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Targeted Inactivation of the *p21*$^{WAF1/cip1}$ Gene Enhances *Apc*-initiated Tumor Formation and the Tumor-promoting Activity of a Western-Style High-Risk Diet by Altering Cell Maturation in the Intestinal Mucosa[1]

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ABSTRACT

Elimination of both alleles of the gene that encodes the cyclin kinase inhibitor *p21*$^{WAF1/cip1}$ increases the frequency and size of intestinal tumors in *Apc1638*$^{+/-}$ mice that inherit a mutant allele of the *Apc* gene, and intermediate effects are seen if a single *p21* allele is inactivated. The increased tumor formation is associated with altered cell maturation in the intestinal mucosa of the *p21*-deficient mice—increased cell proliferation, and decreased apoptosis, and goblet cell differentiation—that is also a function of *p21* gene dosage. Moreover, a Western-style diet that mimics principal risk factors for colon cancer (high fat and phosphate, low calcium and vitamin D) accelerates tumor formation in *Apc1638*$^{+/-}$ mice, and the loss of a single or both *p21* alleles is additive with the tumor-promoting effects of this diet, resulting in more and larger tumors, and a highly significant decrease in survival time. Thus, *p21* normally suppresses *Apc*-initiated tumor formation and is haplo-insufficient in this regard. This is consistent with recent reports that *Apc* initiates tumor formation by up-regulating c-*myc* expression through altered β-catenin-Tcf signaling and that c-*myc* then up-regulates *cdk4*, whose activity is inhibited by *p21*. Decreased expression of *p21* is also a marker of poor prognosis in patients, and the data presented suggest that dietary alterations in patients undergoing treatment for colon cancer might be highly effective in improving outcome.

INTRODUCTION

The *p21*$^{WAF1/cip1}$ gene, a downstream effector of *p53* (1), is an inhibitor of cyclin-dependent kinase activity (2) and is therefore an important regulator of the cell cycle and potentially, of apoptosis and cell differentiation. In the intestinal tract, *p21* is expressed as cells exit the proliferative compartment, and loss of both expression and topological regulation is detected early in colon tumor formation (3, 4). Absence of *p21* is linked to inability of colon tumor cells to arrest in the G_1 phase of the cell cycle (5, 6), and the cell cycle arrest and apoptosis of colon tumor cells stimulated by the short-chain fatty acid butyrate, the nonsteroidal anti-inflammatory drug sulindac, and radiation are all linked to induction of *p21* (6–11).

Despite this evidence for an important role of *p21* in the regulation of intestinal cell maturation and tumor formation, the targeted inactivation of the *p21* gene in mice does not result in an obvious phenotype in the intestinal tract or other organs, although embryonic fibroblasts derived from such mice are defective in G_1 checkpoint arrest (12). However, it is possible that the loss of *p21* is important in the formation of tumors that are initiated by other genetic events. In particular, loss of the wild-type *APC* gene initiates the development of almost all human colon cancers (13), and mice that inherit an inactivated *Apc* allele develop intestinal tumors, principally in the small intestine, when they spontaneously lose or inactivate the remaining wild-type *Apc* allele (14–18). Moreover, because *Apc* up-regulates c-*myc* expression through defective β-catenin-Tcf signaling (19) and c-*myc* then up-regulates *cdk4* (20), a prediction is that *p21*, an inhibitor of *cdk4* activity, should have important effects on the initiation of tumors by *Apc*.

Therefore, to determine whether *p21* could alter intestinal tumorigenesis initiated by loss of *Apc*, we generated mice that inherited a mutant *Apc* allele and that were also either heterozygous or homozygous for loss of *p21* and found that loss of *p21* enhanced tumor formation in a dosage-dependent manner. Further, this increased tumorigenesis was associated with striking effects on cell maturation in the intestinal mucosa. Finally, because a Western-style diet has been shown to act on later stages of tumor promotion in enhancing *Apc*-initiated tumor formation, we investigated whether the loss of *p21* was additive with a Western-style diet. Additive effects on tumor number and size were indeed seen, resulting in a highly significant decrease in life span for the mice. Thus, the combination of *p21* loss and a Western-style diet has a profound impact on survival of mice with *Apc*-initiated tumors, which mimics the poorer prognosis for colon cancer patients whose tumors show decreased *p21* expression (21).

MATERIALS AND METHODS

The *Apc1638* and *p21* mouse models and methods for genotyping have been reported (12, 16–18). *Apc1638*$^{+/-}$ mice were mated with *p21*$^{-/-}$ mice to produce *Apc1638*$^{+/-}$,*p21*$^{+/-}$ offspring (F_1). F_1 mice were mated to produce desired genotypes: *Apc1638*$^{+/-}$,*p21*$^{+/+,\ +/-,\ \text{or}\ -/-}$. At weaning (approximately 3–4 weeks), littermates were randomized to genetic/dietary groups and fed *ad libitum* either AIN76A or a Western-style diet that is formulated on the basis of nutrient density to mimic major risk factors for colon cancer in the Western diet: high in fat and phosphate and low in calcium and vitamin D (22, 23). Diets were from Teklad (Madison, WI).

Mice were weighed weekly and maintained on diet for 36 weeks or until they exhibited significant weight loss or other signs of extensive tumor formation. Mice were killed by CO_2 overdose and cervical dislocation and rapidly dissected for evaluation of tumors and fixation of tissues, as described previously (16, 18). Proliferation and apoptosis were evaluated by staining for proliferating cell nuclear antigen (Zymed, South San Francisco, CA) or TUNEL[3] assay (Trevigen, Gaithersburg, MD), as described previously (24). Goblet cells were detected by staining for mucins with Alcian blue or by immunohistochemical detection of mucins with MCM antibody (Ref. 25; a generous gift of A. Einerhand, The University of Amsterdam, The Netherlands), with detection by immunoperoxidase, using an ABC kit (Vector Laboratories, Burlingame, CA).

RESULTS

Apc1638$^{+/-}$ mice were mated with *p21*$^{-/-}$ mice to produce *Apc*$^{+/-}$,*p21*$^{+/-}$ offspring (F_1). The F_1 mice were then mated to

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[3] The abbreviations used are: TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; MCM, murine colonic mucin.

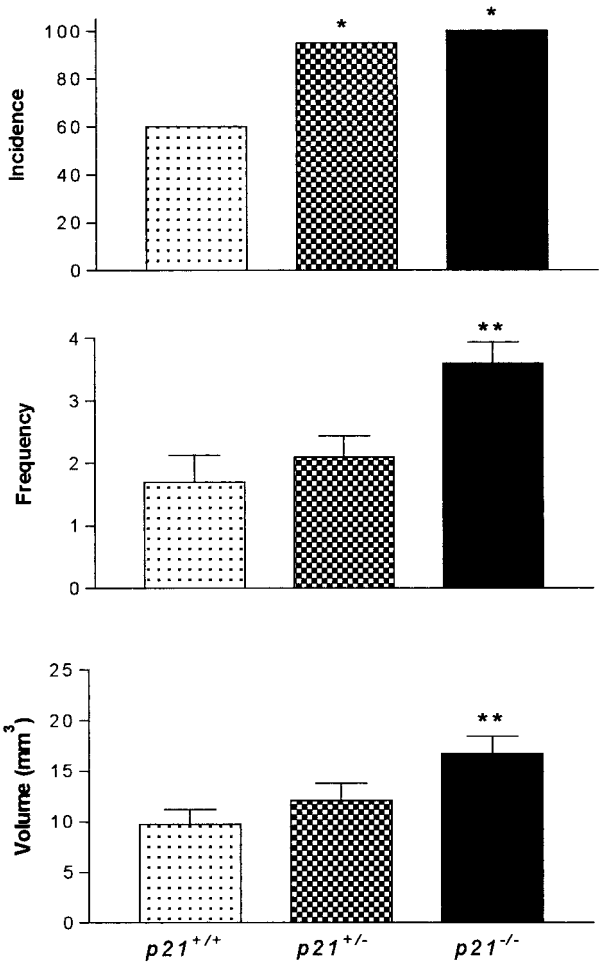

Fig. 1. The incidence, frequency and size of gastrointestinal tumors in $Apc^{+/-}$, $p21^{+/+,\ +/-,\ \text{or}\ -/-}$ mice fed AIN-76A diet for 36 weeks (*, $P < 0.05$, **, $P < 0.01$ comparison with $Apc^{+/-}$, $p21^{+/+}$ mice).

produce mice that were $Apc^{+/-}$,$p21^{+/+,\ +/-,\ \text{or}\ -/-}$ (F_2). By 36 weeks on a defined AIN76A diet, the F_2 $Apc^{+/-}$,$p21^{+/+}$ mice developed intestinal tumors in 60% of the animals, at a frequency of 1.7 tumors per mouse (Fig. 1). This tumor incidence and frequency were identical to that of $Apc1638^{+/-}$ mice on a homogeneous B6 background (18), thus eliminating the possibility that unlinked loci from the *p21* mice had a significant effect on the *Apc*-initiated intestinal tumor formation. However, littermates that were $Apc^{+/-}$ and either $p21^{+/-\ \text{or}\ -/-}$ had a significantly higher tumor incidence of 95 and 100%, respectively. In addition, the tumor frequency per mouse was increased by 23% in the $Apc^{+/-}$,$p21^{+/-}$ mice and by >118% in the $Apc^{+/-}$, $p21^{-/-}$ mice. The effect on tumor size was also striking: tumors in $Apc^{+/-}$,$p21^{+/-}$ mice were 26% larger than the tumors in $Apc^{+/-}$,$p21^{+/+}$ mice at 36 weeks, and in the $Apc^{+/-}$,$p21^{-/-}$ mice, the tumors were 74% larger.

In vitro, the short-chain fatty acid butyrate, a physiological regulator of cell maturation in the intestinal tract (7, 24), elevates *p21* expression in association with stimulation of cell cycle arrest, differentiation markers, and apoptosis (7). Thus, *p21* elevation in cultured intestinal cells mimics the *in vivo* association of elevated *p21* expression with the major pathways of intestinal cell maturation seen after cells exit the proliferative compartment (3, 4). We, therefore, investigated how the targeted inactivation of *p21 in vivo* is associated with alterations in cell maturation pathways in the intestine. In the duodenum, the principal site of tumor formation in these mice regardless of genotype, absence of a single *p21* allele in the $Apc^{+/-}$ mice increased proliferation by 25%, and in the absence of both alleles, proliferation increased by 38%. We also detected decreased apoptosis, measured by TUNEL, of 47% in the $Apc^{+/-}$,$p21^{+/-}$ mice and 68% in the $Apc^{+/-}$,$p21^{-/-}$ mice (Fig. 2). Figure 2 also shows that there is a major effect of the loss of a single or both *p21* alleles on the ratio of proliferating to apoptotic cells in the duodenum. An important observation was that the increased proliferation and decreased apoptosis were not seen in the mucosa of the proximal colon of the $Apc^{+/-}$,$p21^{-/-}$ mice compared with $Apc^{+/-}$ littermates wild type for *p21* (data not shown). Because tumors formed only in the small intestine in these $Apc^{+/-}$ mice, even in the absence of *p21*, the changes in proliferation and apoptosis were tightly associated with the risk for tumor development in the tissue.

We also investigated the effects of *p21* on intestinal cell differentiation. We focused on the goblet or secretory cell lineage, because this lineage is often lost very early in aberrant crypt foci in humans at risk for development of colon cancer (26–28), in mice treated with chemical carcinogens (27), and in $Apc1638^{+/-}$ mice (27). Figure 3 illustrates a comparison of $Apc^{+/-}$,$p21^{-/-}$ mice with the $Apc^{+/-}$,$p21^{+/+}$ mice, showing a substantial decrease in goblet cells in the duodenal mucosa that could be detected by Alcian blue staining (Fig. 3, *A* and *B*) or by immunohistochemistry with MCM antibody, which recognizes mature mucin (Fig. 3, *C* and *D*). Frequencies of Alcian blue- and MCM-positive cells were determined for six mice in each genetic group. Table 1 shows that the decrease in the number of these cells was approximately 25% in the $p21^{+/-}$ heterozygotes and 39% in the $p21^{-/-}$ homozygotes ($P < 0.05$ and $P < 0.001$, respectively,

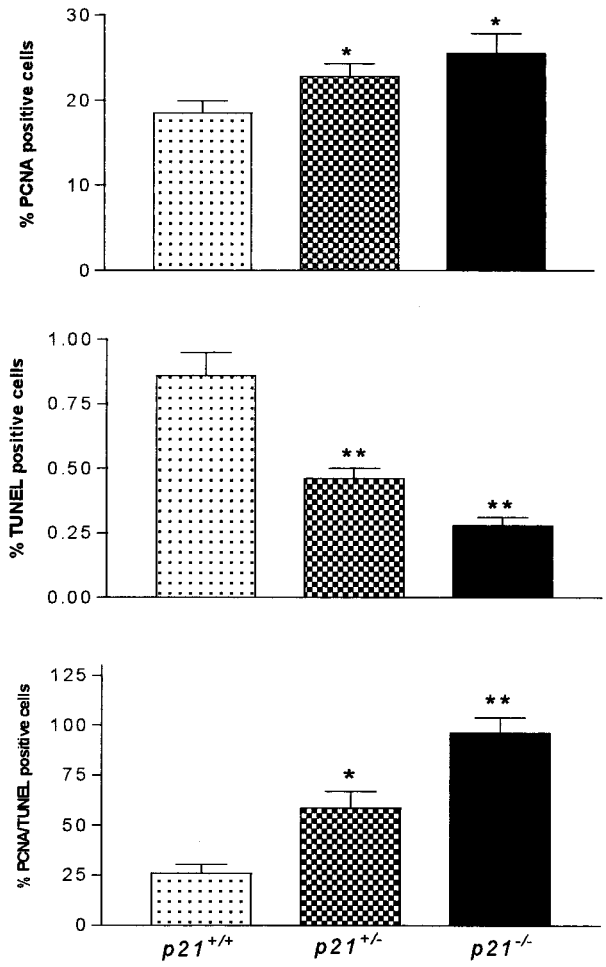

Fig. 2. Proliferation and apoptotic index in the duodenal epithelia of $Apc^{+/-}$,$p21^{+/+,\ +/-,\ \text{or}\ -/-}$ mice fed AIN-76A diet for 36 weeks (*, $P < 0.05$, **, $P < 0.01$ comparison with $Apc^{+/-}$,$p21^{+/+}$ mice).

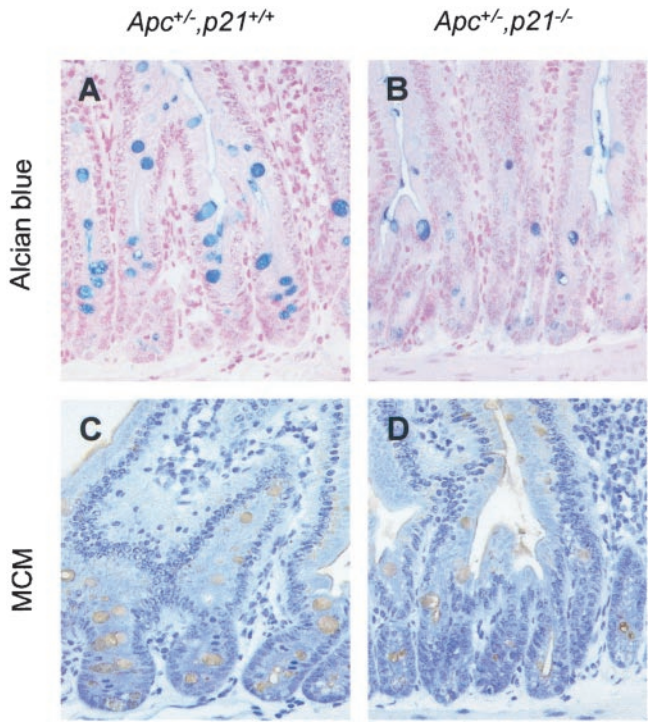

Fig. 3. Identification of goblet cells in the duodenal epithelia of $Apc^{+/-}$,$p21^{+/+ \text{ or } -/-}$ mice fed AIN-76A diet for 36 weeks. Detection of goblet cells was by Alcian blue staining (*A*, *B*) or immunohistochemistry with an antibody (*MCM*; *C*, *D*) that recognizes mature murine mucin.

compared with *p21* wild-type mice). Similar to the results for proliferation and apoptosis, no effect of the loss of *p21* on goblet cell frequency was detected in the large intestine, where tumors do not form (not shown).

The effects of *p21* inactivation in the flat mucosa of the intestine at the anatomical site of tumor formation suggests that loss of *p21* plays a role early in the development of tumors initiated by the *Apc* mutation. A Western-style diet that mimics the human dietary intake of high fat and phosphate and low calcium and vitamin D, which are risk factors for colon cancer, also enhances tumor formation in the $Apc1638^{+/-}$ mouse, but this has been reported to be attributable to later effects on tumor promotion (29, 30).

If the *p21* and a Western-style diet indeed act at different times during *Apc*-initiated tumor formation, we would hypothesize that the effects of the two would be additive and independent. To test this, mice that were $Apc^{+/-}$,$p21^{+/+, +/- \text{ or } -/-}$ were fed the Western-style diet. We found a striking effect on intestinal tumor formation first reflected in the survival of the animals. $Apc1638^{+/-}$ mice that were wild type for *p21* and fed the defined AIN-76A diet developed intestinal tumors (*i.e.*, Fig. 1) but survived to 36 weeks of age (Fig. 4). Littermates that were $Apc^{+/-}$ and either $p21^{+/- \text{ or } -/-}$ fed AIN-76A diet died somewhat earlier, coincident with the increase in tumor number and growth, and this was statistically significant for the $Apc^{+/-}$,$p21^{-/-}$ group compared with $Apc^{+/-}$,$p21^{+/+}$ ($P < 0.004$). However, when fed the Western-style diet, all of the animals showed decreased survival compared with the same genetic groups fed AIN-76A (Fig. 4). For each genetic group, the effects of the Western-style diet in reducing life span were highly significant ($P < 0.02$, 0.003, and 0.015 for $Apc^{+/-}$,$p21^{+/+, +/-, \text{ and } -/-}$, respectively). Most dramatic was the effect of a combination of absence of both alleles of *p21* and the Western-style diet. Fewer than 29% of these mice survived to 36 weeks, whereas for the wild-type *p21* mice on the standard or Western diet, the survival rates were 100 and 75%, respectively. The difference in survival for $Apc^{+/-}$,$p21^{+/+}$ mice fed AIN-76A compared with the $Apc^{+/-}$,$p21^{-/-}$ mice fed the Western-style diet was significant at the $P < 0.0001$ level.

Because many of the animals on the Western-style diet died before 36 weeks of age or had to be killed early because of their tumors, the number and size of tumors for this experiment and a comparison to the mice fed AIN76A is presented only for those mice that survived to 36 weeks of age. Table 2 illustrates that for this subset, the frequency and size of the tumors was much greater for each *p21* genotype fed the Western-style diet compared with those fed AIN76A. The effects of the Western-style diet appeared to be additive with the effects of the *p21* mutation. For each *p21* genetic group (wild type, heterozygotes, and homozygotes), tumor number and size were approximately double for the Western-style diet groups compared with the AIN76A diet groups (Table 2). As a result, the combination of the Western-style diet and the absence of both *p21* alleles increased the number of tumors per mouse by 4.4-fold ($P < 0.004$) compared with *p21*

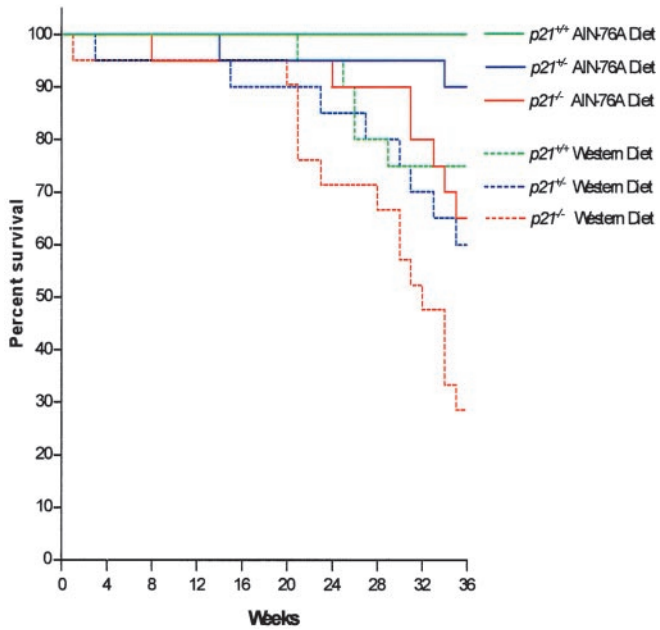

Fig. 4. Survival of $Apc^{+/-}$,$p21^{+/+, +/-, \text{ or } -/-}$ mice fed AIN-76A or Western-style diet.

Table 1 *Alcian blue and MCM staining in the duodenum of* $Apc^{+/-}$, $p21^{+/+}$, $^{+/-}$, *or* $^{-/-}$ *mice fed AIN-76A diet*

All mice $Apc^{+/-}$ and	N^a	Total cells per column[b,c]	Alcian blue (+) cells	Alcian blue (+) cells (%)	MCM (+) cells	MCM (+) cells (%)
$p21^{+/+}$	6	14.7 ± 0.6	1.72 ± 0.22	11.7	1.66 ± 0.18	11.3
$p21^{+/-}$	6	14.6 ± 0.2	1.38 ± 0.27[d]	9.4[d]	1.33 ± 0.40[e]	9.2[e]
$p21^{-/-}$	6	14.8 ± 0.3	1.07 ± 0.18[f]	7.2[f]	1.13 ± 0.17[f]	7.7[f]

[a] *N*, number of mice studied each group.
[b] Values are mean ± SD.
[c] Fifty crypt columns were counted in each mouse.
[d] $P < 0.05$, [e] $P = 0.06$, [f] $P < 0.001$, in comparison to the $Apc^{+/-}$, $p21^{+/+}$ mice (by Student's *t* test).

Table 2 *The frequency and size of gastrointestinal tumors in $Apc^{+/-}$, $p21^{+/+}$, $^{+/-}$, or $^{-/-}$ mice fed AIN-76A diet or Western-style diet*

	Number of tumors per mouse[a]		Volume (mm^3)[a]	
All mice $Apc^{+/-}$ and	AIN-76A diet	Western diet	AIN-76A diet	Western diet
$p21^{+/+}$	1.7 ± 1.9	4.3 ± 1.9[d]	9.6 ± 8.9	17.5 ± 13.0[e]
$p21^{+/-}$	2.1 ± 1.5	6.6 ± 3.9[b,d]	12.1 ± 10.7	23.0 ± 15.2[b,d]
$p21^{-/-}$	3.7 ± 1.4[c]	7.5 ± 4.9[b,f]	16.7 ± 13.3[c]	27.3 ± 20.2[c,d]

[a] Values are means ± SD.
[b] $P < 0.05$, [c] $P < 0.01$, in comparison to the $Apc^{+/-}$, $p21^{+/+}$ mice (by Student's *t* test).
[d] $P < 0.001$, [e] $P < 0.01$, [f] $P < 0.05$, in comparison to mice fed AIN-76A diet (by Student's *t* test).

wild-type animals fed AIN-76A, and the size of the tumors increased by 3-fold ($P < 0.001$). This significant enhancement of $Apc^{+/-}$-initiated tumor formation by a combination of inactivation of *p21* and a Western-style diet led to the highly significant decrease in life span for the mice shown in Figure 4.

DISCUSSION

We have demonstrated that the *p21* gene normally suppresses tumor formation initiated by inactivation of the *Apc* gene. Although the largest effects were seen when both alleles of *p21* were absent, absence of a single allele had intermediate effects, and these effects in the $p21^{+/-}$ mice were significant for tumor incidence in mice fed AIN-76A, for tumor frequency and size in mice fed a Western-style diet, and for proliferation, apoptosis, and goblet cell differentiation in the duodenal mucosa. In this regard, related molecules, such as *p53*, which is a regulator of *p21* expression, and $p27^{kip1}$, like *p21* an inhibitor of cyclin-dependent kinases, have also been reported to have a tumor-enhancing effect upon loss of a single allele (31, 32).

A Western-style high-risk diet increased tumor formation in the $Apc1638^{+/-}$ mice (30), and a high-fat diet also increases tumor formation in *Min* mice (33). In the present study, the combination of loss of *p21* and the consumption of the Western-style diet were additive on tumor formation and together resulted in much more frequent and larger tumors. The result of this was a highly significant decrease in life span of the mice.

The fact that *p21* inactivation and a Western-style diet were additive on *Apc*-initiated tumor formation and that *p21* inactivation had pronounced effects on cell maturation in the duodenal mucosa, whereas a Western-style diet has been reported to affect tumor formation in later stages (30), suggests that the absence of *p21* and the Western-style diet acted at different stages and pathways of tumor development and were independent. A role for *p21* early in *Apc*-initiated tumor development is also consistent with reports that *Apc* inactivation up-regulates c-*myc* through its effects in altering β-catenin-Tcf signaling (19) and that c-*myc* in turn up-regulates *cdk4* (20), whose activity is inhibited by *p21*. Thus, our data support the suggestion that this pathway is important in the initiation of tumor formation by mutations in *Apc*.

These data have important implications for understanding prognosis of human colon cancer. Although *p21* is not frequently lost during the development and progression of human colon tumors, it is down-regulated in expression (3, 4), and low expression of *p21* in the tumors is an independent prognostic factor that is linked to poorer survival in colorectal cancer patients (21). Our data on increased tumor formation with loss of *p21* in mice are consistent with these clinical data, although it should be pointed out that in the mouse genetic model, *p21* is also missing from the surrounding stromal cells, and there are as yet no data suggesting that *p21* down-regulation is a characteristic of nonepithelial cells in tumors. It is potentially important that low *p53* expression was not a prognostic marker in the clinical studies (21), a fact that is perhaps consistent with a role for *p21* in tumor suppression independent of *p53* (34), such as its *p53*-independent role in pathways of cell cycle arrest, differentiation, and/or apoptosis of leukemia cells (35, 36), muscle cells, (37), or colonic cells treated with butyrate (7) or sulindac (9). Moreover, the fact that mouse life span is most significantly reduced by a combination of a tumor-promoting diet and a genetic modifier of *Apc*-initiated tumor formation suggests that dietary alterations in patients undergoing treatment for colon cancer might be effective in improving either disease-free and/or overall survival, especially in an adjuvant setting when patients have been surgically cured of disease and the primary goal is prevention of recurrence or metastasis.

We have found gene dosage-dependent effects of the absence of *p21* on increasing cell proliferation and decreasing apoptosis and on reducing the number of mature goblet cells in the mucosa of $Apc^{+/-}$ mice that are specifically linked to the site of tumor formation (duodenum *versus* proximal colon). We believe the effects on proliferation in the mucosa are more important than the effects on apoptosis, because, as we have previously reported, the number of apoptotic cells is very low in the mucosa, and elimination of apoptosis by genetic elimination of short-chain fatty acid metabolism is ineffective in producing colon tumors (24). However, the percentage change in apoptotic cells with loss of *p21* is larger than the percentage change in proliferating cells, and the ratio between proliferating and apoptotic cells both in the mucosa and in the initiated tumor may be crucial elements in determining how tumors form and respond to dietary factors that modulate tumor formation.

The loss of *p21* expression may be linked to the decrease in goblet cells attributable to the continued proliferation of cells that prevents their differentiation. Alternatively, *p21* could have a more specific role in regulating lineages of differentiation in the intestinal mucosa than that which is presently understood. However, regardless of the mechanism by which goblet cells are depleted by the loss of *p21*, it is particularly significant that loss of this lineage and mucin secretion is a characteristic of early, preneoplastic aberrant crypt foci in patients who are at risk for developing colon cancer, in rodents treated with colon-specific carcinogens, and in the $Apc1638^{+/-}$ mouse and thus may be an important factor in the promotion of these early lesions (24–26).

There are clearly profound interactions between diet and genetics in the development and progression of colorectal cancer (30, 33, 38, 39). Our finding that the absence of *p21* and a Western-style diet can significantly increase tumor formation in combination to a greater extent than either does alone again demonstrates the importance of considering both dietary and genetic factors in tumor formation, in chemoprevention, and in therapy.

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