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Full Paper

Effect of circadian clock and claudin regulations on inulin-induced calcium absorption in the mouse intestinal tract

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Dietary calcium supplementation has been shown to be an effective adjunct therapy in an inflammatory bowel disease model. Soluble dietary fiber reduces intestinal pH and is known to enhance calcium absorption. Although many circadian clock regulations of nutrient absorption in the intestinal tract have been reported, the effects of clock regulation on calcium absorption have yet to be understood. In this study, we investigated the timing of efficient calcium intake by measuring urinary calcium excretion in mice. The diurnal variations in channel-forming tight junctions (claudins) were detected in both the jejunum and ileum. Following 2 days of feeding with a Ca^{2+}-free diet, Ca^{2+}-containing diets with or without soluble fiber (inulin) were fed at specific timings, and urine was subsequently examined every 4 hr. There was an evident increase in urinary calcium concentration when the inulin diet was fed at the beginning of the resting period. The *Claudin 2* (*Cldn2*) expression level also showed a significant day-night change, which seemed to be a mechanism for the increased calcium excretion after inulin intake. This diurnal rhythm and enhanced *Cldn2* expression were abolished by disruption of the suprachiasmatic nucleus, the central clock in the hypothalamus. This study suggests that intestinal calcium absorption might be modulated by the circadian clock and that the intake of inulin is more effective at the beginning of the resting period in mice.

Key words: circadian rhythm, clock gene, claudin, tight junction, prebiotics, calcium absorption

INTRODUCTION

Inflammatory bowel disease (IBD) has been found in increasing numbers of patients worldwide in recent years and is being tackled by a variety of disciplines related to intestinal immunity. It is associated with a significant decline in quality of life (QOL), and concern about its impact on labor productivity is increasing. In 2009, an interesting report showed that dietary calcium supplementation inhibits the development of the disease in a rodent colitis model [1]. Therefore, calcium is not only an essential nutrient for bone formation but also seems to play an important role in immune functions and may possibly be a good target for raising the QOL of IBD patients. When the blood calcium level falls below the acceptable range, the parathyroid hormone level increases, bone resorption accelerates, and calcium is eluted from bone into the blood to maintain the blood calcium level. Therefore, if low calcium levels continue, bone mass will decrease, leading to the development of osteoporosis [2], which IBD patients are at higher risk of developing in addition to osteopenia and fracture compared with non-IBD patients [3]. Thus, proper intake of calcium is very important for maintaining a healthy condition. However, according to a report of the Ministry of Health, Labour and Welfare of Japan, the average intake of calcium in Japanese is far below the recommended intake in most age groups for both men and women. Reasons for this include the fact that the traditional Japanese meal style lacks dairy products as well as the fact that the soft water commonly consumed in Japan has a low calcium content. In addition, the absorption rate of calcium is about 20 to 40% in adults, which is extremely low among nutrients [4]. However, if the intake

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of calcium is drastically increased by supplementation or other method, excretion from the body will be promoted due to the rapid increase in blood calcium concentration [5]. It is also known that a rapid increase in blood calcium level leads to an increased risk of coronary artery disease [6, 7]. Therefore, it is considered that proper intake of a normal diet and improvement of absorption efficiency are important for increasing calcium absorption.

There are two known calcium absorption pathways in the intestinal tract: the intracellular and intercellular pathways [8]. The intercellular pathway exists in the jejunum and the ileum, whose structure is consists of tight junctions including occludin and claudins [8]. Claudin-2 (Cldn2) and Claudin-15 (Cldn15), both of which are channel-forming tight junctions, have been shown to be particularly involved in the transport of cations, such as Ca^{2+} and Na^{+}, using MDCK lines, canine kidney cell lines, but are generally used as a model for epithelial cells. [9, 10]. Studies with knockout of *Cldn2* [11] and the use of siRNA on *Cldn15* [12] showed that both molecules are essential in paracellular intestinal Ca^{2+} absorption. Calcium absorption via the intracellular pathway mainly occurs in the duodenum and colon, with transient receptor potential vanilloid subfamily member 6 (Trpv6) being responsible for calcium transport into cells and Pmca1 transporting calcium into the blood from epithelial cells [13–16]. Recently, the benefits of dietary supplementation with soluble dietary fiber have become evident for enhancing calcium absorption in both humans and mice [17, 18], and this has led to significant growth in the market for prebiotics. Prebiotics, including some types of soluble dietary fiber, enhance microbial fermentation, increase intestinal short-chain fatty acid (SCFA) levels, lower intestinal pH, and improve mineral absorption [19]. However, there have been no studies in which the effect of a prebiotic on calcium absorption was verified by focusing on the intercellular pathways.

The environment of our planet changes in accordance with the 24-hr light and dark cycle. Because of this, our bodies maintain a circadian clock, a 24-hr rhythm, that plays important roles in many physiological functions, such as sleep/wakefulness, hormone secretion, metabolism, and eating behavior [20, 21]. In mammals, the pacemaker-like role of this circadian rhythm is played by the suprachiasmatic nucleus (SCN), which is called the central clock [22]. It is also known that a circadian rhythm mechanism called a peripheral clock is present in almost every organ, including the liver, kidney, heart, and intestine [23], and diurnal oscillations of clock gene expression levels have been detected in almost every cell. Circadian rhythms are created by a negative feedback loop formed by clock genes such as period (*Per*), cryptochrome (*Cry*), brain and muscle ARNT-like protein (*Bmal*), and circadian locomotor output cycles kaput (*Clock*).

The central clock synchronizes with light stimulation, while the peripheral clock synchronizes with food, exercise, and temperature [24–27]. Among these factors, the entrainment effect of the diet is strong, especially that of the diet after a long fast [28, 29]. In the intestine, diurnal variations of clock genes are known to influence the circadian variations in the intestinal absorption of nutrients. For instance, diurnal changes in glucose transporters, drug transporters, and amino acid transporters have been reported [30]. In addition, diurnal rhythms have been observed in intestinal tight junction genes, such as occludin (*Ocdn*) and some claudins (*Cldn1* and *Cldn3*) [31, 32]. This intercellular rhythmicity is abolished in *Clock* mutant mice and causes enhancement of intestinal permeability, which can result in worsening of dextran sulfate sodium (DSS)-induced colitis [31]. However, the diurnal variation of cation-selective tight junction protein and other proteins related to calcium absorption remains unclear. Therefore, the purpose of this study was to elucidate the optimal intake timing for inulin and its underlying mechanism.

MATERIALS AND METHODS

Animals

All animal care and procedures followed the guidelines of the Committee for Animal Experimentation of the School of Science and Engineering at Waseda University and were in compliance with Japanese law (Act No. 105 and notification No. 6 of the Prime Minister's Office). The experiments were approved by the School of Science and Engineering of Waseda University (No. 2018-A033 and 2019-A062). Eight-week-old male ICR mice were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) and housed under laboratory conditions (temperature, 22 ± 2°C; humidity, 60 ± 5%; lights on at 8 AM and off at 8 PM) with food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum* for at least a week before starting the experiment. The beginning of the light period was defined as Zeitgeber time (ZT) 0, and the beginning of the dark period was defined as ZT12.

Treatment

AIN-93 M (Oriental Yeast Co., Ltd.) was used as a control diet; 5% cellulose (insoluble fiber) was included in the control AIN-93 M diet. To produce a Ca^{2+}-free AIN-93 M diet, 1.2495% $CaCO_3$ was replaced with sucrose. An additional 5% cellulose (Oriental Yeast Co., Ltd.) or 5% inulin (Fuji FF; Fuji Nihon Seito Corporation, Tokyo, Japan) was mixed with the AIN-93 M diet for a feeding experiment to measure acute Ca^{2+} absorption and the effect of fiber. The details of the experimental schedules are described in the figures and Results section. Time-restricted feeding was conducted using automated feeding equipment (Natsume Seisakusho Co., Ltd., Tokyo, Japan), as described previously [27]. Since this study assumed that the calcium absorption rate would increase due to dietary improvement, the experiments were conducted by feeding rather than oral administration.

Gene expression analysis

Total RNA was extracted from intestinal tissue with phenol (Omega Bio-Tek, Norcross, GA, USA). Purity of samples was assessed in 50 ng aliquots with NanoVue Plus (GE Healthcare Life Science, Chicago, IL, USA), and real-time reverse transcription polymerase chain reaction (PCR) was performed with a One-Step SYBR RT-PCR Kit (Takara Bio Inc., Kusatsu, Shiga, Japan) and PikoReal PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences used for amplification are shown in Table 1. Quantification was performed by the ΔΔCt method, and relative mRNA expression levels were normalized against TATA-binding protein (*Tbp*).

Measurement of urine calcium, urine creatinine, and cecal pH

The current experiment evaluated the urine Ca^{2+} level determined by the blood Ca^{2+} level to identify intestinal Ca^{2+} absorption, as the blood Ca^{2+} concentration is strongly regulated by the Ca^{2+} homeostasis system, and it is difficult to see changes

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in blood level [8]. Spot urine was collected from mice under a slightly stressful conditions by collecting it into a microtube immediately after grabbing them. Since some mice did not urinate during the sampling period, more mice (n=6 for each group in Figs. 1 and 2, n=20 for each time point in Fig. 3, and n=7 for each group in Fig. 4) were prepared, and the number of samples collected was not the same at each time point (at least n=5 in each group). LabAssay™ creatinine (Wako Pure Chemical

Table 1. Primer sequences

Gene name	Forward (5′ → 3′)	Reverse (5′ → 3′)
Tbp	CAGCCTCAGTACAGCAATCAAC	TAGGGGTCATAGGAGTCATTGG
Per2	CTGCTAATGTCCAGTGAGAG	GTACAGGATCTTCCCAGAAAC
Bmal1	GGGAAATACGGGTGAAATCTAT	GTGTCCTATGTCGTCTTGATG
Cldn2	CGGAGTCATCCTTTGCTTT	AACTCACTCTTGGCTTTGG
Cldn15	CTCTACTTGGGCTGGAGTG	CGTAGAAGGCTTGTAGGGAA
Trpv6	ATGGCTGTGGTAATTCTGGG	AGGAAGAGTTCAAAGGTGCTG
Pmca1	TCTGGCTACGGAACCACCCA	AAAGGCTTCCCGCCAAACTG

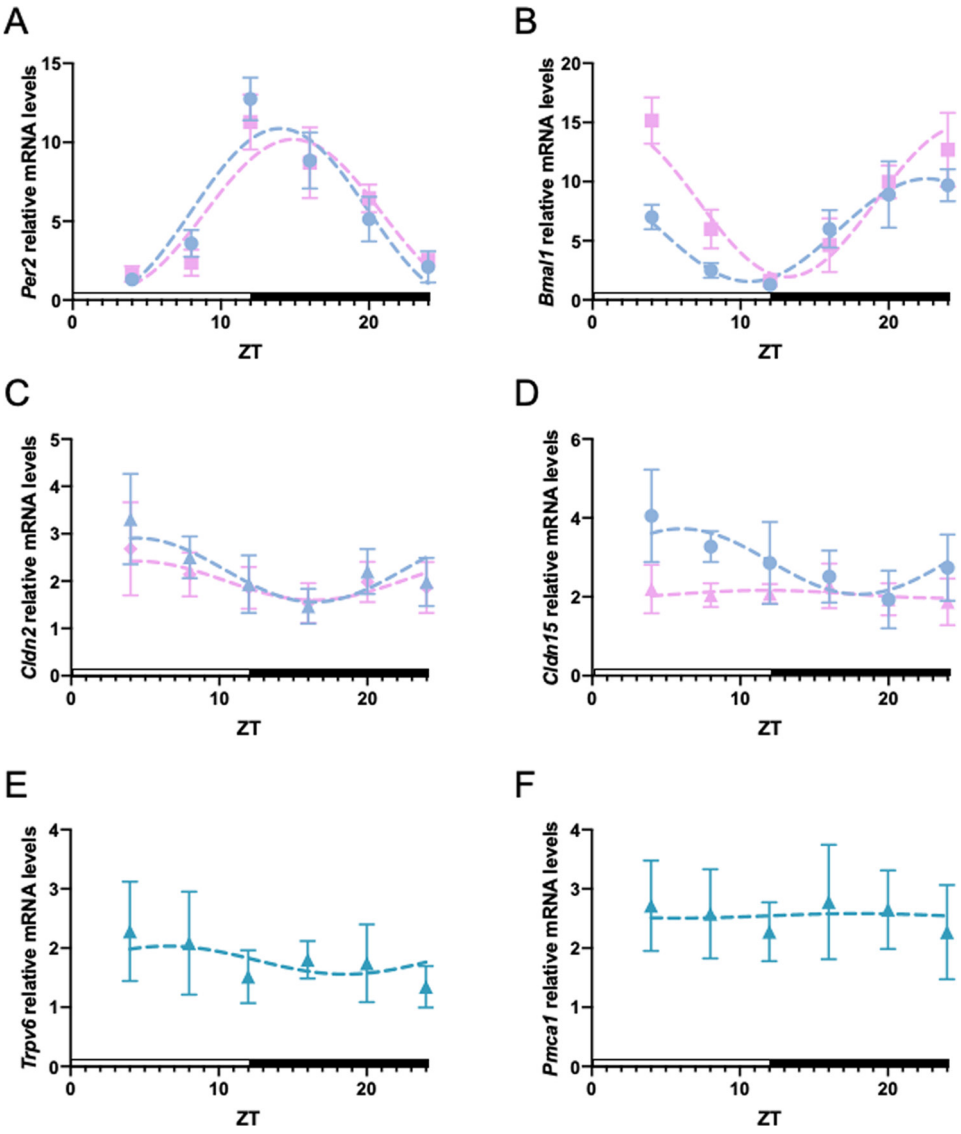

Fig. 1. Diurnal variations of clock genes and calcium absorption-related genes in the intestine.

Daily mRNA expression rhythms of the core clock genes *Per2* (A) and *Bmal1* (B) and intestinal calcium absorption-related genes *Cldn2* (C), *Cldn15* (D), *Trpv6* (E), and *Pmca1* (F) in the intestine (blue, jejunum; pink, ileum; green, colon) by qPCR. Tissue samples were collected every 4 hr over the course of a day under *ad libitum* feeding of control AIN-93 M diet. The white and dark bars at the bottom indicate the light and dark periods, respectively. Data are expressed as mean ± SEM values. N=6 at each time point.

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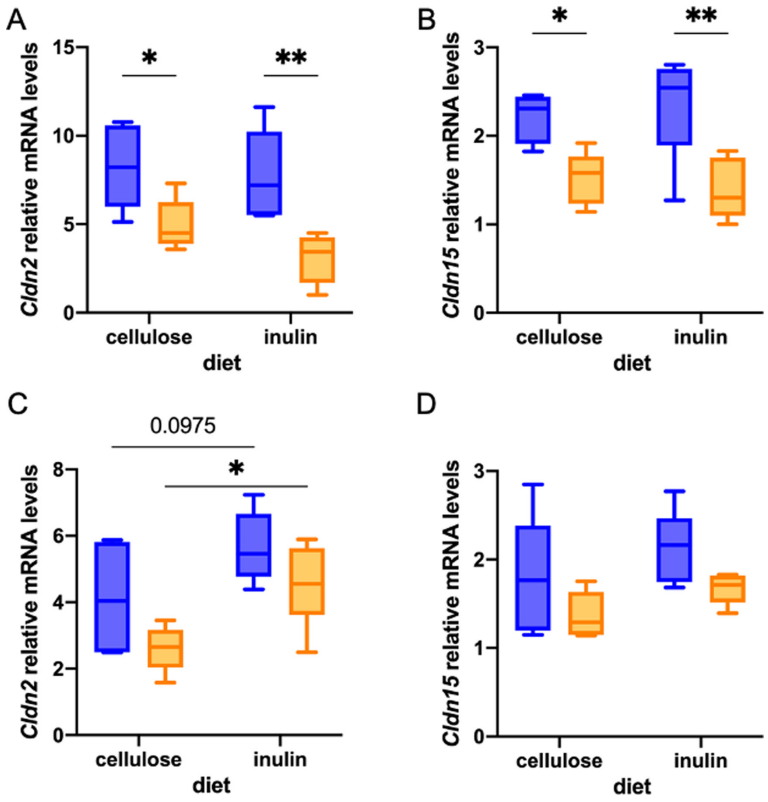

Fig. 2. Elevated *Claudin* expression levels in the ZT0 inulin-fed group.

Day-night changes in *Claudin* expression levels under time-restricted fiber feeding conditions. The mRNA expression levels of *Cldn2* (A, C) and *Cldn15* (B, D) in the intestine (upper, jejunum; lower, ileum) were measured for each experimental condition (5% cellulose and 5% inulin diets; blue, ZT0 feeding; orange, ZT12 feeding) by qPCR. Vertical lines through boxes indicate the median. The whiskers go from each quartile to the minimum or maximum. N=5–6 each. **p<0.01, *p<0.05.

Industries, Ltd., Osaka, Japan) was used to measure the creatinine levels in urine. The Ca^{2+} concentration was measured using a Ca^{2+} assay kit (Metallogenics Co., Ltd., Chiba, Japan) based on the formation of a chelate complex between chlorophosphonazo III and calcium. Cecal pH was measured by inserting a pH meter electrode (pH Spear, Eutech Instruments, Vernon Hills, IL, USA) directly into the cecum after euthanasia.

Time-course calcium excretion assay

Mice were fed 1 g of AIN-93 M after 2 days of feeding with the Ca^{2+}-free diet, and then spot urine was collected every 4 hr. To habituate the mice to the feeding times, the mice were kept on a two-times-a-day feeding schedule at ZT0-4 and ZT12-16 using an automated feeding apparatus. We had two different timings of feeding (ZT0 and 12) and three different diets (Ca^{2+}-free diet, AIN-93 M + 5% cellulose, AIN-93 M + 5% inulin); therefore, a total of six groups were prepared (Supplementary Fig. 1). The creatinine concentration was used as a reference for the Ca^{2+} concentration.

SCN lesion

Bilateral thermal lesions of the SCN were performed stereotaxically (RWD Life Science, Guangdong, China) under midazolam/medetomidine hydrochloride/butorphanol tartrate anesthesia as described previously [27]. A stainless-steel electrode (0.35 mm in diameter) was inserted bilaterally into the SCN (0.1 mm anterior and ± 0.2 mm lateral to the bregma and 5.8 mm from the skull surface) with a thermal lesion generator (RFG-4A; Muromachi Kikai Co., Ltd., Tokyo, Japan). The lesion was made by maintaining a temperature of 55°C for 8 sec using a current path. Sham-operated animals were treated by opening the skull surface but not inserting the electrode. The cage activity was measured with ClockLab software (Actimetrics, Wilmette, IL, USA) using an infrared sensor placed on the cage lid to confirm the SCN lesion. Chi-square analysis was performed to verify the rhythmicity of locomotor activity with a criterion of $p<0.001$. Brains were obtained from euthanized mice and stored in 4% PFA solution for at least two nights and then in 20% sucrose for one night at 4°C. Brain slices (30 µm thickness) were prepared using a cryostat (Leica Biosystems, Wetzlar, Germany) and stained with 0.1% cresyl violet for Nissl staining. Slice photos were taken with a light microscope (Keyence, Osaka, Japan). After checking the slices, we excluded the data from mice with a remaining SCN, as described previously [27].

Data analysis

Data were analyzed using GraphPad Prism (version 9.3.1, GraphPad Software, San Diego, CA, USA). Equal variance and normal distribution tests were performed to select the appropriate statistical approach. Parametric analyses were performed using one-way or two-way analysis of variance (ANOVA) with Tukey's test or Student's t-test for post hoc analysis. Data are expressed

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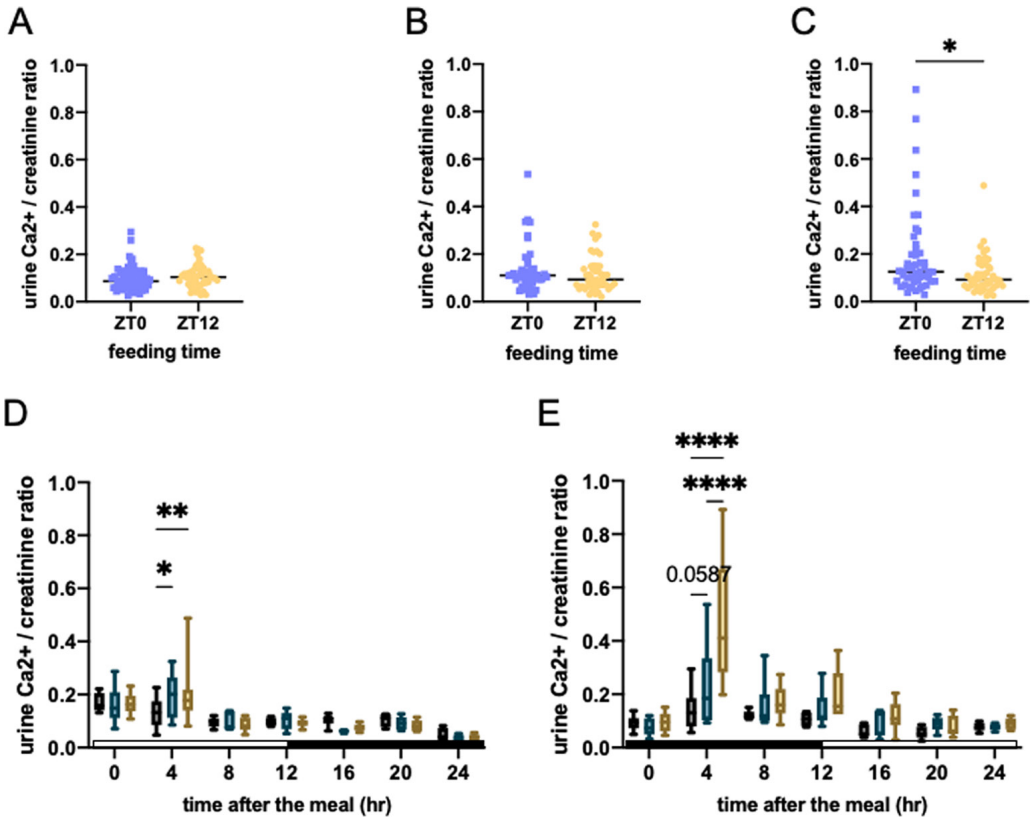

Fig. 3. Inulin intake effectively enhances urine the Ca^{2+}/Creatinine ratio in mice fed at ZT0.

Time-dependent calcium administration and urine excretion in mice under time-restricted feeding conditions. Urine Ca^{2+}/creatinine ratios under each of the experimental conditions, Ca^{2+}-free diet (A), 5% cellulose diet (B), and 5% inulin diet (C), as a total of different timepoints (blue, ZT0 feeding; orange, ZT12 feeding). Comparison of urine Ca^{2+}/creatinine ratios according to duration after each meal (black, Ca^{2+}-free diet; green, 5% cellulose diet; orange, 5% inulin diet) for the different feeding times: ZT0 feeding (D) and ZT12 feeding (E). Vertical lines through boxes indicate the median. The whiskers go from each quartile to the minimum or maximum. Since some mice did not urinate during sampling period, more mice (n=20) were prepared, and the number of collected samples was not the same at each time point (n=5–13 each). ****p<0.0001, **p<0.01, *p<0.05.

as individual plots with the mean ± standard error of the mean (SEM) or box and whiskers charts with the median, minimum, and maximum displayed. Statistical significance was set at $p<0.05$, and $p<0.1$ data are shown.

In order to verify the circadian rhythmicity of gene expression levels, data were also analyzed by nonlinear regression fitting with the cosinor equation using GraphPad Prism [33].

RESULTS

Expression rhythms of clock genes in intestinal segments

In order to detect the diurnal variations in clock genes, mice were divided into six groups every 4 hr over the course of one day. All of the mice had been bred under *ad libitum* feeding of the control AIN-93 M diet as an imposed feeding schedule in order to minimize the peripheral clock by dietary entrainment. Mice sacrificed at ZT0 and ZT12 were given extra care to be killed before the change in light environment takes place, and their intestinal tracts (jejunum, ileum, colon) were collected.

The expression rhythms of the clock genes *Per2* and *Bmal1* were measured by qPCR in time-course samples of jejunum and ileum. Both *Per2* and *Bmal1* showed significant circadian rhythms in the jejunum and ileum (Fig. 1A, 1B). The expression level of *Per2* showed a peak at the beginning of the dark period. On the other hand, *Bmal1* showed a peak at around the end of the dark period, which was antiphase to *Per2*.

These results suggest that intestinal segments are under the control of the circadian clock via major clock genes such as *Per2* and *Bmal1*.

Diurnal variations in tight junction proteins that regulate cation selectivity

Using the same RNA samples as in the above experiment, we verified the expression rhythms of calcium absorption-related genes for both transcellular and paracellular pathways.

The expression rhythms of claudins (*Cldn2*, *Cldn15*), which are known as cation-selective paracellular channels, were determined in time-course samples of jejunum and ileum. In the jejunum, both *Cldn2* and *Cldn15* showed significant diurnal variations, with both showing a peak at around the middle of the light period (Fig. 1C, 1D). In the ileum, *Cldn2* expression level continued to show diurnal oscillation; however, the expression level of *Cldn15* showed no difference throughout the day (Fig. 1C, 1D). We also examined the expression levels of transcellular calcium transporters that play an important role in the colon. *Trpv6*, which is a Ca^{2+} channel in the intestine, showed slight circadian variation but no significant diurnal rhythm (Fig. 1E). Regarding *Pmca1*, which is a major Ca^{2+} pump, cosinor fitting for examining diurnal

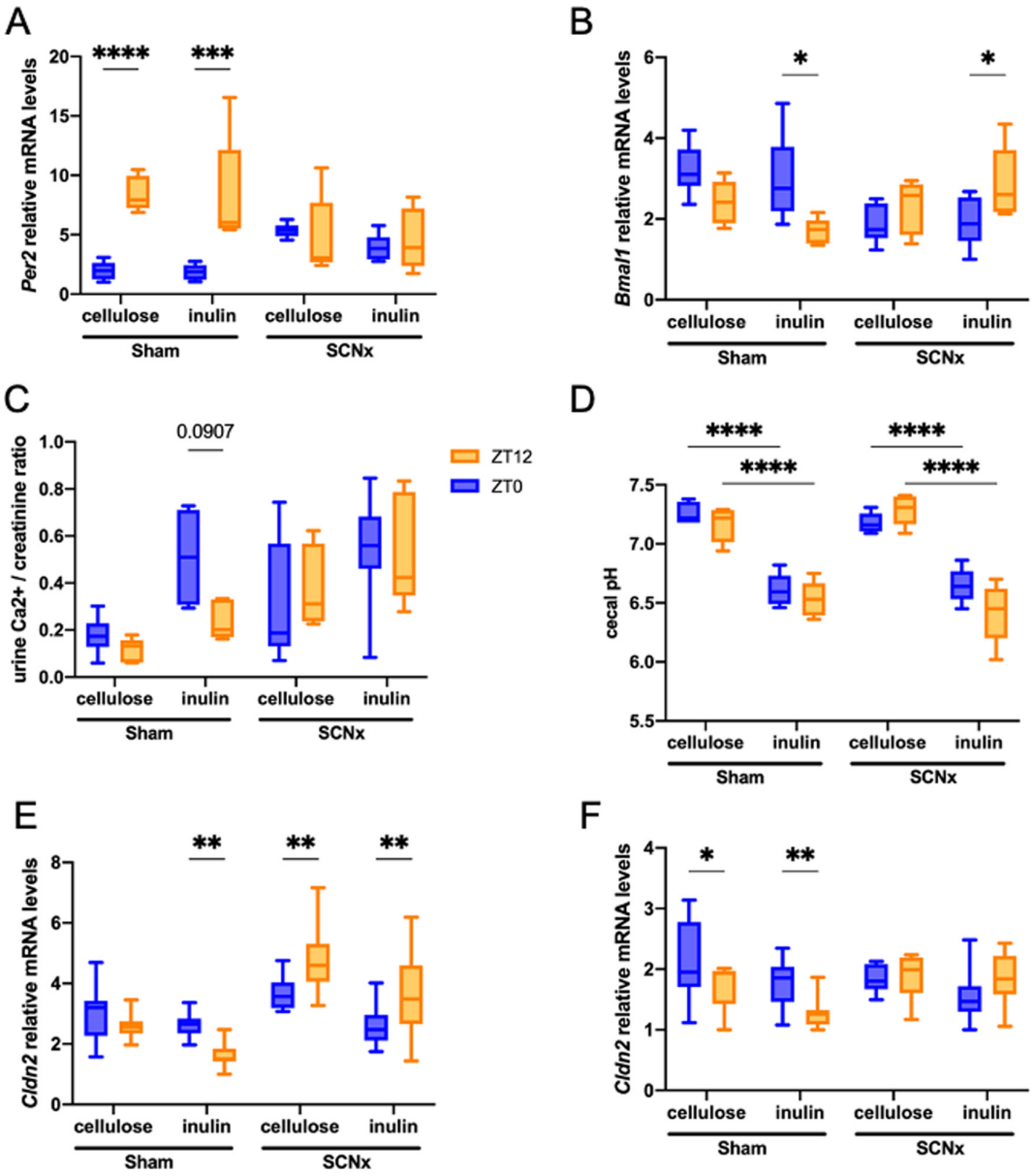

Fig. 4. Effect of SCN lesion on time-dependent Ca^{2+} administration and urine excretion.

Day-night variations of the core clock genes *Per2* (A) and *Bmal1* (B) were detected in the jejunum under each experimental condition (5% cellulose and 5% inulin diets; blue, ZT0 feeding; orange, ZT12 feeding) by qPCR. Urine Ca^{2+}/creatinine ratio (C) and cecal pH (D) under each experimental condition. Day-night variations of *cldn2* were detected in the jejunum (E) and ileum (F) under each experimental condition (5% cellulose and 5% inulin diets; blue, ZT0 feeding; orange, ZT12 feeding) by qPCR. Vertical lines through boxes indicate the median. The whiskers go from each quartile to the minimum or maximum. N=5–7 each. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

rhythm failed to calculate a fitted model (Fig. 1F). A summary of the model fitting is shown in Table 2.

These results indicated that some of the claudins that regulate cation selectivity in a paracellular pathway showed diurnal variations.

Difference in calcium excretion observed according to meal timing and fiber intake

We next examined the difference in the calcium absorption-promoting effect and its persistence according to meal timing as well as fiber intake.

When the time points were compared as a whole, we found a significant increase in urine Ca^{2+}/creatinine ratio after feeding inulin at ZT0 but not at ZT12 (Fig. 3A–3C). Next, we investigated the influence of the duration after meal intake. The highest urine Ca^{2+}/creatinine ratio was observed 4 hr after feeding at ZT0, and this was the only time point showing a significant difference between cellulose and inulin among the time points (Fig. 3D, 3E). Similar trends were observed at 8, 12, and 16 hr after feeding at ZT0, as well as at 4 hr after feeding at ZT12 (Fig. 3D, 3E).

Calcium absorption is thought to correlate with urine calcium excretion. Thus, taken together, our data suggested that inulin

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Table 2. Summary of the data for cosinor model fitting

Intestinal segment	Gene	Amplitude	Acrophase	R^2
jejunum	*Per2*	5.249	13.99	0.796
	Bmal1	4.338	22.59	0.815
	Cldn2	0.676	4.43	0.368
	Cldn15	0.830	6.03	0.336
ileum	*Per2*	4.688	14.88	0.764
	Bmal1	6.107	24.00	0.807
	Cldn2	0.412	4.39	0.210
	Cldn15	0.098	11.72	0.023
colon	*Trpv6*	0.238	6.54	0.067
	Pmca1	n.d.	n.d.	n.d.

Consinor analysis circadian amplitude, acrophase, and R^2 of each target gene in different intestinal segment are listed.
n.d.: not detected.

has a calcium absorption promoting effect and that this is more evident at the beginning of the light phase (= resting period) than during the dark phase (= active period).

Oscillations in tight junction proteins affect calcium excretion at 4 hr after fiber intake

In order to investigate the mechanism responsible for the observed difference in calcium excretion observed, we measured the expression levels of tight junction proteins that were found to exhibit circadian variations above. Compared with the group fed at ZT12, the ZT0 group showed significantly high expression levels of *Cldn2* and *Cldn15* in the jejunum at 4 hr after each feeding (Fig. 2A, 2B). The expression level in *Cldn2* in the ileum was also significantly high in the ZT0-fed group (Fig. 2C). We also determined that inulin intake significantly enhanced *Cldn2* expression level in the ileum, with the difference being significant in the ZT12-fed group (Fig. 2C); a similar result was not detected for *Cldn15* (Fig. 2D).

These results strongly indicated that the tight junction protein *Cldn2* together with *Cldn15* may be involved in the increased calcium absorption caused by inulin intake at ZT0.

SCN-lesioned mice did not show an increase in calcium excretion

Since our results indicate a peripheral clock influence on the increase in calcium absorption caused by inulin, we next performed an experiment in SCN-lesioned mice, the circadian rhythms of which were eliminated. SCN-lesioned mice are known to demonstrate dampened rhythmicity in sleep-wake behavior and clock gene expression in tissues [27, 34]. After recovery from surgery, the arrhythmicity of locomotor activity was evaluated for the success of the SCN lesion (Supplementary Fig. 2A). Considering the possibility of detecting rhythmicity in the peripheral clock if SCN-lesioned mice were fed with restrictive feeding, we chose to feed these mice *ad libitum*. After *ad libitum* feeding of the Ca^{2+}-free diet for 2 days and fasting for 8 hr, the mice were fed AIN-93 M + 5% cellulose or AIN-93 M + 5% inulin (Supplementary Fig. 2B).

When we compared the Ca^{2+}/creatinine ratio at 4 hr after feeding, the SCN-lesioned mice did not show significant day-night differences in either clock genes, while sham-operated mice showed significant differences like those detected in the circadian variations in Fig. 1 (Fig. 4A, 4B). SCN-lesioned mice showed a significant difference in an opposite *Bmal1* expression pattern, higher in ZT12, which clarified that the mice with a damaged central clock had a disrupted peripheral clock (Fig. 4B).

Consistent with Fig. 2C, sham-operated mice fed inulin showed a trend toward day-night changes in Ca^{2+}/creatinine ratio (Fig. 4C). However, these day-night differences disappeared in the SCN-lesioned mice (Fig. 4C). The detected prebiotic effect of inulin on the decrease in cecal pH was similar at both feeding times, and no difference was detected between sham-operated and SCN-lesioned mice (Fig. 4D).

Subsequently, we measured the *Cldn2* expression levels in these mice. While significant day-night differences were detected in the sham-operated group (Fig. 4E, 4F), the SCN-lesioned mice fed either cellulose or inulin showed opposite significant differences in the jejunum (Fig. 4E) and no significant differences were detected in the ileum (Fig. 4F).

Therefore, these results suggested that daily changes in calcium absorption as well as excretion may be regulated by the circadian clock and that at least a portion of them may be regulated by *Cldn2*.

DISCUSSION

This is the first study to report that inulin-induced enhancement of calcium absorption is affected by the circadian clock and that the enhancement of calcium absorption may be caused by the expression of claudins in the jejunum. There are studies that have reported an increase in calcium absorption caused by the intake of soluble dietary fibers [35–37] and a circadian profile of serum calcium concentrations with significant decreases during the dark phase, focusing on transcellular calcium absorption, including the vitamin D receptor signaling pathway [38]. However, there are no chrono-nutritional reports that have investigated the circadian effects of soluble dietary fibers on calcium concentrations.

In this study, we showed the diurnal variations in clock genes as well as in *Cldn2* and *Cldn15* in the jejunum that regulate cation selectivity in a paracellular pathway. The phases of *Per2* and *Bmal1* (Fig. 1A, 1B) were consistent with previous research [39, 40]. In a previous study, rhythmic expression of the *Ocdn* and *Cldn1* genes was reported for epithelial barrier molecules in colon epithelial cells, and these oscillations were lost in a clock mutant [31]. Unlike our data, that study failed to detect diurnal

variation in the *Cldn2* expression level. This may be because the assay in that study was based on isolated colon epithelial cells, whereas the significant diurnal variations in the present study were found in the jejunum and ileum (Fig. 1C). As the core clock gene expression rhythms are consistent in the murine gastrointestinal tract [41], these tight junction genes might be expressed periodically in other parts of the intestinal tract. The claudin proteins have a four-transmembrane structure and show selectivity for incoming substances depending on the amino acid residues in the extracellular loop [10, 42]. In line with this, the presence of negatively charged aspartic acid in the extracellular loop of claudin-2 and claudin-15 helps with the influx of cations, such as Ca^{2+} and Na^{+}. Thus, the higher the expression of *Cldn2* and *Cldn15*, the higher the influx of these cations reported [16, 43]. Therefore, the detected diurnal variations in *Cldn2* and *Cldn15* suggested that calcium absorption may be influenced by these core clock gene expression rhythms.

We then evaluated calcium absorption by changing the timing of calcium intake and measuring urinary calcium excretion, which has been previously reported as an indirect measurement of calcium influx [11, 44]. It was previously reported that there were no differences in the creatinine concentration in spot urine collected from mice at ZT2 and ZT12 [45] and that no rhythms were observed in Ca^{2+}/creatinine ratio in normally housed mice [46]. In our study, no significant diurnal rhythms were observed at ZT4 and ZT16 in the Ca^{2+}-free diet group. Although slight oscillation was observed in the urine Ca^{2+}/creatinine ratio, the observed increase in Ca^{2+}/creatinine ratio was thought to have been caused by calcium administration. Our data showed that a difference in calcium excretion can be caused by a change in meal timing and fiber intake (Fig. 2D, 2E). The intestinal calcium absorption rate is reported to be 20–40% in humans, but it was reportedly increased by inulin, inulin-derived difructose anhydrides III (DFA III), or other soluble dietary fibers [35–37]. In this study, we speculate calcium absorption from urine excretion and did not directly measure the calcium absorption itself. Although a slight oscillation was observed in urine Ca^{2+}/creatinine level, an increase of urine Ca^{2+} level is observed in increase in both calcium fed groups after 4 hr after feeding at ZT0; not in calcium-free diet group nor feeding at ZT12 (Supplementary Fig. 3). In addition, since creatinine level stays within a similar range, the Ca^{2+}/creatinine increase is regarded as influenced by the calcium administration. Granting there remains a possibility that calcium excretion fluctuation interferes with our interpretation, it may fall as our potential study limitation. Consistent with these studies, we also showed that inulin boosted calcium absorption, which became more evident when mice were fed at the beginning of the resting period than during the active period. Moreover, we showed *Cldn2* and *Cldn15* expression levels fluctuate in parallel with calcium excretion (Fig. 3). Considering the time lag from food intake to it reaching the intestine and subsequently mRNA being transcribed upon stimulation, 4 hr after inulin intake is a rather early time point to see an inulin effect. A separate experiment was conducted to determine the time required for ingestion to defecation in mice, and the results showed the 3–6 hr was sufficient (data not shown). Thus, although assessment of the fecal output of calcium together with urine may have supported our data even more strongly, 4 hr after inulin intake is considered to be a relevant timing for observing the effects of intake at the mRNA level. However, since we were focusing on day-night changes in calcium absorption and no diurnal changes were detected in either *Trpv6* or *Pmca1* in the colon, we focused on claudins in the jejunum and ileum, as well as calcium excretion via urine. Kawai *et al.* reported calcium absorption oscillation that peaked in the early resting period in control mice [38], which is similar to our data. In their study, as in our data for the SCN-lesioned mice, they observed a reduction in the peak level of calcium absorption in intestinal-specific-*Bmal1*-deficient mice [38]. Although they did not feed their mice any soluble fibers, their data supports our data. Therefore, it suggested that the increase in calcium absorption caused by inulin can be affected by the oscillations in tight junction proteins, especially for *Cldn2*.

In our final experiment, the data for the SCN-lesioned mice, the circadian rhythms of which were eliminated, strongly indicated that daily changes in calcium absorption as well as excretion may be regulated by the circadian clock. In the mice with both disrupted central and peripheral clocks, we failed to detect a similar day-night change in Ca^{2+}/creatinine ratio, even with inulin intake (Fig. 4C). Since our cecal pH data showed a significant decrease in the inulin-fed group, it confirmed that the intake of inulin was successful [47]. Thus, the failure to detect a similar day-night change in Ca^{2+}/creatinine ratio is thought to be the result of clock disruption. Furthermore, we detected that the day-night change in *Cldn2* expression rhythm was also discomposed. The solubilization of Ca^{2+} by acids is known to promote the function of Ca^{2+} transporters [48–50]. As was mentioned above, it was confirmed that the intake of inulin was successful, since cecal pH did decline in our study after inulin intake, both in daytime and nighttime (Fig. 4D) [47]. Regardless of the decline in cecal pH, the day-night difference detected in urine Ca^{2+}/creatinine ratio is not thought to be caused by the fiber-induced intestinal pH changes. Therefore, these results suggested that daily changes in calcium absorption as well as excretion may be regulated by the circadian clock and that at least a portion of them may be regulated by *Cldn2*. The expression patterns of *Cldn2* in both the jejunum and ileum of sham-operated mice were similar to those in Fig. 3; however, the variation in expression levels between trials seemed somewhat smaller in sham-operated mice. This may be due to the effect of changing the protocol from restrictive feeding to *ad libitum* feeding conditions. Previous studies have shown that the amplitude and phase of the peripheral clock, including the intestinal tract, fluctuates depending on the time of day when limit feeding is applied [41, 51]. The detailed mechanism of the effects of inulin needs to be explored further in a separate experiment; however, a previous study using DFA III showed a possible pathway. DFA III binds to intestinal epithelial cells, phosphorylates myosin through several intracellular signaling pathways, and regulates switching of tight junctions via anchoring junction proteins connected to them [52]. As inulin used in this study is a component of DFA III, and it is conceivable that it would show a similar effect on tight junctions. Thus, it is suggested that inulin may open tight junctions by anchoring junction proteins and that this can be more potent when inulin is fed at the beginning of the resting period in mice.

This study revealed that calcium absorbance can be boosted by the intake of inulin at the beginning of the resting period. The *Cldn2* expression level is upregulated at that point, and this was shown in our SCN-lesioned mouse experiment. Claudin-2 is known as a critical factor for vitamin D-dependent calcium

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absorption [53]. This supports our study, suggesting that claudin-2 may be involved in the upregulation of inulin-promoted calcium absorption. However, the present study assessed *Cldn2* only at the mRNA level, and no vitamin D receptor data was examined. Further studies may be required to clarify the time-dependent mechanism of the calcium absorption-promoting effect of inulin.

To date, several reports have been published on calcium concentrations and sleep. In mice, systems biology studies of the sleep-wake cycle have reported decreased sleep durations due to impairment of Ca^{2+}-dependent K^{+} channels, voltage-gated Ca^{2+} channels, and glutamate-gated calcium-permeable ion channels, such as NMDA receptors [54]. This indicates that Ca^{2+}-dependent neural hyperpolarization pathways are very closely related to slow-wave sleep. In a recent human study, shift workers with higher serum calcium concentrations tended to fall asleep more easily and have shorter total sleep times, whereas no such significant correlation was found in non-shift workers, and a negative correlation was found between the calcium concentration and peak time of daily activity as measured by actigraphy [55]. Therefore, the fact that increased calcium absorption appears at the beginning of the resting period may be an important physiological effect for better quality sleep.

As mentioned at the beginning, osteopenia and osteoporosis are prevalent in IBD patients [3]. Interestingly, high expression levels of claudin-2 can be found in IBD (both Crohn's disease and ulcerative colitis) [56]. This high expression level of claudin-2 is thought to lead to the leaky gut condition, and inactivation of claudin-2 has attenuated IBD symptoms in mice [57]. Although further examinations in an IBD model is required, our data suggest that IBD patients with a high claudin-2 level may be more likely to obtain the benefit of inulin-promoted calcium absorption.

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