Evidence for a circadian effect on the reduction of human growth hormone gene expression in response to excess caloric intake

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*Running title: Diet affects the circadian synthesis of human GH

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Abstract

Rhythmicity of biological functions is fundamental for optimal adaptations to environmental cues. Growth hormone (GH) is a major metabolic homeostatic factor that is secreted with a circadian pattern, but whether it is synthesized rhythmically is unknown. We used transgenic (TG) mice containing the human (h) GH gene (hGH1) locus to investigate the rhythmicity of hGH synthesis and secretion, and showed RNA and secreted protein levels oscillate over a 24-hour cycle. Analysis of hGH1 promoter sequences revealed an enhancer motif (E-box) element that binds the circadian transcriptional machinery (Bmal1 and Clock). Furthermore, Bmal1/Clock were able to transactivate the hGH1 promoter, and mutation of this E-box element adversely affected basal activity after gene transfer. The ability of Bmal1 to bind the hGH1 promoter region containing the E-box element was confirmed in the hGH1 TG mouse pituitary in situ. Occupancy was reduced in mice fed a high fat diet (HFD) during the light (inactive) stage of the daily cycle in mice, and corresponded to a decrease in hGH1 RNA levels. The decreases in occupancy and RNA levels were not seen, however, during the dark (active) stage. A chromatin loop required for efficient postnatal hGH1 expression was negatively affected by the HFD in the light but not dark stage similar to the pattern observed with Bmal1 association with the promoter region. This is the first evidence that hGH synthesis follows a diurnal rhythm, and of dynamic associations of the circadian machinery with a component of a chromosomal structure of the hGH1 locus that is essential for efficient expression.

Introduction

Rhythmicity of biological function including the endocrine system is fundamental for optimal adaptation to environmental cues (1,2). Human (h) growth hormone (GH) secretion is subject to a circadian and diurnal rhythm that follows a sleep pattern (3,4). Specifically, the peak in hGH secretion occurs during sleep in the dark stage of the daily cycle (3,4). If this sleep pattern is disrupted, however, compensatory and largely unpredictable pulses of GH release are observed during the light/awake stage as
demonstrated in shift-workers (5,6). This suggests that GH production is not solely under the control of the sleep/awake cycle, but is regulated at a higher level of complexity and perhaps by the circadian machinery. However, it is not known whether hGH synthesis occurs, like secretion, with a circadian rhythm (7). The difficulty in establishing that hGH synthesis, and specifically the hGH gene (hGH1), is a target for circadian rhythm, is because of a lack of access to pre-mortem human pituitary samples. Animal models are also not optimal. There are distinct differences in structure between human (primate) and murine (non-primate) GH genes, including in both the flanking and coding DNA. As a result, hGH and mouse (m) GH have the potential for different regulatory control and function (8-14).

In this study we used 171hGH/CS transgenic (TG) mice that preferentially express hGH1 in mouse pituitary somatotrophs (15,16). This pattern of tissue/cell specific expression is a result of including the intact hGH gene and locus control region (LCR) in a ~150 kb fragment of human chromosome 17 (15) as part of the transgene (16). The LCR can act as a tissue or cell-specific enhancer and also confer appropriate site of integration-independent expression of a transgene (15-18). These mice grow normally and specifically express (not overexpress) hGH1 in somatotrophs of the anterior pituitary gland (15,16). This property depends on the presence of essential hypersensitive site (HS) regions I-III and V within the LCR. HS I/II is seen in human pituitary somatotrophs, is re-established in the hGH1 TG mouse genome, and is the major determinant of pituitary hGH1 expression (13,19-22). Physical interaction between HS I/II and the local hGH1 promoter and looping of intervening (14.5 kb) chromatin (23-25) is essential for hGH1 activation (13,24) and postnatal expression (23,26).

Microarray studies of transcripts have revealed that 10-30% of the human genome is under the control of circadian molecular clocks (27). Daily rhythms seen in these transcripts are controlled by an endogenous circadian timing system (28,29). These self-directed rhythms are generated by well-characterized intracellular transcriptional feedback loop involving a cis-regulatory element known as an enhancer box (E-box) (28,29). The core players of this molecular transcriptional feedback system, which orchestrates the cyclic expression of clock-controlled genes, are the circadian locomotor output cycles kaput (clock), the brain and muscle ARNT-like 1 (Bma1), the Period proteins (Per 1-3), two Cryptochrome (Cry) proteins and Rev-erba (28-30). This sophisticated and elegant transcriptional clock machinery ensures coordinated and orchestrated molecular and physiological responses by affecting target gene expression with changes in the environment, for example, through changes in diet (31).

Human GH levels are known to fluctuate dynamically under different physiological as well as pathophysiological conditions, including excess caloric intake and obesity. Normal healthy individuals that indulge in short term overeating display a dramatic suppression of serum GH levels. Interestingly, this phenomenon happens when obesity is not an issue (32). We have previously shown that hGH production is extremely sensitive to increased caloric intake within the continuum of obesity progression. Three days of excess caloric intake induced by high fat diet consumption led to a 75% reduction in hGH1 RNA levels and impaired secretion in transgenic mice containing the hGH gene and LCR (33). Unlike hGH1, the endogenous mGH gene (mGh) displayed no
negative response to the hyperinsulinemic conditions induced by acute excess caloric in transgenic and wild type CD1 mice *in vivo* (33). An explanation for this differential response could relate to the presence of an E-box DNA element in the proximal hGH1 promoter region that is absent from equivalent mGh promoter DNA (34). Furthermore, the presence of this E-Box element offers a potential target for Bmal1 and Clock proteins.

Here, we provide evidence that hGH synthesis, and specifically hGH1 expression, is under the control of a circadian rhythm and a target of Bmal1/Clock interaction. Furthermore, we show that excess caloric intake can perturb hGH1 Bmal1/E-box DNA element binding as well as the chromatin loop linking HS I/II and promoter regions. These observations are presented and discussed in the context of the daily light/dark cycle.

**Experimental procedures**

**Animals and diet**

All procedures involving animals, their tissues and cells conform to the Guide for the Care and Use of Laboratory Animals published by the Canadian Council on Animal Care, and was approved by the Animal Protocol Management and Review Committee at the University of Manitoba. Animals were housed with *ad libitum* access to food and water in an environmentally controlled room maintained on a 12-hour light/dark cycle with lights on at zeitgeber time (ZT 0; which refers to the number of hours after the light phase onset of 6 a.m., and lights off at ZT 12 or 6 p.m.). Control regular chow diet (CD; fat, 26 kcal%; carbohydrate, 54 kcal%; protein, 20 kcal% from Prolab – RHM 3000, St. Louis, MO) or high-fat diet (HFD; fat, 60 kcal%; carbohydrate, 20 kcal%; protein, 20 kcal% from Research Diets, New Brunswick, NJ) was supplied as palatable pellets for three days to four-week-old male mice (16,34). Mice were maintained on their respective diets until time of euthanization (cervical dislocation) and assay.

**Blood chemistry**

Trunk blood was collected and allowed to clot at 4 °C for 15 minutes (min) and serum was separated by centrifugation (9300 x g for 5 min). Serum samples were stored at -80 °C until assessed by human GH ELISA (22-HGHHUU-E01, ALPCO).

**RNA preparation and quantitative real-time reverse transcriptase-PCR**

Total RNA was isolated using QIA shredder and RNaseasy Plus Mini Kit (Qiagen, ON, Canada, 79656, 74136). Quality of the extracted RNA was assessed by agarose gel electrophoresis. Total RNA (1 µg) was transformed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, 205314) according to the manufacturer’s instructions. Quantitative real-time reverse transcriptase (RT) -PCR (qPCR) analyses were done in a 7500 system (AB Applied Biosystems, Warrington, UK) with specific primers (Table 1). Reactions (20 µL) included 10 µL of Power SYBR green Master mix (Applied Biosystems), 1µL of forward and reverse primers (0.5 pM) and 0.1 µg of cDNA. Thermal cycling was started with 5 min denaturation at 95 °C, followed by 40 cycles of 95 °C for 30 seconds, annealing at 60 °C for 15 seconds and 72 °C for 30 seconds. Minus RT controls were done using the same PCR primers and conditions as a control for genomic DNA contamination. Specific amplifications were identified by a single peak in the melting curve and a single band in the final PCR product visualized on 1% agarose gel. The gene expression level in each sample was calculated from a standard curve (for each primer set) and normalized to mouse β-actin expression. Tests were run at least in duplicate and on three mice for each experiment.
Cell culture, plasmid constructs and gene transfer

Human embryonic kidney 293 (HEK293) cells were maintained in a monolayer culture in Dulbecco’s modified Eagle’s Medium (DMEM) (Invitrogen Corp., 12100-061, Carlsbad, California) supplemented with 0.5 mM glutamine, 50 µM/L penicillin, 50 µg/mL streptomycin, and fetal bovine serum (FBS) at an appropriate (v/v) percentage in a humidified air/CO2 (19:1) atmosphere, at 37°C (35). Cells were harvested using trypsin-EDTA after a single wash with phosphate buffered saline (PBS). The 0.5 kb of the \( mGh \) promoter region was generated by PCR using primers (forward: 5´-GATCGGGATCCTCCCCAAAAGTTACTCTC-3´, reverse: 5´-CGATCAAGCTTGGAATCTGGACTCTAGGATG-3´) that introduce \( \text{BamH}1 \) (5´-GGATCC-3´) and \( \text{Hin}d\text{III} \) (5´-AAGCTT-3´) sites for subcloning into the \( \text{BamH}1 \) and \( \text{Hin}d\text{III} \) sites of the firefly luciferase vector, pXP1-Luc (36). Mutation of the wild type (WT) E-box element from 5´-CCACGTGACC-3´ to 5´-CCAgcTGACC-3´ in the 0.5 kb \( hGH1 \) promoter region, was done using a two-step PCR reaction approach and use of specific primers as described previously (37). In brief, a double-stranded fragment with a 21-nucleotide overlap of the region to be mutated was generated, and \( \text{BamH}1 \) and \( \text{Hin}d\text{III} \) sites were then introduced for subcloning into pXP1-Luc. The cDNA expression vectors for mouse circadian locomotor output cycles kaput (Clock), previously reported as (Gal4-Clock/UA256), and brain and muscle ARNT-like 1 (Bmal1), previously reported as (Gal4-Bmal1/UA255), were kindly provided by Dr. Urs Albrecht (University of Fribourg, Switzerland) (38). The construct for human period circadian protein homolog 2 (Per2) luciferase construct (pGL3-hPer2-Luc) was kindly provided by Dr. Louis Ptacek (University of California, San Francisco) (39). Trans-IT293 reagent (Mirus Biol Corp., MIR 2700, Madison, Wisconsin) diluted with serum-free DMEM, and containing expression vectors or hybrid reporter genes was used for transient transfection of HEK293 cells. For expression of cDNA vectors, up to 4 µg of plasmid DNA (expression vector) was used per 1x10^6 cells/100 mm plate. For transfection with reporter genes, up to 0.5 µg of reporter gene plasmid and 10 ng pRL-TKp-Luc plasmid (as a control for DNA uptake) was used per 2.5x10^5 cells/well (6-well plate). Cells were treated with DNA/reagent for 24 hours, washed with PBS and harvested and luciferase assays done 48 hours later.

Nuclear protein extraction and detection

The EpiSeeker Nuclear Extraction Kit was used according to the manufacturer’s instructions (Abcam, ab113474, Toronto, ON) (40). Briefly, cells were suspended in hypotonic buffer containing dithiothreitol and protease inhibitors on ice for 15 min. Nuclei were separated by centrifugation (14,000 \( \times \) g for 1 min at 4°C), and the nuclear pellet extracted in a hypertonic lysis buffer for 30 min on ice. Following centrifugation (14,000 \( \times \) g for 10 min at 4°C), the supernatant containing the nuclear fraction was collected, and protein concentration was determined by a Bradford protein assay (Bio-Rad, Richmond, CA, USA).

For detection of Clock and Bmal1, 20 µg of nuclear and cytoplasmic proteins from HEK293 cells transfected with expression vectors were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane and immunoblotted with anti-Clock (Abcam, ab3517) and Bmal1 (Abcam, ab3350) antibodies. Beta (\( \beta \))-tubulin (Santa Cruz, sc-9104, Dallas, Texas) and lamin B (Santa Cruz, sc-6217) were assessed as controls for protein loading for whole cell lysates and nuclear proteins, respectively. The
proteins were visualized using horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) secondary antibody and ECL plus immunoblotting detection reagents (Thermo Fisher Scientific Inc, ON, Canada).

**Electrophoretic mobility shift assay (EMSA)**

EMSA and competition with oligonucleotides was performed essentially as previously described (41). Oligonucleotides (purchased from Invitrogen) were radio-labeled with [γ-32P] ATP (PerkinElmer, Wellesley, MA) using T4 polynucleotide kinase (New England Biolabs). Briefly, 4 µg of HEK293 nuclear extract was incubated with EMSA buffer containing 2-µg poly-dIdC for 5 min. Radiolabeled oligonucleotide probe (1 ng) was then added and the reactions were incubated for a further 10 min at room temperature. For competition, 50-fold molar excess of unlabeled oligonucleotide was added during the pre-incubation period. The DNA-protein complexes were resolved in non-denaturing 5% (w/v) polyacrylamide gels, and visualized by autoradiography.

**Luciferase assay**

Luciferase activity was measured using the Dual-luciferase assay system (Promega Corp., PRE1960, Madison, Wisconsin), with a photon counting luminometer (LUMAT LB9507, EG&G Berthold). In brief, cell pellets were resuspended in 50 µL of lysis buffer (100 mM Tris- HCl pH 7.8, 0.1% Triton X-100) for 10 min on ice, and centrifuged at 15,800 x g for 15 min at 4 °C. Values were normalized through co-transfection with pRL-TKp-Luc and assessment of Renilla luciferase in a dual assay as well as with protein concentration, which was assessed using the Bradford Protein assay.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was done with isolated mouse anterior pituitaries using EZ-Magna ChIP kit (Catalog # 17-10086, Millipore, Etobicoke, Ontario) as reported previously (33). In brief, pituitaries were cross-linked with 1% of formaldehyde at room temperature for 30 min before lysis. Chromatin was fragmented by sonication (100 seconds in 10-second pulses and 100% amplitude). Insoluble material was removed by centrifugation. DNA content was then measured by spectrophotometry. Soluble chromatin was immunoprecipitated with 5 µg of specific antibodies (indicated in figure legends) along with magnetic protein (A/G) beads overnight with rotation at 4 °C. The eluted chromatin separated from magnetic beads was reverse cross-linked at 65 °C overnight, and DNA was isolated using the QIAquick PCR purification kit (Qiagen). Quantitative PCR was done under conditions standardized for each primer set (Table 2). Each qPCR reaction was carried out in duplicate in a 20 µL reaction volume by using 5 µL of the 1% of input DNA and 5µL of pre-amplified (15 cycles) eluted immunoprecipitated DNA and 10 µL of Power SYBR green Master mix (Applied Biosystems). Dissociation curves were analyzed as a mean to ensure the quality of amplicons and to monitor primer dimers. Final PCR products were visualized as a single band on 1% agarose gel. ChIP Enrichment was determined based on a percent input method (42). Briefly, the signals obtained from the immunoprecipitated DNA amplification were divided by the signals obtained from an input sample. Enrichment was calculated based on the formula, 100 x 2^(CT adjusted Input – CT Enriched) and data are presented as percent input and relative fold change compared to the control which is arbitrary set to 1.

**Chromosome conformation capture (3C) assay**

The 3C assay was performed essentially as described (33,43,44). Briefly,
mouse pituitaries (4 pituitary glands/3C test) were homogenized in a tissue nuclei isolation buffer to obtain a cell suspension. The cells were cross-linked for 10 min at room temperature using 1% formaldehyde-HEPES buffer and the reaction was quenched by addition of 0.125 M glycine. The cells were washed with PBS, and lysed in 1 mL of ice-cold lysis buffer (10 mM Tris-Cl; pH 8.0, 10 mM NaCl, 0.2% NP-40) for 30 min at 37°C. Triton X-100 was added to obtain a 1% final concentration, and then the nuclei were incubated for a further 30 min at 37°C. Nuclei were washed with restriction enzyme (BglII) digestion buffer, then cross-linked DNA was digested overnight with BglII. The restriction enzyme was inactivated with 0.1% SDS and incubating at 65°C for 20 min. Two µg of digested chromatin was diluted in a final volume of 0.8 mL containing 1% Triton-X. Ligation reaction was carried out at 16°C for 4 hours and followed by incubation at room temperature for a further 30 min in the presence of T4 DNA ligase (New England Biolabs). Reverse cross-linking of ligated DNA was carried out overnight at 65°C in the presence of proteinase K. The following day, samples were incubated for 30 min at 37°C with RNase A (10 ng/mL) followed by purification and determination of DNA concentration. Nested primers were used with two rounds of PCR to increase sensitivity, which was followed by qPCR using a primer set that encompassed the BglII restriction cut site (Table 3) under the following conditions using the Power SYBR PCR Kit (Applied Biosystems): the initial denaturation for 5 min at 95°C, 30 cycles of 95°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec. The results were normalized to GAPDH in ligated and unligated samples. Interaction between HS I/II and the hGH1 promoter (GHp) was determined by calculating the ligation frequency according to the formula:

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\text{Ligation frequency} = \frac{\text{level of ligation product/loading control}}{\text{level of non-ligation product/loading control}}
\]

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism® software. For the rhythmicity analysis and thus multiple comparisons between each time points, one-way ANOVA was used with the Bonferroni post-test. The diet effect at each time point (single comparisons) was assessed by paired student t-test. A value of p<0.05 is considered statistically significant and represented in figures as: *, p<0.05; ** or ##, p<0.01; *** or ###, p<0.001.

**Results**

**Human GH unlike mouse GH RNA levels oscillate over a 24-hour period**

Anterior pituitary tissue was isolated every four hours over a 26-hour period from 4-5 week-old male hGH/CS-TG mice, fed a regular chow (control) diet (CD) and maintained under a 12-hour light/dark cycle. Levels of hGH1 RNA displayed a circadian pattern with high and comparable levels at ZT 2 and 26 (8 a.m.), that was significantly reduced by >65% at ZT 14 (12 p.m.) (p<0.001, n=6-12 per time point) (Figure 1A). By contrast, mGh RNA levels showed no significant oscillation over the same 26-hour period (Figure 1B).

**Human GH promoter E-box supports Bmal1/Clock heterodimer binding**

We showed previously that the hGH1 promoter region contains a palindromic hexanucleotide (5′-CACGTG-3′) E-box element at position –264 to –259 (34). This E-box is located within the chromatin loop region linking hGH1 promoter and HS I/II regions that is required for hGH1 activation and efficient postnatal expression (26), but is not present in the same region of mGh.
sequences (34). Thus, EMSA was used to test the ability of this sequence to support binding of the E-box factors Bmal1/Clock. Expression of Bmal1 and its partner Clock in transfected HEK293 cells was confirmed by electrophoresis of nuclear and cytosolic extracts and immunodetection (Figure 2A). EMSA was done using nuclear extracts from HEK293 cells overexpressing Bmal1 and Clock proteins with radiolabeled hGH1 promoter E-Box element (nucleotides -278/-250) as a probe. Multiple low to high mobility complexes were seen with nuclear extract from HEK293 cells transfected with an empty vector (Figure 2B). The intensity of these complexes, and particularly the two large low mobility complexes (arrows) were increased with nuclear extract from HEK293 cells expressing Bmal1 and Clock cDNAs (Figure 2B). The low and high mobility complexes were competitively inhibited by (50-100-fold) molar excess unlabeled wild type (WT) hGH1 promoter E-Box element (5′-CACGTG-3′). By contrast, use of two oligonucleotides, Mut1 and Mut2, containing two (5′-CAgcTG-3′) and six (5′-gatcga-3′) base pair mutations of the WT hGH1 promoter E-Box element (45), respectively, were progressively less effective as competitors (Figure 2B).

**Bmal1/Clock overexpression trans-activates a hGH1p-luciferase reporter gene via association with an E-box DNA element**

HEK293 cells were transiently transfected with hybrid luciferase genes driven by 496 bp of hGH1 promoter sequences with a wild type (WT) or mutated (Mut1) E-Box DNA element; the latter introduced by PCR-based site-directed mutagenesis. Both hybrid Luc genes were co-transfected with either control empty vector or a Bmal1/Clock expression vector. A significant increase in expression was detected in the presence of the WT (p<0.001, n=9), but not Mut1 E-box DNA element in response to Bmal1/Clock cDNA expression (Figure 3A). A significant increase by the hGH/CS-TG mice in hGH RNA and protein levels was detected at the time point with the highest levels detected on the CD; specifically during day light hours ZT 0-2 (Figure 4A). Serum hGH levels were also reduced throughout the study period in mice on the HFD compared to CD, and specifically at ZT 2 and 22 (8 a.m. and 4 a.m.) (p <0.05, n=5-6) (Figure 4B). By contrast, no significant changes were detected in mGh transcript levels over the 24-hour period on either diet, or differences between effects of a HFD versus CD at any time point (Figure 4C). Like the hGH/CS-TG mice, there were also no detectable differences seen in mGH RNA levels in wild type mice fed the HFD versus CD for three days at any time point over the 24 hours (n=4) (Figure 4D).
variation during the diurnal cycle with peaks during the onset of daylight hours (ZT 2) and troughs during dark hours (ZT 10-22). HFD consumption for three days led to lack of diurnal variation in Clock RNA levels in the pituitary gland as a decreasing trend was detected during the light phase and an increasing trend during the night cycle when compared to the CD (Figure 5A). However, these differences at each time point did not reach statistical significance (n=12). There was a clear diurnal variation of both Bmal1 and Per2 RNAs with a peak at ZT 2 and ZT 14, respectively, in both CD and HFD groups. The HFD increased Bmal1 RNA levels significantly on the HFD (p<0.05, n=12) at ZT 22 during the dark period (Figure 5B), but had no significant impact on Per2 RNA levels (Figure 5C). Rev-erba transcript levels displayed diurnalvariation in mice on either a CD or HFD, with a peak during the light period (highest levels at ZT 10) and low levels throughout the dark period (Figure 5D). By contrast, the opposite time-dependent cycling was observed for Cry1 and Cry2 RNA levels, which are essential components of the negative limb of the circadian clock feedback loop (46). Diurnal variation in Cry1 RNA levels in the CD group was at its maximum during the dark period (highest levels at ZT 18), whereas Cry2 RNA levels peaked at ZT 10 (Figure 5E and F). The HFD was associated with increased amplitude of Cry1 and a phase shift in Cry2 RNA levels, as peak levels were delayed and appeared during the onset of the dark period (Figure 5E and F). A diurnal expression is described for the c-fos gene in the pituitary gland (47), and rhythmic expression of this gene is sensitive to excess caloric intake (48). As expected, c-fos RNA levels were reduced (70%) at early and late stages of the dark period after three days on a HFD (data not shown).

The effect of a CD versus HFD for three days on binding of Bmal1 to the hGH1 promoter region in mouse pituitary during the light (ZT 2) and dark (ZT 18) stages was assessed by ChiP assay. Association of Bmal1 with the hGH1 promoter region was reduced significantly at ZT 2, but was not decreased at ZT 18 for mice on the HFD versus CD (Figure 6). Furthermore, Bmal1 levels for mice on the HFD were comparable and thus restored to those detected on the CD in the dark stage at ZT 18. However, a comparison of Bmal1 association with the hGH1 promoter region at ZT 2 versus ZT 18 in mice fed a CD, reveals a significant 30% reduction binding (p<0.01, n=3) in the dark stage (Figure 6). No effect of diet was observed on Bmal1 association with an untranscribed region on mouse chromosome 6 (Untr6), which served as a measure of non-specific association of DNA-protein in mouse pituitary at either ZT 2 or 18 (Figure 6).

**A chromatin loop required for efficient postnatal hGH1 expression is negatively affected by a HFD in the light (inactive) but not dark (active) phase of the day in mice**

The effect of a CD versus HFD for three days on hGH1 promoter and HS I/II region interactions (evidence of chromatin looping) in mouse pituitary during the light (ZT 2) and dark (Z T 18) phases was assessed by 3C assay (Figure 7). A significant 65% decrease in ligation frequency of the hGH1 promoter and HS I/II fragment was detected at ZT 2, the light and relatively inactive phase in mice (p<0.001, n=3). This decrease with a HFD was not apparent, however, by ZT 18 (dark and relatively active phase in mice), where levels were comparable to those of mice on a CD at Z 18 (p<0.01, n=3). A reduction in the ligation frequency between the hGH1 promoter and HS I/II regions was also suggested in the pituitaries of mice fed with CD at ZT 18 when compared to ZT 2, but

**Bmal1 association with the hGH1 promoter is reduced by excess caloric intake**
did not reach statistical significance (p=0.07, n=3).

Discussion:

In this study, we show that hGH1 but not mGh is expressed rhythmically and that RNA levels oscillate over a 24-hour period in hGH/CS-TG mice. Analysis of hGH1 proximal promoter sequences revealed the presence of an E-box element that was not present in equivalent mGh DNA (34). We provide evidence that these sequences have the capacity to bind the E-box factors Bmal1 and Clock, and transactivate the hGH1 promoter, using EMSA, ChIP and transient transfection assays. Association of Bmal1 with the E-box element was reduced in male mice fed a HFD during the light stage of the 24-hour period in situ. By contrast, this decrease was not seen during the dark stage, which is associated with a time of greater daily activity in mice. These changes also correlate with a significant negative impact of excess caloric intake on the hGH1 chromosomal structure, involving interaction between remote enhancer and proximal promoter regions that is required for efficient postnatal expression, and thus a negative impact on the hGH production.

Levels of hGH1 RNA displayed a circadian pattern with high and comparable levels at ZT 2 and 26 (8 a.m.), which was significantly reduced at ZT 14 (12 p.m.) in hGH1 transgenic mice. Thus, the hGH1 transcript profile has the appearance of a classic clock-controlled gene with a peak and trough that are 12 hours apart under light and dark conditions (49). By contrast, mGh RNA levels showed no significant oscillation over the same 26-hour period. This difference might be related to the E-box element, with 5′-CACGTG-3′ at its core, located in the hGH1 promoter region that is absent from equivalent mGh sequences (34). Efficient postnatal expression and even activation of hGH1 is linked to pituitary specific transcription factor Pit-1 binding, at both the promoter and remotely 14.5 kb upstream at HS I/II within the hGH1 LCR (50). These Pit-1 binding regions interact through looping out of intervening sequences (50). Evidence presented here and in previous studies indicates that sequences within the chromatin loop, and specifically an E-box DNA element at nucleotide position -264/-259 (relative to the hGH1 transcription initiation site), plays a role in maintaining basal promoter activity (33,34,51). Results from EMSA support the ability of Bmal1/Clock and hGH1 E-box DNA element to participate in a common complex. Furthermore, mutation of two nucleotides at the core of the E-box element significantly impaired basal promoter activity, and deletion of nucleotides -266 to -253 that includes the E-box element completely suppressed hGH1 promoter activity (52). Although the focus here has been on the potential contribution of an E-box element located at nucleotide position –264/–259, we cannot rule out the possibility that other E-box elements play a role in circadian oscillation of hGH1. However, location as in association with hGH1 chromatin loop-related sequences may be as important as the simple presence of an E-box element(s), given the lack of response seen with mGh.

Excess caloric intake had a negative impact on hGH1 RNA levels at only one time point during the day when the highest levels of the transcript were detected (ZT 2). This reduction at ZT 2, however, was associated with significant >40% decreases in hGH detected in the circulation at ZT 2 and 22 (8 a.m. and 4 a.m.), when the highest levels would normally be detected. Thus, our data are consistent with a negative impact of a HFD for three days on rhythmic hGH production during the inactive locomotor phase, considering the nocturnal nature of
mice as opposed to humans. This observation is consistent with the spikes in GH levels in humans during the biological evening and night or sleep/inactive period (53).

The altered hGH1 expression might be related to the reduction seen in Bmal1 association with the hGH1 promoter region for mice on the HFD versus CD at ZT 2, rather than affects on Bmal1 production. This pattern of clock-controlled gene dysregulation by a HFD has been reported in the liver (31). The mechanism involved disruption of Bmal1 association with the chromatin in situ without any significant changes in Bmal1 RNA and/or protein levels (31). This is consistent with our observation that the positive limb of the core clock transcriptional machinery including Clock and Bmal1 were not affected significantly by HFD at most time points assessed. While Bmal1 RNA levels were increased significantly at the late stage of the dark cycle (ZT 22), the HFD had a significant positive effect at this time on the negative arm of the core clock machinery including Cry1. These increases in Bmal1 and Cry1 transcript levels with a HFD have been reported previously (54) and may reflect a disrupted feedback system. Evidence suggests that a physical interaction between Cry1 and Bmal1 is responsible for switching Bmal1 from an activated to a repressive state (55). Thus, earlier appearance of the Cry1 in the pituitaries of hGH1 TG mice on the HFD may lead to repression of the Bmal1/Clock protein complex, which in turn signals an increase in Bmal1 production and increased RNA levels. A positive trend in Clock expression during the dark cycle by HFD was also observed, although it did not reach statistical significance (55).

It is noted that Bmal1/Clock complexes have significantly different chromatin binding properties at different times during the day, possibly due to post-translational modification (56). Thus, binding of these transcription factors to E-box elements follows a circadian pattern in vivo (57). The Bmal1 ChIP analysis supports this notion; there was a difference between the levels of Bmal1 associated with the hGH1 promoter region based on the time of euthanization (light versus dark stage). As the Bmal1/Clock complex does not remain bound to chromatin throughout the day (58), the opportunity for other E-box associated factors to bind and perhaps modulate promoter activity is possible. As a result, a circadian exchange of different transcription factors binding to E-box elements would offer a target for a variety of signaling pathways, perhaps reflecting environmental and/or intrinsic cellular cues to influence transcriptional activity depending on physiological demand.

We have previously shown that the hGH gene (hGH1) promoter region is hypoacetylated in response to excess caloric intake at time points between ZT 2-6 (33). Histone modifications are implicated in the transcriptional regulation of the hGH1 locus. Thus the hGH1 promoter region is condensed and can limit the activator transcription factor such as clock/bmal1 heterodimer accessibility. Furthermore, transcriptional repression is mediated by recruitment of co-repressor complexes, which can lead to chromatin remodeling by directing histone modification enzymes to specific site of regulatory elements of the target gene. Among the most well characterized transcriptional co-repressors of the pituitary gland is the nuclear receptor co-repressor (NCOR). We have previously demonstrated that NCOR can mediate the transcriptional repression of hGH1 in somatotrophs/pituitary from 171hGH/CS TG mice overfed for three days (33). Excess caloric intake resulted in a 4-fold increase in association of NCOR with the hGH1 promoter region in the pituitary as assessed by ChIP assay (33).
A recent study implicated the circadian machinery in determining temporal and spatial cycles of chromosomal organization, and is capable of affecting the physical organization of the nuclear landscape via involvement of E-box elements (59). The diurnal variation of hGH1 transcript levels raises the possibility that the hGH1 locus can transit between an active and inactive chromosomal conformation in a cyclic manner daily. If so, the physical interaction between HS I/II and the proximal promoter chromatin regions may respond to or influence association of E-box transcription factor(s), as seen with Bmal1 and the hGH1 promoter. Intriguingly, the reduction in the HS I/II-hGH1 promoter chromatin loop observed during the light cycle with the HFD regimen was not evident during the dark stage of the cycle, suggesting a recovered and/or intact chromosomal conformation. This observation may reflect an example of transcriptional memory (60). It is anticipated that changes in the availability and accessibility of E-box factors would reverse the reduction in hGH1 RNA levels and associated changes in the hGH1 locus (chromatin remodeling). This would likely involve elimination of repressive factors resulting from HFD consumption during the dark stage (33).

A study of hGH synthesis is not possible in humans, but our studies in TG mice implicate the pituitary as a target for circadian control of hGH1 transcription. To our knowledge, this provides the first evidence for rhythmic hGH1 RNA levels that correlate with the rhythmic hGH secretion reported in humans over a 24 hour cycle. Our data also indicate that this rhythmic hGH1 expression is sensitive to excess caloric intake that can disrupt association of the circadian machinery with the locus.

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Conflict of interest
The authors have declared that no conflict of interest exists.

Author contributions
HV and PAC conceived and coordinated the study and wrote the paper. HV designed, performed and analyzed the experiments. YJ performed the experiments shown in Figures 6 and 7.
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Figure legends:

**Figure 1: Human unlike mouse GH RNA expression oscillates over a 24-hour period**

Anterior pituitary tissues were isolated every four hours over a 24-hour period from 4-5 week-old male mice, fed a regular chow (control) diet (CD) and maintained under a 12-hour light/dark cycle. Total pituitary RNA from hGH/CS TG mice at each time point was assessed by qPCR for (A) human GH and (B) mGH RNA levels. The results are expressed as mean percentage change ± standard error of mean relative to levels detected at zeitgeber time (ZT) 2, which is arbitrarily set to 100%, n=6-12.

**Figure 2: Human GH promoter E-box supports Bmal1/Clock heterodimer binding**

(A) Expression of Bmal1 (~69 kDa) and its partner Clock (~95 kDa) in transfected HEK293 cells was confirmed by electrophoresis of nuclear (NE) and cytosolic (CE) extracts and immunodetection. β-Tubulin (~55 kDa) and lamin B (~66 kDa) were used as loading control for CE and NE proteins, respectively. (B) EMSA was done using NE from HEK293 cells overexpressing Bmal1 and Clock proteins with radiolabeled hGH1 promoter E-Box element (nucleotides -278/-250) as a probe. The effects of molar excess (50 and 100-fold) of unlabeled wild type (WT) hGH1 promoter E-Box element (with 5'-CACGTG-3' core sequence) and two oligonucleotides, Mut1 and Mut2, containing two (5'-CAgcTG-3') and six (5'-gatcga-3') base pair mutations of the WT hGH1 promoter E-Box core sequence were used with their respective WT radiolabeled counterpart. Major low mobility DNA-protein complexes are indicated (open arrowheads).

**Figure 3: Bmal1/Clock overexpression in HEK293 cells trans-activates hGH1p-luciferase reporter via association with an E-box DNA element**

HEK293 cells were transiently transfected with hybrid luciferase (Luc) genes driven by: (A) 496 bp of hGH1 promoter sequences with a WT or Mut1 E-Box DNA element; (B) 0.5 kb mGh promoter and (C) human Per2 promoter sequences. All hybrid Luc genes were co-transfected with either control empty vector or a Bmal1/Clock expression vector. The results are expressed as relative mean fold change ± standard error of the mean, compared to the control value (empty vector). Values were normalized through co-transfection with pRL-TKp-Luc and assessment of Renilla luciferase in a dual assay (n=6). Significant differences are indicated by *** and ### p<0.001, n=6.

**Figure 4: The effect of acute high caloric intake on rhythmic synthesis and secretion of hGH over a 24 hour period**

Human pituitary GH RNA (A) and secreted protein (B) levels were assessed by qPCR and ELISA respectively every 4 hours for 24 hours, after three days on a control or high fat diet. The levels of mGH RNA in (C) hGH/CS TG and (D) wild type (WT) CD1 mice at three days were also assessed as above. The results are expressed as mean percentage change/concentration (ng/mL) ± standard error of mean relative to levels detected at zeitgeber time (ZT) 2, which is arbitrarily set to 100%, n=6-12. Significant differences are indicated by * p<0.05, ** p<0.01 and *** p<0.001.
Figure 5: The effect of acute high caloric intake on the rhythmicity of clock machinery RNA levels in the pituitary over a 24 hour period  
RNA levels of the cellular clock machinery were assessed by qPCR every 4 hours for 24 hours, after three days on a control or high fat diet. The results are expressed as mean percentage change ± standard error of mean relative to levels detected at zeitgeber time (ZT) 2, which is arbitrarily set to 100%, n=6-12. Significant differences are indicated by * p<0.05, ** p<0.01 and *** p<0.001.

Figure 6: Bmal1 association with the hGH1 promoter is reduced by excess caloric intake  
ChIP was performed with an anti-Bmal1 antibody to assess changes in association of Bmal1 with the hGH1 promoter in mouse pituitary during the light (ZT 2) and dark (ZT 18) stages in response to a control diet (CD) versus high fat diet (HFD) for three days. Binding events were normalized to the amount of input chromatin used for the Bmal1 immunoprecipitation reaction and the control untranscribed regions of mouse (Untr6). The results are expressed as relative mean fold change ± standard error of the mean, compared to the control value (control diet), which is arbitrarily set to 1.0. Significant differences were assessed by t-test and are indicated by **, ## P<0.01 and ### p<0.001, n=3.

Figure 7: A chromatin loop required for efficient postnatal hGH1 expression is negatively affected by a HFD in the light (inactive) but not dark (active) stage of the day in mice  
The effect of a control diet (CD) versus high fat diet (HFD) for three days on hGH1 promoter and HS I/II region interactions in mouse pituitary during the light (ZT 2) and dark (ZT 18) stages was assessed by 3C assay. The results are normalized to GAPDH from ligated and unligated samples. Interaction between HS I/II and the hGH1 promoter was determined by calculating the ligation frequency. The results are obtained from 3 independent samples and are expressed as relative mean change ± standard error of the mean. Significance was assessed by t-test, P<0.001. The results are obtained from three independent samples and are expressed as relative mean change ± standard error of the mean. Significance was assessed by t-test, ## P<0.01 and ### P<0.001, n=3.
Table 1: Primers used for qPCR; h = human, m = mouse

| Transcript | Primer sequence                  |
|------------|----------------------------------|
| h GH1      | For: CCTAGAGGAAGGCATCCAAA        |
|            | Rev: GCAGCCCGTAGTTCTTGAGTAG      |
| m GH       | For: ACGCGCTGCTCAAAAAACTAT       |
|            | Rev: CACAGGAGAGTGCAAGGAG         |
| m Clock    | For: ACAGTTTCACGAGGGTGTC         |
|            | Rev: TCCCTACCGCTCTCATCAAGG       |
| m Bmal1    | For: CCACCTCAGACCCATTGATACA      |
|            | Rev: GAGCAGGTTTAGTTCCACTTTGTCT   |
| m Per2     | For: TGTGCGATGATGATCCGTGA        |
|            | Rev: GGTAAGGTACGTTTTGTTTGC       |
| m Rev-erba | For: AGACTTCCCGCTTCAACCAAG       |
|            | Rev: AGCTTCTCGGAATGCTGTTG        |
| m Cry1     | For: TGGGCGAGAAGGTAGATG           |
|            | Rev: CCTCTGTTACCCGGAAAGCTG       |
| m Cry2     | For: CTGGGCGAGAAGGTAGG           |
|            | Rev: GACGAGATTAGCCTTGTGC         |
| m C-fos    | For: GACAGCCTTTTCTACTACCATTCC    |
|            | Rev: GACAGATCTGCGCAAGAGTC        |
Table 2: Primers used for ChIP-qPCR

| Genomic regions | Primer sequence               |
|-----------------|-------------------------------|
| hGH1 promoter   | For: CCCCTTCTCTCCCACTGTTG     |
|                 | Rev: AACCCTCACAACACTGGTGAC    |
| Untr6           | For: TCAGGCATGAACCACCATAC     |
|                 | Rev: AACATCCACACGTCCAGTGA     |
Table 3: Primers used for the 3C assay

| Genomic regions | Primer sequence                      |
|-----------------|--------------------------------------|
| Nested PCR      | HS I/II: ACAGCACCCATGAGGCAACA        |
|                 | GHp: CAGGATAGCCAGTCCTTGAGAC          |
| HS I/II-GH1p    |                                      |
| qPCR            | HS I/II: CCATTCTTAACCTCTCAGTGACCA    |
|                 | GHp: GAAAAAGAAAAAGAAAGATGCCCTGT      |
| HS I/II-GH1p    |                                      |
Figure 1

A

Human GH RNA levels (%)

B

Mouse Gh RNA levels (%)

ZT  2  6  10  14  18  22  26

ZT  2  6  10  14  18  22  26
Figure 2

A

|           | NE | CE |
|-----------|----|----|
| Empty vector | +  | +  |
| cDNA vector | -  | +  |

IB: Bmal1
IB: Clock
IB: Lamin B
IB: β-tubulin

kDa
75
50
100
75
50

B

|          | WT | Mut1 | Mut2 |
|----------|----|------|------|
| Bmal1/Clock NE | -  | +    | +    |
| Empty vector NE | -  | +    | -    |

Arrow symbols point to specific bands or areas in the gel.
Figure 3

A

Clock/Bmal1 cDNA

|   |   |   |
|---|---|---|
| - | + |   |

hGH1p-Luc
WT E-box

hGH1p-Luc
Mut1 E-box

B

Luciferase activity

0

1000

2000

3000

4000

5000

6000

7000

8000

9000

mGhp-Luc

C

Luciferase activity

0

15000

30000

45000

60000

75000

90000

hPer2-Luc
Figure 4

A. hGH/CS-TG

B. hGH/CS-TG

Control diet
High fat diet

C. hGH/CS-TG

D. WT

Human GH RNA levels (%)

Serum hGH (ng/ml)

Mouse GH RNA levels (%)

Mouse GH RNA levels (%)

ZT

2 6 10 14 18 22
Figure 6

![Box plot showing relative Bmal1 binding events for different conditions and time points.](http://www.jbc.org/)

- CD vs. HFD at ZT 2 and ZT 18.
- Untr6 vs. hGH1 promoter.
- Statistical significance indicated by symbols: ***, ##.
Figure 7

Box plots showing the HS III- hGH1 promoter ligation frequency for CD and HFD groups at ZT 2 and ZT 18. The plots indicate a significant difference between the groups, with CD showing lower expression than HFD at both time points, as indicated by the ** and *** symbols.
Evidence for a circadian effect on the reduction of human growth hormone gene expression in response to excess caloric intake
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