Period 2 Mutation Accelerates Apc\(^{Min/+}\) Tumorigenesis

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Abstract

Colorectal cancer risk is increased in shift workers with presumed circadian disruption. Intestinal epithelial cell proliferation is gated throughout each day by the circadian clock. Period 2 (Per2) is a key circadian clock gene. Per2 mutant (Per2\(^{m/m}\)) mice show an increase in lymphomas and deregulated expression of cyclin D and c-Myc genes that are key to proliferation control. We asked whether Per2 clock gene inactivation would accelerate intestinal and colonic tumorigenesis. The effects of PER2 on cell proliferation and \(\beta\)-catenin were studied in colon cancer cell lines by its down-regulation following RNA interference. The effects of Per2 inactivation in vivo on \(\beta\)-catenin and on intestinal and colonic polyp formation were studied in mice with Per2 mutation alone and in combination with an Apc mutation using polyp-prone Apc\(^{Min/+}\) mice. Down-regulation of PER2 in colon cell lines (HCT116 and SW480) increases \(\beta\)-catenin, cyclin D, and cell proliferation. Down-regulation of \(\beta\)-catenin along with Per2 blocks the increase in cyclin D and cell proliferation. Per2\(^{m/m}\) mice develop colonic polyps and show an increase in small intestinal mucosa \(\beta\)-catenin and cyclin D protein levels compared with wild-type mice. Apc\(^{Min/+}\)Per2\(^{m/m}\) mice develop twice the number of small intestinal and colonic polyps, with more severe anemia and splenomegaly, compared with Apc\(^{Min/+}\) mice. These data suggest that Per2 gene product suppresses tumorigenesis in the small intestine and colon by down-regulation of \(\beta\)-catenin and \(\beta\)-catenin target genes, and this circadian core clock gene may represent a novel target for colorectal cancer prevention and control. (Mol Cancer Res 2008;6(11):1786–93)

Introduction

Central and peripheral clocks generate self-sustained circadian rhythms of about 24 hours, which coordinate physiologic processes with the rhythmically changing environment (1-3). The master circadian pacemaker, in the suprachiasmatic nuclei of the brain, is synchronized by the daily light-dark cycle, which in turn synchronizes the organism and peripheral clocks in each cell (4). The molecular mechanisms of circadian oscillation in the suprachiasmatic nuclei and peripheral cells are based on a negative transcriptional-translational feedback loop generated by at least nine core clock genes (1). Circadian clockworks in peripheral tissues coordinate physiologic processes through tissue-specific clock-controlled genes, such as thymidylate synthase, p21, and Wee-1, which gate DNA replication and mitosis and other growth regulators, such as vascular endothelial cell growth factor (2, 5-9). The proliferation of intestinal epithelial cells and the rate of cell migration up the crypt-villus axis are gated throughout each day by the circadian clock (10, 11).

Disruption of circadian rhythms deregulates cell proliferation and increases cancer risk. Period 2 (Per2) is a core circadian clock gene, which also acts as a negative growth regulator and tumor suppressor (12, 13). Mice with homozygous mutation in the Per2 circadian clock gene (Per2\(^{m/m}\)) display altered circadian behavioral rhythms, deregulation of c-Myc and cyclin D RNA levels and daily rhythms, and spontaneous tissue hyperplasias and tumors. Following \(\gamma\)-radiation, these mice have a shortened survival, an abnormal DNA damage response, and increased incidence of lymphoid neoplasia, teratomas, and salivary hyperplasias (12). A recent screen of all mutations in several human breast cancers has identified mutations in the PER2 gene (14). Altered PER2 levels in breast cancers have been associated with altered methylation of the PER2 promoter (15).

Increasing evidence indicates that disruption of circadian rhythms significantly increases the incidence of epithelial cancers. Higher rates of breast, colorectal, and endometrial cancer have been found in shift workers with presumed...
circadian disruption (16-18). Shift work has recently been listed by the IARC as a probable carcinogen (19). Colorectal cancer is a major cause of cancer death in the United States (20).

Mutation in the familial adenomatous polyposis coli (APC) gene and deregulated Wnt-APC-β-catenin signaling pathway contributes to the development of spontaneous and hereditary colorectal cancer. Heterozygous mutations in the germline APC gene causes familial adenomatous polyposis with colon, and sometimes small intestinal, polyps, and, eventually, cancer, following the inactivation of the remaining wild-type APC allele. Inactivation of both APC alleles often occurs in sporadic colorectal adenomas that progress to cancer (21-23). The Min (multiple intestinal neoplasia; Apc<sup>Min/+</sup>) mouse, with a heterozygous mutation of the Apc gene, is a useful model for colorectal cancer (24-26). Loss of Apc function mimics the effect of constitutive Wnt stimulation, resulting in increased β-catenin levels, continuous β-catenin/T-cell factor (TCF) signaling, and increased β-catenin-dependent gene expression (e.g., cyclin D). This leads to excess intestinal epithelial cell proliferation and adenoma formation in the intestine and colon, in addition to extraintestinal proliferation abnormalities (26).

Because of our interests in how the circadian clock influences cellular proliferation and antiproliferative cytotoxic and targeted therapeutic drug responsiveness, we have investigated the effects of Per2 on colon cancer cell proliferation and intestinal and colonic neoplastic changes.

**Results**

**Down-Regulation of PER2 Increases β-Catenin and Cell Proliferation in Colon Cancer Cell Lines**

Because activation of the β-catenin pathway is key to the promotion of intestinal and colonic cell proliferation and tumorigenesis, we asked whether alteration in PER2 expression modulates β-catenin levels and the β-catenin signaling pathways in human colon cancer cell lines. We used small interfering RNA (siRNA) to down-regulate PER2 expression in HCT116 (wild-type APC, mutant β-catenin) and SW480 (mutant APC, wild-type β-catenin) colon cancer cell lines in vitro. In HCT116 cells, with lower endogenous β-catenin protein levels, down-regulation of PER2 increases β-catenin protein concentration (Fig. 1A and B). In SW480 cells, with

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**FIGURE 1.** Down-regulation of PER2 by siRNA in vitro increases β-catenin and cyclin D and accelerates cell growth in colon cancer cell lines compared with control siRNA cultures. Representative Western blots (A and C) and quantitation of protein abundance (B and D), relative to actin content, show that reducing PER2 results in an increase in β-catenin and cyclin D protein in HCT116 and SW480 colon cancer cell lines. Down-regulation of PER2 also accelerates cell growth of HCT116 (E) and SW480 (F) cell lines compared with control siRNA–treated cultures (representative growth experiment). Findings are similar among three experiments. Points, mean; bars, SE. *, P < 0.03, compared with control conditions.
high endogenous β-catenin levels, down-regulation of PER2 further elevates β-catenin protein (Fig. 1C and D). Following down-regulation of PER2, cyclin D protein, a β-catenin target gene associated with cell proliferation, increases in SW480 and HCT116 colon cancer cell lines (Fig. 1). This increase in β-catenin and cyclin D, which occurs with PER2 down-regulation, accelerates cell proliferation in both SW480 and HCT116 cell lines (Fig. 1E and F).

When β-catenin protein induction is prevented by its siRNA-mediated down-regulation, PER2 down-regulation no longer increases cyclin D protein in HCT116 (Fig. 2A and C) and SW480 (Fig. 2B and D) cells and this prevents the

FIGURE 2. Increase in cyclin D and acceleration of cell growth caused by PER2 down-regulation is prevented when β-catenin expression is down-regulated by siRNA in vitro compared with control siRNA cultures. Representative Western blots (A and B) are shown with the siRNA treatment listed at the left top and proteins detected on the right with quantitation of protein abundance (C and D), relative to actin content. Columns, mean of three independent experiments; bars, SE. In HCT116 (A, B, and E) and SW480 (C, D, and F) colon cancer cell lines, down-regulation of PER2 alone increases cyclin D protein and cell growth, and this PER2-associated change in cyclin D and cell growth is prevented when β-catenin expression is simultaneously down-regulated. *, P < 0.01, compared with control conditions.
acceleration in cell proliferation (Fig. 2E and F). Partial down-regulation of β-catenin alone (e.g., 50% reduction), in each of these cell lines under these conditions, has no effect on proliferation compared with control siRNA–treated cultures (data not shown), similar to previous reports with β-catenin down-regulation (27).

Cyclin D expression can be induced by β-catenin/TCF–dependent signaling or through other β-catenin–independent pathways. The effect of PER2 down-regulation and PER2 overexpression on β-catenin/TCF transcriptional activation was investigated using the TOPFLASH luciferase assay. PER2 overexpression, compared with control vector used for over-expression, decreased β-catenin/TCF reporter activity, whereas PER2 down-regulation, compared with control vector used for down-regulation, increased β-catenin/TCF reporter activity (Fig. 3). Therefore, the effect of PER2 on cyclin D seems, at least in part, dependent on β-catenin transcriptional activation.

These effects of PER2 down-regulation on increasing both β-catenin and cell proliferation seem not to be restricted to colon cell lines because similar effects are also seen in HeLa cells (Supplementary Fig. S1).

Per2 Mutation Increases Intestinal Mucosa β-Catenin Levels and Colon Polyps

Because down-regulation of Per2 expression in colon cancer cell lines increases β-catenin levels and β-catenin target gene expression, we asked whether Per2m/m mice with a homozygous inactivation of Per2 would show a de novo increase in intestinal mucosa β-catenin protein at a time of day when wild-type PER2 protein levels are near their daily maximum (mid-activity/mid-dark phase in mice). Small intestinal mucosa β-catenin protein levels in 15-week-old Per2m/m female mice are 2-fold higher compared with levels in small intestinal mucosa of age- and sex-matched wild-type mice (P < 0.05; Fig. 4A and B). This increase in small intestinal mucosa β-catenin in Per2m/m mice is also associated with an increase in cyclin D protein (P < 0.05) compared with wild-type mucosa (Fig. 4A and B).

Because increased β-catenin expression is often associated with polyp formation, we examined Per2m/m mice for intestinal polyps. Fifteen-week-old Per2m/m mice show an increase in the numbers of colonic polyps compared with wild-type mice. Small intestinal mucosa from 15-wk-old wild-type and Per2m/m mice was isolated during the mid-activity phase and homogenized for protein analysis. Representative Western blots of individual mice (A) with quantitation of protein abundance (B), relative to control tubulin content (five mice/genotype), show an increase in β-catenin and cyclin D (β-catenin target gene) proteins in the mucosa of Per2m/m mice. C. Colon polyp numbers and frequency in 15-wk-old wild-type, Per2m/m, and Per2m/+ mice. *, P < 0.05, for Per2m/m compared with wild-type.

FIGURE 4. β-Catenin and cyclin D levels in small intestinal mucosa and colon polyps are increased in Per2m/m mice compared with wild-type mice. Small intestinal mucosa from 15-wk-old wild-type and Per2m/m mice was isolated during the mid-activity phase and homogenized for protein analysis. Representative Western blots of individual mice (A) with quantitation of protein abundance (B), relative to control tubulin content (five mice/genotype), show an increase in β-catenin and cyclin D (β-catenin target gene) proteins in the mucosa of Per2m/m mice. C. Colon polyp numbers and frequency in 15-wk-old wild-type, Per2m/m, and Per2m/+ mice. *, P < 0.05, for Per2m/m compared with wild-type.
Per2 Mutation Increases Apc<sup>Min/+</sup> Intestinal and Colonic Polyp Formation

Because alteration in Per2 modulates β-catenin and β-catenin target gene expression, cell proliferation, and polyp formation, we crossed Per2<sup>+/−</sup> mice with Apc<sup>Min/+</sup> mice to determine whether the Per2 mutation would accelerate intestinal and colonic tumorigenesis in vivo. We compared the number and size of small intestinal polyps and number of colonic polyps in 10-week-old Apc<sup>Min/+</sup> mice with mice homozygous (Apc<sup>Min/+Per2<sup>−/−</sup>/Per2<sup>−/−</sup></sup>) and heterozygous (Apc<sup>Min/+Per2<sup>−/−</sup>/Per2<sup>−/+</sup></sup>) for the Per2 mutation. Compared with age-matched 10-week-old Apc<sup>Min/+</sup> mice, Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice have nearly twice the number of small intestinal polyps (83.9 ± 9.5 versus 46.7 ± 4.4; P < 0.001; Fig. 4A). This is the result of an increase in the number of small-sized (<1 mm) small intestinal polyps in Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice compared with Apc<sup>Min/+</sup> mice (51.7 ± 7.3 versus 21.3 ± 2.1; P < 0.001), whereas the number of larger-sized (>1 mm) small intestinal polyps failed to differ (32.3 ± 5.3 versus 25.4 ± 2.9; P = 0.122). Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice show a significant increase (2- to 3-fold) in total polyps in the second, third, and fourth segments of the small intestine (P < 0.01), whereas polyp numbers failed to differ in the proximal small intestine compared with Apc<sup>Min/+</sup> mice (Fig. 4B).

The number of total colonic polyps is also 2- to 3-fold higher in Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice compared with Apc<sup>Min/+</sup> mice (4.2 ± 1.3 versus 1.6 ± 0.3; P = 0.025; Fig. 4C). These effects require Per2<sup>−/−</sup> homozygosity. Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice that are heterozygous for the Per2 mutation fail to show differences in total small intestinal polyps (43.0 ± 5.5 versus 46.7 ± 4.4; P = 0.31; Fig. 4A) or colonic polyp numbers (2.7 ± 1.1 versus 1.6 ± 0.3; P = 0.14; Fig. 4C) compared with Apc<sup>Min/+</sup> mice. Wild-type, Apc<sup>Min/+</sup>, and Per2<sup>−/−</sup> mice at this age do not show small intestinal polyps.

By histologic examination, the frequency of small intestinal microadenomas is higher in 10- to 15-week-old Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice than in Apc<sup>Min/+</sup> mice (88.9% versus 42.3%; P < 0.001). The majority of these microadenomas in Apc<sup>Min/+</sup> and Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice show low-grade dysplasia. Severe dysplasia/carcinoma in situ is infrequent in both Apc<sup>Min/+</sup> and Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice and did not differ significantly (2 of 26 versus 4 of 27 mice; P = 0.42).

Per2 Mutation Increases Apc<sup>Min/+</sup> Extraintestinal Manifestations

The extraintestinal changes seen in the Apc<sup>Min/+</sup> mouse include progressive anemia, splenomegaly, and extramedullary hematopoiesis (EMH; ref. 26). At 10 weeks of age, Apc<sup>Min/+</sup> mice show lower hematocrits (46.3 ± 1.1% versus 54.6 ± 1.2%; P < 0.001) and larger spleens (higher spleen index, 0.532 ± 0.045% versus 0.375 ± 0.013%; P < 0.006) compared with wild-type mice (Supplementary Table S1). Ten-week-old Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice show acceleration of this anemia (40.6 ± 1.8% versus 46.3 ± 1.1%; P < 0.004) and greater splenomegaly (0.900 ± 0.119 versus 0.532 ± 0.045 spleen index; P < 0.002) compared with Apc<sup>Min/+</sup> mice. Hematocrits and the spleen index of Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice did not differ from Apc<sup>Min/+</sup> mice (Fig. 5).

We have previously shown that the increase in spleen weights in Apc<sup>Min/+</sup> mice is associated with increased splenic EMH, which progresses with increasing age and anemia (26). Apc<sup>Min/+</sup> mice at 10 weeks of age already have splenomegaly and EMH (Supplementary Table S1; Supplementary Fig. S2). Apc<sup>Min/+Per2<sup>−/−</sup></sup> mice, compared with Apc<sup>Min/+</sup> mice, show greater splenic EMH by qualitative histologic scoring (2.97 ± 0.20 versus 1.97 ± 0.21 score; P < 0.001), consistent with the increase in spleen weight. This EHM is composed of subcapsular infiltration of cells of heterogeneous morphology consisting of clusters of myeloid and erythroid precursors at various stages of maturation, along with an increase in megakaryocyte numbers (26). These cellular infiltrates fail to...
stain with B-cell (PAX-5) or pan T-cell (CD3) markers (data not shown). Apc<sup>Min/+</sup>Per2<sup>−/−</sup> and Apc<sup>Min/+</sup> mice also show a decrease in content of mature, nonnucleated erythrocytes in the spleen. Apc<sup>Min/+</sup>Per2<sup>−/−</sup> mice, heterozygous for the Per2 mutation, show similar EMH scores to Apc<sup>Min/+</sup> mice (2.10 ± 0.21 versus 1.97 ± 0.21 score; P = 0.28). These changes in spleen EMH and weights are not seen in Per2<sup>−/−</sup> or Per2<sup>+/+</sup> mice.

With increasing age, the hematocrits of Apc<sup>Min/+</sup>Per2<sup>−/−</sup> mice remain persistently lower than age-matched Apc<sup>Min/+</sup> mice (Supplementary Fig. S3; P < 0.01). Age-dependent progression of anemia in Apc<sup>Min/+</sup>Per2<sup>−/−</sup> mice fails to differ from that in Apc<sup>Min/+</sup> mice.

Discussion

We show that PER2 circadian core clock gene modulates β-catenin signaling in colon cancer cell lines, and other cell types, in vitro. We find that down-regulation of PER2 in cell lines increases β-catenin protein levels and the β-catenin target protein cyclin D and accelerates cancer cell proliferation. PER2-mediated effects apparently require increased β-catenin expression because down-regulation of β-catenin prevents the PER2-associated increase in cyclin D and acceleration of cancer cell proliferation. The effects of PER2 on β-catenin also occur in vivo. Per2<sup>−/−</sup> mice, with homozygous inactivation of Per2, show increased β-catenin and cyclin D protein levels in the mucosa of the small intestine and an increase in colonic polyp formation (number and frequency). Furthermore, we find that genetic inactivation of Per2 in vivo, by crossing Per2<sup>−/−</sup> mice with Apc<sup>Min/+</sup> mice, accelerates intestinal and colonic tumorigenesis, as well as the extraintestinal manifestations, in the Apc<sup>Min/+</sup> mouse model. The effects of PER2 on β-catenin are not restricted to intestinal cell lines and therefore may be relevant to tumorigenesis in other tissues.

We do not yet know how PER2 modulates β-catenin protein levels. PER2 protein is, however, known to function as a transcription factor, which dimerizes with other clock proteins (Cry1 and Cry2) that then modulates transcription through interactions with BMAL1/CLOCK protein heterodimers at E box sites. Both PER2 and β-catenin protein turnover and nuclear translocation are affected by their phosphorylation and ubiquitination (28-30).

Per2 has tumor suppressor properties in mice (12). Decreased PER2 RNA levels have been reported in some human acute leukemias (31). Intratumoral injection of Per2 slows murine tumor growth in vivo (32). PER2 overexpression in cancer cell lines in vitro inhibits growth and causes cell cycle arrest, increased apoptosis, and decreased clonogenicity, supporting the negative growth-regulatory properties of Per2 (31, 33). We show accelerated cell growth following PER2 down-regulation in vitro and increased intestinal and colonic tumorigenesis following Per2 homologous mutation in vivo, further supporting the role of Per2 as a tumor suppressor. We find in colon cancer lines, intestinal mucosa, and Apc<sup>Min+/-</sup> -mediated intestinal and colonic polyp formation that Per2 mediates these effects, at least in part, through modulation of β-catenin and β-catenin signaling. We do not yet know at what level or how Per2 and β-catenin pathways interact.

Per2 mutation can affect the expression of Per1, in addition to other clock genes (Cry1 and Bmal1). Period 1 (Per1) clock gene plays an analogous role in circadian time keeping to Per2, both acting in the negative feedback loop of the clock.

Although homozygous Per1 mutation has, however, not been associated with a tumor-prone or proliferative tissue phenotype in mice, decreased PER1 RNA levels have been reported in some lung, breast, and endometrial cancers compared with adjacent normal tissues (31, 34, 35). One study of human colorectal cancer reports decreased PER1 RNA levels in high-grade colon cancers compared with patient-matched normal tissues (36). In vitro PER1, similar to PER2, can act as negative growth regulator (31, 34). The time of day of tissue sampling has not been qualified or examined in these clinical studies of clock gene expression. This may be critical because the expression of these clock genes in normal tissues, and even in cancers, varies rhythmically throughout each day. Therefore, specifying the time of day of tissue sampling is essential to defining alterations in clock gene expression in preclinical and clinical tumor samples.

We conclude that the Per2 gene product suppresses intestinal and colonic tumorigenesis, in part, by down-regulation of β-catenin and β-catenin signaling pathways. Thereby, the Per2 gene product may be an effective and novel target for colon cancer prevention and/or control.

Materials and Methods

Tissue Culture

Human cancer cell lines HCT116, SW480 (colon), HeLa (cervical), and HEK293 (kidney; American Type Culture Collection) were cultured in RPMI 1640 containing 10% fetal bovine serum at 37°C with 5% CO₂.

siRNA Transfection

Cells were plated in six-well plates the day before to reach ~30% confluency by the time of transfection. Cells were washed with PBS and incubated in 1.5 mL of fresh medium with 10% fetal bovine serum but without antibiotics. siRNA oligo (100 pmol) diluted in 250 μL Opti-MEM (Invitrogen) was mixed with 5 μL of Lipofectamine 2000 diluted in 250 μL OptiMEM. The mixture was added drop wise to the cells after being incubated at room temperature for 15 to 30 min. The knocked down effect was examined 48 to 72 h later. The GFP control siRNA sequence is AAGGCAAGCUGACCCUGAdTT. The sequence of human PER2 siRNA is GCCGUUACCUCUGACCAUdTT. siRNA sequence against human β-catenin is GCCGGGGAUGCAAGCUUdTT.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Growth Assay

Seventy-two hours after siRNA treatment, cells were trypsinized and replated in 96-well plates. Cell growth was measured daily by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, after removing the medium, 100 μL of serum-free medium containing 1 mg/mL MTT (Sigma) were added and incubated at 37°C for 3 h. Then, 100 μL DMSO was added to each well to dissolve the precipitate after removal of MTT/medium. Absorbance was read at 595 nm by microplate reader (Bio-Rad).
Western Blots

 Cultured cells were washed with PBS and incubated in NP40 buffer [0.5% NP40, 150 mmol/L NaCl, 50 mmol/L Tris-Cl (pH 7.4) with protease inhibitors] to isolate total protein. Small intestinal mucosa was processed by mechanical homogenization in homogenization buffer [200 mmol/L Tris-HCl (pH 7.4), 20 mmol/L β-mercaptoethanol, 100 mmol/L NaF] with protease and phosphatase inhibitors to isolate soluble protein. Proteins were then separated by standard SDS-PAGE using Mini-gel system (Bio-Rad) and transferred to nitrocellulose membranes by semidy transfer apparatus (Bio-Rad). Membranes were incubated in blocking buffer (5% fat-free dry milk in TBS-Tween 20 buffer) at room temperature for 1 h. Membranes were then incubated with optimal diluted primary antibodies (1:200-1:5,000) overnight at 4°C. Membranes were washed with TBS-Tween 20 and then incubated in horseradish peroxidase–conjugated secondary antibodies for 1 h at room temperature. After being washed with TBS-Tween 20 thrice, membranes were incubated in TLC chemiluminescence reagent (Bio-Rad) and exposed to X-ray film. Anti-PER2 antibody was from Alpha Diagnostic International and antibodies against β-catenin, cyclin D1, c-MYC, α-tubulin, and glyceraldehyde-3-phosphate dehydrogenase were from Santa Cruz Biotechnology and anti-actin antibody was from Sigma. Films are scanned, bands are quantitated by densitometry (ImageJ software), and samples are expressed as the ratio to control protein for each sample and normalized to a standardized sample on each gel.

β-Catenin/TCF Transcriptional Assay

 Human HEK293 cells were used due to their high transfection rates for optimal signal intensity and assay. Cells were transfected with 0.5 μg of TOPFLASH-Luc and 0.1 μg of cytomegalovirus–β-galactosidase plasmid in combination of 0.5 μg of pcDNA, pcDNA-Per2, 100 pmol of control siRNA, or 100 pmol of PER2 siRNA. Forty-eight hours after transfection, cells were lysed by reporter assay buffer (Promega). Luciferase activities and β-galactosidase activities were determined by Luciferase Assay System and Beta-Glo Assay System (Promega), respectively, according to the manufacturer’s protocol. The ratio of TOPFLASH to β-galactosidase activity represented β-catenin/TCF activity (37).

Animals

 ApcMin/+ mice in the C57BL/6J background (25) were maintained by breeding male mice heterozygous for the Min allele to wild-type C57BL/6J females and care was in accordance with institutional guidelines (38). ApcMin/+ mice were maintained as heterozygotes, as homozygosity in the Min allele is embryonic lethal. Homozygous Per2m/mice (Per2m/m; The Jackson Laboratory) in the C57BL/6 background were crossed to ApcMin/+ mice to generate ApcMin/+ mice with a homozygous mutation of the Per2 gene. The presence of the Min allele and the mutation of the Per2 gene were verified by PCR genotyping using Min- and Per2-specific primers, respectively (The Jackson Laboratory procedures) using genomic DNA from tail snips or ear punches as templates. Both strains were originally purchased from The Jackson Laboratory but were maintained as breeding colonies at the Mouse Core Facility of the Center of Colon Cancer Research of the University of South Carolina Center. The mice were housed four to five per cage, provided free access to food and water, and maintained on a 12-h light/12-h dark schedule. All animal experiments and procedures were approved by the University of South Carolina Center and Veterans Affairs Institutional Animal Care and Use Committee. Equal numbers of male and female 10-wk-old mice of six different genotypes (C57BL/6 wild-type, Per2m/m, ApcMin/+ Per2m/m, and ApcMin/+ Per2m/m) were humanely euthanized for tissue procurement (n = 20-39 mice/genotype). All genotypes were confirmed on sacrifice in the Veterans Affairs Laboratory. Complete blood parameters of peripheral blood from retro-orbital sinus, before sacrifice, were determined using an automated blood cell analyzer (VetScan) at the Mouse Core Facility. Body and spleen weights were recorded and the spleen index was calculated (spleen weight/body weight × 100). For polyp enumeration, small intestines (divided into four segments) and colons (single segment) were removed, flushed with PBS, dissected longitudinally, flattened, and fixed in 10% buffered formalin overnight. Segments were stained with 0.5% methylene blue in saline. Intestinal and colonic polyps were enumerated under a dissecting microscope and small intestinal polyps were classified as small (<1 mm) or large (>1 mm). Serial complete blood parameters were recorded every 2 wk in mice 15 to 23 wk of age (n = 16-27/genotype). For small intestinal mucosal protein studies, 15-wk-old wild-type and Per2m/m female mice (n = 5/genotype) were euthanized in the middle of the activity/dark phase. Small intestines were harvested and rapidly flushed with PBS. The first two thirds of the intestine was opened longitudinally and mucosa was separated by mechanical dislodgement (glass slide) and stored at −80°C.

Tissue Histology Assessments

 Spleens (10-wk-old mice) were fixed in buffered formalin and embedded in paraffin blocks. Sections (5 μm) were cut and histologically assessed on H&E-stained slides. Sections mounted on positively charged slides were stained with antibodies against CD3 pan T cell (Vector Laboratories) and PAX-5 pan B cell (Biocare Medical) and visualized as previously described (2, 5). Slides were examined in an Axioskop (Zeiss) compound microscope and representative areas were captured with a Zeiss Axiocam digital camera using Axiosvision 4.5 software. Spleens were scored for EMH that we previously reported as characteristic of ApcMin/+ mice (26). This subcapsular infiltrative process is characterized by cells that lack CD3 and PAX-5 staining, which are morphologically heterogeneous and consistent with hematopoietic precursors along with increased numbers of scattered megakaryocytes. EMH was scored by depth of infiltration from the splenic capsule (mild, moderate, severe) and extent (focal, diffuse) of involvement of the spleen (0 = none; 1 = mild, focal; 2 = moderate, focal; 3 = moderate, diffuse; 4 = severe, diffuse).

The distal third of the intestine was opened longitudinally and rolled into a Swiss roll, formalin fixed, and paraffin embedded and 5-μm sections were cut and histologically assessed on H&E-stained slides. Microadenomas were counted and dysplasia (low grade versus high grade) was graded.
Statistical Analysis

For each numerical value, mean and SEs were calculated and graphed. Average and standard values were computed across gene mutations and manipulations. Mean comparisons across gene mutations/alterations were assessed through ANOVA, Kruskal-Wallis test, or t test among two genotypes. Repeated measures analysis of hematocrit levels at 10, 15, 18, and 21 wk of age were compared across gene mutations using Proc Mixed in SAS. Significant differences are set at α = 0.05. All statistical tests were two sided.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest.

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References

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