Robust circadian rhythms in organoid cultures from PERIOD2::LUCIFERASE mouse small intestine

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Abbreviations: Per2, PERIOD2; MSIE, mouse small intestinal epithelial cells; Caco-2, human colorectal adenocarcinoma cells; LUC, luciferase; EGF, epidermal growth factor

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SUMMARY
Disruption of circadian rhythms is a risk factor for several human gastrointestinal (GI) diseases, ranging from diarrhea to ulcers to cancer. 4-dimensional tissue culture models that faithfully mimic the circadian clock of the GI epithelium would provide an invaluable tool to understand circadian regulation of GI health and disease. We hypothesized that rhythmicity of a key circadian component, PERIOD2 (PER2), would diminish along a continuum from ex vivo intestine, organoids (epithelial “miniguts”), and nontransformed (MSIE) and transformed (Caco-2) intestinal epithelial cells. Here we show that bioluminescent jejunal explants from PERIOD2::LUCIFERASE (PER2::LUC) mice display robust circadian rhythms for >72 hours post-excision. Circadian rhythms in primary or passaged PER2::LUC jejunal organoids are similarly robust, synchronize with serum shock, and persist beyond 2 weeks in culture. Remarkably, unshocked organoids autonomously synchronize rhythms within 12 hours of recording. The onset of this autonomous synchronization is slowed by >2 hours in the presence of glucocorticoid receptor antagonist RU486 (20 μM). Doubling standard concentrations of organoid growth factors EGF, Noggin, and R-spondin enhances PER2 oscillations, whereas subtraction of these factors individually at 24 hours following serum shock produced no detectable effects. Growth factor pulses induce modest phase delays in unshocked, but not serum-shocked, organoids. Circadian oscillations of PER2::LUC bioluminescence align with \textit{Per2} mRNA expression by qPCR. Concordant findings of robust circadian rhythms in bioluminescent jejunal explants and organoids provide further evidence for a peripheral clock intrinsic to the intestinal epithelium. The rhythmic and organotypic features of organoids should offer unprecedented advantages as a resource for elucidating the role of circadian rhythms in GI stem cell dynamics, epithelial homeostasis, and disease.
INTRODUCTION

Circadian rhythms are biological processes with ~24-hour oscillations that regulate diverse life functions across a wide spectrum of organisms (Panda et al., 2002). These processes include the digestive, immune, and regenerative functions of the gastrointestinal (GI) epithelium necessary for organismal growth and survival (Potten et al., 1977; Hoogerwerf, 2006; Hussain and Pan, 2009; Karpowicz et al., 2013; Yu et al. 2013). In humans, disruption of circadian rhythms is increasingly common and associated with a variety of GI diseases ranging from diarrhea to ulcers to cancer (Savarino et al., 1996; Hoogerwerf, 2009; Xu et al., 2013). Progress in understanding the mechanisms by which circadian rhythms influence GI health and disease has lagged, in part, due to a paucity of in vitro models with appropriate phenotypes and robust circadian rhythms (Hughes et al., 2009). For example, Caco-2 cells are a commonly studied human colorectal adenocarcinoma cell line recently shown to exhibit circadian oscillations of circadian component Per2 (Ballesta et al., 2011; Swanson et al., 2011); however, Caco-2 cells’ limited capacity for differentiation and dampened circadian oscillations over time may hinder extrapolation of in vitro results to functional GI tissue (Hughes et al., 2011). To our knowledge, a tissue culture system that overcomes these limitations has yet to be described.

Intestinal organoids have recently emerged as a powerful platform for understanding adult stem cell dynamics, intestinal epithelial homeostasis, and gut pathophysiology (Sato et al., 2009; reviewed by Sato and Clevers, 2013). Mouse intestinal organoids arise from Lgr5+ intestinal stem cells that differentiate into all lineages present in the epithelium and, strikingly, self-organize into 3-dimensional structures with crypt-, villous- and lumen-like domains (Sato et al., 2009). We hypothesized that rhythmicity of a known circadian component in the small intestine, PERIOD2 (PER2), would diminish in amplitude along a continuum from mouse small intestinal explants, intestinal organoids, a nontransformed mouse small intestinal epithelial (MSIE) cell line (Whitehead et al., 1993), and transformed human colorectal adenocarcinoma (Caco-2) cells. Here
we show that: 1) Jejunal explants and organoids derived from PERIOD2::LUCIFERASE (PER2::LUC) mice (Yoo et al., 2004) display robust circadian rhythms, 2) PER2::LUC organoids synchronize clock in response to serum shock and unshocked organoids autonomously synchronize clock within 12 hours of monitoring, and 3) organoid growth factors and Matrigel™ enhance circadian rhythms.

RESULTS

In initial experiments, jejunal explants from PER2::LUC mice displayed 1.5- to 2-fold circadian oscillations of PER2 protein abundance that persisted for up to ~84 hours (Figure 1), with a notable lengthening of period over time. We next generated organoids derived from the jejunal crypts of PER2::LUC mice. Figures 2A-C show a representative 3-dimensional confocal image reconstruction of a jejunal PER2::LUC organoid 42 hours following serum shock, with PER2 and LUC fluorescent antibody staining throughout all crypt- and villous-like domains. The single plane confocal images in Figures 2D-K show increased PER2 and LUC staining at 42 hours vs. 28 hour following serum shock, indicating a circadian variation in the expression of these proteins. Separately, incubating luminometer real-time measurements of PER2 abundance in PER2::LUC jejunal organoids demonstrated 1.5- to 3-fold circadian oscillations of PER2 abundance for up to 96 hours following serum shock (Figure 3A). In addition to a longer duration of detectable PER2 rhythms, period lengthening over time appeared less pronounced in organoids versus explants.

Mammalian cell lines derived from peripheral tissues such as the gut require an extrinsic cue such as serum shock or steroid pulse to synchronize circadian rhythms (Balsalobre et al., 1998). We compared PER2 oscillations in serum-shocked and unshocked organoids. We found that serum shocked organoids displayed synchronized PER2 oscillations immediately upon initiation of bioluminescent recordings (Figure 3A). Strikingly, unshocked organoids displayed modest PER2
rhythms during the first 8 to 12 hours of recording then spontaneously developed robust PER2 oscillations out of phase with shocked controls (Figure 3A). Steroid receptor antagonist RU486 has been shown to disrupt circadian rhythms in mesenchymal stem cells (So et al., 2009), therefore we tested RU486 effects on organoid PER2 rhythms in the absence of serum shock. Pretreatment of organoids with RU486 (20 μM) significantly delayed the time to first peak of PER2 abundance by 2 hours (\(P<0.0001\); Figures 3B and 3C). Separately, PER2::LUC organoids suspended in media without Matrigel™ showed dramatic reductions in the amplitude and persistence of PER2 oscillations, but maintained circadian rhythms both in the presence and absence of serum shock (Figure 3D). Furthermore, by refreshing organoid media and adding luciferin every 5 days (arrows, Figure 3E), we were able to extend the duration of detectable circadian PER2 oscillations up to 15 days, with a reset of PER2 oscillations following the second media change at day 10.

To optimize conditions favorable for circadian rhythms in organoids, we doubled standard concentrations of EGF, Noggin, and R-spondin in organoid media and found a resulting increase in the amplitude of PER2 oscillations following serum shock (Figure 4A). To test the effects of depletion of individual factors on organoid circadian rhythms, while maintaining organoid viability, we individually subtracting these three growth factors from organoid media at 24 hours following serum shock. We detected no significant differences in either the amplitude or duration of PER2 oscillations (Figure 4B).

Under typical culture conditions, R-spondin removal from organoid growth media induces growth arrest and cell death within 2 to 4 days. We therefore compared the growth of passaged organoids over 5 days under the following conditions: a) standard Sato media following serum shock, b) Sato media with R-spondin removal at 24 hours following serum shock (i.e., the R-spondin-negative experimental condition in Figure 4B), and c) Sato media with R-spondin removal
immediately following serum shock. Organoids maintained in R-spondin-free media immediately following serum shock showed marked reductions in both size and numbers relative to controls by Day 3, indicating growth arrest and death. In contrast, organoids maintained in Sato media with R-spondin removal at 24 hours following serum shock remained similar in size and numbers to controls at Day 5, but exhibited an atypical lengthening of crypt-like domains. When organoids from all 3 conditions were passaged into standard minigut media at the end of Day 5, only control organoids continuously maintained in R-spondin (top panels Figure 4C) prior to passaging formed new organoids. Together, these results suggest that the R-spondin-negative organoids used in Figure 4B were sufficiently viable to allow continued measurements of circadian rhythms, but lacked the regenerative capacity to form new organoids upon passaging.

As noted above, a media change at day 10 of bioluminescent recordings reset PER2 oscillations (Figure 3E), therefore we tested whether a combined pulse of growth factors EGF, Noggin, and R-spondin alters PER2 oscillations at different phases of clock. In serum-shocked organoids (Figures 5A and 5B), growth factor pulses at circadian times (CT) 38 hours and 50 hours did not perturb PER2 oscillations. In contrast, unshocked organoids (Figures 5C and 5D) pulsed with growth factors at CT 36 and 48 hours showed modest phases delays in PER2 oscillations relative to controls, more so following the pulse at CT 48 hours.

To compare circadian rhythms of Per2 gene expression in PER2::LUC organoids, MSIE cells, and Caco-2 cells, we performed qPCR over 48 hours at 4-hour time resolution under standard culture conditions. Serum-shocked PER2::LUC organoids exhibited synchronized circadian oscillations of Per2 gene expression (Figure 6A) over 48 hours. Serum-shocked MSIE and Caco-2 cells exhibited less consistent circadian oscillations of Per2 over 48 hours (Figure 6B and 6C, respectively) relative to organoids.
DISCUSSION

To our knowledge, this study is the first to demonstrate robust oscillations of a core circadian clock component in small intestine explants and organoid preparations, providing additional evidence for a self-regulating, peripheral clock intrinsic to the intestinal epithelium. By identifying a novel organotypic feature of intestinal organoids, this study also provides a new resource to elucidate the role of circadian rhythms in adult stem cell dynamics, intestinal epithelial homeostasis, and gut pathophysiology. As such, bioluminescent organoid platforms offer several potential advantages over 2D intestinal cell culture. First, organoids are a non-transformed cell culture system and retain more in vivo characteristics, including the capacity to differentiate and organize into crypt- and villous-like domains. In addition, the simultaneous presence of intestinal stem cells and their progeny within the organized 3-dimensional structure of the organoids likely provides a more accurate model of the cell-cell contact and communication that coordinate circadian behaviors of the intestinal epithelium in vivo. Finally, luciferase bioluminescent organoids allow real-time measurement of PER2 abundance at high time-density sampling (every 10 minutes). Although highly sensitive and specific, qPCR of homogenates from repeated harvests of PER2::LUC organoids, MSIE and Caco-2 cells is less efficient in comparison for experiments requiring fine time resolution measurements over several days.

We observed a lengthening of PER2 periods over 72 hours of bioluminescent recording in jejunal explants. Period lengthening has previously been demonstrated in PER2::LUC colon explants and is likely due to a compromised tissue viability ex vivo (Malloy et al., 2012). In contrast, jejunal organoids appeared to maintain a ~24-hour period for up to 96 hours of bioluminescent recording, with no change in media.

Our finding that PER2::LUCIFERASE organoids autonomously synchronize circadian rhythms is...
surprising, but correlates well with our finding of PER2 oscillations in unshocked jejunal explants. This distinguishes the circadian behavior of organoids from transformed mammalian cell lines such as NIH 3T3 cells, which require a serum shock to synchronize (Nagoshi et al., 2004). The organoid cultures we studied contained multiple organoids, suggesting that not only do individual organoids self-synchronize, but that populations of organoids within culture achieve synchrony as well. Matrigel™, although not required for organoid synchronization, clearly strengthens the amplitude of PER2 rhythms, perhaps via synchronizing agents contained in Matrigel™ yet to be identified, or by promoting organoid stability and cell-cell communication. Present within organoids, enteroendocrine cells represent a potential source of secreted factors that might entrain organoid rhythms. Enteroendocrine cells produce a variety of gut hormones, including vasoactive intestinal peptide, which has been shown to synchronize clock in neurons of the suprachiasmatic nucleus (Aton et al., 2005). The extent to which enteroendocrine cells or other epithelial cell lineages entrain gut epithelial clocks in organoids and in vivo is unknown and will be an important question for future research.

Interestingly, a doubling of the concentration of growth factors required for organoid culture (EGF, Noggin, and R-spondin) enhanced the amplitude of PER2 rhythms (perhaps by increasing organoid size and numbers); however, these growth factors were not individually required for maintenance of PER2 rhythms once oscillations were established. We speculate that endogenous production of growth factors following their removal from media 24 hours following serum shock may be sufficient to maintain short-term viability and circadian rhythms in established organoid cultures. Our finding that growth factor pulses delay the phase of autonomously synchronized organoids is intriguing. In vivo, Paneth cells provide these growth factors, thereby constituting the intestinal stem niche (Sato et al., 2011). Well-described circadian rhythms of mouse crypt base columnar stem cell proliferation (Potten et al., 1977) suggests that the cellular dynamics of such growth factors is almost certainly under circadian regulation and should thus be further
explored.

The close correlation of PER2 oscillations in jejunal explants and organoids emphasizes potential advantages of intestinal organoids as a relevant experimental platform for understanding temporal regulation of intestinal epithelial homeostasis and stem cell dynamics and differentiation in mammals. In *Drosophila*, Karpowicz and colleagues have recently reported that intestinal stem cell regeneration is rhythmic and regulated by the circadian clock and that clock mutations impair the regenerative capacity of intestinal stem cells (Karpowicz et al., 2013). Work is underway to confirm these findings in mice and we propose that intestinal organoids will provide an ideal 4-dimensional tissue culture system in which to study these phenomena. The circadian properties of organoids will also be relevant to exploring recently uncovered connections between gut microbiota, circadian rhythms, and epithelial homeostasis in isolated intestinal epithelial cells (Mukherji et al., 2013). Finally, recent breakthroughs with the development of intestinal organoids derived from human stem cells and gut tissue should accelerate translation of these findings to improvements in human health (Spence et al., 2011).

**MATERIALS AND METHODS**

*Intestinal explants and organoids*

We obtained PERIOD2::LUCIFERASE (PER2::LUC) mice on a C57Bl/6J background from Jackson Laboratories (Bar Harbor, ME, USA; Yoo et al. 2004). PER2::LUC mice and wild type C57Bl/6j mice were housed in a barrier facility with an ambient temperature of 22°C, a relative humidity ranging from 30 to 70%, and a 14:10-h light-dark cycle. For ex vivo explant experiments, PER2::LUC mice were sacrificed 3 hours after lights on using CO2 inhalation followed by cervical dislocation according to rules and regulations of the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center. The jejunum was harvested and flushed with cold Kreb’s solution before being cut into 3-5mm segments. Tissue
was splayed open and placed lumen side up onto a Millicell™ cell culture insert (EMD Millipore Corporation, Billerica, MA, USA) in a 35mm dish. Within 30 minutes of sacrificing mice, explants were placed in a Kronos Dio™ AB-2550 incubating luminometer (ATTO Corporation, Tokyo, Japan) with DMEM and 200µM Beetle Luciferin Potassium Salt (Promega, Fitchburg, WI, USA) for real-time periodic quantification of PER2 protein abundance by bioluminescence recording (Malloy et al., 2012).

Using the methods of Sato and Clevers (Sato et al., 2009), we prepared intestinal organoids (enteroids) by isolating fresh mid-jejunal crypts from PER2::LUC mice. We sacrificed animals using CO2 inhalation followed by cervical dislocation. Approximately 6 cm of jejunum was dissected from the mouse, flushed with ice cold Phosphate Buffered Saline (PBS), and splayed open with scissors. The jejunal segment was then sliced into 1-cm pieces and transferred to 5 mL of cold PBS on ice. This suspension was placed on a rocking table at 4°C for 5 minutes to remove residual blood or stool from jejunal segments. After rocking, PBS was aspirated and 5 mL of 2mM EDTA chelation buffer was added. The suspension was placed back on a rocking table at 4°C for 30 minutes. Chelation buffer was then removed and 5 mL of shaking buffer (PBS: 43.3mM Sucrose: 54.9mM Sorbitol) was added. Conical tube was gently shaken by hand for 2 minutes. A sample of the crypt suspension was visualized by microscope to ensure crypts had released. If crypts were still attached, samples were gently shaken for an additional 30 seconds to 2 minutes. The intestinal crypt suspension was filtered through a 70 µm cell strainer into a 50 mL conical tube. The filter was rinsed with 5mL of cold shaking buffer. Samples were visualized again and a portion of the suspension was centrifuged for 10 minutes at 4°C. Supernatant was gently poured off ensuring all excess liquid was removed from the tube. Intestinal crypts were resuspended in Matrigel™ (BD Biosciences, San Jose, CA, USA) plus the growth factors R-spondin, mNoggin, and mEGF (R&D Systems, Minneapolis, MN, USA) then plated onto tissue culture plates. The Matrigel™ suspension was allowed to polymerize at 37°C between 15 minutes
and 1 hour before fresh minigut media was supplied. Minigut media plus growth factors was replaced every 3-4 days.

Organoids were maintained in culture for approximately 6-9 days before being propagated into additional wells or used for experimental procedures. Organoids were passaged by rinsing with ice cold PBS, then aspirating and adding additional cold PBS and using a P1000 pipette tip to scrape the Matrigel™ from the vessel surface and break apart organoids. Intestinal organoid-Matrigel™ suspensions were pooled, spun down at 4°C for 10 minutes at 150xg. Organoids were resuspended in 100-250 mL of cold PBS and passed through a syringe needle several times to further break apart crypts.

Organoids were then recentrifuged at 150xg to pellet the suspension. Matrigel™ with growth factors was used to resuspend the crypts, which were then relayed onto tissue culture plates.

Quantitative real-time PCR

PER2::LUC organoids were plated into 12-well plates at the start of the experiment. Each time point was plated into a separate plate to limit manipulation or exposure to possible resetting cues. End of serum shock is indicated by circadian time 0 to compare with bioluminescent recordings done in parallel. RNA was harvested at 4-hour time resolution over 48 hours using 0.25mL/well of TRI Reagent™ RT (Molecular Research Center, Inc., Cincinnati, OH, USA). Suspensions were passed through a syringe needle several times to break up the Matrigel™ and organoids. Samples were immediately stored at -80°C until all time points were collected and could be processed. Total RNA was treated with RQ1 DNaseI (Promega, Fitchburg, WI, USA). 1 μg of total RNA was used for reverse transcription reaction with GoScript Reverse Transcriptase (Promega) according to manufacturer’s instructions. PCR was performed with SYBR Green Master Mix (Qiagen, Germantown, MD, USA). Real-time PCR results were detected with
StepOnePlus System (Life Technologies, Grand Island, NY, USA).

**Bioluminescent recordings**

For Kronos Dio™ incubating luminometer experiments, PER2::LUC organoids were plated into 35mm dishes, allowed to polymerize, and either exposed to a 50% Fetal Bovine Serum shock for 2 hours or immediately overlaid with minigut media. Prior to luminometry, all samples were placed in minigut media plus 200µM Beetle Luciferin Potassium Salt (Promega, Fitchburg, WI, USA). No serum shock was performed on tissue explants. For experiments performed in the absence of Matrigel™, organoids were resuspended and plated in 1.5mL of growth media with Beetle Luciferin Potassium Salt and 10µM Y-27632 (ROCK inhibitor, Sigma Aldrich, St. Louis, MO) to stabilize floating organoids. RU486 (Sigma Aldrich) solubilized in 100% ethanol was added directly to 50µL of Matrigel™/organoid suspension to a final concentration of 20µM. For vehicle controls, an equal volume of 100% ethanol was added in the same manner. For growth factor pulse experiments, a pulse of standard growth factors (500ng/mL R-Spondin, 100ng/mL mNoggin, 50ng/mL mEGF) or an equal volume of PBS (vehicle control) were added to the media at the upslope or downslope of the second peak, with timing calculated as 20 and 32 hours after the first peak.

**R-spondin removal experiments**

PER2::LUC enteroids were plated in Matrigel™ with mNoggin and mEGF. R-spondin was added to the media instead of Matrigel™ to ensure complete washout. Samples were serum shocked for 2 hours. Serum shock was then replaced with minigut media plus R-spondin for both the control and 24-hour washout samples, or minigut media alone for the No R-spondin samples. After 24 hours, all samples were washed with 1mL warm PBS then refreshed with minigut media with Noggin and EGF. R-spondin was added back only to control samples. Brightfield images (4x) were taken immediately following washout, then again on Day 3 and Day 5 to monitor viability.
under the three conditions using the Nikon ECLIPSE TE 200-U microscope.

Intestinal cell lines

Nontransformed, conditionally-immortalized mouse small intestinal epithelial (MSIE, a generous gift from Dr. Robert Whitehead, Vanderbilt University) and transformed Caco-2 cell lines were grown to confluence in their respective media (RPMI 1640 or DMEM/F12) at 37°C (this temperature reverts MSIE cells to a quiescent, wild-type state). At approximately 80% confluence, cells were shocked with 50% fetal bovine serum (FBS) in media for 2 hours then replaced with standard growth media. Start of serum shock is indicated by circadian time 0. RNA was harvested at 4-hour time resolution over 48 hours using Qiagen RNeasy Mini™ kit and QIAshredder™ columns (Qiagen, Germantown, MD, USA). Briefly, RLT plus 2-mercaptoethanol was used to lyse cells and a rubber cell scraper was used to release them from the tissue culture plastic per kit instructions. Each well was collected individually using the RNeasy protocol. Once RNA was isolated and quantified, cDNA was made using the Superscript III First-Strand Synthesis System™ for Real-Time PCR (Invitrogen, Carlsbad, CA, USA). Quantitative Real-Time PCR was performed using Agilent Brilliant III Ultra-Fast SYBR QPCR Mastermix (Agilent Technologies, Santa Clara, CA, USA) with the Stratagene MX3000™ Real-Time PCR machine. The cDNA from all 3 samples per time point was pooled and run in triplicate on 96-well plates using Per2 and GapDH primers (IDT, Coralville, IA, USA).

Confocal microscopy

For whole mount immunofluorescence, organoids were plated onto chamber slides and fixed in 4% paraformaldehyde for 30 minutes. Organoids were permeabilized with PBS- Triton X (0.1%) for 30 minutes and blocked using 10% goat serum for 30 minutes. Organoids were immunostained with primary antibodies specific for PER2 and LUC (Santa Cruz) at a 1:100 dilution at 4°C overnight followed by 4°C overnight incubation with a 1:200 dilution of Alexa
Fluor 594 or 488 goat anti-rabbit IgG (Invitrogen). For nuclear staining, organoids were incubated with Hoechst 33342 (10 μg/ml: Invitrogen) for 20 minutes.

COMPETING INTEREST STATEMENT
No competing interests

AUTHOR CONTRIBUTIONS
Concept and design (all authors), data acquisition (SRM, JP, JV, EA, TM), data interpretation (all authors); writing (all authors); supervision and funding (SRM, CIH)

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FIGURE LEGENDS

Figure 1: Circadian rhythms in PERIOD2::LUCIFERASE (PER2::LUC) jejunal explants. Representative jejunal segments displaying 1.5- to 2-fold circadian PER2 oscillations for up to 3 days following excision, with lengthening of the period of PER2 oscillations over time.

Figure 2: Organotypic PERIOD2::LUCIFERASE (PER2::LUC) jejunal organoids display circadian variation in PER2 and LUC expression. (A-C) Representative 3-dimensional confocal image reconstruction of a PER2::LUC organoid 42 hours following serum shock, with crypt and villous and lumen domains and fluorescent PER2 and LUC antibody staining throughout. Comparison of single plane confocal images at (D-G) 28 hours following serum shock versus (H-K) 42 hours following serum reveal a time-dependent increase in PER2 and LUC staining.

Figure 3. Robust circadian rhythms in PERIOD2::LUCIFERASE jejunal organoids. (A) PER2::LUC organoids display 1.5- to 3-fold circadian oscillations of PER2 abundance following serum shock (blue), as measured by luciferase bioluminescence. In the absence of serum shock (red), PER2::LUC organoids develop synchronized PER2 oscillations within 8-to-12 hours of recording (n=4 per group). (B) Representative traces of detrended luciferase bioluminescence of unshocked organoids treated with steroid antagonist RU486 (20uM, red), control (blue) or an ethanol vehicle control (black). (C) Averaged tracings of detrended luciferase bioluminescence in unshocked PER2::LUC jejunal enteroids treated with either steroid receptor antagonist RU486 (red) or an ethanol vehicle control (blue). RU486 treatment delays the initial peak of PER2
oscillations by a mean of 2.1 hours (*P<0.0001 by Mann-Whitney U test; n=9 samples per group). (D) Representative tracings of detrended luciferase bioluminescence in shocked (blue) and unshocked (red) organoids in the absence of Matrigel™. PER2 oscillations appear more variable and lower amplitude but maintain a clear circadian rhythm. (E) Replenishment of organoid media and luciferin every 5 days enhances and extends PER2 oscillations beyond 2 weeks but resets oscillations after day 10 (representative detrended tracings).

Figure 4. Effects of organoid growth factors on PER2 rhythms

A) Doubling standard concentrations of EGF, Noggin, and R-spondin in organoid media increases the amplitude of PER2 oscillations (representative detrended tracings). (B) Individual subtraction of these growth factors from organoid media 24 hours following serum shock does not dampen PER2 rhythms (representative detrended tracings). (C) Top panels, sequential micrographs (4X) of serum-shocked organoids passaged into standard media over 5 days. Middle panels, organoids with R-spondin washout at 24 hours following serum shock. Bottom three panels, organoids passaged immediately into R-spondin-negative media following serum shock show loss of proliferation and death by day 5.

Figure 5: Growth factors induce phase shifts in PER2 oscillations in unshocked organoids. Serum-shocked organoids (A, B) show no response to growth factors (red) pulsed at either an (A) upslope or (B) downslope of PER2 oscillations vs. a PBS vehicle control (blue). In contrast, unshocked enteroids pulsed with growth factors (red) at both an (C) upslope or (D) downslope of PER2 oscillations show phase shifts by day 5 of recording (representative detrended tracings).

Figure 6: Circadian oscillations of Per2 mRNA expression in PER2::LUC organoids and cell lines. (A) Per2 mRNA expression over 48 hours in mouse small intestinal organoids (n=3 per time point). (B) Per2mRNA expression over 48 hours in nontransformed mouse small intestinal
epithelial (MSIE) cells under quiescent conditions (n=3 per time point). (C) *Per2* mRNA expression over 48 hours in transformed human colorectal adenocarcinoma Caco-2 cells (n=3 per time point). Errors bars represent standard error.

**TRANSLATIONAL IMPACT**

**Clinical issue:** Circadian rhythms influence the risk and outcomes for several gastrointestinal (GI) diseases, ranging from diarrhea to ulcers to cancer. Circadian rhythms also mediate the absorption and GI side effects of numerous drugs. 4-dimensional tissue culture models that reliably reproduce the circadian rhythms of the epithelial lining of the GI tract would help advance a basic understanding of how circadian rhythms regulate GI health and disease.

**Results:** We collected small intestinal jejunal segments from mice with a bioluminescent luciferase marker for a key circadian clock component, PERIOD2 (PER2). PER2::LUCIFERASE (PER2::LUC) intestinal segments showed robust circadian rhythms of PER2 protein abundance for up to 3 days following excision from mice. Separately, we generated intestinal organoids (or epithelial “mini-guts”) by culturing jejunal crypts derived from PER2::LUC mice. Bioluminescent PER2::LUC organoids showed structural features typical of the intestinal epithelium and exquisite circadian rhythms. We found that organoid circadian rhythms synchronize with serum shock, a typical external cue designed to align the clocks of mammalian cells in culture. In the absence of serum shock, PER2::LUC organoids demonstrated the unusual ability to self-synchronize circadian rhythms after 12 hours in culture. Lastly, we found modest circadian patterns of *Per2* gene expression in serum shocked intestinal epithelial cell lines from mice (MSIE, mouse small intestine epithelial) and humans (Caco-2, colorectal adenocarcinoma) over 48 hours.

**Implications and future directions:** Because of their robust circadian rhythms and other
organotypic features, bioluminescent organoids offer an exciting new tool to understand the
interplay of circadian rhythms with stem cell dynamics and intestinal epithelial homeostasis. Such
interplay may have broad implications for disease modeling, stem cell-based therapies,
personalized medicine, and drug development. Questions to guide future directions abound. Do
disrupted circadian rhythms enhance organoid susceptibility to epithelial injury or neoplasm, as
they do in humans? Do circadian rhythms gate the once daily (circadian) division of slowly
cycling Lgr5+ stem cells in the crypt base and twice daily (ultradian) divisions of rapidly cycling
intestinal stem cells in the crypt neck? If so, might chronotherapy translate to novel strategies to
prevent and treat GI cancers or enhance GI wound healing? For pharmacotherapy, do the intrinsic
circadian rhythms of organoids provide a more sensitive in vitro assay for the absorption,
mechanisms, and GI toxicity of new drugs? Our key finding that intestinal organoids possess
robust circadian rhythms sets the stage for studies to address these critical questions.
FIGURE 3D
FIGURE 3E
FIGURE 6A