Circadian Expression of Clock Genes in Human Oral Mucosa and Skin

Association with Specific Cell-Cycle Phases

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We studied the relative RNA expression of clock genes throughout one 24-hour period in biopsies obtained from the oral mucosa and skin from eight healthy diurnally active male study participants. We found that the human clock genes *hClock*, *hTim*, *hPer1*, *hCry1*, and *hBmal1* are expressed in oral mucosa and skin, with a circadian profile consistent with that found in the suprachiasmatic nuclei and the peripheral tissues of rodents. *hPer1*, *hCry1*, and *hBmal1* have a rhythmic expression, peaking early in the morning, in late afternoon, and at night, respectively, whereas *hClock* and *hTim* are not rhythmic. This is the first human study to show a circadian profile of expression for all five clock genes as documented in rodents, suggesting their functional importance in man. In concurrent oral mucosa biopsies, thymidylate synthase enzyme activity, a marker for DNA synthesis, had a circadian variation with peak activity in early afternoon, coinciding with the timing of S phase in our previous study on cell-cycle timing in human oral mucosa. The major peak in *hPer1* expression occurs at the same time of day as the peak in G_1 phase in oral mucosa, suggesting a possible link between the circadian clock and the mammalian cell cycle. (*Am J Pathol 2001, 158:1793–1801*)

In mammals, most physiological, biochemical, and behavioral processes vary in a regular and predictable periodic manner with respect to time of day (endogenous circadian rhythm).[1] The suprachiasmatic nuclei (SCN) of the anterior hypothalamus are the site of the circadian pacemaker in mammals.[2] Such evolutionary distant taxa as *Synechococcus*, *Neurospora*, *Drosophila*, and mammals share a common mechanism and in some cases homologous genes for circadian clock control.[3] Five mammalian clock genes, *Clock*,[4] *Per*,[5,6] *Bmal1*,[7] *Tim*,[8] and *Cry*[9] have been cloned and their interactions in an intracellular transcriptional/translational feedback loop studied.[3,10] Clock genes have a characteristic circadian expression in the SCN of nocturnally active rodents. The three mouse *mPer* genes ('m' prefix for mouse)[11] peak at different times, with *mPer1* peaking soon after subjective dawn (early activity period) and *mPer3* and *mPer2* peaking 2 hours and 4 hours later, respectively. *mBmal1* RNA levels have a circadian rhythm with a peak after subjective dusk (late activity period).[12] *mCry1* oscillates in the SCN with a rhythm similar to the phase of *mPer2*.[10] *mClock*[5,6] and *mTim* RNA levels do not oscillate in the SCN.[8] Clock genes are also expressed in rodent extraneural tissues with a phase delay of 4 to 6 hours with respect to the SCN rhythm.[3,10,11,13,14] The human circadian pacemaker has been shown to be as stable and precise in measuring time as that of other mammals.[15] This suggests that the molecular mechanisms regulating the circadian rhythm in rodents should also be detectable in humans.

We previously reported that the nuclear expression of cell-cycle protein markers varies in an orderly and sequential manner throughout the day in the oral mucosa of diurnally active human study participants.[16] Specifically,

a marker of late G_1 phase (p53), is maximal at 11:00 hours, whereas markers of G_1 to S phase (cyclin E), G_2 phase (cyclin A), and M phase (cyclin B) are maximal at 15:00 hours, 16:00 hours, and 21:00 hours, respectively. The normal physiological progression throughout time for the peak expression of these proteins is consistent with previous rodent and human data showing a circadian variation in cell proliferation in gastrointestinal tract mucosa.[17–20]

Thymidylate synthase (TS) catalyzes the reductive methylation of deoxyuridine-5′-monophosphate to deoxythymidine-5′-monophosphate, the only pathway for *de novo* synthesis of deoxythymidine-5′-monophosphate and subsequent DNA synthesis.[21] TS activity is highest during S phase, and can therefore be used to mark this event in the cell cycle.[22–24]

We hypothesized that the human ('*h* ' prefix for human) clock genes *hPer1, hCry1, hBmal1, hClock*, and *hTim*, would be expressed in human oral mucosa and skin with a circadian profile analogous to that reported for rodent peripheral tissues (relative to the activity/rest cycle). Based on the known circadian coordination of proliferation in oral mucosa, we hypothesized that TS enzyme activity in oral mucosa would have a circadian rhythm with peak activity in early afternoon. We hypothesized also that there might be a predictable association between clock gene expression and cell-cycle phases.

Materials and Methods

Study Participants, Tissue Biopsies, and Serum Samples

The Sunnybrook Health Science Center Ethics Review Board approved the study protocol, and all study participants signed an informed consent. All study participants were healthy males, mean age 27.3 years (range, 22 to 33 years). The average time of sleep onset was 23:30 hours (range, 22:30 to 01:00 hours) and that for awakening was 07:15 hours (range, 06:30 to 09:00 hours). No attempt was made to synchronize the sleeping habits of the study participants before the study. The study participants slept during the night of the procedure except for the 30 minutes required to perform the biopsies at midnight and at 04:00 hours. All study participants had lunch at 12:30 hours and dinner at 20:30 hours. Snacks and cold drinks were allowed at the study participant's discretion at other times. The six biopsies, from each of the eight study participants, were obtained during a single 24-hour period beginning at 08:00 hours on December 5, 1998.

Buccal mucosa and skin biopsies for RNA studies were obtained under local anesthesia using a 3-mm dermatological punch at 4-hour intervals beginning at 08:00 hours. Skin biopsies were obtained from the right hip, in an area not exposed to sun. All tissues were immediately snap-frozen in liquid nitrogen and stored at −70°C. A second biopsy was taken from the oral mucosa from all study participants at the same times for a separate study of TS enzyme activity. Serum was separated from blood and stored at −70°C until assayed for cortisol (microparticle enzyme immunoassay, Abbot Diagnostics) and melatonin.[25]

RNA Isolation

Tissues were thawed and then minced using a homogenizer. Total RNA was extracted using Trizol (Gibco BRL) according to the manufacturer's specifications. cDNA was generated by reverse transcription (Gibco BRL) of 2 μg of total RNA in a volume of 20 μl of buffer containing 2.5 mmol/L DTT, 20 U RNase inhibitor, 50 pmol random primer, 100 nmol dNTP, and 200 U of reverse transcriptase. The mix was incubated for 1 hour at 37°C, followed by three times dilution, and inactivation at 65°C for 10 minutes before storage at −20°C.

Polymerase Chain Reaction (PCR)

Primers were designed based on published data on human homologues of the clock genes in GenBank as follows: *hClock*: forward (f) AAGTTAGGGCTGAAAGACGACG, reverse (r) GAACTCCGAGAAGAGGCAGAAG, product size 171 bp. *hPer1*: f-CTGAGGAGGCCGAGAGGAAAGAA, r-AGGAGGAGGAGGCACATTTACGC, 132 bp; *hBmal1*: f-AAGGATGGCTGTTCAGCACATGA, r-CAAAAATCCATC-TGCTGCCCTG, 132 bp; *hTim*: f-GGAGAAAGCTCAGCAG-CATGATGA, r-TGCTCAATGAAGTGGAAGGTACGG, 138 bp; *hCry1*: f-ATCTAGCCAGGCATGCAGTT, r-CTCCAA-TCTGCATCAAGCAA, 132 bp; *β-actin*: f-GGGGCTGTGCT-GTGGAAGCTAA, r-GTGCCAGGGCAGTGATCTCCTT, 208 bp; *TS*: f-CCAAAGCTCAGGATTCTTCG, r-GCACCCTAAA-CAGCCATTTC, 119 bp.

Duplex PCR was performed in a 25-μl reaction mixture containing 1 μl of cDNA, 0.2 μmol/L of each primer, 200 μmol/L dNTP, 2 U of *Taq* polymerase (Gibco BRL), and buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L potassium chloride, 1.5 mmol/L magnesium chloride, and 1% Triton X-100). Each PCR reaction consisted of one cycle at 94°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 90 seconds, with an additional 7-minute cycle at 72°C at the end of the reaction. First, primers for *hClock* were co-amplified with *β-actin*. After showing that there was no variation in the expression of *hClock* throughout a 24-hour period, primers for *hBmal1, hTim, hPer1, hCry1*, and *TS* were amplified together with *hClock* in a duplex PCR. Products were analyzed by the electrophoresis of 10 μl of PCR product through 2% agarose gel. The relative intensity of each band was compared using computer densitometry, and the results expressed as a ratio of the density for the gene of interest to that of the control gene in each sample.

TS Enzyme Activity

TS enzyme activity was studied only in oral mucosa to allow us to correlate the clock gene data with our previous data on cell-cycle progression in oral mucosa. Frozen tissues were thawed and homogenized in fixed volumes of ice-cold 200 mmol/L Tris-HCl, pH 7.4, with 100

mmol/L NaF, 20 mmol/L β-mercaptoethanol, and a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) by mechanical homogenization using 40 complete strokes with a glass Dounce homogenizer. The homogenates were centrifuged at 4°C at 12,000 × g for 10 to 20 minutes, the supernatant fluid recovered, and protein content determined by the dye-binding method of Bradford.[26]

TS catalytic activity was determined using a tritium release assay by the method of Armstrong and Diasio[27] with modifications. TS activity was determined on 35-μl freshly prepared 12,000 × g tissue supernatants in a final volume of 50 μl in 200 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaF, 20 mmol/L β-mercaptoethanol, protease inhibitors, 5 μl of cofactor solution (6.5 mmol/L tetrahydrofolic acid, 65 mmol/L NaHCO3, 65 mmol/L formalin, 0.25 mol/L β-mercaptoethanol, and 40 mmol/L sodium ascorbate, pH 7.0) and 10 μl of 50 μmol/L [5-³H]-2'-deoxyuridine 5'-monophosphate (20 Ci/mmol; Moravek Biochemical, Brea, CA) and 25 mmol/L CMP. Linearity was established for the working range of protein concentrations and incubation times at 37°C. Samples were incubated in triplicate at 37°C. At one time point, the radioactivity in the acid soluble fraction, after acid charcoal treatment, was determined. Background counts were consistently <5% of total input counts and standard deviations of triplicate determinations were <15%.

Data Analysis

The single cosinor method[28] was used to analyze for circadian rhythm individually and as a group, using both original data and data normalized from 0 to 1 (the six original data points for each variable throughout 24 hours divided by the largest value). This inferential method involves fitting a curve of a predefined period(s) by the method of least squares. The rhythm characteristics and their dispersions standard error (SE) and 95% confidence interval (CI)) estimated by this method include the mesor (middle value of the fitted cosine representing a rhythm-adjusted mean), the amplitude (half the difference between the minimum and maximum of the fitted cosine function), and the high point or acrophase (time of peak value in the fitted cosine function expressed as the lag in hours and minutes from midnight). The waveform of a time series may sometimes be more accurately approximated by the least squares fit of a multiple-component cosine model involving a concomitant fit of two or more components (ie, 24 hours plus 12 or 8 or 3 hours, etc). Each time series was tested for a circadian rhythm by the fit of a 24-hour single cosine and a 24- and 12-hour composite cosine model. The latter model was informative (ie, the 12-hour component significantly decreased residual error estimates beyond that of the 24-hour component alone) only for the analysis of *hPer1*. Detection of rhythm was achieved by rejection of the zero amplitude hypothesis with 95% certainty as reflected by the *P* value resulting from a comparison of residuals before and after the cosine curve fit. Rhythm characteristics (mesor, amplitude, acrophase) for each variable were compared
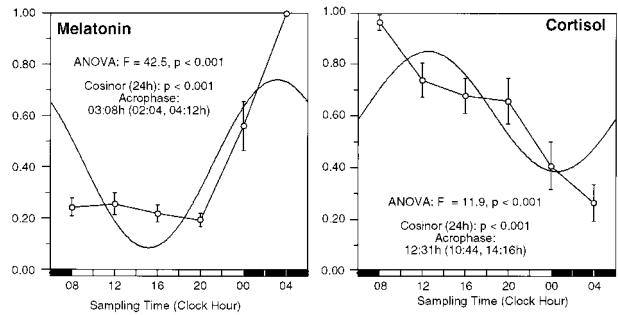

Figure 1. Melatonin and cortisol rhythms during the 24-hour biopsy period. Normalized data for the serum melatonin and cortisol in eight study participants during the 24-hour biopsy period are shown. Time point means (normalized value) and SE are depicted along the 24-hour time scale. The best fitting 24-hour single component cosine curve is shown. The time for the acrophase (95% confidence limits), *P* value from cosinor analysis, and time-effect by analysis of variance are shown.

between sites (skin *versus* oral mucosa) by a parameter test.[29] The six sampling time-normalized means were also analyzed for time-effect by one-way analysis of variance (*F* and *P* values).

Results

Circadian Coordination of Study Participants

Cortisol and melatonin rhythms were studied in each individual during the biopsy period to further objectively document normal circadian time keeping and complement the daily patterns of reported sleep and activity. During the 24-hour biopsy period, all study participants showed the expected circadian variation in serum cortisol and melatonin (Figure 1) with peak values early in the day for cortisol (12:31 hours, cosinor, $P < 0.001$; analysis of variance: $F = 11.9$, $P < 0.001$) and at night for melatonin (03:08 hours, cosinor, $P < 0.001$; analysis of variance: $F = 42.5$, $P < 0.001$).

The peak time for cortisol by the cosinor curve fit is later than expected because of 12-hour components that are particularly prominent in two individuals (study participants 7 and 8). The raw data for cortisol show a peak at 08:00 hours for study participants 1 to 6. Study participant 7 has a primary peak at 0800 hours and a secondary peak at 2000 hours. Study participant 8 has a primary peak at 1200 hours and a secondary peak at 0800. When a 12-hour component is added to the model for cortisol, the peak is at 0924 hours. The raw data for melatonin show a peak at 0400 hours for all study participants.

Circadian Pattern of Clock Gene Expression

Figure 2, A through F, shows representative panels for the duplex PCR with *hClock* co-amplified with β-actin, and *hPer1*, *hCry1*, *hBmal1*, *hTim*, and *TS*. There was no significant 24-hour variation in *hClock* expression using β-actin as control (analysis of variance: $F = 0.7$, $P = 0.593$; cosinor: $P = 0.162$). Therefore, *hClock* was used in subsequent experiments as control for *hBmal1, hPer1, hTim, hCry1*, and *TS*. The data are summarized in Tables 1, 2, and 3.
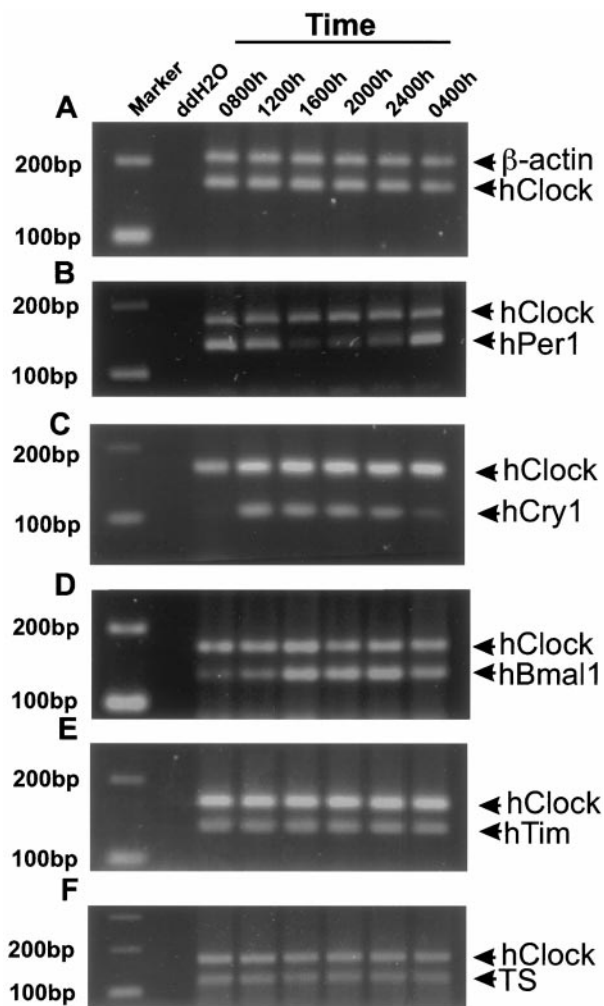
Figure 2. Examples of the results for duplex PCR for gene expression in human oral mucosa. Primers for *hClock* were co-amplified with β-*actin* (**A**). After showing that there was no significant variation throughout 24 hours in the expression of *hClock versus* β-*actin*, *hPer1* (**B**), *hCry1* (**C**), *hBmal1* (**D**), *hTim* (**E**), and *TS* (**F**) were co-amplified with *hClock*. **A** to **F** show the relative expression of all four clock genes throughout 24 hours, a control lane (ddH₂O), and a marker lane.

hClock and hTim

There was no significant 24-hour variation in *hClock* expression (Figure 3) in oral mucosa (analysis of variance: $F = 0.7$, $P = 0.593$; cosinor, $P = 0.162$) or skin (analysis of variance: $F = 0.6$, $P = 0.722$; cosinor, $P = 0.293$). The expression of

hTim (Figure 3) throughout 24 hours was also nonrhythmic in both oral mucosa (analysis of variance: $F = 0.7$, $P = 0.638$; cosinor, $P = 0.265$) and skin (analysis of variance: $F = 0.8$, $P = 0.638$; cosinor, $P = 0.294$).

hPer1

Although there is a significant 24-hour variation in the expression of *hPer1* in both oral mucosa and skin (Tables 1 and 2), a 12/24-hour composite cosinor model, explains a greater amount of the variance in the data and predicts better the times of day for peak values than the 24-hour single cosinor model (Table 3 and Figure 4). With the 12/24-hour composite cosine model, two significant daily peaks in *hPer1* expression were detected in both tissues. The first and dominant peak was at 0758 hours (95% CI, 0628 to 0928 hours; cosinor, $P < 0.001$) and 1051 hours (95% CI, 09:40 to 1202 hours; cosinor, $P = 0.013$) in oral mucosa and skin, respectively. The predicted peak times for the major peaks in oral mucosa and skin are now much closer in time than with the 24-hour fit, but the timing of the peaks is still significantly different ($P < 0.001$ from parameter test). The second and smaller peak in *hPer1* expression was at 1958 hours (95% CI, 1828 to 2128 hours; cosinor, $P = 0.045$) and 2251 hours (95% CI, 21:40 to 00:02 hours, cosinor, $P = 0.01$) in oral mucosa and skin, respectively. The timing of these peaks was also significantly different ($P = 0.036$ from parameter test). The percent amplitude of the primary peak for *hPer1* was significantly lower in skin than in oral mucosa (65.5% *versus* 31.1%; $P = 0.0182$). In the oral mucosa, all eight study participants had peak measured (raw data) *hPer1* expression at 0800 hours. In the skin, five of eight study participants had peak measured *hPer1* expression at 1200 hours, with the remaining three peaking between 0000 and 0800 hours.

hBmal1 and hCry1

There was a significant 24-hour variation (Figure 4) in the expression of *hBmal1*, in both oral mucosa (analysis of variance: $F = 25.7$, $P < 0.001$) and skin (analysis of variance: $F = 6.6$, $P < 0.001$). A 24-hour cosinor fit to these data demonstrates a significant circadian rhythm with peak expression after dusk in both oral mucosa (2140 hours; 95% CI, 2100 to 2220 hours; cosinor, $P <$

Table 1. Normalized Data for the Expression of Clock Genes, TS mRNA, and TS Activity in Human Oral Mucosa

Variable	Mesor, ± SE	Amplitude, ± SE	Amplitude, %	Acrophase and 95% CI for 24 hour fit (hour:min)	Cosinor P value (24 hour)	Analysis of variance P value
hPer1	0.55 ± 0.03	0.29 ± 0.04	52.7	08:29 (07:16 to 09:40)	<0.001	<0.001
hCry1	0.66 ± 0.03	0.22 ± 0.04	33.3	17:04 (15:36 to 18:36)	<0.001	<0.001
hBmal1	0.65 ± 0.02	0.35 ± 0.03	53.8	21:40 (21:00 to 22:20)	<0.001	<0.001
hClock	0.77 ± 0.03	0.07 ± 0.04	—	—	0.162	0.593
hTim	0.78 ± 0.03	0.06 ± 0.04	—	—	0.265	0.638
TS mRNA	0.83 ± 0.02	0.04 ± 0.03	—	—	0.497	0.218
TS Activity	0.67 ± 0.03	0.16 ± 0.05	23.9	13:27 (11:00 to 16:00)	0.008	0.076

SE, standard error; CI, confidence interval for the best fitting 24-hour single component cosine curve.

Table 2.   Normalized Data for the Expression of Clock Genes in Human Skin

Variable	Mesor ± SE	Amplitude ± SE	Amplitude, %	Acrophase and 95% CI for 24 hour fit (hour:min)	Cosinor P value (24 hour)	Analysis of variance P value
hPer1	0.74 ± 0.03	0.13 ± 0.05	17.6	14:44 (11:44 to 17:44)	0.024	0.005
hBmal1	0.75 ± 0.02	0.16 ± 0.03	21.3	22:14 (20:48 to 23:40)	<0.001	<0.001
hClock	0.78 ± 0.03	0.06 ± 0.04	—	—	0.293	0.722
hTim	0.84 ± 0.02	0.05 ± 0.03	—	—	0.294	0.562

SE, standard error; CI, confidence interval for the best fitting 24-hour single component cosine curve.

0.001) and skin (2214 hours; 95% CI, 2048 to 2340 hours; cosinor, $P < 0.001$). The timing of the peak for *hBmal1* in oral mucosa was identical to that in skin ($P = 0.462$ for difference). The percent amplitude of the variation in *hBmal1* was significantly lower in skin than in oral mucosa (21.3% *versus* 53.8%; $P < 0.0001$). In all eight study participants, peak measured *hBmal1* expression occurred between 2000 hours and 0000 hours both in the oral mucosa and skin.

There was a significant 24-hour variation (Figure 4) in the expression of *hCry1* in oral mucosa with peak expression at 17:04 hours (95% CI, 1536 to 1836 hours; cosinor, $P < 0.001$; analysis of variance: $F = 8.7$, $P < 0.001$). In seven of eight study participants peak measured *hCry1* expression occurred between 1200 hours and 1600 hours, with one study participant peaking at 2000.

Circadian Pattern of TS Activity and TS mRNA Levels in Oral Mucosa

There was a significant circadian variation in TS activity (Figure 5) in oral mucosa with peak values in early afternoon at 1327 hours (95% CI, 1100 to 1600 hours, cosinor, $P = 0.008$; analysis of variance: $F = 2.2$, $P = 0.076$). The mean value for oral mucosal TS activity in the eight study participants was 8.5 ± 0.73 pmol/min/mg with a range of individual's mean values of 6.7 to 16.6 pmol/minute/mg. Within individuals, oral mucosa TS activity varied 1.4- to 5.1-fold (mean, 3.2-fold) throughout the day, with values varying from 1.5 to 27.8 pmol/minute/mg. In all eight study participants, peak measured TS activity occurred between 0800 hours and 1600 hours, with six study participants having peak TS activity between the 4-hour time span from 1200 hours to 1600 hours. Protein yields in the tissue supernatant preparations failed to vary with time of day suggesting that these time of day differences in TS activity are not the result of differential dilution by variable amounts of total protein throughout the day.

There was no circadian variation in TS mRNA expression in oral mucosa (cosinor, $P = 0.497$; analysis of variance: $F = 1.5$, $P = 0.218$).

Discussion

Clock Gene Expression in Human Tissues

This is the first study to show a rhythmic expression of clock genes in peripheral human tissue throughout 24 hours. Study participants had a normal circadian activity profile as documented by their reported sleep onset/awakening times and their rhythm in cortisol and melatonin. We have shown that *hClock*, *hTim*, *hPer1*, *hCry1*, and *hBmal1* are expressed in human oral mucosa and skin, with a circadian profile that is consistent with that found in the SCN and peripheral tissues of mice and rats.[3] *hPer1*, *hCry1*, and *hBmal1* show a rhythmic expression, peaking early in the morning (early activity), in late afternoon and at night (late activity), respectively, whereas *hClock* and *hTim* are not rhythmic. In the mouse SCN, *mPer1* peaks soon after subjective dawn (early activity), *mCry1* peaks later in the day, and *mBmal1* peaks after subjective dusk (late activity), whereas *mClock* and *mTim* expression is not rhythmic. In rodent peripheral tissues the clock gene expression follows the same pattern, but *Per1*, *Cry1*, and *Bmal1* peak 4 to 6 hours later than in the SCN.[10,11] These data suggest that the human clock genes may be functionally important for the molecular control of the human circadian pacemaker, thus extending the homology that exists between the clock genes in *Drosophila* and rodents to humans. The functional importance of the clock genes in man is further suggested by the recent finding of *hClock* expression in the human SCN[30] and that a *hClock* polymorphism is associated with an inherited human diurnal preference (eveningness).[31]

When the data for *hPer1* expression in oral mucosa and skin are analyzed using a 24/12 composite cosine model, two significant peaks (a major and a minor one)

Table 3.   Normalized Data for the Expression of *hPer1* in Human Oral Mucosa and Skin (12/24 Composite Cosinor Model)

Variable	Mesor ± SE	Amplitude ± SE	Amplitude, %	Acrophase and 95% CI for 24/12 hour fit (hour:min)	Cosinor P value (24/12 hour)	Analysis of variance P value
Mucosa *hPer1/pp*	0.55 ± 0.03	0.36	65.5	07:58 (06:28 to 09:28)	<0.001	<0.001
Skin *hPer1/pp*	0.74 ± 0.03	0.23	31.1	10:52 (09:40 to 12:04)	0.013	0.005

SE, standard error; CI, confidence interval for the best fitting 24-hour single component cosine curve; pp, primary peak.
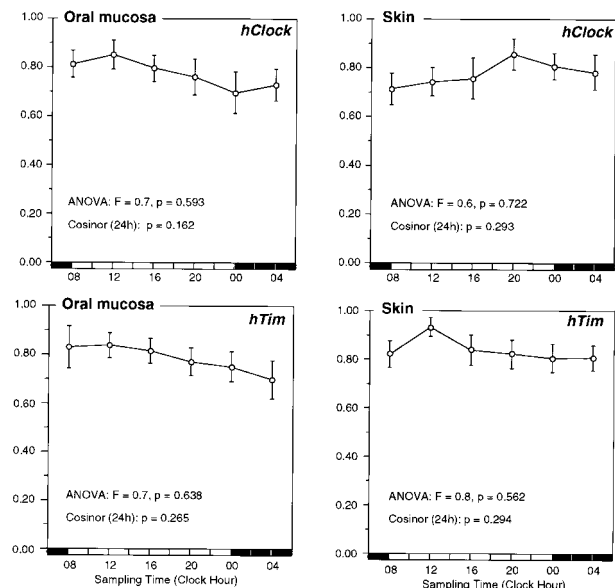
Figure 3. The timing of *hClock* and *hTim* expression in oral mucosa and skin. Normalized data for the circadian expression of *hClock*, and *hTim* in human oral mucosa and skin of eight study participants. Time point means (normalized values) and SE are depicted along the 24-hour time scale.

are detected in both tissues. In rodents, *Per1*, *Per2*, and *Per3* expression peaks several hours apart during a 4- to 6-hour period within a given tissue.[11] Previous reports of *per* gene expression in peripheral tissues of rodents have documented one major 24-hour peak for each *per* gene. Some of these studies, however, used a limited number of sampling times and analysis for multiple rhythm components were not performed. Although the two daily peaks for *hPer1*, seen here, could represent hybridization of the *hPer1* primers with more than one variant of *hPer* with different times of peak expression, a truly biphasic expression of *hPer1* is possible and warrants further study.

Sampling every 4 hours is used commonly in human studies and rodent studies in chronobiology. Although more frequent sampling might allow for a better detection of rhythms with a shorter period than 24 hours, it could also interfere with and distress the study participants to the extent of masking the circadian rhythm. In discussions with our ethics committee, every 4-hour sampling was seen as the most we could expect our study participants to endure. This sampling frequency proved adequate to detect the expected circadian variation in clock gene expression.

TS in Oral Mucosa

This is the first study to look at the enzyme activity of TS in human tissue throughout 24 hours. The finding of a significant circadian rhythm in this S-phase marker further supports our previous finding of a circadian coordination of cell-cycle events in oral mucosa.[16] The timing of the peak of TS activity correlates well with the timing of S phase in the previous study (Figure 6, A and B3). A circadian variation has been demonstrated in rodent tis-
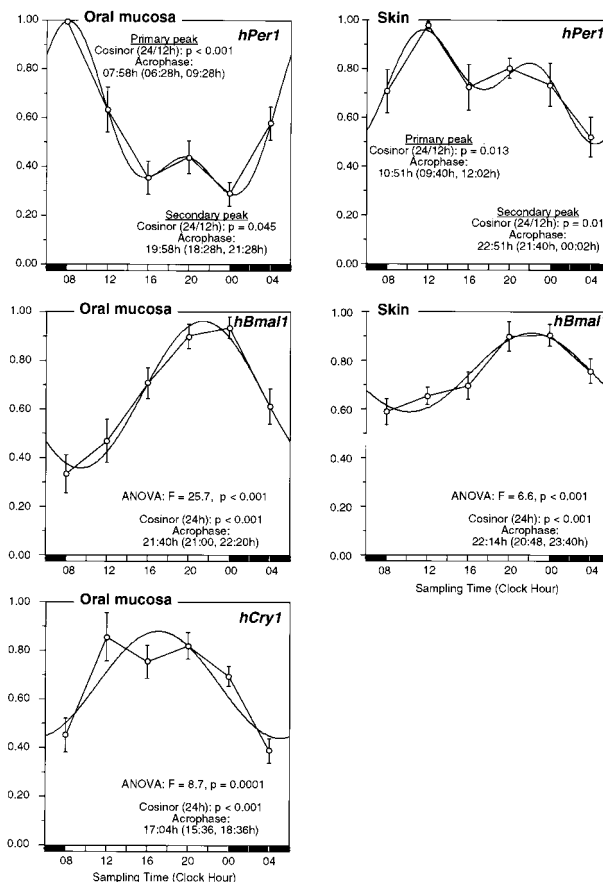

Figure 4. The timing of *hPer1*, *hBmal1*, and *hCry1* expression in oral mucosa and skin. Normalized data for the circadian expression of *hPer1*, *hCry1*, and *hBmal1* in human oral mucosa and skin of eight study participants. Time point means (normalized values) and SE are depicted along the 24-hour time scale. For *hBmal1* and *hCry1*, a 24-hour single-component cosine curve is shown along with the predicted acrophase (95% confidence limits) and *P* values from cosinor analysis and time-effect by analysis of variance. For *hPer1*, a 12/24 hours composite cosinor curve is shown along with the predicted acrophases for each peak (95% confidence limits) and *P* values for each rhythm component from cosinor analysis.

sue TS enzyme activity[32] and rodent and *in vitro* studies have shown that peak TS activity coincides with S phase.[22–24]
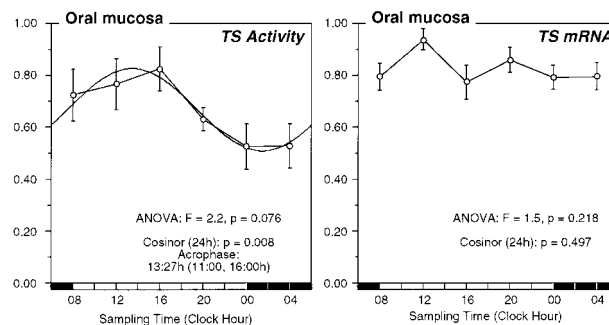

Figure 5. Circadian TS activity and TS mRNA expression in oral mucosa. Normalized data for the circadian variation in TS enzyme activity and *TS* mRNA expression in human oral mucous in eight study participants. Time point means (normalized value) and SE are depicted along the 24-hour time scale. The best fitting 24-hour single-component cosine curve is shown. The time for the acrophase (95% confidence limits), *P* value from cosinor analysis, and time-effect by analysis of variance are shown.
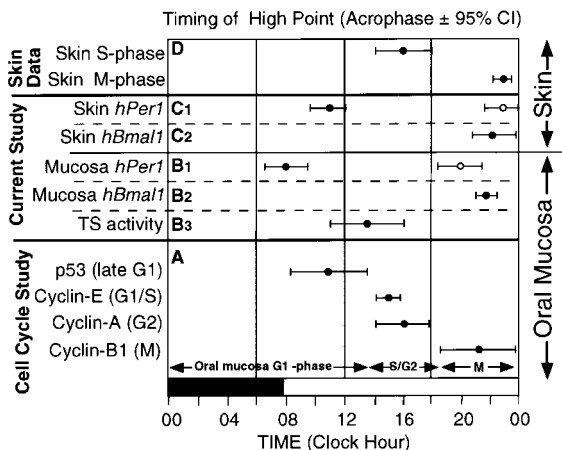
Figure 6. Association of circadian clock gene expression with the timing of cell-cycle phases. **A:** The high points for the best-fitting cosine for p53, cyclin-E, cyclin-A, and cyclin-B1 are depicted along the 24-hour time scale based on our previous study on human oral mucosa. The 95% confidence limits for each variable are shown. The cell-cycle phase, for which each protein is a marker, is shown in parentheses on the *y* axis and on the graph. **B:** The acrophases for the major (**filled circle**) and minor (**open circle**) peaks of expression for *hPer1* (**B1**) and for the single peak in expression of *hBmal1* (**B2**) in oral mucosa are depicted along the 24-hour time scale. In **B3**, the acrophase for the peak activity of TS (S phase) in oral mucosa is depicted along the 24-hour time scale. The 95% confidence limits for each variable are shown. **C:** The acrophases for the major (**filled circle**) and minor (**open circle**) peaks of expression for *hPer1* (**C1**) and for the single peak in expression of *hBmal1* (**C2**) in skin are depicted along the 24-hour time scale. The 95% confidence limits for each variable are shown. **D:** The acrophases for S phase and M phase in human skin based on pooled data from 12 studies looking at M phase and 14 studies looking at S phase throughout 24 hours are depicted along a 24-hour time scale. The 95% confidence limits for each variable are shown.

There was no rhythm in the expression of TS mRNA in oral mucosa, implying that the control of the rhythmic TS enzyme activity is at the posttranscriptional level. This is consistent with several studies showing that the acute induction of TS as cells enter S phase is controlled primarily at the translational level.[23,24,33,34]

TS is the target for chemotherapy drugs such as 5-FU, FdUrd, and methotrexate, and also for several new folate-based TS inhibitors and multi-targeted antifolates. Studies of the circadian timing of FdUrd and 5-FU in rodents and humans show large differences in gastrointestinal toxicity and antitumor response depending on the time of day of therapy.[35–37] In a prospective randomized clinical trial, optimal timing (for reduced toxicity) of 5-FU-based chemotherapy reduced fivefold (14% *versus* 76%, $P < 0.0001$) the rate of severe oral mucosal toxicity.[37] Our findings of a circadian variation in both cell-cycle progression[16] and TS enzyme activity in human oral mucosa may partly explain this significant time-dependent incidence of 5-FU-induced oral mucosal toxicity.

Human Cell-Cycle Progression May Be Gated by the Circadian Clock

Gating of the cell division cycle by the circadian clock has been demonstrated in several unicellular organisms including *Euglena, Cyanobacteria, Chlamydomonas, Paramecium, Tetrahymena*, and *Gonyaulax polyedra*.[38–43] The data on the TS enzyme activity in oral mucosa allow

timing of the peak in S phase in relation to the peak expression of the clock genes (Figure 6, B3). The timing of the TS peak in early afternoon agrees well with the time of peak S-phase markers in our previous study (Figure 6A) of cell-cycle progression in human oral mucosa.[16] In oral mucosa, the major peak in *hPer1* expression coincides with the peak in a G_1 phase marker (p53) but precedes the peak in markers of S phase (cyclin E and TS). The peak for *hBmal1* coincides with an M-phase marker (cyclin B1).

The information on human skin proliferation (Figure 6D) is based on our cosinor analysis of pooled data from 14 studies looking at the timing of S phase (peak, 16:06 hours; 95% CI, 14:04 to 18:08 hours), and 12 studies looking at the timing of M phase (peak, 22:54 hours; 95% CI, 22:12 to 23:36 hours).[44] Although we have not measured proliferation markers in the skin samples in this study, the coincidence of peaks in specific cell-cycle stages in skin compared to the oral mucosa is striking. With these limitations in mind, the major peak in *hPer1* expression in skin may also coincide with the peak in G_1 phase, whereas the peak in *hBmal1* may coincide with the peak in M phase (Figure 6, C and D).

Our correlation of the timing of clock gene expression in oral mucosa with the timing of S phase (TS activity), suggests that the circadian clock may, in part, control the timing of cell-cycle events in tissues undergoing continuous circadian rhythms in proliferation (gastrointestinal mucosa, skin, and bone marrow). That the cell cycle may be influenced by the circadian rhythm, is supported by the observation that phase-shifting of mice leads to a corresponding shift (throughout 3 to 4 weeks) in the timing of cell-cycle events both in gut and bone marrow.[45,46] Such phase-shifting has recently been shown to be associated with a corresponding rapid shift (1 day) in the circadian expression of *Per1* in rat SCN and a delayed shift (≥ 6 days) in the *Per1* rhythm in peripheral tissues.[47] *Per1* peak expression has been found to coincide with the peak expression of clock-controlled genes that control downstream circadian processes.[48,49] In our study, the *hPer1* peak coincides with the peak in oral mucosa G_1 phase, where the main restriction point controlling progression through the cell cycle resides.[50] Although no cause and effect relationships between clock gene expression and cell proliferation can be claimed by our results, these data set the stage for a testable hypothesis to relate these two fundamental processes.

Many mouse and rat organs that are not actively proliferating (muscle, kidney, etc) have been shown to have a circadian expression of *Per, Bmal1*, and *Cry*.[10,11,13,14] Active tissue proliferation is therefore not a requisite for synchronous clock gene expression. In these tissues the clock genes may provide clock control of nonproliferative metabolic and physiological processes via the expression of clock-controlled genes.[51]

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