Period 2 Mutation Accelerates *Apc*$^{Min/+}$ Tumorigenesis

Patricia A. Wood,[1,2,4,8] Xiaoming Yang,[1,8] Andrew Taber,[1] Eun-Young Oh,[1] Christine Ansell,[1] Stacy E. Ayers,[1] Ziad Al-Assaad,[3] Kevin Carnevale,[4] Franklin G. Berger,[6,8] Maria Marjorette O. Peña,[6,8] and William J.M. Hrushesky[1,2,5,7,8]

[1]Medical Chronobiology Laboratory, Dorn Research Institute, [2]Department of Oncology and Hematology, and [3]Department of Pathology, WJB Dorn Veterans Affairs Medical Center; Departments of [4]Pathology, Microbiology, and Immunology and [5]Cell Developmental Biology and Anatomy, School of Medicine, [6]Department of Biological Sciences, College of Arts and Sciences and [7]Department of Epidemiology and Biostatistics, Arnold School of Public Health, and [8]Center for Colon Cancer Research, University of South Carolina, Columbia, South Carolina

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 β-catenin were studied in colon cancer cell lines by its down-regulation following RNA interference. The effects of *Per2* inactivation *in vivo* on β-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 β-catenin, cyclin D, and cell proliferation. Down-regulation of β-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 β-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 β-catenin and β-catenin target genes, and this circadian core clock gene may represent a novel target for colorectal cancer prevention and control.

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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). Individual cells within tissues and even cancer cells have circadian clocks composed of the same set of clock genes (2, 5). 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 γ-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

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Requests for reprints: Patricia A. Wood, Medical Chronobiology Laboratory, WJB Dorn Veterans Affairs Medical Center, Building 9, 6439 Garners Ferry Road, Columbia, SC 29209. Phone: 803-776-4000, ext. 5599; Fax: 803-647-5656. E-mail: patricia.wood2@va.gov

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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; $Apc^{Min/+}$) 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

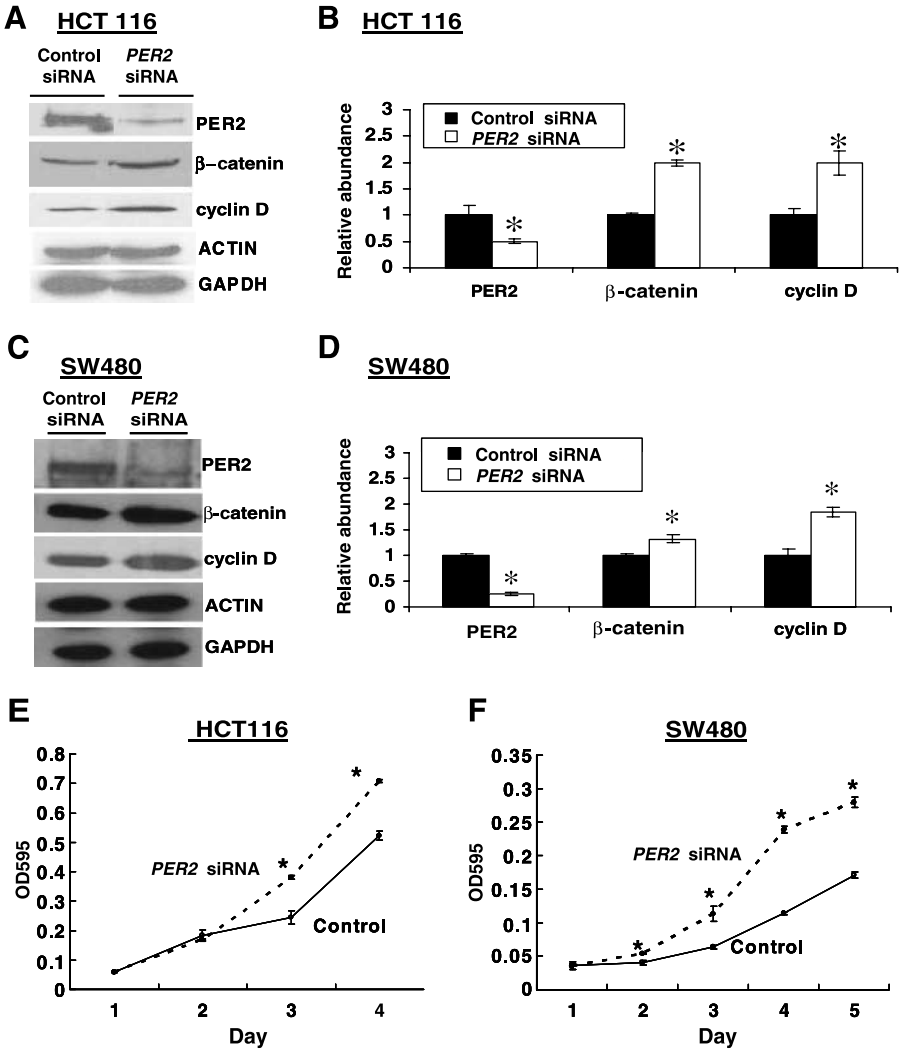

FIGURE 1. Down-regulation of *PER2* by siRNA *in vitro* increases β-catenin and cyclin D and accelerates cell growth in colon cancer cell lines compared with control siRNA cultures. Representative Western blots (**A** and **C**) and quantitation of protein abundance (**B** and **D**), relative to actin 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.

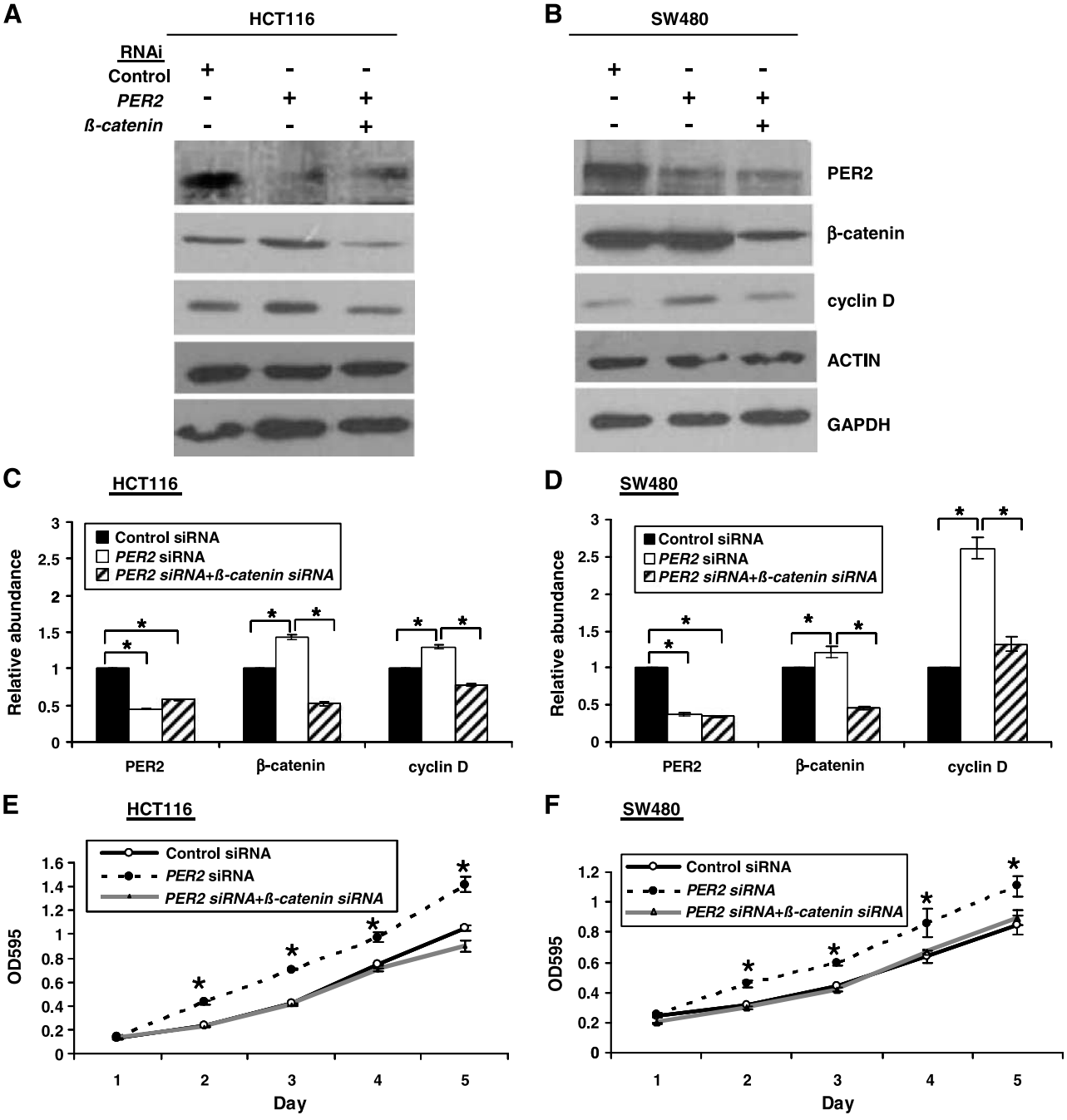

FIGURE 2. Increase in cyclin D and acceleration of cell growth caused by *PER2* down-regulation is prevented when β-catenin expression is down-regulated by siRNA *in vitro* compared with control siRNA cultures. Representative Western blots (**A** and **B**) are shown with the siRNA treatment listed at 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.

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

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 overexpression, 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 $Per2^{m/m}$ 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 $Per2^{m/m}$ 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 $Per2^{m/m}$ mice is also associated with an increase in cyclin D protein ($P < 0.05$) compared with wild-type mucosa (Fig. 4A and B).

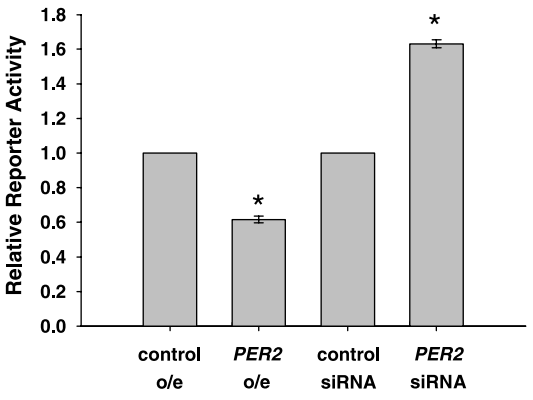

FIGURE 3. β-Catenin transcriptional activity by TOPFLASH assay in human HEK293 kidney cells. *PER2* overexpression (*o/e*) decreased and *PER2* down-regulation (*siRNA*) increased β-catenin/TCF transcriptional activity compared with control cultures (equal to one). Means of four to five experiments are shown. *, $P < 0.05$.

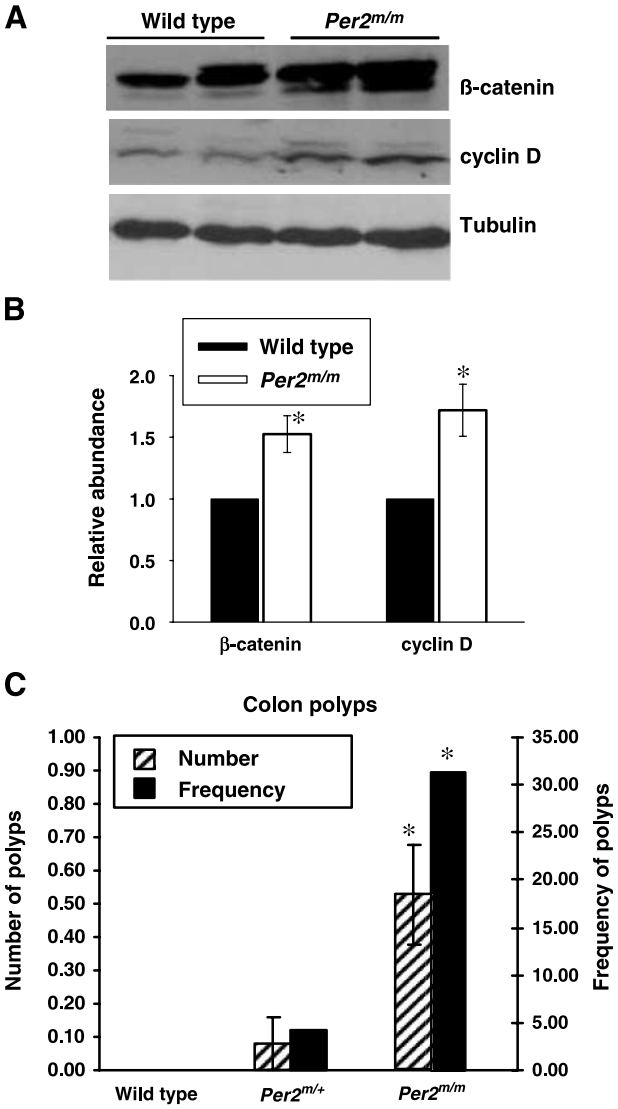

FIGURE 4. β-Catenin and cyclin D levels in small intestinal mucosa and colon polyps are increased in $Per2^{m/m}$ mice compared with wild-type mice. Small intestinal mucosa from 15-wk-old wild-type and $Per2^{m/m}$ mice was isolated during the mid-activity phase and homogenized for protein analysis. 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 $Per2^{m/m}$ mice. **C.** Colon polyp numbers and frequency in 15-wk-old wild-type, $Per2^{m/+}$, and $Per2^{m/m}$ mice. *, $P < 0.05$, for $Per2^{m/m}$ compared with wild-type.

Because increased β-catenin expression is often associated with polyp formation, we examined $Per2^{m/m}$ mice for intestinal polyps. Fifteen-week-old $Per2^{m/m}$ mice show an increase in the numbers of colonic polyps compared with wild-type mice [$Per2^{m/m}$, 0.6 ± 0.16 (range, 0-2/mouse) versus 0 polyps in wild-type mice; $P = 0.012$; $n = 18$-32 mice/genotype], which occurs at a frequency of 31.3% ($Per2^{m/m}$, 10/32 versus wild-type 0/18 affected mice/total mice; $P = 0.008$; Fig. 4C). Colon polyp numbers and frequency are not increased in $Per2^{m/+}$ heterozygous mice. Small intestinal polyps were not seen in 15-week-old wild-type, $Per2^{m/+}$, or $Per2^{m/m}$ mice.

Per2 *Mutation Increases* Apc$^{Min/+}$ *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 $Per2^{m/m}$ mice with $Apc^{Min/+}$ 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 $Apc^{Min/+}$ mice with mice homozygous ($Apc^{Min/+}Per2^{m/m}$) and heterozygous ($Apc^{Min/+}Per2^{m/+}$) for the *Per2* mutation. Compared with age-matched 10-week-old $Apc^{Min/+}$ mice, $Apc^{Min/+}Per2^{m/m}$ 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 $Apc^{Min/+}Per2^{m/m}$ mice compared with $Apc^{Min/+}$ 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; $P = 0.122$). $Apc^{Min/+}Per2^{m/m}$ 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 $Apc^{Min/+}$ mice (Fig. 4B).

The number of total colonic polyps is also 2- to 3-fold higher in $Apc^{Min/+}Per2^{m/m}$ mice compared with $Apc^{Min/+}$ mice (4.2 ± 1.3 versus 1.6 ± 0.3; $P = 0.025$; Fig. 4C). These effects require $Per2^{m/m}$ homozygosity. $Apc^{Min/+}Per2^{m/+}$ 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 $Apc^{Min/+}$ mice. Wild-type, $Per2^{m/+}$, and $Per2^{m/m}$ 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 $Apc^{Min/+}Per2^{m/m}$ mice than in $Apc^{Min/+}$ mice (88.9% versus 42.3%; $P < 0.001$). The majority of these microadenomas in $Apc^{Min/+}$ and $Apc^{Min/+}Per2^{m/m}$ mice show low-grade dysplasia. Severe dysplasia/carcinoma *in situ* is infrequent in both $Apc^{Min/+}$ and $Apc^{Min/+}Per2^{m/m}$ mice and did not differ significantly (2 of 26 versus 4 of 27 mice; $P = 0.42$).

Per2 *Mutation Increases* Apc$^{Min/+}$ *Extraintestinal Manifestations*

The extraintestinal changes seen in the $Apc^{Min/+}$ mouse include progressive anemia, splenomegaly, and extramedullary hematopoiesis (EMH; ref. 26). At 10 weeks of age, $Apc^{Min/+}$ 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 $Apc^{Min/+}Per2^{m/m}$ 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 $Apc^{Min/+}$ mice. Hematocrits and the spleen index of $Apc^{Min/+}Per2^{m/+}$ mice did not differ from $Apc^{Min/+}$ mice Fig. 5.

We have previously shown that the increase in spleen weights in $Apc^{Min/+}$ mice is associated with increased splenic EMH, which progresses with increasing age and anemia (26). $Apc^{Min/+}$ mice at 10 weeks of age already have splenomegaly and EMH (Supplementary Table S1; Supplementary Fig. S2). $Apc^{Min/+}Per2^{m/m}$ mice, compared with $Apc^{Min/+}$ 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 EMH 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

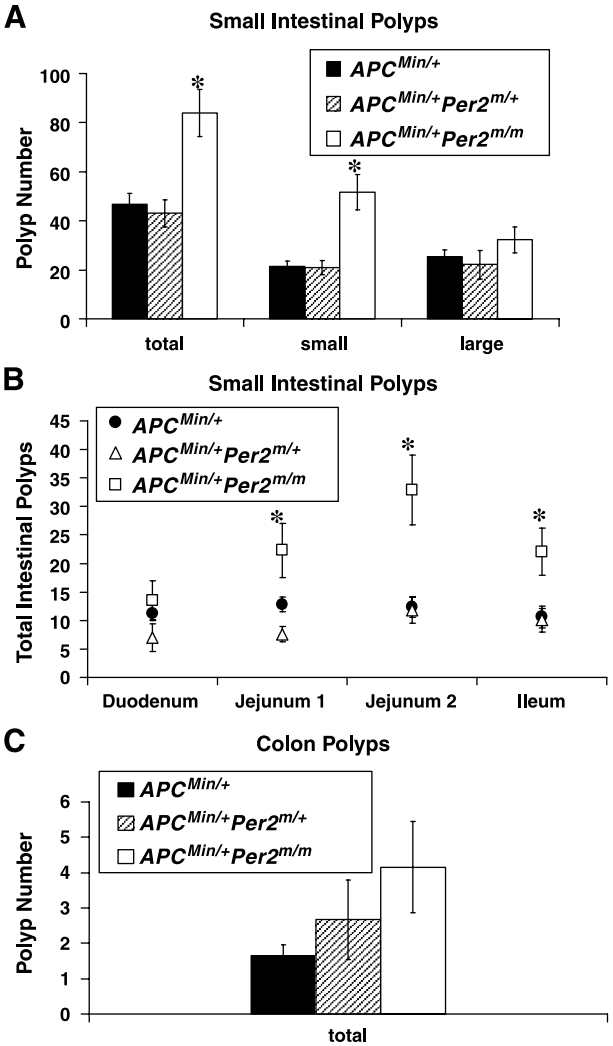

FIGURE 5. Homozygous *Per2* inactivation increases small intestinal polyp and colonic polyp numbers in 10-wk-old $Apc^{Min/+}$ mice. **A.** Total and small-sized (<1 mm) small intestinal polyps, but not large-sized (>1 mm) small intestinal polyps, are higher in $Apc^{Min/+}Per2^{m/m}$ mice compared with $Apc^{Min/+}$ mice. *, $P < 0.001$. **B.** Distribution of total small intestinal polyps throughout the proximal (duodenum), first and second jejunum, and distal (ileum) segments of the small intestine shows higher numbers in $Apc^{Min/+}Per2^{m/m}$ compared with $Apc^{Min/+}$ mice in the second, third, and fourth segments of the intestine. *, $P < 0.01$. **C.** Total colonic polyp numbers are higher in $Apc^{Min/+}Per2^{m/m}$ compared with $Apc^{Min/+}$ mice ($P = 0.025$). Small intestinal polyp numbers and distribution, and colonic polyp numbers did not differ between $Apc^{Min/+}Per2^{m/+}$ and $Apc^{Min/+}$ mice. Columns, mean; bars, SE.

stain with B-cell (PAX-5) or pan T-cell (CD3) markers (data not shown). $Apc^{Min/+}Per2^{m/m}$ and $Apc^{Min/+}$ mice also show a decrease in content of mature, nonnucleated erythrocytes in the spleen. $Apc^{Min/+}Per2^{m/+}$ mice, heterozygous for the *Per2* mutation, show similar EMH scores to $Apc^{Min/+}$ 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 $Per2^{m/m}$ or $Per2^{m/+}$ mice.

With increasing age, the hematocrits of $Apc^{Min/+}Per2^{m/m}$ mice remain persistently lower than age-matched $Apc^{Min/+}$ mice (Supplementary Fig. S3; $P < 0.01$). Age-dependent progression of anemia in $Apc^{Min/+}Per2^{m/+}$ mice fails to differ from that in $Apc^{Min/+}$ 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*. $Per2^{m/m}$ 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 $Per2^{m/m}$ mice with $Apc^{Min/+}$ mice, accelerates intestinal and colonic tumorigenesis, as well as the extraintestinal manifestations, in the $Apc^{Min/+}$ 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* homozygous mutation *in vivo*, further supporting the role of *Per2* as a tumor suppressor. We find in colon cancer lines, intestinal mucosa, and $Apc^{Min/+}$-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% CO_2.

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 Lipofectmine 2000 diluted in 250 μL Opti-MEM. 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 AAGGCAAGCUGACCCUGAAdTT. The sequence of human *PER2* siRNA is GCGUUACCUCUGAG-CACAUdTT. siRNA sequence against human β-catenin is GCUGGUGGAAUGCAAGCUUdTT.

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 total soluble protein. Proteins were then separated by standard SDS-PAGE using Mini-gel system (Bio-Rad) and transferred to nitrocellulose membranes by semidry 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

$Apc^{Min/+}$ 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). $Apc^{Min/+}$ mice were maintained as heterozygotes, as homozygosity in the *Min* allele is embryonic lethal. Homozygous $Per2^{m/m}$ mice ($Per2^{tm1/Brd}/J$; The Jackson Laboratory) in the C57BL/6 background were crossed to $Apc^{Min/+}$ mice to generate $Apc^{Min/+}$ 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, $Per2^{m/+}$, $Per2^{m/m}$, $Apc^{Min/+}$, $Apc^{Min/+}$ $Per2^{m/+}$, and $Apc^{Min/+}$ $Per2^{m/m}$) 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 $Per2^{m/m}$ 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 Axiovision 4.5 software. Spleens were scored for EMH that we previously reported as characteristic of $Apc^{Min/+}$ 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 $\alpha = 0.05$. All statistical tests were two sided.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest.

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