A). This targeted mutation was designed to recapitulate a mutation in the eighth exon that was found in a Gardner's syndrome family (Nishisho et al., 1991).

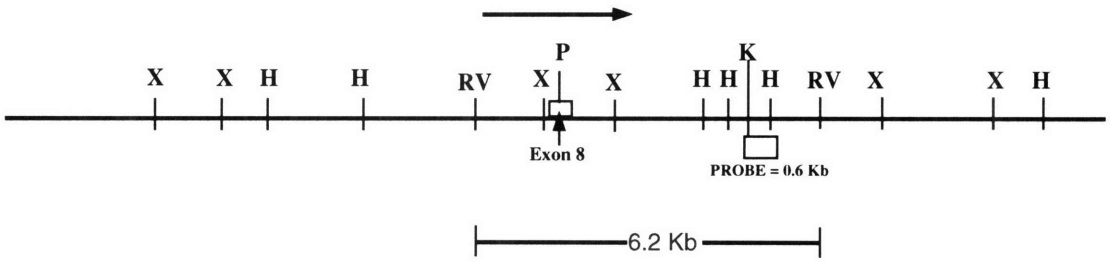
The *Apc*^{8} targeting vector (Fig. 1A) was introduced into 129/Sv D3 embryonic stem (ES) cells (Gossler et al., 1986; Mansour et al., 1988; Robertson, 1987). The resulting ES cell colonies were screened for those in which alteration of an *Apc* gene had occurred through homologous recombination with this vector. Of two hundred G418- and gancyclovir-resistant ES cell clones screened, three had targeted a copy of the neomycin transferase (*neo*) gene into the *Apc* locus by homologous recombination (data not shown). Mouse chimeras generated by two of the heterozygous ES cell clones transmitted the mutant *Apc* through their germline (Fig. 1B). Breeding of resulting heterozygous *Apc*$^{8/+}$ mice resulted in no viable homozygous offspring, indicating that the functioning of the *Apc* gene is essential for normal development. As described below, homozygous mutant embryos die between days 9.5 and 12.5 of development.

Figure 1

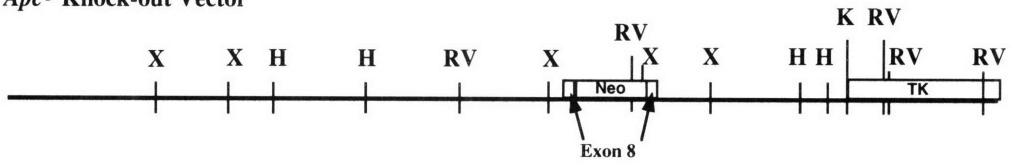
(A) The *Apc*^{8} targeting vector was constructed using fragments of murine *Apc* gene extending from intron 7 to intron 8. The regions of homology consist of a 9.5 Kb and a 3.4 Kb fragments. The *pgk-neo* -polA expression cassette was inserted at the Pml-I site in the eighth exon of *Apc* leading to the generation of a new Eco RV restriction site within the eighth exon of a targeted gene. The HSV-*tk* cassette (also under the control of the *pgk* promoter) was inserted adjacent to the sequences from the eighth *Apc* intron. The HSV-*tk* and *pgk-neo* -polA cassettes are transcribed in the same transcriptional orientation as the *Apc* gene (shown with an arrow). X (Xba I), H (Hind III), RV (Eco RV), P (Pml I) and K (Kpn I) are restriction endonuclease sites. (B) Germ-line transmission of *Apc*^{8}. Southern analysis on Eco-RV-digested DNA from *Apc*$^{8/+}$ mice using a probe from intron 8 displayed an *Apc*^{8}-specific 5.0 Kb restriction fragment in addition to the 6.2 Kb fragment corresponding to the wild-type allele. Lanes 2, 5, 6, 7, and 9 correspond to heterozygote mice. Lane 10 is DNA size marker.

A

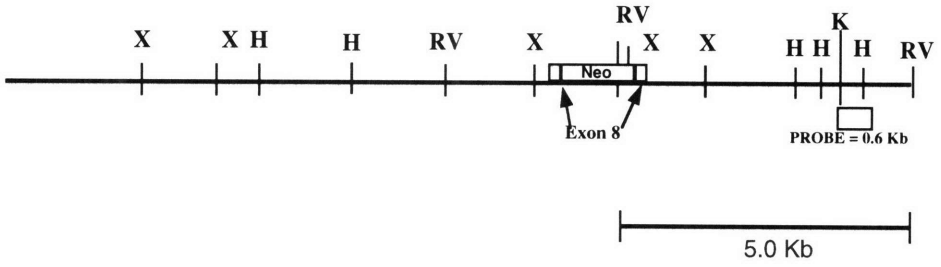
Wild Type Allele



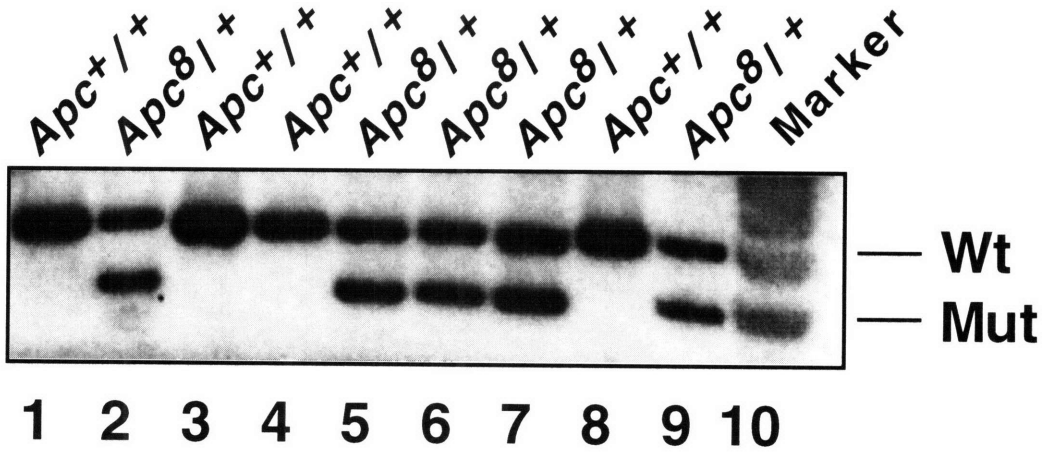
***Apc*^{8} Knock-out Vector**



***Apc*^{8} Allele**



B



Expression of Targeted *Apc*$^{\Delta 8}$ Allele

The APC protein is expressed ubiquitously in most if not all human cell types (Groden et al., 1991; Kinzler et al., 1991). To examine the effect of the targeted mutation on *Apc* expression, we examined the expression of the mouse APC protein in homozygous *Apc*$^{8/8}$ embryonic fibroblasts (EF) using a monoclonal antibody that recognizes the N-terminus of human APC (Smith et al., 1993). The *Apc*$^{8/8}$ fibroblasts were prepared from embryos at day 11.5 of gestation. Embryo fibroblasts were used in this experiment because cultures of *Apc*$^{8/8}$ intestinal epithelial cells were difficult to establish.

While protein species corresponding to the full-length 300 kd APC protein were present in the immunoprecipitates derived from both wild-type and heterozygous cells, neither a truncated nor a full-length APCprotein was detectable in the lanes corresponding to the*Apc*$^{8/8}$ embryo fibroblasts upon initial western immunoblot analysis (Fig. 2A). However, upon extended exposure of lanes containing several-fold more protein from *Apc*$^{8/8}$ cells, an apparently full-length APC protein was detectable. Densitometric analysis of three independently prepared homozygous mutant and control cell lysates showed expression of an apparently full-length APC protein in homozygous mutant *Apc*$^{8/8}$ cells at significantly reduced levels compared to those seen in wild-type cells (Fig. 2A).We estimated from densitometric analysis of these gels that intensity of the APC protein band from the homozygous mutant cells were at most one percent of that seen in the wild-type cells.

Figure 2

Comparison of the effects of *Apc*^{8} and *Apc*^{Min} mutations on *Apc* expression. (A) The APC protein was western blotted using an anti-N-terminal antibody (Smith et al., 1993) . Lanes 1 and 2 are control wild-type 129 embryonic fibroblasts and lanes 3 and 4 correspond to fibroblasts from homozygous mutant *Apc*$^{8/8}$ littermates. The location of electrophoretic migration of wild-type APC (300 Kd) and the truncated Min (100 Kd) protein are indicated by arrows. Lanes 5 and 6 correspond to C57BL/6 *Apc*$^{Min/+}$ and *Apc*$^{Min/Min}$ embryonic stem cells. (B) Products of PCR reactions on reverse-transcribed RNA from 129Sv *Apc*$^{+/+}$ control and 129Sv *Apc*$^{8/8}$ embryonic fibroblasts are shown in Lanes 1 and 2, respectively. The wild type and the mutant cells each express two mRNA species. In each lane, the larger species correspond to the mRNA molecules containing exon 9a and the smaller species correspond to the mRNA molecules without exon 9a . Both species in *Apc*$^{8/8}$ cells also lack the 99 nucleotides corresponding to exon 8 into which the *pgk-neo* cassette was targeted. The exons contained in each mRNA species are displayed graphically next to them. The mRNA species spanning exons 7-15 of *Apc* were amplified using *Apc* exon7-2 (5'^{798}AAGCAACACCGCAGCCTCCAGTAGT^{822}3') and *Apc* exon 15 (5'^{2296}GCTGAGCATCTAGTCTAGCTTCTAG^{2272}3') primers. From each population of cells, equal amounts of RNA were used in the reverse transcription and the follow-up PCR reactions.

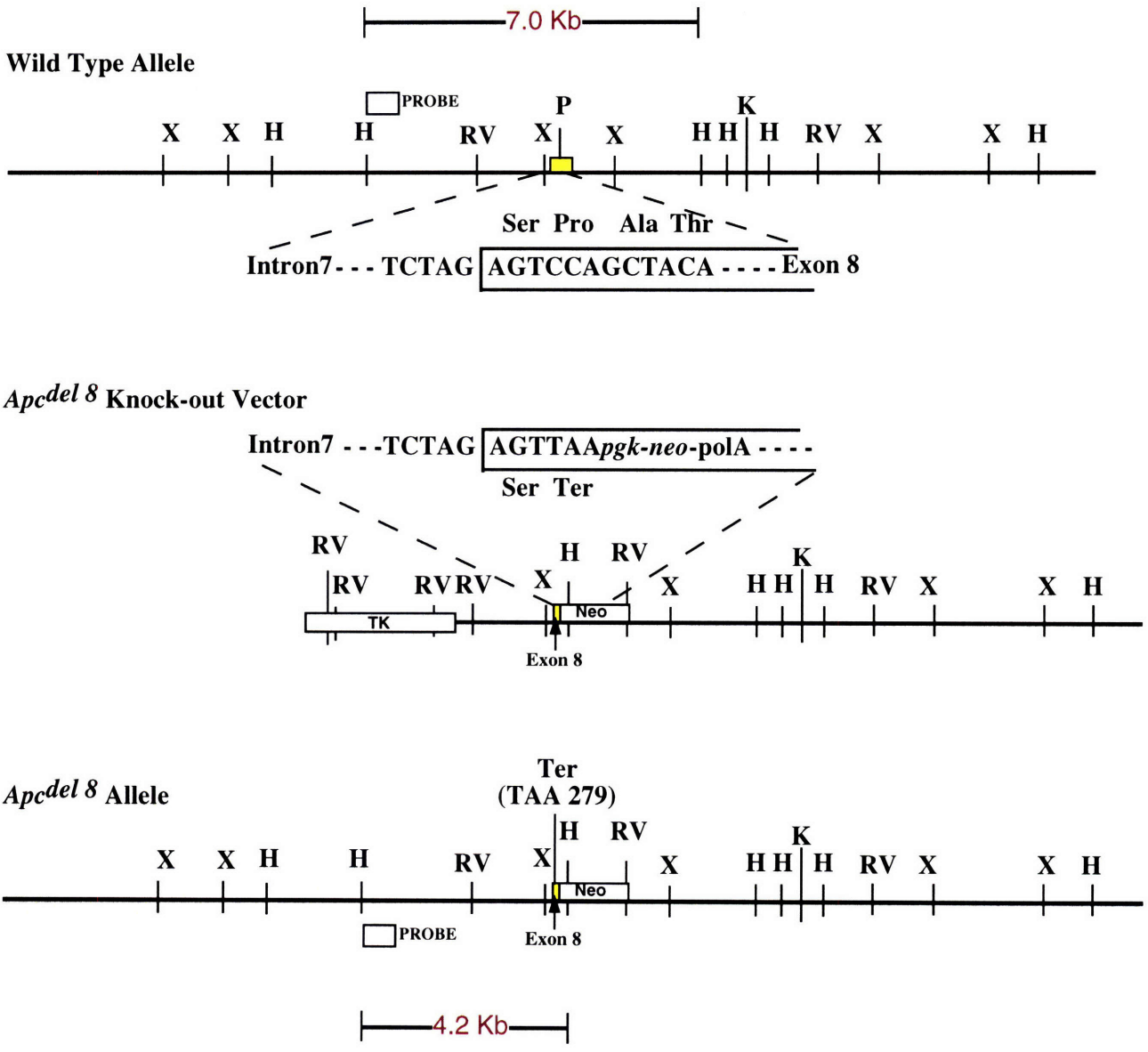
Reverse transcription of total cellular RNA followed by polymerase chain reaction analysis (RTPCR) using primers to several exons of *Apc* (including exons 1, 3, 5, 6, 7, 9, 9a, 10, 14 and 15) showed that all known alternatively spliced *Apc* mRNA species were expressed at reduced levels in the $Apc^{8/8}$ cells and that the mRNA species contained all exons of the *Apc* gene save the eighth (Fig. 2B). This exon is composed of 99 nucleotides; hence, upon translation, an mRNA resulting from deletion of this 8th exon would yield a polypeptide 33 amino acids shorter than its wild-type counterpart. This truncated protein would not be resolvable from the wild-type APC protein in our western blot analyses.

We concluded that the mutation that we created by homologous recombination in the *Apc* gene has two effects on its expression: the fusion of the 7th directly to the 9th exon of the processed mRNA and a reduction in the level of this RNA. Together, these effects resulted in the synthesis of APC protein molecules lacking the residues encoded by the 8th exon that are expressed at a small fraction (<1%) of the wild type level.

The functionality of this minimally truncated APC protein has been addressed by other investigators by engineering a second distinct mutant allele of *Apc* that we have termed $Apc^{del\ 8}$ and also results in the precise deletion of the eighth exon from the *Apc* mRNA. To create this allele, oligonucleotide insertion was used to introduce a translation termination codon at codon 270 (Fig. 3). The mutation also deletes a 1.0 Kb fragment of *Apc* that includes codons 282-311 of exon 8 and the 5' portion of intron 8, and replaces it with a copy of the *pgk-neo* -polA cassette (Fig. 3). PCR-amplification of the reverse-transcribed products of RNA from homozygous $Apc^{del\ 8}$ embryonic fibroblasts and colonic tissues, followed by sequencing of the PCR products confirmed, as was the case with the Apc^{8} allele described above, the direct fusion of the seventh to the ninth exon of the mRNA species (data not shown). However, in contrast to Apc^{8}, the $Apc^{del\ 8}$ allele yields mRNA and protein at levels that are equivalent to those associated with the wild-type *Apc* gene (data not shown).

Figure 3

The *Apc*$^{del\ 8}$ targeting vector was constructed by introducing a termination codon at the second codon of exon 8 by oligonucleotide insertional mutagenesis. The remaining sequences of the eighth exon and a 0.9 Kb portion of intron 8 were deleted and replaced by the *pgk-neo* -polA expression cassette. The *neo* and the HSV-*tk* cassettes were inserted in the same orientation as the *Apc*. The presence of the targeted *Apc*$^{del\ 8}$ allele in the genomic DNA was detected by Southern analysis on Hind III-digested genomic DNA using a probe from intron 7. This probe detects a 4.2 Kb band in the heterozygote cells in addition to the wild-type 7.0 Kb fragment.


7.0 Kb
Wild Type Allele
PROBE
P
K
X X H H RV X X H H H RV X X H
Ser Pro Ala Thr
Intron7- - - TCTAG AGTCCAGCTACA - - - - Exon 8
Apcdel 8 Knock-out Vector
Intron7 - - -TCTAG AGTTAApgk-neo-polA - - - -
Ser Ter
RV
RV RV RV X H RV X H H H RV X X H
TK
Neo
Exon 8
Apcdel 8 Allele
Ter
(TAA 279)
X X H H RV X H RV X H H H RV X X H
Neo
PROBE
Exon 8
4.2 Kb

Mice homozygous for the $Apc^{del\ 8}$ allele develop normally, do not develop polyps, and are indistinguishable from wild-type mice for the first 2 years of age. The normal development of $Apc^{del8/del8}$ embryos and the absence of tumors in the adult $Apc^{del8/+}$ and $Apc^{del8/del8}$ mice demonstrated that the slightly truncated APC protein lacking the residues encoded by the 8th exon is fully functional. Hence, we deduce that the Apc^{8} allele expresses essentially wild-type protein but at a severely reduced level and that any phenotypic effects associated with the Apc^{8} allele must be ascribed to its very low level of expression.

We also examined the effect on protein expression of the Apc^{Min} allele, which carries a nonsense mutation in the fifteenth exon of the mouse *Apc* gene (Apc^{Min}, codon 850) that was induced by chemical mutagenesis (Moser et al., 1990; Su et al., 1992). $Apc^{Min/Min}$ embryos die by E7.5, making it impossible to generate $Apc^{Min/Min}$ embyonic fibroblasts. Instead, the effect of the Apc^{Min} mutation on APC expression was analyzed in the embyonic stem cells. Western blotting analyses on $Apc^{Min/+}$ and homozygous $Apc^{Min/Min}$ ES cells revealed that, in contrast to Apc^{8}, the Apc^{Min} allele encodes a truncated protein (100 Kd); this Apc^{Min} -encoded protein is coexpressed with wild-type protein (300 Kd) at comparable levels in the $Apc^{Min/+}$ cells (Fig. 2A).

Spontaneous Polyp Initiation in The $Apc^{8/+}$ Mice

In human carriers of mutant *APC* alleles, the adenomatous polyps in the colon usually become apparent only by 20-30 years of age (Groden et al., 1991; Nishisho et al., 1991; Spirio et al., 1993). For this reason, we followed 129Sv/C57BL6 (129Sv/B6) F1 $Apc^{8/+}$ mice and their wild-type littermates for a period of two years, screening at different ages for the presence of gastrointestinal polyps. Intestinal adenomas were found in $Apc^{8/+}$ mice only when they reached one-and-a-half to two years of age (Table 1 & Fig. 4A). Even at this advanced age, forty percent of the heterozygotes were free of polyps. The number of polyps in the heterozygotes ranged between zero and seven with an average of one adenoma per mouse (Table 1). We also examined fifty-eight

wild-type 129Sv/B6 F1 mice aged between one-and-a-half and two years and found no adenomas in any of these control mice (Table 1).

Table 1

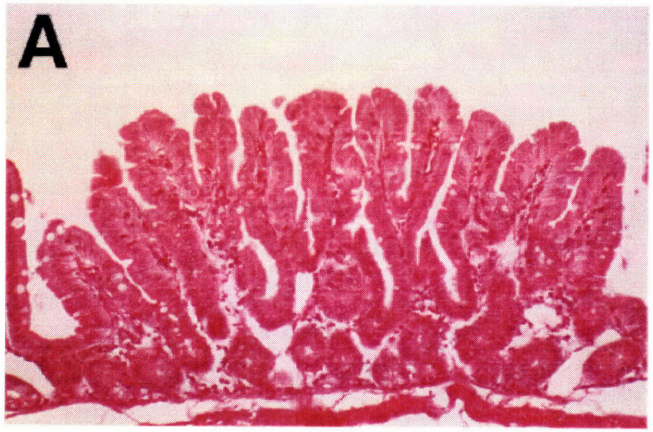
Intestinal Adenomas (Polyps). The effect of germline inheritance of the *Apc*$^{\delta}$ and *Apc*^{Min} mutations on intestinal adenoma formation in 129/B6 F1 mice were compared by counting the number of adenomas in the intestine of *Apc*$^{\delta/+}$ and *Apc*$^{Min/+}$ mice under a dissecting microscope.

Effects of alleles of Apc on formation of intestinal polyps

Mouse	# of Mice	Age (mo)	Average # of Polyps per Mouse	Range
129sv/B6 F1 *Apc*$^{\delta}$/+	63	18-24	1	0-7
129sv/B6 F1 *Apc*^{Min}/+	20	5-10	110	45-135
129sv/B6 F1 *Apc*$^{+}$/+	58	18-24	0	NA

Figure 4

Tumors in *Apc*^{8} mice. (A) An intestinal adenoma from a 129Sv/B6 F1 *Apc*$^{8/+}$ mouse (93X magnification). (B) The arrow points to a unilateral facial osteoma in a 12-month-old 129Sv *Apc*$^{8/+}$ *p53*$^{+/-}$ mouse. (C) Foci of squamous cell metaplasia of the salivary gland (arrows) in a 129Sv/B6 F1 *Apc*$^{8/+}$ mouse (400X magnification).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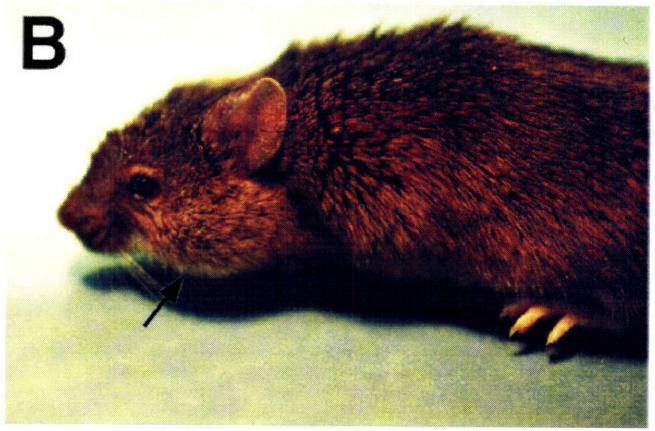
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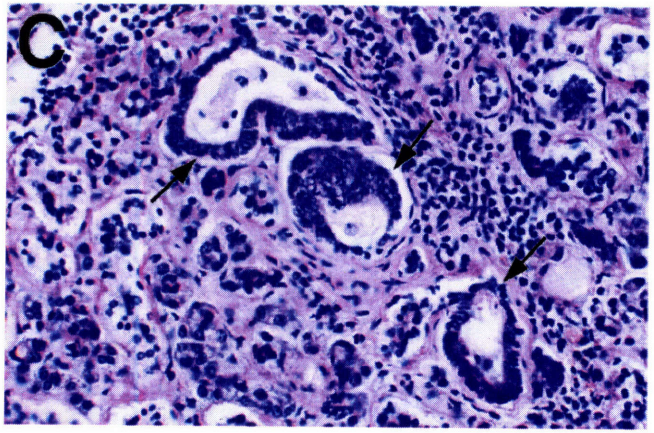
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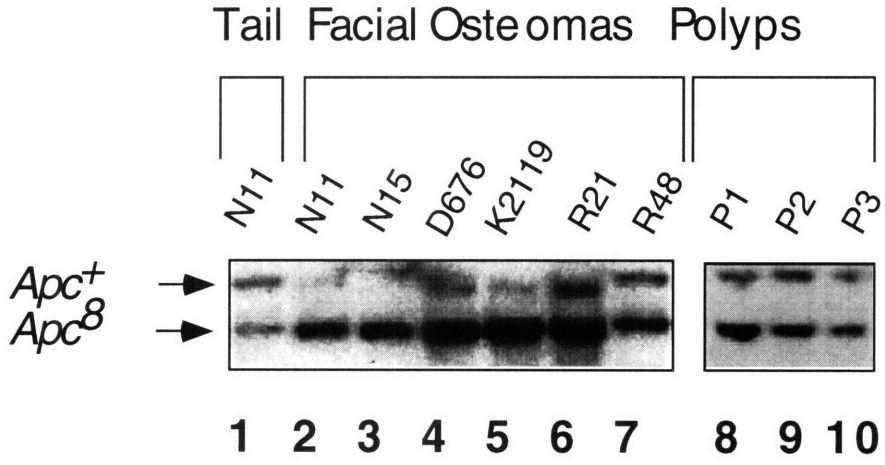
The hallmark of a tumor suppressor gene is the inactivation of both of its alleles in a tumor cell (Weinberg, 1991). For this reason, we undertook Southern blot analysis of the genomic DNA of polyps removed from $Apc^{8/+}$ mice. These polyps were composed of both epithelium-derived adenomatous cells and contaminating mesenchymal cells including fibroblasts, macrophages and other blood cells. When compared to the constitutional DNA of the heterozygotes, genomic DNA from the great majority of these adenomas displayed a reduced ratio of the wild-type to the mutant Apc^{8} allele; Fig. 5A represents some examples of analysis of these adenomas. These results suggest that the wild type *Apc* allele was lost from many and perhaps all of these polyps. This in turn suggests that the Apc^{8} allele has minimal effect on cell phenotype when present in a heterozygous configuration and that it acts in a recessive manner on intestinal epithelial cells.

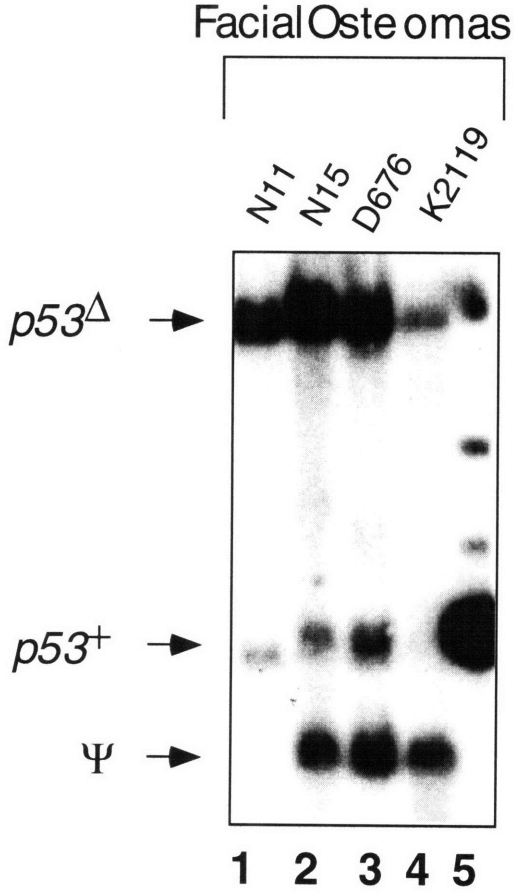
Figure 5

Loss of heterozygosity (LOH) in tumors. (A) LOH at the *Apc* locus in the intestinal adenomas and the facial osteomas from mice carrying the Apc^{8} allele. Lane 1 is control tail DNA from a heterozygous 129Sv $Apc^{8/+}$ $p53^{+/-}$ mouse. Lanes 2-7 are facial osteomas from 129Sv $Apc^{8/+}$ $p53^{+/-}$ mice. Lanes 8-10 are three intestinal adenomas from a 129Sv/B6 F1 $Apc^{8/+}$ mouse. The arrows depict the 6.2 Kb wild-type *Apc* and the 5.0 Kb mutant Apc^{8} fragments. (B) LOH at the *p53* locus in the facial osteomas. Lanes 1-4 are facial osteomas from 129Sv $Apc^{8/+}$ $p53^{+/-}$ mice. The Top arrow points to the mutant 3.0 Kb band and the middle arrow to the 1.3 Kb wild-type band. The lowest arrow marks the 1.0 Kb fragment which appears due to cross-hybridization of the probe to DNA corresponding to a *p53* pseudogene. Lane 5 is DNA size marker.

A


Tail
Facial Osteomas
Polyps
N11
N11
N15
D676
K2119
R21
R48
P1
P2
P3
Apc+
Apc8
1 2 3 4 5 6 7 8 9 10

B


Facial Osteomas
N11
N15
D676
K2119
p53Δ
p53+
Ψ
1 2 3 4 5

We then compared these $Apc^{8/+}$ mice with those of the Min strain. The Apc^{Min} mutation is similar to those mutations in humans that result in the classical polyposis characterized by the formation of hundreds of adenomas. This class of mutations encode truncated proteins that retain the oligomerization repeats. In stark contrast to the Apc^{8} heterozygotes, mice carrying the Apc^{Min} mutation in an identical genetic background and raised in the same colony developed an average of 110 adenomas within 150 days (Table 1). These Min mice died by 5-10 months of age from intestinal obstruction (Table 1), confirming earlier reports of others (Moser et al., 1990). Therefore, as is the case in humans, distinct mutant alleles of the *Apc* gene cause the development of profoundly different numbers of adenomatous polyps in mice.

Fifteen percent of all 129Sv/B6 mice developed lymphomas, leukemia, sarcomas or lung adenomas by 24 months of age regardless of their genotype at the *Apc* locus. Since, the $Apc^{+/+}$ mice developed the same frequency of these tumors as the $Apc^{8/+}$ mice did (data not shown), we concluded that these extra-colonic lesions arose due to the intrinsic cancer predisposition of mice having a 129Sv/B6 genetic background.

Effect of ENU Mutagenesis on Polyp Initiation in $Apc^{8/+}$ Mice

The effects of the Apc^{8} mutation on intestinal polyp formation was also gauged by exposing an F1 litter, derived from a cross of a 129Sv $Apc^{8/+}$ heterozygote with a wild-type C57BL/6 mouse, to the alkylating carcinogen ethylnitrosourea (ENU). These mice were exposed to a single ENU dose of 50 micrograms per gram of body weight at 105 days of age. The animals were not genotyped at the time of injection to ensure identical treatment of wild-type and heterozygous mice. A total of 60 polyps were found in six heterogzygous mice (average of 10 with a range of 8-12 polyps per mouse) while none were found in their five wild type littermates.

Southern blot analysis of the genomic DNA of ten polyps removed from ENU-treated $Apc^{8/+}$ mice showed the presence of the bands of equal intensities corresponding to both the Apc^{8} and the wild-type allele of *Apc* (data not shown). These results suggested, though did not prove,

that ENU-mediated inactivation of the wild-type *Apc* allele had accelerated the formation of polyps in the $Apc^{8/+}$ mice. More importantly, they demonstrated that the Apc^{8} allele can predispose to greatly increased rates of polyp formation under certain experimental conditions.

Extracolonic Tumors in The $Apc^{8/+}$ Mice

Members of some human FAP families display extracolonic tumors, most commonly facial osteomas, epidermoid cysts, or one of a variety of fibromas, a condition known as Gardner's syndrome (Bulow, 1990; Gregory and Ho, 1992; Gurbuz et al., 1994; Leppard and Bussey, 1975; Leppard, 1974). In such families, some carriers develop both colonic and extracolonic abnormalities while others exhibit only growths in the colon (Nishisho et al., 1991). This suggests the involvement of yet other genes that influence the development of the extracolonic tumors.

In the 129Sv or 129Sv/B6 mice carrying the Apc^{8} mutation, such extracolonic growths were not observable. In order to ascertain whether the Apc^{8} allele might cause such extracolonic tumors in another genetic background, we crossed 129Sv $Apc^{8/+}$ mice with mice lacking one copy of the *p53* tumor suppressor gene (129Sv $p53^{+/-}$) (Jacks et al., 1994). As documented by others, mice inheriting a mutant *p53* gene are predisposed to a variety of tumors (Donehower et al., 1992; Jacks et al., 1994). In a cohort of seventeen 129Sv $Apc^{8/+}$ $p53^{+/-}$ mice aged between 12 to 18 months, fifteen mice developed unilateral and the remaining two mice developed bilateral osteomas of the facial bones. These affected the maxillary and mandibular bones and occasionally the orbital bones and those of the skull (Fig. 4B & Table 2). Southern blot analysis of the DNA extracted from these facial osteomas confirmed that the tumors had lost the wild type allele of both *Apc* and *p53* and had retained the targeted alleles of both genes (Fig. 5, A and B).

Table 2

Facial Osteomas. Mice with mutations of *Apc*^{8} or *Apc*^{Min} with or without mutations of *p53* were monitored for up to two years for signs of facial osteomas. C57BL/6 *Apc*$^{Min/+}$ or 129 *Apc*$^{8/+}$ mice were crossed to 129 *p53*$^{+/-}$ mice and their F1 and F2 progeny were monitored closely.

Effects of alleles of Apc on formation of facial osteomas

Mouse	Mice with Facial Osteoma / Mice Generated	Age (mo)
129sv $Apc^{8}/+p53+/-$	17/17	12-18
129sv $Apc^{8}/+$	0/77	18-24
129sv/B6 F1 $Apc^{8}/+$	0/63	18-24
129sv/B6 F1 $Apc^{Min}/+$	0/55	5-10
129sv/B6 F1 $Apc^{Min}/+$ $p53+/-$	0/24	5-10
129sv/B6 F2 $Apc^{Min}/+$ $p53-/-$	0/35	2-6
129sv $p53+/-$	0/46	12-18
129sv/B6 F1 $p53+/-$	0/43	12-18
129sv/B6 F2 $p53-/-$	0/38	2-6

We have not observed this unusual tumor in a series of 46 129Sv *p53* $^{+/-}$ or 38 other 129Sv/B6 *p53* $^{-/-}$ mice (Table 2). This observation indicates that *Apc*^{8} mutation can also predispose mice to an unusual extracolonic tumor seen in humans carrying mutations at the *APC* locus. We also observed that these facial osteomas had a high propensity of metastasizing into the lung and the liver. We have not detected any facial osteomas in a cohort composed of 55 (129Sv/B6 F1) *Apc*$^{Min/+}$, 24 (129Sv/B6 F1) *Apc*$^{Min/+}$ *p53*$^{+/-}$ and 35 (129Sv/B6 F2) *Apc*$^{Min/+}$ *p53*$^{-/-}$ mice, perhaps because these mice die from intestinal obstruction or *p53*-specific tumors within 2-10 months (Table 2).

P53 dosage reduction did not affect polyp initiation as measured by the number of intestinal adenomas or polyp progression as judged by increase in size, degree of dysplasia or the acquisition of an invasive phenotype in either the *Apc*$^{8/+}$ or *Apc*$^{Min/+}$ mice. We concluded from this study that, when in heterozygous configuration, *Apc*^{8} allele predisposes mice to one of the extracolonic tumors of Gardner's syndrome.

Aberrant Differentiation in Adult *Apc*$^{8/+}$ Mice

Seventy percent of *Apc*$^{8/+}$ mice also developed squamous cell metaplasia of the salivary glands (Fig. 4C). These lesions were composed of keratin-filled cysts surrounded by one or a few layers of squamous cells. Such lesions reflect abnormal differentiation of the salivary gland epithelial cells in that this gland is normally devoid of squamous cells. Whether these lesions have lost the wild type allele of *Apc* was not determined due to the difficulty in isolating them and their microscopic size. In two instances, the *Apc*$^{8/+}$ mice were affected by squamous cell carcinomas of the salivary gland. These two tumors were composed of large numbers of keratin cysts surrounded by squamous cells (data not shown). This suggests that these squamous lesions of the salivary gland have a propensity to progress to tumors. Moreover, the Harderian gland of approximately 10% of the *Apc*$^{8/+}$ mice also contained similar squamous cysts. We also note that none of their the *Apc*$^{+/+}$ littermates developed these squamous lesions.

APC And Embryonic Development

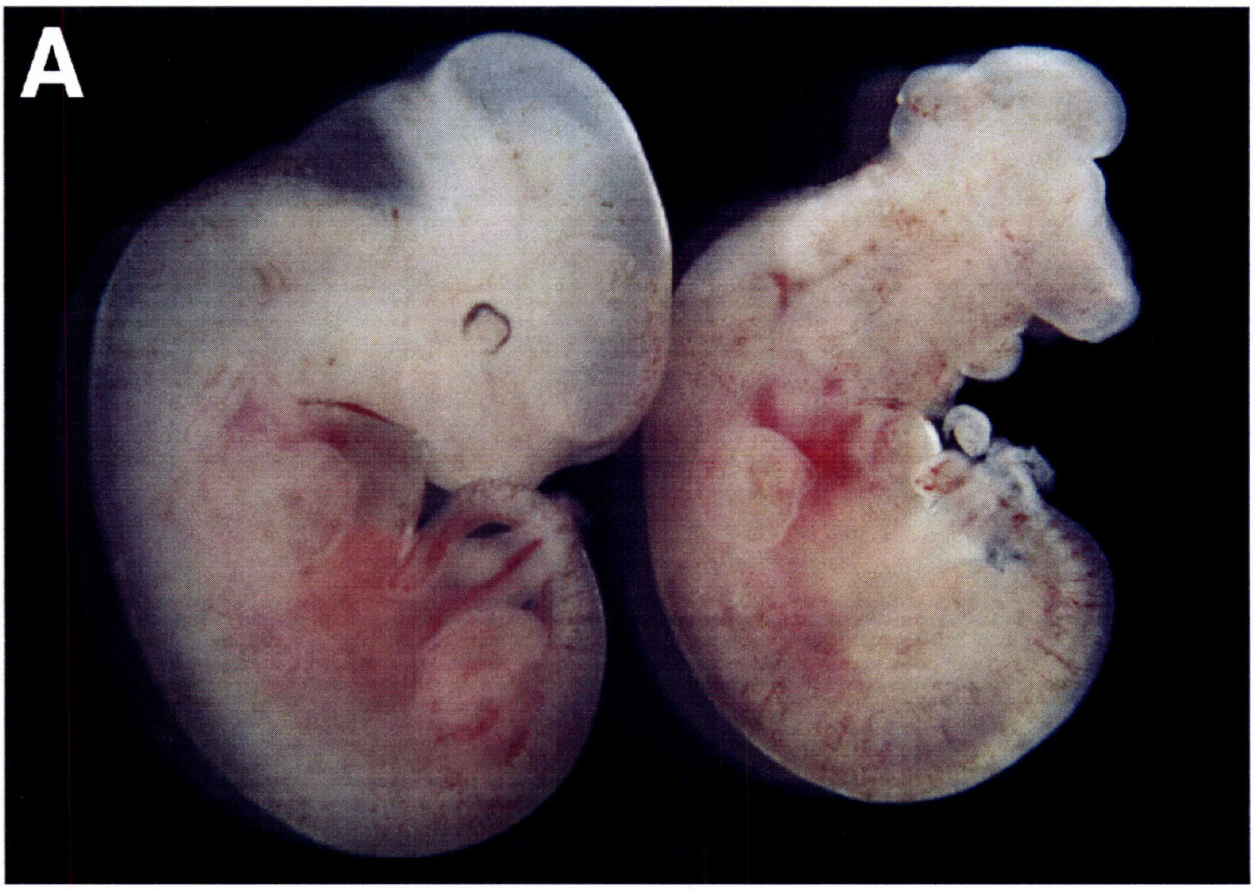
As mentioned above, breeding of heterozygous $Apc^{8/+}$ mice resulted in no viable homozygous offspring, confirming the reports of others that the functioning of the *Apc* gene is essential for normal development (Moser et al., 1995). To understand the effects of Apc^{8} on mouse development, we examined litters from the crosses between $Apc^{8/+}$ mice during E 8.5 and E 17.5 of gestation. One fourth of the embryos were homozygous mutant at E9.5 of gestation but the fraction of the homozygous mutants decreased in later stages of development. Only two homozygous mutant embryos were found in a group of 40 embryos isolated at E12.5 and no mutant embryos were obtained thereafter. The phenotype of $Apc^{8/8}$ embryos is less severe than the phenotype of the $Apc^{Min/Min}$ embryos, which die by E 7.5 (Moser et al., 1995).

The organs of the homozygous mutant embryos caudal to the brachial region had a normal appearance (Fig. 6, A & C). Histological examination of embryos at E 11.5 revealed that the viscera including the primitive gut had developed normally although this was not confirmed by immnuohistochemical analysis using differentiation-specific markers.

The $Apc^{8/8}$ embryos displayed clear defects in craniofacial development (Fig. 6C). While closure of the neural tube in the wild type and the heterozygous embryos was complete by E 9 of gestation, the rostral end of the neural tube remained open in all homozygous mutant $Apc^{8/8}$ embryos. Furthermore, the embryonic mesenchyme that is the progenitor of the bones of the face and the skull was severely malformed (arrow in Fig. 6C).Based on these observations, we speculated that normal APC is required for the normal development of the bones in the head and the face, the same bones that are inflicted with osteomas in adult Gardner's syndrome patients. This notion was reinforced through the creation of the chimeras described below.

Figure 6

Effect of *Apc*^{8} on embryonic development. (A) An *Apc*$^{+/+}$ and its *Apc*$^{8/8}$ littermate (right) at E11.5. (B) Mid-sagittal section of an *Apc*$^{+/+}$ at E11.5. (C) Mid-sagittal section of an *Apc*$^{8/8}$ at E11.5. Embryonic mesenchyme (arrow) that is the progenitor of the facial bones in the adult mouse develops abnormally.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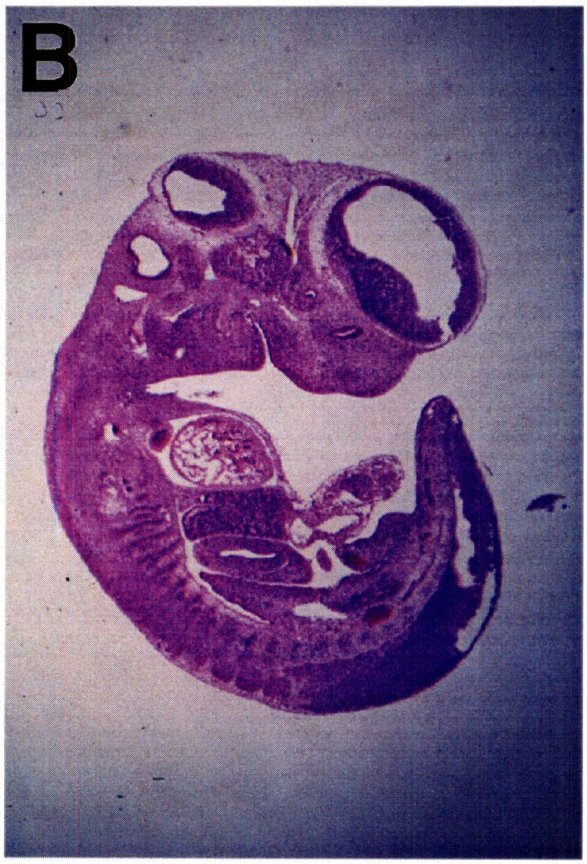
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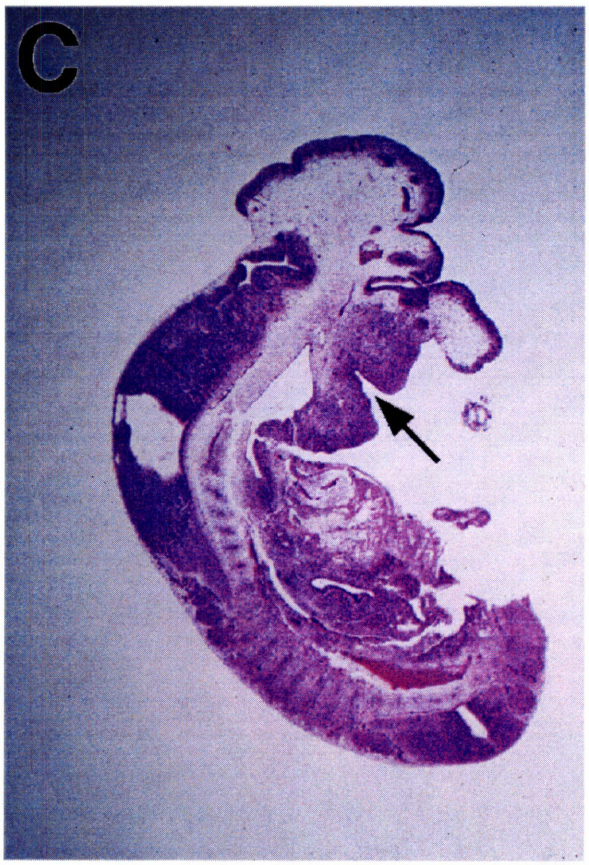
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APC And Tissue Formation

The early death *in utero* of Apc^{8} homozygotes prevented us from assessing the full effects of loss of *Apc* on the development and differentiation in most organ systems. To circumvent this difficulty, we generated chimeric embryos composed in part of homozygous mutant cells. We first created homozygous mutant $Apc^{8/8}$ ES cells by subjecting the 129Sv heterozygous $Apc^{8/+}$ ES cells to high concentrations of the drug G418 (Mortensen et al., 1992). These homozygous cells as well as heterozygous control ES cells that had retained the wild-type *Apc* following selection in high G418 were injected into wild-type C57BL/6 (B6) blastocysts.

Control chimeras created by injection of three independent clones of heterozygous $Apc^{8/+}$ ES cells that had remained heterozygous following G418 selection appeared normal at birth and for the one year that followed, even when these ES cells contributed to as much as 90% of the coat color in the chimeras. Descendants of these introduced heterozygous ES cells contributed to the germline tissue of chimeric mice, indicating that the earlier exposure of ES cells to high concentration of G418 did not have any deleterious effect on them.

In contrast, all chimeric mice derived from blastocysts injected with homozygous mutant $Apc^{8/8}$ cells died hours after birth. Injection of as few as two $Apc^{8/8}$ cells per 64-cell blastocyst resulted in the formation of craniofacial abnormalities in the newborns. Some pups were anencephalic while others were born with an open cranium and cleft palate (data not shown). Moreover, 2/20 of the embryos developed craniopharyngiomas (Fig. 7A), which in humans is seen as a rare childhood brain tumor.

In addition, the homozygous mutant chimeras developed numerous skin fibromas termed alternatively acrocorns or cutaneous tags (Fig. 7, C & D). These benign lesions, often present in several dozen per animal, were sometimes several millimeters in length and were found more commonly on the extremities and the face. The cells of hair follicles also formed abnormal structures termed epidermoid hair cysts (Fig. 7E). We observed the largest number of the skin

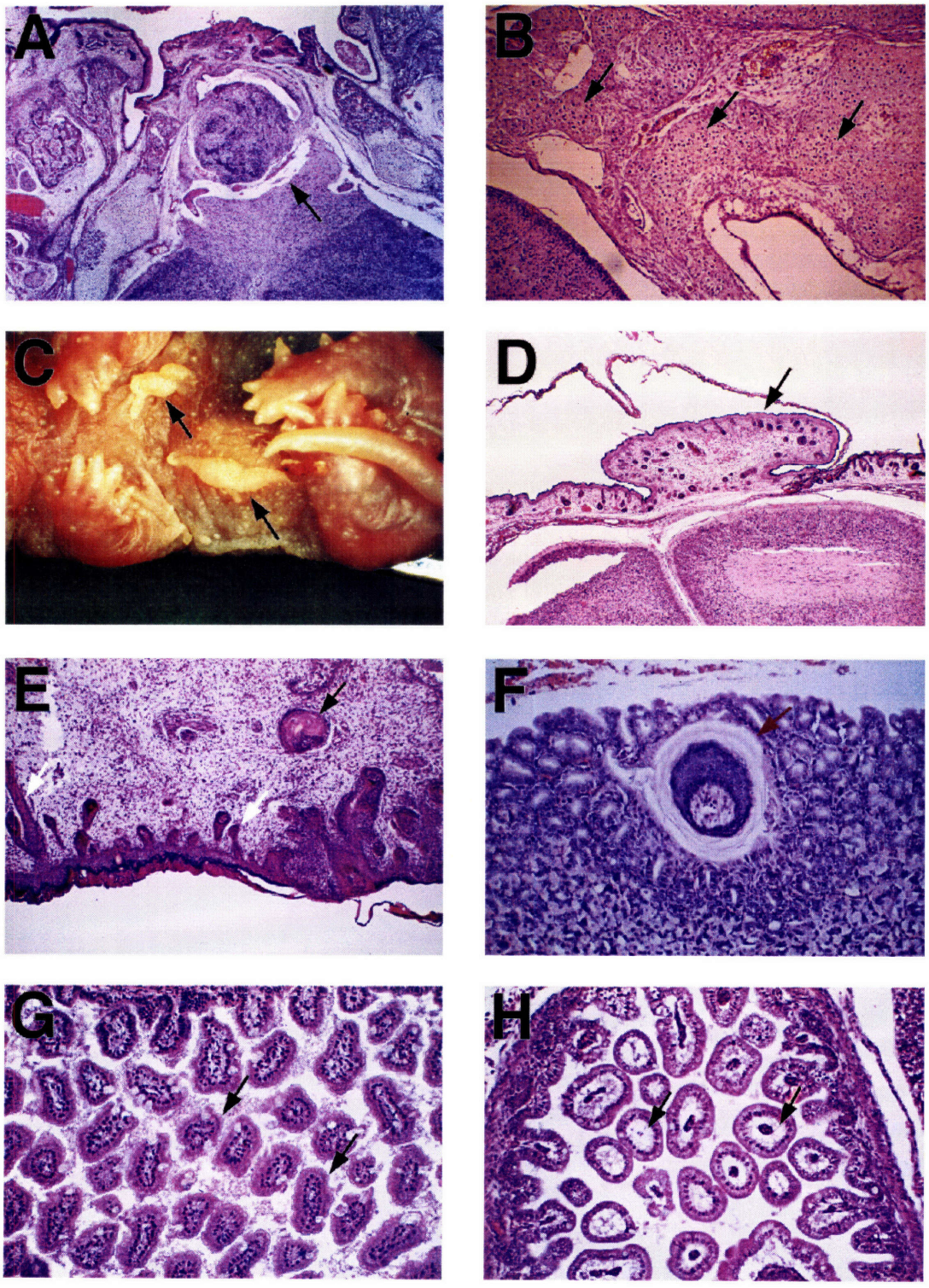
fibromas in those chimeras that we generated through injection of 10-20 homozygous mutant cells per embryo. Glucose phosphate isomerase (GPI) enzyme analysis, which shows the relative ratio of the isomers expressed by the 129 or B6 cells in a chimeric tissue, of several of these fibromas showed that they were composed of 40% 129Sv $Apc^{8/8}$ cells and 60% B6 $Apc^{+/+}$ cells (data not shown).

The newborn $Apc^{8/8}$ chimeras also displayed squamous cell metaplasia of the salivary and the harderian glands which was indistinguishable from that observed in the adult $Apc^{8/+}$ mice (Fig. 7F). Furthermore, a large fraction of the villi in the intestine of these chimeras had a dilated appearance. The connection between the formation of these dilated villi and the formation of adenomatous polyps remains unclear at present.

Finally, the facial bones in the $Apc^{8/8}$ chimeras looked overgrown upon gross examination. Histological examination revealed that all intramembraneous bones in the homozygous mutant $Apc^{8/8}$ chimeric newborns were dysplastic and displayed early signs of bone tumors (Fig. 7B). However, the endochondral bones that include the majority of the bones in the body displayed normal morphology when examined histologically (data not shown).

Figure 7

Effect of *Apc*^{8} on tissue formation and differentiation in newborn chimeras composed of C57BL/6 *Apc*$^{+/+}$ and 129Sv *Apc*$^{8/8}$ cells. (A) Craniopharyngioma (arrow, 50X magnification). (B) A dysplastic facial intramemraneous bone (arrows, 200X magnification). (C) Superficial view of skin fibromas (arrows, 6X magnification). (D) Histology of a skin fibroma (arrow, 20X magnification). (E) Epidermoid hair cyst (black arrow) and the finger-like projections of epithelial cells into dermal mesenchyme (white arrow) in skin (200X magnification). (F) Squamous cell metaplasia of the salivary gland (arrow, 400X magnification). (G) Transverse section of the normal villi in the intestine of newborn chimeras (arrows, 100X magnification). (H) Transverse section of the dilated villi in the intestine of newborn chimeras composed of C57BL/6 *Apc*$^{+/+}$ and 129Sv *Apc*$^{8/8}$ cells (arrows, 100X magnification).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Discussion

Hypomorphic Alleles of APC As The Cause of Human Attenuated Polyposis

In the present study, we have compared the effects on polyp formation of two alleles of the murine homologue of the *Adenomatous Polyposis Coli* gene (*Apc*), one causing a drastic reduction in the expression of *Apc* and another encoding a truncated APC protein. We observed that mice expressing wild-type APC protein at levels slightly more than 50% of normal, as seen in heterozygous $Apc^{8/+}$ mutants, developed an average of one polyp after 18 months, while other mice expressing 50% wild-type protein and 50% truncated protein, as seen in the $Apc^{Min/+}$ heterozygotes, developed an average of 110 polyps in five months (Fig. 2A & Table 1). It is unlikely that the observed difference in polyp number in the $Apc^{8/+}$ and $Apc^{Min/+}$ mice was caused by loci other than *Apc* because the effects of genetics and environment were controlled by expressing both alleles in mice of common genetic background and raising the mice in the same environment.

The findings of the present study are subject to two interpretations. One proposes that an allele encoding a truncated form of APC (Apc^{Min}) capable of oligomerization creates many polyps while an allele making very little full length protein (Apc^{8}) is unable to do so.While our results are suggestive of and are compatible with a dominant-negative mechanism of action of truncated forms of the APC protein, they do not provide definitive evidence in favor of it.

Instead, our findings show that another mechanism, that proposed by Varesco *et al.*, can explain the low polyp number in the AAPC families. Our findings support the notion that hypomorphic alleles of *APC* that reduce but do not completely eliminate APC expression can suppress polyp formation and cause attenuated polyposis when transmitted through the germline. According to this interpretation, the mutant Apc^{8} allele studied here, through expressing approximately 1% of an essentially wild type protein, is able to regulate cell proliferation in a

relatively normal fashion, even when present in a homozygous configuration. This in turn would suppress the tendency of the intestinal epithelium to spawn polyps.

APC And Differentiation of Intramembraneous Bones

The present study indicates that APC plays an important role in both normal differentiation and in the suppression of cellular proliferation in developing intramembraneous bones. The mesenchyme that is the embryonic progenitor of the bones in the face and the skull develops abnormally in $Apc^{8/8}$ embryos (Fig. 6). Moreover, by the last day of embryonic development, these embryonic tissues form bones that are dysplastic and display early signs of tumor phenotype (Fig. 7B). This suggests that mutant alleles of *APC* compromise normal differentiation and simultaneously initiate benign growths in the facial bones. Moreover, osteomas that progress to tumors arise in the bones of the face and the skull of 129 $Apc^{8/+}$ $p53^{+/-}$ mice (Fig. 4B & Table 2), the same small group of bones whose differentiation is abrogated by the loss of APC in development. These effects in turn may explain the predisposition of these bones to subsequent transformation, resulting in the osteomas that are seen in patients suffering from Gardner's syndrome. Based on these observations, we propose that APC-deficient cells in these tissues fail to participate in terminal differentiation, and that this failure in turn places them at risk for subsequent events leading to neoplasms.

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CHAPTER 3

Suppression of Intestinal Neoplasia by DNA Hypomethylation in Mice

Introduction

Methylation of mammalian DNA is found as the covalent modification of the fifth carbon position of the pyrimidine ring of cytosines in CpG dinucleotides (Adams and Burdon, 1982; Antequera and Bird, 1993; Gardiner-Garden and Frommer, 1987). Newly replicated DNA lacks this methylation in the nascent strand. Shortly after passage of the replication fork, a maintenance DNA methyltransferase methylates CpG dinucleotides on the newly synthesized strand at sites opposite to those methylated in the parental strand, thereby recreating the spectrum of methyl groups that existed prior to replication. To date, only one mammalian DNA (cytosine-5) methyltransferase enzyme (EC 2.1.1.37) has been identified and characterized (Bestor et al., 1988; Leonhardt and Bestor, 1993). The carboxy-terminal part of this protein shares sequence similarity with the conserved catalytic domain of prokaryotic DNA (cytosine-5) methyltransferases (Kumar et al., 1994; Lauster et al., 1989; Posfai et al., 1989). The mammalian enzyme has an additional large amino-terminal extension lacking sequence similarity to other known DNA methyltransferases. This amino-terminal region is thought to be a regulatory domain and is known to contain signals necessary for the targeted localization of the enzyme to replication foci in the nucleus (Leonhardt et al., 1992).

Changes in the pattern of DNA methylation have been correlated with a number of different processes in mammals. These include the expression level of genes (Yeivin and Razin, 1993), chromatin structure (Kass et al., 1993; Keshet et al., 1986; Tazi and Bird, 1990), the timing of DNA replication (Selig et al., 1988), genomic imprinting (Barlow, 1993; Li et al., 1993) and somatic X-chromosomal inactivation in females (Monk and Grant, 1990; Singer-Sam and Riggs, 1993). Whether DNA methylation is a cause or consequence of these processes remains to be resolved. Nevertheless, it is clear that DNA methylation is an essential process in mammalian development, since mouse embryos deficient for the known DNA methyltransferase do not survive past mid-gestation (Li et al., 1992).

Several observations implicate DNA methylation in cancer pathogenesis. Changes in the pattern of DNA methylation are commonly seen in human tumors (Baylin et al., 1991; Jones and Buckley, 1990; Laird and Jaenisch, 1994; Spruck et al., 1993). Some loci tend to show increased levels of DNA methylation (Baylin, 1992; Issa et al., 1994; Ohtani-Fujita et al., 1993), while others are hypomethylated (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1983; Goelz et al., 1985; Hanada et al., 1993; Rao et al., 1989; Vorce and Goodman, 1991). The global level of DNA methylation is generally lower in tumor cells than in normal cells (Feinberg et al., 1988; Gama-Sosa et al., 1983). This decrease in the overall level of DNA methylation is curious in light of the normal to high levels of DNA methyltransferase expression usually seen in tumor cells (El-Deiry et al., 1991; Issa et al., 1993; Kautiainen and Jones, 1986).

Some investigators have proposed that changes in DNA methylation contribute to oncogenesis by affecting the expression levels of proto-oncogenes and tumor-suppressor genes. Thus, DNA hypomethylation would allow increased expression of oncogenes; alternatively, DNA hypermethylation would help to silence tumor-suppressor genes. However, there is no direct evidence that either mechanism is operative in oncogenesis. Indeed, it is not even clear whether the changes in DNA methylation play a causal role in oncogenesis or whether they are merely a consequence of the transformed state of the tumor cell.

We have designed experiments in an attempt to distinguish between these alternatives. Using a combination of genetics and pharmacology, we have manipulated the levels of functional DNA methyltransferase in mice and determined the consequences for early steps of the neoplastic process. To do so, we used mice heterozygous for the Apc^{Min} mutation (*Min* mice) as a model system to assess the role of DNA methylation in early neoplastic transformation (Luongo et al., 1993; Luongo et al., 1994; Moser et al., 1990; Su et al., 1992). *Min* mice develop multiple intestinal adenomas within the first few months of life (Luongo et al., 1994; Moser et al., 1992; Moser et al., 1990). The number of intestinal polyps present in these mice at specific ages offered us a readily quantifiable measure of any effects that changes in DNA methylation might have on the incidence or rate of polyp formation.

Min mice carry a germline point mutation in the *Apc* tumor-suppressor gene, the mouse homolog of the human *APC* gene, which, when present in mutant form in the human germ line, results in the syndrome of familial adenomatous polyposis coli (FAP) (Su et al., 1992). FAP patients develop as many as a thousand benign colorectal polyps, some of which progress to malignancy if they are not removed (Groden et al., 1991; Ichii et al., 1992; Joslyn et al., 1991; Miyoshi et al., 1992; Nakamura, 1993; Powell et al., 1992). Similarly, mice carrying the *Min* allele of the *Apc* gene develop one hundred or more intestinal polyps in the first 6 months of life. These polyps thus provide a model system for the early stages of human colorectal cancer, for which changes in DNA methylation patterns and in methyltransferase expression have been well documented (El-Deiry et al., 1991; Feinberg et al., 1988; Gama-Sosa et al., 1983; Issa et al., 1993; Silverman et al., 1989).

Mice carrying germ-line mutations in the DNA methyltransferase gene have previously been reported (Li et al., 1993; Li et al., 1992). Mouse embryos homozygous for either the $Dnmt^N$ allele (Li et al., 1992) or the stronger $Dnmt^S$ allele (Li et al., 1993) die before birth. Heterozygous mice have approximately half wild-type levels of DNA methyltransferase expression, which is apparently sufficient to maintain normal levels of DNA methylation (Li et al., 1992).They appear otherwise phenotypically indistinguishable from their wild-type littermates.

In the present study, we further perturbed methyltransferase activity through use of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC), which is known to titrate out the enzyme activity by a covalent trapping mechanism (Jüttermann et al., 1994; Santi et al., 1983; Santi et al., 1984). The combination of *Dnmt* heterozygosity and treatment with 5-aza-dC might be predicted to reduce the functional levels of DNA methyltransferase to below the threshold needed for the maintenance of normal DNA methylation patterns. In this paper we employ this combined strategy to study the effects of reduced DNA methyltransferase activity on the number of intestinal adenomas appearing in mice carrying the *Min* allele of the *Apc* gene.

Materials and Methods

Mice

Mice were maintained in the facilities of the Whitehead Institute for Biomedical Research and were fed *ad libitum*. Their diet consisted of Agway Prolab Rat, Mouse and Hamster 3000 chow, which has a crude protein content of at least 22%, a crude fat content of at least 5% and a crude fiber content of at most 5%. C57BL/6 $Apc^{Min/+}$ mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. 129/SV $Dnmt^{S/+}$ mice were generated in our facilities (Li et al., 1993; Li et al., 1992).

5-aza-dC Injections

129/SV $Dnmt^{S/+}$ female mice were crossed to C57BL/6 $Apc^{Min/+}$ males. Progeny were weighed on a Mettler balance and injected weekly with either 5 μg 5-aza-dC per 5 g body weight (rounded to the nearest multiple of 5 g) or with its solvent, phosphate-buffered saline (PBS). 5-aza-dC (Sigma Chemical Co., St. Louis, MO, catalog number A-3656) was prepared as a 2.5 mg/ml solution in PBS and stored in aliquots at -70°C. Injections were performed with a 100 μl Hamilton 700 series syringe with a Luer tip and a PB600 dispenser attachment, allowing injection of 50 discrete 2 μl volumes. The injections were started on day 7 of age and continued for 100 days (14 injections), after which the mice were monitored for an additional 80 days and then sacrificed.

Polyp Analysis

The entire intestine was excised immediately following sacrifice, fixed and washed with Bouin's fixative and subjected to a systematic microscopic screen for polyp formation along the entire length of the intestine. Adenomas were counted if they had reached a size of at least several villi. The investigator counting the adenomas was blind to both *Apc* and *Dnmt* genotypes. Adenomas

destined for DNA analysis were micro-dissected from intestines that had been washed with PBS, before being fixed with Bouin's fixative.

DNA Methylation Analysis

DNA was prepared from frozen tissue samples as described previously (Laird et al., 1991). Five μg of genomic DNA was digested with the restriction enzyme HpaII (New England Biolabs, Beverly, MA) as specified by the manufacturer. After agarose gel electrophoresis followed by Southern blotting, the filters were hybridized with a probe containing a

Allelic Loss Analysis

The murine *Apc* cDNA sequence was obtained from GenBank (accession no. M88127) (Su et al., 1992). A 619-bp fragment spanning the site of the *Min* mutation was amplified from genomic DNA samples by PCR using oligonucleotides MAPC-15 (*Apc* Sequence 2859 - 2841) (5' -TTCCACTTTGGCATAAGG C-3') and MAPC-9 (APC Sequence 2241 - 2258) (5'-GCCATCCCTTCACGTTAG-3'). The PCR products were gel-fractionated on a 2% agarose gel, Southern blotted and hybridized with three probes in sequential hybridizations. The three probes were Min-Sense(*Apc* Sequence 2542 - 2556) (5'-AGAAGTTAGGAGAGA-3'), Apc-Sense (*Apc* Sequence 2542 - 2556) (5'-AGAAGTTTGGAGAGA-3') and Apc-Uni (*Apc* Sequence 2530-2544) (5'-TCTGAGAAAGACAGA-3'). Min-Sense hybridizes specifically to the Apc^{Min} mutant allele while Apc-Sense hybridizes specifically to the wildtype allele. Apc-Uni hybridizes to both alleles. The hybridization signals were quantitated using a Fuji phosphoimager. The signal levels in the Apc-Uni hybridization were used to normalize the amount of DNA in each lane.

Results

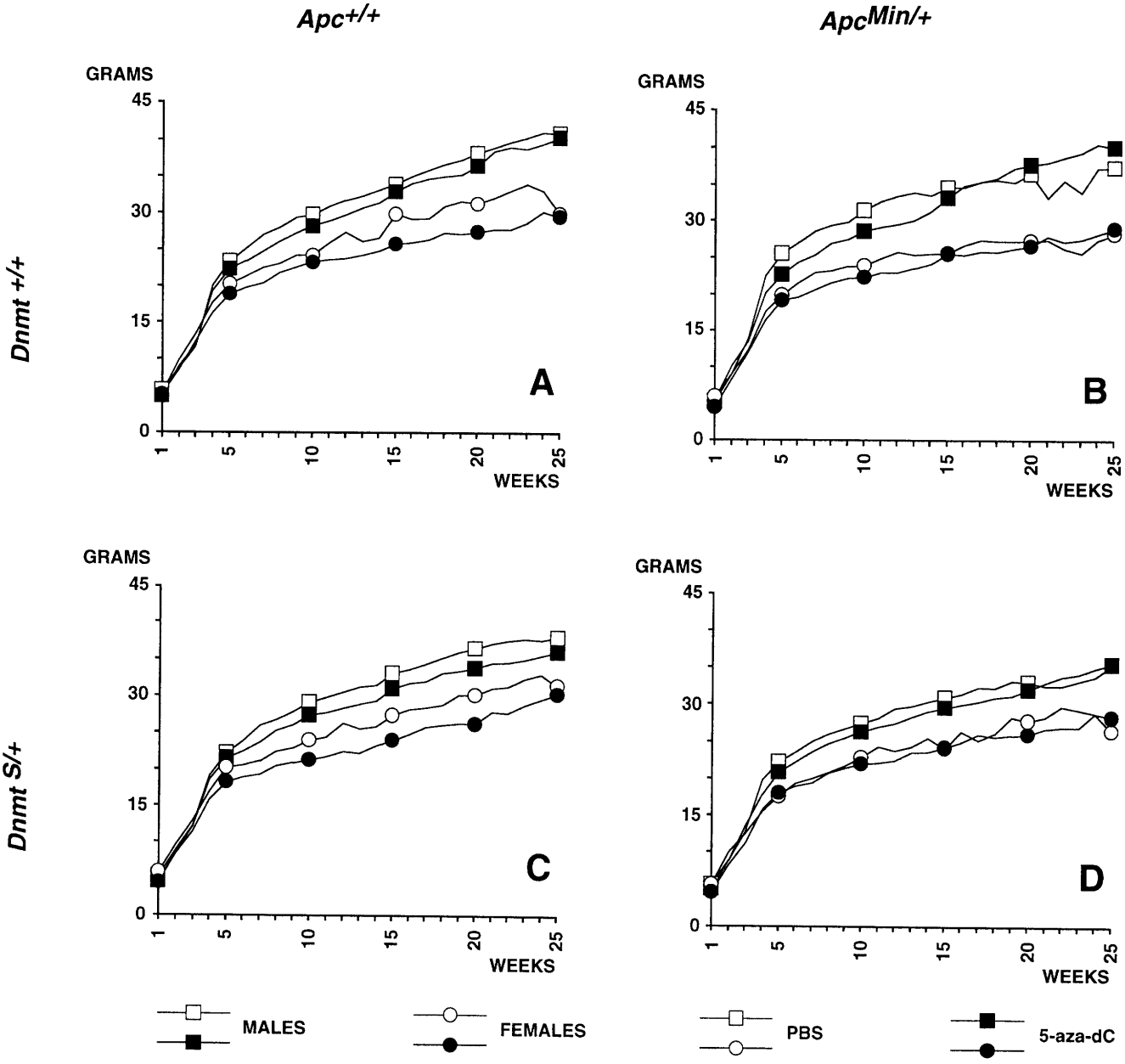
Effects of Decreased DNA Methyltransferase Activity on the Number of Intestinal Adenomas

In order to study the effects of DNA methyltransferase levels on intestinal polyp formation, we crossed 129/SV *Dnmt*$^{S/+}$ female mice with C57BL/6 *Apc*$^{Min/+}$ males. In addition, we determined the maximal dose of 5-aza-dC that mice could tolerate during an extended treatment protocol starting at one week of age. This dose, 5 μg of 5-aza-dC per 5 grams body weight, was given weekly by subcutaneous injection to the progeny of the cross described above. Control offspring were injected with phosphate-buffered saline (PBS) instead of 5-aza-dC. The injections were performed by investigators blind to the genotypes of the animals.

Previous experience indicated that at 100 days of age, intestinal polyps are just at the threshold of detectability in this strain background. During the subsequent 80 days, such polyps grow to a size that is readily visible. For this reason, mice were injected for 100 days (14 injections) . The drug treatment was then discontinued and the number of polyps was monitored 80 days later, allowing us to gauge the effects of DNA methyltransferase levels on polyp initiation and early growth during the first 100 days of life. In doing so, we avoided any cytotoxic or cytostatic effects that 5-aza-dC might have on the expansion of cell populations in the polyps during the growth period from 100 to 180 days. Figure 1 shows growth curves for the four different genotypes generated in this cross. As is apparent, the treatment with 5-aza-dC did not have a statistically significant effect on the body weights of the mice.

Figure 1

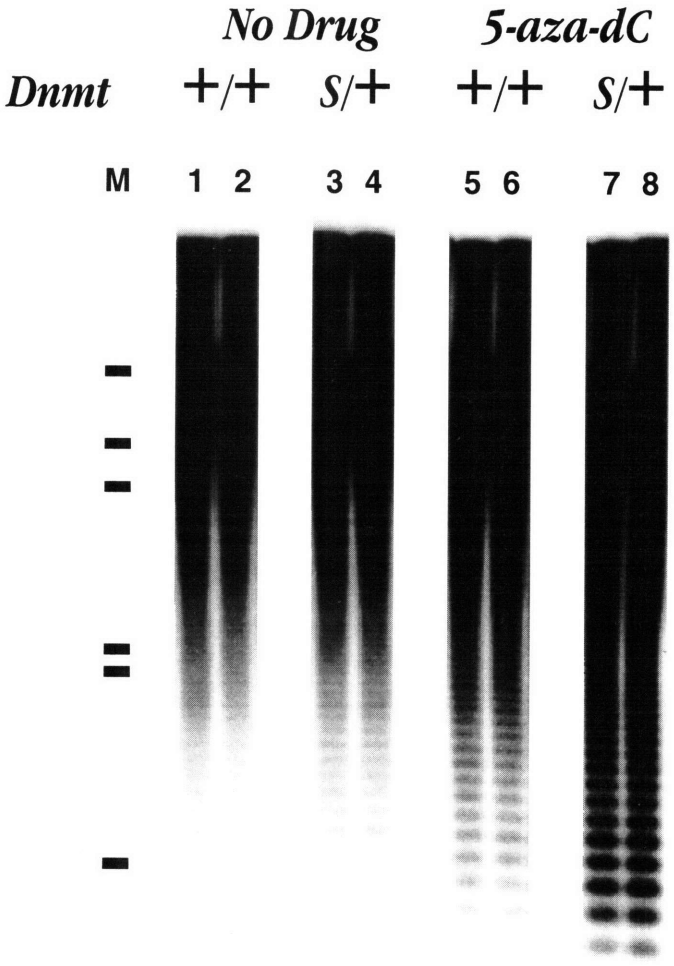
Growth curves of mice treated weekly with either PBS or 5-aza-dC. 129/SV *Dnmt*$^{S/+}$ female mice were crossed to C57BL/6 *Apc*$^{Min/+}$ males. Ungenotyped progeny were weighed and injected weekly with either 5 μg 5-aza-dC per 5 g bodyweight or with its solvent, phosphate-buffered saline (PBS). The injections were continued for 100 days (14 injections), after which the mice were monitored for an additional 80 days and then sacrificed. The average weights were calculated for each of the genotypes, sexes and treatments. The weight measurements were plotted by week. However, the symbols are indicated only every five weeks to preserve clarity. Squares indicate males. Circles indicate females. Open symbols indicate PBS treatment. Closed symbols indicate 5-aza-dC treatment. Panel A shows *Dnmt*$^{+/+}$, *Apc*$^{+/+}$ mice. Panel B shows *Dnmt*$^{+/+}$, *Apc*$^{Min/+}$ mice. Panel C shows *Dnmt*$^{S/+}$, *Apc*$^{+/+}$ mice. Panel A shows *Dnmt*$^{S/+}$, *Apc*$^{Min/+}$ mice.


Apc+/+
ApcMin/+
Dnmt +/+
Dnmt S/+
GRAMS
45
30
15
0
1
5
10
15
20
25
WEEKS
A
B
C
D
MALES
FEMALES
PBS
5-aza-dC

A subset of animals was sacrificed at 100 days to determine the DNA methylation levels in the colon immediately following 5-aza-dC treatment. Figure 2 shows an analysis of DNA methylation levels of genomic colon DNA derived from the four categories of experimental animals. We compared DNA methylation levels by Southern blot analysis of restriction digests with the DNA methylation-sensitive enzyme HpaII, hybridized with a centromeric satellite repeat probe (Sanford et al., 1987) (see Figure 2). In this assay, reduced levels of DNA methylation are revealed by a relative increase in lower-molecular-weight bands. The analysis in Figure 2 shows that both *Dnmt* heterozygosity and 5-aza-dC treatment have a synergistic effect in causing hypomethylation of on colon DNA. The effect of 5-aza-dC alone appears to be stronger than that of *Dnmt* heterozygosity.

Figure 2

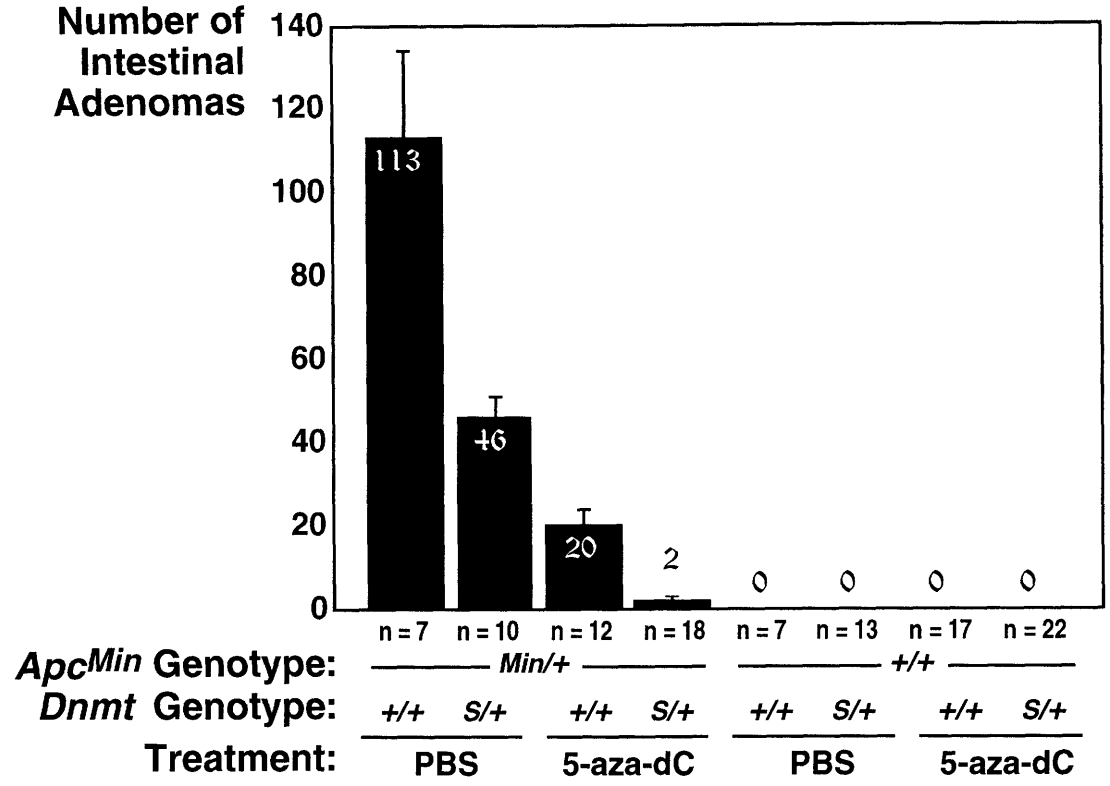
DNA Methylation Analysis of Genomic Colonic DNA from Mice Treated Weekly with or without 5-aza-dC. Mice derived from a cross between 129/Sv *Dnmt*$^{S/+}$ females and a C57BL/6 male were injected weekly with 5-aza-dC for 100 days (14 weeks) or untreated. Genomic DNA from colons collected from 100-day-old mice was isolated as described previously(Laird et al., 1991) . Analysis of DNA methylation levels involved digestion of genomic DNA by the methylation-sensitive restriction enzyme HpaII, followed by hybridization with a centromeric satellite repeat probe (Chapman et al., 1984) for a reflection of the global DNA methylation status The *Dnmt* genotypes and treatments are indicated above the lanes.


No Drug
5-aza-dC
Dnmt
+/+
S/+
+/+
S/+
M
1 2
3 4
5 6
7 8

The number of intestinal polyps at sacrifice at 180 days was determined by examination of the mucosa over the entire length of the intestine under a dissecting microscope. Figure 3 shows the averaged results obtained for the various experimental groups. Mice wild-type at the *Apc* locus did not develop intestinal polyps with any combination of *Dnmt* genotype or drug treatment. $Apc^{Min/+}$ heterozygous mice developed an average of 113 polyps in the $Dnmt^{+/+}$ PBS control group. $Dnmt^{S}$ heterozygosity reduced the number to a statistically significant lower average of only 46 polyps. In addition, adenomas in $Dnmt^{S/+}$ mice were on average smaller than those in the $Dnmt^{+/+}$ Min cohort (data not shown). A more pronounced effect was seen in the$Apc^{Min/+}$ heterozygous mice treated with 5-aza-dC. Treatment of *Dnmt* wild-type mice with 5-aza-dC reduced the number of polyps from 113 to an average of 20. The combined effects of *Dnmt* heterozygosity and 5-aza-dC treatment resulted in only 2 intestinal adenomas.

Figure 3

The number of intestinal polyps in mice treated weekly with either PBS or 5-aza-dC. 129/SV *Dnmt*^{S} /+ female mice were crossed to C57BL/6 *Apc*^{Min} /+ males. Ungenotyped progeny were weighed and injected weekly with either 5 μg 5-aza-dC per 5 g bodyweight or with its solvent, phosphate-buffered saline (PBS). The injections were continued for 100 days (14 injections), after which the mice were monitored for an additional 80 days and then sacrificed. The entire intestine was excised immediately following sacrifice, fixed and washed with Bouin's fixative and subjected to a systematic microscopic screen for polyp formation along the entire length of the intestine. The investigator counting the polyps was blind to both *Apc* and *Dnmt* genotypes. The average number of polyps for each group is shown as a bar with the average also indicated above or within the bar. The standard error of mean for each value is indicated by the thin error bars. The treatments, genotypes of the mice, and number of mice in each experimental group are indicated below the bars. All mice for this experiment were generated by the same limited number of parents and were maintained in the same room with the same food and cage conditions.

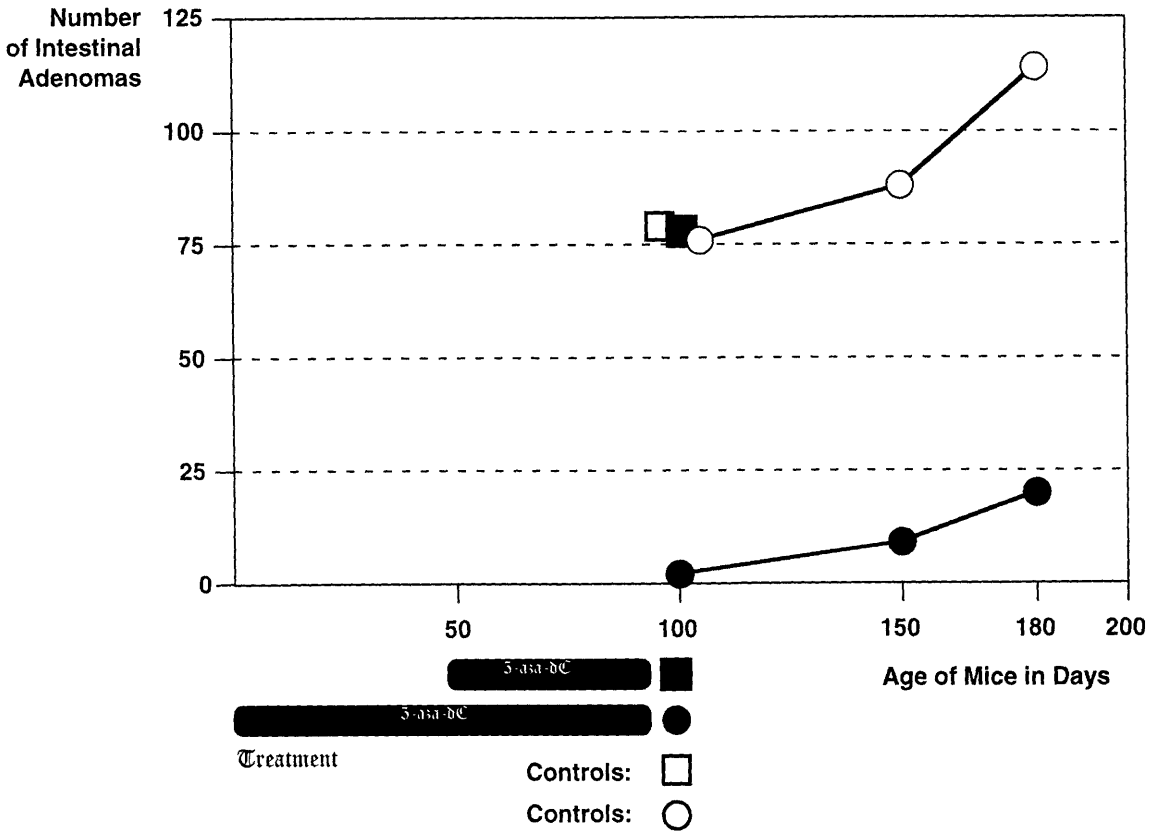

Number of Intestinal Adenomas
140
120
100
80
60
40
20
0
113
46
20
2
0
0
0
0
n = 7
n = 10
n = 12
n = 18
n = 7
n = 13
n = 17
n = 22
ApcMin Genotype:
Min/+
+/+
Dnmt Genotype:
+/+
S/+
+/+
S/+
+/+
S/+
+/+
S/+
Treatment:
PBS
5-aza-dC
PBS
5-aza-dC

Early Administration of 5-aza-dC is Required for its Effect on Polyp Number

While treatment of *Min* mice with 5-aza-dC had a dramatic effect on the number of intestinal polyps, the available evidence did not provide strong indications whether the effect was attributable to an early influence on the rate of initiation of adenoma formation or, alternatively, to a later cytostatic or cytotoxic influence of 5-aza-dC on already initiated adenomas. An indication that 5-aza-dC affects polyp initiation came from experiments in which drug treatment was delayed until day 50 in a cohort of C57BL/6 *Min* mice. In this strain background, the majority of polyps become visible between day 50 and day 100 of age (A.F., unpublished data, and (Moser et al., 1992; Moser et al., 1990)).

Figure 4

The number of intestinal polyps in mice with various timed treatments and time of sacrifice. C57BL/6 *Apc*$^{Min/+}$ males were crossed to either 129/SV (circles) or to C57BL/6 female mice (squares). The progeny were started on weekly injections of 5 μg 5-aza-dC per 5 g bodyweight at either 50 days (closed squares) or 7 days of age (closed circles). Control mice (open symbols) were either injected with PBS or monitored without injection. In all cases, the injections were continued until the mice were 100 days old. The mice were sacrificed at either 100, 150 or 180 days of ages, as indicated and subjected to an intestinal polyp count as described for Figure 3. The sizes of the experimental groups were as follows: open squares: n=6; closed squares: n=9; open circles: n=2, n=5 and n=13 for 100, 150 and 180 days respectively; closed circles: n=3, n=2 and n=12 for 100, 150 and 180 days respectively. The treatments are indicated schematically at the bottom of the figure.


Number of Intestinal Adenomas
125
100
75
50
25
0
50
100
150
180
200
Age of Mice in Days
5-ASA-DC
5-ASA-DC
Treatment
Controls:
Controls:

Loss of Heterozygosity at the *Apc* Locus

The precise nature of the interactions of DNA methylation and the mutant Apc^{Min} allele in tumor development were not made clear by these analyses. The paradigm developed from extensive study of the *Apc* and similarly behaving tumor suppressor genes dictates that loss of the wildtype *Apc* allele should accompany tumor development (Boynton et al., 1992; Brewster et al., 1994; Ichii et al., 1992; Levine, 1993; Nakamura, 1993; Scrable et al., 1990; Tamura et al., 1994; Weinberg, 1991). We wished to ascertain that this genetic mechanism operated in all of the experimental groups studied, regardless of the status of their *Dnmt* locus or their history of 5-aza-dC treamtent.

We tested polyps from the different cohorts for loss of heterozygosity at the site of the Apc^{Min} mutation. A segment of DNA encompassing the mutation was amplified from DNA derived from polyps and from adjacent normal tissue. The PCR products were hybridized consecutively with oligodeoxynucleotide probes specific for the Apc^{Min} allele, the Apc^{+} allele and finally with a probe recognizing both alleles. The relative strengths of the signals derived from the Apc^{Min}- and Apc^{+}-specific probes were calculated after quantitation on a phospho-imager and subsequent normalization to the signal deriving from the probe recognizing both alleles. Loss of heterozygosity in polyps should reveal a reduction in the relative level of the Apc^{+} signal and an increase in the relative level of the Apc^{Min} signal in polyps compared to normal adjacent tissue.

Figure 5 shows the analysis of a representative series of polyps from *Dnmt* wild-type mice, along with control DNAs derived from tail biopsies from $Apc^{Min/+}$ and $Apc^{+/+}$ mice. The results obtained for all polyps analyzed are summarized in Table 1. Loss of the wild-type allele was seen in the majority of polyps derived from all experimental groups. These results are in agreement with the loss of heterozygosity at the *APC* locus seen as an early event in human cancer (Boynton et al., 1992; Brewster et al., 1994; Ichii et al., 1992; Nakamura, 1993; Tamura et al., 1994). The high frequency of loss of heterozygosity at the *Apc* locus is close to the 100% loss of *Apc* heterozygosity in Apc^{Min}-induced intestinal adenomas found by Luongo *et al.* (Luongo et al., 1994).

Figure 5

Analysis of allelic loss at the *Apc* locus in colon adenomas. Intestines from randomly chosen mice from the experiment shown in Figures 1 and 2 were washed in PBS. Large distinct adenomas were excised and processed for DNA isolation as described previously (Laird et al., 1991). In addition, normal intestinal mucosa adjacent to the polyp was excised and is shown immediately to the right of each polyp in the figure. The normal tissue in lane 9 is the control for both lanes 7 and 8. Lanes 10 and 11 contain samples derived from tail biopsies as control DNAs. Lane 10 is from an $Apc^{+/+}$ mouse. Lane 11 is from an $Apc^{Min/+}$ mouse. PCR reactions were performed as described in Experimental Procedures. The PCR products were resolved by gel electrophoresis, blotted, and hybridized consecutively with oligodeoxynucleotide probes specific for the Apc^{Min} allele (Panel A), the Apc^{+} allele (Panel B) and finally with a probe recognizing both alleles (Panel C).

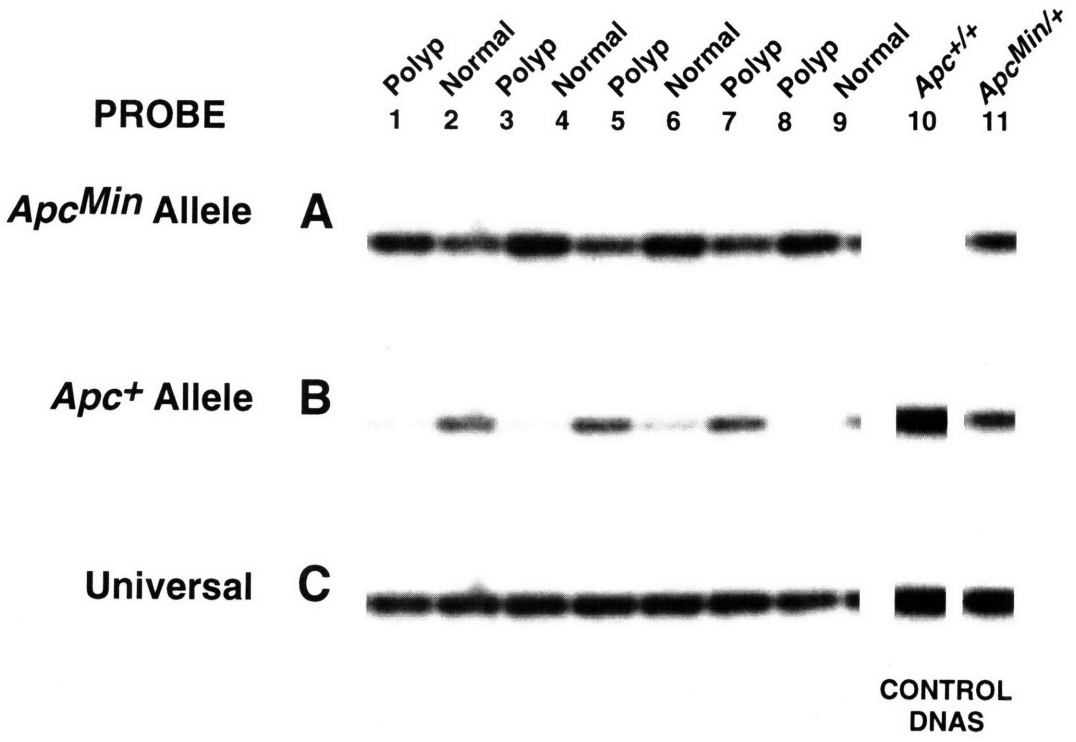

Polyp
Normal
Polyp
Normal
Polyp
Normal
Polyp
Polyp
Normal
Apc+/+
ApcMin/+
PROBE
1 2 3 4 5 6 7 8 9 10 11
ApcMin Allele A
Apc+ Allele B
Universal C
CONTROL DNAS

Table 1

Analysis of allelic loss at the *Apc* locus in intestinal adenomas. Intestines from randomly chosen mice from the experiment shown in Figures 1 and 2 were washed in PBS. Large distinct adenomas were excised and processed for DNA isolation as described previously (Laird et al., 1991). In addition, normal intestinal mucosa adjacent to the polyp was excised as a reference standard. PCR reactions were performed as described in Experimental Procedures. The PCR products were resolved by gel electrophoresis, blotted, and hybridized consecutively with oligodeoxynucleotide probes specific for the Apc^{Min} allele, the Apc^{+} allele and finally with a probe recognizing both alleles. The relative hybridization signals were quantitated with a phospho-imager. Signals were first normalized using the hybridization signal specific for both alleles (Apc-Uni probe). Subsequently, the ratio of Apc^{Min} to Apc^{+} hybridization was calculated. Most polyps had ratios of more than 2.0. The four polyps defined as not showing loss of heterozygosity had ratios between 0.9 and 1.1, within the range seen for all normal adjacent tissues. *WT* control = adenomas from 129Sv/B6 $Apc^{Min/+}$ $Dnmt^{+/+}$; *HET* control= adenomas from 129Sv/B6 $Apc^{Min/+}$ $Dnmt^{S/+}$; *WT* 5-aza-dC (0-100)= adenomas from 129Sv/B6 $Apc^{Min/+}$ $Dnmt^{+/+}$ treated with aza-dC for a 100 days after birth; *WT* 5-aza-dC (50-100)= adenomas from 129Sv/B6 $Apc^{Min/+}$ $Dnmt^{+/+}$ treated with aza-dC for days 50-100 after birth; *HET* 5-aza-dC (0-100)= adenomas from 129Sv/B6 $Apc^{Min/+}$ $Dnmt^{S/+}$ treated with aza-dC for a 100 days after birth.

Experimental Group	**Number of Polyps Analyzed**	**Polyps with Loss of Heterozygosity**	**Polyps with no Loss of Heterozygosity**
WT Control	12	11	1
HET Control	6	5	1
WT 5-aza-dC (0-100)	5	5	0
WT 5-aza-dC (50-100)	4	3	1
HET 5-aza-dC (0-100)	5	4	1
Total	32	28	4

We found several polyps that lacked any sign of loss of heterozygosity (See Figure 5 and Table 1). Such cases do not seem to be over-represented in any particular cohort. It is possible that these polyps have lost the function of the wild-type allele through another mechanism, such as the acquisition of a mutation elsewhere in the *Apc* gene. Alternatively, loss of function of the wild-type Apc protein may not be an absolute requirement for polyp formation. In support of this, we have recently obtained evidence for a dominant mode of action for the truncated protein product of the Apc^{Min} allele by comparing the phenotypes of mice heterozygous for different *Apc* mutations (for a discussion see Chapter 1).

Discussion

DNA Methyltransferase is an Oncogenic Determinant in *Min*-Induced Intestinal Neoplasia

Mice carrying the *Min* allele of the *Apc* gene are predisposed to intestinal polyps which become readily observable at several months of age (Luongo et al., 1993; Luongo et al., 1994; Moser et al., 1992; Moser et al., 1990; Su et al., 1992). As demonstrated here, the number of these polyps can be reduced in two ways - through treatment with the drug 5-aza-dC or through introduction of a mutant allele of the DNA methyltransferase gene. These two perturbants are also united by a common biochemical mechanism of action. 5-aza-dC, when incorporated into DNA, causes the formation of stable covalent adducts between DNA methyltransferase and the DNA, resulting in the depletion of free, active enzyme from the cell (Jüttermann et al., 1994; Santi et al., 1983; Santi et al., 1984). The $Dnmt^{S}$ mutation achieves the same end result through inactivation of a chromosomal gene copy encoding this enzyme. Indeed, both Dnmt heterozygosity and 5-aza-dC treatment result in a reduction of genomic DNA methylation levels as represented by an analysis of centromeric repeat sequences.

The genesis of these polyps depends upon the frequency with which they are initiated and their subsequent ability to proliferate to a size that makes them visible under the dissecting

microscope. Thus, the present phenomena need to be interpreted in light of these two events governing observed polyp number. The initiation of polyp formation would appear to depend upon low frequency stochastic events, in that the number of distinct clones of polyps is many orders of magnitude lower than the number of cells in the target tissue. This stochastic event could involve the loss of the wild-type *Apc* allele that initially coexists with the mutant Apc^{Min} allele in all cells of the intestinal epithelium. This is a possibility that could be neither proven nor refuted by the present data.

The decrease in polyp number in mice having reduced levels of active DNA methyltransferase was opposite to the result expected for a model in which global DNA hypomethylation contributes to the neoplastic process in colorectal cancer. The effect of DNA methyltransferase on *Min*-induced intestinal neoplasia appeared to be on both polyp number and size but not morphology. The adenomas in the $Dnmt^{S/+}$ heterozygous mice appeared to be somewhat smaller, but this was not be confirmed by a statistical morphometric analysis. We did not find any evidence for a difference in the state of differentiation or progression of the adenomas from the different experimental groups as assessed by histological examination of the polyps in *Min* mice from all four cohorts.

Significantly, a substantial reduction in polyp number was achieved in the absence of 5-aza-dC treatment. This decrease, seen in the context of $Apc^{Min/+}$ heterozygosity, is the first example of an observed phenotypic difference between *Dnmt* wild-type mice and $Dnmt^{S/+}$ heterozygotes, which appear to have normal levels of DNA methylation (Li et al., 1992). Furthermore, these results demonstrate that *Dnmt* is a genetic modifier of the Min phenotype. It is the second such modifier to be described, the first being the AKR/J allele of *Mom-1* (Dietrich et al., 1993; Moser et al., 1992). *Dnmt* and *Mom-1* are distinct loci, however. *Mom-1* maps to mouse chromosome 4 (Dietrich et al., 1993), while *Dnmt* maps to mouse chromosome 9 (Copeland et al., 1993).

As can be seen in Figure 4, the delay in the start of 5-aza-dC treatment abolished its inhibitory effect on the number of polyps counted at day 100. Furthermore, there was no readily

apparent difference in the size of polyps from mice that had received a delayed 5-aza-dC treatment compared to their PBS-treated littermates. These results suggested that the initiation of polyp formation occurs prior to day 50 of age and is followed by a period of expansion of cell populations in already initiated polyps in the subsequent 50 day period. In order to exert its effects on polyp number, 5-aza-dC must be present during this critical formative period rather than during the subsequent proliferative phase. As such, we consider it unlikely that 5-aza-dC acts through a cytotoxic or cytostatic effect on the cells in already formed adenomatous cell clones.

It is also apparent from Figure 4 that the majority of polyps at day 180 in mice treated from day 0 to 100 appeared only after cessation of drug treatment. These adenomas may represent rapidly growing polyps initiated after day 100, since the experiments described above argue against an inhibitory effect of 5-aza-dC on the expansion of previously initiated polyps. This raises the possibility that continued treatment with 5-aza-dC could lead to the virtual elimination of polyp formation.

An early, rate-limiting stochastic event rather than a later proliferative process would appear to be the target of inhibition by the 5-aza-dC that we have administered to these mice. We conclude this from our observations of the comparative effects of this drug administered at weekly intervals beginning either at the first week of life or only at day 50. The first protocol involving early administration severely depresses polyp number; delayed administration in contrast has no effects on polyp number or size. During days 50 to 100 of life, these polyps undergo a size proliferation that causes them to become visible in the dissecting microscope. Therefore, we conclude that 5-aza-dC has minimal effects on proliferation but profound effects on the earlier, initiation, which must occur well before day 50.

Consistent with this conclusion is the work that documents the cytostatic/cytotoxic effects of 5-aza-dC treatment. It was found that these anti-proliferative effects are not mediated through loss of DNA methylation *per se*, but rather through the formation of stable covalent complexes between the DNA methyltransferase enzyme and the incorporated cytosine analog (Jüttermann et al., 1994; Santi et al., 1983; Santi et al., 1984). Consequently, cells that have lower levels of DNA

methytransferase are less sensitive to growth inhibition by 5-aza-dC (Jüttermann et al., 1994). In the present study, we observed the exact opposite: the ability of 5-aza-dC to suppress polyp formation was stronger in mice with levels of DNA methyltransferase reduced due to the *Dnmt*^{S} mutation.

DNA Methyltransferase Levels and Polyp Formation - Possible Mechanisms

Studies on DNA methylation changes in human cancer cells have presented a somewhat confusing picture. Hypotheses to explain the involvement of DNA methylation in cancer fall broadly into two classes. The first class of models propose that changes in DNA methylation contribute to the oncogenic process through effects on gene expression. The other class propose that the major role of DNA methylation in cancer is mediated through the disproportionately high rate of mutation of methylated cytosine residues.

Among the models that address gene expression-mediated effects of DNA methylation in oncogenesis, two opposing mechanisms have been proposed. One model proposes that the commonly observed global DNA hypomethylation in human tumors is of selective advantage to the tumor cell through facilitated proto-oncogene expression (Feinberg and Vogelstein, 1983) (Hanada et al., 1993; Rao et al., 1989; Vorce and Goodman, 1991). In the alternative model, high expression of the DNA methyltransferase is thought to drive hypermethylation of specific loci such as tumor-suppressor genes or genes specific to differentiated cell types (Baylin et al., 1991; El-Deiry et al., 1991; Greger et al., 1989; Issa et al., 1994; Issa et al., 1993; Kautiainen and Jones, 1986; Ohtani-Fujita et al., 1993; Silverman et al., 1989). This would allow outgrowth of undifferentiated neoplastic cells. The suppression of polyp formation by the reduction of DNA methyltransferase and DNA methylation levels is inconsistent with the first mechanism, which favors on oncogenic effect of DNA hypomethylation. Our results are consistent with the tumor-suppressor gene hypermethylation model, although one would have to argue that reduction of

global DNA methyltransferase levels would affect local DNA hypermethylation. It is not clear how regional DNA hypermethylation is achieved in the face of global DNA hypomethylation.

In contrast to the gene-expression-mediated models discussed above, the mutation-mediated models propose that DNA methylation contributes to oncogenesis through a disproportionately high mutation rate of 5-methylcytosine residues (Jones et al., 1992; Laird and Jaenisch, 1994; Spruck et al., 1993). Deamination at C-4 of both methylated and unmethylated cytosine residues is known to occur spontaneously to yield thymine and uracil, respectively (Ehrlich et al., 1986; Frederico et al., 1990; Shen et al., 1994; Wang et al., 1982). A uracil residue is ultimately substituted by thymine if it is not repaired prior to DNA replication. The occurrence of C-to-T transition mutations is considerably higher for 5-methylcytosine than for unmethylated cytosine residues. In fact, mutations of this sort are of significance in human tumorigenesis. Approximately 47 % of the characterized point mutations in the p53 tumor-suppressor gene in colorectal tumors are nucleotide transitions within CpG dinucleotides, the target for DNA methylation (Greenblatt et al., 1994). This frequency contrasts with the fact that CpG dinucleotides occur within the coding region of the wild-type human p53 gene at a frequency of 3.3% (39 out of 1179). The target size for mutagenesis at either nucleotide within CpG dinucleotides is therefore 6.6%. Mutations at CpG dinucleotides are thus over represented approximately 7-fold in human colorectal tumors (Greenblatt et al., 1994). Reduced cellular DNA methylation levels could lead to a lower rate of spontaneous C-to-T transitions. If rate-limiting mutational events, in addition to the loss of the wild-type *Apc* allele, were required for polyp formation, then a reduced rate of C-to-T transitions could result in a lower number of intestinal polyps.

The high mutation rate of methylated cytosine residues might also be achieved through a different mechanism that directly involves the DNA methyltransferase enzyme (reviewed in: (Laird and Jaenisch, 1994)). The covalent enzyme adduct formed between DNA methyltransferase and its substrate cytosine has been found to destabilize the amine group of the latter, resulting in an enhanced rate of deamination. Normally, this adduct is formed only ephemerally; the presence of the methyl donor S-adenosyl methionine (SAM) results in the rapid completion of the reaction and

the disappearance of this destabilized intermediate. But in the absence of sufficient amounts of SAM, the half-life of this intermediate may be substantially extended, resulting in turn in a great increase in cytosine deamination. This has been demonstrated experimentally for a prokaryotic DNA methyltransferase (Shen et al., 1992). If such a mechanism were also applicable to the mammalian enzyme, limiting levels of SAM in the intestinal epithelium could result in a mutagenic process with an activity correlating with DNA methyltransferase levels. We note in passing that diets deficient in methionine have been reported to predispose humans to increased numbers of colonic polyps (Giovannucci et al., 1993). Whether this is only an adventitious association or derives from a mechanism associated with DNA methyltransferase-mediated deamination and mutation is not revealed by presently available evidence. Experiments in rodents have shown that methyl-deficient diets, which lead to SAM depletion, enhance liver tumorigenesis (Mikol et al., 1983; Shivapurkar et al., 1986).

Recently, the laboratories of Peter Jones and Richard Roberts have found evidence for yet another mechanism through which DNA methyltransferase enzymes could exert an effect on cytosine mutation rates (Yang et al., personal communication and Klimasauskas and Roberts, personal communication). Both groups have demonstrated that a prokaryotic DNA methyltransferase is capable of high-affinity binding to G:U mismatches. In addition, the enzyme is then capable of converting the uracil residue to thymine by the transfer of a methyl group. G:U mismatch repair would be inhibited directly by the binding of the DNA methyltransferase as well as indirectly by the conversion of U to T, which is less efficiently repaired in a mismatch with G (Brown and Brown, 1989; Shenoy et al., 1987). Whether the mammalian DNA methyltransferase exhibits similar properties remains to be seen.

Future experiments should address the precise effects of 5-aza-dC treatment and the inactivated *Dnmt* allele on the mutational rate of defined genes within specific tissues of the mouse. Such experiments should indicate whether these perturbants directly suppress mutation rate and have profound effects on the rate of polyp initiation in the *Min* mice.

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Chapter 4

Phenotype of Mice Lacking Functional *Deleted in Colorectal Cancer* (*DCC*) Gene

Introduction

Deleted in Colorectal Cancer (*DCC*)was isolated as a candidate tumor suppressor gene from the region of chromosome 18q that is frequently subjected to LOH (Fearon et al., 1990). *DCC* encodes a group of structurally similar 175-190 Kilodalton (kDa) transmembrane proteins which are members of the immunoglobulin gene superfamily. These proteins are characterized by the presence of four immunoglobulin domains and six fibronectin type III repeats in their extracellular domains (Fearon et al., 1990). Some members of this family of proteins are implicated in neural morphogenesis, including the neural cell adhesion molecule (NCAM) (Doherty and Walsh, 1992).

Several studies of *DCC* expression have provided results that would seem at odds with the proposed tumor suppressor role of this gene. First, high level expression of the *DCC* gene product might be expected in the intestine if DCC functions to regulate intestinal differentiation or proliferation. However, the level of *DCC* RNA in the normal colonic tissue, composed of both epithelial and non-epithelial cells, is at the threshold of detection by the polymerase chain reaction (PCR) (Fearon et al., 1990), and it is unclear whether this RNA is translated into protein, as some investigators have had difficulty detecting DCC protein expression in colonic cells (Turley et al., 1995).

Second, as described above, the hallmark of a tumor suppressor gene is its homozygous inactivation in tumor cells (Cavenee et al., 1983; Weinberg, 1991). The *DCC* gene represents an anomaly in this regard. To date, analysis of the *DCC* alleles retained in human colonic tumors has led to the discovery of mutant alleles in only 2 of 60 colonic tumors affected by 18q LOH (Cho et al., 1994). An additional 12% of the tumors carry insertions in one of the *DCC* introns (Fearon et al., 1990), but the effect of these insertions on *DCC* function is not known.

The absence of detectable mutations in *DCC* in most colonic tumors is unusual for a tumor suppressor gene but does not exclude a role for DCC in tumor suppression. For example, the cells in an adenomatous polyp may employ a mechanism other than mutation of the *DCC* gene to reduce or eliminate its product. Evidence for this comes from the observation that *DCC* RNA is either

drastically reduced or completely lost in these tumors (Fearon et al., 1990; Kikuchi-Yanoshita et al., 1992) but is detectable by PCR in RNA pools prepared from whole colonic tissue that includes several cell types including the epithelial cells (Fearon et al., 1990). A study of a group of 18 colonic tumors showed that RNA spanning the 3' end of *DCC* was absent in 10 tumors and RNA spanning the 5' end of *DCC* was not detectable in any of these tumors (Thiagalingam et al., 1996).

Although initial studies of DCC function focused on its possible involvement in intestinal development and pathogenesis (reviewed in (Cho and Fearon, 1995)), it was evident from its initial isolation that one of the major sites of DCC expression was in the nervous system (Fearon et al., 1990), in particular on axonal processes (Hedrick et al., 1994). The idea that DCC might function in neural development was further reinforced by the identification of a structurally-related protein, neogenin, as a transmembrane protein expressed by retinal axons during the growth of these axons to their targets in the chick embryo (Vielmetter et al., 1994).

Recently, studies have suggested that DCC may function as a component of a receptor complex that mediates the effects of the axonal chemoattractant netrin-1 on the commissural axons of the spinal cord of vertebrates (Keino-Masu et al., 1996). Commissural neurons differentiate in the dorsal portion of the spinal cord and pioneer a ventrally-directed circumferential pathway that leads them to a specialized group of cells at the ventral midline of the spinal cord termed floor plate cells (Colamarino and Tessier-Lavigne, 1995). During development of the nervous system, these migrating axons are guided to the floor plate at least partly by a chemoattractant, netrin-1, secreted by floor plate cells (Kennedy et al., 1994; Serafini et al., 1994). Netrin-1 is a member of a phylogenetically conserved family of laminin-related molecules that have been implicated in axonal guidance in *Caenorhabditis elegans*, *Drosophila melanogaster* and vertebrates (Harris et al., 1996; Ishii et al., 1992; Kennedy et al., 1994; Mitchell et al., 1996; Serafini et al., 1994; Wadsworth et al., 1996). Netrin-1 can promote the outgrowth of spinal cord commissural axons into collagen matrices and reorient the growth of these axons *in vitro* (Kennedy et al., 1994; Serafini et al., 1994). Moreover, in the developing mouse embryo, *netrin-1* mRNA is expressed at high levels by floor plate cells and at lower levels in the ventral two-thirds of the spinal cord; these distributions

have led to the hypothesis that there is an increasing dorsal-to-ventral gradient of netrin-1 protein in the spinal cord that serves to guide the migration of commissural axons to the floor plate (Kennedy et al., 1994).

An involvement of DCC-related proteins in mediating the axonal guidance effects of netrins was evidenced through complementary studies in worms, flies and vertebrates. Loss-of-function phenotypes and genetic interactions in *C. elegans* and *Drosophila* have suggested that homologues of the DCC protein function in the response pathway of netrin family members (Chan et al., 1996; Kolodziej et al., 1996), but direct biochemical interactions between the ligands and the putative receptors have been reported in those species. In vertebrates, a biochemical interaction between netrin-1 and DCC has been demonstrated (Keino-Masu et al., 1996), and DCC has been implicated in mediating netrin effects on spinal commissural axons *in vitro* through antibody perturbation studies, but the significance of this interaction for guidance of axons by netrin-1 *in vivo* has not been addressed.

To help elucidate the functions of the *DCC* gene in mice, we have inactivated its homologue (*Dcc*) in the mouse genome through use of homologous recombination and have examined the effects of this inactivation on both the intestine and the developing nervous system. The observed phenotypes of the mice are consistent with the hypothesis that DCC is part of a receptor for netrin-1 *in vivo* both in the spinal cord and in the developing brain but fail to provide support for the hypothesis that *DCC* controls cell proliferation in the normal or neoplastic mouse intestine.

Materials and Methods

Generation And Analysis of Mice with Dcc^{X3} Mutation

Embryonic stem cell culture, generation of chimeric mice, Southern and western blot analyses were performed according to standard procedures.

Tumor Analysis

All mice in this study were maintained in the facilities of the Whitehead Institute for Biomedical Research and were given Agway Prolab Rat, Mouse and Hamster 3000 chow *ad libitum*. The chow has a crude protein content of at least 22%, a crude fat content of at least 5%, and a crude fiber content of at most 5%. After euthanization, the entire gastrointestinal tract was removed, dissected along the cephalocaudal axis, and washed in phosphate buffered saline. Adenomas were at least twice as wide as a villus, at most 5-6 millimeters in diameter and were readily visible under a dissecting microscope. Adenomas were removed surgically, fixed in Bouin's fixative, dehydrated in graded alcohols, embedded in paraffin, sectioned at 6 mm, and stained with hematoxylin and eosin (H&E). For LOH analysis, adenomas were removed surgically and DNA was prepared. C57BL/6 $Apc^{Min/+}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine).

Generation of Compound Heterozygous Apc^{Min} Dcc^{-}/Apc^{+} Dcc^{+} *Mice*

C57BL/6 $Apc^{Min/+}$ and 129/Sv $Dcc^{+/-}$ animals were crossed to generate 129Sv/B6 F1 $Apc^{Min/+}$ $Dcc^{+/-}$ mice. These F1 mice were then backcrossed to 129/Sv mice; the compound heterozygote progeny of this latter cross had the mutant allele of both the *Apc* and the *Dcc* gene in the cis configuration.

Immunohistochemical Analysis of Postnatal Day 1 (P1) Intestines

Single and multilabel immunohistochemical analyses of postnatal day 1 (P1) 129/Sv $Dcc^{-/-}$, $Dcc^{+/-}$ and $Dcc^{+/+}$ mice were conducted in a single blinded fashion. Each member of a litter of thirteen P1 mice, derived from a cross of 129/Sv $Dcc^{+/-}$ animals, received an intraperitoneal injection of 5'-bromo-2'-deoxyuridine (BrdU; Sigma; 120 mg/kg body weight) and 5-fluoro-2'-deoxyuridine

(Sigma; 12 mg/kg) 90 minutes prior to sacrifice. Following sacrifice, an incision was made in the abdominal wall. Each mouse was then placed in Bouin's solution for 12 h followed by 70% ethanol. The gastrointestinal tract was removed *en bloc*, displayed so that its entire cephalocaudal axis could be surveyed on a single slide, and then embedded in paraffin. 5 mm-thick sections were cut. Sections from each animal were initially stained with hematoxylin and eosin to define epithelial architecture and cellular morphology.

Some antigens or glycoconjugates could only be detected after enzymatic unmasking. This unmasking involved incubation of sections for 15 minutes (min) at 37^{o}C in a 1 mg/ml solution of chymotrypsin (prepared in 7 mM $CaCl_2$, pH 7.8). All sections, whether pre-treated with protease or not, were then incubated with blocking buffer [bovine serum albumin (1%, wt/vol), powdered skim milk (0.2%; omitted for lectin staining), Triton X-100 (0.3%) in PBS] for 15 min at room temperature. After an overnight incubation at 4^{o}C with primary antisera, antigen-antibody complexes were visualized using: (i) gold-labeled secondary antibodies with silver enhancement (Amersham; final dilution in PBS blocking buffer = 1:40), (ii) Cy3-labeled donkey anti-goat or rabbit secondary antibodies (Jackson Immunoresearch; 1:500) or (iii) Cy3-labeled sheep anti-digoxigenin (1:1000). Lectins were used as fluorescein isothiocyanate- (FITC), peroxidase- or biotin-labeled conjugates. Bound biotinylated lectins were visualized with FITC-conjugated strepavidin (1:100; Sigma) while peroxidase-labeled lectins were detected with metal-enhanced 3'-3'diaminobenzidine. Slides were washed in PBS, incubated with bis-benzimide (Sigma; diluted 1:20,000 in PBS) for 10 min at room temperature to visualize nuclei, washed several times with PBS, and were then mounted in PBS/glycerol (1:1 v/v) or counterstained with hematoxylin, dehydrated, and mounted in Permount (Fischer).

A panel of 11 antisera were used. They included (i) goat anti-BrdU (specificity = S-phase cells; final dilution = 1:3000), (ii) rabbit anti-rat liver fatty acid binding protein (L-FABP; specificity in developing and adult normal 129/Sv mouse intestine = villus enterocytes; 1:1000) ; (iii) rabbit anti-intestinal fatty acid binding protein (I-FABP; 1:1000; enterocytes); (iv) rabbit anti-C/EBPa (1:500; nonproliferating epithelial cells); (v) rabbit anti-pan-cadherin (1:2000; epithelial

cadherins; Sigma), (vi) rabbit anti-laminin (1:1000, Sigma), (vii) rabbit anti-collagen IV (1:1000; Collaborative Biomedical Products); (viii) rabbit anti-serotonin (1:4000); (ix) rabbit anti-substance P (1: 1000); (x) rabbit anti-cryptdin (1:25; Paneth cells); and (xi) rabbit anti-enhancing factor (1:200; Paneth cells).

Nine lectins were used, all at a final concentration of 5 mg/ml: (i) Jacalin (*Artrocarpus integrifolia* agglutinin (stains small intestinal enterocytes (primarily Golgi and brush border) plus a subset of enteroendocrine and goblet cells); (ii) Peanut agglutinin (stains brush border of jejunal enterocytes, small intestinal enteroendocrine cells and a subset of goblet cells, a subset of colonic enterocytes); (iii) *Tirchosantes kirilowii* (stains Paneth and goblet cells plus a subset of enterocytes in the small intestine plus all three epithelial cell lineages in the colon); (iv) *Dolichos biflorus* agglutinin (stains all four principal small intestinal epithelial cell lineages; enterocytes and subset of goblet cells in the colon); (v) *Helix pomentia* agglutinin (stains Paneth cells, brush border of enterocytes, subset of goblet cells located in the lower villus or in the nascent P1 crypt, rare enteroendocrine cells, subset of colonic enterocytes, goblet cells in the lower half of colonic crypts); (vi) *Griffonia simplifolica*, stains Paneth cells and subset of small intestinal goblet cells, subset of colonic goblet cells; (vii)*Ulex europaeus* agglutinin type I (stains a majority of small intestinal goblet cells, proliferating and nonproliferating cells in the P1 intervillus epithelium, colonic enterocytes and subset of colonic goblet cells); (viii) Cholera toxin B subunit (stains proximal half of small intestine = goblet cells, enteroendocrine cells, weak staining of brush border and Golgi apparatus of enterocytes; distal half of small intestine = subset of goblet cells, strong staining of enterocytic brush border and Golgi apparatus, colon = enterocytes); and (ix) *Sambucus nigra* agglutinin (stains all goblet cells in small intestine and colon).

The number of goblet cells was defined in the proximal, mid and distal thirds of the small intestine and in the colon. PAS/Alcian Blue-stained goblet cells in 20 well-oriented, nascent crypt-villus units were counted/region/mouse (n = 2 animals/genotype). Paired Student's T test was performed to assess whether any differences in the total number of goblet cells/region of $Dcc^{-/-}$, $Dcc^{+/-}$ and $Dcc^{+/+}$ mice were statistically significant.

Generation of *Dcc*$^{-/-}$ Embryonic Stem Cells

Homozygous mutant *Dcc*$^{-/-}$ ES cells were generated from 129/Sv *Dcc*$^{+/-}$ ES cells using a previously described method (Mortensen et al., 1992). 10^4 or 10^5 heterozygous cells were plated on gelatinized 10-cm dishes and 24 h later medium containing 400-600 mg/ml G418 was added. In each dish, an average of 10 colonies survived two weeks of culturing in the G418 medium. These colonies were picked, passaged, and genotyped. Fifty percent of the colonies had lost the wild type allele of *Dcc* and showed only the mutant allele by Southern analysis (data not shown). We assumed that these colonies had lost the wild type *Dcc* allele, duplicated the mutant *Dcc* allele and its PMCI-*neo*-polA cassette, and thus became resistant to high concentrations of G418 by virtue of having two copies of *neo*. The colonies that survived high G418 but remained *Dcc*$^{+/-}$ served as the control for the homozygous mutant cells. The homozygous mutant or the control heterozygous ES cells were injected into B6 blastocysts to generate chimeric mice. Some of the chimeras created by injection of three independent clones of homozygous *Dcc*$^{-/-}$ cells displayed the same behavioral abnormalities as the constitutionally homozygous mutant pups. Others appeared normal at birth, lived to adulthood, and displayed less severe behavioral abnormalities such as abnormal limb flexure. The *Dcc*$^{-/-}$ cells in these viable chimeras contributed to the germline: when bred with wild type mice, these *Dcc*$^{-/-}$ chimeras gave rise almost exclusively to *Dcc*$^{+/-}$ progeny. Interbreeding of the resulting *Dcc*$^{+/-}$ mice resulted in *Dcc*$^{-/-}$ pups with a phenotype identical to the *Dcc*$^{-/-}$ pups generated from the heterozygous chimeras.

Whole-Mount Immunostaining of B6 *Dcc*$^{+/+}$<->129/Sv *Dcc*$^{-/-}$ intestines

Intestinal wholemounts were prepared from adult C57BL/6 *Dcc*$^{+/+}$<->129Sv *Dcc*$^{-/-}$chimeric mice (Hermiston and Gordon, 1995; Hermiston et al., 1993) and incubated with peroxidase-conjugated

UEA1. Peroxidase activity was visualized using metal enhanced DAB. Note that some B6 goblet cells express glycoconjugates recognized by the UEA1 lectin.

Brain histology and spinal cord histochemistry

Brains were processed for histochemistry by standard procedures. Immunohistochemistry on 20 μm sections of spinal cord was performed with antibodies to TAG-1 (4D7, gift of M. Yamamoto) and NF-M (gift of V. Lee). For TAG-1 staining, sections were blocked in 1% heat-inactivated normal goat serum, 0.1% Triton X-100 and 0.2% fish skin gelatin in phosphate buffered saline (PBS), incubated with 1:200 dilution of 4D7 supernatant overnight, washed with blocking solution (6 x 30 min), incubated overnight with 1:250 dilution of Cy3 conjugated anti mouse IgM (Jackson ImmunoResearch), washed as above, and mounted in Fluoromount G (Fisher) (all steps performed at 4°C). Staining with anti-NF-M (1:5,000 dilution; 1:250 dilution of Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch) used as secondary antibody) was performed in the same way except that sections were boiled in 150 mls of citric acid buffer (0.1% citric acid sodium salt dihydrate, pH 6) for 10 minutes prior to use. For whole-mount immunohistochemistry, E11.5 spinal cords (without dorsal root ganglia) were removed from the vertebral column, fixed in 4% paraformaldehyde in PBS at 4°C overnight, blocked for 3 hr in PHT (PBS / 1% heat-inactivated goat serum / 0.1% Triton X-100), incubated with 4D7 (1:100 dilution in PHT) for 2 days, washed with 20 changes of PHT over 2 days, incubated with Cy3-conjugated secondary antibody (1:700 dilution in PHT) for 2 days, washed with 30 changes of PHT over 3 days, and mounted in Fluoromount G. All steps were performed at 4°C, except for the blocking which was performed at room temperature. Dye-tracing experiments were performed as described previously (Stoeckli and Landmesser, 1995).

Results

Inactivation of the mouse homologue of *DCC*

To extend our understanding of the human *DCC* gene, we inactivated its homologue (*Dcc*) in the mouse genome. The Dcc^{X3} targeting vector (Fig. 1A) was introduced into 129/Sv D3 embryonic stem (ES) cells (Gossler et al., 1986). Southern blot analysis demonstrated that three of the 200 gancyclovir- and G418-resistant ES cell clones screened had acquired a copy of the neomycin transferase (*neo*) gene in the mouse *Dcc* locus by homologous recombination (Fig. 1B). We term this mutant allele Dcc^{-}.

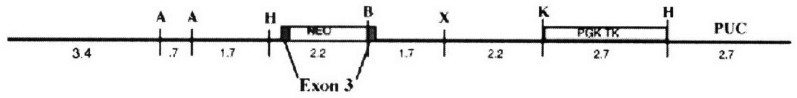
Mouse chimeras generated from two of these $Dcc^{+/-}$ ES cell clones transmitted the Dcc^{-} allele through their germline. Interbreeding of the resulting heterozygous $Dcc^{+/-}$ mice produced homozygous offspring that appeared to be grossly normal at birth but died within 24 hours. In one study, twenty-two of eighty-eight newborn mice died within the first day of life. All that died were found to be $Dcc^{-/-}$ when genotyped. The littermates that survived were either $Dcc^{+/+}$ or $Dcc^{+/-}$ in the expected Mendelian ratio. The $Dcc^{-/-}$ neonatal mice homozygous for the Dcc^{-} (null) allele exhibited striking behavioral phenotypes, including the inability to suckle, labored respiration, abnormal body posture, and abnormal limb flexion in response to pinch stimuli, suggesting that DCC might be essential for normal development of the nervous system (see below).

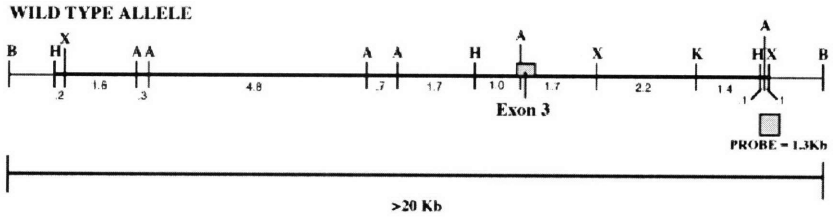
Immunoblots of proteins extracted from the brains of the $Dcc^{-/-}$ pups and their normal littermates were probed with a previously characterized antibody that recognizes the intracellular domain of the DCC protein (Ekstrand et al., 1995). The results confirmed that the Dcc^{X3} mutation caused complete loss of DCC protein expression in the homozygous mutant mice (Fig. 1C).

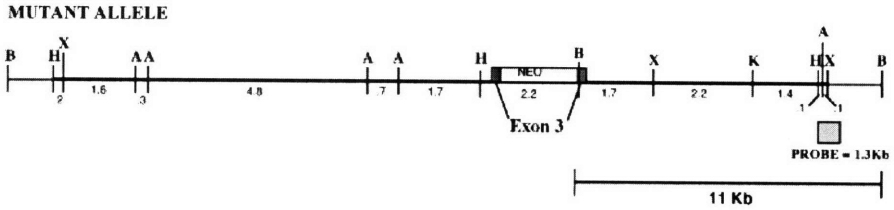
Figure 1

Construction of Dcc^{X3} Mutation. (A) The Dcc^{X3} targeting vector was constructed using fragments of the 129/Sv mouse *Dcc* gene, extending from intron two to intron three. The third exon of *Dcc* spans most of the protein's second immunoglobulin-like domain and contains codons 138-232. The PMCI-*neo*-polA expression cassette was inserted at the Afl-II site in the third exon of *Dcc* leading to the generation of a new Bam-HI restriction site within the third exon of a targeted gene. The HSV-*thymidine Kinase* (*tk*) cassette, under the control of the *pgk* promoter, was inserted adjacent to sequences from the third *Dcc* intron. The HSV-*tk* and PMCI-*neo*-polA cassettes are transcribed in the same transcriptional orientation as the *Dcc* gene. X (Xba I), H (Hind III), A (Afl II), B (Bam HI), K (Kpn I) and N (Not I) are restriction endonuclease sites.
(B) Targeting *Dcc* in the ES cells. Southern analysis of Bam-HI-digested DNA from ES cell clones using a probe from intron 3. Some clones contain a Dcc^{X3}-specific 11-Kb restriction fragment in addition to the >20 Kb fragment corresponding to the wild-type *Dcc* allele.
(C) Protein Analysis. DCC was immunoprecipitated from total brain protein ($Dcc^{-/-}$ in lanes 1 & 2; $Dcc^{+/+}$ in lane 3) using an antibody directed to C-terminus of DCC and western blotted as described previously (Ekstrand et al., 1995). The full-length 190 kDa DCC protein is indicated by arrow. Lane 4 represents a (+) control which is a DCC-deficient human cell line transfected with a DCC-expressing construct and Lane 5 is a (-) control which is the same cell line before transfection (Ekstrand et al., 1995).

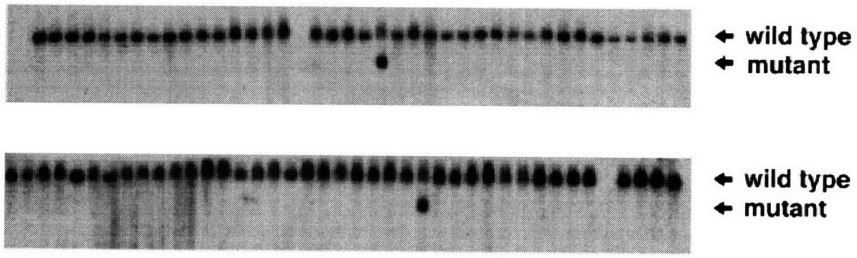
A.


A A H B X K H
NEO
PGK TK
PUC
3.4 .7 1.7 2.2 1.7 2.2 2.7 2.7
Exon 3

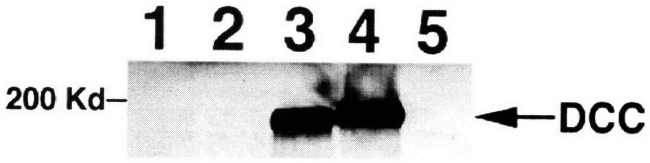

WILD TYPE ALLELE
B H X A A A A H A X K H X B
.2 1.6 .3 4.8 .7 1.7 1.0 1.7 2.2 1.4 1 1
Exon 3
PROBE = 1.3Kb
>20 Kb


MUTANT ALLELE
B H X A A A A H NEO B X K H X B
.2 1.6 .3 4.8 .7 1.7 2.2 1.7 2.2 1.4 1 1
Exon 3
PROBE = 1.3Kb
11 Kb

B.


wild type
mutant
wild type
mutant

C.


1 2 3 4 5
200 Kd–
DCC

Tumor predisposition in *Dcc*$^{+/-}$ mice

To determine if heterozygosity at the *Dcc* locus causes tumor predisposition in mice, we followed a cohort of nearly two-hundred 129Sv/C57BL6 (129Sv/B6) F1 *Dcc*$^{+/-}$ mice for two years. *Dcc*$^{+/-}$ animals were monitored closely and screened at different ages for the presence of tumors in their gut and extra-intestinal tissues. Only three *Dcc*$^{+/-}$ mice displayed signs of sickness, each being observed at ~18 months of age. One of these animals had six adenomas in its duodenum. The other two mice had brain neoplasms, one an astrocytoma, the other a meningioma. While these observations suggested that aged *Dcc*$^{+/-}$ mice have a predisposition to tumors, whether heterozygosity at the *Dcc* locus contributed to their formation could not be determined unless the the incidence of tumors in aged *Dcc*$^{+/-}$ mice were compared to that of aged *Dcc*$^{+/+}$ littermates.

For this purpose, we performed autopsies on ninety-eight *Dcc*$^{+/-}$ mice and thirty-six *Dcc*$^{+/+}$ littermates, aged 1.5 to 2 years. In addition, the entire intestine of each animal was examined for the presence of adenomas. The heterozygous and the wildtype mice developed adenomas at a low frequency (Table 1). A total of 22 adenomas were found in 9 mice from the *Dcc*$^{+/-}$ cohort (one mouse with seven adenomas, two with four adenomas, one with two adenomas, two with one adenoma and eighty-nine mice with no adenomas). Examination of thirty-six *Dcc*$^{+/+}$ mice revealed adenomas in six mice, one in each. This study showed that *Dcc*$^{+/-}$ mice had no significant increase in predisposition to adenoma formation when compared to wild type controls (Table 1). Thirty percent of the animals had leukemias, soft tissue sarcomas, adenomas or adenocarcinomas of tissues outside of the gastrointestinal tract or lymphomas; the frequency of these lesions was not affected by the presence of the mutant allele of *Dcc* either. Most of these tumors had not resulted in overt signs of sickness in the mice at the time of sacrifice and were detected only during histological examination of the removed tissues. Finally, no brain tumors were found either in the *Dcc*$^{+/-}$ or in the *Dcc*$^{+/+}$ cohort.

Table 1

Tumors in *Dcc*$^{+/-}$ mice. $^{+}$Mice are 18-24 month-old F1 and F2 129Sv/B6; *n is the number of mice autopsied; $^{\#}$number and fraction (inside the parantheses) of mice that had a given class of tumor or neoplasia. indicate the number of mice with each . Gastrointestinal lesions are adenomas and were detected by examination of the gastrointestinal lumen under a dissecting microscope. A total of 22 adenomas were found in 9 mice from the *Dcc*$^{+/-}$ cohort (one mouse with seven adenomas, two with four adenomas, one with two adenomas, two with one adenoma and eighty-nine mice with no adenomas). Examination of thirty-six *Dcc*$^{+/+}$ mice revealed adenomas in six mice, one in each. The majority of the lesions in liver (hepatomas or hepatocarcinomas), lung (adenomas or adenocarcinomas), uterus (adenomas), the hematological lesions and sarcomas were detected only by histological & microscopic examination of the tissues removed at autopsy.

Table 1 Tumors in *Dcc*$^{+/-}$ mice

Genotype+	*Dcc*$^{+/-}$ (n=98)*	*Dcc*$^{+/+}$ (n=36)*
Epithelial		
Gastrointestinal	9 (9%)#	6 (17%)#
Liver, lung & uterine	11 (11%)	7 (19%)
Mesenchymal		
Leukemia & lymphoma	11 (11%)	4 (11%)
Sarcoma	8 (8%)	2 (6%)

If loss of DCC played a causative role in the development of the intestinal adenomas described above, the wild type *Dcc* allele would be expected to be lost in the tumors recovered from $Dcc^{+/-}$ mice. Southern blot analysis of genomic DNA prepared from four adenomas from the above cohort of 18 month-old heterozygotes (including one from the mouse with six such lesions) and a brain tumor indicated that both the wild-type and the mutant *Dcc* alleles had been retained (data not shown). Similar analysis, described below, indicated that our analytical procedures were easily able to detect loss of the wild-type *Dcc* alleles in other adenomas of comparable size. Although it appears unlikely that these lesions arose due to the *Dcc* mutation, we cannot rule out the possibility that expression of the wild type *Dcc* allele was repressed in these lesions through mechanisms other than LOH.

Effect of *Dcc* inactivation on the growth and progression of adenomatous polyps

Loss of *Dcc* expression could affect the progression of intestinal or colonic adenomas previously initiated by the partial or complete loss of *Adenomatous polyposis coli* (*Apc*) function. This notion is supported by the observation in humans that LOH of 18q correlates with an increase in the size of colonic adenomas (Fearon and Vogelstein, 1990; Vogelstein et al., 1988). Moreover, some studies have linked the loss of *Dcc* expression to morphological changes such as the acquisition of a more dysplastic phenotype or to the appearance of adenomas that are of a more villus than tubular appearance (Fearon, 1995).

To examine this possible interaction, we introduced the null *Dcc* allele into the germline of the Min mouse (Moser et al., 1990) which carries a mutant *Apc* allele (Apc^{Min}) in its germline that predisposes it to develop small intestinal and colonic adenomas (Moser et al., 1992; Moser et al., 1990; Su et al., 1992). The intestinal pathology of the Min mouse is similar to that of humans in which germline *APC* mutations result in familial adenomatous polyposis (Groden et al., 1991; Nishisho et al., 1991). In mice, both the *Apc* gene and the *Dcc* gene are on chromosome 18 (Luongo et al., 1993). The frequency of polyp formation and the phenotype of the adenomas in

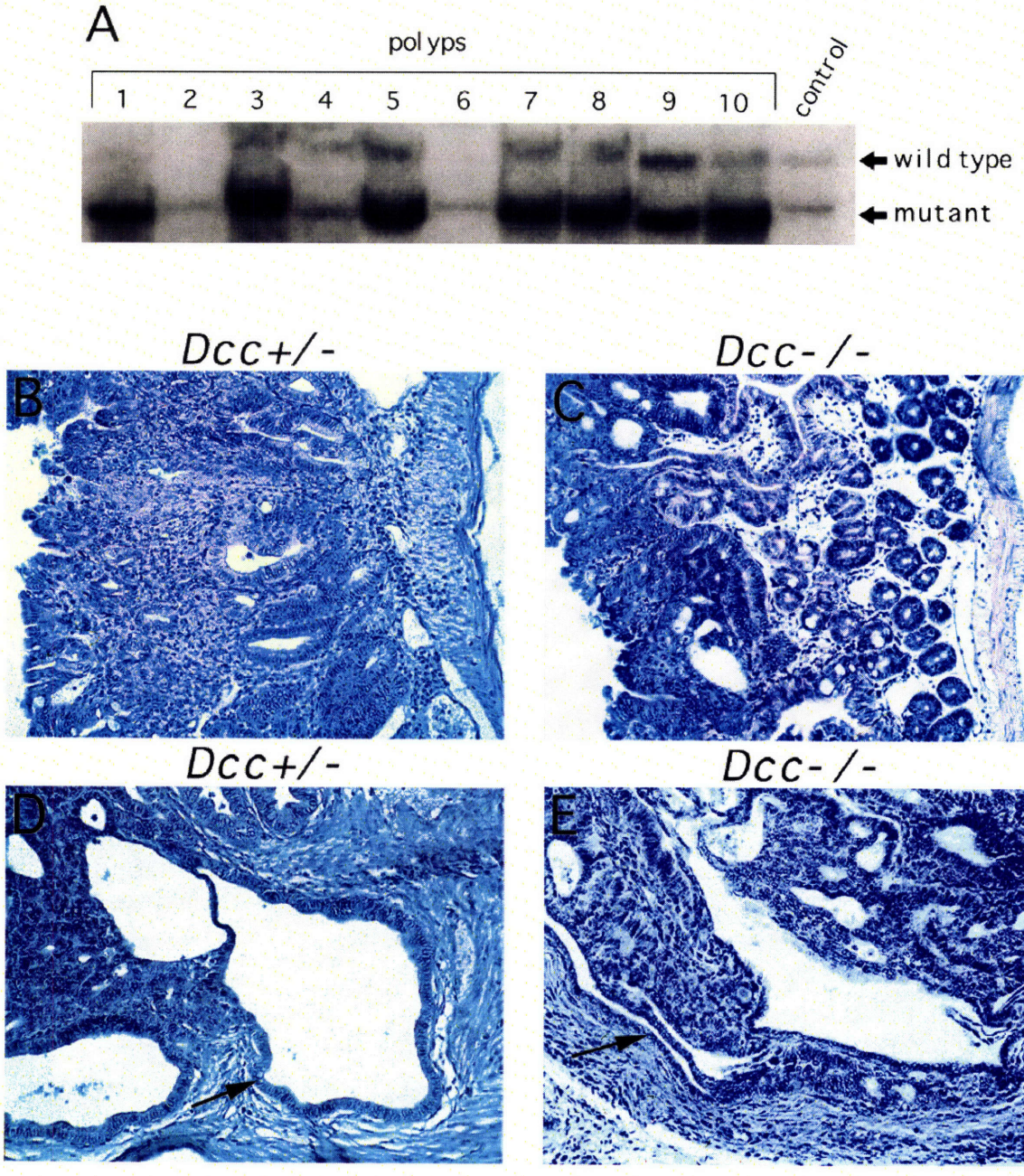
mice carrying the Min allele of *Apc* was not affected by the presence of the Dcc^- allele on opposite chromosomes, i.e., in *trans* configuration (data not shown).

Prior studies had demonstrated that nearly all adenomas in intestines of $Apc^{Min/+}$ mice lose not only the wild type allele of *Apc* but also the entire chromosome 18 harboring the wild type *Apc* allele (Luongo et al., 1994). This loss affects any heterozygosity that may exist at the *Dcc* locus, which happens to be located within 30 centimorgans of the *Apc* gene on chromosome 18 (Luongo et al., 1993). We took advantage of this linkage by introducing the mutant *Dcc* allele into the chromosome carrying the Apc^{Min} mutation. We then bred mice having a genotype in which this doubly mutant chromosome was present opposite a fully wild-type chromosome 18.

A cohort of forty-seven Apc^{Min} Dcc^-/Apc^+ Dcc^+ mice was established that carried the mutant alleles of these two genes in a *cis* configuration. A cohort of twelve Apc^{Min} Dcc^+/Apc^+ Dcc^+ littermates was used as control. LOH analysis on the DNA of adenomas from Apc^{Min} Dcc^-/Apc^+ Dcc^+ mice followed by densitometric analysis showed that 8/10 of the adenomas in the *cis* compound heterozygous mice had lost the wildtype *Dcc* allele (adenomas numbered 1-3, 5-8, and 10 in Fig. 2A), as is seen for the linked wild-type *Apc* in virtually all adenomas in Min mice (Laird et al., 1995; Levy et al., 1994; Luongo et al., 1994).

Figure 2

Effect of *Dcc* on tumor progression in the mouse intestine. (A) Southern blot analysis of adenomas extracted from the intestines of compound heterozygote (Apc^{Min} Dcc^{-}/Apc^{+} Dcc^{+}) mice. Controls are constitutional DNA of tissues from $Dcc^{+/-}$ mice. Representative histological section of two of these adenomas are shown in panels C & E. The band corresponding to the wildtype DNA transfers less effeciently in Southern blots due to its larger size (>20 Kb) but a change in the ratio of the intensity of the two bands can be determined using densitometric analysis (B) A benign adenomatous polyp from a 6 month-old $Apc^{Min/+}$ $Dcc^{+/+}$ mouse stained, as in subsequent panels, with hematoxylin and eosin. (C) A benign adenomatous polyp from a 6 month-old Apc^{Min} Dcc^{-} /Apc^{+} Dcc^{+} mouse. (D) An invasive adenocarcinoma from a 6 month-old 129 $Apc^{Min/+}$ $Dcc^{+/+}$ mouse. Arrow points to carcinomatous cells that have invaded the muscularis mucosa. (E) An adenocarcinoma from a 6 month-old 129 Apc^{Min} Dcc^{-}/Apc^{+} Dcc^{+} mouse. Arrow points to the site of invasion in the muscularis mucosa by carcinomatous cells.


A
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1
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control
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mutant
Dcc+/-
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C
Dcc+/-
D
Dcc-/-
E

Germline heterozygosity for the mutant *Dcc* allele in the *cis* configuration with respect to the Apc^{Min} allele did not affect initiation of tumorigenesis in the intestine as assessed by a polyp count (Table 2). Moreover, the absence of DCC had no effect on the average size of the adenomas (Table 2). Histological analysis failed to reveal a change in the morphology of the adenomas; for example, they showed no tendency to progress to a more villous phenotype (Fig. 2 B & C). An equal proportion (approximately 1/20) of the adenomas in both groups of mice exhibited local invasion, with extension of cyst- and finger-like projections of adenomatous cells deep into muscularis mucosa (Fig. 2 D & E). Thus, loss of the wild type allele of the *Dcc* gene occurred in the majority of intestinal adenomas in the *cis* compound heterozygous mice but it could not be correlated with tumor progression (Fig. 2).

Table 2

Effect of *Dcc* inactivation on formation of Apc^{Min}-induced intestinal adenomas and their growth. +Mice are first, second, or third-generation backcross of 129Sv/B6 F1 into 129/Sv; age = 6-12 months. * n is the number of mice analyzed. # n is the number of adenomas analyzed in mice of the same age (6 months old).

Effect of Dcc Inactivation on Polyp Number and Size

Genotype+	Average Number of Adenomas per mouse	Average Size (mm)
Apc$^{Min/+}$ *Dcc*$^{+/-}$ (n=47)*	56	2.66 (n=452)#
Apc$^{Min/+}$ *Dcc*$^{+/+}$ (n=12)*	59	2.69 (n=335)#

Intestinal epithelial proliferation and differentiation in newborn *Dcc*$^{-/-}$ mice

To determine whether DCC had any detectable role in regulating the normal proliferation and differentiation programs of the self-renewing intestinal epithelium, we compared this tissue prepared from five newborn (P1) *Dcc*$^{-/-}$ mice with those from three *Dcc*$^{+/+}$ and five *Dcc*$^{+/-}$ littermates. Inspection of hematoxylin- and eosin-stained sections prepared along the length of the duodenal-ileal axis revealed no differences in villus height or cellular architecture (Fig. 3 A & B).

Figure 3

Multilabel Immunocytochemical Studies of the Intestinal Epithelium of P1 129/Sv *Dcc*$^{-/-}$ Mice

(A, B) Sections, prepared from *Dcc*$^{-/-}$ and *Dcc*$^{+/+}$ mouse jejunum (panels A and B, respectively), were stained with hematoxylin and eosin. The villi were cut either parallel or perpendicular to the crypt-villus axis. Arrows point to M-phase cells in the intervillus epithelium.

(C) Jejunum from a P1 *Dcc*$^{-/-}$ mouse that had been injected with BrdU 90 minutes prior to sacrifice. The section was incubated with goat anti-BrdU sera (visualized with Cy3-labeled donkey anti-goat Ig) and biotin-conjugated *Dolicos biflorus* agglutinin (DBA, visualized with FITC-conjugated strepavidin). Closed arrowheads point to proliferating, BrdU^{+} (red) cells in the intervillus epithelium. Rare BrdU^{+} lymphocytes are evident in the lamina propria (open arrowheads). DBA recognizes a glycoconjugate present on the enterocyte brush border (open arrow) and in the mucus globules of goblet cells (closed arrow).

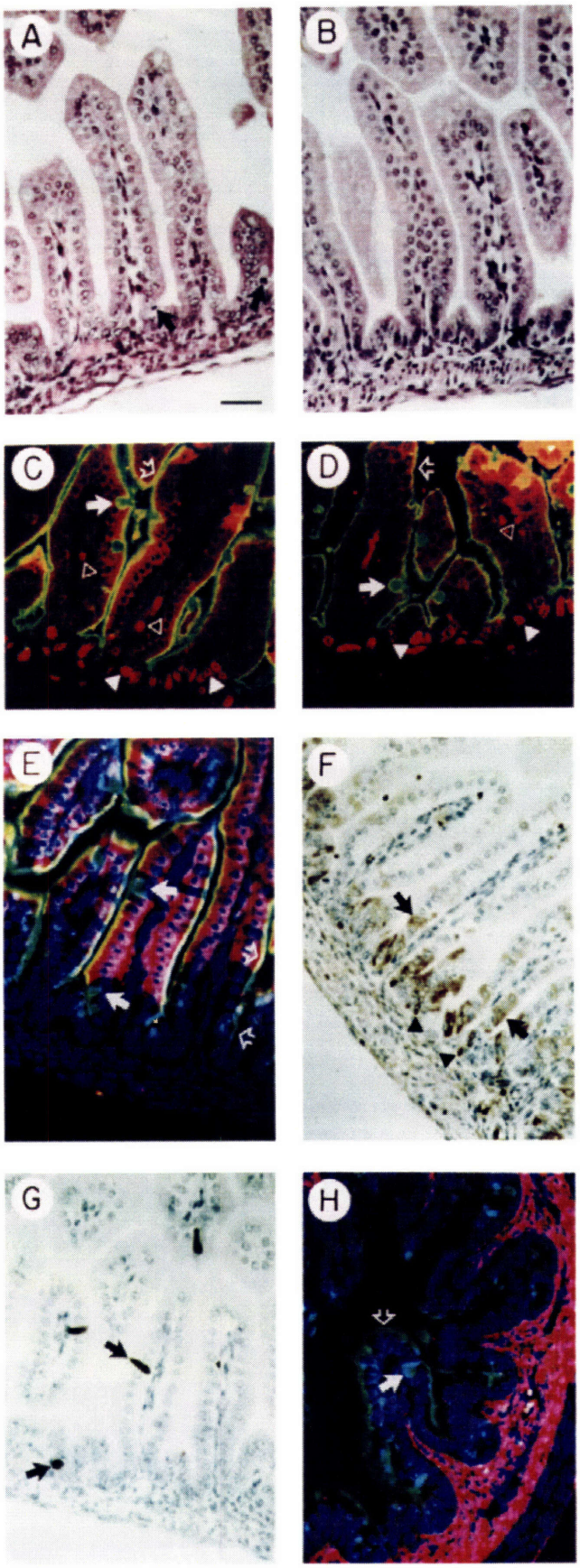
(D) Comparable segment of 129/Sv *Dcc*$^{+/+}$ jejunum stained as in Panel C.

(E) Triple label of a jejunal segment from a *Dcc*$^{-/-}$ mouse. The section was incubated with rabbit anti-liver fatty acid binding protein (L-FABP) sera (visualized with Cy3-donkey anti-rabbit Ig), FITC-conjugated CholeraToxin B subunit (CTB), and bis-benzimine (dark blue nuclear stain). L-FABP (red-purple) is present in villus enterocytes. CTB binds to goblet cells (green; closed arrows) and to the brush border of enterocytes located on the villus and in nascent crypts (open arrow).

(F) Section of duodenum from a *Dcc*$^{-/-}$ mouse incubated with rabbit anti-CEBPa sera and gold-labeled goat anti-rabbit Ig. Antigen-antibody complexes were visualized by silver staining (IGSS). Nuclei were counterstained with hematoxylin. CEBPa-positive enterocytes are present on the lower half of the villi (closed arrows) and in nascent crypts (closed arrowheads).

(G) Jejunum from a *Dcc*$^{-/-}$ mouse stained with rabbit anti-serotonin sera (visualized with IGSS). Nuclei were counterstained with hematoxylin. Immunoreactive enteroendocrine cells (black, arrows) are present in the nascent crypt and in the villus epithelium.

(H) Triple labeling of a section of ileum from a *Dcc*$^{-/-}$ mouse. Collagen IV in the extracellular matrix (red) was stained with rabbit anti- collagen IV sera (visualized with Cy3-donkey anti-rabbit Ig). FITC-conjugated *Helix pomentia* agglutinin (HPA) labeled glycogonjugates located in the enterocytic brush border (green, open arrow) and in the mucus globules of goblet cells (green, closed arrow). Nuclei were visualized with bis-benzimine. Bar = 25 mm.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We examined the proliferative status of intestinal epithelial cells in the newborn *Dcc*$^{-/-}$, *Dcc*$^{+/-}$, and homozygous wild type mice by injecting them with a bolus of bromodeoxyuridine (BrdU) 90 minutes prior to sacrifice. S-phase cells were confined to the intervillus epithelium in all animals. Moreover, the number and distribution of BrdU-positive cells within the intervillus epithelium were identical between all groups of littermates (Fig. 3C & D).

Expression of the CCAAT enhancer-binding protein (C/EBPa) also provides a sensitive index of the proliferative state of enterocytes, the principal intestinal epithelial cell lineage (Chandrasekaran and Gordon, 1993). C/EBPa was present at highest concentrations in epithelial cells located in the intervillus epithelium and the lower half of duodenal and jejunal villi in a pattern that was identical in the epithelia of *Dcc*$^{-/-}$, *Dcc*$^{+/-}$, and *Dcc*$^{+/+}$ mice (Fig. 3F). Together, these findings indicated that loss of *Dcc* does not affect intestinal epithelial proliferation on the first day after birth.

The role played by DCC in epithelial differentiation was examined using a panel of lectins and antibodies. Glycoconjugate production is a sensitive reporter of perturbations in intestinal epithelial differentiation (Falk et al., 1994). Changes in glycoconjugate production can be monitored by noting the cellular patterns of lectin binding to intestinal sections (Falk et al., 1994). Loss of *Dcc* produced no apparent changes in the pattern of lectin binding to the enterocytic lineage (e.g., Fig. 3C & E). Substance P- and serotonin-producing enteroendocrine subpopulations are good markers of the spatial complexity of this lineage's differentiation program along the crypt-villus and duodenal-ileal axes (Roth and Gordon, 1990). Loss of *Dcc* did not have any detectable qualitative or quantitative effects on either of these subpopulations (Fig. 3G).

Some immunochemical and *in situ* hybridization studies of the adult human colon have suggested that DCC is a cell surface protein most prominently represented in goblet cells (Hedrick et al., 1994). Spatial and developmental changes in goblet cell differentiation are readily detected by lectin binding patterns (Falk et al., 1994). Our panel of 9 lectins (see Methods) revealed no differences in goblet cell subpopulations between *Dcc*$^{-/-}$, *Dcc*$^{+/-}$ and wild type littermates (Fig. 3C, E & H). Periodic Acid Schiff (PAS) and Alcian Blue together stain essentially all goblet cells

in the P1 mouse intestine. There was no statistically significant difference ($p>0.05$) in the number of PAS/Alcian Blue-positive goblet cells in the proximal, mid, or distal small intestine or in the colon of *Dcc*$^{-/-}$ mice compared to their *Dcc*$^{+/-}$ or *Dcc*$^{+/+}$ littermates.

The Paneth cell lineage is confined to the base of small intestinal crypts and undergoes a characteristic developmental stage-specific sequence of expression of marker gene products from E15 to P28 (Bry et al., 1994). Loss of *Dcc* did not produce any precocious induction of these markers at P1 (data not shown).

The basement membrane underlying the intestinal epithelium receives contributions from both epithelial and mesenchymal cell populations (Louvard et al., 1992). Laminin and collagen IV are prominent components of this basement membrane. Loss of *Dcc* did not affect the amount or distribution of these proteins along the nascent crypt-villus or duodenal-colonic axis (Fig. 3H). Together, these studies indicate that *Dcc* does not have any observable effect on the development of the intestinal epithelium of newborn mice.

Proliferation, migration and differentiation in the intestinal epithelium of adult B6 *Dcc*$^{+/+}$<->129/Sv *Dcc*$^{-/-}$ chimeric mice

The early death of *Dcc*$^{-/-}$ homozygous mice at P1 prevented us from assessing the function of *Dcc* in the adult intestine. To circumvent this difficulty, we generated chimeras composed in part of *Dcc*$^{-/-}$ cells. First, we produced homozygous mutant 129/Sv *Dcc*$^{-/-}$ ES cell lines by subjecting 129/Sv *Dcc*$^{+/-}$ ES cells to high concentrations of G418 (Mortensen et al., 1992). Cells from three independent *Dcc*$^{-/-}$ ES cell clones were then injected into normal C57BL/6 (B6) *Dcc*$^{+/+}$ embryos. Hereafter, such chimeras are identified by the notation B6 *Dcc*$^{+/+}$ <-> 129/Sv *Dcc*$^{-/-}$.

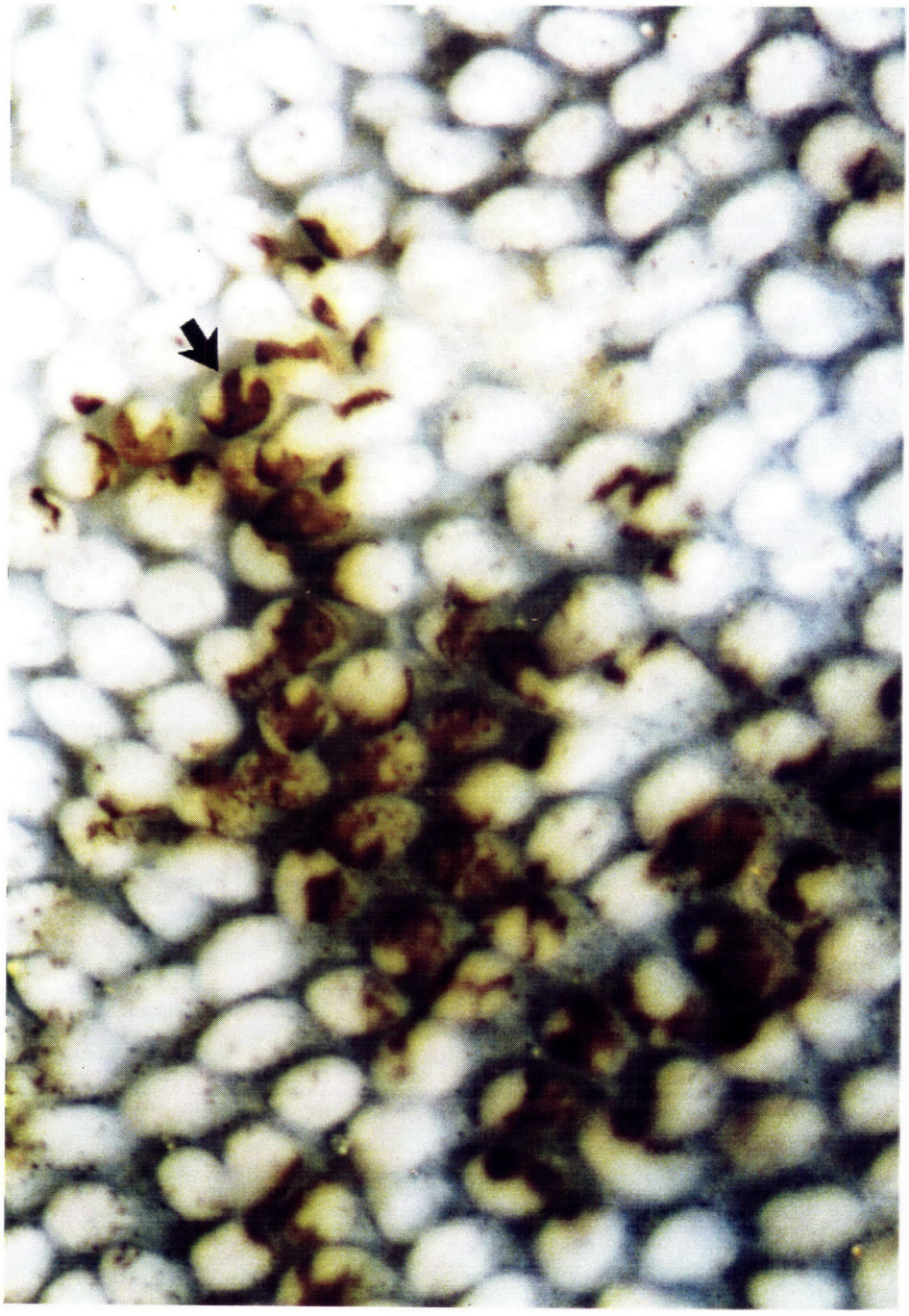
Work of others has shown that crypts in the intestine of adult mice are monoclonal (reviewed in (Gordon et al., 1992)). Thus, in B6<->129/Sv chimeric mice, the crypts are populated by either B6 or 129/Sv cells but not by a mixture of both (Hermiston and Gordon, 1995; Hermiston et al., 1993). However, each villus is populated by cells originating in several adjacent

crypts (reviewed in (Gordon and Hermiston, 1994)) and may therefore be polyclonal in origin. Hence, a B6<->129/Sv chimeric intestine contains patches of 129/Sv and B6 crypt-villus units. These can be readily distinguished because 129/Sv but not B6 enterocytes bind the lectin *Ulex europeaus* agglutinin type 1 (UEA1) (Hermiston and Gordon, 1995; Hermiston et al., 1993); & Fig. 3).

A portion of the B6 $Dcc^{+/+}$<->129/Sv $Dcc^{-/-}$ chimeras appeared normal at birth and lived to adulthood. Intestinal wholemounts were prepared from eight-week old B6 $Dcc^{+/+}$ <->129/Sv $Dcc^{-/-}$ chimeras in which the 129/Sv contribution to coat color was about 50%. These were then stained with the UEA1 agglutinin. Some of the villi encountered at the border of ES cell- and host blastocyst-derived epithelium were polyclonal, being composed of cells originating from both 129/Sv-$Dcc^{-/-}$ and B6-$Dcc^{+/+}$ crypts. These polyclonal villi contained vertical coherent columns of wholly UEA1-positive 129/Sv ($Dcc^{-/-}$) enterocytes and adjacent columns of wholly UEA1-negative B6 ($Dcc^{+/+}$) enterocytes (Fig. 4).

Figure 4

Wholemount of An Adult Chimeric B6 ($Dcc^{+/+}$) <->129/Sv ($Dcc^{-/-}$) Small Intestine. A segment of jejunum from the wholemount preparation is shown, viewed from the luminal surface. Villi are apparent as finger-like projections. The arrow points to a representative polyclonal villus. This villus contains 3 columns of UEA1-positive (brown) $Dcc^{-/-}$ enterocytes. These columns of cells extend from adjacent crypts to the tip of the villus. The columns of $Dcc^{-/-}$ cells are separated by columns of UEA1-negative $Dcc^{+/+}$ enterocytes (white) supplied by B6 crypts.



The presence of these two easily distinguished cell populations on a single villus provided us with an opportunity to precisely assess the effects of *Dcc* loss. The ES-derived $Dcc^{-/-}$ $UEA1^{+}$ cellular columns extended from the base to the tip of each polyclonal villus. The border between ES and B6 $Dcc^{+/+}$ columns was well defined (Fig. 4), indicating that the absence of *Dcc* function did not affect the normal orderly migration of cells from the crypt to the villus tip. Moreover, the two sides of such polyclonal villi were indistinguishable morphologically. Villi that were composed exclusively of UEA^{+} $Dcc^{+/+}$ B6 cells had the same height as adjacent villi composed of wholly $Dcc^{-/-}$ 129/Sv cells (Fig. 4). These similarities in height indicated that the absence of *Dcc* did not perturb the rate of cell production in crypts and/or the rate of cell loss from villi.

Finally, no intestinal adenomas or tumors of other tissues were found in a cohort of ten two-year-old chimeric mice at the time of their sacrifice. In these chimeric mice, the 129/Sv $Dcc^{-/-}$ cells contributed to at least 90% of the coat color. We presume that these mutant cells constituted a similar percentage of the colonic epithelium as well. An indication of significant contribution by 129/Sv $Dcc^{-/-}$ ES cells to the internal organs of these chimeric mice came from breeding of four of these chimeras to C57BL/6 mice. Virtually all resulting progeny over a period of one year had agouti coat color and were $Dcc^{+/-}$ indicating that the large majority of germ cells in these mice were the descendants of 129/SV $Dcc^{-/-}$ ES cells.

Defects in spinal commissural axon projections in $Dcc^{-/-}$ mice

To investigate the function of DCC in the nervous system, we first examined whether loss of DCC function affected the growth of commissural axons in the developing spinal cord, because of previous results from *in vitro* studies which indicated that DCC is involved in mediating the effects of netrin-1 on these axons (Keino-Masu et al., 1996). In the mouse, commissural neurons differentiate in the dorsal spinal cord between embryonic day 9.5 (E9.5) and E11.5 (E0 = day of vaginal plug), and extend their axons to the floor plate (Colamarino and Tessier-Lavigne, 1995). We have found that DCC is expressed by commissural axons in the mouse at these stages

(assessed by immunohistochemistry, data not shown; see also (Cooper et al., 1995)), as it is at comparable developmental stages in the rat (E11-E13) (Keino-Masu et al., 1996).

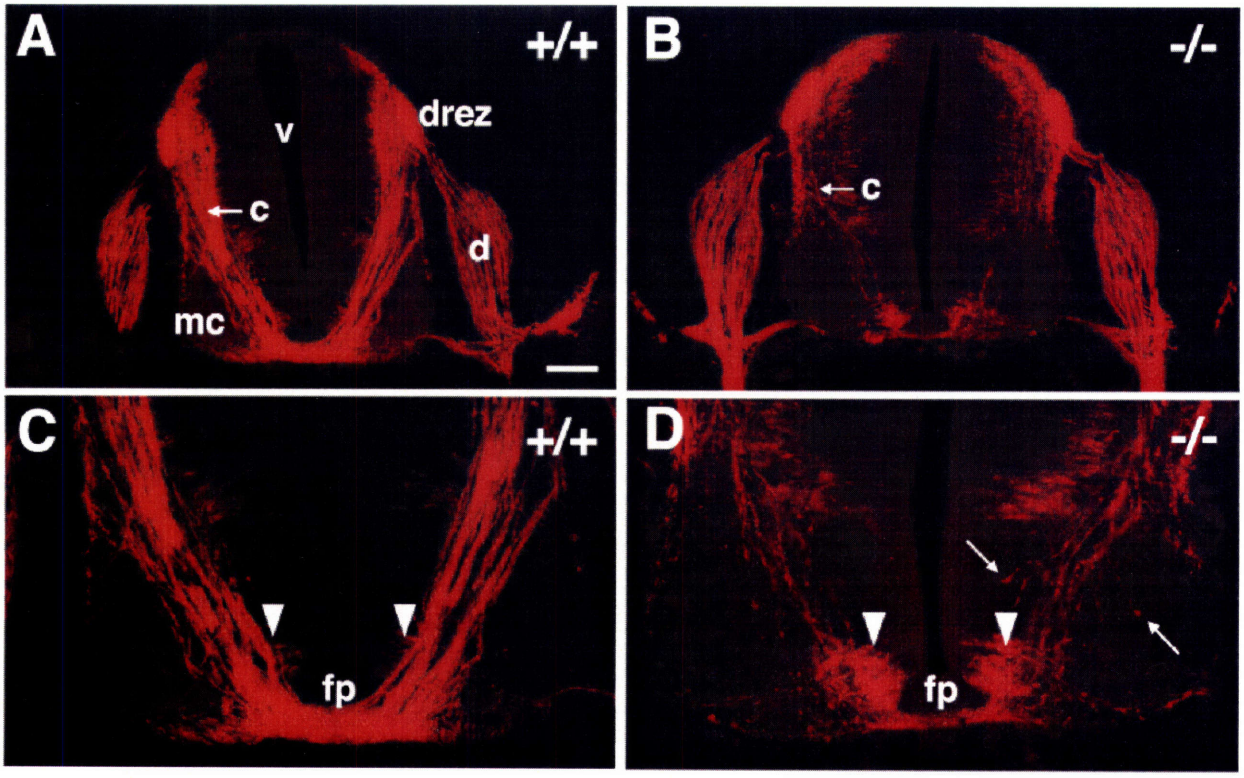
To assess the trajectories of commissural axons at E9.5-E11.5 in the mouse, we labeled these axons in transverse sections of spinal cord at forelimb levels using an antibody to TAG-1, a surface antigen that is expressed by commissural axons as they project to the floor plate (Dodd et al., 1988). In wildtype embryos and embryos heterozygous for the *Dcc*$^-$ allele, commissural axons were seen to take their normal dorsal-to-ventral trajectory. As illustrated for an E11.5 embryo (Fig. 5A), commissural axons initially extend in the dorsal spinal cord along a dorsal-to-ventral trajectory near the edge of the spinal cord until they arrive at the level of the developing motor column. At early stages when the motor column is still small (E9.5), some of the axons continue to grow along the edge of the spinal cord all the way to the ventral midline (Wentworth, 1984). But as the motor column increases in size, most of the commissural axons that reach the motor column break away from their trajectory along the lateral edge of the spinal column and project medially to the motor column or through the motor column in a ventromedial direction toward the floor plate (Fig. 5A).

Figure 5

Defects in Commissural Axon Projections in *Dcc*$^{-/-}$ Embryos. Trajectories of commissural axons in sections of a wildtype (A, C) and *Dcc*$^{-/-}$ (B, D) E11.5 embryos, visualized by immunohistochemistry using an antibody to TAG-1. In wildtype embryos, commissural axons (c) extend along a dorso-ventral trajectory in the dorsal spinal cord (A), then project along a ventromedially-directed trajectory to the floor plate (fp) at the ventral midline of the spinal cord (C). In *Dcc*$^{-/-}$ embryos, commissural neurons are present (see TAG-1^{+} cell bodies), but fewer axons extend in the dorsal spinal cord (B) and fewer yet extend into the ventral spinal cord (D). Many of those that do project along aberrant trajectories within the ventral spinal cord (arrows in D). Projections of sensory axons, and the ventral roots, appear largely normal (B). Arrowheads in (C) and (D) indicate a population of TAG-1^{+} cells adjacent to the floor plate.

Additional abbreviations: d, dorsal root ganglion; drez, dorsal root entry zone; mc, motor column; v, ventricle.

Scale bars are 100 μm in (A, B), 50 μm in (C, D).


A
+/+
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D
-/-
fp

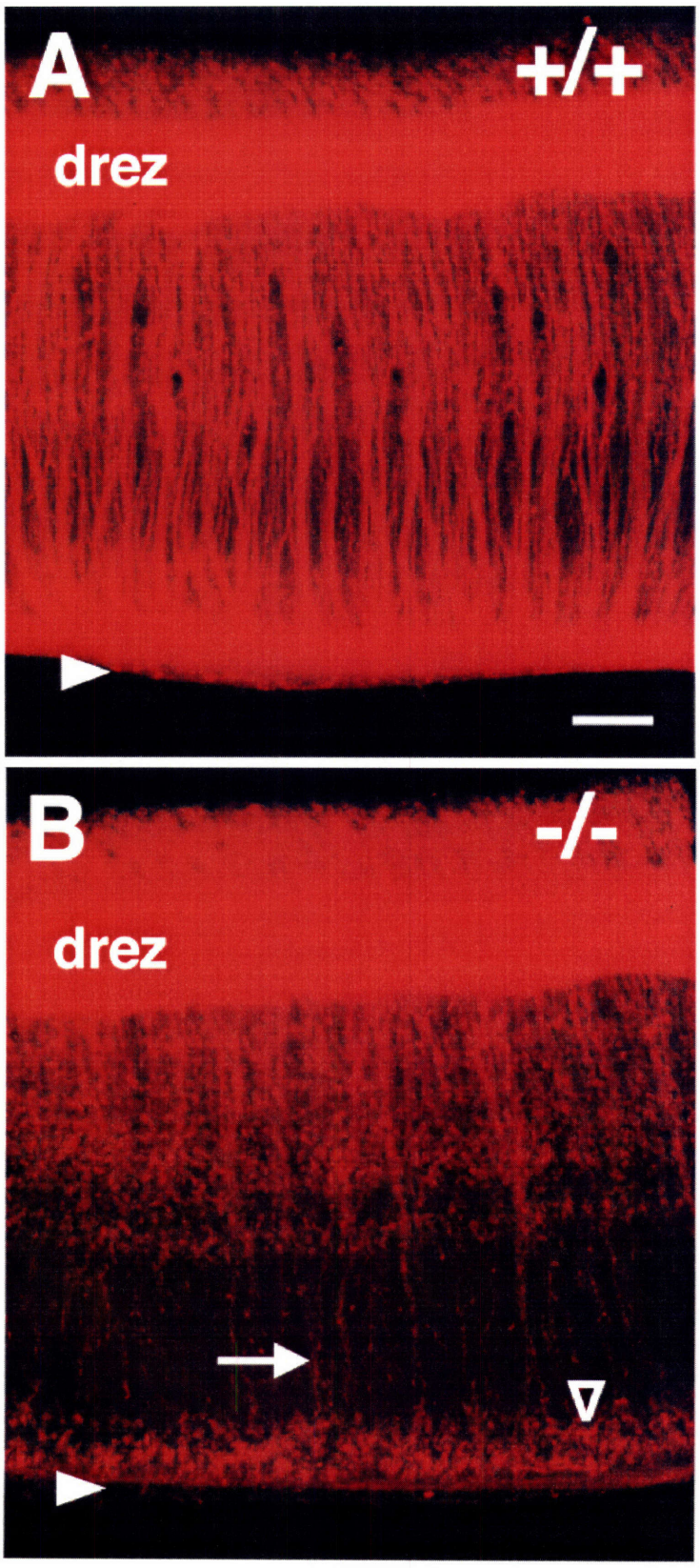
In E9.5-E11.5 embryos homozygous for the null *Dcc* allele, the projections of commissural axons were markedly disrupted (Fig. 5 and data not shown). In the dorsal spinal cord, there was a reduction in the number of commissural axons, though those that did extend appeared to adopt a normal dorsal-to-ventral trajectory. There was also a significant reduction in the number of axons that penetrated the ventral spinal cord. Interestingly, in some sections (see Fig. 5B and 5D), some axons were observed that projected all the way to the floor plate. In other sections, however, no axons were seen reaching the floor plate (data not shown). This heterogeneity along the rostrocaudal axis was evident is sagittal views of the spinal cord (Fig. 6), where small numbers of axons, often clustered at particular locations along the rostro-caudal axis, can be seen reaching the floor plate in some regions (arrowheads in Fig. 6B), while many regions are devoid of such axons. In addition to this reduction in the number of commissural axons projecting into the ventral spinal cord, there appeared to be a misrouting of many of the axons that did project into this region. Whereas in the wildtype spinal cord, the axons that enter the ventral half of the spinal cord are largely directed toward the ventral midline (Fig. 5C), in the $Dcc^{-/-}$ embryos the axons appeared more randomly directed, with some projecting more medially and others more laterally (Fig. 5D). These defects revealed by TAG-1 staining were fully penetrant, as they were observed in all $Dcc^{-/-}$ embryos examined (over 10 transverse sections of n = 8 embryos, and whole-mounts of n = 8 embryos), but in none of the wildtype littermates (n = 6 and 5, respectively).

Figure 6

Few Commissural Axons Reach the Floor Plate in *Dcc*$^{-/-}$ Embryos.

Sagittal views (side views) of spinal cords from a wildtype (A) and a *Dcc*$^{-/-}$ (B) E11.5 embryo that were subjected to whole-mount immunohistochemistry with an antibody to TAG-1 to visualize commissural axon trajectories. Dorsal is up, ventral down (arrowhead indicates floor plate) (drez indicates sensory axons coursing longitudinally in the dorsal root entry zone). The characteristic microsegmented projections of commissural axons along a dorso-ventral trajectory to the floor plate is visible in the wildtype embryo (A). In contrast, in the mutant (B) many fewer axons extend within the dorsal spinal cord, and only a few reach the floor plate (arrow). Open arrowhead indicates TAG-1^{+} cells located adjacent to the floor plate (see also Figure 5D).

Scale bar is 100 μm.


A
+/+
drez
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drez

The defects in commissural axon projections did not appear to reflect a major reduction in the number of commissural neurons, the presence of which could be demonstrated through their expression of the cell surface marker TAG-1. The distribution of TAG-1$^+$ cell bodies in the spinal cord in the mutant appeared similar to that observed in wildtype and heterozygous littermates, though the cell bodies in the latter case were obscured by the numerous TAG-1$^+$ axons that were present (compare Fig. 5A and 5C with Fig. 5B and 5D). One difference, however, was the observation that in the mutant spinal cords TAG-1 staining was stronger in locations closer to the dorsal midline (roof plate) than was in the corresponding regions of wild-type or heterozygous littermates (compare Fig. 5A and 5B). The distribution of TAG-1$^+$ cell bodies in mutants was actually very similar to the distribution of *Dcc* transcripts at these stages (see (Keino-Masu et al., 1996), Figure 5D), reflecting the distribution of the cell bodies of commissural neurons. TAG-1 staining is not normally observed on the cell bodies of the dorsal-most commissural neurons, because TAG-1 expression is usually downregulated on cell bodies as neurons extend axons (Dodd et al., 1988). Thus, the continued expression of TAG-1 in the most dorsal regions correlates with and may be caused by the reduced extension of commissural axons.

This analysis assumed that TAG-1 is a reliable marker of commissural axons as they grow to the floor plate not just in wildtype but also in *Dcc*$^{-/-}$ embryos. However, since DCC and TAG-1 are normally coexpressed on these axons, it also seemed possible in principle that a change in the distribution of TAG-1 immunoreactivity in the spinal cord of mutant embryos could reflect not a change in the growth of commissural axons but rather a change in the expression of TAG-1 by these axons caused by the absence of DCC protein. To address this possibility, we examined the trajectories of commissural axons by injecting the fluorescent lipophilic dye DiI into the cell bodies of these neurons in the dorsal spinal cord, which diffused down their axons. The pattern of axonal projections visualized in this way at E11.5 was very similar to that observed by TAG-1 immunoreactivity both in wildtype embryos (compare Fig. 5A, 5C and Fig. 7A, 7B), and in *Dcc*$^{-/-}$ embryos (compare Fig. 5B, 5D with Fig. 7D-F), indicating that TAG-1 is in fact a reliable marker

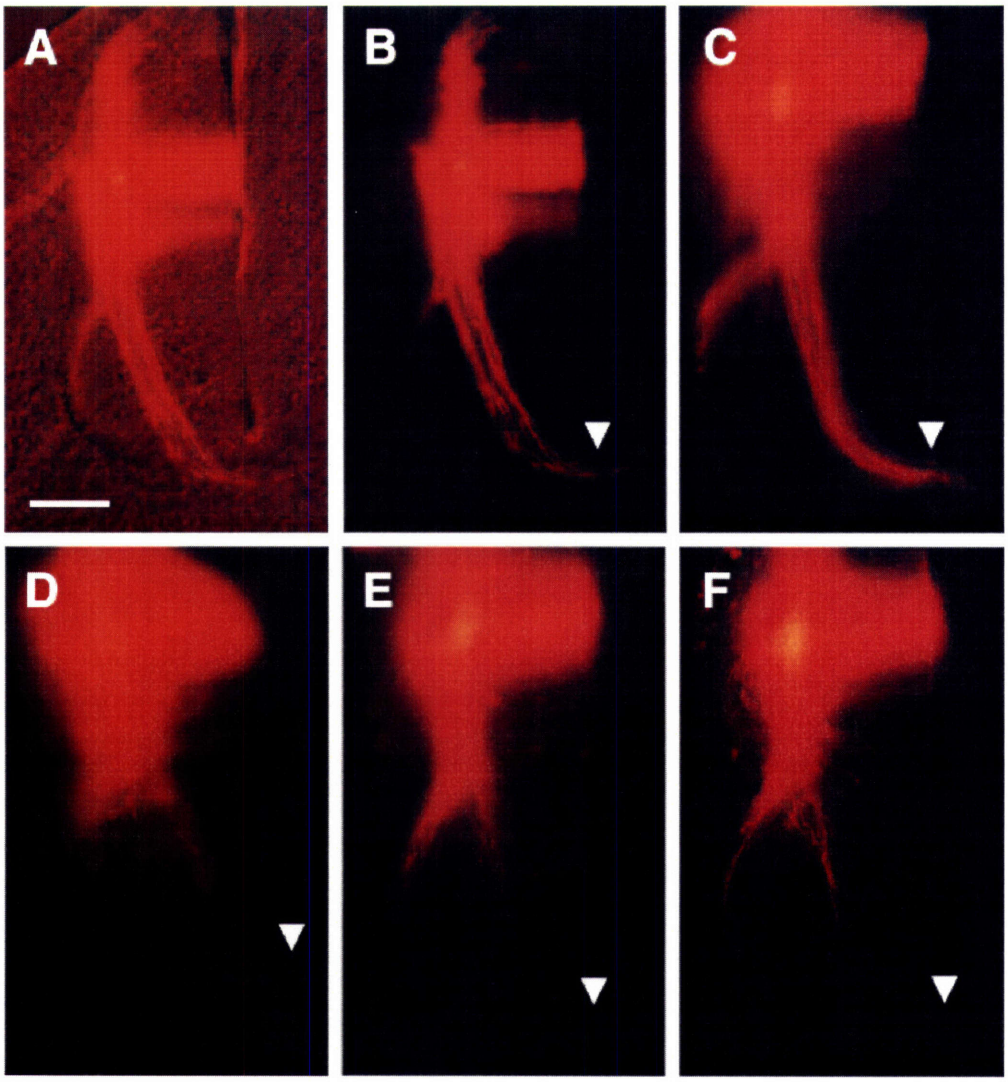
of commissural axons in the mutant embryos. Again, the defects appeared fully penetrant, as they were observed in 9/9 *Dcc*$^{-/-}$ embryos.

Figure 7

Defects in Commissural Axon Projections Revealed by Dye-tracing in the Spinal cord of Wildtype and *Dcc*$^{-/-}$ Embryos. DiI was injected into the dorsal spinal cord of wildtype (A, B), heterozygous (C), and homozygous mutant *Dcc*$^{-/-}$ E11.5 embryos (D-F) and allowed to diffuse down commissural axons. Axonal projections were visualized in transverse vibratome sections from these embryos. Note that less DiI was injected in the wildtype embryo than in the other embryos shown.

(A, B, C). Combined phase contrast and fluorescence micrograph (A), and fluorescence micrograph (B), of a wildtype embryo, showing the normal trajectory of commissural axons to the floor plate (arrowhead in (B)). Note the presence of ipsilaterally projecting axons originating in the dorsal spinal cord, which do not express TAG-1 (see Figure 5). A similar pattern of projection is observed in *Dcc*$^{+/-}$ embryos (C) (n = 12/12).

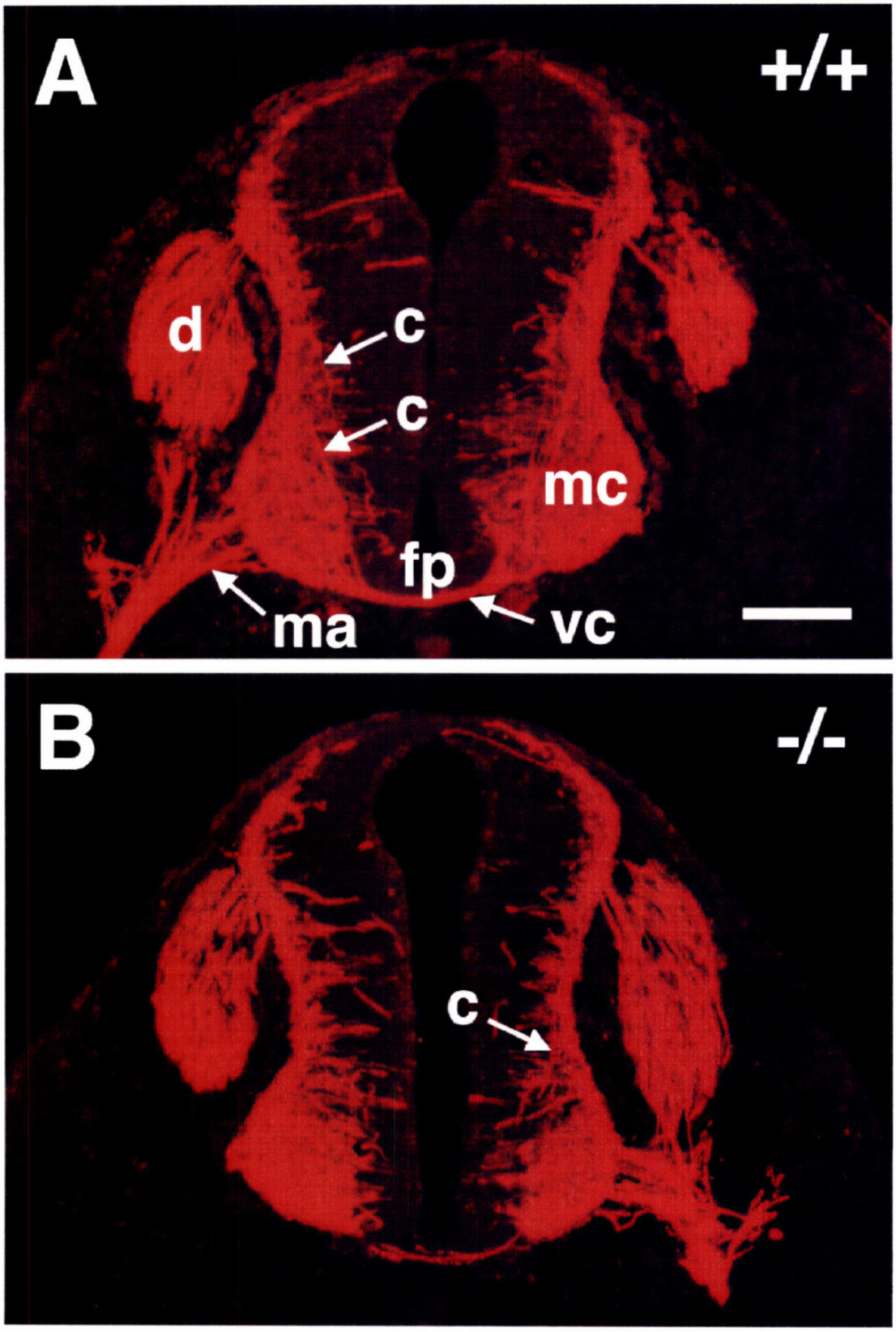
(D - F). Trajectory of axons labelled from the dorsal spinal cord in 3 different *Dcc*$^{-/-}$ embryos. The trajectory of presumptive commissural axons is similar to that seen by TAG-1 labeling (Figure 5): many fewer axons project into the ventral spinal cord; some axons project ventromedially but are not particularly directed towards the floor plate (E, F), and many axons wander within the motor column (G). Few axons reach the floor plate (arrowheads in (D -F)). Axons projecting along the lateral edge of the motor column, which are presumptive ipsilaterally projecting axons, are observed in all cases. Similar results were observed in 9/9 embryos.


A
B
C
D
E
F

The defects in commissural axon projections appeared to be specific to this class of axons, inasmuch as the pattern of TAG-1 expression did not reveal obvious defects in the development of the axons associated with both sensory and motor neurons, the latter of which express DCC (Keino-Masu et al., 1996) (Fig. 5A and 5B). This was further confirmed by staining sections using an antibody to neurofilament protein, which labels all axons present in the spinal cord. As was the case with the TAG-1 antibody, this staining failed to reveal any obvious defects within the spinal cord in the projections of axons other than commissural axons (Fig. 8). It also revealed a clear reduction in the size of the ventral commissure though not its complete absence (Fig. 8 and data not shown), consistent with the projection of some commissural axons to the floor plate in the mutant animals.

Figure 8

Neuronal Differentiation in Wildtype and *Dcc*$^{-/-}$ Embryos Visualized Using Anti-Neurofilament Antibodies. Axonal trajectories were visualized by immunohistochemistry using an antibody to NF-M in sections of caudal spinal cord from wildtype (A) and *Dcc*$^{-/-}$ (B) E11.5 embryos, revealing a reduction in the number of commissural axons (c) projecting to the floor plate (fp) as well as a reduction in the size of the ventral commissure (vc) in the mutant, but no obvious defects in pattern of motoneurons in the motor column (mc) and of their axons (ma), or of sensory neurons in the dorsal root ganglia (d) (see also Figure 5).
Scale bar is 100 μm.


A
+/+
d
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c
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-/-
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Defects in brain development in *Dcc*$^{-/-}$ mice

The defects in commissural axon projections in the spinal cord were similar to those observed in *netrin-1*-deficient animals, though more severe (Serafini et al., 1996; see Discussion). In addition to defects in spinal cord development, *netrin-1*-deficient animals show multiple defects in brain development: the absence of corpus callosum and hippocampal commissure, a reduction in the anterior commissure, the presence of an unusual commissure at the junction of midbrain and hindbrain, and the absence of pontine nuclei in the rostral hindbrain (Serafini et al., 1996). We therefore examined whether any defects were apparent in morphogenesis or axonal projections in horizontal sections of brains from newborn *Dcc*$^{-/-}$ mice.

Striking defects were indeed observed in these mutants which were similar to those observed in *netrin-1*-deficient animals. The corpus callosum and hippocampal commissure appeared to be completely absent in the *Dcc*$^{-/-}$ mice (Fig. 9A and 9B). The axons that normally form these commissures appeared to be present but failed to cross the midline and remained ipsilateral, projecting to aberrant locations and forming tangles ("Probst bundles"). The anterior commissure in *Dcc*$^{-/-}$ mice was also severely reduced; the large anterior and posterior limbs of the anterior commissure (Fig. 9C) did not form and join near the midline (data not shown), although in most mutant animals we detected a reduced commissure at the midline which, based on its horizontal projection pattern, might correspond to a portion of the posterior component of the anterior commissure. Figure 9D shows the largest such commissure that was detected. As in *netrin-1*-deficient animals, these defects did not reflect a generalized defect in commissure formation, as the habenular and posterior commissures both appeared to be intact in these animals (Fig. 9E and 9F).

In addition to these forebrain defects, two phenotypes in the brainstem were observed in the *Dcc*$^{-/-}$ mice. First, an enlarged commissure that is not normally present was observed in the anterior medullary velum which forms the roof of the fourth ventricle in the junctional region between hindbrain and midbrain (Fig. 9G and 9H). Second, the pontine nuclei at the base of the

rostral hindbrain appeared to be absent in the mutant animals (Fig 9I and 9J). Thus, the defects seen in the *Dcc* -deficient animals were similar to those observed in *netrin-1*-deficient animals (Serafini et al., 1996). The various defects observed in the homozygous animals were not observed in heterozygous littermates (data not shown).

Figure 9

Defects in Brain Commissures and Absence of Pontine Nuclei in *Dcc*$^{-/-}$ Embryos. Horizontal sections from brains of wildtype or heterozygous neonatal pups (A, C, E, G, I), and homozygous mutant pups (B , D, F, H, J) stained with hematoxylin and eosin (rostral is up in each).

(A, B). Sections through the region of the corpus callosum (CC) and hippocampal commissure (HC) of a wildtype (A) and *Dcc*$^{-/-}$ (B) pup, showing the absence of these commissures in the mutant. Axons that normally form the commissures appear to be present in the mutant but to coalesce in aberrant swirls (Probst bundles, arrowheads). These commissures were both present in the heterozygous pups (data not shown).

(C, D) Sections through the region of the anterior commissure (AC) showing both the anterior limb (aAC) and posterior limb (pAC) of this commissure in a wildtype pup (C). The anterior commissure is much reduced in the homozygous *Dcc*$^{-/-}$mutant (D). The anterior limb is not observed approaching the posterior limb in any section; instead, an aberrant bundle of axons (arrow in D) was observed approaching the midline at more rostral levels, which may consist of misrouted axons that normally form the anterior limb. The posterior limbs were much reduced but not, apparently, completely absent, and in most mutants a reduced commissure was observed (arrowhead). The anterior commissure appeared normal in heterozygous pups (data not shown).

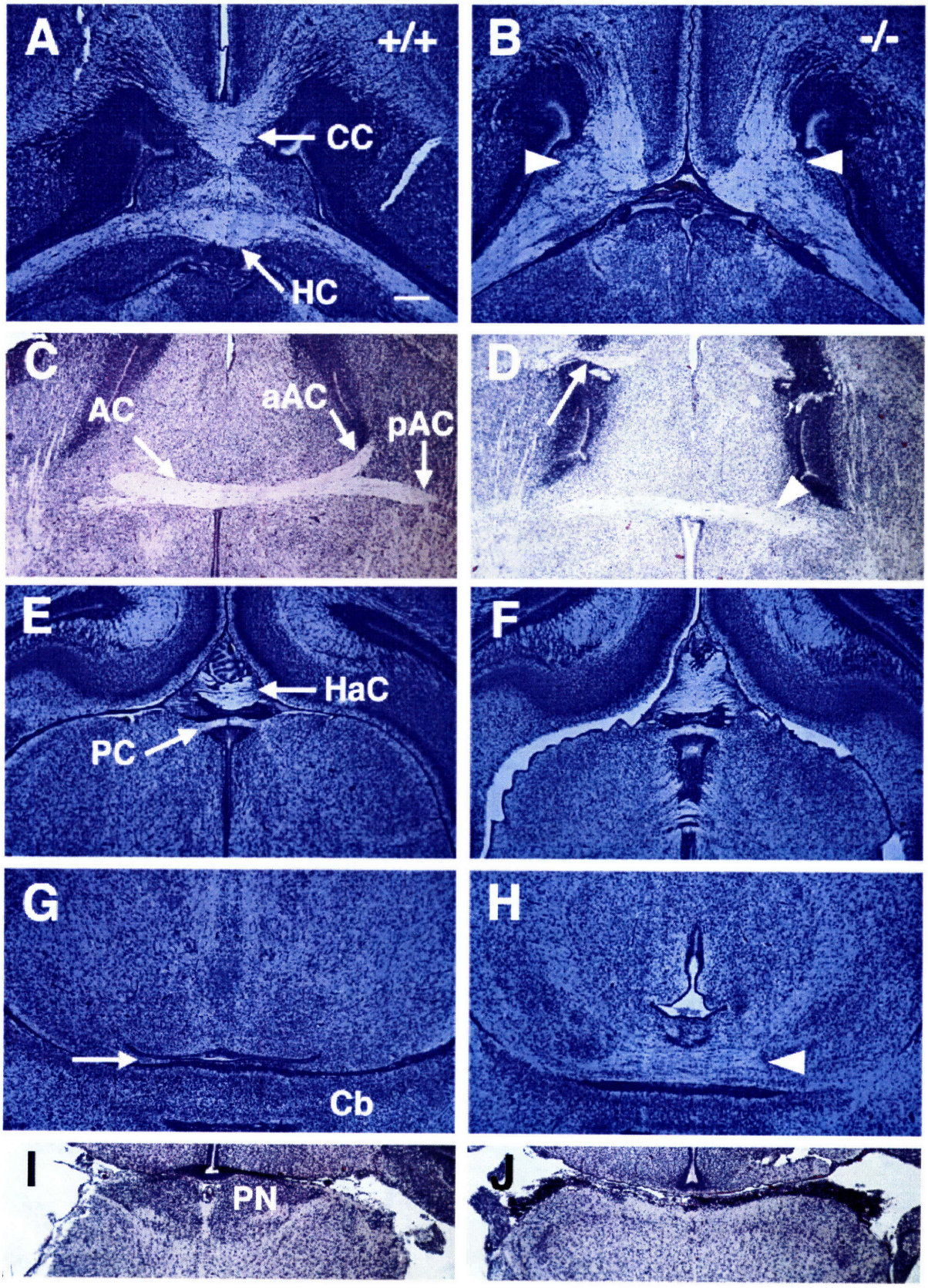
(E, F) The habenular commissure (HaC) and posterior commissure (PC) are present in wildtype (data not shown), heterozygous mutant (E), and homozygous mutant (F) pups. Note that the caudal extension of the posterior commissure shown in (F) is also observed in appropriate sections in wildtype and heterozygous pups (data not shown).

(G, H) In *Dcc*$^{-/-}$pups (H), a thick commissure (arrowhead) is observed in the roof of the fourth ventricle in the region of the hindbrain-midbrain junction, that is not observed in wildtype pups (G) or heterozygous pups (data not shown) in this region (arrow in (G)).

(I, J) The pontine nuclei (PN) at the base of the rostral hindbrain in wildtype (data not shown) and heterozygous (I) pups are not detected in *Dcc*$^{-/-}$pups (J).

Additional abbreviation: Cb, cerebellum.

Scale bars are 175μm in (A, B, E, F), 135μm in (C, D), 150μm in (G, H), 200 μm in (I, J).


A
+/+
CC
HC
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PN
J

Discussion

Candidacy of *DCC* as a Tumor Suppressor Gene in Colon Cancer

The experiments described here were initially designed to assess the role of the human *DCC* gene in colonic tumorigenesis through manipulation of its mouse homologue. At the outset of these experiments, we anticipated that mice carrying a null allele of the *Dcc* gene would exhibit an increased susceptibility to tumorigenesis. While we observed a small number of tumors in several tissues in aged $Dcc^{+/-}$ mice, this number was no greater than that observed in wild-type mice (Table 1), and those lesions examined genetically gave no evidence of loss of the remaining wild type *Dcc* allele. These observations argue against a causal role for the mutant allele in the pathogenesis of the tumors that were observed.

A potentially more sensitive test of a tumor suppressor role of *DCC* involved inactivation of both alleles of *Dcc* in mouse intestinal adenomas, since loss of DCC has been implicated to play a causative role in the progression of early adenomas to intermediary adenomas (Boland et al., 1995) and intermediary adenomas to advanced adenomas (Boland et al., 1995; Fearon et al., 1990; Vogelstein et al., 1988) in human colon. Mice of the Apc^{Min} genotype are predisposed to developing large numbers of intestinal polyps (Moser et al., 1990; Su et al., 1992), a condition very similar to familial adenomatous polyposis seen in humans (Groden et al., 1991; Nishisho et al., 1991). Moreover, somatic mutation of *APC* occurs in an early stage in the majority of sporadic human colonic tumors (Powell et al., 1992). Since *DCC* has been described as a tumor suppressor gene whose expression is eliminated after *APC* inactivation during colonic tumor progression (Boland et al., 1995; Fearon, 1995; Fearon and Vogelstein, 1990; Vogelstein et al., 1988), we expected that its loss in the mice would accelerate the progression of or modify the phenotype of polyps initiated in the $Apc^{Min/+}$ mice. We observed no such effect in adenomas that carried only the inactivated allele of *Dcc*. (Table 2 and Fig. 2).

A third line of investigation addressed any part played by the *Dcc* gene in intestinal morphogenesis in the mouse. We reasoned that if DCC played an ongoing role in the control of proliferation of intestinal epithelial cells, then its absence from the embryonic gut or adult intestine would, with great likelihood, have a readily observable effect on proliferation or the development of colonic crypts, villi, and the associated epithelial cell populations. The absence of *Dcc* had no effect on proliferation or intestinal morphogenesis (Fig. 3 and 4).

Taken together, these three lines of investigation argue that *Dcc* plays little if any part in the physiology of the intestinal epithelium in the mouse. The implications of these findings for the human *DCC* gene and its role in human colorectal tumorigenesis are less clear, however. Of importance, the evidence from other lines of work has failed to implicate *DCC* conclusively as a tumor suppressor gene in colon cancer.

One line of evidence has come from tissue culture experiments and suggests that DCC can reverse the tumorigenic phenotype (Narayanan et al., 1992). In those experiments, high levels of anti-DCC RNA appeared to cause a non-specific cytostatic effect in Rat-1 cells and thus the cells had to be passaged in culture to select for clones able to tolerate anti-DCC RNA (Narayanan et al., 1992). The selected clones were found to be more tumorigenic than the parental cells, but the long term culture of these cells may have inadvertantly selected for those with increased tumorigenicity. Moreover, the rationale underlying the mechanism of action of an anti-sense RNA on cells that do not express detectable levels of a sense transcript is unclear.

In a second study, DCC-overexpression in an immortalized human keratinocyte cell line caused poor growth *in vitro* and reduced tumor growth *in vivo* (Klingelhutz et al., 1995). While compatible with a physiologic role for DCC in constraining cell proliferation, this latter study is difficult to interpret for two reasons. First, high levels of DCC protein (which is not normally expressed in these cells) may, like many ectopically expressed proteins, exert non-specific cytostatic effects on cells. Second, these studies were not undertaken in cell lines generated from human colon carcinomas in which a tumor suppressive function for DCC is proposed.

The strongest evidence in support of the role of *DCC* as a tumor suppressor stems from observations of the reduction or loss of *DCC* RNA in cell lines or xenografts derived from human colon carcinomas (Fearon et al., 1990; Kikuchi-Yanoshita et al., 1992; Thiagalingam et al., 1996). Even this evidence is not conclusive however. A similar LOH of 18q and loss of *DCC* RNA expression can be observed in many pancreatic tumors (Hohne et al., 1992), in which another gene on chromosome 18q21, termed *DPC4,* has recently been identified as a tumor suppressor (Hahn et al., 1996). Its importance in pancreatic tumorigenesis is strongly supported by the discovery of a number of inactivating mutations affecting this gene in a series of tumor samples (Hahn et al., 1996). Hence, in these tumors, loss of *DCC* expression may be a consequence of events affecting a linked gene rather than the primary target of inactivation during tumor progression.

Thus, the evidence accumulated to date supporting a role of *DCC* as a tumor suppressor operating in the human colon is far from conclusive. The findings of the present study in mouse also fail to add support to the candidacy of DCC as a tumor suppressor gene. In contrast, the role played by *DCC* in another, quite distinct tissue site has been demonstrated definitively by a confluence of recent studies of *C. elegans, Drosophila melanogaster*, rat (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996) and the mouse (this study), which indicate that the products of the *DCC* gene and its homologs serve as receptors to morphogens that guide axonal migration during the development of the nervous system.

These disparate observations in humans, rodents and invertebrates can be rationalized in at least two ways. It is possible that the mouse and human genes have assumed distinct roles during the divergent evolution that has separated rodents from primates. If this were so, then the lack of an effect of *DCC* loss on the growth, differentiation and neoplastic transformation of cells in the mouse intestine would provide little insight into the role of *DCC* in human colon cancer pathogenesis. Arrayed against this possibility of such divergent roles of *DCC* in humans and mice is the evidence that DCC plays a highly conserved function in axonal guidance in organisms as divergent as worms, fruit flies and vertebrates (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996) (and this study). Such highly conserved function for DCC homologs in

these organisms suggests that in humans, as in other species, the major role of DCC is in axonal guidance. Also, some investigators have had difficulty verifying the presence of DCC protein in the human colonic epithelium (Turley et al., 1995) while the DCC protein is readily detectable in the developing human brain (Hedrick et al., 1994). Nevertheless, the fact that DCC does not act as a tumor suppressor gene in mice does not completely rule out a role for DCC in tumor suppression in humans.

The second, alternative possibility is that in human colon cancer, as is seemingly the case in pancreatic cancer, the loss of DCC expression is a consequence of events affecting a linked gene and that *DCC* is not the primary target of inactivation during tumor progression. According to this scenario, the observed LOH of 18q21 affects not only the *DCC* gene but other neighboring genes as well, one or more of which is the bonafide target of inactivation during colon tumor progression. The definitive proof of this possibility can only come from the identification of a gene on 18q that undergoes LOH and whose retained allele is also mutated in the majority of colonic tumors. This linked gene is unlikely to be either *DPC4* or *JV18* because their retained alleles appear to be mutated in only a fraction of human colon tumors that suffers 18q LOH (Thiagalingam et al., 1996).

DCC is required for commissural axon projections in the spinal cord

Whereas *Dcc*$^{-/-}$ mice show no obvious phenotypes in intestinal development or increased tumor susceptibility, the present results indicate profound effects of loss of DCC protein on the development of axonal projections in the central nervous system. These defects are observed in the same classes of axons that are affected in *netrin-1*-deficient animals (Serafini et al., 1996). Our observations are therefore consistent with the hypothesis that DCC is a component of a receptor mechanism for netrin-1, a model that was suggested by the finding that DCC is a netrin-1-binding protein expressed on spinal commissural axons whose function is required to mediate the outgrowth-promoting effects of netrin-1 on these axons *in vitro* (Keino-Masu et al., 1996).

Commissural axon projections are defective in the spinal cord of *Dcc*$^{-/-}$ embryos, as they are in *netrin-1*-deficient animals (Serafini et al., 1996). Although the similarity in phenotypes is striking, commissural axons appear more foreshortened in the *Dcc*$^{-/-}$ than in the *netrin-1*-deficient mice (compare this study and Serafini et al., 1996). One possible explanation for this difference is that the *netrin-1* allele that was studied is not a complete null allele, and residual netrin-1 function is present in those animals (discussed in Serafini et al., 1996). This residual netrin function could account for the less severe phenotype that is observed in that study. Hence, it is possible that the phenotype of mice lacking all *netrin-1* function would be identical to the *Dcc* null phenotype reported here. Such an observation would indicate that netrin-1 is the sole ligand for DCC in the spinal cord, and that DCC is absolutely required for netrin-1 effects on commissural axons.

An alternative possibility is that DCC is required not just to mediate responses to netrin-1, but also to mediate the responses of commissural axons to other cues that collaborate with netrin-1 to guide these axons. In *C. elegans*, although the DCC homologue UNC-40 is involved primarily in migrations that depend on the netrin homologue UNC-6, it is also required for certain migrations that do not require UNC-6 function (Hedgecock et al., 1990). This raises the possibility that UNC-40 has ligands other than UNC-6. By extension, it seems plausible that DCC has ligands other than netrin-1, accounting for the more severe phenotype observed in the spinal cord of *Dcc*-deficient animals.

Whichever of these models is correct, it is important to note that the defect in axon growth in the spinal cord of *Dcc*$^{-/-}$ mice is selective for commissural axons. In particular, motor axons, which also express DCC but do not respond to netrin-1 *in vitro* (Keino-Masu et al., 1996; unpublished observations), do not show projection defects like those observed for commissural axons in the *Dcc*$^{-/-}$ embryos. This observation provides an argument against a model in which DCC is simply a structural component of axons required for their extension, and instead supports the idea that the defects in commissural axon growth in the *Dcc*$^{-/-}$ embryos reflect the inability of this class of axons to transduce environmental signals, including netrin-1.

We note that some commissural axons do in fact succeed in reaching the floor plate in the *Dcc*-deficient mice (Serafini et al., 1996). Taken together, these observations point to the existence of a DCC-independent mechanism for guidance of commissural axons to the floor plate. It is possible that netrin-1 itself can function via a DCC-independent mechanism to guide commissural axons. Perhaps just as likely is the existence of cues distinct from netrin-1 that guide commissural axons toward the floor plate and function via a DCC-independent mechanism. One candidate for such a cue is a second chemoattractant distinct from netrin-1 made by floor plate cells, whose existence was suggested through analysis of the in vitro guidance activities of floor plate cells from *netrin-1*-deficient animals (Serafini et al., 1996).

DCC as a mediator of netrin-1 effects in the brain

The finding of defects in brain morphogenesis in $Dcc^{-/-}$ animals that are similar to those observed in *netrin-1* deficient animals suggests that DCC is in the response pathway for netrin-1 effects in the brain. It is not known how the defects in the brain arise in either the *netrin-1*- or the *Dcc*-deficient animals, although the distribution of *netrin-1* mRNA in relation to the axons and cells that are affected has suggested that netrin-1 might function as a guidance cue whose absence is the direct cause of the misroutings that are observed (Serafini et al., 1996). This is true not just for the defects in axonal projections that are observed, but also for the absence of pontine nuclei, which normally arise from a long-range migration of postmitotic pontine neuronal cell bodies. The absence of the nuclei could therefore conceivably reflect a failure of migration of these neurons (discussed in Serafini et al., 1996). Whatever the precise role played by netrin-1 in these events, however, our results suggest that its effects are mediated by DCC or a receptor complex of which DCC is a necessary component. If the defect in formation of pontine nuclei does in fact arise from a failure of cell migration, this would indicate that DCC functions to guide cell migrations in addition to axonal migrations.

Our results do not address whether netrin-1 has other functions in the brain that are mediated by receptors other than DCC. One candidate for a distinct netrin-1 receptor in the brain is the DCC-related protein neogenin (Vielmetter et al., 1994), which can also bind netrin-1 (Keino-Masu et al., 1996). Conversely, as discussed above, our results also do not exclude the possibility that some DCC functions in the brain are mediated by ligands other than netrin-1.

Nonetheless, our results showing a similarity in phenotypes of *Dcc*$^{-/-}$ and *netrin-1*$^{-/-}$ mice parallel the finding of interactions between netrin and DCC homologues in invertebrates that are implied by similarities of phenotypes of mutations in ligands and receptors in those species (Chan et al., 1996; Harris et al., 1996; Ishii et al., 1992; Kolodziej et al., 1996; Mitchell et al., 1996), and support the hypothesis that DCC is a component of a netrin receptor mechanism. Taken together, these studies provide strong support for the hypothesis that the wiring of the nervous system involves rules and mechanisms that are strongly conserved among nematodes, insects and vertebrates (Goodman, 1994).

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Chapter 5

Role of *p53* in Apoptosis in Colon cancer

Introduction

Studies using cultured cells have ascribed several distinct cell physiologic functions to the *p53* tumor suppressor gene. Its product, the p53 protein, may respond to DNA damage by triggering either growth arrest during the G1 (Lane, 1992) or G2 (Agarwal et al., 1995; Stewart et al., 1995) phases of the cell cycle or, alternatively, programmed cell death (Lane, 1992). In this manner, p53 may protect the normal cell from proceeding to replicate damaged DNA (Lane, 1992). Conversely, inactivation of p53 function may render a cell's genome more mutable, thereby accelerating the accumulation of mutations that represent rate-limiting steps in tumor progression.

Mechanistically related to the role of p53 in genomic surveillance may be its role in mediating cellular senescence, which is hypothesized to be triggered by telomeric collapse. This senescence prevents cells from progressing to an immortalized state. The inactivation of p53 function during tumor progression allows evolving premalignant cell populations to circumvent senescence, thereby facilitating progression to a state of immortality (Bond et al., 1994; Harvey and Levine, 1991; Harvey et al., 1993; Metz et al., 1995; Shay et al., 1995).

Apoptotic death may also be triggered in cells in response to the activities of certain oncogenes. Several studies have demonstrated that oncogenes such as *myc* and E1A trigger an apoptotic response in a variety of cultured cells (Evan et al., 1992; Qin et al., 1994; Rao et al., 1992; Wu and Levine, 1994) that represents an obstacle to the further clonal expansion of these oncogene-bearing cells. In some cells, this apoptotic response has been shown to be p53-dependent (Hermeking and Eick, 1994; Lowe et al., 1994; Lowe et al., 1993; Qin et al., 1994; White, 1994; White et al., 1992; Wu and Levine, 1994). Hence, an inactivating mutation of *p53* may enable cell populations carrying an oncogenic mutation to avoid attrition through apoptosis and thus allow them to benefit from the growth impetus provided by the mutant oncogene. Evidence supporting this model comes from studies in mice showing that normal p53, by activating apoptosis, is able to suppress the progression of tumors initiated by the expression of

viral oncogenes (Howes et al., 1994; Kim et al., 1993; Morgenbesser et al., 1994; Symonds et al., 1994).

Finally, p53-dependent apoptosis is also observed in cells that are anoxic, as might occur in tumors that are inadequately perfused by vasculature (Graeber et al., 1996). Together, these reports indicate that p53 is an important regulator that enables normal cells to halt growth or to enter apoptosis in response to a variety of physiologic stresses.

We focus here on the contribution of *p53* inactivation to colon cancer. In many human colon carcinomas, loss of *p53* heterozygosity (LOH) occurs as the last in a defined succession of mutations affecting the adenomatous polyposis coli gene (*APC*), the N- or Ki-*ras* proto-oncogene, a presumed tumor suppressor gene on chromosome 18q, and mutation of one of the two alleles of *p53* gene (Fearon and Vogelstein, 1990; Vogelstein et al., 1988). This LOH of the *p53* gene in human colonic adenomas coincides with the appearance of highly dysplastic cells within less dysplastic, intermediate grade adenomas (Boland et al., 1995). Such highly dysplastic adenomatous cells are presumably the immediate precursors of colon carcinomas.

This loss of p53 function during a defined step in colonic tumor progression must be rationalized in terms of the known biochemical and physiologic functions of the wild type *p53* gene and protein. For example, if p53 prevented colonic cells from replicating damaged DNA, one might predict that *p53* inactivation would occur relatively early in tumor progression, thereby increasing the overall rate of mutation and accelerating the advance of colonic cell clones through subsequent stages of this process.

On the other hand, if the role played by normal p53 in colonic adenomas were to induce apoptosis in response to the cumulative effects of mutations affecting the *APC*, *ras* and other genes, then inactivation of *p53* would benefit tumor progression only at a late stage. Alternatively, if p53 were responsible for inducing senescence following extensive cell division, loss of this gene would be expected only at a late stage in tumor progression. With these models in mind, we examined the effects of *p53* loss on an early and an intermediate stage of colonic progression.

Materials and Methods

Polyp Count

After euthanization, the entire gastrointestinal tract of a mouse was removed, dissected along the cephalo-caudal axis, and washed in phosphate buffered saline. Adenomas ranged in size from at least twice the width of an average size villus to at most 5-6 millimeter in diameter and were readily visible in the intestines upon inspection under a dissecting microscope at 60X magnification. For histological analysis, the entire intestine was removed surgically, fixed in Bouin's fixative, dehydrated in graded solutions of alcohol, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E).

All mice in this study were maintained in the facilities of the Whitehead Institute for Biomedical Research and were fed *ad libitum*. Their diet consisted of Agway Prolab Rat, Mouse and Hamster 3000 chow, which has a crude protein content of at least 22%, a crude fat content of at least 5% and a crude fiber content of at most 5%.

Genotyping And Southern Blot

C57BL/6 $Apc^{Min/+}$ mice were purchased from the Jackson laboratory (Bar Harbor, Maine). Min progenies were genotyped by PCR (initial step of 10 min. @ 95°C, 3 min. on ice, followed by addition of dNTP's and Taq enzyme, then one cycle of 2 min. @ 94°C; followed by 35 cycles of 2 min. @ 95°C, 2 min. @ 55°C, 3 min. @ 72°C; followed by one cycle of 5 min. @ 72°C) in two independently run reactions with each reaction used to genotype a mouse's constitutional DNA with regard to either the Min (Apc^{Min}) or the wild-type (Apc^{+}) allele of *Apc*. The reaction used to genotype for the Min allele contained the Apc^{Min}-specific primer ($5'^{2532}$TGAGAAAGACAGAAGTTA$^{2549}3'$) and the universal downstream primer ($5'^{2859}$TTCCACTTTGGCATAAGGC$^{2841}3'$) (Luongo et al., 1994).The reaction used to

genotype for the wild-type allele contained the *Apc*$^{+}$-specific primer (5'^{2241}GCCATCCCTTCACGTTAG^{2258}3') and the universal downstream primer (Luongo et al., 1994). These primers are readily available from Research Genetics Inc. Huntsville, AL using the order numbers: MGS 28566 (*Apc*^{Min}), MGS 28568 (universal) and MGS 28567 (*Apc*$^{+}$).

A colony of 129 *p53*$^{+/-}$ mice was started using the gift of one 129 *p53*$^{+/-}$ mouse from Tyler Jacks (Center for Cancer Research and the Howard Hughes Medical Institute, M.I.T.). Mice were genotyped for the *p53* locus by PCR (initial step of 10 min. @ 95°C, 3 min. on ice, followed by addition of dNTP's and Taq enzyme, then one cycle of 1 min. @ 94°C; followed by 35 cycles of 30 seconds @ 92°C, 1 min. @ 55°C, 1 min @ 72°C; followed by one cycle of 5 min 72°C) in two independently run reactions with each reaction used to genotype a mouse's constitutional DNA with regard to either the mutant (*p53*$^{-}$) or the wild-type (*p53*$^{+}$) allele. The reaction used to genotype for the wild-type allele contained the *p53*X6S primer (5'TTATGAGCCACCCGAGGT3') and the down stream primer *p53*X7AS (5'TATACTCAGAGCCGGCCT3'). The reaction used to genotype for the mutant allele contained the *p53*$^{-}$-specific primer *Neo* 18.5 (5'TCCTCGTGCTTTACGGTATC3') and the down stream primer *p53*X7AS. These primers are readily available from Research Genetics Inc. Huntsville, AL using the order numbers: MGS 24828 (*p53*X6S), MGS 24829 (*p53*X7AS) and MGS 31359 (*Neo* 18.5). PCR reactions for both *Apc* and *p53* loci tested were initially attempted by multi-plexing the three PCR primers together, but superior results were obtained by amplifying the two alleles in independent reactions.

For loss of heterozygosity (LOH) analysis, adenomas were removed surgically and DNA was prepared from them using standard methods. Southern blottings were performed as described previously (Jacks et al., 1994).

Statistical Analysis

Polyp multiplicities were compared using a modification of the Wilcoxon's Sum of Ranks Test as described by H. Mann and D. Whitney (Zar, 1974). The incidence of apoptotic cells in adenomas was compared using the Student t-test.

Gamma-Irradiation

Mice were subjected to 8 grays of gamma-irradiation using a cesium source. Eight hours later the mice were euthanized and their intestines fixed in fomalin and prepared for cell death analysis.

Quantifying The Apoptotic Cells

TUNEL assay was performed on 5-6 micron paraffin-embedded sections of adenomas or normal intestines. To do this, the sections were deparaffinized in Xylenes (Sigma) (2X8 min) and dehydrated through sequential steps of 100% ethanol (2X3 min), 90% ethanol (3 min), 70% ethanol (3 min) and distilled H_2O (3 min). Each tissue section was then treated with 100 microliters of pepsin (Sigma) (0.5% V/W in H_2O), incubated at 37°C (60 min in humid air) and washed in phosphate buffered saline (PBS^-, 3X3 min). The samples were then treated with Saponin (0.055% V/W in H_2O, 30 min) at room temperature and washed with PBS^- (3X3 min). Then, the samples were incubated in 3% H_2O_2 at room temperature for 60 min and washed in PBS^- (3X3 min). The samples were treated with the terminal transferase mix [Terminal deoxytransferase enzyme (TdT, Gibco-BRL), TdT buffer (Gibco-BRL), biotin-16-dUTP (Boehringer Mannheim) and H_2O] and incubated at 37°C (60 min in humid air). The reactions were terminated by adding the TdT reaction termination buffer (15 min) and washing the samples in PBS^- (3X3 min). The nuclei that had incorporated the biotinylated dUTP were detected using the DAB (SK4100, Vector Laboratories) and the ABC Kits (PK4000, Vector labs). The samples were washed in PBS^- (2X3min), dehydrated through sequential steps of 70% and 100% ethanol (3 min each), dried and mounted.

Counts of the apoptotic cells were performed using a light microscope at 1000X magnification. Only the TUNEL-positive epithelial cells were counted; the mesenchymal cells were excluded from our analyses. The fractions of the sloughed cells that remained in the tissue preparation varied form one preparation to the next and were therefore excluded from the counts. Only the apoptotic epithelial cells that remained a part of the solid tissue were counted. The TUNEL-positive cells had the appearance of apoptotic cells described previously (Anilkumar et al., 1992). The apoptotic cells appeared shrunken and fragmented and often had pyknotic nuclei. A 'halo' was often seen around the TUNEL positive cells.

Human Colonic Adenomas

Adenomas in Table 2 were obtained from sequential lists of resected tumors from the Ann Arbor Veterans Affairs Medical Center and the Henry Ford Hospital (Detroit, Michigan) and fixed in formalin soon after removal. Microallelotyping of these adenomas using markers on chromosome 17p were reported (Boland et al., 1995). These tumors and the detailed clinical and histopathological data regarding these tumors are available upon request from R. S. Bresalier.

Results

p53 and initiation of mouse intestinal adenomas

In order to examine the effect of *p53* inactivation on colonic epithelial cells that are still at a relatively early stage in tumor progression, we used mice of the Min strain (Moser et al., 1990), in which the first of the mutational steps involved in colonic tumor progression - mutation of the *Apc* gene - is already present in all colonic epithelial cells. Because of the germline Apc^{Min} mutation carried by these mice (Su et al., 1992), these mice are strongly predisposed to intestinal adenomas (polyps) (Moser et al., 1992; Moser et al., 1990) of the type seen in humans who carry germline

mutations of the *APC* gene and suffer from familial adenomatous polyposis (Groden et al., 1991; Nishisho et al., 1991). In order to determine the effects of mutations of *p53* on the initiation and development of adenomas in these mice, we bred a mutant allele of the *p53* gene (Jacks et al., 1994) into the germline of $Apc^{Min/+}$ mice.

Thus, 129 $Apc^{+/+}$ $P53^{+/-}$ (Jacks et al., 1994) mice were crossed to C57BL/6 $Apc^{Min/+}$ $p53^{+/+}$ (Moser et al., 1990) mice to generate 129/B6 F1 $Apc^{Min/+}$ $p53^{+/-}$ progeny. F2 and F3 progeny were generated by breeding F1 or F2 siblings, respectively. Because there was no statistically significant difference ($p>0.2$) between the average number of adenomas among the F1, F2 and F3 $Apc^{Min/+}$ $p53^{+/+}$ mice (FIG. 1), the effect of *p53* dosage reduction on polyp incidence was gauged in mice of a mixed 129/B6 genetic background.

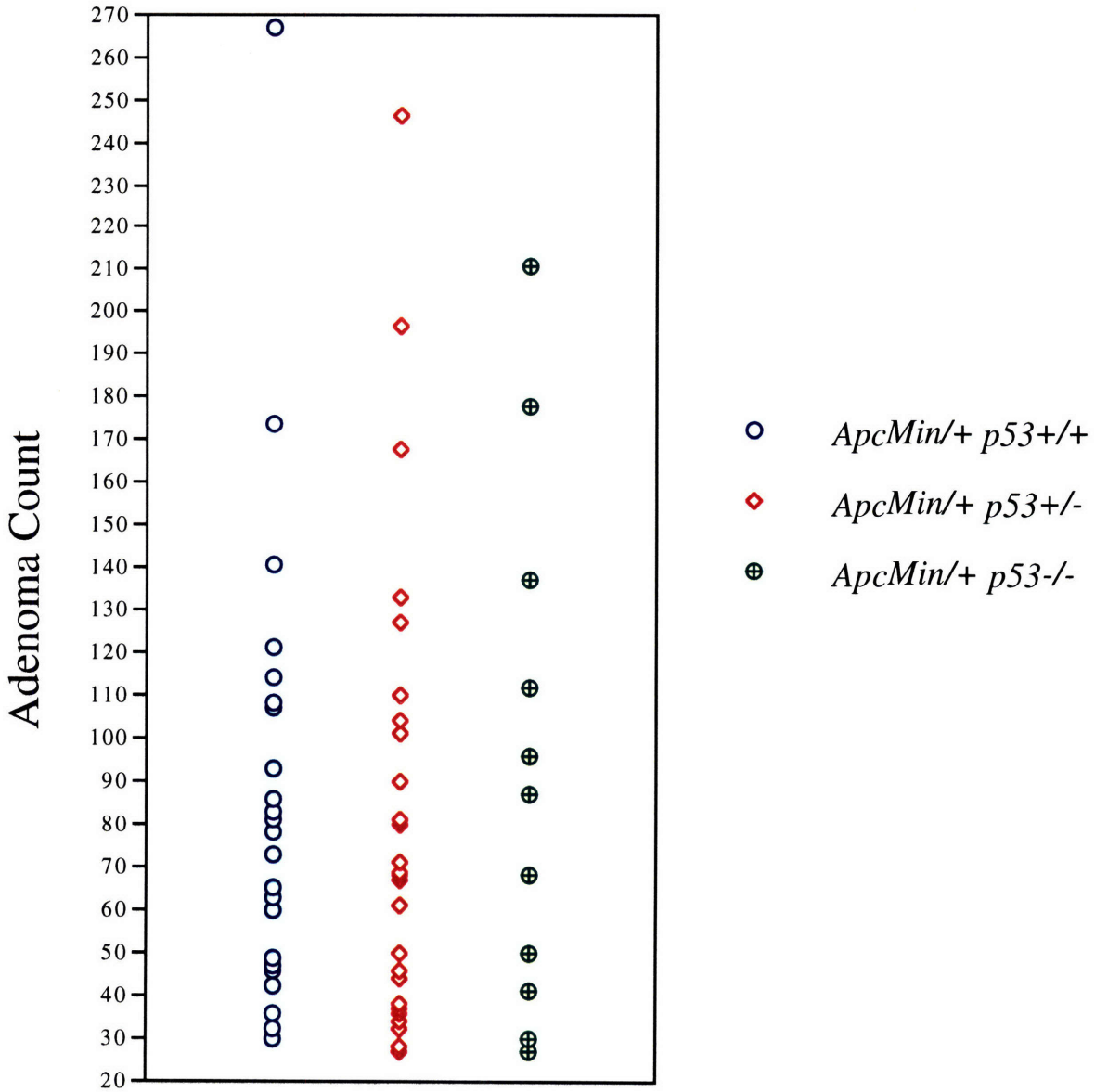
The effects of *p53* inactivation on the incidence, size and progression of polyps in mouse intestine were then ascertained. As part of this analysis, we examined the entire length of the gastrointestinal tract of these mice, registering both the size and the number of polyps. The great majority of the adenomas in these $Apc^{Min/+}$ mice were found to be localized to the small intestine regardless of the genotype of these mice at the *p53* locus (data not shown). Min mice in this colony rarely developed colonic or gastric adenomas.

Homozygosity for a germline inactivating mutation of *p53* did not enhance polyp initiation in the small intestines of the Min mice (FIG. 1) confirming previous reports (Clarke et al., 1995). Statistical analysis indicated that the number of adenomas did not increase after 80 days of age in any of the three Min groups in which the Apc^{Min} mutation was present in a $p53^{+/+}$, $p53^{+/-}$ *or* $p53^{-/-}$ background. Thus, we counted polyps at various ages beyond 80 days and assumed that these numbers accurately reflected the cumulative number of polyps arising in the mice. The $Apc^{Min/+}$ $P53^{-/-}$ mice showed a mean number of adenomas per mouse (94 adenomas, n=11) similar to the $Apc^{Min/+}$ $p53^{+/-}$ (83 adenomas, n= 26) and $Apc^{Min/+}$ $p53^{+/+}$ mice (86 adenomas, n=29) (Fig. 1). The difference between the $Apc^{Min/+}$ $P53^{-/-}$ mice and other Min cohorts was not statistically significant ($p=0.2$). The number of adenomas varied from 30 to 267 in the $Apc^{Min/+}$

p53$^{+/+}$ and from 27 to 197 in *Apc*$^{Min/+}$ *p53*$^{+/-}$ cohort and from 27 to 211 in the *Apc*$^{Min/+}$ *p53*$^{-/-}$ cohort.

Figure 1

Effect of *p53* dosage reduction on polyp initiation in the Min mice. Mice are 129Sv/B6 F1, F2 and F3. The Y-axis shows the number of adenomas observed along the entire length of intestine of mice of 80-days of age and older. Number of adenomas was determined by counting the adenomas in the intestine of each mouse under a dissecting microscope as described in the methods section.


Adenoma Count
270
260
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
ApcMin/+ p53+/+
ApcMin/+ p53+/-
ApcMin/+ p53-/-

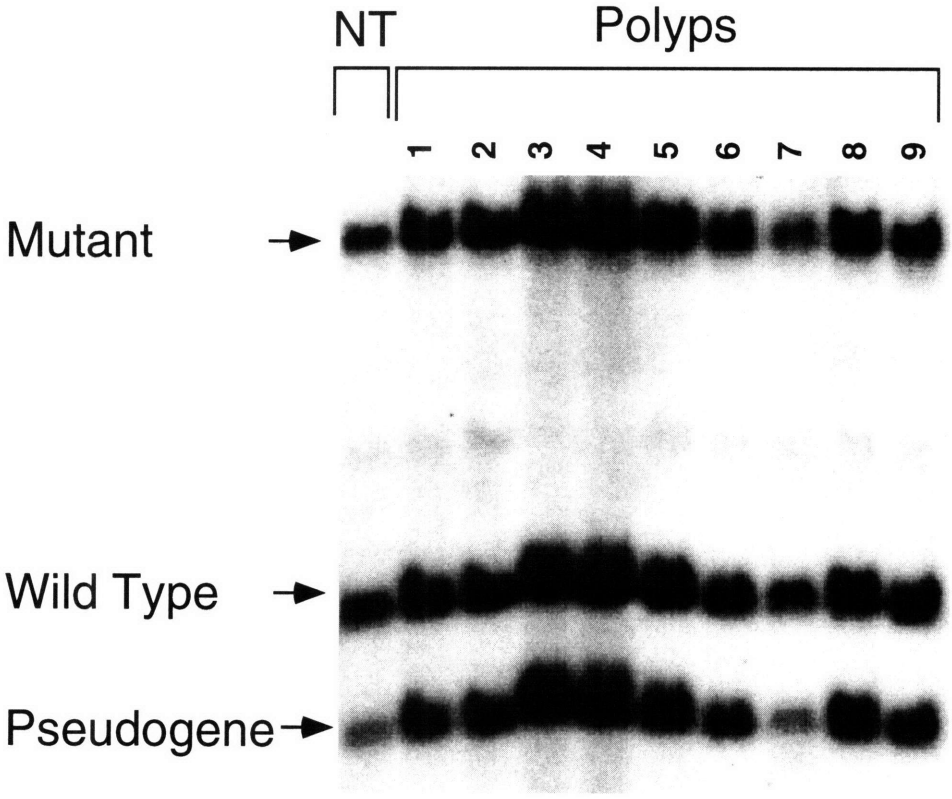
Approximately two-thirds of the *Apc*$^{+/+}$ *p53*$^{-/-}$ progeny also developed a single adenoma at the ileocecal junction but none of the *Apc*$^{+/+}$ *p53*$^{+/+}$ or *Apc*$^{+/+}$ *p53*$^{+/-}$ mice developed such adenomas (data not shown). We did not further study this effect of *p53* inactivation on the rate of initiation of adenomas at this particular anatomical site. Moreover, since we focused on the role of p53 in intestinal polyp initiation in this study, possible cooperative effects of *Apc*^{Min} allele and the *p53* mutation on tumor initiation in other organs were not examined.

We determined the extent of progression of the adenomas that did appear by examining their size and histological and morphological appearance (Vogelstein et al., 1988). Significantly, the absence of a functional *p53* gene afforded no growth advantage to the adenomas once they were initiated. We detected no difference in the size of the *p53*$^{-/-}$, *p53*$^{+/-}$ or *p53*$^{+/+}$ adenomas in age-controlled Min mice upon macroscopic examination. Furthermore, histological analysis showed that all adenomas in the Min mice were benign, similar in morphology, and characterized by a similar degree of dysplasia regardless of their genotype at the *p53* locus (data not shown). In fact, we did not observe any histological difference among any of the adenomas in *p53*$^{-/-}$ mice examined between the ages of 80 and 132 days and of any of the adenomas in the *P53*$^{+/+}$ and *P53*$^{+/-}$ groups examined between 80 and 210 days of age.

The above experiments suggested that elimination of p53 function had little effect either on the initiation of adenomas or their subsequent progression from early to intermediate stage polyps. This notion was reinforced by Southern blot analysis performed on DNA samples prepared from twenty adenomas of *Apc*$^{Min/+}$ *p53*$^{+/-}$ mice. This analysis showed that the adenomas in *Apc*$^{Min/+}$ *p53*$^{+/-}$ mice retained both the mutant and the wild type allele of *p53* even up to 150 days of age (FIG. 2). This retention of the wild type *p53* allele contrasts with previous analysis of the fate of the wild type *Apc* allele in adenomas of *Apc*$^{Min/+}$ mice, which showed that the wild type *Apc* allele is invariably discarded as adenomas progress (Laird et al., 1995; Levy et al., 1994; Luongo et al., 1994). Hence, elimination of the surviving wild type *p53* allele conferred no growth advantage on adenomatous cells while loss of the wild type *Apc* function was indeed advantageous.

Figure 2

LOH analysis of the *p53* locus in adenomas from $Apc^{Min/+}$ $p53^{+/-}$ mice. Lane 1 is the constitutional DNA from the normal tissue (NT) of a $Apc^{Min/+}$ $p53^{+/-}$ mouse. Lanes 2-10 are adenomas from five-month old $Apc^{Min/+}$ $p53^{+/-}$ mice. The top arrow points to the mutant 3.0 Kb band and the middle arrow to the 1.3 Kb wild-type band. The lowest arrow marks the 1.0 Kb fragment that appears due to hybridization of the probe to DNA corresponding to a *p53* pseudogene.


NT
Polyps
1
2
3
4
5
6
7
8
9
Mutant
Wild Type
Pseudogene

In sum, germline inactivation of *p53* neither increased the rate of initiation of adenomas in the $Apc^{Min/+}$ intestines nor did it appear to affect their size. We concluded that any effects that loss of p53 function may have on the growth properties of adenomatous cells were not sufficient to affect either the size or morphology of adenomas in the early stages of colon tumor progression in the mouse.

p53-dependent apoptosis in Apc^{Min}-induced adenomas

While *p53* inactivation had no apparent effect on the overall rate of progression of adenomatous polyps in the Min mice, it remained possible that *p53* loss influenced adenoma development in more subtle ways by affecting the rate of apoptosis of the cells in the polyps. For this reason, we examined the incidence of apoptotic cell death in the *p53*-wild type and *p53*-deficient benign mouse adenomas.

In the normal intestine, the proliferative zone is found in the crypts and in the base of the villi while the apoptotic zone is largely in the tips of the villi (Gordon and Hermiston, 1994). In addition, a small fraction of epithelial cells in the proliferative zone are also seen to be in the process of undergoing apoptotic cell death (Anilkumar et al., 1992; Clarke et al., 1994; Merritt et al., 1994). These apoptotic cells of the proliferative zone, to the extent that they can be observed, are largely confined to the crypts of the intestinal epithelium (Anilkumar et al., 1992; Clarke et al., 1994; Merritt et al., 1994). The presence of the apoptotic cells of intestine can be demonstrated on thin paraffin-embedded sections of this tissue using the TUNEL, (terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labeling) assay (Gavrieli et al., 1992).

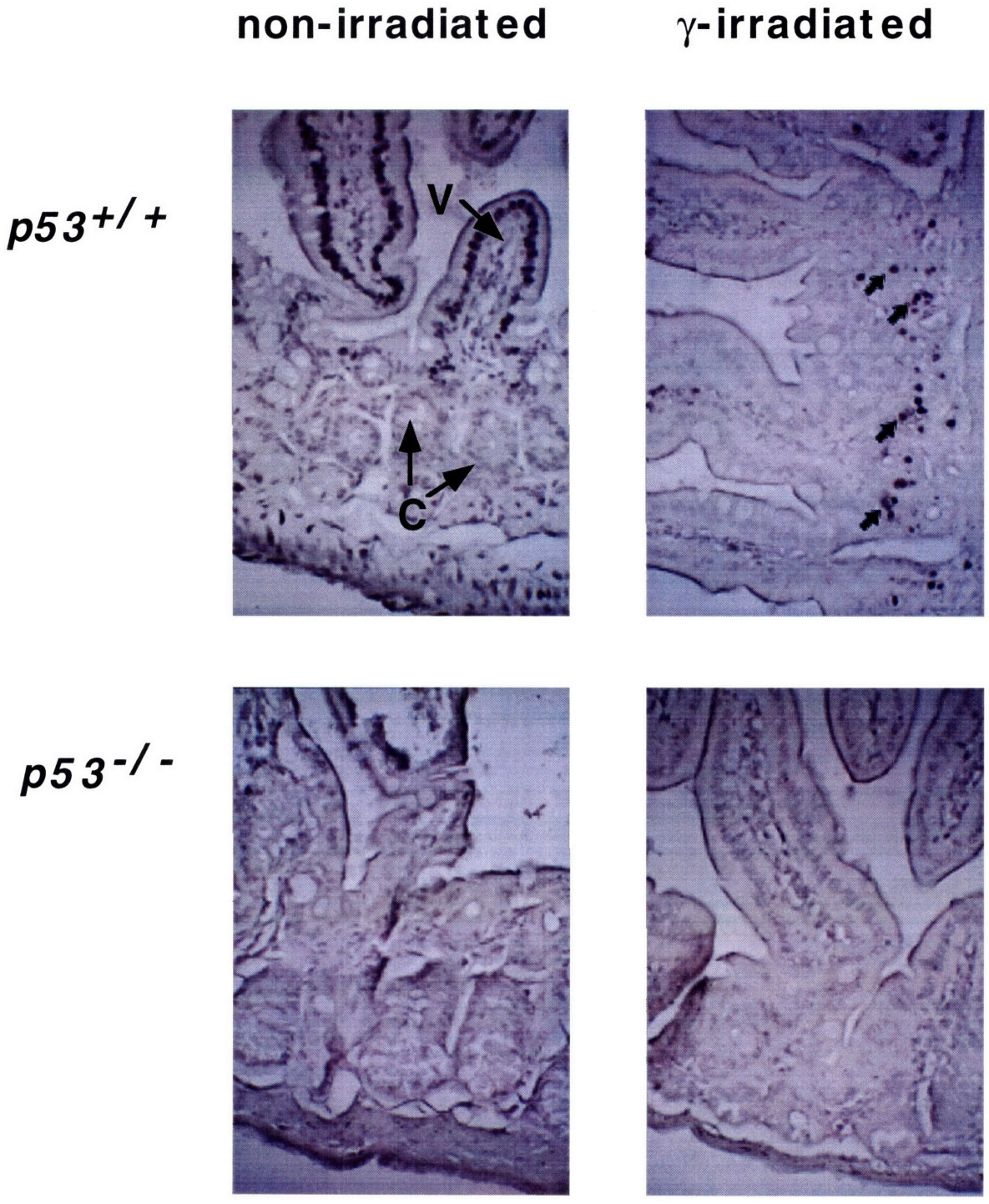
We first tested the sensitivity of the TUNEL assay, as used by us, in two circumstances in which apoptosis is a frequent occurrence. Macrophages that have just engulfed apoptotic cells can be readily identified upon microscopic examination of the Peyer's patches of the mouse intestine (Earnshaw, 1995). All nuclei of the apoptotic cells that were engulfed by macrophages registered

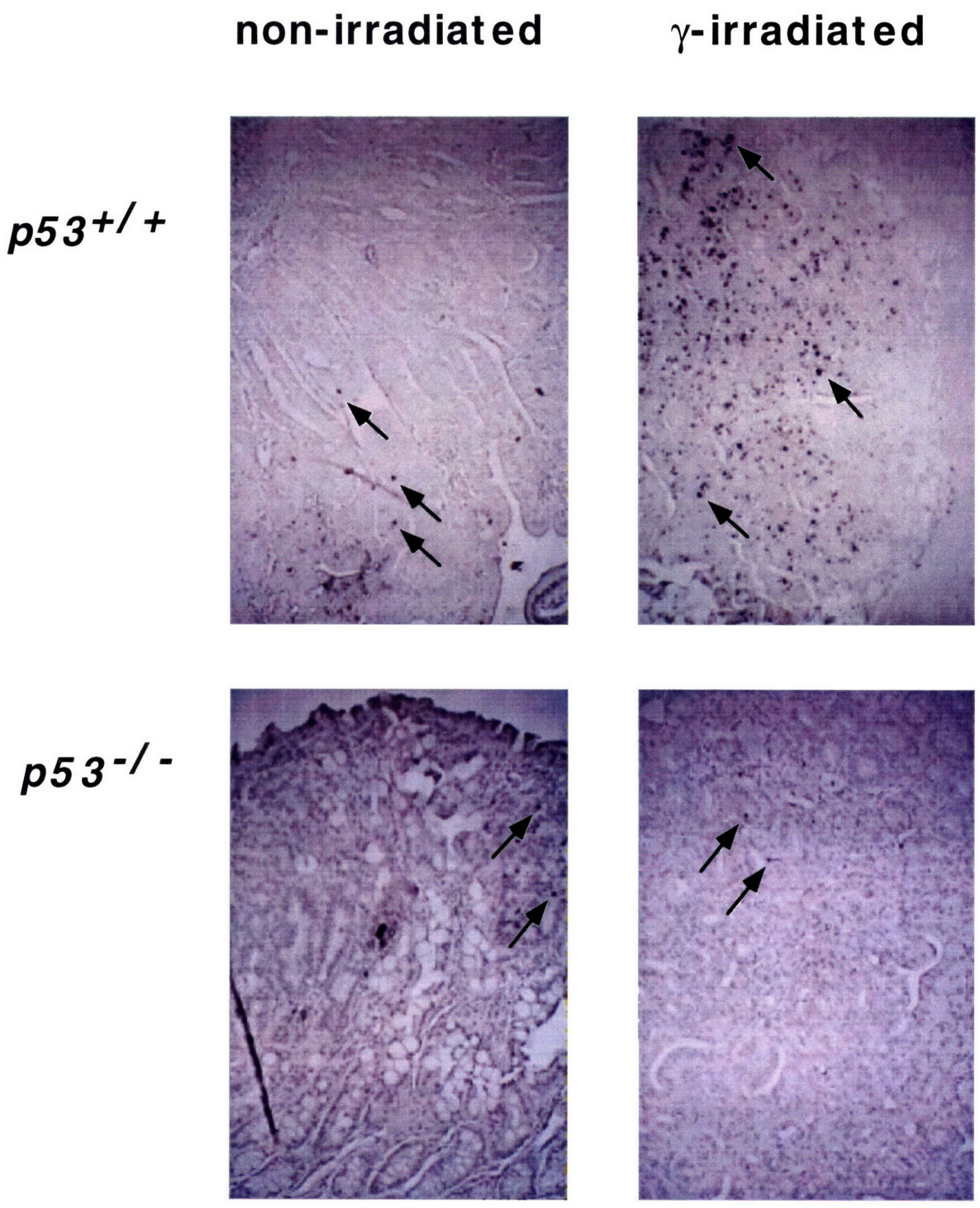
positive in our TUNEL assay in several formalin-fixed sections of Peyer's patches (data not shown).

We also verified the sensitivity of our assay by examining the apoptotic cells in irradiated intestines. Gamma-irradiation of $p53^{+/+}$ intestines is known to result in the appearance of many cells with pyknotic nuclei in the normal crypts (Clarke et al., 1994; Merritt et al., 1994).In several experiments, all of the intestinal epithelial cells having pyknotic nuclei following 8 grays of ionizing radiation registered positively in our TUNEL assay (FIG. 3A & B). We also extended the reports of others (Clarke et al., 1994; Merritt et al., 1994) that this gamma-irradiation-induced apoptosis is p53-dependent, in that both the crypts and the adenomas of $Apc^{Min/+}$ $p53^{-/-}$ intestines displayed a low rate of apoptosis similar to that of non-irradiated controls (Table 1, FIG. 3A & B).

Figure 3

Effects of p53 inactivation and gamma-irradiation on apoptosis in intestine of the Min mice. (A) Normal small intestine (400X magnification). (B) Small intestinal adenomas (200X magnification). Intestines were prepared and the apoptotic cells were identified using the TUNEL assay. "C" and "V" refer to the crypt and the villus respectively. Arrows mark some of the apoptotic cells.




non-irradiated
γ-irradiated
p53+/+
p53-/-

We then used the TUNEL assay, as validated above, to gauge apoptotic rates in *p53*$^{+/+}$ and *p53*$^{-/-}$ adenomas. Our analyses showed that on average, 1.5% of the epithelial cells of adenomas were undergoing the process of apoptotic cell death at any time (Table 1). In contrast to the effects of p53 on radiation-induced apoptosis, the presence or absence of p53 had no obvious effect on the incidence of apoptotic cells in the non-irradiated early adenomas (p=0.3, 1.5% in *p53*$^{+/+}$ vs 1.6% in *p53*$^{-/-}$, Table 1 & FIG. 3B). Thus, *p53* loss did not affect development of early adenomas through its ability to suppress apoptosis. This was in consonance with our earlier observations indicating that *p53* loss had no effect on the growth rate or histopathology of these early adenomas.

Table 1

Effect of *p53* inactivation on gamma-irradiation-induced apoptosis in mouse intestinal adenomas. [a]

Numeric values represent mean percentage of epithelial cells that were apoptotic as determined by a combination of the TUNEL assay and histological analysis. Percentage apoptotic cells was measured for each segment of normal intestine or each adenoma by examining five independent non-overlapping regions.

[b] n is the number of adenomas or number of segments of the normal intestine where apoptotic cells were measured.

Rate of apoptosis in normal crypts and adenomas in both gamma-irradiated and non-irradiated mice.

P53 Genotype	Normal crypts non-irradiated	Normal crypts gamma-irradiated	Adenomas non-irradiated	Adenomas gamma-irradiated
+/+	1.4%[a] (n=10)[b]	11.3% (n=10)	1.5% (n=9)	17% (n=11)
-/-	0.5% (n=17)	0.9% (n=8)	1.6% (n=25)	2.7% (n=12)

P53 inactivation and apoptosis in late-stage human colonic adenomas

In human colonic adenomas, LOH at the *p53* gene coincides with the appearance of highly dysplastic cells within adenomas. These highly dysplastic nodules are often found to arise within large, less dysplastic, intermediate grade adenomas. This observation has suggested that *p53* mutation plays an important role in a relatively late step of colon cancer progression by enabling intermediate grade adenomas to progress to those having a highly dysplastic phenotype. We wished to gauge the possible role played by *p53* inactivation in modulating apoptosis at this late stage of colonic tumor progression. The highly dysplastic regions of polyps, which are presumably the immediate precursors of colon carcinomas, are not frequently encountered in mice. For this reason, we turned to human biopsy samples and compared highly dysplastic nodules with closely apposed, less dysplastic precursors, hoping to gauge the influence, if any, of *p53* loss on this progression.

Loss of p53 function in colonic tumors involves two genetic steps. In the first of these, p53 function is partially lost through the appearance of a mutant, dominant-negative allele (Hollstein et al., 1991; Nigro et al., 1989); the protein product of such an allele has lost its ability to mediate wild-type p53 signaling but gained the ability to compromise most but not all of the functioning of the wild-type protein, which continues to be expressed in heterozygous cells. Residual wild-type p53 function can then be totally eliminated through the loss of the surviving wild type allele and duplication of the already mutant allele. This second step is characterized by the LOH of chromosome 17p on which the *p53* gene is located (Baker et al., 1989; Baker et al., 1990; Nigro et al., 1989).

LOH at the *p53* locus is detectable in the highly dysplastic regions of about 40% of human colonic adenomas (Boland et al., 1995). By the time the subsequent conversion to carcinoma is complete, LOH of the *p53* gene is detectable in an additional 20% of colonic growths (Boland et al., 1995). We focused our attentions on the subset of colonic growths in which LOH of *p53* gene

accompanied the transition to a highly dysplastic phenotype and asked whether *p53* inactivation was accompanied by a change in apoptotic rates.

We measured the incidence of apoptotic cell death in a panel of 8 adenomas having distinct regions within them that displayed high grades of dysplasia (Table 2A). Previous published microallelotyping analyses (Boland et al., 1995) had demonstrated that in this panel of 8 adenomas, LOH of markers on chromosome 17p (including the *p53* gene) had occurred in the regions with high grades of dysplasia while the regions with low grades of dysplasia were still heterozygous for loci on chromosome 17p. These adenomas had been fixed immediately after surgical or endoscopic excision to minimize necrosis.

TUNEL analysis showed no statistically significant difference between the incidence of apoptotic cell death before and after the LOH at chromosome 17p in these adenomas ($p=0.14$, Table 2). Thus, two of these adenomas showed an increase, two showed no change and four showed a decrease in the incidence of apoptosis following the loss of *p53*.

As mentioned above, LOH of the *p53* gene is detectable in only 40% of the adenomas during the transition to the highly dysplastic phenotype. Analysis of a panel of 6 adenomas from the group with no detectable *p53* LOH revealed that the incidence of apoptosis decreased in 5 of these adenomas during the transition to a highly dysplastic phenotype even though the *p53* LOH could not be detected during this transition (Table 2). Indeed, the degree of reduction of apoptosis was comparable to that seen in the adenomas that did suffer LOH. In sum, the transition of adenomas to a highly dysplastic phenotype is often associated with a decrease in the incidence of apoptosis but this change could not be correlated with LOH of the *p53* gene.

Table 2

Effect of LOH of *p53* on the incidence of apoptosis in human colonic adenomas. [a] (+) indicates that LOH of loci on chromosome 17p including the *p53* gene was detected in the highly dysplastic regions (HGD or high grade dysplasia) of adenoma but not in the neighboring regions of the same adenomas with lower degree of dysplasia (LGD or low grade dysplasia) in a previously reported study (Boland et al., 1995). (-) indicates that LOH of chromosome 17p was not detectable in either the regions with high or regions with low grade of dysplasia.

[b] The numeric values represent mean percentage of the epithelial cells that are were apoptotic. Percentage apoptotic cell was determined for ten independent non-overlapping segments of the adenoma or the carcinoma regions using a combination of TUNEL and histological characterization performed on two tissue sections of each tumor.

Table 2. Effect of loss of *p53* on the incidence of apoptotic cell death in human colonic carcinomas

Tumor Name in Boland *et al.*	17p LOH[a]	Apoptotic Cells[b] LGD	HGD
2, right	+	0.60%	0.51%
4, right	+	0.85%	3.35%
11, left	+	0.53%	0.63%
8, left	+	1.20%	0.51%
13 left	+	0.46%	0.82%
17, left	+	1.38%	0.38%
22, left	+	0.95%	0.57%
29, left	+	1.70%	0.57%
3, right	-	2.35%	1.30%
15, left	-	0.91%	0.87%
10, left	-	2.17%	1.11%
28, left	-	4.48%	0.35%
20, left	-	1.30%	0.97%
21, left	-	2.04%	1.52%

Discussion

Mice predisposed to adenomatous polyposis through mutation of the *Apc* gene sustain multiple intestinal adenomas in the first three months of life (Moser et al., 1990). Our initial studies attempted to gauge the effects, if any, of inactivation of p53 function on the appearance and subsequent development of these adenomatous polyps. Our results indicate that polyp number and growth were not affected by the presence or absence of functional alleles of the *p53* gene in the four-month life span of the $p53^{-/-}$ mice. We concluded that, to the extent that *p53* mutation plays a role in colon cancer progression, such role only becomes important in later stages.

P53 mutations rarely occur in cells of colonic adenomas having a low to intermediate degree of dysplasia but frequently occur in adenomas as they progress to a high grade of dysplasia (Boland et al., 1995). This inactivation of *p53* at a relatively late stage of tumor progression might be explained as a necessary response to mutations sustained previously in other growth-regulating genes including proto-oncogenes and tumor suppressor genes. Thus, earlier mutations in these growth-regulating genes might provoke apoptosis in colonic epithelial cells, as has been shown for other types of cells (Hermeking and Eick, 1994; Howes et al., 1994; Kim et al., 1993; Lowe et al., 1994; Lowe and Ruley, 1993; Morgenbesser et al., 1994; Qin et al., 1994; Symonds et al., 1994; White et al., 1992; Wu and Levine, 1994), which may then be countered by the inactivation of their *p53* gene.

The results of the present study argue against such a model. We have gathered evidence from human adenomas that causes us to propose that avoidance of apoptosis, as mediated by *p53* mutation, is not the primary effect of the loss of *p53* in intestinal adenomas. Our data show that the transition of intermediary adenomas to late-stage adenomas often correlates with suppression of apoptosis, but this reduced incidence of cell death occurs independent of LOH of the *p53* gene and in some tumors loss of *p53* correlates with an increase in the incidence of apoptosis. These data suggest that *p53* status is not the sole determinant of apoptosis in intestinal adenomas.

The conclusion that *p53* may be only one of several genes that determine the incidence of apoptosis in colonic tumors is supported by the studies of Polyak *et al* that showed that the status of the P21 gene determine the effect of p53 on apoptosis in colon carcinomas (Polyak et al., 1996). Another gene that is likely to affect apoptosis in intestinal adenomatous cells and colonic carcinomas is the BAX gene which is mutated in fifty percent of colon carcinomas with the microsatellite mutator phenotype [Rampino, 1997 #152]. One study showed that colon cancer cell lines with BAX mutations belong to different apoptosis complementation groups [Rampino, 1997 #152]. This further supports the idea that apoptosis in colon cancer is determined by genes of several different pathways [Casares, 1995 #151].

Together with the data in the present study, these observations re-direct attention to other functions ascribed to normal p53, including notably p53's ability to mediate cellular senescence. One attractive possiblity is that the mutation of *ras* or other growth-regulating genes that occurs early in colon tumor progression might provoke a senescence in adenomatous cells that may be countered by the subsequent inactivation of the *p53* gene. Such *p53* inactivation may then confer other benefits. Thus, the *p53*-dependent senescence associated with the excessive generational doublings may be circumvented, permitting cells to proceed to crisis and immortalization. Moreover, *p53* inactivation may allow other consequences of *p53* loss such as increased angiogenic potential (Dameron et al., 1994), an uncoupling of DNA synthesis from mitosis [Waldman, 1996 #153] and increased genemoic instability (Boland et al., 1995; Offerhaus et al., 1992) to conspire to accelerate the progression leading to malignant colonic tumors.

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CHAPTER 6

Summary And Implications

Implications of this study

In 1990 a team of scientist at Johns Hopkins University proposed that human colon cancer is caused by a succession of defined genetic alterations including mutation of the *Adenomatous Polyposis Coli* (*APC*) tumor suppressor gene, reduced DNA methylation, mutation of the N- or Ki-*ras* proto-oncogene, inactivation of the *Deleted in Colorectal Cancer* (*DCC*) gene, and mutation of the *p53* tumor suppressor gene (Fearon and Vogelstein, 1990). To test the veracity of this genetic model of colon cancer, the technique of gene targeting was used to create genetic alterations in mice similar to those observed in human colonic tumors and examine their effect on the biology of normal and neoplastic intestine in mice.

In the first experiment, mice were created that are constitutionally heterozygous for a mutation in the mouse homologue of the *APC* gene (*Apc*). This targeted mutation, named Apc^{δ}, reduces the level of APC protein by a factor of a hundred fold. Mice heterozygous for this mutation ($Apc^{\delta/+}$ mice) are predisposed to intestinal adenomas and facial osteomas. However, the low incidence and the late onset of intestinal adenomas in $Apc^{\delta/+}$ mice caused by this hypomorphic allele of *Apc* contrasted sharply with the rapid development of large numbers of adenomas induced by another mutation of *Apc*, Apc^{Min}, that encodes a truncated APC protein. This study showed that mutation of a gene that is mutated in an early step in human colonic tumors causes intestinal adenomas in mice and suggested that mice may be used to study human colon cancer. Moreover, this study showed that, as it is the case in human, distinct mutant alleles of the *Apc* gene cause the development of profoundly different numbers of adenomatous polyps in mice.

In the second study, mice that are predisposed to intestinal adenomas were used to address a controversy regarding human colon cancer. The Hopkins model hypothesized that DNA hypomethylation plays an early and causative role in the formation of colonic tumors. To examine the effects of reduced DNA methylation on formation of intestinal adenomas, a combination of genetics and pharmacology was used. A reduction in the DNA methyltransferase activity in the Apc^{Min} mice due to heterozygosity of the DNA methyltransferase gene in conjunction with a

weekly dose of the DNA methyltransferase inhibitor 5-aza-deoxycytidine reduced DNA methylation and the average number of adenomas from 113 in the control mice to only 2 polyps in the treated heterozygotes. This study proved that DNA methyltransferase activity contributes substantially to tumor development in this mouse model of intestinal neoplasia, and that contrary to the prediction of the Hopkins model, reducing DNA methylation in cells of intestine suppresses the formation of adenomas instead of promoting it.

In the next study, another controversial component of the Hopkins model regarding the role of the *Deleted in Colorectal Cancer* (*DCC*) gene in colon cancer was tested. The *DCC* gene was first identified as a candidate for a tumor suppressor gene on human chromosome 18q, loss of which contributes to the progression of colorectal cancers. However, the accumulated evidence to date has failed to implicate *DCC* conclusively as the colorectal tumor suppressor gene on chromosome 18q. *In vitro* experiments have also failed to provide conclusive proof that *DCC* is a tumor suppressor gene. Moreover, more recent *in vitro* studies in rodents had suggested a role for DCC in a process that appeared to be unrelated to colon cancer, i.e. axon guidance, and provided evidence that DCC might function as a receptor for the axonal chemoattractant netrin-1.

To help elucidate the functions of the *DCC* gene in mice, its homologue (*Dcc*) was inactivated in the mouse genome through use of homologous recombination and the effects of this inactivation on both the intestine and the developing nervous system was examined . $Dcc^{-/-}$ newborn mice and adult chimeric mice produced from $Dcc^{-/-}$ embryonic stem cells had no detectable intestinal pathology. Moreover, $Dcc^{+/-}$ mice did not show any increased predisposition to intestinal adenomas. When present together with a germline mutation predisposing to adenomatous polyps, the mutant *Dcc* allele had no effect on adenoma formation. Furthermore, adenomas that carried only the mutant allele of *Dcc* showed no signs of progression to more advanced lesions. In contrast, $Dcc^{-/-}$ mice did show defects in the formation of commissures in the spinal cord and the brain that were strikingly similar to those observed in *netrin-1*-deficient mice. These observations failed to provide support for a tumor suppressor function for *Dcc*, but

were consistent with the hypothesis that DCC is a component of a receptor for netrin-1 in the developing spinal cord and brain.

The *p53* gene is mutated in colon tumorigenesis at the time of transition of a benign adenoma to a carcinoma. The reason for loss of both alleles of *p53* at a relative late stage in the progression leading to colonic tumors is not known. Two different models have attempted to predict the consequences of the loss of *p53* in a tumor cell. The guardian of the genome model predicts that a *p53*-deficient tumor cell is unable to detect DNA damages that would normally lead to a cell cycle arrest. The second apoptosis model states that a *p53*-deficient cell is unable to activate p53-induced cell death when it receives conflicting growth stimulatory and growth inhibitory signals. According to both models, a p53-deficient cell would continue dividing in spite of having suffered DNA damages. The continual growth of such a cell would lead to the propagation of genetic alterations to the descending cells over time. This in turn would cause the progression of a benign tumor to a carcinoma.

In order to test the validity of the apoptosis model, the effects of inactivation of the *p53* tumor suppressor gene on the incidence of apoptotic cell death were examined in two stages of the adenoma-to-carcinoma progression in the colon: in early adenomas where *p53* mutations are rare and in highly dysplastic adenomas where loss of *p53* occurs frequently. Homozygosity for an inactivating germline mutation of *p53* had no effect on the incidence, the rate of progression, or the frequency of apoptosis in the cells of $Apc^{Min/+}$ induced adenomas in mice. To examine the effect of *p53* loss on apoptosis in late-stage adenomas, the incidence of apoptotic cell death was compared before and after the appearance of highly dysplastic cells in human colonic adenomas. The appearance of highly dysplastic cells, which usually coincides during tumor progression with loss of heterozygosity at the *p53* locus, did not correlate with a reduction in the incidence of apoptosis. These studies suggest that wild type *p53* retards the progression of benign colonic adenoma to malignant carcinomas by mechanism(s) other than the promotion of apoptosis.

In conclusion, the genetic model for colon cancer proposed by Fearon and Vogelstein (Fearon and Vogelstein, 1990) should be revised to incorporate the findings of the present study

regarding the roles of DNA methylation and the *DCC* gene in colon cancer (Figure 1). First, the results of this study suggest that alterations in DNA methylation and not DNA hypomethylation *per se* are important for formation of adenomas. Second, our data do not support the candidacy of *DCC* as a colon tumor suppressor gene and suggest that the loss of DCC expression may be a consequence of events affecting a linked gene. According to this scenario, the observed LOH of 18q21 affects not only the *DCC* gene but other neighboring genes as well, one or more of which is the *bona fide* target of inactivation during colon tumor progression. This controversy will not be resolved completely until another gene on 18q is identified that undergoes LOH and whose retained allele is also mutated in the majority of colonic tumors.

Are mice good model for studying human cancer?

To date, fifteen *bona fide* tumor supressor genes have been identified whose mutation or deletion are associated with one or more types of human cancer (Weinberg, 1996). They include the *retinoblastoma* gene (*Rb*) (Friend et al., 1986), the *p53* gene (Malkin et al., 1990), the *Wilms' tumor gene-1* (*WT1*) (Haber et al., 1990), the *breast cancer genes-1 & 2* (*BRCA1* and *BRCA2*) (Futreal et al., 1994; Miki et al., 1994; Wooster et al., 1994), the *von Hippel Lindau* (*VHL*) gene (Latif et al., 1993), the *adenomatous polyposis coli* (*APC*) gene (Groden et al., 1991; Nishisho et al., 1991), the *neurofibromatosis type-1* (*NF1*) (Xu et al., 1990), and *neurofibromatosis type-2* (*NF2*) genes (Trofatter et al., 1993), the mismatch repair genes (*PMS1, PMS2, MSH2, MUTL & MLH1*) (Bronner et al., 1994; Nicolaides et al., 1994; Papadopoulos et al., 1994), the *multiple tumor susceptibility gene-1* (*MTS1*) (Kamb et al., 1994), the *patched* (*PTC*) gene (Gailani et al., 1996; Hahn et al., 1996; Johnson et al., 1996; Stone et al., 1996), *MMAC1/PTEN* gene (Liaw, 1997; Steck, 1997), and the *tuberous sclerosis-2* (*TSC2*) gene (Consortium, 1993).

Inheritance of a mutant allele of each of these tumor suppressor genes is linked to a familial form of human cancer. For instance, patients who are born with a mutant allele of the *Rb* gene develop retinoblastoma (Friend et al., 1986). Similarly, the *p53* gene is mutated in the Li-Fraumeni

families (Malkin et al., 1990), *WT1* in the Wilms' tumor patients (Haber et al., 1990), *BRCA1* and *BRCA2* in some breast cancer families (Castilla et al., 1994; Miki et al., 1994; Wooster et al., 1994), *VHL* in the von Hippel Lindau disease (Latif et al., 1993), *APC* in the familial adenomatous polyposis (FAP) disease (Groden et al., 1991; Nishisho et al., 1991), *NF1* and *NF2* in patients with familial neurofibromatosis (Bourn et al., 1994; Rouleau et al., 1993; Trofatter et al., 1993; Xu et al., 1990), mismatch repair genes in patients with hereditary non-polyposis colon cancer or the Lynch syndromes (Bronner et al., 1994; Fishel et al., 1993; Hemminki et al., 1994; Leach et al., 1993; Nicolaides et al., 1994; Papadopoulos et al., 1994), *MTS1* in some families with inherited melanoma (Ranade et al., 1995; Zuo et al., 1996), the *patched* gene in familial nevoid basal cell carcinoma (Gailani et al., 1996; Hahn et al., 1996), *MMAC1/PTEN* in the Cowden disease (Liaw, 1997; Nelen et al., 1996), and *TSC2* in patients with familial tuberous sclerosis (Consortium, 1993; Wilson et al., 1996).

In addition, a candidate tumor suppressor gene has been identified and named deleted in pancreatic cancer-4 (DPC4) (Hahn et al., 1996). *DPC4* is the only example of a tumor suppressor gene that has not been linked to any familial form of human cancer but a proposed role for it in tumor suppression is supported by the observation that both of its alleles are either mutated or deleted in fifty percent of sporadic (non-familial) pancreatic carcinomas (Hahn et al., 1996).

Tumor suppressor genes can be classified into two groups: the care takers and the gate keepers (Kinzler and Vogelstein, 1997). Caretakers function to help maintain the integrity of the DNA of normal cells. This group includes the *p53*, *BRCA1, BRCA2* and the mismatch repair genes. For instance, the product of the *p53* gene, i. e. , the P53 protein can eliminate cells with damaged DNA from tissues by activating the cells' suicide program (Lane, 1992). *BRCA1* and *BRCA2* are hypothesized to participate in the *Rad51*-dependent DNA repair of double-strand breaks (Scully et al., 1997; Sharan, 1997). Finally, the mismatch repair genes encode components of a protein machinery that identifies and corrects DNA mismatches in normal cells by excision repair (Mellon et al., 1996). Loss-of-function mutations of care taker genes are suspected to accelerate cancer progression by allowing the accumulation of growth deregulating mutations.

The gate keepers are usually master genes that regulate the expression of other genes. This class of tumor suppressor genes includes *Rb*, *WT1*, *VHL*, *APC*, *MTS1*, *PTC* and *DPC4*. When these genes are mutated, tumors are formed because large numbers of growth promoting genes are turned on and growth inhibitory genes are turned off. For instance, the normal product of the *Rb* gene inhibits the progression of cells from the G1 to the S phase of cell cycle by inhibiting the activity of the E2F family of transcription factors (Weinberg, 1996; Weinberg, 1995). *MTS1* encodes another cell cycle check point. The protein encoded by this genes, P16INK4A inhibits the entry of cells into the S phase of the cell cycle by inhibiting the activity of the cyclin-dependent kinase (CdK)-4 that inactivates the product of the retinoblastoma gene by phosphorylation (Serrano et al., 1995; Serrano et al., 1993). VHL interacts with transcription elongation factors elongin B and C to repress transcription elongation (Duan et al., 1995; Kibel et al., 1995). The APC protein binds to and degrades beta-catenin in the cytoplasm which when accumulated in the cytoplasm can leak into the nucleus and interact with the Lef/Tcf family of transcription factors and activate gene transcription (Behrens et al., 1996; Molenaar et al., 1996; Rubinfeld et al., 1993; Su et al., 1993). Similarly, *WT1* is a transcription factor that suppresses tumor growth by affecting the transcription of other genes (Madden et al., 1991; Rauscher, 1993; Wang et al., 1992).

Two other gate keepers, *NF1* and *TSC2* are members of the GTPase-activating family of molecules. These proteins enhance the GTPase activity of small GTPase molecules such as ras in the cytoplasm and convert them to the inactive or the GDP-bound form (Basu et al., 1992; Boguski and McCormick, 1993; Bollag et al., 1996; Largaespada et al., 1996). For example, loss of NF1 activity in the cytoplasm causes the Ras protein to remain in the active GTP-bound form which in turn leads to the activation of other signalling molecules in the cytoplasm and eventually lead to the activation of growth promoting genes in the nucleus. The *patched* (*PTC*) gene encodes a transmembrane receptor that represses transcription of genes encoding transforming growth factor (TGF)-beta and Wnt class of signaling proteins as well as *PTC* itself (Stone et al., 1996). *DPC4* is a candidate tumor suppressor gene that encodes a gene similar in sequence to the *Drosophila*

melanogaster Mad gene and is suspected to be a component of the signalling pathway of transforming growth factor (TGF)-beta-like proteins (Hahn et al., 1996).

Three tumor suppressor genes, the *p53* gene, *BRCA1* and *BRCA2* function both as transcription factors and care takers (Chapman and Verma, 1996; Gowen et al., 1996; Lane, 1992; Scully et al., 1997; Sherr, 1996). Some domains of both BRCA1 and BRCA2 are able to activate transcription when linked to the DNA-binding domain of other transcription factors (Chapman and Verma, 1996; Milner, 1997). Likewise, the normal but not the mutant forms of p53 protein can activate transcription in a sequence-specific fashion (Chen et al., 1993; Crook et al., 1994; Farmer et al., 1992; Raycroft et al., 1990; Thut et al., 1995). The NF2 protein belongs to a family of molecules that link actin cytoskeleton to transmembrane protein and MMAC1/PTEN is a phosphatase that is deleted in sporadic glioblatomas. However, the mechanism of tumor suppression by the products of these two tumor suppressor genes is not elucidated yet.

To date, knock outs of the mouse homologues of eleven tumor suppressor genes have been reported. They have reported mutations in the germline of mice in the mouse homologues of the *Rb* (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992), *p53* (Donehower et al., 1992; Jacks et al., 1994), *WT1* (Kreidberg et al., 1993), *BRCA1 & 2* (Gowen et al., 1996; Hakem et al., 1996; Liu et al., 1996), *APC* (Moser et al., 1990; Su et al., 1992), *NF1* (Brannan et al., 1994; Jacks et al., 1994), *NF2* (Jacks, 1996), mismatch repair genes (*PMS1*, *PMS2*, *MSH2*) (Baker et al., 1995; Baker et al., 1996; de Wind et al., 1995; Edelmann et al., 1996; Reitmair et al., 1995), and *MTS1/p16INK4A* genes (Serrano et al., 1996). In addition, a knock out of the rat homologue of *TSC2* (the Eker rat model) (Kobayashi et al., 1995) has been reported.

While the cells and tissues from mice with mutations of the mouse homologues of human tumor suppressors genes have beeen very useful in deciphering the biochemical and cell physiological functions of the proteins encoded by tumor suppressor genes, the mutant mice often do not model the human diseases. For instance, $Rb^{+/-}$, $WT1^{+/-}$, $BRCA1^{+/-}$, $BRCA2^{+/-}$, $NF1^{+/-}$, $NF2^{+/-}$ and mice with germline mutations of mismatch repair genes do not model the humans with similar mutations. Moreover, to date there are no reports of increased tumor predisposition in

WT1$^{+/-}$, *BRCA1*$^{+/-}$ and *BRCA2*$^{+/-}$ mice. *Rb*$^{+/-}$ mice do not develop retinoblastomas and neither *NF1*$^{+/-}$ nor *NF2*$^{+/-}$ mice develop neurofibromatosis. Likewise, mice with mismatch repair mutations do not develop colon cancer and *MTS1*$^{-/-}$ mice do not develop melanoma.

The difference between the phenotype of the mice and humans that carry germline mutations of homologues of a tumor suppressor gene may be due to many reasons but two are worth mentioning here. One explanation is that the wild type allele of the tumor suppressor gene is not mutated or deleted in the cells of the heterozygous mouse at sufficiently high rate to form tumors. This appears to be the reason why *NF1*$^{+/-}$ mice do not develop neurofibromas (Jacks, 1996). If the second allele of *NF1* is mutated in embryonic stem cells and these cells are then used to generate chimeric mice, the newborn mice composed partly of homozygous mutant cells develop tens to hundreds of neurofibromas (Jacks, 1996). Thus the rate of loss of the second allele of *NF1* in the neuronal precursor cells appears to be the rate-limiting step for the formation of neurofibromas in mice. This lower rate of loss of or mutations of the *NF1* gene may be due to a better ability of the mouse cells to repair such mutations or because the mouse *NF1* locus has a lower rate of mutation.

In the case of some tumor suppressor genes, the rate-limiting step in the formation of tumors in mice may not be loss of the second wildtype allele of the tumor suppressor gene. For instance, loss of the second copy of *Rb* is not sufficient for formation of retinoblastoma in mice (Maandag et al., 1994; Williams et al., 1994). In this instance, the mouse tissue appears to have other genes whose functions are redundant with those of the *Rb* gene and has the ability to eliminate those cells that have lost RB protein by apoptosis (for an expanded discussion see Jacks, 1996). Evidence in support of this hypothesis comes from the observation that transgenic expression in the mouse retinal cells of the large T antigen, which binds and inactivates Rb and its close relative p107 and p130 as well as the cellular p53 protein, causes the formation of retinoblastomas (O'Brien et al., 1990; Windle et al., 1990).

However, most mouse strains with mutations of tumor suppressor genes show increased tumor predisposition in some tissues. For example, *Rb*$^{+/-}$ mice develop pituitary tumors (Jacks et

al., 1992; Lee et al., 1992). *NF1*$^{+/-}$ mice develop pheochromocytoma and myeloid leukemia (Brannan et al., 1994; Jacks et al., 1994) and *NF2*$^{+/-}$ mice are predisposed to sarcomas (Jacks, 1996). Those with a germline mutation of *p53* do not develop the full spectrum of the tumors observed in the Li-Fraumeni families but the *p53*$^{+/-}$ mice are predisposed to lymphomas, leukemias and sarcomas and *p53*$^{-/-}$ mice die within six months from tumors (Donehower et al., 1992; Jacks et al., 1994). Mice with mismatch repair mutations develop lymphoid tumors and *MTS1*$^{-/-}$ mice (which are also deficient in the overlapping gene *p19*^{ARF}) develop lymphomas, leukemias and soft tissue sarcomas (Serrano et al., 1996).

As yet, there are no reports of mice that carry germline mutations of five of the tumor suppressor genes, i. e. , *VHL*, *PTC*, *MMAC1/PTEN*, *TSC2* and *DCP4* although a rat model of TSC2 (the Ekert rat) exists (Kobayashi et al., 1995). The phenotype of this rat strain is different from the tuberous sclerosis disease in human although the rats are predisposed to renal tumors (Kobayashi et al., 1995).

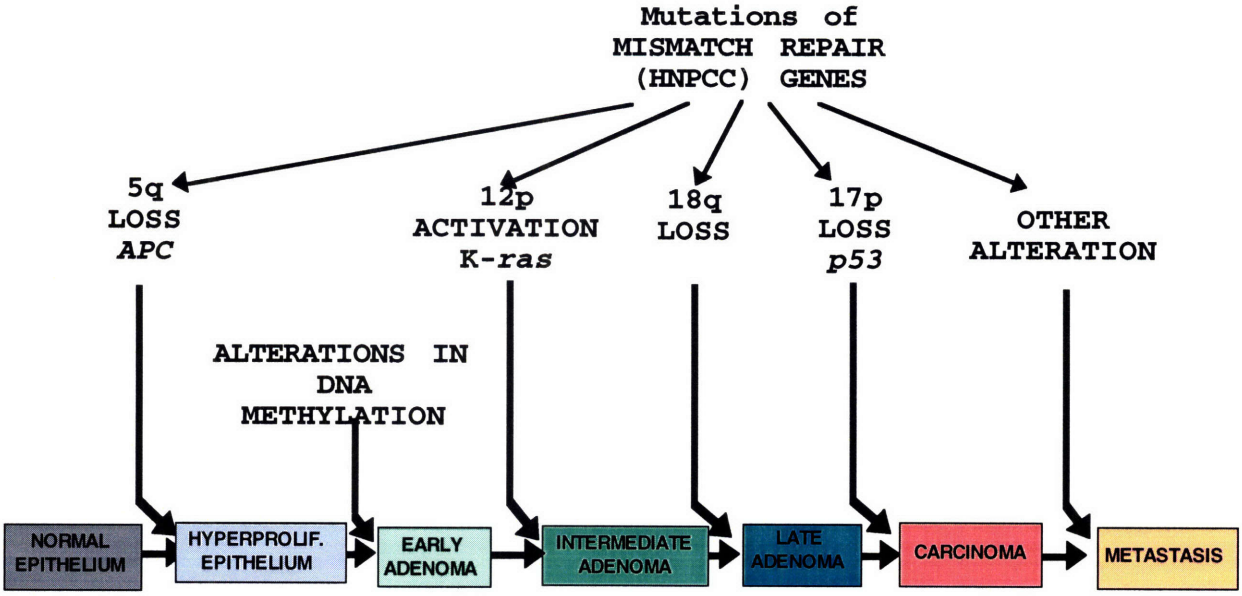
Thus, mice with mutations of homologues of most tumors suppressor genes often have increased tumor predisposition in tissues different from those seen in human and therefore, and in this respect do not provide good models for human inherited cancer syndromes. However, there are two notable exceptions. Mutations of the *APC* gene and inactivation of the functions of the *Patched* tumor suppressor gene create diseases in mice that are very similar to familial adenomatous polyposis and nevoid basal cell carcinomas (NBCC) of skin in human, respectively, A knock out of the *Patched* gene is not reported yet but overexpression of its ligand sonic hedgehog (Shh), which in *Drosophila* mimics loss of PTC function, leads to the development of skin lesions in mice that very similar to human nevoid basal cell carcinoma. As described above, mutations of the *APC* gene result in benign growths or polyps in the intestinal tract of mouse that are very similar to adenomas that are the precursors of colon cancer in human. However, the site of adenomas caused by *APC* mutations differ in the human and the mouse intestinal tract. In *Apc*$^{+/-}$ mice adenomas primarily appear in the small intestine while in *APC*$^{+/-}$ humans most adenomas are colonic.

There are other similarities between intestinal tumor formation in human and mouse. As shown in this study, different mutations of *Apc* have profoundly different effects on polyp number in the familial adenomatous polyposis in human and mouse. C-terminal truncations of APC protein lead to a larger number of adenomas than the N-terminal mutations. Moreover, alterations in DNA methylation appear to be an important step in the formation of adenomas of intestine in both human and mouse. Finally, inactivation of cyclooxogenase-2 enzyme using non-steroidal anti-inflammatory drugs such as sulindac leads to polyp suppression in both human and mouse (Beazer-Barclay et al., 1996; Boolbol et al., 1996; Du Bois, 1995; Giardiello et al., 1996; Oshima et al., 1996).

The conserved role of APC as the gate keeper for intestinal adenomas in both mouse and human provides hope for the possibility that other events that occur as late steps in the progression leading to colon cancer in human could also be important in the progression of mouse intestinal adenomas. It remains to be seen whether mutations of *Apc*, *ras*, the mouse counterpart of the tumor suppressor gene on human chromosome 18q (and/or the TGF-beta type II receptor which is mutated in tumors from Lynch syndrome patients) and *p53* in a correct temporal order would cause the progression of benign intestinal adenomas to carcinomas in the mouse intestine.

Figure 1

Revised genetic model of colon cancer


Mutations of MISMATCH REPAIR (HNPCC) GENES
5q LOSS APC
12p ACTIVATION K-ras
18q LOSS
17p LOSS p53
OTHER ALTERATION
ALTERATIONS IN DNA METHYLATION
NORMAL EPITHELIUM
HYPERPROLIF. EPITHELIUM
EARLY ADENOMA
INTERMEDIATE ADENOMA
LATE ADENOMA
CARCINOMA
METASTASIS

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