

## Circadian Expression of Clock- and Tumor Suppressor Genes in Human Oral Mucosa

Derek Zieker<sup>1,2,\*</sup>, Isabel Jenne<sup>2,\*</sup>, Ingmar Koenigsrainer<sup>1</sup>, Marty Zdichavsky<sup>1</sup>, Kay Nieselt<sup>3</sup>, Katharina Buck<sup>3</sup>, Judith Zieker<sup>2,4</sup>, Stefan Beckert<sup>1</sup>, Joerg Glatzle<sup>1</sup>, Rainer Spanagel<sup>5</sup>, Alfred Koenigsrainer<sup>1</sup>, Hinnak Northoff<sup>2</sup> and Markus Loeffler<sup>1,2</sup>

<sup>1</sup>Department of General, Visceral and Transplant Surgery, <sup>2</sup>Department of Transfusion Medicine, <sup>3</sup>Department of Information and Cognitive Sciences, Center for Bioinformatics Tuebingen, <sup>4</sup>Department of Psychiatry, University of Tuebingen, Tuebingen; <sup>5</sup>Central Institute of Mental Health, University of Mannheim, Mannheim, \*The first two authors contributed equally to this work

### Key Words

Circadian Gene Expression • Clock Gene • Tumor Suppressor • Oral Mucosa • Microarray

### Abstract

**Purpose:** Circadian rhythms are daily oscillations of multiple biological processes driven by endogenous clocks. Imbalance of these rhythms has been associated with cancerogenesis in humans. To further elucidate the role circadian clocks have in cellular growth control, tumor suppression and cancer treatment, it is revealing to know how clock genes and clock-controlled genes are regulated in healthy humans. **Materials and Methods:** Therefore comparative microarray analyses were conducted investigating the relative mRNA expression of clock genes throughout a 24-hour period in cell samples obtained from oral mucosa of eight healthy diurnally active male study participants. Differentially expressed selected genes of interest were additionally evaluated using qRT-PCR. **Results:** Microarray analysis revealed 33 significant differentially regulated clock genes and clock-controlled genes, throughout a one day period (6.00h, 12.00h, 18.00h, 24.00h). Hereof were 16 clock genes and 17 clock-

controlled genes including tumor suppressor- and oncogenes. qRT-PCR of selected genes of interest, such as hPER2, hCRY1, hBMAL1, hCCRN4L and hSMAD5 revealed significant circadian regulations. **Conclusion:** Our study revealed a proper circadian regulation profile of several clock- and tumor suppressor genes at defined points in time in the participants studied. These findings could provide important information regarding genes displaying the same expression profile in the gastrointestinal tract amounting to a physiological expression profile of healthy humans. In the future asynchronous regulations of those genes might be an additional assistant method to detect derivations distinguishing normal from malignant tissue or assessing risk factors for cancer.

Copyright © 2010 S. Karger AG, Basel

### Introduction

Circadian rhythms, synchronized to external environmental cycles such as day and night, are daily oscillations of multiple biological processes driven by

### KARGER

Fax +41 61 306 12 34  
E-Mail [karger@karger.ch](mailto:karger@karger.ch)  
[www.karger.com](http://www.karger.com)

© 2010 S. Karger AG, Basel  
1015-8987/10/0262-0155\$26.00/0

Accessible online at:  
[www.karger.com/cpb](http://www.karger.com/cpb)

Dr. Derek Zieker,  
Department of General, Visceral and Transplant Surgery  
University of Tuebingen, Hoppe-Seyler-Strasse 3, D-72076 (Germany)  
Tel. +49-7071-2981658, Fax +49-7071-295459  
E-mail [derek.zieker@med.uni-tuebingen.de](mailto:derek.zieker@med.uni-tuebingen.de)

endogenous clocks. 'Circadian' is a Latin word, meaning 'about a day'. Thus, the circadian rhythm of approximately 24 hours is a fundamental physiological principle in mammals and a successful evolutionary strategy. Molecular-genetic analyses in vertebrates have led to evidence that constant changes of gene expression are crucial in the generation of circadian rhythmicity (clock genes) and controlling further output genes (clock-controlled genes) [1-3]. In humans circadian rhythms regulate various physiological functions, such as sleep and wake, vigilance, digestive secretion and immune activity. Therefore imbalance of these rhythms can exert a major influence on human health. The disruption of circadian rhythms was linked to cardiovascular diseases, psychiatric diseases and cancer in humans [4-8]. In epidemiological studies a correlation between disrupted diurnal rhythms and elevated incidence of gastrointestinal cancers has been described [9]. Investigations in animal studies and human tumor samples have revealed that the disruption of circadian rhythms is a crucial endogenous factor contributing to carcinogenesis in mammals [10-13].

Since circadian clock genes were related to tumor suppression, we were interested in physiological regulations and similarities in clock- and clock-controlled gene expression of healthy humans. Knowing the common regulation of those genes in healthy humans, differences between normal and malignant tissues concerning asynchronous gene regulation could be distinguished. In the gastrointestinal tract, cancer development mainly accounts for diseases in elderly persons beyond 50 years of age. Hence, within our study we decided to study male participants in the age range of 50 - 60 years, to gain an impression of clock gene- and tumor suppressor gene expression in the digestive tract. Thus we investigated diurnal changes in mRNA-levels in cell samples obtained from oral mucosa of eight healthy male study participants, due to its easy availability. Furthermore, in former research [14, 15] evidence for an expression wave present from oral mucosa to rectum was shown for a single clock gene and coherent cell proliferation. Investigations in oral mucosa thus may allow certain conclusions concerning downstream gastrointestinal tissues.

## Material and Methods

### *Study Participants and cell samples*

The study included eight healthy males aged between 50 and 60 years. All participants were working in the same company at the time assessed with regular daytime working

hours omitting shift work. In the course of the study no participant suffered from acute or chronic disease and based on anamnestic data all of the participants had a blank history for dysplasias or cancer. Further, no participant took any medication and all of them were non smokers. Any attempts during or before the study to synchronize daily activities or sleeping habits were omitted. Anthropometric and health status data of participants are given in Table 1. Tissue samples were obtained from oral mucosa during a two day period every six hours (6.00h, 12.00h, 18.00h, 24.00h) using a standard toothbrush in a time interval from 28<sup>th</sup> June 2007 to 11<sup>th</sup> July 2007. The participants took cell material from both buccal sides and the tongue and converted it immediately to 25ml of RNeasy (Qiagen, Hilden, Germany) which was stored at 4°C until subsequent RNA-extraction. Furthermore the participants were instructed not to brush their teeth prior to sampling and to flush their mouth with water thoroughly immediately prior to sample taking.

The study was designed accordant with the Declaration of Helsinki in its latest available version and the study protocol was evaluated and accepted by the local ethics committee previous to the beginning of the study (112-2007). After participant instruction all of them provided written informed consent to participate in the study and data were handled due to institutional and ethical guidelines.

### *RNA extraction and isolation*

Preliminary testing evaluating the extraction method and storage in RNeasy (Qiagen) as described above was performed and RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), with satisfactory results. RNA-extraction was performed using the RNeasy® Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA quantity was assessed using a Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies). Samples of corresponding time points were pooled and further processed for microarray application.

### *Clock genes and clock associated genes*

Based on literature and homology search we identified 20 clock genes and 70 clock-associated genes in humans. 65-mer oligonucleotides were designed by (Sigma-Aldrich, Taufkirchen, Germany) for the custom-generated microarray.

### *Microarray data extraction and statistical analysis*

Microarray data were generated using 65mer oligonucleotide microarrays produced at the department of transfusion medicine in Tuebingen, Germany, as described previously [16]. Our custom-designed array has a total of 2304 spots, including transcripts, buffers, controls and empty spots. The array contained 789 gene transcripts in total, containing every feature at least in duplicate. Further details concerning the array can be obtained from the National Center for Biotechnology Information (<http://www.ncbi.nlm.gov/geo/>) under GPL 6865.

Study Participant	Age, yr	Weight, kg	Height, cm	Medication	Acute infections	Chronic infections	Alcohol (beer, wine)	Nicotine
1	59	81	178	Diclofenac (occasionally)	no	Herpes labialis	3-4 times/week	no
2	56	70	182	no	no	Herpes labialis	daily	no
3	53	81	173	ASA (occasionally)	no	Herpes labialis	2-3 times/week	no
4	51	85	183	no	no	no	4-5 times/week	no
5	54	80	178	no	no	Herpes labialis	2 times/week	no
6	55	98	184	no	no	no	4-5 times/week	no
7	55	82	180	no	no	no	1-2 times/week	no
8	54	--	--	no	no	Hypertonia	4 times/week	no

**Table 1.** Anthropometric data of the participants. Body and health characteristics and habits of all male study participants.

Gene product	Accession Number	Sense primer	Antisense primer
Cryptochrome 1(CRY1)	NM_004075	CATCCGCTGCGTCTACATCC	CATCAAGATCCTCAAGACACTGAAG
Aryl hydrocarbon receptor nuclear translocator-like (ARNTL/ BMAL1)	NM_001178	CTGCATCCTAAAGATATTGCCAAAG	GTCGTGCTCCAGAACATAATCG
Period homolog 2 (PER2)	NM_022817	TGGATGAAAGGGCGGTCCCT	ACTGCAGGATCTTTTTGTGGA
SMAD family member 5 (SMAD5)	NM_005903	CTTGTGAAGATAAATGTTACTCCTC	AACTCAAAGTCAGTGGCTAC
Nocturnin (CCRN4L)	NM_012118	CGATTCAAGCTAGTCAACAGTGC	CTTTAGATGGGTAACAGCGATGC
Actin, beta (ACTB)	NM_00101	CCGCAAAGACCTGTACGCCAAC	GGAGCCGCCGATCCACACG

**Table 2.** Primer sequences (human) in qRT-PCR (5'-3').

An indirect reference design was chosen with Cy3 labeled uniRNA (Stratagene, La Jolla, CA) and Cy5 labeled sample RNA. Amplification of RNA was performed using the Amino Allyl Message Amp II™ aRNA Amplification Kit (Ambion Inc., Austin, TX) together with Amersham CyDye post-labeling Reactive Pack™ (GE Healthcare, Buckinghamshire, UK) for fluorochrome labeling following manufacturer's protocols. Dye incorporation was assessed using a NanoDrop® ND-1000 spectrophotometer (NanoDrop) and an online calculator (<http://www.ambion.com/dye>). RNA fragmentation was performed (Ambion's fragmentation reagents, Ambion) followed by a hybridization step for 14h at 48°C. Subsequent scanning was conducted using the hybridized and washed microarray slides employing a microarray scanner (Affymetrix Inc., Santa Clara, CA). The photomultiplier tube voltage was set to 100% for both green and red channels. The resulting green and red images were overlaid using ImaGene 5.0 (Biodiscovery Inc., El Segundo, CA) for raw data collection. All array data has been deposited at GEO under GSE 11469.

#### Microarray data preprocessing

Raw data preprocessing and normalization of the microarray data was done using the limma (Linear models for microarray) package for R from the bioconductor project [17]. As a first step in signal extraction, for each channel we used the mean of the pixel distribution for the foreground signal and the median for the background of each spot as estimators for the raw signal values. Raw data were normalized using median-

normalization for intra-array normalization on the normexp-background corrected expression values followed by inter-array quantile normalization across arrays [18]. After normalization the mean expression value of each feature was computed from expression values of the corresponding technical replicates.

#### Identification of periodic gene expression

The aim of our study was to find statistical evidence of cyclicity or periodicity, and to identify this subset of genes that is responsible for this behaviour.

Altogether we used 4 different published methods to identify significant periodically expressed genes. The first is Fisher's g-statistic as described by Wichert et al. [19] followed by the method of FDR as multiple comparison procedure [20]. We used the author's R implementation GeneTS, which has been replaced by GeneCycle (available from the CRAN repository).

The second test is the cyclohedron test which computes a permutation count as a test statistic. To compute periodicity of genes based on the cyclohedron test we used the R implementation topoGraph.R as distributed by the authors of the cyclohedron test.

As a third test we used an adaptation of a method based on cosine curves correlation [21]. We empirically tested for statistical significant cross-correlation between the time point courses of each gene and cosine curves of defined period and phases. We used the cosine curve  $\{t_i, v2\cos(2(t_i-\phi)/24)\}$  with a phase  $\phi$  ( $0 \leq \phi \leq 24h$ ) in increments of 10 min, and calculated the

**Table 3.** Accession Number, periodic time and p-values of circadian regulated genes detected by microarray analysis. Microarray analysis revealed 33 significant differentially regulated clock genes and clock controlled genes throughout a two day period (6.00h, 12.00h, 18.00h, 24.00h) in samples obtained from oral mucosa of eight healthy male participants in the age range from 50-60 years. Thereof were 16 clock genes and 17 clock- controlled genes containing tumor suppressor and oncogenes. Here, all circadian regulated genes with a periodic time (T) and p-value <0.1 are listed.

	Gene Name	Accession Number	Periodic Time (T)	P-value
Clock Genes	PER1	NM_002616	24	0,059
	PER2	NM_022817	24	0,087
	ARNTL/ BMAL1	NM_001178	23,143	0,074
	CRY1	NM_004075	24	0,067
	CRY2	NM_021117	14,087	0,048
	NR1D1	NM_021724	24	0,065
	NR1D2	NM_005126	24	0,086
	CSNK1D	NM_001893	24	0,095
	CSNK1E	NM_001894	24	0,070
	DEC1	NM_017418	24	0,066
	BHLHE41/DEC2	NM_030762	24	0,070
	CCRN4L/ NOC	NM_012118	24	0,064
	PRKCA	NM_002737	23,143	0,063
	MTNR1B	NM_005959	24	0,062
	RASD1	NM_016084	23,143	0,065
	KCNMA1	NM_002247	24	0,078
Tumor suppressor and oncogenes	SMAD5	NM_001001419	24	0,096
	RARRES1 (V1)	NM_206963	22,345	0,063
	RARRES1 (V2)	NM_002888	24	0,071
	HDAC8	NM_018486	21,6	0,086
	MMP9	NM_004994	20,25	0,080
Transcription Factors	RFX4	NM_213594	24	0,073
	HSF2	NM_004506	24	0,092
NADPH-Oxidases	NOX1	NM_013955	24	0,079
	NOX4	NM_016931	24	0,080
Other Genes	SH3BP4	NM_014521	24	0,094
	ACO1	NM_002197	24	0,063
	NAP5	NM_207481	24	0,066
	SLC40A1	NM_014585	20,25	0,075
	HTR7	NM_019860	19,636	0,090
	PTMA	NM_002823	24	0,095
	COL5A1	NM_000093	20,903	0,066
	COL4A1	NM_001845	24	0,093

Pearson correlation value of the best-fitting cosine curve for each gene *i*. We selected the genes with correlation values above the cut off value of 0.8. To compute p-values we employed a permutation test and chose genes with p-value <0.05.

As a fourth test to detect significant periodic gene expression patterns we used the combination of a Lomb-Scargle test statistic for periodicity [22] and a multiple hypothesis testing procedure with controlled false discovery rate.

Because of the low resolution of the time series (4 data points in 24h) we used an interpolation using means to increase the number of time points.

From each method we extracted all genes which showed a significant periodic expression according to a prechosen p-value. Finally we computed a weighted average p-value from all four p-values, and chose those genes with an average weighted p-value less than 0.1. This threshold ensured the best cutoff between sensitivity and specificity according to

simulation studies.

#### *Quantitative real-time PCR (qRT-PCR)*

For affirming microarray results as well as to concentrate on collective gene expression and to minimize the effect of possible outliers, sample RNA was pooled in two random groups of four participants each, of accordant points in time of two subsequent days. Afterwards sample RNA was transcribed using the Transcriptor First Strand cDNA Synthesis Kit™ and random hexamer primers (Roche, Mannheim, Germany). Selected cDNAs were quantified by qRT-PCR using a Light Cycler® I instrument (Roche). For quantification of amplicons the SYBR® Green Jump Start Taq ReadyMix™ and sequence specific primers (Sigma Aldrich, St. Louis, MO) were used adhering to manufacturer's protocols. Specificity of PCR conditions was assured determining the melting temperatures of products. Primer sequences and accession numbers can be referred to in Table 2.

### Statistical analysis and graphical depiction

Relative quantification by qRT-PCR to assess diurnal trends of mRNA expression was performed using REST 2008 (Relative Expression Software Tool) [23]. Sample ratios were calculated using previous points in time as a calibrator value, thus relative changes can be presented. For normalization beta-actin transcripts were measured and reaction efficiencies were assessed employing dilution series of the respective PCR-products and employed in calculations. Differences in between samples and respective previous calibrators were calculated employing a pair wise fixed reallocation randomization test [23]. The significance level was set to p-values <0.01. Respective p-values, standard error ranges and relative expression values are displayed (Table 4).

For graphical presentation (Fig. 1) of expression trends of clock- and clock-controlled genes, the ratio of beta-actin mRNA amount relative to each target gene transcript was calculated at respective points in time [14, 24, 25]. Trends of both pools mentioned and the respective mean values are given separately. The highest mean ratio in the course of day was arbitrarily set to 100.

Relative expression values obtained by REST were calculated to get diurnal mRNA expression profiles (Fig. 2). As sample data 6h, 12h and 18h were employed whereas 24h was set as a calibrator, normalized by beta-actin as a reference gene transcript.

The respective sinus functions were calculated using a calculator (CASIO fx 9860G SD) based on REST data to obtain circadian mRNA oscillations. Concerning hBMAL1 expression a sinus regression was uncalculable.

## Results

Microarray analysis revealed 33 significant differentially regulated clock genes and clock-controlled genes throughout a one day period (6.00h, 12.00h, 18.00h, 24.00h) in cell samples obtained from oral mucosa of eight healthy male study participants in the age range from 50-60 years. Hereof were 16 clock genes and 17 clock-controlled genes, tumor suppressor- and oncogenes. All circadian regulated genes with circadian periodicity and a periodic time (T) and p-value <0.1 are listed in Table 3. Most of these differentially regulated genes showed a periodic time regulation of 24 hours. The clock genes human clock (hCLOCK) and human timeless homolog (hTIM) did not reveal any circadian regulation in our investigations.

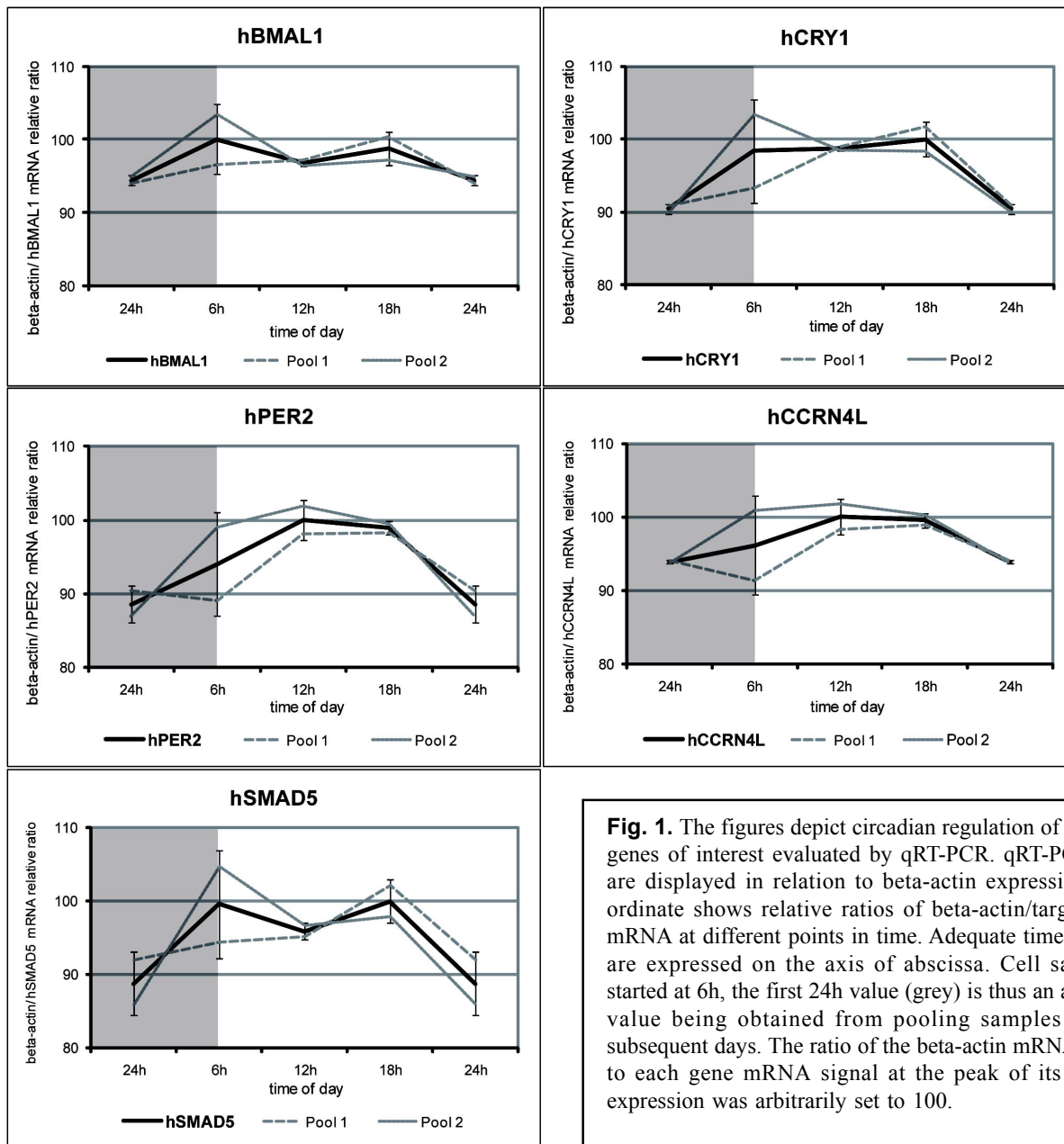
Selected genes of interest were further evaluated in their regulation using qRT-PCR. These genes were human clock genes human Period homolog 2 (hPER2), human Cryptochrome 1 (hCRY1), human Bmal1 (hBMAL1) and human Nocturnin (hCCRN4L) and the tumor suppressor gene human mothers against

Gene product	Control (Calibrator)	Time (sample)	Result (relative expression) SE range	p-value
<b>hBMAL1</b>	6h	12h	<b>0,528</b> 0,359 – 0,79	0,339
	12h	18h	<b>1,417</b> 1,139 – 1,799	0,341
	18h	24h	<b>0,789</b> 0,631 – 0,986	0,49
	24h	6h	<b>1,679</b> 1,151 – 2,503	<b>&lt;0,01 *</b>
<b>hCRY1</b>	6h	12h	<b>1,232</b> 0,556 – 2,759	0,661
	12h	18h	<b>1,056</b> 0,945 – 1,192	0,341
	18h	24h	<b>0,255</b> 0,221 – 0,299	<b>&lt;0,01 *</b>
	24h	6h	<b>3,009</b> 1,352 – 6,793	<b>&lt;0,01 *</b>
<b>hPER2</b>	6h	12h	<b>4,282</b> 2,020 – 9,667	<b>&lt;0,01 *</b>
	12h	18h	<b>0,523</b> 0,410 – 0,691	0,169
	18h	24h	<b>0,135</b> 0,068 – 0,277	<b>&lt;0,01 *</b>
	24h	6h	<b>3,305</b> 1,855 – 9,173	0,321
<b>hCCRN4L/ hNOC</b>	6h	12h	<b>2,415</b> 1,479 – 4,058	<b>&lt;0,01 *</b>
	12h	18h	<b>0,886</b> 0,746 – 1,060	0,51
	18h	24h	<b>0,556</b> 0,461 – 0,673	<b>&lt;0,01 *</b>
	24h	6h	<b>0,84</b> 0,512 – 1,397	0,661
<b>hSMAD5</b>	6h	12h	<b>0,606</b> 0,271 – 1,360	0,83
	12h	18h	<b>1,814</b> 1,319 – 2,508	<b>&lt;0,01 *</b>
	18h	24h	<b>0,190</b> 0,074 – 0,536	<b>&lt;0,01 *</b>
	24h	6h	<b>4,79</b> 2,354 – 17,497	0,66

**Table 4.** Selected genes of interest were further evaluated in their regulation using qRT-PCR. Their p-value and standard-error range (SE range) using REST (relative expression software tool) for identifying changes between time points are given. The significance level was set to p-values <0,01. The mean factors for relative expression results are given in brackets, using  $\beta$ -actin as a reference. Relative expressions are given using previous points in time as a calibrator. Ratios >1 indicate an increase in between points in time; ratios <1 indicate decreases in between points in time. The respective p-values are given in the right column, marked by \* when significantly different. Graphical depiction of the expression levels of all genes are given in Fig. 1. Both, regulation analysis and graphical analysis correspond to each other. Ratios >1 represent an augmented expression comparing sample value to control (previous point in time), thus the expression trend in Fig. 1 will rise and vice versa.

decapentaplegic homolog 5 (hSMAD5).

Diurnal trends of mRNA expression of these genes and significant expression differences between distinct points in time are given in Fig. 1 and Table 4.

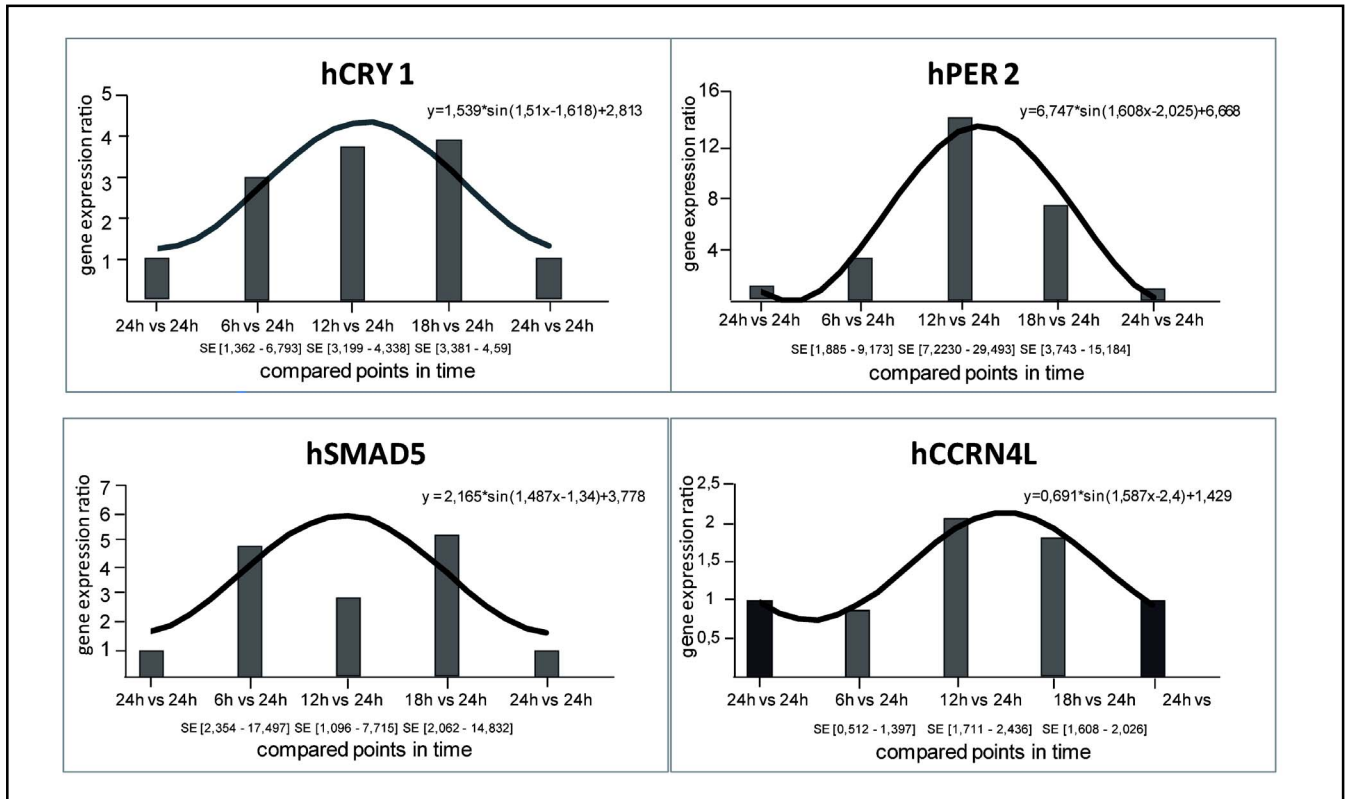


**Fig. 1.** The figures depict circadian regulation of selected genes of interest evaluated by qRT-PCR. qRT-PCR data are displayed in relation to beta-actin expression. The ordinate shows relative ratios of beta-actin/target gene mRNA at different points in time. Adequate times of day are expressed on the axis of abscissa. Cell sampling started at 6h, the first 24h value (grey) is thus an assumed value being obtained from pooling samples of two subsequent days. The ratio of the beta-actin mRNA signal to each gene mRNA signal at the peak of its diurnal expression was arbitrarily set to 100.

hBMAL1 was significantly up-regulated at 6h ( $p < 0.01$ ). Concerning hCRY1, a significant down-regulation was detected at 24h ( $p < 0.01$ ), an expression increase was found between points in time 24h and 6h ( $p < 0.01$ ). We revealed a significant regulation for hPER2 at 12h (up-regulation;  $p < 0.01$ ) and 24h (down-regulation;  $p < 0.01$ ). For hCCRN4L, we were able to detect a significant up-regulation at 12h ( $p < 0.01$ ) and down-regulation at 24h ( $p < 0.01$ ). The tumor suppressor gene hSMAD5 was significantly up-regulated at 18h ( $p < 0.01$ ) and down-regulated at

24h ( $p < 0.01$ ).

Further comparisons of gene expression of hCRY1, hPER2, hSMAD5 and hCCRN4L at 6h, 12h and 18h and expression at 24h commonly show an up-regulation (ratio  $> 1$ ) during the wake phase (6h, 12h, 18h) of individuals. In the following, the respective factor for up-regulation is given in brackets hCRY1, 6h (3.009), 12h (3.707) and 18h (3.915), hPER2 at 6h (3.305), 12h (14.151) and 18h (7.406), hSMAD5 at 6h (4.79), 12h (2.901) and 18h (5.263) as well as hCCRN4L at 12h (2.029) and 18h (1.798).



**Fig. 2.** Diurnal expression profiles of selected genes. The bar chart of each gene shows the gene expression ratio comparing two points in time. As a reference 24h was always compared with 6h, 12h or 18h (comparisons of 24h with 24h were necessary as assumed values). The ordinate presents the expression ratio while the points in time compared to each other are given on the axis of abscissa. The standard errors of the comparisons (SE under the axis of abscissa) are also given. Thus ratios >1 represent up-regulation relative to 24h (24h vs 24h: =1). Furthermore the figures show sine-regressions (sine function is given) for each gene obtained from the gene expression ratio data.

The sinus curve demonstrates the diurnal oscillation of periodically regulated genes. The respective peaks are always detected during day time (Fig. 2). Comprehensively the results as described above exhibit a trend towards up-regulated gene expressions at daytime.

## Discussion

### *Clock Gene Expression in Human Oral Mucosa*

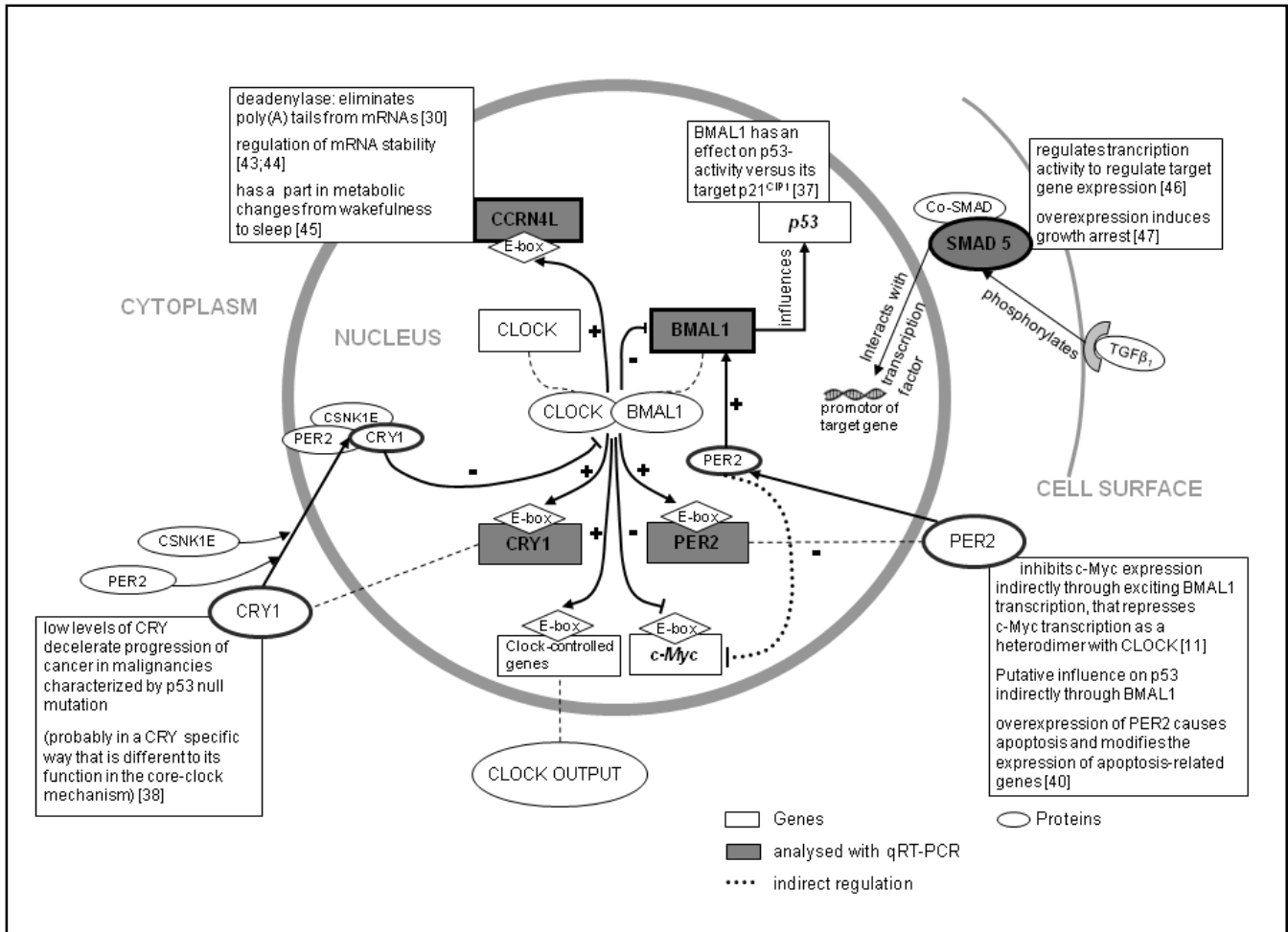
This is the first study intending to investigate physiological regulations of clock-, clock-controlled- and tumor suppressor genes in healthy humans.

All study participants had normal circadian activity profiles, with regular working hours, without any shift work. Their sleeping habits were not synchronized.

To analyse the expression profiles of the above

mentioned genes we performed a microarray screening of cell samples obtained from the oral mucosa throughout a 24 hour period. A large number of differentially regulated clock-, clock-controlled- and tumor suppressor genes was detected. Selected genes of interest exhibiting a differential regulation, such as hBMAL1, hCRY1, hPER2, hCCRN4L and hSMAD5 were further evaluated using qRT-PCR. Thus, we were able to detect common expression profiles of those genes in the study participants.

Analysing our genes in synopsis with the regulations described in the current literature, hCLOCK, hTIM, hCRY1 and hPER2 showed concordant expression patterns corroborated by the literature from studies in human oral mucosa and other peripheral tissues [26, 27]. hCLOCK and hTIM exhibited no circadian rhythmicity according to our microarray analysis as previously demonstrated by Bjarnason et al. [26].



**Fig. 3.** Evaluated clock genes associated with putative tumorigenic pathways. Clock genes with positive and negative regulation pathways are shown. CLOCK and BMAL1 gene transcripts are positive elements in the circadian clock feedback-pathways and therefore predominantly enhance gene transcription. Exceptions are BMAL1-transcription which is inhibited by its own gene product and the inhibition of c-Myc transcription. CLOCK and BMAL1 proteins form heterodimers and bind to E-box-elements which promote the transcription of PER and CRY as well as the transcription of CCRN4L. PER and CRY both form heterodimers being consecutively phosphorylated by CSNK1E. CRY stabilizes PER as well as CSNK1E gene transcripts which phosphorylate PER to stabilize PER-protein. The molecular clock-mechanism has effects on several processes verifiably associated with tumorigenesis. It influences the p53 pathway. CLOCK-BMAL1 heterodimers, whose amount is positively influenced by PER2, inhibit the transcription of c-Myc and regulate other *clock-controlled genes*, which are putative components of vulnerable pathways controlling cell fate. The “Clock output” consists of all rhythmic processes molecularly represented by cycling levels of nucleotides, proteins and enzymes.

Previous analyses of hCRY1 detected peak gene expression in oral mucosa during the afternoon [26]. Thus our results are consistent with these findings. hPER2 was described to peak near 12h [27], which is affirmed by our results.

Furthermore our results support the theory of the circadian transcriptional-translational feedback loop, where the heterodimer CLOCK/BMAL1 induces the transcription of hPER2, hCRY1 and the clock-controlled

genes, such as hCCRN4L [4, 28]. hBMAL1 maximum expression was observed in our results at 6h followed by the peaks of hPER2 and hCCRN4L at 12h and the plateau of hCRY1 from 6h until 18h.

In qRT-PCR findings hCRY1 shows a constant up-regulation during day-time with its peak at 18h, hPER2 and hCCRN4L peaks were detected at 12h and the troughs at 24h. The rhythm of hBMAL1 was nearly anti-cyclical to this. It was at its trough at 12h/24h

and peaked at 6h.

The described anti-cyclical expression profile of hBMAL1 and the hPERs [29] is accredited by the literature.

CCRN4L/NOC showed significant circadian oscillations in peripheral tissue as previously described [30, 31]. The expression is under control of CLOCK/BMAL1 and shows an identical regulation to hPER1 which was reported previously [28]. The parallel expression profile revealed in hCCRN4L and hPER2, whose circadian regulation is quite similar to that of hPER1 [27], therefore matches well with precedently published results and underlines our findings.

Additionally our current findings display that hSMAD5 shows a distinct circadian rhythmicity. Its peaks were determined at 6h and 18h, the troughs at 12h and 24h.

For hSMAD5 no investigation of circadian gene expression profiles has been performed, hence our results are the first evidence that this tumor suppressor [32-34] is also under circadian clock control.

Owing to the fact that our data are obtained from healthy subjects without disruption in circadian rhythm, as well as being generally concordant with former results of circadian gene expression analyses in oral mucosa and other peripheral tissues, we can assume the detected expressions comply with the physiological expressions of these genes in healthy humans.

#### *Dysregulations and down-regulations of circadian regulated genes enhance cancer susceptibility*

About 7% of all identified human clock-controlled genes regulate either cell proliferation or apoptosis. Both mechanisms play a crucial role in cancerogenesis. The associated clock-controlled genes imply the oncogene c-Myc, the tumor suppressor gene p53 (Fig. 3) alongside cell-cycle genes like caspases, cyclins and transcription factors [35]. Concerning oral mucosa Bjarnason et al. showed synchronous circadian oscillation patterns of distinct core clock genes and different cyclins as well as p53. These findings connect endogenous clocks to cell cycle events and tumor suppression [26, 36].

Hence dysregulations of the circadian clock might render individuals prone to tumorigenesis and therefore a physiological clock-, clock-controlled- and tumor suppressor gene expression profile might be suitable for recognizing aberrant profiles and therefore subjects at risk of cancerogenesis.

In respect of our study results it is a relevant finding that there is an association between hBMAL1 and the p53 pathway [37].

The core clock gene hBMAL1 influences the activity of p53 towards its target p21<sup>CIP1</sup>, a critical effector of cell cycle arrest. Knockdown of BMAL1 anticipates the tumor suppressor function of p53 and allows cells to proliferate in spite of p53 activation [37].

Moreover, for the examined CRY1 it was shown that CRY1<sup>-/-</sup> mutations in p53 mutant mice postponed cancer onset and lengthened their life span. CRY1 importantly influences cell proliferation and loss of CRY1 could be a protective factor in cancer development [38, 39].

There is evidence for CRY-specific influences on tumor suppressors apart from the normal function of the gene in the core-clock mechanism [38].

hPER2 examined in this study acts as a tumor suppressor gene. This assumption is supported by cancer-prone mPER2<sup>-/-</sup> mice that present increased tumor susceptibility and reduced apoptosis in thymocytes. Moreover the expression levels of PER2 are diminished in different cancer types. The following model demonstrates the role of hPER2 in tumor suppression: when hPER2 is deficient normal stimulation of BMAL1 transcription is omitted, in turn the expression level of BMAL1 and hence the level of BMAL1/CLOCK heterodimers, which suppress c-Myc transcription through E-box mediated reactions are diminished. This leads to the activation of c-Myc transcription and the overexpression of c-Myc favors tumor development [11, 40, 41].

On the other hand overexpression of mPER2 induces apoptosis in cancer cells, exhibiting a significant cell-growth-inhibition and influence on the expression of apoptosis-related genes like the transcription factor c-Myc (down-regulation) and the tumor suppressor p53 (up-regulation) [35].

hSMAD5 is an important component of the intracellular TGF- $\beta$ -pathway, which in turn plays a crucial role in many different tumor types. Detecting a circadian expression profile of this tumor suppressor gene in our study, the TGF- $\beta$  pathway might also be linked to circadian regulation mechanisms, possibly even to the circadian clock [46, 47].

The consideration that loss of the tumor suppressor SMAD5 amongst others is a reason for juvenile granulosa cell tumors and their dissemination [33], which could indicate an important role for up-regulation of SMAD5, especially during times of stress factor impact.

The studied CCRN4L encodes a deadenylase removing poly(A) tails from mRNA and thus destabilizes or prevents it from transcription [42]. Its targets have to be investigated further [31], especially in vivo and with respect to oncogenes and rhythmic cell-cycle genes. Thus CCRN4L could eventually exert certain tumor suppressor functions. The detected circadian expression profile of CCRN4L could constitute a protective feature during daytime up-regulation of the gene, in case a connection between CCRN4L and tumor promoting genes proves true.

#### *Up-regulation of tumor suppressor genes during the wake period*

During the wake period different stress factors, produced in metabolism or externally have an impact on every single cell and can cause cancer-relevant mutations. During sleeping periods those stressors are obviously reduced.

As various stressors do have a comparatively higher impact when protective factors do not attenuate them, we believe the main contribution of physiological circadian rhythms is the impact of protective gene regulation. If circadian rhythms are asynchronous their protective function on cells could be diminished. On the one hand this might result when doing shift work [9] or living a lifestyle which does not permit a regular synchronized clock gene activity. Hence individuals are awake and physically active during times of low protective gene expression (especially at night time).

On the other hand long-time shift work could cause totally asynchronous rhythms with low protective gene expression during vulnerable periods which could persist even if the individual sleeps regularly during night times again. Thus protective gene regulation could be diminished during daytime and individuals are at risk of cancerogenesis during a normally secure period.

The asynchronous manner of clock gene regulation will not be responsible for the malignant potential as such, but lacking protection will permit harmful factors to affect cells in a more vulnerable state.

Hence the assumption that tumor suppressor genes should normally be up-regulated during highest impact of stress factors in wake-time is obvious. Our examination of the existing theory showed an up-regulation of the tumor suppressors hPER2 and hSMAD5 during day-time, thus that assumption can be supported. Furthermore hCRY1 and hCCRN4L also show up-regulations during daytime. Hence, both genes could likewise influence distinct tumor suppressive

features, which need further investigation.

In summary, the tumor suppressor function of several genes investigated here (hPER2, hSMAD5, eventually hCRY1 and hCCRN4L), reach their respective maxima during daytime since the expression peaks of those genes were detected in this time. We therefore conclude that humans are well protected from cell-damaging influences during this period.

The increased risk of cancer development in people doing shift work [9, 13] could be explained by disturbed circadian rhythms with down-regulation instead of up-regulation of tumor suppressor genes during wake times. Thereby the probability to be awake and physically active during a period of low protective gene expression rises. Therefore it is more likely that tumorigenic factors exert their impact during vulnerable phases of diminished tumor suppressor gene regulation and accordingly have an increased risk of cancer.

As asynchronous gene regulation can be seen as a factor contributing to the risk profile in malignant processes, knowledge of physiological gene regulations of relevant genes might enable detection of those alterations. Thus on the one hand persons with alterations in gene expression might be able to normalize them again by keeping with a regular daily routine omitting night-time activity. On the other hand patients suffering from malignancy exhibiting altered gene expression might profit more from adapted treatment regimes taking into account circadian differences, thus rendering medicamentous therapy more effective.

A good starting point to further evaluate the approach would be the establishment of standard expression profiles in important clock-, clock-controlled-, tumor suppressor genes and oncogenes, based on our results, in a more comprehensive group of healthy humans, which could be compared with expression profiles represented in mucosa of humans with anamnestic risk factors or cancer.

### **Acknowledgements**

We would like to thank the volunteers who participated in this study. In addition, we thank Mrs. Melanie Hauth for helping with mRNA isolation, Mrs. Sarah Bühler for her generous helpfulness especially with qRT-PCR and Mr. Tobias Jenne for his support with calculations.

## References

- 1 Aronson BD, Johnson KA, Loros JJ, Dunlap JC: Negative feedback defining a circadian clock: autoregulation of the clock gene frequency. *Science* 1994;263:1578-84.
- 2 Hall JC: Tripping along the trail to the molecular mechanisms of biological clocks. *Trends Neurosci* 1995;18:230-40.
- 3 Wilsbacher LD, Takahashi JS: Circadian rhythms: molecular basis of the clock. *Curr Opin Genet Dev* 1998;8:595-602.
- 4 Fu L, Lee CC: The circadian clock: pacemaker and tumour suppressor. *Nat Rev Cancer* 2003;3:350-61.
- 5 Hastings MH, Reddy AB, Maywood ES: A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci* 2003;4:649-61.
- 6 Reppert SM, Weaver DR: Coordination of circadian timing in mammals. *Nature* 2002;418:935-41.
- 7 Roenneberg T, Merrow M: The network of time: understanding the molecular circadian system. *Curr Biol* 2003;13:R198-R207.
- 8 Schibler U, Sassone-Corsi P: A web of circadian pacemakers. *Cell* 2002;111:919-22.
- 9 Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Fuchs CS, Colditz GA: Night-shift work and risk of colorectal cancer in the nurses' health study. *J Natl Cancer Inst* 2003;95:825-8.
- 10 Filipinski E, King VM, Li X, Granda TG, Mormont MC, Liu X, Claustrat B, Hastings MH, Levi F: Host circadian clock as a control point in tumor progression. *J Natl Cancer Inst* 2002;94:690-7.
- 11 Fu L, Pelicano H, Liu J, Huang P, Lee C: The circadian gene *Period2* plays an important role in tumor suppression and DNA damage response in vivo. *Cell* 2002;111:41-50.
- 12 Hansen J: Increased breast cancer risk among women who work predominantly at night. *Epidemiology* 2001;12:74-7.
- 13 Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Colditz GA: Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. *J Natl Cancer Inst* 2001;93:1563-8.
- 14 Pardini L, Kaeffer B, Trubuil A, Bourreille A, Galmiche JP: Human intestinal circadian clock: expression of clock genes in colonocytes lining the crypt. *Chronobiol Int* 2005;22:951-61.
- 15 Scheving LE, Tsai TH, Scheving LA: Chronobiology of the intestinal tract of the mouse. *Am J Anat* 1983;168:433-65.
- 16 Northoff H, Symons S, Zieker D, Schaible EV, Schafer K, Thoma S, Loffler M, Abbasi A, Simon P, Niess AM, Fehrenbach E: Gender- and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise. *Exerc Immunol Rev* 2008;14:86-103.
- 17 Smyth GK: Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004;3:1027.
- 18 Bolstad BM, Irizarry RA, Astrand M, Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;19:185-93.
- 19 Wichert S, Fokianos K, Strimmer K: Identifying periodically expressed transcripts in microarray time series data. *Bioinformatics* 2004;20:5-20.
- 20 Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 1995;57:289-300.
- 21 Lemos DR, Downs JL, Urbanski HF: Twenty-four-hour rhythmic gene expression in the rhesus macaque adrenal gland. *Mol Endocrinol* 2006;20:1164-76.
- 22 Glynn EF, Chen J, Mushegian AR: Detecting periodic patterns in unevenly spaced gene expression time series using Lomb-Scargle periodograms. *Bioinformatics* 2006;22:310-6.
- 23 Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002;30:e36.
- 24 Yano A, Fujii Y, Iwai A, Kageyama Y, Kihara K: Glucocorticoids suppress tumor angiogenesis and in vivo growth of prostate cancer cells. *Clin Cancer Res* 2006;12(10):3003-9.
- 25 Yamamoto T, Nakahata Y, Soma H, Akashi M, Mamine T, Takumi T: Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. *BMC Mol Biol* 2004;5:18.
- 26 Bjarnason GA, Jordan RC, Wood PA, Li Q, Lincoln DW, Sothorn RB, Hrushesky WJ, Ben-David Y: Circadian expression of clock genes in human oral mucosa and skin: association with specific cell-cycle phases. *Am J Pathol* 2001;158:1793-801.
- 27 Dunlap JC: Molecular bases for circadian clocks. *Cell* 1999;96(2):271-90.
- 28 Li R, Yue J, Zhang Y, Zhou L, Hao W, Yuan J, Qiang B, Ding JM, Peng X, Cao JM: CLOCK/BMAL1 regulates human nocturnin transcription through binding to the E-box of nocturnin promoter. *Mol Cell Biochem* 2008;317:169-77.
- 29 Reppert SM, Weaver DR: Molecular analysis of mammalian circadian rhythms. *Annu Rev Physiol* 2001;63:647-76.
- 30 Douris N, Green CB: NOC out the fat: a short review of the circadian deadenylase Nocturnin. *Ann Med* 2008;40(8):622-6.
- 31 Wang Y, Osterbur DL, Megaw PL, Tosini G, Fukuhara C, Green CB, Besharse JC: Rhythmic expression of Nocturnin mRNA in multiple tissues of the mouse. *BMC Dev Biol* 2001;1:9.
- 32 Zavadil J, Brezinova J, Svoboda P, Zemanova Z, Michalova K: Smad5, a tumor suppressor candidate at 5q31.1, is hemizygotously lost and not mutated in the retained allele in human leukemia cell line HL60. *Leukemia* 1997;11:1187-92.
- 33 Middlebrook BS, Eldin K, Li X, Shivasankaran S, Pangas SA: Smad1-Smad5 ovarian conditional knockout mice develop a disease profile similar to the juvenile form of human granulosa cell tumors. *Endocrinology* 2009;150:5208-17.
- 34 Pangas SA, Li X, Umans L, Zwijsen A, Huylebroeck D, Gutierrez C, Wang D, Martin JF, Jamin SP, Behringer RR, Robertson EJ, Matzuk MM: Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. *Mol Cell Biol* 2008;28(1):248-57.
- 35 Hua H, Wang Y, Wan C, Liu Y, Zhu B, Yang C, Wang X, Wang Z, Cornelissen-Guillaume G, Halberg F: Circadian gene *mPer2* overexpression induces cancer cell apoptosis. *Cancer Sci* 2006;97:589-96.
- 36 Bjarnason GA, Jordan RC, Sothorn RB: Circadian variation in the expression of cell-cycle proteins in human oral epithelium. *Am J Pathol* 1999;154:613-22.
- 37 Mullenders J, Fabius AW, Madiredjo M, Bernards R, Beijersbergen RL: A large scale shRNA barcode screen identifies the circadian clock component ARNTL as putative regulator of the p53 tumor suppressor pathway. *PLoS One* 2009;4:e4798.
- 38 Ozturk N, Lee JH, Gaddameedhi S, Sancar A: Loss of cryptochrome reduces cancer risk in p53 mutant mice. *Proc Natl Acad Sci U S A* 2009;106:2841-6.
- 39 Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H: Control mechanism of the circadian clock for timing of cell division in vivo. *Science* 2003;302:255-9.
- 40 Winter SL, Bosnoyan-Collins L, Pinnaduwa D, Andrusis IL: Expression of the circadian clock genes *Per1* and *Per2* in sporadic and familial breast tumors. *Neoplasia* 2007;9:797-800.

- 41 Yang X, Wood PA, Oh EY, Du-Quiton J, Ansell CM, Hrushesky WJ: Down regulation of circadian clock gene *Period 2* accelerates breast cancer growth by altering its daily growth rhythm. *Breast Cancer Res Treat* 2009;117:423-31.
- 42 Baggs JE, Green CB. Nocturnin, a deadenylase in *Xenopus laevis* retina: a mechanism for posttranscriptional control of circadian-related mRNA. *Curr Biol* 2003;13:189-98.
- 43 Garbarino-Pico E, Niu S, Rollag MD, Strayer CA, Besharse JC, Green CB: Immediate early response of the circadian polyA ribonuclease nocturnin to two extracellular stimuli. *RNA* 2007;13:745-55.
- 44 Green CB, Douris N, Koijima S, Strayer CA, Fogerty J, Lourim D, Keller SR, Besharse JC: Loss of Nocturnin, a circadian deadenylase, confers resistance to hepatic steatosis and diet-induced obesity. *Proc Natl Acad Sci USA* 2007;104:9888-93.
- 45 Ramsey KM, Bass J: *Lean* gene and the clock machine. *Proc Natl Acad Sci USA* 2007;104:9553-4.
- 46 Attisano L, Lee-Hoeflich ST: The Smads. *Genome Biol* 2001;2:reviews 3010.1-3010.8.
- 47 Gemma A, Hagiwara K, Vincent F, Ke Y, Hancock AR, Nagashima M, Bennett WP, Harris CC: *hSmad5* gene, a human *hSmad* family member: its full length cDNA, genomic structure, promotor region and mutation analysis in human tumors. *Oncogene* 1998;16:951-6.