Intestinal Tumorigenesis in Compound Mutant Mice of both *Dpc4* (*Smad4*) and *Apc* Genes

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Summary

The *DPC4* (*SMAD4*) gene plays a key role in the TGFβ signaling pathway. We inactivated its mouse homolog *Dpc4* (*Smad4*). The homozygous mutants were embryonic lethal, whereas the heterozygotes showed no abnormality. We then introduced the *Dpc4* mutation into the *Apc*^{Δ716} knockout mice, a model for human familial adenomatous polyposis. Because both *Apc* and *Dpc4* are located on chromosome 18, we constructed compound heterozygotes carrying both mutations on the same chromosome by meiotic recombination. In such mice, intestinal polyps developed into more malignant tumors than those in the simple *Apc*^{Δ716} heterozygotes, showing an extensive stromal cell proliferation, submucosal invasion, cell type heterogeneity, and in vivo transplantability. These results indicate that mutations in *DPC4* (*SMAD4*) play a significant role in the malignant progression of colorectal tumors.

Introduction

About 90% of human pancreatic carcinomas, and 30%–65% of colorectal tumors have allelic loss at chromosome 18q (Vogelstein et al., 1988; Hahn et al., 1996), where several candidates for tumor suppressor genes were proposed (Kinzler and Vogelstein, 1996). Among them, *DCC* has been demonstrated to be a receptor for the axonal chemoattractant netrin-1, rather than a tumor suppressor (Friess et al., 1993; Fazeli et al., 1997), leaving *DPC4* (*SMAD4*) and *JV18–1* or *MADR2* (*SMAD2*) as likely candidates. *DPC4* (*D*eleted in *p*ancreatic *c*arcinoma, or *SMAD4*, and formerly hMAD-4) was isolated from the convergent site of homozygous deletions on 18q in a panel of pancreatic carcinomas (Hahn et al., 1996; Zhang et al., 1996). On the other hand, *SMAD2* was isolated as a gene related to *Drosophila Mad* (*M*others *a*gainst *D*pp) or human *MADR1* (*SMAD1*) (Eppert et al.,

1996; Riggins et al., 1996). Both *DPC4* (*SMAD4*) and *SMAD2* belong to the *SMAD* gene family involved in the signal transduction pathways activated through the TGFβ family receptors (Massague et al., 1997).

Although TGFβ is one of the most potent inhibitors of normal cell growth, many malignancies of epithelial and hematopoietic origins are resistant to TGFβ, suggesting that developing resistance to TGFβ plays an important role in tumorigenesis (Polyak, 1996). Tumors acquire resistance to TGFβ relatively late during tumorigenesis, and this usually correlates with developing invasiveness (Filmus and Kerbel, 1993; Fynan and Reiss, 1993). Tumors can become resistant to TGFβ in many different ways (Polyak, 1996). Loss of both of the TGFβ receptors (type I and type II) occurs infrequently in tumors but has been observed in retinoblastomas and certain colon carcinoma cell lines (Coffey et al., 1987; Kimchi et al., 1988). Decreased expression of either type of receptor has been frequently observed in human tumor cell lines (Filmus et al., 1992). TGFβ type II receptor mutations were found to be associated with microsatellite instability in colorectal carcinoma cell lines (Lu et al., 1995; Markowitz et al., 1995). Resistance to TGFβ can also be caused by mutations that result in signaling defects (Filmus and Kerbel, 1993). While *SMAD1* is a bone morphogenetic protein (BMP) signal transducer, *SMAD2* and *SMAD3* are TGFβ/activin signal transducers. *DPC4* (*SMAD4*), on the other hand, plays a pivotal role because it is a shared and obligate partner of pathway-restricted *SMAD*s (Zhang et al., 1996; Lagna et al., 1996; Massague et al., 1997). Both *DPC4* (*SMAD4*) and *SMAD2* are candidate tumor suppressor genes whose inactivation may play roles in pancreatic, colorectal, and possibly other human cancers (Eppert et al., 1996; Hahn et al., 1996; Riggins et al., 1996). In addition to pancreatic carcinomas, homozygous mutations of *DPC4* (*SMAD4*) were found in up to 30% of colorectal cancers as well (Thiagalingam et al., 1996; Moskaluk and Kern, 1996). However, homozygous *DPC4* (*SMAD4*) mutations appear to be relatively uncommon (<10%) in other carcinomas (Schutte et al., 1996).

To determine the role of the *DPC4* mutation in carcinogenesis, we constructed knockout mice in which its homolog *Dpc4* was inactivated by homologous recombination. We then introduced the mutation into the *Apc*^{Δ716} knockout mice, a model for human familial adenomatous polyposis (FAP) (Oshima et al., 1995, 1997). Because both *Dpc4* and *Apc* are located on mouse chromosome 18 (Chr 18), we constructed compound heterozygotes by meiotic recombination in which both mutations were brought onto the same chromosome in the *cis*-configuration. In the tumors of these mice, both *Apc* and *Dpc4* were homozygously inactivated by loss of heterozygosity (LOH). This LOH appears to be due to the loss of the entire Chr 18 that contained the wild-type alleles of *Apc* and *Dpc4*, followed by reduplication of the remaining chromosome with the knockout alleles for both genes. Accordingly, we have created viable conditional mutant mice in which both *Apc* and *Dpc4* are totally inactivated only in the tumor cells, despite

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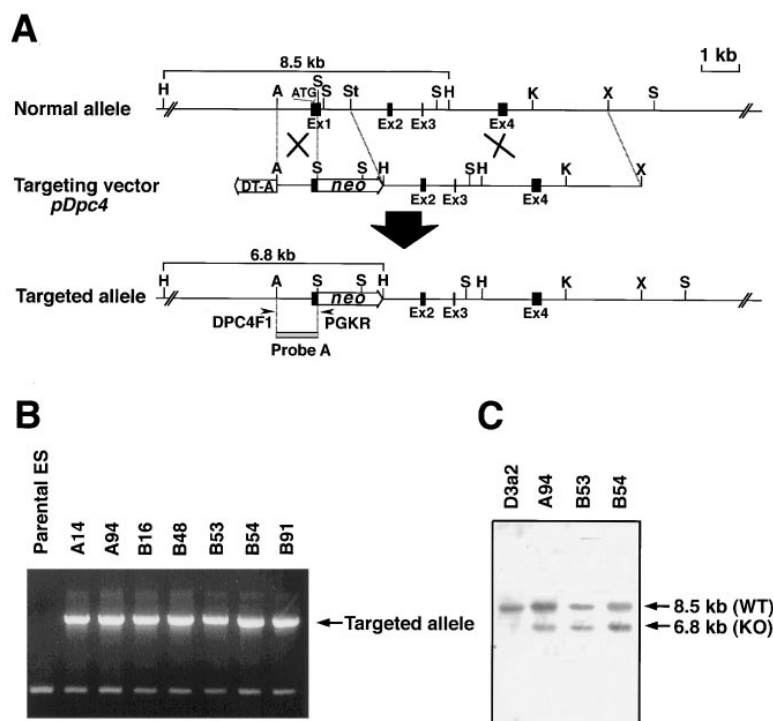
Figure 1. Construction of *Dpc4*(+/−) Knock-out Mice

(A) Strategy for targeting *Dpc4* (*Smad4*). The structures of the normal allele, targeting vector *pDpc4*, and targeted allele are shown. Open boxes *neo* and DT-A indicate the neomycin resistance gene and diphtheria toxin α-subunit gene cassettes, respectively, derived from plasmid pGK-neo-bpA (Soriano et al., 1991). Exons are shown as filled boxes, Ex1 (containing the translation initiation site, ATG), Ex2, Ex3, and Ex4, whereas other genomic segments are shown in solid lines. Only relevant restriction sites are shown: A, ApaI; H, HindIII; K, KpnI; S, SacI; St, StuI; and X, XhoI.

(B) PCR data for homologous recombinant ES cell clones. The positions of primers DPC4F1 and PGKR used for amplification are shown as arrowheads in (A). The names of the recombinant ES cell clones are shown on top of the lanes, whereas the position of the amplified fragment is shown on the right (1.2 kb; arrow).

(C) Confirmation of homologous recombination in ES cell clones by Southern hybridization. The positions of the hybridized HindIII bands for the wild-type (8.5 kb; WT) and knockout (6.8 kb; KO) alleles are shown, respectively (arrows). The locations of the hybridization probe (probe A) and the hybridizable HindIII fragments are shown in (A).

the fact that systemic homozygous inactivation of either of these genes causes lethality in the early embryonic life.

Although it has been proposed to use unified nomenclature for the *Smad* gene family members (Derynck et al., 1996), we have modified the symbols to meet the standard formats for human and mouse gene symbols, respectively. Namely, the human genes are shown in all upper case, whereas the mouse genes are shown with intial capital only. While gene symbols are italicized, upper case Roman letters are used to indicate the protein products of these genes. We have also used *DPC4*/*Dpc4* because of the historical reason and its prevalent use in cancer research, with *SMAD4*/*Smad4* in parentheses when appropriate.

Results

Homozygous Inactivation of *Dpc4* (*Smad4*) Causes Early Embryonic Lethality, whereas Heterozygous Mutants Appear Normal

A targeting vector was constructed (Figure 1A) in which exon 1 of *Dpc4* (*Smad4*) was interrupted by a neomycin resistance gene cassette (PGK-Neo-bpA) and was expected to truncate the peptide with 75 residues, accordingly (Experimental Procedures). It was introduced by electroporation into 129/Sv embryonic stem cell line D3a2 (Shull et al., 1992). About 200 G418 resistant clones were isolated, and seven homologous recombinant candidates were identified by a PCR screening (Figure 1B). They were all verified by Southern hybridization to be

genuine homologous recombinants (Figure 1C). Three such clones were injected into C57BL/6 blastocysts, and germline chimeras were generated. The *Dpc4*(+/−) mice were viable and fertile, and appeared normal in their morphology and behavior. We have made extensive histological examinations of the pancreas and the intestines of these mice at various ages from 4 weeks to 1 year. However, we could not find any noticeable difference from the wild-type littermates. This was the case with the mice not only in the F_1 (129/Sv × C57BL/6) background, but also in their N_5 (C57BL/6) backcross generation.

When the progeny of the heterozygous intercrosses was analyzed, no *Dpc4*(−/−) pups were found, with only the wild-types and the heterozygotes at the ratio of 1:2, indicating that the homozygous embryos died in utero (data not shown). To determine the stage of lethality, embryos were dissected at various ages of gestation, and their genotypes were determined: *Dpc4*(−/−) embryos of the normal appearance were found only before 7 dpc (data not shown). Homozygous mutants were found at the Mendelian ratio when the preimplantation embryos at 3.5 dpc were placed into culture and genotyped by PCR using the proliferated inner cell mass cells (data not shown).

In *Dpc4* and *Apc*$^{\Delta 716}$ *cis*-Compound Heterozygote Intestinal Tumors, Chromosome 18 Carrying the Wild-Type Alleles Is Lost whereas that Carrying the Knockout Mutations Is Reduplicated

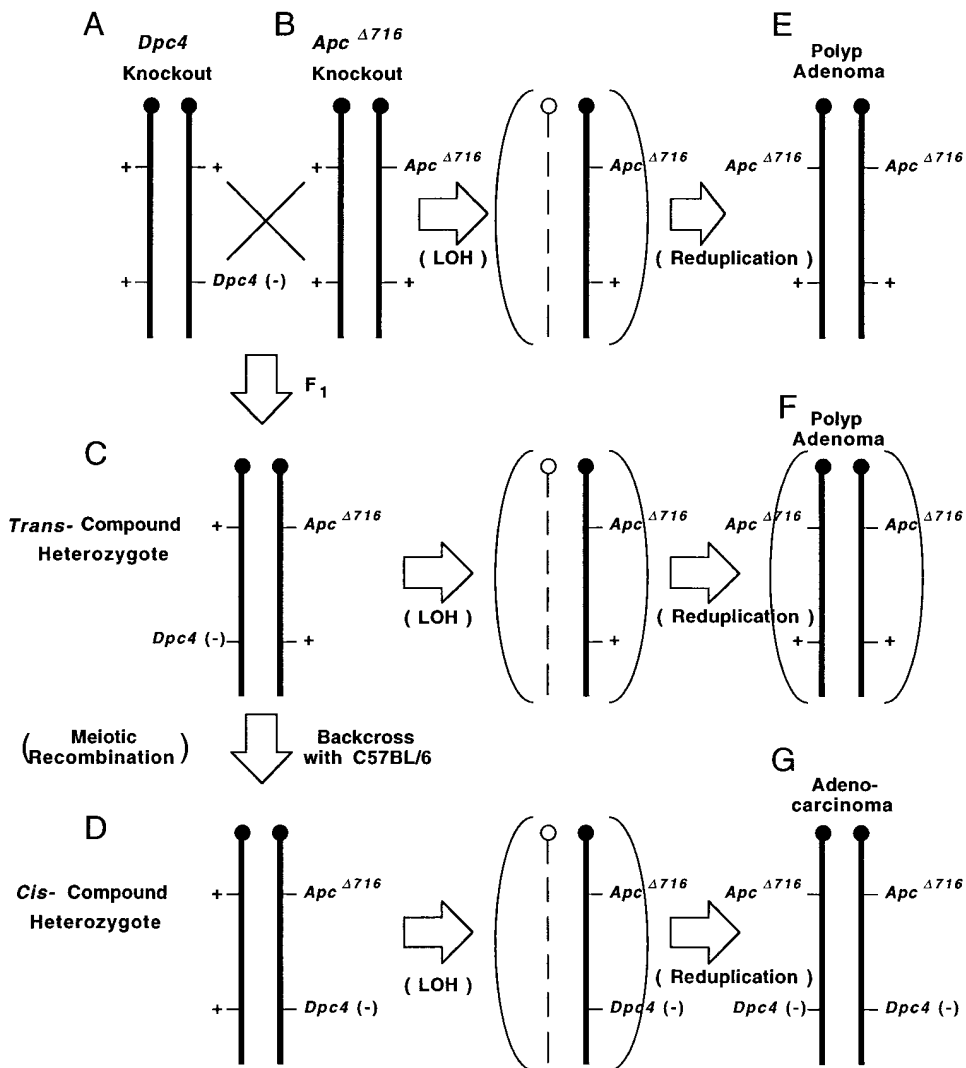Earlier, we constructed a mouse strain carrying knockout mutation *Apc*$^{\Delta 716}$, which develops numerous polyps

Figure 2. Strategy for Construction of $Apc^{\Delta716}(+/-)$ $Dpc4(+/-)$ Compound Heterozygotes

Only relevant alleles on Chr 18 are shown for simplicity.

(A) $Dpc4(+/-)$ simple heterozygote.

(B) $Apc^{\Delta716}(+/-)$ simple heterozygote.

(C) *trans*-compound heterozygote in the $[Dpc4(+/-) \times Apc^{\Delta716}(+/-)]$ F_1. Note the two mutations on separate chromosomes.

(D) *cis*-compound heterozygote in the $[Dpc4(+/-) \times Apc^{\Delta716}(+/-)]$ $F_1 \times$ C57BL/6 backcross offsprings. Note the two mutations on the same chromosome.

(E) *Apc* gene LOH caused by loss of the wild-type-carrying Chr 18, followed by reduplication of the mutation-carrying Chr 18 in the $Apc^{\Delta716}(+/-)$ simple heterozygote polyps.

(F) *Apc* gene LOH caused by loss of the wild type-carrying Chr 18, followed by reduplication of the mutation-carrying Chr 18 in the *trans*-compound heterozygote. Note that the polyp genotype after the chromosomal loss and reduplication here should be the same as that in the $Apc^{\Delta716}$ simple heterozygote in (E).

(G) LOH in both the *Apc* and *Dpc* genes by loss of the wild-type-carrying chromosome, followed by reduplication of the mutation-carrying Chr 18 in the *cis*-compound heterozygote polyps. Experimental evidence has not yet been obtained for genotypes shown in parentheses.

in the intestines (Oshima et al., 1995, 1997)—a mouse model for human FAP. While human *APC* is located on 5q21 (Groden et al., 1991; Kinzler et al., 1991), its mouse homolog *Apc* is on proximal Chr 18 (Luongo et al., 1993). Although human *DPC4* is on 18q21, its mouse homolog, *Dpc4*, has recently been mapped to Chr 18 (Anna and Devereux, 1997). We also mapped *Dpc4* using a panel of interspecific backcross mice and confirmed the results;

Dpc4 mapped on mouse Chr 18, about 30 centimorgans (cM) distal to *Apc*. The linkage distances ± SE between the markers were: *Adrb2*—5.3 ± 2.1 cM—*D18Mit9*—1.8 ± 1.2 cM—*Dpc4*—2.6 ± 1.5 cM—*D18Mit8*. This result is consistent with the data of human and mouse homology between these regions of the chromosomes (Johnson and Davisson, 1997). It indicates the unique situation that the two mouse genes *Apc* and *Dpc4* are located
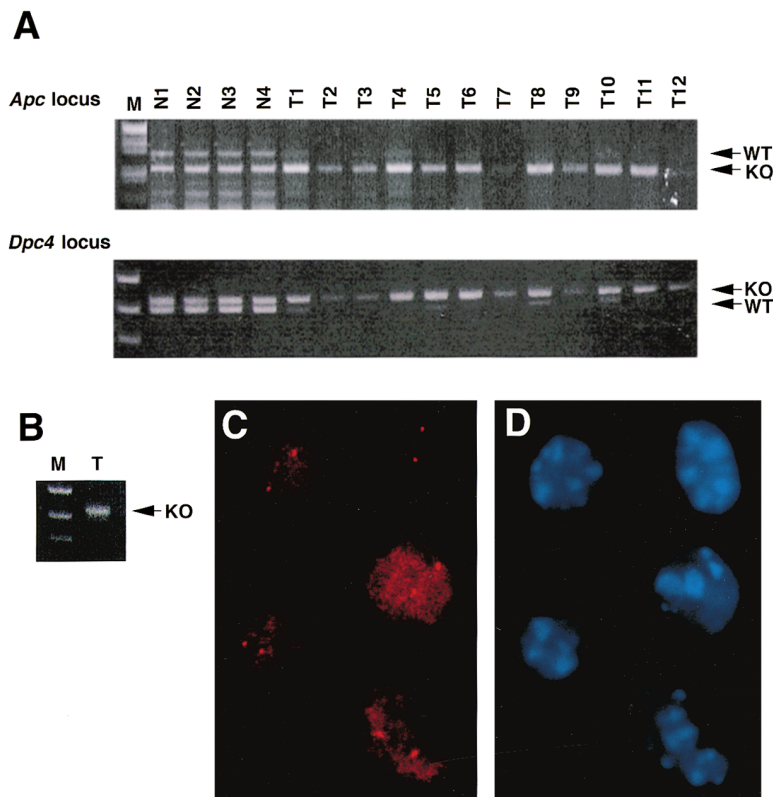
A

Apc locus

WT
KO

Dpc4 locus

KO
WT

B

M T

KO

C

D

on the same chromosome, while their human homologs are on separate chromosomes. Accordingly, we constructed compound heterozygotes in which the two mutations were brought on the same Chr 18 in the cis-configuration, and determined the effects of the Dpc4 knockout mutation in the $Apc^{\Delta716}$ mutant mice. This strategy is shown in Figure 2 together with the results of the chromosomal reduplication. First, the respective heterozygotes (Figures 2A and 2B) were crossed, and F$_1$ mice were obtained (Figure 2C). These compound heterozygotes in the trans-configuration were then backcrossed with the wild-type C57BL/6 mice. Their offspring contained not only simple heterozygotes [55 $Apc^{\Delta716}$(+/−) and 41 Dpc4(+/−); i.e., 96 pups combined], but also compound heterozygotes in the cis-configuration [Cis-$Apc^{\Delta716}$(+/−) Dpc4(+/−); 19 pups] (Figure 2D) and the wild-type mice (23 pups) caused by meiotic recombination. Because the Apc and Dpc4 genes are separated by about 30 cM, this recombination frequency of 30.4% [(19 + 23)/(19 + 23 + 96)] precisely matches their linkage distance.

As we demonstrated earlier (Oshima et al., 1995, 1997), LOH in the Apc gene is always associated with the formation of intestinal and colonic polyps in the $Apc^{\Delta716}$ knockout mice, and it is likely to be the triggering event in tumorigenesis. This was confirmed for the polyp microadenomas in the cis-compound heterozygotes as well. As shown in Figure 3A (top), the PCR band for the wild-type Apc allele was not detected in the polyps

(T1–T12), whereas it remained in the normal intestinal epithelium (N1–N4). We then determined the Dpc4 genotype in the same polyp microadenomas as those tested for Apc. As shown in Figure 3A (bottom), the full-length wild-type Dpc4 band was also missing in the polyps (T1–T12), although it remained in normal parts of the intestinal epithelium (N1–N4)—indicating LOH for Dpc4 as well. These results strongly suggest that a wide range of Chr 18 that carried the wild-type alleles was missing in the polyp microadenoma cells. To investigate the nature of the Apc and Dpc4 LOH further, we placed some polyp microadenomas from the cis-compound heterozygotes into culture and obtained primary cells. We confirmed by PCR genotyping that these cultured cells were of the tumor epithelial origin, but not of the stromal origin because of their LOH in the Dpc4 gene (Figure 3B). Such interphase cells were analyzed by FISH using a recombinant phage P1 probe which covered an ∼100 kb region in the Apc locus. As shown in Figure 3C, we detected two hybridizing spots per cell, essentially in all cells. The same result was obtained with another phage P1 probe for the Dpc4 locus (data not shown). Another recombinant phage P1 probe for Chr 11 also showed two spots, confirming the diploid nature of the cultured tumor cells (data not shown). These results, taken together, strongly suggested that the whole Chr 18 carrying the wild-type Apc and Dpc4 alleles in the cis-compound heterozygotes was lost, followed by reduplication of the Chr 18 with the knockout alleles. This
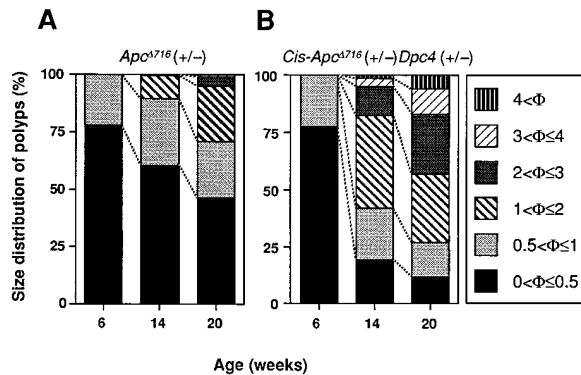
A **B**

Figure 4. Polyp Size Distribution in the Small Intestine of the *cis*-Compound *Apc*$^{\Delta 716}$ *Dpc4* Heterozygotes Compared with that of the Simple *Apc*$^{\Delta 716}$ Heterozygotes

(A) Polyp size distribution in the *Apc*$^{\Delta 716}$ heterozygotes.
(B) Polyp size distribution in the *cis*-compound heterozygotes. The bar patterns for the respective size classes are shown in the box on the right (Φ, diameter in mm), whereas the ages are shown on the bottom. Mean values from 6 mice per group are shown as percentages for the respective size classes. (For the mice of 6 weeks, sample number was 2 per group.)

process is shown schematically in Figure 2G. Accordingly, the polyp adenoma cells in the *cis*-compound heterozygotes resulted in conditional homozygous mutants for both *Apc* and *Dpc4* genes, with the rest of the genes on Chr 18 remaining unchanged from the background (see Discussion).

In *Dpc4* and *Apc*$^{\Delta 716}$ *cis*-Compound Heterozygotes, Polyp Sizes Increase in the Small and Large Intestines

As shown in Figure 4, the polyps in the small intestine of the *cis*-compound heterozygotes were much larger than those in the *Apc*$^{\Delta 716}$ heterozygotes at the age of 14 weeks. The modal size was ~1.0–2.0 mm in diameter compared with <0.5 mm in the *Apc*$^{\Delta 716}$ heterozygotes. This difference in the polyp size was not observed at 6 weeks but became more apparent with age. Some colonic polyps in the *cis*-compound heterozygotes of both 14 and 20 weeks developed to larger sizes that were never found in the *Apc*$^{\Delta 716}$ heterozygotes (e.g., Φ = 7 mm). This increase in the polyp size and the change in the size distribution were confirmed with the *cis*-compound heterozygotes derived from *Dpc4* heterozygotes of further backcross generations as well (N$_5$ in C57BL/6), but were not observed with the 20-week-old *trans*-compound heterozygotes (data not shown). Regarding the polyp numbers, the *cis*-compound heterozygotes had much fewer polyps in the small intestine than the simple *Apc*$^{\Delta 716}$ heterozygotes. At 14 weeks, for example, the *cis*-compound heterozygotes had only 82 polyps, corresponding to 12% of the 676 polyps in the *Apc*$^{\Delta 716}$ heterozygotes. In contrast, the number of colonic polyps had a tendency to increase in the *cis*-compound heterozygotes at 14 and 20 weeks, although no colonic polyps were found at 6 weeks in either mutant (see Discussion).

In *cis*-Compound Heterozygotes, Polyps Develop into Malignant Adenocarcinomas with Marked Desmoplasia and Submucosal Invasion

In addition to the difference in the polyp number and size, the morphology of the polyps was significantly different in the *cis*-compound heterozygotes from that of the *Apc*$^{\Delta 716}$ heterozygotes. As we published previously (Oshima et al., 1995, 1997), most intestinal polyps in the *Apc*$^{\Delta 716}$ heterozygotes were limited in the mucosal layer (e.g., Figure 5A). On the other hand, the small intestinal polyps in the *cis*-compound heterozygotes showed more proliferation of the stromal (interstitial) cells than in the *Apc*$^{\Delta 716}$ heterozygotes, and discernible thickenings of the smooth muscle layer were observed as early as 6 weeks of age (Figure 5B). To confirm the loss of the wild-type *Dpc4* allele determined by PCR (Figure 3A), we stained the small intestinal polyps in the *cis*-compound heterozygotes with an antibody against the human DPC4 protein. As shown in Figure 5C, the normal epithelium of the villi as well as the crypts was stained. In contrast, the tumor epithelium of the polyps was not stained, while some staining was found in the stromal cells. When the nascent DNA was labeled with BrdU and stained with its antibody, only crypts were stained in the normal epithelium whereas the tumor cells were stained all over the polyp (Figure 5D). Although the *Apc*$^{\Delta 716}$ adenomas consisted of essentially enterocyte-like cells without mucinous differentiation (Figure 5E), the tumors in the *cis*-compound heterozygotes developed into adenocarcinomas containing much more heterogeneous types of cells, including mucinous cells, and showed marked submucosal invasions (Figure 5F). Such mucinous cells were often found in more advanced intestinal tumors with extensive submucosal invasions, showing the so-called "signet ring cell" appearance (Figures 5G and 5H) (Fenoglio-Preiser et al., 1990). The malignant nature of these mucinous adenocarcinomas is also exemplified by a very high labeling index with BrdU (Figure 5I). In addition to the mucinous cells, some other types of cells, such as dysplastic Paneth's cells were also observed (data not shown). Most *cis*-compound heterozygotes became moribund before 14–20 weeks, and often (5/20) died of intussusception of the small intestine, whereas such a complication was not found among 30 simple *Apc*$^{\Delta 716}$ heterozygotes.

The histopathological pictures were similar in the colonic tumors developed in the *cis*-compound heterozygotes, except that a much more extensive proliferation of the stromal cells (desmoplasia) and an irregular architecture of the adenocarcinomas were observed. Figure 6 shows a comparison of age-matched colonic tumors between the *Apc*$^{\Delta 716}$ mice and *cis*-compound heterozygotes. While the *Apc*$^{\Delta 716}$ polyps contained essentially homogeneous cells resembling colonocytes with sparse stromal cells (Figures 6A and 6C), the *cis*-compound heterozygote polyps showed an extensive desmoplasia with a more irregular arrangement of the tumor glands (Figures 6B and 6D). Although not evident upon hematoxylin and eosin (H & E) staining, the mucinous cells were demonstrated in the *cis*-compound heterozygote polyps by Alcian blue staining (Figure 6F compared with 6E, respectively). In more advanced tumors in the *cis*-compound heterozygotes, not only the cell type, but
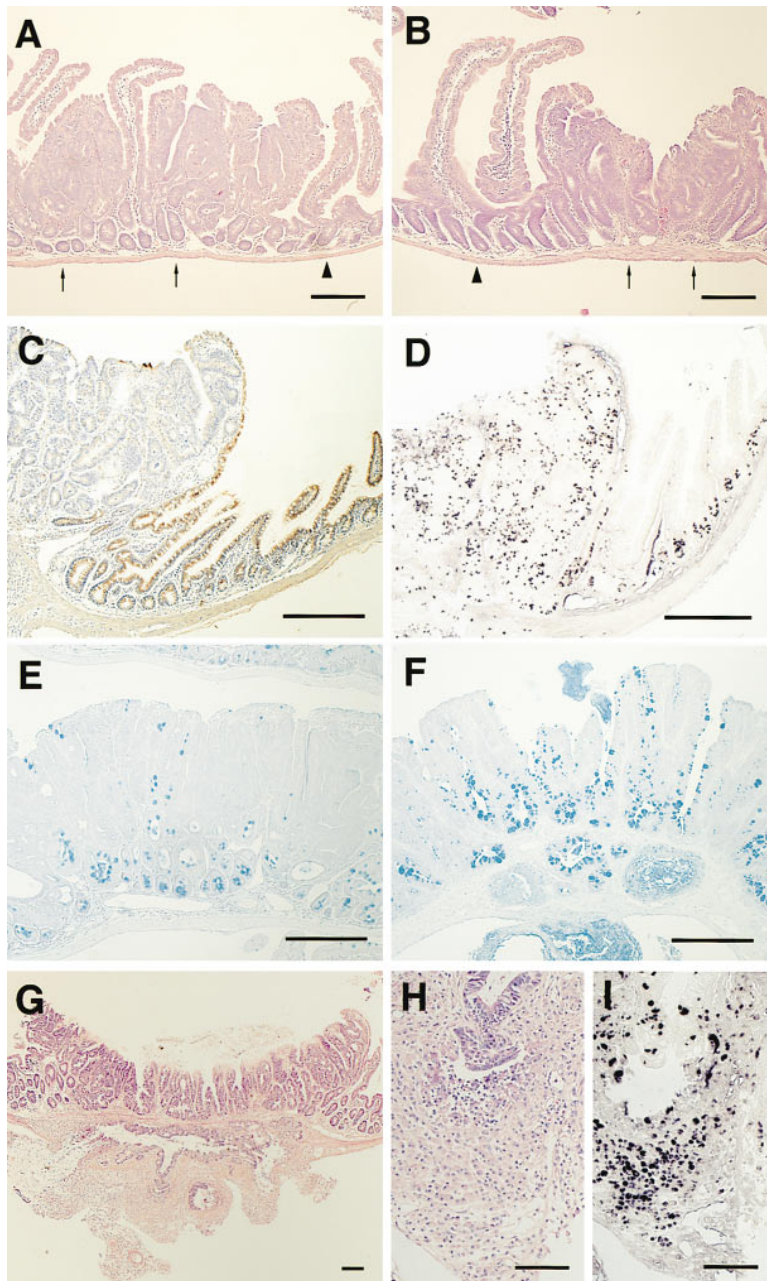
Figure 5. Histopathology of Small Intestinal Polyps in the *cis*-Compound Heterozygotes, Compared with Those in the Simple $Apc^{\Delta716}$ Heterozygotes

(A) An early polyp adenoma in a 6-week-old $Apc^{\Delta716}$ heterozygote (control). Note that no thickening of the smooth muscle layer is observed underneath the polyp (arrows) compared with the normal part (arrowhead). (H & E.)

(B) An early polyp adenoma in a 6-week-old *cis*-compound heterozygote. Note the thickening of the smooth muscle layer and stromal cell proliferation underneath the polyp (arrows) compared with the normal part (arrowhead). (H & E.)

(C) Immunohistochemical staining of a tumor for the DPC4 protein in a 20-week-old *cis*-compound heterozygote. A part of the tumor is shown with the adjoining normal small intestinal tissue. (Hematoxylin counterstaining.)

(D) BrdU labeling of the nascent DNA stained with a specific antibody in a tumor of a *cis*-compound heterozygote. A section adjoining that used in (C). (Hematoxylin counterstaining.)

(E) A polyp adenoma in a 20-week-old $Apc^{\Delta716}(+/-)$ mouse stained with Alcian blue for sulfomucin-producing cells. Note that the stained cells in the polyp are of the remaining normal villus epithelium and crypts. (Hematoxylin counterstaining.)

(F) A polyp adenocarcinoma in a 20-week-old *cis*-compound heterozygote stained with Alcian blue. Note that many tumor cells are stained not only in the mucosa, but also in the muscle layer. (Hematoxylin counterstaining.)

(G) A more advanced tumor in a 20-week-old *cis*-compound heterozygote with an extensive submucosal invasion. (H & E.)

(H) A higher magnification of (G) showing "signet ring cells." (H & E.)

(I) A section adjoining (H) stained for nascent DNA by BrdU labeling.

Scale bars: (A)–(F), 250 μm; (G)–(I), 125 μm.

also the adenocarcinoma glands were heterogeneous. For example, mucinous cell glands, colonocyte glands, and glands with both types of cells were found in single tumors (Figure 6G). Moreover, the submucosal invasion was much more marked in the *cis*-compound tumors, often into the muscle layer (Figure 6H). Some of these cells were examined further by electron microscopy and their cell type identities were confirmed (data not shown). The histology of the polyps in the *trans*-compound heterozygotes was not significantly different from that in the simple $Apc^{\Delta716}$ heterozygotes (data not shown).

To assess the invasiveness of the tumors in the *cis*-compound heterozygotes, we scored the submucosal invasion at 14, 17, and 20 weeks of age. As shown in Table 1, the invasive patterns in randomly sampled tumors increased with age in the *cis*-compound heterozygotes and reached more than half (55%) of the tumors at 20 weeks. In the $Apc^{\Delta716}$ polyps, on the other hand, such invasive patterns were not found at all at 14 or 17 weeks of age, and only 2 were found out of 66 tumors at 20 weeks. It is worth noting that these invasive tumor cells incorporated BrdU extensively and were not stained by an antibody against the human DPC4 protein (data not shown).

We also collected some of these tumors at the age of 18 weeks, and transplanted them into nude mice subcutaneously. Neither of the two $Apc^{\Delta716}$ polyps
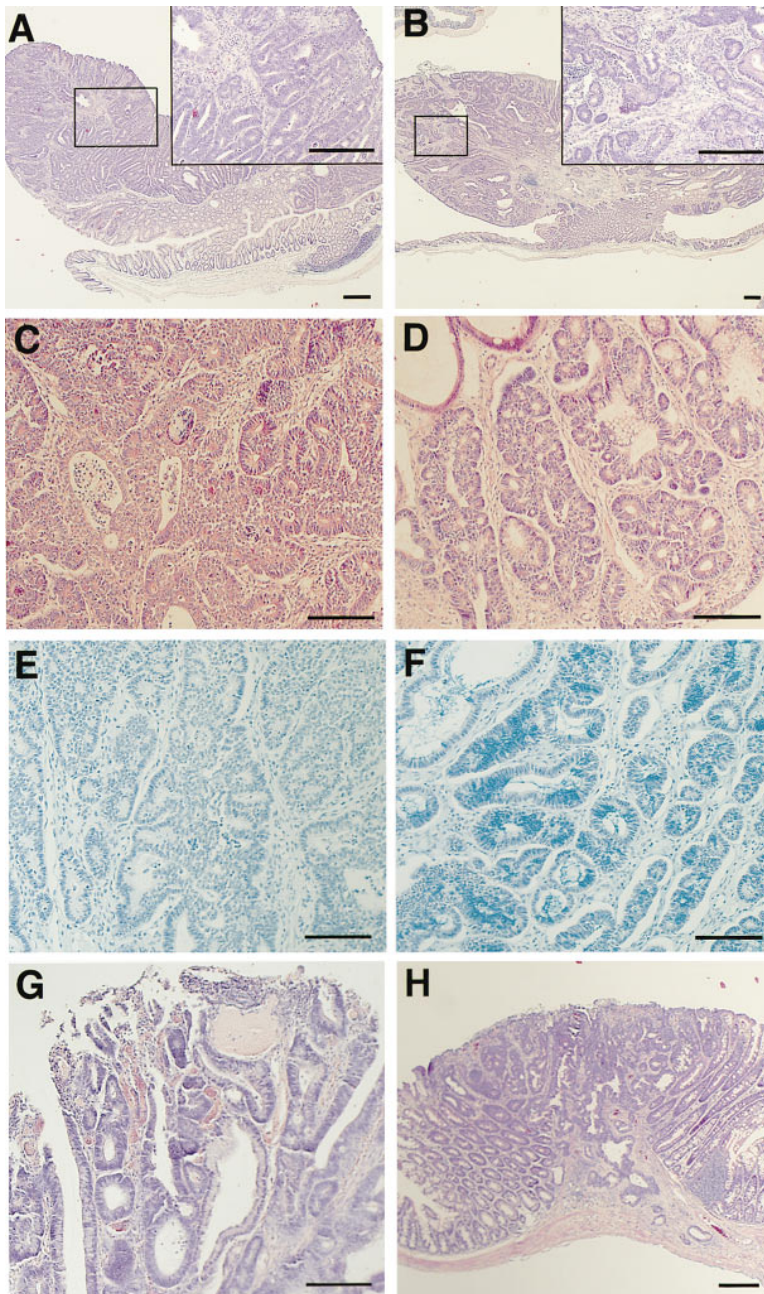
Figure 6. Histopathology of Colonic Polyps in the *cis*-Compound Heterozygotes, Compared with Those in the *Apc*[Δ716] Heterozygotes

(A) One of the most representative tumors developed in a 14-week-old *Apc*[Δ716] simple heterozygote. Inset: a higher magnification. (H & E.)

(B) One of typical polyps in a 14-week-old *cis*-compound heterozygote. Note that the polyp contains much more stromal cells (areas with less staining) compared with the tumor in (A). Also note the difference in the size of the polyps, based on the scale bar lengths. Inset: a higher magnification. (H & E.)

(C) A higher magnification of an *Apc*[Δ716] simple heterozygote polyp (20-week-old). (H & E.)

(D) A higher magnification of a *cis*-compound heterozygote polyp (20-week-old). Note extensive proliferation of the stromal cells not tightly adhering to the tumor epithelium. (H & E.)

(E) Alcian blue staining of the same tumor as in (C).

(F) Alcian blue staining of the same tumor as in (D). Note that some tumor cells are stained for sulfomucin, a sign of mucinous adenocarcinoma.

(G) A tumor in a 14-week-old *cis*-compound heterozygote. Note the tissue heterogeneity of the adenocarcinoma glands consisting of mucinous cells, and colonocytes, respectively, as well as glands with both types of cells.

(H) A tumor in a 14-week-old *cis*-compound heterozygote. Note the pedunculate growth of the submucosal layer with an extensive invasion by the adenocarcinoma cells. (H & E.)

Scale bars: (A) and (B), 250 μm; (C)–(G), 125 μm; and (H), 250 μm.

formed tumors even after two months, whereas all three polyps from the *cis*-compound heterozygotes developed into tumors. Histological examinations of these tumors showed a variety of adenocarcinomas including the signet ring cell type (data not shown).

Table 1. Submucosal Invasion of Intestinal Tumors[a]

Genotype	Age (wk)			
	14	17	20	Total
Cis-Apc[Δ716](+/−) *Dpc4*(+/−)	1/4	2/4	26/45	29/53
Apc[Δ716](+/−)	0/11	0/12	2/43	2/66

[a] Number of tumors with submucosal invasion / number of tumors examined.

To determine other possible genetic changes associated with this malignant phenotype in the *cis*-compound heterozygotes, we placed the tumor cells into culture and determined the DNA sequences for the K-*ras* gene (*Kras2*). Among 7 cell lines derived from 7 independent tumors, none of them showed mutations in the *Kras2* hot spots corresponding to codon 12, 13, or 61 (data not shown).

Although examined extensively, no signs of metastasis were found in the *cis*-compound heterozygotes by the time these mice became moribund at 14–20 weeks.

These results collectively indicate that inactivation of the *Dpc4* gene results in the malignant progression of the intestinal and colonic polyps initiated by LOH in the *Apc* gene (see Discussion).
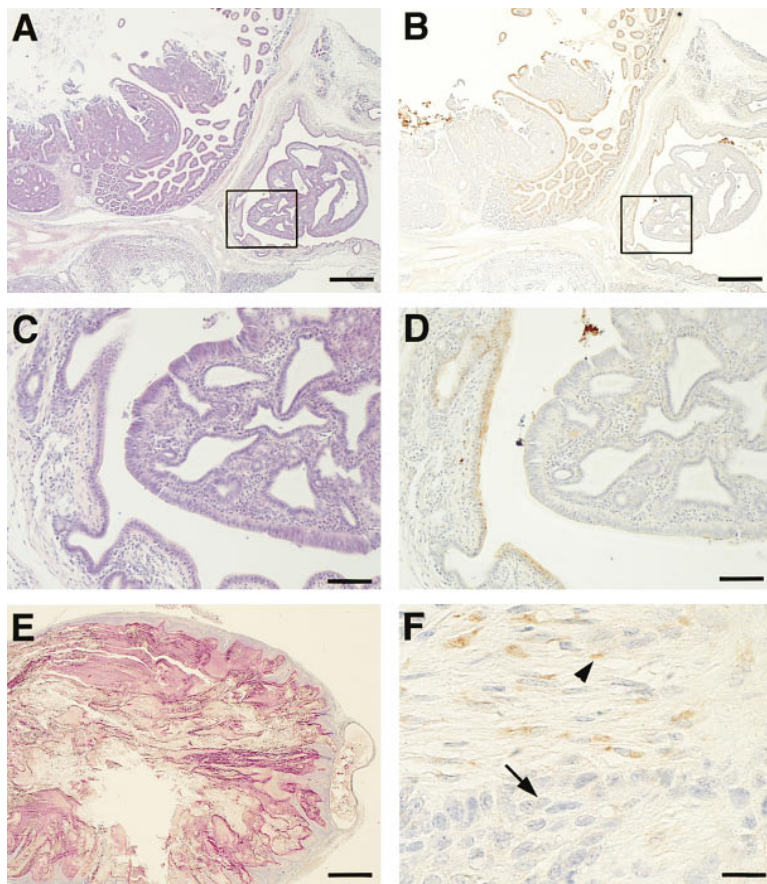
Figure 7. Histopathology of a Duodenal Ampullary Tumor and of a Skin Epidermoid Cyst in the *cis*-Compound Heterozygotes

(A–D) Histological sections of an ampullary tumor in the duodenal papilla in a *cis*-compound heterozygote. (A and C) Boxed area in (A) is shown in (C) at a higher magnification. (H & E.) (B and D) Immunohistochemical staining for the DPC4 protein. Boxed area in (B) is shown in (D) at a higher magnification. (Hematoxylin counterstaining.)
(E and F) Histological sections of a skin epidermoid cyst in a *cis*-compound heterozygote. (E) H & E staining. (F) Immunohistochemical staining for the DPC4 protein. Note that the tumor epidermal cells (arrow) are not stained whereas the surrounding fibroblasts (arrowhead) are stained by the antibody.
Scale bars: (A), (B), and (E), 250 μm; (C) and (D), 62.5 μm; and (F), 10 μm.

Ampullary Tumors Develop in Duodenal Papilla of Vater in *cis*-Compound Heterozygotes

Interestingly, 3 of the 15 *cis*-compound heterozygotes autopsied at 14–20 weeks had adenocarcinomas of significant sizes in the ampullary region of the pancreatic duct in the major duodenal papilla (Figures 7A and 7C). Their histology was similar to that of the tumors often found in FAP patients (see Discussion). Immunohistochemical staining of such tumors showed lack of the *Dpc4* protein (Figures 7B and 7D), suggesting the same genetic changes as in the intestinal tumors, i.e., the loss of the Chr 18 that carried the wild-type alleles, followed by reduplication of Chr 18 that carried the knockout *Apc* and *Dpc4* alleles. On the other hand, none of the $Apc^{\Delta716}$ mice had such tumors up to about 20 weeks of age, though they were examined extensively.

Extra-GI Phenotypes in *cis*-Compound Heterozygotes

As the simple *Dpc4* heterozygotes did not develop any tumors in the pancreas, thorough histological examinations of the pancreas of the *cis*-compound heterozygotes did not reveal any malignant tumors at least up to the age of 20 weeks, when most mice became moribund due to the tumors of the intestines.

In 8 out of 15 (i.e., 53%) of the *cis*-compound heterozygotes older than 10 weeks of age, skin epidermoid cysts were found in the left axillary region and/or the ventral side of the neck. The multiplicity of the macroscopically visible cysts varied from one to two dozen per mouse,

and they were found in both male and female mice. A representative histological picture is shown in Figure 7E (see Discussion). No staining for the DPC4 protein was found in the cysts (Figure 7F), suggesting a mechanism to similar that causing intestinal tumors, i.e., LOH in both *Apc* and *Dpc4*.

Discussion

We have inactivated the mouse *Dpc4* (*Smad4*) gene by homologous recombination in ES cells and established germ-line transmitted mutant mice. Because *Dpc4* (*Smad4*) is involved not only in the TGFβ/activin pathway, but also in the BMP-mediated pathway in early embryogenesis (Massague et al., 1997), it is not surprising that the homozygous *Dpc4* embryos are lethal around 7 dpc, when BMP-2/4 plays critical roles in mesoderm formation during gastrulation. It is worth noting that the homozygous knockout mutation in the type I receptor gene for BMP (*Bmpr*) shows an embryonic lethal phenotype around 7 dpc (Mishina et al., 1995).

Taking advantage of the fact that *Dpc4* and *Apc* are 30 cM apart on Chr 18, we then constructed by meiotic recombination, compound heterozygotes that carried the two knockout mutations on the same chromosome (the *cis*-compound heterozygotes). In the tumor epithelium developed in such mice, the wild-type allele of not only *Apc*, but also *Dpc4* was missing, suggesting loss of the entire Chr 18. Because FISH analyses of the tumor

cells showed two hybridizing spots per cell with either probe for the *Apc* or *Dpc4* locus, reduplication of the mutant allele-carrying Chr 18 was strongly suggested. Accordingly, both *Apc* and *Dpc4* were totally inactivated by the knockout mutations only in the tumor epithelium, maintaining the diploid state for other genes. Thus, we excluded the possibility that the malignant tumor characteristics in the *cis*-compound heterozygotes were synergistic effects of the *Apc/Dpc4* inactivation and the haploinsufficiency of other genes on Chr 18, which would be expected if no reduplication occurred. This reduplication is not the result of the malignant change in the *cis*-compound heterozygote tumors, because we found the same reduplication also in the adenoma cells of the *Apc*$^{\Delta 716}$ simple heterozygotes.

Using F$_1$ hybrid mice between the C57BL/6 and AKR or C3H genetic backgrounds, the polyp adenomas in the Min mice and *Apc*^{1638N} knockout mice were shown to have lost the entire Chr 18 that carried the wild-type allele (Luongo et al., 1994; Smits et al., 1997). In the Min mice, however, a PCR determination suggested that the Chr 18 is not reduplicated, but remains monosomic (Luongo et al., 1994). Although the difference between their data and our results awaits further investigation, it is worth noting that reduplication of the remaining chromosome is a commonly observed phenomenon (Cavenee and Hansen, 1986).

We accelerated the LOH in both *Dpc4* and *Apc* by a single event of Chr 18 loss, followed by reduplication. Accordingly, we demonstrated the malignant progression of the intestinal tumors at a much earlier stage in the *cis*-compound heterozygotes than expected in the simple *Apc*$^{\Delta 716}$ heterozygotes by natural accumulation of such genetic changes. Recently, we reported that no hot spot mutations were found in the K-*ras* gene in the *Apc*$^{\Delta 716}$ polyp adenomas (Oshima et al., 1997). Likewise, no K-, N- or H-*ras*, or *p53* gene (*Trp53*) mutations were found in the polyp adenomas of *Apc*^{1638N} knockout mice (Smits et al., 1997). In the present study, we found no mutations in the K-*ras* gene hot spot at codon 12, 13, or 61 in the malignant adenocarcinoma cells from the *cis*-compound mice, either. These results suggest that the polyp adenomas initiated by *Apc* LOH can become malignant with only additional inactivation of the *Dpc4* gene. It is worth noting that Markowitz et al. reported that a TGFβ-resistant and malignant derivative of a benign cultured human colon adenoma cell line expressed wild-type *p53* (*TP53*) transcripts (Markowitz et al., 1994). Mutations in genes such as *ras* and *p53* may contribute to further changes such as metastatic characteristics. We have not observed any metastatic foci in any organs of the *cis*-compound heterozygotes, at least in their short life span of about 20 weeks.

Considering the role of *Dpc4* in the TGFβ pathway (Massague et al., 1997), similar malignant phenotypes can be expected in the *Apc*$^{\Delta 716}$ polyps by an additional homozygous inactivation in one of other genes in the pathway than *Dpc4* as well—namely, the gene for ligand TGFβ1, -β2, or -β3; TGFβ receptor type I or II; or signaling proteins such as Smad2 or -3. Mouse *Smad2* will be particularly interesting in this regard because its human homolog is linked to *DPC4* (*SMAD4*), only 4 cM proximal on 18q21 (Eppert et al., 1996). In fact, Eppert et al. described four inactivating missense mutations of the

SMAD2 gene in sporadic colorectal carcinomas, two of which displayed LOH (Eppert et al., 1996).

Although TGFβ inhibits the growth of the normal cells, many malignancies of epithelial and hematopoietic origins are resistant to TGFβ (Polyak, 1996). It can even stimulate the proliferation of some advanced stage tumors (Mulder et al., 1988; Jennings et al., 1991). We have demonstrated that shutting off the TGFβ signaling pathway by the mutation of *Dpc4* in the *Apc*$^{\Delta 716}$ polyp adenomas made their growth much more malignant in nature. While most of the data we have presented in this report were collected from the mice of the F$_1$ generation regarding the *Dpc4* mutation, essentially the same results have been obtained with the N$_4$ (C57BL/6) backcross generation as well, except for higher intestinal polyp numbers. Moreover, *Apc*$^{\Delta 716}$ heterozygous segregants from the *cis*-compound mutants of the F$_1$ generation obtained by backcrosses with C57BL/6 mice, had similar numbers of polyps to the *cis*-compound parents; much fewer than the *Apc*$^{\Delta 716}$ heterozygotes of the N$_{12}$ backcross generation. However, the polyp size distribution and histopathology of the segregant *Apc*$^{\Delta 716}$ and the *trans*-compound heterozygotes were essentially the same as in the *Apc*$^{\Delta 716}$ heterozygotes in the N$_{12}$ generation. Accordingly, the malignant phenotype we observed in the *cis*-compound mutants was not due to other genes in the genetic background, whereas the polyp number was reduced by the background gene(s) brought in from the 129/Sv strain.

It is interesting that some of the *cis*-compound heterozygotes developed ampullary carcinomas in the duodenal papilla of Vater, while such tumors were not found in the simple *Apc*$^{\Delta 716}$ heterozygotes. In human FAP, the ampullary tumors are serious complications after the colectomy operations (Jagelman et al., 1988). Duodenal polyps occur in 45%–91% of FAP patients and are considered as premalignant lesions (Beckwith et al., 1991). Most of them are clustered in the periampullary region, and they often become malignant (Sugihara et al., 1982; Jagelman et al., 1988). In a large genetic study, Sanabria et al. reported recently a statistically significant familial segregation for the incidence and severity of periampullary neoplasia. Although there was no correlation between specific *APC* germline mutations and periampullary polyp frequency or severity, age was also a statistically significant variable. They predicted as-yet-unidentified modifier genes or perhaps common environmental factors. However, the pedigree linkage of the periampullary carcinomas was relatively weak, though statistically significant (Sanabria et al., 1996). Considering the fact that *DPC4* (on 18q) and *APC* (on 5q) are on different chromosomes in humans, it is possible that some FAP kindreds already carry *DPC4* mutations and that *DPC4* LOH is responsible for the malignant change of the ampullary adenomas. The age factor may also be explained by the time taken for the homozygous inactivation of *DPC4*, or one of the other genes in the TGFβ signaling pathway. On the other hand, in the *cis*-compound heterozygote mice, this takes place in a single event of Chr 18 loss followed by reduplication.

We also observed that more than half of the *cis*-compound heterozygotes developed epidermoid cysts of the skin. In Min mice, intraperitoneal injection of the

alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) was reported to increase the incidence of epidermoid cysts up to about 60% (Shoemaker et al., 1995). It is conceivable that mutations introduced into the *Dpc4* gene were responsible for the change.

We did not observe any neoplasms in the pancreas of the *cis*-compound heterozygote mice. Given the fact that *DPC4* is homozygously inactivated in about a half of pancreatic cancers (Hahn et al., 1996), the lack of pancreatic cancers in the *Apc/Dpc4 cis*-compound heterozygotes was somewhat surprising. This result may be explained by two possibilities. First, LOH in *Apc* and *Dpc4* by loss and reduplication of Chr 18 may be a very rare event in the mouse pancreas and does not take place before the *cis*-compound heterozygotes die of the intestinal tumors. Second, in most pancreatic cancers, the triggering event may be mutation(s) in a gene(s) other than *Apc*. Although *APC* mutations are found in some pancreatic cancers, such mutations appear to be heterozygous (Horii et al., 1992). At the same time, *DPC4* mutations are likely to affect the progression of the pancreatic tumors after the initiation process is triggered. The high frequency of *DPC4* mutation in pancreatic cancers is consistent with their histological characteristics of marked desmoplasia (Kloppel, 1993), which we have demonstrated in the intestinal adenocarcinomas developed in the *Apc/Dpc4 cis*-compound heterozygotes.

In conclusion, we have demonstrated that homozygous inactivation of the *Dpc4* gene causes *Apc*$^{\Delta 716}$ polyp adenomas to progress into malignant and invasive adenocarcinomas without additional mutations.

Experimental Procedures

Dpc4 (*Smad4*) and *Apc*$^{\Delta 716}$ Knockout Mice

Dpc4 (*Smad4*) knockout mice were constructed according to the strategy shown in Figure 1. A 380 bp mouse cDNA fragment was amplified by PCR, using oligonucleotide primers corresponding to human *DPC4* cDNA codons 25–31 (5′-TGCCATAGACAAGGTGG AGA-3′) and codons 145–151 (5′-TTACTCTGCAGTGTTAATCC-3′; Hahn et al., 1996). The fragment was cloned and used as the probe for screening a mouse genomic DNA library (Stratagene). A 6.8 kb StuI-XhoI fragment containing exons 2, 3, and 4 was isolated for the long homology arm, another fragment containing exon 1 and the 5′-upstream of the coding region (ApaI-SacI) was isolated for the short homology arm. They were cloned into a double cassette containing the neomycin resistance gene and diphtheria toxin gene both under the control of the PGK promoter, respectively (Soriano et al., 1991; Oshima et al., 1995), resulting in targeting vector *pDpc4*. This construct should encode 66 N-terminal amino acids of *Dpc4* elongated by 9 extra residues (PAFVPFSEG) from the vector. The truncated peptide, however, did not appear to be synthesized in detectable amounts in the *cis*-compound mice because N-terminal- and C-terminal-specific antibodies did not show any difference in immunohistochemical staining.

pDpc4 was linearized at the single NotI site, electroporated into the ES cells, and homologous recombinant (HR) clones were identified by PCR using the following primers: DPC4F1 (5′-GAGGCTGAT ACAGGGGTATTG-3′), and PGKR (5′-CTAAAGCGCATGCTCCAG ACT-3) (Figure 1). After incubation at 94°C (2 min), PCR were performed for 30 cycles at: 94°C (30 s), 60°C (1 min), and 72°C (1 min); followed by 72°C (8 min). The HR clones were injected into C57BL/6 blastocysts and germline chimeras were produced as described (Oshima et al., 1995).

Construction of the *Apc*$^{\Delta 716}$(+/−) mice have been described previously (Oshima et al., 1995).

Apc$^{\Delta 716}$ and *Dpc4* (*Smad4*) Compound Heterozygous Mice

Construction of the *cis*-compound heterozygotes are described in Results and shown schematically in Figure 3.

Mapping of the *Dpc4* (*Smad4*) on the Mouse Genome

The informative RFLVs for *Dpc4* were defined using TaqI; the C3H allele showed a band of 7.4 kb, whereas that of *M. spretus* was 11.0 kb. RFLVs and the segregation of *Adrb2* have been described in Oakey et al., 1991, whereas *D18Mit8* and *D18Mit9* have been described in Gariboldi et al., 1995. The chromosomal locations of the genes were determined, using a panel of DNA samples from an interspecific backcross between C3H/HeJ-*gld* and (C3H/HeJ-*gld* × *M. spretus*) F$_1$ (Seldin et al., 1988). This panel has been characterized for over 1100 genetic markers throughout the genome.

Polyp Scoring

The number and size of the polyps were scored by a single examiner (K. T.) as described (Oshima et al., 1995, 1996).

PCR Analysis for *Apc* and *Dpc4* LOH

PCR primers and conditions for *Apc* LOH determination have been described previously (Oshima et al., 1995, 1997). *Dpc4* LOH was determined using the following PCR primers; DPC4F1 described above and DE1R1 corresponding to the wild-type exon (5′-TATGGT GACACACTTGCTAGGATG-3′). The knockout allele genotype was determined using the DPC4F1 and PGKR as described above.

Histopathological and Immunohistochemical Analyses

Tissue specimens were prepared according to the standard protocol as described (Oshima et al., 1996, 1997). Polyclonal antibody against human DPC4 protein was purchased from Santa Cruz Biotechnology, CA. To determine the invasion score, polyps larger than 2 mm in diameter from 9 randomized mice were sectioned for each group, stained with H & E, and scored. The BrdU labeling experiments were performed according to the method described previously (Oshima et al., 1997) using a kit from Boehringer Mannheim. Electron microscopic samples were prepared, examined, and photographed by BML, Tokyo.

FISH Analysis for *Apc* and *Dpc4* Loci in Interphase Tumor Cells

Interphase chromosomes were analyzed as described (Inazawa et al., 1992).

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